STUDY OF EXTRACTION PROCESS AND CHARACTERIZATION OF SOYBEAN EXTRACTS FOR TRANSDERMAL DELIVERY AS MICROEMULSIONS

By

Miss Parapat Sobharaksha

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree

Doctor of Philosophy Program in Pharmaceutical Technology

Graduate School, Silpakorn University

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การศึกษากระบวนการสกัดและคุณลักษณะของสารสกัดถั่วเหลืองเพื่อนำส่งผ่านผิวหนังในรูปแบบไมโครอิมัลชัน

โดย

นางสาวปารภัทร โศภารักษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรดุษฎีบัณฑิต

สาขาวิชาเทคโนโลยีเภสัชกรรม

บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร

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The Graduate School, Silpakorn University has approved and accredited the Thesis title of “Study of extraction process and characterization of soybean extracts for transdermal delivery as microemulsions ” submitted by Miss Parapat Sobharaksha as a partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmaceutical Technology.

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Soybean has numerous health benefits derived from their functional contents including isoflavones, especially daidzein and genistein, natural antioxidant and estrogen-like molecules and also known for its various skin benefits. The purpose of this research was to develop the delivery of a soybean extract, namely genistein and daidzein, by incorporating it into the microemulsion system in order to enhance the permeation properties. When comparing the extraction operated by different methods, it was found that the extraction conducted by the classical method, i.e. at temperature of 65°C, provided the highest amount of concentration of daidzein and genistein. It was selected to evaluate the effects of several excipients for spray drying on the chemical and biological properties of soybean extract. The extracted soybean powder without any excipient possessed the highest stability, retaining the highest concentration of genistein and daidzein for a three years period of storage, at room temperature. The antioxidant activities of the soybean extracts were evaluated by free radical scavenging (DPPH and ABTS) assays. Total phenolic and isoflavone substances were also determined by using Folin-Ciocalteau reagent and developed HPLC methods, respectively. The results showed that the IC50 values of daidzein, genistein and reference standard, trolox, by DPPH radical scavenging assay were 0.41, 0.39 and 0.28 mg/ml, respectively. Daidzein and genistein were found to scavenge the ABTS+ with the IC50 values of 0.55 and 0.53 mg/ml, respectively, and their activities were also higher than trolox (IC50 = 0.88 mg/ml). The extracts were then formulated into microemulsion using pseudo-ternary phase diagrams to construct for proper ratio of components for various microemulsion formulations. As a result, the formulations, which were prepared by using lemon oil, lavender oil and peppermint oil as the oily phase; a mixture of Tween 20® with Tween 80® (1:1 ratio) as surfactants; and ethanol as a cosurfactant, were suitable among 15 simple studied oils. The 30%, 40% and 50% of oils were selected considering from the wide region of missibility among oil, surfactant and co-surfactant. All microemulsions were evaluated for their chemical and physical stabilities when stored at 40°C for three months. F06le formulation showed the best microemulsions, consisting of 30% lemon oil, 10% Tween 20®, 10% Tween 80® and, 40% ethanol and DI-water. It presented a clear microemulsions, no drug precipitation or separation and the amounts of genistein and daidzein remained high level all through three months. Further study was on skin permeation using Yucatan micro pig skin which possessing a similar property to human skin. F06le provided significantly the highest skin permeation parameters. The cumulative amount at 48 hours *Q48* (genistein = 5.12 µg/cm^2, daidzein = 1.08 µg/cm^2) and the steady state flux, *Jss,* (genistein = 0.0020±0.91 and daidzein = 0.00058±0.74 µg/cm^2/h), when compared to other formulations. The lag time (*Tlag)* of genistein and daidzein was 22.01±0.4 hours and 36.79±0.12, respectively. These parameters demonstrated that the isoflavones had high potential for skin delivery as well as high permeation properties. The study showed the beneficial effect of soybeans for their antioxidant activities and the application of drying process to improve the soybean stability. It is proved that the soy isoflavone extracts, rich in effective estrogen-like agents, can be loaded into micoremulsion to increase the skin permeation. The research could then be extended to the development of other natural products for nutraceutic or cosmetic manufacturing and for the delivery system using the application of microemulsion system.

Program of Pharmaceutical Technology Graduate School, Silpakorn University
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สารสำคัญที่ได้จากการสกัดถั่วเหลืองมีประโยชน์มากมายต่อสุขภาพ สารสำคัญคือเจนิสเทอีนและไดเซอีน ซึ่งเป็นสารในกลุ่มไอโซฟลาโวน มีลักษณะโครงสร้างคล้ายกับเอสโตรเจนในร่างกายมนุษย์ มีประโยชน์ต่อสุขภาพเช่น มีฤทธิ์การต้านอนุมูลอิสระ รวมไปจนถึงคุณสมบัติที่ดีต่อผิวหนัง วัตถุประสงค์ของงานวิจัยนี้เพื่อพัฒนาการนำส่งเจนิสเทอีนและไดเซอีนจากการสกัดถั่วเหลือง และนำส่งทางผิวหนังในรูปแบบของไมโครอิมัลชัน จากการเปรียบเทียบการสกัดด้วยวิธีการต่างๆ พบว่าการสกัดด้วยวิธีดั้งเดิมที่อุณหภูมิ 65 องศาเซลเซียสให้เจนิสเทอีนและไดเซอีนในปริมาณที่สูงสุด สารสกัดที่ได้จากวิธีการนี้ถูกเลือกมาทำการศึกษาต่อโดยทำให้อยู่ในรูปแบบผงแห้งโดยวิธีการพ่นแห้ง โดยใช้สารช่วยต่างชนิดกัน เพื่อศึกษาผลของการใช้สารช่วยต่อสมบัติต่าง ๆ พบว่าผงแห้งที่ไม่ใส่สารช่วยมีความคงตัวมากที่สุด ตลอดระยะเวลา 3 ปีที่อุณหภูมิห้องโดยพิจารณาจากปริมาณของเจนิสเทอีน และไดเซอีน การศึกษาคุณสมบัติในการต้านอนุมูลอิสระ ศึกษาด้วยวิธี DPPH และ ABTS ปริมาณสารประกอบฟีนอลและไอโซฟลาโวนด้วยวิธี Folin-Ciocalteau และ HPLC ผลการทดลอง พบว่าไดเซอีน เจนิสเทอีนและ trolox มีค่า IC50 เมื่อทดสอบด้วยวิธี DPPH assay เท่ากับ 0.41, 0.39 และ 0.28 มก/มล. โดยลำดับ และเมื่อทดสอบด้วยวิธี ABTS assay ไดเซอีนและเจนิสเทอีนมีค่า IC50 เท่ากับ 0.55 และ 0.53 มก/มล. ตามลำดับ ซึ่งมีค่าสูงกว่าเมื่อเปรียบเทียบกับสารมาตรฐาน trolox ที่มีค่า IC50 เท่ากับ 0.88 มก/มล. จากนั้นได้หาสัดส่วนที่เหมาะสมที่สุดในการเกิดระบบไมโครอิมัลชัน โดยการใช้ pseudo ternary phase diagram พบว่าน้ำมันสามชนิดคือ น้ำมันเลมอน น้ำมันลาเวนเดอร์ และน้ำมันเปปเปอร์มินท์ เป็นน้ำมันที่เหมาะสมจากน้ำมันที่หาง่ายจำนวน15 ชนิดและใช้สารลดแรงตึงผิวคือ ทวีน80 และ ทวีน20 (อัตราส่วน 1:1) โดยมีเอธานอลเป็นสารลดแรงตึงผิวร่วม ซึ่งพบว่าสัดส่วนของน้ำมันที่ 30%, 40% และ 50% มีความเข้ากันได้ดีระหว่างน้ำมัน สารลดแรงตึงผิว และสารลดแรงตึงผิวร่วม ศึกษาสูตรตำรับที่มีความคงตัวทางเคมีและกายภาพที่ 40 องศาเซลเซียสเป็นเวลา 3 เดือน พบว่าตำรับไมโครอิมัลชัน F06le เป็นตำรับที่ดีสุด ประกอบด้วยน้ำมันเลมอน 30% ทวีน20 10% ทวีน80 10% เอธานอล 40% และน้ำ โดยไม่มีการเปลี่ยนสภาพ ไม่มีการแยกชั้น หรือตกตะกอนของสารสำคัญและสารสำคัญ มีปริมาณที่สูงตลอดระยะเวลาสามเดือน จากนั้นศึกษาการซึมผ่านผิวหนังของสารสำคัญโดยใช้ผิวหนังของหมู Micro-Yucatan ซึ่งมีคุณสมบัติใกล้เคียงกับผิวหนังมนุษย์ การสะสมตัวยาในผิวหนังนอกร่างกาย พบว่าไมโครอิมัลชัน F06le มีการซึมผ่านสูงสุดเมื่อเทียบกับตำรับอื่น มีค่าการซึมผ่านผิวหนังที่ 48 ชั่วโมง เท่ากับ 5.12 ไมโครกรัมต่อพื้นที่ผิว 1 ตารางเซนติเมตรสำหรับเจนิสเทอีน และ 1.08 ไมโครกรัมต่อพื้นที่ผิว 1 ตารางเซนติเมตรสำหรับเจนิสเทอีน มีค่า Steady-state fluxหรือ *Jss* ของเจนิสเทอีนท่ากับ 0.0020±0.91 ไมโครกรัมต่อพื้นที่ผิว 1 ตาราง เซนติเมตรต่อชั่วโมง และของไดเซอีนเท่ากับ 0.00058±0.74 ไมโครกรัมต่อพื้นที่ผิว 1 ตาราง เซนติเมตรต่อ และมีค่าระยะเวลาในการเริ่มซึมผ่านหรือ *Tlag* ของตำรับเท่ากับ 22.01±0.4 ชั่วโมงสำหรับเจนิสเทอีน และ 36.79±0.12 สำหรับไดเซอีน ผลจากการศึกษานี้แสดงให้เห็นว่าตำรับยาทาภายนอกที่เตรียมได้มีคุณสมบัติในการนำส่งไอโซฟลาโวนและเพิ่มการซึมผ่านเข้าสู่ผิวหนัง จากการศึกษาครั้งนี้แสดงให้เห็นถึงประโยชน์จากถั่วเหลืองในการต้านอนุมูลอิสระ และการทำแห้งเพื่อเพิ่มความคงตัว รวมทั้งการใช้ไมโครอิมัลชันเพื่อประสิทธิภาพในการนำส่งไอโซฟลาโวนผ่านผิวหนังซึ่งมีคุณโครงสร้างคล้ายเอสโตรเจน ซึ่งข้อมูลที่ได้มีประโยชน์ต่อการพัฒนาผลิตภัณฑ์ทางธรรมชาติ อุตสาหกรรมเครื่องสำอาง รวมทั้งประโยชน์ต่อการพัฒนาระบบนำส่งที่มีประสิทธิภาพโดยอาศัยระบบไมโครอิมัลชัน

สาขาวิชาเทคโนโลยีเภสัชกรรม บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร

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ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์ 1. ..... 2. .....

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%w/w	percent weight by weight
°C	degree Celsius
μg	microgram(s)
μL	microliter(s)
μm	micrometer(s)
θ	theta
Ave.	average
cc	cubic centimeter(s)
cm	centimeter(s)
cm^{-1}	wavenumbers
cm^2	square centimeter(s)
Conc.	concentration
e.g.	exemplī grātiā (Latin); for example
Eq.	equation
et al.	and others
etc.	et cetera (Latin); and other things/ and so forth
g	gram(s)
h	hour(s)
i.d.	inner diameter
i.e.	id est (Latin); that is
kDa	kilodalton
kg	kilogram(s)
L	liter(s)
mA	milliampere(s)
MC	moisture content
mg	milligram(s)
min	minute(s)
mL	milliliter(s)

mm	millimeter(s)
mM	millimolar(s)
ms	millisecond(s)
MW	molecular weight
ng	nanogram(s)
nm	nanometer(s)
nmol	nanomole(s)
o.d.	outer diameter
PBS	phosphate buffer solution
pH	potentia hydrogenii (Latin); power of hydrogen
R^2	coefficient of determination
rpm	revolutions per minute
s	sec(s)
SC	stratum corneum
S.E.	standard error
V	volt(s)

CHAPTER 1

INTRODUCTION

1.1 Statement and significance of the research problems

Soybeans and soy based products have attracted increased attention owing to their nutritional and health-related beneficial aspects. Soy isoflavones are phytochemicals of prominent interest for beneficial health effects [1-2]. In addition to isoflavones, soybeans contain a large number of bioactive phytochemicals such as phytosterols, protease inhibitors and inositol hexaphosphates [1-3]. Isoflavones are a subclass of flavonoids and are also called phytoestrogen compounds due to their weak estrogen activity with potentially protective effect against some hormone related diseases [4-5]. The main isoflavones found in soybeans are in the form of aglycones (genistein, daidzein, glycitein) and their respective conjugated forms with glucose, malonyl glucose and acetyl glucose [5-9]. These three conjugated of genistein, daidzein and glycitein are found in an approximate ratio of 6: 3: 1, respectively [10]. Several studies have been shown that soy isoflavones play an important role on the reduction in cardiovascular disease risk as well as prevention of several hormonally influenced cancers, menopausal symptoms and osteoporosis. Their abilities to act as antioxidants may also serve for prevention of oxidative damage in living tissue [10-13]. Earlier studies indicated that soy isoflavones, especially genistein, have the antiphotocarcinogenic properties by blocking both the initiation and promotion of skin carcinogenesis via the prevention of DNA- adducted formation and inhibition of various oxidative events. Genistein can also significantly decrease UV-induced cutaneous erythema and skin ulceration in human skin [14-17].

Further, isoflavones in soymilk can reduce hair growth and hair follicle dimensions [18]. These findings have encouraged soy isoflavones as possible topical alternative agent and surge of interest for the cosmetic industry.

Different researchers have deployed various techniques such as stirring, shaker, pressurized liquid extractor and supercritical fluid extractor for extraction of isoflavones from soybeans [3-4,10-11]. All analyses were determined on different samples using a wide variation of solvent composition for extraction. There were reports of different yields obtained from different methods for another substances. However, none of the studies had investigated on the effect of various extraction techniques on the yield of isoflavones which were performed on a homogenous sample obtained by grinding soybeans procured from a single source. In addition, other studies showed some drawbacks of quantification of daidzein and genistein due to long retention time and not simple solvents used [1-2,6,10,14,18].

Several studies have been shown that the biological activity of isoflavones may depend on the type of soy food and its processing and storage conditions. The temperature, during drying and extraction, affect the activity and stability of compounds due to chemical and enzymatic degradations, losses by volatization or thermal decomposition. The factors have been suggested to be the main mechanism causing the reduction in polyphenol content. Nowadays, the development of new technologies to obtain standardized dried plant and food extracts is an important research subject. The advantages of the dried extract over conventional liquid forms are lower storage costs, higher concentration, and stability of active substances. The spray drying has been adopted for manufacturing of powders due to its ability to generate a product with precise quality of specifications and continuous operation [3].

Common carriers for spray-drying process including carbohydrates, gums, semisynthetic cellulose derivatives and synthetic polymers are required during spray drying process. A carrier can increase drying rates causing decrease in powder moisture. Each carrier has advantages and disadvantages in terms of properties, cost and encapsulation efficiency. Currently, maltodextrins, soluble modified starch derivatives, are used alone or in combination with other materials in food and drug processing of plant extracts, aromatic additives, carotenoids and vitamins. Maltodextrins have multifaceted functions including bulking, film formation properties and binding ability of flavour and fat, reduction in oxygen permeability to the wall matrix. However, its low glass transition temperature, leading to crystal formation under increasing temperature, may induce disruption of the structural integrity of wall matrix, and produce agglomeration or caking of microparticulate powders. In addition, the release of encapsulated actives and degradation/oxidation process may occur during the storage period. Therefore, a more efficient and stable carrier for natural products needs to be explored as an important criteria for the development of food industry [19].

The studies on “oxidative stress and its adverse effects on human health” has become a subject of immense interest. In this respect, understanding the possible role of free radicals on human health and its prevention is gaining interest. Antioxidants have gained more importance because of their positive involvement as health promoters of many conditions such as cardiovascular problems, atherosclerosis, anti-tumor activities and the ageing. Many antioxidant compounds, naturally occurring in plant sources have been identified as free radical scavengers

[20-21]. Numerous studies have focused on natural antioxidants in terrestrial plants and their application in food systems to prevent oxidation.

Free radicals, other reactive oxygen species, are generated by exogenous chemicals or endogenous metabolic process in food systems or the human body. The radicals may cause oxidative damage by oxidizing biomolecules and resulting in cell death and tissue damage. However, ingestion of antioxidative supplements, or foods containing antioxidants, may reduce the oxidative damage on a human body. An increasing body of epidemiological research has associated high soy intake with a lower risk for certain types of cancer.

Drug degradation and possible first-pass effect by the enzymatic system existed in the gastrointestinal tract and liver associated with oral delivery are the main obstacles that inhibit the development of drug substance into an oral formulation. Injection, compared with other routes of administration, is the fastest and effective way to deliver the drugs throughout the body. Patients, however, are not typically able to self-administer and suffering from pain as well as risk of infection. Transdermal drug delivery has emerged over many years as an alternative pathway for systemic drug delivery which overcomes the difficulties occurred by the conventional drug delivery systems as mentioned above and provides controlled delivery of drugs over the period of time. Because of its great advantages, it has become one of the highly studied research field among the various drug delivery systems.

The skin provides the formulator with a vast area for applying drugs. Transdermal drug delivery offers many advantages over other traditional routes of drug delivery. Unfortunately, the barrier nature of the skin caused the difficulty in delivering of most drugs through the skin [22]. Many strategies have been employed

to enhance dermal and transdermal delivery. These include the use of chemical penetration enhancers [23], preparation of supersaturated drug delivery systems, electrically driving molecules into or through the tissue employing iontophoresis [24], physically disrupting the skin structure, for example, by electroporation or sonophoresis [25-26] or encapsulating the drug in vesicular delivery systems. Among these methods, vesicular delivery system is the widely used. Microemulsion, a vesicular system, provides another promising alternative for dermal and transdermal delivery of both hydrophilic and lipophilic drugs. Microemulsion is a thermodynamically stable transparent, single optically isotropic liquid system of water, oil and surfactants. Microemulsions can be considered as ideal liquid vehicles for drug delivery as they have most of the requirements including the thermodynamic stability, ease of formulation, low viscosity, high solubilization capacity and small droplet size. The latter characteristic provides a better chance for adherence to biological membranes and transporting drugs in a controlled manner. The role of penetration enhancers is dependent on the amphilic components and the internal mobility of drugs within the vesicle

Microemulsions are transparent systems of two immiscible fluids, stabilized by an interfacial film of surfactant or a mixture of surfactants, frequently in combination with a cosurfactant. These systems could be classified as water-in-oil, bicontinuous and oil-in-water depending on their microstructure which is influenced their physicochemical properties and the extent of their ingredients. Microemulsions are characterized by ultra low interfacial tension between the immiscible phases and offering several advantages such as spontaneous formation, thermodynamic stability, simplicity of manufacturing, solubilization capacity of lipophilic, hydrophilic and

amphiphilic solutes, improved solubilization and bioavailability of hydrophobic drugs, the large area per volume ratio for mass transfer, and the potential for permeation enhancement. The development of delivery systems has had a huge impact on treatment of numerous diseases. To attain the highest pharmacological effects with least side effects of drugs, drugs should be delivered to target sites without a significant distribution to non-target areas. Microemulsion systems have emerged as novel vehicles for drug delivery which allows sustained or controlled release for transdermal, topical, oral, nasal, intravenous, ocular, parenteral and other administration routes of drugs. Microemulsion drug delivery is a practical delivery platform for improving target specifity, therapeutic activity, and reducing toxicity of drugs. Owing to the existence of different domains of variable polarity in the microemulsion systems, they show an enormous potential to be used as delivery vehicles for a diversity of drugs. In this review article, we have attempted to present a broad view over the past five- year research on microemulsions as solubilization and dissolution enhancers for poorly soluble drugs, as a medium for generating new drug delivery systems and as delivery systems themselves [27].

The objective of the present study was to develop the delivery of a soybean extract by incorporating into the microemulsion system in order to enhance the permeation properties.

1.2 Objectives of research

1.2.1 To evaluate the method of extraction from soybean to obtain the high amount of genistein and daidzein.

1.2.2 To investigate the influence of diluents on physical properties of soybean extracts in powder.

1.2.3 To examine the antioxidant activities of the soybean extracts.

1.2.4 To formulate genestein and daidzein loaded microemulsion with the high stability for transdermal drug delivery systems using the phase diagramme

1.2.5 To investigate the skin permeation of genistein and daidzein from microemulsions.

1.3 Hypothesis of research

1.3.1 The yield of genistein and daidzein is dependent on extraction method.

1.3.2 The stability of extract active ingredient is storage condition and dosage form dependence.

1.3.3 Micoemulsion can be used as a carrier for loading genistein and daidzein and can increase skin penetration.

CHAPTER 2

LITERATURE REVIEWS

2.1 History and Origin of Soybean

The first domestication of soybean has been traced back to the early eleventh century B.C. in the eastern half of North China. Soybean production was initially localized in China until the Japanese began to import soybean oil cake for use as fertilizer during the Chinese-Japanese war of 1894-1895. Shipments of soybeans were made to Europe about 1908, and the soybeans attracted world-wide attention. Europeans had been aware of soybeans as early as 1712 through the writing of a German botanist. Some soybean seeds may have been sent from China by missionaries as early as 1740 and planted in France.

The first use of the word "soybean" in U.S. literature was in 1804. However, it is thought that soybean was first introduced into the American Colonies in 1765 as "Chinese vetches". Early authors mentioned that soybeans appeared to be well adapted to Pennsylvania soil. In 1879, the Rutgers Agricultural College in New Jersey was the first reference that reported the study of soybeans at a scientific agricultural school in the United States. For many years, most of the references were reported by people from eastern and southeastern United States where it was first popular. Initially, most of the U.S. soybeans were used as a forage crop rather than a harvested seed. Most of the early introductions planted in these areas were obtained from China, Japan, India, Manchuria, Korea and Taiwan.

For many years, soybean acreage increased very slowly. There were only 1.8 million acres in the United States in 1924 when the first official estimation was available. At that time, most of the crop was used for hay. It was not until the 1920's that soybean acreage expanded to any great quantity in the U.S.

Before World War II, the U.S. imported more than 40% of its edible fats and oils. Disruption of trade routes during the war resulted in a rapid expansion of soybean acreage in the U.S. as the country looked for alternativesforthe imports. Soybean was one of the only two major new crops introduced into the U.S. in the twentieth century. The other major crop, Canola was initially developed in Canada and is now in U.S. Soybean was a new successful crop because there was an immediate need for soybean oil and meal [28].

Soy has been used in traditional Chinese medicine for thousands of years because of its healthy and nutritional benefits. Soybeans have specific, active, non-denatured components that have documented benefits for skin. Soy was among the first crops grown in ancient China. Soybean nutrition discovered in ancient times was rediscovered, elucidated and quantified by modern nutritionists, doctors and agriculturalists [29].

Soy is a rich source of protein, lipids, carbohydrates and vitamins, which provides moisturization and antioxidant benefits for the skin. Whole soy also contains a number of small soy proteins and large soy protein. The small soy protein such as the serine protease inhibitors, Soybean Trypsin Inhibitor (STI) and Bowman-Birk Inhibitor (BBI), is to promote skin tone while the large proteins is to smooth and soften the skin.

Soy was regarded as having medicinal values and was used as a preventative medicine in some countries. It was also stated in medical writings to be a specific remedy for proper functioning of heart, liver, kidney and stomach, and bowels. It was also used as a stimulant for the lungs, eradication of poison from the system, improving complexion by cleaning the impurities of skin. It is also used by Chinese doctors to strengthen the blood [29].

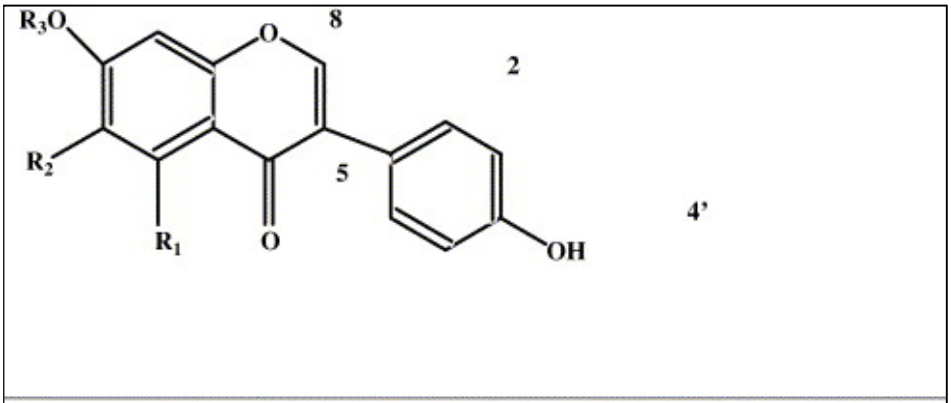
In the early 90's, scientists noticed that Asian workers in soybean factories, who handled soy regularly, had especially smooth and even-toned hands. Previously, the refining process could destroy the bean's complex proteins and loss some properties. The scientists, then, devised method to retain the soybean's naturally active benefits. This unique processing system ensures minimal loss of the natural soybean's active benefits and provides a stable, concentrated form of the whole soybean.Theproprietary Total Soy complex which captures the multiple cutaneous benefits of the natural soybean for skin care was then created.

2.1.1 Components of soybean

Soybeans are known to contain a large number of bioactive phytochemicals such as isoflavones, saponins, phytosterols, protease inhibitors [30-34], inositol hexaphosphates, sphingolipids, phenolic acids, and Bowman-Birk trypsin inhibitors. Isoflavones enriched extracts have been evaluated in the prevention of a wide range of health problems associated with menopause, cardiovascular disease, osteoporosis, and breast, prostate, and colon cancers [35-36]. Isoflavones are widely distributed in the plant kingdom, but accumulated predominantly in plants of the Leguminosae family. The best natural source of isoflavones is soybeans, which have been a major part of the traditional diet for eastern Asian populations for centuries.

The global annual consumption of soybeans has increased from 114 to 170 million tons during the past decade [37]. Soybeans contain 1.2–2.4 mg of total isoflavones per gram of sample [10]. The hundred percent variation in isoflavones contents in soybeans is due to variation in genotypes, environment, location, post-harvest storage, and assay procedures.

Isoflavones are oxygen heterocycles containing a 3-phenylchroman skeleton that is hydroxylated at 4′ and 7 positions (Figure.1) [5]. Based on the substitution pattern on carbons 5 and 6, three aglycon forms of isoflavones commonly found in soybeans are daidzein, genistein, and glycitein. These three isoflavones can also exist in conjugated forms with glucose (daidzin, geinstin and glycitin), malonylglucose (malonyldaidzin, malonylgeinstin and malonylglycitin), and acetylglucose (acetyldaidzin, acetylgeinstin and acetylglycitin) units. Thus 12 free and conjugated forms of isoflavones have been isolated from different soybean samples (Figure 2.1).



Name	R_1	R_2	R_3
Daidzein	H	H	H
Glycitein	H	OCH_3	H
Genistein	OH	H	H
Daidzin	H	H	Glu
Glycitin	H	OCH_3	Glu
Genistin	OH	H	Glu
Acetyldaidzin	H	H	Glu-$COCH_3$
Acetylglycitin	H	OCH_3	Glu-$COCH_3$
Acetylgenistin	OH	H	Glu-$COCH_3$
Malonyldaidzin	H	H	Glu-$COCH_2COOH$
Malonylglycitin	H	OCH_3	Glu-$COCH_2COOH$
Malonylgenistin	OH	H	Glu-$COCH_2COOH$

Figure 1 Chemical structures of 12 isoflavones isolated from soybeans.

Source : Peñalvo, J.L., T. Nurmi, and H. Adlercreutz. (2004). "A simplified HPLC method for total isoflavones in soy products." **Food Chemistry** 87(2): 297-305.

2.2 Extraction techniques of plants

Extraction, as the term is used pharmaceutically, involves the separation of medicinally active portions of plant or animal tissues from the inactive or inert components by using selective solvents in standard extraction procedures. The products so obtained from plants are relatively impure liquids, semisolids or powders intended only for oral or external use. These include classes of preparations known as decoctions, infusions, fluid extracts, tinctures, pilular (semisolid) extracts and powdered extracts. Such preparations popularly have been called galenicals, named

after Galen, the second century Greek physician. The purposes of standardized extraction procedures for crude drugs are to attain the therapeutically desired portion and to eliminate the inert material by treatment with a selective solvent known as menstruum. The extract obtained may,thus, be ready for use as a medicinal agent in the form of tinctures and fluid extractsand further proceed to be incorporated in any dosage form such as tablets or capsules.It may be fractionated to isolate individual chemical entities such as ajmalicine, hyoscine and vincristine, which are modern drugs. Thus, standardization of extraction procedures contributes significantly to the final quality of the herbal drug.

2.2.1 Extraction Methods of Medicinal Plants

In traditional herbal medicine systems, herbal are prepared in several rather standardized ways which usually vary based upon the plant utilized and sometimes what condition is being treated. There are several methods to extract the plant. Simple traditional to advanced technologies conforming to official procedures are being used to manufacture different types of preparations popularly known as Galenicals. Such class of preparations includes, maceration, digestion, decoction, percolation, infusion, etc. The best extraction depends on the many factors. Yield almost all the constituents present in the plant when select the suitable method. Some of the methods that have been used are described as follows.

2.2.1.1 Maceration

In this process, the whole or coarsely powdered crude drug is placed in a stoppered container with the solvent and allowed to stand at room temperature for a period of at least 3 days with a frequent agitation until the soluble matter has been dissolved. The mixture is then saturated, the marc (the damp solid

material) is pressed, and the combined liquids are clarified by filtration or decantation after standing [38-40].

2.2.1.2 Infusion

Fresh infusions are prepared by macerating the crude drug for a short period of time with cold or boiling water. These are dilute solutions of the readily soluble constituents of crude drugs.

2.2.1.3 Digestion

It is a form of maceration which gentle heat is used during the process of extraction. It is used when moderately elevated temperature is not objectionable. The solvent efficiency of the menstruum is thereby increased

2.2.1.4 Decoction

In this process, the crude drug is boiled in a specified volume of water for a defined time; it is then cooled and strained or filtered. This procedure is suitable for extracting water-soluble, heatstable constituents. This process is typically used in the preparation of Ayurvedic extracts called “quath” or “kawath”. The starting ratio of crude drug to water is fixedatthe volume of 1:4 or 1:16; and is reducedto one-fourth of its original volume by boiling during the extraction procedure. Then, the concentrated extract is filtered and used as such or processed further.

2.2.1.5 Percolation

The most frequently used to extract active ingredients in the preparation of tinctures and fluid extracts is percolation. A percolator (a narrow, cone-shaped vessel open at both ends) is generally used. The solid ingredients are

moistened with an appropriate amount of the specified menstruum and allowed to stand for approximately 4 h in a well- closed container. The mass is obtained, and then packed and the top of the percolator is closed. Additional menstruum is added to form a shallow layer above the mass, and the mixture is allowed to macerate in the closed percolator for 24 h. The outlet of the percolator is then opened and the liquid contained therein is allowed to drip slowly [Figure.2]. Additional menstruum is added as required, until the percolate measures about three-quarters of the required volume of the finished product. The marc is then pressed and the expressed liquid is added to the percolate. Sufficient menstruum is added to produce the required volume, and the mixed liquid is clarified by filtration or by standing followed by decanting [38-44].

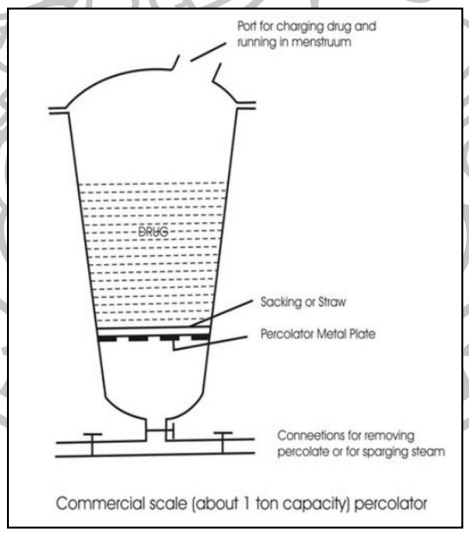


Figure 2 Percolation Extraction

2.2.1.6 Hot Continuous Extraction (Soxhlet)

Normally a solid material containing some of the desired compound is placed inside a thimble made from thick filter paper, which is loaded

into the main chamber of the Soxhlet extractor. The Soxhlet extractor is placed onto a flask containing the extraction solvent. The Soxhlet is then equipped with a condenser. The solvent is heated to reflux. The solvent vapour travels up a distillation arm and floods into the chamber housing the thimble of solid. The condenser ensures that any solvent vapour cools and drips back down into the chamber housing the solid material. The chamber containing the solid material slowly fills with warm solvent. Some of the desired compound will then dissolve in the warm solvent. When the Soxhlet chamber is almost full, the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask. This cycle may be allowed to repeat many times, over hours or days. During each cycle, a portion of the non-volatile compound dissolves in the solvent. After many cycles the desired compound is concentrated in the distillation flask [Figure.3].

The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled. After extraction the solvent is removed, typically by means of a rotary evaporator, yielding the extracted compound. The non-soluble portion of the extracted solid remains in the thimble, and is usually discarded.

The advantage of this method, compared to previously described methods, is less solvent used to extract large amount of drug. This method is saving time, energy andcost. At small scale, it is employed as a batch process only, but it becomes much more economical and viable when it is converted into a continuous extraction procedure on medium or large scale [45,46].

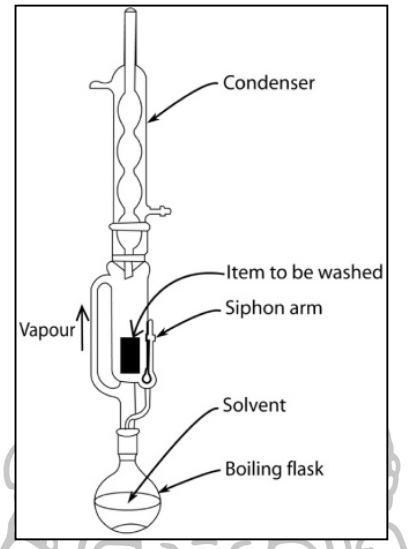


Figure 3 Soxhlet apparatus for hot extraction

2.2.1.7 Aqueous Alcoholic Extraction by Fermentation

Some medicinal preparations of Ayurveda (like asava and arista) has adopted the technique of fermentation for extracting the active principles. The extraction procedure involves soaking the crude drug, in the form of either a powder or a decoction (kasaya), for a specified period of time, during which it undergoes fermentation and generates alcohol in situ; this facilitates the extraction of the active constituents contained in the plant material. The alcohol thus generated also serves as a preservative. Water should first be boiled in the vesselwhenearthenvesselis used for the fermentation. For large-scale manufacture, wooden vats, porcelain jars or metal vessels are used in place of earthen vessels [47,48].

2.2.1.8 Counter-current Extraction

In counter-current extraction (CCE), wet raw material is pulverized using toothed disc disintegrators to produce a fine slurry. In this process, the material to be extracted is moved in one direction (generally in the form of a fine slurry) within a cylindrical extractor where it comes in contact with extraction solvent. The further the starting material moves, the more concentrated the extract becomes. Complete extraction is thus possiblyobtainedwhen the quantities of solvent and material and their flow rates are optimized. The process is highly efficient, requiring little time and posing no risk from high temperature. Finally, sufficiently concentrated extract comes out at one end of the extractor while the marc (practically free of visible solvent) falls out from the other end [49].

This extraction process has significant advantage [38-40,48,49]

i. A unit quantity of the plant material can be extracted with much smaller volume of solvent as compared to other methods like maceration, decoction, and percolation.

ii. CCE is commonly done at room temperature, which spares the thermolabile constituents from exposure to heat which is employed in most other techniques.

iii. As the pulverization of the drug is done under wet conditions, the heat generated during comminution is neutralized by water. This again spares the thermolabile constituents from exposure to heat.

iv. The extraction procedure has been rated to be more efficient and effective than continuous hot extraction.

2.2.1.9 Ultrasound Extraction (Sonication)

The procedure involves the use of ultrasound with frequencies ranging from 20 kHz to 2000 kHz; this increases the permeability of cell walls and produces cavitation. Although the process is useful in some cases, like extraction of rauwolfia root, its large-scale application is limited due to the higher costs. One of the main disadvantages of the procedure is the occasionallydeleterious effect of ultrasound energy (more than 20 kHz) on the active constituents of medicinal plants causing the formation of free radicals and consequently undesirable changes in the drug molecules [38-40,50-52].

2.2.1.10 Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) is an alternative sample preparation method with general goals of reduced use of organic solvents and increased sample throughput. The factors to consider are temperature, pressure, sample volume, modifier (cosolvent) addition, flow and pressure control, and restrictors. Generally, cylindrical extraction vessels are used for SFE and their performance is good beyond any doubt. The collection of the extracted analyte following SFE is another important step.Thesignificantloss can occur during this step, leading to the poor actual efficiency.

There are many advantages to the use of CO_2 as the extracting fluid. In addition to its favorable physical properties, carbon dioxide is inexpensive, safe and abundant, butit possesses several polarity limitations. Solvent polarity is applied when polar solutes and strong analyte-matrix interactions are

present. Organic solvents are frequently added to the carbon dioxide extracting fluid to alleviate the polarity limitations. Argon is being used recently because it is inexpensive and more inert. The component recovery rates generally increase with increasing pressure or temperature.The highest recovery rate in case of argon was obtained at 500 atm and 150°C [53-55].

The extraction procedure possesses distinct advantages:

i The extraction of constituents at low temperature, which strictly avoids damage from heat and some organic solvents.

ii No solvent residues.

iii Environmental friendly extraction procedure.

2.3 Identification of Medical plants

Due to the fact that plant extracts usually occur as a combination of various types of bioactive compounds or phytochemicals with different polarities, their separation still remains as big challenge for the process of identification and characterization of them. It is a common practice in isolation of these bioactive compounds that a number of different separation techniques such as Thin-Layer Chromatography (TLC), column chromatography, flash chromatography, Sephardim chromatography and High-Performance Liquid Chromatography (HPLC), should be used to obtain pure compounds

High-performance liquid chromatography (or High pressure liquid chromatography, HPLC) is a specific form of column chromatography generally used in biochemistry and analyzed to separate, identify, andquantify the active compounds [56]. HPLC consists of a column for a packing material as a stationary phase, a pump

to move the mobile phase(s) through the column, and a detector showingthe retention times of the molecules. Retention time varies depending on the interactions between the stationary phases, the molecules being analyzed, and the solvents used [57]. The sample to be analyzed is introduced to asmall volume of the stream of mobile phase and is retarded by specific chemical or physical interactions with the stationary phase. The amount of retardation depends on the nature of the analyte and composition of both stationary and mobile phase. The time at which a specific analyte elutes (comes out at the end of the column) is called the retention time. Common solvents used include some miscible combinations of water or organic liquids (the most common are methanol and acetonitrile) [57,58]. Seperation has been done to vary the mobile phase composition during the analysis; this is known as a gradient elution [58]. The gradient can separate the analyte mixtures as a function of the affinity between the analyteandthe mobile phases. The choice of solvents, additives and gradient depend on the nature of the stationary phase and the analyte.

2.3.1 Types of HPLC

Types of HPLC generally depend on phase system used in the process [58, 59]. Types of HPLC generally used in analysis as the followings:

2.3.1.1 Normal phase chromatography

This method is also known as Normal phase HPLC (NP-HPLC). The separationis based on polarity of analytes. NP-HPLC uses a polar stationary phase and a non-polar mobile phase. The polar analyte interacted with and is retained by the polar stationary phase. Adsorption strengths increase with increased analyte polarity, and the interaction between the polar analyte and the polar stationary phase increases the elution time.

2.3.1.2 Reversed phase chromatography

Reversed phase HPLC (RP-HPLC or RPC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. RPC operates on the principle of hydrophobic interactions, which results from repulsive forces between a polar eluent, the relatively non-polar analyte, and the non-polar stationary phase.

Reversed-phase chromatography is the most commonly used separation technique in HPLC due to its broad application range. It is estimated that over 65% of all HPLC separations are carried out in the reversed phase mode. The reasons for this include the simplicity, versatility, and scope of the reversed-phase method as it is able to handle compounds of a diverse polarity and molecular mass [40,41], for example, to identify secondary plant metabolites.

2.3.1.3 Size exclusion chromatography

Size exclusion chromatography (SEC), also called as gel permeation chromatography or gel filtration chromatography mainly separates particles on the basis of size. It is also useful for determining the tertiary structure and quaternary structure of proteins and amino acids. This technique is widely used for the molecular weight determination of polysaccharides.

2.3.1.5 Ion exchange chromatography

Ion-exchange chromatography, retention is based on the attraction between solute ions and charged sites bound to the stationary phase. Ions of the same charge are excluded. [58,59]

2.3.1.5 Bio-affinity chromatography

Separation is based on specific reversible interaction of proteins with ligands. Ligands are covalently attached to solid support on a bio-affinity matrix, retaining proteins with interaction to the column-bound ligands.

2.3.2 Parameters of HPLC

For the accurate analysis of a compound, there are some parameters which are used as a standard for a particular compound. If there is a change occured in the parameters the result may be affected greatly. The most commonly used parameters are internal diameter, particle size, pore size and pump pressure. For different compounds the parameters can be modified according to their nature and chemical properties.

2.3.2.1 Internal diameter

The internal diameter (ID) of HPLC column is a critical aspect that determines quantity of analyte. Larger columns are usually seen in industrial applications such as the purification of a drug product for later use. Low ID columns have improved sensitivity and lower solvent consumption of loading capacity.

2.3.2.2 Particle size

Most traditional HPLC is performed with the stationary phase attached to the outside of small spherical silica particles (very small beads). Smaller particles generally provide more surface area and better separations, but the high pressure is required for optimum linear velocity.

2.3.2.3 Pore size

Many stationary phases are porous providing greater surface area. Small pores provide greater surface area while larger pore size has better kinetics especially for larger analytes. Pore size defines an ability of the analyte molecules to penetrate inside the particle and interact with its inner surface.

2.3.2.4 Pump pressure

Pumps have various pressure capacities, but their performance is measured on their ability to yield a consistent and reproducible flow rate. Modern HPLC systems have been improved to work at much higher pressures, therefore it can be used at much smaller particle sizes of the columns.

2.3.3 Intrumentation of HPLC

2.3.3.1 Injection of the sample

Sample can be injected when the mobile phase is flowing or it is stopped. A new advanced rotary valve and loop injector can be used to produce reproducible results.

2.3.3.2 The detector

There are several ways to detect a substance. Generally UV spectroscopy is attached, which detect the specific compounds. Many organic compounds absorb UV light atvarious wavelengths. The amount of light absorbed will depend on the amount of a particular compound that is passing through the beam at the time.

2.3.3.3 Interpreting the output from the detector

The output is recorded as a series of peaks, each one representing a compound in the mixture passing through the detector and absorbing

UV light. The area under the peak is proportional to the amount of substance, which is passed through detector. The area can be calculated automatically by the computer linked to the detector.

2.3.4 Application of HPLC

The information including identification, quantification, and resolution of a compound can be obtained using HPLC. Preparative HPLC refers to the process of isolation and purification of compounds

2.3.4.1 Chemical Separations

The separation is based on property of compound possessing different migration rates. The extent or degree of separation is dependent on the choice of stationary phase and mobile phase.

2.3.4.2 Purification

Purification is defined as the process of separation or extraction of the target compound from a mixture of compounds or contaminants. Each compound showed a characteristic peak under certain chromatographic conditions. The widely different migration rate of the compounds and contaminants through the column is required to purify the desired compound without any incurring undesired compound.

2.3.4.3 Identification

The requirements for identification of compound are clean peak from the chromatograph of the known sample, a reasonable retention time and well separation from extraneous peaks at the detection levels.

2.3.5 Other applications of HPLC: Other applications of HPLC are as the followings;

2.3.5.1 Pharmaceutical applications [60-63]

i. Tablet dissolution study of pharmaceutical dosage form

ii. Shelf-life determinations of pharmaceutical products

iii. Identification of active ingredients of dosage forms

iv. Pharmaceutical quality control

2.3.5.2 Environmental applications [64-67]

i. Detection of phenolic compounds in drinking water

ii. Identification of diphenhydramine in sedimented samples

iii. Bio-monitoring of pollutant

2.3.5.3 Forensics [68-70]

i. Quantification of the drug in biological samples.

ii. Identification of anabolic steroids in serum, urine, sweat, and hair

iii. Forensic analysis of textile dyes.

iv. Determination of cocaine and metabolites in blood

2.3.5.4 Clinical [71-74]

i. Quantification of ions in human urine analysis of antibiotics in blood plasma.

ii. Estimation of bilirubin and bilivirdin in blood plasma in case of hepatic disorders.

iii. Detection of endogenous neuropeptides in extracellular fluids of brain.

2.3.5.5 Food and Flavor [75]

i. Ensuring the quality of soft drink and drinking water.

ii. Analysis of beer.

iii. Sugar analysis in fruit juices.

iv. Analysis of polycyclic compounds in vegetables.

v. Trace analysis of military high explosives in agricultural crops.

2.4 Antioxidants

The adverse effects of oxidative stress on human health have become a serious issue. The World Health Organization (WHO) has estimated that 80% of the earth's inhabitants rely on traditional medicine for their primary health care needs and most of this therapy involves the use of plant extracts and their active components [76]. Under stress, our bodies produce more reactive oxygen species (ROS) (e.g., superoxide anion radicals, hydroxyl radicals and hydrogen peroxide) than enzymatic antioxidants (e.g., superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase) and non-enzymatic antioxidants (e.g., ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione, carotenoids, and flavonoids). This imbalance leads to cell damage and health problems [77-83]. A lack of antioxidants, which can quench the reactive free radicals, facilitates the development of degenerative diseases [84], including cardiovascular diseases, cancers [85], neurodegenerative diseases, Alzheimer's disease [86] and inflammatory diseases [87]. One solution to this problem is to supplement the diet with antioxidant compounds that are contained in natural plant sources [88]. These natural plant antioxidants can therefore serve as a type of preventive medicine. Recent reports indicate that there is an inverse relationship between the dietary intake of antioxidant-rich foods and the incidence of

human disease. However, synthetic antioxidants, such as butylatedhydroxytoluene (BHT) and butylatedhydroxyanisole (BHA), have been widely used as antioxidants in the food industry and may be responsible for liver damage and carcinogenesis [89]. For this reason, interest in the use of natural antioxidants has increased. Plants have been the basis of traditional medicines throughout the world for thousands of years and continue to provide newremedies to humankind; a great deal of effort has therefore focused on using available experimental techniques to identify natural antioxidants from plants. Several authors have reviewed the beneficial uses of these plant species [90,91]. Recently, some researchers [92] reviewed twenty-four medicinal Indian herbs possessing a highly potential antioxidant activity. This review covers medicinal species from various countries (Africa, Algeria, The United States of America, Australia, Brazil, Bulgaria, China, India, Iran, Italy, Japan, Malaysia, Poland, Portugal, Thailand and Turkey).

Oxygen is absolutely essential for the life of aerobic organism but it may become toxic at the higher concentrations. Dioxygen in its ground state is relatively inactive; its partial reduction gives rise to active oxygen species (AOS) such as singlet oxygen, super oxide radicalanion, hydrogen peroxide etc. This is partly due to the oxidative stress that is basically the adverse effect of oxidant on physiological function. Free oxygen radicals plays cardinal role in the etiology of several diseases like arthritis, cancer, atherosclerosis etc. The oxidative damage to DNA may play vital role in aging [93] and the presence of intracellular oxygen also can be responsible to initiate a chain of in advertent reaction at the cellular level. These reaction causedamage to critical cell biomolecules. These radicals are highly toxic and thus generate oxidativestress in plants. Plants and other organisms have provided

a wide range of mechanism to combat with the free radical problems. Free radicals are an atom or molecule bearing an unpaired electron and they are extremely reactive and capable of engaging in rapid change reaction [Figure 4]. This leads to destabilize other molecules and generate many more free radicals. In plants and animals, these free radicalsare deactivated by antioxidants.

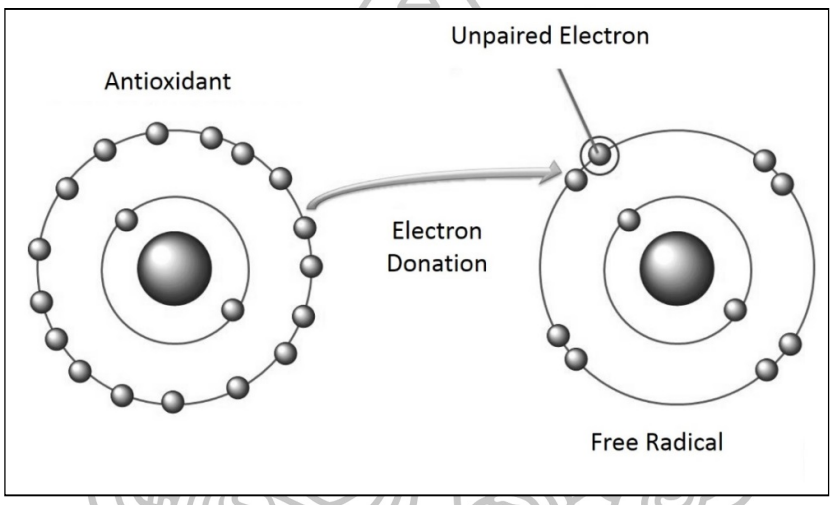


Figure 4 How antioxidants reduce the free radicals

Plants have developed an array of defense strategies (antioxidant system) to cope with oxidative stress. The antioxidative system includes both enzymatic and non-enzymatic systems. The examples of non enzymatic system are ascorbic acid (vitamin C)ά-tocopherolandcartenes etc. and those of enzymatic system are superoxide dismutase (SOD), catalase (CAT), peroxidase (POX),ascorbate peroxidase (APX), glutathione reductase (GR) and polyphenol oxidase (PPO) etc. The function of this antioxidant system is to scavenge the toxic radicals produced during oxidativestress and thus help the plants to survive through such conditions.

Spices and herbs in food as medicine is a current hot trend that is capturing everyone'simagination with an image of a new *magic bullet or fountain of youth*. The intake of antioxidant compounds present in food is an important health-protecting factor. Natural antioxidants present in foods and other biological materials have attracted considerable interest because of the presumed safety and potentially nutritional and therapeutic effects. Because extensive and expensive testing of food additives is required to meet safety standards, synthetic antioxidants have generally been eliminated from many food applications. The increasing interest in thesearch for natural replacements of synthetic antioxidants has led to the antioxidant evaluation of a number of plant sources.

It is well known that Mediterranean diet, which is rich in natural antioxidants, leads to a limited incidence of cardio- and cerebrovascular diseases [94]. It is known that compounds belonging to several classes of phytochemical components such as phenols, flavonoids, and carotenoids are able to scavenge free radical such as $O2^{\cdot}$, $OH^{\cdot}$, or lipid peroxyl radical $LOO^{\cdot}$ in plasma [95]. The effective intake of single food antioxidants and their fate in the human body have been defined only for a few compounds [96,97]. It is reasonable that the higher the antioxidant content in foods is, the higher the intake by the human body will be. Natural antioxidants occur in all parts of plants. These antioxidants are carotenoids, vitamins, phenols, flavonoids, dietary glutathione, and endogenous metabolites. Plant-derived antioxidants have been shown to function as singlet and triplet oxygen quenchers, free radical scavengers, peroxide decomposers, enzyme inhibitors, and synergists. The most current research on antioxidant action focuses on phenolic compounds such as flavonoids. Fruits and vegetables contain different antioxidant

compounds, such as vitamin C, vitamin E and carotenoids, their activities have been established in recent years.

2.4.1 An overview of the assay methods used to estimate antioxidant content

Antioxidants, from phenolic compounds (e.g., flavonoids, phenolic acids and tannins), have diverse biological effects, such as anti-inflammatory, anti-carcinogenic and anti-atherosclerotic effects, as a result of their antioxidant activity [98]. The antioxidant extracts were evaluated in terms of their total phenols (TP), total flavonoids (TFA), total flavonols (TFO), phenolic acids, catechins, lignans and tannins [99,100]. The antioxidant properties were evaluated using the following methods: 1,1-diphenyl-2-picrylhydrazine (DPPH) radical scavenging assay [101-105], β-carotene linoleic acid bleaching assay [106-108], inhibition of linoleic acid peroxidation [109], ferric reducing antioxidant power (FRAP) [110-112], total radical trapping antioxidant potential (TRAP) assay [113-115], oxygen radical absorbance capacity (ORAC) assay [116-118], 15-lipoxygenase inhibition, lipid peroxidation (LPO) method [119], nitro blue tetrazolium (NBT) reduction assay or superoxide anion scavenging activity [120-122], hydroxyl radical scavenging activity [123-125] or non-site and site-specific deoxyribose degradation assay, hydrogen peroxide scavenging activity, enzymatic and non-enzymatic *in vitro* antioxidant assay, 2,2-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging method [126-128], reducing power assay, 50% inhibition of a particular assay (IC50), Briggs Rauscher (BR) method [129], Trolox equivalent antioxidant capacity (TEAC) method [130,131], phenazine methosulfate–nicotinamide adenine dinucleotide reduced (PMS-NADH) system superoxide radical scavenging [132], linoleic acid peroxidation,

ammonium thiocyanate (ATC) method [133], ferric thiocyanate (FTC) method, thiobarbituric acid (TBA) method [134] and luminol-photochemiluminescence (PCL) assay. The phenolic concentration was determined using the Folin-Ciocalteau (FTC) method [135-137], while the total phenol content [138-139], the total flavonoid content [140-142], the tannin content [143] and the total flavanol content [144] were also determined by differences methods. Some of method were described as below. Each method has its own merits and drawbacks, it is important to employ a consistent and rapid method. Although many methods are available to determine antioxidant activity, the most common and reliable methods are the ABTS and DPPH methods. Both methods have been modified and improved in recent years.

2.4.1.1 DPPH method

The 1,1-diphenyl-2-picrylhydrazine (DPPH) radical scavenging assay was first described by Blois in 1958 [145,146] and was later modified slightly by numerous researchers. It is one of the most extensively used antioxidant assays for plant samples. DPPH, a stable free radical, canreact with compounds attributing to a hydrogen atom. This method is based on the radical scavenging capability of DPPH through the addition of a radical species causing decolourization of the DPPH solution. The antioxidant activity is then measured by the decrease in absorption at 515 nm.

2.4.1.2 ABTS method

The ABTS radical scavenging method was first developed by Rice- Evans and Miller in 1994 [126] and modified by Re et al. in 1999 [127]. The modification is based on the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS•+ producing a radical cation. The ABTS radical cation is

generated by the oxidation of ABTS with potassium persulfate, and its reduction in the presence of hydrogen-donating antioxidants is measured by spectrophotometer at 734 nm. This decolourisation assay measures the total antioxidant capacity in both lipophilic and hydrophilic substances. The antioxidant activity is determined based on antioxidant concentration and the duration of the inhibition of the radical cation. Trolox, a water-soluble analog of Vitamin E, is used as a positive control. The activity is expressed in terms of the Trolox-equivalent antioxidant capacity of the extract (TEAC/mg).

2.4.1.3 ORAC assay

The ORAC assay uses beta-phycoerythrin (PE) as an oxidizable protein substrate and 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) as a peroxyl radical generator or a $Cu2+H_2O_2$ system as a hydroxyl radical generator. To date, it is the only method that takes the free radical reaction and uses an area-under-the-curve (AUC) technique for quantification, thereby combining both the inhibition percentage and the length of the inhibition time of the free radical's action into a single quantity. The assay has been widely used in many recent studies of plants.

2.4.1.4 PCL assay

The PCL assay measures the antioxidant capacity of a compound against the superoxide radical in lipid (ACL) and aqueous (ACW) phases. This method can quantify the antioxidant capacity of both hydrophilic and lipophilic substances either as pure compounds or as a component in a complex matrix from various origins, including synthetic, vegetable, animal, or human sources. The PCL method can provide approximately 1000-fold acceleration of the oxidative reactions

in vitro when an appropriate photosensitizer is used. The PCL method is a very quick and sensitive method of measurement. Using the PCL assay, researchers have determined the antioxidant properties of marigold flowers.

2.4.1.5 Folin-Ciocalteu method

All phenolic compounds contained in plants are oxidized by Folin-Ciocalteu reagent. This reagent is formed from a mixture of phosphotungsticacid, $H_3PW_{12}O_{40}$, and phosphomolybdic acid, $H_3PMo_{12}O_{40}$. The oxidation of the phenolscause a reduction of a mixture of blue oxides of tungsten, W_8O_{23}, and molybdenum, Mo_8O_{23}. The blue coloration produced has a maximum absorption in the region of 750 nm, and is proportional to the total quantity of phenolic compounds originally present [135].

2.5 Spray dryer

The spray dryer is a device used to produce dried powder. It consists of spraying column, nozzle and collector in a drum or cyclone. The liquid input stream is sprayed through a nozzle into a hot vapor stream and vaporized. The solid powder is formed as moisture contents quickly leave the droplets. The solid is then collected in a drum or cyclone. A nozzle is usually used to make the droplets as small as possible to maximize the heat transfer and rate of water vaporization. The spray dryers can dry a product very quickly compared to other methods of drying. They also turn a solution or slurry into a dried powder in a single step, which can be the advantage for maximizing the profit and minimize the process [147,148].

2.5.1 Principle of spray drying technique

The first spray dryers were manufactured in the USA in 1933. Spray drying is one of the best drying methods to convert directly the fluid materials into solid or semi-solid particles [148]. Spray drying is a unit operation by which a liquid product is atomized in a hot gas current to instantaneously obtain a powder. The gas generally used is air or more rarely an inert gas, particularly nitrogen gas. The initial liquid feeding can be a solution, an emulsion or a suspension [149]. It can be used to both heat-resistant and heat sensitive products. Various factors of spray drying such as process of spraying, apparatus and feed parameters have an influence on the quality of final product [150]. The spray drying process can produce a good quality final product with low water activity and reduce the weight, resulting in easy storage and transportation. The physicochemical properties of the final product mainly depend on inlet temperature, air flow rate, feed flow rate, atomizer speed, types of carrier agent and their concentration. Spray drying is often selected as it is a rapid processprovidingthecontrolled particle size distribution [151].

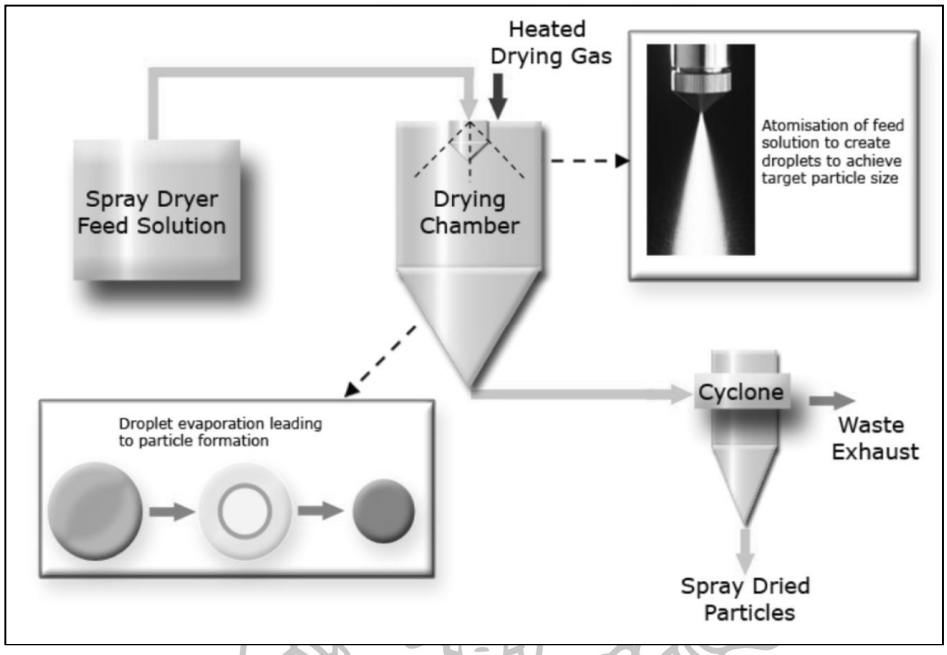


Figure 5 Schematic Diagram of Spray Drying Process

2.5.2 Schematic Diagram of Spray Drying Process

Spray drying is a unit operation in which a liquid stream (solution, suspension or emulsion) is continuously divided into very fine droplets (a process known as atomization) into a drying chamber. Once the droplets meet the warm air in the drying chamber they rapidly evaporate to form dry particles, which are separated from the drying gas using a cyclone or a bag-filter. Hence the spray drying process can be described as consisting of four events:

i. atomization of the liquid into droplets

ii. contact of the droplets with the warm drying gas

iii. rapid evaporation of the droplets to form dry particles

iv. recovery of the dry particles from the drying gas, using a cyclone/filter

The schematic (Figure 5), the key features of the continuous spray drying processisshown as the followings: A liquid is atomized into a drying chamber using a 2-fluid nozzle (powered by compressed air) and exhaust gases are vented to the atmosphere. Other available type of spray dryer is a closed loop system which the exhaust gas is re-cycled (through a condenser to remove solvents) prior to reentering the drying chamber. When flammable solvents are incorporated, nitrogen gas will be applied in closed loop system rather than air in order to minimize the risk of explosions.

2.5.3 Key features of spray dried powders

Spray drying is one of the most flexible processing technologies in the pharmaceutical industry. It has a remarkable capability to manipulate powder properties such as particle size, morphology and density. This technology can produce various particle sizes from very fines to large particles. They canbe used in a variety of powder formulations such as inhalation, solid dispersions, microcapsules for drug protection or controlled release. In addition spray drying is also suitable for difficult-to crystallize materials and temperature sensitive compounds such as proteins, peptides and monoclonal antibodies (the relatively short exposure to temperature makes it suitable for even the most sensitive materials).

2.5.4 Basic steps of spray drying

2.5.4.1 Concentration

Generally, the concentrated feedstock is introduced into the spray dryer. The concentration of juice has been increased and liquid solvent is evaporated until solid powder is obtained. Generally, the concentration of feedstock in conventional large scale before introducing into spray dryeris around 50%-60%

whereas the small scale laboratory spray dryer is more diluteddue to easiness in clogging [148,152].

2.5.4.2 Atomization

Atomization is the process of conversion of bulk liquid into a spray or mist, often by passing the liquidthrough a nozzle. The sprayed liquid through nozzle will increase the surface area of liquid which it is later contacted to hot air and then dried into a powder. The nozzle size may differ according to the size of spray dryer. Droplet size ranges from 20 μm to 180 μm and it depends on the nozzle. The aim of this stage is to create a maximum heat transferring surface between the dry air and the liquid, in order to optimize heat and mass transfers. The choice upon the atomizer configuration depends on the nature and viscosity of feed and desired characteristics of the dried product [153,154].

2.5.4.3 Droplet-air contact

The important component of spray dryer is the chamber; here the sprayed droplet is contacted with the hot air and the drying process begins. Air is heated by the heating element then entering the chamber to a predefined temperature depending upon the characteristics of the feed fluid. The hot air is brought in contact with the spray droplets in the following ways through the air distributor.

Co-current-air and particles move in the same direction.

Counter-current-air and particles move in the opposite direction.

Mixed flow - particles are subjected to co-current and counter-current phase.

The thermal energy of the hot air is used for evaporation and the cooled air pneumatically conveys the dried particles in the system. The contact time of the hot air is only a few seconds, once the drying is achieved and the air temperature of air drops instantaneously. The nozzle increases the contact area of droplet and hot air influences the huge heat transfer between droplet and hot air. The hot air evaporates moisture content in the droplet and changes into powder form. In co-current process the liquid is sprayed in the same direction as the flow of hot air through the apparatus, hot air inlet temperature is typically 150-220°C, evaporation occurs instantaneously and then dry powders will be exposed to moderate temperatures (typically 50-80°C) which limits the thermal degradations. In counter current drying, the liquid is sprayed in the opposite direction from the flow of hot air the high temperature process. Thermo-sensitive products are usually restricted to in this process. However, the main advantage of this process is considered as more economic in term of consuming energy [148,149,153,154]

2.5.4.4 Droplet drying

At the stage of droplets - hot air contacts between the liquid and gas phases and balances the temperature and established the vapor partial pressure. Heat transfer is carried out from the air towards the product and thus induces the difference in temperature. Water transfer is carried out in the opposite direction due to the vapor pressure difference. Based on the drying theory, three successive steps can be distinguished. Just after the hot air-liquid contact, heat transfer majorly causes the increase of droplets temperature up to a constant value. This value is defined as the air drying humid thermometer temperature; after that, the evaporation of water droplet is carried out at a constant temperature and water vapor

partial pressure. The diffusion rate of water from the droplet core to its surface is usually considered as constant and equal to the surface evaporation rate. A dry crust is formed at the droplet surface when the water content reaches a critical value, The drying rate, then, rapidly decreases with the drying front progression and becomes dependent on the water diffusion rate through this crust. Drying process is finished when the particle reaches the air temperature. Each product has different particle-forming characteristics such as expansion, contraction, fracture or disintegration. The resulting particles may be relatively uniform and hollow spheres, or porous and irregular shape [148,149,153,154].

2.5.4.5 Separation of dried particles

The separation is often done through a cyclone, which is placed outside the dryerforreduction of product loss in the atmosphere. The dense particles are recovered at the bottom of the drying chamber while the finest ones pass through the cyclone separated from the humid air. In addition to cyclones, spray dryers are commonly equipped with the filters, called ‘‘bag houses’’ for removing the finest powder, and the chemical scrubbers for removingthe remaining powder or any volatile pollutants (e.g. Flavourings). The drop of water and gas content is depending on the composition and these particles can be compact or hollow [149,153,155].

2.5.4.6 Carrier agent

Knowledge of food properties is essential to know and thus will help to optimize the processes, functionalities, to reduce costs, mainly in the case of powders produced or used in pharmaceutical and food industries. Properties such as moisture content and water activity are essential for powder stability and storage. Bulk density is important for packaging and shipping considerations. Particle size

distribution is having major role in processing, handling and shelf life and the microstructure is related to powders functionality, stability and flowability.

The powder obtained by spray drying might have some problems with their property, such as stickiness, hygroscopic and solubility, due to the presence of low molecular weight of sugars (sucrose, glucose and fructose) and acids. The low molecular weight of sugars hasa low glass transition temperature [147,156]. The glass transition temperature (Tg), is the temperature at which the amorphous phase of the polymer is converted between rubbery and glassy states. Thus, these substances can stick on the dryer chamber wall during drying, leading to low product yield and operational problems. The low glass transition temperature (Tg), high hydroscopic, low melting point, and high water solubility of the dry solids can produce highly sticky products. Roos and Karel [157] stated that these solid materials are very hygroscopic in amorphous state and loose flowing character at high moisture content. These problems can be solved by the addition of some carrier agents, like polymers and gums, prior to spraying. Moreover, carrier agent is also used for microencapsulation. Carrier agent can protect sensitive food components against unfavorable ambient conditions and it canmask or preserve flavours and aromas, reduce thevolatility and reactivity and provide additional attractiveness for the merchandising of food products [156]. The common carrier agents are maltodextrins and gum Arabic [158,159]. Maltodextrins are products of starch hydrolysis, consisting of D-glucose units linked mainly by $\alpha(1\rightarrow4)$ glycosidic bonds. They are described by their dextrose equivalence (DE), which is inversely related to their average molecular weight. Maltodextrins are low cost and very useful for spray drying process on food materials. Gum Arabic is natural plant exudates of Acacia

trees, which consists of a complex heteropolysaccharide with highly ramified structure. It is the only gum used in food products which has the high solubility and low viscosity in aqueous solution, making it easier for spray drying process [160, 161]. The uses of different carrier agents and drying conditions produces the different physicochemical properties of powders.

2.5.5 Factors influencing the properties of powder produced by spray drying technique

Spray drying technique is widely used in food and pharmaceutical industries to produce food and drug powders due to its effectiveness under the optimum condition [156,158]. The spray drying parameters such as inlet temperature, air flow rate, feed flow rate, atomizer speed, types of carrier agent and their concentrations haveinfluences on particle size, bulk density, moisture content, yield and hygroscopicity in spray dried foods [147,152,162]. The detail of all these parameters is described belows.

2.5.5.1 Inlet temperature

Powder properties such as moisture content, bulk density, particle size, hygroscopicity and morphology are affected by inlet temperature. Normally, the inlet temperature of spray drying technique for food powder is 150-220°C. Chegini and Ghobadian [152] studied the effect of inlet temperature (110-190°C) on the moisture content of orange juice powder. It was found that at a constant feed flow rate, an increase in the inlet air temperature reduced the residual moisture content. The similar observation was obtained in different fruit juice powders such as watermelon juice [163], tomato juice [164], acai juice [165] and pineapple juice [156]. Moisture content decreased with the increase in drying

temperature, due to the faster heat transfer between the product and drying air. At higher inlet air temperatures, there is a greater temperature gradient between the atomized feed and drying air resulting in the greatest driving force for water evaporation. Additionally, the inlet temperature affects the bulk density of powder. Tonon et al. [166] studied the effect of inlet temperature (140, 170, 200ºC) on the bulk density of acai juice powder and found that the increased temperature caused the reduction in bulk density. This leads to the formation of vapor-impermeable films on the droplet surface, followed by the formation of vapor bubbles and, consequently the droplet expansion [152,165,166]. Walton [167] reported the increase in drying air temperature generally causes the decrease in bulk and particle densities and provides the greater tendency to get the hollow particle. In addition, the particle size was affected by inlet temperature as reported by Tonon et al. [166]. The use of higher inlet air temperature leads to the production of larger particles and causeing the higher swelling. The similar finding was also obtained by other authors [152,168]. Reineccius [169] reported that drying at higher temperatures resulted in faster drying rates leading to an early formation of a structure and not allowingshrink of particles during drying. When the inlet air temperature is low, the particle remains shrunken and smaller than the higher inlet temperature. Nijdam and Langrish [168] reported the similar results in the production of milk powder at 120°C and 200°C. Chegini and Ghobadian [152] also obtained similar results in the production of orange juice powder. An increase in inlet air temperature often results in a rapid formation of dried layer at the droplet surface.

The inlet air temperature has a role on the hygroscopicity of the powder. Tonon et al. [165] studied the effect of inlet temperature (140, 170,

200°C) on the hygroscopicity of acai juice powder. The higher drying temperature is lower the moisture content and increase its hygroscopicity (its capacity to absorb ambient moisture). It was in agreement with the work of Goula et al. on the spray drying of tomato pulp [164]. Moreover, the effect of inlet temperature on process yield of acai juice powder was investigated by Tonon et al. [165]. The increase in inlet temperatures has given the higher yield due to the greater efficiency of heat and mass transfer processes occurred when the higher inlet air temperatures were used. On the other hand, the lower yield was reported with the increase in inlet air temperature as a result of melting of the powder and cohesiveness to wall of spraying chamber [147,170,171,172]. Furthermore, the inlet temperature also influenced the morphology of acai juice powder as reported by Tonon et al. [165].

2.5.5.2 Air dry flow rate

The moisture content in tomato powder increased with an increase in flow rateofdrying air [173,174]. Generally, the energy used for evaporation is varied according to the amount of drying air. The rate of air flow must be at a maximum in all cases. The movement of air is decided the rate and degree of droplet. Evaporation by inducing, the passage of spray through the drying zone. The concentration of product in the region of the dryer walls and finally extent the semi-dried droplets and thus re-enter the hot areas around the air disperser. A lower flow rate of drying air causes an increase in the halting time of productin drying chamber and enforcement in the circulatory effects [175-177]. The increased residence time leads to the greater degree of moisture removal. As a result, the increase in the flow rateof drying air decreases the residence time of the product in the drying chamber leading to higher moisture contents gained. In addition, the effect of drying- air flow

rate of bulk density depends on its effect on moisture content due to the sticky nature of the product. The higher moisture content in the powder leads to stick together and consequently leaving more interspaces between them and it results the larger bulk volume. Therefore, the raise of air flow rate leads to an increase of moisture content in the powder and decreased in powder bulk density. Masters [176] reported that, the increasing residual moisture content was increased the bulk density of dry product. In addition, powder solubility was influenced by air dry flow rate. The effect of drying air flow rate on powder solubility depends on its effect on powder moisture content, as low moisture content seemed to be associated with the fast dissolution. The rising of air flow rate was led to the increased of powder moisture content and decrease in powder solubility [178].

2.5.5.3 Atomizer speed

Chegini and Ghobadian [179] studied the effect of atomizer speed (10,000-25,000 rpm) on the properties of an orange juice powder. The residual moisture content decreased withthe increase in the atomizer speed. At higher atomizer speed, the smaller droplets were produced and more moisture was evaporated resulting from an increased contact surface. This was correlated with the findings of Knipschildt [180]. The higher atomizer speed resulted in a smaller particle size and quicker drying due to the larger surface area and consequently preventing the ‘‘skinning’’ over the droplets. The increased atomizer speed contributed to the smaller droplet and particle size spreading into thin film layer. Additionally, the higher atomizer speedthe higher bulk density was. However, the increase in atomizer speed might cause the spread on a large surface resulting in the reduction in particle size and bulk density [181].

2.5.5.4 Feed flow rate

The feed flow rate has an influence on the moisture content and product yield. Higher flow rates give a shorter contact time between the feed and drying air and causing a less efficiency in the heat transfer and thus the lower water evaporation. The higher feed flow rate shows a negative effect on yield, resulting in the decreased heat, mass transfer and the lower yield [166]. In addition, this might be due to a dripping inside the main chamber resulting in an unatomized liquid and lower yield is obtained. Toneli et al. [182] also verified an increase on mass production rate with increasing air temperatures and decreasing pump speeds in the spray drying of innulin, The higher yield was shown from this study.

2.5.5.5 Type of carrier agent

The addition of high molecular weight additives to the product before atomizing is widely used as an alternative way to increase Tg of powder [183]. The use of carrier agents such as maltodextrins, gum Arabic, waxy starch, and microcrystalline cellulose influenced the properties and stability of the powder. Powders in crystalline and amorphous forms show differences in particle size, shape, bulk density, physicochemical properties, chemical stability, water solubility and hygroscopicity [162]. The common carrier agents are maltodextrins and gum Arabic. Goula and Adamopoulos [175] studied the effect of maltodextrin (maltodextrin 6DE, 12DE, 21DE) on the properties of tomato powder. The result showed that the higher maltodextrin dextrose equivalent (DE) causedthe higher moisture content in the powder. This was probably due to chemical structure of high-DE maltodextrins, possessing a high number of ramifications with hydrophilic groups, contributing to easily bind with water molecules from the ambient air during powder

handling. Additionally, higher maltodextrin DE showed the increase in bulk density due to its stickiness.

2.5.5.6 Concentration of carrier agent

The concentration of the carrier agent also affected the powder properties. Low concentration of carrier agent may give the stickiness powder. Investigation of the effect of maltodextrin concentrations (0, 3 and 5%) on the properties of the watermelon juice powder [163]. The result showed that there was no accumulated powders in the collector if maltodextrin was notused. The particles produced were very sticky and mainly deposited onto the wall of drying chamber and cyclone and it could not, thus, be recovered. The addition of 5% maltodextrin to the feed appeared to give better results than addition of 3% maltodextrin. These results showed that the maltodextrin was a useful drying aid in the spray drying process of watermelon juice resulting in improving the yield of product. The addition of maltodextrin could increase the total solid content in the feed and thus, reduce the moisture content of the product. It was suggested that maltodextrin could alter the surface stickiness of low molecular weight sugars such as glucose, sucrose and fructose and organic acids, therefore, it could facilitate drying and reducing stickiness of the spray- dried product. However, if the maltodextrin was more than 10%, the resulted powders lost their attractive red-orange colour [163].

The concentration of the carrier agent also affected the moisture content of powder. Moisture content of powder had increased with the increase in maltodextrin concentration as it was reported [175]. The similar observation was attained in the maltodextrin addition on drying kinetics and stickiness of sugar and acid-rich foods during convective drying and it also lowered the drying

rate [184]. This study was in accordance with the production of orange juice powder [185]. In contrast, the moisture content of spray dried watermelon juice powders decreased when the increased addition of maltodextrin. The addition of the drying additives has increased the total solids of the feed and reduced the amount of water evaporation. This was in accordance with the findings of Jittanit*et al*. [156]. Jittanit reported that the increase in maltodextrin concentration resulted in the decreased moisture content of pineapple juice powder. It was due to the maltodextrin has the capability to hurdle the sugars in the fruit powder that have the highly hygroscopic nature of absorbing the humidity in the surrounding air [186].

2.6 Skin

The skin is the largest organ of the body, with a total area of about 20 square feet. The skin protects us from microbes and the elements, helps regulate body temperature, and permits the sensations of touch, heat, and cold.

2.6.1 The structure and function of skin

Skin is the outermost layer of the human organism separating the internal from the external environment. It is the largest organ of the body, accounting for more than 10% of body mass [187]. Itweighs an average of 4 kg and covers an area of 2 m^2. It acts as a barrier, protecting the body from harsh external conditions and preventing the loss of important body constituents, especially water. One of its most important purposes is to function as a barrier; i.e., to prevent the entry of foreign materials and invasion of pathogens into and the loss of endogenous substances such as water out of the body. Adeath from destruction of skin, as in a burn or intoxic epidermal necrolysis and the miseryof unpleasant acne, remind us of its many

importantfunctions, which range from the vital to the cosmetics (Table.1). The skin has three layers. The outer one is the epidermis, which is firmly attached to, and supportedby connective tissue in the underlying dermis. Beneaththe dermis is a loose connective tissue, the subcutis/hypodermis, which usually contains abundant fat (Figure. 6). It would be very useful to predict the penetrate rate of materials to access potentially toxicological hazards improving transdermal administration. The structure of human skin is illustrated in Figure 8. The major skin layers, from inside to outside, comprise the fatty subcutaneous layer (hypodermis), the dermis of connective tissue and the stratified avascular cellular epidermis [188].

Table 1 Functions of the skin.

Function	Structure/cell involved
Protection against:	
- chemicals, particles	Horny layer
- ultraviolet radiation	Melanocytes
- antigens, haptens	Langerhans cells
- microbes	Langerhans cells
Preservation of a balanced internal environment	Horny layer
- Prevents loss of water, electrolytes and macromolecules	Horny layer
- Shock absorber Strong, yet elastic and compliant	Dermis and subcutaneous fat
- Temperature regulation	Blood vessels
Insulation	Eccrine sweat glands
Sensation	Subcutaneous fat
Lubrication	Specialized nerve endings
Protection	Sebaceous glands
Calorie reserve	Nails
Vitamin D synthesis	Subcutaneous fat
Body odour/pheromones	Keratinocytes
Psychosocial, display	Apocrine sweat glands
	Skin, lips, hair and nails

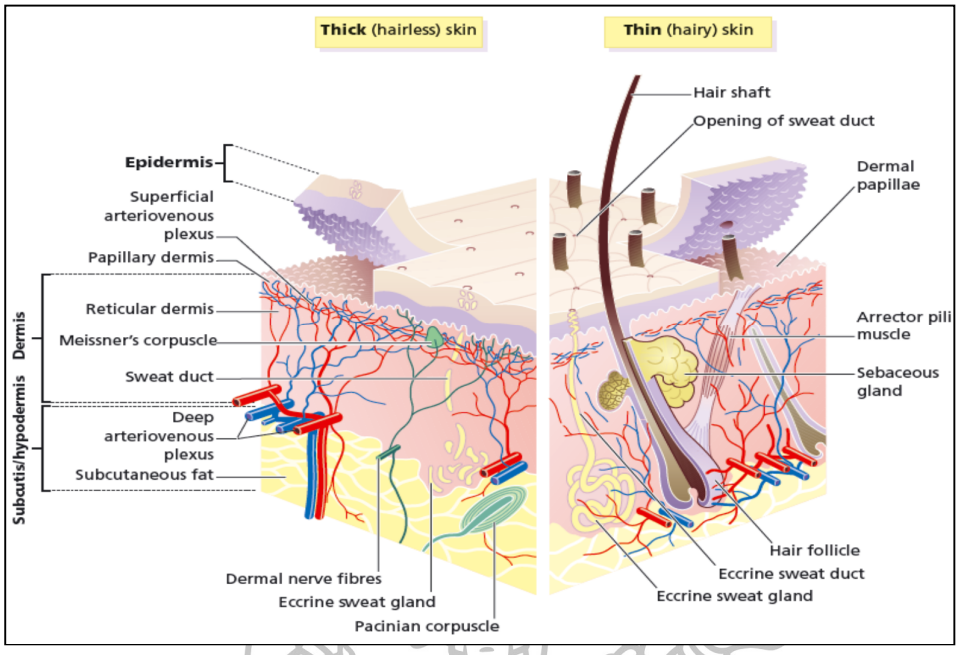


Figure 6 Three-dimensional diagram of the skin, including a hair follicle.

Source : Three-dimensional diagram of the skin, including a hair follicle. Available from http://wiley-vch.e-bookshelf.de/products/reading-epub/product-id/3863329/title/clinical+dermatology.html

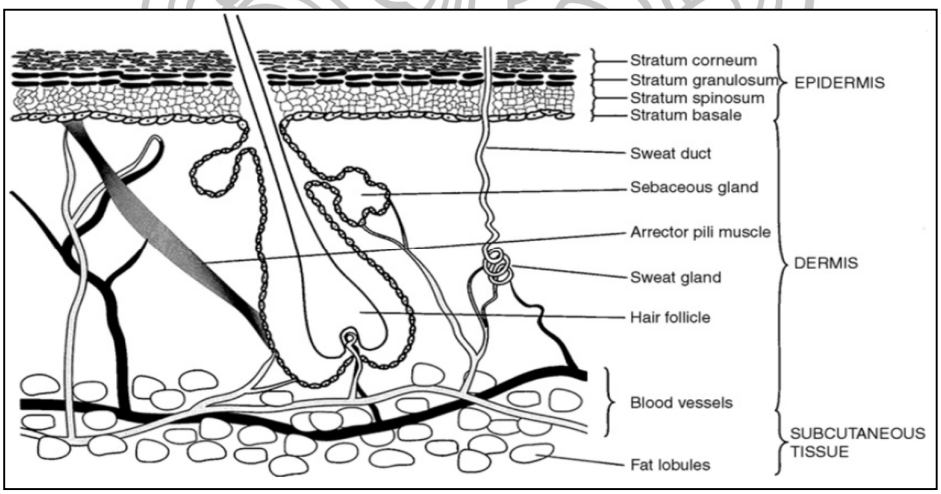


Figure 7 Diagrammatical representation of a cross-section through human skin showing the different cell layers and appendages [188].

Source : Maghraby E., et al. (2008). "Liposomes and skin: From drug delivery to model membranes." **European Journal of Pharmaceutical Sciences** 34: 203–222.

2.6.1.1 The subcutaneous fat layer

The subcutaneous fat layer or hypodermis is the deepest layer of the skin and consisting of the subcutaneous tissue filled with fat cells, fibroblasts, and macrophages [189]. This layer bridges between the overlying dermis and the underlying body constituents. In most areas of the body this layer is relatively thick, typically in the order of several millimeters [190]. This layer serves as a heat insulator, a shock absorber, and an energy storage region.

2.6.1.2 The dermis

The dermis is typically 3–5 mm thick and is the major component of human skin. It is composed of a network of connective tissue, predominantly collagen fibrils (70%) providing support and cushioning, and elastic connective tissue providing flexibility, embedded in a semigel matrix of mucopolysaccharides [190,191]. In terms of transdermal drug delivery, this layer is often viewed as essential gell water, providing a minimal barrier to the delivery of most polar drugs [168]. There are numerous structures embedded within the dermis; blood and lymphatic vessels, nerve endings, pilosebaceous units (hair follicles and sebaceous glands), and sweat glands, as shown in Figure 6. The hair follicles and sweat ducts open directly into the environment at the skin surface and provide the so-called appendageal route of skin permeation [188]. In addition, this layer is filled with a sparse cell population of scattered fibroblasts, macrophages, leukocytes, and mast cells [189].

2.6.1.3 The epidermis

The epidermis is a complexly multiply layered membrane, consisting of several types of cell (corneocytes, melanocytes, Langerhans cells, and

Merkel cells) and a variety of catabolic enzymes (esterases, phosphatases, proteases, nucleotidases, and lipases) [189]. The epidermis consists of many layers of closely packed cells, and the stratified squamous epithelium is the most superficial, flattening filled with keratins. It adheres to the dermis partly by the interlocking of its downward projections (*epidermal ridges* or *pegs*) with upward projections of the dermis (*dermal papillae*) (Figure. 7). The epidermis contains no blood vessels.

The stratified epidermis ,about 100–150 μm thick, comprises five histologically distinct layers, which from inside to outside are the stratum germinativum (basal layer), stratum spinosum (spinous layer), stratum granulosum (granular layer), stratum lucidum and the stratum corneum (SC, horny layer) [188,190]. Because the SC cells are dead, the epidermis without the SC is usually termed the viable epidermis. The viable epidermis is made up of keratinocytes at various stages of differentiation. Typically, it takes 14 days for a daughter cell from the stratum basal to differentiate into a SC cell, and the SC cells are typically retained for a further 14 days prior to shedding [190]. The phospholipid content of skin decreases while the sphingolipid and cholesterol content gradually increases as the cells differentiate during their migration to the surface [189].

The SC is the heterogeneous outermost layer of skin and is approximately 10–20 μm thick. It is nonviable epidermis and it consists of 15–25 flattened, stacked, hexagonal, and cornified cells embedded in a mortar of intercellular lipid, representing as a ‘brick and mortar’ model [190,191]. The SC is recognized as a rate limiting barrier in transdermal permeation of most molecules and it is often viewed as a separate membrane in topical and transdermal drug delivery studies [190]. The barrier nature of the SC depends critically on its unique

constituents ie. 75–80% of protein, 5–15% of lipid, and 5–10% unidentified constituent on a dry weight basis [190]. The majority of the intracellular protein in the SC is composed of insoluble keratin filaments (around 70%) and the components of the cornified cell envelope (around 5%). The intercellular lipids of the SC exist as a continuous lipid phase and arrange in multiple lamellar structure. A remarkable feature is the lack of phospholipids and the predominance of ceramides (41%) and choresterol (27%), together with free fatty acids (9%), cholesteryl esters (10%), and cholesteryl sulfate (2%) [187]. Many studies reveal that relatively polar lipids play a critical role in maintaining the barrier integrity of the SC [189]. The composition of the SC intercellular lipids is summarized in Table 2.

Table 2 Lipid content of the stratum corneum intercellular space.[a] [191]

Lipid	% (w/w)	mol %
Cholesterol esters	10.0	7.5[b]
Cholesterol	26.9	33.4
Cholesterol sulfate	1.9	2.0
Total cholesterol derivatives	38.8	42.9
Ceramide 1	3.2	1.6
Ceramide 2	8.9	6.6
Ceramide 3	4.9	3.5
Ceramide 4	6.1	4.2
Ceramide 5	5.7	5.0
Ceramide 6	12.3	8.6
Total ceramides	41.1	29.5
Fatty acids	9.1	17.0[b]
Others	11.1	10.6[c]

[a]Walters and Roberts, 2002: 20. [b]Based on C_{16} alkyl chain. [c]Based on MW of 500.

2.6.2 The transdermal permeation process

The process of percutaneous absorption involves multiple potential steps from a molecule's first application to the skin surface until it appears in the systemic circulation. The two key determinants for a solute crossing a membrane are solubility and diffusivity [192]. The relative solubility of a solute in two domains determines its partition coefficient and, therefore, the likelihood of the solute being taken up into the SC from a vehicle. The diffusivity is a measurement of traverse of solute through a given barrier and it is affected by binding, viscosity of the environment, and the tortuosity of the path.

In the first step of transport process, the drug is typically applied to the skin. The molecule adjacent to the SC surface will partition into the membrane dependent on their physicochemical properties. Only molecules adjacent to the skin can partition from the vehicle into the membrane. The additional consideration is taken for suspended particles within vehicle. For poorly water-soluble drugs delivered from an aqueous system, dissolution of drug particles to maintain a saturated solution may be the rate-limiting step for transdermal drug delivery [190].

Once the permeant has partitioned into the outer layer of the SC, the drug then diffuses through the SC. At the SC/viable epidermis junction there is another partitioning step as the molecules move into the viable epidermis before further diffusion through the epidermis/dermis junction. Again, there is partitioning followed by diffusion through the dermis tissue where the systemic absorption can take place. In addition to these multiple partitioning and diffusion processes for transdermal drug delivery, there are other potential fates for molecules entering human skin. They are the binding effect of permeants with various elements of the

skin providing a reservoir, drug degradation or activation at metabolic sites, and the deep partition into the subcutaneous fatty layer or muscles not the systemic circulation depending on nature of drug [168].

2.6.3 Transport pathways through the stratum corneum

There are three potential pathways postulated for the diffusion of permeants through the intact SC: (a) intercellular (paracellular) pathway; (b) transappendageal pathway; and (c) transcellular pathway [190,17] (Figure. 8). Typically, all molecules traverse the SC by a combination of all three routes. The relative contributions of these pathways to the total flux will depend on the molecules physicochemical characteristics. An additional potential pathway (d) that enabling the large molecular transport via micron-scale holes in skin created by microneedles (see Section 2.5) and thermal poration is also illustrated in Figure 8.

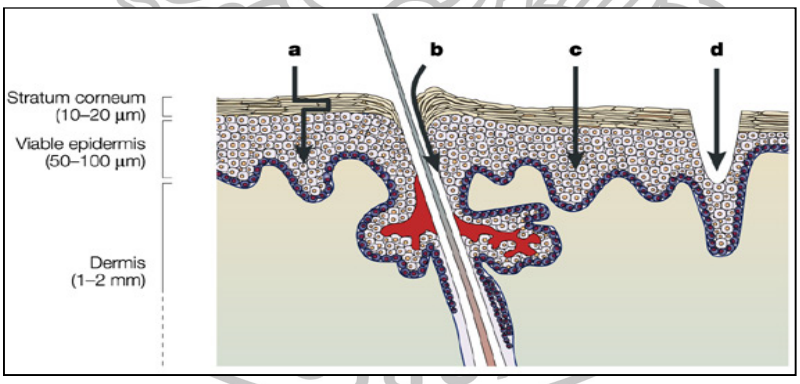


Figure 8 The possible drug permeation pathways through the SC: (a) Intercellular pathway; (b) Transappendageal pathway; (c) Transcellular pathway; (d) Micropores generated by microneedles and thermal poration to provide pathways for enhancing drug transport. [193]

Source : Prausnitz, M. R., S. Matragotri, and R. Langer. (2004). “Current status and future potential of transdermal drug delivery.” **Nature Reviews Drug Discovery** 3, 2: 115–124.

2.6.3.1 Intercellular pathway

The intercellular pathway is a continuous and highly tortuous way through the intercellular lipid domain. It is now generally accepted that, most molecules penetrate through skin via this intercellular microroute and therefore various enhancing techniques aim to disrupt, possibly in the presence of a chemical enhancer, or bypass its well-organized molecular architecture [194]. The pathlength for intercellular permeation has been proposed ranging from 150 to 500 μm, which is considerably greater than the SC thickness.

2.6.3.2 Transappendageal pathway

The transappendageal route is also known as a shunt route, including the permeation through the sweat glands and across the hair follicles with their associated sebaceous glands. Because of the low fractional appendageal area (around 0.1% of the total skin surface), this pathway usually contributes negligibly to steady state drug flux [194]. However, with finite dosing and at short time periods after drug application, the relative contribution of the appendages will be significantly greater since molecules will not have had time to cross the bulk of the SC [190]. Transappendageal pathway may also be important for ions and large polar molecules that poorly traverse the intact SC. Moreover, these shunt routes are also important for delivering vesicular structures to the skin and for targeting to the pilosebaceous units. One of the most considerable determinants of targeted follicular transport is the particle size of applied materials in which the degree of follicular penetration was inversely dependent on the particle size and the optimum size at which microspheres selectively entered the follicles was 3–10 μm [192,195]. Low-voltage electrical

enhancement by iontophoresis can provide the transport pathways through hair follicles and sweat ducts more accessible [193].

2.6.3.3 Transcellular pathway

The transcellular pathway can be defined as the pathway where molecules permeate across the intact SC through the keratinized cells. The transport process faces numerous repeating hurdles because the keratinocytes are bound to a lipid envelop that attaches to the intercellular multiply bilayered lipid domain. Thus, the molecules require not only partitioning into and diffusion through the hydrated keratin but also into and across the intercellular lipids. It is obvious that the process of multiple partitioning and diffusion steps between hydrophilic and hydrophobic domains is generally unfavorable for most drugs [190]. Moreover, the keratin also provides the potential binding sites for the solutes. For highly hydrophilic molecules, the transcellular route may predominate at pseudo-steady state. However, the rate-limiting barrier for permeation via this pathway remains the multiply intercellular lipid bilayers [190]. High-voltage enhancement by electroporation has been shown to occur via the transcellular pathway by disrupting the lipid bilayers [193].

2.6.4 Percutaneous penetration enhancers

The range of drugs that can be delivered in therapeutic doses via the skin for both local and systemic therapies is limited by a highly efficient and effective barrier of the SC. The simple equation for steady-state flux is useful when considering factors controlling the SC permeation rates. The ideal properties needed for a molecule to penetrate the SC well can be accessed. There are: low molecular mass, preferably less than 600 Da, adequate solubility in oil and water, high but

balanced (optimal) partition coefficient, and low melting point, correlating with good solubility as predicted by ideal solubility theory [194]. If the permeant does not possess appropriate partitioning, diffusivity or solubility properties, then the use of penetration enhancers are favorably. Figure 9 summarizes some techniques for overcoming the barricade offered by an intact SC [196]. Drug permeation across the skin could be enhanced by adapting one or more of these several strategies.

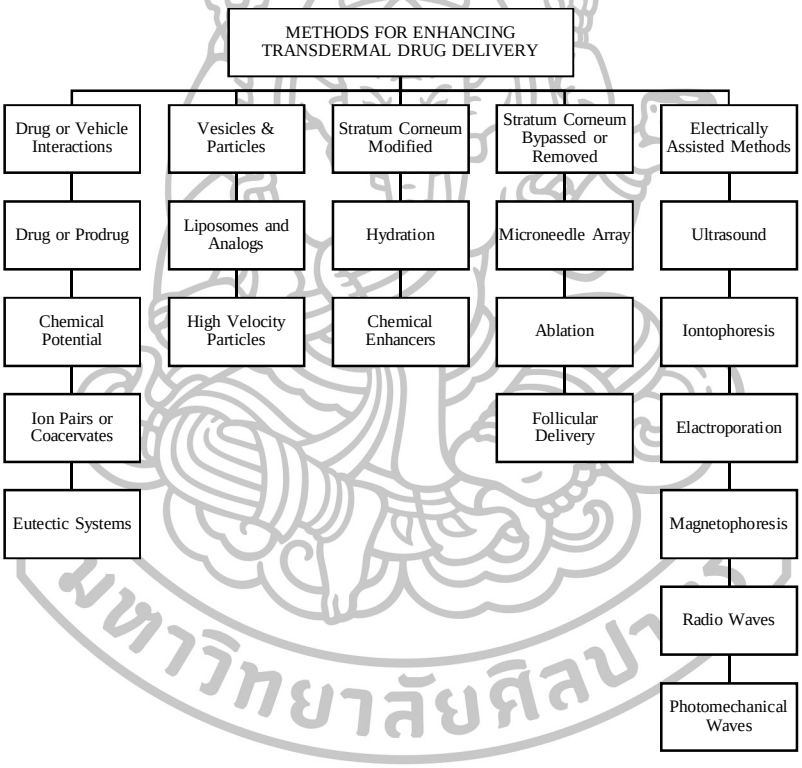


Figure 9 Some methods for enhancing transdermal drug therapy.[196]

Source : Barry, B. W. (2006). “Penetration Enhancer Classification.” **In Percutaneous Penetration Enhancers**, 3–16. Edited by Eric W. Smith and Howard I. Maibach. New York: Taylor & Francis Group

Permeation enhancement methods showing in Figure 10 can be classified roughly into two categories; the chemical enhancers and the physical enhancers.

2.6.4.1 Chemical enhancers

Substances that help promoting drug diffusion through the SC and epidermis are referred to as chemical penetration enhancers. The literature reveals numerous classes of chemical compounds that have been accessed for their ability to promote or enhance the permeation of biomolecules across the skin [197]. Various elegant formulations that may contain substances which have penetration enhancing activity are also addressed. For example, vesicles are often prepared from phospholipids; phospholipids themselves have some penetration enhancing activity. Likewise, penetration enhancers have been formulated into eutectic systems or into slow or sustained release delivery systems. Table. 3 provides an overview of some of different chemical classes that have been used as penetration enhancers and examples of substances within each specific chemical class [197].

Table 3 Some of chemical classes used as penetration enhancers.*[197]

Chemical Class	Examples
Sulfoxides	Dimethlsulfoxide, decylmethylsulfoxide
Alcohols	Alkanol: ethanol, propanol, butanol, pentanol, hexanol, octanol, nonanol, decanol, 2-butanol, 2-pentanol, benzyl alcohol Fatty alcohol: caprylic, decyl, lauryl, 2-lauryl, myristyl, cetyl, steryl, oleyl, linoleyl, linolenyl alcohol
Fatty acids	Linear: valeric, heptanoic, pelagonic, caproic, capric, lauric, myristic, stearic, oleic, caprylic Branched: isovaleric, neopentanoic, neoheptanoic, neononanoic, trimethylhexanoic, neodecanoic, isostearic
Fatty acid esters	Aliphatic-isopropyl *n*-butyrate, isopropyl *n*-hexanoate, isopropyl *n*-decanoate, isopropyl myristate, isopropyl palmitate, octyldodecylmyristate Alkyl: ethyl acetate, butyl acetate, methyl acetate, methyl valerate, methyl propinoate, diethyl sebacate, ethyl oleate
Polyols	Propylene glycol, polyethylene glycol, ethylene glycol, diethylene glycol, triethylene glycol, dipropylene glycol, glycerol, propanediol, butanediol, pentanediol, hexanetriol
Amides	Urea, dimethylacetamide, diethyltoluamide, dimethylformamide, dimethyloctamide, dimethyldecamide Biodegradable cyclic urea: 1-alkyl-4-imidazolin-2-one Pyrrolidone derivatives: 1-methyl-2-pyrrolidone, 2-pyrrolidone, 1-lauryl-2-pyrrolidone, 1-methyl-4-carboxy-2-pyrrolidone, 1-hexyl-4-carboxy-2-pyrrolidone, 1-lauryl-4-carboxy-2-pyrrolidone, 1-methyl-4-methoxycarbonyl-2-pyrrolidone, 1-hexyl-4-methoxycarbonyl-2-pyrrolidone, 1-lauryl-4-methoxycarbonyl-2-pyrrolidone, *N*-cyclohexylpyrrolidone, *N*-dimethylaminopropylpyrrolidone, *N*-cocoalkylpyrrolidone, *N*-tallowalkylpyrrolidone

Table 3 (continued) Some of chemical classes used as penetration enhancers.*

Chemical Class	Examples
Amides	Biodegradable pyrrolidone derivatives: Fatty acid esters of *N*-(2-hydroxyethyl)-2-pyrrolidone
	Cyclic amides: 1-dodecylazacycloheptan-2-one (Azone®), 1-geranylazacycloheptan-2-one, 1-farnesylazacycloheptan-2-one, 1-geranylgeranylazacycloheptan-2-one, 1-(3,7-dimethyloctyl)azacycloheptan-2-one, 1-(3,7,11-trimethyldodecyl)azacyclohaptan-2-one, 1-geranylazacyclohexan-2-one, 1-geranylazacyclopentan-2,5-dione, 1-farnesylazacyclopentan-2-one
	Hexamethylenelauramide and its derivatives
	Diethanolamine, triethanolamine
Surfactants	Anionic: Sodium laurate, sodium lauryl sulfate
	Cationic: Cetyltrimethyl ammonium bromide, Tetradecyltrimethylammonium bromide, benzalkonium chloride, octadecyltrimethylammonium chloride, cetylpyridinium chloride, dodecyltrimethylammonium chloride, hexadecyltrimethylammonium chloride
	Nonionics: Poloxamer (231, 182, 184), Brij (30, 93, 96, 99), Span (20, 40, 60, 80, 85), Tween (20, 40, 60, 80), Myrj (45, 51, 52), Miglyol 840
	Bile salts: Sodium cholate, sodium salts of taurocholic, glycolic, desoxycholic acids
	Lecithin

Table 3 (continued) Some of chemical classes used as penetration enhancers.*

Chemical Class	Examples
Terpenes	Hydrocarbons: D-Limonene, α-pinene, β-carene Alcohols: α-Terpineol, terpinen-4-ol, carvol Ketones: Carvone, pulegone, piperitone, menthone Oxides: Cyclohexene oxide, limonene oxide, α-pinene oxide, cyclopentene oxide, 1,8-cineole Oils: Ylangylang, anise, chenopodium, eucalyptus
Alkanones	*N*-heptane, *N*-octane, *N*-nonane, *N*-decane, *N*-undecane, *N*-dodecane, *N*-tridecane, *N*-tetradecane, *N*-hexadecane
Organic acids	Salicylic acid and salicylates (including their methyl, ethyl, and propyl glycol derivatives), citric and succinic acid

Although many chemicals have been evaluated as penetration enhancers in human or animal skins, to date none has proven to be ideal. The ideal penetration enhancers should have the following characteristics [189,197,198].

i.Be both pharmacologically and chemically inert and chemically stable

ii.A high degree of potency with specific activity and reversible effects on the skin properties

iii.Be both predictable and reproducible on the activity and duration of action

iv.Show compatibility with formulation and system components

v.Work rapidly and uni-directionally, i.e. should allow therapeutic agents into the body whilst preventing the loss of endogenous material from the body

vi.Be non-irritating, non-sensitizing, non-allergenic, non-toxic, and non-comedogenic

vii.Be odorless, tasteless, colorless, and cosmetically acceptable

It is difficult to select rationally a penetration enhancer for a given permeant. Penetration enhancer potencies appear to be drug specific, or at best may be predictive for a series of compounds that have the similar physico-chemical properties [198]. The exact mechanism(s) by which many chemical penetration enhancers function remains to be clearly elucidated. It is almost certain that they will have multiple effects once absorbed into the SC. The potential mechanisms of action of the enhancers can range from direct effects on the skin to modification of the formulation. By directly acting on the skin, enhancers can act by altering skin lipids and/or proteins and/or by affecting partitioning behavior; termed the lipid–protein partititioning theory as depicted in Figure. 10. In addition, chemical enhancers can act indirectly by modification of the thermodynamic activity of the vehicle and solubilising the permeant in the donor solution (e.g. with surfactants) [198].

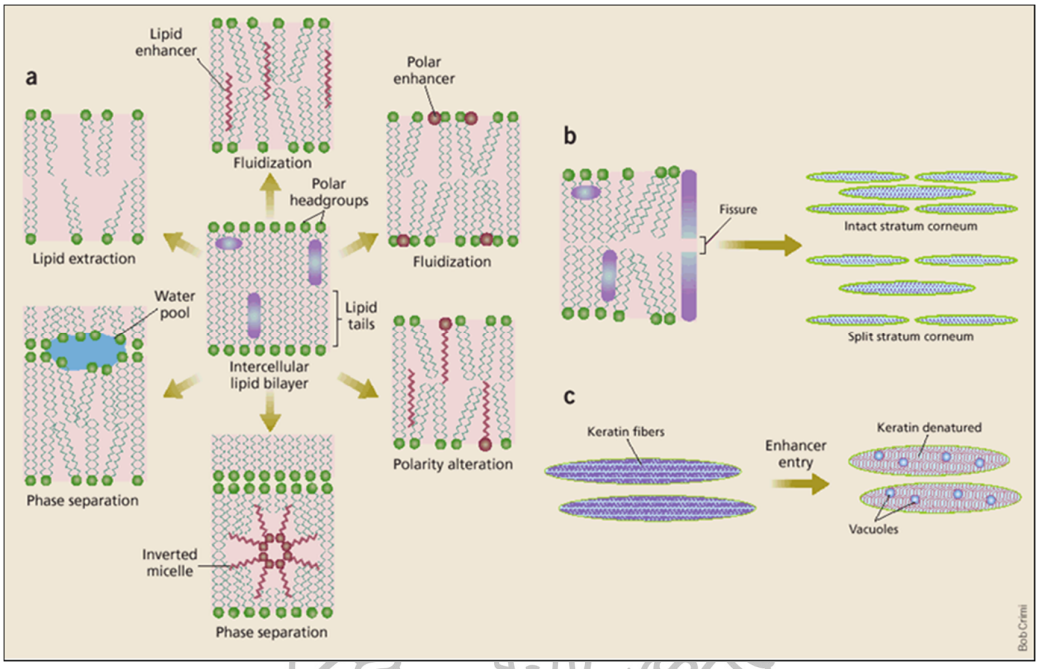


Figure 10 Some actions of penetration enhancers on the human SC: (a) Action at intercellular lipids. Some of the ways by which chemical penetration enhancers attack and modify the lamellar intercellular lipid domain of the SC; (b) Action at desmosomes and protein structures. Such dramatic disruption by enhancers (particularly potent solvents) as they split the SC into additional squames and individual cells would be clinically unacceptable; (c) Action within corneocytes. Swelling, further keratin denaturation and vacuolation within individual horny layer cells would not beso drastic but would usually be cosmetically challenging [198].

Source : Barry, B. W. (2004). “Breaching the skin's barrier to drugs.” **Nature Biotechnology** 22: 165–167.

2.6.4.2 Physical enhancers

The uncertainty of the chemical means of penetration enhancement makes the use of the physical approaches attractive. By the physical means, the SC can be modified by bypassing or removing this tissue layer via microneedles and thermal ablation. Thermal ablation or thermal poration refers to the selective removal of the SC without damaging the deeper tissue layers by heating the skin surface briefly (e.g. << 1 s). The formation of micropores of 30 μm diameter and 70 μm depth and the absence of necrosis in surrounding tissue has been addressed using selective ablation techniques [199]. Electrically assisted methods, i.e. iontophoresis, ultrasound, and electroporation, are also promising. Ionized drugs and complex macromolecules such as proteins or peptides may be induced to penetrate the SC at a faster rate than normal by an iontophoresis technique. A small electrical current (approximately 0.5 mA/cm^2) is applied across the membrane. The charged permeant is repelled from the electrode of similar polarity into the SC, which acts as the electrical conduit to the companion electrode [200]. Iontophoretic flux is obtained not only by electrorepulsion but also electroosmotic solvent flow. The application of ultrasound (at low frequency) on skin makes the intercellular and transcellular pathways more permeable by cavitation and fluidizing the SC lipids and/or increasing the convective flow. This method is also known as phonophoresis or sonophoresis. High-voltage (50–500 V) enhancement at short time (< 1 s) by electroporation has been shown to occur via transcellular pathways by disorganizing the SC lipid structure. The creation and/or the enlargement of aqueous pathways during electroporation has been extensively proposed and observed in many studies [180]. Table 4 shows the comparative efficacy of some delivery methods in terms of

increase in drug transport, sustained drug release, pain sensation, and complexity [201]. Although all these methods have been individually shown to enhance the transdermal drug transport, their combinations have been widely studied and found to enhance the transdermal transport more effectively than each of them alone [202].

2.7 Microemulsions

One of the best definitions of microemulsions is from Danielsson and Lindman [203] "a microemulsion is a system of water, oil and an amphiphile which is a single optically isotropic and thermodynamically stable liquid solution". In some respects, microemulsions can be considered as small-scale versions of emulsions, i.e., dispersed droplet of either oil-in-water (o/w) or water-in-oil (w/o), with a size range of 5-50 nm in drop radius. Such a description, however, lacks precision there are significant differences between microemulsions and ordinary emulsions (or macroemulsions). In particular, in emulsions the average drop size grows continuously with time until phase separation ultimately occurs under gravitational force, i.e., they are thermodynamically unstable and their formation requires input of work. The drops of the dispersed phase are generally large (> 0.1 μm) so that they often show a milky, rather than a translucent appearance. For microemulsions, once the conditions are right, spontaneous formation occurs.

2.7.1 Phase diagrams and types of microemulsions

As for simple aqueous systems, microemulsion formation is dependent on surfactant type and structure. If the surfactant is ionic and contains a single hydrocarbon chain (e.g., sodium dodecylsulphate, SDS) microemulsions are only formed if a co-surfactant (e.g., a medium size aliphatic alcohol) and/or

electrolyte (e.g., 0.2 M NaCl) are also present. With double chain ionics (e.g., Aerosol-OT) and some non-ionic surfactants a co-surfactant is not necessary. This results from one of the most fundamental properties of microemulsions, that is, an ultra-low interfacial tension between the oil and water phases, $\gamma_{o/w}$. The main role of the surfactant is to reduce $\gamma_{o/w}$ sufficiently i.e., lowering the energy required to increase the surface area so that spontaneous dispersion of water or oil droplets occurs and the system is thermodynamically stable. Ultra-low tensions are crucial for the formation of microemulsions and depend on system composition.Microemulsions were not really recognized until the work of Hoar and Schulman in 1943, who reported a spontaneous emulsion of water and oil on addition of a strong surface-active agent [204]. The term “microemulsion” was first used even later by Schulman et al. [205] in 1959 to describe a multiphase system consisting of water, oil, surfactant and alcohol, which forms a transparent solution. There has been much debate about the word “microemulsion” to describe such systems [206]. Although not systematically used today, some prefer the names “micellar emulsion” [207] or “swollen micelles” [208]. Microemulsions were probably discovered well before the studies of Schulmann: Australian housewives have used since the beginning of last century water/eucalyptus oil/soap flake/white spirit mixtures to wash wool, and the first commercial microemulsions were probably the liquid waxes discovered by Rodawald in 1928. Interest in microemulsions really stepped up in the late 1970's and early 1980's when it was recognized that such systems could improve oil recovery and when oil prices reached levels where tertiary recovery methods became profit earning. Nowadays this is no longer the case, butothermicroemulsion applications were

discovered, e.g., catalysis, preparation of submicron particles, solar energy conversion, liquid−liquid extraction (mineral, proteins, etc.). Together with classical applications in detergency and lubrication, the field remains sufficiently important to continue to attract a number of scientists. From the fundamental research point of view, a great deal of progress has been made in the last 20 years in understanding microemulsion properties. In particular, interfacial film stability and microemulsion structures can now be characterized in detail owing to the development of new and powerful techniques such as small-angle neutron scattering. The following sections deal with fundamental microemulsion properties, i.e., formation and stability, surfactant films, classification and phase behaviour.

The term of microemulsion applies to a mixture with at least three components; an oily phase, an aqueous phase and a surface active species, so called surfactants. Sometimes the forth component i.e., co-surfactant can/must be present [209,210]. Depending on the ratios between the components, in the two extremes the microstructureof the microemulsions vary from a very tiny water droplets dispersed in oil phase (w/omicroemulsion) to a oil droplets dispersed in water phase (o/w microemulsion). Themicrostructure of the mixture changes continuously from one to another extreme, namely, froma spherical to cylindrical, tubular and nterconnected continuous oil and water phasesseparatedwith a very thin layer of surfactant molecules, in the middle, which is defined as bicontinuesmicroemulsion [211]. The microemulsions of each kind are thermodynamicallystableand transparent solutions. There are main differences between emulsions and microemulsionsin terms of structure and stability. In contrast to the microemulsions, the emulsions are unstablesystems and without agitation, phase separation will occur in them. The other

difference is thatthe size of droplets in emulsions are in the range of micrometers, while in microemulsionsthesize of micelles are in the range of 5-100 nm, depending on the some parameters such assurfactant type and concentration, the extent of dispersed phase [212-214]. Hence, sometimes the microemulsion term is misleading, because it doesn’treflect the size of dispersed phase droplets in the system which, are in the nanometer range.Depending on the type of the surfactants employed in the preparation of the microemulsion,another important parameter that affects the main characteristics of a microemulsion is thepresence of electrolytes in the aqueous phase.

2.7.2 Phase diagrams and types of microemulsions

The formation of the thermodynamically stable microemulsions require that an adequateamount of the corresponding components must be mixed. Determination of these properproperties. For this purpose, one must prepare mixtures with different compositions of thecomponents, and check them regarding the type and number of phases present in thesystem. The resulting diagrams, showing the number/or type of phases present in thesystem associated with each specific composition, are called phase diagrams. From theindustrial and application point of view, this process is called formulation, which indicates thespecific compositions of the components giving a stable mixture effective in the concernedproperty. A number of different methodologies have been used for determination of the phasediagrams. Almost the earliest studies about the phase diagrams of the microemulsions can befound in the 1960s [215]. Using the phase diagrams, it has been confirmed thatthe Schulman's so-called micromulsion is not an emulsion but a solubilized solution [216]. The mechanism of the microemulsion formation has been studied inconnection with the phase diagrams and the relation between the amounts of componentsrequired

to form a clear microemultion has been understood from the phase diagrams [217]. They have studied the phase diagrams of different systems with anionic,compositions is an important issue in this field to obtain the microemulsions with requiredcationic and non-ionic surfactants, and could obtain maximum solubillization with theoptimum ratio of the surfactant and co-surfactant. By a detailed investigation onpseudoternary phase diagrams of two microemulsion systems it has been evidenced that agreat variety of phases is present. They have concluded that the interaction between water andoil domains is an important parameter affecting the stability of microemulsions [218]. The phase diagrams of the ternary system containing water-sodium alkylbenzenesulfonate (NaDBS)-hexanol and their quaternary system with xylene have been prepared atthree different temperatures. The formation of different phases, such as microemulsionphase, reverse micelle phase was observed which have been qualitatively examined by optical (phasecontrast and polarizing) microscopy or low angle X-ray diffraction. According to the resultsthe amount of microemulsion phase was decreased by increasing of the temperature atsurfactant concentrations of lower than 15% [219]. The phase diagrams of thesystems with alkyl polyether surfactants have been studied extensively in different aspects, [220-226].The effect of addition of inorganic salts into the aqueous phase of the microemusionshavebeen studied using phase diagrams. It has been observed that the added salts has a greatinfluence on the solubilisation ability of the microemulsion system [206,228,235]. As an example, it has been observed that the additionof salt shifts the fish diagram towards more hydrophobic oil systems and higher surfactantconcentrations will be required [228]. Determination of the phasediagrams has been used also as the bases for the applications of the microemulsion for

thepreparation of the nanoparticles [224,236]. Here, thephase diagrams have been used to select the proper compositions of the microemulsionstoget spherical well defined micelles, and consequently resulting nanoparticles.

2.7.3 Predicting microemulsion type

A well-known classification of microemulsions is that of Winsor [237] who identified four general types of phase equilibria:

2.7.3.1 Type I

The surfactant is preferentially soluble in water and oil-in-water (o/w) microemulsions form (Winsor I). The surfactant-rich water phase coexists with the oil phase where surfactant is only present as monomers at small concentration.

2.7.3.2 Type II

The surfactant is mainly in the oil phase and water-in-oil (w/o) microemulsions form. The surfactant-rich oil phase coexists with the surfactant-poor aqueous phase (Winsor II).

2.7.3.3 Type III

A three-phase system where a surfactant-rich middle-phase coexists with both excess water and oil surfactant-poor phases (Winsor III or middle-phase microemulsion).

2.7.3.4 Type IV

A single-phase (isotropic) micellar solution, that forms upon addition of a sufficient quantity of amphiphile (surfactant plus alcohol).

Depending on surfactant type and sample environment, types I, II, III or IV form preferentially, the dominant type being related to the molecular

arrangement at the interface (see below). As illustrated in Figure 3.2, phase transitions are brought about by increasing either electrolyte concentration (in the case of ionic surfactants) or temperature (for non-ionics). Table.4 summarizes the qualitative changes in phase behaviour of anionic surfactants when formulation variables are modified [238]. Various investigators have focused on interactions in an adsorbed interfacial film to explain the direction and extent of interfacial curvature. The first concept was that of Bancroft [239] and Clowes [240] who considered the adsorbed film in emulsion systems to be duplex in nature, with an inner and an outer interfacial tension acting independently [241]. The interface would then curve such that the inner surface was one of higher tension. Bancroft's rule was stated as "that phase will be external in which the emulsifier is most soluble"; i.e., oil-soluble emulsifiers will form w/o emulsions and water-soluble emulsifiers o/w emulsions. This qualitative concept was largely extended and several parameters have been proposed to quantify the nature of the surfactant film. They are briefly presented in this section. Further details concerning the thesemicroemulsion types and their location in the phase diagram will be given in next Section.

Table 4 Qualitative effect of several variables on the observed phase behaviour of anionic surfactants. [238]

Scanned variables (increase)	Ternary diagram transition
Salinity	I ⌫III ⌫II
Oil: Alkane carbon number	II ⌫III ⌫I
Alcohol: low M.W.[a]	I ⌫III ⌫II
high M.W.[b]	I ⌫III ⌫II
Surfactant: lipophilic chain length	I ⌫III ⌫II
Temperature	II ⌫III ⌫I

2.7.4 The R-ratio

The R-ratio was first proposed by Winsor [218] to account for the influence of amphiphiles and solvents on interfacial curvature. The primary concept is to relate the energies of interaction between the amphiphile layer and the oil and water regions. Therefore, this R-ratio compares the tendency for an amphiphile to disperse into oil, to its tendency to dissolve in water. If one phase is favoured, the interfacial region tends to take on a definite curvature. A brief description of the concept is given below, and a full account can be found elsewhere [242]. In micellar or microemulsion solutions, three distinct (single or multicomponent) regions can be recognized: an aqueous region, W, an oil or organic region, O, and an amphiphilic region, C. As shown in Figure. 11, it is useful to consider the interfacial zone as having a definite composition, separating essentially bulk-phase water from bulk-phase oil. In this simple picture, the interfacial zone has a finite thickness, and will contain, in addition to surfactant molecules, some oil and water.

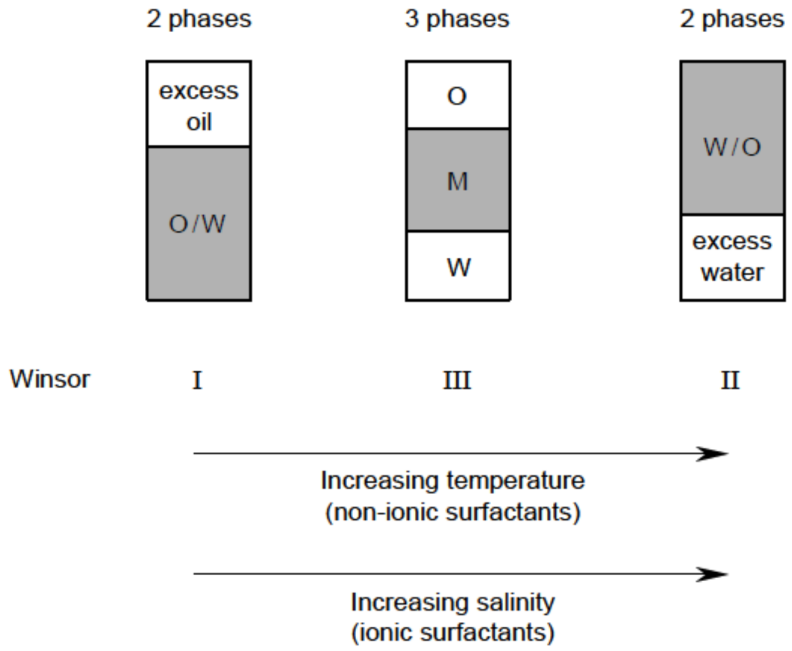


Figure 11 Winsor classification and phase sequence of microemulsions encountered as temperature or salinity is scanned for non-ionic and ionic surfactant respectively. Most of the surfactant resides in the shaded area. In the three-phase system the middle-phase microemulsion (M) is in equilibrium with both excess oil (O) and water (W).

2.7.5 Surfactants

The surfactants are molecules with at least two parts, one part soluble in polar solvents (hydrophilic) and the other part insoluble in the polar solvent (hydrophobic). Because of thisdouble character, the term amphiphile is also used as synonym with surfactant [243]. The polar part of the surfactant molecule is referred as head, and the non-polarpart of the molecule as tail. Having these two parts with opposing solubilisationabilities,gives the surfactant molecules unique capabilities,

such as tendency to adsorb at the surfacesand interfaces, which results in the decrease of the surface tension, and also formation of the aggregates inside the solutions, resulting in the formation of the microemulsions. Thisdouble character of the surfactant molecule enables it to orient in desired way while incontact with the two phases with different hydro/lypophilic properties, or to makeaggregates inside of the solution with hydro- or lypophilic parts directed towards themedia. Such aggregates can solubilize an oil in aqueous phase (micelles) or water in the oilyphase (reversed micelles).The polar nature of the head group of surfactants vary from non-ionic to ionic character.Depending on the nature of this part, the surfactants are categorized into non-ionic,anionic, cationic and amphoteric (zwitterionic) surfactants [244-246]. Versatile types of functional groups have been utilized as the head groupfor the surfactants. Among them carboxylates, sulphates, phosphates, sulfonates, quaternary amines, polyethers have a great importance in many different applications. Commercially used surfactants can be obtained from synthetic or natural resources

2.7.6 Uses and applications of microemulsions

2.7.6.1 Microemulsions in cosmetics

In many cosmetic applications such as skin care products, emulsions are widely used with water as the continuous phase. It is believed that microemulsion formulation will result in a faster uptake into the skin. Cost, safety (as many surfactants are irritating to the skin when used in high concentrations), appropriate selection of ingredients (i.e. surfactants, cosurfactants, oils) are key factors in the formulation of microemulsions. Microemulsions as skin care products have been reported [247]. In these formulations, sodium alkyl sulfate, tetraethylene glycol monododecyl ether, lecithin, dodecyl oligoglucoside, alkyl dimethyl amine

oxide, propanol, hexadecane, isopropyl myristate have been used as surfactants, cosurfactants and oils respectively. Unique microemulsions as hair care products have been prepared. They contain an amino-functional polyorganosiloxane (a nonionic surfactant) and an acid and/or a metal salt. Solubilization of fragrance and flavoured oils can be achieved in microemulsions. Cosmetic microemulsions (transparent and translucent) of silicone oils, produced by emulsion polymerization have been reported. They are, however, not thermodynamically stable products because of low solubility of silicone oil in the surfactants. Ultrafine emulsions prepared by condensation method have some advantages in cosmetic and medical products, as they have excellent stability and safety and their droplet size can be readily controlled. Ultrafine emulsions can be regarded as thermodynamically unstable microemulsions, as they are o/w emulsions with droplet size similar to microemulsion. Cosmetic formulations for skin care products using commercial nonionic surfactants and oils usually used in cosmetics are also investigated. There are patents on the formulation (by dispersion method) of skin care cosmetic products using ionic surfactants. Silicone oil ultrafine emulsions have been reported. Tokuoka*et al*. [248] have studied the solubilization of several systems consisting of water, surfactant and synthetic perfumes (viz. d-limonene, *a*-ionone, benzyl accetate, linalol, eugenol and *a*-hexylcinnamaldehyde), clarifying (a) the influence of fragrance structure on the phase regions in a water/nonionic surfactant systems, (b) the distribution co-efficient between micelles and the bulk phase, and (c) the partition between dissolved and solubilized perfume components on their volatility. In this regard, the phase equilibria in water, lecithin, soybean oil and vanillin have been studied. The conditions of preparation-related function of a fragrance in a personal care product have been

studied and this field has been reviewed [249]. According to them, the variation of the fragrance vapour pressure over time is an essential factor both for consumer acceptance and economic point of view. The systems are water/AOT/cyclohexane, water/Brij 30/phenethyl alcohol, water/Brij 30/linalol.

2.7.6.2 Microemulsions in food

Certain foods contain natural microemulsions. Microemulsions as a functional state of lipids have been, therefore, used in the preparation of foods. Microemulsions form in the intestine during the digestion and absorption of fat. The possibility of producing microemulsion on purpose and using them as tools in food production is, however, a neglected field in food technology. Excellent component solubilization, enriched reaction efficiency and extraction techniques have considerable potential in the area of food technology. The major differences between food and other microemulsions are in the composition of the oil component and food grade surfactants. In foods, the oil is a triglyceride, whereas in other microemulsions the oil is a hydrocarbon, often a mineral oil. The triglyceride molecule is itself surface active, which in turn implies that triglycerides are not capable of forming separate oil domain in an amphiphile–water system in the same way as mineral oils. Therefore, the composition range in the oil–water–surfactant systems that allows microemulsions to form when the oil is a triglyceride is much smaller than the range allowing microemulsion formation when the oil is a hydrocarbon. Food grade surfactants, viz. phosphatidycholine (lecithin), AOT and sorbatinmonostearate/monolaurate (Tweens) have been extensively studied with regard to the formation of o/w and w/o microemulsions [249]. Although microemulsion have a host of promising application in the food industry, there are

still very few reports in the literature [250]. Recently, Current information on o/w and w/o microemulsion formed using food-grade materials, complex food mixture (liquid crystal, gels), possibilities of incorporating food ingredients (such as flavour, preservatives and vitamins) within microemulsions, reactions carried out in microemulsions media and potential of microemulsions for extracting food components from a complex mixture. Larsson *et al.* [251] have focused on the cereal and edible lipid systems that form microemulsions and their potentialities. Recent research has shown that microemulsions of carnauba wax form better protective coatings on citrus fruit than shellac, wood resin, oxidized polyoxyethylene or mixtures of these substances with caranuba wax. The protective coatings minimize weight loss as well as internal oxidation. The fruit coated with the microemulsions of caranauba wax maintains a better appearance than other coatings after washing and drying. Microemulsions have also been used to produce glycerides for application in food products. An important application of microemulsion is to provide improved antioxidation effectiveness because of the possibility of a synergistic effect between hydrophilic and lipophilic antioxidants. It is known that soybean oil is effectively protected when contained within an L2-phase produced by the addition of monoglycerides (sunflower oil monoglycerides) to water. An approximately 1:5 ratio of monoglycerides to triglycerides is needed to get enough water into the L2-phase (about 5 wt%). In such a system, 200 ppm of tocopherol in the oil and 5% ascorbic acid in the reverse micelles give a dramatic antioxidant effect compared to conventional methods of dissolving or dispersing antioxidants in oils. In fish oils, the same microemulsion-based method to achieve an antioxidant protective effect has also been used. Glycerol has been used instead of water for further improvement of

the protectivity. The effect of adding various lipids and propylene glycol to monoolein (a common food emulsifier)–water system and the cubic liquid crystal thus formed undergoing a transition to a sponge or L3-phase have been reported. The structure of the spongy cubic phase has been described as a ‘melted’ bicontinuous cubic phase. Although considerable research has been conducted to show the usefulness of microemulsions in foods, the application and technology require further work.

2.7.6.3 Microemulsion in pharmaceuticals

Liquid crystalline, miceller and emulsion forming systems are widely used in pharmaceutical preparations. Low solubilization capacity of micelles and instability of emulsions are disadvantageous. The easy formation, remarkable environment independent stability, excellent solubilization capacity, etc. favourmicroemulsions to be a better roposition over other compartmentalized systems. The dispersed phase, lipophilic or hydrophilic (o/w or w/o type) can act as a potential reservoir of lipophilic or hydrophilic drugs that can be partitioned between the dispersed and the continuous phases. Coming in contact with a semipermeable membrane, such as skin or mucous membrane, the drug can be transported through the barrier. Both lipophilic and hydrophilic drugs can be administered together in the same preparation. Low viscous formulations using microemulsions with suitable protein compatible surfactants can be used as injection solutions, for they are miscible with blood in any ratio. In contrast to emulsions, microemulsions cause minimum immuno reactions or fat embolism. Proteins are not denatured in microemulsions although they are unstable at high or low temperatures. The total dose of the drug can be reduced when applied through the microemulsion route and thus side effects can be

minimized. Toxicity, bioincompatibility of surfactants and cosurfactants, requirement of high concentrations for formulations and other relevant factors such as maintenance of thermodynamic stability in the temperature range between 0° and 40°C, salinity, constant pressure during storage, low solubilizing capacity for high molecular weight drug (and oil), etc. limit the uses of microemulsions in the pharmaceutical and medicinal fields. An interesting and specific practical application of o/w microemulsion in the pharmaceutical industry is the use of strongly hydrophobic fluorocarbons (as oils) to produce short-time blood plasma substitutes to maintain the supply of oxygen in the living systems. The components to be used must have low allergic potential, good physiological compatibility and high biocompatibility. The biocompatibility requirements of the amphiphiles are fulfilled by lecithins, non-ionic surfactants (Brijs, Arlacel 186, Spans, Tweens and AOT). Microemulsions are promising delivery systems [252,253] to allow sustained or controlled drug release for percutaneous, peroral, topical, transdermal, ocular and parenteral administration. Enhanced absorption of drugs, modulation of the kinetics of the drug release and decreased toxicity are several advantages in the delivery process. Microemulsions as drug delivery systems for different types of drugs, viz. antineoplastics/antitumour agents (doxorubicin, idarubicin, tetrabenzamidine derivative), peptide drugs (cyclosporine, insulin, vassopressin), sympatholytics (bupranolol, timolol, levobunolol, propanolol), local anesthetics (lidocaine, benzocaine, tetracaine, heptacaine), steroids (testosterone, testosterone propionate, testosterone enanthate, progesterone, medroxyprogestorane acetate), anxiolytics(benzodiazepines), antiinfective drugs(cloitrimazole, ciclopiroxolamine, econazole nitrate, tetracycline hydrochloride), vitamins(menadione, ascorbic acid),

anti-inflammatory drugs(butibufen, indomethacin), and dermological products(tyrocine, azelaic acid, octyl dimethyl PABA, 2-ethyl hexyl *p*-methoxycinnamate). Enzyme doped silica nanoparticles (ceramic drug carrier) in the aqueous core of reverse micelles and microencapsulation of diospyrin, a plant-derived bisnapthoquinol of potential chemotherupic activity have been very recently reported. [254]

CHAPTER 3

MATERIALS AND METHODS

1. Materials

1. Soybean (Glycine max (L.) Merr)(Rhai-Thip Co.Ltd, Thailand)
2. Genistein (Lot No. 086K4101, Sigma-Aldrich Chemical, Germany)
3. Daidzein (LotNo. 046K13diss12, Sigma-Aldrich Chemical, Germany)
4. Isopropanol (Lot No. K40861440 010, Merck KGaA, Germany)
5. Ethanol (Lot No.14010154, RCI Labscan, Thailand)
6. Methanol (Lot No. K36193607627, Merck KGaA, Germany)
7. Dichloromethane (Lot No. M83G1A, Merck KGaA, Germany)
8. Ethyl acetate (Lot No. 0893234, RCI Labscan, Thailand)
9. Glacial acetic acid (Lot No. AX0076-3, Merck KGaA, Germany)
10. Mannitol (Lot No. M205435206, Merck KGaA, Germany)
11. Maltrodextrin (Lot No. MKBB3308, Sigma-Aldrich Chemical, Germany)
12. Modified starch (National starch and Chemical, Thailand)
13. FA6008 (Siam Modified Starch Co., Ltd., Thailand).
14. Flomax-8 (Lot No. MGB2164, National starch and Chemical, Thailand)
15. 2,2,-Azino-bis(3-ethylbenzothizoline-6-sulfonic acid, diammonium salt (ABTS) (Lot No. 128K1082, Sigma-Aldrich Chemical, Germany)

16. 1,1-diphenyl 2-picryl hydrazyl (DPPH) (Lot No. 29K1376, Sigma-Aldrich Chemical, Germany) 6-Hydroxy-2,5,7,8-tetra-methylchromane-2-carboxylic acid (trolox) (Lot No. AC218940010 , Fisher Scientific Company, Canada)

17. 2,4,6-Tri (2-pyridyl)-s-triazine (TPTZ) (Lot No. WA11291, Sigma-Aldrich Chemical, Germany)

18. Folin-Ciocalteu's phenol (Lot No 65296CM, Sigma-Aldrich Chemical, Germany)

19. Sodium Carbonate (Lot No 46703, School-Tech Inc, USA)

20. Gallic acid (Lot No 91215, FlukaChemika, Switzerland)

21. Potassium Persulfate (Lot No. 118H0560, Sigma-Aldrich Chemical, Germany)

22. Iron(III) Chloride Hexahydrate (Lot No. 10025-77-1, Merck KGaA, Germany)

23. Ferrous Sulphase Heptahydrate (Lot No. 7782-63-0, Merck KGaA, Germany)

24. Polysorbate20 (Tween 20) (Sigma-Aldrich Chemical C0., St. Louis, MO)

25. Polysorbate80 (Tween 80) (Sigma-Aldrich Chemical C0., St. Louis, MO)

26. Olive Oil

27. Peppermint Oil (Lot No. 784672651226, Majestic pure cosmeceuticals™., San diego, USA)

28. Jojoba Oil (Leven Rose Co.,Ltd., colorado, USA)

29. Light mineral Oil (Lot No. A093093, Restek, Bellefonte, PA, USA)

30. Apricot Oil (Lot No. X000OTOFl1, Renewalize®, Miami, FL, USA)

31. Castor Oil (Lot No. 182650, Heritage store Ltd., USA)

32. Lavender Oil (Lot No. X000RG1YRV, Pure Luxe LLC, Houston, USA)

33. Sweet almond Oil (Lot No. 820103300250, SCB Marketing Co., Awendaw SC, USA)

34. Eucalyptus Oil (Lot No. 0312-140302, Nikko Chemicals Co., LTD, Japan)

35. Sunflower Oil (King - Thai Edible Oil Co., Ltd., Thailand)

36. Coconut Oil (Lot No. SS54/06177-01, Tropicana oil Ltd., Thailand)

37. Cinnamon Oil (Plant Therapy Ltd., USA)

38. Lemon Oil (Lot No. 784672651257, Majestic pure cosmeceuticals™., San diego, USA)

39. Glycerol, ACS reagent (Lot No. 23296EM, Sigma-Aldrich Chemical, Germany)

40. Diethyl Ether (Lot No. A41B19, J.T.Baker, USA)

41. Propylene Glycol (Lot No. 8804003073, S.Tong Chemicals Co., LTD, Thailand)

42. n-Hexane 99% AR (Lot No. 040110999, CRI Labscan, Thailand)

43. Ethylene Diamine Tetra Aacetic Acid (Lot No.2004494, FlukaChemika, Switzerland)

44. Butylated Hydroxy Toluene (BHT) (Lot No. 20131009, Namsiang Co., LTD., Thailand)

45. Di-Sodium Hydrogen Phosphate 12 – water(Nikko Chemicals, Japan)

46. Sodium Dihydrogen Phosphate Dihydrate (Nikko Chemicals, Japan)

47. Sodium Chloride (Nikko Chemicals,Tokyo, Japan)

48. Kanamycin Sulfate (Lot No. IB02120, Universal Medicals, USA)

2. Equipment

1. High performance liquid chromatography (HPLC)

1.1 C18 column (250 x 4.6 mm I.D., particle size 10 μm) (A PhenomenexINC, CA, USA)

1.2 A Finnigan modular LC system (Thermo Fisher Scientific, IL, USA)

1.3 Pressure pump, Model P4000 dual pump (Thermo Fisher Scientific, IL, USA)

1.4 Rheodyne 7725i injector (Sigma-Aldrich Chemical C0., St.Louis, MO)

1.5 UV absorbance detector, Model UV 6000 photodiode array detector (Jusco, MD, USA)

1.6 Software ChromQuest (Thermo Fisher Scientific, IL, USA)

2. Mesh Sieve

3. Bench Coffee Grinder

4. Horizontal shaker (HS 501 digital, Kika Labortechnik, Staufen, Germany)

5. Ultrasonic bath (Branson 3510, Ultrasonic Corporation, Danbury, CT, USA)

6. Soxlet apparatus

7. Daigger Vortex Genie 2 (Scientific Industries, Inc., NY, USA)
8. Magnetic stirrer and Magnetic bar(Mettler-toledo GmbH, Germany)
9. Centrifuge (Universal 320r, Hettich, Germany)
10. Syringe filter
11. Fume Hood
12. Freezer
13. Spray Drying (Mini Spray Dryer B-290, Buchi, Switzerland)
14. pH meter (Mettler Toledo seveneasy, Switzerland)
15. Desiccators (Biologix Reseach Company, USA)
16. FTA 1000 drop shape instrument (First Ten Angstroms, USA)
17. Differential scanning calorimeter (Sapphire, PerkinElmer, USA)
18. FT-IR spectrophotometer (Magna-IR system 750, Nicolet Biomedical Inc., USA)
19. Hot air oven (Heraeus, Germany)
20. Light microscope (CX41RF, Olympus, Japan)
21. Loss on drying mesurement (Sartorious Moisture balance, Germany)
22. Magnetic stirrerand Magnetic bar(Mettler-toledo GmbH, Germany)
23. pH meter (Mettler Toledo seveneasy, Switzerland)
24. Particle size analyzer (LA-950, Horiba, Japan), (nano ZS, Malven, England)
25. Powder X-Ray diffractrometer (D8, Bruker, Germany)
26. Scanning electron microscope (SEM; MX2000, Camscan, UK)
27. Shaker incubator (SL SHEL LAB, SI4-2, USA)
28. UV-Spectrophotometer (Lamda2, Perkin-Elmer, USA)

29. Zeta-potential analyzer (Zeta Plus, Brookhaven, USA), (nano ZS, Malven, England)

30. Petri Dishes

31. Funnel

32. Scissors

33. Grater

34. Cellophane Tape

35. Hi Scotoron (NS-50, Nichion, Chiba, Japan)

36. Microprocessor conductometer (LF 2000, WTW).

37. Ultra-high Resolution Cryo-Scanning Electron Microscope (S-4800, Hitachi, Japan)

38. High Frequency Low Stress Rheometer (Gemini 150, Bohlin, Malvern, England)

39. Dynamic light scattering Zetasizer (HS 1000, Nano ZS 3600, Malvern, England)

40. Differential Scanning Calorimeter (Micro DSC III, DSC VII, Setaram, England)

41. Beaker 150, 250, 400, 600, 1000 ml (SCHOTT, Duran, Germany)

42. Centrifuge tubes-Sterile 15 ml, 50 ml (Biologix Research Company)

43. Eppendorf tubes

44. Glass bottle 100, 250, 500, 1000 ml (SCHOTT, Duran, Germany)

45. Micropipette 20-100 µl, 100-1000 µl, 1-5 ml, 1-10 ml (masterpette ; Bio-Active Co.,Ltd.)

46. Petri Dish (Lot No. D131102C, Grenier Bio-One, Thailand)

47. Thermometer

48. Water bath (W38, Grant Instruments, UK)

49. Weighing balance (CP224S, Sartorius, Germany)

50. Watch glass

3. Methods

Soybeans was extracted by different methods. The amount of genistein and daidzein obtained were then identified by TLC and HPLC methods and the comparison was made. The method which gave the highest amount of genistein and daidzein was selected for drying process by spray dryer. The stability and physiochemical properties including antioxidant activities were carried out. The stable sample of genistein and daidzein was formulated into microemulsion dosage form. The physicochemical properties and permeation study were then performed. All studies were summarized in the Figure 12.

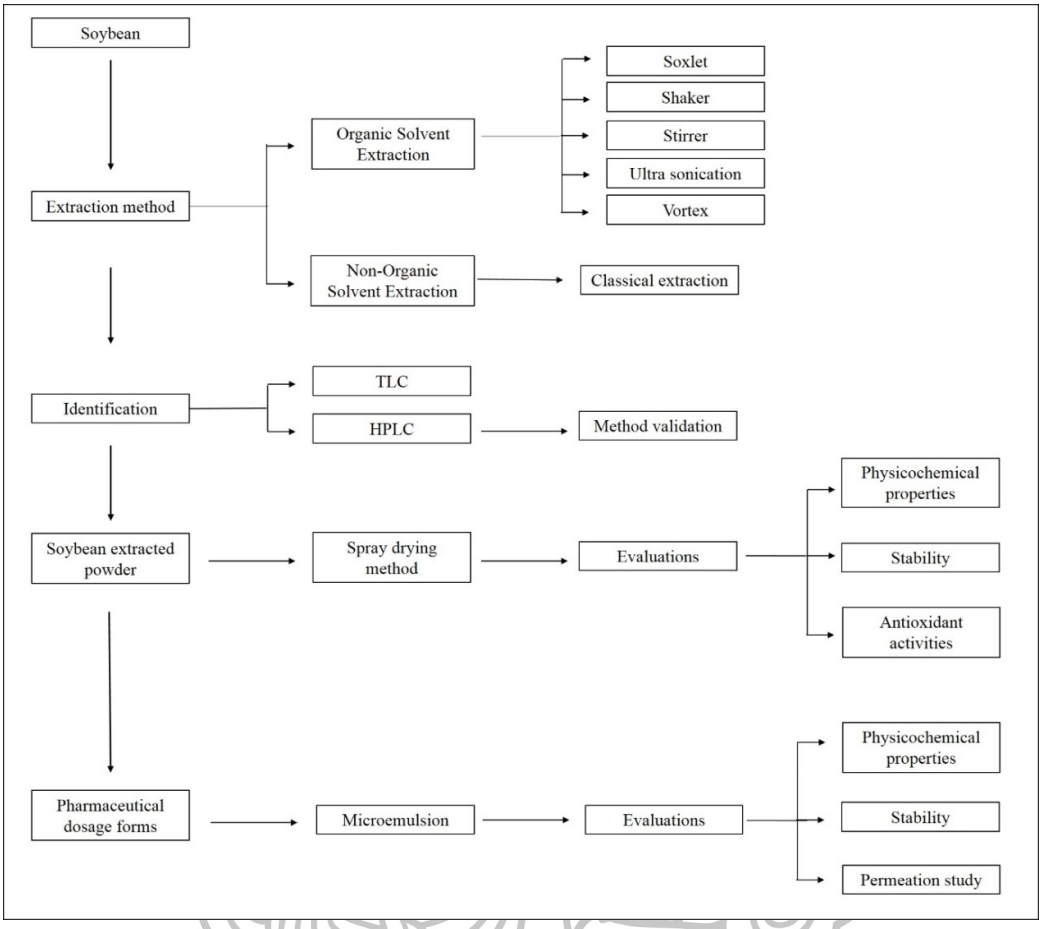


Figure 12 Diagram of the studies

3.1 Extraction method

3.1.1 Organic solvent extraction.

Sample Preparation; The dried soybean seeds were ground in a bench coffee grinder. The ground material was then passed through a standard mesh sieve (particle size < 0.4287 mm), mixed thoroughly and stored in a freezer until extraction. Prior to extraction, the ground soybean seeds were defatted by isopropanol (10 mL/g of sample) using a magnetic stirrer (500 rpm) for 2 hours. The mixture was then centrifuged at 4500xg for 10 minutes and the supernatant was discarded. The

remaining material was dried at room temperature under fume hood and used as a sample for the experimental extraction**.**

Solvent Extraction Methods; The soybean isoflavones were extracted with 85% ethanol by using five different methods: soxhlet, shaker, stirring, ultrasonication, and vortexing extractions. 1-gram of ground soybean seeds using 85% ethanol at the solid to solvent ratio of 1:2 were carried for all extraction methods. The extraction time of all methods was 1 hr.

3.1.1.1 Extraction by Using a Soxhlet Apparatus.

An exact amount of 10 grams of ground soybean seeds was placed in a thimble inside soxhlet extraction apparatus. The extraction was carried out using 85% ethanol as solvent (10 mL/g of sample) for 1 hour. The crude extract was concentrated by reducing pressure of evaporation at 45°C and then centrifuged at 2140xg for 10 minutes (Centrifuge, Universal 320r, Hettich, Germany). The supernatant was taken into 10 mLvolumetric flask. Two millilitres of the extract was filtered through 0. 45 µm PTFE (polytetrafluoroethylene) syringe filtering for the analysis of isoflavone by using HPLC. Three replicate HPLC analyses of the extracts were carried out.

3.1.1.2 Extraction by Using a Shaker; Stirring; Ultra Sonication; and Vortexing Procedures.

In each method, 1-g of sample was weighed and 10 mL of 85% ethanol was added to each flask. For extraction by a shaker (Horizontal shaker, HS 501 digital, Kika Labortechnik, Staufen, Germany), sample was vigorously shaken on a horizontal shaker at a speed of 200 rpm for 1 hour. Stirring method (Magnetic stirrer and Magnetic bar, Mettler-toledo GmbH, Germany), extraction was

carried out by placing extraction flask on a magnetic stirrer for 1 hour. Extraction with ultra sonication (Ultrasonic bath, Branson 3510, Ultrasonic Corporation, Danbury, CT, USA), sample flask was placed in an ultrasonic bath for 1 hour. For the method of vortexing procedure (Daigger Vortex Genie 2, Scientific Industries, Inc., NY, USA), 20 mL tube with sample was placed on a Daigger Vortex Genie 2 for 1 hour.

The crude extracts from each procedures were centrifuged at 2140xg for 10 minutes. The supernatant was taken from 10 mL volumetric flask. Two milliliters of the extract was filtered through 0.45 µm PTFE syringe filtering for analysis of isoflavone by using HPLC. Three replicate HPLC analysis of each extract was performed.

3.1.2 Non-organic solvent extraction

3.1.2.1 Extraction by using classical method

Water was selected for a classical extraction. The temperature might be the main effect for amount of genistein and daidzein. This study focus on the temperature effects of extraction. The soybeans were macerated in water for 8 hours and then blended with water (soybeans 1 g / water 12 ml.). The crude extract was filtered and boiled for 1 hour at different temperature ranges; 26-30, 31-35, 36-40, 41-45, 46-50, 51-55, 56-60, 61-65, 66-70, 71-75, 76-80, 81-85, 86-90, 91-95 and 96-100°C. Water was removed by using spray drying. The dried extract was used for the assessment of antioxidant activity. The isoflavones content of the extracts were determined by using high performance liquid chromatography in comparison to standard genistein and daidzein.

3.2 Identification of soy isoflavone contents

The method were used to analyze amount of genistein and daidzein from both organic solvent and non-organic solvent

3.2.1 Thin Layer Chromatography method

The extracted solution from various methods was screening for genistein and daidzein by Thin Layer Chromatography. To determine soy isoflavone contents by using developed thin-layer chromatography (TLC) method, soybean extract solution was fractionated by TLC. 10 µl of the extract were placed on the aluminium plates coated with silica gel 60 and the mixture of dichloromethane, ethyl acetate and glacial acetic acid at the ratio of 6:3:1,v/v were used as a mobile phase.

3.2.2 Determination of soyisoflavone content by using developed high performance liquid chromatography (HPLC)

After preliminary screening by TLC, HPLC was used for further identification and determination of the content of genistein and daidzein from soybean extracts. A Finnigan modular LC system with a Model P4000 dual pump equipped with a Rheodyne 7725i injector linked to a 20 µl loop and a Model UV 6000 photodiode array detector was used for analysis by liquid chromatography. A Phenominex C18 column (250 x 4.6 mm I.D., particle size 10 µm) was used for chromatographic separations. The chromatographic data were obtained by a PC system, and a software **ChromQuest** from Thermo Fisher Scientific was used to acquire and process the data. The gradient elution was needed for completed separation of the analysis. The mobile phase consisted of two eluents: (A) 0.1% acetic acid in deionized water and (B) 0.1% acetic acid in methanol. Solvent A and Solvent B were mixed to 100% along the process. The analyses were performed

under gradient elution conditions. The system was maintained at 50% B for 5 minutes with the flow rate of 1 ml/min, then, increased to 80% for 5 minutes with the flow rate of 0.5 ml/min and held at 80% for another 7 minutes with the flow rate of 1 ml/min. At the end, the system was set to increase solvent B from 80% to 100% within 2 minutes, holding these conditions for 11 minutes and then returned to the original condition, 50% B, for 10 minutes. Total run time was 40 minutes including 10-minute stabilization time. The chromatographic analysis was performed at an ambient temperature and detection wavelength was set to 254 nm. Injection of 20 µl was effected with a SGE Analytical Science 100 µl syringe. The HPLC conditions was confirmed by validation. Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use.

3.3 Preparation of soybean extracted powder

3.3.1 Influence of diluents on physical properties of soybean extracted powder

The best temperature condition from non-organic solvent extraction (classical method) was then chosen for further study. The extract was prepared in dried powder form by spray drying (Mini Spray Dryer B-290, BUCHI, Switzerland) at a spraying rate of 500 mL/hour, the inlet and outlet temperature of 180°C and 80°C, respectively. Four carrier agents were applied during process of spray drying. They were mannitol (Sample a.), maltrodextrin (Sample b.), and 2 types of modified starch, FA 6008 (Sample c.) and Flomax-8 (Sample d.). The extracted powders were compared with soybean extracted power without a carrier agent (Sample e.) The best carrier of dried extract was then stored at a room temperature in a desiccator at room

temperature for 0, 0.25, 0.5, 1, 2, 4, 8, 12, 16, 20, 24, 28, 32 and 36 months for stability and characterization studies.

3.3.2 Determination of physicochemical properties study

Soybean extract powder were determined a physicochemical properties at the initial time as follows;

3.3.2.1 Identification of genistein and daidzein

The TLC and HPLC were used to detect the effect of storage condition on the identification of soybean extract of all samples. The standard of genistein and daidzein were compared with the samples.

3.3.2.2 Amount of genistein and daidzein

The amount of soy isoflavone content can be measured by our developed HPLC method as previously described (3.2.1).

3.3.2.3 pH determination

The pH value of soybean extracted powders was determined for pH by blending 5- g powder with 25 mL deionized water at the ambient temperature, using the pH meter (pH meter, Mettler Toledo seveneasy, Switzerland). All the samples were performed in triplicates.

3.3.2.4 Flowability

The angle of repose was determined by pouring a pre-defined mass of spray-dried powder through a funnel located at a fixed height on a graph paper, flat horizontal surface and measuring the height (h) and radius (r) of the conical pile formed. The tangent of the angle of repose is given by the h/r ratio. All the samples were performed in triplicates.

3.3.2.5 Moisture content (MC)

Powder moisture content was determined by a moisture analyzer with moisture balance (Sartorious Moisture balance, Germany). All the samples were performed in triplicates.

3.3.2.6 Particle size distribution

Particle size distribution of soybean extracted powder was measured using a laser light diffraction instrument, (LA950, Horiba, Japan). Average particle size was expressed as D, mean volume diameter. All the samples were performed in triplicates.

3.3.2.7 Particle morphology

Morphological examinations of the samples were performed by Scanning electron microscope (SEM, VE-7800, KEYENCE company, Japan). Samples were mounted on metal stubs and coated with a fine gold layer under vacuum before obtaining the micrographs. The acceleration voltage used was 5 kV.

3.3.2.8 X-ray diffraction (XRD)

XRD patterns were recorded using a X-ray diffraction system (D8, Bruker, Germany). The anode X-ray tube was operated at 30 kV and 40 mA. Measurements were taken from 5° to 45° on the 2θ scale at a step size of 2°/min using Cu K radiation wavelength of 1.5406 Å.

3.3.3 Stability analysis of soybean extracts

Figure 13 shows process of stability studies. The effect of temperature and the physical form of extract were investigated. The solution form was kept at -20°C and 5°C for one month while the dried powder form was kept at room temperature in a desiccator for 3 years. Both physical forms were detected for

identification and amount of genistein and daidzein at certain time according to the Figure 13. The powder form was determined at initial for their physicochemical properties. They were pH, dispersion time, bulk density, solubility, flowability, moisture content, particle size distribution, particle morphology, X-ray diffraction and differential scanning calorimetry. For solution form at both temperatures, the identification and amount of genistein and daidzein were sampling weekly for 1 month while the dried form were detected at 0, 1, 2 weeks, 1 , 2, 4, 8, 12, 16, 20, 24, 28, 32 and 36 months.

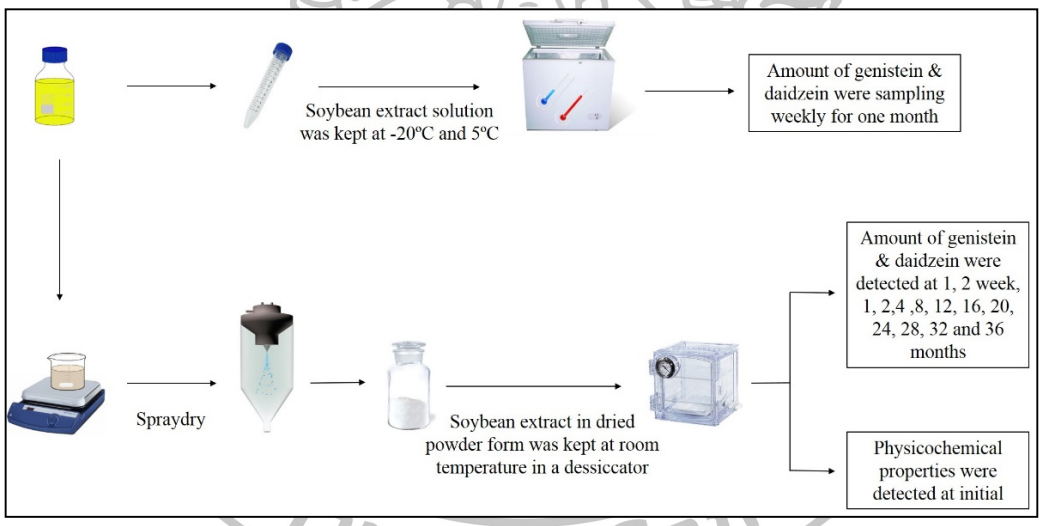


Figure 13 Process of stability studies

3.3.4 Determination of antioxidant activities of the soybean extracts.

Soybean extract in dried powder form at initial was used for the assessment of antioxidant activity. Purification of genistein and daidzein isoflavones content of the extracts were determined by using high performance liquid chromatograph. The antioxidant activity study by several methods as the followings:

3.3.4.1 Free radical-scavenging activity of soybean extract using DPPH (1,1-diphenyl 2-picryl hydrazyl)

The free radical-scavenging activity of the soybean extract was measured in terms of hydrogen donating on radical-scavenging ability using the stable radical DPPH. Reaction of $DPPH^{\bullet}$ with antioxidant (AH) or radical species ($R^{\bullet}$) as shown in Eq. 1-2.

$$DPPH^{\bullet} + AH \text{ ---------- } DPPH\text{-}H + A^{\bullet} \quad \text{…….. (Eq.1)}$$

$$DPPH^{\bullet} + R^{\bullet} \text{ ---------- } DPPH\text{-}R \quad \text{…….. (Eq.2)}$$

0.20 mM solution of DPPH in ethanol was prepared and 950 μl of this solution was added to 50 μl of extract solution at different concentrations (3-100 mg/ml). The mixture was shaken vigorously and was allowed to stand for 60 minutes at room temperature. The absorbance of the resulting solution was measured at 517 nm. The inhibition percentage of free radical by the studied sample was calculated using the following formula:

$$\% \text{ inhibition} = \frac{\text{A control} - \text{A sample}}{\text{A control}} \times 100 \quad \text{……..……..…..……(Eq.3)}$$

where $A_{control}$ was the absorbance of the control (blank, without extract) and A_{sample} was the absorbance in the presence of the extract.

All the tests were performed in triplicate and the graph was plotted with the mean values. Radical-scavenging ability was calculated as IC_{50} (concentration causing 50% inhibition) and expressed as Trolox equivalent antioxidant capacity (TEAC) in mg trolox per g extract as follows:

$$\text{TEAC (mg trolox/g)} = \frac{\text{IC50(trolox)}}{\text{IC50(sample)}} \times 10^3 \quad \text{.....(Eq.4)}$$

3.3.4.2 ABTS cation radical scavenging assay

The antioxidant activity was measured by $ABTS^+$(2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid free radical cation) decolorisation assay. Reaction of antioxidant (AH) with ABTS●+ as shown in Eq 5-6

$$OH^\bullet + ABTS \text{ ---------- } ABTS^{\bullet+} + H_2O \quad \text{..... (Eq.5)}$$

$$ABTS^{\bullet+} + AH \text{ ---------- } ABTS + A \quad \text{..... (Eq.6)}$$

The stock solution of 7 mM ABTS and 140 mM potassium persulfate ($K_2S_2O_8$) were mixed for 16-18 hours in dark and low temperature. The solution was diluted with ethanol to give 0.7±0.05 absorbance at 750 nm. The soybean extract was dissolved in ethanol to give an appropriate concentration. An aliquot of 20 μl of each ethanolic extract solution was added to 980 μl of $ABTS^+$ radical cation solution. The mixture was shaken vigorously and the absorbance was measured at 750 nm. The radical scavenging assay was calculated using the following formula:

$$\text{\% remaining ABTS} = \frac{\text{A control} - \text{A sample}}{\text{A control}} \times 100 \quad \text{.....(Eq.7)}$$

where $A_{control}$ was the absorbance of the control (blank, without extract) and A_{sample} was the absorbance in the presence of the extract.

3.3.4.3 Ferric reducing antioxidant power or FRAP method

Antioxidant activity assessed as reduction of Fe(3+)-TPTZ to Fe(2+)-TPTZcomplex. FRAP reagent was prepared by mixing 10mM 2,4,6-tripyridylstriazine (TPTZ) solution with 20mM $FeCl_3.6H_2O$ in acetate buffer pH 3.7. Ethanolic solutions of 5-50 μM $FeSO_4.7H_2O$ were used for standard curve. 50 μl of the soybean extract was mixed with 950 μl solution of FRAP reagent and the absorbance at 595 nm was determined. The results were expressed as mM equivalent of ferric per weight of sample. All measurements were taken in triplicate and the mean values were calculated.

3.3.4.4 Determination of total phenolic content

The Folin–Ciocalteu reagent (FCR) or Folin's phenol reagent or Folin–Denis reagent, also called the gallic acid equivalence method (GAE), is a mixture of phosphomolybdate and phosphotungstate used for the colorimetric in vitro assay of phenolic and polyphenolic antioxidants. The reaction as eq. 8

$$Na_2WO_4/Na_2MoO_4 \text{ ---------- (phenol} - MoW_{11}O_{40})^{-4} \text{ ……..…(Eq.8)}$$

Total phenols in the soybean extracts were determined by Folin–Ciocalteau reagent using gallic acid as a standard. 100 μl solution of soybean extract was added to 500 μl of Folin–Ciocalteau reagent. After 10 minutes, 400 μl of 7.5% Na_2CO_3 was added and the mixture was incubated at 50°C for 5 minutes. The absorbance of the blue color that developed was read at 760 nm. The content of total phenols was expressed as gallic acid equivalents (GAE) in mg per g of extract. All analyses were run in triplicate and mean values were calculated.

3.4 Pharmaceutical dosage form containing soybean extract.

The term of microemulsion applies to a mixture with at least three components; an oily phase, an aqueous phase and a surface active species, so called surfactants. The co-surfactant may be required. As for simple aqueous systems, microemulsion formation is dependent on surfactant type and structure.

3.41 Determination of surfactant miscibility with different oils.

To determine the miscibility, different oils was mixed with a selected surfactants by using binary phase diagram. Tween 20 and Tween 80 were selected for a surfactant. Different commonly used oils were olive oil, peppermint oil, jojoba oil, light mineral oil, apricot oil, castor oil, lavender oil, sweet almond oil, eucalyptus oil, sunflower oil, coconut oil cinnamon oil, fish oil, lemon oil and rice bran oil. The concentration of surfactants was fixed at 50% and mixed with varying ratio of oils and deionized water. The samples were centrifuged at 8000 rpm for 10 minutes and kept for 1 week before it was recorded. The volume ratio of various oils and surfactants and deionized water were shown in table 5. The resulting mixture was then examined for cloudiness or turbidity. The condition of non separation, free of cloudiness or turbidity, was defined as miscible. The oil which showed the widest region in binary phase diagram was selected for the formulation.

Table 5 Various weight ratios of oil: surfactant: deionized water

Formulas	Oil* : Surfactant** : Deionized water (%weight)
01	0 : 50 : 50
02	5 : 50 : 45
03	10 : 50 : 40
04	15 : 50 : 35
05	20 : 50 : 30
06	25 : 50 : 25
07	30 : 50 : 20
08	35 : 50 : 15
09	40 : 50 : 10
10	45 : 50 : 5
11	50 : 50 : 0

* Oil in the formulation including olive oil (ol), peppermint oil (pp), jojoba oil (jo), light mineral oil (li), apricot oil (ap), castor oil (ca), lavender oil (la), sweet almond oil (sw), eucalyptus oil (eu), sunflower oil (su), coconut oil (co), cinnamon oil (ci), fish oil (fi), lemon oil (le) and rice bran oil (ri)

** Surfactant in a formulation including Tween 20 (A) and Tween 80 (B)

For example of code, A01ol standท for a ratio from a formulation 01, using Tween 20 as a surfactant and olive oil as an oil.

3.4.2 Solubility determination

Solubility of soybean extract was determined in different solvents. Soybean extract was added in excess to different solvents and stirred with magnetic stirrer for 24 hours. The samples were centrifuged at 8000 rpm for 10 minutes and the content of active compound from the supernatant was analysed by HPLC. The solubilities were calculated. The solvents having different dielectric constants, including acetone, iso-propanol, ethanol, methanol, glycerol, diethyl ether, propylene glycol, hexane, ethyl acetate, and water were selected for this study. The best solvent was then selected for the formulation as a co-surfactant.

3.4.3 Pseudo ternary phase diagram

Microemulsions are basically thermodynamically stable, isotropically clear dispersions of two immiscible liquids such as oil and water stabilized by the interfacial film of any surfactant and/or cosurfactant. Pseudo ternary phase diagram were constructed to find the area of microemulsion existence. Tween 20 and Tween 80 were selected for surfactant and ethanol was selected for a co-surfactant. Lavender oil, lemon oil, peppermint oil and coconut oil were chosen from above study for the investigation of pseudo ternary phase diagram. The weight ratio of surfactant varied as 100% of Tween 20 (A), 100% of Tween 80 (B), mixture between 50% Tween 20 and 50% Tween 80 (C) and ratio of surfactant/co surfactant varied as 2:1 (D), 1:1 (E) and 1:2 (F). The solution existence region of the system containing soybean extract, oil, ethanol and aqueous solution of surfactant was determined by constructing of pseudo-ternary phase diagram. The volume ratios of oil : deionized water : aqueous solution of surfactant were indicated in Table 6.

Firstly, soybean extract powder was dissolved with absolute ethanol. Different oils and ethanol were mixed in a test tube until clear solution was obtained. Then, aqueous solution of surfactant (Tween 80 and Tween 20) and deionized water were added according to the ratio indicated in Table and then mixed thoroughly by vortex mixer. All samples were vigorously stirred. The weight ratio of surfactant to co-surfactant was varied as 1:2, 1:1 and 2:1. The samples were centrifuged at 8000 rpm for 10 minutes and clearness, cloudiness or phase separation were checked under light versus a dark background,. The samples were allowed to equilibrate at 25ºC for at least 24 h before they were examined. All samples which remained transparent and homogeneous after vigorous vortexing, were considered as a monophasic area in the phase diagram.

Table 6 Various weight ratios of oil: surfactant/co-surfactant: deionized water

Formulas	*Oil : **Surfactant/Co-Surfactant : Deionized water (% w/w)
01	10 : 80 : 10
02	10 : 70 : 20
03	20 : 70 : 10
04	10 : 60 : 30
05	20 : 60 : 20
06	30 : 60 : 10
07	10 : 50 : 40
08	20 : 50 : 30
09	30 : 50 : 20

Formulas	*Oil : **Surfactant/Co-Surfactant : Deionized water (% w/w)
10	40 : 50 : 10
11	10 : 40 : 50
12	20 : 40 : 40
13	30 : 40 : 30
14	40 : 40 : 20
15	50 : 40 : 10
16	10 : 30 : 60
17	20 : 30 : 50
18	30 : 30 : 40
19	40 : 30 : 30
20	50 : 30 : 20
21	60 : 30 : 10
22	10 : 20 : 70
23	20 : 20 : 60
24	30 : 20 : 50
25	40 : 20 : 40
26	50 : 20 : 30
27	60 : 20 : 20
28	70 : 20 : 10
29	10 : 10 : 80
30	20 : 10 : 70
31	30 : 10 : 60

Formulas	*Oil : **Surfactant/Co-Surfactant : Deionized water (% w/w)
32	40 : 10 : 50
33	50 : 10 : 40
34	60 : 10 : 30
35	70 : 10 : 20
36	80 : 10 : 10

* Oil in the formulation including peppermint oil (pp), lavender oil (la), coconut oil (co) and lemon oil (le)

** Surfactant/Co-Surfactant in a formulation as follow ;

A = Tween 20 ,

B = Tween 80 ,

C = Tween 20 : Tween 80 = 1 : 1

D = Tween 20 : Tween 80 : EtOH = 1 : 1 : 1

E = Tween 20 : Tween 80 : EtOH = 0.5 : 0.5 : 1

F = Tween 20 : Tween 80 : EtOH = 0.5 : 0.5 : 2

For example of code,

- A02la stand for a formulation 02 by using only Tween 20 as a surfactant and lavender oil as an oil. This preparation without a co-surfactant.

- D01pp stand for a formulation 01 by using Tween 20 : Tween 80 : EtOH in a ratio 1 : 1 : 1 as a surfactant/co-surfactant and peppermint oil as an oil.

3.4.4 Preparation of dosage form containing soybean extract Formula

Soybean extract was dissolved in Ethanol as a co-surfactant, and then mixed with surfactant, oil and water following a ratio from pseudo-ternary phase diagram. The microemulsions was homogeneous after vigorous vortexing. The effect of type and amount of other excipients was also further investigated as exemplified in Table 7 .

Table 7 Formula of dosage forms

Material	Amount
Oil	30-50(% w/w)
Absolute ethanol	20-40(% w/w)
Surfactant/Co-Surfactant	20(% w/w)
Soybean extract	0.1-0.3(% w/w)
BHT (butylated hydroxytoluene)	0.01(% w/w)
EDTA (ethylenediaminetetraacetic acid)	0.01 (% w/w)
Water qs to	100

3.4.5 Characterization of microemulsion

The prepared microemulsion was characterized as bellows:

3.4.5.1 Visual observation

Microemulsion samples from established phase diagrams were visually observed after an 1-week equilibration time, upon shaking. Clearly one phase liquids were defined as microemulsions.

3.4.5.2 Particle size and zeta potential

The particle size and zeta potential of soybean extract loaded microemulsion formulation were measured by dynamic light scattering at 25ºC using Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd., Worcestershire, England). The particle size was expressed as an average particle size of droplets in the system and polydispersity index (PDI) which indicated the width of the size distribution. All experiments were performed in triplicate.

3.4.5.3 Measurement of pH and refractive index of microemulsion

The pH of microemulsions was using the Mettler Toledo 320 pH meter (Switzerland), the pH values of the samples were measured at 25 ± 1ºC. The measurements were carried out in triplicate.

3.4.5.4 Refractive index measurement

A refractometer (Abbe refractometer, ATAGO, Japan) was used to determine the refractive indices of the formulations in triplicate at 25ºC.

3.4.5.5 Rheological measurements

The rheological properties of the microemulsions were investigated using a Bohlin CVOR (Creep, Viscosity, Oscillation, Relaxation) rheometer (Malvern Instruments, UK) equipped with Bohlin software and thermostatic control system (Bohlin KTB30, Malvern, UK). The rheometer consisted of a thermostatically controlled cone and plate tool. The temperature was maintained at 25 ± 0.5 °C throughout the experiment. Sample weight was 15 g and test was carried out in triplicate. Flow properties were investigated by measuring the dynamic

viscosity (η, Pa/s) as a function of time for 60 min in addition to measurment of viscosity as a function of shear rate (ranging from 0.1 s−1 to 200 s−1).

3.4.5.6 Surface tension

Surface tensions measurements were performed using a KRÜSS Digital Tensiometer K10, with a Platinum Wilhelmy plate.

3.4.5.7 Microscopic evaluation

Each microemulsion was visualized for droplet appearances by polarizing microscopes (Polarizing microscope with LED illumination, Leica DM2700 P, Germany)

3.4.6 Stability study

The optimized microemulsion was stored at three different temperatures 5 ± 2◦C, 25 ± 2◦C and 40 ± 2◦C for a period of 3 months. The samples were centrifuged at 13,000 rpm for 30 min to evaluate the physical instability indicating phase separation.

3.4.7 Ex vivo model for skin permeation study.

A piece of Yucatan micropig skin (YMP skin set, Charles River Japan, Yokohama) was thawed at room temperature for approximately 30 min, followed by removal of the adhering fat layer using scissors and a grater. Stripped skin was obtained by removing the stratum corneum using adhesive cellophane tape (Washitake et al., 1973). The skin was cut to an appropriate size and mounted on modified Franz-type diffusion cells (1.1 cm^2). The receptor compartment was filled with pH 7.1 isotonic phosphate buffered saline (PBS) containing 0.01% kanamycin and kept at 37 °C with stirring at 600 rpm. An aliquot (3 ml) of the drug solution was applied to the donor compartment. A glass ball occluded the upper portion of the

donor phase. At appropriate time intervals, 0.2 ml of the receptor phase was withdrawn, and the same volume of fresh PBS was added to the cell to keep the volume constant [Figure 14]. Each experiment was carried out for 30 h. After 48 hours, the skin was removed from the diffusion cell apparatus and wiped with purified water. The epidermis, consisting of the stratum corneum and viable epidermis, was separated from the dermis by a heat separation technique [255]. The separated tissues were then minced with scissors and homogenized at 4,000 rpm. by using a Hiscotoron NS-50 homogenizer (Nichion, Chiba, Japan) following by the addition of 75% methanol. After centrifugation, the supernatant of each sample was injected into an HPLC system for detection of the amount of genistein and daidzein. For permeation data analysis, the cumulative amount of genistein and daidzein permeated (*Qt*, $\mu g/cm^2$) was plotted as a function of time. The steady state flux (*Jss*) was calculated from the slope of linear portion of the plot. The lag time (*Tlag*) for microemulsion to permeate through Yucatan micro pig skin before reaching the receptor fluid was calculated from the X-intercept of the plot. The permeability coefficient (*Kp*) of the microemulsion through Yucatan micro pig skin was calculated from the equation;

$$Kp = Jss/C0, \quad \text{.....(Eq.4)}$$

where *C0* is the initial concentration of genistein and daidzein in the donor compartment.

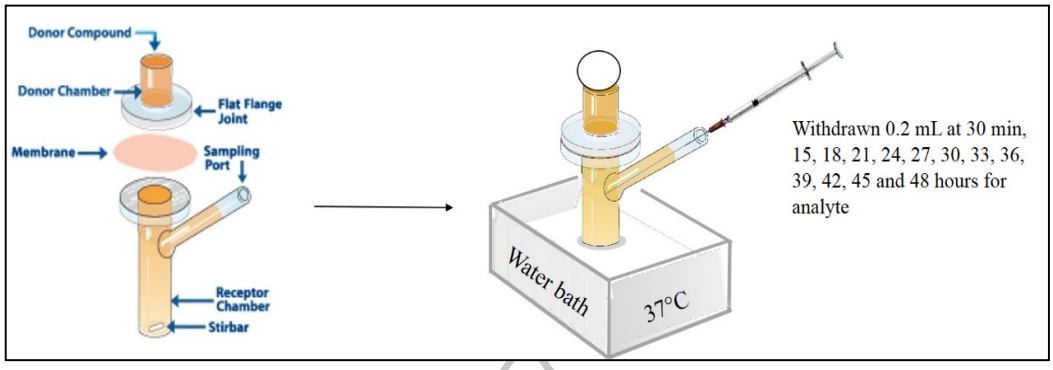


Figure 14 Franz diffusion cell for permeation study

3.5 Data analysis

Data were expressed as the means ± standard deviation (SD) of the mean. A one-way analysis of variance (ANOVA) was carried out as a statistical analysis. A value of $P<0.05$ was considered as statistically significant. The number of samples were in triplicate for all experiments.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Determination of soybean extract

4.1.1 Identification of soybean extract by TLC method

Thin layer chromatography (TLC) is one of the simple tools for identification of substances in the complex mixtures, determining their purity and following the progress of a reaction. In comparison with column chromatography, it requires a small amount of the compound and takes a relatively short period of time [256]. In this study, to screen the active compounds of the soybean extracts, the TLC chromatogram analysis method was applied for detection of the genistein and daidzein. The mixture of dichloromethane, ethyl acetate and glacial acetic acid at the ratio of 6:3:1, v/v were used as a mobile phase. Figure 15 displays the TLC chromatogram of the soybean extracts from various methods. Genistein and daidzein obtained from all extraction methods (soxhlet (C), shaker (D), stirring (E), ultra sonication (F) and vortex (G)) were identified and compared with standard genistein (A), standard daidzein (B). As shown in Figure 15, the R_f values of standard genistein and standard daidzein were 0.37 and 0.55, respectively. The results suggested that the genistein and daidzein from various methods could be detected by TLC showing the same R_f values by using mixture of dichloromethane, ethyl acetate and glacial acetic acid at the ratio of 6:3:1, v/v solvent. All the TLC finger prints from each method showed a similar pattern. The results suggested that this solvent mixture can be used

for screening the substances from soybean extracts. The genistein and daidzein can be detected from all the extraction methods. Nevertheless, TLC is only a preliminary step for qualitative analysis, further studies for the determinations of both substances are required as the method is limited to identification not the determination of amount of substances. Therefore, other characterization techniques need to be performed as would be described in the following sections.

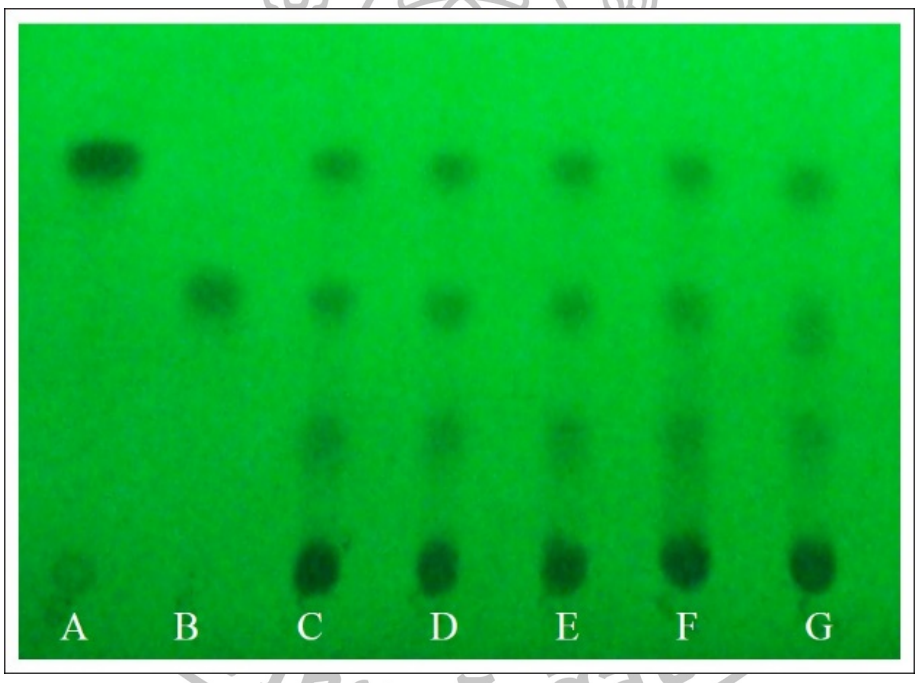


Figure 15 A-G, The fingerprint of standard genistein (A) and standard daidzein (B), soybean extract solution from soxhlet(C), shaker(D), stirring(E), ultra sonication (F) and vortex (G),

4.1.2 Identification of isoflavones in soybeans from solvent extraction methods by HPLC

The HPLC chromatogram of the isoflavone standards, genistein and daidzein, is presented in Figure 16(a). The use of a Phenominex C18 column with gradient elution consisting of 0.1% acetic acid and 0.1% acetic acid/methanol as binary mobile phase, resulted in a good resolution of standards about within 30 minutes. The chromatograms of the isoflavone extracts from ground soybean seeds by using different methods, *i.e.* soxhlet, shaker, stirring, ultrasonication, and vortex (sample A-E), showed differences in the isoflavone profile (Figure 16(b)–2(f)). The retention times of genistein and daidzein obtained from samples were identified and compared with pure standard. The retention time of all extraction methods was 13.8 and 14.9 minutes for genistein and daidzein, respectively. The results indicated that genistein and daidzein could be found in all extraction methods and the developed HPLC method could be used to identify them.

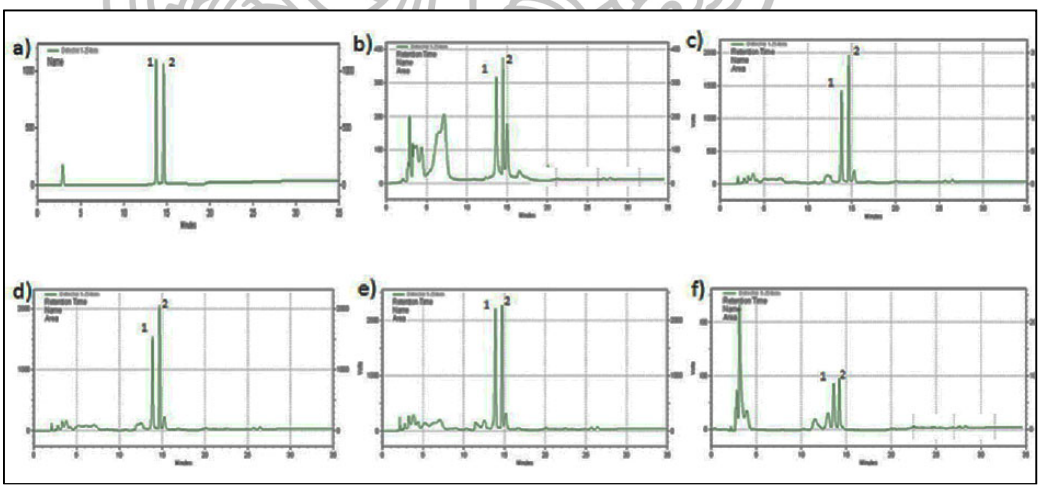


Figure 16 HPLC chromatograms of isoflavones using different extraction methods, standard mixture (a) of genistein (1) and daidzein (2), using soxhlet (b), shaker (c), stirring (d), ultrasonication (e) and vortexing procedures (f).

4.1.3 Organic solvent extraction ; Study on Quantification of Isoflavones in Soybeans from Various Extraction Methods by HPLC

The extraction efficiency of soybean isoflavones with single extraction solvent mixture (85% ethanol in deionized water) with 1:2 solid-to-solvent ratio was carried out by using five common extraction methods (soxhlet, shaker, stirring, ultrasonication, and vortex) . The contents of individual isoflavones (genistein and daidzein) were calculated from peak areas of compounds as listed in Table 8 (A-E). The results indicated that highest yields of genistein and daidzein were obtained with ultrasonication procedure, which showed the amounts of 272.27± 6.83 and 343.53 ± 6.18 µg/g, respectively; while the lowest yields of genistein (101.45 ± 5.75 µg/g) and daidzein (165.64 ± 0.89 µg/g) were obtained by using soxhlet and vortexing procedures, respectively. Only shaker and stirring methods were not significantly different. The highest yield of ultrasonication was the result of high frequency of ultrasound causing the penetration into liquid mediums. This had a potential to give stream waves and bubbles called "*cavitation*". Cavitation is the phenomenon of formation of very low size air bubbles, approximately 1 in 1 million meter (micron), produced by a flowing liquid, generating a high power of energy, accordingly [257]. The production of highest energy allowing a greater penetration of solvent into the sample matrix, in comparison to other techniques, possibly attributed to the optimum isolation of essential substances from soybean. Another reason for the improvement of reactions in a pulsed field can be the disintegration of the stable agglomerations of cavitation bubbles or prevention of disintegration. The bubbles inside the cluster are shielded from the ultrasound field by the bubbles at the outside

part of the cluster and act strongly on each other. The pulse modulation of the ultrasound prevents the clustering and this can favor the clarification of the cavitation zone and thus maximization of the process yield as reported by some authors [258]

The developed HPLC method, hence, could be proved to determine the essential substances of soybean obtained from various extraction methods.

Table 8 Amount of isoflavones (genistein and daidzein) extracted from soybean seeds using different extraction methods

Extraction Methods	Genistein (μg/g)	CV	Daidzein (μg/g)	CV
Soxhlet (A)	101.45 ± 5.75	5.67	186.11 ± 6.31	3.39
Shaker (B)	242.24 ± 8.42	3.48	235.48 ± 10.22	4.34
Stirring (C)	248.69 ± 5.12	2.06	251.01 ± 3.54	1.41
Ultrasonication (D)	272.27± 6.83	2.51	343.53 ± 6.18	1.80
Vortex (E)	173.27 ± 5.65	3.26	165.64 ± 0.89	0.54

Values were expressed as means ± SD (n=3); values differing at $p \leq 0.05$ were mostly considered significant with each other by least significant difference test.

4.1.4 Non-Organic solvent extraction ; Study on the effect of temperatures on the amount of genistein and daidzein extracted from aqueous system.

The effect of temperature on the amount of both extracted isoflavones was studied. The extraction temperature of the aqueous system was from 25-100°C. Figure 17 shows the concentration of genistein and daidzein obtained under different

temperatures. The results indicated that the amount of genistein and daidzein increased significantly with the increased temperature ($p \leq 0.05$). The highest yields of genistein and daidzein were obtained at the temperature range of 61-65°C (439.37 µg/g and 300.70 µg/g for genistein and daidzein, respectively). The isoflvones content increased with the increase in the temperature from 25°C to 65°C. The result was due to the increase in temperature can disrupt the strong solute-matrix interactions caused by van der Waals forces, hydrogen bonding and dipole attractions of the solute molecules and active sites on the matrix [259]. However, further increase in temperature over 65°C genistein and daidzein showed a continually significant decrease (91.23 µg/g and 81.1 µg/g for genistein and daidzein, respectively at the range of 96-100°C, $p < 0.05$). The reduction was a result of the degradation of the substances at very high temperature. The result was correlated with the study of GA Akuwuahet. al., [260] which reported the effect of different extraction temperature on total soluble solids and total phenolic contents of *Gynuraprocumbens* leaf. The extraction above 60^{o} C showed a significantly lower total phenolic content compared with the extractions at 40^{o} C and 50^{o} C. The value of the extract obtained at 60^{o} C was significantly lower than that of the extractions at 40^{o} C and 50^{o} C. The content of total phenolic of all the extracts at the studied temperatures decreased in the order 40°C > 50^{o} C > 60^{o} C > 80^{o} C and 100^{o} C. The strong negative correlation between extraction temperatures and total genistein and daidzein suggested that extraction at elevated temperature is partly responsible for significant degradation of isoflavone contents.

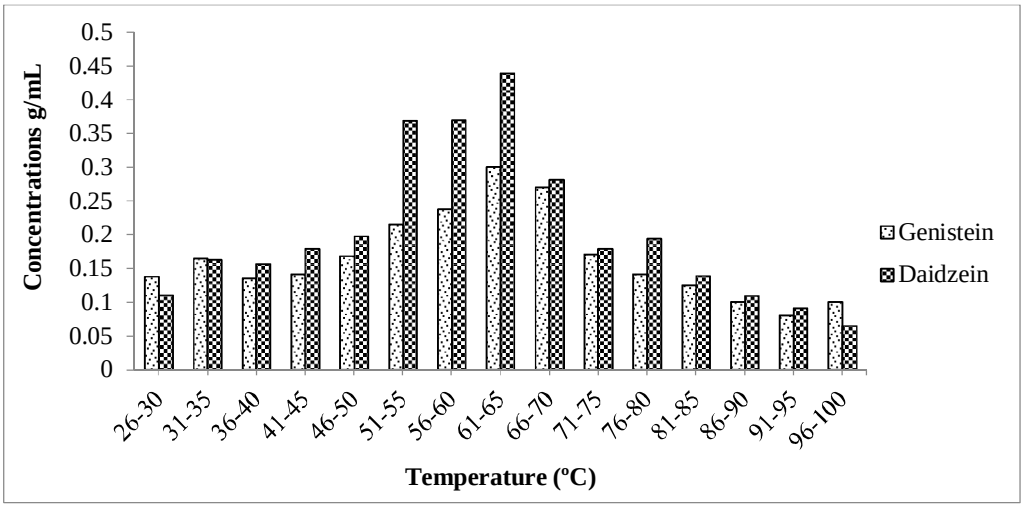


Figure 17 Concentration of genistein and daidzein from soybean extract under different extraction temperatures

4.2 Validation of Analytical Method.

Among all extraction methods ultrasonication method was chosen for validation study because of soybean extracts from ultrasonication method showed the highest value of isoflavones when compared with all organic solvent extraction methods. The accuracy and precision were performed. The accuracy of the method was evaluated by recovery assay by adding the known amounts of each standard isoflavone to a known amount of sample D (extraction by ultrasonication), to obtain three different levels (75, 100, and 125 percent) of addition. Spiked amounts were ranged from 100.41-167.35 µg/g for genistein and 106.27-177.12 µg/g for daidzein. Each sample was analysed quantitatively in triplicate. The mean recovery and %RSD were calculated. The expected values for isoflavones and the recoveries for each level are summarized in Table 9. The average recoveries of spiked isoflavones were ranged from 94.72 to 97.84 percent for genistein and daidzein, respectively. No considerable

differences had been found between recoveries at different spiked levels, which indicated a good accuracy of the method. The obtained results of genistein and daidzein had shown the recoveries between 90-107 percent, which was within the range of AOAC (The Association of Official Agricultural Chemists) guidelines [261].

To assess the precision of the method, six replicates of the sample D were determined on the same day (intraday precision) and one time injection on five consecutive days (inter-day precision). The results showed acceptable precision with the developed HPLC method as revealed by coefficient of variation (CV) data presented in table 9. Intra- and interassay CVs were 1.56 and 1.59 percent for genistein and 1.61and 1.59 percent for daidzein, respectively, with no differences in CVs between compounds at high or low concentrations. The data showed less than ± 2.0% RSD (Relative standard deviation), and hence complyed with the evaluation criterion of AOAC guidelines [261].

The linearity was determined using five concentrations of both standard solutions of genistein and daidzein in the range of 5-30 µg/mL (n=3). The regression equations were found by plotting the peak area (y) versus the isoflavone concentration (x) expressed in µg/mL. The equation showns a good linearity with calibration curves for genistein and daidzein were $Y = 6.0806x10^8 + 354440$ and $Y = 4.8209x10^8 + 200815$, respectively. The correlation coefficient (r^2) for genistein and daidzein was 0.9998 and 0.9999, respectively, as shown in Table 10 and Figure 18. The (r^2) demonstrated the excellent relationship between peak area and concentration of each isoflavone standard. The developed method was successfully applied to quantitatively assay the soy isoflavones from the extraction of different methods. Among all

different extraction methods, non-organic solvent extraction method exhibited a maximum yield of genistein and daidzein.

Table 9 Accuracy and precision of the developed HPLC method

Isoflavone	Concentration (μg/g)	Accuracy				Precision	
		Added (μg/g)	Found (μg/g)	Recovery (%)	CV	Intraassay CV (n=6)	Interassay CV (n=6)
Genistein	133.88	100.41	221.75	94.65	3.23	1.56	1.59
		133.88	265.78	99.26	1.09		
		167.35	271.30	90.06	2.27		
Daidzein	141.69	106.27	247.51	99.82	0.21	1.61	1.59
		141.69	284.11	100.25	6.15		
		177.12	297.90	93.44	0.49		

Table 10 peak areas of standard isoflavones at 5 concentrations, CV and r^2

Isoflavones	Concentration (μg/g)	Peak Area			Average	CV	r^2
		n1	n2	n3			
Genistein	5.00	3396729	3361784	3340710	3366408	0.8405	0.9998
	8.00	5115110	5127858	5133449	5125472	0.1834	
	10.00	6345273	6600104	6619991	6521789	2.3489	
	20.00	12393783	12975419	12485599	12618267	2.4780	
	30.00	18466908	18595508	18522613	18528343	0.3480	
Daidzein	5.00	2611420	2701351	2642957	2651909	1.7206	0.9999
	8.00	4031109	4059774	4120455	4070446	1.1207	
	10.00	4996506	4862336	5126701	4995181	2.6463	
	20.00	9900767	10031130	9363960	9765286	3.6211	
	30.00	14677211	14752713	14710353	14713426	0.2572	

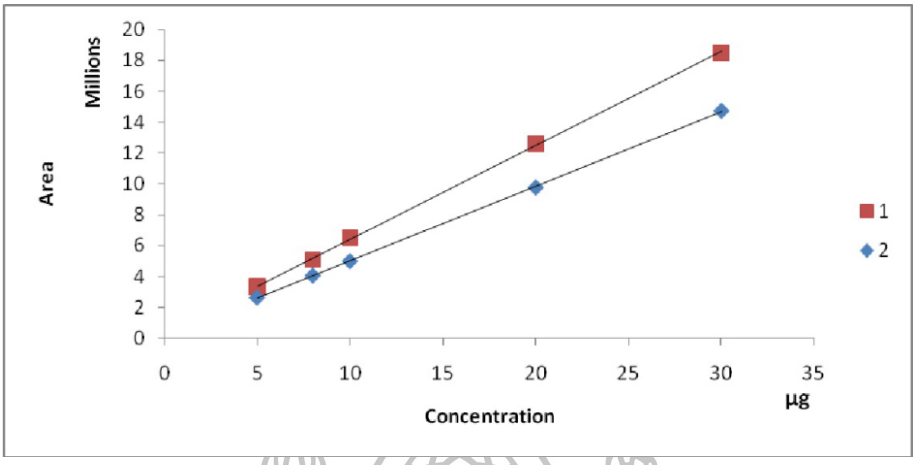


Figure 18 The calibration curves of standard genistein (1) and daidzein (2) determined by HPLC system

4.3. Preparation of soybean extracted powder

4.3.1 Influence of carrier agents on physical properties of soybean extracted powder

Four carrier agents were applied during process of spray drying. They were mannitol (Sample a.), maltrodextrin (Sample b.), and 2 types of modified starch, FA 6008 (Sample c.) and Flomax-8 (Sample d.) The extracted powders were compared with soybean extracted powder without a carrier agent (Sample e.) All samples were characterized for their flowability, particle morphology, particle size distribution and moisture content.

4.3.2 Determination of physicochemical properties

4.3.2.1 Flowability, pH, particles morphology, particles size distribution and moisture content

Flow properties of dried products are directly related to their behavior during storage, handling, and processing [262]. According to USP 36-

NF 31[263], the flowability of powder can indicate by angle of repose. The powder is defined as excellent, good, fair and poor at the angle repose of <30°, 31-35°, 35-40° and >40°, respectively. As shown in Table 11. sample a showed passable flowing property, sample e showed good flowing property and sample b, c and d showed the excellent flowing property. The best angle of repose was obtained from sample b (α=12±2°). The moisture content of soybean extract powder was in the range of 6.21 ± 0.12 and 8.6 ± 1.18% as shown in the table 11. The moisture content of natural dry powder should not over 12% for prevention from bacterial growth on the product.

The result of flowing property of all sample can be proved by particle morphology from SEM. The morphological analyses were carried out for the dried extracts obtained at a temperature of 150°C. Figure 19 presents typical SEM photomicrographs of the dried product magnified at 500 times. The result of flowability was in agreement with the photomicrograph. Samples b-d showed smooth surface with a wide size distribution surfaces and the excellent flow was noted (Figure 19b, 19c and 19d). Although smooth surface was obtained for sample a, the poor flow was reported (Figure 19a). This might be a result of the low moisture content of sample a. rendering the poor flow. The MC was reported to play a role of enhancement of flow property [264]. The optimum content was required to gain a good flow, the too high MC will give an opposite result. The result was in agreement with sample e which indicated the agglomeration. The addition and type of carrier tend to affect the flow property of soybean extract dried powder.

All the samples showed unimodal particle size distributions (Table 11), being the mean volume particle diameter values between 4.2963±0.05 and 33.5001±2.74 μm. Particle size of sample e showed the biggest size due to the

agglomeration of the sample. The effect of pH was not significant when using different carriers. The pH value of all samples showed about 5 to 6.5, its similarity with the pH values of pure carrier [265]. From the result of various diluents, a soybean extract powder with carrier agent was shown a good properties at the initial time. After keeping for 1 week, all samples with carrier agent were melted. Only a soybean extract powder without carrier remained stable form so a soybean extract powder without carrier agent was selected for the stability study in section 4.3.3.

Table 11 pH, angle of repose, Particle size distribution, moisture content (values are mean ± standard error.) of sample with different carrier agents, mannitol (sample a.), maltrodextrin (sample b.), FA 6008 (sample c.) and Flomax-8 (sample d.) soybean extracted powder without a carrier agent (sample e.)

	Sample a.	Sample b.	Sample c.	Sample d.	Sample e.
pH	5.3	6.5	5.5	5	6.2
α (º)	41 ± 1	12 ± 2	19 ± 1	30 ± 1	35 ± 2
Size Distribution (μm)	5.95 ± 0.31	5.50 ± 0.22	4.29 ± 0.05	15.46 ± 2.15	33.50 ± 2.74
Moisture Content (%)	6.2 ± 0.12	7.5 ± 1.39	7.5 ± 0.47	7.8 ± 0.23	8.6 ± 1.18

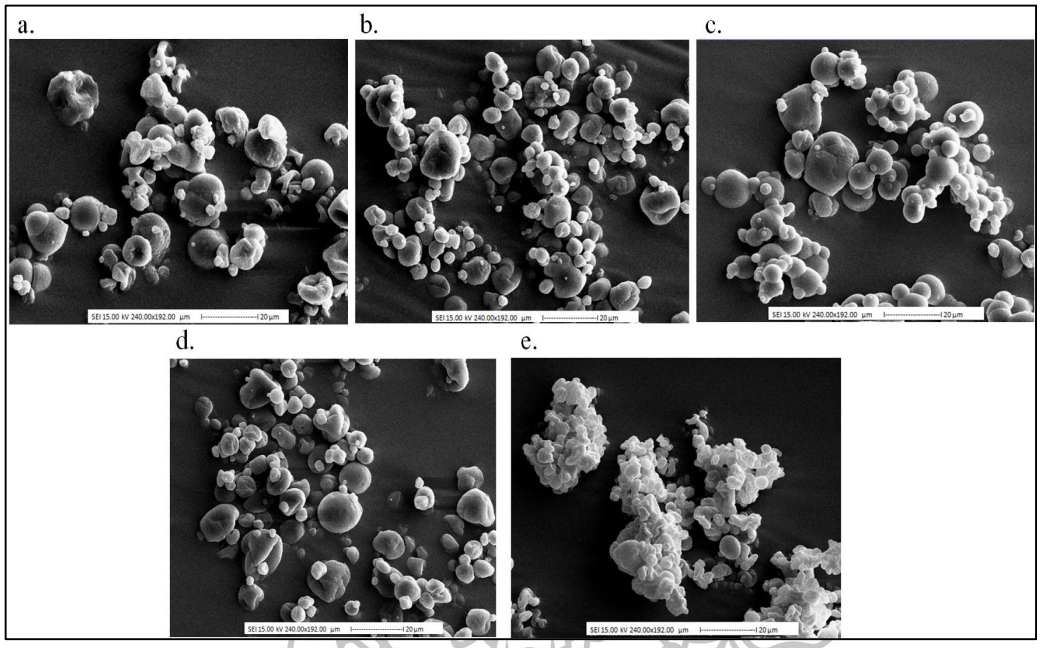


Figure 19 SEM photomicrographs of sample with different carrier aids, mannitol (a.), maltrodextrin (b.), FA 6008 (c.) and Flomax-8 (d.) soybean extracted powder without a carrier agent (e.)

4.3.2.2 Powder X-ray diffraction (PXRD)

XRD measurements were taken for all samples. The samples b, c, d and e. revealed a completely amorphous state, as it can be confirmed by the presence of broad non-defined peaks with abundant noises while sample a crystallinity was seen due to mannitol characteristic (Figure. 20) [8]. However, the other physical properties were in a similarity. This could prove that the carrier aid had an effect on the crystallinity form.

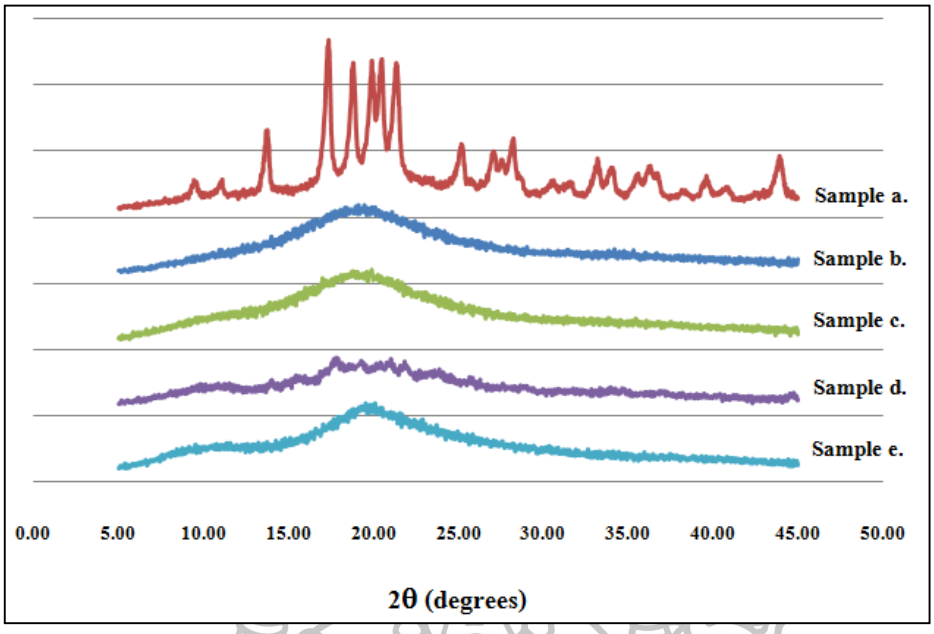


Figure 20 PXRD patterns of sample a, b, c, d and e.

4.3.3 Stability analysis of soybean extracts

Figure 21 shows the effect of temperature on the concentration of isoflavone. Soybean extracts in solution form at 5˚C was compared to -20˚C. The concentrations of genistein at 5˚C was reduced to 80.731% and 13.289%, while daidzein was reduced to 75.854% and 0.524% after storing for 1 week and 2 weeks, respectively. The concentration of both isoflavones was significantly reduced ($p<0.05$). Further storage for 3 weeks, 0% both genistein and daidzein was not seen, (Fig 21a). The improvement in stability was reported at lower temperature. The concentrations of genistein and daidzein at -20˚C was reduced to 89.701%, 66.445%, 23.256% and 19.934% , and to 75.171%, 68.337%, 9.112% and 2.278% after storing for 1 week, 2 weeks, 3 weeks and 4 weeks, respectively.(Figure 21(b)). Similar results were reported by Fischer et al. [266]) on the investigation of thermal stability of

anthocyanin from three pomegranate juices. The protection was due to the frozen temperature resulting in the reduction in easy accessibility of hydrolysis and the high loss is noted.

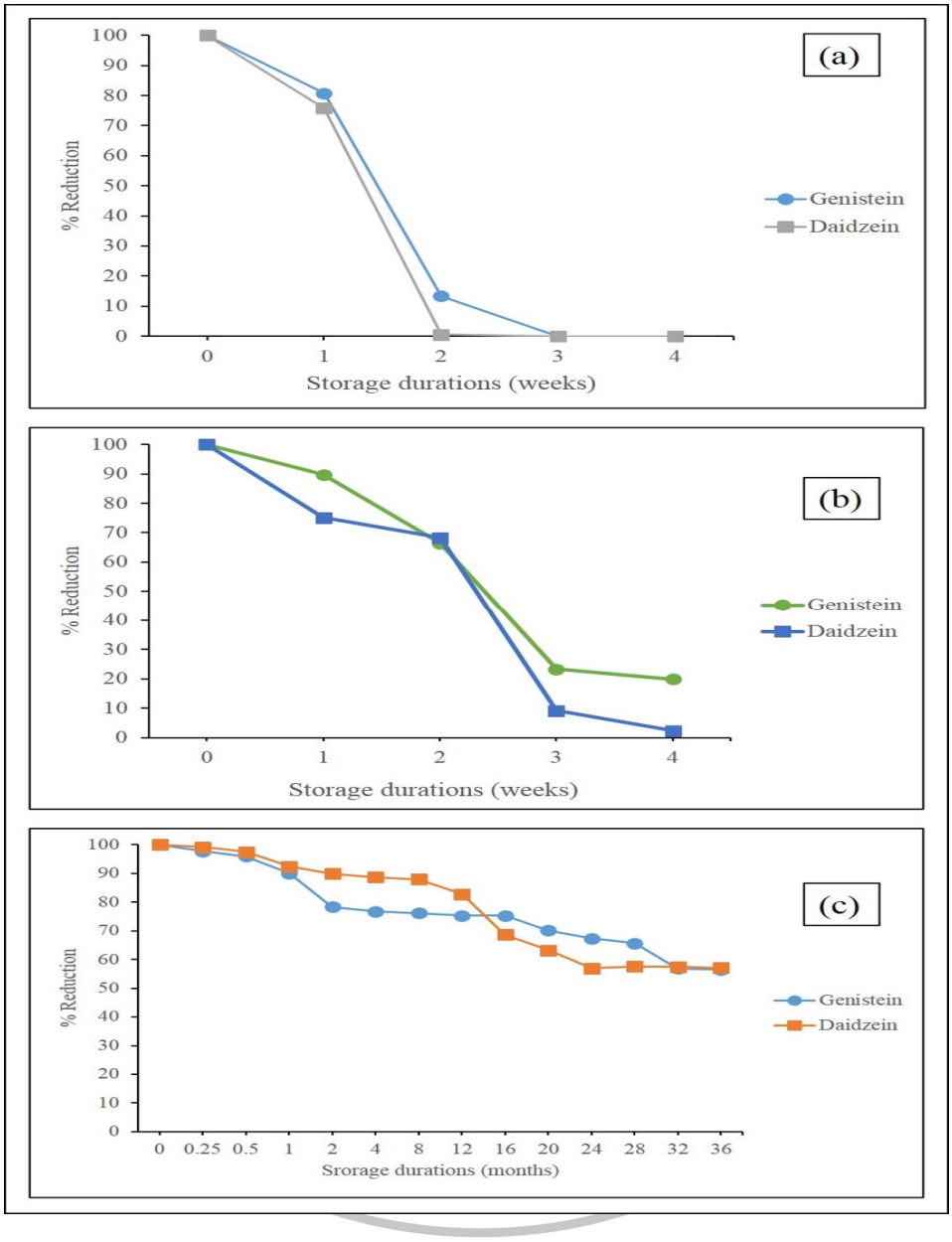


Figure 21 (a); Concentrations of genistein and daidzein solutions, kept in the refrigerator at 5°C for0, 1, 2, 3 and 4 weeks. (b); Concentrations of genistein and daidzein solutions, kept in the freezer -20°C at 0, 1, 2, 3 and 4 weeks. (c); Concentrations of genistein and daidzein extracted powder, kept in the desiccators at room temperature for 0, 0.25, 0.5, 1, 2, 4, 8, 12, 16, 20, 24, 28, 32 and 36 months

Although the lower temperature tended to decrease the degradation process, the high extent of loss was still reported after a longer storage. The loss of isoflavones are widely known due to oxidation and degradation process [9], inhibiting them from the use as functional raw materials for food and pharmaceutical purposes [9]. However, the stability of soybean extract was improved by drying process in this study. The concentrations of genistein and daidzein from the soybean extract powder reduced to 56.534% and 57.047% when they were kept for 36 months. It is notable that after the drying process under the deployment of a spray dry technology, the concentrations of the isoflavone contents decreased insignificantly ($p>0.05$). This indicates that the drying process did not cause any deterioration of isoflavones. The moisture content plays a crucial effect on the stability of the extracts, the dry extract is more stable than the solution form. It can be concluded that the isoflavones (daidzein and genistein) were degraded when exposed to the high moisture content and high temperature. The stability of isoflavone proved to be temperature and physical form dependence. The concentration of the soybean extracted powder can retain the high concentration of genistein and daidzein for the storage of 3 years (Figure 21(c)). Hence, for further formulation, soybean extract should be kept in a dry powder form.

4.3.4 Determination of antioxidation activities

4.3.4.1 The scavenging effects of soybean extract on DPPH

The DPPH radical had been used widely in the model system to investigate the scavenging activities of several natural compounds such as phenolic compounds, anthocyanins, or crude extracts of plants. DPPH radical was scavenged by antioxidants through the donation of hydrogen, forming the reduced

DPPH-H•. The extracts were allowed to react with a stable free radical, 2,2-diphenyl-1-picryl hydrazyl radical (DPPH•). The free radical chain reaction is widely accepted as a common mechanism of lipid peroxidation. Radical scavengers may directly react with quench peroxide radicals to terminate the peroxidation chain reaction improving the quality and stability of food products. DPPH• is a stable free radical and can accept an electron or hydrogen radical to become a stable diamagnetic molecule [15-16]. The reduction in capability of DPPH• induced by antioxidants is determined by the decrease in its absorbance at 517 nm. The ability of samples to scavenge DPPH• radical was determined on the basis of the concentration of 50% inhibition (IC50). Table 12 shows the radical-scavenging activity of soybean extracts. The scavenging effect of soybean extract on the concentration of 50%inhibition (IC50) was compared with IC50 of trolox. A higher DPPH• radical-scavenging activity is associated with a lower IC50 value. The results showed that the DPPH• radical scavenging activity of soybean extracts was found to increase in a dose dependent manner (Figure 22). The main phenolic substances, daidzein and genistein, in the soybean extracts at the used concentrations displayed a noticeable effect on DPPH• radical scavenging activity. The amount of daidzein and genistein in the soybean extract, which providing potential effect of DPPH• activity at 50% of free radicals inhibition, were quantified by the HPLC assay with the values of 0.41 and 0.39 mg, respectively (Figure 22a,b). Daidzein and genistein isoflavones in the soybean extracts showed the similar scavenging activity to trolox, The IC50 of trolox was showed at 0.28 mg (Figure 22c). Antioxidant activities of daidzein and genistein were expressed in the terms of TEAC values as shown in Table 12. TEAC is a measurement of the effective antioxidant activity of the extract, -the higher TEAC would imply the greater antioxidant activity

of the sample. Soybean extracts, significant differences in scavenging activity against the DPPH radical were recorded ($p \leq 0.05$). The antioxidant activity of the plant extracts is correlated with their phenolic content [267]. The DPPH assay indicated that both isoflavones had a relatively high TEAC. Genistein had the higher antioxidant activity than daidzein as shown in Figure 22.

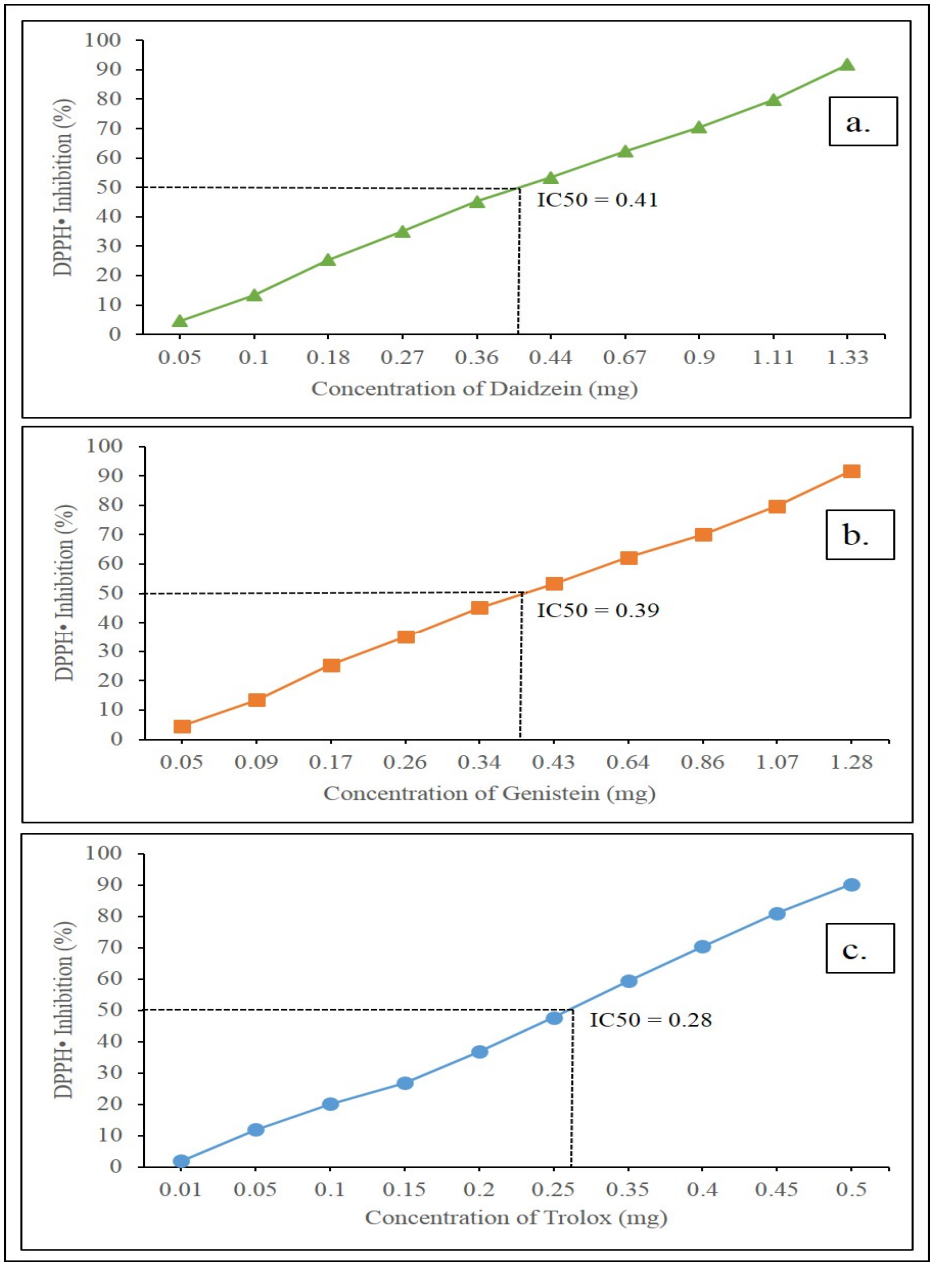


Figure 22 Free radical scavenging activity of daidzein and genistein, compare with trolox, by DPPH method. (Results are means ±SD of three replicates).

a. Free radical scavenging activity of daidzein by DPPH method.

b Free radical scavenging activity of genistein by DPPH method.

c Free radical scavenging activity of trolox by DPPH method.

Table 12 Antioxidant activities of the soybean extracts and its major components (daidzein and genistein) in DPPH and ABTS radical scavenging activities.

Sample	DPPH assay		ABTS assay	
	IC_{50} (50mg)	TEAC	IC_{50} (50mg)	TEAC
Daidzein	0.41	682.90	0.55	1600.00
Genistein	0.39	717.95	0.53	1660.30
Trolox	0.28	-	0.88	-

TEAC = Trolox equivalent antioxidant capacity

IC50 = Concentration causing 50% inhibition

4.3.4.2 The scavenging effects of soybean extract on ABTS

The ABTS assay was employed to measure the antioxidant activity of the soybean extract because it does not require sophisticated analytical equipment and provides a good estimation of the antioxidant activity of pure compounds and complex matrices [268]. The soybean extract displayed antioxidant activities as it was able to scavenge the ABTS•+ radical cation. The soybean extract had a scavenging activity on ABTS•+ radicals in a dose dependent manner (0.03-1 g). Nonetheless, when compared to trolox, the ABTS radical scavenging effect of the extract was found to be low. As shown in Figure 23, the scavenging power of all the soybean extracts increased with increasing amount of sample. The concentrations of 50% inhibition of daidzein and genistein in the soybean extract quantified by the HPLC assay were 0.55 and 0.53 mg, respectively. The scavenging effect of the extract and isoflavones were determined in the terms of TEAC values as shown in Table12. It

is interesting to find that daidzein and genistein had relatively high TEAC and exhibited antioxidant potential greater than trolox.

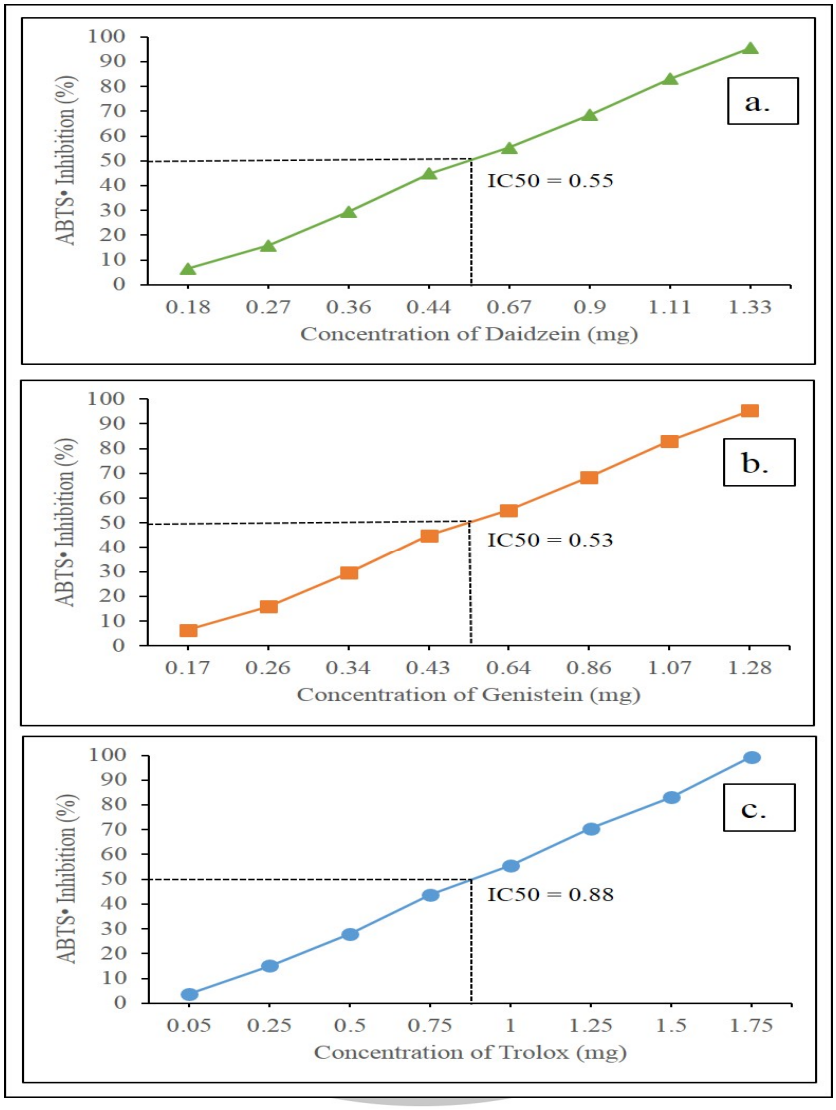


Figure 23 Free radical scavenging activity of genistein and daidzein, compare to trolox, by ABTS method. (Results are means ± SD of three replicates).

a. Free radical scavenging activity of daidzein by ABTS method.

b. Free radical scavenging activity of genistein by ABTS method.

c. Free radical scavenging activity of trolox by ABTS method.

4.3.4.3 FRAP assay

FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method, employing and easily reduced oxidant system present in stoichiometric excess. The ferric reducing antioxidant power (FRAP) method is based on the reduction in a ferroin analog, the Fe^{3+} complex of tripyridyltriazine [$Fe(TPTZ)^{3+}$], to the intensely blue coloured Fe^{2+} complex [$Fe(TPTZ)^{2+}$] by antioxidants in acidic medium. Results are obtained with an increase in absorbance at 593 nm and it can be expressed as micromolar of Fe^{2+} equivalents or relative to an antioxidant standard.

The result of activity present by Fe^{3+}/g extract of sample. In one gram of soybean extract, the amounts of isoflavones, daidzein and genistein, in soybean extract, were 1.28±1.24 and 1.33±1.67 mg per g extract, respectively and 53.53±0.43 µM Fe^{3+} equivalent per g extract of ferric was detected.

4.3.4.4 Amount of total phenolic constituents

The Folin-Ciocalteu reagent is a mixture of phosphomolybdate and phosphotungstate used for the colorimetric assay of phenolic and polyphenolic antioxidant. It works by measuring the amount of the substance being tested needed to inhibit the oxidation of the reagent [269]. It is considered that the phenolic compounds contributed to overall antioxidant activities of soybean extract. Total phenolic contents of soybean extract was assessed using the Folin-Ciocalteu reagent, which is a mixture of phosphomolybdate and phosphotungstate. In one gram of soybean extract, the amounts of isoflavones, daidzein and genistein, in soybean extract, were 1.28±1.24 and 1.33±1.67 mg per g extract, respectively and 4.79±0.43 mg gallic acid equivalent per g extract of phenols was detected. The results

indicated that both isoflavones account for about half part of total phenolics in soybean extracts.

From the studies above, it could be concluded that soybean, an abundant natural resources, providing a high antioxidant substances will be of value for use in pharmacy and phytotherapy. The extraction method had an effect on the obtained phenolic compound. The classical aqueous method at a controlled heating temperature exhibited the highest concentration of phenolic compounds at the temperature of 61-65ºC. Further studies on in vivo or in vitro studies should be performed in order to prepare a natural pharmaceutical products of high value.

4.4 Pharmaceutical dosage form containing soybean extract.

Liquid dosage forms are the popular dosage form since they offer several advantages such as good absorption, and no requirement of shaking before use, designing for any route of administration [270]. However, the formulation presents some technical problem, including solubilization of active ingredients. Generally, oils are composed of non-polar compounds which have the limited water solubility so they are difficult to incorporate into aqueous dosage forms, Therefore the attempt to formulate soybean extracts in to microemulsion dosage forms was made to tackle the problem of oil solubility. Several formulation parameters were investigated to find the suitable components of soybean extract preparations.

4.4.1 Determination of surfactant miscibility with different oils.

Surfactant miscibility is an important parameter for a microemulsion dosage forms, Tween 20 and Tween 80 were mixed with different oils. Binary phase diagram was used for the preparation. The surfactant at 50% w/v was

fixed in the system and a volume of oil and deionized water were varied. The clear region or the miscible area, in which the oil could be solubilized, was selected for the microemulsion formulations.

The choosing criteria of different oils was considered by the miscibility of the system. The system shows a one phase totally or a widely clear area was selected for the next study.

The systems used were peppermint oil, lavender oil, cinnamon oil and fish oil shown a totally clear region for both Tween 20 and Tween 80 as a surfactant were shown in Figures 24-27. Although the system containing cinnamon and fish oil displayed a clear region, they were not selected for the next formulation because of their strong scent. The system containing jojoba oil showed totally immiscible for both Tween 20 and Tween 80 so it was rejected for the next study (Figure 28). The system which exhibited two phase for one surfactant and partially clear region for the second surfactant would also be discarded. They were light mineral oil, apricot oil, castor oil, almond oil, sun flower oil and rice brand oil as shown in Figures 29-34. The totally or partially clear region for both surfactants will be considered for further formulation. They were system containing olive oil, eucalyptus oil, coconut oil and lemon oil as shown in Figures 35-38.

The results suggested that binary phase diagram can be applied for screening oils for microemulsion preparation. The commonly used surfactants were Tween 20 and Tween 80 could be used. The system containing Tween 80 gave a wider clear region than Tween 20. The microemulsions could be prepared by incorporation of Tween 20 and Tween 80 as a surfactant with the following oils:

peppermint oil, lavender oil, coconut oil and lemon oil as an oil in the pseudo ternary phase diagram.

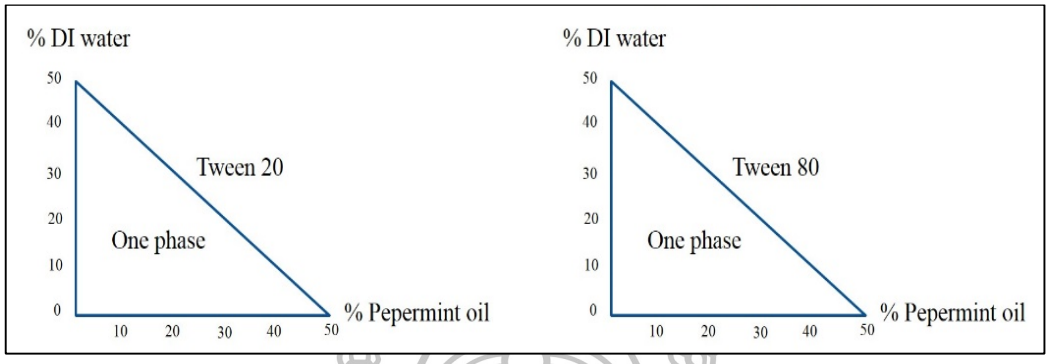


Figure 24 Miscibility of peppermint oil with 50% Tween 20 and Tween 80

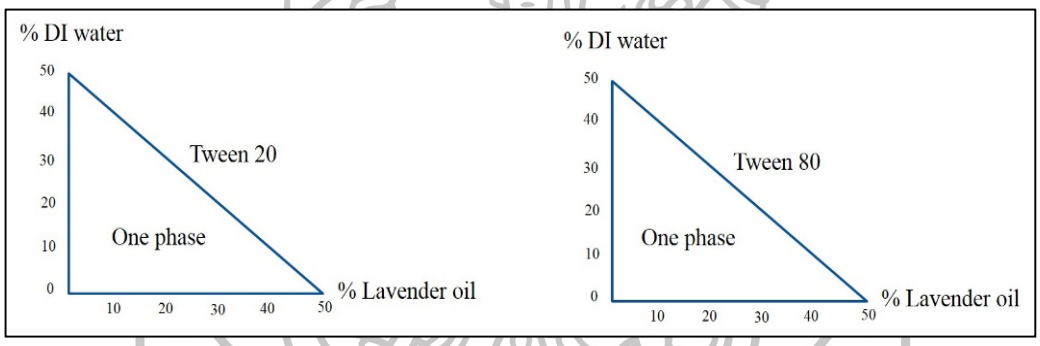


Figure 25 Miscibility of lavender oil with 50% Tween 20 and Tween 80

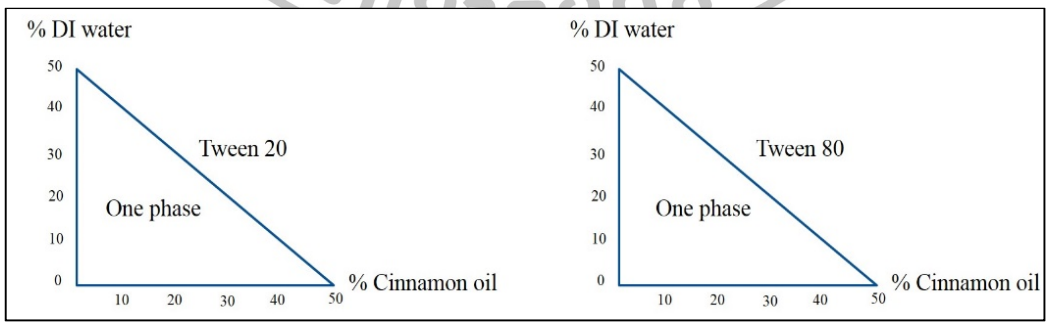


Figure 26 Miscibility of cinnamon oil with 50% Tween 20 and Tween 80

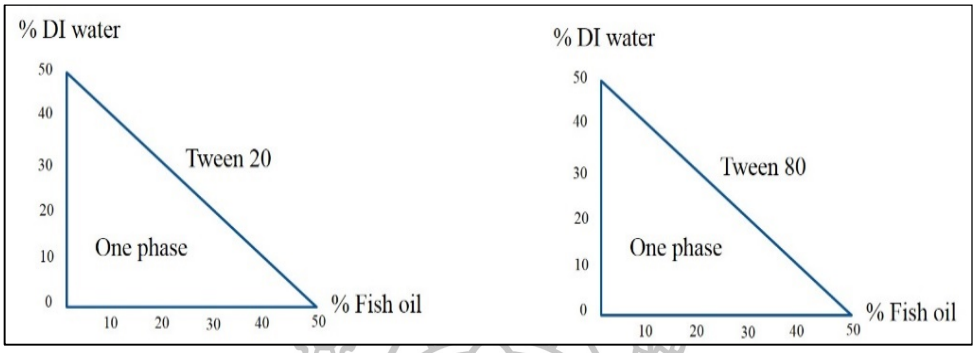


Figure 27 Miscibility of fish oil with 50% Tween 20 and Tween 80

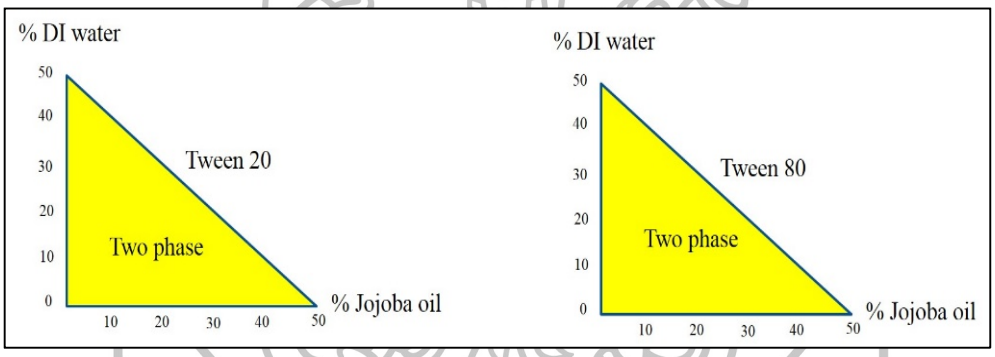


Figure 28 Miscibility of jojoba oil with 50% Tween 20 and Tween 80

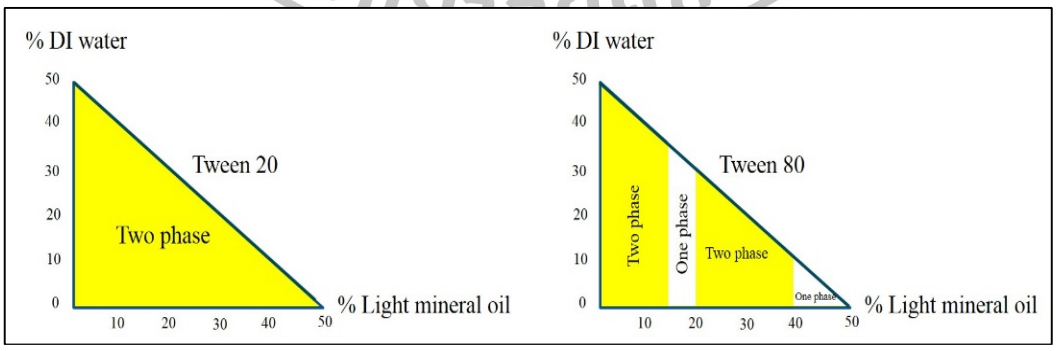


Figure 29 Miscibility of light mineral oil with 50% Tween 20 and Tween 80

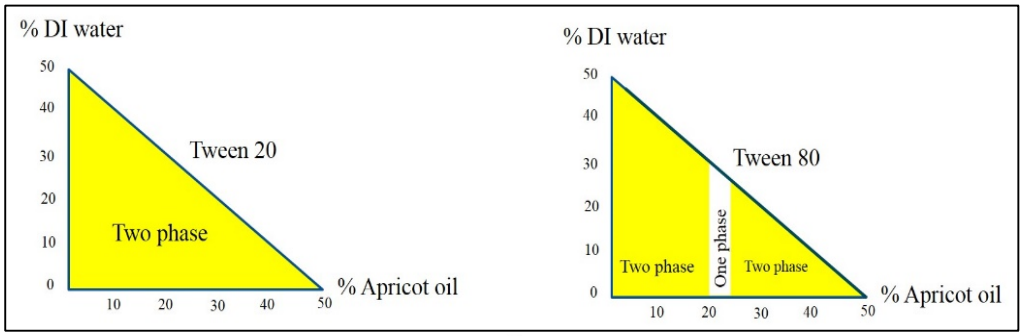


Figure 30 Miscibility of apricot oil with 50% Tween 20 and Tween 80

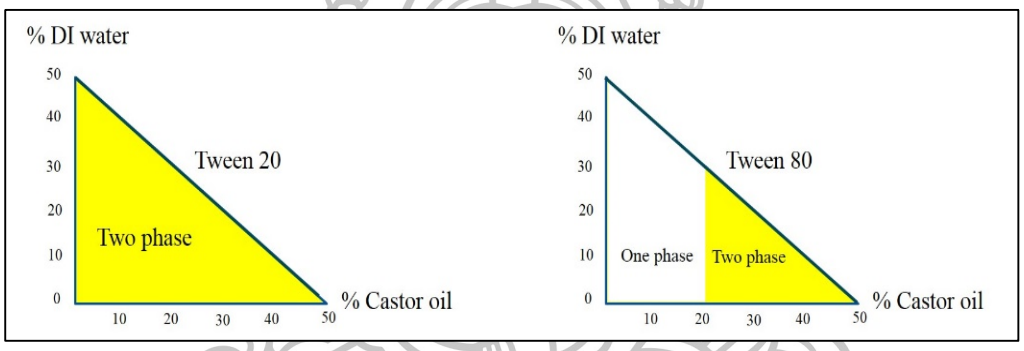


Figure 31 Miscibility of castor oil with 50% Tween 20 and Tween 80

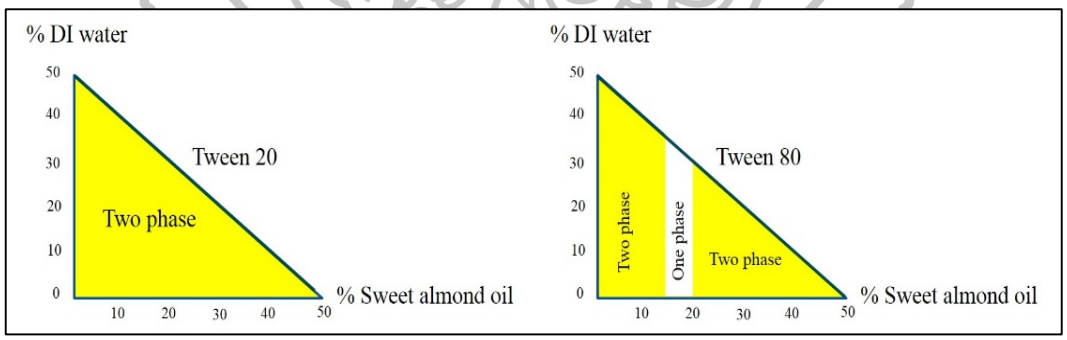


Figure 32 Miscibility of sweet almond oil with 50% Tween 20 and Tween 80

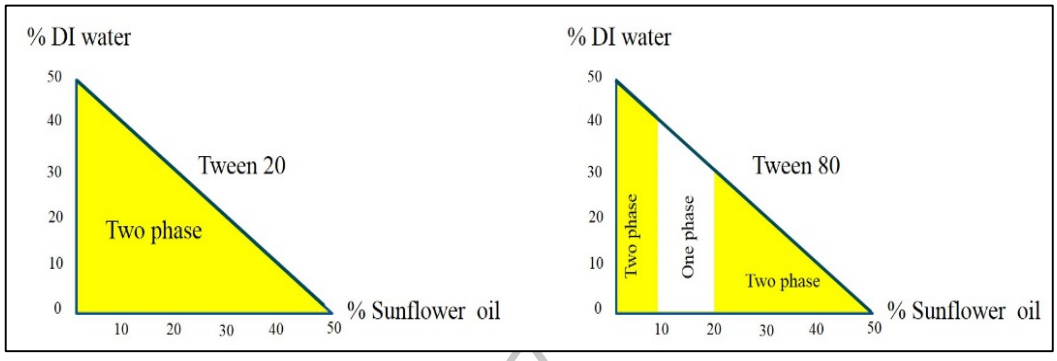


Figure 33 Miscibility of sunflower oil with 50% Tween 20 and Tween 80

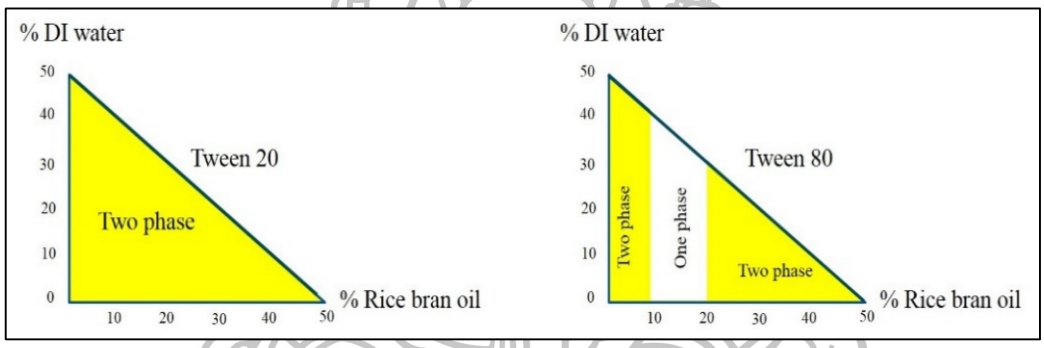


Figure 34 Miscibility of rice brand oil with 50% Tween 20 and Tween 80

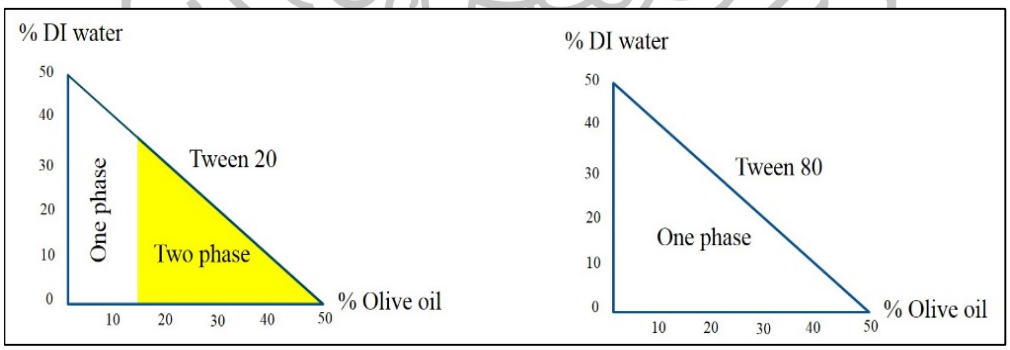


Figure 35 Miscibility of olive oil with 50% Tween 20 and Tween 80

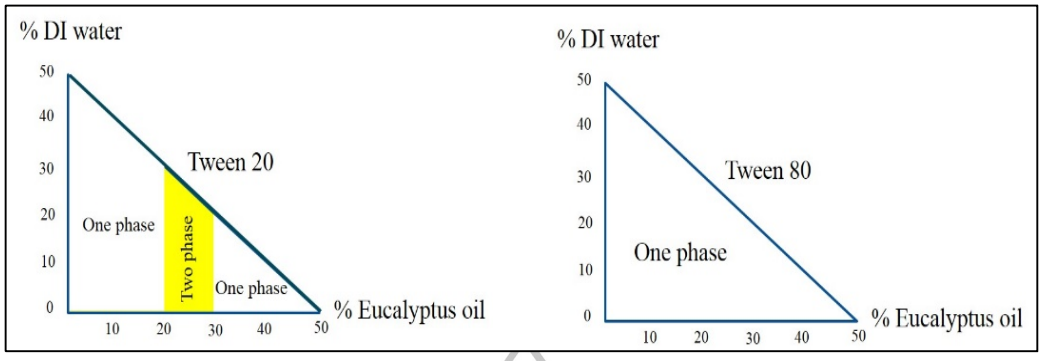


Figure 36 Miscibility of eucalyptus oil with 50% Tween 20 and Tween 80

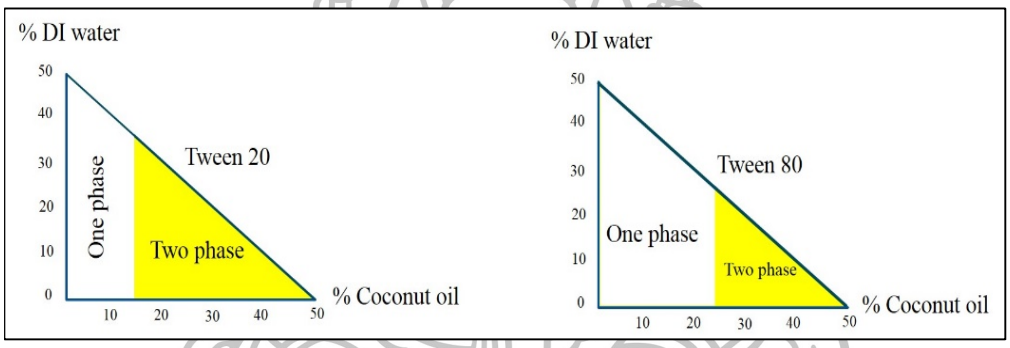


Figure 37 Miscibility of coconut oil with 50% Tween 20 and Tween 80

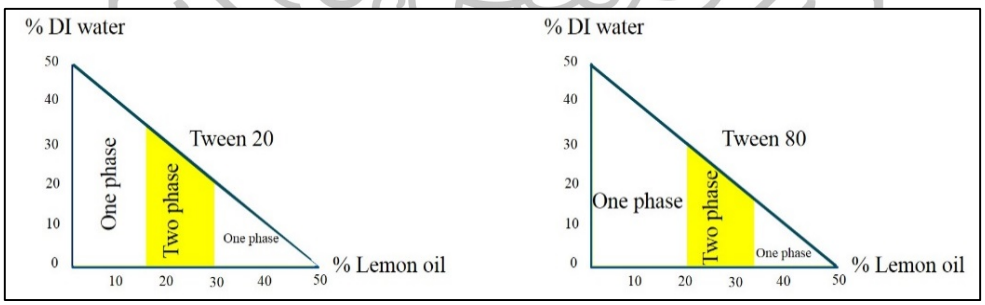


Figure 38 Miscibility of lemon oil with 50% Tween 20 and Tween 80

4.4.2 Soybean solubility determination

Solubility is a main measurement to determine the degree of dissolved solute in a solvent at a given temperature. For this study, the different dielectric constant solvents were used for the study at room temperature. To determine the polarity, the solubility of soybean extracted powder with various solvents was investigated (Figure. 39). The result indicated that a soybean extracts was well soluble with low polarity solvent having the dielectric constant ranging from 18 (isopropanol) to 32.7 (methanol). However, soybean extracts was slightly soluble with high polarity solvent possessing the dielectric constant over 42.5 (glycerol) and 80.3 (deionized water). The result suggested that the isopropanol, acetone, ethanol, propylene glycol and methanol showed the high solubility with soybean extracts. However, isopropanol and acetone are high vapor pressure and they are easily vaporized so they are not a good characteristic for the microemulsions system [271]. When compare a propylene glycol with ethanol, many report shows the ethanol can be successfully used as potential vehicles in developing transdermal therapeutic system and can be used for enhancing solubilization in microemulsions system [238,244]. It was also noteworthy that alcohols were suitable as the co-solvents for soybean extracts. Therefore ethanol was selected for further study.

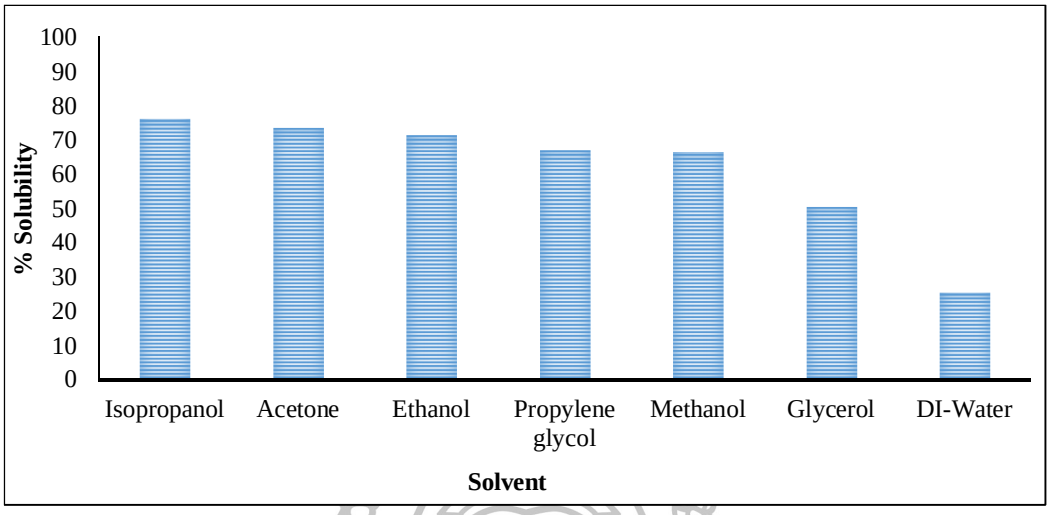


Figure 39 Soybean solubility with different solvents

4.4.3 Physical appearance of microemulsions evaluated by construction of pseudo-ternary phase diagram

The formation of microemulsion systems is indicated by the shaded area in the phase diagrams while the turbid liquid with multiphase systems is depicted by the area outside the shaded area. Pseudo-ternary phase diagrams are commonly used to study the behavior of the liquid mixtures after changing the fraction of the components, i.e. oil, water and surfactants/co-surfactant in the system [272,273].

From the binary phase diagram study, olive oil, coconut oil, lemon oil and eucalyptus oil were selected to mix with Tween 80 by using pseudo-ternary phase diagram. Additionally, the upper shade area in which the sample contained relatively low percentage of Tween 80 and high percentage of oil were observed. The results suggested that the coconut oil liquid preparations could be prepared by incorporation of Tween 80 (Figure 41). The similar result was observed in the system containing Tween 20 and Tween 80 at the ratio of 1:1 (Figure 40-42). The transparent

region was observed at the area containing high percentage of oil. In addition, the lower amount of surfactant was used to achieve the transparent liquid mixture. To achieve the transparent wider region with a lower amount of surfactant used, the combination of 2 surfactant was used. For example, the transparent liquid mixture containing 10% of mixture of Tween 20 and Tween 80 can be prepared at the level of 40% aqueous solution of lemon oil (Figure 43), lavender oil (Figure 44) and peppermint oil (Figure 45) while it cannot be prepared at the same amount in the system of Tween 80 with olive oil (Figure 40), coconut oil (Figure 41) and eucalyptus oil (Figure 42).

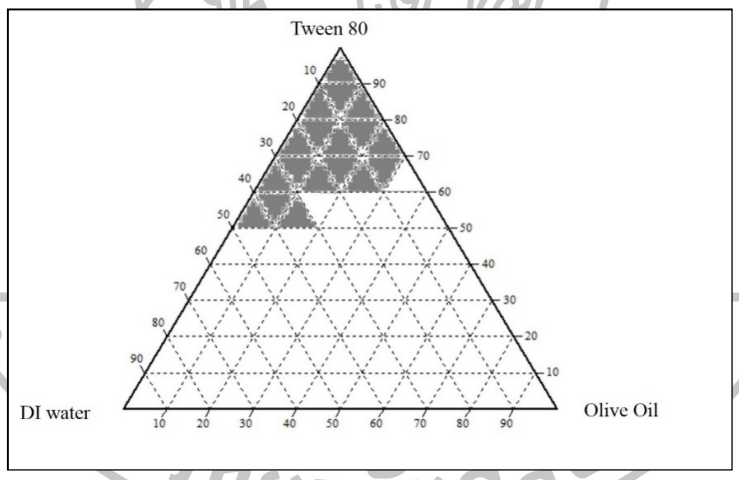


Figure 40 Ternary phase diagram of system containing olive oil and Tween 80

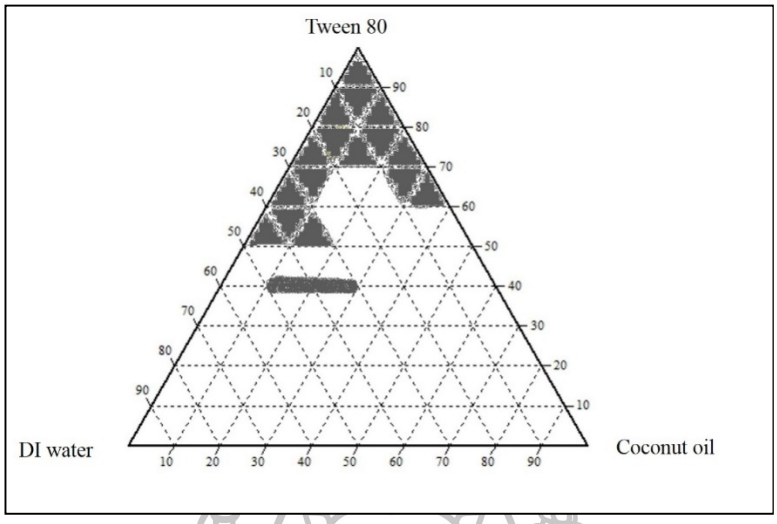


Figure 41 Ternary phase diagram of system containing coconut oil and Tween 80

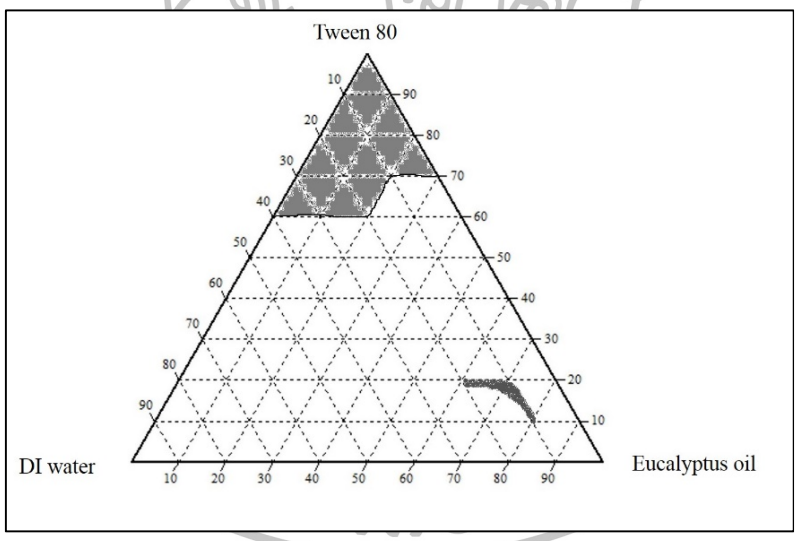


Figure 42 Ternary phase diagram of system containing eucalyptus oil and Tween 80

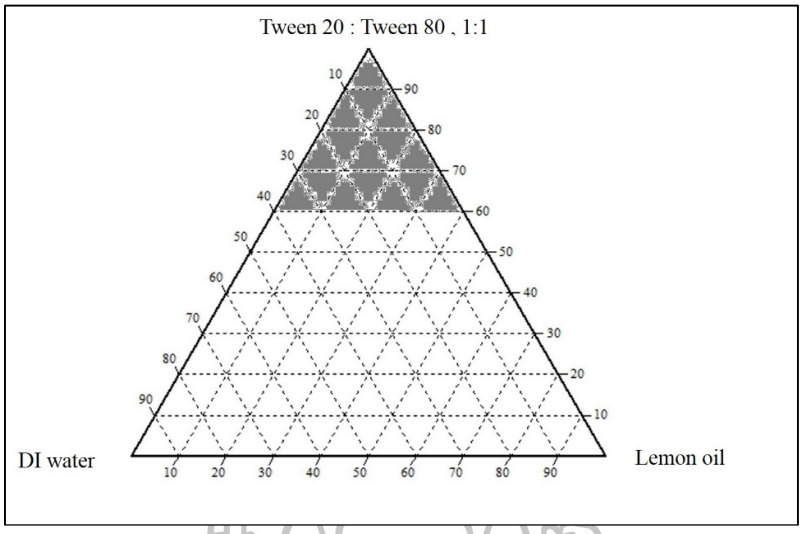


Figure 43 Pseudo ternary phase diagram of system containing lemon with Tween 20 and Tween 80 at ratio 1:1

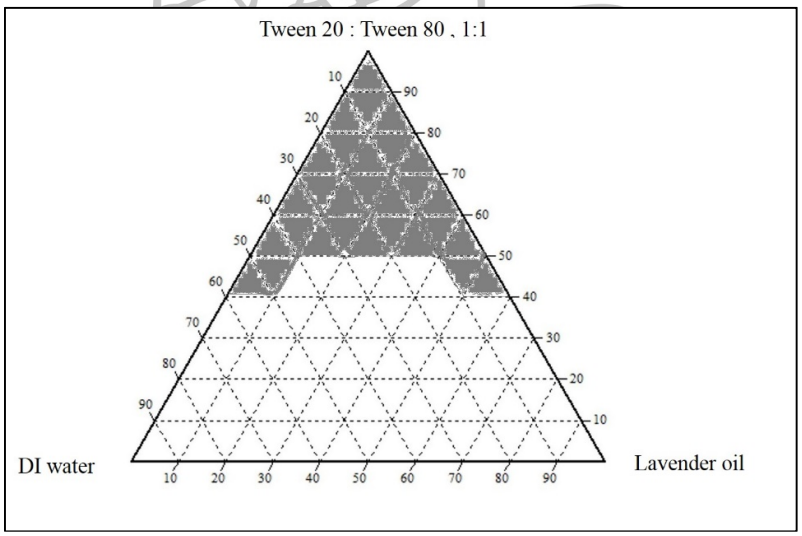


Figure 44 Pseudo ternary phase diagram of system containing lavender oil with Tween 20 and Tween 80 at ratio 1:1

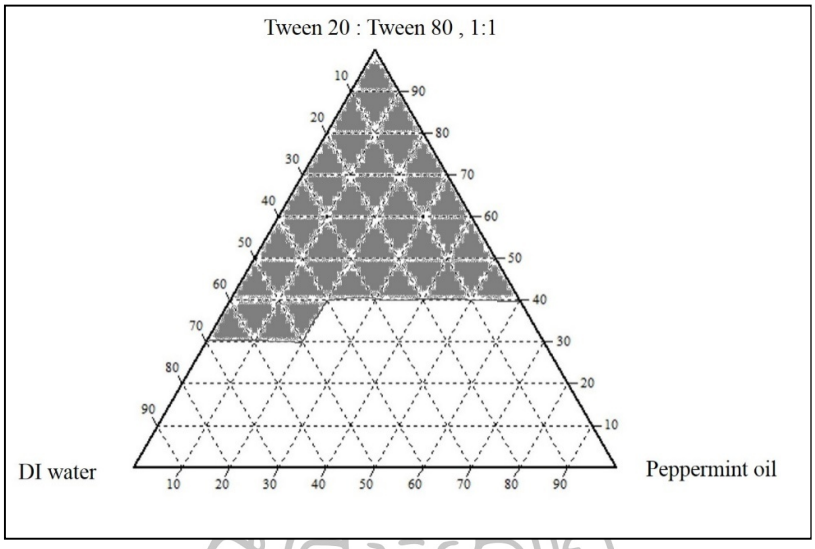


Figure 45 Pseudo ternary phase diagram of system containing peppermint oil with Tween 20 and Tween 80 at ratio 1:1

From the above results, the binary phase diagram system is proven to be an acceptable system to use for a simple screening of oil. Diagrams above showed that a binary surfactant was appropriate to use than single surfactant due to a lager transparent area. Microemulsions are basically thermodynamically stable, isotropically clear dispersions of two immiscible liquids such as oil and water stabilized by the interfacial film of any surfactant and easier to be stabilized by using co-surfactant [273]. Ethanol was selected for a co-surfactant. The suitable ratio of surfactant and co-surfactant was studied by pseudo ternary phase diagram. Lavender oil, lemon oil and peppermint oil shows the wider clear region more than olive oil coconut oil and eucalyptus oil. Lavender oil, lemon oil and peppermint oil were chosen from above study for the investigation of pseudo ternary phase diagram. The

weight ratio of surfactant/co surfactant (Smix) was varied at the ratio of 2:1, 1:1 and 1:2.

The constructed pseudo ternary phase diagrams are presented in Figures 46 – 48. The pseudo ternary phase diagrams showed that the zone of microemulsion (upper zone) was largest in the systems prepared with surfactant - co/surfactant mixture at 1:2 ratio [Figure 46F), 47F), 48F)]. The systems prepared with surfactant – co/surfactant mixtures (Smix) of 1:2, 1:1 and 2:1 ratios remained as microemulsions (Figures 46 – 48). Although formulations containing Smix of 1:1 and 2:1 had a slightly smaller zone at areas of higher oil content compared to those with 1:2 Smix, however at areas of higher surfactant concentration and lower oil content, the former had a larger microemulsion zone.

Different microemulsions can be prepared by selecting appropriate oil, surfactant, co-surfactant and water concentrations within the microemulsion area. However this present study involved the use of pre-concentrates consisting of oil and surfactants and the pseudo ternary phase diagram was only used to select the appropriate oil, surfactant and co-surfactant mixtures.

This results from one of the most fundamental properties of microemulsions, that is, an ultra-low interfacial tension between the oil and water phases, $\gamma o/w$. The main role of the surfactant is to reduce $\gamma o/w$ sufficiently − i.e., lowering the energy required to increase the surface area − so that spontaneous dispersion of water or oil droplets occurs and the system is thermodynamically stable.

Pseudo ternary phase diagram is a good system to find suitable component for microemulsions. The commonly used oils and surfactants which were available in laboratory were investigated in this study. By applying co-surfactant,

ethanol, to reduce the use of surfactant, the wider area of microemulsion could be obtained.

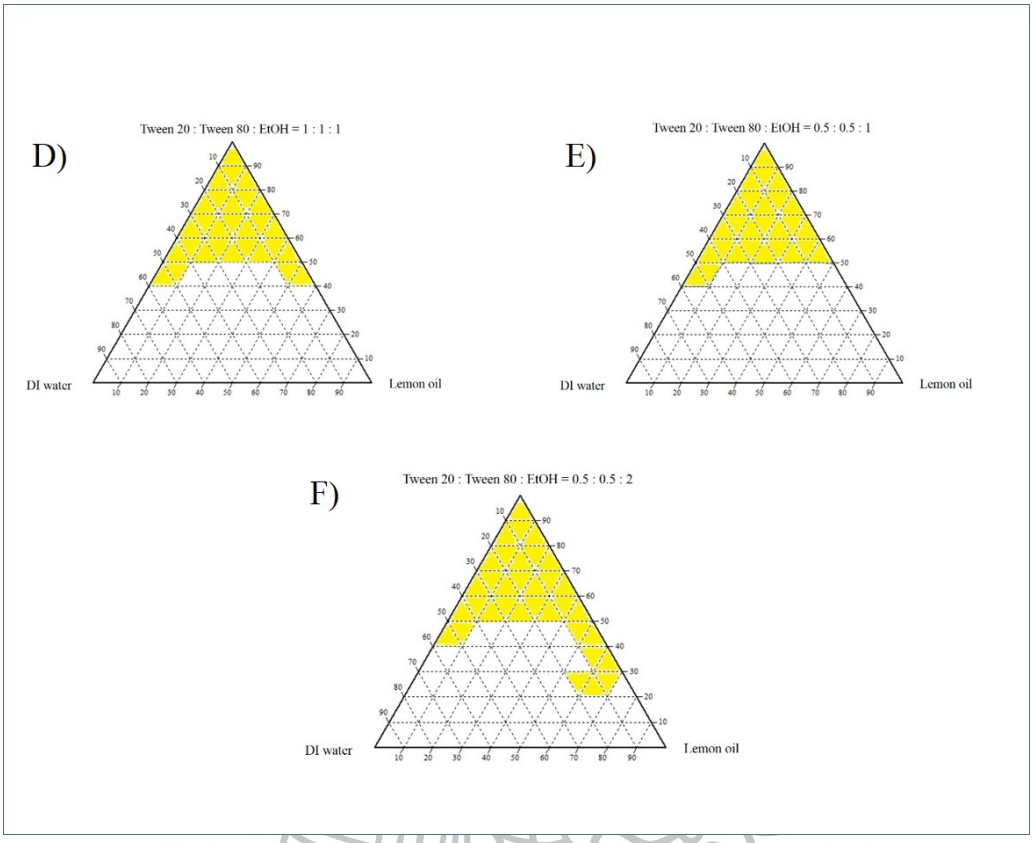


Figure 46 Pseudo ternary phase diagram of system containing lemon oil with different ratio of Smix.

D) Pseudo ternary phase diagram of system containing lemon oil with ratio of Smix at 2:1

E) Pseudo ternary phase diagram of system containing lemon oil with ratio of Smix at 1:1

F) Pseudo ternary phase diagram of system containing lemon oil with ratio of Smix at 1:2

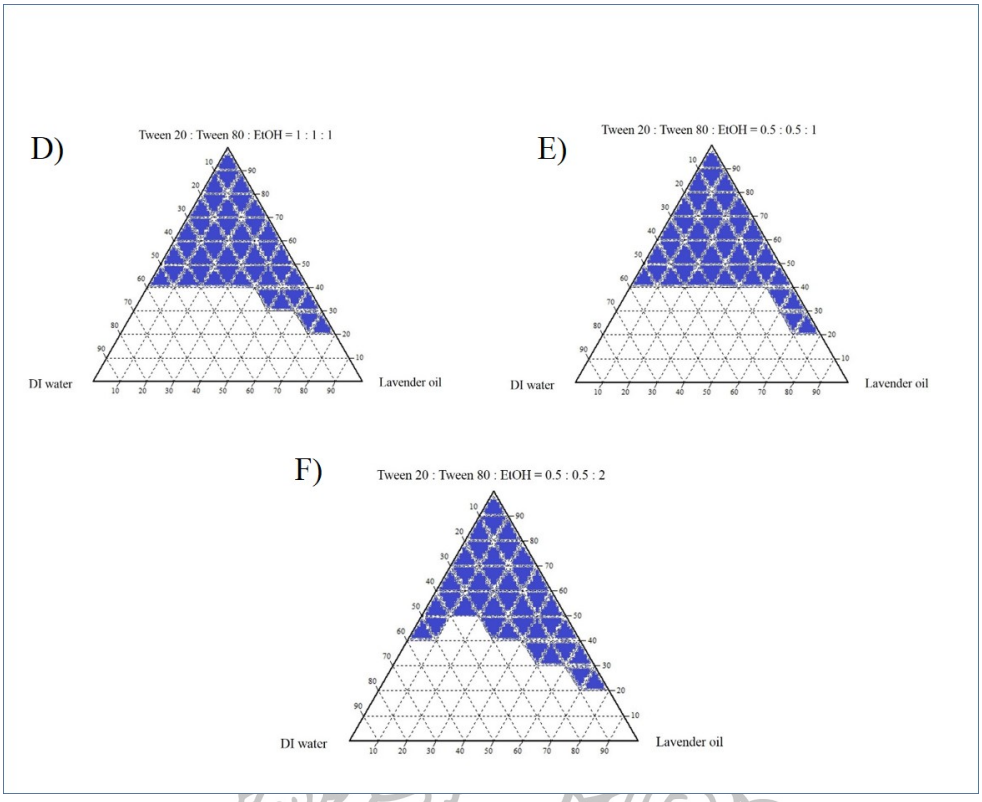


Figure 47 Pseudo ternary phase diagram of system containing lavender oil with different ratio of Smix.

D) Pseudo ternary phase diagram of system containing lavender oil with ratio of Smix at 2:1

E) Pseudo ternary phase diagram of system containing lavender oil with ratio of Smix at 1:1

F) Pseudo ternary phase diagram of system containing lavender oil with ratio of Smix at 1:2

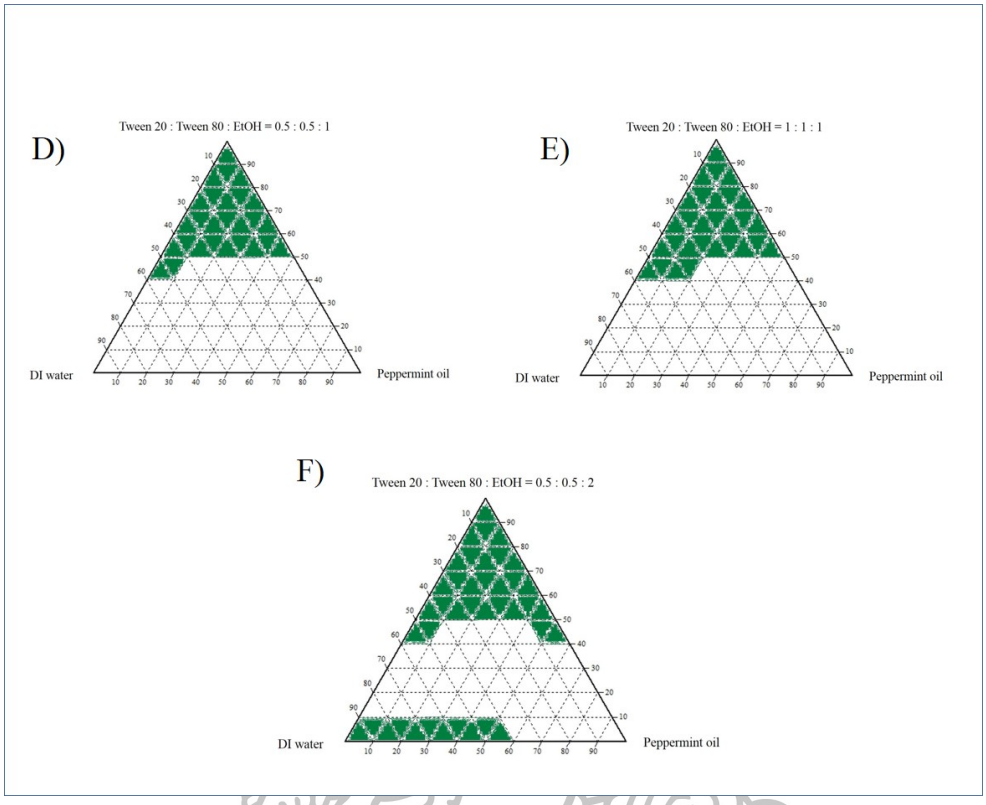


Figure 48 Pseudo ternary phase diagram of system containing lavender oil with different ratio of Smix.

D) Pseudo ternary phase diagram of system containing lavender oil with ratio of Smix at 2:1

E) Pseudo ternary phase diagram of system containing lavender oil with ratio of Smix at 1:1

F) Pseudo ternary phase diagram of system containing lavender oil with ratio of Smix at 1:2

4.4.4 Preparation of dosage form containing soybean extract Formula

As described in sections 4.4.2 and 4.4.3, ethanol was suitable co/surfactant for the formulation. Soybean extract was dissolved in ethanol as a co-surfactant, then mixed with surfactant, oil and water following a ratio from pseudo-ternary phase diagram. Lemon oil, peppermint oil and lavender oil were given more range of transparent region in pseudo ternary phase diagram. The selected formulae (as shown in Table 13) which obtained from the transparent region in the pseudo ternary phase diagram were prepared and comparatively evaluated for their physical properties and permeation properties. Three same points in pseudo ternary phase diagram of three types of oil were selected to compare their physical properties and permeation studies. All the selected formulae composite with high volume of ethanol (25%-40% by weight), the co-solvent that loaded soybean extract were chosen for further study. In the permeation study, the high amount of genistein and daidzein isoflavones was considered for permeation through the skin so the microemulsion system with high amount of soybean extract was selected. BHT (butylated hydroxytoluene) and EDTA (ethylene diamine tetraacetic acid) were used to prevent oxidation in the formulations. Furthermore, the stability after storage for 3 months of each formula was also investigated.

Table 13 Formula of the selected microemulsions

	F06le	F10le	F15le	F06la	F10la	F15la	F06pe	F10pe	F15pe
Oil	30	40	50	30	40	50	30	40	50
Tween 20	10	8.335	6.67	10	8.335	6.67	10	8.335	6.67
Tween 80	10	8.335	6.67	10	8.335	6.67	10	8.335	6.67
Absolute ethanol	40	33.34	26.66	40	33.34	26.66	40	33.34	26.66
Soybean extract	0.189	0.189	0.189	0.189	0.189	0.189	0.189	0.189	0.189
BHT	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
EDTA	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Water qs to	100	100	100	100	100	100	100	100	100

4.4.5 Evaluation of microemulsions

4.4.5.1 Visual observation

The phases were identified by visual inspection. A microemulsion is optically clear and transparent. A clear single phase as shown in the Figure 49 and a clear two-phase as shown in the Figure 50.

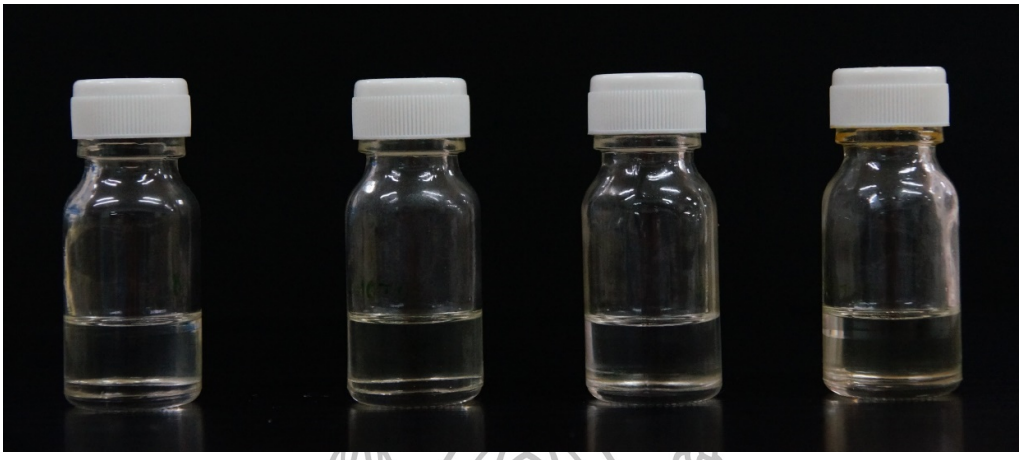


Figure 49 The example of apparently clear single phase formulations

Figure 50 The example of apparently two-phase formulations

4.4.5.2 Droplet size

Droplet size and size distribution can affect the flocculation or coalescence of microemulsion. Normally, the smaller droplet size, the more stable the microemulsions is formed, even though there is a limited size which cannot provide the acceptable curvature of interface [274]. Droplet size under 150 nm confirms the formation of transparent microemulsions [273]. In this study, the droplet

size as well as distribution was used as a tool for comparative characterization of microemulsions prepared from different compositions.

Table 14 and Figure 51 show the mean droplet sizes of microemulsions (F06le, F10le, F15le, F06la, F10la, F5la, F06pe, F10pe and F15pe). Increase in oil concentration caused a considerable change in the mean droplet size. The mean droplet size of microemulsions containing 30%, 40% and 50% lemon oil were about15.24 ± 3.69, 65.02 ± 0.66 and 103.37 ± 1.22 nm, respectively. The mean droplet size of microemulsions containing 30%, 40% and 50% lavender oil were about 14.12 ± 0.87, 47.58 ± 3.97 and 141.50 ± 1.67 nm, respectively. The mean droplet size of microemulsions containing 30%, 40% and 50% peppermint oil were about18.55 ± 2.23, 66.15 ± 3.41 and 115.47 ± 1.05nm, respectively. The result indicated the formation of transparent microemulsions since the droplet of internal phase was observed and the average droplet size was less than 150 nm [273]. In addition, the droplet size had tendency to increase with lowering the amount of Smix. The mean droplet size of microemulsions containing 60% and 40% w/v Smix (F15la and F06la) decreased from 141.50 ± 1.67 to 14.12 ± 0.87 nm. They were in agreement with previous work which has shown that the mean droplet size rapidly decreases with the increase in surfactant concentration [275].

Polydispersity index (PDI) is one of the basic tools for indicating the size distribution and the tendency of phase separation after storage. The PDI is range from 0 (monodisperse) to 1 (completely polydisperse). Practically, it should be less than 0.5 since the polydisperse system has a greater tendency to aggregate as compared to monodisperse system [276-278]. In this study, all

microemulsions showed the PDI less than 0.5, suggesting the good physical stability (Table 14).

Table 14 Particle size, poly dispersity index and zeta potential of selected microemulsions

Formula	Particle size (nm)	Polydispersity index	Zeta potential (mV)
F06le	15.24 ± 3.69	0.3334	-31.1 ± 0.07
F10le	65.02 ± 0.66	0.2776	-37.1 ± 0.14
F15le	103.37 ± 1.22	0.2261	-28.4 ± 0.11
F06la	14.12 ± 0.87	0.2563	-15.5 ± 0.05
F10la	47.58 ± 3.97	0.3293	-29.2 ± 0.04
F15la	141.50 ± 1.67	0.4456	-40.1 ± 0.08
F06pe	18.55 ± 2.23	0.4693	-11.4 ± 0.21
F10pe	66.15 ± 3.41	0.489	-18.3 ± 0.16
F15pe	115.47 ± 1.05	0.2878	-37.6 ± 0.23

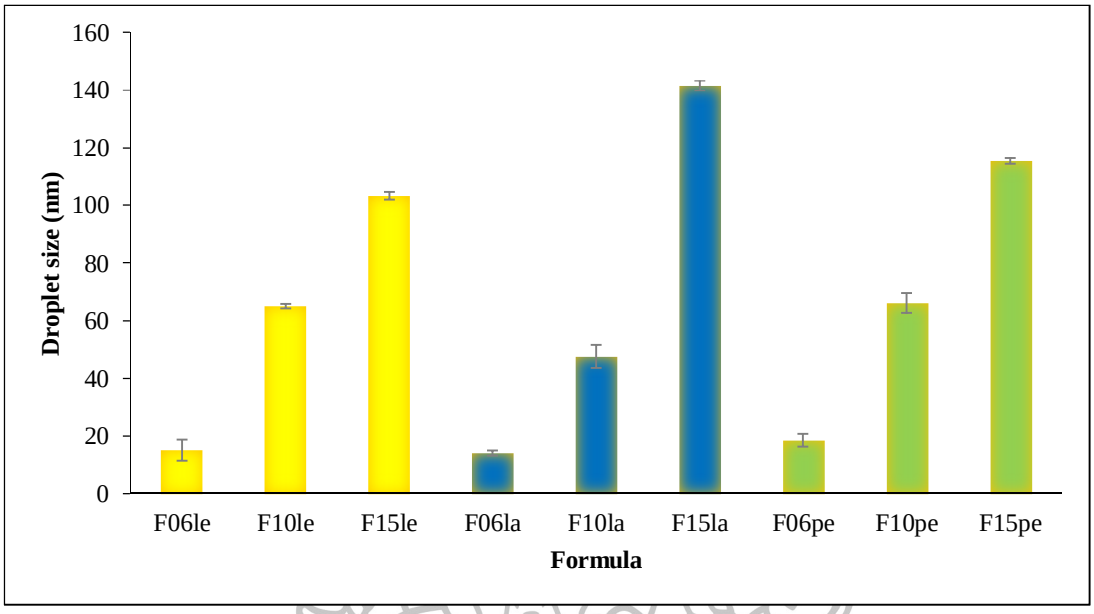


Figure 51 Droplet size of selected microemulsions

Zeta potential represents the net charge on the surface of particles or oil droplets which is one of the key parameter indicating the stability of the colloidal system [279,280]. In order to study the influence of composition on the physical stability of microemulsions, the zeta potential values of different oils of microemulsions were also comparatively evaluated (Table 14). The result showed that the zeta potential values of all microemulsions were almost same value. Additionally, the values were almost zero regardless of amount of microemulsions. However, the very small negative values from -11.4 to -37.1 mV were observed in all microemulsions. The result suggested that oil droplets of microemulsions might almost completely covered with the non-ionic surfactant which possessed no surface charge.

The magnitude of zeta potential indicates the degree of electrostatic repulsion of charged particles or droplets. When the value is small, the attractive forces may overcome this repulsion and therefore the particles or droplets may flocculate or coalescence. So, colloids with high zeta potential (negative or positive) are electrically stabilized and preferred for the stable system [281]. In contrast with this study, the microemulsions showed the good physical stability even the zeta potential was very low. In this case, it might be possible that steric effect of surfactant on the surface of oil droplets demonstrated more pronounced effect on stabilization than effect of surface charge. The finding was also supported by other studies [282,283].

4.4.5.3 pH

The pH value of microemulsions has to adjust to be pharmaceutically acceptable. Generally, slightly acidic formulation is preferred to skin since the pH of human skin is in ranges from 4 to 6 [284]. As shown in Figure 52, all microemulsions slightly decreased from 5.65 to 5.08 as the amounts of oil increased from 30% to 50% w/v.

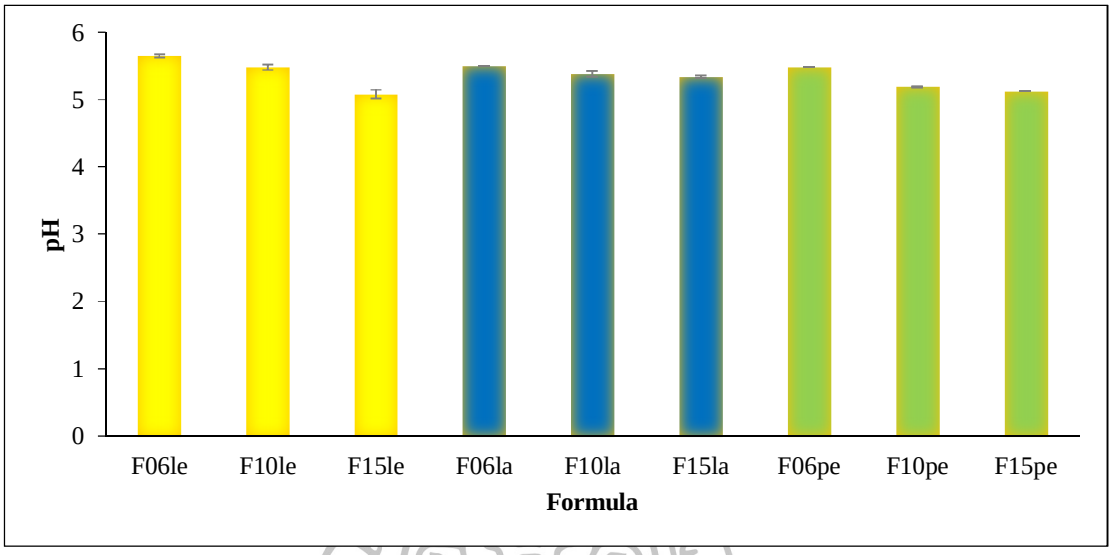


Figure 52 pH of selected micromulsions

4.4.5.4 Viscosity

Figure 51 demonstrated the effect Smix on the viscosity of microemulsions. The viscosity tended to increase as oil content increased. The result agreed well with that previously reported by Kistis [285]. The viscosity of emulsions formed from polysorbate 20, polysorbate 80, oil and water was dependent on concentration of oil volume fraction, and mass ratio of surfactant : co/surfactant [Figure 53]. Dynamic viscosity depends mainly on the microemulsion type, i.e., the microstructure and shape of aggregates, and interactions between dispersed droplets. Therefore, it can be of use to understand the structural transformation or the stage of transition [286, 287].

The viscosity continues to decrease as the nano droplets decrease in number and size, and become more and more diluted in aqueous phase. The progressive dilution decreases the interdroplet interactions.

The very low viscosity of all preparations indicated that the w/o structure might be formed since the bicontinuous structure possessed high viscosity. As a consequence of their low viscosity which was not so different as compared to solutions, such systems might be considered suitable as substitutes for common solutions.

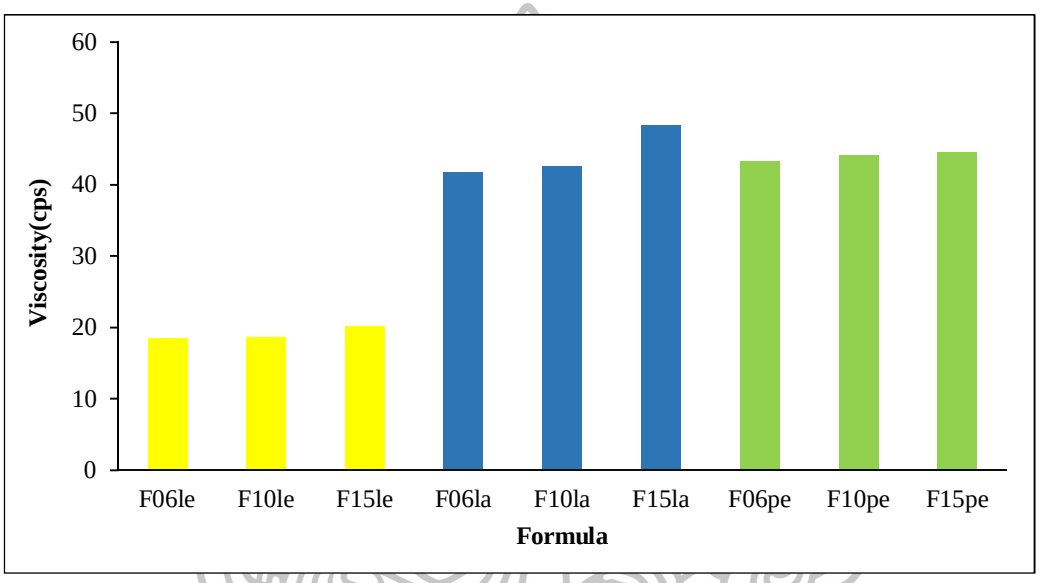


Figure 53 Viscosity of selected micromulsions

4.4.5.5 Reflactive index

The physicochemical data for selected microemulsions are given in Table 15. pH ranged from 5.08 to 5.65, while the refractive indices values of samples gradually decreased as the concentration of water increased and the oil phase decreased as shown in Figure 54. At the same time, the refractive indices for the samples (which are w/o microemulsion type) were higher than those of o/w and bicontinuous microemulsions and this is caused by the higher refractive index as external pseudophase, compared to that of water as external pseudophase.

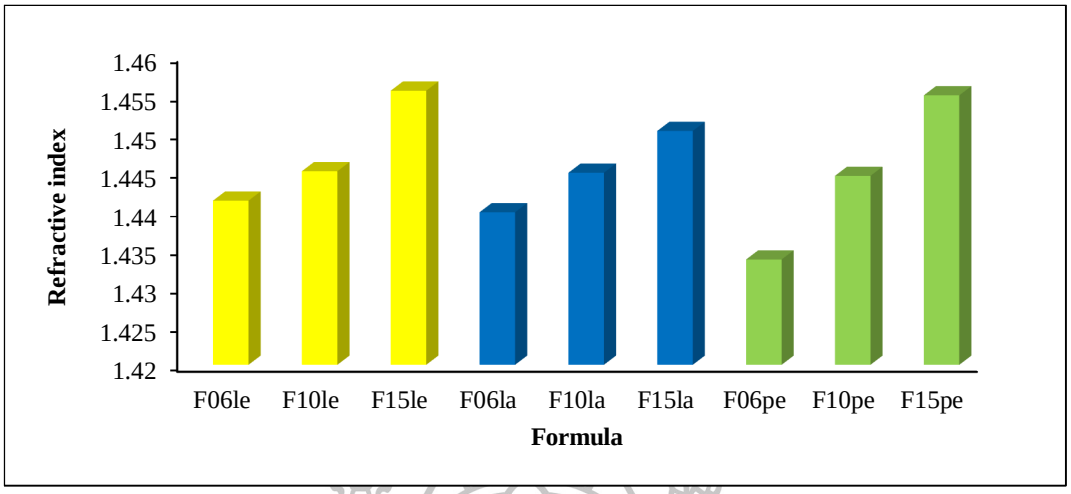


Figure 54 Refractive index of selected micromulsions

4.4.5.6 Surface tension

In microemulsions, the co-surfactant can reduce the surface tension and fluidize the interfacial surfactant film as well as providing the proper hydrophile-lipophile balance in the interfacial layer [288]. F06 had a lower surface tension than F10, F15 due to the increase in alcohol. Short chain alcohol, are the most widely used co-surfactants, and the solubilization of water and phase behavior of microemulsions substantially depends on chain of co-surfactant. For this study, The microemulsion containing ethanol had a higher phase-forming capacity than that non containing ethanol because the spontaneity of the microemulsification increased with increasing co-surfactant. The surface tension values of samples gradually decreased as the concentration of ethanol increased but the change was not significant ($p > 0.05$) as shown in Table 15.

Table 15 pH, Refractive index, Surface tension and Viscosity of selected microemulsions.

Formula	pH	Refractive index	Surface tension (dyne/cm)	Viscosity(cps)
F06le	5.65 ± 0.08	1.4412 ± 0.002	27.5 ±0.002	18.51 ± 2.34
F10le	5.48 ± 0.12	1.4450 ± 0.008	28.4 ± 0.002	18.57 ± 1.45
F15le	5.08 ± 0.76	1.4554 ± 0.004	31.3 ± 0.008	20.14 ± 4.56
F06la	5.5 ± 0.14	1.4397 ± 0.019	28.9 ± 0.007	41.72 ± 2.78
F10la	5.38 ± 0.12	1.4448 ± 0.01	30.0 ±0.012	42.71 ± 3.44
F15la	5.34 ± 0.08	1.4502 ± 0.002	31.4 ± 0.005	48.3 ± 1.23
F06pe	5.48 ± 0.65	1.4336 ± 0.012	27.8 ± 0.016	43.3 ± 2.66
F10pe	5.19 ± 0.11	1.4444 ± 0.01	28.3 ± 0.004	44.23 ± 4.74
F15pe	5.12 ± 0.1	1.4548 ± 0.07	28.9 ± 0.011	44.53 ± 3.34

4.4.5.7 Microscopic evaluation

Polarized light microscopy can distinguish between isotropic and anisotropic materials. Microemulsion is an isotropic system, and birefringence is not found in it. A black background under polarized light is a key distinctive property of microemulsion. The samples exhibited no birefringence, and a black background under polarized light indicated that microemulsions were formed for all selected formulae. Figure 55 shows our microemulsion picture under polarized light microscopy.

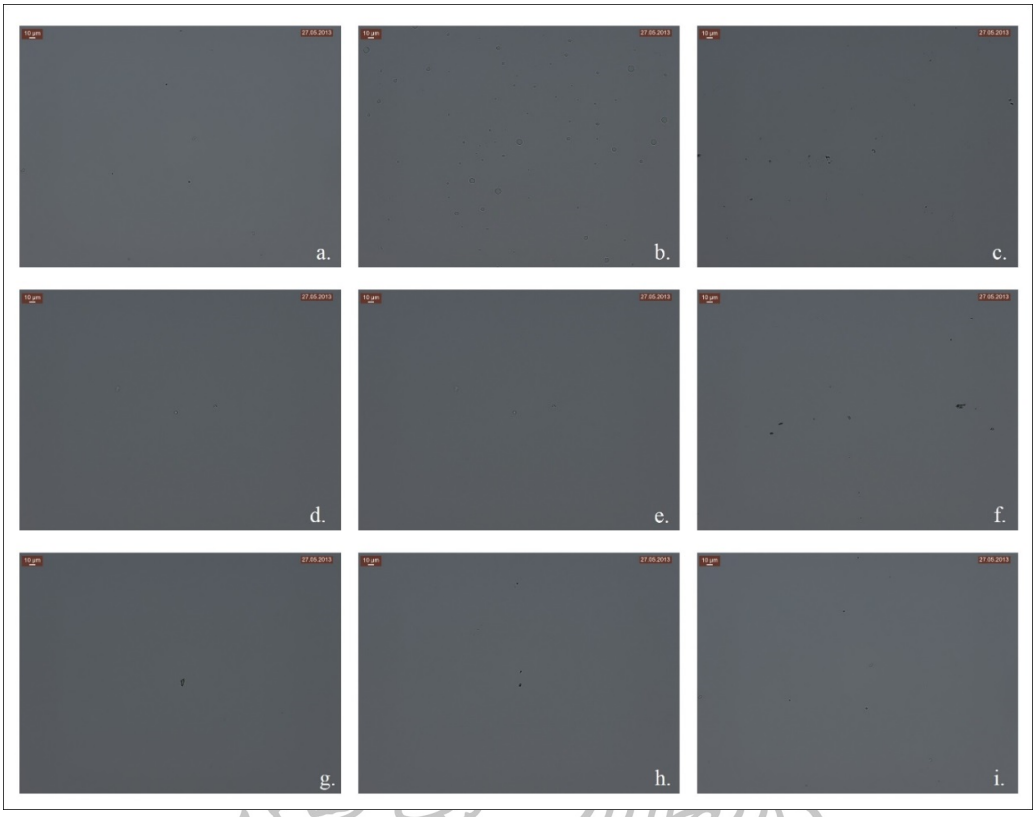


Figure 55 Black background of microemulsion under polarized light microscopy.

a. Black background of F06le microemulsion under polarized light microscopy. b. Black background of F10le microemulsion under polarized light microscopy. c. Black background of F15le microemulsion under polarized light microscopy. d. Black background of F06la microemulsion under polarized light microscopy. e. Black background of F10la microemulsion under polarized light microscopy. f. Black background of F15la microemulsion under polarized light microscopy. g. Black background of F06pe microemulsion under polarized light microscopy.

h. Black background of F10pe microemulsion under polarized light microscopy. i. Black background of F15pe microemulsion under polarized light microscopy.

4.4.6 Stability test of microemulsions

Microemulsions are the thermodynamically stable isotropic system which can easily prepared by simple mixing of the components. The microemulsion is proved to be stable upon storage compared with conventional emulsion. The size does not change and incorporation of drug does not cause the change in stability. In our study, to confirm the formation of stable microemulsions, all formulation were stored at accelerated conditions (40°C, 75% RH) for 3 months. The samples were periodically withdrawn and comparative evaluated for their appearance, droplet size, zeta potential, pH value, viscosity and reflective index. After storage, the physical appearance of all microemulsions did not clearly change. The clear isotropic liquid mixtures were still observed even storage up to 3 months.

The droplet size of each microemulsions was monitored during storage for 3 months as indicated in Figures 56, 57 and 58. The result indicated that the mean droplet size of microemulsions containing various amounts of selected oil and Smix was in nano range and relatively unchanged. All microemulsions were still stable as indicated by clear isotropic characteristic after storage for 3 months. These three types of oil and their compositions could form a stable microemulsion in term of size for a period of 3-month storage. The finding was also supported by other researches [289,290].

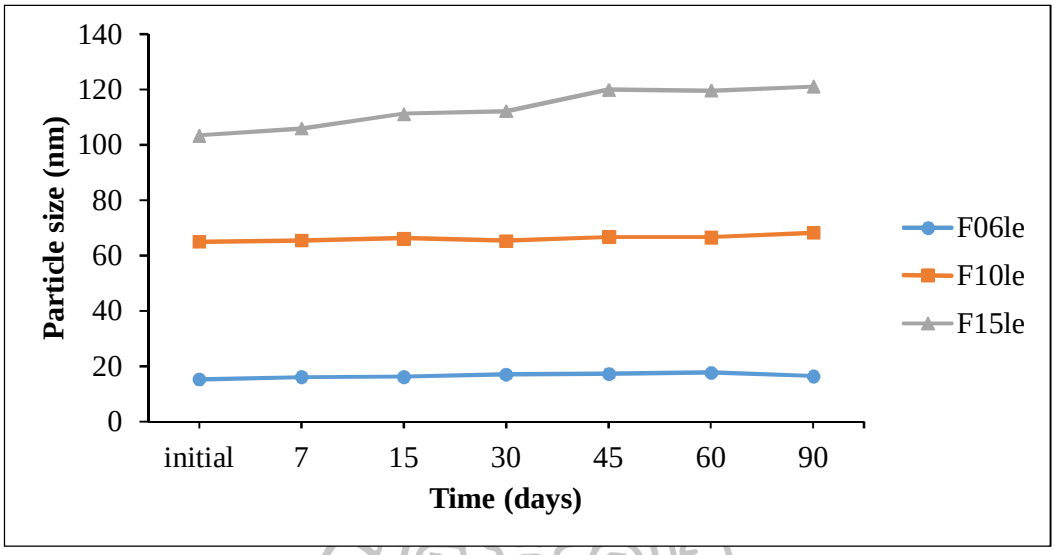


Figure 56 Change in droplet size of microemulsions containing lemon oil (F06le, F10le and F15le) during storage at 40° C, 75% RH for 3 months

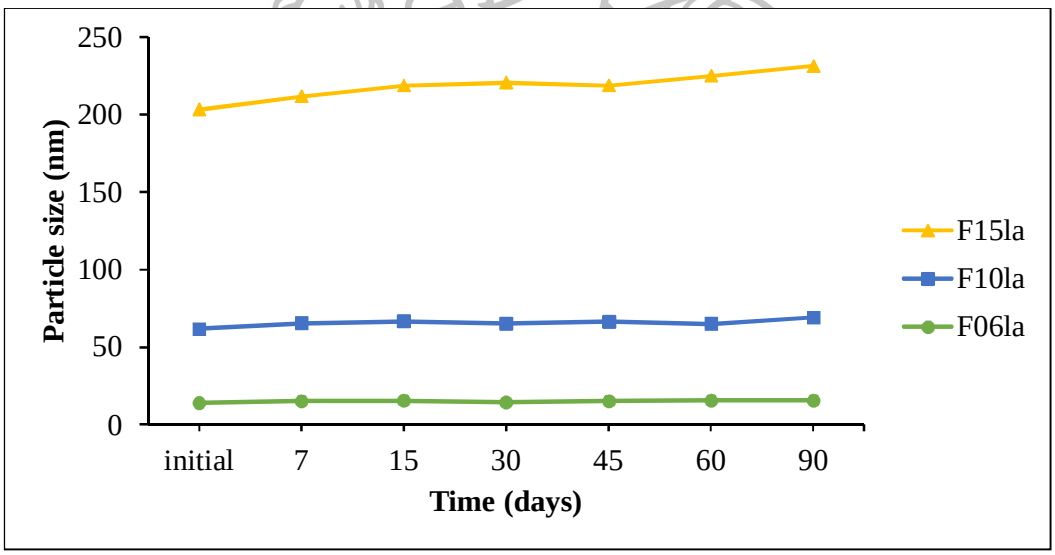


Figure 57 Change in droplet size of microemulsions containing lavender oil (F06la, F10la and F15la) during storage at 40° C, 75% RH for 3 months (mean ± SD)

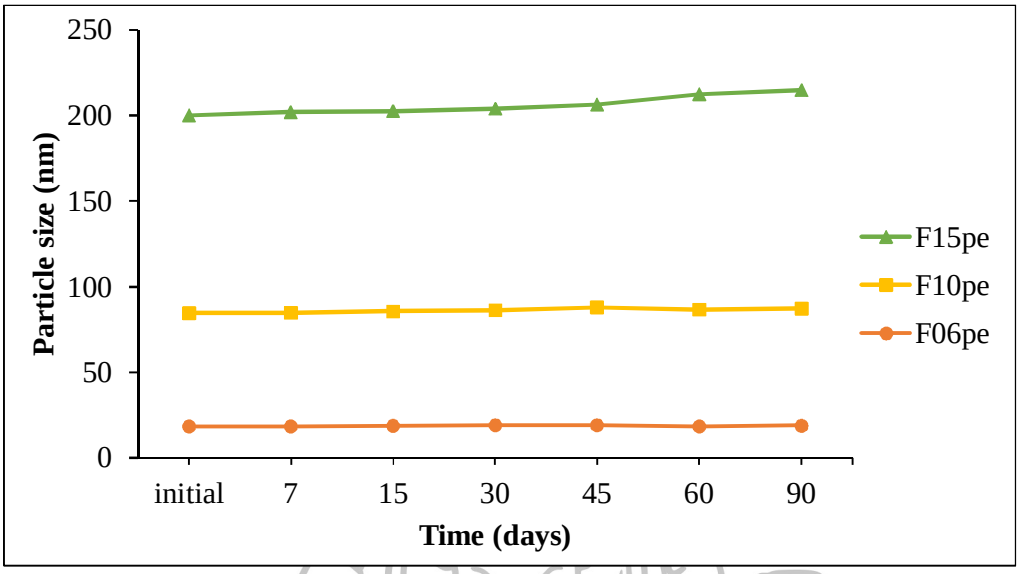


Figure 58 Change in droplet size of microemulsions containing peppermint oil (F06pe, F10pe and F15pe) during storage at 40° C, 75% RH for 3 months (mean ± SD)

The zeta potential measurement was also employed for monitoring change in surface charge of oil droplets in each microemulsion during storage. Tables 16, 17 and 18 show zeta potential values of microemulsions with different amount of selected oil and Smix. The result demonstrated that zeta potential values of all microemulsions were almost the same value and relatively unchanged even after stability test. As previously described, the negatively charge of microemulsions was assumed to be due to the presence of pe, le, la and related in oil droplets. Nevertheless, all microemulsions exhibited the negative charge values from -11.4 to -40.1 and remained negative charge after storage. For microemulsions that are very small, a high zeta potential will confer stability. Particles with zeta potentials more positive than +30 mV or more negative than -30 mV are normally considered stable.

The result suggested that oil droplets were still almost completely covered with non-ionic surfactant although the increment of droplet size was observed in the formulation with low amount of Tween 20.

Table 16 Zeta potential of microemulsions containing lemon oil (F06le, F10le and F15le) during storage at 40° C, 75% RH for 3 months (mean ± SD)

Time (days)	Zeta potential (mV)		
	F06le	F10le	F15le
initial	-31.1 ± 0.07	-37.1 ± 0.14	-28.4 ± 0.11
7	-29.4 ± 0.04	-28.9 ± 0.07	-33.3 ± 0.14
15	-27.8 ± 0.08	-33.6 ± 0.14	-31.7 ± 0.07
30	-33.1 ± 0.11	-34.5 ± 0.12	-26.5 ± 0.07
45	-24.4 ± 0.06	-25.5 ± 0.04	-30.3± 0.12
60	-26.7 ± 0.08	-22.2 ± 0.07	-26.7 ± 0.02
90	-21.4 ± 0.15	-21.4 ± 0.07	-27.1 ± 0.12

Table 17 Zeta potential of microemulsions containing lavender oil (F06la, F10la and F15la) during storage at 40° C, 75% RH for 3 months (mean ± SD)

Time (days)	Zeta potential (mV)		
	F06la	F10la	F15la
initial	-15.5 ± 0.05	-29.2 ± 0.04	-40.1 ± 0.08
7	-21.3 ± 0.10	-33.3 ± 0.11	-36.5 ± 0.09
15	-16.7 ± 0.09	-33.2 ± 0.14	-34.4 ± 0.17
30	-23.3 ± 0.19	-29.9 ± 0.07	-39.7 ± 0.07
45	-19.8 ± 0.21	-31.7 ± 0.20	-31.8 ± 0.11
60	-20.3 ± 0.01	-34.5± 0.12	-39.8 ± 0.12
90	-21.2 ± 0.04	-27.8 ± 0.07	-34.7 ± 0.12

Table 18 Zeta potential of microemulsions containing peppermint oil (F06pe, F10pe and F15pe) during storage at 40° C, 75% RH for 3 months (mean ± SD)

Time (days)	Zeta potential (mV)		
	F06pe	F10pe	F15pe
initial	-11.4 ± 0.21	-18.3 ± 0.16	-37.6 ± 0.23
7	-19.9 ± 0.08	-20.4 ± 0.07	-33.3 ± 0.17
15	-20.1± 0.08	-21.3± 0.16	-35.6 ± 0.07
30	-21.1 ± 0.12	-29.1 ± 0.16	-31.7 ± 0.07
45	-19.8 ± 0.09	-26.5 ± 0.12	-35.6 ± 0.15
60	-22.4 ± 0.12	-24.5 ± 0.18	-36.7 ± 0.12
90	-26.5 ± 0.12	-25.5 ± 0.04	-38.7 ± 0.11

The pH values of microemulsions with different amounts of oil and Smix after stability test are shown in Figure 59, 60 and 61. The pH of all microemulsions was slightly acidic and did not significantly change during storage ($p > 0.05$).

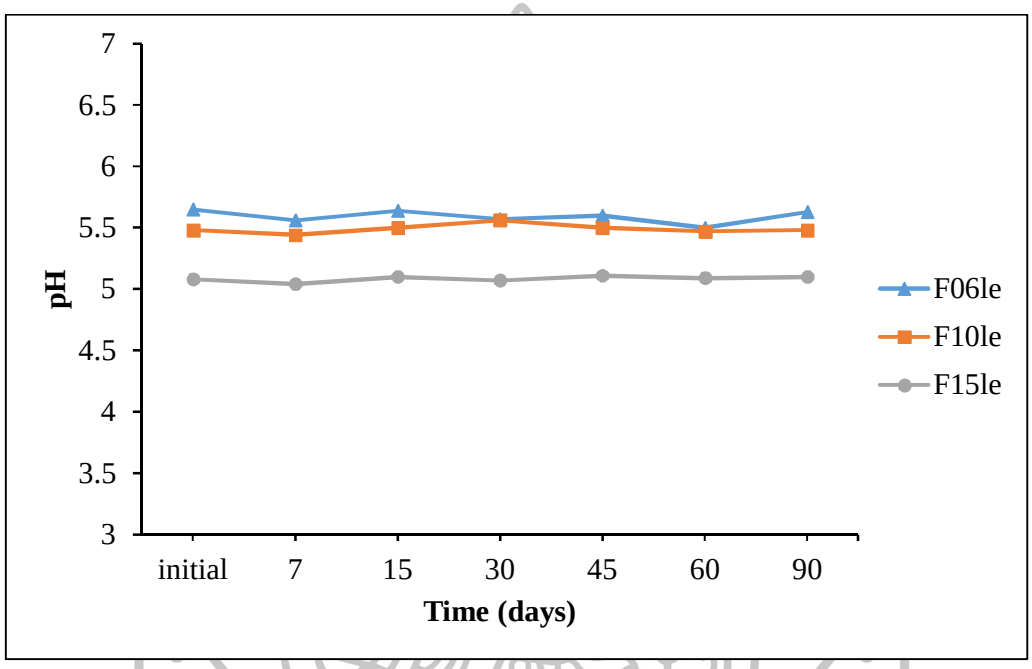


Figure 59 Change in pH of microemulsions containing lemon oil (F06le, F10le and F15le) during storage at 40° C, 75% RH for 3 months

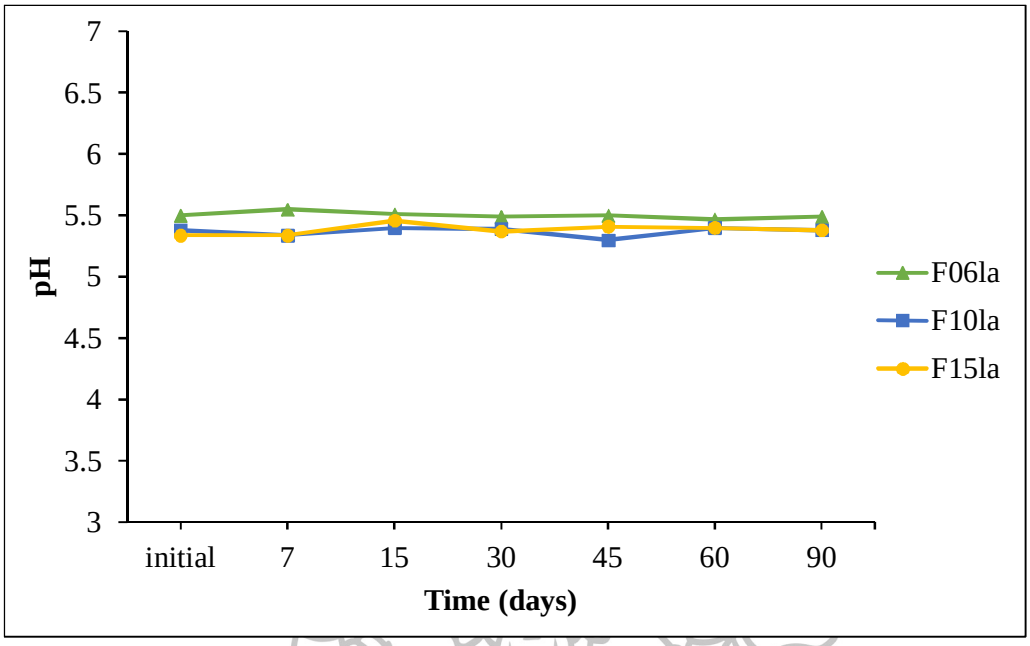


Figure 60 Change in pH of microemulsions containing lavender oil (F06la, F10la and F15la) during storage at 40° C, 75% RH for 3 months (mean ± SD)

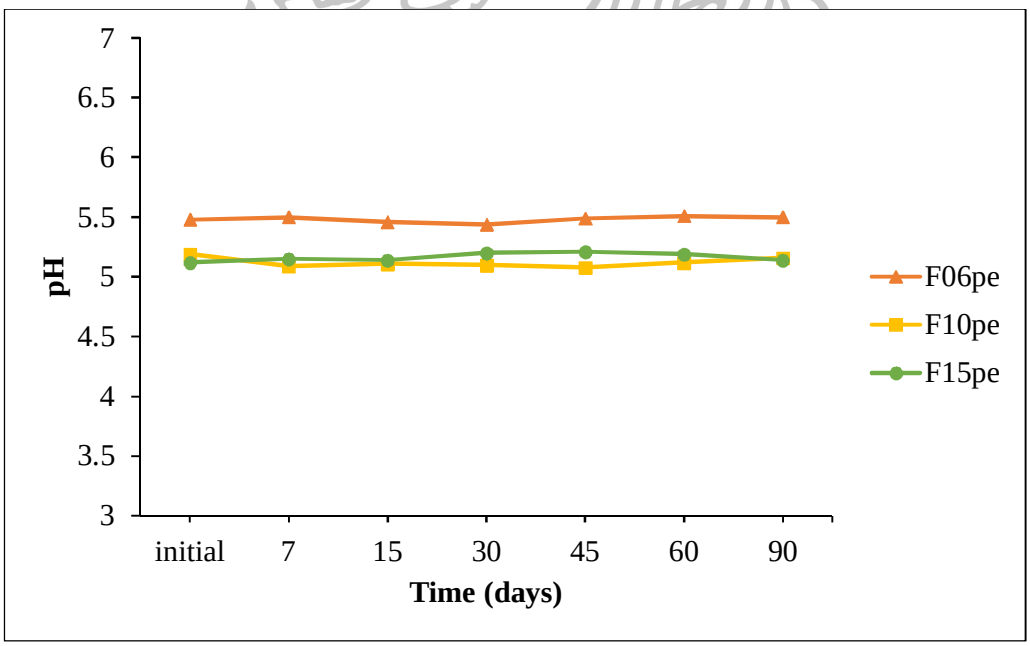


Figure 61 Change in pH of microemulsions containing peppermint oil (F06pe, F10pe and F15pe) during storage at 40° C, 75% RH for 3 months

Viscosity and surface tension of microemulsions containing different amounts of selected oil and Smix is illustrated in Figure 62 and 63. The results suggested that the rheology behavior and surface tension of microemulsions were relatively unchanged even after stability test.

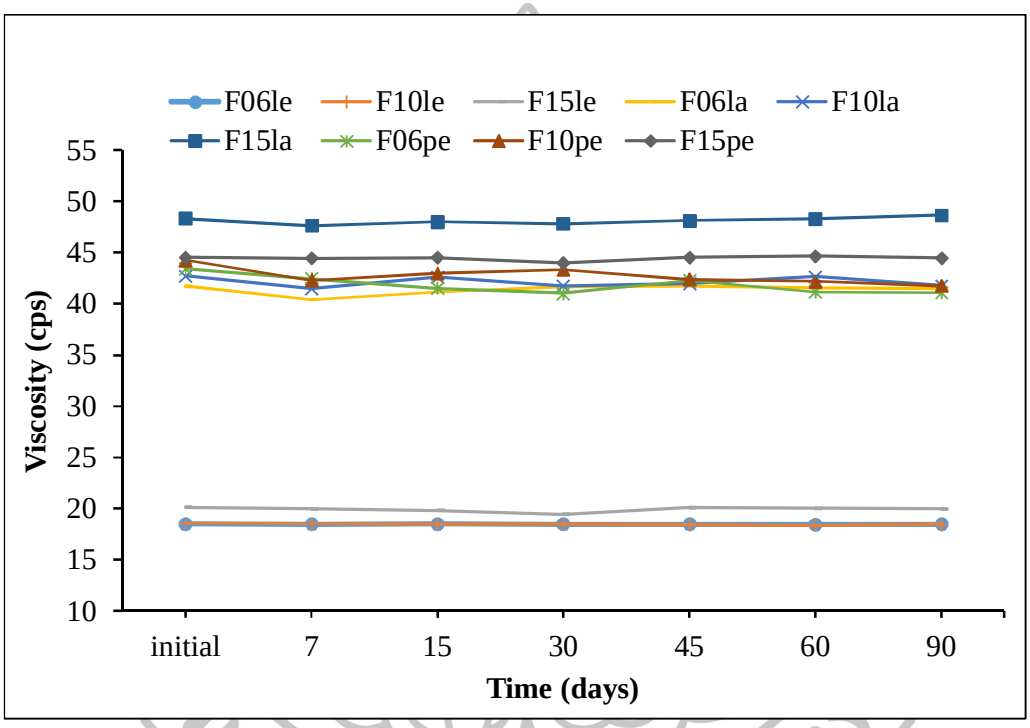


Figure 62 Change in viscosity of microemulsions containing different oils (F06le, F10le, F15le, F06la, F10la, F15la, F06pe, F10pe and F15pe) during storage at 40° C, 75% RH for 3 months

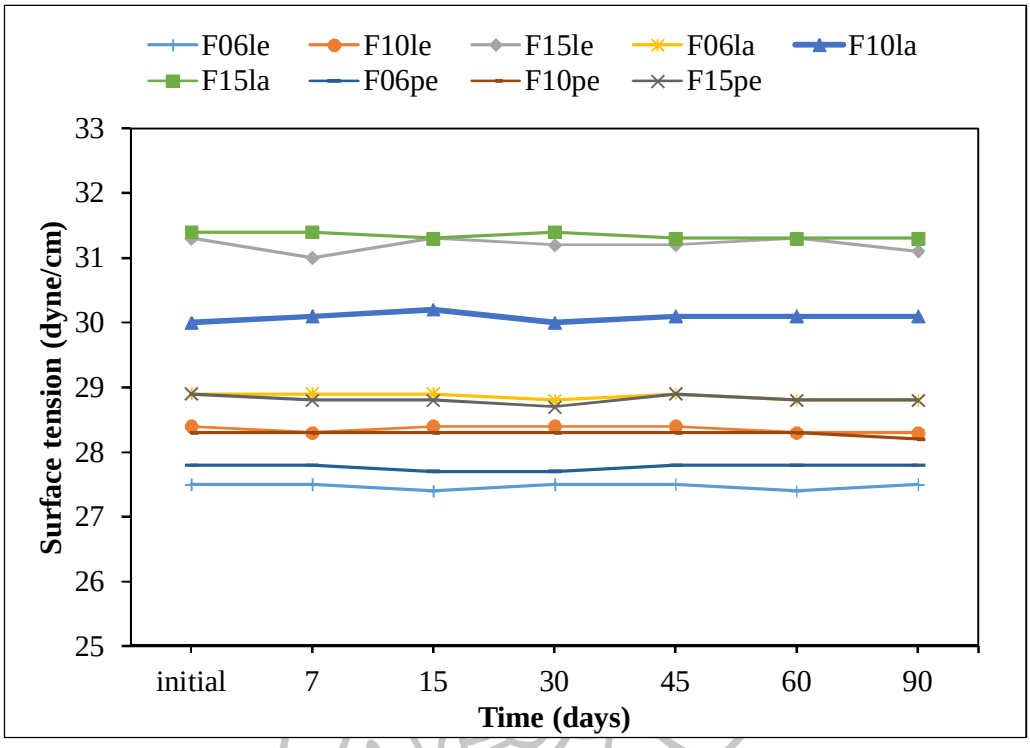


Figure 63 Change in surface tension of microemulsions containing different oils (F06le, F10le, F15le, F06la, F10la, F15la, F06pe, F10pe and F15pe) during storage at 40° C, 75% RH for 3 months

As a result, it is conclude that, no significant change ($p > 0.05$) was found in the initial droplet size, zeta potential, pH, viscosity and surface tension of the microemulsions upon storage at 40° C, 75% RH for 3 months; therefore, it is assumed that no droplet coalescence and/or aggregation occurred. Moreover, no color change and phase separation were observed, indicating that these microemulsions were stable on storage. From all the results mentioned above, it could be reasonable to conclude that the microemulsions with good stability were easily fabricated through the specific combination among lemon oil or lavender oil or peppermint oil with surfactant and

ethanol. For this study, the microemulsions containing with lemon oil shows the best properties of microemulsions as a smaller droplet size, higher zeta potential and lower viscosity.

4.4.7 Permeation from microemulsions

To evaluate the effect of concentration parameter, concentrations of soybean extract solution were loaded into the microemulsions and study by franz diffusion cell methods.

Yucatan micropig (YMP) skin was used as a model skin due to its similarities with human skin [191,192]. The ability of microemulsion as a carrier of topical dosage form for delivery of genistein and daidzein through Yucatan micro pig skin was evaluated. The skin permeation and retention of soybean extract at the same concentration were tested and compared between microemulsion and soybean extract solution (SES).

Figures 62-64 show the cumulative amount of genistein and daidzein released per unit area of skin over 48 hours of permeation study. The cumulative amount of genistein and daidzein permeated from its microemulsion in various oils was determined. The permeation parameters e.g. steady state flux *Jss*, permeation coefficient *Kp* and lag time *Tlag* of this study were shown in Table 19. It could be seen clearly in the permeability profile that throughout the assay period of 48 hours. The cumulative amount at 48 hours (Q_{48}) of genistein from F106e, F10le and F15le formula permeated through Yucatan micro pig skin were 5.12 μg/cm^2, 3.64 μg/cm^2 and 2.75 μg/cm^2, respectively while those of daidzein were 1.08 μg/cm^2, 0.88 μg/cm^2 and 0.87 μg/cm^2, respectively. (Figure 62).

For the permeation studies, only the microemulsion containing lemon oil could be detected both genistin and daidzein while only the genistein could be detected for the microemulsions containing lavender oil and peppermint oil (Figures 63, 64). The high amount of lemon oil showed a high skin penetration into the skin (F06le>F10le>F15le). The Q_{48} of genistein from F06le formula was 1.40-fold higher than that from the F10le formula (3.64 μg/cm^2) and 1.86-fold higher than that from the F15le formula (2.75 μg/cm^2). For genistein and daidzein from SES, the genistein and daidzein could not be detected in receptor fluid throughout the experiment. This incident could be confirmed that the genistein and daidzein as a lipophilic substances, which has a slight solubility in water, it might still remain on stratum corneum lipid and not penetrate into the skin. The result was in accordance with the result of the steady state flux *Jss* (0.0020±0.91 μg/cm^2/h) and the skin permeation coefficient *Kp* (1.32±0.09x10^{-3}cm/h). The F06le formula was significantly higher (p<0.05) than other formulations. Furthermore, the lag time of F06le formula (22.01±0.04 hours) was longer than other formulations. From the skin permeation study, these results were proved that the lower oil content, F06le formula system, was more effective for delivering genistein and daidzein than other formulations. The system could deliver genistein in a higher extent than daidzein.

The permeation flux of genistein and daidzein microemulsions containing lemon oil as oil phase was comparatively higher than that of microemulsions containing lavender oil and peppermint oil as oil phase, when all other excipients were the same. It was also apparent that the genistein and daidzein permeation increased with the increase in the amount of surfactant phase and aqueous phase in their composition. This could be attributed to skin permeation enhancement

capacity by the surfactants. Surfactants can loosen or fluidize the lipid matrix of the stratum corneum. The principal diffusional barrier of the skin and act as skin permeation enhancer. In addition, other components such as lemon oil, which were used as oil phase in these formulated microemulsions, have the capacity as skin permeation enhancers and they could add the skin permeation enhancement of genistein and daidzein from formulated microemulsions. Therefore, lemon oil exhibited highest amount of permeation, lowest lag time and highest partition coefficient.

From the above results, soybean extracts microemulsion formulation F06le (30% of lemon oil as oil phase, 10% of aqueous phase, and 60% of surfactant phase containing Tween 80, Tween 20 and Ethanol) was selected as best formulation based on its higher permeation flux through the excised Yucatan micro pig skin than other formulations in this investigation. The soybean extract microemulsions F06le was further studied for determination of droplet size, polydispersity index, and zeta potential. The average droplet diameter (z-average diameter) of best microemulsion formulation was 15.24 ± 3.69 nm and smaller than other formulations. Due to its small droplet size high surface areas wereobtined and giving a close contact with the skin, providing high concentration gradient and improving permeation of drug from microemulsions. The zeta potential of microemulsion F06le was -31.1 ± 0.07 mV. The skin has also slight negative charge. Therefore, the negative zeta potential of microemulsion F06le might cause an influence in improving drug permeation through the skin due to electrostatic repulsion between the same charge of the skin surface and the microemulsion. The polydispersity index has been found to be 0.3334, which means that the globules are homogeneously distributed.

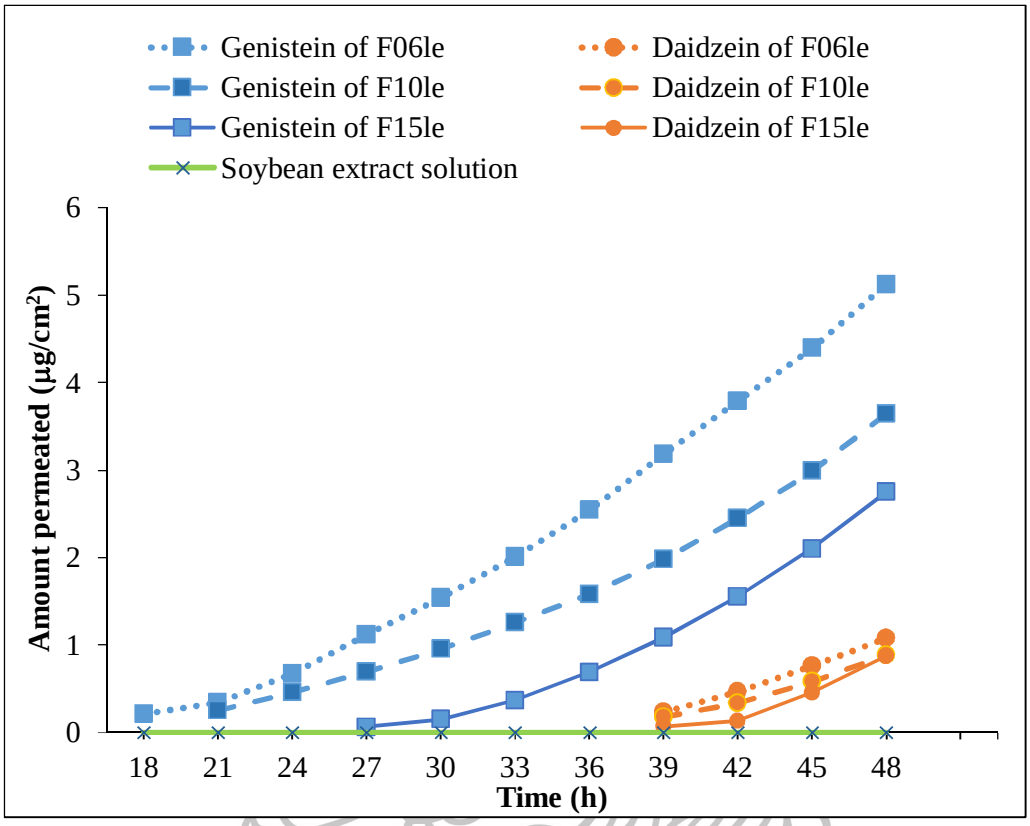


Figure 62 Permeation profiles of genistein and daidzein from microemulsions containing with lemon oil through excised Yucatan micro pig skin.

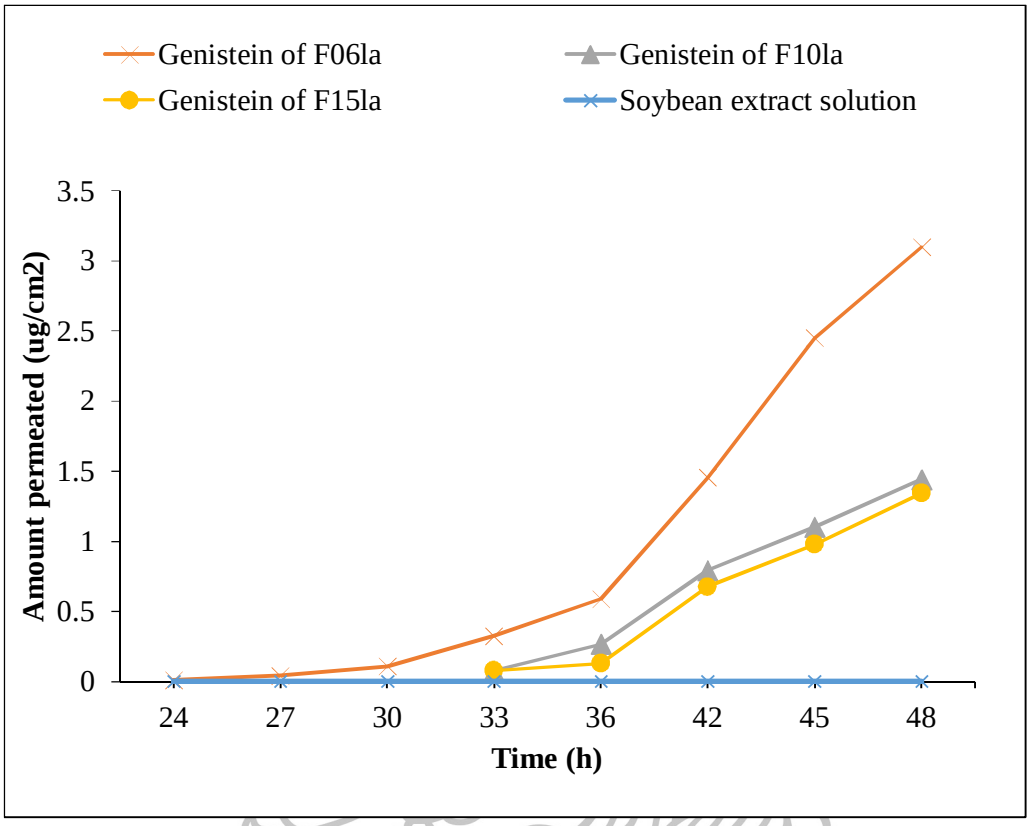


Figure 63 Permeation profiles of genistein from microemulsions containing with lavender oil through excised Yucatan micro pig skin.

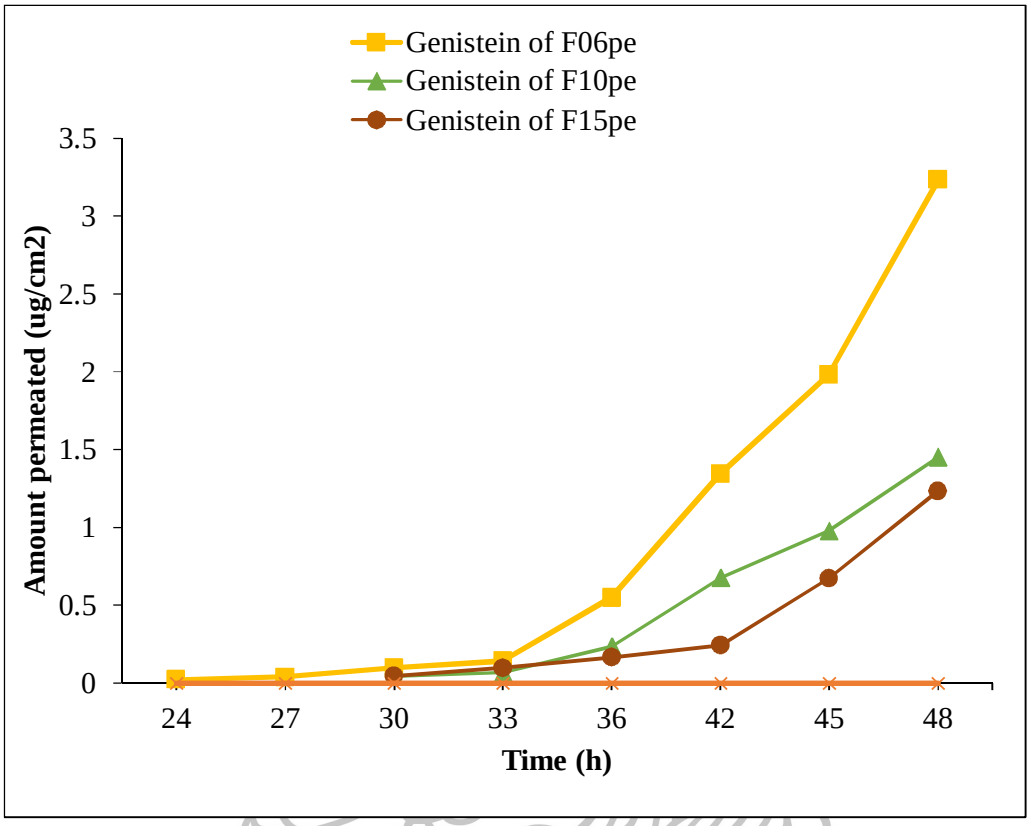


Figure 64 Permeation profiles of genistein from microemulsions containing with peppermint oil through excised Yucatan micro pig skin.

Table 19 *In vitro* skin permeation parameters of genistein and daidzein from microemusion system containing with different oils and the aqueous solutions.

Formula		Lag time (T_{lag}) (h)	Flux (J_{ss}) (μg/cm^2/h)	Patition coefficient (Kp) (x10^{-3} cm/h)
F06le	Genistein	22.01 ± 0.4	0.0020±0.91	1.32±0.09
	Daidzein	36.79 ± 0.12	0.00058 ±0.74	0.36±0.06
F10le	Genistein	25.83 ± 0.3	0.00184±0.3	0.80±0.08
	Daidzein	31.48 ± 0.6	0.00051 ±0.63	0.34±0.06
F15le	Genistein	37.22 ± 0.9	0.00179±0.41	0.80±0.04
	Daidzein	41.05 ± 0.6	0.00054±0.31	0.33±0.06
F06la	Genistein	27.95 ± 0.3	0.00184±0.26	0.74±0.03
	Daidzein	N.D.	N.D.	N.D.
F10la	Genistein	32.72 ± 0.8	0.00177±0.64	0.71±0.08
	Daidzein	N.D.	N.D.	N.D.
F15la	Genistein	34.91 ± 0.7	0.00155±0.54	0.64±0.1
	Daidzein	N.D.	N.D.	N.D.
F06pe	Genistein	25.44 ± 0.5	0.00194±0.32	0.76±0.04
	Daidzein	N.D.	N.D.	N.D.
F10pe	Genistein	29.45 ± 0.13	0.00168±0.87	0.71±0.08
	Daidzein	N.D.	N.D.	N.D.
F15pe	Genistein	31.22 ± 0.3	0.00151±0.87	0.72±0.07
	Daidzein	N.D.	N.D.	N.D.
SES	Genistein	N.D.	N.D.	N.D.
	Daidzein	N.D.	N.D.	N.D.

Each data represents the mean±SD (n = 6). *N.D., non-detected

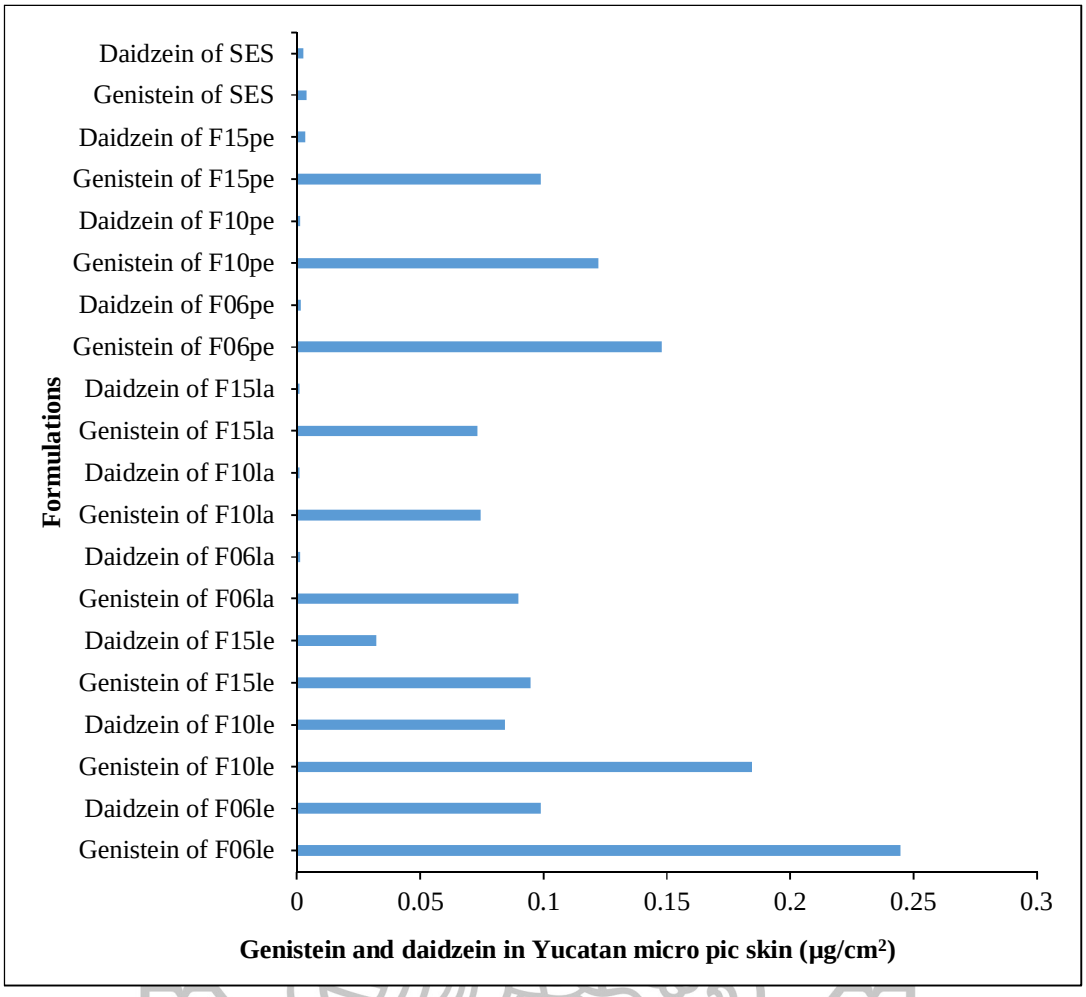


Figure 48 *In vitro* amount of genistein and daidzein accumulated in Yucatan micro pig skins after 48-hours *in vitro* skin permeation experiment.

In the skin retention study, the amount of the genistein and daidzein accumulated in the Yucatan micro pig skin was analyzed after the end of the skin permeation at 48 hours (Figure 65). All microemulsions system gave comparable amount of genistein and daidzein accumulated in the Yucatan micro pig skin. The F06le formula accumulated significantly more genistein and daidzein in Yucatan micro pig skin than that from the others formulations. These results indicated that F06le was effective in delivering genistein and daidzein through the skin and the systemic circulation. The better permeability of the microemulsions carriers may be the result of the addition of ethanol into the formulation as a co/surfactant with a suitable oil ratio. Ethanol has long been used as a skin permeation enhancer for topical and transdermal delivery. It could increase genistein and daidzein solubility leading to enhance the entrapment efficiency of the formulation, alteration of stratum corneum's barrier property and increasing in thermodynamic activity due to evaporation of ethanol. However, the skin permeation not only effect the ethanol but also depending on phospholipids and skin lipids. Touitou,. et al. [293] suggested a hypothetical model for enhancing the penetration of drug through the stratum corneum lipid. It was explained that the better skin permeation of system may be due to the synergistic mechanism between high concentration of ethanol, oil, surfactant vesicles and skin lipids. The stratum corneum lipid at physiological temperature are densely packed and highly ordered conformation. Ethanol can interact with lipid molecules in the polar head group region results in increasing fluidity and hence leading to increase membrane permeability. In addition, it may provide the vesicles with soft flexible characteristics which are easy to penetrate into deeper layers of the skin. The microemulsions vesicle can forge paths in the disordered stratum corneum.

The release of drug could be the result of fusion of microemulsion with skin lipids and drug release at various points along the penetration pathway.

CHAPTER 5

CONCLUSION

Soy as most commonly known in the form of soybean is the plant of multipurpose use. It is not only the source of protein as it is famous of; but it is also rich in other nutrients such as vitamins, carbohydrates, oils, fibers, etc. Apart from this its end products after the extraction process are useful in pharmaceutical industries. It is economical but its use is not without controversy as it may lead to increase in cancer risk in post-menopausal women. Its excessive use may cause constipation in some patients but a lot of benefits than risk as it can prevent various cancers, lower the bad cholesterol level, prevent hair loss, its anti-ageing property etc. Although it has some negative effects but if it is properly exploited for its beneficial use it can become the treasure of future for the nutritionist, medical practitioner and pharmaceutical industries as well as for the common human.

The study was categorized into 3 major parts: (1) the extraction and validation of soybean, (2) the evaluation and stability of soybean extract, (3) the preparation and characterization of soybean extract loaded microemulsions .

In the first part, the study was aimed to extract and validate the method of HPLC to determine an amount of genistein and daidzein isoflavones from soybean. Extraction with organic solvent by using sonication, shaker, vortex, soxhlet were compared to extract the soybean. Sonication method showed a high amount of

genistein and daidzein. Extraction with non-organic solvent was proven to be temperature dependence. The highest amount of genistein and daidzein obtained wasat 61-65°C. The isoflavone extraction methods presented in this thesis has been developed by using one simple single step and avoiding the use of special technique that could be used routinely for extraction a broad range of concentrations of the isoflavones from soybeans and soy products. The most suitable extraction method for isoflavones proved to be the ultrasonication. The developed HPLC method for quantification of genistein and daidzein has been validated and shown to be reliable, accurate, precise, and linear (in the concentration range of 5-30 µg/mL). The validity of the method has met the requirement of AOAC guidelines. Therefore the developed HPLC method can be used as an accurate routine procedure for the quantification of genistein and daidzein from soybeans and soy products due to its short retention times (13.8 and 14.5 minutes respectively) and simple.

The aim of the second part was to evaluate and characterize the soybean extract powder. The soybean extract powder was successfully prepared by spray drying method. The soybean extract powder were characterized for their physiochemical properties such as pH, flowability, moisture content, particle size distribution, particle morphology and x-ray diffraction. Antioxidant activities by DPPH, ABTS, Folin-Ciocalteu and phenolic content method were also investigated. The results showed that the soybean extracts still contained high level of total phenolic compounds, including daidzein and genistein, and were capable of inhibiting free radicals to terminate the radical chain reaction. Furthermore, a significant and linear relationship was found between the antioxidant activities and phenolic contents, indicating that phenolic compounds could be major contributors for antioxidant

activity. The main phenolic compounds, daidzein and genistein, in soybean extracts showed strong antioxidant activity by inhibiting ABTS•+ stronger than DPPH• when compared with standard trolox. Therefore, the soybean extracts obtained by classical extraction can be used as an easily accessible source of natural antioxidants with consequent health benefits. The stability of soybean extract were compared with solution form and dried powder form. The soybean extract powder could be stored for 3 years with high amount of genistein and daidzein isoflavones remained.

The aim of last part was to formulate soybean extract with the various common oils and surfactants into microemulsion. To improve the skin permeation efficiency of soybean extract, the permeation enhancing substance carrier named microemulsions was explored in this research. For this experiment, nine formulations of microemulsions loading soybean extracts were compared with the composition such as lavender oil, peppermint oil, lemon oil with Tween 80, Tween 20 and ethanol. The different formulations of soybean extract microemulsions were formulated and evaluated for their physiochemical property such as the appearance of formulation (non-sedimentation, non-separation), particle size and viscosity. This study had proposed to select the suitable physicochemical properties of formulations for stability study. Thus, nine suitable formulations showed the range of particle size of 14.12 ± 0.87 to 115.47 ± 1.05 nm. Soybean extract loaded microemulsions was stable after storage at accelerated conditions (40°C, 75% RH) for 3 months. Nine formulations of soybean extract loaded microemulsions were selected for skin permeation study comparison to soybean extract solution. It was found that, compositions of formulations affected the physicochemical property of microemulsions. The significantly high potential for in vitro skin permeation was reported for soybean

extract in microemulsion form rather than solution. The best formulation consisted of 30% of lemon oil, Tween 20, Tween 80 and ethanol. The skin permeation parameters of the best genistein microemulsion such as Q_{48}, Jss, Kp, and lag time were 5.12 µg/cm^2, 0.002 ± 0.91 µg/cm^2/h, 1.32±0.09x10^{-3} cm/h, and 22.01±0.4 h, respectively while for diadzein microemulsion were 1.08 µg/cm^2, 0.00058±0.74 µg/cm^2/h, 0.36±0.06x10^{-3} cm/h and 36.79±0.12 h, respectively. These all values were higher than those of soybean extract solution. Additionally, the in vitro skin permeation of soybean extract microemulsions containing 30% of lemon oil gave significantly higher amount when compared with soybean extract microemulsion at different ratios of lemon oil (40 and 50%) and different types of oil (lavender and peppermint oil). It is indicated that the microemulsion could be used as a carrier to enhance the skin penetrations of soybean isoflavone (genistein and daidzein). However, the in vivo and clinical evaluation should be achieved to conform the clinical efficiency. The final part showed either improved technological characteristics and enhanced permeation properties. Thus, microemulsions loaded soy isoflavones extract could serve as adequate ingredient, rich of effective estrogen-like agents, for nutraceutic or cosmetic manufacturing, providing multiple health benefits.

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APPENDIX

Table 20. Concentration of genistein and daidzein from soybean extract under different extraction temperature.

Temperature (ºC)	Concentrations (µg/mL)	
	Genistein	Daidzein
26-30	0.13846	0.1105
31-35	0.1652	0.16356
36-40	0.13594	0.15682
41-45	0.1421	0.17975
46-50	0.16893	0.19826
51-55	0.2157	0.3689
56-60	0.23842	0.37045
61-65	0.30071	0.43937
66-70	0.2705	0.28146
71-75	0.17107	0.17975
76-80	0.1421	0.19476
81-85	0.1261	0.13917
86-90	0.1008	0.10976
91-95	0.08105	0.09123
96-100	0.10101	0.06531

Table 21 Concentrations of genistein and daidzein solutions, stored in the refrigerator at 5ºC for 0, 1, 2, 3 and 4 weeks.

Storage Durations (Months)	Concentrations (µg/mL)	
	Genistein	Daidzein
0	0.301	0.439
1	0.27	0.33
2	0.04	0.0023
3	0	0
4	0	0

Table 22 Concentrations of genistein and daidzein solutions, stored in the freezer at -20ºC for 0, 1, 2, 3 and 4 weeks.

Storage Durations (Months)	Concentrations (µg/mL)	
	Genistein	Daidzein
0	0.301	0.439
1	0.27	0.33
2	0.2	0.3
3	0.07	0.04
4	0.06	0.01

Table 23 Concentrations of genistein and daidzein solutions, stored in desiccators at the room temperature for 0, 0.25, 1, 2, 3, 4, 8, 12, 16, 20, 24, 28, 32 and 36 weeks.

Storage Durations (Months)	Concentrations (µg/mL)	
	Genistein	Daidzein
0	2.344	1.892
0.25	2.291	1.876
0.5	2.248	2.11
1	2.11	1.846
2	1.837	1.792
4	1.800	1.784
8	1.784	1.664
12	1.767	1.566
16	1.76	1.29
20	1.644	1.198
24	1.578	1.078
28	1.54	1.089
32	1.333	1.088
36	1.325	1.08

Table 24 Free radical scavenging activity of daidzein by DPPH method.

Concentration of daidzein (mg)	DPPH inhibition (%)
0.05	4.65
0.1	13.4
0.18	25.4
0.27	35.1
0.36	45.2
0.44	53.4
0.67	62.3
0.9	70.5
1.11	79.8
1.33	91.8

Table 25 Free radical scavenging activity of genistein by DPPH method.

Concentration of genistein (mg)	DPPH inhibition (%)
0.05	4.65
0.09	13.4
0.17	25.4
0.26	35.1
0.34	45.2
0.43	53.4
0.64	62.3
0.86	70
1.07	79.8
1.28	91.8

Table 26 Free radical scavenging activity of trolox by DPPH method.

Concentration of trolox (mg)	DPPH inhibition (%)
0.01	1.92
0.05	11.9
0.1	20.1
0.15	26.8
0.2	36.8
0.25	47.7
0.35	59.3
0.4	70.4
0.45	81
0.5	90.2

Table 27 Free radical scavenging activity of daidzein by ABTS method.

Concentration of daidzein (mg)	DPPH inhibition (%)
0.18	6.46
0.27	15.8
0.36	29.5
0.44	44.9
0.67	55.2
0.9	68.4
1.11	83
1.33	95.4

Table 28 Free radical scavenging activity of genistein by ABTS method.

Concentration of genistein (mg)	DPPH inhibition (%)
0.17	6.46
0.26	15.8
0.34	29.5
0.43	44.9
0.64	55.2
0.86	68.4
1.07	83
1.28	95.4

Table 29 Free radical scavenging activity of trolox by ABTS method.

Concentration of trolox (mg)	DPPH inhibition (%)
0.05	3.64
0.25	15
0.5	27.9
0.75	43.8
1	55.6
1.25	70.5
1.5	83
1.75	99.3

Table 30 Change in droplet size of microemulsions containing lemon oil, lavender oil and peppermint oil during storage at 40ºC, 75% RH for 3 months

Time	Particle size (nm)								
(days)	F06le	F10le	F15le	F06la	F10la	F15la	F06pe	F10pe	F15pe
initial	15.24	65.02	103.37	14.12	47.58	141.5	18.55	66.15	115.47
7	16.12	65.4	105.87	15.21	50.33	146.2	18.56	66.4	116.98
15	16.23	66.21	111.21	15.44	51.21	152.1	18.87	66.89	116.74
30	17.1	65.34	112.1	14.51	50.77	155.47	19.12	67.13	117.78
45	17.25	66.74	119.85	15.23	51.24	152.28	19.22	68.81	118.43
60	17.74	66.65	119.56	15.57	49.48	159.87	18.48	68.1	125.76
90	16.4	68.21	121.02	15.64	53.59	162.2	19.11	68.27	127.4

Table 31 Change in pH of microemulsions containing lemon oil, lavender oil and peppermint oil during storage at 40ºC, 75% RH for 3 months

Time (days)	F06le	F10le	F15le	F06la	F10la	F15la	F06pe	F10pe	F15pe
initial	5.65	5.48	5.08	5.5	5.38	5.34	5.48	5.19	5.12
7	5.56	5.44	5.04	5.55	5.34	5.34	5.5	5.09	5.15
15	5.64	5.5	5.1	5.51	5.4	5.46	5.46	5.11	5.14
30	5.57	5.56	5.07	5.49	5.39	5.37	5.44	5.1	5.2
45	5.6	5.5	5.11	5.5	5.3	5.41	5.49	5.08	5.21
60	5.5	5.47	5.09	5.47	5.4	5.4	5.51	5.12	5.19
90	5.63	5.48	5.1	5.49	5.38	5.38	5.5	5.16	5.14

Table 32 Change in pH of microemulsions containing lemon oil, lavender oil and peppermint oil during storage at 40ºC, 75% RH for 3 months

Time (days)	F06le	F10le	F15le	F06la	F10la	F15la	F06pe	F10pe	F15pe
initial	18.51	18.57	20.14	41.72	42.71	48.3	43.4	44.23	44.53
7	18.48	18.52	19.99	40.4	41.48	47.6	42.45	42.28	44.44
15	18.51	18.49	19.84	41.14	42.59	47.98	41.5	42.98	44.51
30	18.5	18.5	19.43	41.67	41.74	47.77	41	43.33	43.98
45	18.49	18.44	20.11	41.74	41.98	48.12	42.28	42.39	44.53
60	18.47	18.38	20.05	41.55	42.65	48.27	41.14	42.18	44.65
90	18.5	18.47	19.98	41.48	41.77	48.64	41.09	41.74	44.47

Table 33 Change in surface tension of microemulsions containing lemon oil, lavender oil and peppermint oil during storage at 40ºC, 75% RH for 3 months

Time (days)	F06le	F10le	F15le	F06la	F10la	F15la	F06pe	F10pe	F15pe
initial	27.5	28.4	31.3	28.9	30	31.4	27.8	28.3	28.9
7	27.5	28.3	31	28.9	30.1	31.4	27.8	28.3	28.8
15	27.4	28.4	31.3	28.9	30.2	31.3	27.7	28.3	28.8
30	27.5	28.4	31.2	28.8	30	31.4	27.7	28.3	28.7
45	27.5	28.4	31.2	28.9	30.1	31.3	27.8	28.3	28.9
60	27.4	28.3	31.3	28.8	30.1	31.3	27.8	28.3	28.8
90	27.5	28.3	31.1	28.8	30.1	31.3	27.8	28.2	28.8

Table 34 Permeation profiles of genistein and daidzein from microemulsions containing with lemon oil through excised Yucatan micro pig skin.

Time	F15le		F10le		F06le	
(h)	Genistein of F06le	Daidzein of F06le	Genistein of F10le	Daidzein of F10le	Genistein of F15le	Daidzein of F15le
18	0.21345					
21	0.3467657		0.2599			
24	0.676453		0.46485			
27	1.124234		0.698		0.06739	
30	1.545343		0.957		0.153167	
33	2.009797		1.2636		0.36932	
36	2.546537		1.5867		0.6924	
39	3.189765	0.23455	1.98012	0.17896	1.0858	0.06788
42	3.7897676	0.466487	2.45159	0.34057	1.55728	0.13455
45	4.398655	0.767576	2.99663	0.58921	2.10232	0.458976
48	5.12344	1.083498	3.645598	0.88953	2.75128	0.878675

Table 35 Permeation profiles of genistein and daidzein from microemulsions containing with lavender oil through excised Yucatan micro pig skin.

Time (h)	Genistein of F06la	Genistein of F10la	Genistein of F15la
24	0.010970121		
27	0.040693423		
30	0.10729773		
33	0.32346035	0.07458	0.07756
36	0.589335098	0.26708	0.12983
42	1.454211611	0.792312	0.67654
45	2.451317262	1.10114	0.97858
48	3.098563647	1.439263	1.343597

Table 36 Permeation profiles of genistein and daidzein from microemulsions containing with peppermint oil through excised Yucatan micro pig skin.

	Genistein of F06pe	Genistein of F10pe	Genistein of F15pe
24	0.023444221		
27	0.040982322		
30	0.098424234	0.0453542	0.0456344
33	0.142346035	0.0674532	0.09798675
36	0.549335098	0.2354355	0.16545322
42	1.344211611	0.6764533	0.24214321
45	1.983423421	0.9787532	0.67364322
48	3.234081512	1.4532313	1.23423566

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