DEVELOPMENT OF BENZOYL PEROXIDE EMULGEL CONTAINING *KELULUT* HONEY FOR ACNE TREATMENT

BY

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A thesis submitted in fulfilment of the requirement for the degree of Master of Science in Pharmacy (Pharmaceutical Technology).

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MAY 2024

ABSTRACT

Acne vulgaris affects people globally with negative psychological impacts. Benzoyl peroxide has widely been used as an effective topical treatment for acne. However, it can cause skin irritation such as drying, itchiness and redness. These side effects may increase the concern among the patients to use benzoyl peroxide as an acne treatment. Hence, this study aimed to formulate a benzoyl peroxide emulgel incorporating stingless bee honey for acne vulgaris treatment. This is because stingless bee honey possesses excellent moisturising properties which are believed can help to counter the side effect of benzoyl peroxide. Besides, stingless bee honey also has antibacterial properties which may enhance the bactericidal (antiacne) effect of benzoyl peroxide. The objectives of this study are to formulate and characterise the physicochemical properties of the benzoyl peroxide and stingless bee honey emulgel formulations, to determine the minimum inhibitory concentration (MIC) with minimum bactericidal concentration (MBC) of the stingless bee honey, to evaluate the in vitro antiacne activity of the formulated emulgel and also to assess the skin hydration, transepidermal water loss (TEWL) and skin erythema of human volunteers after the application of the formulated benzoyl peroxide and stingless bee honey emulgel. Four formulations with the same excipients were prepared which are benzoyl peroxide with stingless bee honey emulgel (F1), benzoyl peroxide emulgel (F2), stingless bee honey emulgel (F3) and base emulgel (F4). All of these formulations underwent characterisation encompassing organoleptic properties, centrifugation, pH, droplet size, polydispersity index (PDI), zeta potential, spreadability, and microbial limit count study. They were further subjected to stability study at two different conditions (30ºC/75%RH and 40ºC/75%RH) for three months and the formulations' characterisation was measured in weeks 2, 4 and 12 of the study. Besides, the MIC and MBC of stingless bee honey against *P.acnes* were evaluated to determine the effective concentration of stingless bee honey to be incorporated into the formulation. Meanwhile, the antiacne study of all formulations was conducted to assess the formulations' effectiveness against *P.acnes* bacteria. Lastly, *an in vivo* study on 26 volunteers was conducted to assess the effectiveness of stingless bee honey in reducing the side effects of benzoyl peroxide and the safety of the formulated emulgel on human skin. All formulations showed good characterisation and were considered stable since all the parameters remained in the acceptable range during the three-month stability study although there were some changes observed for the pH, droplet size, PDI, zeta potential and spreadability study. Based on the MIC and MBC results, it showed that stingless bee honey could inhibit and kill *P.acnes* bacteria with concentrations of 12.5% and 15% respectively. Hence, a 15% concentration was incorporated into the formulation. Since F3 showed some inhibition zone and the inhibition zone of F1 was significantly larger than F2, hence this proved that stingless bee honey could still exhibit its antiacne effect when in a dosage form. All formulations were able to improve the skin erythema, hydration and TEWL of the volunteers except the F2. F2 worsened these three parameters which were signs of impaired skin barrier. In conclusion, the combination of stingless bee honey with benzoyl peroxide emulgel was successfully developed and effective in reducing the side effects of benzoyl peroxide.

ملخص البحث

يؤثر حب الشباب عالمياً على الأفراد، كما أن له تأثيرات نفسية سلبية، وكعلاج موضعي فعال لعلاج حب الشباب يستخدم بنزويل بيروكسايد على نطاق واسع، لكنه يمكن أن يسبب تهيج للجلد كجفافه واحمراره والإصابة بالحكة. هذه الآثار الجانبية قد تزيد القلق بين المرضى لاستخدام بنزويل بيروكسايد كعلاج. بالتالي، هدفت هذه الدراسة إلى صياغة مستحلب بنزويل بيروكسايد الذي يتضمن عسل النحل الغير لاسع (stingless bee honey) لعلاج حب الشباب. وذلك لأن عسل النحل الغير لاسع يمتلك خصائص ترطيب ممتازة والتي يعتقد أنها يمكن أن تساعد في مواجهة التأثير الجانبي للبنزويل بيروكسايد. بالإضافة إلى ذلك، يحتوي عسل النحل غير اللاسع أيضاً على خصائص مضادة للجراثيم. كانت أهداف هذه الدراسة هي صياغة وتوصيف الخواص الفيزيائية والكيميائية لتركيبات مستحلب عسل النحل الغير لاسع والبنزويل بيروكسايد، ولتحديد الحد الأدنى للتركيز المثبط (MIC) مع الحد الأدنى من التركيز المبيد للجراثيم (MBC)، ولتقييم النشاط المضاد لحب الشباب في المختبر للمستحلب المحضر وأيضاً لتقييم ترطيب الجلد وفقدان الماء عبر البشرة (TEWL) واحمرار جلد المتطوعين. تم تحضير أربع تراكيب وهي البنزويل بيروكسايد مع مستحلب العسل (F1)، مستحلب البنزويل بيروكسايد (F2)، مستحلب العسل (F3) والمستحلب (F4). خضعت كل هذه التركيبات للتوصيف الذي يشمل الخواص الحسية والطرد المركزي ودرجة الحموضة وحجم القطرة ومؤشر التشتت المتعدد (PDI)، وإمكانات زيتا، وقابلية الانتشار، ودراسة عدد الحدود الميكروبية. كما تم إخضاعهم لدراسة الاستقرار في حالتين مختلفتين (30 درجة مئوية، 75% رطوبة نسبية و40 درجة مئوية، 75% رطوبة نسبية) لمدة ثلاثة أشهر وتم قياس توصيف التركيبات في الأسابيع: 2 و4 و12 من الدراسة. علاوة على ذلك، تم

تقييم MIC وMBC لعسل النحل غير اللاسع ضد حب الشباب لتحديد التركيز الفعال للعسل الذي سيتم دمجه في التركيبة. وفي الوقت نفسه، أجريت دراسة مقاومة حب الشباب لجميع التركيبات لتقييم فعالية التركيبات ضد بكتيريا البروبيونية العدية (*P.acnes*). وأخيراً، أجريت دراسة على 26 متطوعاً لتقييم فعالية العسل في تقليل الآثار الجانبية للبنزويل بيروكسيد وسلامة المستحلب على جلد الإنسان. أظهرت جميع التركيبات توصيفاً جيداً واعتبرت مستقرة نظراً لأن جميع المعلمات ظلت في النطاق المقبول خلال دراسة الثبات لمدة ثلاثة أشهر على الرغم من وجود بعض التغييرات الملحوظة في درجة الحموضة وحجم القطرة وPDI وإمكانات زيتا ودراسة قابلية الانتشار. العسل يمكن أن يثبط ويقتل بكتيريا البروبيونية العدية بتركيزات 12.5% للMIC و15% للMBC. ومن ثم، تم دمج تركيز 15% في التركيبة. نظراً لأن F3 أظهر بعض التثبيط وكانت منطقة التثبيط لF1 أكبر بكثير من F2، فقد أثبت هذا أن عسل النحل غير اللاسع ما يزال بإمكانه إظهار تأثيره المضاد لحب الشباب عندما يكون في شكل جرعة. كانت جميع التركيبات قادرة على تحسين احمرار الجلد والترطيب وTEWL للمتطوعين باستثناء F2. أدى F2 إلى تفاقم هذه العوامل الثلاثة التي كانت علامات على ضعف حاجز الجلد. في الختام، تم تطوير مزيج عسل النحل الغير لاسع مع مستحلب بنزويل بيروكسايد بنجاح وكان فعالاً في تقليل الآثار الجانبية للبنزويل بيروكسايد.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science in Pharmacy (Pharmaceutical Technology)

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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Signature………………………………… Date 30th April 2024……………………

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ACKNOWLEDGEMENTS

First and foremost, I would like to begin by expressing my heartfelt gratitude to Allah, the Almighty, whose guidance, blessings and unwavering presence have been the cornerstone of my master's journey through the pursuit of knowledge.

I would like to thank my parent and family for their boundless support, encouragement and sacrifices during this journey. Their constant love and encouragement have been the bedrock of my achievement today. I am profoundly grateful for your unwavering belief in me.

I would like to extend my deepest appreciation to my dedicated and insightful supervisor, Assoc. Prof. Dr Hazrina Ab Hadi. Your unwavering guidance, expertise and mentorship have been invaluable throughout my research, shaping me into a better researcher. Besides, I am also grateful to my co-supervisor, Assoc. Prof. Dr. Juliana Md Jaffri for her assistance in this research.

I would also like to extend my appreciation to the lab staff from the Pharmaceutical Technology Department, Basic Medical Science Department and Pharmaceutical Chemistry Department especially Sr Hazan Haryanti, Br Faris, Sr Zailillah, Br Dzadil, Br Hanif, Sr Salmi, Br Radziff and Sr Fatimah for their invaluable assistance and expertise in providing the necessary resources and technical support for my experiments. Also, special thanks to staff from IKOP Sdn. Bhd especially Sr Shahida, Sr Ika, Sr Shamimi and Br Akbar who always lend a hand that eases my work.

I would like to thank my labmates and friends, both within and outside academia especially Sr Shakirah, Sr Syuhaida, Sr Shima, Br Rizal and Br Safwan. Your companionship, encouragement, support and help have made the research process both enjoyable and enlightening.

Lastly, to all those who have contributed to my growth directly or indirectly, I am truly grateful for your support and encouragement. Thank you all for being an integral part of my academic journey and for helping me reach this milestone.

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CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

Skin disease is a skin condition that often occurs in children and adults which can lead to severe disability. Globally, the combination of skin disorders including acne problems denotes the fourth greatest cause of non-fatal disability, especially in resource-poor areas. In 2013, among 306 diseases and injuries, skin diseases were anticipated to contribute to 1.79% of the total global burden of disease (GBD) measured in disability-adjusted life years (DALYs) (Bragazzi et al., 2019). In fact, acne vulgaris has been recognised as the second greatest cause of DALYs after dermatitis, resulting in a loss of productivity for patients and caregivers similar to the cost of skin cancer productivity. Acne also has been placed as the eighth-most frequent disease worldwide and it affects almost 9.4% population in the world (Alkhabbaz et al., 2020). Although acne is not a life-threatening condition, but it can lead to psychological comorbidities such as depression, social anxiety and even suicidal thoughts. This is because patients feel embarrassed, frustrated and have low self-esteem to interact with other people especially those with severe facial acne (Costeris et al., 2021). Hence, they tend to seek treatment to cure their acne problem.

Nowadays, there are various conventional acne treatments including topical medications, oral medications and therapies. According to the Federal Food, Drug and Cosmetic in Code of Federal Regulations Title 21 (Volume 5), the active ingredients for over-the-counter topical acne treatment are 2.5% to 10% benzoyl peroxide, 2% of resorcinol, 2% of resorcinol combined with 3% to 8% sulfur, 3% of resorcinol monoacetate combined with 3% to 8% of sulfur, 0.5% to 2% salicylic acid, 3% to 10% of sulfur (FDA., 2020). All of these basically are keratolytic agents that can treat acne by breaking down the keratin plugs and disrupting the adhesions between the keratinocytes (Jadhav et al., 2018). This is because keratolytic agents can dissolve the intercellular cement. Benzoyl peroxide, salicylic acid and sulfur are also able to possess bactericidal and anti- inflammatory properties (Marubayashi et al., 2021). However, these keratolytic agents may cause adverse effects like skin dryness and irritation in some patients, especially for benzoyl peroxide. Hence, these side effects may reduce the patient's compliance with the treatment and negatively impact therapeutic outcomes (Brigitte Dréno et al., 2020).

Honey has been used as traditional medicine for a long period in many cultures. Honey is basically a food source for bees that are rich in carbohydrates. It is an acidic mixture that contains at least 200 constituents with sugar and water as the main compounds. Other components including organic acids, proteins, minerals such as copper and calcium and also vitamins such as vitamin B6, niacin, riboflavin, thiamine and pantothenic acid (Martinotti et al., 2019). Honey has natural bacteriostatic and bactericidal properties due to its low pH, high sugar content and high total phenolic and flavonoid compounds. Hence, it could be an active compound as an antibacterial against acne-forming bacteria, *Propionibacterium acnes*. Honey also possesses anti-inflammatory and antioxidant activities that can decrease the redness of acne (Julianti et al., 2017). Furthermore, honey also exhibits a moisturising and soothing effect on the skin. This is because the high sugar content in honey acts as a natural humectant and emollient that increases the water content and reduces dryness in the skin even after they have been removed (Kaur et al., 2020).

1.2 PROBLEM STATEMENT

Acne vulgaris is one of the most prevalent dermatologic diseases that can affect all people but predominantly in adolescents. It can be considered a serious disease since it can have a negative impact on psychosocial functioning and lead to psychological comorbidities. Many studies reported that adolescents with acne tend to have lower self-esteem and life satisfaction compared to controls. Not only that, but some of them also exhibit self-injurious behaviour (Özyay et al., 2018). These psychological effects encourage people to seek out ways to treat acne. Benzoyl peroxide which is a keratolytic agent is readily available in the market and widely used as acne treatment since they are over-the-counter products. However, it possesses some adverse effects which is skin irritation. Many studies stated that benzoyl peroxide causes skin irritation including dryness, redness, burning, pruritis, peeling of skin (desquamation) and tingling sensation at the application area (Grobel & Murphy, 2018; Iijima & Tsunoda, 2019). All of these adverse effects had been reported in three commercialised benzoyl peroxide products which are 0.1% adapalene and 2.5% benzoyl peroxide combination gel (adapalene/BPO gel) (Epiduo®, Maruho Co), 1% clindamycin (CLDM) and 3% benzoyl peroxide combination gel (CLDM/BPO gel) (Duac®, Pola Pharma Co) and 2.5% benzoyl peroxide gel (Bepio®, Maruho Co) during the products' approval. The adverse effects were developed in 10.8%, 30.6% and 43.7% patients for Epiduo®, Duac® and Bepio® respectively (Iijima & Tsunoda, 2019).

A study conducted by Iijima & Tsunoda (2019) in Japan found that 4.5% of acne patients experienced allergic contact dermatitis when being treated with topical benzoyl peroxide gels. In another study conducted by Kawashima et al. (2017), the percentage of benzoyl peroxide-related adverse effects developed by the patients using 2.5% and 5% benzoyl peroxide were 37.3% and 38.7% respectively. The incidences of adverse effects in both of these 2.5% and 5% benzoyl peroxide groups were not significantly difference (Kawashima, Sato, et al., 2017). Meanwhile, the adverse effects were detected in 20.9% of moderate and severe facial acne patients treated with adapalene 0.3%/benzoyl peroxide 2.5% gel with the most frequent treatment-related adverse effects were skin irritation and pain skin (Dreno, Bissonnette, et al., 2018). Based on the Galderma Global Safety Database from 1992 to 15th October 2021, 558 adverse effect reports were identified related to topical acne drug products comprising solely benzoyl peroxide as the active ingredient with most of them assessed to have a nonserious outcome except for one case (Szymanski & Arekapudi, 2022). Due to all of these side effects, most of the patients discontinued the benzoyl peroxide treatment (Kawashima, Nagare, et al., 2017; Lam Hoai et al., 2021). Hence, this makes the incorporation of natural humectant such as honey into the conventional formulation is reasonable to counter the adverse effect. This is because honey is a natural humectant that possesses an excellent moisturising effect (Said et al., 2020). Moreover, honey may enhance the bactericidal effect of benzoyl peroxide since it possesses antimicrobial activity too (Julianti et al., 2017). Therefore, honey is considered the recommended ingredient to be incorporated in the benzoyl peroxide emulgel.

1.3 RESEARCH OBJECTIVES

1.3.1 General objective

To develop a benzoyl peroxide and stingless bee honey emulgel for acne vulgaris treatment.

1.3.2 Specific objectives

1. To formulate a benzoyl peroxide emulgel incorporating stingless bee honey and characterise the physicochemical properties of the formulation.
2. To determine the minimum inhibitory concentration (MIC) with minimum bactericidal concentration (MBC) of the stingless bee honey and evaluate the *in vitro* antiacne activity of the formulated emulgel.
3. To assess the skin hydration, transepidermal water loss (TEWL) and skin erythema of human volunteers after the application of the formulated benzoyl peroxide and stingless bee honey emulgel (human study).

1.4 RESEARCH HYPOTHESIS

The stingless bee honey with benzoyl peroxide emulgel formulation is expected to have fewer side effects (skin irritation) compared to the benzoyl peroxide emulgel.

1.5 EXPECTED OUTCOME

A combination of stingless bee honey with benzoyl peroxide is expected to have lesser skin irritation side effects than benzoyl peroxide alone in treating acne.

CHAPTER TWO

LITERATURE REVIEW

2.1 SKIN

Skin is basically the largest and most important organ for human life. It covers the whole-body surface which makes it the main interaction site with the external environment. It plays an important role in maintaining the fluid balance of the organism and performing as a mechanical barrier against external injury either physical, chemical or biological. Skin is also involved in thermoregulation and immunological surveillance (Ribeiro et al., 2017). The structure of the skin is composed of a few layers which are the epidermis (outermost layer), dermis (middle layer) and hypodermis (innermost layer). The epidermis functions as a barrier to the external environment and prevents water loss. It mostly consists of keratinocytes and has five layers which are stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and stratum basale. However, stratum lucidum is only present in thick skin such as the hands' palms and the feet' soles. Other than keratinocytes, there are also melanocytes, Langerhans' cells and Merkel cells (Sandra Lawton, 2019). The human skin structure and the layers of the epidermis are illustrated in Figure 2.1.

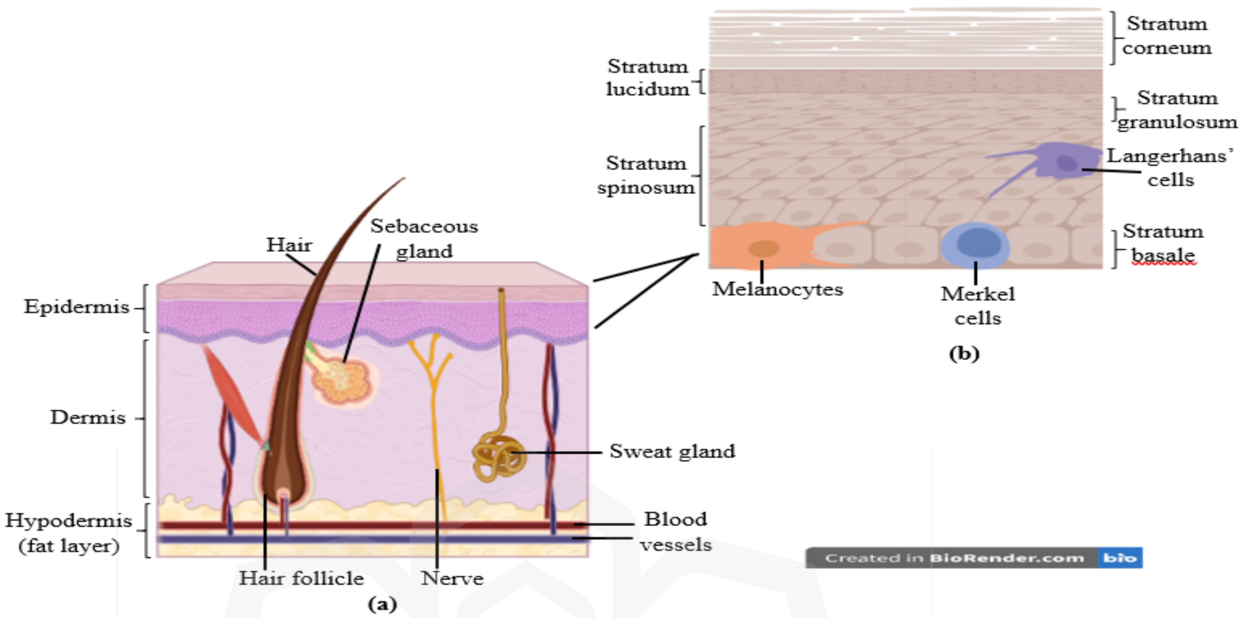


Figure 2.1 a) Human skin structure b) Epidermis layers.

The dermis is the thickest layer of the skin that is made up of papillary dermis and reticular dermis. It comprises cellular and interstitial components but mainly is the collagen (~70%). There are also muscles, blood vessels, lymphatic channels, sensory nerves, sweat glands and hair follicles in the dermis (Lai-Cheong & McGrath, 2017). The combination of hair follicles and sebaceous gland with the arrector pili muscle will form a pilosebaceous unit. This pilosebaceous unit is the one that will be associated with acne development since sebaceous glands produce sebum when the encoded sebocyte differentiates and dies (Plewig et al., 2019b). Sebum is composed of sebaceous lipids including triglycerides and fatty acid breakdown products, wax esters, squalene, cholesterol esters and cholesterol (Bhat et al., 2017) functions to reduce moisture permeation through the outer skin layers, protect skin from abrasion (Masterson, 2018) and UVB irradiation (photoprotection) and also possess lipophilic antioxidants that involve in the healing process (Plewig et al., 2019b). Alterations in the composition of the lipid, amount of sebum and the oxidant/antioxidant ratio of the skin surface lipids characteristic can lead to acne formation (Bhat et al., 2017; Masterson, 2018).

Normally, comedones are developed in the distal hair follicle near the pore opening which is known as the infundibulum. Meanwhile, the isthmus which is distal to the infundibulum is the area that attaches the sebaceous gland and hair follicle through a short keratinised canal. This sebaceous gland linked to the hair follicle region is also called as junctional zone (Clayton et al., 2019). Lastly, the hypodermis is located below the dermis that is also known as subcutaneous fascia. It is the deepest layer of skin and contains adipose lobules along with some skin appendages like in the dermis such as hair follicles, sensory neurons and blood vessels (Yousef et al., 2020).

2.2 ACNE VULGARIS

Acne vulgaris is the main inflammatory disease that involves the pilosebaceous unit (Solomon & Zaenglein, 2018). It is a chronic skin disease that commonly starts during puberty and affects both teenagers and adults. However, adolescents are more likely to get acne with a prevalence of up to 94% among this population (Lu et al., 2017). It can occur all over the body since the pilosebaceous unit is present in almost all the skin areas except the lips, palmar and plantar surfaces (Yousef et al., 2021). However, the most common skin areas associates with acne are the face, shoulders, chest and upper back in which all of these areas are sebaceous gland–rich regions (Solomon & Zaenglein, 2018). Acne can affect both genders but studies show a high prevalence in males during adolescence, meanwhile during adulthood, females are more associated with acne compared to males. Yet, the prevalence of acne during adulthood is still low compared to in adolescents for both genders (Skroza et al., 2018).

Acne vulgaris is manifested by multifarious lesions that can be categorised as non-inflammatory lesions and inflammatory lesions. Non-inflammatory lesions are characterised by open and closed comedones meanwhile inflammatory lesions are described as papules, pustules, nodules and cysts (Jusuf et al., 2020). Open comedones also known as blackeads occur due to ploughing of the pilosebaceous unit by sebum on the skin surface meanwhile closed comedones that are also called whiteheads are due to plugging of the pilosebaceous unit under the skin surface by keratin and sebum (Sutaria et al., 2021).

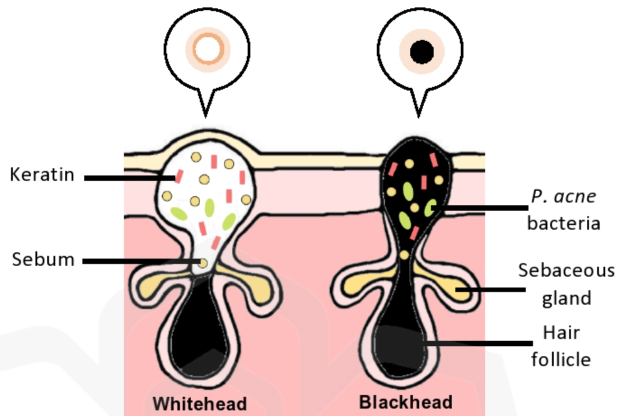


Figure 2.2 Illustration of whitehead and blackhead formation.

Papules and pustules are almost the same in which papules only appear as raised lesions on the skin that are smaller than 1 cm in diameter, meanwhile pustules are inflamed and filled with pus (Heng & Chew, 2020). Moreover, a nodule lesion is hard and has a dome or irregular shape same as a papule but it is inflamed and can extend into deeper layers of the skin that may cause damage to the tissue. Meanwhile, cysts manifest as large red and white bumps that occur due to the clogging of the pore by a combination of sebum, dead skin cells and also bacteria. These cysts also can extend into deeper layers of the skin even below the surface than nodules. Both nodules and cysts can cause a painful sensation when being touched and are most likely to scar (Syal et al., 2020). These lesions can be graded from Grade 1 until Grade 4 based on their severity either comedonal, mild, moderate, or severe acne (Kamra & Diwan, 2017). Comedones are considered as Grade 1 meanwhile Grade 2 and Grade 3 are demonstrated as a small papule with erythema and pustules respectively. In addition, many pustules that are combined together to form nodules and cysts are considered Grade 4 (Sutaria et al., 2021).

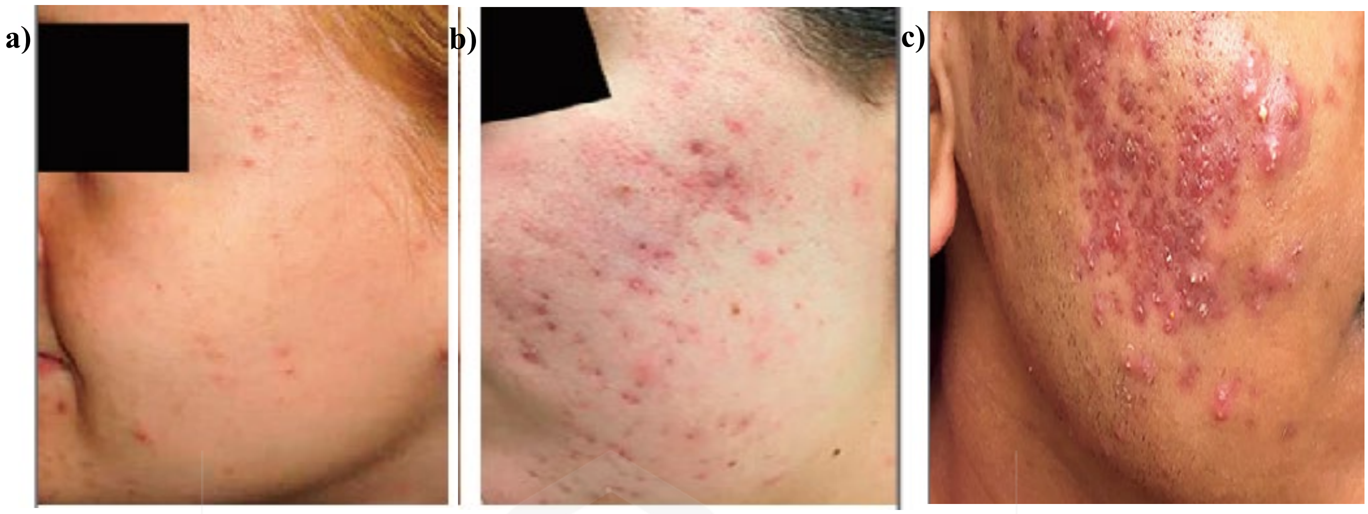


Figure 2.3 a) Mild acne b) Moderate acne c) Severe acne (Solomon & Zaenglein, 2018).

2.3 ETIOLOGY OF ACNE VULGARIS

The etiology of acne vulgaris is multifactorial including genetic, hormone, diet, psychological stress and environmental factors. Genetic is one of the main factors for acne occurrence. Many genetic studies show a high prevalence rate of acne among first-degree relatives and twins (Heng & Chew, 2020). It is found that acne develops earlier even in the pre-puberty period and is more severe in those with a positive family history of acne vulgaris (Alshammrie et al., 2020). Besides, a retrospective study through non-standardised medical records that involve 930 pairs of twins of the Kaiser - Permanente Twin Registry shows a high concordance of acne in monozygotic twins. Also, the severity of acne is more commensurate in monozygotic twins (Lichtenberger et al., 2017).

Other than that, the hormone also is one of the causes of acne vulgaris which is mainly contributed by the androgen level. Androgen is basically a sex hormone that is present in both males and females and its level rises during puberty. This high level of androgen during puberty will enhance the size of the sebaceous glands and trigger sebum production which consequently leads to the formation of acne (Hassan et al., 2019). For example, testosterone which is an androgen hormone will be catalyzed by a 5α-reductase

(5-ARD) enzyme to form dihydrotestosterone (DHT), a more potent form of androgen hormone that can cause excess sebum production. Although androgen hormones have always been related to the formation of acne, but most studies have found that the level of androgen hormones is normal in acne patients. Furthermore, low estradiol hormone level in women also tends to be associated with acne problems (Akdogan et al., 2018). This may explain the formation of acne flares during premenstrual in women (Mohiuddin, 2019).

Dietary intake has been shown to affect the sebum production on the skin and this will lead to the growth of acne. There is a positive relationship between the consumption of dairy products with the occurrence of acne. This is because dairy products such as milk and cheese will boost the serum insulin growth factor-1 (IGF-1) concentration as they contain IGF-1 and casein. The IGF-1 contained in the dairy products could not be hydrolyzed by the digestive enzymes meanwhile the casein will trigger the release of IGF-1 from the liver. This will lead to the rise of plasma IGF-1 concentration that consequently stimulates sebocytes and acne occurrence. Many studies show that IGF-1 will reduce Forkhead box transcription factor (FoxO1) expression that plays a role in regulating the acne-related genes and secretion of the sebaceous gland (Kara & Ozdemir, 2020); Aghasi et al., 2019; Claudel et al., 2018). In addition, IGF-1 also enhances androgen activity and stimulates follicle cell development as it elevates the formation of androgen receptors, testosterone and dihydrotestosterone (DHT) that subsequently cause the development of acne (Aghasi et al., 2019). Other than dairy products, carbohydrate-rich intake that consists of a high glycemic load also triggers acne formation. This is because carbohydrates will stimulate insulin release and IGF-1 production consequently inhibiting the FoxO1 expression (Kara & Ozdemir, 2020; Younas et al., 2020).

Although acne can induce psychological stress but stress itself can aggravate acne formation. In a hospital-based study conducted in Kerala, India, there were 32.7% of acne patients had acne exacerbation during emotional stress periods (George & Sridharan, 2018). Besides, the prevalence of acne among medical students can be up to 97.9% due to stress, gender differences, and lifestyle choice factors (Sachdeva et al., 2021). Exposure to stress will activate the hypothalamus-pituitary-adrenal (HPA) axis and the sympathetic nervous system. This will subsequently stimulate the release of many kinds of neurotransmitters, hormones and immune cell- like cytokines that can act on skin receptors and exacerbate several skin disorders including acne (Jović et al., 2017).

Basically, the exact mechanisms of stress-inducing acne exacerbation have not yet been fully understood, but several mechanisms have been suggested (Jović et al., 2017). Activated immune cells cause the response of pro-inflammatory or anti-inflammatory on the skin. Moreover, stress also will induce the secretion of corticotropin-releasing hormone, glucocorticoids and adrenal androgens. This will lead to the conversion of androgen to testosterone and decrease skin permeability while increasing sebaceous hyperplasia (Aslan Kayiran et al., 2020). Due to these, the development of acne will be accelerated. Other than that, stress also increases the eating habit especially as it helps relieve the stress (Dreno, Bagatin, et al., 2018). As explained earlier, dietary may affect acne development due to the high insulin and IGF-1 levels and also the differentiation of sebocytes.

2.4 PATHOGENESIS OF ACNE VULGARIS

One of the pathogenesis for the development of acne vulgaris is an increased production of sebum. Essentially, this heightened sebum production is regulated by the levels of androgen hormones within the pilosebaceous unit. The potential mechanism behind this excessive sebum production can be attributed to either a direct increase in the production of androgen hormones or an enhanced sensitivity of the sebaceous gland to the normal levels of androgens (Masterson, 2018). During puberty, there is a surge in the levels of androgen hormones, such as testosterone and 5α-dihydrotestosterone (DHT). This surge leads to the enlargement of sebaceous glands, an increase in the synthesis of sebaceous lipids, and the differentiation of sebocytes (Stefania Briganti et al., 2020). These differentiated sebocytes exhibit a significant number of nuclear androgen receptors and peroxisome proliferator-activated receptors (PPARs), rendering the sebaceous glands highly responsive to androgens (Cong et al., 2019). Furthermore, the elevated sebum within sebaceous follicles provides a nutrient-rich and anaerobic environment conducive to the growth of *Propionibacterium acne* (Soleymani et al., 2020).

The second pathophysiology of acne is the follicular hyperkeratinisation of the follicle. Numerous factors are believed to contribute to the occurrence of follicular hyperkeratinisation. These factors include a decrease in the concentration of linoleic acid in sebaceous secretions, heightened androgen activity, inflammation and *P.acnes* biofilms formation. The diminished concentration of linoleic acid in sebaceous secretions is thought to increase the permeability of the skin to inflammatory substances that can trigger acne, thereby disrupting the skin's barrier function (da Cunha et al., 2018; Li et al., 2017). Generally, follicular hyperkeratinisation refers to the abnormal and rapid shedding of skin cells within the follicular infundibulum and sebaceous gland (Lambrechts et al., 2018; Sharma et al., 2021). This excessive proliferation and accumulation of keratinocyte cells at the base of the hair follicle obstruct the pilosebaceous duct, leading to the formation of microcomedones. Microcomedones can manifest as either closed comedones, where the duct is nearly completely blocked and may rupture, or open comedones, where the duct remains open and exposed to the air (Figure 2.2). Open comedones typically appear dark and are called as blackheads due to the oxidation of lipids and melanin (Brown, 2020).

Moreover, it is proposed that *P. acnes* plays a role in the development of acne. Notably, the nomenclature for *P. acnes* has recently been revised to *Cutibacterium acnes* (Platsidaki & Dessinioti, 2018). Basically, *P. acnes* is one of the microorganisms residing on the skin, serving as an initial defence against external pathogens. *P. acnes* has the capacity to generate antimicrobial peptides and antimicrobial short-chain fatty acids. Both these peptides and short-chain fatty acids collaborate with the host's innate protective mechanisms to uphold the overall health and balance of the skin (Ramasamy et al., 2019).

However, certain free fatty acids produced by the lipase activity of *P. acnes* can act as potent inducers of comedogenicity, *P. acnes* biofilm formation and inflammation (Plewig et al., 2019a). The lipase secreted by *P. acnes* breaks down the triglycerides present in sebum into glycerol and free fatty acids. While glycerol can serve as a nutrient source for bacterial growth, free fatty acids promote the development of comedones, oxidative stress, inflammatory responses, and tissue damage (Soleymani et al., 2020). Additionally, aside from triglycerides, lipase also oxidises squalene found in sebum. This oxidised squalene triggers the generation of pro-inflammatory cytokines like IL-1α, IL-6 and IL-8, and activates peroxisome proliferator-activated receptors (PPARs). Furthermore, oxidised squalene enhances the innate immune responses of keratinocytes and sebocytes, thereby inducing an inflammatory process (da Cunha et al., 2018).

Lastly, inflammation is considered a pivotal factor in the development of acne. The enlargement of microcomedones, which results from the accumulation of keratin, sebum, and bacteria, eventually leads to the rupture of the follicular wall. As a consequence, the contents are expelled into the dermis rather than remaining on the skin surface. This event triggers an inflammatory response, giving rise to the formation of inflammatory acne lesions like papules and pustules (Greydanus et al., 2021; Kanwar et al., 2018). Other than that, inflammation is predominantly mediated by the actions of *P. acnes* through both innate and adaptive immune responses. The impact of *P. acnes* on the innate immune system initiates the activation of Toll-like receptor-2 (TLR-2) on neutrophils and monocytes, leading to the production of proinflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin-12 (IL-12), and interleukin-8 (IL-8) (Soleymani et al., 2020). Meanwhile, *P. acnes* induces an adaptive immune response that triggers the secretion of interleukin-17A (IL-17A) and interferon-gamma (IFN-gamma) from CD4+ T cells (Tan et al., 2018). The summary of the pathophysiology of acne vulgaris is illustrated in Figure 2.4.

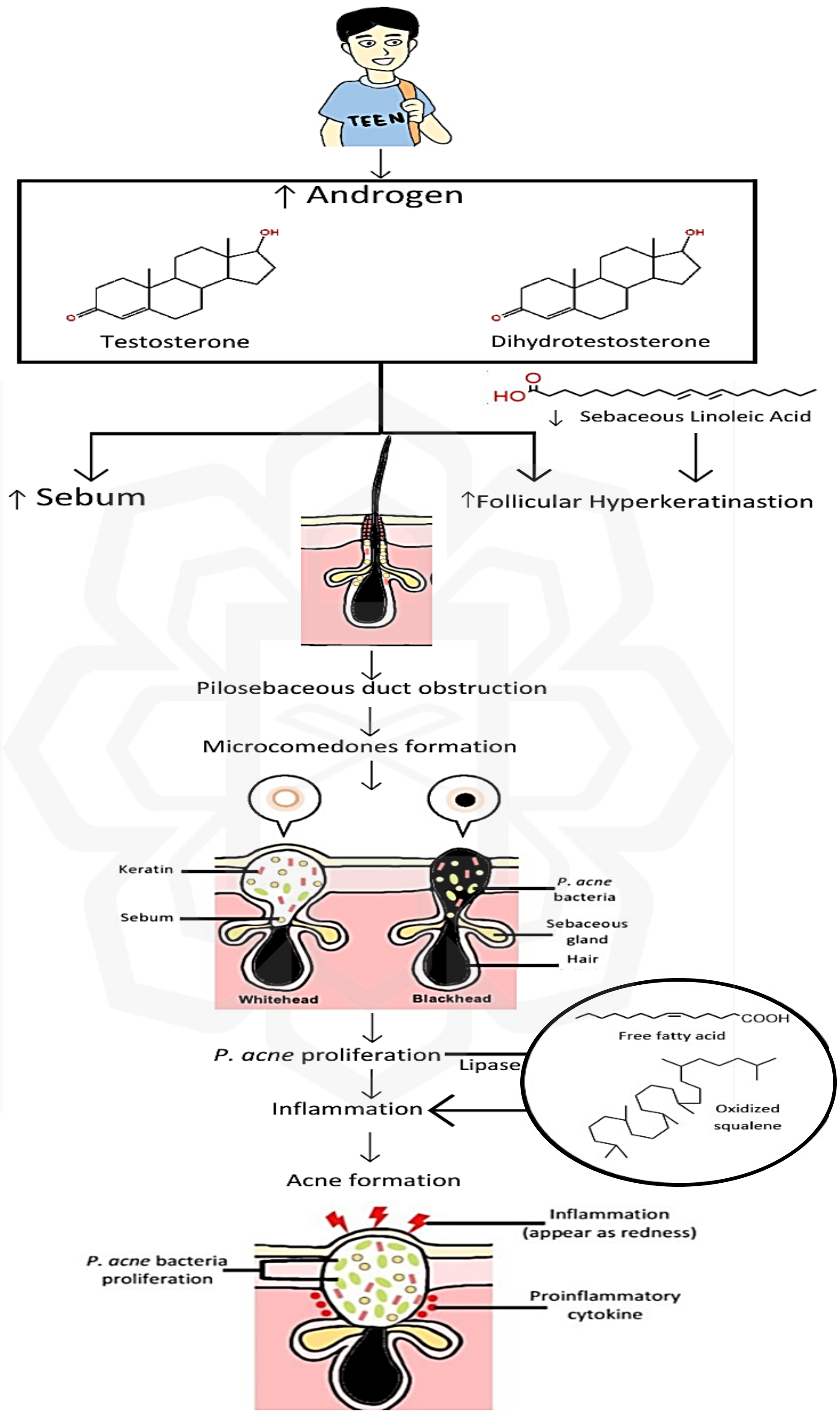


Figure 2.4 Summary of the pathophysiology of acne vulgaris.

2.5 TOPICAL DELIVERY SYSTEMS

Topical delivery is one of the treatment administration routes for both local and systemic diseases. It is considered an effective treatment for a local cutaneous disorder such as fungal and bacterial infection as the drug can act directly to the site of action. It is normally used to treat skin problems where other administration routes such as oral, parental, rectal and sublingual are not appropriate. The topical application enables the drug to penetrate deeper into the skin which consequently provides a better absorption (Patil et al., 2019). Other than skin, it is also being applied for the treatment of the external ear lining, nasal mucosa, cornea, vagina, urethral membrane, rectal tissue and buccal tissue. Topical application has many advantages such as convenience and ease of application, easy to be removed when required, the drug being delivered specifically to a particular site (Maqbool et al., 2018), escape from the first-pass metabolism and gastrointestinal incompatibility and also less toxic and side effects. It will be less toxic and side effects due to the bilayer conformation and arrangement of the skin which makes only a small amount or none of the drugs go into the systemic. As it is convenient and easy to apply, it will enhance patient compliance and be appropriate to use for self-medication. The main disadvantages of topical application are skin irritation and allergy (Patil et al., 2019).

2.6 EMULGEL

As the name suggests, emulgel is a combination of emulsion and gel. An emulsion is one of the colloid systems in which both dispersed and continuous phases are in liquid form (David & Akhondi, 2022). It is a blend of two or more immiscible liquids where one liquid is dispersed into another liquid (Costa et al., 2019; David & Akhondi, 2022). The dispersed phase which is also called as internal or discontinuous phase refers to the droplets in the emulsion meanwhile continuous phase is the surrounding or external phase where the droplets are dispersed (Akbari & Nour, 2018; Costa et al., 2019). The dispersed droplet size normally ranges between 100 nm to 100 µm (Costa et al., 2019). Emulsion basically is formed through an emulsification process in which this process is dynamic and nonspontaneous. Mechanical energy is required to break up the droplet of the dispersed phase liquid into smaller sizes in the continuous phase. This emulsification process can be done by different techniques such as homogeniser (either rotor-stator or high-pressure), ultrasonicator and liquid injection through porous membranes (Akbari & Nour, 2018). An emulsion can be categorised as oil-in-water (O/W) or water-in-oil (W/O) based on what kind of liquid forms the dispersed and continuous phases. These types of emulsions are considered simple emulsions. There are also multiple emulsions such as water-in-oil-in-water (W/O/W) and oil-in-water-in-oil (O/W/O) emulsions. These multiple emulsions are more complex since they are like emulsions of emulsions (Akbari & Nour, 2018; Costa et al., 2019).

Meanwhile, gels are homogenous semisolid preparation that comprises of small inorganic particles or large organic molecules dispersion that are entrapped and interpenetrated by a liquid phase (Maqbool et al., 2017). It basically has a great aqueous constituent that allows better drug dissolution and eases drug migration through liquid vehicles compared with the ointment or cream base. These make gels superior in terms of ease of use and patient acceptance. Despite their benefits, gels have a significant constraint in the delivery of hydrophobic medicines. Therefore, emulgel is produced to make up for this limitation and allow hydrophobic therapeutic moiety to enjoy the unique properties of gel (Phad et al., 2018).

The presence of a gelling agent or polymer in the water phase converts a classical emulsion into an emulgel. In an emulsion, either O/W or W/O type, the drug droplets are incorporated in the internal phase acting as a drug reservoir from where the drug passes through the external phase and to the skin and gets absorbed in order to provide the controlled effect. Thus, emulgel is basically the approach using the benefits of both emulsion and gel properties, thereby exhibiting improved stability and elegance. Due to its dual nature, emulgel formulations have gained significant attention in the pharmaceutical and cosmetic industries. Besides, emulgel also possesses some favourable properties for dermatological use such as a high ability to penetrate the skin, being thixotropic, greaseless, easily spreadable, easily removable, emollient, non-staining, water-soluble, longer shelf life, bio- friendly, transparent and pleasing appearance (Kumar Yadav et al., 2017; Phad et al., 2018; Sah et al., 2017).

Several studies have explored the formulation and characterisation of emulgel systems for various topical and transdermal applications. In a study by Manian et al. (2022), the researchers investigated the development of diclofenac sodium with different semi-solid formulations including emulgel, emulsion, gel and ointment. The result showed that emulgel and gel demonstrated the highest release rate among all the formulations. However, the emulgel formulation exhibited favourable skin permeation and retention. This stresses the potential of emulgel systems for improved topical drug delivery. In another study, Reena et al. (2023) conducted a comparative evaluation of emulgel and gel formulations containing curcumin and tea tree oil for transdermal delivery. The emulgel formulation demonstrated superior drug content percentages, high drug release and better spreadability compared to the gel formulation. Besides, psoriasis symptoms healed faster when being applied with the emulgel formulation compared to the gel. These findings underscore the potential of the emulgel as a promising drug delivery platform and display the synergistic advantages of combining tea tree oil and curcumin as an innovative therapeutic approach for psoriasis.

Furthermore, eight formulations of clotrimazole emulgel were developed based on a 2^3 factorial design incorporating qualitative factors and levels including the type of gelling agent, concentration of natural permeation enhancers and concentration of emulsifying agent. Most of the emulgel formulations displayed a better clotrimazole release *in vivo* (5 out of 8 emulgel formulations) and *ex vivo* (7 out of 8 emulgel formulations) than the innovator brand (clotrimazole cream). Besides, the antimicrobial activity of all emulgel formulations was also better than the innovator brand since the inhibitory effect of the innovator brand against *Candida albicans* was the lowest among the clotrimazole emulgel formulations (Ilomuanya et al., 2018). Meanwhile, a study was done by Pravallika & Reddy (2019) to develop aceclofenac emulgel formulations using different types of gelling agents.

The carbopol emulgel formulation showed enhanced drug release, drug content and stability compared to the sodium alginate, sodium carboxymethylcellulose (Na CMC) and hydroxypropylmethylcellulose (HPMC) emulgel formulations. Hence, this carbopol formulation was designated as an optimised formulation. Given that emulgel has emerged as a novel and new method for topical drug administration, it holds significant promise for the effective delivery of hydrophobic drugs.

In addition, a research was conducted to examine the effects of creams and emulgels containing immortelle extract and hemp oil on healthy human volunteers' skin (*in vivo* study). The outcomes of this study indicated that the application of all formulations resulted in increased skin hydration and reduced transepidermal water loss (TEWL) without inducing irritation or disrupting the skin's natural pH. Additionally, this study highlighted the significance of the carrier system with emulgels being notably favoured by the panelists for their superior sensory attributes. Besides, this study also showed that the skin hydration improvement of the emulgel containing immortelle extract and hemp oil was significantly higher compared to the placebo emulgel, which this case was not observed with the creams. These findings suggested an augmented delivery of herbal active components via emulgels as opposed to creams (Reena et al., 2023).

All of these studies collectively highlight the versatility and potential applications of emulgel formulations in topical drug delivery, offering improved drug release, skin permeation, stability and therapeutic efficacy compared to conventional formulations. Since emulgel formulation can offer these kinds of benefits, hence emulgel formulation also has emerged as a promising vehicle for delivering anti-acne agents topically. Several studies have investigated the formulation and evaluation of emulgel systems for the treatment of acne vulgaris.

For instance, a study was run to develop an emulgel consisting of metronidazole and niacinamide for the rosacea treatment an this emulgel formulation was compared to the commercially marketed formulations (two creams and a gel). The outcome demonstrated that this emulgel facilitated a high delivery of both active pharmaceutical ingredients (APIs), exhibiting a quicker release of metronidazole compared to the marketed cream formulations, albeit marginally slower than the marketed gel formulation. This affords a practical timeframe for drug delivery aligning with the anticipated duration of adherence of the adhesive emulgel onto the affected facial region (Torregrosa et al., 2020). In the meantime, an adapalene emulgel formulation with a combination of 0.5 % Carbopol 934 and 0.5 % HPMC K100M polymers as the gelling agents and 4% emulsifiers were observed to possess an amplified release profile compared to the marketed formulation *in vitro*. Besides, this emulgel formulation produced no skin irritation when performed on albino rabbits. Hence, topical adapalene emulgel might serve as a preferable alternative as a topical drug delivery system for acne treatment (Goyani et al., 2018).

Furthermore, an optimised clindamycin phosphate emulgel formulation developed by Ranjan et al. (2019) also was observed to possess higher drug release compared to the marketed clindamycin gel. The drug release from the clindamycin emulgel was significantly slower, hence this validated the slight prolonged drug release rate of emulgel. This can be seen as the drug release cumulative percentage of marketed clindamycin gel was 94.46% at 60 minutes meanwhile the drug release cumulative percentage of the optimised clindamycin emulgel was 98.89% at 240 minutes. Also, the optimised clindamycin emulgel possesses better antiacne properties against *Propionibacterium acne* than the marketed clindamycin gel as the zone of inhibitions were larger for all three concentrations (10 μg/mL,20 μg/mL and 30 μg/mL). Lastly, four benzoyl peroxide gellified emulsions were prepared using four different vegetable oils (almond oil, jojoba oil, sesame oil and wheat germ oil) by Thakur et al. (2012). All of these four formulations cause lesser skin irritation in male albino rats compared to the marketed benzoyl peroxide gel. This is because only the histopathological examination of the skin specimens treated with the marketed benzoyl peroxide gel showed both dysplastic changes and basal vacuolar changes in the epidermal layer which were similar to the untreated one.

Overall, these studies communally stress the potential of emulgel formulations as effective and well-tolerated options for delivering anti-acne agents, paving the way for innovative topical treatments in acne management.

2.7 BENZOYL PEROXIDE

Benzoyl peroxide is an organic peroxide obtained from the byproduct of coal tar (Bandyopadhyay, 2021). It is an organic compound in the peroxide family that appears as a colourless crystalline solid. It contains two benzoyl groups connected by a peroxide link. Its structural formula is $[C_6H_5C(O)]_2O_2$ (Kamra et al., 2018). The chemical structure of benzoyl peroxide is shown in Figure 2.5 below. Its IUPAC name is benzoyl benzenecarboperoxoate with a molecular weight of 242.23 g/mol (Emmerich et al., 2021). Its melting point is between 104°C and 106°C meanwhile its boiling point is 107°C. It is poorly soluble in water with a solubility value of 9.1 mg/lit at 25°C. It can be dissolved properly in alcohol, ether and other organic compounds (Aarthi et al., 2018). Its partition coefficient (logP) between *n*-octanol and water is 3.42 which means it is hydrophobic (Ancheria et al., 2019). In general, logP less than or equal to 0 indicates high hydrophilicity meanwhile logP more than 0 indicates increased lipophilicity (Roy & Patel, 2023). Benzoyl peroxide can be categorised as highly lipophilic since its logP value is more than 3 (Lindsley, 2014).

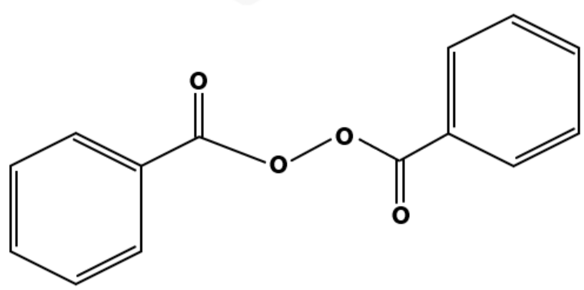


Figure 2.5 Chemical structure of benzoyl peroxide.

Benzoyl peroxide has been used since the early 1900s due to its antimicrobial activity. Nowadays, it has been listed as one of the essential medicines by the World Health Organisation. It is widely been used as a topical treatment for acne vulgaris (Aarthi et al., 2018) due to its bactericidal, keratolytic, comedolytic and slightly anti-inflammatory properties (Clinical Practice Guideline, 2022; Kosmadaki & Katsambas, 2017). Since the antimicrobial property of benzoyl peroxide is possessed by various modes of action, thus it is insensitive to bacterial resistance mechanisms. The FDA-approved concentration used for acne treatment is 2.5% to 10% benzoyl peroxide (Kosmadaki & Katsambas, 2017) but the common concentrations used in the formulation are 2.5%, 5% and 10% (Clinical Practice Guideline, 2022; Kosmadaki & Katsambas, 2017). Benzoyl peroxide can penetrate the stratum corneum and enter the pilosebaceous duct due to its lipophilic feature. It will rapidly degrade to benzoic acid and hydrogen peroxide, producing oxidative free radicals. This will eventually lead to bacteria death. Low concentrations of benzoyl peroxide also can destroy neutrophils, preventing their release of reactive oxygen species. This will consequently inhibit the release of pro-inflammation signals that are involved in acne formation (Kosmadaki & Katsambas, 2017).

It has been suggested that topical benzoyl peroxide monotherapy or in combination with other topical anti-acne agents should be used in mild to moderate acne vulgaris (Clinical Practice Guideline, 2022; Ly et al., 2023). Benzoyl peroxide also is recommended for predominantly inflammatory lesions. Once the acne is under control, topical benzoyl peroxide is one of the treatments that has been proposed as the maintenance therapy. Benzoyl peroxide needs to be applied to the affected area once or twice daily. It is safe to be used in adolescents (Clinical Practice Guideline, 2022). It is also one of the safe treatment choices for pregnant and lactating women as there are no harmful effects on fetal development (teratogenic effects) reported (Clinical Practice Guideline, 2022; Ly et al., 2023). Applying a maximum concentration of 5% benzoyl peroxide twice a day is regarded as a safe and acceptable treatment for acne throughout all stages of pregnancy. Regardless of the limited data available, benzoyl peroxide is considered low-risk due to its minimal absorption into the bloodstream and rapid excretion by the kidneys (Ly et al., 2023).

It has been advised to start with a low concentration of benzoyl peroxide first and increase gradually to a higher concentration if no improvement. The treatment needs to be stopped first if skin irritation occurs and once the irritation has subsided, the treatment can be restarted on the alternate day. The common side effects of benzoyl peroxide are dryness, redness, burning sensation, skin exfoliation, swelling, increased sensitivity and also contact dermatitis. Therefore, it is advisable to put on moisturiser in order to enhance tolerability. However, most of these side effects are mild to moderate. Besides, precaution needs to be taken when applying benzoyl peroxide as it may bleach the clothes and hair (Clinical Practice Guideline, 2022).

2.8 STINGLESS BEE HONEY

In this research, the stingless bee honey type is being utilised in the formulation. Stingless bees typically originate from tropical and sub-tropical regions such as Malaysia, Brazil, and Australia. More than 500 species of these bees have been identified in the Afrotropical, Indo-Australian, and Neotropical provinces (Fletcher et al., 2020). In Malaysia specifically, over 38 species of stingless bees have been identified, but only four species are commercially cultivated which are *Heterotrigona itama, Geniotrigona thoracica, Tetragonula leviceps* and *Lepidotrigona terminate* (Mustafa et al., 2018). These bees can be classified into two genera, namely Melipona and Trigona (Zulkhairi Amin et al., 2018), with the primary distinction being the size of the bees. Melipona bees are larger when compared to Trigona bees (Figure 2.6). Stingless bee honey is known as *Kelulut* in Malaysia. Similar to other honeybee species, stingless bees also consist of a single queen and worker bees that coexist permanently. The worker bees are responsible for collecting pollen and nectar to feed the larvae and store honey in the hive (Fletcher et al., 2020).

a)  **b)** 

Figure 2.6 **a)** Melipona genera **b)** Trigona genera (Pimentel et al., 2022).

Stingless bee honey has a long history of traditional use in various societies, including Malay, Chinese, Arab, Indian, Hebrew, Persian, and Roman cultures. It is composed of carbohydrates, phenolic compounds, organic acids, minerals, vitamins, proteins (including amino acids and enzymes) and lipids (Fatima et al., 2018). The primary constituents of stingless bee honey are fructose and glucose, which together make up approximately 65% of its sugar content, with only a minimal amount of hydroxymethylfurfural (HMF). Conspicuously, the phenolic compound content of stingless bee honey is around ten times higher than that of other types of honey (Mustafa et al., 2018). The composition of stingless bee honey is summarised in Table 2.1.

‘

Table 2.1 The composition of stingless bee honey.

Composition	Amount	References
Carbohydrates (g/100g) • Glucose (%) • Fructose (%)	68.33-72.25 8.10–31.00 31.11–40.20	(Ismail et al., 2021; Lemos et al., 2018; Lim et al., 2019; Zulkhairi Amin et al., 2018)
Phenolic compounds (mg GAE/ 100 g)	33.20-60.20	(Ismail et al., 2021; Lemos et al., 2018; Zulkhairi Amin et al., 2018)
Protein (µg/g) • Amino acid (g/100g)	335-682.7 20-300	(Ismail et al., 2021; Lemos et al., 2018; Zulkhairi Amin et al., 2018)
Organic acids (g/kg)	0.23-3.12	(Wong et al., 2019)
Minerals (mg/kg)	1411.77-1539.24	(Cheng et al., 2019)
Vitamins C (mg/100g)	6.49–13.58	(Agussalim et al., 2022)
Lipid	Negligible	(Lim et al., 2019)
Hydroxymethylfurfural, HMF (mg/kg)	8.80–69.00	(Ismail et al., 2021; Lemos et al., 2018; Zulkhairi Amin et al., 2018)

Due to its elevated phenolic compound content, stingless bee honey has the potential to be used in the treatment of various medical conditions, including hepatic and cardiac diseases, owing to its antioxidant properties. Additionally, it possesses a range of other beneficial properties such as anti-acne, anti-fungal, anti-bacterial and anti-cancer activities (Ramli et al., 2019). Stingless bee honey is a preferred choice in Malaysia due to its greater availability when compared to common honey (Manuka and Tualang honey). The limited production of Manuka honey by *Apis mellifera* honeybees was influenced by the *Varroa* destructor mite outbreak in 1996. On the other hand, hives of sting bees such as Tualang bee (*Apis dorsata*) are predominantly located in jungle areas and at elevated heights, making the procurement of Tualang honey a more challenging process (Mustafa et al., 2018). In contrast, stingless bees typically construct their hives within existing chambers or hollow spaces in trees or buildings (Fletcher et al., 2020).

Figure 2.7 Honey hive of stingless bee in Bukit Kuin, Kuantan, Pahang.

These three types of honey exhibit distinct physicochemical characteristics and bioactive compounds, as outlined in Table 2.2. Notably, their appearance differs significantly, with stingless bee honey having an amber-brown colour, while common honeybee-produced honey appears as dark brown (Tualang honey) and light-dark brown (Manuka honey). In terms of acidity, stingless bee honey is the most acidic, as it has the lowest pH value. This acidity suggests that stingless bee honey may possess potent antimicrobial properties as acidity contributes to these characteristics. Additionally, stingless bee honey contains the highest percentage of moisture content compared to Tualang honey and Manuka honey, giving it excellent moisturising properties (Rao et al., 2016; Zulkhairi Amin et al., 2018).

Table 2.2 Comparison of physicochemical characteristics and bioactive compounds between stingless bee honey, Tualang honey and Manuka honey.

Analysis	**Stingless bee honey**	**Tualang honey**	**Manuka honey**	**References**
Appearance	Amber brown	Dark brown	Light dark brown	(Ismail et al., 2021; Adalina et al., 2020; Lemos et al., 2018; Nascimento et al., 2018; Zulkhairi Amin et al., 2018)
pH	3.15-4.56	3.29-4	3.2-4.52	
Moisture content	24.45-35.11	17.38-23.3	17.28-18.7	
Total reducing sugars (%)	54.9–87	67.6	75.8	
Glucose (%)	8.1–31	29.5-37.56	31.55-37.6	
Fructose (%)	31.11–40.2	29.6-49.29	37.77-39.29	
Protein (µg/g)	335-682.7	351.1- 495.5	274.4-1058.9	
Hydroxymethylfurfural, HMF (mg/kg)	8.8–69	46.17	400	
Ash (%)	0.01-0.9	0.2-0.29	0.1-0.7	
Total Phenolic Content, TPC (mg GAE/ 100 g)	33.2-60.2	22.3-46.3	12.6-47.1	
Total Flavanoid Content, TFC (mg QE/100 g)	43.2-65.9	24.4-70.3	14.5-48.1	

2.9 MECHANISM OF HONEY AS ACNE TREATMENT

Honey exhibits anti-acne properties through its antimicrobial, anti-inflammatory, and antioxidant attributes. The antibacterial effect of honey is attributed to the presence of hydrogen peroxide (H_2O_2), its acidity, and high osmolarity (Albaridi, 2019). The formation of hydrogen peroxide is facilitated by the glucose oxidase enzyme which catalyses the oxidation of glucose (Equation 2.1). Research has demonstrated that even at low concentrations, H_2O_2 can effectively inhibit various types of bacteria (Febriyenti et al., 2019). H_2O_2 has been observed as a very potent antimicrobial agent for *P. acnes.* Even at low concentrations, it possesses both bacteriostatic and rapid bactericidal effects (Hernandez et al., 2019). This may be due to its ability to degrade the protein and damage the cells of the bacteria (Ab Hadi et al., 2016) by producing destructive hydroxyl free radicals (CDC, 2016). Besides, H_2O_2 will boost the cytokine production for the inflammatory response to kill the bacteria (Jalil et al., 2017). It was found that the MBC for hydrogen peroxide solution was 1% and complete obliteration of *P. acnes* in vitro within five minutes of contact with H_2O_2. Besides, there was no regrowth of *P. acnes* for up to a week after the treatments with H_2O_2 (Hernandez et al., 2019).

$$(Glucose \xrightarrow[oxidase]{Glucose} Gluconic\ acid\ +\ Hydrogen\ peroxide) \qquad \text{Equation 2.1}$$

Besides, honey's acidic pH, typically ranging between 3.2 and 4.5 creates an unfavourable environment for bacterial growth as bacteria tend to thrive in neutral or slightly alkaline conditions (Achermann et al., 2014; Minden-Birkenmaier & Bowlin, 2018). A study conducted by Ng et al. (2020) tested the antibacterial activity of hydrochloric acid solution (pH 3.3) in order to mimic the acidity of honey against *E. coli*. However, this hydrochloric acid solution failed to display any inhibition zone. Regardless of this finding, in this study, stingless bee honey with the lowest pH showed more potent antimicrobial activity, thus it was proposed that the antimicrobial activity of stingless bee honey could be influenced by acidity in combination with other aspects (Ng et al., 2020). Hence, it can be deduced that the lower the pH, the higher the ability of the honey to inhibit the growth of bacteria. Therefore, stingless bee honey can be presumed to pose potent antimicrobial activity since it has the lowest value of pH when compared to other types of honey such as *Tualang* honey and *Acacia* honey (Fatima et al., 2018; Muhammad & Sarbon, 2021).

Furthermore, it has been hypothesised that an alternative way to inhibit the growth of *P. acnes* is by targeting the environment in which it grows. Basically, some of the environmental parameters that can be changed are osmolarity and ionic strength which are related to water availability, pH or oxygen tension. It had been conjectured that reducing the water availability is a safe therapeutic approach for acne management (Eady et al., 2013). The hyperosmolality of honey due to its high sugar content will constrain the water intake by bacteria for growth and even draw water out from the bacteria (Almasaudi, 2020). This makes the bacterial cells dehydrated and incapable of growing and proliferating in hypertonic sugar solution (Ardhany et al., 2021). Moreover, it had been observed that the individual sugar types such as glucose, fructose, sucrose and maltose displayed significant antimicrobial properties at high concentrations. However, monosaccharides (fructose and glucose) were found to be more potent in inhibiting bacteria compared to disaccharides (sucrose and maltose) (Mizzi et al., 2020). Nevertheless, hyperosmolality is not the main antibacterial factor in honey. This is because when honey and 'artificial honey' with the same concentration were tested for antibacterial activity through the agar well diffusion method, the 'artificial honey' was unable to give the same outcome as the honey (Ng et al., 2020).

Other than that, the antibacterial properties of honey are also attributed to its non-peroxide activity. The antibacterial activity of non-hydrogen peroxide in the honey sample has garnered more attention from researchers. The presence of a unique or innovative bioactive component in honey is responsible for its antibacterial activity, which is not attributed to hydrogen peroxide. According to a study conducted by Jibril et al. (2020), it has been noted that honey samples that consist of solely non-hydrogen peroxide are still capable of exhibiting antibacterial therapeutic properties. The non-peroxide antibacterial action of honey is influenced by polyphenols specifically phenolic and flavonoid components. This is because polyphenols contain saturated side-chain and benzene ring substitutions in their chemical structure, which produce antibacterial action (Tuksitha et al., 2018). It has been suggested that honey with the highest phenolic compounds has the lowest MIC against several bacterial types. This showed that the high phenolic content of the honey makes the antibacterial effect more potent. In addition, the enhanced antibacterial activity of honey may be attributed to its elevated levels of flavonoids such as catechin, chrysin and rutin. Flavonoids possess the capacity to disrupt the structural and functional characteristics of bacterial membranes, resulting in an increased permeability of these membranes to protons. As a result, the cellular integrity will be compromised, leading to the release of cytoplasmic contents (Sousa et al., 2016). Many studies have demonstrated that stingless bee honey consists of high levels of phenolic and flavonoid contents (Mustafa et al., 2018; Ranneh et al., 2018). This postulated that stingless bee honey possesses potent antibacterial activity due to high non-peroxide activity with other factors.

However, the antibacterial activity of honey may differ based on the phytogeographic regions, flower sources and seasons/ weather which leads to different constituents and characteristics of the honey (Mahmood et al., 2021; Nishio et al., 2016). A study by Mahmood et al. (2021) displayed that flower sources and seasons did influence the antimicrobial activity of the stingless bee honey. Stingless bee honey with multi-floral sources (more than two flowers) and collected during dry weather showed higher antimicrobial activity compared to those with two flower sources and during rainy weather respectively. This is because multiflora stingless bee honey exhibited more phytoconstituents that contribute to its antimicrobial properties. Meanwhile, rainy weather limits foraging activity and pollen sustainability as the wind is too strong and the water vapour is high. This will hamper the antimicrobial effect of stingless bee honey since the quality of the stingless bee honey will be reduced. Besides, the high humidity is presumed to negatively affect the pH of stingless bee honey which consequently diminishes the antimicrobial effect of stingless bee honey (Mahmood et al., 2021).

Other than antibacterial activity, the antioxidant and anti-inflammatory effects possessed by the honey also help in reducing acne formation. Honey possesses potent antioxidant capability owing to its high content of phenolic compounds (Angie et al., 2019). Oxidative stress resulting from reactive oxygen species (ROS) is recognised as a factor that can directly or indirectly lead to various diseases. In the context of acne, the irritation during infection is related to the formation of free radicals specifically hydroxyl, superoxide and nitrous oxide. These free radicals are primarily present in sebum when the follicular walls of sebaceous glands rupture (Vora et al., 2018). Furthermore, oxidative stress is also triggered by the generation of free fatty acids in the pathogenesis of acne. The antioxidative properties of honey contribute to its anti-inflammatory effects, potentially reducing inflammation during the formation of acne. This is because honey's anti-inflammatory effects can lead to a decrease in the secretion of pro-inflammatory cytokines induced by *P. acnes* (Djakaria et al., 2020).

2.10 STABILITY OF HONEY IN TOPICAL DELIVERY SYSTEM

The stability of honey in formulation dosage forms for topical application had been explored previously, giving insights into various factors influencing their stability and efficacy. One critical aspect examined in previous studies is the effect of pH on honey stability. Alvarez-Suarez et al. (2014) noted that acidic pH levels can enhance the stability of honey formulations by inhibiting microbial growth and enzymatic degradation. Similarly, stingless bee honey which naturally possesses a lower pH compared to other honey may exhibit improved stability under acidic conditions (da Silva et al., 2016). This finding emphasises the importance of formulating honey-based products at optimal pH levels to ensure their stability and efficacy over time.

Temperature also plays a crucial role in determining the stability of honey in formulations. High temperatures can lead to the degradation of heat-sensitive compounds in honey such as enzymes and phenolic compounds, hence compromising their therapeutic properties (da Silva et al., 2016; Yalçin, 2021). Therefore, careful temperature control during formulation processing and storage is essential to preserve the bioactivity of honey. Furthermore, the interaction between honey and other formulation ingredients has been explored in previous research. da Silva et al. (2016) also highlighted the importance of antioxidants and chelating agents in preventing the oxidative degradation of honey constituents. Additionally, emulsifiers and stabilisers play a crucial role in maintaining the physical stability of emulsion-based formulations containing honey (Rosdi et al., 2021; Wiyani et al., 2020). These judgments stress the significance of selecting compatible ingredients and optimising formulation strategies to enhance the stability of honey-based formulations.

Several studies investigated the long-term stability of honey in topical dosage forms by subjecting the formulations to real or accelerated stability testing storage conditions. The changes in physical appearance, chemical composition and efficacy were evaluated over time. These studies provide valuable perceptions into the shelf-life and storage requirements of honey-based products, guiding formulation optimisation and quality control practices. For example, the stability of topical antibacterial formulation consisting of Malaysian *Kelulut* honey as the active ingredient and guar gum as the gelling agent was determined over six months of storage under long-term (25°C ± 2/60% ± 5 relative humidity (RH)) and accelerated conditions (40°C ± 2/75% ± 5 RH). The results showed a very minimal reduction in pH (4% reduction) and a significant change in the viscosity of the formulation over six months of storage. However, other physicochemical properties (colour and homogeneity) and also antibacterial efficacy (against *S. aureus, E. faecalis ATCC 29212, S. pyogenes, E. coli, K. pneumonia* and *E. aerogenes*) of the formulation remained unchanged in both storage conditions over six months (Mohd-Aspar et al., 2021).

Besides, Abd Jalil (2020) developed several PVA-natural biopolymer hydrogel formulations incorporated with stingless bee honey for wound healing. Hydrogel formulation with 10% w/v PVA, 6% PEG w/v, 0.25% agar, 40% stingless bee honey and three freeze and thaw cycles exhibited the best characterisation overall. Thus, this stingless bee honey hydrogel formulation was subjected to a stability study for three months with a storage condition of 25°C ± 2/50% ± 5 RH. The result showed that this stingless bee honey hydrogel was stable since the physicochemical properties of the formulation were maintained during the three months of storage. These were proved by the slight and insignificant reduction of the swelling ratio and gel-fraction of the hydrogel during the stability study.

Additionally, a study by Rozman et al. (2021) evaluated the stability of stingless bee honey nanoemulsion and reported no significant alterations in its formulation characterisations (particle size and visual appearance) on days 0, 7 and 14 at room temperature, 4°C and 40°C. Lastly, the optimised alginate-based honey-loaded topical formulations (pre-gel solution, wet sheet, and dry sheet) were stored over 6 months at 5 ºC, 30 ºC and 40ºC for stability testing. There was a minor decrease observed in methylglyoxal (MGO) levels over time and a slight increase in the spreadability of the pre-gel solution formulation. However, other characteristics such as pH, moisture content, dimension and tensile strength of the formulations were retained throughout the stability study. Besides, the determination of specific honey constituents through HPTLC analysis showed that these compounds were present in the formulations at over 97% of the levels detected in the original honey. This discovery indicated that the manufacturing process did not cause a notable reduction in these components (Hossain et al., 2023).

Although research on the stability of honey in topical formulations remains relatively limited, the studies discussed earlier have contributed valuable insights. Collectively, these findings suggest that honey remains stable in various topical preparations with most assessed parameters remaining consistent throughout the stability study particularly under low-temperature storage conditions. However, further research is warranted to comprehensively evaluate the stability of honey in diverse formulations since both storage conditions and formulation ingredients influence the overall stability of the honey formulation.

CHAPTER THREE

PREPARATION AND CHARACTERISATION OF BENZOYL PEROXIDE WITH STINGLESS BEE HONEY EMULGEL

3.1 INTRODUCTION

Topical drug delivery is a drug dosage form that applies to the skin and mucous membrane. It is the preferred dosage form to treat cutaneous conditions including acne as the drug can act directly on the site of action. Topical drug delivery system can be categorised into several groups which are solid, liquid, semisolid and miscellaneous (Patel et al., 2021; Sah et al., 2017; Sreevidya, 2019) Semisolid is the most common and widely used topical dosage form. Semisolid basically mixes the behaviours of liquid and solid. It acts more like a solid at rest as it is stable against sedimentation and permits a prolonged residence time at the application site. With the presence of force, it will liquefy and can be spread uniformly on the skin (Herbig et al., 2023). There are various semisolid preparations available such as cream, ointment, gel, paste and emulgel. Emulgel is a mixture of emulsion and gel that can be used for hydrophilic and lipophilic drug delivery. This is because emulgel consists of both aqueous and non-aqueous phases. Emulgel has a dual control release system since it possesses both gel and emulsion properties. Besides, it also has been discovered to have better drug loading capacity and stability (Patel et al., 2021). Therefore, an oil-in-water emulgel formulation was chosen to be developed and formulated in this study since benzoyl peroxide is hydrophobic meanwhile stingless bee honey is hydrophilic.

However, emulsions basically are not thermodynamically stable. This is because over time, the dispersed and continuous phases can separate back into two distinct phases of oil and aqueous due to their different densities (Maphosa & Jideani, 2018). Besides, the free energy required to break down the emulsion is lower than the energy needed for emulsification (Liu et al., 2019). There are several mechanisms that lead to the separation of the emulsion such as flocculation, coalescence, gravitational separation, phase inversion and Ostwald ripening (Liu et al., 2019; Maphosa & Jideani, 2018). Hence, a stability study needs to be conducted in order to assess the stability of the emulsion over time. According to ASEAN (2013), stability is an important factor of the quality, efficacy and safety of a product. Inadequate stability of a product can lead to alteration of physical, chemical and microbiological characteristics of the product. Stability studies involve the evaluations of the product characteristics that are vulnerable to change during storage and are expected to affect the products' quality, efficacy and safety. Different dosage forms will have different evaluations. For emulsions, it needs to be tested for organoleptic properties, phase separation, pH, microbial limits, droplet size, polydispersity index (PDI) and drug content. Besides, the shelf-life of the product also can be predicted through a stability study (ASEAN, 2013).

Hence, this chapter aimed to formulate a benzoyl peroxide emulgel incorporating stingless bee honey and characterise the physicochemical properties of the formulation. The characterisation of the formulation that had been assessed included benzoyl peroxide content, organoleptic properties, centrifugation, pH, droplet size, polydispersity index (PDI), zeta potential, spreadability, microbial limit count study and also stability study.

3.2 MATERIALS

Dibenzoyl peroxide, 97% (dry wt.), wet with 25% water was bought from Alfa Aesar (Mexico), stingless bee honey was obtained from Kuantan, Pahang (Desa Kelulut Bukit Kuin) (Figure 3.1), 1,2-propanediol, sabouraud 4% dextrose agar, centrimide agar, tryptic soy agar, tryptic soy broth, methanol gradient grade for liquid chromatography and acetonitrile, for HPLC, mobile phase were purchased from Merck (Darmstadt, Germany), mannitol salt agar was received from Oxoid (United Kingdom), tween 20 was purchased from Sigma Aldrich (United Kingdom), phosphate buffer saline was procured from Sigma

Aldrich (United States), sodium hydroxide was gotten from Sigma Aldrich (Sweden), squalane was purchased from Kono Chem Co. Ltd. (Xi'an, China), Vitamin E acetate was bought from Alfa Laboratory (Germany), span 60® for synthesis was received from Sigma Aldrich (Darmstadt, Germany), Carbopol® 940 was bought from Acros Organics (New Jersey, USA), euxyl® PE 9010 was purchased from schülke (Germany), triethanolamine was obtained from R&M Chemicals, distilled water and purified water.

Figure 3.1 Desa Kelulut Bukit Kuin's stingless bee honey

3.3 METHODS

3.3.1 High Performance Liquid Chromatography Analysis

3.3.1.1 Instrumentation and chromatographic condition

The chromatographic separation was performed on a Reverse-Phase HPLC Prominence instrument (Shimadzu Corp., Tokyo, Japan). This HPLC system consisted of an integrated controller, an autosampler (SIL-20S HT), a quaternary pump (LC-20AT), a solvent degasser unit (DGU-20A SR), a column oven (CTO-10AS VP) and a photodiode array detector (SPD-M20A). An Agilent Eclipse Plus C18 (4.6 x 250 mm, 5-micron) column (Agilent Technologies, USA) with a guard-column cartridge (4.6 x 12.5 mm, 5-micron) (Agilent Technologies, USA) was used as the stationary phase for the chromatographic separation. The analytical column and the guard column were operated at ambient temperature (25 ºC). All the chromatographic data were analysed through the Lab Solution software (version 5.54 SP5). The HPLC procedure conducted in this experiment was adapted from the methods developed by Chen et al. (2015) and Barange et al. (2018) with some modifications in terms of the type and ratio of the mobile phase. The mobile phase used consisted of acetonitrile: deionised water mixture (80:20, v/v). Both acetonitrile and deionised water were filtered by using a nylon membrane filter (47 mm diameter, 0.45 µm pore size). The elution was carried out isocratically at a flow rate of 1 mL/min. The injection volume of the sample was 20 µL and the detection was performed at wavelength of 270 nm. The total running time for each sample was 8 minutes.

3.3.1.2 Preparation of standard stock solution and working standard solution

A standard stock solution of 2 mg/mL was prepared by dissolving 100 mg of benzoyl peroxide drug in 50 mL of HPLC-grade methanol. Meanwhile, the working standard solution was prepared by further diluting the standard stock solution with HPLC-grade methanol to obtain target concentrations of 0.7, 0.8, 0.9, 1.0, 1.1 and 1.2 mg/mL.

3.3.1.3 HPLC method validation

The HPLC method was validated for linearity, specificity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ) according to the guidelines for assay methods of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) (Chen et al., 2015; Singh et al., 2018).

3.3.1.3.1 Linearity

To evaluate the linearity, six standard solutions in the concentration range of 0.7 mg/mL to 1.2 mg/mL were prepared from stock standard solution. All of these concentrations were filtered through a 0.45 µm syringe filter before being transferred into a 2 mL HPLC vial. Each concentration was injected into the HPLC and run in triplicate at a wavelength of 270 nm to develop a calibration curve by plotting the peak areas versus the concentrations. From the plotted graph, a regression correlation coefficient was acquired.

3.3.1.3.2 Accuracy

Accuracy was carried out by preparing three different standard concentrations which are 0.8, 1.0 and 1.2 mg/mL in three replicate samples and injected them into HPLC. The accuracy result was expressed by the percentage recovery of the drug. The percentage recovery of the drug can be calculated as Equation 3.1.

$$Percentage\ recovery\ (\%) = \frac{\text{Measured concentration}}{\text{Theoretical concentration}} \times 100 \qquad \text{Equation 3.1}$$

Where the measured concentration is the benzoyl peroxide amount recovered meanwhile theoretical concentration is the benzoyl peroxide amount being prepared.

3.3.1.3.3 Precision

Similar to accuracy, the precision of the developed method was determined by analysis of three replicates of standard solution in three different concentrations (0.8, 1.0 and 1.2 mg/mL). There were two assays which are intra-day and inter-day precisions conducted in order to assess the precision. The intra-day and inter-day assays were considered as repeatability and intermediate precision respectively. The intra-day assay was performed when three standard concentrations in triplicate samples were injected and run in HPLC on the same day with a short time interval at the same condition. Meanwhile, the inter-day assay was conducted the same as the intra-day but on three different days (day 1, day 2 and day 3). The precision was calculated as the relative standard deviation (RSD). The RSD was calculated as Equation 3.2 and the acceptable RSD was below than 2% (USP41-NF36, 2018).

$$RSD = \frac{\text{Standard deviation (SD)}}{\text{Mean}} \times 100 \qquad \text{Equation 3.2}$$

3.3.1.3.4 Specificity

Specificity was determined to ensure there was no interference with the analyte elution. Blank, standard solution, placebos and real samples that had been filtered with 0.45 µm syringe filter and put in 2 mL HPLC vial were injected into HPLC at the same condition to examine any interference at the standard solution's peak. The blank used in this HPLC study was methanol meanwhile the placebos were stingless bee honey emulgel (F3) and base emulgel (F4). The real samples were stingless bee honey with benzoyl peroxide emulgel (F1) and benzoyl peroxide emulgel (F2). The preparation of the placebos was similar to the real samples preparation which was further described in Section 3.3.4 later.

3.3.1.3.5 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The limit of detection (LOD) is the lowest amount of the analyte that can be measured by the HPLC equipment. Meanwhile, the limit of quantitation (LOQ) is the smallest analytical concentration in the standard curve that was quantified with precision and accuracy. Based on the calibration curve obtained earlier from the linearity part, the LOD and LOQ were calculated using the following equations.

$$LOD = 3.3x\ \sigma/S \qquad \text{Equation 3.3}$$

$$LOQ = 10x\ \sigma/S \qquad \text{Equation 3.4}$$

Where σ is the standard deviation of the response and S is the slope of the calibration curve.

3.3.2 Identification test and compatibility study of benzoyl peroxide with stingless bee honey

Identification test and compatibility study of benzoyl peroxide and stingless bee honey were conducted by using FTIR spectroscopy. Spectra of Formulation 1 (refer to Section 3.3.3), raw benzoyl peroxide and stingless bee honey were taken individually and in combination. The FTIR analysis was carried out by using approximately 10 mg of benzoyl peroxide and stingless bee honey for individual testing. Meanwhile, for combination, 5 mg of benzoyl peroxide and 5 mg of stingless bee honey were combined (1:1 ratio) for the FTIR analysis. For Formulation 1, a 10 mg sample was taken for the testing. The raw benzoyl peroxide was dried for 10 minutes before testing since it was wet with 25% water. All the samples were placed directly into the instrument and ATR-FTIR spectra were recorded at the mid-frequency range (4000-400 cm^{-1}) at 4 cm^{-1} resolution (Shettigar et al., 2021).

3.3.3 Preparation of benzoyl peroxide with stingless bee honey emulgel formulation

Table 3.1 shows the composition and amount of each ingredient used in the emulgel formulation. This emulgel formulation involved a few steps which were the preparation of the (1) Oil-in-water emulsion and (2) Base gel, followed by the addition of the emulsion into the base gel. Benzoyl peroxide was dissolved in propylene glycol to form a benzoyl peroxide solution. The oil phase of the emulsion was prepared by dissolving the squalane in Span 60 (lipophilic surfactant). For the aqueous phase, Tween 20 (hydrophilic surfactant) was dissolved in distilled water. Both the oil and aqueous phases were heated up to 65°C separately in a water bath for 15 minutes. Then, the oil phase was added to the aqueous phase and homogenised at a speed of 8000 rpm for 5 minutes to form an emulsion. Then, the emulsion was cooled down. After that, stingless bee honey, benzoyl peroxide solution, phenoxyethanol and tocopherol acetate were added to the emulsion and homogenised at a speed of 8000 rpm for 8 minutes.

The base gel was prepared by cold mechanical method (El-Kased et al., 2017). 1 g of carbopol was mixed with distilled water and homogenised at a speed of 8000 rpm for 6 minutes in order to swell the carbopol. Then, sodium hydroxide was added to the carbopol solution and continued homogenised for 2 minutes and form the base gel. Lastly, the obtained emulsion was added to the base gel with a 1:1 ratio and homogenised at a speed of 8000 rpm for 8 minutes to form the emulgel. All of these steps were summarised in Figure 3.2 and the composition of the formulations prepared were tabulated as in Table 3.1.

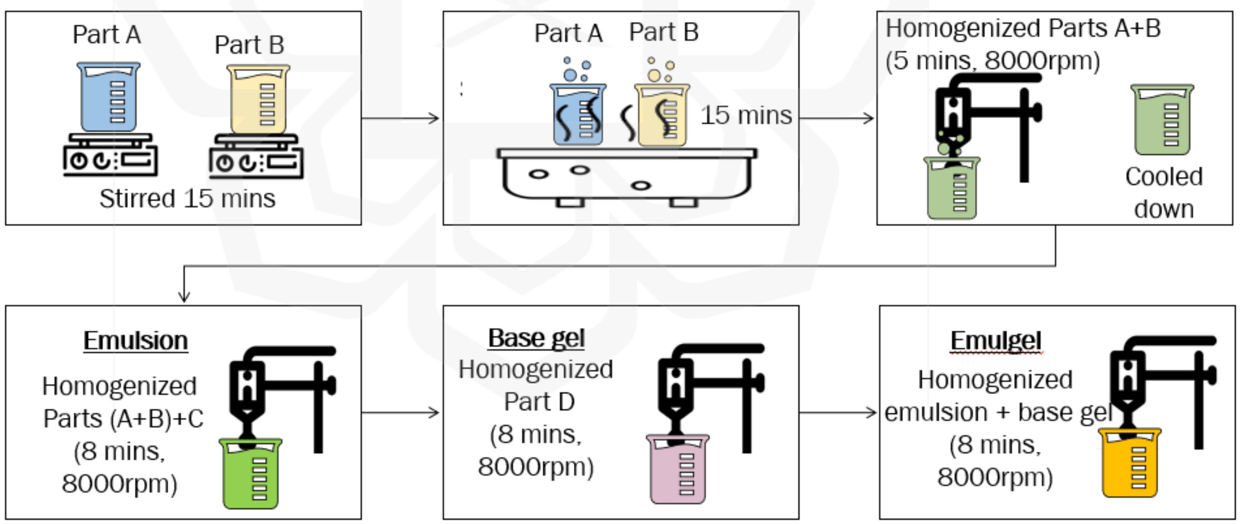


Figure 3.2 Illustration for emulgel formulation preparation.

Table 3.1 Composition of benzoyl peroxide with stingless bee honey emulgel formulation (Thakur et al., 2012).

Formulation	F1	F2	F3	F4
(1) Oil-in-water emulsion				
Part A (Aqueous phase)				
Tween 20 (% w/w)	2	2	2	2
Distilled water	q.s	q.s	q.s	q.s
Part B (Oil phase)				
Span 60 (% w/w)	2.5	2.5	2.5	2.5
Squalane (% w/w)	6	6	6	6
Part C (Cold phase)				
Stingless bee honey, SBH (% w/w)	15	-	15	-
Benzoyl peroxide, BPO (% w/w)	5	5	-	-
Propylene glycol (% w/w)	10	10	10	10
Phenoxyethanol & ethylhexylglycerine (% w/w)	0.5	0.5	0.5	0.5
Tocopherol acetate (% w/w)	2	2	2	2
(2) Base gel				
Part D (Gel)				
Carbopol 940 (% w/w)	1	1	1	1
Sodium hydroxide, NaOH (% w/w)	0.2	0.2	0.2	0.2
Distilled water	q.s	q.s	q.s	q.s
Total (%)	100	100	100	100

3.3.4 Benzoyl peroxide content analysis

Quantification of benzoyl peroxide content in formulation by using High-Performance Liquid Chromatography (HPLC) was conducted based on the HPLC method that had been validated before. Sample stock solution was prepared by mixing 5 g emulgel in 50 mL of methanol (5 mg/mL) in a volumetric flask. Then, the solutions were sonicated for 15 minutes. After that, 2 mL of the solution was diluted with methanol in a 10 mL volumetric flask (1 mg/mL). The prepared solution was filtered through a 0.45 µm syringe filter and transferred into a 2 mL HPLC vial. The sample solutions were then injected three times, and the chromatogram was detected at a wavelength of 270 nm. The benzoyl peroxide content in the emulgel formulation was calculated by the following formula.

$$Benzoyl\ peroxide\ content\ (\%) = \frac{\text{Measured concentration}}{\text{Actual concentration}} \times 100 \qquad \text{Equation 3.5}$$

Where measured concentration is the benzoyl peroxide amount recovered meanwhile the actual concentration is the benzoyl peroxide amount put in the emulgel.

3.3.5 Characterisation of Emulgel Formulation

3.3.5.1 Organoleptic properties and forced centrifugation study

For, organoleptic properties, the appearance of all formulations was observed visually for the colour, homogeneity, transparency, texture and odour. Meanwhile, for the forced centrifugation study, 5 g of the emulgel formulation was placed in a centrifugal tube and centrifuged at 5000 rpm for 10 min at 25 ºC. Then, the occurrence of phase separation was observed (Rafique & Akhtar, 2019).

3.3.5.2 pH measurement

1 g of the emulgel formulation was weighed and dissolved in 25 mL of distilled water. The pH of the mixture was determined using a pH meter. The pH meter was calibrated with buffer solutions 4.0, 7.0 and 10.0 before each use. The test was repeated in triplicate and the average result was reported (El-Kased et al., 2017).

3.3.5.3 Droplet size and polydispersity index analysis (PDI)

Droplet size analysis and polydispersity index analysis (PDI) of the formulation were determined by laser droplet size analyser instrument (Malvern, UK) at 25 ºC. The formulated emulgel was diluted in distilled water in 1:1000. The sample was sonicated for 10 to 20 minutes before placing it in a disposable cuvette and placed in the instrument for the droplet size and PDI measurement. The sample was run in triplicate to obtain an average result (Omar et al., 2021; Purnima & Reddy, 2021).

3.3.5.4 Zeta potential measurement

Zeta potential can determine the stability of the formulation. It basically measures the degree of electronic repulsion or attraction between droplets (Bachhav, 2017). The Zeta potential of the formulation was measured by using Zetasizer (Malvern, UK) at 25ºC. The formulated emulgel was diluted in distilled water in 1:1000 and sonicated for 10 to 20 minutes. Then, the sample was put in a disposable cuvette and placed in the instrument for the zeta potential measurement. The sample was run in triplicate.

3.3.5.5 Spreadability

0.1 g was put on the pre-marked centre at the front side of a glass slide and covered it by a second glass slide. Weight (500 g) was applied over the glass slide for 5 minutes. The length (in cm) of each spread circle area of the formulation was measured and repeated three times. The spreadability was calculated by using the following formula (Javed et al., 2018):

$$\text{Spreadability} = \text{Weight applied over the glass slide(kg)} \times \text{Length of the glass (cm)} \times \text{Time taken in minutes} \qquad \text{Equation 3.6}$$

3.3.5.6 Microbial limit test

For the determination of Total Aerobic Microbial Count (TAMC) and Total Yeast and Mold Count (TYMC), a dilution of 1:10 of the emulgel in phosphate buffer saline solution (PBS) pH 7.2 was prepared. The pour-plate method was conducted where 1 mL of the diluted sample was added to a petri dish and 15–20 mL of tryptic soy agar for TAMC was added. The plate was then incubated at 30–35 ºC for 5 days or less. Similar steps were repeated on Sabouraud dextrose agar for TYMC. The plate for TYMC was then incubated at 20–25 ºC for 5 days or less. Meanwhile, for the determination of *Staphylococcus aureus* and *Pseudomonas aeruginosa* growth, a dilution of 1:10 of the emulgel in PBS solution pH 7.2. 10 mL of the diluted sample was mixed with 10 mL of tryptic soy broth (TSB) and then incubated at 30–35 ºC for 18–24 hours. The sample was then subcultured on a mannitol salt agar plate and incubated for 18–72 hours at 30–35 ºC to check the presence of *Staphylococcus aureus*. The same steps were repeated but subcultured on cetrimide agar and incubated for 18–72 hours at 30–35 ºC for *Pseudomonas aeruginosa* determination (Omar et al., 2021). These microbial limit test procedures were illustrated in Figure 3.3.

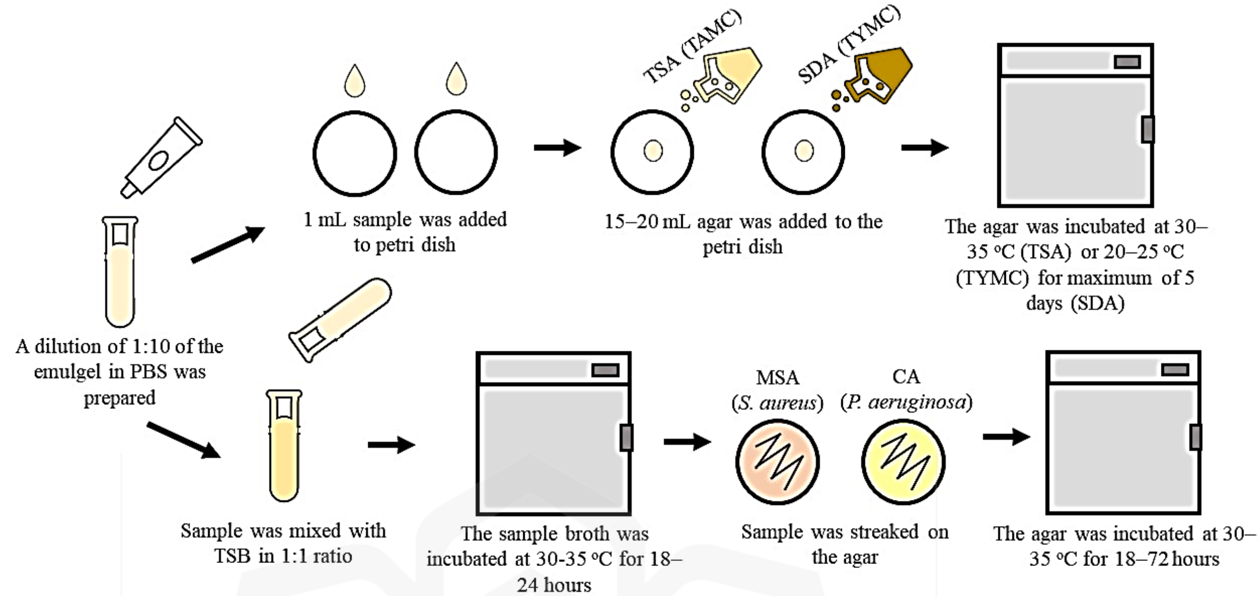


Figure 3.3 Illustration for microbial limit test procedures.

3.3.6. Stability Study

A stability study was conducted by incubating the optimised formulation for 3 months at temperatures of 30 ± 2°C (real-time chamber) and 40 ± 2°C (accelerated-time chamber) with a relative humidity (RH) of 75 ± 5% (ICH, 2003). All the organoleptic properties, phase separation, pH, droplet size, PDI, zeta potential, spreadability and microbial limit were evaluated at week 0 (baseline), week 2, week 4 and week 12. The benzoyl peroxide content also was measured for each time point by using the following formula.

$$Benzoyl\ peroxide\ content\ (\%) = \frac{\text{Benzoyl peroxide content at each time point}}{\text{Initial benzoyl peroxide content}} \times 100 \qquad \text{Equation 3.7}$$

3.4 RESULTS AND DISCUSSION

3.4.1 HPLC Method Validation

3.4.1.1 Linearity

A standard calibration curve was plotted between peak areas versus six concentrations of benzoyl peroxide ranging between 0.7 mg/mL to 1.2 mg/mL. The method demonstrated a good linearity with a correlation coefficient (R^2) of 0.9988. In general, a value of R^2 more than 0.998 is considered an acceptable fit for the regression line data (Alquadeib, 2019). Based on the calibration curve, the regression equation obtained was y = 5064341x – 289081.

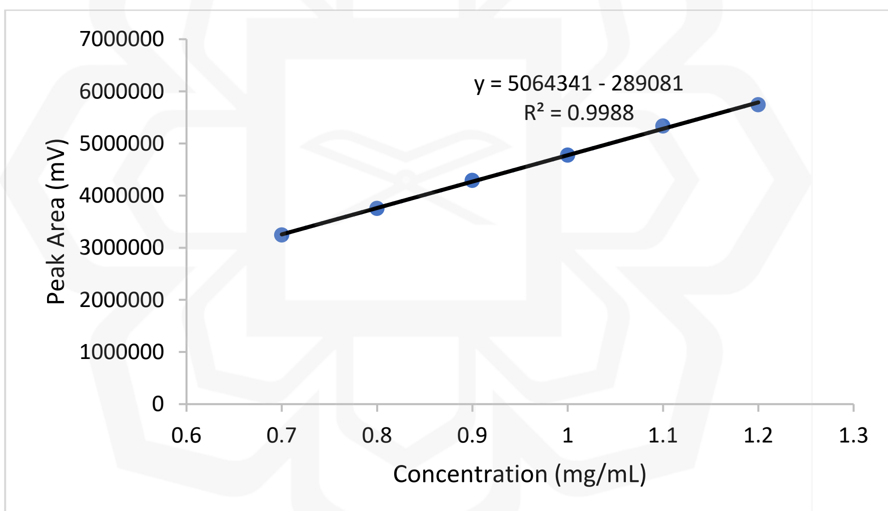


Figure 3.4 Standard calibration of six concentration levels consisting of 0.7, 0.8, 1.0, 1.1 and 1.2 mg/mL benzoyl peroxide.

3.4.1.2 Accuracy and Precision

The results for accuracy and precision are shown in Table 3.2. The accuracy was expressed as percentage recovery and the percentage recoveries for all three concentrations (0.8, 1.0

and 1.2 mg/mL) were within the acceptance range of 90 – 125% (USP41-NF36, 2018). Hence, the method validated is accurate. Meanwhile, precision expressed as percentage relative standard deviation (% RSD) was found in the range of 0.52% to 0.99% and 0.77% to 1.27% for intra-day and inter-day assays, respectively. The percentage relative standard deviations for both assays were within the acceptance criteria of ±2 % (USP41-NF36, 2018).

Table 3.2 Result for accuracy and precision of HPLC (n=9).

	Accuracy	Precision	
Theoretical Concentration (mg/mL)	Percentage Recovery (%)	Intra-day Assay (% RSD)	Inter-day Assay (% RSD)
0.8	100.23 ± 0.64	0.63	1.27
1.0	99.41 ± 0.98	0.99	0.77
1.2	100.57 ± 0.53	0.52	1.17

3.4.1.3 Specificity

The specificity of the HPLC was validated by comparing the chromatograms of the methanol as the blank, standard benzoyl peroxide, samples which are the emulgel that contained benzoyl peroxide and also placebos which emulgel that contained all excipients without benzoyl peroxide. There were two samples and two placebos being assessed which are stingless bee honey with benzoyl peroxide emulgel, benzoyl peroxide emulgel, stingless bee honey emulgel and base emulgel. Based on Figure 3.5, there was no peak interference of the blank and placebos at the peak of benzoyl peroxide with a retention time of 5.595 minutes. Hence, this indicates that this HPLC method was specific.

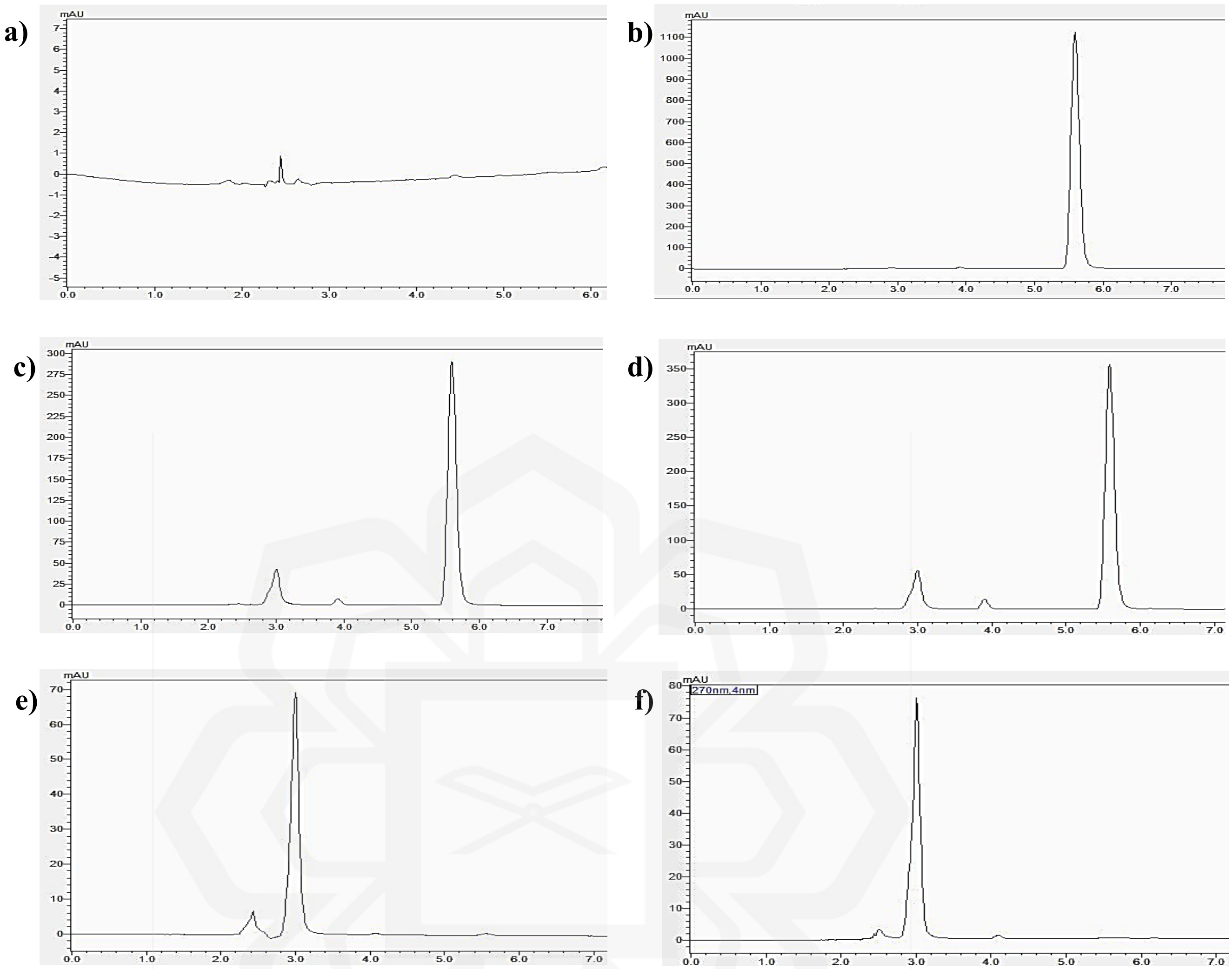


Figure 3.5 HPLC chromatograms of a) blank, b) standard benzoyl peroxide, c) benzoyl peroxide with stingless bee honey emulgel, d) benzoyl peroxide emulgel, e) stingless bee honey emulgel and f) base emulegel.

3.4.1.4 Limit of Detection (LOD) and Limit of Quantification (LOQ)

Based on the standard deviation of the response and the slope of the calibration curve, the lowest concentration of benzoyl peroxide that was detected was 0.12 mg/mL. Meanwhile, the lowest quantification concentration of benzoyl peroxide was 0.37 mg/mL.

3.4.2 Identification test and compatibility study of benzoyl peroxide with stingless bee honey

Benzoyl peroxide is composed of two benzyl rings that are connected by a peroxide group. Based on Figure 3.6, the peaks that represented the functional group of benzoyl peroxide in this study were around 1700 cm^{-1} (C=O stretching), 1500 cm^{-1} (C=C stretching), 1400 cm^{-1} (C=C stretching), 1200 cm^{-1} (C-O stretching), 1100 cm^{-1} (C-O stretching), 1000 cm^{-1} (C-H bending, C-O stretching) and 990 cm^{-1} (C-H bending, C-O stretching). These results were in accordance with the results found by Aarthi et al. (2018), Minhas et al. (2016) and Guo et al. (2016).

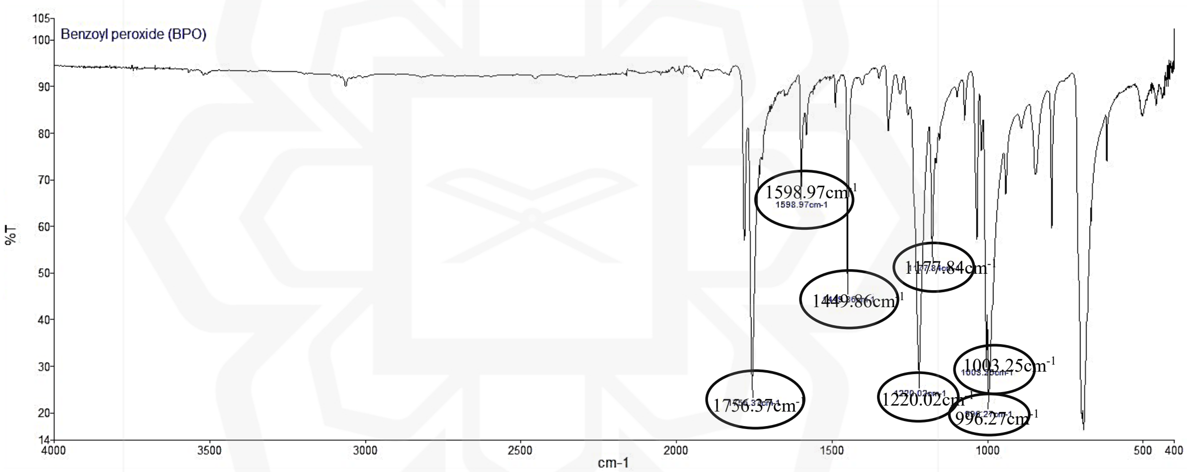


Figure 3.6 IR fingerprint results for benzoyl peroxide

Referring to Figure 3.7, the FTIR spectra of stingless bee honey obtained in this study were the same as the literature (Mail et al., 2019; Md Zin et al., 2019; Rozman et al., 2021). The main peaks acquired were around 3000 cm^{-1} (O-H stretching), 2900 cm^{-1} (C-H stretching), 1600 cm^{-1} (O-H bending), 1000 cm^{-1} (C-O, C-OH stretching) and 920 cm^{-1} (C-O, C-C stretching). All of these main peaks represented the five main functional groups of honey. The O-H stretching was basically characterised by carbohydrates, water and organic acids meanwhile C-H stretching denoted to carbohydrates and carboxylic acids. There was also water O-H bending, carbohydrates and organic acids C-O, C-OH stretching and carbohydrates C-O, C-C stretching (Mail et al., 2019; Md Zin et al., 2019; Rozman et al., 2021).

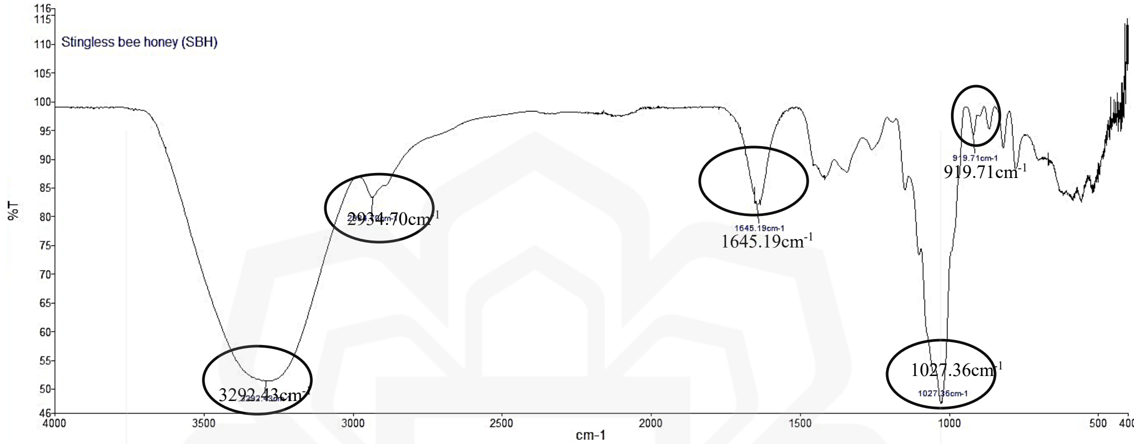


Figure 3.7 IR fingerprint results for stingless bee honey

As shown in Figure 3.8, there was no deviation of principle peaks observed in the spectra of benzoyl peroxide when mixed with stingless bee honey and also in Formulation 1. In addition, there was a band around 3000 cm^{-1} (O-H stretching) appeared in the mixture. This may be contributed by stingless bee honey constituents of carbohydrates, water and organic acids. Besides, the O-H stretching was prominent in Formulation 1 compared to the mixture of benzoyl peroxide and stingless bee honey. This may be due to undried raw benzoyl peroxide incorporated in the formulation. Hence, high water content contributed to the O-H stretching (Md Zin et al., 2019; Veras et al., 2019). This result suggested that there were no chemical incompatibilities between benzoyl peroxide and stingless bee honey (Gill & Nanda, 2020; Shettigar et al., 2021; Veras et al., 2019).

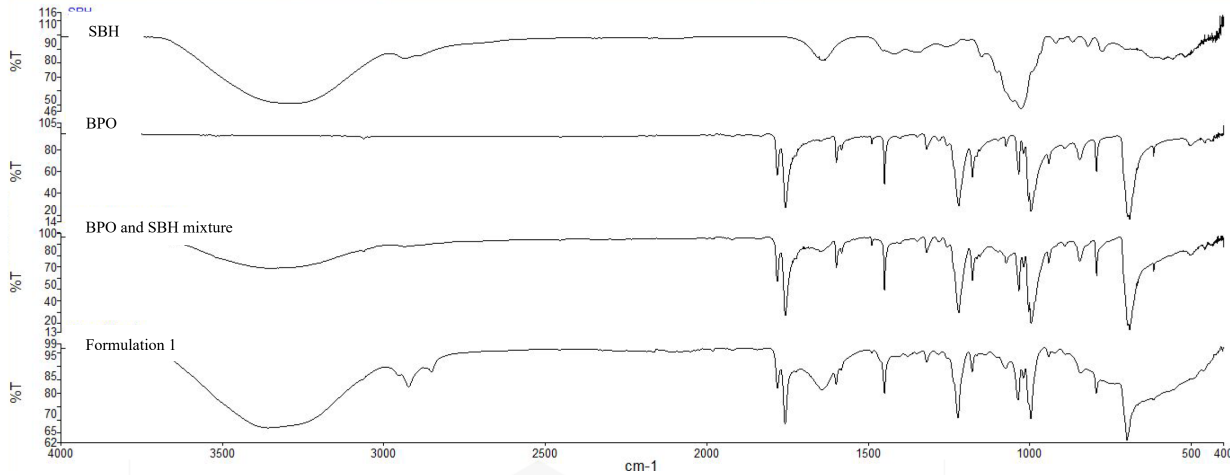


Figure 3.8 Comparison IR fingerprint results for benzoyl peroxide, stingless bee honey, mixture of benzoyl peroxide and stingless bee honey and Formulation 1.

3.4.3 Formulation of Emulgel

In this study, an oil-in-water (O/W) emulsion type was formulated with 78% of the aqueous phase and 22% of the oil phase before it was combined with the gel to form the emulgel. O/W emulsion was chosen because it is less greasy and easy to wash off. These factors make it more cosmetically acceptable and tolerable since it is more comfortable to be applied to the skin especially the face (Mohiuddin, 2019). Generally, there are many constituents used in order to form an emulgel. The constituents normally will include active ingredients, aqueous materials, oil materials, emulsifiers, gelling agents and also pH adjusting agents (Sreevidya, 2019).

As the aim of this emulgel formulation was for acne treatment, hence benzoyl peroxide and stingless bee honey were incorporated as the active ingredients. Most studies have shown that a lower concentration of benzoyl peroxide is as effective as a higher concentration with fewer side effects of skin irritation. Hence, there is no need to use a concentration higher than 5% since a higher concentration is associated with more skin irritation occurrence (Grobel & Murphy, 2018). A study conducted to assess the minimum contact time of different concentrations of benzoyl peroxide for a bactericidal effect against *Propionibacterium acnes* showed that both 5% and 10% concentrations of benzoyl peroxide demonstrated rapid bactericidal activity within 30 seconds. This indicated that the effectiveness of a 5% concentration of benzoyl peroxide was equivalent to a 10% concentration. Meanwhile, a 2.5% concentration of benzoyl peroxide required a longer contact time (15 minutes) for it to show bactericidal effects (Boonchaya et al., 2022). Therefore, a 5% concentration of benzoyl peroxide was selected to be incorporated in this research. Meanwhile, the concentration of stingless bee honey used in the formulation was decided based on the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) results that will be discussed in Chapter 4 later. In this study, there were four formulations that had been prepared where F1 was the main formulation that contained both benzoyl peroxide and stingless bee honey. F2 consisted of benzoyl peroxide only and F3 composed of solely stingless bee honey as the active ingredient. Meanwhile, F4 did not include any active ingredient and regarded as base emulgel.

Based on Table 3.1, there were many constituents or excipients that had been incorporated into the emulgel with different functions. Distilled water was used as a solvent for the aqueous phase of the emulsion and also the base gel. Meanwhile, propylene glycol was incorporated as a solvent for the benzoyl peroxide. Propylene glycol also can act as a penetration enhancer that can improve benzoyl peroxide permeation through the skin from the emulgel preparations (Carrer et al., 2020). Squalane in the formulation functions as an emollient and also a penetration enhancer (Sarango-Granda et al., 2022). Emollients have commonly been included in topical formulations to provide a skin conditioning effect and improve the consistency of semisolid preparation (Kar et al., 2019). Squalene is considered an excellent emollient since it will rapidly and effectively be absorbed into the skin. Hence, the incorporation of squalane in topical formulations is believed can promote the permeation of the drug through the skin either locally or systemically (Kar et al., 2019; Sarango-Granda et al., 2022). Other than emollient, a preservative is usually added to the formulation as primary prevention or inhibition of microbial growth (Alvarez-Rivera et al., 2018). Phenoxyethanol and ethylhexylglycerine are commonly used as a preservative in cosmetic formulations since phenoxyethanol possesses a broad spectrum of antimicrobial activity (Dréno et al., 2019).

In addition, an antioxidant is also important to be added in the formulation in order to prevent oxidative degradation (Gabrič et al., 2022). Hence, tocopherol acetate was included in the emulgel formulation. Carbopol 940 was incorporated in the formulation as the gelling agent. Gelling agents basically function as a thickener, suspender or stabiliser that alters the flow characteristic of the formulation. Carbopol is a good thickening agent even when used in low concentrations (Mulyani & Hartanto, 2018). It is a hydrophilic and crosslinked polyacrylic acid polymer that has a very high molecular weight. It is also recognised as a pH-dependent polymer as its state will change according to pH. For instance, it will liquefy (liquid form) in acidic pH but it will produce low viscosity gel (semisolid form) in alkaline pH (Mackiewicz et al., 2019). Hence, due to this characteristic of carbopol, pH adjusting agents or buffers are crucial to be included in the formulation. Besides, the incorporation of pH adjusting agents in the formulation also can alter the formulation's pH to the pH that suits the skin. pH adjusting agent that was used in this study was sodium hydroxide. Sodium hydroxide is considered safe and has been widely used as a pH-adjusting agent in cosmetic and pharmaceutical products (Burnett et al., 2021).

Lastly, an emulsifier or surfactant plays a vital role in the emulsion as it can lower the interfacial tension between oil and aqueous phases via absorption at the liquid-liquid interface. This consequently will stabilise the system (Anarakdim et al., 2020). Emulsifiers can be categorised based on the hydrophilic-lipophilic balance (HLB) value. HLB value ranges between 0 to 20 with the low value representing an emulsifier that is substantially more soluble in oil than in water. In contrast, a high HLB value means the emulsifier is more soluble in water than oil. It has been identified that emulsifiers with HLB values between 9 and 12 stabilise O/W emulsion meanwhile emulsifiers with HLB values around 3 to 8 stabilise W/O emulsion (Rahaman et al., 2023). A stable emulsion can be achieved by incorporating an emulsifier or emulsifier blend with a HLB value that is compatible with the required HLB (rHLB) value of the oils used (Park & Kim, 2021). The rHLB values for all oil components were tabulated in Table 3.3 and it had been calculated by using the following equation:

$$rHLB_{total} = \frac{(C_1 x rHLB_1)+(C_2 x rHLB_2)\ldots}{C_{total}} \qquad \text{Equation 3.8}$$

Where $rHLB_{total}$ is the required HLB value of oil components used in O/W emulsion, $C_{1,2\ldots}$ is the composition of respectively oily ingredients, r $HLB_{1,2\ldots}$ is the required HLB of respectively oily ingredients and C_{Total} is the total oily ingredients composition.

Table 3.3 Calculation on total required HLB (rHLB) values of oil components used in O/W emulsion (Mohd Hanif, 2018).

Oil components	% (w/w)	rHLB value of the oil component	rHLB value of oil components used in O/W emulsion
Squalene	6	11.4	6x11.4/8 = 8.55
Tocopherol acetate	2	6	2x6/8 = 1.5
Total rHLB value of oil components used in O/W emulsion			10.1

O/W emulsion prepared in this study used two non-ionic emulsifiers which were tween 20 (hydrophilic surfactant) and span 60 (lipophilic surfactant) with HLB values of 16.9 and 4.7 respectively (Suyanto et al., 2019). The use of an emulsifier blend would enhance the stability of the emulsion than using a single emulsifier (Restuinjaya et al., 2019). This emulsifier blend was used to obtain the predetermined rHLB value of 10.1. The concentration of tween 20 required to mix with span 60 to reach the rHLB was calculated as equation below (Alam et al., 2020). Based on the following equations, the concentration of Tween 20 and Span 60 were 45% and 55% respectively.

$$\% \, Tween \, 20 = \frac{100(rHLB - HLB_{Span \, 60})}{HLB_{Tween \, 20} - HLB_{Span \, 60}} \qquad \text{Equation 3.9}$$

$$\% \, Span \, 60 = 100 - \% \, Tween \, 20 \qquad \text{Equation 3.10}$$

3.4.4 Benzoyl peroxide content analysis

Based on the regression equation from the calibration curve obtained before (y = 5064341x – 289081), the benzoyl peroxide content in both F1 and F2 was analysed. Referring to Table 3.4, the benzoyl peroxide content for both formulations was within the acceptance range of 90 – 125% (USP41-NF36, 2018). Based on the unpaired t-test analysis, there was no significant difference in benzoyl peroxide content in both F1 and F2 ($p = 0.1977$) with a mean difference of 0.4%.

Table 3.4 Benzoyl peroxide content in Formulations 1 and 2 (Mean ± SD, n = 3, $p < 0.05$).

Benzoyl peroxide content (%)	
F1	F2
105.2 ± 0.12	105.6 ± 0.43

3.4.5 Characterisation of Emulgel Formulation

3.4.5.1 Organoleptic properties and forced centrifugation test

The organoleptic characteristic of a product is an important parameter since it can affect the interest and compliance of the consumers (Alam et al., 2020). The results for the organoleptic properties of all four formulations are tabulated in Table 3.5. It was found that all formulations had a smooth texture, opaque appearance and homogenous dispersion with no signs of phase separation after being centrifuged (Figure 3.9). The absence of phase separation with no creaming and foaming indicated that all formulations were stable. This may be due to proper homogenisation and the presence of adequate amount of emulsifier (Alam et al., 2020). In terms of colour and odour, there were differences between F1 and F3 with F2 and F4. Both F1 and F3 had cream-white colour with a honey odour meanwhile F2 and F4 were white in colour without any odour. This is because only F1 and F3 consist of stingless bee honey in the formulation which affects the colour and odour of the formulation.

Table 3.5 Physical analysis of Formulations 1, 2, 3 and 4.

Properties	**F1**	**F2**	**F3**	**F4**
Colour	Cream-white	White	Cream-white	White
Homogeneity	Homogenous			
Transparency	Opaque			
Texture	Smooth			
Odour	Honey smell	Odourless	Honey smell	Odourless
Phase separation	Absent			

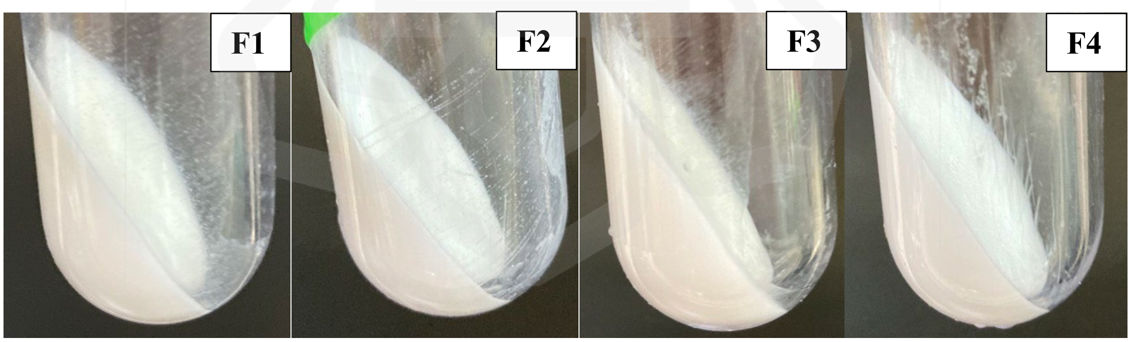


Figure 3.9 Observation of F1, F2, F3 and F4 that showed no phase separation after centrifuged.

3.4.5.2 pH measurement

It is important to maintain the acidic pH of the skin surface since it is a regulating factor for the maintenance of the stratum corneum homeostasis and barrier permeability. The changes in the pH of the skin may damage the healthy skin condition (Luki et al., 2021). In addition, it has been reported that stratum corneum and skin protein will swell significantly when exposed to alkaline pH solutions. The stratum corneum lipids also will be affected by an alkaline pH solution since it will ionise the fatty acids in the lipid bilayers. This will lead to lipid bilayer destabilisation and hence impair the skin barrier (Hwang et al., 2022). Therefore, topical formulation should be acidic and have pH values in the range of 4 to 6 (Luki et al., 2021). Based on the one-way analysis of variance (ANOVA) result, the pH of all formulations was significantly different ($p < 0.0001$). F1 and F3 have lower pH compared to F2 and F4 (Figure 3.10). This may be due to the presence of stingless bee honey that is acidic. The pH value of stingless bee honey used in this study was found to be 3.14 ± 0.01. However, the pH of all formulations was in the normal skin and topical formulation's pH range which is between 4 to 6, hence they were safe to be applied on the skin and would not cause skin irritation (Khan et al., 2022).

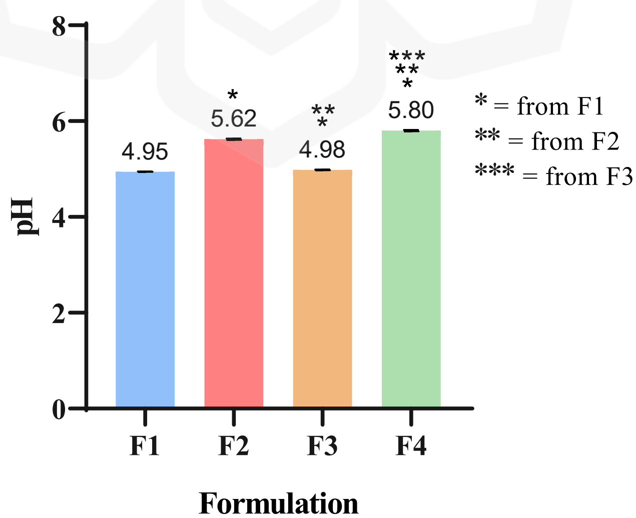


Figure 3.10 pH for Formulation 1, 2, 3 and 4 (Mean ± SD, n = 3, (*) $p < 0.05$).

3.4.5.3 Droplet size analysis

Droplet size is a crucial parameter that can influence emulsion stability. Reduced droplet size will make the emulsion more stable since more droplets will be dispersed at the interface. Meanwhile, a larger droplet-size emulsion has a higher tendency to undergo phase separation as the droplet will coalesce (Venkataramani et al., 2020). In addition, reduced droplet size emulsions were also found to have better skin absorption and drug permeation due to larger surface area (Ding et al., 2018; Venkataramani et al., 2020). The droplet size of the emulsion is inversely proportional to the concentration of the surfactant. This is because due to the large surface area of the reduced droplet size, thus higher concentrations of emulsifiers are needed to cover all the small droplets (Iyer et al., 2015). The droplet size can be categorised into three categories which are coarse (> 10 µm), fine (1-10 µm) and ultrafine (0.1–1 µm) (Omar et al., 2021). Based on Figure 3.11, the droplet size of all formulations was classified as ultrafine since they were below than 1 µm. However, the overall result for droplet size for all formulations was significantly different ($p < 0.0001$). The droplet sizes of F1 and F3 were higher compared to F2 and F4. This may be due to the addition of stingless bee honey which comprises mainly fructose and glucose in those formulations that make the droplet size a bit larger. A study conducted by Liang et al. (2014) revealed that the inclusion of sugars such as glucose and maltose in the emulsion makes the droplet size slightly increase. It also appeared that the droplet size of liquid whole eggs which is a type of emulsion increased with the increase in glucose concentration (Hu et al., 2022).

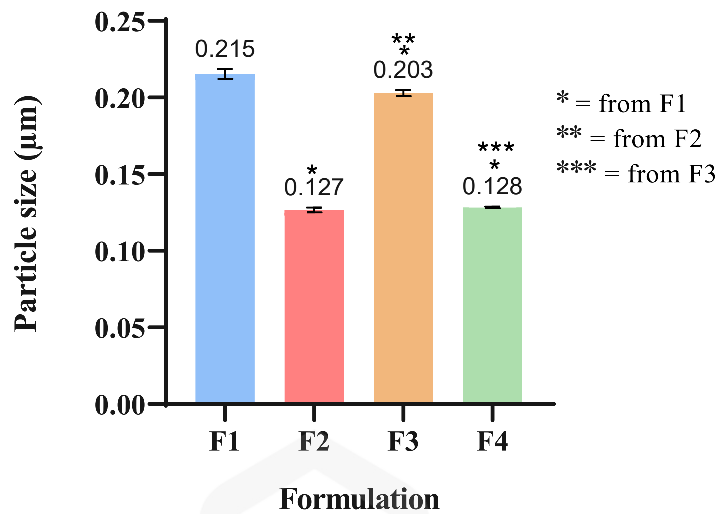


Figure 3.11 Droplet size for Formulation 1, 2, 3 and 4 (Mean ± SD, n = 3, (*) $p < 0.05$).

3.4.5.4 Polydispersity index (PDI)

The polydispersity index (PDI) value represents the homogeneity and uniformity of the droplet size in the emulsion. It ranges between 0 to 1 and low PDI value has greater uniformity. Emulsion with a low PDI value suggests a monodispersed system since the droplet size distribution is homogenous. Meanwhile, an emulsion with a high PDI value is called a highly dispersed system due to heterogeneous droplet size distribution. Generally, a PDI value less than 0.5 is considered a narrow size distribution, hence it possesses a good size distribution (Ding et al., 2018; Lino et al., 2020). A uniform and narrow size distribution of droplet size will make the emulsion more stable due to lower interfacial tension (Ding et al., 2018). All formulations posed good size distribution and monodispersed system since the PDI value for all formulations was less than 0.5 (Figure 3.12). The statistical analysis showed that the overall PDI value results for all formulations were significantly different ($p < 0.0001$). Similar to the droplet size result, the PDI values of F1 and F3 were higher compared to F2 and F4. It was relevant because larger droplet sizes tend to have higher PDI values (Iyer et al., 2015)

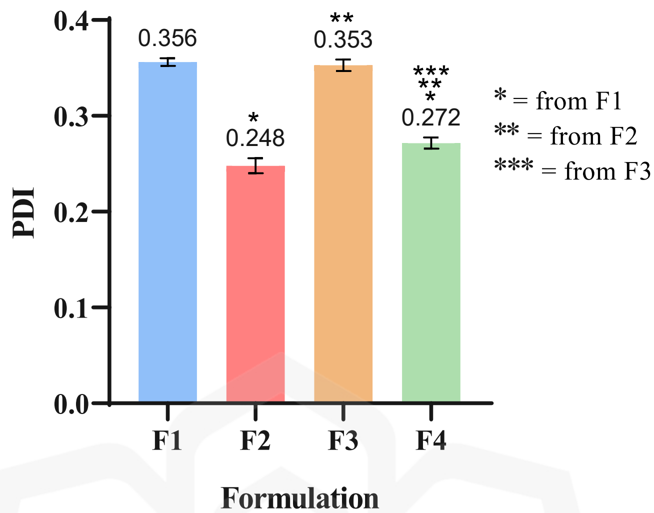


Figure 3.12 Polydispersity index (PDI) for Formulation 1, 2, 3 and 4 (Mean ± SD, n = 3, (*) $p < 0.05$).

3.4.5.5 Zeta potential measurement

Zeta potential is also one of the attributes that can determine the stability of the emulsion. It is the quantification of the electric charge on the surface of the droplets. Emulsions with a higher positive or negative value of zeta potential will have good stability due to electrostatic repulsion between the droplets. Meanwhile, a lower zeta potential value will make the opposite charge droplets attract to each other which consequently form aggregates and flocs. Zeta potential value other than -30 mV to +30 mV is considered ideal for good stabilisation of dispersion (Joseph & Singhvi, 2019; Selvamani, 2018). This is because zeta potential values other than -30 mV to +30 mV will possess adequate electrostatic repulsion to prevent droplet agglomeration from occurring (Joseph & Singhvi, 2019). The zeta potential value of all formulations was more than −30 mV (Figure 3.13) which means all formulations had a good stabilisation of dispersion. The zeta potential value of F1 and F2 is slightly lower than F3 and F4. The zeta potential values for F1 (-39.1 mV) and F2 (-39.0

mV) which contained benzoyl peroxide were similar to the literature. It was found that the zeta potential values for lemongrass oil nanoemulgel incorporating benzoyl peroxide nanoparticles formulations with different concentrations of carbopol (0.4% to 1%) as gelling agent were also between -35 mV to -40 mV (Eid et al., 2023). Based on the statistical analysis, there was a significant difference in the overall result of zeta potential values for all formulations (p = 0.0023).

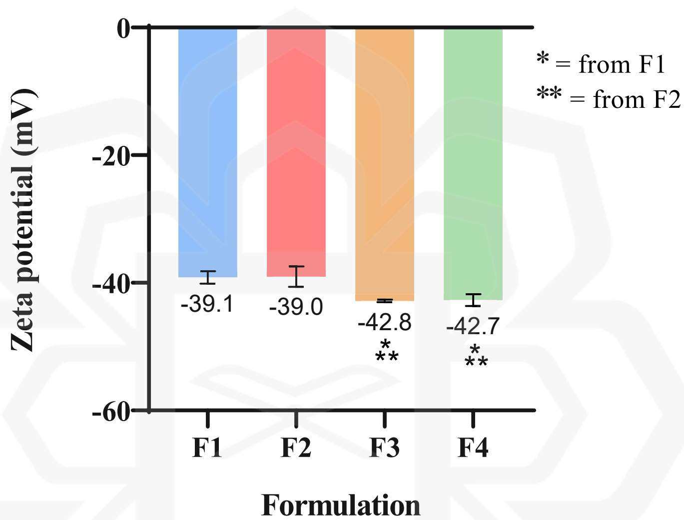


Figure 3.13 Zeta potential values for Formulation 1, 2, 3 and 4 (Mean ± SD, n = 3, (*) $p < 0.05$).

3.4.5.6 Spreadability

Spreadability is described as the force needed to make the formulation flow. It is an important characteristic that represents the applied force required for the formulation to flow out from its bottle and form a uniform application to the skin. Thus, good spreadability will aid the topical formulation to attain a uniform application. Good spreadability is determined by less time taken to spread with high credibility (Algahtani et al., 2020). Spreadability also can be interconnected with viscosity. It shows that the lower the viscosity, the higher the spreadability of the formulation. This is because the low viscosity of the formulation will have low surface tension, thus the formulation can easily spread and be absorbed into the skin. However, too low viscosity and consistency (high spreadability) of the formulation may be associated with the leaking problem (Adejokun & Dodou, 2020). This leaking problem will reduce the residence time and concentration of the formulation being applied to the affected area which consequently will affect the effectiveness of the formulation.

In this study, the spreadability of all formulations was between 5.78 g cm/s to 5.94 g cm/s in which F4 had the greatest spreadability followed by F3, F1 and F2 (Figure 3.14). However, the ANOVA test presented that there was no significant difference in the overall result of spreadability for all formulations ($p = 0.4099$). Based on the study conducted by Surekha et al., (2021), the spreadability of ibuprofen emulgel that consisted of 0.5%, 1% and 1.5% carbopol were 4.2 g cm/s, 5.2 g cm/s and 5.7 g cm/s respectively. Meanwhile, the spreadability of six bisoprolol hemifumarate emulgel prepared with 1% carbopol by Gul et al. (2022) was observed to be between 4.2 g cm/s to 5.8 g cm/s. In another study, the spreadability range of all *Carthamus tinctorius* L. oil emulgels developed with 2% carbopol 940 as the gelling agent was about 4.3 ± 0.1 g cm/s to 4.7 ± 0.1 g cm/s (Saeed et al., 2023). The spreadability of these *Carthamus tinctorius* L. oil emulgels was a bit lower compared to the results obtained here may be due to a higher concentration of carbopol 940 incorporated. This is because a high concentration of carbopol will increase the viscosity of the formulation and spreadibility is inversely proportional to viscosity (Safitri et al., 2021).

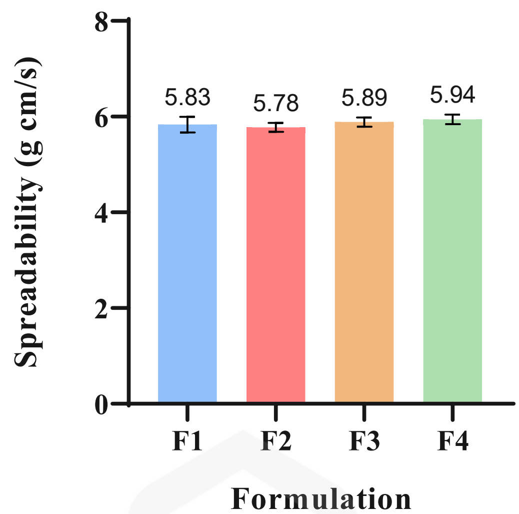


Figure 3.14 Spreadability for Formulation 1, 2, 3 and 4 (Mean ± SD, n = 3, $p < 0.05$).

3.4.5.7 Microbial limit test

This test basically was performed to check the presence of certain types of bacteria in the formulation. This is because formulations that consist of high amounts of water and nutrients are prone to microbial contamination under appropriate physicochemical conditions. Microbial contamination will reduce the quality and stability of the formulation. Besides, the formulation also will no longer be safe to be applied and may affect the health of the consumers (Halla et al., 2018; Kim et al., 2020). Based on Table 3.6, the results showed no growth of microorganisms for all formulations. This may be due to a sufficient amount of preservative that has been included in the formulations (Halla et al., 2018).

Table 3.6 Microbial test observation of Formulation 1, 2, 3 and 4 (n = 3).

Microbial type	**F1**	**F2**	**F3**	**F4**
Total Aerobic Microbial count (TAMC)	Absent			
Total Yeast and Mould count (TYMC)	Absent			
Staphylococcus aureus	Absent			
Pseudomonas aeruginosa	Absent			

3.4.6 Stability Study

3.4.6.1 Benzoyl peroxide content analysis

The benzoyl peroxide content in F1 and F2 emulgels for every sampling period in both real-time and accelerated-time chambers was carried out to observe the percentage left in the emulgel over time. Referring to Figure 3.15, the percentage of benzoyl peroxide content for both F1 and F2 from week 0 until week 12 showed a decreasing trend for both real-time and accelerated-time chambers. In the real-time chamber, the drug content of F1 and F2 reduced from 100% to 95.43% and 95.47% respectively. Meanwhile, the drug content of F1 and F2 declined from 100% to 94.03% and 93.57% respectively in the accelerated-time chamber. The percentage reduction was higher in the accelerated-time chamber compared to the real-time chamber for both formulations. When analysed via repeated measure one-way ANOVA, all the decrements for both formulations in both chambers were significant ($p < 0.05$).

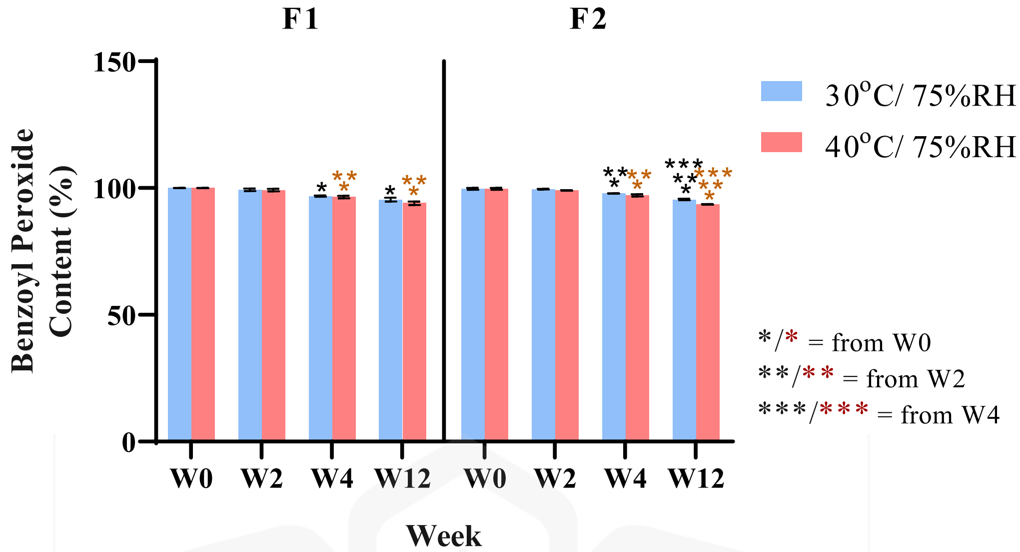


Figure 3.15 Benzoyl peroxide content percentage of Formulation 1 and 2 stored in 30ºC/ 75%RH and 40ºC/ 75%RH at week 0, week 2, week 4 and week 12 (Mean ± SD, n = 3, (*) $p < 0.05$).

These results were similar to the study conducted by Thakur et al. (2012) in which the benzoyl peroxide content in four gellified emulsion preparations was reduced from 100-104% to 96-99% after 3 months of storage at 25 ºC. In another study, the benzoyl peroxide content in foamable emu oil emulsion decreased after 28 days of storage from 98.07 ± 0.41% to 97.56 ± 1.16% and 96.5 ± 1.07% at 25 ºC and 40 ºC respectively (Shatalebi & Roostaei, 2015). There are many factors that can affect the degradation of active substances in a formulation such as oxidation, thermolysis, hydrolysis and photolysis (Bhangare et al., 2022). The degradation of benzoyl peroxide may be due to thermal degradation in which higher temperatures increase the decomposition (Tan et al., 2020). The main degradation product of benzoyl peroxide is benzoic acid which also possesses antimicrobial activity (CHMP, 2019). Based on CFR - Code of Federal Regulations Title 21, benzoic acid is presented as an active ingredient in over-the-counter topical drug products for acne. Nevertheless, considering the existing evidence, there is insufficient data to confirm widespread acknowledgement of the safety and efficacy of this ingredient for treating acne (FDA, 2023). Although the benzoyl peroxide content for both formulations decreased over time, but benzoyl peroxide concentrations were still within the acceptance range of 90 – 125% (USP41-NF36, 2018). Besides, drug degradation below than 10% of the initial amount is considered acceptable and stable (Agrahari et al., 2015).

3.4.6.2 Organoleptic properties and forced centrifugation test

Table 3.7 showed that all organoleptic properties including colour, homogeneity, transparency, texture and odour for all formulations remained the same for 12 weeks of storage at both temperatures. There was also no phase separation and formation of creaming and foaming after centrifuging for all formulations for every sampling time point during 12 weeks of storage. This indicated that all formulations were physically stable. This may be contributed by the presence of Tween 20 and Span 60 as emulsifiers blend with HLB values similar to the rHLB of the oil components. The use of an emulsifier blend can improve the stability of the emulsion as it will enhance the repulsive forces between the droplets in the emulsion (Park & Kim, 2021).

Besides, it is important to use an emulsifier with a HLB value same or near to the rHLB value of the oil components. This is because HLB highly influences the balance of the interfacial tension between the aqueous and oil phases which affects the stability and homogeneity of the formulation (Alam et al., 2020). A study conducted by Alam et al. (2020) observed that all emulsions that incorporated emulsifiers with a HLB value less than the rHLB value of the oil components were unable to form a stable and homogenised emulsion. In addition, the use of carbopol which has a very high molecular weight (Mackiewicz et al., 2019) as the gelling agent also can improve the stability of the emulgel. This is because high molecular weight polymers can function as thickeners and enhance the viscosity of the continuous phase. This high viscosity of the continuous phase will make the emulsion resist gravity stress (Huang et al., 2018). Moreover, the droplet mobility also be reduced in a viscous continuous phase, hence the occurrence of droplet collision also will be decreased (Tesch & Schubert, 2002).

Table 3.7 Organoleptic properties and phase separation observation of Formulation 1, 2, 3 and 4 stored in 30ºC/ 75%RH and 40ºC/ 75%RH at week 0, week 2, week 4 and week 12.

Properties	Storage	F1				F2				F3				F4			
		W0	W2	W4	W12	W0	W2	W4	W12	W0	W2	W4	W12	W0	W2	W4	W12
Colour	30ºC/ 75%RH	CW	CW	CW	CW	W	W	W	W	CW	CW	CW	CW	W	W	W	W
	40ºC/ 75%RH	CW	CW	CW	CW	W	W	W	W	CW	CW	CW	CW	W	W	W	W
Homogeneity	30ºC/ 75%RH	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
	40ºC/ 75%RH	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
Transparency	30ºC/ 75%RH	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
	40ºC/ 75%RH	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
Texture	30ºC/ 75%RH	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	40ºC/ 75%RH	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Odour	30ºC/ 75%RH	HS	HS	HS	HS	OL	OL	OL	OL	HS	HS	HS	HS	OL	OL	OL	OL
	40ºC/ 75%RH	HS	HS	HS	HS	OL	OL	OL	OL	HS	HS	HS	HS	OL	OL	OL	OL
Phase separation	30ºC/ 75%RH	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	40ºC/ 75%RH	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Abbreviation: W0 is Week 0; W2 is Week 2; W4 is Week 4; W12 is Week 12; CW is Cream-white; W is White; H is Homogenous; O is Opaque; S is Smooth; HS is Honey smell; OL is Odourless; X is Absent.

3.4.6.3 pH measurement

Based on the repeated measure one-way ANOVA analysis (Figure 3.16), the pH of each formulation decreased significantly over 12 weeks ($p < 0.05$). In real-time and accelerated-time chambers, the pH of F1 reduced from 4.95 to 4.68 and 4.26 respectively meanwhile pH of F2 declined from 5.62 to 5.12 and 4.49 respectively. The pH of F3 and F4 were in the range of 4.98 to 4.71 and 5.80 to 5.63 respectively in the real-time chamber. Meanwhile in the accelerated-time chamber, the pH of F3 and F4 were in the range of 4.98 to 4.68 and 5.80 to 5.58 respectively.

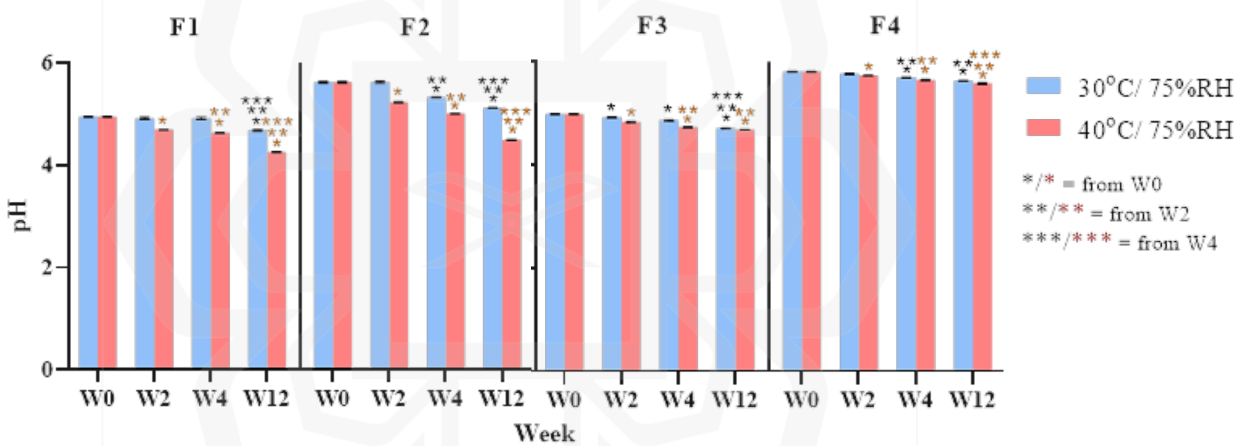


Figure 3.16 pH measurement of Formulation 1, 2, 3 and 4 stored in 30ºC/ 75%RH and 40ºC/ 75%RH at week 0, week 2, week 4 and week 12 (Mean ± SD, n = 3, (*) $p < 0.05$).

These pH reduction results basically were in accordance with a study by Khan et al. (2022) and Smaoui et al. (2017) that detected a decrement in the pH of their emulgel and cream formulations respectively during three months of stability study. pH reduction of the formulation may occur due to the oxidation of oil elements of the emulsion at high temperatures which consequently form aldehydes and organic acids (Khan et al., 2022). Besides, high temperature also may affect the stability of the emulsion through the hydrolysis process (Smaoui et al., 2017). Among these four formulations, F4 has higher pH values overall. This may be due to the absence of benzoyl peroxide and stingless bee honey. As explained before, benzoyl peroxide degrades to benzoic acid which is acidic over time, hence reducing the pH of F1 and F2. Besides, the acidity of honey also increased under long-term storage and higher temperatures (Chou et al., 2020). Therefore, the pH of F1 and F3 decreased over time and significantly in the accelerated-time chamber. Overall, the pH of F1 was the lowest and this may be due to the presence of both benzoyl peroxide and stingless bee honey. Although the pH of all formulations decreased, however their pH values were still acidic and in the normal range of 4 to 6 (Luki et al., 2021). Thus, they were safe to be applied and would not irritate the skin.

3.4.6.4 Droplet size analysis

Referring to Figure 3.17, all droplet sizes for all formulations increased throughout the three-month storage period in both real-time and accelerated-time chambers. The droplet size measurement for all formulations varied from 0.127 μm to 0.296 μm over time. The increment of droplet size was significant ($p < 0.05$) for all formulations at both storage conditions (30ºC/ 75%RH and 40ºC/ 75%RH). The increment was prominent in the accelerated-time chamber compared with the real-time chamber and started to become obvious in week 4 of the storage. The increase in droplet size may be due to flocculation and coalescence. Flocculation happens when two or more droplets gather and make contact to form clusters without coalescing. After some time, the smaller size of droplets tends to shift and bind to the larger size of droplets (Pawignya et al., 2019). This happens because the attractive forces between the droplets such as hydrophobic, van der Waals and depletion forces are higher than the repulsion forces (steric and electrostatic forces) (Liu et al., 2019).

Meanwhile, coalescence is the aftermath of flocculation where the droplets attach and merge together to form a bigger droplet (Pawignya et al., 2019) due to the rupture of the layer and the domination of the attractive inter-droplet forces between the droplets

(Cerff et al., 2021). In high temperatures, the droplets will move perpetually and tend to collide with each other more frequently. This enhances the tendency of flocculation and coalescence occurrence between the droplets and consequently produces large droplets in order to reduce the frequency of collisions between the droplets. However, large droplet size will lead to instability of the emulsion (Sobhaninia et al., 2017). Even though the droplet size of all the formulations was increased during the storage time, they all were still in the ultrafine droplet size range (0.1–1 μm) category. Besides, there was no sign of physical instability observed throughout the storage period.

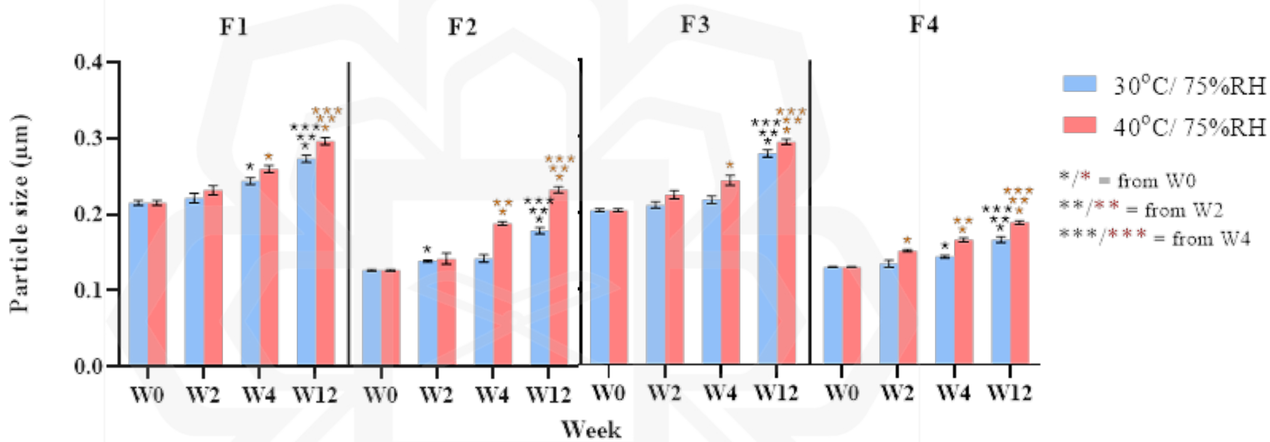


Figure 3.17 Droplet size of Formulation 1, 2, 3 and 4 stored in 30ºC/ 75%RH and 40ºC/ 75%RH at week 0, week 2, week 4 and week 12 (Mean ± SD, n = 3, (*) $p < 0.05$).

3.4.6.5 Polydispersity Index (PDI)

Based on Figure 3.18, all polydispersity index (PDI) values for all formulations increased throughout the three-month storage period in both 30ºC/ 75%RH and 40ºC/ 75%RH storage conditions. The PDI values for all formulations varied from 0.236 to 0.412. Based on repeated measures of one-way ANOVA, the rise of PDI was significant ($p < 0.05$) for all formulations except F4 at both storage conditions. The PDI values of F4 in both real-time ($p = 0.2337$) and accelerated-time ($p = 0.2947$) chambers did not change significantly.

Similar to droplet size, the increment was obvious in the accelerated-time chamber compared with the real-time chamber and started to become noticeable in week 4 of the storage. This increment in PDI may be due to an increase in droplet size that may contribute to various droplet size distributions. Besides, larger droplet size also tends to have higher PDI values (Iyer et al., 2015). This result was similar to a study that found that the PDI value of the emulsion increases with an increase in droplet size (Schachner-Nedherer et al., 2019). Nonetheless, all formulations still maintained their PDI values below than 0.5 which means they still possess good size distribution and stable (Ding et al., 2018; Lino et al., 2020).

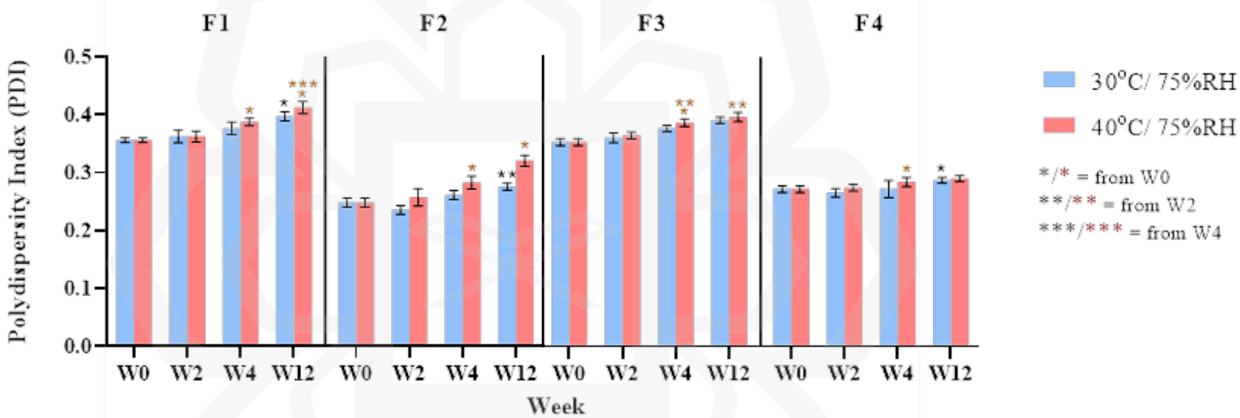


Figure 3.18 Polydispersity Index (PDI) of Formulation 1, 2, 3 and 4 stored in 30ºC/ 75%RH and 40ºC/ 75%RH at week 0, week 2, week 4 and week 12 (Mean ± SD, n = 3, (*) $p < 0.05$).

3.4.6.6 Zeta potential measurement

Zeta potential for all formulations for every sampling time point ranges between -33.57 mV to -48.7 mV. According to Figure 3.19, over time, the zeta potential for all formulations in both 30ºC/ 75%RH and 40ºC/ 75%RH storage conditions were fluctuated. Most of the zeta potential changes were not significant ($p > 0.005$) for most of the formulations in both real-time and accelerated-time chambers except for F1 in both chambers (p = 0.0217, 0.0232) and F3 in the accelerated-time chamber (p = 0.0492). However, based on Tukey's multiple comparisons test, there was no significant difference for each of the weeks for F1 in the real-time chamber and F3 in the accelerated-time chamber. Only the zeta potential of F1 in week 12 was significantly lower from the baseline (p = 0.0265) and week 2 (p = 0.0255) in 40ºC/ 75%RH storage condition. Besides, it had been observed that the zeta potential values for all formulations were decreased in week 12 compared to the baseline. This zeta potential value reduction may be due to the decrease in the pH of the formulations. This is because pH does affect the zeta potential. It has been observed that zeta potential becomes less negative in a lower pH due to high H+ ions concentration (Mahani et al., 2017; Smith et al., 2017). Although the zeta potential values were reduced in week 12, they were still more than -30 mV. Hence, this signified that all formulations maintained good stabilisation of dispersion throughout the three-month storage period. This may be also attributed to the presence of the non-ionic surfactant blends of Tween 20 and Span 60 was sufficient to stabilise the system for a long time by creating a coating over the droplets' surface and inducing electrostatic repulsion to avoid droplet aggregation (Joseph & Singhvi, 2019; Selvamani, 2018).

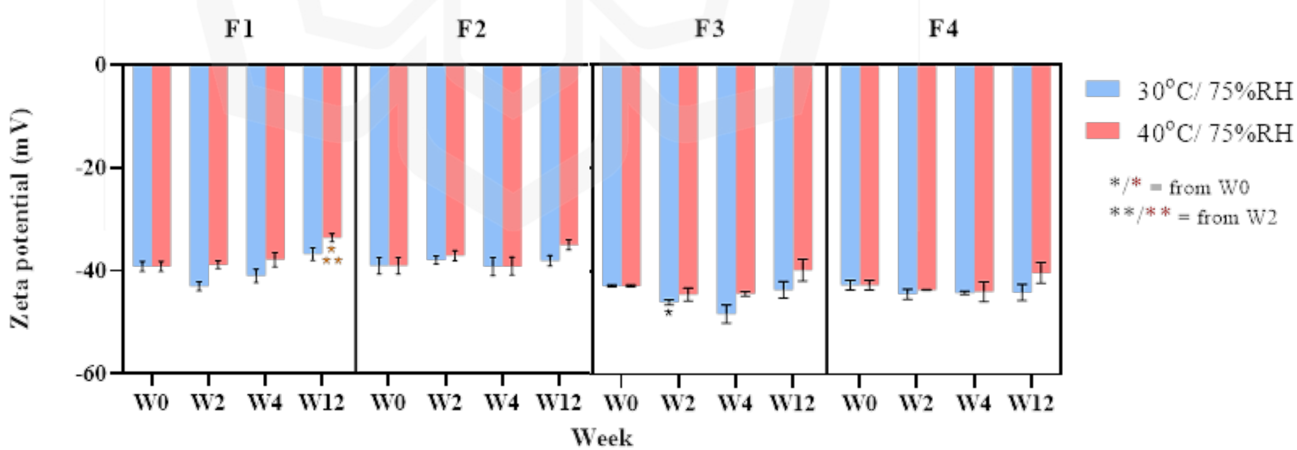


Figure 3.19 Zeta potential measurement of Formulation 1, 2, 3 and 4 stored in 30ºC/ 75%RH and 40ºC/ 75%RH at week 0, week 2, week 4 and week 12 (Mean ± SD, n = 3, (*) $p < 0.05$).

3.4.6.7 Spreadability

Based on Figure 3.20, the spreadability of all formulations increased throughout the three-month storage period in both 30ºC/ 75%RH and 40ºC/ 75%RH storage conditions. In real-time and accelerated-time chambers, the spreadability of F1 increased from 5.83 g cm/s to 6.38 g cm/s and 6.68 g cm/s respectively meanwhile spreadability of F2 raised from 5.78 g cm/s to 6.28 g cm/s and 6.56 g cm/s respectively. The spreadability of F3 and F4 were in the range of 5.89 g cm/s to 6.28 g cm/s and 5.94 g cm/s to 6.06 g cm/s respectively in the real-time chamber. Meanwhile in the accelerated-time chamber, the spreadability of F3 and F4 were in the range of 5.89 g cm/s to 6.44 g cm/s and 5.94 g cm/s to 6.28 g cm/s respectively. However based on the repeated measure one-way ANOVA analysis, the overall significant increment was observed only for F1 in the accelerated-time chamber ($p = 0.0377$), F2 in both chambers ($p = 0.0362$ - 30ºC/ 75%RH) ($p = 0.0052$ - 40ºC/ 75%RH) and F3 in the accelerated-time chamber ($p = 0.0232$). F1 in the real-time chamber showed no overall significant difference throughout the 12 weeks of study but there were significant increment in week 12 from the baseline ($p = 0.0229$) and week 4 ($p < 0.0001$).

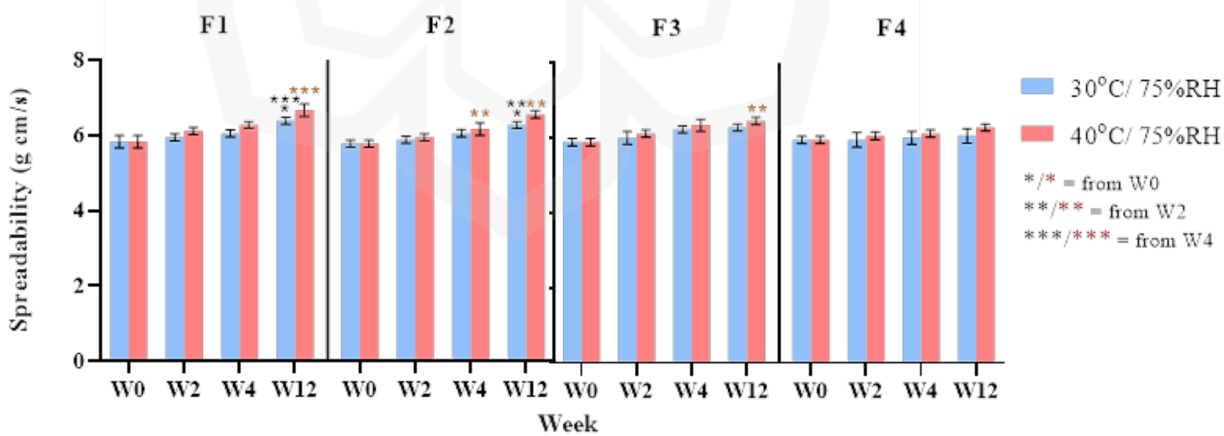


Figure 3.20 Spreadability measurement of Formulation 1, 2, 3 and 4 stored in 30ºC/ 75%RH and 40ºC/ 75%RH at week 0, week 2, week 4 and week 12 (Mean ± SD, n = 3, (*) $p < 0.05$).

The increment in spreadability of all emulgel over time basically was similar to the result discovered by Safitri et al. (2021) in which the spreadability of carbopol 940 gel increased with decreasing viscosity after four weeks of stability study. Besides, the firmness of the fat or oil phase is reduced at high temperatures (Ziarno et al., 2023) due to oxidation (Khan et al., 2022). This will consequently increase the spreadability of the emulgel formulation. In addition, the spreadability increase was prominent in week 12 especially for F1 and F2. This may be due to the presence of an active ingredient which is benzoyl peroxide in those formulations. This is because over time and under high temperatures, the benzoyl peroxide will degrade into benzoic acid. The formation of benzoic acid will make the pH of the formulation decrease. Lower pH will make the viscosity of the emulgel gel decrease and subsequently increase the spreadability due to the pH-dependent feature of carbopol (CHMP, 2019). Although the spreadability of all emulgel increased in the stability chambers, but the physical characteristics of all formulations remained the same. Hence, all formulations can be concluded to be stable.

3.4.6.8 Microbial limit test

Based on Table 3.8, the results showed no growth of microorganisms for all formulations stored in 30ºC/ 75%RH and 40ºC/ 75%RH at week 0, week 2, week 4 and week 12. This may be due to the adequate amount of preservative that has been incorporated in the formulations (Halla et al., 2018). As mentioned before, phenoxyethanol possesses a broad spectrum of antimicrobial activity (Dréno et al., 2019). The addition of ethylhexylglycerin will further enhance the preservative activity of phenoxyethanol as it will disrupt the cell membrane of the microbe (Langsrud et al., 2016). No growth of microorganisms proved that all formulations were stable and safe to be applied.

Table 3.8 Microbial test observation of Formulation 1, 2, 3 and 4 stored in 30ºC/ 75%RH and 40ºC/ 75%RH at week 0, week 2, week 4 and week 12 (n=3).

Properties	Storage	F1				F2				F3				F4			
		W0	W2	W4	W12	W0	W2	W4	W12	W0	W2	W4	W12	W0	W2	W4	W12
TAMC	30ºC/ 75%RH	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	40ºC/ 75%RH	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
TYMC	30ºC/ 75%RH	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	40ºC/ 75%RH	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Staphylococcus aureus	30ºC/ 75%RH	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	40ºC/ 75%RH	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Pseudomonas aeruginosa	30ºC/ 75%RH	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	40ºC/ 75%RH	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Abbreviation: W0 is Week 0; W2 is Week 2; W12 is Week 12; TAMC is Total Aerobic Microbial count; TYMC is Total Yeast and Mould count; X is Absent.

3.5 CONCLUSIONS

An isocratic reversed phased HPLC method for benzoyl peroxide was validated for the determination of benzoyl peroxide concentration in the sample. The parameters that had been well validated were linearity, specificity, accuracy, precision, LOD and LOQ. There were four formulations emulgel were developed that differed in terms of active ingredients with the same excipients. F1 was the main formulation that contained both benzoyl peroxide and stingless bee honey. F2 consisted of benzoyl peroxide only and F3 consisted of solely stingless bee honey as the active ingredient. Meanwhile, F4 did not include any active ingredient and was regarded as base emulgel. The benzoyl peroxide content in both F1 and F2 were within the USP acceptable range of 90% to 125%. All the emulgels formulated possess good organoleptic properties with no phase separation after being centrifuged. The pH of all emulgel formulations was safe to be applied to the skin since they are acidic and categorised under the normal range for the pH of topical formulation. Also, all formulations had ultrafine droplet size which was below than 1 μm and acceptable PDI and zeta potential values were in the normal range which were less than 0.5 and more than -30 mV respectively. The spreadability for all formulations was in the range of 5.78 to 5.94 g cm/s. For the microbial limit study, no growth was observed in Total Aerobic Microbial Count, Total Yeast and Mold Count, *Staphylococcus aureus* and *Pseudomonas aeruginosa* tests. This showed that the formulations were microbiological stable.

For the stability study, the organoleptic properties including colour, homogeneity, transparency and odour for all formulations remained the same throughout three-month storage in both 30ºC/ 75%RH and 40ºC/ 75%RH conditions. There was also no phase separation after centrifuging for all formulations for every sampling time point during 12 weeks of storage. The benzoyl peroxide content in both F1 and F2 reduced significantly in both chambers but the concentration was still in acceptable ranges and the drug degradation was below than 10%. Besides, in both chambers, the pH, droplet size, PDI, zeta potential and spreadability of all formulations were changed throughout the three-month stability study and most of the changes were statistically significant. The prominent changes basically were observed in an accelerated-time chamber compared to the real-time chamber. However, the values were still in the acceptable range and no physical instability was observed during the stability study. All formulation also maintained their microbiological stability as no growth was observed for every sampling time point. Hence, it can be concluded that all formulations were stable in terms of physical, chemical and microbiological throughout three months of storage in both 30ºC/ 75%RH and 40ºC/ 75%RH conditions. Due to more prominent changes in the accelerated-time chamber, hence the suitable storage condition of all emulgel formulations was at room temperature (22 ºC to 31 ºC) instead of high temperature. Also, the emulgel formulations need to be avoided from sunlight or any direct heat sources.

From this chapter, it can be concluded that the benzoyl peroxide with stingless bee honey emulgel formulation has been successfully developed. The effectiveness of this formulation in exhibiting its antiacne property was evaluated and discussed in the next chapter. Besides, the selection of stingless bee honey concentration to be incorporated in the formulation also was explained in the next chapter.

CHAPTER FOUR

IN VITRO EVALUATION OF ANTIACNE PROPERTY OF STINGLESS BEE HONEY AND FORMULATED EMULGEL

4.1 INTRODUCTION

Gram staining is an important staining technique utilised in the field of microbiology for the purpose of identifying and classifying bacteria based on their kind and morphology. The nomenclature of this technique is derived from the Danish bacteriologist Hans Christian Gram, who initially presented it in 1882 with the primary objective of identifying microorganisms responsible for pneumonia. The initial stage of the gram staining procedure often entails the utilisation of crystal violet or methylene blue as the main dye. Gram-positive organisms are a classification of organisms that have the property of retaining their primary colour and displaying a purple hue when observed under a microscope. Meanwhile, gram-negative organisms are a type of microorganism that does not retain primary stains and exhibit a pink hue when observed under a microscope (Tripathi & Sapra, 2022). According to Mohanna (2016), there are variations in bacterial cell shapes (Figure 4.1) such as:

1. Cocci - spherical or oval shaped cells
2. Bacilli - rod shaped cells
3. Vibrios - comma shaped curved rods cells
4. Spirilla - rigid spiral shaped cells
5. Spirochetes - flexuous spiral shaped cells
6. Actinomycetes - branching filamentous shaped cells
7. Mycoplasmas - cell with no cell wall, thus, its morphology is not stable. Normally, appear as round or oval shapes and as interlacing filaments.

However, spherical, rod and spiral are the most common bacterial shapes. Other than the shape, bacteria also can be classified based on the cell arrangement. The cells may be arranged in pairs (diplo), groups of four (tetrad), groups of eight (sarcina), chains (strepto), and grape-like clusters (staphylo) (Mai & Ishibashi, 2021; Mohanna, 2016).

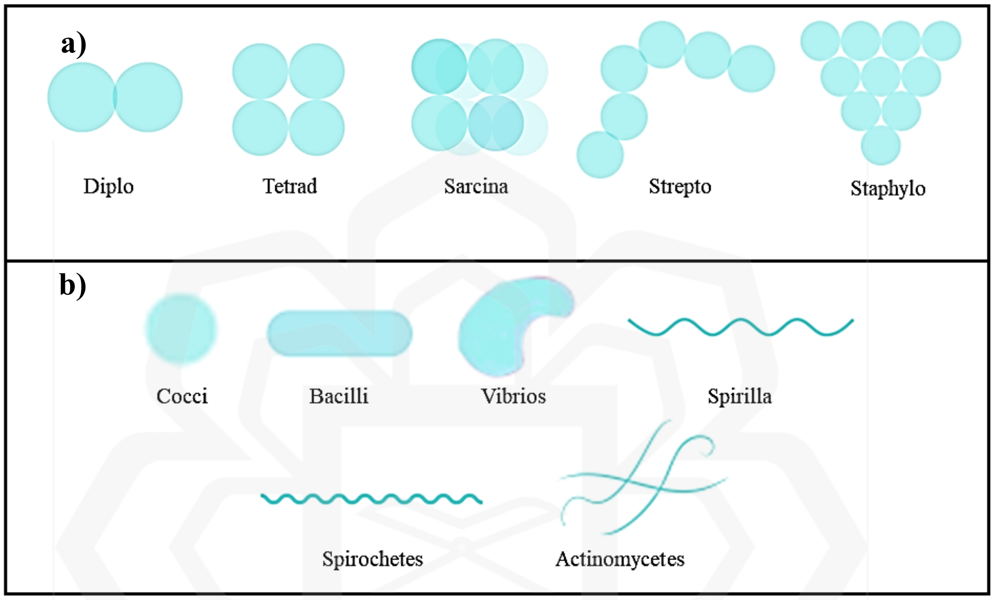


Figure 4.1 **a)** Arrangement and **b)** shape of bacterial cell.

The are many methods that can be conducted to evaluate the antibacterial activity of antimicrobial agents or natural ingredients. One of them is the broth dilution method. The broth dilution method is one of the basic and reliable antimicrobial susceptibility testing techniques for natural ingredients including honey. It can be performed for both anaerobic and aerobic bacteria types (Hossain et al., 2022). Depending on the volume of the sample used in the study, it can be called specifically either broth macrodilution or broth microdilution method. As the name itself, this method can be done by diluting the sample in broth through serial dilution. Normally, it is done in two-fold serial dilution (Akinduti et al., 2019). One of the advantages of this method is it able to discover the bacteriostatic and bactericidal activity of the sample. Bacteriostatic activity is expressed by the minimum inhibitory concentration (MIC) of the sample meanwhile bactericidal activity is indicated by the minimum bactericidal concentration (MBC) of the sample. MIC and MBC basically are portrayed by the lowest concentration of the sample that inhibited and killed bacterial growth respectively evaluated by clear with no turbidity well. Besides, various samples' concentrations can be tested and the results obtained are reproducible (Akinduti et al., 2019; Hossain et al., 2022; Schumacher et al., 2018). However, too pale turbidity can be misinterpreted as MIC and MBC which leads to an inaccurate result. Therefore, the addition of spectrophotometry used in this method is beneficial as the inhibition rate can be calculated. Hence, inaccurate results can be avoided (Akinduti et al., 2019).

Another common antimicrobial susceptibility testing technique is the diffusion method. It is also called as Kirby–Bauer method (Schumacher et al., 2018). There are two types of diffusion methods which are agar well and disk diffusion method. The principle for both methods is based on the capability of the sample to diffuse and move from a high gradient to a lower gradient in order to show the antimicrobial effect. In the agar well diffusion method, the bacteria-seeded agar will be bored in order to form a well for placing the sample in it (Akinduti et al., 2019). Meanwhile, a paper disc impregnated with the sample will be put on the bacteria-seeded agar in the disc diffusion method (Akinduti et al., 2019; Schumacher et al., 2018). This diffusion method can provide qualitative results since it can screen the samples with and without antibacterial activity based on the inhibition zone size. The advantage of this method is it is easy to conduct as it is simple and does not involve any special tool (Osés et al., 2016; Schumacher et al., 2018). Hence, it can be considered as rapid and inexpensive antimicrobial assay.

Therefore, this chapter aimed to examine the MIC and MBC of the stingless bee honey through the broth dilution method in order to determine the stingless bee honey concentration that would be incorporated in the formulation. Besides, this chapter also intended to evaluate the *in vitro* antiacne activity of the formulated emulgel via the agar well diffusion method. Basically, the MIC and MBC parts were done before the formulation development.

4.2 MATERIAL

Brain Heart Infusions (BHI) agar, Brain Heart Infusions (BHI) broth and sodium chloride were purchased from Merck (Darmstadt, Germany), Gram stain set that consisted of Crytal Violet (0.5% Aqueous Solution), Gram's Iodine (Staining Solution), ethyl alcohol 95% v/v min (Decoloriser) and SafranineO (1.0% Aqueous Solution) were procured from R&M Chemicals (Essex, UK), *Cutibacterium acnes* subsp. acnes (DSM 1897) acquired from Apical Scientific, distilled water and stingless bee honey was obtained from Kuantan, Pahang (Desa Kelulut Bukit Kuin).

4.3 METHODS

4.3.1 Reviving and cultivation of freeze-dried *P. acnes* bacteria

The freeze-dried *P. acnes* bacteria was rehydrated with 0.5 mL of Brain Heart Infusions (BHI) broth for 30 minutes. The *P. acnes* bacteria suspension was mixed properly by repetitive pipetting (repeatedly dispensing and withdrawing the suspension). Approximately half of the whole amount of the *P. acnes* bacteria suspension was transferred to 5 mL of BHI broth meanwhile the other half of it was streaked onto Brain Heart Infusions (BHI) agar. The liquid and agar cultures were incubated at 37 °C in an anaerobic environment for 3-5 days and 5-7 days respectively. These procedures basically were done in an anaerobic chamber.

4.3.2 Bacterial Identification Test

A bacterial identification test was done through colony morphology observation and gram staining (Figure 4.2). Gram staining procedures involved bacterial fixation for the first step. Bacterial fixation was prepared by putting a drop of distilled water on a glass slide and a colony of *P. acnes* bacteria taken from the BHI agar was placed onto the glass slide. Then, the *P. acnes* bacteria colony was smeared on the glass slide and fixed by heat. Meanwhile, for bacteria from the broth, the bacterial fixation was prepared by putting a drop of *P. acnes* bacterial broth on the glass slide. Then, the *P. acnes* bacterial broth was smeared and fixed on the glass slide by heat. The heat-fixed bacterial smear slide was taken and flooded with crystal violet and allowed to stand for 60 seconds and then washed with distilled water. The slide then was again flooded with Gram's Iodine solution and allowed to stand for 60 seconds, then washed with distilled water. Ethyl alcohol was then dropped onto the slide and immediately washed off with distilled water to discolourise the bacterial smear slide. A drop of SafraninO was put in and allowed to stay for 60 seconds, then washed off with distilled water. The bacterial smear slide was blotted and air-dried before being observed under a 100x objective lens.

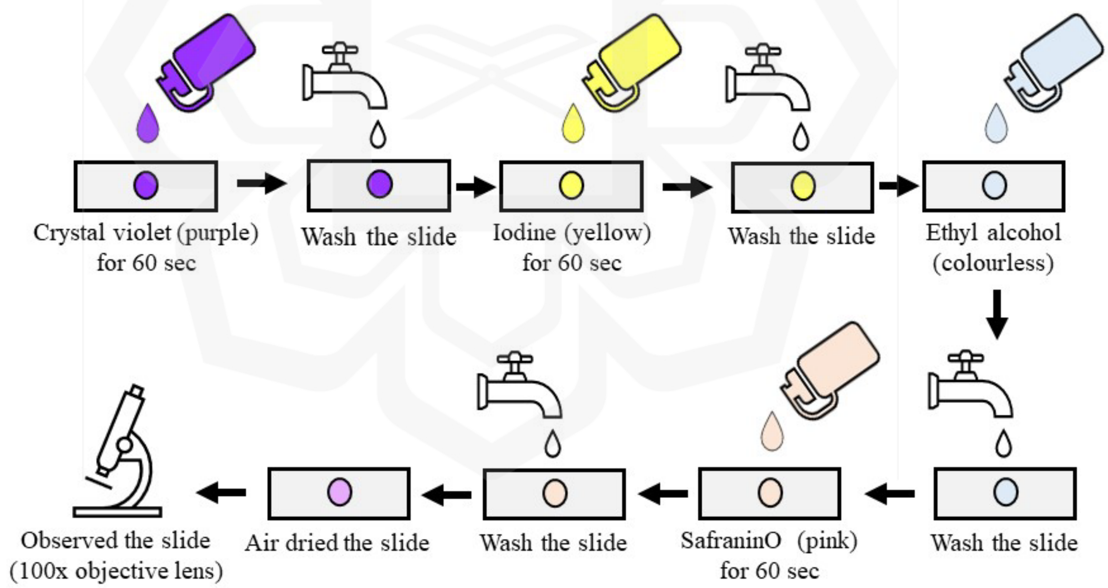


Figure 4.2 Illustration of Gram staining procedures.

4.3.3 Preparation of *P.acnes* bacterial suspension

0.85% normal saline solution was prepared by dissolving 0.85 g sodium chloride in 100 mL distilled water. Then, this normal saline solution was autoclaved to make it sterile. *P. acnes* bacteria were streaked on the surface of the BHI agar and then incubated in an anaerobic environment for 3-5 days at 37 °C. *P. acnes* colonies were taken using an inoculation loop and suspended in sterile normal saline. The bacterial turbidity was measured using a densitometer to get a 0.5 McFarland unit. This 0.5 McFarland unit was equivalent to a bacterial cell suspension with a concentration of 1.5 x 10^8 CFU/mL. (Kusuma et al., 2018).

4.3.4 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of stingless be honey against *P.acnes*

The MIC was conducted by using the broth microdilution method with microplates 96 wells. Each column was filled with 100 µL BHI broth except for the negative control column. The negative control column was filled with 200 µL BHI broth. Columns 1 until 6 were used as the test column, column 7 as a positive control, column 8 as a negative control and column 11 as a sterility control. Then, 100 µL of stingless bee honey was suspended in column 1 and column 11 and they were homogenised by pipetting. From column 1, 100 µL was taken and put into column 2 to obtain a honey concentration half than the honey concentration in column 1. Thus, until column 6 and last, 100 µL of test media suspension was removed from column 6, resulting in all columns containing only 100 µL of test media suspension. From this step, a concentration series of 1.56%, 3.125%, 6.25%, 12.5%, 25% and 50% stingless bee honey was obtained. Then, 100 µL suspensions of the *P. acnes* bacteria were inoculated into all columns except columns 8 and 11. The microplate was closed and incubated in an anaerobic environment at 37 °C for 18-24 hours. The lowest concentration with a clear culture observed was detected as the MIC (Kusuma et al., 2018).

For determination of the MBC, all samples in the clear wells, a sample with a concentration less than MIC, positive control, negative control and sterility control were transferred as much as 10 μL into the new 96 well plate. Then, 100 μL BHI broth and 100 μL 12.5% stingless bee honey were added to columns 1 to 6 and column 7 respectively. Then, all the samples were reincubated in an anaerobic environment at 37°C for 24 hours. The lowest sample concentration that was still clear after the second incubation was chosen as the MBC. Sample with a concentration lower than the MBC and the positive control were streaked onto BHI agar. The agar plates were then incubated in an anaerobic environment at 37 ºC for 48 hours. This step was performed basically to affirm the turbidity of the wells was due to the growth of *P. acnes* bacteria instead of contamination (Djakaria et al., 2020; Kusuma et al., 2018). All of these MIC, MBC and contamination examination procedures were illustrated in Figure 4.3. The MIC or MBC test will be repeated with different concentrations of stingless bee honey if the MIC or MBC value is more than 22%. In this study, the MBC test was repeated with 13% to 20% stingless bee honey concentration. The inhibition rate of the MIC and MBC were calculated by using Equation 4.1 (Abed Savaya et al., 2020).

$$Inhibition\ rate\ (\%) = 100 - \left[\left(\frac{\text{Absorbance of test well} - \text{Absorbance of blank (negative control)}}{\text{Absorbance of bacterial growth (positive control)}}\right) \text{x } 100\right]$$ Equation 4.1

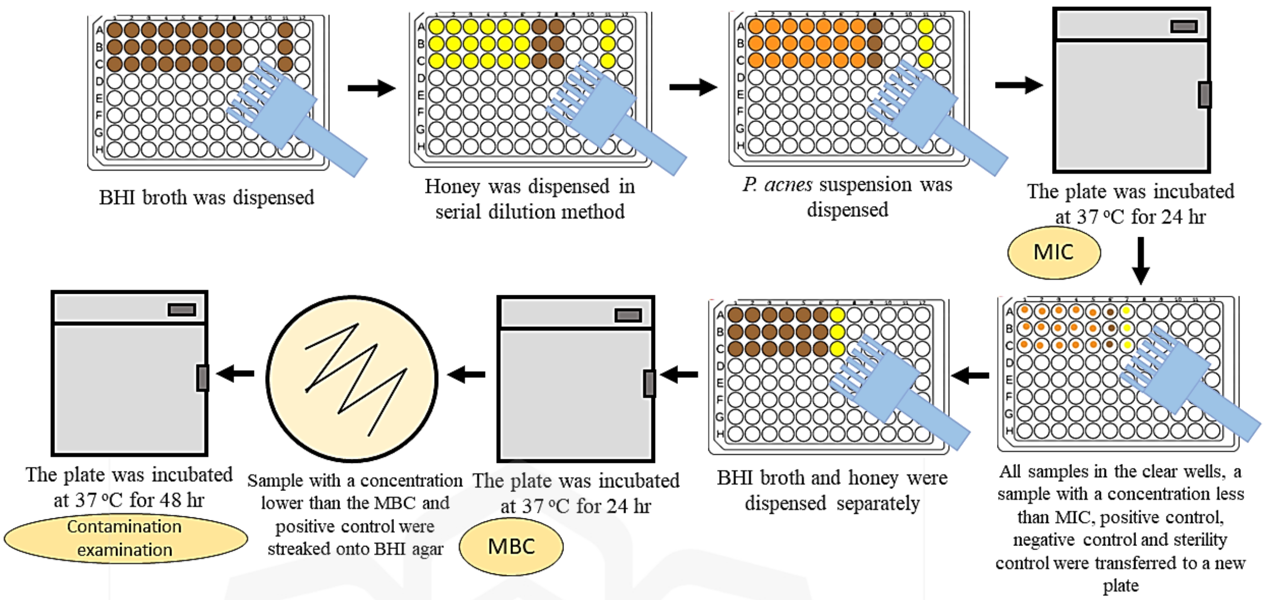


Figure 4.3 Illustration of MIC, MBC and contamination examination procedures.

4.3.5 Antibacterial study of formulated emulgel

The antibacterial activity was conducted for all four formulations that had been developed. F1 and F3 were the test formulations meanwhile F2 and F4 were positive and negative control respectively. A modified agar well diffusion method was applied in this antibacterial study. In this method, BHI agar was streaked with *P. acne*s broth culture that had been incubated for 24 hours. The agar was allowed to dry for 5 to 10 minutes. Four holes with a diameter of 8 mm were punched aseptically with a sterile tip. 0.2 g of each formulation was introduced into the hole. The agar was incubated at 37 ºC. The antibacterial activities were evaluated by measuring the diameter of zones of inhibition (in mm) This test was done in triplicate (Soni & Bharadwaj, 2020; Jantarat et al., 2018). The antibacterial activity was also assessed at 2 weeks, 4 weeks and 12 weeks of stability studies in order to determine whether the formulations were capable of retaining their antibacterial potential over some time during storage (Nawarathne et al., 2019).

4.4 RESULT AND DISCUSSION

4.4.1 Characterisation of *P. acnes* bacteria and gram staining

Colony morphology refers to the observable cultural traits exhibited by a bacterial colony when grown on an agar plate. The ability to visually analyse colony morphology is a crucial competency employed in the field of microbiology for microbe identification. In order to accurately monitor and analyse the characteristics of colonies, it is important to ensure their effective isolation from neighbouring colonies. This isolation facilitates the examination of key attributes including but not limited to size, shape, colour and opacity (CDC, 2021). Based on Figure 4.4, the colony morphology of *P. acnes* bacteria cultured was small, circular in shape, opaque and creamy in colour. This result was in line with the observation by Polugari et al. (2016). It was observed that the size of *P. acnes* colonies can vary from pinhole-sized to 0.5 mm in diameter size (Zhou & Li, 2015).

Figure 4.4 Colony morphology of *P. acnes* bacteria on BHI agar.

Meanwhile, gram staining results showed that *P. acnes* bacteria possess the characteristic of rod shape and purple in colour (Figure 4.5). This signifies that *P.acnes* bacteria belong to the Gram-positive Bacilli group. This result was in accordance with the study performed by Chandra (2023) that observed *P .acnes* bacteria as stick shape with purple colour. Generally, gram-positive bacteria appeared as purple due to their cell walls which consist of mainly peptidoglycan. This peptidoglycan of gram-positive bacteria will retain the purple colour of the crystal violet solution (Chandra, 2023). This crystal violet solution basically will dissociate to produce CV^+ and chloride (Cl^-) ions. Both ions will then enter the cell wall and cell membrane of the bacteria and the CV^+ ion will bind with negatively charged elements of the bacterial cells and dye the cells purple. After crystal violet, iodine was added as a substance that strengthened the affinity of the cell for crystal violet so that the crystal violet would be hard to be eliminated from the cell (Sari et al., 2018). This is because iodide (I^- or I^{-3}) interacts with CV^+ and forms large complexes of crystal violet and iodine (CV–I) within the inner and outer of the cell layers that will avoid the elimination of the CV–I complex (Sandle, 2016).

When ethyl alcohol which is the decolouriser was applied, gram-positive bacteria will not really be affected due to low lipid content. In contrast, the outer membrane of gram-negative bacteria which is composed of mainly lipopolysaccharide will be broken by the alcohol and the thin peptidoglycan layer inside will no longer be protected. Hence, the CV–I complexes are removed from the cell together with the outer membrane. In addition, the alcohol also will make the gram-positive bacterial cell wall dehydrated and reduce pore size. This will hinder the CV–I complex from leaving the gram-positive bacterial cell wall and the cell will remain purple (Sandle, 2016; Sari et al., 2018). However, the duration of the decolourisation step in gram staining is an important parameter as prolonged contact with a decolourising chemical can diminish the purple pigments from both types of bacteria (Tripathi & Sapra, 2022). The last step of the gram staining basically is a counterstain This basically gives the decolourised gram-negative bacteria a pink to light red colour (Sandle, 2016; Tripathi & Sapra, 2022).

a) b)

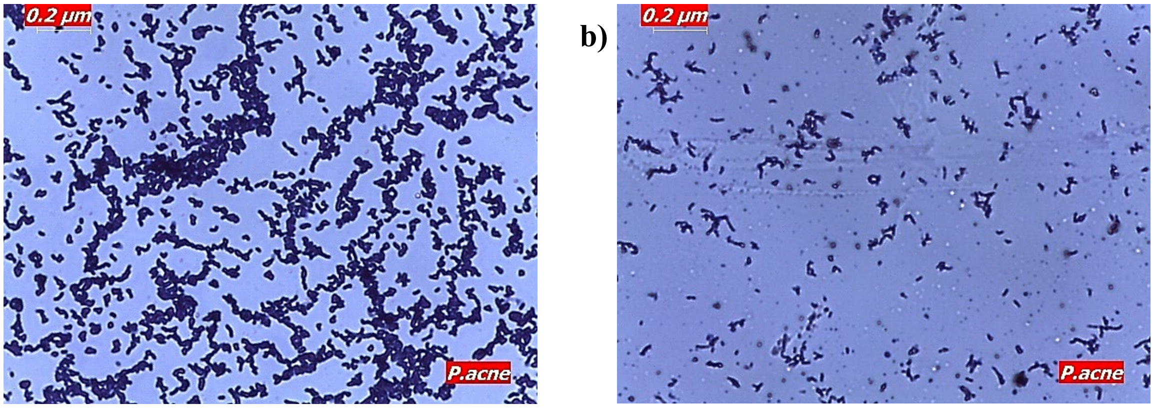


Figure 4.5 Gram staining of *P. acnes* bacteria from a) BHI agar b) BHI broth under light microscope magnified 100x.

4.4.2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of stingless be honey against *P. acnes* bacteria

MIC is the lowest concentration of an antimicrobial agent that is bacteriostatic which means it prevents the visible growth of bacteria. It can also be determined by a 90% inhibition rate. Meanwhile, MBC is the lowest concentration of antibacterial agent required to kill 99.9% of the initial bacterial population. MIC and MBC assay can be used to evaluate or screen the in vitro antimicrobial activity of a pure compound (Hassounah et al., 2016). It also can determine the potential concentration of the compound that will be incorporated in the formulation for it to exert the effect. Many studies have shown that stingless bee honey has a broad-spectrum antibacterial effect that covers both gram-positive and gram-negative bacteria. However, gram-positive bacteria are more susceptible to stingless bee honey compared to gram-negative ones. The hindrance of access and prevention of bioactive chemical disruption to the cell wall in gram-negative bacteria can be attributed to the presence of an outer membrane. The increased resistance of gram-negative bacteria to honey's inhibitory effects is likely attributed to the presence of the lipopolysaccharide outer membrane which comprises a core polysaccharide known as lipid A and an O-side chain. In contrast, the cell wall of gram-positive bacteria which consists predominantly of peptidoglycan (90 to 95%) facilitates the more accessible penetration of hydrophobic compounds present in stingless bee honey into the bacterial cells (Syed Yaacob et al., 2020).

However, the bioactivity of some constituents in the honey may be organism-specific. Therefore, it is needed to test the antibacterial activity of that specific bacteria (Syed Yaacob et al., 2020). Although gram-positive bacteria are more susceptible to stingless bee honey compared to gram-negative, many studies revealed that *Salmonella spp.* is the most sensitive towards stingless bee honey compared to *Staphylococcus aureus, Listeria monocytogenes, Pseudomonas aeruginosa* and *Escherichia coli.* These results are notable because *Salmonella spp*. is a gram-negative bacterium and gram-negative bacteria usually possess resistance to antimicrobial agents compared to gram-positive bacteria (Mahmood et al., 2021; Sousa et al., 2016).

A study was carried out to examine the disruption of cell architecture in four distinct bacterial species (gram-positive: *Bacillus subtilis* and *Staphylococcus aureus* and gram-negative: *Escherichia coli* and *Pseudomonas aeruginosa*) using scanning electron microscopy (SEM) after direct exposure to raw stingless bee honey (*Heterotrigona itama spp.*). The findings of this investigation indicate that the morphological structures of bacterial cells for all four bacterial species were significantly modified after a 4-hour incubation in raw stingless bee honey at the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The micrographs displayed notable elongation in the cells of *B. subtilis*, deflagellation in *P. aeruginosa*, membrane disturbance and leakage of intracellular contents in *E. coli*, as well as the presence of non-dividing septated cells in *S. aureus*. This indicated that the mode of action of stingless bee honey in inhibiting bacterial growth involves the disruption of the cell membrane, interaction with a cytosolic target, interference with septum development and impede DNA and protein syntheses (Syed Yaacob et al., 2020). Therefore, this work proves that stingless bee honey exhibits antibacterial activities against both gram-positive and gram-negative microorganisms.

Table 4.1 MIC and MBC results of stingless bee honey against *P. acnes*

Assay	MIC	MBC	
Result (%)	12.5	25	15
Inhibition rate (%)	91.54 ± 0.4	100.04 ± 0.4	100.00 ± 0.3

Based on Table 4.1 and Appendix I, the result proved that stingless bee honey could inhibit the growth of *P. acnes* bacteria at a minimum concentration of 12.5% with an inhibition rate of 91.54 ± 0.4 %. It was also able to kill *P. acnes* bacteria at a minimum concentration of 25% with an inhibition rate of 100.04 ± 0.4%. These results were similar to the study by Djakaria et al. (2020) which found that MIC and MBC of stingless bee honey were 12.5% and 25% respectively. However, the maximum concentration of honey that can be incorporated into cosmetics was only 22% (Cosmetic Ingredient Review, 2019). Hence, the MBC assay was repeated by using stingless bee honey concentration between 13% to 20% in order to check whether the MBC was lower than 25% or not. The result displayed that MBC values of stingless bee honey against *P. acnes* were lower than 25% since clear wells were obtained for a 15% concentration of stingless bee honey. The inhibition rate for 15% stingless bee honey concentration was 100.00 ± 0.3%. Therefore, a 15% stingless bee honey concentration was selected and incorporated into the emulgel formulation. These MIC and MBC results confirmed that the antimicrobial property of stingless bee honey does include *P. acnes* as well and it can be a potential antiacne agent.

Besides, the bacteria's colony morphology of the sample with a concentration lower than the MBC cultured on the BHI agar was the same as the positive control bacteria (Figure 4.6). These observations proved that there was no contamination and the results obtained were true. This may be due to proper sterilisation techniques practised during the experiment. All the autoclavable equipment was autoclaved first before being used in this experiment. An autoclave is a steam sterilisation procedure that has the capability to deactivate all organisms through a 6-log reduction (Holmes, 2019). This is due to its ability to utilise thermal energy from high-pressure saturated steam to break the DNA and enzymes of bacteria. As a result, the bacteria present on device surfaces are effectively eradicated

(Tranquillo et al., 2022). Nevertheless, it has been found that its effectiveness in eliminating prions and endotoxins is limited (Holmes, 2019). The utilisation of high temperature (121 $^{\circ}$C) and humidity in this procedure makes it unsuitable for equipment with melting points, softening points or glass transition temperatures that are below or close to the sterilising temperatures (Tranquillo et al., 2022). Steam sterilisation is often regarded as the ideal approach due to its efficacy, reliability, lack of toxicity and corrosiveness, ease of usage and the ability to be monitored and validated (Holmes, 2019).

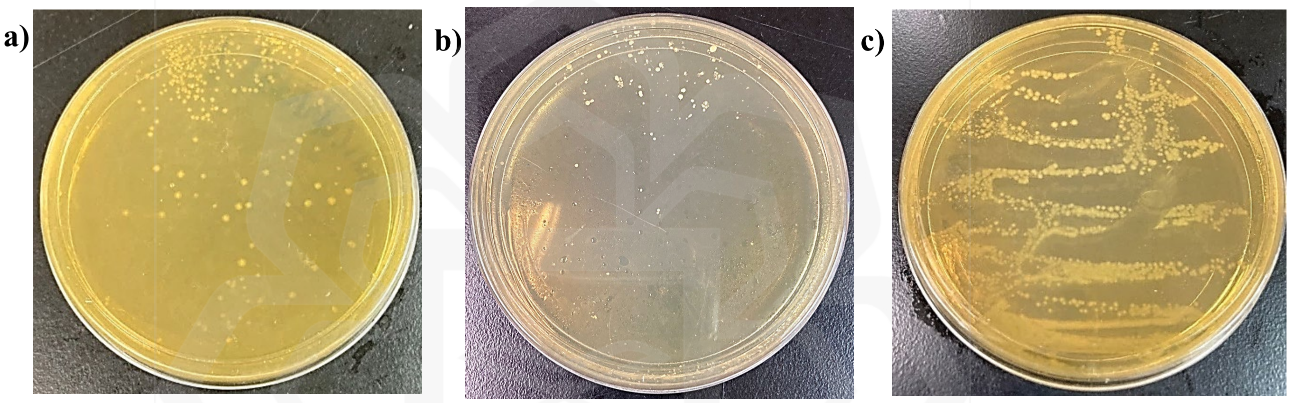


Figure 4.6 Colony morphology of *P.acnes* cultured from a sample of a) 12.5% dilution concentration of stingless bee honey, b) 14% dilution concentration of stingless bee honey and c) positive control.

In addition, the anaerobic chamber and un-autoclavable equipment were sterilised using 70% ethanol and ultraviolet (UV) radiation before being utilised. Ethanol and UV radiation have been widely practised as disinfection techniques. This is because ethanol solutions with concentrations between 60% to 90% in water and UV radiation with wavelengths between 240 nm to 280 nm are able to kill the microbes (Horakova et al., 2020). The antimicrobial activity of ethanol is influenced by the ability of the ethanol to denature the proteins and inhibit the metabolite production that is necessary for the cell division of the microbes. It was also observed that proteins will denature rapidly in the presence of water compared to in pure ethanol. However, ethanol is lack of sporicidal activity (Rutala & Weber, 2019). Meanwhile, UV radiation will damage the DNA of the microbe irreversibly by dimerising the thymine molecules. This consequently will kill the microbe (Rudhart et al., 2022; Rutala & Weber, 2019; Turnbull et al., 2008). UV radiation was found to effectively kill most bacteria, viruses and certain types of spores (Pineau et al., 2022; Turnbull et al., 2008). The combination use of ethanol and UV radiation in sterilisation treatment was found to have a synergy effect in reducing the number of microbes compared to ethanol or UV radiation alone (Tao et al., 2021).

4.4.3 Antiacne study of formulated emulgel against *P.acnes*

An antiacne study was conducted to determine the inhibition zone of all formulated emulgel against *P. acnes* in order to check the efficacy of the formulation. Based on one-way ANOVA, the overall inhibition zone of F1, F2 and F3 were significantly different ($p < 0.0001$). F1, F2 and F3 showed inhibition zones of 13.3 mm, 12.3 mm and 9.5 mm respectively (Figure 4.7). F4 did not show any zone of inhibition since it did not have any active ingredient and acted as a negative control role, hence it was excluded from one-way ANOVA analysis. F1 and F2 presented a bigger zone of inhibition compared to F3. This may be due to the presence of benzoyl peroxide which is effective against *P. acnes*.

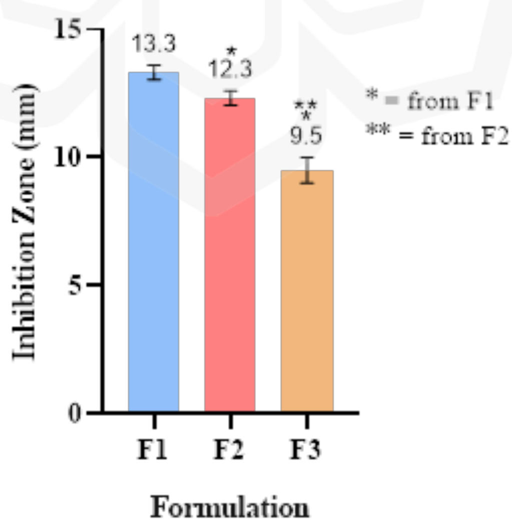


Figure 4.7 Inhibition zone (mm) for Formulation 1, 2 and 3 (Mean ± SD, n = 3, (*) $p < 0.05$).

Benzoyl peroxide is a strong oxidising agent that can destroy essential bacterial cell elements such as cell walls and cytoplasmic membranes (Bandyopadhyay, 2021). This is because benzoyl peroxide will decompose into benzoic acid and hydrogen peroxide (H_2O_2) and produce free radicals which are benzoyloxy radicals that exert a bactericidal action. These benzyloxy radicals basically generate from the cleavage of the peroxide bond and act on bacterial proteins which subsequently disrupt the bacterial function and compromise their viability (Hew et al., 2023). That is why it is effective in treating antibiotic-resistant *P. acnes* and can inhibit the emergence of antibiotic-resistant *P. acnes* when used in combination with topical antibiotics (Bandyopadhyay, 2021). Research conducted by Okamoto et al. (2021) observed the condition of *P. acnes* under transmission electron microscopy after being treated with benzoyl peroxide. Based on the observation, there was reduced electron density and some bacterial cell wall damage that led to bacterial death.

Other than its bactericidal effect, benzoyl peroxide also treats acne through its mild keratolytic, comedolytic and anti-inflammatory properties. It has been proposed that benzoyl peroxide possesses keratolytic and comedolytic properties by loosening the intercellular cohesion in the stratum corneum, hence speeding up the peeling of the stratum corneum (Okamoto et al., 2016). Moreover, the radicals formed by benzoyl peroxide also can break down the keratin (Cruz et al., 2023). A study conducted by Waller et al. (2006) found that 2% benzoyl peroxide exhibits stronger keratolytic effects within a few hours of administration in contrast to 0.05% retinoic acid or 2% salicylic acid. These three components exhibit comparable effectiveness in keratolysis after a 6-hour treatment period as both retinoic acid and salicylic acid demonstrate similar capabilities in eliminating stratum corneum comparable to the efficacy of benzoyl peroxide. The study of tape strips demonstrated that benzoyl peroxide has greater efficacy when applied to deeper layers of the stratum corneum. The potential of benzoyl peroxide to induce loosening of the stratum corneum at deeper layers may be associated with the efficacy of benzoyl peroxide in addressing more severe acne lesions characterised by pustules (Waller et al., 2006).

Meanwhile, the mild anti-inflammatory action of benzoyl peroxide is exerted by its ability to block the production of ROS in neutrophils and polymorphonuclear leukocytes in a dose-dependent manner (Bowe & Logan, 2010; Jalian et al., 2006). Besides, it had been observed that the free fatty acid in sebum was reduced after the application of benzoyl peroxide (Kanlayavattanakul & Lourith, 2011; Mueller, 2008). This is probably due to its antibacterial activity since lipases produced by *P.acnes* play a crucial role in the generation of free fatty acids (Mueller, 2008; Plewig et al., 2019a). Free fatty acids generated by *P.acnes* basically can promote oxidative stress and inflammatory reactions in acne pathogenesis (Soleymani et al., 2020). Hence, reducing free fatty acid will consequently reduce inflammation.

In addition, based on Figure 3.10, the zone of inhibition for F1 is significantly higher than F2 ($p = 0.0383$) although the difference is only about 1 mm. These results were in line with the study by Ng et al. (2020) that showed that the inhibition zone against *E.coli* bacteria for the combination of stingless bee honey and drug (either ampicillin or gentamicin) was larger compared to the drug alone. F3 which consists of only stingless bee honey did show a zone of inhibition although it was very small. A study conducted by Julianti et al. (2017) evaluated the antibacterial activity of raw honey (100%) against *P.acnes* and the result showed an inhibition zone of 16.2 mm. Up till now, there were no studies that assessed the antibacterial activity of stingless bee honey in dosage form against *P.acnes*. This study proved that stingless bee honey could still exhibit its antibacterial effect when it is in dosage form.

The antimicrobial property of honey is described to be influenced by its acidity, high osmolarity and hydrogen peroxide content (Albaridi, 2019). Bacteria generally grow in neutral or slightly alkaline environments with a pH range of 6 to 7. This makes the acidic pH of honey will give an unconducive environment for the growth of the bacteria (Achermann et al., 2014; Minden-Birkenmaier & Bowlin, 2018). Besides, the acidity environment provided by honey is depicted to change the bacteria metabolism by intervening with the enzymatic activities and destroying plasma membrane integrity. This is because a low pH environment enhances the diffusion of short-chain fatty acids such as acetic acid and formic acid into the bacterial membrane which leads to dissipation of proton motive force. This dissipation of proton motive force will cause rapid loss of microbial cell viability and microbial death (Jin & Kirk, 2018).

Furthermore, refer to Figure 4.8 and Table 4.2, the findings appeared that the antimicrobial property of these emulgel formulations against *P.acnes* had retained during the three-month storage period in the both real-time and accelerated-time chamber as evidenced by insignificant different zones of inhibition for F1, F2 and F3 ($p > 0.05$). This may be due to the low degradation of benzoyl peroxide content throughout the three-month storage in both chambers. Besides, benzoyl peroxide was degraded into benzoic acid which this benzoic acid also possesses antimicrobial properties (CHMP, 2019).

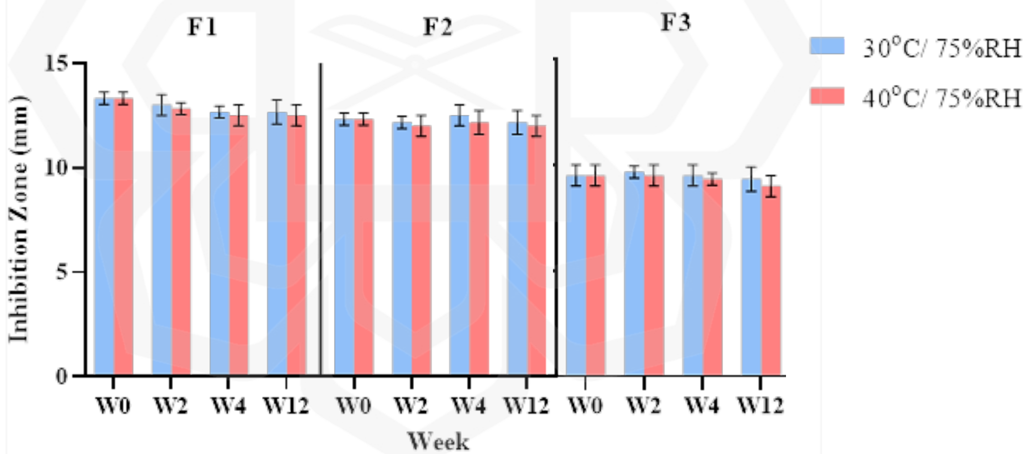
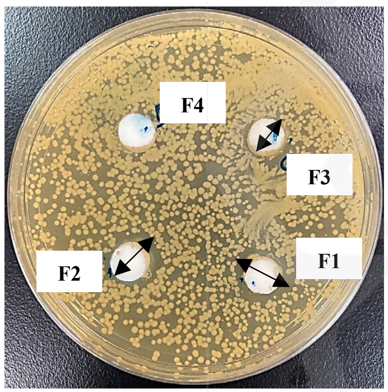
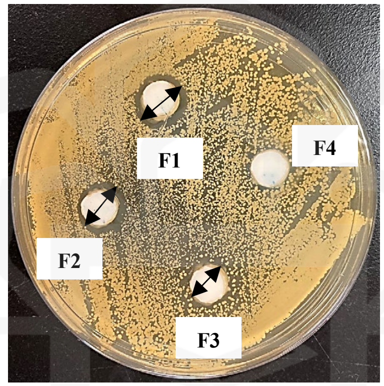
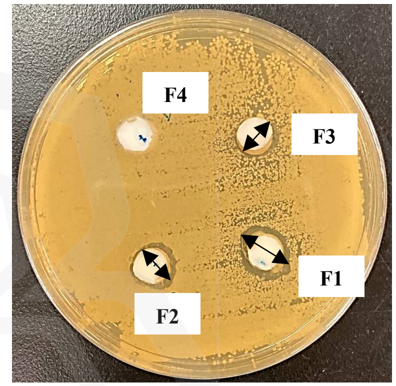
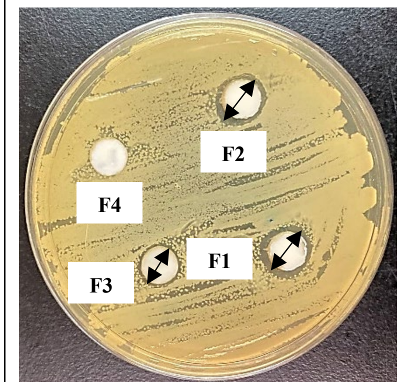
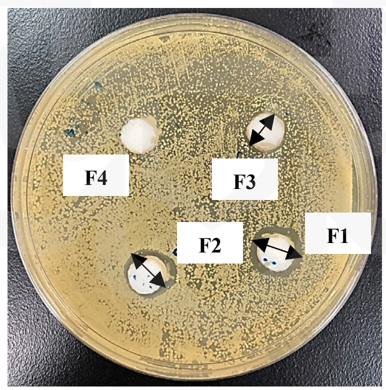
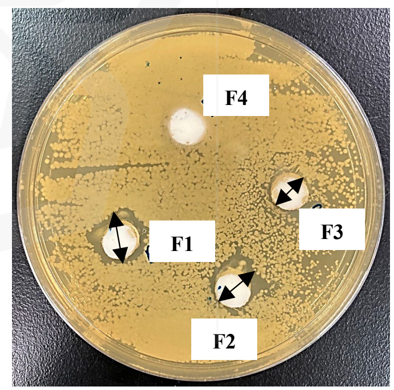
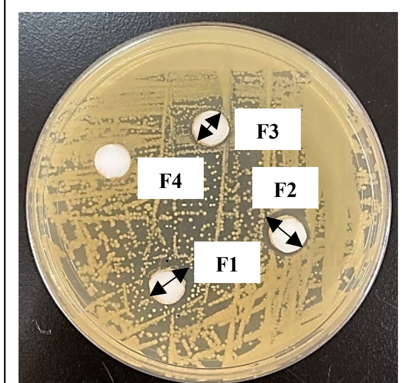


Figure 4.8 Inhibition zone of Formulation 1, 2, 3 and 4 stored in 30ºC/ 75%RH and 40ºC/ 75%RH at week 0, week 2, week 4 and week 12 (Mean ± SD, n = 3, $p < 0.05$).

Benzoic acid is basically a weak organic acid that is commonly used as a preservative (Bensid et al., 2022). Its antibacterial activity is pH-dependent which it is more effective in acidic environments. This is because in acidic environments, it remains in its undissociated (protonated and uncharged) (Arcari et al., 2020; Ben Braïek & Smaoui, 2021) which is a lipophilic form (Ben Braïek & Smaoui, 2021; Bensid et al., 2022). Hence, it can easily penetrate the bacterial cell membrane and enter the cytoplasm. After entering the bacterial cytoplasm, benzoic acid molecules encounter a relatively higher pH, leading to their dissociation into charged anions and protons. These dissociated species are unable to re-enter the plasma membrane. The buildup of H^+ ions subsequently lowers the intracellular pH, creating adverse physiological conditions that can result in cellular damage and alterations in the activity of enzymes, structural proteins and DNA (Ben Braïek & Smaoui, 2021). Moreover, the accumulation of dissociated acid anions within the cell leads to the occurrence of turgor stress resulting from an elevated osmotic pressure (Arcari et al., 2020). These accumulated anions also have the potential to interfere with cellular processes, inhibit metabolic reactions, disrupt the coupling of the proton motive force, reduce cellular mobility, disturb the membrane's functionality and ultimately cause cell death (Arcari et al., 2020; Ben Braïek & Smaoui, 2021).

Table 4.2 Antiacne effect of Formulation 1, 2, 3 and 4 against *P.acnes* stored in 30ºC/ 75%RH and 40ºC/ 75%RH at week 0, week 2, week 4 and week 12.

Condition/Week	Week 0	Week 2	Week 4	Week 12
30ºC/ 75%RH				
40ºC/ 75%RH				

4.5 CONCLUSIONS

Based on the MIC and MBC results, it showed that stingless bee honey had the ability to inhibit and kill *P. acnes* bacteria with concentrations of 12.5% and 15% respectively. This indicated that the antimicrobial property of stingless bee honey does include *P. acnes* as well. Besides, an antibacterial study through the agar well diffusion method proved that stingless bee honey could still exhibit its antiacne effect when in a dosage form. Besides, the inhibition zone by F1 was significantly larger than F2 although the difference was only approximately 1 mm. Hence, it can be presumed that stingless bee honey can give some additional bactericidal activity of the benzoyl peroxide. In addition, the antimicrobial properties of F1, F2 and F3 against *P. acnes* remained the same after three months of storage in both real-time and accelerated-time chambers which means they can still exert the same efficacy after three months. Lastly, proper sterilisation techniques were practised during conducting the experiment since no contamination was observed. Hence, the results produced were positive and true.

Henceforth, this chapter conclude that benzoyl peroxide with stingless bee honey emulgel formulations were effective in inhibiting and killing *P. acnes* bacteria. Other than the effectiveness in treating acne, the safety and effectiveness of the formulations to reduce the side effects of benzoyl peroxide also were assessed since the main purpose of incorporating stingless bee honey in the benzoyl peroxide formulation was to reduce the benzoyl peroxide side effects. These parts were examined and discussed in the following chapter.

CHAPTER FIVE

HUMAN STUDY (SKIN HYDRATION, TRANSEPIDERMAL WATER LOSS (TEWL) AND SKIN ERYTHEMA) OF THE FORMULATED EMULGEL

5.1 INTRODUCTION

In vivo study whether on human or animal is important to identify the safety and efficacy of the topical formulation. *In vivo* studies using animals have been widely used, however the structural differences between animal to human skin make it a disadvantage in providing the exact effect as in human skin (Basson et al., 2019). This is because different species have different skin permeability which will affect the percutaneous absorption. Animal skin has been found to be more permeable compared to human skin which means animal skin will have more percutaneous absorption (Abd et al., 2016). Besides, *in vivo* study using animals for cosmetic products was banned in the European Union in March 2013 (Basson et al., 2019). This makes *in vivo* studies using humans a better option for assessing the topical formulation since it will be more relevant. Besides, it has been suggested if the ingredients in the formulation are well-studied and have a history of safe use in humans, the need for animal testing may be reduced (Archibald et al., 2019). Benzoyl peroxide is a drug that has been extensively studied and its safety profile is well-documented (FDA, 2011). It has been investigated and classified as non-carcinogen to humans (WHO, 1999). Therefore, it has been long used and marketed as an acne treatment (FDA, 2011).

Nowadays, non-invasive techniques are preferable compared to invasive techniques since it is safer, cheaper and the result produced was comparable with invasive techniques for both model and human studies. Invasive techniques will restrict the investigators from repeating the test since it is destructive. Hence, the results produced may not be significant. Testing of cosmetic products is most informative when conducted with human volunteers, thus supporting the utilisation of non-invasive analytical tools. The skin barrier is important in cosmetic dermatology since all cosmetic products may impact the skin barrier either positively or negatively. Positive effects may be portrayed through skin hydration and transepidermal water loss (TEWL) improvement. Conversely, the negative effect can be seen through skin hydration reduction, TEWL deterioration and also irritant-induced contact dermatitis occurrence (Duffy et al., 2017). DermaLab Combo® is one of the non-invasive instruments used for skin properties assessments that is easy and convenient to operate (Hua et al., 2017). It can assess a variety of skin parameters including skin sebum, thickness, elasticity, TEWL, hydration, colour, melanin content and erythema index. Besides, DermaLab Combo® also able to visualise and augment the skin surface using polarised or non-polarised white light through the videoscope probe (Cortex Technology, 2012; Hua et al., 2017).

Hence, this chapter was designed to assess the skin hydration, transepidermal water loss (TEWL) and skin erythema of human volunteers after the application of the formulated benzoyl peroxide and stingless bee honey emulgel.

5.2 MATERIALS

Dibenzoyl peroxide, 97% (dry wt.), wet with 25% water was purchased from Alfa Aesar (Mexico), stingless bee honey was bought from Kuantan, Pahang (Desa Kelulut Bukit Kuin), 1,2-propanediol was received from Merck (Darmstadt, Germany), tween 20 was obtained from Sigma Aldrich (United Kingdom), sodium hydroxide was attained from Sigma Aldrich (Sweden), squalane was acquired from Kono Chem Co. Ltd. (Xi'an, China), Vitamin E acetate was achieved from Alfa Laboratory (Germany), span 60® for synthesis was purchased from Sigma Aldrich (Darmstadt, Germany), Carbopol® 940 was bought from Acros Organics (New Jersey, USA), euxyl® PE 9010 was procured from schülke (Germany), triethanolamine was attained from R&M Chemicals and distilled water.

5.3 METHODS

5.3.1 Ethical approval

Ethical approval for this study was obtained from the IIUM Research Ethics Committee (IREC) with identification number IREC 2022-064. This study also was registered with the National Medical Research Register (NMRR) with research identification number RSCH ID-22-02516-XLY.

5.3.2 Study design and setting

The study design that was applied was an *in vivo* study that assessed the effectiveness of stingless bee honey to reduce the side effects of benzoyl peroxide and the safety of the formulated emulgel on human skin. There were three skin parameters that were assessed which are hydration, TEWL and erythema index. Skin hydration and erythema index were assessed since drying and redness are some of the benzoyl peroxide side effects. Moreover, skin hydration and TEWL are important to skin properties since they reflect skin barrier function (Mehta et al., 2018). Besides, a questionnaire that include two items which are skin irritation assessment and the acceptability and tolerability of the formulations' characteristics was given to the participants. In the skin irritation assessment part, the participants were allowed to report and rate the skin irritation that they experienced throughout the study such as dryness, redness, itchiness and pain sensation. Meanwhile, in the acceptability and tolerability of the formulation's characteristic part, there were five characteristics were evaluated which were appearance, colour, stickiness, spreadability, absorption and odour. The setting of the study was in Kulliyyah of Pharmacy, International Islamic University Malaysia, Kuantan, Pahang.

5.3.3 Test formulations

There were four formulations that were tested in this study as follows:

a. Stingless bee honey and benzoyl peroxide emulgel formulation (F1)

b. Stingless bee honey emulgel formulation (F2)

c. Benzoyl peroxide emulgel formulation (F3)

d. Base emulgel formulation (F4)

These formulations were prepared based on the composition in Table 3.1 in Chapter 3.

5.3.4 Recruitment of participants

Recruitment of participants was done through advertisement on social media by using a poster and 'Google Form' for registration or directly approaching the potential participants. Participants that volunteered to join were contacted by the investigator and the aim of this study was explained in detail to them. Informed Consent Form (ICF) that contains a patient information sheet (demographic characteristics) and consent form was given to all volunteered participants for them to read for further understanding and to give consensus. Participation was utterly voluntary, and they could withdraw from the study at any time. At first, a total of 30 volunteers (male and female) with healthy skin were recruited for this study. However, four of them dropped out of the study. Hence, the final total number of volunteers recruited was 26. The inclusion and exclusion criteria of the volunteers that were recruited were described in Table 5.1 below. The volunteers were randomly divided into two groups in which each group had 13 participants. This sample size was relevant and significant as it is more than 12 subjects. This is because a minimum of 12 subjects per group was recommended for a pilot study to ensure both feasibility and precision in estimating parameters critical for designing future studies (Julious, 2005). Group 1 was given F1 and F2 meanwhile Group 2 was given F3 and F4.

Table 5.1 Inclusion and exclusion criteria of the recruited volunteers (Alves et al., 2021; Omar et al., 2021; Plyduang et al., 2022).

Inclusion criteria	Exclusion criteria
• Volunteers of both genders aged between 20 and 40 years old • Normal skin condition • Able to comply with daily use of the test samples • Able to come for the skin assessment every week for a month	• Having any skin disease such as eczema, psoriasis and allergy • Having skin disease in the past three months and was treated with medication • Impaired skin around the study site • Using any product at the area of study and not willing to stop

5.3.5 Application of test formulation on the study site

All participants were informed not to apply any topical products on the test sites a week before the baseline skin condition assessment. Participants were only allowed to have basic shower creams or soap at the test site. Each participant was given two test formulations to be applied at the left and right upper volar forearm twice daily. Table 5.2 shows the site of application for each test formulation. Two 1 mL syringes and two containers of different formulations were given to each participant for 4 weeks of study duration. Participants were instructed to withdraw 0.1 mL of each test formulation by using a 1 mL syringe and applied onto the study site twice daily. The area of the study site was 20 cm^2. To ensure that the emulgel was applied to the same area each time, as well as to make sure that the emulgel had been applied to the tested area, a tool illustrated in Figure 5.1 was provided to aid the process. A weekly skin condition assessment was performed on the participants for 4 weeks to determine the effects of the formulation. The analysis was performed to find a significant difference in the parameters measured ($p < 0.05$) before and after the application of the emulgel (Omar et al., 2021).

Table 5.2 Application area for each test formulation.

Group	Formulation	Application area	
		Left volar forearm	Right volar forearm
1	1	/	
	2		/
2	3	/	
	4		/

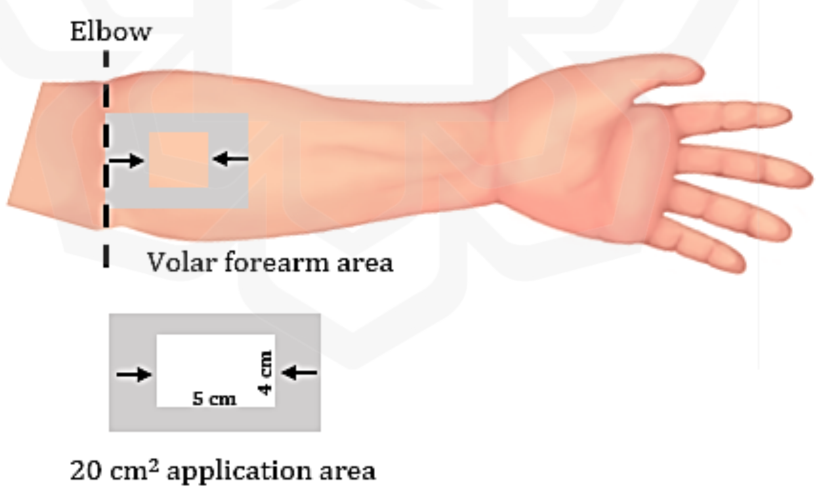


Figure 5.1 Emulgel application area and application tool.

5.3.6 Skin assessment

Skin assessments were conducted for all participants prior to the study and at the end of weeks 1, 2, 3, and 4. Participants were requested to stay in the testing room five to fifteen minutes before the assessment to ensure full skin adaptation to the room temperature (25 ± 2°C). There were three skin assessments that were performed which are erythema index, hydration and transepidermal water loss (TEWL) by using DermaLab® Combo (Cortex Technologies) (Figure 5.2).

Figure 5.2 DermaLab® Combo (Cortex Technologies).

5.3.6.1 Erythema index

The erythema index was measured by using the skin tone probe (Figure 5.3). The erythema measurement of the DermaLab® Combo is based on the principle of narrow-band reflectance spectrophotometry (550 nm ± 30 nm for haemoglobin). This probe has an optical focus on a 7 mm diameter target area with a clear front for accurate positioning and is irradiated by two angled white light-emitting diode lights (Cortex Technology, 2012). The probe was calibrated first before being used to ensure the accuracy of the measurement. Then, the probe was pressed onto the study site at three different places and the reading for each place including the average value was shown on the screen.

Figure 5.3 Skin tone probe.

5.3.6.2 Hydration pin probe

A hydration pin probe (Figure 5.4) was used to measure the hydration. This measurement of skin moisture will apply the conductance principle. A pin probe with eight-pin electrodes was used to facilitate dry skin application. This pin probe has a spring-loaded design feature that can initiate the measurement at the skin surface (Hadi et al., 2016). The probe also was calibrated first before being utilised. Then, the pin hydration probe was pressed onto the study site at three different places and the measurements for each place including the average value appeared on the screen. Based on the hydration values, the result could be classified as mentioned in Table 5.3 below.

Table 5.3 Skin moisture classification (Cortex Technology, 2012).

Reading (uSiemens)	**Moisture**
< 150	Very dry
150- - 300	Dry
> 300	Sufficiently moistured

Figure 5.4 Hydration pin probe.

5.3.6.3 TEWL open chamber probe

Transepidermal water loss (TEWL) open chamber probe of DermaLab® Combo (Figure 5.5) applies the vapour diffusion gradient principle in which the amount of condensed water that passes through a fixed area of stratum corneum to the skin surface per unit time (Hadi et al., 2016). The probe was placed on the study site in which the open end was in contact with the skin and the 'START' button was pressed. Then, the TEWL measurement was displayed on the screen and expressed as $g/m^2/hour$. The normal range of TEWL values is between 2 to 10 $g/m^2/hour$ (Cortex Technology, 2012).

Figure 5.5 TEWL open chamber probe.

5.3.7 Questionnaire

In the last week of the study, a questionnaire that included two items which are skin irritation assessment and the acceptability and tolerability of the formulations' characteristics was given to the participants. In the skin irritation assessment part, the participants were allowed to report and rate the skin irritation that they experienced throughout the study such as dryness, redness, itchiness and pain sensation. They could rate the intensity of the irritation from 0 to 5 in which 0 = none, 1 = mild, 2 = moderate, 3 = severe, 4 = very severe and 5 = worst possible irritation. Meanwhile, in the acceptability and tolerability of the formulation's characteristic part, there were five characteristics were evaluated which were appearance, colour, stickiness, spreadability, absorption and odour. They could rate the formulation's characteristics from 1 to 5 with 1 = completely unacceptable/ very poor, 2 = unacceptable/ very poor, 3 = neutral, 4 = acceptable/ good and 5 = very acceptable/ excellent.

5.3.8 Data collection and statistical analysis

The data were collected and filled into the template that had been formed. The demographic data were described statistically as frequency and percentage by using SPSS version 29. The results for skin assessments were presented as mean ± standard error of the mean (SEM). Variance values analysis was analysed using a two-way analysis of variance (ANOVA) then followed by post-hoc analysis using a Tukey test with $p < 0.05$ as the minimum level of significance using PRISM 8.0 software (Graph Pad, San Diego, CA, USA). Meanwhile, the questionnaire data was analysed by using the Kruskal-Wallis test with $p < 0.05$ as the minimum level of significance using SPSS version 29.

5.4 RESULTS AND DISCUSSION

5.4.1 Demographic data

A total of 26 participants were recruited and adhered to the formulation application and skin assessments for four weeks of the study. The majority of the participants were female (n = 26, 88.5%) and three (11.5%) were male. 100% of the participants were Malay. Most of the participants were between 20-24 years old (n = 9, 34.6%) and 25-29 years old (n =

9, 34.6%) with a majority of them being IIUM students (n = 17, 65.4%). Also, most of the participants were non-smokers (n = 24, 92.3%). The characteristics of the participants were summarised in Table 5.4 below.

Table 5.4 Demographic characteristics of the participants (n = 26).

Characteristics		**Frequency**	**Percentage (%)**
Gender	Male	3	11.5
	Female	23	88.5
Age (years old)	20-24	9	34.6
	25-29	9	34.6
	30-34	6	23.1
	35-39	2	7.7
Race	Malay	26	100
	Chinese	0	0
	Indian	0	0
	Others	0	0
Position	IIUM Student	17	65.4
	IIUM Staff	5	19.2
	Others	4	15.4
Smoker	No	24	92.3
	Yes	2	7.7

5.4.2 Skin assessments data

5.4.2.1 Erythema index

Generally, there was no significant difference for skin erythema when comparing between four formulations from week to week (p = 0.2783) and also between weeks within each formulation (p = 0.5040). However, the overall erythema index result for the interaction between these two variables displayed a significant difference (p = 0.0044). Based on Figure 5.6 a), it showed that there was no significant difference between all four formulations for each week. However, the erythema index for F2 was the highest for every week. Referring to Figure 5.6 b), the erythema index of F1, F3 and F4 were more or less the same for each week and had a mean difference of -0.3000, 0.3462 and 0.4000 respectively between week 4 and the baseline. These showed that at the end of the study, the skin erythema of F1 vaguely increased meanwhile the skin erythema of F3 and F4 slightly decreased. Only the erythema index for F2 was constantly increased throughout the study and there were significant increments in week 2 (p = 0.0419) and week 3 (p = 0.0286) when compared with the baseline.

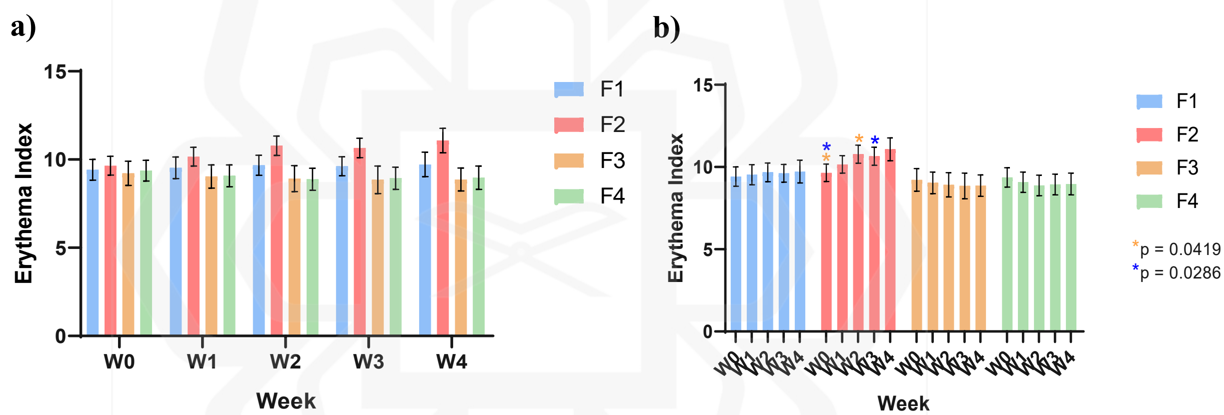


Figure 5.6 Skin erythema index for Formulations 1, 2, 3 and 4 at different time intervals that are arranged and displayed by **a)** week **b)** formulation (Mean ± SEM, *n* = 52, (*) $p < 0.05$).

Erythema is basically skin reddening that presents as a rash or flare caused by injury or any condition that can trigger inflammation (Abdlaty & Fang, 2021; Moy & Tunnell, 2016). The intensity and size of the erythema will depend on the type and intensity of the stimulus (Abdlaty & Fang, 2021). Erythema which is a sign of irritation is one of the side effects of benzoyl peroxide and this side effect is dose-dependent. This means that the irritation worsens with higher concentrations (Kaewsanit et al., 2021). Skin irritation from benzoyl peroxide basically may occur due to its ability to trigger an inflammatory response through oxidative stress, alongside with its antibacterial properties (Briganti & Picardo, 2003). Besides, benzoyl peroxide also disturbs the antioxidant defence system of the skin since it has been illuminated to decrease endogenous epidermal vitamin E (Mills et al., 2016). It has been observed that vitamin E oxidation and subsequent lipid peroxidation occur in the HaCaT keratinocyte cell line when exposed to benzoyl peroxide. This process alters the permeability of the cell membrane, hence promoting the cellular uptake of the drug. Within the cellular cytoplasm, benzoyl peroxide interacts with water-soluble antioxidants which result in the depletion of reduced glutathione (GSH) and elevation in the intracellular ratio of oxidised glutathione (GSSG) to GSH forms. The modification of the cellular redox environment leads to the activation of gene expression related to the production of pro-inflammatory cytokines such as IL-1α (Briganti & Picardo, 2003).

Based on the result, it does show that F2 which consists of benzoyl peroxide cause the skin erythema to increase throughout the study. Although the concentration of benzoyl peroxide incorporated in the formulation was only 5% which is a medium concentration, the consistent application of the formulation twice daily for 4 consecutive weeks may enhance the possibility of irritation to occur. F1 also contains benzoyl peroxide in it, however the skin erythema only increases vaguely. This may indicate the effectiveness of stingless bee honey in reducing the side effects associated with benzoyl peroxide since it possesses anti-inflammatory properties. The anti-inflammatory properties seem to be associated with the high phenolic compound in the stingless bee honey. Many studies found that the anti-inflammatory properties of stingless bee honey were presented by its ability to suppress nitric oxide (NO) production in lipopolysaccharide (LPS)-induced RAW 264.7 cells (Biluca et al., 2020; Chong et al., 2021; Ooi et al., 2021).

NO is a transient and highly reactive molecule that functions as a signalling molecule in various physiological and pathological processes. NO is produced by the catalysis of arginine by nitric oxide synthases (NOS), which are present in three distinct isoforms which are endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). The dysregulation of NO production has been implicated in various clinical disorders, including inflammation. The excessive production of NO can serve as an indicator for assessing the extent of acute and chronic inflammation. Hence, the regulation of NOS activity is a viable strategy for mitigating inflammation (Król & Kepinska, 2021). Other than NO, the anti-inflammatory action of stingless bee honey also is contributed by its capability to reduce pro-inflammatory cytokines such as TNF-α, IL-6, MCP-1, IL-12p70, INF-γ and IL-10 (Biluca et al., 2020).

Besides, the anti-inflammatory effects of stingless bee honey were partially due to its potent antioxidant properties since it can scavenge reactive oxygen species. This consequently reduces oxidative stress and halts the inflammation progression (Ooi et al., 2021). Research has demonstrated that honey with high total phenolic levels exhibits a higher capacity to scavenge $ABTS^+$ cation radicals. This finding suggests a clear correlation between phenolic content and the antioxidant properties of honey. Stingless bee honey possesses high antioxidative and anti-inflammatory properties since its polyphenolic compound is approximately ten times greater than other honey types (Mustafa et al., 2018). Both research on the stingless bee (*Trigona and Hypotrigona sp*.) in Malaysia and Nigeria indicated that the honey produced consists of a higher level of flavonoids and total antioxidant activity compared with the honey produced *by Apis dorsata* and *Apis mellifera* respectively (Nweze et al., 2017; Ranneh et al., 2018). The maintained erythema indexes every week for F3 and F4 proved that the excipients used in the formulation including stingless bee honey were safe as they did not irritate and inflame the skin of the participants.

5.4.2.2 Hydration

The skin moisture for each formulation was maintained as dry skin classification throughout the four study weeks. The overall result of skin hydration exhibited no significant difference comparing between four formulations from week to week (p = 0.0685), between weeks within each formulation (p = 0.0509) and also the interaction between these two variables (p = 0.6322). Referring to Figure 5.7, skin hydration for F1, F3 and F4 fluctuated throughout the study. However, all their hydration values in weeks 1, 2, 3 and 4 were higher compared to the baseline except in week 3 for F4 which was slightly lower than the baseline (Mean difference: 3.769 uSiemens). Only hydration values of F2 showed a decreasing trend. The skin hydration of F2 also was the lowest for every week except in week 0 (baseline). Based on Tukey's multiple comparisons test, it disclosed that there was a significant rise in skin hydration for F3 in weeks 1 (p = 0.0251) and 2 (p = 0.0141) compared to the baseline. F3's hydration in week 2 also was significantly higher when compared to F2 (p = 0.0340).

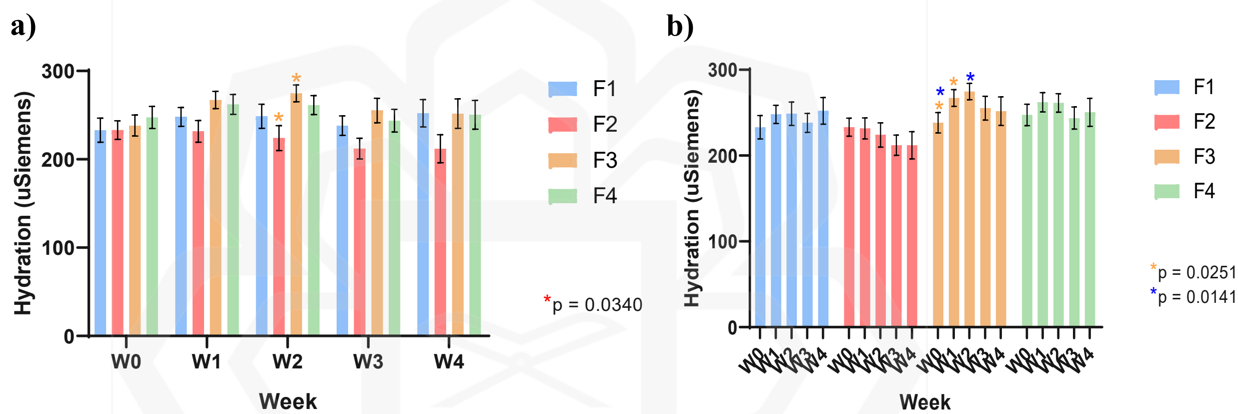


Figure 5.7 Skin hydration for Formulations 1, 2, 3 and 4 at different time intervals that are arranged and displayed by **a)** week **b)** formulation (Mean ± SEM, *n* = 52, (*) $p < 0.05$).

Skin drying is also one of the side effects of benzoyl peroxide and this was demonstrated by F2 which makes the skin hydration decreased throughout the study. Although there is an emollient in the formulation which is squalane, this proved that it is not enough to counter back the drying side effect. The higher skin hydration for F1 that consists of both benzoyl peroxide and stingless bee honey in weeks 1, 2, 3 and 4 compared to the baseline confirmed that stingless bee honey was effective in countering the skin drying side effect of benzoyl peroxide. Significant improvement of skin hydration in weeks 1 and 2 for F3 which contains stingless bee honey as the main ingredient strengthens the evidence that stingless bee honey does possess an excellent moisturising effect. The result was in line with the study by Athirah et al. (2018) that showed the lip moisture of all volunteers was improved when applying the lip balm formulation containing stingless bee honey. Besides, it had been studied that formulations containing higher concentrations of honey moisturised the skin more effectively (Pavlačková et al., 2020).

This moisturising effect of stingless bee honey may be attributed to its composition such as sugars, amino acids and lactic acid (Fletcher et al., 2020; Jalil et al., 2017) and also high moisture content which makes it a natural humectant (Kaur et al., 2020; Rao et al., 2016; Said et al., 2020; Zulkhairi Amin et al., 2018). A humectant is basically a substance that acts like a sponge that draws water into the skin. It will attract water from the deeper epidermis and dermis and make the skin smoother by filling holes in the stratum corneum through swelling (Draelos, 2018). The significant moisture content in stingless bee honey can prevent skin dryness by inducing an osmotic effect which gradually retains moisture within the dry skin (Nur Eszaty et al., 2022). Besides, the presence of hydroxyl groups in honey (Fletcher et al., 2020; Jalil et al., 2017) makes it have a water-binding ability and form hydrogen bonds with the water molecules. Hence, the hydrated skin condition can be preserved since the hydrogen bonds help to retain water in the stratum corneum by averting water loss through evaporation (Hadi et al., 2015). The mechanisms of stingless bee honey preserve skin hydration by attracting water from the dermis to the epidermis and making a hydrogen bond with water molecules were portrayed in Figure 5.8. Moreover, the presence of vitamins B, E and K together with a diverse range of vital minerals like potassium, phosphorus and calcium play a significant role in the moisturising properties exhibited by stingless bee honey (Nur Eszaty et al., 2022). However, skin hydration may not depend solely on formulations. There are many external factors that may affect skin hydration such as water intake, relative humidity or weather and also bathing (Iizaka, 2017) which make the hydration values fluctuate throughout the four weeks of the study.

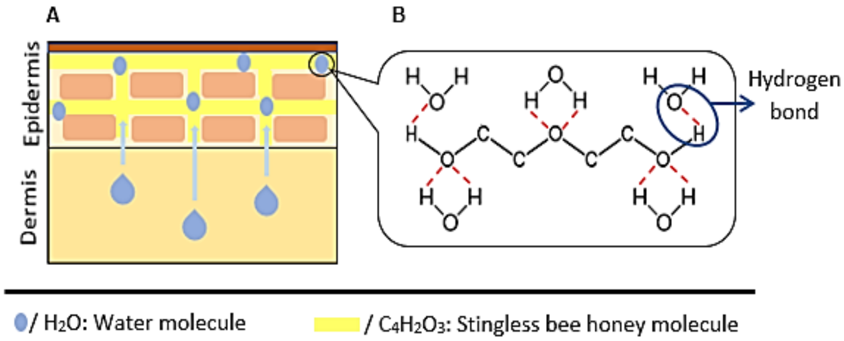


Figure 5.8 Illustration of how stingless bee honey preserves skin hydration by (A) attracting water from the dermis to the epidermis and (B) making a hydrogen bond with water molecules.

5.4.2.3 Transepidermal water loss (TEWL)

Based on the transepidermal water loss result (TEWL) result, it showed that the skin barriers of all participants were maintained within the normal range every week for each formulation. The changes of TEWL for all formulations within the group itself were not significant from baseline to week 4 ($p = 0.1019$). However, when comparing between four formulations from week to week ($p = 0.0005$) and also the interaction between those two variables ($p = 0.0104$), there were significant differences. Similar to skin hydration, TEWL for formulations 1, 3 and 4 fluctuated throughout the study (Figure 5.9). However, at the end of the study, the TEWL measurements for F3 and F4 were lower than the baseline (MD: 0.5538, 0.2538) meanwhile TEWL measurement of F1 was a bit higher compared to the baseline (MD: -0.4154). TEWL reading for F2 showed an increasing pattern from baseline to week 4 (MD: -1.200) and there was a significant increment in weeks 3 ($p = 0.0244$) and 4 ($p = 0.0420$) compared to the baseline. TEWL for F2 was the highest in every week except the baseline and its measurement was significantly higher when compared to F3 in weeks 2 ($p = 0.0029$), 3 ($p = 0.0006$) and 4 ($p = 0.0094$). When compared to F4, the TEWL for F2 was also significantly higher in weeks 3 ($p = 0.0019$) and 4 ($p = 0.0259$).

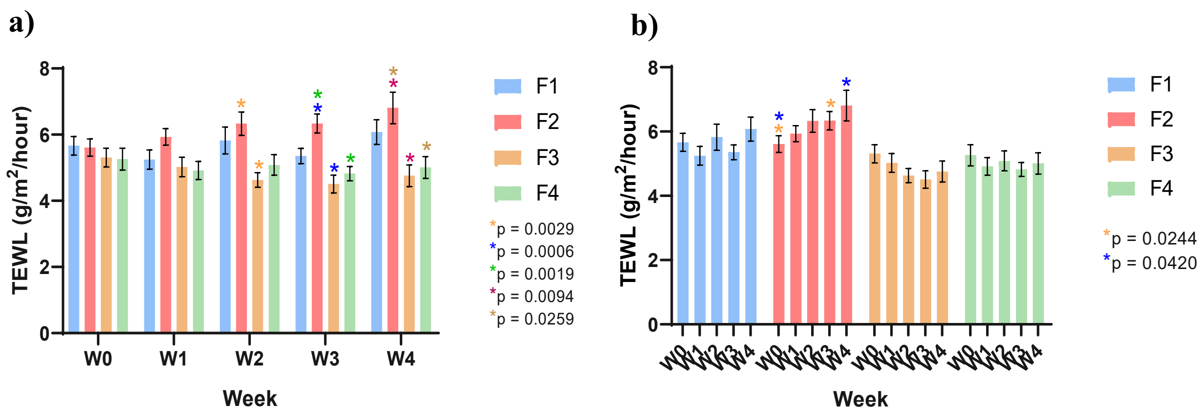


Figure 5.9 TEWL for Formulations 1, 2, 3 and 4 at different time intervals that are arranged and displayed by **a)** week **b)** formulation (Mean ± SEM, *n* = 52, (*) $p < 0.05$).

TEWL measurement could reflect skin barrier conditions either healthy or diseased conditions. A lower TEWL value was considered a good skin barrier since the stratum corneum could prevent water loss from the skin (Anthonissen et al., 2013). The amount of water loss from the skin to the external environment is based on the water vapour pressure gradient on both sides of the skin barrier. TEWL has commonly been analysed to measure the possible irritation or protective effect of topical formulation since it is a sensitive indicator for skin irritation (Honari & Maibach, 2014). The rise of the TEWL for F2 throughout the study showed that the skin barrier was disrupted and there was skin irritation occurrence. This is consistent with its result for skin erythema and hydration which displayed an increment and decrement respectively. This is because all the side effects related to benzoyl peroxide such as drying, skin peeling, itchiness, burning, and redness are signs of skin barrier impairment (Grobel & Murphy, 2018; Iijima & Tsunoda, 2019). This result was similar to a study conducted by Zhou et al. (2021) in which the TEWL of the patients significantly increased after applying topical treatment with 5% benzoyl peroxide gel for 12 weeks.

Furthermore, benzoyl peroxide may cause elevated TEWL due to its deeper keratolytic ability which disrupts the stratum corneum and water barrier to a significant extent (Waller et al., 2006). TEWL for F1 fluctuated throughout the study in which the TEWL values were higher and lower in certain weeks compared to the baseline. Although the TEWL values were higher in some weeks, but F1 did not exhibit a similar pattern as F2 which increased continuously. This may be due to the presence of stingless bee honey that increases skin hydration due to its moisturising effect, hence reducing water loss. Studies revealed that the correlation between skin hydration and TEWL was negative, which means that an increase in skin hydration will decrease the TEWL level (Prakoeswa et al., 2022). Besides, since the accuracy of TEWL measurements also could be influenced by environmental factors such as humidity, temperature and ventilation (Honari & Maibach, 2014), hence the fluctuation result may be due to this reason. TEWL reduction for F3 and F4 indicated that the excipients used in the formulation including stingless bee honey did not impair the skin barrier. In fact, both formulations help to maintain and strengthen the skin barrier.

5.4.3 Questionnaire Data

5.4.3.1 Skin Irritation Assessments

<u>*5.4.3.1.1 Dryness*</u>

Skin irritation including dryness, redness, pruritis and pain sensation at the application area is a known side effect of benzoyl peroxide (Grobel & Murphy, 2018; Iijima & Tsunoda, 2019). Based on the Kruskal-Wallis test, it showed that there were significant differences between each formulation regarding the dryness side effect. This significant difference ($p = 0.005$) was shown due to the highest percentage of F2 for mild and moderate dryness compared to other formulations. F2 caused more participants to experience dryness (6 out of 13 participants) compared to F1 (3 out of 13 participants). Moderate dryness also was higher for F2 (3 out of 13) than for F1 (2 out of 13). The participants reported experiencing this dryness side effect mostly during week 3 and 4 application and the occurrence was not frequent. All participants that applied F3 and F4 did not experience any dryness side effects.

Table 5.5 Dryness experienced by participants (different formulations) throughout the study (expressed in percentages, n = 52, (*) $p < 0.05$).

Formulation	**Dryness experienced by participants (expressed in percentages)**						***P-Value**
	0 (None)	**1 (Mild)**	**2 (Moderate)**	**3 (Severe)**	**4 (Very severe)**	**5 (Worst possible irritation)**	
F1	76.9	7.7	15.4	0	0	0	0.005
F2	53.8	23.1	23.1	0	0	0	
F3	100	0	0	0	0	0	
F4	100	0	0	0	0	0	

5.4.3.1.2 Redness

Similar to the dryness side effect, the redness side effect exhibited significant differences between each formulation. This significant difference ($p < 0.001$) was portrayed due to the highest percentage of moderate redness for F2 compared to other formulations. More participants experienced redness (8 out of 13 participants) after applying F2 compared to F1 (6 out of 13 participants). 6 out of 8 participants who experienced redness for F2 rated the intensity as moderate meanwhile only two participants had moderate redness for F1. The occurrence of the redness varied for each participant in which some of them reported the redness occurred two weeks after application meanwhile some of them stated that it happened in week 3 or week 4. There were two participants informed that the redness appeared gradually from the first time using the F2. Meanwhile, the others reported that redness happened occasionally. Several participants mentioned that the redness resolved after 1 hour. Generally, most of the participants described that redness for F2 appeared more severe and faster than for F1. There was no redness side effect that occurred for F3 and F4.

Table 5.6 Redness experienced by participants (different formulations) throughout the study (expressed in percentages, n = 52, (*) p < 0.05).

Formulation	**Redness experienced by participants (expressed in percentages)**						***P-Value**
	0 (None)	**1 (Mild)**	**2 (Moderate)**	**3 (Severe)**	**4 (Very severe)**	**5 (Worst possible irritation)**	
F1	53.8	30.8	15.4	0	0	0	< 0.001
F2	38.5	15.4	46.2	0	0	0	
F3	100	0	0	0	0	0	
F4	100	0	0	0	0	0	

5.4.3.1.3 Itchiness

The result presented that there were significant differences between each formulation for itchiness side effects. This significant difference ($p = 0.012$) was revealed due to the highest percentage of F2 for moderate itchiness compared to other formulations. F2 also caused more participants to experience itchiness (7 out of 13 participants) than F1 (6 out of 13 participants). Although there was only one participant different, however F2 caused more severe itchiness in which five of them experienced moderate itchiness and one of them rated the itchiness as severe. F1 only caused moderate itchiness with two participants experiencing it meanwhile the rest were under the mild itchiness category. Most of the participants reported experiencing this itchiness side effect during weeks 3 and 4 of application. There were three participants informed that the itchiness happened two weeks after usage of F2 and getting worse. Overall, most of the participants mentioned that the itchiness occurrence for F2 was more severe and faster than for F1. Both F3 and F4 caused moderate itchiness to only a participant. This participant mentioned that it rarely happened but it happened usually during hot weather and might be due to sweating and hand sock usage.

Table 5.7 Itchiness experienced by participants (different formulations) throughout the study (expressed in percentages, n = 52, (*) $p < 0.05$).

Formulation	**Itchiness experienced by participants (expressed in percentages)**						***P-Value**
	0 (None)	**1 (Mild)**	**2 (Moderate)**	**3 (Severe)**	**4 (Very severe)**	**5 (Worst possible irritation)**	
F1	53.8	30.8	15.4	0	0	0	0.012
F2	46.2	7.7	38.5	7.7	0	0	
F3	92.3	0	7.7	0	0	0	
F4	92.3	0	7.7	0	0	0	

5.4.3.1.4 Pain sensation

The result showed that there was no significant difference between each formulation for pain sensation side effects since all participants for all formulations did not experience it except for one participant. This participant experienced mild pain sensations after applying F1 and F2 in week 3.

Table 5.8 Pain sensation experienced by participants (different formulations) throughout the study (expressed in percentages, n = 52, $p < 0.05$).

Formulation	**Pain sensation experienced by participants (expressed in percentages)**						**P-Value**
	0 (None)	**1 (Mild)**	**2 (Moderate)**	**3 (Severe)**	**4 (Very severe)**	**5 (Worst possible irritation)**	
F1	92.3	7.7	0	0	0	0	0.564
F2	92.3	7.7	0	0	0	0	
F3	100	0	0	0	0	0	
F4	100	0	0	0	0	0	

The result of this study was similar to the literature as most of the side effects were experienced by participants who applied formulations that consisted of benzoyl peroxide which are F1 and F2. A study conducted by Kawashima et al. (2017) suggested that the side effects with a potential causal relation with benzoyl peroxide drug were exfoliation of the skin (19.1-23.5%), redness at the application area (10.8-13.7%), irritation at the application area (8.3-12.3%), itchiness at the application area (2.5-3.4%) and contact dermatitis (1.5-2.5%). Besides, the most frequently reported side effects were dry skin (15%), skin burning sensation (9%), redness (8%), skin desquamation (5%), skin irritation (5%,), itchiness (4%), rash (3%) and pain of skin (3%) according to the Galderma Global Safety Database from 1992 to 15th October 2021 (Szymanski & Arekapudi, 2022). Meanwhile, the most frequent side effects associated with adapalene 0.3%/benzoyl peroxide 2.5% gel were skin irritation (14.9%) and pain of the skin (3%) (Dreno, Bissonnette, et al., 2018).

Side effects due to topical cutaneous medications or formulations can lead to a significant number of patients discontinuing their treatment, which has implications for the quality and cost of healthcare. It has been exhibited that optimised formulations incorporating moisturising components such as humectants and emollients are able to mitigate dryness and irritation in acne patients which subsequently improves tolerability. This improvement in tolerability may enhance patient adherence to treatment regimens and consequently give a better result (Gold et al., 2023).

Based on the side effect results described before, in general, many participants experienced more side effects when using F2 compared to F1. Besides, more severe side effects were also associated with F2 instead of F1. These qualitative data were consistent with the skin assessment data which proved that the incorporation of stingless bee honey which is a natural humectant in the benzoyl peroxide formulation did reduce the side effects of the benzoyl peroxide. Clinical Practice Guideline (2022) has suggested the application of moisturiser along with benzoyl peroxide in order to improve the tolerability. Hence, incorporating stingless bee honey in the benzoyl peroxide formulation also may increase patient adherence by facilitating the patients to make a routine (Yosipovitch et al., 2019). This is because the patients only need to apply one formulation instead of several formulations. F3 and F4 did not cause any side effects since the side effects reported for these two formulations were almost none. This demonstrated that the excipients used in the formulation including stingless bee honey were safe and non-irritant as they were no side effects associated with F3 and F4.

5.4.3.2 Acceptability and Tolerability of the Formulations' Characteristic

Based on Table 4.5, there was no significant difference between all formulations for each parameter assessed. This indicated that relative agreement among all volunteers that all formulations had almost the same score for all the parameters assessed. The majority of volunteers found that all characteristics including appearance, colour, stickiness, spreadability, absorption and odour for all formulations were good or excellent. There was none of the volunteers thought the characteristics for F1 were very poor or poor. This is similar to F2 except for the stickiness characteristic. Only one volunteer felt that the stickiness of F2 was poor. F3 also was categorised as poor by only one volunteer for stickiness, spreadability, absorption and odour meanwhile F4 was considered as poor by one volunteer for stickiness, absorption and odour. This suggested that the characteristics of all formulations were acceptable and tolerable. This was in line with studies by Oliveira & Almeida (2023) and Oliveira et al. (2022) that found emulgel had good skin tolerance since their constituents had large amounts of water or aqueous phase, low oil phase and well-tolerated polymers.

It is important to assess the patients' or consumers' acceptability and tolerability of the formulation characterisations because they also can affect the medication or product adherence. Medication adherence is widely recognised as a crucial issue in healthcare systems as poor adherence is associated with negative health outcomes and higher patient costs (Gold et al., 2023; Oliveira et al., 2022). Poor adherence has been reported for several dermatological conditions. The World Health Organisation (WHO) acclaims that the factors of non-adherence are classified into five main types which are socioeconomic factors, healthcare and system-related factors, therapy-related factors, condition-related factors, and patient-related factors. Topical medications are widely used in dermatology and are the most commonly used therapeutic approaches (Menditto et al., 2020; Oliveira & Almeida, 2023). Many patients were not adhering to the topical medication formulation mainly due to the unpleasant texture of the formulation. Some of the unpleasant characteristics of the formulation that hinder adherence are the texture is too greasy and sticky, hard to be absorbed and spread and too much residues are left out (Savary et al., 2019). Hence, this shows that patient-centric perception needs to be taken into account in the pharmaceutical drug design process as it can improve the adherence of the patients to topical medication (Oliveira & Almeida, 2023; Oliveira et al., 2022).

Table 5.9 Acceptability and Tolerability of the Formulations' Characteristic (expressed in percentages, n = 52, p < 0.05).

Formulation	F1 (N=13)					F2 (N=13)					F3 (N=13)					F4 (N=13)					P-Value
Acceptability and tolerability of the formulations' Characteristics (expressed in percentages)	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	
Appearance	0	0	0	61.5	38.5	0	0	0	69.2	30.8	0	0	7.7	30.8	61.5	0	0	7.7	46.2	46.2	0.643
Colour	0	0	0	46.2	53.8	0	0	7.7	53.8	38.5	0	0	7.7	46.2	46.2	0	0	7.7	38.5	53.8	0.809
Stickiness	0	0	15.4	69.2	15.4	0	7.7	7.7	53.8	30.8	0	7.7	30.8	46.2	15.4	0	7.7	38.5	30.8	23.1	0.449
Spreadability	0	0	23.1	46.2	30.8	0	0	23.1	46.2	30.8	0	7.7	23.1	30.8	38.5	0	0	30.8	38.5	30.8	0.995
Absorption	0	0	15.4	53.8	30.8	0	0	23.1	53.8	23.1	0	7.7	15.4	38.5	38.5	0	7.7	15.4	46.2	30.8	0.945
Odour	0	0	7.7	38.5	53.8	0	0	0	53.8	46.2	0	7.7	30.8	15.4	46.2	0	0	46.2	15.4	38.5	0.336

1 = Completely unacceptable/ very poor, 2 = Unacceptable/ poor, 3 = Neutral, 4 = Acceptable/ good,

5 = Very acceptable/ excellent

* Using Kruskal-Wallis Test

5.5 CONCLUSIONS

There were three skin parameters that were assessed in this in vivo study which are hydration, TEWL and erythema index. Skin hydration and erythema index were assessed since drying and redness are some of the benzoyl peroxide side effects. Moreover, skin hydration and TEWL are important to skin properties since they reflect skin barrier function. From the result of the study, the skin erythema of F1 was maintained and the TEWL values fluctuated throughout the study. However, the fluctuation was not significant. Besides, the skin hydration for F1 was improved throughout the study. Meanwhile, the skin erythema, hydration and TEWL for F2 were aggravated throughout the study. This means F2 might impair the skin. On the contrary, the skin erythema, hydration and TEWL for F3 and F4 were improved throughout the study. This was consistent with the skin irritation assessment since most skin irritations occurred after the F2 application. This result proved that stingless bee honey is able to reduce the side effects of benzoyl peroxide. In addition, the characteristics of all formulations including appearance, colour, stickiness, spreadability, absorption and odour were acceptable and tolerable since most of the volunteers categorised the characteristics of all formulations as good and excellent. In brief, this chapter proved the safety and effectiveness of F1 in reducing the skin irritation side effects of benzoyl peroxide.

CHAPTER SIX

GENERAL CONCLUSIONS AND FUTURE WORKS

6.1 GENERAL CONCLUSIONS

The present study aims to develop a benzoyl peroxide with stingless bee honey emulgel for acne vulgaris treatment. In order to achieve this objective, four different emulgel formulations were developed with the same excipients. F1 contained both benzoyl peroxide and stingless bee honey, F2 consisted of only benzoyl peroxide, F3 comprised solely stingless bee honey and F4 served as the base emulgel without any active ingredients. The preparation involved creating an oil-in-water emulsion and a base gel, and then combining the two. The benzoyl peroxide content in F1 and F2 was 105.20 ± 0.09% and 105.60 ± 0.11% respectively. All formulations had uniform, opaque and smooth textures. F1 and F3 had a cream-white colour and a honey smell while F2 and F4 were white and odourless. No separation occurred in any formulation after centrifugation. The pH values ranged from 4.8 to 5.9 and droplet sizes were below 1 µm for all formulations. The formulations had good polydispersity index (PDI) and zeta potential values which were less than 0.5 and more than -30 mV respectively. The spreadability was consistent among all formulations in the range of 5.78 to 5.94 g cm/s. Microbial limit tests showed no growth in Total Aerobic Microbial Count (TAMC), Total Yeast and Mold Count (TYMC), *Staphylococcus aureus* and *Pseudomonas aeruginosa* tests which indicated microbiological stability.

During the stability study, all formulations were stored in both real-time (30ºC/75%RH) and accelerated-time (40ºC/75%RH) chambers for three months. Various characteristics of the formulations such as benzoyl peroxide content, organoleptic properties, phase separation, pH, droplet size, PDI, zeta potential, spreadability and microbial limit count were evaluated at weeks 0, 2, 4 and 12 of the study. The benzoyl peroxide content in F1 and F2 decreased significantly in both chambers and the reduction was more pronounced in the accelerated-time chamber. However, the remaining benzoyl peroxide content of both formulations in both chambers was still within the acceptable levels. The organoleptic properties including colour, homogeneity, transparency and odour remained consistent for all formulations over the three-month storage in both chambers. No phase separation, creaming or foaming occurred after centrifugation at any sampling time point. Changes in pH, droplet size, PDI, zeta potential and spreadability were observed throughout the study with most changes being statistically significant especially in the accelerated-time chamber. However, these values remained within acceptable ranges and no physical instability observed during the study. Microbial growth also was not observed at any sampling time point. Overall, all four emulgel formulations exhibited stability in terms of physical, chemical and microbiological properties over three months of storage under both 30ºC/75%RH and 40ºC/75%RH conditions. However, room temperature storage condition was deemed more suitable for the emulgel formulations in order to preserve the formulations' stability.

Additionally, the antiacne property of raw stingless bee honey and all formulations were assessed against *P. acnes.* The antiacne activity of raw stingless bee honey was determined using the broth microdilution method to establish the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The concentration of stingless bee honey incorporated into the formulations was determined based on these MIC and MBC values. The results indicated that stingless bee honey could inhibit and eradicate *P. acnes* bacteria at concentrations of 12.5% and 15% respectively, affirming its antimicrobial potential against *P. acnes*. Therefore, a concentration of 15% stingless bee honey was included in the formulations. The antibacterial evaluation of all formulations was carried out using the agar well diffusion method. As anticipated, only F4 which lacked active ingredients did not exhibit any inhibition zone. F1 and F2 demonstrated larger inhibition zones compared to F3, likely due to the presence of benzoyl peroxide which is effective against *P. acnes.* The presence of an inhibition zone for F3 indicated that stingless bee honey retained its antibacterial activity in the formulated dosage form. This underscores the additional bactericidal activity of benzoyl peroxide when combined with stingless bee honey as evidenced by the significantly larger inhibition zone of F1 compared to F2.

Since stingless bee honey has been proposed as the ingredient to counter back the benzoyl peroxide side effects, hence the effectiveness needs to be assessed. Therefore, skin condition assessment by using DermaLab® Combo was performed in order to evaluate the effectiveness of stingless bee honey in reducing the side effects of benzoyl peroxide and the safety of the formulated emulgel on human skin. Three skin parameters which were hydration, transepidermal water loss (TEWL) and erythema index were evaluated. These parameters were chosen as benzoyl peroxide side effects often include drying and redness, hence hydration and erythema index were assessed. Additionally, skin hydration and TEWL reflect important aspects of skin barrier function. In this study, skin erythema remained stable and the TEWL values fluctuated insignificantly throughout the study for F1. This suggested that no disruption in skin barrier function. Moreover, skin hydration for F1 improved throughout the study, representing enhanced moisture levels in the skin. Meanwhile, skin erythema, hydration and TEWL worsened over the study period for F2 suggesting potential impairment to the skin. Conversely, improvements in skin erythema, hydration and TEWL were observed for F3 and F4. This proved the safety of the formulation's excipients including stingless bee honey. This finding was consistent with skin irritation assessments where most irritations occurred following F2 application, highlighting the role of stingless bee honey in mitigating benzoyl peroxide side effects. Furthermore, all formulations demonstrated acceptable characteristics in terms of appearance, colour, stickiness, spreadability, absorption and odour as most volunteers rated them as good or excellent. In short, the combination of stingless bee honey with benzoyl peroxide emulgel was successfully developed and effective in reducing the side effects of benzoyl peroxide.

6.2 FUTURE WORKS AND RECOMMENDATIONS

For future works, animal studies can be conducted to further evaluate the efficacy and safety of the formulated treatment for acne. This study should involve the application of the emulgel on animal models induced with acne-like conditions in order to closely mimic the human physiological response. These experiments would allow for a thorough assessment of the formulation's potential in treating acne lesions, its impact on inflammatory markers and its overall tolerance in vivo. This can be achieved by evaluating the number of acne lesions and histopathological assessment before and after the treatment. Furthermore, as part of the future research agenda, consideration should be given to upscaling the formulation since the stability of large scale may differ. Hence, a full characterisation and stability study of the optimised upscale formulation can be done to ensure the consistency and quality of the formulation on a large scale.

REFERENCES

Aarthi, R., Ramalingam, S., Periandy, S., & Senthil Kannan, K. (2018). Molecular structure-associated pharmacodynamic investigation on benzoyl peroxide using spectroscopic and quantum computational tools. *Journal of Taibah University for Science*, *12*(1), 104–122. https://doi.org/10.1080/16583655.2018.1451116

Ab Hadi, H., Omar, S. S. S., & Awadh, A. I. (2016). Honey, a Gift from Nature to Health and Beauty: A Review. *British Journal of Pharmacy*, *1*(1), 46–54. https://doi.org/10.5920/bjpharm.2016.05

Abd, E., Yousef, S. A., Pastore, M. N., Telaprolu, K., Mohammed, Y. H., Namjoshi, S., Grice, J. E., & Roberts, M. S. (2016). Skin models for the testing of transdermal drugs. *Clinical Pharmacology: Advances and Applications*, *8*, 163–176. https://doi.org/10.2147/CPAA.S64788

Abd Jalil, M. A. (2020). Development of PVA-natural biopolymer hydrogel incorporated with stingless bee honey for wound healing [International Islamic University Malaysia]. In *Doctoral Dissertation, International Islamic University Malaysia*. http://studentrepo.iium.edu.my/handle/123456789/10032

Abdlaty, R., & Fang, Q. (2021). Skin erythema assessment techniques. *Clinics in Dermatology*, *39*(4), 591–604. https://doi.org/10.1016/j.clindermatol.2021.03.006

Abed Savaya, N. S., Issa, R. A., & Talib, W. H. (2020). In vitro evaluation of the antioxidant, anti-Propioni bacterium acne and antityrosinase effects of Equisetum ramosissimum (Jordanian horsetail). *Tropical Journal of Pharmaceutical Research*, *19*(10), 2147–2152. https://doi.org/10.4314/tjpr.v19i10.19

Achermann, Y., Goldstein, E. J. C., Coenye, T., & Shirtliffa, M. E. (2014). Propionibacterium acnes: From Commensal to opportunistic biofilm-associated implant pathogen. *Clinical Microbiology Reviews*, *27*(3), 419–440. https://doi.org/10.1128/CMR.00092-13

Adejokun, D. A., & Dodou, K. (2020). Quantitative sensory interpretation of rheological parameters of a cream formulation. *Cosmetics*, *7*(2), 1–12. https://doi.org/10.3390/cosmetics7010002

Administration, F. and D. (2023). *CFR - Code of Federal Regulations Title 21*. U.S. Food and Drug Administration. https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm?fr=310.545

Aghasi, M., Golzarand, M., Shab-Bidar, S., Aminianfar, A., Omidian, M., & Taheri, F. (2019). Dairy intake and acne development: A meta-analysis of observational studies. *Clinical Nutrition*, *38*(3), 1067–1075. https://doi.org/10.1016/j.clnu.2018.04.015

Akbari, S., & Nour, A. H. (2018). Emulsion types, stability mechanisms and rheology: A review. *International Journal of Innovative Research and Scientific Studies*, *1*(1), 11–17. https://doi.org/10.53894/ijirss.v1i1.4

Akdogan, N., Dogan, S., Atakan, N., & Yalçin, B. (2018). Association of serum hormone levels with acne vulgaris: Low estradiol level can be a pathogenetic factor in female acne. *Our Dermatology Online*, *9*(3), 249–256. https://doi.org/10.7241/ourd.20183.4

Akinduti, P. A., Motayo, B., Idowu, O. M., Isibor, P. O., Olasehinde, G. I., Obafemi, Y. D., Ugboko, H. U., Oyewale, J. O., Oluwadun, A., & Adeyemi, G. A. (2019). Suitability of spectrophotometric assay for determination of honey microbial inhibition. *Journal of Physics: Conference Series*, *1299*(1). https://doi.org/10.1088/1742-6596/1299/1/012131

Alam, S., Algahtani, M. S., Ahmad, M. Z., & Ahmad, J. (2020). Investigation utilizing the HLB concept for the development of moisturizing cream and lotion: In-vitro characterization and stability evaluation. *Cosmetics*, *7*(2). https://doi.org/10.3390/COSMETICS7020043

Albaridi, N. A. (2019). Antibacterial Potency of Honey. *International Journal of Microbiology*, 1–10. https://doi.org/https://doi.org/10.1155/2019/2464507

Algahtani, M. S., Ahmad, M. Z., & Ahmad, J. (2020). Nanoemulgel for improved topical delivery of retinyl palmitate: Formulation design and stability evaluation. *Nanomaterials*, *10*(5), 848. https://doi.org/10.3390/nano10050848

Alkhabbaz, M., Saeed, M., & Albatineh, A. N. (2020). Predictors of Acne Vulgaris among Adolescents in Kuwait. *Medical Principle and Practice*, *29*, 310–317. https://doi.org/10.1159/000503711

Alquadeib, B. T. (2019). Development and validation of a new HPLC analytical method for the determination of diclofenac in tablets. *Saudi Pharmaceutical Journal*, *27*(1), 66–70. https://doi.org/10.1016/j.jsps.2018.07.020

Alshammrie, F. F., Alshammari, R., Alshammari, R., Khan, F. H., & Khan, F. H. (2020). Epidemiology of Acne Vulgaris and Its Association With Lifestyle Among Adolescents and Young Adults in Hail, Kingdom of Saudi Arabia: A Community-Based Study. *Cureus*, *12*(7), 1–15. https://doi.org/10.7759/cureus.9277

Alvarez-Rivera, G., Llompart, M., Lores, M., & Garcia-Jares, C. (2018). Preservatives in Cosmetics: Regulatory Aspects and Analytical Methods. In *Analysis of Cosmetic Products: Second Edition*. Elsevier B.V. https://doi.org/10.1016/B978-0-444-63508-2.00009-6

Alvarez-Suarez, J. M., Gasparrini, M., Forbes-Hernández, T. Y., Mazzoni, L., & Giampieri, F. (2014). The composition and biological activity of honey: A focus on manuka honey. *Foods*, *3*(3), 420–432. https://doi.org/10.3390/foods3030420

Alves, E., Gregório, J., Baby, A. R., Rijo, P., Rodrigues, L. M., & Rosado, C. (2021). Homemade kefir consumption improves skin condition—a study conducted in healthy and atopic volunteers. *Foods*, *10*(2794). https://doi.org/10.3390/foods10112794

Anarakdim, K., Gutiérrez, G., Cambiella, Á., Senhadji-Kebiche, O., & Matos, M. (2020).

The effect of emulsifiers on the emulsion stability and extraction efficiency of Cr(Vi) using emulsion liquid membranes (ELMS) formulated with a green solvent. *Membranes*, *10*(4). https://doi.org/10.3390/membranes10040076

Ancheria, R. K., Jain, S., Kumar, D., Soni, S. L., & Sharma, M. (2019). Devlopment and In vitro Evaluation of Nanosuspension Gel of Benzoyl Peroxide. *Asian Journal of Pharmaceutical Research and Development*, *7*(4), 49–55. https://doi.org/http://dx.doi.org/10.22270/ajprd.v7i4.515

Angie, E., Abdullah, H., Martinus, A. R., & Ikhtiari, R. (2019). The Potential of Forest Honeybee on Decreasing Malondialdehyde Levels in Adult with Mild Acne Vulgaris. *International Conference on Health Informatics and Medical Application Technology*, 309–316. https://doi.org/10.5220/0009490803090316

Anthonissen, M., Daly, D., Fieuws, S., Massagé, P., Van Brussel, M., Vranckx, J., & Van Den Kerckhove, E. (2013). Measurement of elasticity and transepidermal water loss rate of burn scars with the Dermalab®. *Burns*, *39*(3), 420–428. https://doi.org/10.1016/j.burns.2012.07.026

Arcari, T., Marie-Lucie Feger, Guerreiro, D. N., Wu, J., & O'Byrne, C. P. (2020). Comparative Review of the Responses of Listeria monocytogenes and Escherichia coli to Low pH Stress Talia. *Genes*, *11*(1330). https://doi.org/10.3390/genes11111330

Archibald, K., Coleman, R., & Drake, T. (2019). Replacing Animal Tests to Improve Safety for Humans. *Human-Animal Studies*, *22*, 417–442. https://doi.org/10.1163/9789004391192_019

Ardhany, S. D., Putra, C. D., & Novaryatiin, S. (2021). Modification of Anti-acne Bawang Dayak (Eleutherine bulbosa (Mill.)Urb.) Cream to Propionibacterium acnes. *Journal of Advanced Pharmaceutical Technology & Research*, *12*(1), 94–98. https://doi.org/https://dx.doi.org/10.4103%2Fjaptr.JAPTR_107_20

ASEAN. (2013). ASEAN guideline on stability study of drug product. *20th ACCSQ-PPWG Meeting, Bali, Indonesia*, *May*, 1–40.

Aslan Kayiran, M., Karadag, A. S., & Jafferany, M. (2020). Psychodermatology of acne: Dermatologist's guide to inner side of acne and management approach. *Dermatologic Therapy*, *33*(6). https://doi.org/10.1111/dth.14150

Athirah, A., Yusof, B., Ajit, A. B., Sulaiman, A. Z., & Naila, A. (2018). Production of Lip Balm From Stingless Bee Honey. *The Maldives National Journal of Research*, *1*(1), 57–72.

Bachhav, J. K. (2017). Formulation and Evaluation of Topical Emulgel of Ketoconazole By Cubosomal Technique. *World Journal of Pharmaceutical Research*, *6*(10), 567–588. https://doi.org/10.20959/wjpr201710-9221

Bandyopadhyay, D. (2021). Topical Antibacterials in Dermatology. *Indian Journal of Dermatology*, *6*(22), 117–125. https://doi.org/10.4103/ijd.IJD_99_18

Barange, H., Asghar, S., & Sheikh, U. (2018). Development of Analytical Method for Simultaneous Estimation of Adapalene and Benzoyl Peroxide in Gel Formulation by

RP-HPLC. *Global Journal of Pharmacy & Pharmaceutical Sciences*, *4*(3), 1–7. https://doi.org/10.19080/gjpps.2018.04.555639

Basson, R., Baguneid, M., Foden, P., Al Kredly, R., & Bayat, A. (2019). Functional Testing of a Skin Topical Formulation in Vivo: Objective and Quantitative Evaluation in Human Skin Scarring Using a Double-Blind Volunteer Study with Sequential Punch Biopsies. *Advances in Wound Care*, *8*(5), 208–219. https://doi.org/10.1089/wound.2018.0864

Ben Braïek, O., & Smaoui, S. (2021). Chemistry, Safety, and Challenges of the Use of Organic Acids and Their Derivative Salts in Meat Preservation. *Journal of Food Quality*, *6653190*. https://doi.org/10.1155/2021/6653190

Bensid, A., El Abed, N., Houicher, A., Regenstein, J. M., & Özogul, F. (2022). Antioxidant and antimicrobial preservatives: Properties, mechanism of action and applications in food–a review. *Critical Reviews in Food Science and Nutrition*, *62*(11), 2985–3001. https://doi.org/10.1080/10408398.2020.1862046

Bhangare, D., Rajput, N., Jadav, T., Sahu, A. K., Tekade, R. K., & Sengupta, P. (2022). Systematic strategies for degradation kinetic study of pharmaceuticals: an issue of utmost importance concerning current stability analysis practices. *Journal of Analytical Science and Technology*, *13*(1). https://doi.org/10.1186/s40543-022-00317-6

Bhat, Y. J., Latief, I., & Hassan, I. (2017). Update on etiopathogenesis and treatment of Acne. *Indian Journal of Dermatology, Venereology and Leprology*, *83*(3), 298–306. https://doi.org/10.4103/0378-6323.199581

Biluca, F. C., da Silva, B., Caon, T., Mohr, E. T. B., Vieira, G. N., Gonzaga, L. V., Vitali, L., Micke, G., Fett, R., Dalmarco, E. M., & Costa, A. C. O. (2020). Investigation of phenolic compounds, antioxidant and anti-inflammatory activities in stingless bee honey (Meliponinae). *Food Research International*, *129*, 108756. https://doi.org/10.1016/j.foodres.2019.108756

Boonchaya, P., Rojhirunsakool, S., Kamanamool, N., & Khunkhet, S. (2022). 10 % Benzoyl Peroxide for a Bactericidal Effect Against Cutibacterium acnes. *Clinical, Cosmetic and Investigational Dermatology*, *15*, 403–409.

Bowe, W. P., & Logan, A. C. (2010). Clinical implications of lipid peroxidation in acne vulgaris : old wine in new bottles. *Lipids in Health and Disease*, *9*(141), 1–11. https://doi.org/10.1186/1476-511X-9-141

Bragazzi, N. L., Sellami, M., Salem, I., Conic, R., Kimak, M., Daniele, P., Pigatto, M., & Damiani, G. (2019). Fasting and Its Impact on Skin Anatomy , Physiology , and Physiopathology : A Comprehensive Review of the Literature. *Nutrients*, *11*(249), 1–15. https://doi.org/10.3390/nu11020249

Briganti, S., & Picardo, M. (2003). Antioxidant activity, lipid peroxidation and skin diseases. What's new. *Journal of the European Academy of Dermatology and Venereology*, *17*(6), 663–669. https://doi.org/10.1046/j.1468-3083.2003.00751.x

Briganti, Stefania, Flori, E., Mastrofrancesco, A., & Ottaviani, M. (2020). Acne as an altered dermato-endocrine response problem. *Experimental Dermatology*, *29*(9), 833–839. https://doi.org/10.1111/exd.14168

Brown, L. (2020). Acne and its management. *South African Pharmaceutical Journal Pharmaceutical Journal*, *87*(5), 37A-37H.

Burnett, C. L., Bergfeld, W. F., Belsito, D. V., Hill, R. A., Klaassen, C. D., Liebler, D. C., Marks, J. G., Shank, R. C., Slaga, T. J., Snyder, P. W., Gill, L. J., & Heldreth, B. (2021). Safety Assessment of Inorganic Hydroxides as Used in Cosmetics. *International Journal of Toxicology*, *40*(2_suppl), 16S-35S. https://doi.org/10.1177/10915818211018381

Carrer, V., Alonso, C., Pont, M., Zanuy, M., Córdoba, M., Espinosa, S., Barba, C., Oliver, M. A., Martí, M., & Coderch, L. (2020). Effect of propylene glycol on the skin penetration of drugs. *Archives of Dermatological Research*, *312*(5), 337–352. https://doi.org/10.1007/s00403-019-02017-5

CDC. (2016). *Chemical Disinfectants*. Centers for Disease Control and Prevention. https://www.cdc.gov/infectioncontrol/guidelines/disinfection/disinfection-methods/chemical.html#:~:text=Hydrogen peroxide works by producing,and other essential cell components.

CDC. (2021). Colonial characteristics. In *CDC. gov*. https://www.cdc.gov/labtraining/docs/job_aids/biochemicals_gram_positive_organism_id/Colonial_Characteristics_Branded_508.pdf

Cerff, B., Key, D., & Bladergroen, B. (2021). A review of the processes associated with the removal of oil in water pollution. *Sustainability (Switzerland)*, *13*(22). https://doi.org/10.3390/su132212339

Chandra, M. A. (2023). Identification of bacterial morphology and catalase coagulation test on propionibacterium acnes bacteria. *Journal of Health Management and Pharmacy Exploration*, *1*(2), 45–50. https://doi.org/https://doi.org/10.52465/johmpe.v1i2.152

Chen, Y. C., Tsai, P. J., Huang, Y. Bin, & Wu, P. C. (2015). Optimization and validation of high-performance chromatographic condition for simultaneous determination of adapalene and benzoyl peroxide by response surface methodology. *PLoS ONE*, *10*(3), 1–9. https://doi.org/10.1371/journal.pone.0120171

Chong, K. Y., Chin, N. L., Yusof, Y. A., & Fakurazi, S. (2021). Anti-inflammatory activity of raw and processed stingless bee honey. *Food Research*, *5*, 201–208. https://doi.org/10.26656/fr.2017.5(S1).007

Chou, W.-M., Liao, H.-C., Yang, Y.-C., & Peng, C.-C. (2020). Evaluation of Honey Quality with Stored Time and Temperatures. *Journal of Food and Nutrition Research*, *8*(10), 591–599. https://doi.org/10.12691/jfnr-8-10-8

Claudel, J. P., Auffret, N., Leccia, M. T., Poli, F., & Dréno, B. (2018). Acne and nutrition: hypotheses, myths and facts. *Journal of the European Academy of Dermatology and Venereology*, *32*(10), 1631–1637. https://doi.org/10.1111/jdv.14998

Clayton, R. W., Göbel, K., Niessen, C. M., Paus, R., van Steensel, M. A. M., & Lim, X. (2019). Homeostasis of the sebaceous gland and mechanisms of acne pathogenesis. *British Journal of Dermatology*, *181*(4), 677–690. https://doi.org/10.1111/bjd.17981

Clinical Practice Guideline. (2022). *Management of Acne Vulguris* (2nd ed.). Malaysian Health Technology Assessment Section (MaHTAS).

Cong, T. X., Hao, D., Wen, X., Li, X. H., He, G., & Jiang, X. (2019). From pathogenesis of acne vulgaris to anti-acne agents. *Archives of Dermatological Research*, *311*(5), 337–349. https://doi.org/10.1007/s00403-019-01908-x

Cortex Technology. (2012). *DermaLab® Series SkinLab Combo Instruction Manual*. 38.

Cosmetic-Ingredient-Review. (2019). *Safety Assessment of Honey Derived-Ingredients as Used in Cosmetics*.

Costa, C., Medronho, B., Filipe, A., Mira, I., Lindman, B., Edlund, H., & Norgren, M. (2019). Emulsion formation and stabilization by biomolecules: The leading role of cellulose. *Polymers*, *11*(10), 1–18. https://doi.org/10.3390/polym11101570

Costeris, C., Petridou, M., & Ioannou, Y. (2021). Psychological Impact of Skin Disorders on Patients ' Self-esteem and Perceived Social Support. *Journal of Dermatollogy & Skin Science*, *3*(1), 14–22.

Cruz, S., Vecerek, N., & Elbuluk, N. (2023). Targeting Inflammation in Acne: Current Treatments and Future Prospects. *American Journal of Clinical Dermatology*, *24*(5), 681–694. https://doi.org/10.1007/s40257-023-00789-1

da Cunha, M. G., Daza, F., Filho, C. D. A. M., da Veiga, G. L., & Fonseca, F. (2018). The relevance of sebum composition in the etiopathogeny of acne. *European Journal of Biological Research*, *8*(1), 21–25. https://doi.org/http://dx.doi.org/10.5281/zenodo.1184139

da Silva, P. M., Gauche, C., Gonzaga, L. V., Costa, A. C. O., & Fett, R. (2016). Honey: Chemical composition, stability and authenticity. *Food Chemistry*, *196*, 309–323. https://doi.org/10.1016/j.foodchem.2015.09.051

David, M. N. V., & Akhondi, H. (2022). Emulsions. *Emulsions*, 1–735. https://doi.org/10.1016/C2015-0-01030-9

Ding, Z., Jiang, Y., & Liu, X. (2018). Nanoemulsions-based drug delivery for brain tumors. In *Nanotechnology-Based Targeted Drug Delivery Systems for Brain Tumors*. Elsevier Inc. https://doi.org/10.1016/B978-0-12-812218-1.00012-9

Djakaria, S. A., Batubara, I., & Raffiudin, R. (2020). Antioxidant and Antibacterial Activity of Selected Indonesian Honey against Bacteria of Acne. *Jurnal Kimia Sains Dan Aplikasi*, *23*(8), 267–275. https://doi.org/10.14710/jksa.23.8.267-275

Dreno, B., Bagatin, E., Blume-Peytavi, U., Rocha, M., & Gollnick, H. (2018). Female type of adult acne: Physiological and psychological considerations and management. *JDDG - Journal of the German Society of Dermatology*, *16*(10), 1185–1194. https://doi.org/10.1111/ddg.13664

Dreno, B., Bissonnette, R., Gagne-Henley, A., Barankin, B., Lynde, C., Kerrouche, N., & Tan, J. (2018). Prevention and Reduction of Atrophic Acne Scars with Adapalene 0 . 3 %/ Benzoyl Peroxide 2 . 5 % Gel in Subjects with Moderate or Severe Facial Acne : Results of a 6-Month Randomized , Vehicle-Controlled Trial Using Intra-Individual Comparison. *American Journal of Clinical Dermatology*, *19*(2), 275–286. https://doi.org/10.1007/s40257-018-0352-y

Dréno, B., Zuberbier, T., Gelmetti, C., Gontijo, G., & Marinovich, M. (2019). Safety review of phenoxyethanol when used as a preservative in cosmetics. *Journal of the European Academy of Dermatology and Venereology*, *33*(S7), 15–24. https://doi.org/10.1111/jdv.15944

Dréno, Brigitte, Araviiskaia, E., Kerob, D., Andriessen, A., Anfilova, M., Arenbergerova, M., Forero Barrios, O. L., Bukvić Mokos, Z., Haedersdal, M., Hofmann, M. A., Khamaysi, Z., Kosmadaki, M., Lesiak, A., Roó, E., Zbranca-Toporas, A., Wiseman, M. C., Zimmo, S., Guerin, L., & Fabbrocini, G. (2020). Nonprescription acne vulgaris treatments: Their role in our treatment armamentarium—An international panel discussion. *Journal of Cosmetic Dermatology*, *19*(9), 2201–2211. https://doi.org/10.1111/jocd.13497

Duffy, E., De Guzman, K., Wallace, R., Murphy, R., & Morrin, A. (2017). Non-invasive assessment of skin barrier properties: Investigating emerging tools for in vitro and in vivo applications. *Cosmetics*, *4*(4). https://doi.org/10.3390/cosmetics4040044

Eady, E. A., Layton, A. M., & Cove, J. H. (2013). A honey trap for the treatment of acne: Manipulating the follicular microenvironment to control propionibacterium acnes. *BioMed Research International*, *679680*, 1–8. https://doi.org/10.1155/2013/679680

Eid, A. M., Naseef, H., Jaradat, N., Ghanim, L., Moqadeh, R., & Yaseen, M. (2023). Antibacterial and Anti-Acne Activity of Benzoyl Peroxide Nanoparticles Incorporated in Lemongrass Oil Nanoemulgel. *Gels*, *9*(3), 1–12. https://doi.org/10.3390/gels9030186

El-Kased, R. F., Amer, R. I., Attia, D., & Elmazar, M. M. (2017). Honey-based hydrogel: In vitro and comparative in vivo evaluation for burn wound healing. *Scientific Reports*, *7*(1), 1–11. https://doi.org/10.1038/s41598-017-08771-8

Emmerich, V. K., Purvis, C. G., & Feldman, S. R. (2021). An overview of adapalene and benzoyl peroxide once-daily topical gel as a therapeutic option for acne. *Expert Opinion on Pharmacotherapy*, *22*(13), 1661–1667. https://doi.org/10.1080/14656566.2021.1939678

Fatima, I. J., Mohd Hilmi, A. B., Salwani, I., & Lavaniya, M. (2018). Physicochemical characteristics of Malaysian stingless bee honey from Trigona species. *IIUM Medical Journal Malaysia*, *17*(1), 187–191. https://doi.org/https://doi.org/10.31436/imjm.v17i1.1030

FDA. (2011). *Guidance for Industry Topical Acne Drug Products for Over-the-Counter Human Use-Revision of Labeling and Classification of Benzoyl Peroxide as Safe and Effective Small Entity Compliance Guide*.

http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htm

Febriyenti, F., Lucida, H., Almahdy, A., Alfikriyah, I., & Hanif, M. (2019). Wound-Healing Effect of Honey Gel and Film. *Journal of Pharmacy & Bioallied Sciences*, *11*(2), 176–180. https://doi.org/https://dx.doi.org/10.4103%2Fjpbs.JPBS_184_18

Fletcher, M. T., Hungerford, N. L., Webber, D., Jesus, M. C. De, Zhang, J., Stone, I. S. J., Blanchfield, J. T., & Zawawi, N. (2020). Stingless bee honey, a novel source of trehalulose : a biologically active disaccharide with health benefits. *Scientific Reports*, *10*(1), 1–8. https://doi.org/10.1038/s41598-020-68940-0

Gabrič, A., Hodnik, Ž., & Pajk, S. (2022). Oxidation of Drugs during Drug Product Development: Problems and Solutions. *Pharmaceutics*, *14*(2). https://doi.org/10.3390/pharmaceutics14020325

George, R. M., & Sridharan, R. (2018). Factors Aggravating or Precipitating Acne in Indian Adults: A Hospital-Based Study of 110 Cases. *Indian Journal of Dermatology*, *63*(4), 328–331. https://doi.org/https://dx.doi.org/10.4103%2Fijd.IJD_565_17

Gill, V., & Nanda, A. (2020). Preparation and characterization of etodolac bearing emulsomes. *International Journal of Applied Pharmaceutics*, *12*(5), 166–172. https://doi.org/10.22159/ijap.2020v12i5.38842

Gold, L. S., Kwong, P., Draelos, Z., Arekapudi Krysten, Levy-Hacham, O., Erlich, M., & Desai, S. R. (2023). Impact of topical vehicles and cutaneous delivery technologies on patient adherence and treatment outcomes in acne and rosacea. *Journal of Clinical and Aesthetic Dermatology*, *16*(5), 26–34.

Goyani, M., Akbari, B., Chaudhari, S., & Jivawala, R. (2018). FORMULATION AND EVALUATION OF TOPICAL EMULGEL OF ANTIACNE AGENT. *International Journal of Advanced Research and Review*, *3*(7), 52–68. www.ijarr.in

Greydanus, D. E., Azmeh, R., Cabral, M. D., Dickson, C. A., & Patel, D. R. (2021). Acne in the first three decades of life: An update of a disorder with profound implications for all decades of life. *Disease-a-Month*, *67*(4), 101103. https://doi.org/10.1016/j.disamonth.2020.101103

Grobel, H., & Murphy, S. A. (2018). Acne Vulgaris and Acne Rosacea. In D. Rakel (Ed.), *Integrative Medicine* (Fourth). Elsevier Inc. https://doi.org/10.1016/B978-0-323-35868-2.00077-3

Gul, R., Khan, Y., & Aman, T. (2022). Formulation and Evaluation of Bisoprolol Hemifumarate Emulgel for Transdermal Drug Delivery. *Dissolution Technologies*, *29*(1), 38–44. https://doi.org/10.14227/DT290522P38

Guo, X. X., Hu, W., Liu, Y., Sun, S. Q., Gu, D. C., He, H., Xu, C. H., & Wang, X. C. (2016). Rapid determination and chemical change tracking of benzoyl peroxide in wheat flour by multi-step IR macro-fingerprinting. *Spectrochimica Acta - Part A: Molecular and Biomolecular Spectroscopy*, *154*, 123–129. https://doi.org/10.1016/j.saa.2015.10.017

Hadi, H, Awadh, A. I., Hanif, N. M., Sidik, N. F. A. M., Rani, M. R. N. M., & Suhaimi, M. S. M. (2016). The investigation of the skin biophysical measurements focusing on daily activities, skin care habits, and gender differences. *Skin Research and Technology*, *22*(2), 247–254. https://doi.org/10.1111/srt.12257

Hadi, Hazrina, Razali, S. N. S., & Awadh, A. I. (2015). A comprehensive review of the cosmeceutical benefits of vanda species (orchidaceae). *Natural Product Communications*, *10*(8), 1483–1488. https://doi.org/10.1177/1934578x1501000842

Halla, N., Fernandes, I. P., Heleno, S. A., Costa, P., Boucherit-Otmani, Z., Boucherit, K., Rodrigues, A. E., Ferreira, I. C. F. R., & Barreiro, M. F. (2018). Cosmetics preservation: A review on present strategies. *Molecules*, *23*(7), 1–41. https://doi.org/10.3390/molecules23071571

Hassan, R. A., Nofal, A. A., & Hussien, N. N. (2019). Prevalence and Risk Factors of Acne Vulgaris in Adolescents in Belbis City, Sharkia Governorate, Egypt: A Cross-Sectional Study. *Egyptian Family Medicine Journal (EFMJ)*, *3*(1), 1–14.

Hassounah, I. A., Shehata, N. A., Kimsawatde, G. C., Hudson, A. G., Sriranganathan, N., Joseph, E. G., & Mahajan, R. L. (2016). Designing and testing single tablet for tuberculosis treatment through electrospinning. In *Fabrication and Self-Assembly of Nanobiomaterials: Applications of Nanobiomaterials*. Elsevier Inc. https://doi.org/10.1016/B978-0-323-41533-0.00011-8

Heng, A. H. S., & Chew, F. T. (2020). Systematic review of the epidemiology of acne vulgaris. *Scientific Reports*, *10*(1), 1–29. https://doi.org/10.1038/s41598-020-62715-3

Herbig, M. E., Evers, D.-H., Gorissen, S., & Köllmer, M. (2023). Rational Design of Topical Semi-Solid Dosage Forms-How Far Are We? *Pharmaceutics*, *15*(7), 1822. https://doi.org/10.3390/pharmaceutics15071822

Hernandez, P., Sager, B., Fa, A., Liang, T., Lozano, C., & Khazzam, M. (2019). Bactericidal efficacy of hydrogen peroxide on Cutibacterium acnes. *Bone & Joint Research*, *8*(1), 3–10. https://doi.org/10.1302/2046-3758.81.bjr-2018-0145.r1

Hew, S. Y., Goh, Y. C., & Tan, H. Y. (2023). Effects of Lactobacillus plantarum TAR4, TAR7 and TAR8 Isolated From Tapai On The Growth Of Cutibacterium acnes. *International Journal of Application on Sciences, Technology and Engineering*, *1*(1), 23–27. https://doi.org/10.24912/ijaste.v1.i1.23-27

Holmes, S. (2019). An overview of current surgical instrument and other medical device decontamination practices. In *Decontamination in Hospitals and Healthcare* (Second Edi, Vol. 2017). Elsevier Ltd. https://doi.org/10.1016/B978-0-08-102565-9.00020-0

Honari, G., & Maibach, H. (2014). Skin Structure and Function. In H. Maibach (Ed.), *Applied Dermatotoxicology* (pp. 1–10). Elsevier Inc. https://doi.org/10.1016/B978-0-12-420130-9.00001-3

Horakova, J., Klicova, M., Erben, J., Klapstova, A., Novotny, V., Behalek, L., & Chvojka, J. (2020). Impact of Various Sterilization and Disinfection Techniques on Electrospun

Poly-ϵ-caprolactone. *ACS Omega*, *5*(15), 8885–8892. https://doi.org/10.1021/acsomega.0c00503

Hossain, M. L., Lim, L. Y., Hammer, K., Hettiarachchi, D., & Locher, C. (2022). A Review of Commonly Used Methodologies for Assessing the Antibacterial Activity of Honey and Honey Products. *Antibiotics*, *11*(7). https://doi.org/10.3390/antibiotics11070975

Hossain, M. L., Lim, L. Y., Hammer, K., Hettiarachchi, D., & Locher, C. (2023). Design, Preparation, and Physicochemical Characterisation of Alginate-Based Honey-Loaded Topical Formulations. *Pharmaceutics*, *15*(5), 1–26. https://doi.org/10.3390/pharmaceutics15051483

Hu, W., Wu, Y., Chen, H., Gao, J., & Tong, P. (2022). Effects of Glucose and Homogenization Treatment on the Quality of Liquid Whole Eggs. *Foods*, *11*(16). https://doi.org/10.3390/foods11162521

Hua, W., Fan, L. M., Dai, R., Luan, M., Xie, H., Li, A. Q., & Li, L. (2017). Comparison of two series of non-invasive instruments used for the skin physiological properties measurements: the DermaLab® from Cortex Technology vs. the series of detectors from Courage & Khazaka. *Skin Research and Technology*, *23*(1), 70–78. https://doi.org/10.1111/srt.12303

Huang, X., Sun, J., Lv, K., Liu, J., & Shen, H. (2018). An alternative method to enhance w/o emulsion stability using modified dimer acid and its application in oil based drilling fluids. *RSC Advances*, *8*(46), 26318–26324. https://doi.org/10.1039/c8ra02293c

Hwang, J. H., Lee, S., Lee, H. G., Choi, D., & Lim, K. M. (2022). Evaluation of Skin Irritation of Acids Commonly Used in Cleaners in 3D-Reconstructed Human Epidermis Model, KeraSkinTM. *Toxics*, *10*(10). https://doi.org/10.3390/toxics10100558

Iijima, S., & Tsunoda, T. (2019). Twenty cases of allergic contact dermatitis due to benzoyl peroxide in acne patients in Japan. *Journal of Cutaneous Immunology and Allergy*, *2*(4), 108–112. https://doi.org/10.1002/cia2.12069

Iizaka, S. (2017). Skin hydration and lifestyle-related factors in community-dwelling older people. *Archives of Gerontology and Geriatrics*, *72*(January), 121–126. https://doi.org/10.1016/j.archger.2017.05.016

Ilomuanya, M. O., Ogbewele, G. E., Osei, Y., Ubani-Ukoma, U. C., & Sowemimo, A. (2018). Optimisation of clotrimazole emulgel using essential oils of Citrus sinensis and Zingiber officinale as permeation enhancers. *West African Journal of Pharmacy*, *29*(2), 19–33. https://www.researchgate.net/publication/342007741

International, C. on H. (ICH). (2003). *Guidance for industry Q1A(R2) stability testing of New Drug Substances and Products*.

Ismail, N. I., Kadir, M. R. A., Zulkifli, R. M., & Mohamed, M. (2021). Comparison of physicochemical, total protein and antioxidant profiles between Malaysian apis and trigona honeys. *Malaysian Journal of Analytical Sciences*, *25*(2), 243–256.

Iyer, V., Cayatte, C., Guzman, B., Schneider-Ohrum, K., Matuszak, R., Snell, A., Rajani, G. M., McCarthy, M. P., & Muralidhara, B. (2015). Impact of formulation and particle size on stability and immunogenicity of oil-in-water emulsion adjuvants. *Human Vaccines and Immunotherapeutics*, *11*(7), 1853–1864. https://doi.org/10.1080/21645515.2015.1046660

Jadhav, K. L., Kapare, P. R., Khairmode, D. V, Vhatkar, N., Raut, S., & Mali, A. S. (2018). Development of Cosmeceutical Cream for Dermatitis and Acne vulgaris. *Research Journal of Topical and Cosmetic Sciences*, *9*(1), 33. https://doi.org/10.5958/2321-5844.2018.00007.9

Jalian, H. R., Takahashi, S., & Kim, J. (2006). Overview of dermatological diseases. *Comprehensive Medicinal Chemistry II*, *7*, 935–955. https://doi.org/10.1016/b0-08-045044-x/00235-2

Jalil, M. A. A., Kasmuri, A. R., & Hadi, H. (2017). Stingless Bee Honey , the Natural Wound Healer : A Review. *Skin Pharmacology*, *30*, 66–75. https://doi.org/10.1159/000458416

Jantarat, C., Sirathanarun, P., Chuchue, T., Konpian, A., Sukkua, G., & Wongprasert, P. (2018). In vitro antimicrobial activity of gel containing the herbal ball extract against propionibacterium acnes. *Scientia Pharmaceutica*, *86*(1), 1–9. https://doi.org/10.3390/scipharm86010008

Javed, H., Shah, S. N. H., & Iqbal, F. M. (2018). Formulation Development and Evaluation of Diphenhydramine Nasal Nano-Emulgel. *American Association of Pharmaceutical Scientists*, *19*(4), 1730–1743. https://doi.org/10.1208/s12249-018-0985-4

Jibril, F. I., Hilmi, A. B. M., & Aliyu, S. (2020). Effect of Non-hydrogen Peroxide on Antibacterial Activity of Malaysian Meliponini Honey against Staphylococcus aureus. *Jpurnal of Pharmacy & Bioallied Sciences*, *12*(2), :S831-S835. https://doi.org/10.4103/jpbs.JPBS_280_19

Jin, Q., & Kirk, M. F. (2018). pH as a primary control in environmental microbiology: 1. thermodynamic perspective. *Frontiers in Environmental Science*, *6*, 1–15. https://doi.org/10.3389/fenvs.2018.00021

Joseph, E., & Singhvi, G. (2019). Multifunctional nanocrystals for cancer therapy: A potential nanocarrier. In *Nanomaterials for Drug Delivery and Therapy*. Elsevier Inc. https://doi.org/10.1016/B978-0-12-816505-8.00007-2

Jović, A., Marinović, B., Kostović, K., Čeović, R., Basta-Juzbašić, A., & Mokos, Z. B. (2017). The impact of pyschological stress on acne. *Acta Dermatovenerologica Croatica*, *25*(2), 133–141.

Julianti, E., Rajah, K. K., & Fidrianny, I. (2017). Antibacterial Activity of Ethanolic Extract of Cinnamon Bark , Honey , and Their Combination Effects against Acne-Causing Bacteria. *Scientia Pharmaceutica*, *85*(19), 1–8. https://doi.org/10.3390/scipharm85020019

Julious, S. A. (2005). Sample size of 12 per group rule of thumb for a pilot study.

Pharmaceutical Statistics, *4*(4), 287–291. https://doi.org/10.1002/pst.185

Jusuf, N. K., Putra, I. B., & Sari, L. (2020). Differences of microbiomes found in non-inflammatory and inflammatory lesions of acne vulgaris. *Clinical, Cosmetic and Investigational Dermatology*, *13*, 773–780. https://doi.org/10.2147/CCID.S272334

Kaewsanit, T., Chakkavittumrong, P., & Waranuch, N. (2021). Clinical comparison of topical 2.5% benzoyl peroxide plus 5% niacinamide to 2.5% benzoyl peroxide alone in the treatment of mild to moderate facial acne vulgaris. *Journal of Clinical and Aesthetic Dermatology*, *14*(6), 35–41.

Kamra, M., & Diwan, A. (2017). Acne: current perspective. *Journal of Applied Pharmaceutical Research*, *5*(3), 1–7. https://doi.org/10.18231/2348-0335.2017.0001

Kamra, M., Diwan, A., & Sardana, S. (2018). Development and Validation of Reversed-phase High-performance Liquid Chromatography Method for the Simultaneous Estimation of Benzoyl Peroxide and Resveratrol. *International Journal of Pharmaceutical & Biological Archives*, *9*(2), 113–121. www.ijpba.info

Kanlayavattanakul, M., & Lourith, N. (2011). Therapeutic agents and herbs in topical application for acne treatment. *International Journal of Cosmetic Science*, *33*(4), 289–297. https://doi.org/10.1111/j.1468-2494.2011.00647.x

Kanwar, I. L., Haider, T., Kumari, A., Dubey, S., Jain, P., & Soni, V. (2018). Models for acne: A comprehensive study. *Drug Discoveries & Therapeutics*, *12*(6), 329–340. https://doi.org/10.5582/ddt.2018.01079

Kar, M., Chourasiya, Y., Maheshwari, R., & Tekade, R. K. (2019). Current Developments in Excipient Science : Implication of Quantitative Selection of Each Excipient in Product Development. In *Basic Fundamentals of Drug Delivery*. Elsevier Inc. https://doi.org/10.1016/B978-0-12-817909-3.00002-9

Kara, Y. A., & Ozdemir, D. (2020). Evaluation of food consumption in patients with acne vulgaris and its relationship with acne severity. *Journal of Cosmetic Dermatology*, *19*(8), 2109–2113. https://doi.org/10.1111/jocd.13255

Kaur, M., Mohan, G., Kumar, S., Sharma, O. P., & Pruthi, S. (2020). Uses of Bee Honey: Traditional and Modern. *Biotica Research Today*, *2*(9), 946–949.

Kawashima, M., Nagare, T., & Doi, M. (2017). Clinical efficacy and safety of benzoyl peroxide for acne vulgaris: Comparison between Japanese and Western patients. *Journal of Dermatology*, *44*(11), 1212–1218. https://doi.org/10.1111/1346-8138.13996

Kawashima, M., Sato, S., Furukawa, F., Matsunaga, K., Akamatsu, H., Igarashi, A., Tsunemi, Y., Hayashi, N., Yamamoto, Y., Nagare, T., & Katsuramaki, T. (2017). Twelve-week, multicenter, placebo-controlled, randomized, double-blind, parallel-group, comparative phase II/III study of benzoyl peroxide gel in patients with acne vulgaris: A secondary publication. *Journal of Dermatology*, *44*(7), 774–782. https://doi.org/10.1111/1346-8138.13798

Khan, B. A., Ahmad, S., Khan, M. K., Hosny, K. M., Bukhary, D. M., Iqbal, H., Murshid,

S. S., Halwani, A. A., Alissa, M., & Menaa, F. (2022). Fabrication and Characterizations of Pharmaceutical Emulgel Co-Loaded with Naproxen-Eugenol for Improved Analgesic and Anti-Inflammatory Effects. *Gels*, *8*(10). https://doi.org/10.3390/gels8100608

Kim, H. W., Seok, Y. S., Cho, T. J., & Rhee, M. S. (2020). Risk factors influencing contamination of customized cosmetics made on-the-spot: Evidence from the national pilot project for public health. *Scientific Reports*, *10*(1), 1–9. https://doi.org/10.1038/s41598-020-57978-9

Kosmadaki, M., & Katsambas, A. (2017). Topical treatments for acne. *Clinics in Dermatology*, *35*(2), 173–178. https://doi.org/10.1016/j.clindermatol.2016.10.010

Król, M., & Kepinska, M. (2021). Human nitric oxide synthase—its functions, polymorphisms, and inhibitors in the context of inflammation, diabetes and cardiovascular diseases. *International Journal of Molecular Sciences*, *22*(1), 1–18. https://doi.org/10.3390/ijms22010056

Kumar Yadav, S., Kumar Mishra, M., Tiwari, A., & Shukla, A. (2017). Emulgel: a New Approach for Enhanced Topical Drug Delivery. *International Journal of Current Pharmaceutical Research*, *9*(1), 15–19. https://doi.org/10.22159/ijcpr.2017v9i1.16628

Kusuma, S. A. F., Abdassah, M., & Valas, B. E. (2018). Formulation and evaluation of anti acne gel containing citrus aurantifolia fruit juice using carbopol as gelling agent. *International Journal of Applied Pharmaceutics*, *10*(4), 147–152. https://doi.org/10.22159/ijap.2018v10i4.26788

Lai-Cheong, J. E., & McGrath, J. A. (2017). Structure and function of skin, hair and nails. *Medicine (United Kingdom)*, *45*(6), 347–351. https://doi.org/10.1016/j.mpmed.2017.03.004

Lam Hoai, X. L., De Maertelaer, V., & Simonart, T. (2021). Real-world adherence to topical therapies in patients with moderate acne. *JAAD International*, *2*, 109–115. https://doi.org/10.1016/j.jdin.2020.12.006

Lambrechts, I. A., de Canha, M. N., & Lall, N. (2018). Exploiting medicinal plants as possible treatments for acne vulgaris. In N. Lall (Ed.), *Medicinal Plants for Holistic Health and Well-Being* (pp. 117–143). Elsevier Inc. https://doi.org/10.1016/B978-0-12-812475-8.00004-4

Langsrud, S., Steinhauer, K., Lüthje, S., Weber, K., Goroncy-Bermes, P., & Holck, A. L. (2016). Ethylhexylglycerin impairs membrane integrity and enhances the lethal effect of phenoxyethanol. *PLoS ONE*, *11*(10), 1–16. https://doi.org/10.1371/journal.pone.0165228

Lemos, M. S., Venturieri, G. C., Dantas Filho, H. A., & Dantas, K. G. F. (2018). Evaluación de los parámetros físico-químicos y constituyentes inorgánicos de mieles de la región amazónica. *Journal of Apicultural Research*, *57*(1), 135–144. https://doi.org/10.1080/00218839.2017.1338120

Li, X., He, C., Chen, Z., Zhou, C., Gan, Y., & Jia, Y. (2017). A review of the role of sebum in the mechanism of acne pathogenesis. *Journal of Cosmetic Dermatology*, *16*(2), 168–173. https://doi.org/10.1111/jocd.12345

Liang, Y., Matia-Merino, L., Patel, H., Ye, A., Gillies, G., & Golding, M. (2014). Effect of sugar type and concentration on the heat coagulation of oil-in-water emulsions stabilized by milk-protein-concentrate. *Food Hydrocolloids*, *41*, 332–342. https://doi.org/10.1016/j.foodhyd.2014.04.022

Lichtenberger, R., Simpson, M. A., Smith, C., Barker, J., & Navarini, A. A. (2017). Genetic architecture of acne vulgaris. *J Eur Acad Dermatol Venereol*, *31*(12), 1978–1990. https://doi.org/10.1111/jdv.14385

Lim, D. C. C., Abu Bakar, M. F., & Majid, M. (2019). Nutritional composition of stingless bee honey from different botanical origins. *IOP Conference Series: Earth and Environmental Science*, *269*(1). https://doi.org/10.1088/1755-1315/269/1/012025

Lindsley, C. W. (2014). Encyclopedia of Psychopharmacology. *Encyclopedia of Psychopharmacology*, 6–11. https://doi.org/10.1007/978-3-642-27772-6

Lino, R. C., de Carvalho, S. M., Noronha, C. M., Sganzerla, W. G., Nunes, M. R., da Rosa, C. G., & Barreto, P. L. M. (2020). Development and Characterization of Poly-ε-caprolactone Nanocapsules Containing β-carotene Using the Nanoprecipitation Method and Optimized by Response Surface Methodology. *Brazilian Archives of Biology and Technology*, *63*. https://doi.org/10.1590/1678-4324-2020190184

Liu, Q., Huang, H., Chen, H., Lin, J., & Wang, Q. (2019). Food-grade nanoemulsions: Preparation, stability and application in encapsulation of bioactive compounds. *Molecules*, *24*(23), 1–37. https://doi.org/10.3390/molecules24234242

Lu, L. Y., Lai, H. Y., Pan, Z. Y., Wu, Z. X., Chen, W. C., & Ju, Q. (2017). Obese/overweight and the risk of acne vulgaris in Chinese adolescents and young adults. *Hong Kong Journal of Dermatology and Venereology*, *25*(1), 5–12.

Luki, M., Pantelic´, I., & Savic´, S. D. (2021). Towards Optimal pH of the Skin and Topical Formulations : From the Current State of the Art to Tailored Products. *Cosmetics*, *8*(69). https://doi.org/https://doi.org/10.3390/cosmetics8030069

Ly, S., Kamal, K., Manjaly, P., & Barbieri, J. S. (2023). Treatment of Acne Vulgaris During Pregnancy and Lactation : A Narrative Review. *Dermatology and Therapy*, *13*(1), 115–130. https://doi.org/10.1007/s13555-022-00854-3

Mahani, H., Keya, A. L., Berg, S., & Nasralla, R. (2017). Electrokinetics of carbonate/brine interface in low-salinity waterflooding: Effect of brine salinity, composition, rock type, and pH on ζ-potential and a surface-complexation model. *SPE Journal*, *22*(1), 53–68. https://doi.org/10.2118/181745-pa

Mahmood, A. L., Lani, M. N., Hassan, Z., Razak, S. B. A., & Ahmad, F. T. (2021). Antioxidant and antimicrobial properties of Indo-Malayan stingless bee (Heterotrigona itama) honey from different seasons and distribution of flowers. *Food*

Research, *5*(2), 498–507. https://doi.org/10.26656/fr.2017.5(2).546

Mai, D. T., & Ishibashi, K. (2021). Bacteria Shape Classification using Small-Scale Depthwise Separable CNNs. *Proceedings of the Annual International Conference of the IEEE Engineering in Medicine and Biology Society, EMBS*, *December*, 2940–2943. https://doi.org/10.1109/EMBC46164.2021.9630658

Mail, M. H., Ab Rahim, N., Amanah, A., Khawory, M. H., Shahudin, M. A., & Seeni, A. (2019). FTIR and elementary analysis of Trigona honey, Apis honey and adulterated honey mixtures. *Biomedical and Pharmacology Journal*, *12*(4), 2011–2017. https://doi.org/10.13005/bpj/1833

Manian, M., Jain, P., Vora, D., & Banga, A. K. (2022). Formulation and Evaluation of the In Vitro Performance of Topical Dermatological Products Containing Diclofenac Sodium. *Pharmaceutics*, *14*(9). https://doi.org/10.3390/pharmaceutics14091892

Maphosa, Y., & Jideani, V. A. (2018). Factors Affecting the Stability of Emulsions Stabilised by Biopolymers. In S. Karakuş (Ed.), *Intech* (pp. 65–81). IntechOpen Limited. https://doi.org/0.5772/intechopen.75308

Maqbool, M. A., Mishra, M. K., Pathak, S., Kesharwani, A., & Kesharwani, A. (2018). Semisolid dosage forms manufacturing: Tools, critical process parameters, strategies, optimization and recent advances. *Indo American Journal of Pharmaceutical Research*, *7*(11), 882–889.

Martinotti, S., Laforenza, U., Patrone, M., Moccia, F., & Ranzato, E. (2019). Honey-mediated wound healing: H2O2 try through AQP3 determines extracellular Ca2+ influx. *International Journal of Molecular Sciences*, *20*(3), 764. https://doi.org/10.3390/ijms20030764

Marubayashi, B. A. dos S., Simoni, S. E., & Fariña, L. O. de. (2021). Topical treatments for acne: a bibliographic review/Tratamentos tópicos para acne: uma revisão bibliográfica. *Brazilian Journal of Health Review*, *4*(1), 3910–3922. https://doi.org/10.34119/bjhrv4n1-310

Masterson, K. N. (2018). Acne Basics: Pathophysiology, Assessment, and Standard Treatment Options. *Journal of the Dermatology Nurses' Association*, *10*(1), S2–S10. https://doi.org/10.1097/JDN.0000000000000361

Md Zin, N. B., Azemin, A., Mohd Rodi, M. M., Mat Rashid, Z., & Mohd, K. S. (2019). Application of FTIR fingerprints coupled with chemometric for comparison of stingless bee propolis from different extraction methods. *Malaysian Journal of Fundamental and Applied Sciences*, *15*(2–1), 350–355. https://doi.org/10.11113/mjfas.v15n2-1.1553

Mehta, H. H., Nikam, V. V, Jaiswal, C. R., & Mehta, H. B. (2018). A cross-sectional study of variations in the biophysical parameters of skin among healthy volunteers. *Indian Journal of Dermatology, Venereology and Leprology*, *84*(4), 521. https://doi.org/10.4103/ijdvl.ijdvl_1151_15

Menditto, E., Orlando, V., De Rosa, G., Minghetti, P., Musazzi, U. M., Cahir, C., Kurczewska-Michalak, M., Kardas, P., Costa, E., Lobo, J. M. S., & Almeida, I. F. (2020). Patient centric pharmaceutical drug product design—the impact on medication adherence. *Pharmaceutics*, *12*(1). https://doi.org/10.3390/pharmaceutics12010044

Mills, O. H., Criscito, M. C., Schlesinger, T. E., Verdicchio, R., & Szoke, E. (2016). Addressing free radical oxidation in acne vulgaris. *Journal of Clinical and Aesthetic Dermatology*, *9*(1), 25–30.

Minden-Birkenmaier, B. A., & Bowlin, G. L. (2018). Honey-based templates in wound healing and tissue engineering. *Bioengineering*, *5*(2), 1–27. https://doi.org/10.3390/bioengineering5020046

Minhas, M. U., Ahmad, M., Khan, S., Ali, L., & Sohail, M. (2016). Synthesis and characterization of β-Cyclodextrin hydrogels: Crosslinked polymeric network for targeted delivery of 5-Fluorouracil. *Cellulose Chemistry and Technology*, *50*(2), 233–242.

Mizzi, L., Maniscalco, D., Gaspari, S., Chatzitzika, C., Gatt, R., & Valdramidis, V. P. (2020). Assessing the individual microbial inhibitory capacity of different sugars against pathogens commonly found in food systems. *Letters in Applied Microbiology*, *71*(3), 251–258. https://doi.org/10.1111/lam.13306

Mohanna, M. T. . (2016). Morphology and Classification of Bacteria. In *Microbiology* (Issue October 2016, pp. 225–238).

Mohd-Aspar, M. A. S., Edros, R. Z., & Hamzah, N. A. (2021). Optimisation and evaluation of antibacterial topical preparation from Malaysian Kelulut honey using guar gum as polymeric agent. *Pertanika Journal of Science and Technology*, *29*(1), 565–592. https://doi.org/10.47836/pjst.29.1.30

Mohd Hanif, N. (2018). *Development and Characterization of Cosmetic Cream Formulation incorporating Piper Bitle Linn. Extract*. International Islamic University Malaysia.

Mohiuddin, A. (2019). A Comprehensive Review of Acne Vulgaris. *Journal of Clinical Research in Dermatology*, *6*(2), 1–34. https://doi.org/10.15226/2378-1726/6/2/00186

Mohiuddin, A. K. (2019). Skin Care Creams: Formulation and Use. *Dermatology Clinics & Research*, *5*(1), 238–27. www.scitcentral.com

Moy, A. J., & Tunnell, J. W. (2016). Diffuse Reflectance Spectroscopy and Imaging. In *Imaging in Dermatology*. Elsevier Inc. https://doi.org/10.1016/B978-0-12-802838-4.00017-0

Mueller, R. S. (2008). Topical dermatological therapy. In *Small Animal Clinical Pharmacology* (Second Edi). Elsevier Ltd. https://doi.org/10.1016/B978-070202858-8.50026-9

Muhammad, N. I. I., & Sarbon, N. M. (2021). Physicochemical profile, antioxidant activity and mineral contents of honey from stingless bee and honey bee species. *Journal of Apicultural Research*, 1–8. https://doi.org/10.1080/00218839.2021.1896214

Mustafa, M. Z., Yaacob, N. S., & Sulaiman, S. A. (2018). Reinventing the honey industry: Opportunities of the stingless bee. *Malaysian Journal of Medical Sciences*, *25*(4), 1–5. https://doi.org/10.21315/mjms2018.25.4.1

Ng, W. J., Sit, N. W., Ooi, P. A. C., Ee, K. Y., & Lim, T. M. (2020). The antibacterial potential of honeydew honey produced by stingless bee (Heterotrigona itama) against antibiotic resistant bacteria. *Antibiotics*, *9*(12), 1–16. https://doi.org/10.3390/antibiotics9120871

Nishio, E. K., Ribeiro, J. M., Oliveira, A. G., Andrade, C. G. T. J., Proni, E. A., Kobayashi, R. K. T., & Nakazato, G. (2016). Antibacterial synergic effect of honey from two stingless bees: Scaptotrigona bipunctata Lepeletier, 1836, and S. postica Latreille, 1807. *Scientific Reports*, *6*(February), 1–8. https://doi.org/10.1038/srep21641

Nur Eszaty, F. E., Mohamed, N. M. A., Saiful, I. A. R., Norjihada, I. I., Norhana, J., Nurliyana, A. Z., Mohamad Ikhwan, J., Sagadevan, S., & Nadirul, H. M. N. (2022). A Review on Recent Progress of Stingless Bee Honey and Its Hydrogel-Based Compound for Wound Care Management. *Molecules*, *27*, 1–20.

Okamoto, K., Kanayama, S., Ikeda, F., Fujikawa, K., Fujiwara, S., Nozawa, N., Mori, S., Matsumoto, T., Hayashi, N., & Oda, M. (2021). Broad spectrum in vitro microbicidal activity of benzoyl peroxide against microorganisms related to cutaneous diseases. *Journal of Dermatology*, *48*(4), 551–555. https://doi.org/10.1111/1346-8138.15739

Okamoto, K., Tamura, R., Tabara, K., Kitano, T., Kanayama, S., Kido, H., Sasaki, T., Moroki, T., Ikeda, F., Matsumoto, T., & Ishii, R. (2016). Keratolytic and comedolytic effects of topically applied benzoyl peroxide in hairless mice and rhino mice. *Journal of Dermatological Science*, *84*(1). https://doi.org/https://doi.org/10.1016/j.jdermsci.2016.08.492

Oliveira, R., & Almeida, I. F. (2023). Patient-Centric Design of Topical Dermatological Medicines. *Pharmaceuticals*, *16*(4). https://doi.org/10.3390/ph16040617

Oliveira, R. S., Ferraz, D., Mota, S., Garrido, J., Garrido, E. M., Sousa, M., & Almeida, I. F. (2022). Design of an Emulgel for Psoriasis Focused on Patient Preferences. *Apllied Sciences*, *12*(3260). https://doi.org/https://doi.org/10.3390/ app12073260

Omar, S. S. S., Hadi, H., Hanif, N. M., Ahmad, H. M. A., & Ng, S.-F. (2021). Lightening Effect of Skin Lightening Cream Containing Piper betle L. Extract in Human Volunteers. *Cosmetics*, *8*(32). https://doi.org/https://dx.doi.org/10.3390/cosmetics8020032

Ooi, T. C., Yaacob, M., Rajab, N. F., Shahar, S., & Sharif, R. (2021). The stingless bee honey protects against hydrogen peroxide-induced oxidative damage and lipopolysaccharide-induced inflammation in vitro. *Saudi Journal of Biological Sciences*, *28*(5), 2987–2994. https://doi.org/10.1016/j.sjbs.2021.02.039

Osés, S. M., Pascual-maté, A., Fuente, D. De, Pablo, A. De, Fernández-muiño, M. A., Sancho, M. T., Osés, S. M., Pascual-maté, A., Fuente, D. De, Pablo, A. De, Fernández-muiño, M. A., Sancho, M. T., Osés, S. M., Pascual-maté, A., Fuente, D. De, Pablo, A. De, Sancho, M. T., & Fernández-mui, M. A. (2016). Comparison of methods to determine antibacterial activity of honeys against Staphylococcus aureus Comparison of methods to determine antibacterial activity of honeys against Staphylococcus aureus. *NJAS - Wageningen Journal of Life Sciences*, *78*, 29–33. https://doi.org/10.1016/j.njas.2015.12.005

Özyay, F., Aktepe, E., & Erturan, İ. (2018). The evaluation of psychiatric comorbidity , self - injurious behavior , suicide probability , and other associated psychiatric factors (lonileness , self - esteem , life satisfaction) in adolescents with acne : A clinical pilot study. *Journal of Cosmetic Dermatology*, 1–6. https://doi.org/10.1111/jocd.12708

Park, Y. H., & Kim, H. J. (2021). Formulation and stability of horse oil-in-water emulsion by HLB system. *Food Science and Biotechnology*, *30*(7), 931–938. https://doi.org/10.1007/s10068-021-00934-8

Patel, B. M., Kuchekar, A. B., & Pawar, S. R. (2021). Emulgel Approach to Formulation Development: A Review. *Biosciences Biotechnology Research Asia*, *18*(3), 459–465. https://doi.org/10.13005/bbra/2931

Patil, P. B., Datir, S. K., & Saudagar, R. B. (2019). A Review on Topical Gels as Drug Delivery System. *Journal of Drug Delivery & Therapeutics*, *9*(3), 989–994. https://doi.org/http://dx.doi.org/10.22270/jddt.v9i3-s.2930

Pavlačková, J., Egner, P., Slavík, R., Mokrejš, P., & Gál, R. (2020). Hydration and barrier potential of cosmetic matrices with bee products. *Molecules*, *25*(11), 1–13. https://doi.org/10.3390/molecules25112510

Pawignya, H., Kusworo, T. D., & Pramudono, B. (2019). Kinetic modeling of flocculation and coalescence in the system emulsion of water-xylene-terbutyl oleyl glycosides. *Bulletin of Chemical Reaction Engineering &Amp; Catalysis*, *14*(1), 60–68. https://doi.org/10.9767/bcrec.14.1.2594.60-68

Phad, A. R., Dilip, N. T., & Ganapathy, S. (2018). Emulgel : A Comprehensive Review for Topical Delivery of Hydrophobic Drugs. *Asian Journal of Pharmaceutics*, *12*(2), S382–S393.

Pimentel, T. C., Rosset, M., de Sousa, J. M. B., de Oliveira, L. I. G., Mafaldo, I. M., Pintado, M. M. E., de Souza, E. L., & Magnani, M. (2022). Stingless bee honey: An overview of health benefits and main market challenges. *Journal of Food Biochemistry*, *46*(3), 1–21. https://doi.org/10.1111/jfbc.13883

Pineau, L., Radix, C., & Weber, D. J. (2022). Comparison of the sporicidal activity of a UV disinfection process with three FDA cleared sterilants. *American Journal of Infection Control*, *50*(12), 1316–1321. https://doi.org/10.1016/j.ajic.2022.02.027

Platsidaki, E., & Dessinioti, C. (2018). Recent advances in understanding Propionibacterium acnes (Cutibacterium acnes) in acne [version 1; referees: 2 approved]. *F1000Research*, *7*(1953), 1–12. https://doi.org/10.12688/f1000research.15659.1

Plewig, G., Melnik, B., & Chen, W. (2019a). Acne Pathogenesis. In G. Plewig, B. Melnik, & W. Chen (Eds.), *Plewig and Kligman´s Acne and Rosacea* (4th ed., pp. 45–61).

Springer International Publishing. https://doi.org/10.1007/978-3-319-49274-2

Plewig, G., Melnik, B., & Chen, W. (2019b). Pilosebaceous Follicles: Structure, Biochemistry, and Function. In G. Plewig, B. Melnik, & W. Chen (Eds.), *Plewig and Kligman´s Acne and Rosacea* (4th ed., pp. 1–34). Springer International Publishing. https://doi.org/10.1007/978-3-319-49274-2

Plyduang, T., Atipairin, A., Yoon, A. S., Sermkaew, N., Sakdiset, P., & Sawatdee, S. (2022). Formula development of red palm (Elaeis guineensis) fruit extract loaded with solid lipid nanoparticles containing creams and its anti-aging efficacy in healthy volunteers. *Cosmetics*, *9*(3). https://doi.org/10.3390/cosmetics9010003

Polugari, R., Marla, S. R., & Shailaja, D. (2016). Isolation and molecular characterization of acne causing Propionibacterium acnes. *International Journal of Scientific and Research Publications*, *6*(6), 809–2250. www.ijsrp.org

Prakoeswa, C. R. S., Damayanti, Anggraeni, S., Umborowati, M. A., Febriana, S. A., Oginawati, K., & Tanziha, I. (2022). Profile of Transepidermal Water Loss (TEWL), Skin Hydration, and Skin Acidity (pH) in Indonesian Batik Workers. *Dermatology Research and Practice*, *2022*, 1–7. https://doi.org/10.1155/2022/7014004

Pravallika, A., & Reddy, A. P. (2019). Formulation and Evaluation of Aceclofenac Topical Emulgel. *Nternational Journal of Advanced Technology and Innovative Research*, *10*(2), 43–48.

Purnima, T. N., & Reddy, M. S. (2021). Formulation and Evaluation of Topical Cubosomal Emulgel of an Antifungal Drug: Itraconazole. *Journal of Advanced Scientific Research*, *12*(2), 263–275. http://www.sciensage.info/journal/1359303580JASR_3006121.pdf

Rafique, S., & Akhtar, N. (2019). Development and in-vitro Evaluation of Cosmetic Emulgels Loaded with Persia Americana Extract. *Pakistan Journal of Pharmacology*, *36*, 1–7.

Rahaman, S. M., Bhattarai, A., Kumar, D., Singh, B., & Saha, B. (2023). Application of biosurfactants as emulsifiers in the processing of food products with diverse utilization in the baked goods. In Inamuddin & C. O. Adetunji (Eds.), *Applications of Next Generation Biosurfactants in the Food Sector* (1st ed., pp. 203–237). Academic Press. https://doi.org/https://doi.org/10.1016/C2019-0-05486-6

Ramasamy, S., Barnard, E., Dawson, T. L., & Li, H. (2019). The role of the skin microbiota in acne pathophysiology. *British Journal of Dermatology*, *181*(4), 691–699. https://doi.org/10.1111/bjd.18230

Ramli, N. Z., Chin, K. Y., Zarkasi, K. A., & Ahmad, F. (2019). The beneficial effects of stingless bee honey from Heterotrigona itama against metabolic changes in rats fed with high-carbohydrate and high-fat diet. *International Journal of Environmental Research and Public Health*, *16*(24), 1–17. https://doi.org/10.3390/ijerph16244987

Ranjan, P., Jain, V., Shende, S., & Jain, K. (2019). Formulation Development and Evaluation of Emulgel of Clindamycin Phosphate for Effective Treatment of Acne.

Journal of Drug Delivery and Therapeutics, *9*(4), 202–207. http://jddtonline.info

Ranneh, Y., Ali, F., Zarei, M., Akim, A. M., Hamid, H. A., & Khazaai, H. (2018). Malaysian stingless bee and Tualang honeys: A comparative characterization of total antioxidant capacity and phenolic profile using liquid chromatography-mass spectrometry. *LWT - Food Science and Technology*, *89*, 1–9. https://doi.org/10.1016/j.lwt.2017.10.020

Rao, P. V., Krishnan, K. T., Salleh, N., & Gan, S. H. (2016). Biological and therapeutic effects of honey produced by honey bees and stingless bees: A comparative review. *Revista Brasileira de Farmacognosia*, *26*(5), 657–664. https://doi.org/10.1016/j.bjp.2016.01.012

Reena, K., Mittal, S., Faizan, M., Jahan, I., Rahman, Y., Khan, Singh, R. L., Alhalmi, A., M. Noman, O., & Alahdab, A. (2023). Enhancement of Curcumin's Anti-Psoriatic Efficacy via Formulation into Tea Tree Oil-Based Emulgel. *Gels*, *9*(973). https://doi.org/https://doi.org/10.3390/gels9120973

Restuinjaya, L. A., Susanty Simaremare, E., & Pratiwi, R. D. (2019). Optimization of Tween 80 and Span 60 on Cream Ethanol Extract the Leaves Matoa *(Pometia Pinnata)* as an Antioxidant. *Journal of Advances in Pharmacy Practices*, *1*(2), 11–21. http://doi.org/10.5281/zenodo.3247411

Ribeiro, C. S., Leal, F., & Jeunon, T. (2017). Skin Anatomy, Histology, and Physiology. *Daily Routine in Cosmetic Dermatology*, 1–10. https://doi.org/10.5005/jp/books/12699_2

Rosdi, F. N., Mohd Nor, M. Z., Wan Mohamad, W. A. F., & Wan Mokhtar, W. M. F. (2021). Physical properties of kelulut honey emulsions with different oil types and emulsifier concentrations. *Journal Of Agrobiotechnology*, *12*(1S), 92–100. https://doi.org/10.37231/jab.2021.12.1s.274

Roy, D., & Patel, C. (2023). Revisiting the Use of Quantum Chemical Calculations in LogPoctanol-water Prediction. *Molecules*, *28*(2). https://doi.org/10.3390/molecules28020801

Rozman, A. S., Hashim, N., Maringgal, B., & Abdan, K. (2021). Optimisation of stingless bee honey nanoemulsions using response surface methodology. *Foods*, *10*(9). https://doi.org/10.3390/foods10092133

Rudhart, S. A., Günther, F., Dapper, L. I., Gehrt, F., Stuck, B. A., & Hoch, S. (2022). Analysis of bacterial contamination and the effectiveness of UV light-based reprocessing of everyday medical devices. *PLoS ONE*, *17*(11 November), 1–13. https://doi.org/10.1371/journal.pone.0268863

Rutala, W. A., & Weber, D. J. (2019). Guideline for Disinfection and Sterilization in Healthcare Facilities. In *Centers for Disease Control and Prevention*. https://doi.org/10.1016/j.idc.2021.04.004

Sachdeva, M., Tan, J., Lim, J., Kim, M., Nadeem, I., & Bismil, R. (2021). The prevalence, risk factors, and psychosocial impacts of acne vulgaris in medical students: a literature review. *International Journal of Dermatology*, *60*(7), 792–798. https://doi.org/10.1111/ijd.15280

Saeed, J., Hussain Shah, S. N., Javed, H., Aslam, A., Ali, A., Siddique, F., Zahra, T., Bin Jardan, Y. A., Wondmie, G. F., Nafidi, H. A., & Bourhia, M. (2023). Experimental and in silico evaluation of Carthamus tinctorius L. oil emulgel: a promising treatment for bacterial skin infections. *Frontiers in Cellular and Infection Microbiology*, *13*(September), 1–19. https://doi.org/10.3389/fcimb.2023.1253095

Safitri, F. I., Nawangsari, D., & Febrina, D. (2021). Overview: Application of Carbopol 940 in Gel. *Advances in Health Sciences Research*, *34*, 80–84. https://doi.org/10.2991/ahsr.k.210127.018

Sah, S. K., Badola, A., & Nayak, B. K. (2017). Emulgel: Magnifying the application of topical drug delivery. *Indian Journal of Pharmaceutical and Biological Research*, *5*(1), 25–33. https://doi.org/10.30750/ijpbr.5.1.4

Said, P., Pashte, V., & Pashte, S. (2020). Nutraceutical properties of natural honey to fight health issues: A comprehensive review. *Phytopathology*, *9*(5), 234–242. https://doi.org/10.22271/PHYTO.2020.V9.I5D.12220

Sandle, T. (2016). Microbial identification. *Pharmaceutical Microbiology*, 103–113. https://doi.org/10.1016/b978-0-08-100022-9.00009-8

Sandra Lawton. (2019). Skin 1: the structure and functions of the skin. *Nursing Times*, *115*(12), 30–33. https://doi.org/10.1097/00000441-183411000-00032

Sarango-Granda, P., Espinoza, L. C., Díaz-Garrido, N., Alvarado, H., Rodríguez-Lagunas, M. J., Baldomá, L., & Calpena, A. (2022). Effect of Penetration Enhancers and Safety on the Transdermal Delivery of Apremilast in Skin. *Pharmaceutics*, *14*(5). https://doi.org/10.3390/pharmaceutics14051011

Sari, N. P., Sari, R., & Untari, E. K. (2018). Antibacterial Activity Test of Bacteriocin from Lactobacillus brevis, Lactobacillus casei and Lactobacillus plantarum Against Gram Positive Pathogenic Bacteria. *Journal of Tropical Biodiversity and Biotechnology*, *3*(3), 85. https://doi.org/10.22146/jtbb.38138

Savary, G., Gilbert, L., Grisel, M., & Picard, C. (2019). Instrumental and sensory methodologies to characterize the residual film of topical products applied to skin. *Skin Research and Technology*, *25*(4), 415–423. https://doi.org/10.1111/srt.12667

Schachner-Nedherer, A. L., Werzer, O., & Zimmer, A. (2019). A Protocol to Characterize Peptide-Based Drug Delivery Systems for miRNAs. *ACS Omega*, *4*(4), 7014–7022. https://doi.org/10.1021/acsomega.8b03562

Schumacher, A., Vranken, T., Malhotra, A., Arts, J. J. C., & Habibovic, P. (2018). In vitro antimicrobial susceptibility testing methods: agar dilution to 3D tissue-engineered models. *European Journal of Clinical Microbiology and Infectious Diseases*, *37*(2), 187–208. https://doi.org/10.1007/s10096-017-3089-2

Selvamani, V. (2018). Stability Studies on Nanomaterials Used in Drugs. In *Characterization and Biology of Nanomaterials for Drug Delivery: Nanoscience and*

Nanotechnology in Drug Delivery. Elsevier Inc. https://doi.org/10.1016/B978-0-12-814031-4.00015-5

Sharma, S., Sharma, S., Saahil, A., & Sucheta. (2021). Potential Application of Probiotics in the Treatment of Acne : A Review. *Annals of the Romanian Society for Cell Biology*, *25*(6), 19680–19699.

Shatalebi, M. A., & Roostaei, M. (2015). Preparation and physicochemical evaluation of benzoyl peroxide 5% foamable emu oil emulsion. *Jundishapur Journal of Natural Pharmaceutical Products*, *10*(3), 3–8. https://doi.org/10.17795/jjnpp-16229

Shettigar, P., Koland, M., Sindhoor, S. M., & Prabhu, A. (2021). Formulation and Evaluation of Clarithromycin Loaded Nanostructured Lipid Carriers for the Treatment of Acne. *Journal of Pharmaceutical Research International*, *33*(40B), 26–38. https://doi.org/10.9734/jpri/2021/v33i40b32260

Skroza, N., Tolino, E., Mambrin, A., Zuber, S., Balduzzi, V., Marchesiello, A., Bernardini, N. T., Proietti, I., & Potenza, C. (2018). Adult acne versus adolescent acne: A retrospective study of 1,167 Patients. *Journal of Clinical and Aesthetic Dermatology*, *11*(1), 21–25.

Smaoui, S., Ben Hlima, H., Ben Chobba, I., & Kadri, A. (2017). Development and stability studies of sunscreen cream formulations containing three photo-protective filters. *Arabian Journal of Chemistry*, *10*, S1216–S1222. https://doi.org/10.1016/j.arabjc.2013.02.020

Smith, M. C., Crist, R. M., Clogston, J. D., & McNeil, S. E. (2017). Zeta potential: a case study of cationic, anionic, and neutral liposomes. *Analytical and Bioanalytical Chemistry*, *409*(24), 5779–5787. https://doi.org/10.1007/s00216-017-0527-z

Sobhaninia, M., Nasirpour, A., Shahedi, M., & Golkar, A. (2017). Oil-in-water emulsions stabilized by whey protein aggregates: Effect of aggregate size, pH of aggregation and emulsion pH. *Journal of Dispersion Science and Technology*, *38*(9), 1366–1373. https://doi.org/10.1080/01932691.2016.1224719

Soleymani, S., Farzaei, M. H., Zargaran, A., Niknam, S., & Rahimi, R. (2020). Promising plant-derived secondary metabolites for treatment of acne vulgaris: a mechanistic review. *Archives of Dermatological Research*, *312*(1), 5–23. https://doi.org/10.1007/s00403-019-01968-z

Solomon, C. G., & Zaenglein, A. L. (2018). Acne vulgaris. *The New England Journal of Medicine*, *379*(14), 1343–1352. https://doi.org/10.1056/NEJMcp1702493

Soni, S., & Bharadwaj, A. (2020). Formulation, characterization and evaluation of Isotretinoin gel for treatment of Acne Vulgaris. *International Journal of Pharmaceutical Sciences and Research*, *11*(2), 873–883. https://doi.org/10.13040/IJPSR.0975-8232.11(2).873-83

Sousa, J. M., de Souza, E. L., Marques, G., Meireles, B., de Magalhães Cordeiro, Â. T., Gullón, B., Pintado, M. M., & Magnani, M. (2016). Polyphenolic profile and antioxidant and antibacterial activities of monofloral honeys produced by Meliponini in the Brazilian semiarid region. *Food Research International*, *84*, 61–68. https://doi.org/10.1016/j.foodres.2016.03.012

Sreevidya, V. S. (2019). An Overview on Emulgel. *International Journal of Pharmaceutical and Phytopharmacological Research (EIJPPR)*, *9*(1), 92–97. https://doi.org/10.15761/god.1000122

Surekha, M., Fathima, S. R., & Ganesh, A. (2021). Design And Characterisation of Ibuprofen Transemulgel. *Research Journal of Pharmaceutical , Biological and Chemical Sciences*, *12*(5), 16–24. https://doi.org/https://doi.org/10.33887/rjpbcs/2021.12.5.3

Sutaria, A. H., Masood, S., & Schlessinger, J. (2021). *Acne Vulgaris*. StatPearls.

Suyanto, A., Noor, E., Rusli, M. S., & Fahma, F. (2019). Nano-emulsion and nano-encapsulation of fruit flavor: Review. *IOP Conference Series: Earth and Environmental Science*, *292*(1). https://doi.org/10.1088/1755-1315/292/1/012025

Syal, S., Pandit, V., & Ashawat, M. S. (2020). Traditional Herbs to treat Acne Vulgaris. *Asian J. Pharm. Res.*, *10*(3), 195–201. https://doi.org/10.5958/2231-5691.2020.00034.9

Syed Yaacob, S. N., Wahab, R. A., Huyop, F., Lani, M. N., & Zin, N. M. (2020). Morphological alterations in gram-positive and gram-negative bacteria exposed to minimal inhibitory and bactericidal concentration of raw Malaysian stingless bee honey. *Biotechnology and Biotechnological Equipment*, *34*(1), 575–586. https://doi.org/10.1080/13102818.2020.1788421

Tan, J. K. L., Stein Gold, L. F., Alexis, A. F., & Harper, J. C. (2018). Current concepts in acne pathogenesis: Pathways to inflammation. *Seminars in Cutaneous Medicine and Surgery*, *37*(3 S), S60–S62. https://doi.org/10.12788/J.SDER.2018.024

Tan, Y., Xu, Y., Shang, Y., Wang, H., Li, W., & Cao, W. (2020). Thermal Decomposition Behavior and Thermal Hazard of Benzoyl Peroxide under Different Environmental Conditions. *ChemistrySelect*, *5*(17), 5049–5054. https://doi.org/10.1002/slct.201904896

Tao, M., Ao, T., Mao, X., Yan, X., Javed, R., Hou, W., Wang, Y., Sun, C., Lin, S., Yu, T., & Ao, Q. (2021). Sterilization and disinfection methods for decellularized matrix materials: Review, consideration and proposal. *Bioactive Materials*, *6*(9), 2927–2945. https://doi.org/10.1016/j.bioactmat.2021.02.010

Tesch, S., & Schubert, H. (2002). Influence of increasing viscosity of the aqueous phase on the short-term stability of protein stabilized emulsions. *Journal of Food Engineering*, *52*(3), 305–312. https://doi.org/10.1016/S0260-8774(01)00120-0

Thakur, N. K., Bharti, P., Mahant, S., & Rao, R. (2012). Formulation and characterization of benzoyl peroxide gellified emulsions. *Scientia Pharmaceutica*, *80*(4), 1045–1060. https://doi.org/10.3797/scipharm.1206-09

Torregrosa, A., Ochoa-Andrade, A. T., Parente, M. E., Vidarte, A., Guarinoni, G., & Savio, E. (2020). Development of an emulgel for the treatment of rosacea using quality by design approach. *Drug Development and Industrial Pharmacy*, *46*(2), 296–308. https://doi.org/10.1080/03639045.2020.1717515

Tranquillo, J., Goldberg, J., & Allen, R. (2022). Chapter 8 - Detailed Design. In *Biomedical Engineering Design* (pp. 235–285). Elsevier Inc. https://doi.org/https://doi.org/10.1016/B978-0-12-816444-0.00008-0

Tripathi, N., & Sapra, A. (2022). *Gram Staining*. StatPearls Publishing.

Tuksitha, L., Chen, Y. L. S., Chen, Y. L., Wong, K. Y., & Peng, C. C. (2018). Antioxidant and antibacterial capacity of stingless bee honey from Borneo (Sarawak). *Journal of Asia-Pacific Entomology*, *21*(2), 563–570. https://doi.org/10.1016/j.aspen.2018.03.007

Turnbull, P. C. B., Reyes, A. E., Chute, M. D., & Mateczun, A. J. (2008). Effectiveness of UV Exposure of Items Contaminated with Anthrax Spores in a Class 2 Biosafety Cabinet and a Biosafety Level 3 Laboratory Pass-Box. *Applied Biosafety*, *13*(3), 164–168. https://doi.org/10.1177/153567600801300306

U.S., F. and D. A. (2020). *CFR - Code of Federal Regulations Title 21*. https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=333&showFR=1&subpartNode=21:5.0.1.1.14.4

Use, C. for M. P. for H. (2019). *Assessment report Basiron AC and associated names* (Vol. 31, Issue 1234). file:///C:/Users/username/Downloads/basiron-ac-article-13-referral-assessment-report_en.pdf

USP41-NF36. (2018). *Benzoyl Peroxide Gel*.

Venkataramani, D., Tsulaia, A., & Amin, S. (2020). Fundamentals and applications of particle stabilized emulsions in cosmetic formulations. *Advances in Colloid and Interface Science*, *283*, 102234. https://doi.org/10.1016/j.cis.2020.102234

Veras, K. S., Fachel, F. N. S., Pittol, V., Garcia, K. R., Bassani, V. L., dos Santos, V., Henriques, A. T., Teixeira, H. F., & Koester, L. S. (2019). Compatibility study of rosmarinic acid with excipients used in pharmaceutical solid dosage forms using thermal and non-thermal techniques. *Saudi Pharmaceutical Journal*, *27*(8), 1138–1145. https://doi.org/10.1016/j.jsps.2019.09.010

Vora, J., Srivastava, A., & Modi, H. (2018). Antibacterial and antioxidant strategies for acne treatment through plant extracts. *Informatics in Medicine Unlocked*, *13*, 128–132. https://doi.org/10.1016/j.imu.2017.10.005

Waller, J. M., Dreher, F., Behnam, S., Ford, C., Lee, C., Tiet, T., Weinstein, G. D., & Maibach, H. I. (2006). "Keratolytic" properties of benzoyl peroxide and retinoic acid resemble salicylic acid in man. *Skin Pharmacology and Physiology*, *19*(5), 283–289. https://doi.org/10.1159/000093984

WHO. (1999). Re-evaluation of Some Organic Chemicals, Hydrazine and Hydrogen Peroxide. In *Iarc Monographs On The Evaluation Of Carcinogenic Risks To Humansarc Monographs On The Evaluation Of Carcinogenic Risks To Humans* (Vol. 71, p. 345). World Health Organization International Agency for Research on Cancer.

http://monographs.iarc.fr/ENG/Monographs/vol83/mono83-1.pdf

Wiyani, L., Aladin, A., Mustafiah, Abriana, A., & Rahmawati. (2020). Characteristics of Virgin Coconut Oil Emulsion with Honey and Citric Acid. *Advances in Engineering Research*, *194*, 246–250. https://doi.org/10.2991/aer.k.200325.048

Yalçin, G. (2021). Effects of Thermal Treatment, Ultrasonication, and Sunlight Exposure on Antioxidant Properties of Honey. *Turkish Journal of Pharmaceutical Sciences*, *18*(6), 776–780. https://doi.org/10.4274/tjps.galenos.2021.53810

Yosipovitch, G., Misery, L., Proksch, E., Metz, M., Ständer, S., & Schmelz, M. (2019). Skin barrier damage and itch: Review of mechanisms, topical management and future directions. *Acta Dermato-Venereologica*, *99*(13), 1201–1209. https://doi.org/10.2340/00015555-3296

Younas, M., Mushtaq, S., Aslam, M., Zahid, I., Mazhar, H., Jafar, M., Azhar, L., Khalid, S., & Kamran, H. (2020). Journal of Dermatology Sciences Research Reviews & Reports Association Between Dietary Practices and Acne in Young Adults ; A Review. *Journal of Dermatology Research Reviews & Reports*, *1*(4), 1–8. https://doi.org/doi.org/10.47363/JDMRS/2020(1)114

Yousef, H., Alhajj, M., & Sandeep Sharma. (2020). *Anatomy, Skin (Integument), Epidermis*. StatPearls Publishing.

Yousef, H., Miao, J. H., Alhajj, M., & Badri, T. (2021). *Histology, Skin Appendages*. StatPearls.

Zhou, L., Chen, L., Liu, X., Huang, Y., Xu, Y., Xia Xiong, & Deng, Y. (2021). The influence of benzoyl peroxide on skin microbiota and the epidermal barrier for acne vulgaris. *Dermatologic Therapy*, *35*(3). https://doi.org/10.1111/dth.15288

Zhou, X., & Li, Y. (2015). Subgingival Microbes. In X. Zhou & Y. Li (Eds.), *Atlas of Oral Microbiology* (pp. 67–93). Academic Press. https://doi.org/10.1016/B978-0-12-802234-4.00004-5

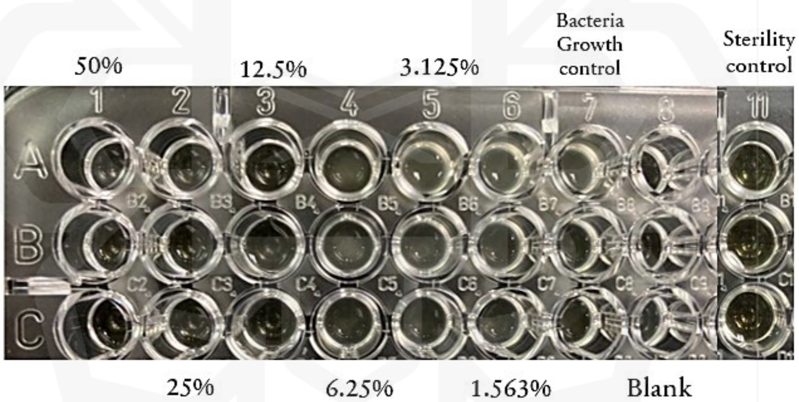
Ziarno, M., 2, D. D., Florowska, A., & Szymanska, I. (2023). Comparison of the Spreadability of Butter and Butter Substitutes. *Applied Sciences*, *13*(4), 2600. https://doi.org/https://doi.org/10.3390/app13042600

Zulkhairi Amin, F. A., Sabri, S., Mohammad, S. M., Ismail, M., Chan, K. W., Ismail, N., Norhaizan, M. E., & Zawawi, N. (2018). Therapeutic properties of stingless bee honey in comparison with european bee honey. *Advances in Pharmacological Sciences*, *2018*, 1–12. https://doi.org/10.1155/2018/6179596

APPENDIX I: Microplate Reader Results for MIC and MBC of Stingless Bee Honey against *P. acnes*

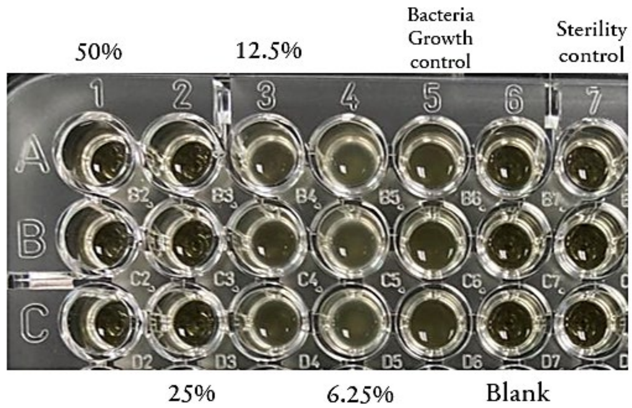
Microplate reader results for MIC determination of stingless bee honey (1.56%-50%) against *P.acnes* using the broth microdilution method.

	Stingless bee honey concentration (%)						Bacterial growth control (%)	Blank (%)
	50	25	12.5	6.25	3.125	1.563		
Optical density (OD) values	0.0998	0.1075	0.12	0.1244	0.1294	0.1384	0.402	0.088
	0.0989	0.1069	0.1123	0.1252	0.1310	0.1399	0.4228	0.0752
	0.0995	0.108	0.1158	0.1248	0.1287	0.1397	0.416	0.0799



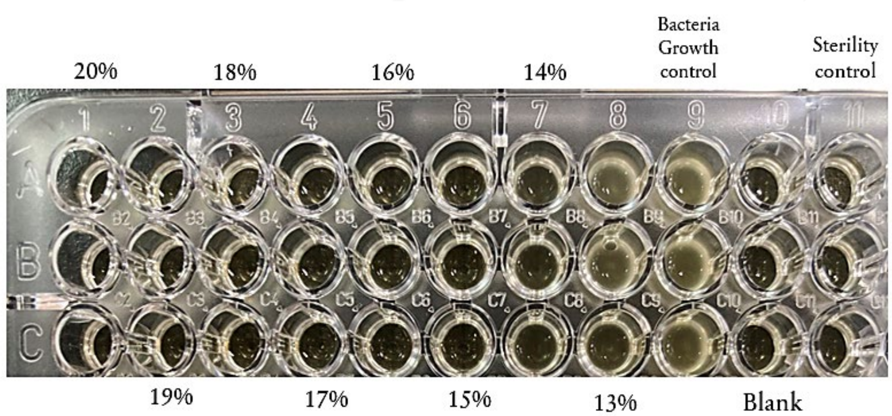
Microplate reader results for MBC determination of stingless bee honey (6.25%-50%) against *P.acnes* using the broth microdilution method.

	Stingless bee honey concentration (%)				Bacterial growth control (%)	Blank (%)
	50	25	12.5	6.25		
Optical density (OD) values	0.0497	0.0554	0.0626	0.0993	0.3676	0.0558
	0.0513	0.0540	0.0631	0.1016	0.3784	0.0555
	0.0543	0.0564	0.0642	0.1002	0.3583	0.055



Microplate reader results for MBC determination of SBH (13%-20%) against *P.acnes* using the broth microdilution method.

		Optical density (OD) values		
Stingless bee honey concentration (%)	20	0.0498	0.049	0.0498
	19	0.0512	0.0507	0.0499
	18	0.0515	0.051	0.0518
	17	0.0521	0.0517	0.525
	16	0.0527	0.0531	0.526
	15	0.0536	0.0540	0.0537
	14	0.0625	0.0636	0.0629
	13	0.0823	0.0858	0.0853
Bacterial growth control (%)		0.3967	0.0433	0.3998
Blank (%)		0.0539	0.0527	0.0546



APPENDIX 1I: Ethics Approval by The IIUM Research Ethics Committee (IREC)

LEADING THE WAY | SUSTAINABILITY INSTITUTION OF THE YEAR

RESEARCH MANAGEMENT CENTRE (RMC)

Our Ref. : IIUM/504/14/11/2/ IREC 2022-064
Date : 17 May 2022

Assoc. Prof. Dr. Hazrina binti Ab Hadi (Principal Investigator)
Kulliyyah of Pharmacy, IIUM Kuantan Campus
25200 Kuantan Pahang

Dear Assoc. Prof. Dr.,

The IIUM Research Ethics Committee (IREC) has reviewed your study protocol as mentioned below:-

ID NO.	:	**IREC 2022-064**
RESEARCH TITLE	:	**Evaluation of Stingless Bee Honey And Benzoyl Peroxide Emulgel Formulation on Healthy Human Volunteers**
REGISTRATION DATE	:	**28 Apr 2022**
CO-INVESTIGATOR	:	**Nuraqilah binti Zulkifli** **Asst. Prof. Dr. Rasimah binti Ismail**
STUDY SITE	:	**Dermatopharmaceutics Research Lab, Level 4, Kulliyyah of Pharmacy, IIUM Kuantan, Pahang.**
SAMPLE SIZE	:	**15**
ETHICAL EXPIRY DATE	:	**14 May 2023**

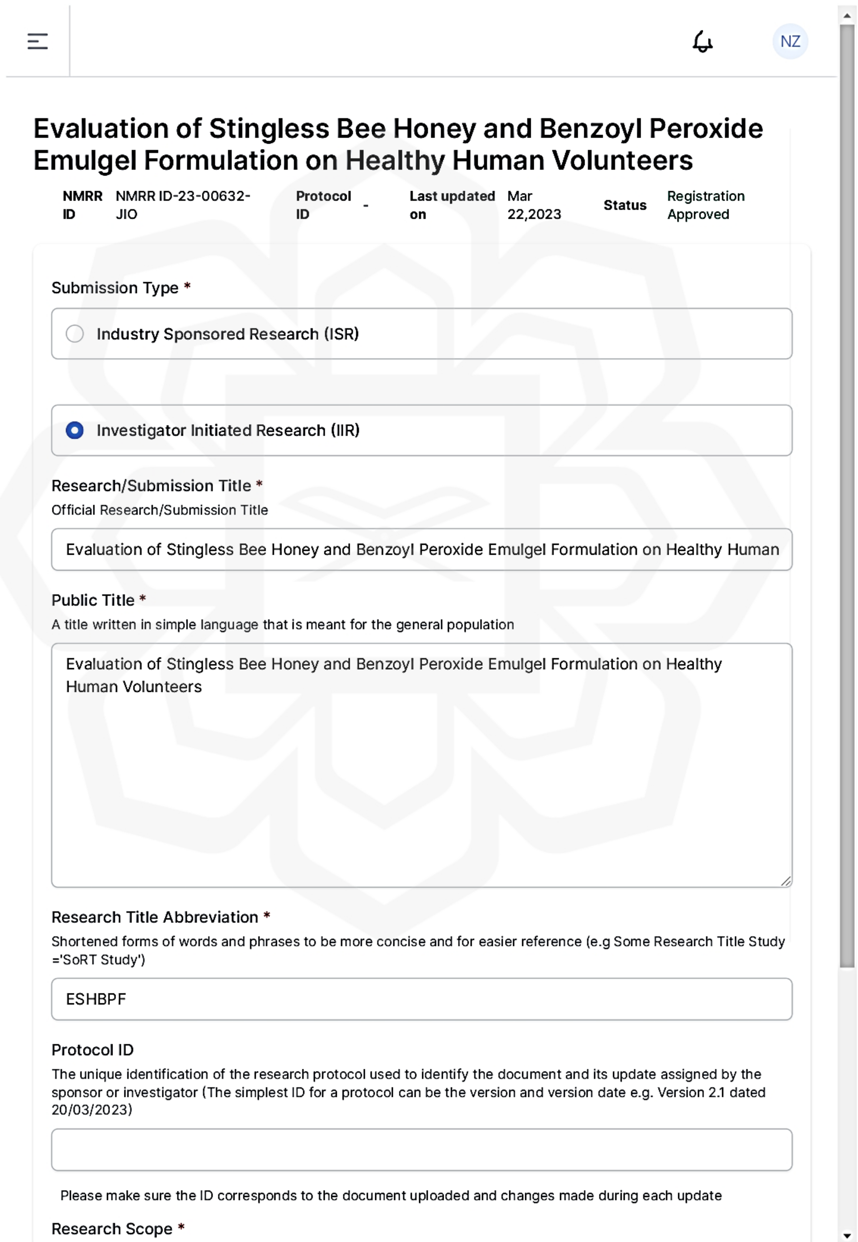
The IIUM Research Ethics Committee (IREC) operates in accordance to the Declaration of Helsinki, International Conference of Harmonization Good Clinical Practice Guidelines (ICH-GCP), Malaysia Good Clinical Practice Guidelines and Council for International Organizations of Medical Sciences (CIOMS) International Ethical Guidelines

The following documents have been received and reviewed to the above study:-

1. Study Proposal/Protocol: Version 1, dated 24 Mar 2022
2. Informed Consent Form (ICF) –
   i. Information Sheet (English) –Version 1, dated 24 Mar 2022
   ii. Consent Form (English) –Version 1, dated 24 Mar 2022
3. Data Collection - Version 1, dated 24 Mar 2022
4. Approval Letter from Kulliyyah of Pharmacy, IIUM
5. Principal Investigator's CV

Research Management Centre
International Islamic University Malaysia, Jalan Gombak, 53100 Kuala Lumpur
Telephone: (+603) 6421 5002 / 5010 | Fax: (+603) 6421 4862
Email: rescentre@iium.edu.my | Website: https://www.iium.edu.my/centre/rmc

APPENDIX III: Registration to The National Medical Research Register (NMRR)



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Evaluation of Stingless Bee Honey and Benzoyl Peroxide Emulgel Formulation on Healthy Human Volunteers

NMRR ID	NMRR ID-23-00632-JIO	Protocol ID	-	Last updated on	Mar 22,2023	Status	Registration Approved

Submission Type *

( ) Industry Sponsored Research (ISR)

(•) Investigator Initiated Research (IIR)

Research/Submission Title *

Official Research/Submission Title

Evaluation of Stingless Bee Honey and Benzoyl Peroxide Emulgel Formulation on Healthy Human

Public Title *

A title written in simple language that is meant for the general population

Evaluation of Stingless Bee Honey and Benzoyl Peroxide Emulgel Formulation on Healthy Human Volunteers

Research Title Abbreviation *

Shortened forms of words and phrases to be more concise and for easier reference (e.g Some Research Title Study ='SoRT Study')

ESHBPF

Protocol ID

The unique identification of the research protocol used to identify the document and its update assigned by the sponsor or investigator (The simplest ID for a protocol can be the version and version date e.g. Version 2.1 dated 20/03/2023)

Please make sure the ID corresponds to the document uploaded and changes made during each update

Research Scope *