Development and Characterization of Cosmetic Microemulsions Containing Arbutin Nanoparticles

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ชื่อวิทยานิพนธ์	การพัฒนาและการแสดงลักษณะเฉพาะของไมโครอิมัลชันทางเครื่องสำอางที่ประกอบด้วยอาบูตินนาโนพาร์ทิเคิล
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บทคัดย่อ

การศึกษาครั้งนี้มีเป้าหมายเพื่อพัฒนาและประเมินศักยภาพของไมโครอิมัลชันทางเครื่องสำอางที่ใช้ไคโตซานนาโนพาร์ทิเคิลในการนำส่งอาบูติน โดยมีส่วนประกอบของไมโครอิมัลชันคือ น้ำมันมะพร้าวบริสุทธิ์ เป็นวัฏภาคน้ำมัน Labrasol®/Plurol® Oleique เป็นสารลดแรงตึงผิว/สารลดแรงตึงผิวร่วม และไคโตซานชนิดละลายน้ำ/ไตรพอลิฟอสเฟต (WSC/TPP) เป็นวัฏภาคน้ำ เฟสไดอะแกรมไตรภาคเทียมของไมโครอิมัลชันที่ประกอบด้วยอาบูติน 0.5% โดยน้ำหนัก ถูกสร้างและประเมินหาขนาดอนุภาค ความคงตัวทางเคมีและกายภาพ การปลดปล่อยอาบูติน การซึมผ่านผิวหนังของอาบูติน และการกักอาบูตินในผิวหนัง รวมทั้งการประเมินผลฤทธิ์ทำให้ขาวของไมโครอิมัลชันเปล่า โดยตรวจสอบแอกติวิตีของเอนไซม์ไทโรซิเนส และปริมาณเมลานินในเซลล์ melan-a melanocytes พบว่าอัตราส่วนระหว่างสารลดแรงตึงผิวต่อสารลดแรงตึงผิวร่วม (K_m) ที่เหมาะสมเป็น 2:1 การตรวจสอบ differential scanning calorimetry และการนำไฟฟ้า แสดงระบบไมโครอิมัลชันชนิดน้ำในน้ำมัน ได้ทำการปรับปรุงความคงตัวทางเคมี-กายภาพของไมโครอิมัลชันที่มี K_m เป็น 2:1 โดยการเติม WSC/TPP ในวัฏภาคน้ำ พบว่าตำรับ F4 ที่เตรียมจาก WSC น้ำหนักโมเลกุล 1 แสนดาลตัน มีขนาดอนุภาคใกล้เคียงกันที่ 200 นาโนเมตร ในขณะที่ตำรับ F7 ที่เตรียมจาก WSC น้ำหนักโมเลกุล 2 แสนดาลตัน มีขนาดอนุภาคในช่วงการกระจายที่กว้างระหว่าง 200 นาโนเมตร ถึง 2 ไมโครเมตร การปลดปล่อยและการซึมผ่านผิวหนังของอาบูตินจากตำรับ F4 ช้ากว่าตำรับ F7 และ F10 (ไม่มี WSC/TPP) ตามลำดับ ส่วนการกักอาบูตินในผิวหนัง พบว่าการกักอาบูตินในผิวหนังสูงสุดในตำรับ F4 การประเมินความคงตัวของอาบูตินในตำรับไมโครอิมัลชัน โดยเก็บรักษาที่อุณหภูมิห้อง 45 องศาเซลเซียส และ 4 องศาเซลเซียส เป็นเวลานาน 1, 2 และ 3 เดือน พบว่าตำรับ F4 มีความคงตัวทางเคมี-กายภาพได้อย่างน้อย 3 เดือน การเก็บที่อุณหภูมิห้อง และที่ 4 องศาเซลเซียส โดยมีปริมาณอาบูตินเป็น 99.02% และ 100.76% ตามลำดับ การเก็บรักษาตำรับที่อุณหภูมิ 45 องศาเซลเซียส เป็นเวลา 3 เดือน พบปริมาณอาบูตินในตำรับ F4 เป็น 94.57% ซึ่งสูงกว่าในสารละลายอาบูติน และตำรับ

F7 และ F10 โดยมีปริมาณของอาบูตินที่เหลืออยู่เป็น 80.66%, 90.02% และ 88.46% ตามลำดับ นอกจากนี้พบว่าตำรับ F4 เปล่า ที่ความเข้มข้น 0.1% เข้ากันได้กับเซลล์ melan-a melanocytes ผลของตำรับ F4 เปล่า ต่อแอกติวิตีของเอนไซม์ไทโรซิเนสเทียบเท่ากับสารละลายอาบูตินที่ความเข้มข้น 250 มคก. ต่อ มล. โดยมีแอกติวิตีของเอนไซม์ไทโรซิเนสเป็น 84.32% และ 82.92% ตามลำดับ พบว่าตำรับ F4 เปล่า สามารถลดปริมาณเมลานินได้ดีกว่าสารละลายอาบูตินที่ความเข้มข้น 250 มคก. ต่อ มล. อย่างมีนัยสำคัญโดยเป็น 75.99% และ 95.99% ตามลำดับ ดังนั้นจะเห็นได้ว่าตำรับ F4 ที่ประกอบด้วยไมโครอิมัลชันชนิดน้ำในน้ำมัน ที่มีอาบูตินและ WSC/TPP นาโนพาร์ทิเคิล เป็นระบบนำส่งที่น่าสนใจอย่างยิ่งสำหรับการนำส่งอาบูตินทางเครื่องสำอางเพื่อประยุกต์ใช้ในผลิตภัณฑ์ทำให้ผิวขาว

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ABSTRACT

The objectives of this study were to develop and evaluate the potential of cosmetic microemulsions containing chitosan nanoparticles for arbutin delivery. Pseudo-ternary phase diagrams were plotted to identify the microemulsification regions. The microemulsions consisted of virgin coconut oil as oil phase, Labrasol®/Plurol® Oleique as surfactant/co-surfactant phase and water phase containing water soluble chitosan (WSC)/tripolyphosphate (TPP). The microemulsions containing 0.5%w/w arbutin were evaluated for the particle size, physico-chemical stability, arbutin release, skin permeation, and skin retention. Furthermore, skin whitening activity (effects on tyrosinase activity, and melanin content of melan-a melanocytes) was investigated in plain microemulsions. The appropriate surfactant to co-surfactant mass ratio (K_m) of the systems was found as 2:1. Determination of differential scanning calorimetry (DSC) and conductivity of the systems indicated water-in-oil (w/o) type microemulsions. Addition of WSC/TPP in the water phase was attempted to improve physico-chemical stability of the microemulsions. The particle size of F4 with medium molecular weight WSC (100 kDa) was 200 nm, while F7 with high molecular weight WSC (200 kDa) was

polydispersion (200 nm-2 μm). The slowest release of arbutin was found in F4, followed by F7, and F10 (without WSC/TPP), respectively. Skin penetration through full thickness newborn pig skin was more sustained in F4, followed by F7 and F10. Moreover the highest skin retention of arbutin was observed in F4. Stability of arbutin in the microemulsions was determined after storage at room temperature, 45°C, and 4°C for 1, 2, and 3 months. The stability studies indicated that F4 were stable for 3 months when stored at room temperature and 4°C (99.02%, and 100.76% arbutin content, respectively). At the elevated temperatures, improved stability was observed in F4 (94.57% arbutin content), compared with the arbutin content of 80.66%, 90.02%, and 88.46% in the arbutin solution, F7, and F10, respectively. Compatibility of plain F4 to melan-a melanocytes was found at the concentrations up to 0.1%. Effects of 250 μg/ml arbutin solution and plain F4 on tyrosinase activity were similar (84.32% and 82.92%, respectively). Significant stronger activity in reduction of melanin content was found in plain F4 (75.99% of control), compared with the 250 μg/ml arbutin solution (95.99% of control). Thus, F4 (w/o arbutin microemulsion with WSC/TPP nanoparticles) appeared to be an interesting delivery system of arbutin for skin whitening application.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
CO_2	Carbondioxide
r^2	correlation coefficient
et al.	Et alii, and others
FBS	Fetal bovine serum
g	Gram (s)
h	Hour (s)
i.e.	Id est (it is or that is)
kg	Kilogram (s)
ku	Kilounit
µg	Microgram (s)
µl	Microliter (s)
µm	Micrometer (s)
mg	Milligram (s)
mM	Millimolar
M	Molar
MW	Molecular weight
nm	Nanometer (s)
o/w	Oil in water
%	Percent
pH	The negative logarithm of the hydrogen ion concentration

LIST OF ABBREVIATIONS (continued)

rpm	Revolutions per minute
s	Second (s)
S.D.	Standard deviation
S.E.M.	Standard error of mean
K_m	Surfactant to co-surfactant mass ratio
TPP	Tripolyphosphate
VCO	Virgin coconut oil
v/v	Volume by volumne
w/o	Water in oil
WSC	Water soluble chitosan
w/w	Weight by weight

CHAPTER I

INTRODUCTION

Arbutin (hydroquinone-D-glucopyranoside), a glycosylated hydroquinone, has been used for the treatment of postinflammatory hyperpigmentation and as a skin whitening agent in pharmaceutical and cosmetic fields. High hydrophilicity of arbutin (logP value of -1.49) makes it difficult to penetrate across the stratum corneum, and reaches its site of action in the basal layer of epidermis, i.e. melanocytes (Wen *et al*., 2006). Attempts in development of delivery systems of arbutin have been published (Wen *et al*., 2006; Cho *et al*., 2007). Entrapment efficiency of liposomal formulations containing arbutin (between 4.35% and 17.63%) was depended on the lipid content of liposome formulation (Wen *et al*., 2006). Stabilization of liposomes in emulsion base was improved by addition of polymer (Cho *et al*., 2007). It was demonstrated that stability of phosphatidylcholine-cholesterol liposomes (PC-Chol liposomes) containing arbutin in o/w emulsion was improved by the addition of poly (methacrylic acid-co-stearyl methacrylate). It was revealed that after mixing of the polymer with liposomes in the o/w emulsion with different weight ratios, the size distribution of the resulting vesicle mixtures was determined to specify the stability of such liposomes. Moreover, skin permeation of arbutin from PC-Chol liposomes was higher than that of polymer-associated liposomes when compared with arbutin in phosphate buffer (Cho *et al*., 2007). However, the report did not cover the study of long-term stability of such systems, and the depigmentation effect of arbutin at the effective concentration in the skin.

Microemulsions are thermodynamic stable systems composed of oil, water, and surfactants, frequently in combination with co-surfactants (Kogan and Garti, 2006). They are of transparency, thermodynamic stability, easy formation, very small droplet size (typically<150 nm) and low viscosity (Kreilgaard, 2002). Recently, microemulsions have gained attentions more interesting attractive in both pharmaceutical and cosmetic fields as delivery systems of active substances via several routes such as oral (Gao *et al*., 1998), transdermal (Park *et al*., 2005) and topical (Peltola *et al*., 2003; Spiclin *et al*., 2001; Spiclin *et al*., 2003) delivery. Numerous advantages of microemulsions as the effective vehicle for skin have been reported (Delgado-Charro *et al*., 1997). Firstly, the microemulsions can improve the permeation of active compounds by their components acting as penetration enhancers. Moreover, they also provide the high entrapment efficiency of the actives in either oil or water phase (Dini *et al*., 2003). The microemulsion components in this study are non-irritant and pharmaceutical acceptable (Sintov and Shapiro, 2004).

Numerous evidences have focused on the application of polymer(s) in microemulsion systems (Biruss and Valenta, 2007; Biruss *et al*., 2007). Significant enhanced chemical stability of the progesterone in microemulsions after the addition of polymer (silicone dioxide and polymeric emulsifier) has been reported (Biruss and Valenta, 2007). The polymeric emulsifier improved the skin permeation and decreased the skin retention of progesterone compared to pure microemulsion (Biruss and Valenta, 2007). Moreover, Biruss *et al*. (2007) presented the results of the addition of polymeric emulsifier (Pemulen TR1) in microemulsions in improving the skin permeation rate and chemical stability of selected steroid hormones.

Chitosan is a biocompatible, bioadhesive, and non-toxic biopolymer. It has been widely known as the useful polymer in both pharmaceutical and cosmetic applications (Agnihotri *et al*., 2004). Currently, chitosan nanoparticles have widely been used as drug delivery carriers because of their improved efficacy, better stability, reduced toxicity, improved patient compliance, and mild process of preparation (Agnihotri *et al*., 2004). There have been many methods in preparation of chitosan nanoparticles, i.e. ionotropic gelation (Campos *et al*., 2001), emulsion-droplet coalescence technique (Tokumitsu *et al*., 1999), water-in-oil nanoemulsion system (Jia *et al*., 2005), and microemulsion method (Andersson and Lofroth, 2003). The microemulsion method was applied by Andersson and Lofroth (2003) to produce heparin/chitosan nanoparticles in water-in-oil (w/o) microemulsion for oral administration. The heparin/chitosan nanoparticles were occurred by the interaction between the positively charged chitosan and negatively charged heparin (Andersson and Lofroth, 2003).

Our study was focused on the development of microemulsions containing chitosan nanoparticles as novel delivery systems of arbutin for topical applications. The systems of water-in-oil (w/o), and bicontinuous type microemulsions containing arbutin, with and without water soluble chitosan (WSC, medium/high molecular weight) were developed, compared with the w/o/w emulsions. The microemulsion formulations were composed of virgin coconut oil, PEG-8 caprylic/capric glycerides (Labrasol®), polyglyceryl-6-dioleate (Plurol® Oleique), water soluble chitosan (WSC) and tripolyphosphate (TPP). Labrasol® and Plurol® Oleique have been reported as penetration enhancers to facilitate penetration of active compounds through the stratum corneum (Spiclin *et al*., 2001; Spiclin *et al*.,

2003; Djordjevic *et al*., 2004; Djordjevic *et al*., 2005). Chitosan and TPP in the inner aqueous phase may form a hydrophilic matrix by ionic gelation and, therefore, provide a temporary macromolecular network which the controlled release of active ingredient (s) may occurred (Dini *et al*., 2003). The scopes of this study were:

1. To develop the cosmetic microemulsions of arbutin containing chitosan nanoparticles.

2. To evaluate physico-chemical properties, arbutin release, skin permeation, and skin retention of cosmetic microemulsions of arbutin containing chitosan nanoparticles.

3. To evaluate the whitening activity of cosmetic microemulsions containing chitosan nanoparticles.

CHAPTER II

LITERATURE REVIEWS

1. Microemulsions

1.1 Phase behavior and properties of microemulsions

Microemulsions are the liquid delivery system composed of oil, water, and surfactants, frequently in combination with co-surfactants (Kogan and Garti, 2006). Such compositions are demonstrated in a phase diagram called ternary or pseudo-ternary phase diagram which contains three or more than three components, respectively (Narang *et al*., 2007). Fig. 1 shows the phase diagram of the oil/surfactant/water system on microemulsion phases in which the different structures are formed (Lawrence and Rees, 2000). In the microemulsion area, there are three basic types of microemulsions, i.e. water-in-oil (w/o), bicontinuous and oil-in-water (o/w) microemulsion as shown in Fig. 2. The w/o microemulsion is formed at the volume of water is low and water droplets are surrounded with surfactant dispersed in continuous oil phase. While the o/w microemulsion is formed at the volume of oil is low and oil droplets are coated with surfactant dispersed in continuous water phase. Whereas, the bicontinuous type microemulsion is formed at the volumes of water and oil are similar and contained dynamic intertwined networks of oil and water, with surfactants monolayers at the interfaces (Lawrence and Rees, 2000; Sathishkumar *et al*., 2008). Moreover, with the features of microemulsions, they are of transparence, thermodynamic stability, easy formation, very small droplets in size (typically < 150 nm) and usually with low viscosity, that caused them more interesting as drug delivery system (Kreilgaard, 2002).

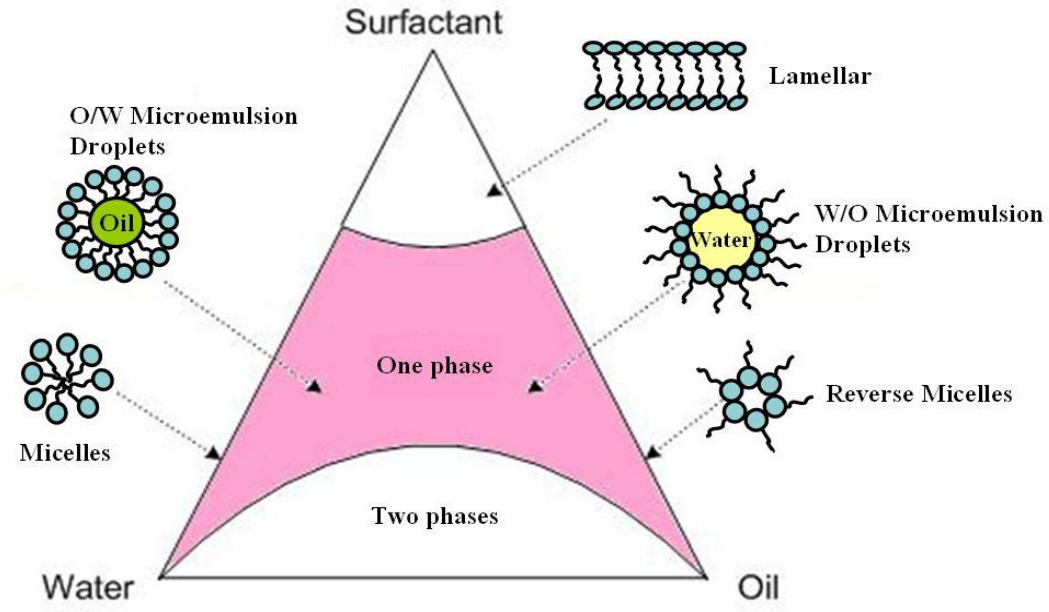


Figure 1 Hypothetical phase diagram of oil/surfactant/water systems on microemulsion and emulsion phases (adapted from Lawrence and Rees, 2000)

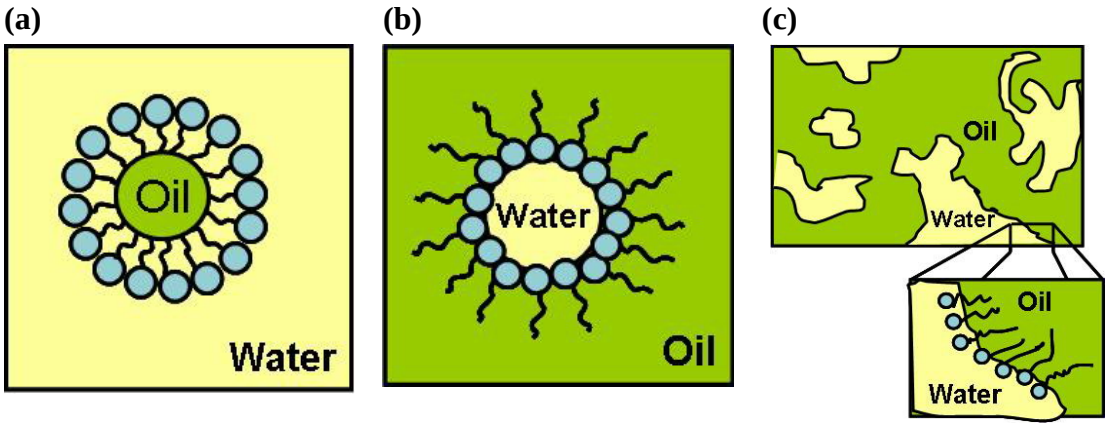


Figure 2 Schematic representations of microemulsions; (a) oil-in-water, (b) water-in-oil, and (c) bicontinuous microemulsions (adapted from Lawrence and Rees, 2000)

1.2 Microemulsions as transdermal and topical delivery system

Recently, in both pharmaceutical and cosmetic fields, many researchers reported the microemulsions as oral (Gao *et al*., 1998), parenteral (Nornoo *et al*., 2008), ocular (Chan *et al*., 2007), transdermal (Kweon *et al*., 2004; Park *et al*., 2005) and topical (Peltola *et al*., 2003; Spiclin *et al*., 2001; Spiclin *et al*., 2003) drug delivery system. Transdermal and topical delivery using microemulsions have gained more attention than other routes of administration because they offer numerous advantages such as patient compliance, reducing systemic side effect (Kweon *et al*., 2004; Park *et al*., 2005) and avoiding the hepatic first-pass metabolism (Chen *et al*., 2006). Several mechanisms have been reported for transportation of numerous substances via topical and transdermal route i.e., a large amount of drug can be included in the microemulsions system due to its high solubilization capacity, an increase in the transdermal flux can be expected in the affinity of the drug in microemulsion that can be modified to favor partitioning into the stratum corneum, the surfactant and co-surfactant in the microemulsion may act as penetration enhancer to reduce the diffusional barrier of the stratum corneum (Delgado-Charro *et al*., 1997; Park *et al*., 2005).

There are numerous substances, both hydrophilic and lipophilic substances, studied for transdermal and topical delivery using microemulsions. For the lipophilic substances, mostly, they are incorporated in the oily dispersed phase of o/w microemulsions; in contrast, the hydrophilic substances are included in the water dispersed phase of w/o type microemulsions. Whereas, in the case of bicontinuous type microemulsion, the hydrophilic part of the substances is diffused in the water phase and the lipophilic part is diffused in the oil phase (Sathishkumar *et al*., 2008).

Many studies reported the advantages of microemulsions as transdermal and topical delivery base on the types of microemulsions (w/o, bicontinuous and o/w microemulsion) that were implied to the specific type of microemulsions influencing to their properties as a drug delivery system (Bolzinger *et al*., 1998).

W/O type microemulsion is composed of water dispersed in the oily continuous phase. The advantage of this type is water phase can incorporate several hydrophilic substances either in dispersed or continuous phase. Spiclin *et al*. (2003) reported the incorporation of sodium ascorbyl phosphate either in the internal or external aqueous phase of w/o and o/w type microemulsions, respectively. Its stability was tested in both types of microemulsions. The result revealed that it was stable in both microemulsions with no significant influence of the location in the carrier system (Spiclin *et al*., 2003). The use of w/o type microemulsion as topical delivery is not only skin delivery but ocular delivery has been reported. Pilocarpine hydrochloride was incorporated in water dispersed phase of microemulsions as topical ocular delivery (Chan *et al*., 2007).

Furthermore, there were many studies reported the use of o/w type microemulsion as a potential topical/transdermal drug delivery system. Biruss *et al*. (2007) used polymeric emulsifier to improve the chemical stability of selected steroid hormones (Biruss *et al*., 2007). Rhee *et al*. (2001) reported that the different compositions of microemulsions effected to the permeation rate of ketoprofen (Rhee *et al*., 2001). Chen *et al*. (2004) presented that triptolide-loaded microemulsions showed the controlled, sustained and prolonged delivery compared with an aqueous solution. Additionally, a new o/w microemulsion system composed of 10% oleic acid, 60% Labrasol®/ethanol (1:5) as oil phase and surfactant/co-surfactant, respectively,

showed the improvement of skin permeation of piroxicam across the rat skins (Park *et al*., 2005). Moreover, the o/w microemulsion system consisting lauryl alcohol (oil), Labrasol® (surfactant) and ethanol (co-surfactant) was developed as transdermal delivery system of diclofenac diethylammonium and the percutaneous absorption of diclofenac diethylammonium was enhanced deu to the increasing of lauryl alcohol and water content (Kweon *et al*., 2004).

Many researchers often focused on the conventional microemulsions, w/o and o/w, that have the spherical shape, the certainly small droplet in size, and can incorporate the hydrophilic or lipophilic drugs in the inner droplets coated with surfactant. Whereas, bicontinuous type microemulsion is composed of both water and oil continuous phase that can incorporate the hydrophilic part of the substance in the water phase and lipophilic part in the oil phase (Sathishkumar *et al*., 2008). Bicontinuous type microemulsion offers numerous advantages such as the large amounts of water and oil can be stabilized by the small amounts of surfactants (Bolzinger *et al*., 1998), the drug loading might be increased due to the large amounts of water and oil in the system (Bolzinger *et al*., 1998), the toxicity of the system might be lowered due to the small amounts of surfactants (Sripriya *et al*., 2007) and such system can enhance the transdermal permeation by the wetting property of surfactants (Bolzinger *et al*., 1998). Bicontinuous type microemulsion-based transdermal and topical delivery system has been reported. Carlfors *et al*. (1991) reported the enhancement of lidocaine through the skin using bicontinuous type microemulsion (Carlfors *et al*., 1991). Bicontinuous type microemulsion containing various sucrose esters as co-surfactant was investigated. Such system showed very low interfacial tension that associated to the wetting properties facilitating to the

transdermal drug delivery system (Thevenin *et al*., 1996). Bolzinger *et al*. (1998) showed that niflumic acid was concentrated in the interfacial film and the results revealed that the 1% niflumic acid based bicontinuous type microemulsion was as effective as the 3% niflumic acid ointment (Nifluril®) (Bolzinger *et al*., 1998). Thus, the bicontinuous type microemulsion could offer the transdermal and topical drug delivery system.

2. Skin

The skin which presents more than 10% of body mass is the largest part of body. It consists of the epidermis, the dermis and the subcutis or subcutaneous tissue. Additionally, each layer is connected with appendages i.e. hair follicles, sweat ducts and sebaceous glands. The major function of the skin is to prevent the loss of endogenous substances such as water to the environment and to protect the skin from the exogenous substances (Glombitza and Muller-Goymann, 2002). The structure of mammalian skin is shown in Fig.3 and described as follows (Mills and Cross, 2006);

2.1 The epidermis

The epidermis is composed of keratinocyte and non-keratinocytes cells. Among the keratinocyte cells, it is divided into five layers which are arranged from the innermost to the uppermost layer of epidermis as follows; stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum. The new keratinocyte cells are produced in the stratum basale, and then differentiated and migrated towards the surface (stratum corneum). Thereafter, they exhibit a turnover every two to three weeks. Whereas, the non-keratinocyte cells contained in the epidermis are melanocyte cells, Langerhans cells and Merkel cells. Moreover, the arrangement of sphingolipids between keratinocyte cells prevents water loss from skin to the circumstance and the penetration of substances through skin.

2.2 The dermis

The dermis which is inner than the epidermis is composed of connective tissue, blood vessels, nerve endings, hair follicles, and sweat and oil glands. There are various cell types in the dermis, especially fibroblast cells. Moreover, mast cells, inflammatory cells and immune cells are also found in the

dermis. The connective tissue gives the skin flexible and strength due to the presence of collagen and elastin. Additionally, blood vessels provide nutrients to the skin and help the regulation of body temperature. Furthermore, the nerve endings are sensitive to pain, touch, pressure and temperature and their function is to response to such stimulation.

2.3 The subcutis

The subcutis or subcutaneous tissue or hypodermis is the fat layer containing mostly adipocytes to help the body avoiding from the change in temperatures. Moreover, it carries the vessels and nerves to supply the skin and connects to the dermis via collagen and elastin fibers.

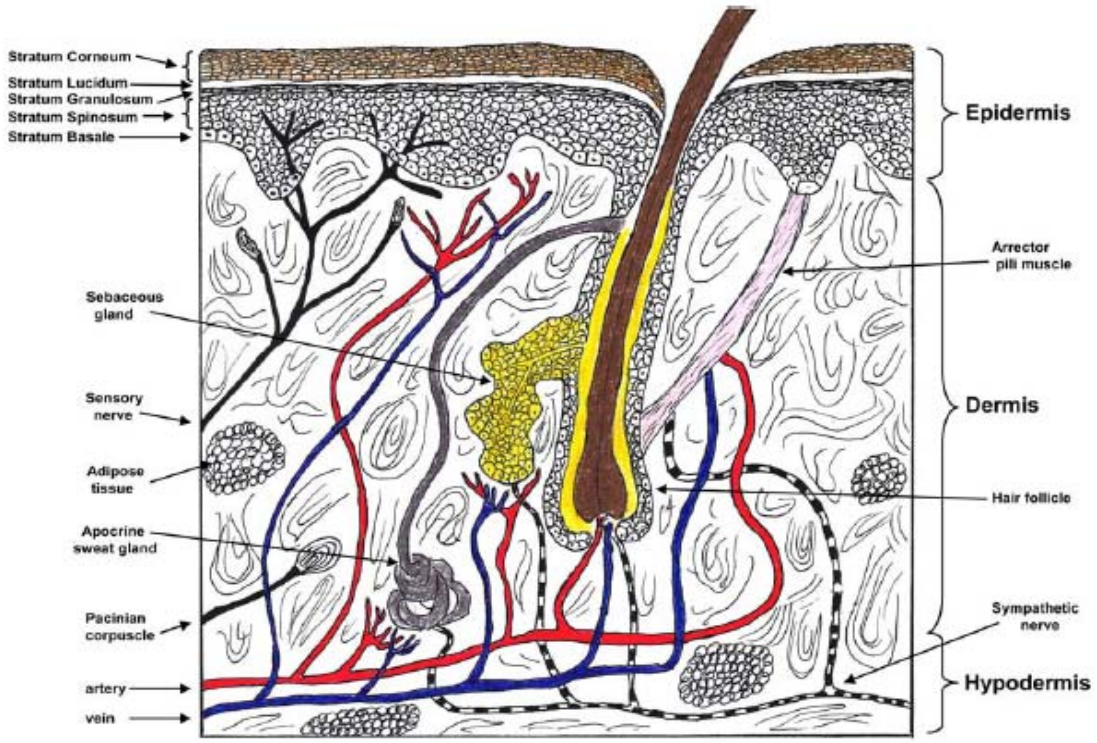


Figure 3 Structure of mammalian skin (Mills and Cross, 2006)

3. Skin whitening agents

In recent year, skin whitening agents have gained more attention since hyperpigmentation is an important problem associated with the exposure to ultraviolet ray and drugs or chemicals. The depigmenting mechanism of skin whitening agents can be generally divided into three types as follows: the suppression of the formation of tyrosinase (Action A), the inhibition of the activity of tyrosinase (Action B) and the direct reduction of melanin (Action C) as shown in Fig.4 (Zuidhoff and Rijsbergen, 2001). The suppression of the formation of tyrosinase is shown as the activity of a few active ingredients i.e. L(+) lactic acid and its lactate salts. Furthermore, there are many skin whitening agents that inhibit the tyrosinase activity. Mostly, they are extracted from natural sources i.e. bearberry leaves (arbutin), licorice root (glabridin), citrus fruits (ascorbic acid) and fermented carbohydrate (kojic acid). Finally, the activity of skin whitening agent to the direct reduction of melanin is presented in hydroquinone which shows the denaturation and death of pigment cells. Such skin whitening agents as demonstrated previously is discussed again as follows;

L (+) lactic acid belongs to the group of alpha hydroxyl acids. It is changed to its salt form (sodium lactate or other salts of lactic acid) in the lower pH (approximately 5-5.5). It has been reported to suppress the formation of tyrosinase (Smith, 1999; Zuidhoff and Rijsbergen, 2001). In addition to such activity, the lower pH (5-5.5) as mentioned previously shows the exfoliate action of lactic acid and its salt form by accelerating the transit of epithelial cells, some may carry melanin, from the lower epidermis to the surface resulting in the reduction of melanin in the epidermis (Smith, 1999; Zuidhoff and Rijsbergen, 2001). Not only the exfoliate action, moreover, Usuki *et al*. (2003) reported the activity of lactic acid with the

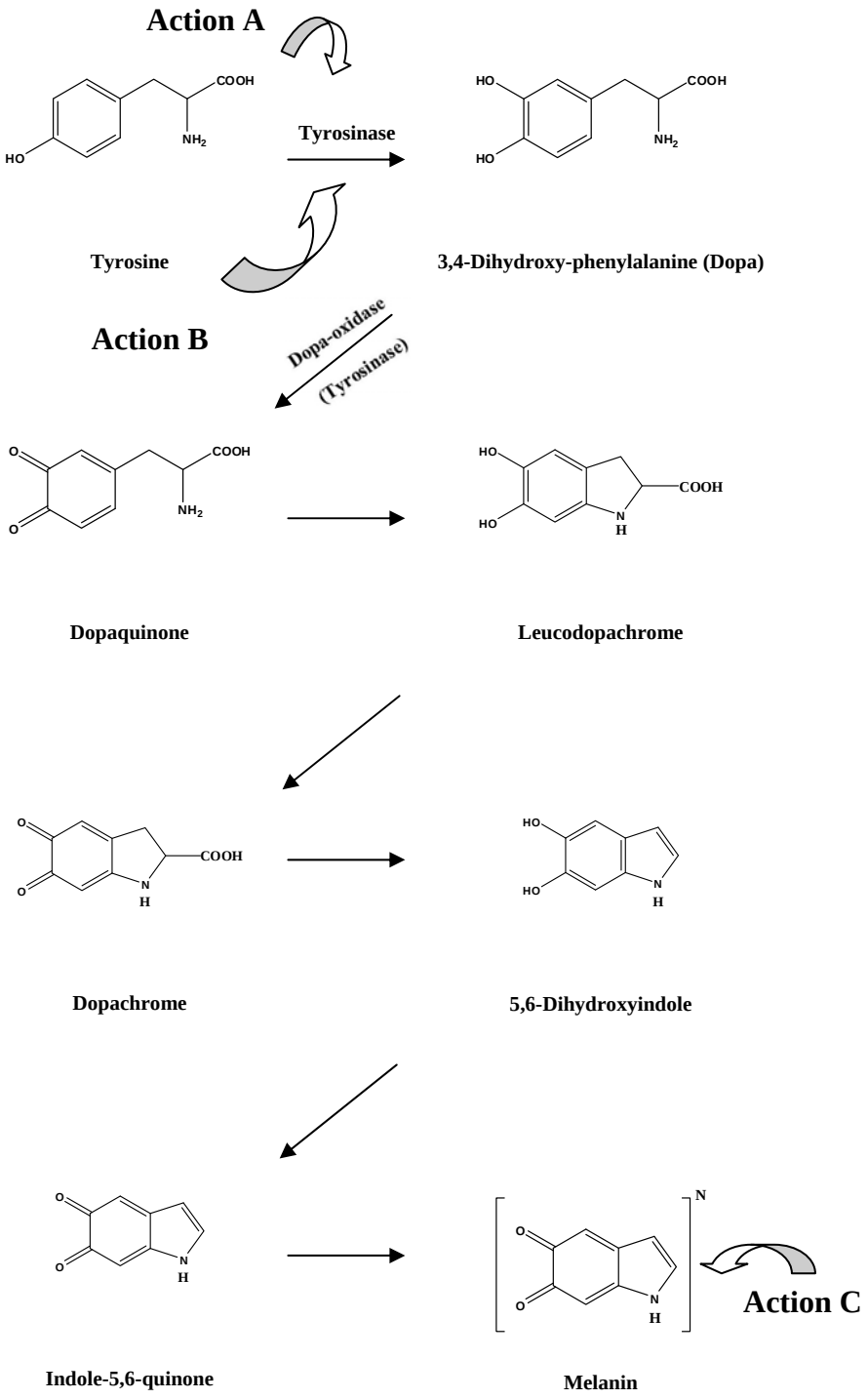


Figure 4 Mechanism of skin whitening action (Zuidhoff and Rijsbergen, 2001)

various actions as follows; direct inhibitors of melanin formation without affecting cell growth, inhibition of tyrosinase activity without affecting the mRNA and protein expression or molecular size of tyrosinase, TRP-1 and TRP-2, direct inhibition of tyrosinase activity without the influence of acidic condition and suppression of melanin formation by directly inhibiting tyrosinase activity (Usuki *et al*., 2003).

Licorice extract is obtained from the root of *Glycyrrhia Glabra Linneva* and its active ingredient is glabridin. Glabridin inhibits the melanin formation by inhibiting tyrosibase activity. The depigmenting effect of glabridin has been reported to be 16 times greater than that of hydroquinone, however, the inhibition of tyrosinase activity of licorice extract has been showed to be higher than that of glabridin extract (Zuidhoff and Rijsbergen, 2001; Rendon *et al*., 2005; Parvez *et al*., 2006).

Ascorbic acid or vitamin C has been used as cosmetic and pharmaceutical ingredient since it has variety of actions on human health. As skin whitening agent, vitamin C effects the melanin production by interacting with copper ions at the active site of tyrosinase enzyme resulting in the inhibition of tyrosinase activity. In addition to its role in skin, vitamin C shows the antioxidant activity by scavenging the reactive oxygen species (ROS). Moreover, vitamin C improves the skin elasticity and reduces wrinkles by stimulating collagen systhesis (Zuidhoff and Rijsbergen, 2001; Spiclin *et al*., 2003; Rendon *et al*., 2005).

Kojic acid is a natural substance produced by some species of fungi. Its tyrosinase inhibiting effect is due to the chelating copper at the active site of tyrosinase enzyme. In addition to the iron-chelating activity of kojic acid, it is found that kojic acid showed the anti-wrinkle activity of the skin which is due to chronic

photodamage. Moreover, kojic acid has been reported to be the free radical scavenger which such reactive oxygen species are produced from cells, tissue or blood (Mitani *et al*., 2001; Rendon *et al*., 2005; Parvez *et al*., 2006).

Hydroquinone is obtained from various plants such as coffee, tea, beer and wine. It is the most effective skin whitening agent but it shows the serious side effects which make hydroquinone is prohibited in several countries, inclusively in Thailand. Its common side effects are skin irritation or contact dermatitis and its serious side effects are the development of ochronosis, a sooty hyperpigmentation in the treatment area which may be difficult to reverse. Therefore, many physicians use hydroquinone combined with steroid drug after such side effects have occurred. Moreover, hydroquinone showed the cytotoxicity to melanocytes, thus, it should be more considerable as a potent melanocytes cytotoxic agent (Rendon *et al*., 2005; Parvez *et al*., 2006).

The active ingredient used in this study was arbutin. Arbutin (hydroquinone-D-glucopyranside), as shown in Fig.5, is a glycosylated hydroquinone and extracted from bearberry leaves. Its chemical formula is $C_{12}H_{16}O_7$, its molecular weight is 272.25, its stable pH is around 5-7 and its maximum wavelength is 267 nm (Couteau and Coiffard, 2000). It is a yellow powder and soluble in water (clear to very slightly hazy colorless to faint yellow solution at 200 mg plus 4 ml of water). It is not an irritant for skin and eyes. Moreover, it is a photostable but a thermodegradable substance which can be incorporated in various formulations at room temperature (Couteau and Coiffard, 2000). In both the pharmaceutical and cosmetic fields, it has been used as the treatment of postinflammatory hyperpigmentation and the skin whitening agent, respectively. Maeda and Fukada

(1996) showed the depigmenting mechanism of arbutin in humans involves inhibition of melanosomal tyrosinase activity, rather than suppression of the expression and synthesis of tyrosinase (Maeda and Fukuda, 1996). Moreover, arbutin inhibits the tyrosinase activity of cultured human melanocytes at non-cytotoxic concentrations and is much less cytotoxic than hydroquinone to cultured human melanocytes (Maeda and Fukuda, 1996). Furthermore, arbutin can act as antioxidant by scavenging free radicals (Ioku *et al*., 1992). Additionally, arbutin shows the high hydrophilicity (logP value, -1.49) (Wen *et al*., 2006) that makes it difficult to pass through the barrier of skin, stratum corneum, and reach its site of action, melanocytes that is in the basal layer of epidermis as shown in Fig.6. There are many researchers attempted to develop the delivery system for arbutin. Wen *et al*. (2006) developed the liposomal formulations contained arbutin. The result showed that its entrapment efficiency was low (between 4.35% and 17.63% depended on the lipid content of each formulation). Additionally, Cho *et al*. (2007) demonstrated that the stability of phosphatidylcholine (PC)-cholesterol (Chol) liposomes containing arbutin in o/w emulsion could be improved by the addition of polymer, poly (methacrylic acid-*co*-stearyl methacrylate). The result revealed that after mixing each type of such liposome in the o/w emulsion with different weight ratio, the difference of size distribution of the resulting vesicle mixtures was determined to specify the stability of such liposome. Moreover, the skin permeation of arbutin from PC-Chol liposomes was higher than that of polymer-associated liposomes when compared with arbutin in phosphate buffer (Cho *et al*., 2007).

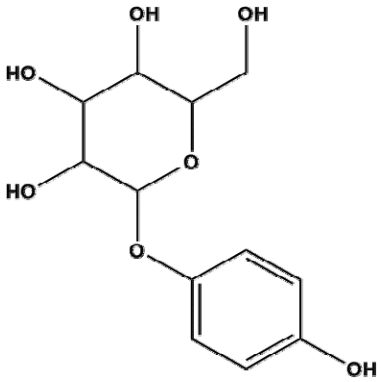


Figure 5 Chemical structure of arbutin

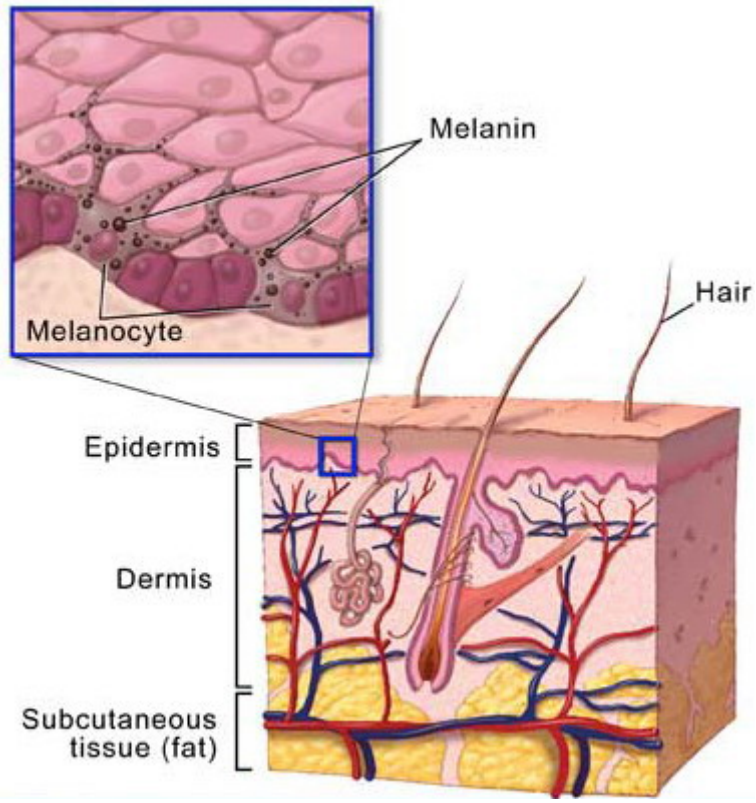


Figure 6 Melanocytes in the basal layer of epidermis (http://www.revolutionhealth.com/conditions/skin/skin-care/sun-exposure/sun-damage)

4. Chitosan

Chitosan which is poly[β-(1–4)-2-amino-2-deoxy-D-glucopyranose] is a linear cationic polymer consisting of β (1–4)-linked 2-amino-2-deoxy-D-glucose (D-glucosamine) and 2-acetamido-2-deoxy-D-glucose (N-acetyl-D-glucosamine) units, as shown in Fig.7a. It has shown that the properties, biodegradability and biological role of chitosan are due to the proportions of N-acetyl-D-glucosamine and D-glucosamine residues (George and Abraham, 2006). The process to obtain chitosan is shown in Fig.8 (Hejazi and Amiji, 2003). Briefly, chitosan is obtained by treating chitin with concentrated alkali solution resulting in the conversion of acetamide group of chitin to amino group of chitosan. The physicochemical and biological properties of chitosan are summarized in Table 1 (Hejazi and Amiji, 2003). Chitosan is a cationic polymer and is insoluble in water in neutral or basic pH depending on its free amino groups. While, in acidic pH, the amino groups of chitosan are protonated and makes it soluble in water. Hence, a number of free amine groups are effected for cross-linking, since cations allow for ionic cross-linking of anions (Agnihotri *et al*., 2004). Additionally, it is a biocompatible, bioadhesive and non-toxic biopolymer which is widely known as the useful polymer in both pharmaceutical and cosmetic applications. Chitosan shows low oral toxicity with the LD_{50} in rats of 16 g/kg. The pharmaceutical requirements of chitosan are: particle size < 30 μm, density between 1.35 and 1.40 g/cm^3, pH 6.5-7.5, insoluble in water, and partially soluble in acids (Hejazi and Amiji, 2003).

Currently, chitosan nanoparticles have been widely used as drug carriers because of their improved efficacy, better stability, reduced toxicity, improved patient compliance and simple and mild preparation method (Agnihotri *et*

al., 2004; Wu *et al*., 2005). There are many methods to prepare chitosan nanoparticles such as ionotropic gelation (Campos *et al*., 2001), emulsion-droplet coalescence technique (Tokumitsu *et al*., 1999), water-in-oil nanoemulsion system (Jia *et al*., 2005), complex coacervation process (Mao *et al*., 2001) and microemulsion method (Andersson and Lofroth, 2003). However, the ionotropic gelation is the most preferable method, since it is a simple and mild method. Tripolyphosphate (TPP) has been widely used as cross-linking agent because TPP is a non-toxic cross-linking agent preferred over other toxic cross-linking agents (formaldehyde or gluteraldehyde) (Desai and Park, 2005; Deai *et al*., 2006). The obtained result is the cross-linked hydrophilic polymers provide a temporary or permanent macromolecular network through which the diffusional release of active ingredients occurs (Dini *et al*., 2003). In addition, chitosan may be cross-linked with TPP through its amino groups as shown in Fig.7b (Lee *et al*., 2001). The numerous advantages of chitosan nanoparticles prepared by ionotropic gelation method have been published as drug delivery vehicle. Campos *et al*. (2001) prepared cyclosporin A-loaded chitosan nanoparticles as a new vehicle for ocular drug delivery (Campos *et al*., 2001). The oral adsorption of ammonium glycyrrhizinate was improved upon the incorporation of ammonium glycyrrhizinate into the stable chitosan nanoparticles for the treatment of chronic hepatitis (Wu *et al*., 2005). Katas and Alpar (2006) showed that chitosan-TPP nanoparticles were much potential as siRNA delivery vehicle for diseases with genetic defects (Katas and Alpar, 2006). For nasal application, chitosan nanoparticles could improve the systemic adsorption of insulin compared with insulin-loaded chitosan solution (Fernandez-Urrusuno *et al*., 1999). Moreover, tetanus toxoid was entrapped within chitosan nanoparticles for nasal vaccine delivery (Vila *et al*., 2004).

Tang *et al*. (2007) presented the advantage of chitosan nanoparticles as the immobilization of neutral lipase enzyme (Tang *et al*., 2007). Additionally, the caspase inhibitor, Z-DEVD-FMK, was encapsulated within chitosan nanoparticles for targeted delivery to the CNS (Aktas *et al*., 2005).

Table 1 Physicochemical and biological properties of chitosan (Hejazi and Amiji, 2003)

Physicochemical property	Biological property
Cationic polyamine	Biocompatibility
High charge density at pH<6.5	Natural polymer
Adheres to negatively charged surfaces	Biodegradable to normal body
Forms gels with polyanions	Safe and non-toxic
High molecular weight linear	Hemostatic, bacteriostatic, and fungistatic
Viscosity, high to low	Spermicidal
Chelates certain transitional metals	Anticancerogen
Amiable to chemical modification	Anticholesteremic
Reactive amino/hydroxyl groups	Reasonable cost

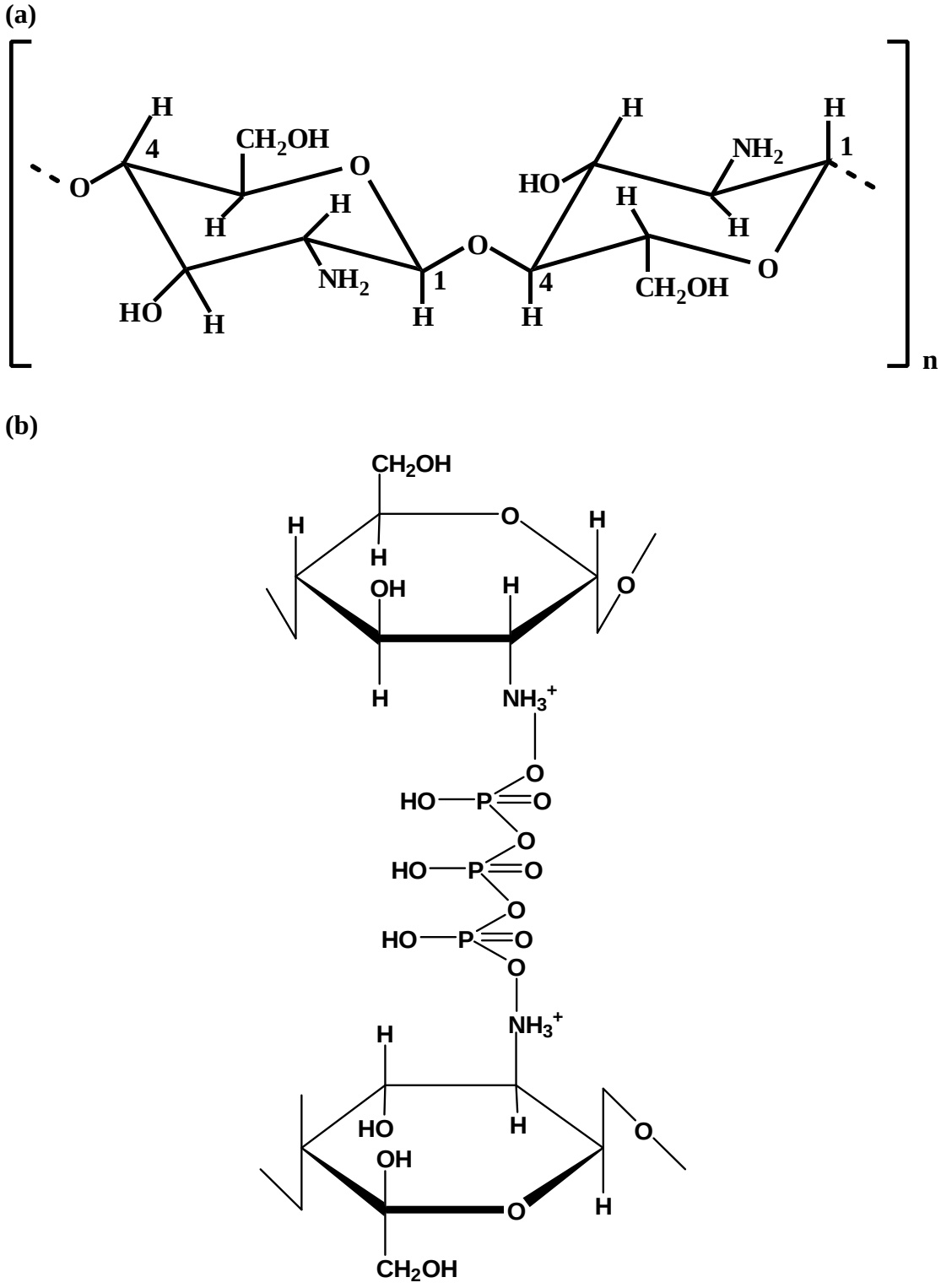


Figure 7 (a) Chemical structure of chitosan (George and Abraham, 2006) and (b) Ionic gelation of chitosan and tripolyphosphate (Lee *et al*., 2001)

Shellfish wastes from food processing (shrimp, crab, squid, lobster)

↓

Deproteination in 3-5%w/v NaOH aqueous solution at 80-90°C for a few hours or at room temperature overnight

↓

Decalcification in 3-5%w/v HCl aqueous solution at room temperature giving a white to beige colored sample of chitin

↓

Deacetylation in an aqueous 40–45%w/v NaOH solution at 90-120°C for 4-5 hours resulting in *N*-deacetylation of chitin (chitosan)

↓

Determination of the polymer molecular weight and the degree of deacetylation by dissolving in aqueous 2%w/v acetic acid to give a clear supernatant solution

↓

Neutralization with NaOH solution resulting in a purified sample of chitosan as a white precipitate

↓

Preparation of medical and pharmaceutical grade chitosan by further purification

Figure 8 Preparation of chitin and chitosan (adapted from Hejazi and Amiji, 2003)

5. Virgin coconut oil (VCO)

Coconut oil contains mostly saturated fatty acids and is usually obtained from copra by dry process. The copra will expose to very high temperatures or sunlight for several days until the moisture is all removed. The exposure to sunlight or high temperatures during dry process may inactivate the minor components such as provitamin A, tocophenols, tocotrienols and polyphenols. Whereas, VCO is obtained from coconut milk by wet process under a controlled temperature. Thus, VCO may have more beneficial effects than coconut oil because of its unsaponifiable components (Nevin and Rajamohan, 2004; Nevin and Rajamohan, 2008). The fatty acid composition of VCO is shown in Table 2.

Table 2 Fatty acid composition of virgin coconut oil (Nevin and Rajamohan, 2008)

Common name of Fatty acids	Fatty acids	Percentage amounts
Caprylic acid	C8:0	8.05
Capric acid	C10:0	5.42
Lauric acid	C12:0	45.51
Myristic acid	C14:0	19.74
Palmitic acid	C16:0	7.83
Stearic acid	C18:0	3.14
Oleic acid	C18:1	4.70
Linoleic acid	C18:2	1.88
Arachidic acid	C20:0	0.086
Eicosenic acid	C20:1	0.027
Behenic	C22:0	0.016
Lignoceric	C24:0	0.032

6. Surfactants and co-surfactants

As the surfactant forms a film at the interface that separates the water and oil domains, the presence of the co-surfactant is often required to lower the interfacial tension of this interface because a low interfacial tension is essential for the formation of microemulsions (Mehta and Bala, 2000). The hydrophobic interactions between the phases, thereafter, are reduced and a stable emulsion is obtained (Lee *et al*., 2003). Additionally, they can be divided into two types, ionic surfactants and non-ionic surfactants. Non-ionic surfactant is selected to minimize skin irritation and charge disruption of the system (Lee *et al*., 2003).

Surfactant and co-surfactant used in this study was PEG-8 caprylic/capric glycerides (Labrasol®) and polyglyceryl-6-dioleate (Plurol® Oleique), respectively. The specification of Labrasol®and Plurol® Oleique is summarized in Table 3 and 4, respectively. Labrasol® is a mixture of 30% mono-, di and triglycerides of saturated C_6-C_{14} fatty acids (predominantly C_8 and C_{10} fatty acids), 50% of mono- and di-esters of poly(ethylene glycol) (PEG 400) and 20% of free PEG 400. (Kreilgaard *et al*., 2000; Djekic and Primorac, 2008). It is oily liquid and it shows high tolerance and low toxicity in animals (LD_{50} 22 g/kg in rats, Hu *et al*., 2001). Whereas Plurol® Oleique is viscous liquid; its specified pH is 7.0-9.5 and it shows LD_{50} more than 20 g/kg (Gattefosse® data sheet, 2002). They are non-ionic surfactants that are selected to minimize skin irritation and charge disruption of the system (Lee *et al*., 2003). Moreover, microemulsions containing Labrasol® and Plurol® Oleique as surfactant and co-surfactant, respectively, have been reported as the composition for topical formulation (Spiclin *et al*., 2001; Spiclin *et al*., 2003; Djordjevic *et al*., 2004; Djordjevic *et al*., 2005).

Table 3 Specification of Labrasol®

Test	Specifications
Appearance	Oily liquid
Odour	Faint
Colour (Gardner scale)	< 2.5
Specific gravity at 20°C (D20/4)	1.060 to 1.070
Refractive index at 20°C	1.450 to 1.470
Viscosity at 20°C	80 to 110 mPa.S
Acid value	< 2.00 mgKOH/g
Saponification value	85 to 105 mgKOH/g
Iodine value	< 2.0 gI_2/100g
Hydroxyl value	170 to 205 mgKOH/g
Peroxide value	< 6.0 meqO_2/Kg
Alkaline impurities	< 80 ppm NaOH
Water content	< 1.00%
Free glycerol content	< 5.0%
Caproic acid (C6)	< 2.0%
Caprylic acid (C8)	50.0 to 80.0%
Capric acid (C10)	20.0 to 50.0%
Lauric acid (C12)	< 3.0%
Myristic acid (C14)	< 1.0%
Total ashes content	< 0.10%
Heavy metals content	< 10 ppm
Ethylene oxide content	< 1 ppm
1.4 Dioxane content	< 10 ppm

Table 4 Specification of Plurol® Oleique

Test	Specifications
Appearance	Viscous liquid
Colour (gardner scale)	< 14.0
Refractive index at 20°C	1.470 to 1.490
pH (at 10% in water)	7.0 to 9.5
Acid value	< 6.00 mgKOH/g
Saponification value	110 to 140 mgKOH/g
Iodine value	50.0 to 70.0 gI_2/100g
Peroxide value	< 12.5 $meqO_2$/Kg
Water content	< 1.00%
Sulphated ashes content	< 1.0%
Heavy metals content	< 10 ppm Pb

CHAPTER III

MATERIALS AND METHODS

MATERIALS

1. Acetonitrile (J.T.Baker, Inc., USA)
2. Arbutin (4-Hydroxyphenyl-β-D-glucopyranoside) (Sigma®, Inc., USA)
3. Cellulose acetate membrane (Spectra/Por®3 Dialysis Membrane, MWCO 3,500 Dalton, USA)
4. 3,4-Dihydroxyl-L-phenylalanine (L-DOPA) (Sigma-Aldrich®, Inc., USA)
5. Dimethyl sulfoxide (Sigma®, Inc., USA)
6. di-Sodium hydrogen orthophophate anhydrous (Ajax Finechem®, Australia)
7. Fetal bovine serum (FBS) (Gibco™, Inc., USA)
8. Labrasol® [caprylocaproyl macrogolglycerides (polyoxylglycerides)] (Gattefosse, France)
9. Methanol (J.T.Baker, Inc., USA)
10. Pentasodium tripolyphosphate hexahydrate (TPP) (Sigma-Aldrich®, Inc., USA)
11. Phenylthiourea (PTU) (Sigma®, Inc., USA)
12. Phorbol 12-myristate 13-acetate (PMA) (Sigma®, Inc., USA)
13. Plurol® Oleique (polyglyceryl-6-dioleate) (Gattefosse, France)
14. Potassium dihydrogen orthophosphate (Ajax Finechem®, Australia)
15. RPMI medium 1640 (Gibco™, Inc., USA)
16. Sodium bicarbonate (Sigma®, Inc., USA)

17. Sodium chloride (Labscan Asia Co., Ltd., Thailand)
18. Sodium dihydrogen orthophosphate (Ajax Finechem®, Australia)
19. Sodium hydroxide (Ajax Finechem®, Australia)
20. Thiazolyl blue tetrazolium bromide (MTT) (Sigma-Aldrich®, Inc., USA)
21. 0.25% Trypsin/EDTA (Gibco™, Inc., USA)
22. Tyrosinase from mushroom (Sigma®, Inc., USA)
23. Water soluble chitosan (MW 100 and 200 kDa)

EQUIPMENTS

1. Autoclave (HV-110, Hirayama Co., Ltd., Japan)
2. Centrifuge (Hermle®, model Z323K, Germany)
3. Chromatography column (Water µBondapak™ C18, 3.9 mm ¥ 300 mm, Ireland)
4. Conductivity meter (Kyoto Electronics, model CM-115, Japan)
5. Differential Scanning Calorimeter (Perkin Elmer®, model TGA7, USA.)
6. Disposable syringe 5 ml (Nipro®, Thailand)
7. Electrical balance (Satorious BP 300 S, Germany)
8. Eppendorf (Easypet® 4421, Germany)
9. Eppendorf (Eppendorf Multipette® 4780, Germany)
10. High pressure liquid chromatography
    - Binary pump (Waters™ 600 HPLC Pump, USA.)
    - Auto-injector (Waters 717 plus Auto-sampler, USA.)
    - UV-VIS detector (Waters™ 486 Tunable Absorbance Detector, USA.)
    - Data printer (Waters 746 Data Module, USA.)
11. Homogenizer (Polytron®, model PT-DA 3012, Switzerland)
12. Laminar air flow hood (Jouan, Thailand)
13. Micropipette (Pipetman®, France) (1000, 200 and 20 µl)
14. Microplate reader (Biotek, model Power Wave X, USA.)
15. Modified Franz diffusion cell (Hanson® model 57-6M, USA.)
16. Multichanel micropipette (Socorex, Swisszerland)
17. Multiple stirrer (IKA®-Werke, model RO5 Power, Germany)

18. pH meter (Orion®, model SA520, USA.)
19. Single use syringe filter (Minisart Sartorious, Germany)
20. Transmission electron microscope (Jeol®, model JEM 2010, Japan)
21. UV spectrophotometer (Spectronic® Genesys™ 5, USA.)

METHODS

1. Preparation of virgin coconut oil

Virgin coconut oil was prepared by cold press method following Oungbho *et al*. (2007) (Oungbho *et al*., 2007). The virgin coconut oil was qualified following the Thai National Standards (Bureau of Product Standards) for coconut oil.

2. Microemulsions preparation

2.1. Construction of pseudo-ternary phase diagrams

The pseudo-ternary phase diagrams were constructed using virgin coconut oil, Labrasol®, Plurol® Oleique and chitosan solution as oil, surfactant, co-surfactant and water phase, respectively. The ratio of Labrasol®:Plurol® Oleique was firstly fixed with 1:1 and then varied from 1:1 to 2:1, 3:1 and 4:1, respectively, to find the appropriate ratios of Labrasol®:Plurol® Oleique. Labrasol® and Plurol® Oleique were weighed as desirable ratios into a screw-cap glass vial. The mixture of surfactant and co-surfactant was vortexed and left for 1 hour for reaching equilibrium. Chitosan (water soluble chitosan (WSC); medium (100 kDa) and high (200 kDa) MW) solution at the concentration of 0.5% was used as water phase. Then, virgin coconut oil was weighed with the ratios of virgin coconut oil:the mixture of surfactant and co-surfactant from 1:9 to 9:1 (1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1) and vortexed for 1 minute. The mixture of virgin coconut oil, surfactant and co-surfactant was called oil phase. Oil phase was titrated with 0.5% WSC solution at room temperature until the isotropic solution presented. The results were plotted in pseudo-ternary phase diagram by SigmaPlot version10.0 (Systat Software, Inc., Germany).

2.2. Preparation of nanoparticle-based microemulsions

After the microemulsion regions in the phase diagrams were identified, the nanoparticle-based microemulsions were formulated. The compositions of such formulations are summarized in Table 5.

Table 5 Compositions of microemulsion systems

Formulation	System	K_m value	Phase composition				Water phase		
		Labrasol:Plurol oleique	Surfactant	Water	VCO	F10	Chitosan [%]	TPP [%]	MW of chitosan
F 1	w/o ME	2:1	75	10	15	0	0.250	0.083	medium
F 2	w/o ME	3:1	75	10	15	0	0.250	0.083	medium
F 3	w/o ME	4:1	75	10	15	0	0.250	0.083	medium
F 4	w/o ME	2:1	65	15	20	0	0.250	0.083	medium
F 5	w/o ME	2:1	65	15	20	0	0.175	0.058	medium
F 6	w/o ME	2:1	65	15	20	0	0.100	0.033	medium
F 7	w/o ME	2:1	65	15	20	0	0.250	0.083	high
F 8	w/o ME	2:1	65	15	20	0	0.175	0.058	high
F 9	w/o ME	2:1	65	15	20	0	0.100	0.033	high
F10	w/o ME	2:1	65	15	20	0	0.000	0.000	-
F11	bicontinuous ME	2:1	60	30	10	0	0.100	0.033	medium
F12	bicontinuous ME	2:1	60	30	10	0	0.000	0.000	-
F13	w/o/w emulsions	2:1	60	30	0	10	0.100	0.033	medium
F14	w/o/w emulsions	2:1	60	30	0	10	0.000	0.000	-

The types of selected formulations were w/o type microemulsion, bicontinuous type microemulsion and w/o/w emulsions. Firstly, w/o type microemulsion was formulated as shown in F1 to F10. The ratio of surfactant:oil and 0.5% WSC (medium and high MW) solution was fixed as described in Table 5. The WSC solution was firstly dissolved together with arbutin in the internal aqueous phase. Thereafter, tripolyphosphate (TPP) was used as cross-linking agent and added at the chitosan:TPP ratio of 3:1 under magnetic stirring. The preparation of w/o type microemulsion containing arbutin nanoparticles in the inner aqueous phase is shown in Fig.9. Arbutin (4-Hydroxyphenyl-β-D-glucopyranoside) was added in the inner

aqueous phase with a final concentration of 0.05%w/w (for *in vitro* release study) and 0.5%w/w (for other studies). It has to be emphasized that the efficiency of the encapsulated arbutin in the microemulsions is expected to be 100% because of arbutin that is a hydrophilic substance thus completely insoluble in the oil phase (Dini *et al*., 2003).

Moreover, bicontinuous type microemulsion was prepared as presented in F11 and F12. The compositions of such system are shown in Table 5. The order of mixing was as similar as the preparation of w/o type microemulsion presented in Fig.9. Briefly, WSC (medium and high MW) solution and arbutin concentration of 0.05%w/w (for *in vitro* release study) and 0.5%w/w (for other studies) were added in the aqueous phase after the equilibrium of the mixture of oil and surfactants was reached. Then, TPP was added under magnetic stirring to form WSC nanoparticles.

Finally, the preparation of w/o/w emulsions as represented in F13 and F14 is shown in Fig.10. Its compositions are presented in Table 5. After the w/o type microemulsions occurred, the w/o type microemulsions was assumed as the oil phase dispersing in water phase of bicontinuous type microemulsion for preparing the w/o/w emulsions.

Additionally, Fig.11 shows the schematic of pure w/o and bicontinuous type microemulsions (Fig.11a and b, respectively), and w/o and bicontinuous type microemulsions containing chitosan nano/microparticles (Fig.11c and d, respectively). In the case of pure microemulsions (Fig.11a and b), it is attributed that the phenol ring of arbutin prefers to insert in hydrophobic tail of non-ionic surfactant and the sugar part of arbutin prefers to exist in hydrophilic head group of non-ionic surfactant (Hincha *et al*., 1999). Whereas, microemulsions containing chitosan

nano/microparticles are hypothesized that arbutin, a hydrophilic substance, prefers to incorporate within hydrophilic matrix of chitosan nano/microparticles as shown in Fig.11c and d.

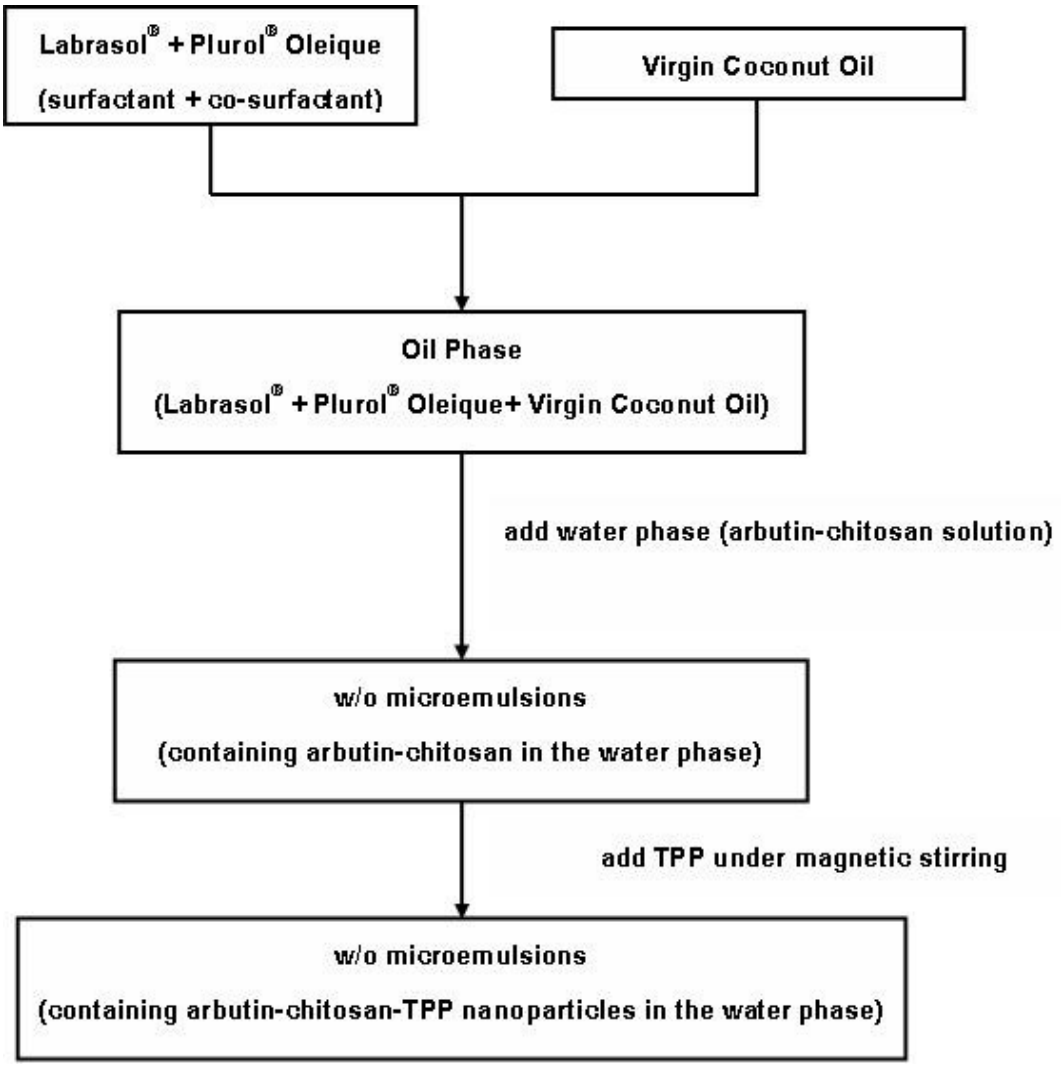


Figure 9 Preparation of w/o type microemulsions containing arbutin nanoparticles

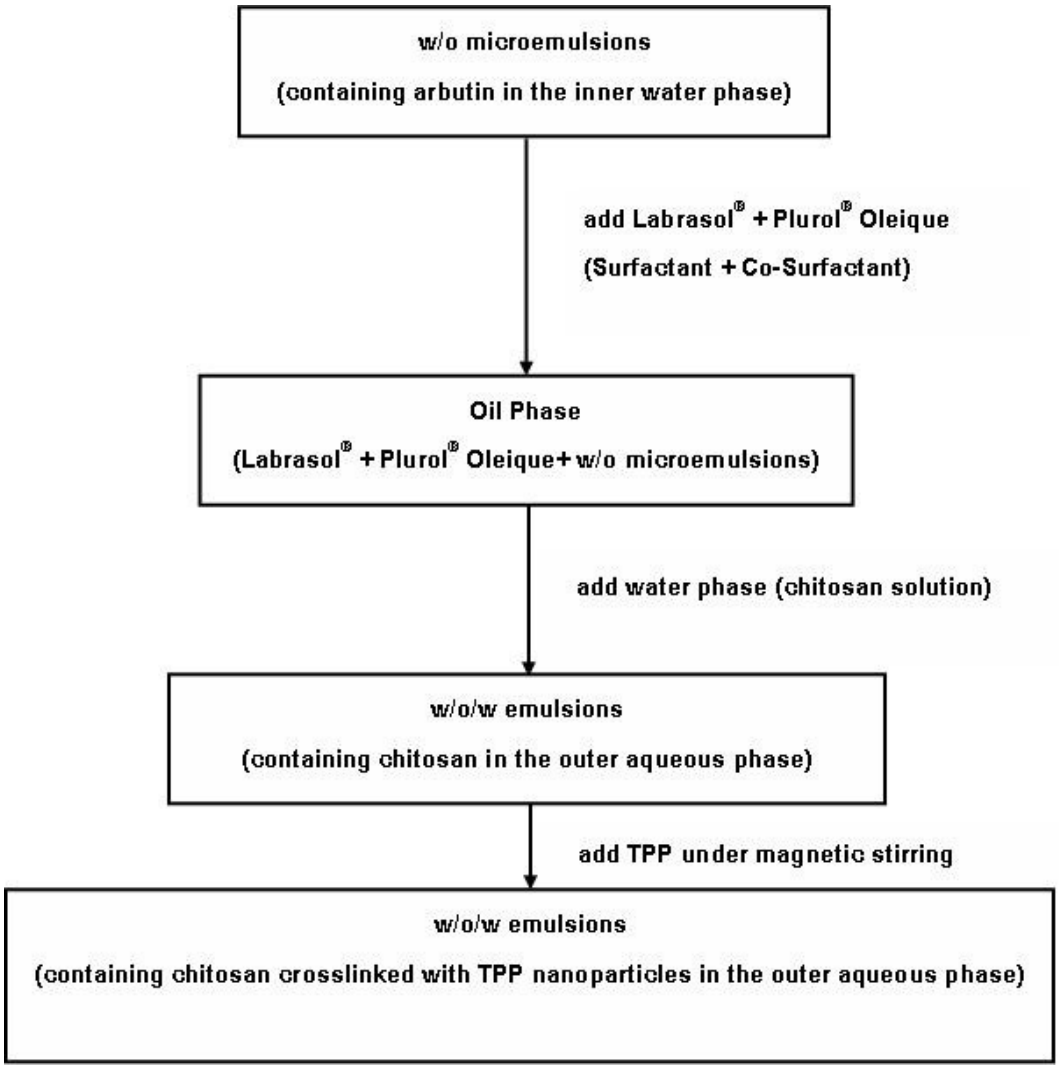


Figure 10 Preparation of w/o/w emulsions containing arbutin nanoparticles

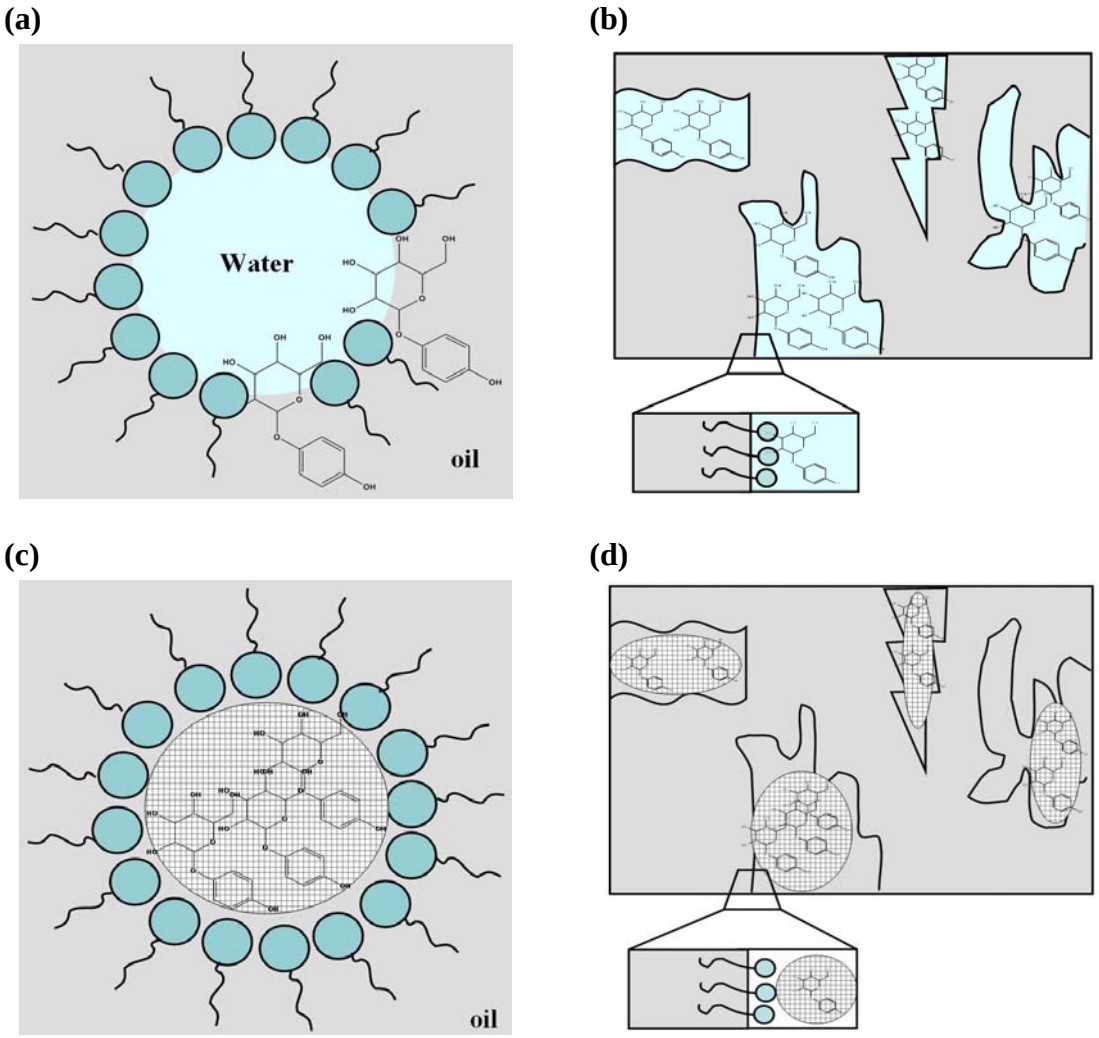


Figure 11 Schematic of (a) pure w/o type microemulsions, (b) pure bicontinuous type microemulsions, (c) w/o type microemulsions containing chitosan nano/microparticles, and (d) bicontinuous type microemulsions containing chitosan nano/microparticles

3. Characteristics of microemulsions

The type of microemulsion was determined using two methods as follows;

3.1 Differential scanning calorimetry (DSC)

DSC measurements were performed under previous reported condition (Boonme *et al*., 2006). Briefly, samples were cooled from 25°C to -50°C at a cooling rate of 5°C /min, held for 3 minutes at -50°C and then heated to 50°C at the heating rate of 10°C /min.

3.2 Conductivity measurements

Conductivity values were used for monitoring the phase transition. The conductivity values increase for the systems containing a higher percentage of water (Delgado-Charro *et al*., 1997). The conductivity was measured using conductivity meter with a cell constant of 0.109 cm^{-1} at 25°Cand repeated in triplicates.

4. Characterization of nanoparticle-based microemulsions

To characterize nanoparticle-based microemulsions (F1-F14), the following method was used.

4.1 Differential scanning calorimetry (DSC)

DSC measurements were performed as described in section 3.1.

4.2 Conductivity measurements

Conductivity values were measured as described in section 3.2

4.3 pH

The pH of all formulations was determined using pH meter and repeated in triplicates.

4.4 Determination of droplet size and morphology

Particle size and morphology were examined using transmission electron microscopy (TEM). The samples were dropped on the grid and dried at room temperature.

4.5 Stability of arbutin microemulsions

4.5.1 Chemical stability

The chemical stability of arbutin in all formulations was analyzed at 0, 1, 2 and 3 months using HPLC. During the observation period, all formulations were stored in centrifuge tubes under 4°C, room temperature and accelerated temperature (45°C) (Biruss and Valenta, 2007; Kittikaiwan *et al*., 2007). All samples were performed in three experiments.

4.5.2 Physical stability

All formulations were stored in centrifuge tubes and its physical stability was observed visually at 4°C, room temperature and accelerated temperature (45°C) (Biruss and Valenta, 2007; Kittikaiwan *et al*., 2007). All samples were performed in three experiments.

5. The *in vitro* release of arbutin microemulsions

1) The modified Franz diffusion cell was used for the *in vitro* release study.

2) The cellulose acetate membranes were soaked in isotonic phosphate buffer. pH 7.4 for 1 hour before they were used. After the modified Franz diffusion cell was set at 37°C, 12 milliliters of degassed isotonic phosphate buffer, pH 7.4 was poured into the receptor compartment. Later, the cellulose acetate membranes were mounted between the donor and receptor compartments. The diffusion surface area of the membrane was 1.77 cm^2.

3) One milliliter of samples was applied on the cellulose acetate membranes after the equilibration of the cellulose acetate membrane for 30 minutes. The donor compartments were covered with Parafilm™ in order to prevent evaporation.

4) At suitable time intervals (0.5, 1, 2, 3, 4, 6, 8, 10, 12 and 24 hours), five hundred microliters of receptor solutions was drawn from the sampling port of modified Franz diffusion cell for arbutin detection. The receptor compartments were immediately refilled with degassed isotonic phosphate buffer, pH 7.4. The amount of arbutin in the receptor solutions was detected by HPLC.

5) The cumulative amount of arbutin release was calculated as follows (Zhao *et al*., 2006; Songkro *et al*., 2003a; Songkro *et al*., 2003b):

$$Q_S = \frac{V}{S} \times C_n + \sum_{n-i}^{n-1} \frac{V_i}{S} C_i$$

Where C_n = drug concentration of the receptor solution at each sampling time; C_i = drug concentration of the i^{th} sample; V and V_i = volumes of the receptor compartment and the sample, respectively; S = diffusional surface area (1.77 cm^2).

6. The *in vitro* skin permeation and retention of arbutin microemulsions

6.1 Preparation of full-thickness newborn pig skin

Newborn pigs that had died of natural causes shortly after birth were used in the current study. Newborn pig skin has been used in several skin permeation studies (Danyi *et al*., 1989; Songkro *et al*., 2003a). Although not a perfect model for human skin, the use of newborn pig skin is a practical alternative to the use of human skin for percutaneous adsorption studies (Songkro *et al*., 2003a). The details of the experiment were described in the following procedures.

1) The epidermal hair at the flank area of newborn pig was clipped with an electric hairs clipper as close as possible to the skin without damaging it and excised with a blade.

2) The subcutaneous fat and underlying tissues were carefully dissected from the dermal surface.

3) The skin was rinsed with isotonic phosphate buffer, pH 7.4, blotted dry, wrapped with aluminum foil and stored at -20°C for no longer than a month. This condition has been reported as a satisfactory method of storage for human skin (Songkro *et al*., 2003a; Songkro *et al*., 2003b)

6.2 Procedure of *in vitro* skin permeation

1) The modified Franz diffusion cell set at 37°C was used in the current study.

2) The skin samples were cut into 4.5 x 4.5 cm^2 pieces, placed in isotonic phosphate buffer, pH 7.4 and hydrated at room temperature for 1 hour.

3) The receptor compartment was filled with 12 milliliters of isotonic phosphate buffer, pH 7.4. The diffusional surface area of the skin was 1.77 cm^2.

4) The skin pieces were mounted between the donor and receptor compartments of the modified Franz diffusion cell. The skin sample was placed with the epidermal side facing toward the donor compartment and the dermal side bathing into the receptor solution.

5) After equilibration of the skin for 30 minutes, one milliliter of formulation was applied onto the skin surface and covered in order to prevent evaporation.

6) At suitable time intervals (0.5, 1, 2, 3, 4, 6, 8, 10, 12 and 24 hours), five hundred microliters of the receptor solution was removed from the receptor compartment and immediately replaced with an equal volume of degassed isotonic phosphate buffer, pH 7.4 (37°C).

7) The amount of arbutin in the receptor solution was determined by HPLC.

8) The cumulative amount of arbutin permeated was calculated as follows (Zhao *et al*., 2006; Songkro *et al*., 2003a; Songkro *et al*., 2003b):

$$Q_S = \frac{V}{S} \times C_n + \sum_{n-i}^{n-1} \frac{V_i}{S} C_i$$

Where C_n = drug concentration of the receptor solution at each sampling time; C_i = drug concentration of the i^{th} sample; V and V_i = volumes of the receptor compartment and the sample, respectively; S = diffusional surface area (1.77 cm^2).

The permeation rate of arbutin at steady state (J_S, $\mu g/cm^2/h$) through newborn pig skin is calculated from the slope of linear portion of the cumulative amount permeated through the newborn pig skin per unit area versus time plot. The permeability coefficient K_P (cm/h) is calculated as follows:

$$K_p = \frac{J_S}{C_0}$$

Where K_P = the permeability coefficient; J_S ($\mu g/cm^2/h$) = the permeation rate of arbutin at steady state; C_0 = the initial concentration of arbutin in the donor compartment.

6.3 Skin retention of arbutin

Skin retention experiment was performed following the method of Peira *et al*. (2007) with our modification (Peira *et al*., 2007).

1) After 24 hours of *in vitro* skin permeation experiment, the skin samples were removed from the modified Franz diffusion cells and washed briefly in water in order to remove excess arbutin from the skin surface.

2) Skin samples were then cut into small pieces and homogenized in 10 milliliters of water using homogenizer at 17,000 rpm for 5 minutes.

3) The obtained solution was centrifuged at 5,000 rpm at 25°C for 5 minutes. The amount of arbutin in the supernatants was determined by HPLC. Each experiment was repeated in triplicate.

7. Determination of arbutin using high performance liquid chromatography (HPLC)

The amount of arbutin was determined by HPLC following the published method of Huang *et al*. (2004) with our modification (Huang *et al*., 2004). A mixture of 0.05M KH_2PO_4 buffer and methanol was used as mobile phase (92:8 v/v) with flow rate of 1 ml/min. The detecting wavelength was 280 nm. The injection

volume was 20 µl. Each arbutin sample was injected in triplicates. The details of HPLC validation are described in Appendix A.

8. Melan-a melanocytes culture (Virador *et al*., 1999)

Melan-a has a characteristic that closely resembles the melanocytes in vivo and has been widely used as a suitable substitute for normal primary mouse melanocytes in melanin metabolism tests (Zhong *et al*., 2006). The melan-a melanocytes were cultured following the method of Virador *et al*. (1999) (Virador *et al*., 1999).

1) Melan-a melanocytes were derived from C57BL/6 mice. Cells were grown in a humidified atmosphere with 5% CO_2 at 37°C in complete RPMI 1640 medium.

2) For the cell passage, cells were rinsed with PBS twice and then harvested by treatment with trypsin/EDTA. Cells were resuspended in complete RPMI 1640 medium at a desired volume.

9. Cytotoxicity (Wang *et al*., 2006)

Cytotoxicity is determined using the MTT method and calculated as follows:

$$\text{Cell viability (\%)} = \frac{\text{absorbance of sample treated cells}}{\text{absorbance of control}} \times 100$$

1) Cells in complete RPMI 1640 medium were seeded with the volume of 100 µl in 96-wells plate and allowed to attach for 1 day (24 hours). The number of cells/well was around $5x10^4$ cells.

2) After the 24-hour incubation time, the complete RPMI 1640 medium was removed and the cells were washed with 100 µl of PBS twice.

3) Samples were added in fresh complete RPMI 1640 medium and this medium was added to the cells. Complete RPMI 1640 medium was added to the cells as controlled. Cells were incubated with 5% CO_2 at 37°C for 1 day (24 hours).

4) After the 24-hour incubation time, the samples were sucked off. Cells were washed with 100 µl of PBS twice. Then, 90 µl of PBS and 10 µl of MTT (5 mg/ml) were added and incubated at 37°C for 3-4 hours.

5) MTT was sucked off and 100 µl of Dimethyl sulfoxide (DMSO) was added. The formazan precipitates were quantitated by absorbance at 570 nm using the microplate reader.

10. Determination of whitening activity

10.1 Assay of mushroom tyrosinase activity (Jones *et al.*, 2002; Hori *et al.*, 2004)

1) Mushroom tyrosinase was prepared in 50 mM potassium phosphate buffer, pH 6.8 at 1000 U/ml as stock solution and stored at -20°C.

2) For use in assays, stock enzyme solutions were thawed and diluted to 200 U/ml with 50 mM potassium phosphate buffer, pH 6.8.

3) L-DOPA (3,4-Dihydroxy-L-phenylalanine, Sigma) used as substrate was prepared in 50 mM potassium phosphate buffer, pH 6.8 at the concentration of 2 mM.

4) Samples were diluted in 50 mM potassium phosphate buffer, pH 6.8.

5) Assays were performed in 96 well-plate and the reaction volume was 200 µl. Firstly, 100 µl of samples were incubated with 50 µl of 200 U/ml mushroom

tyrosinase at 25°C for 10 minutes. Then, 50 µl of 2 mM L-DOPA was added and this solution was immediately determined for the dopachrome formation against blank (solution without enzyme) at 475 nm using microplate reader.

6) The percent inhibition of tyrosinase activity was calculated as follows:

Mushroom tyrosinase activity (%) = [B/A] x 100

Where A = absorbance at 475 nm without test sample and B = absorbance at 475 nm with test sample.

10.2 Melanin assay in melanocytes

Melanin content was determined using modification of previously published method (Ando *et al*., 1999; Curto *et al*., 1999; Wang *et al*., 2006).

1) Cells in complete RPMI 1640 medium were seeded in 12 wells plate and allowed to attach for 1 day (24 hours). The number of cells/well was around $1x10^6$ cells.

2) After the 24-hour incubation time, the complete RPMI 1640 medium was removed and cells were rinsed with PBS twice.

3) Samples added in fresh complete RPMI 1640 medium were added to the cells. Complete RPMI 1640 medium was added to the cells as controlled. Cells were incubated with 5% CO_2 at 37°C for 1 day (24 hours).

4) After the 24-hour incubation time, the samples were sucked off. Cells were washed with PBS twice. Cells were treated with 500 µl of trypsin/EDTA for 15 minutes at 37°C.

5) Then, cells were transferred to centrifuge tubes and centrifuged at 1,000 g at 37°C for 10 minutes. Supernatant was decanted to obtain cell pellets.

6) Cell pellets were lysed with 1 ml of 1 N NaOH boiled in water for 30 minutes. Then, they were vortexed until dissolving completely.

7) Cell extracts were centrifuged at 10,000 g at 25°C for 30 minutes. The optical density (O.D.) of each supernatant was measured at 450 nm using UV spectrophotometer.

8) The melanin content per cell was calculated as follows;

$$\text{Melanin content (\%)} = \frac{\text{absorbance of sample treated cell at 450 nm x 100}}{\text{absorbance of control at 450 nm}}$$

11. Statistic

Results were calculated and expressed as mean ± standard deviation (S.D.) or standard error of mean (S.E.M.). Mean and S.D. or S.E.M. of the results from at least three experiments were calculated using Microsoft Excel®. The chemical stability of arbutin, arbutin release profile, arbutin permeated profiles, arbutin retained in full thickness newborn pig skin, cytotoxicity and mushroom tyrosinase activity and melanin formation assay were studied statistically by means of the Statistical Package for the Social Sciences (SPSS) software version 16.0 for Windows (SPSS Inc., USA) using One-Sample T Test and One-Way analysis of variance (ANOVA) followed by a pairwise mean multiple comparison Turkey test. The significant difference were considered at the level of $p<0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

1. Pseudo-ternary phase diagrams

Pseudo-ternary phase diagrams generated from the system containing virgin coconut oil, Labrasol®, Plurol® Oleique and WSC solution as oil, surfactant, co-surfactant and water phase, respectively, are presented in Fig.12. Microemulsions regions from such system were observed at room temperature. The surfactant to co-surfactant mass ratio (K_m) was varied from 1:1, 2:1, 3:1 and 4:1. The microemulsion regions were obtained at 1:1, 2:1, 3:1 and 4:1 K_m values (Fig.12a to d, respectively). Either Labrasol® or Plurol® Oleique alone was not able to give microemulsions region under our conditions. The result showed that at the lowest K_m value of 1:1, as shown in Fig.12a the smallest microemulsions region was observed. This result was explained that as the K_m value was increased from 1:1 to 2:1, the amount of surfactant was also increased resulting in the greater interfacial area was formed and the dispersed phase was attributed among a greater number of micelles (Junyaprasert *et al*., 2007). While, increasing the K_m values from 2:1 to 4:1 resulted in decreasing the microemulsion regions. It has been reported that as K_m value increases, microemulsion regions failed to form because there is not enough amount of the co-surfactant in the system to enable an interfacial film to be formed (Junyaprasert *et al*., 2007). It might be from the enough amount of Plurol® Oleique resulting in the enough quantity of hydrophilic chain length of polyglycerol esters to form hydrogen bonding with surrounding water. As shown in previous study, increasing in number of glycerol

groups in hydrophilic chain increases the affinity of the co-surfactant to form hydrogen bonds with surrounding water molecules (Djekic and Primorac, 2008). Thus, the appropriate K_m value was 2:1 for the microemulsions containing virgin coconut oil, Labrasol®, Plurol® Oleique and chitosan solution as oil, surfactant, co-surfactant and water phase, respectively.

As Djekic and Primorac (2008) attempted to use vegetable oils, mineral oil and olive oil, to form the microemulsions based on Labrasol® and Plurol® Oleique as surfactant and co-surfactant, respectively. Their attempt was not successful because olive oil contains long-chain triglyceride that is too high to form microemulsions. Additionally, it has been reported that medium chain triglycerides were able to form microemulsions more easily than long chain triglycerides (Djekic and Primorac, 2008). VCO, the recently popular vegetable oil, was used and successful to form microemulsions in our work. The VCO is a mixture of medium and long chain triglycerides in which the mostly favorable saturated fatty acid is lauric acid, the 12 carbon-atoms (about 45%) (Nevin and Rajamohan, 2008). In addition, molecular weight of VCO is not too high to form microemulsions.

For the following experiments, the K_m value was fixed at 2:1 as two reasons. Firstly, this K_m was appropriate to give the broadest microemulsions region as previously mentioned. Secondly, the high concentration of Labrasol® can be potentially irritant to the human skin. Since, the hemolytic activity testing in human erythrocytes is an alternative method *in vivo* testing. It has been used as a potential screening method to evaluate irritant potential of microemulsions for possible application in pharmaceutical and cosmetic formulations (Aparicio *et al*., 2005).

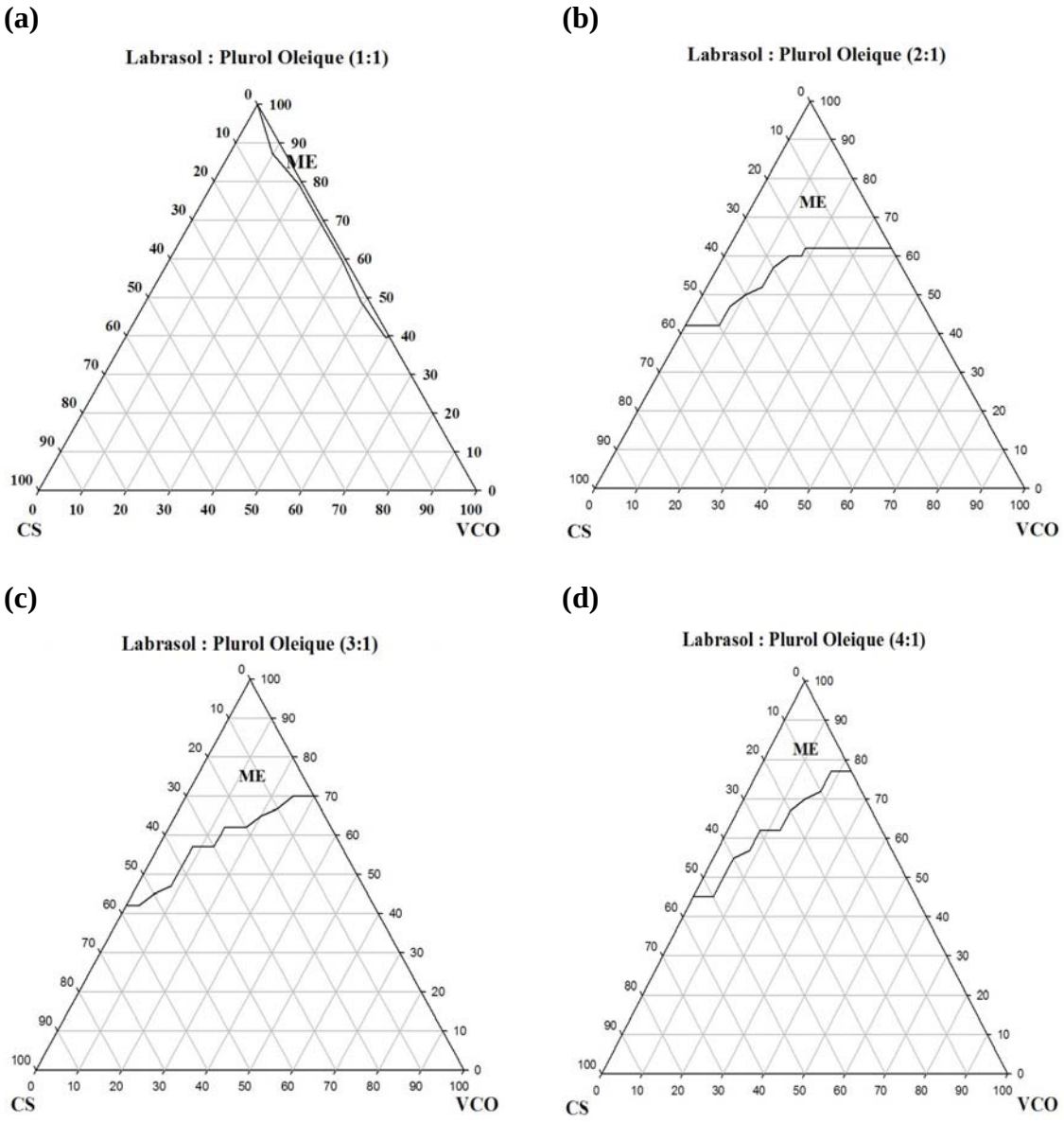


Figure 12 Pseudo-ternary phase diagrams of the microemulsion systems composed of virgin coconut oil/ chitosan/ labrasol:plurol oleique with K_m values of (a) 1:1, (b) 2:1, (c) 3:1, and (d) 4:1

2. Characterization of microemulsions

2.1 Differential scanning calorimetry (DSC)

DSC was used to clarify the types of the microemulsions (w/o, bicontinuous or o/w). In our study, the cooling part of the DSC curve did not reveal the important knowledge, thus, only the heating part was presented. The chosen K_m value for DSC experiment was 2:1 because such value was used to incorporate the selected active substance, arbutin, for preparing the cosmetic formulation.

In Fig.13, the DSC thermograms show a transition from w/o to bicontinuous type microemulsion. The large endothermic peaks were observed at approximately 25^{o}C and 5^{o}C of VCO and chitosan solution, respectively. The endothermic peak of water phase began to appear above 20% of water phase indicating that the microemulsions system of such water phase concentration performed the transition from w/o to bicontinuous type microemulsion. Above 20% of water phase, the endothermic peak shifted towards the higher temperature and gradually approached the melting point of chitosan upon the increasing water phase concentration. It is attributed that the chitosan solution as water phase strongly bound to surfactant becomes less tightly bound upon the increasing water phase concentration as the peak shifts towards to the melting point of chitosan (Podlogar *et al*., 2004; Graf *et al*., 2008). Thus, the water phase concentration at above 20% indicated the bicontinuous type microemulsions. In contrast, the endothermic peak of VCO slightly decreased upon the increasing water phase concentration and eventually disappeared at 50% of water phase (no VCO in the system). The endothermic peak that presented at approximately -10^{o}C represented the surfactant/co-surfactant mixture

as the sample did not contain any water phase (0% of water phase) (Podlogar *et al*., 2004; Graf *et al*., 2008).

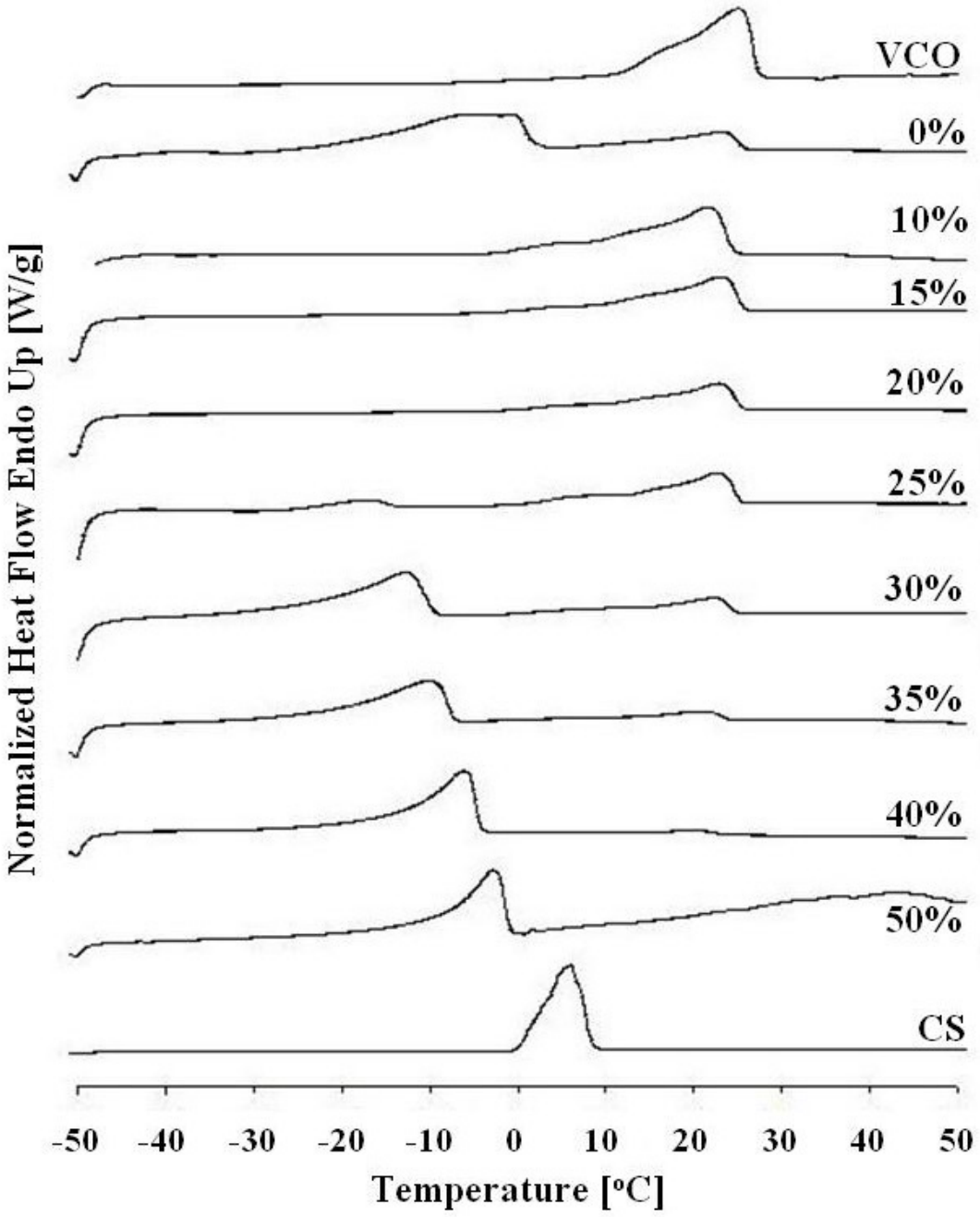


Figure 13 DSC thermograms of microemulsion systems with K_m value of 2:1 and 0-50% water phase

2.2 Conductivity

The conductivity was carried out to confirm the types of microemulsions (w/o, bicontinuous or o/w type microemulsions) after they were determined using DSC. As the concentration of water increased, the conductivity also increased exponentially. The change in conductivity of the microemulsions occurred at the percolation threshold. Below the percolation threshold, the conductance values remained low indicating that the water droplets were isolated from each other and presented in continuous oil phase exhibiting very low conductance values. However, as the water concentrations reach to the percolation threshold, the conductance values increased rapidly. This can be explained that the conductive water droplets began to contact closely to each other as providing in clusters and the counter ions were transferred from one droplet to another through water channels opening between droplets during the merging of droplets. Above the percolation threshold, the number of such clusters increased as the increasing in water concentrations, indicating that the electrical conductivity raised sharply along the curve as being the bicontinuous structure and finally water presents as continuous phase (Mehta and Bala, 2000; Podlogar *et al*., 2004; Graf *et al*., 2008). The presence of electrolyte in non-ionic microemulsions is important for the observation of percolation threshold (Podlogar *et al*., 2004). Our microemulsions system contained chitosan solution with positive charge which could present the electrical conductivity, thus, it was not necessary to add other electrolytes into the microemulsions system.

The microemulsions systems comparing the WSC, VCO, and Labrasol®/Plurol® Oleique of the K_m value of 2:1, 3:1 and 4:1 were prepared on the studied lines shown in Fig.14 and their conductivity were determined. The water

phase was 0.5% aqueous solution of WSC, medium MW. At K_m value of 2:1, the conductance values were low (less than approximately 75 μS/cm) at the water phase of less than 20%, then increased rapidly to 50% with increasing water phase concentrations. This indicated the bicontinuous structure within microemulsions system with the K_m value at 2:1. The conductance values increased sharply which demonstrated bicontinuous structure above the percolation threshold. Whereas, at K_m value of 3:1 and 4:1 with increasing concentration of Labrasol®, slightly lower the percolation thresholds were observed as shown in Fig.15. The percolation thresholds were approximately 18% and 16% of water phase in the systems containing the K_m value at 3:1 and 4:1, respectively. The decreased percolation thresholds may be due to the increase in the interdroplet interactions strength (Mehta and Bala, 2000). The electrical conductivity profiles revealed the slightly lowered percolation threshold resulting from the increasing concentration of Labrasol®. It is attributed that increasing Labrasol® ratios might increase the strength of the interdroplet interactions of microemulsions and cause the droplets to form cluster rapidly compared with the system containing lower Labrasol® ratios.

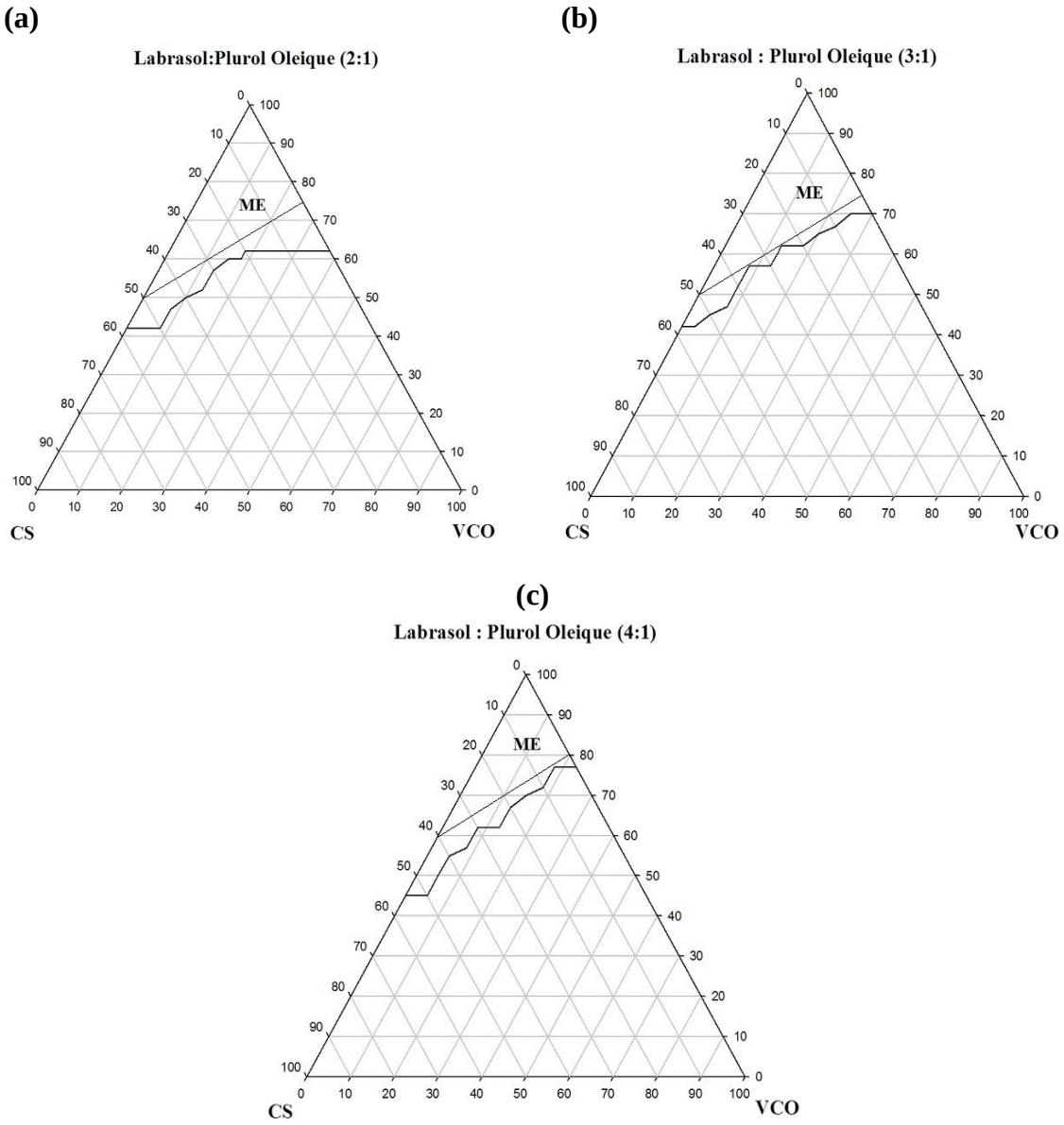
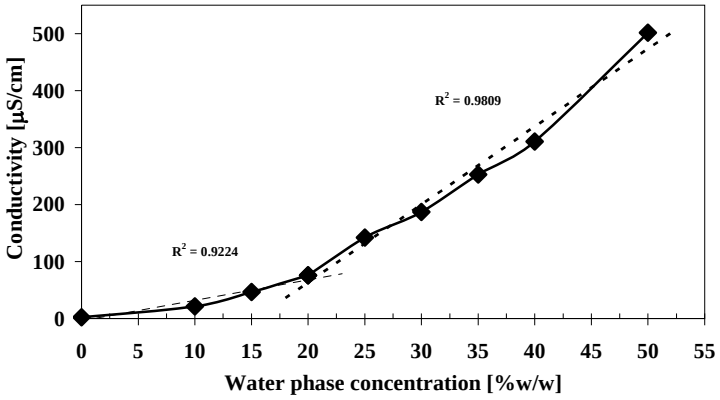
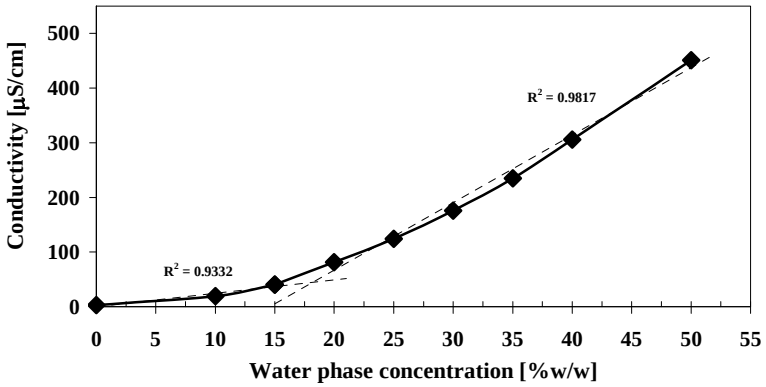


Figure 14 Phase diagrams for investigation of microemulsion types with K_m values of (a) 2:1, (b) 3:1 and (c) 4:1

(a)



(b)



(c)

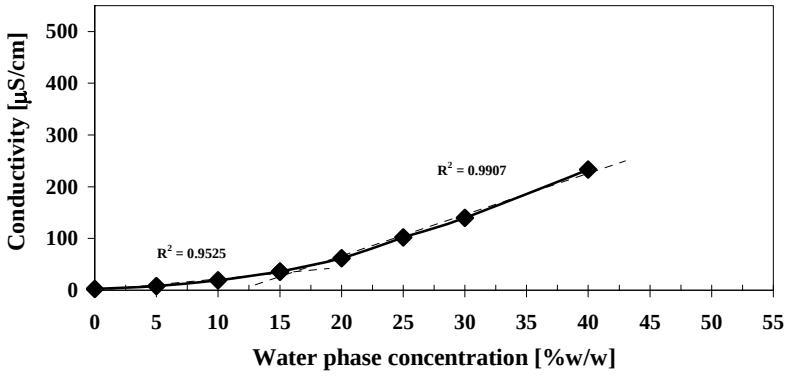


Figure 15 Effects of K_m values on conductivity of microemulsions; (a) 2:1, (b) 3:1, and (c) 4:1

3. Characterization of nanoparticle-based arbutin microemulsions

3.1 Differential scanning calorimetry (DSC)

DSC was determined the types of microemulsions in F4 and F11 used to prepared chitosan nanoparticles. The DSC thermograms was used to confirm the w/o and bicontinuous type microemulsions of F4 and F11, respectively, as shown in Fig.16. Since DSC thermogram of F4 did not present the endothermic peak of water phase, it confirmed the w/o behavior of such system. Moreover, DSC thermogram of F11 indicated the bicontinuous structure, since it presented the endothermic peak of both water phase and VCO.

3.2 Conductivity

All formulations were prepared using the K_m value at 2:1. The conductivity of these systems was investigated to confirm the types of microemulsions as presented in Table 6. F1 to F10 were exhibited as w/o type microemulsions since their conductance values were less than the conductance value exhibited at the percolation threshold (75μS/cm). Whereas, the conductance values of F11 to F14 were higher than the conductivity of percolation threshold exhibiting the bicontinuous type microemulsions.

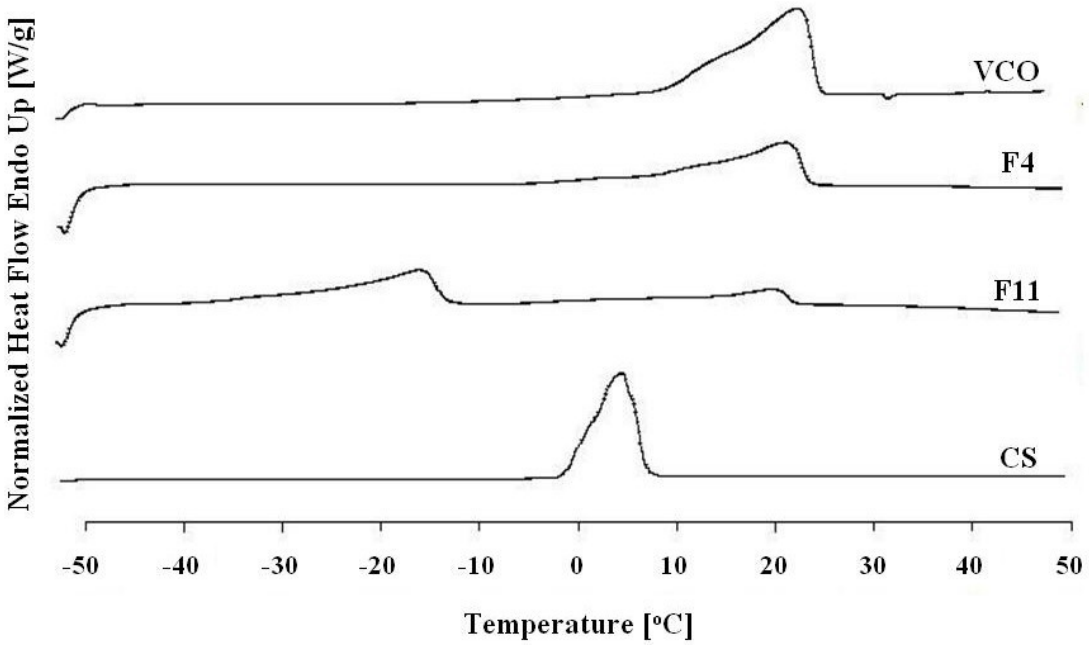


Figure 16 DSC behavior of w/o (F4) and bicontinuous type (F11) microemulsions containing medium MW chitosan

3.3 pH

In present study, lactic acid was used to adjust pH values to 5-5.5. Since, at basic pH (approximately 9), arbutin was decomposed four-times higher than at acidic pH (approximately 5) (Couteau and Coiffard, 2000). Therefore, pHs of all formulations were adjusted to be appropriate for skin and arbutin stability (Couteau and Coiffard, 2000). Moreover, whitening activity of lactic acid has been reported, which the mechanism is due to remodeling of the epidermis and accelerated desquamation (Smith, 1999; Usuki *et al*., 2003). The pH values of all formulations are presented in Table 6.

3.4 Transmission electron microscopy (TEM)

As the size of particles is one of the important factor influenced the penetration of particles through skin, thus, the estimated size of the formulations was investigated using TEM. TEM micrographs of F4 and F7 were determined as the representative formulations for the present study. They were demonstrated as w/o systems that contained the aqueous cores which the hydrophilic reagents could be dissolved and produced the reaction within that cores of droplets (Poulsen *et al*., 2007).

The molecular weight of chitosan affected to the size of particles. As medium MW WSC resulted in the smaller size of chitosan nanoparticles compared with the high MW WSC, therefore, it could be hypothesized that the particles produced from medium MW WSC could pack denser than that from high MW WSC. As the estimated size of F4 prepared by medium MW chitosan was approximately

200 nm, whereas F7 prepared by high MW WSC exhibited the larger size (approximately 200 nm-2 μm) as shown in Fig.17.

Moreover, the morphology of chitosan nano/microparticles was also reckoned using TEM. The rather spherical shape of chitosan nano/microparticles generated from both of medium and high MW WSC was shown in Fig.17.

Table 6 Physicochemical characteristics of microemulsions

Formulation	**pH (mean±S.D.)**	**Conductivity [µS/cm] (mean±S.D.)**
F1	5.33 ± 0.04	19.40 ± 3.04
F2	5.07 ± 0.04	15.80 ± 0.46
F3	5.08 ± 0.04	15.83 ± 0.55
F4	5.27 ± 0.11	35.17 ± 3.27
F5	5.27 ± 0.09	36.47 ± 3.04
F6	5.30 ± 0.14	39.53 ± 2.49
F7	5.35 ± 0.12	34.33 ± 4.24
F8	5.29 ± 0.12	30.00 ± 5.94
F9	5.33 ± 0.05	34.57 ± 4.63
F10	5.26 ± 0.22	34.47 ± 2.30
F11	5.06 ± 0.04	151.00 ± 7.00
F12	5.14 ± 0.06	172.67 ± 5.77
F13	5.10 ± 0.12	146.67 ± 6.43
F14	5.07 ± 0.04	163.00 ± 4.58

The results are the mean ± S.D. of three experiments.

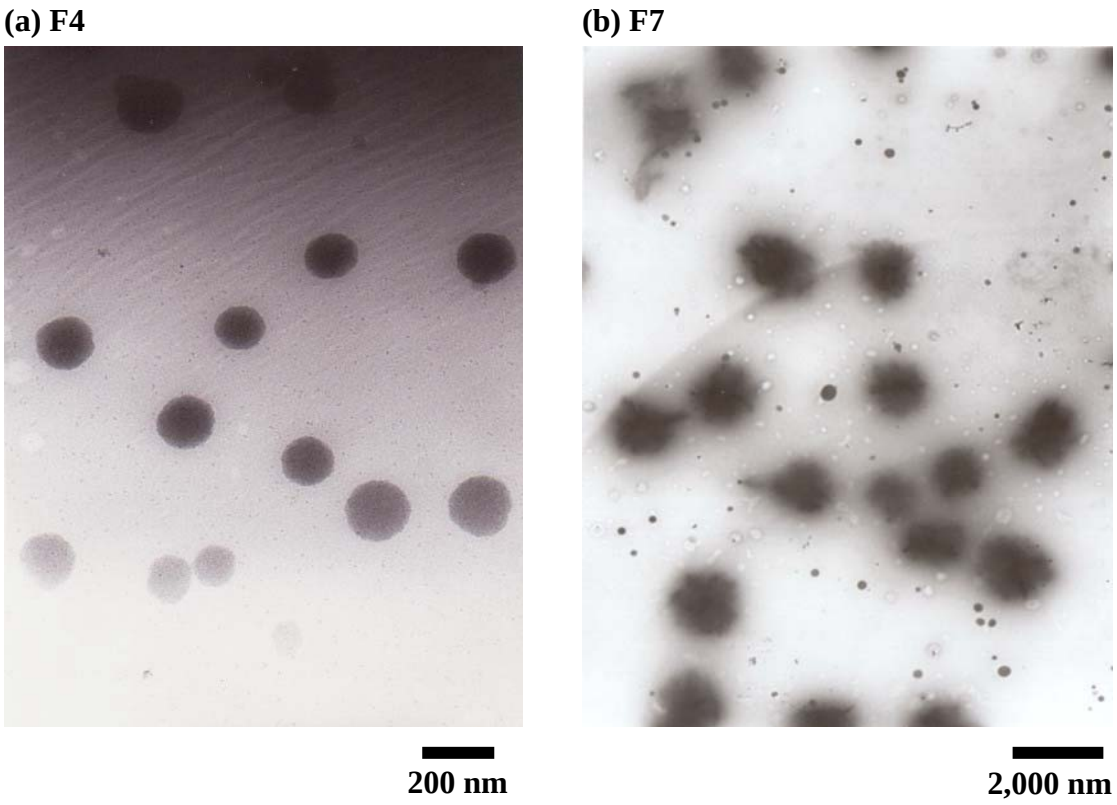


Figure 17 TEM micrographs of w/o type microemulsions containing medium MW chitosan (F4) and high MW chitosan (F7)

3.5 Stability

3.5.1 Chemical stability

The chemical stability of arbutin in the microemulsions was determined at 0, 1, 2 and 3 months using HPLC and the amount of arbutin at initial day was expected as 100%. The storage temperatures were room temperature (about 25°C), 45°C and 4°C (Biruss and Valenta, 2007; Kittikaiwan *et al*., 2007).

Approximately 20% of arbutin in solution as control degraded after 3 months within the storage condition at room temperature and 45°C, except at 4°C which could keep the amount of arbutin as initial day. Since arbutin has been reported as a thermodegradable substance (Coiffard *et al*., 1999), thus, it was degraded upon the increasing in temperature as expected.

Fig.18 shows the undegraded arbutin of F1 to F4 compared with the solution. At room temperature for three months, only F3 showed the slight degradation of arbutin (approximately 10%). This result revealed that the enough amounts of co-surfactant and surfactant were necessary to keep arbutin stable until three months. It is possible that the K_m value at 2:1 (F1) and 3:1 (F2) seem to form stronger interfacial film than that of 4:1 (F3) which produced more degradable product upon the long-period storage. In contrast, they did not show the difference of undegraded arbutin between each formulation at room temperature and 4°C upon the 3-month storage. Furthermore, the influence of different percentage of surfactant (F1 and F4) was discussed. There was no significant difference in undegraded arbutin between such formulations at all temperatures for three months. Garti *et al*. (2006) reported that the higher amount of surfactants are more densely and tightly packed and allows less incorporation of drug at the interfacial film (Garti *et al*., 2006), which

was in contrast to our study, the packing parameter of the higher amount of surfactant was not observed (Garti *et al*., 2006). It might be from the slight difference in percentage of surfactant between F1 and F4, as composed of 70 and 65% surfactant, respectively.

The influence of MW of WSC to the stability of arbutin iss presented in Fig.19. At all storage temperatures, each formulation (F4 to F10) showed its similar tendency of undegraded arbutin. It is likely that medium MW WSC (F4 to F6) showed the good protection to arbutin stability even at high temperature compared with high MW WSC (F7 to F9). It is possible that medium MW WSC exhibited the denser matrix providing smaller in size than high MW WSC, as shown in TEM photograph. The denser matrix seems to keep arbutin in hydrophilic matrix, while the looser matrix as generated from high MW WSC seems to lose arbutin preferably from such matrix to the interfacial film of surfactant. Hincha *et al*. (1999) reported that the phenol ring of arbutin prefers to insert in hydrophobic tail of liposome membrane and the sugar part of arbutin prefers to exist in head group of liposome membrane (Hincha *et al*., 1999). In our case, phenol ring of arbutin seems to embed in hydrophobic tail of non-ionic surfactant, while its sugar part seems to exist in hydrophilic head group of non-ionic surfactant as shown in Fig.11. Moreover, since non-ionic surfactants are affected by temperature, especially which in their head group size (Flanagan *et al*., 2006). Thus, it is possible that all substances incorporating in their head group might be easily impaired by temperature, especially the thermodegradable substances. Therefore, it is possible that arbutin existing at interfacial film might be easily destroyed by temperature. As shown in F10 (without WSC/TPP), it gave less undegraded arbutin than F4 to F6 which had the chitosan matrix for protecting arbutin

from temperatures, while such matrix was not presented in F10. Furthermore, the influence of difference concentration of WSC is reported (F4 to F6 and F7 to F9). The similarity of amount of undegraded arbutin was observed within the difference concentration of WSC either medium or high MW. This result revealed that either high or low concentration of WSC did not have any effects to the chemical stability of arbutin.

The chemical stability of arbutin in F11 to F14 as shown in Fig.20 is discussed. All such formulations were bicontinuous type microemulsion. It is generally recognized that non-ionic surfactants are affected by the change in temperature. Garti *et al*. (2006) reported that the arranged surfactant molecules at interfacial film will be looser upon the increasing in temperature (Garti *et al*., 2006). Especially, bicontinuous type microemulsions that exhibit the low interfacial tension can be destroyed easily by the change in temperature (Bolzinger *et al*., 1998). From this reason, it is possible that arbutin incorporated in interfacial film of bicontinuous type microemulsion might be degraded easier than that of w/o type microemulsion. The degradation of arbutin at room temperature and 45°C might be discussed as mentioned above. However, at 4°C, it is not understand the mechanism of arbutin degradation yet since this temperature could keep arbutin stable in the solution.

In conclusion, the formulations that contained chitosan nanoparticles generated from medium MW WSC at either high or low WSC concentrations were the appropriate formulations based on the chemical stability of arbutin, since they gave higher undegraded arbutin as initial day than control (no chitosan nanoparticles) and formulations containing high MW WSC nano/microparticles.

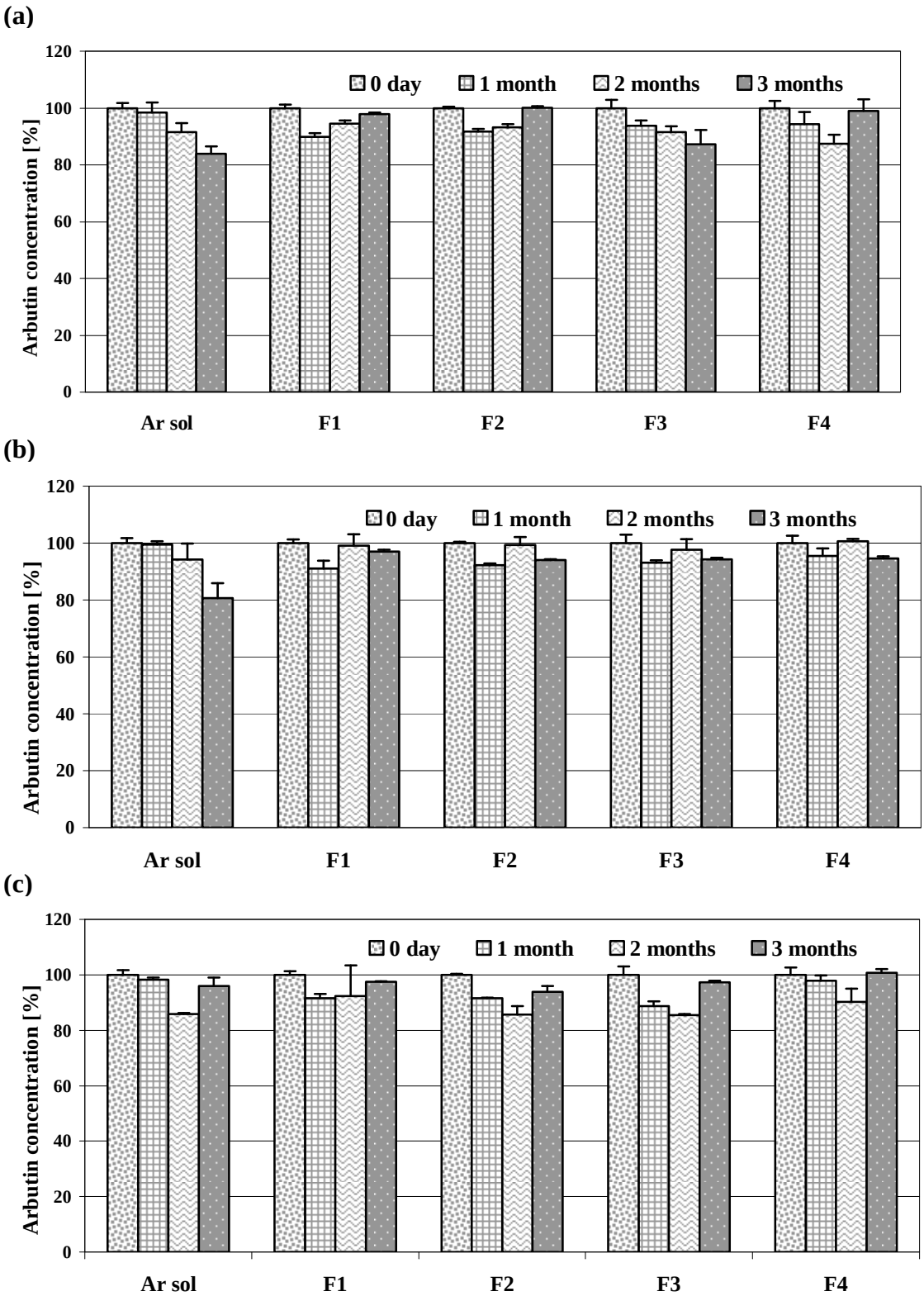


Figure 18 Chemical stability of arbutin microemulsions (F1-F4) at (a) room temperature, (b) 45^{o}C, and (c) 4^{o}C (mean ± S.D.; n=3)

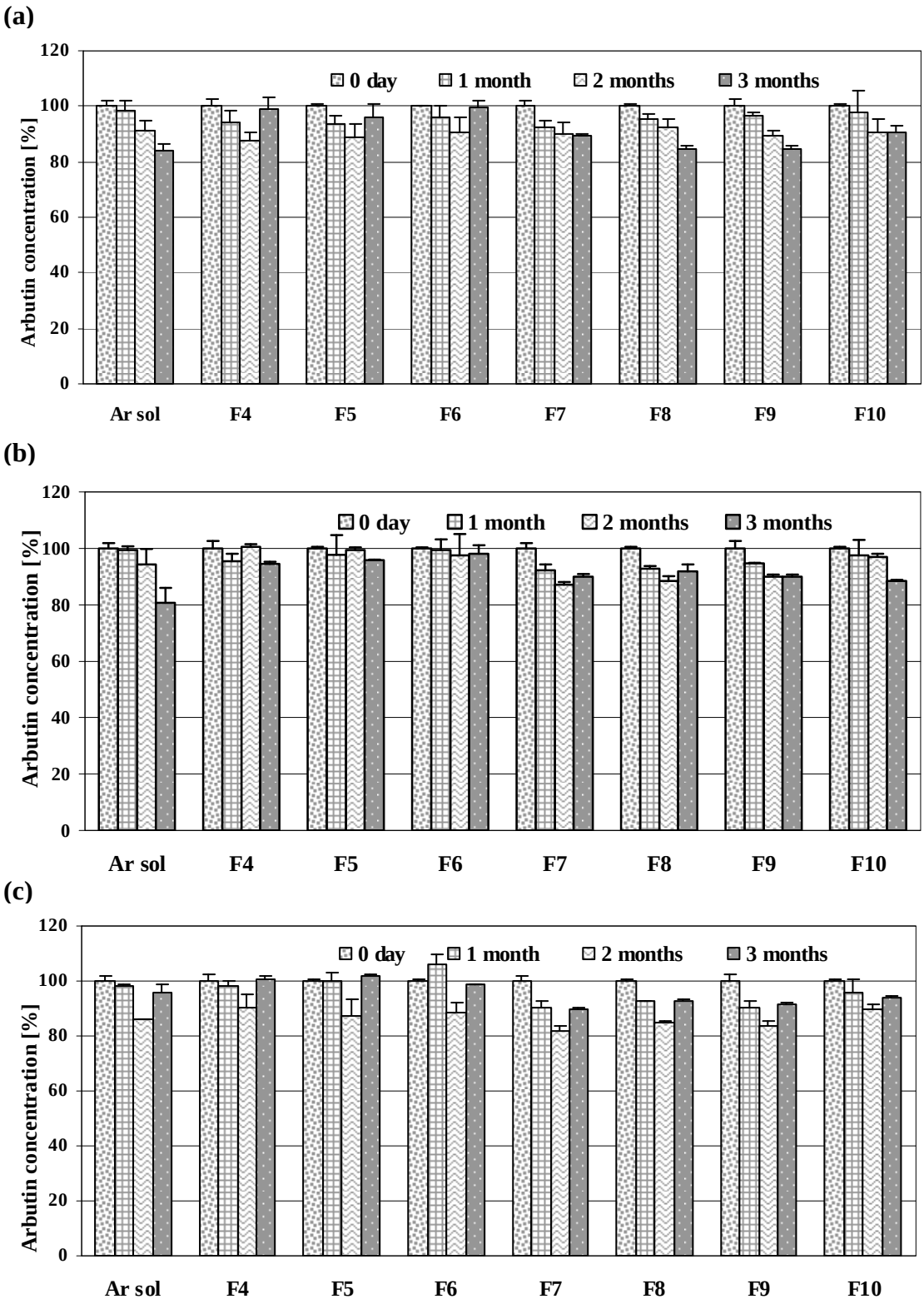


Figure 19 Chemical stability of arbutin microemulsions (F4-F10) at (a) room temperature, (b) 45^{o}C, and (c) 4^{o}C (mean ± S.D.; n=3)

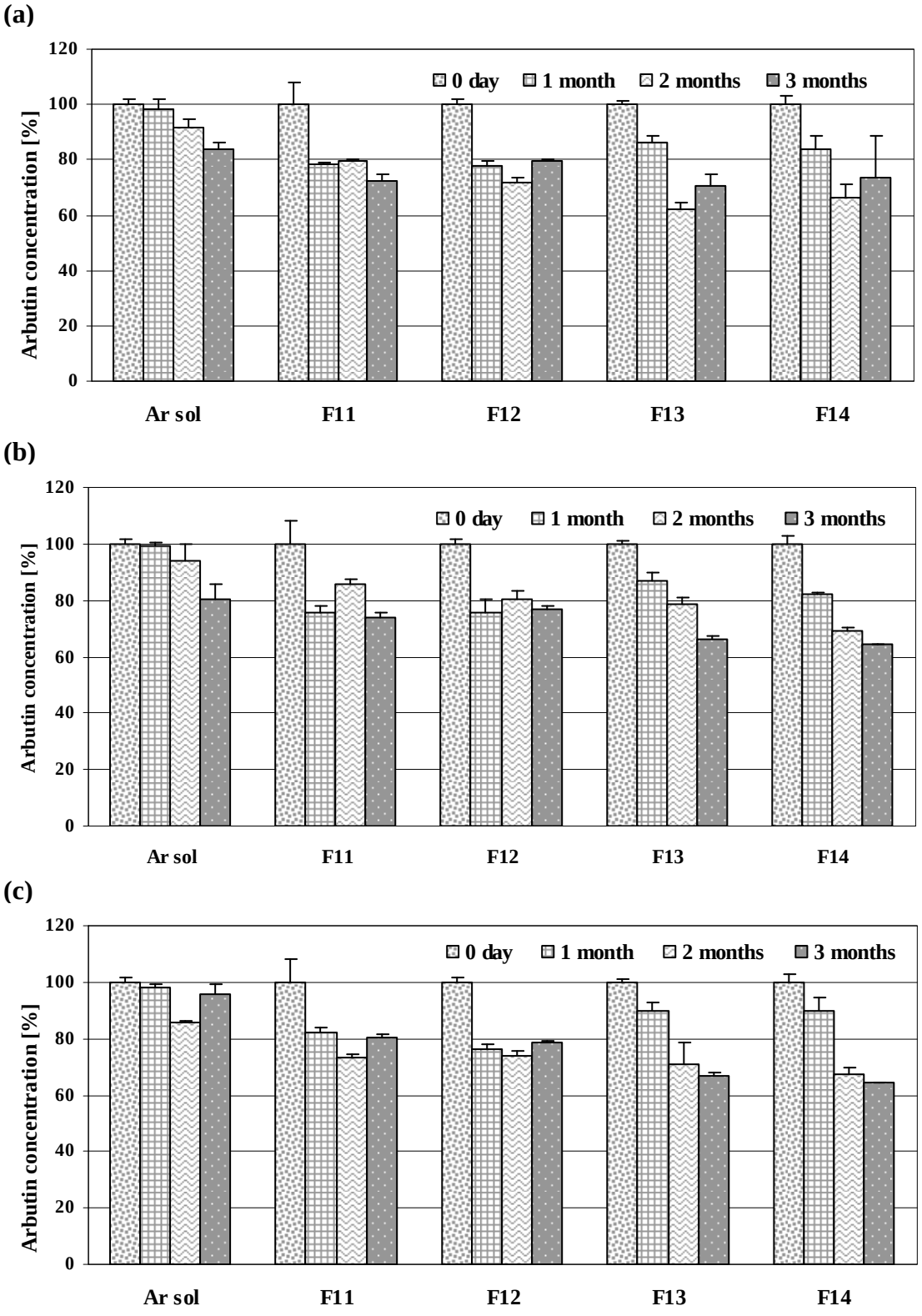


Figure 20 Chemical stability of arbutin microemulsions (F11-F14) at (a) room temperature, (b) 45^{o}C, and (c) 4^{o}C (mean ± S.D.; n=3)

3.5.2 Physical stability

The physical stability was observed visually at room temperature, 45°C and 4°C for three months and that stability of all formulations is summarized in Table 7. After one month, the F11, 12, 13 and 14 was changed in physical stability at 45°C, whereas such formulations at room temperature and 4°C did not exhibit these occurrences. As shown in Fig.21a, F11 as particles suspended in bicontinuous type microemulsion showed the aggregation of the chitosan nanoparticles, while F13 was turbid without precipitation (Fig.21c). F12 and F14 showed the impaired structure of microemulsions as phase separation (Fig.21b and d, respectively). Moreover, F1 to F10 as w/o type microemulsions were stable at least three months even within high temperature. This result revealed that based on our system, w/o type microemulsions were more stable than the bicontinuous type microemulsion at accelerated temperature (45°C). It has been reported that the low interfacial tension (10^{-2} mN/m) of bicontinuous structure that caused the highly dynamic character can make such structure easy to be destroyed by high temperature (Bolzinger *et al*., 1998). However, the w/o type microemulsion is probably influenced by the inert circumstance of continuous oil phase (Chen *et al*., 2004) that makes it more stable than bicontinuous type microemulsion.

After storage at accelerated temperature (45°C) for three months, the structure of F1 to F10 was impaired. The turbidity occurred within F1 to F3 as shown representatively in Fig.22a. The aggregation occurred within F4 to F9 as shown representatively in Fig.22b and c, and F10 showed the phase separation as shown in Fig.22d. Whereas, these systems were stable at room temperature and 4°C. It is likely that the longer storage time influenced the change in characteristic of microemulsion

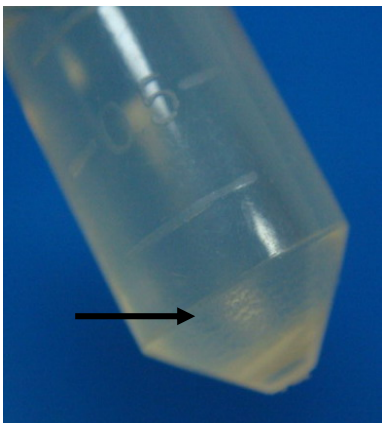
i.e. the inert continuous oil phase was destroyed and changed its physicochemical property causing the chitosan nanoparticles released from dispersed water droplets and settled down. Moreover, Labrasol® and Plurol® Oleique, a non-ionic surfactant, might be sensitive to the changes in temperature (Peltola *et al*., 2003; Flanagan *et al*., 2006) which made the formulation unstable at the higher temperature.

Table 7 Physical stability of microemulsions

Formulation	0 day			1 month			2 months			3 months		
	RT	45°C	4°C	RT	45°C	4°C	RT	45°C	4°C	RT	45°C	4°C
Arb sol	-	-	-	-	-	-	-	-	-	-	-	-
1	-	-	-	-	-	-	-	-	-	-	T	-
2	-	-	-	-	-	-	-	-	-	-	T	-
3	-	-	-	-	-	-	-	-	-	-	T	-
4	-	-	-	-	-	-	-	-	-	-	A	-
5	-	-	-	-	-	-	-	-	-	-	A	-
6	-	-	-	-	-	-	-	-	-	-	A	-
7	-	-	-	-	-	-	-	-	-	-	A	-
8	-	-	-	-	-	-	-	-	-	-	A	-
9	-	-	-	-	-	-	-	-	-	-	A	-
10	-	-	-	-	-	-	-	-	-	-	S	-
11	-	-	-	-	A	-	-	A	-	-	A	-
12	-	-	-	-	S	-	-	S	-	-	S	-
13	-	-	-	-	T	-	-	T	-	-	T	-
14	-	-	-	-	S	-	-	S	-	-	S	-

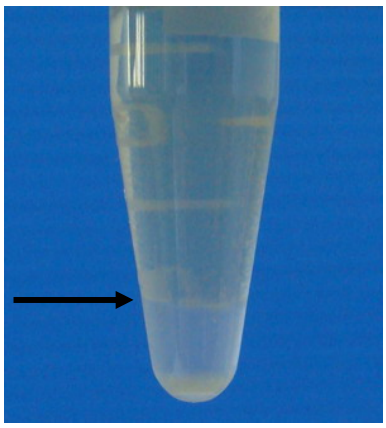
- ; no change, A ; Aggregation, S ; Separation, T ; Turbidity

(a) F11



Aggregation

(b) F12

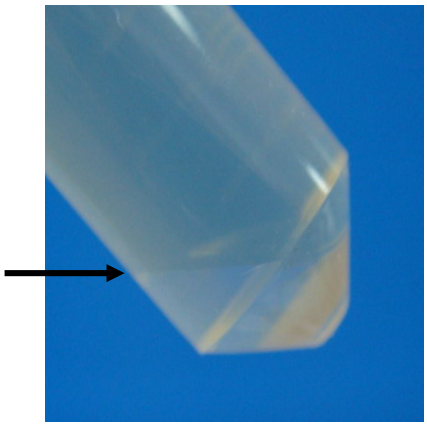


Phase separation

(c) F13

Turbidity

(d) F14



Phase separation

Figure 21 Appearance of microemulsions after storage at 45°C for 1 month; (a) F11, (b) F12, (c) F13, and (d) F14

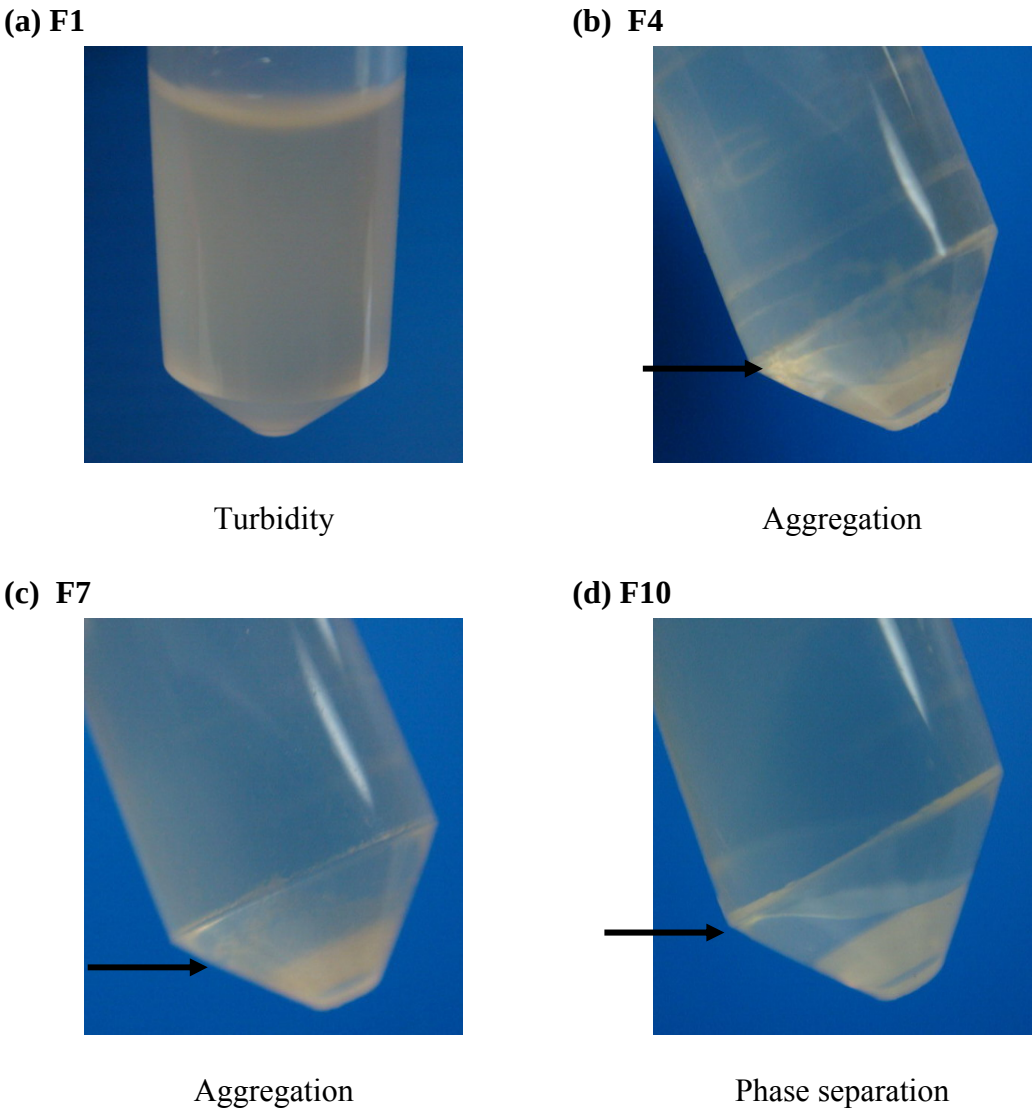


Figure 22 Appearance of microemulsions after storage at 45oC for 3 months; (a) F1, (b) F4, (c) F7, and (d) F10

4. *In vitro* release of arbutin microemulsions

All formulations were studied to the *in vitro* release experiment using modified Franz diffusion cell and the amount of arbutin released through cellulose acetate membrane was detected using HPLC. Cellulose acetate membrane (Spectra/Por®3) was used as the model synthetic membrane. It consists of small pores filled by the aqueous phase (Gilmore *et al*., 2006). Therefore, the small hydrophilic molecules, such as arbutin, could pass easily through this membrane.

First, we studied the influence of different K_m values to the release of arbutin as shown in Fig.23. F1 to F3 contained WSC nanoparticles generated from medium MW WSC at the concentration of 0.25%w/w. The ratio of water, oil and surfactant phase was 10, 15 and 75%w/w, respectively, except with the different K_m values (2:1, 3:1 and 4:1, respectively). The data revealed that the different K_m values did not influence the release of arbutin from the formulations. This result could be confirmed by their release rates as shown in Table 8. The release rates of F1 to F3 were not difference significantly (One-Way ANOVA, $p>0.05$). As Part 1 shows the reasons for the selection of K_m value at 2:1, therefore, this result supports such reasons that there is no difference in the selection of K_m value for our system based on the release data.

Thereafter, the influence of different MW of WSC (medium, 100 kDa and high, 200 kDa MW WSC) to the release of arbutin from the formulations was studied as in Fig.24. F4 and F7 contained WSC nanoparticles generated from WSC concentration of 0.25%w/w, K_m value at 2:1, and the ratio of water, oil and surfactant phase at 15, 20 and 65%w/w, respectively, except with the different MW of WSC (medium and high MW, respectively). The result revealed that the different MW of

WSC influenced the release of arbutin, since the release rate of F4 was less than that of F7 significantly (One-Sample T-Test, $p<0.05$) as shown in Table 8. Moreover, as shown in Fig.24, the release of arbutin from F4 after 24 hours was significantly lower than that of F7 and F10, respectively (One-Way ANOVA, $p<0.05$). It is likely that the release of arbutin decreased with the increase in MW of WSC. It might be from the high MW WSC generated chitosan particles (approximately 2 μm) higher in size than that of medium MW WSC (approximately 200 nm) as shown in TEM photograph. It is attributed that the denser matrix might be more useful than looser matrix in controlled release of arbutin which in agreement with the result of Dini *et al* (2003). They showed that chitosan cross-linking with glutaraldehyde prepared by the suspension cross-linking method could form the chitosan matrix for the controlled release of hydroquinone. It is possible that the release of the drug was affected by the polymer degradation due to the hydrolysis of cross-linking bonds of the chitosan matrix (Dini *et al*., 2003).

Furthermore, the study of different concentration (%w/w) of WSC influencing the release of arbutin is presented in Fig.25. F4 to F6 and F7 to F9 were studied. F4 to F6 contained WSC nanoparticles generated from medium MW WSC with different concentration at 0.25, 0.175 and 0.1%w/w, respectively. They were composed of K_m value at 2:1 and the ratio of water, oil and surfactant phase at 15, 20 and 65%w/w, respectively. F7 to F9, whereas, contained all similar components to F4 to F6 except WSC particles generated from high MW. Arbutin released from the lower concentration of WSC either medium or high MW was faster than that of the higher concentration. It could be confirmed by their release rates as shown in Table 8. The release rates of F4 to F6 and F7 to F9 were gradually increased following the

decrease in WSC concentration. It is possible that the low density structure is more degradable than the high density structure (Desai and Park, 2005) and provides the faster release of drug than the high one.

Moreover, the release profiles of bicontinuous type microemulsion were studied and presented in Fig.26. F11 and F12 exhibited the bicontinuous type microemulsion. They contained K_m value at 2:1 and the ratio of water, oil and surfactant phase at 30, 10 and 60%w/w, respectively. F11 contained WSC nanoparticles generated from medium MW WSC concentration of 0.1%w/w, while, pure bicontinuous type microemulsion was presented as F12. The result revealed that the release of arbutin from pure bicontinuous type microemulsions was faster than those containing WSC nanoparticles. Moreover, the release rates of F11 and F12 as shown in Table 8 could support that result. The release rate of F12 was higher than that of F11 which contained WSC nanoparticles in its system and the release rate of either F11 or F12 was higher than that of other formulations. This result revealed that bicontinuous type microemulsion exhibited the highest release rate compared with w/o type microemulsion. It is possible that the dynamic intertwined networks of bicontinuous structure were easily destroyed and, thereafter, arbutin was released from that networks conveniently compared with the spherical dispersed water droplets of w/o type microemulsion which hindered the release of arbutin by the surfactant around water droplets.

Moreover, the release of arbutin from the w/o/w emulsions was reported as shown in Fig.27. The w/o/w emulsions were characterized as the inner water droplets dispersed in the dynamic intertwined networks of oil and water of bicontinuous type microemulsion. For the presence of WSC nanoparticles in the

w/o/w emulsions, the w/o type microemulsion containing arbutin was firstly prepared. Thereafter, the bicontinuous type microemulsion was prepared as continuous phase. WSC was then soluble in the part of intertwined networks of water and TPP as cross-linking agent was added to form the WSC nanoparticles. This situation was presented in F13 and F14. They were composed of K_m value at 2:1 which presented in both inner water phase of first w/o type microemulsion and outer intertwined networks of water phase of latter bicontinuous type microemulsion. Their ratio of water, oil and surfactant phase was 15, 20 and 65%w/w, respectively, for the inner layer as w/o type microemulsion and 30, 10 and 60%w/w, respectively, for the construction of continuous phase as bicontinuous structure. F13 contained WSC nanoparticles generated from medium MW WSC concentration of 0.1%w/w, while, pure w/o/w emulsions was presented as F14. The results revealed that the release of arbutin from pure w/o/w emulsions was faster than w/o/w emulsions containing WSC nanoparticles. Moreover, the release rate of F13 and F14 were presented in Table 8. F13 provided significantly lower release rate than F14 (One-Sample T-Test, $p<0.05$). It is likely that the presence of the WSC nanoparticles in the intertwined networks of bicontinuous structure could provide the controlled release of arbutin.

It might be concluded that F4 seems to be the appropriate formulation based on the *in vitro* release, because it gave the lowest release rate resulting in the controlled release of arbutin. Moreover, F4 gave the higher undegraded arbutin at room temperature, 4^{o}C and even 45^{o}C as shown in Part 3.5.1.

Table 8 Release rates of arbutin from microemulsions

Formulations	Release rate [μg/cm^2/h] (from 1-12 hr)
F1	12.22 ± 0.57
F2	14.33 ± 0.80
F3	12.62 ± 0.33
F4	11.71 ± 2.39
F5	13.72 ± 0.88
F6	13.57 ± 0.91
F7	13.07 ± 0.34
F8	14.09 ± 0.86
F9	14.50 ± 0.20
F10	14.38 ± 0.02
F11	17.58 ± 0.46
F12	20.29 ± 0.81
F13	15.13 ± 0.53
F14	16.95 ± 0.59

The results are the mean ± S.E.M. of three experiments.

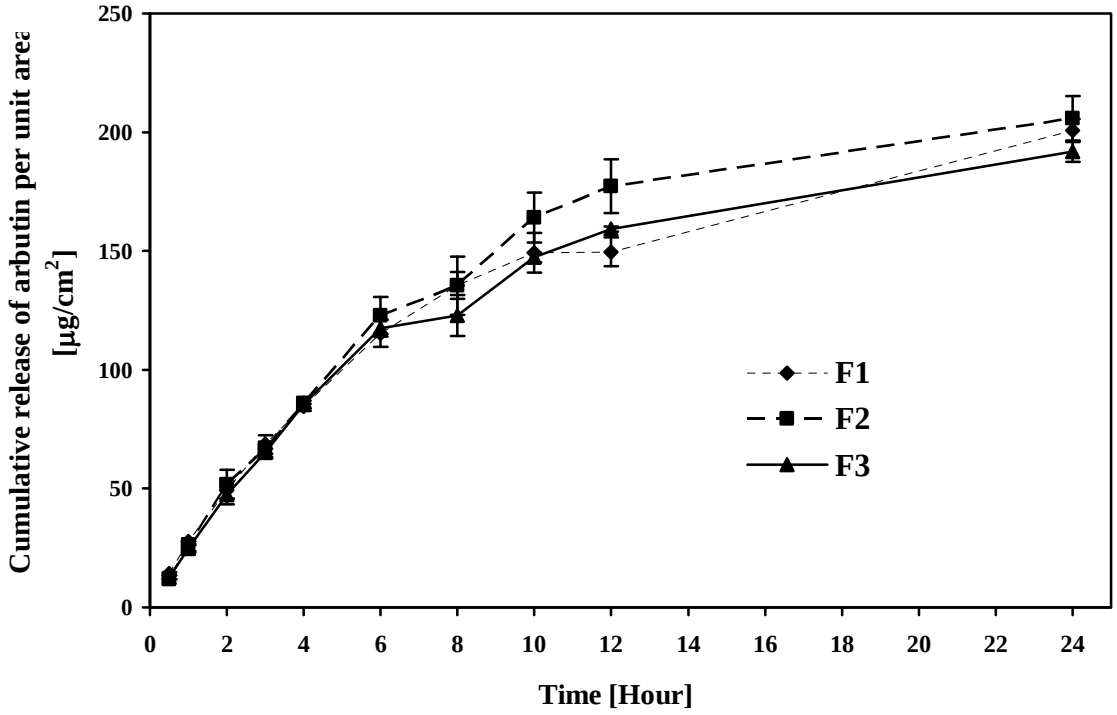


Figure 23 Release profiles of arbutin from w/o type microemulsions with various K_m values (2:1 in F1, 3:1 in F2, and 4:1 in F3) (each point represents mean ± S.E.M.; n=3)

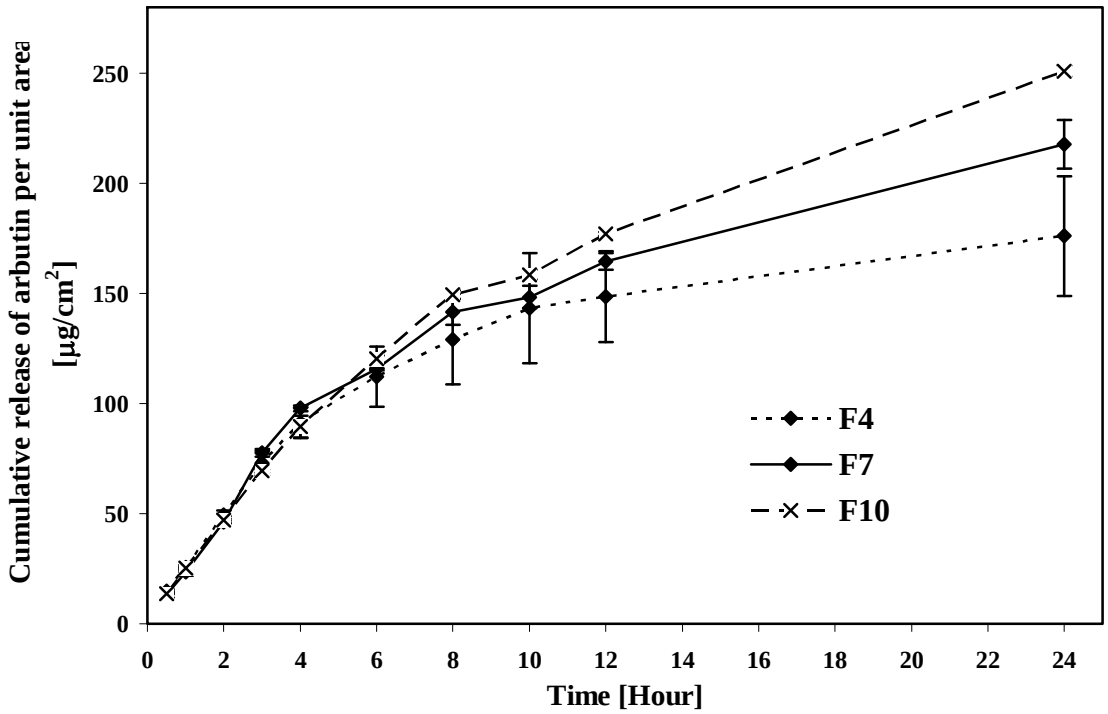


Figure 24 Release profiles of arbutin from w/o type microemulsions containing medium MW chitosan (F4) and high MW chitosan (F7) compared with without chitosan (F10) (each point represents mean ± S.E.M.; n=3)

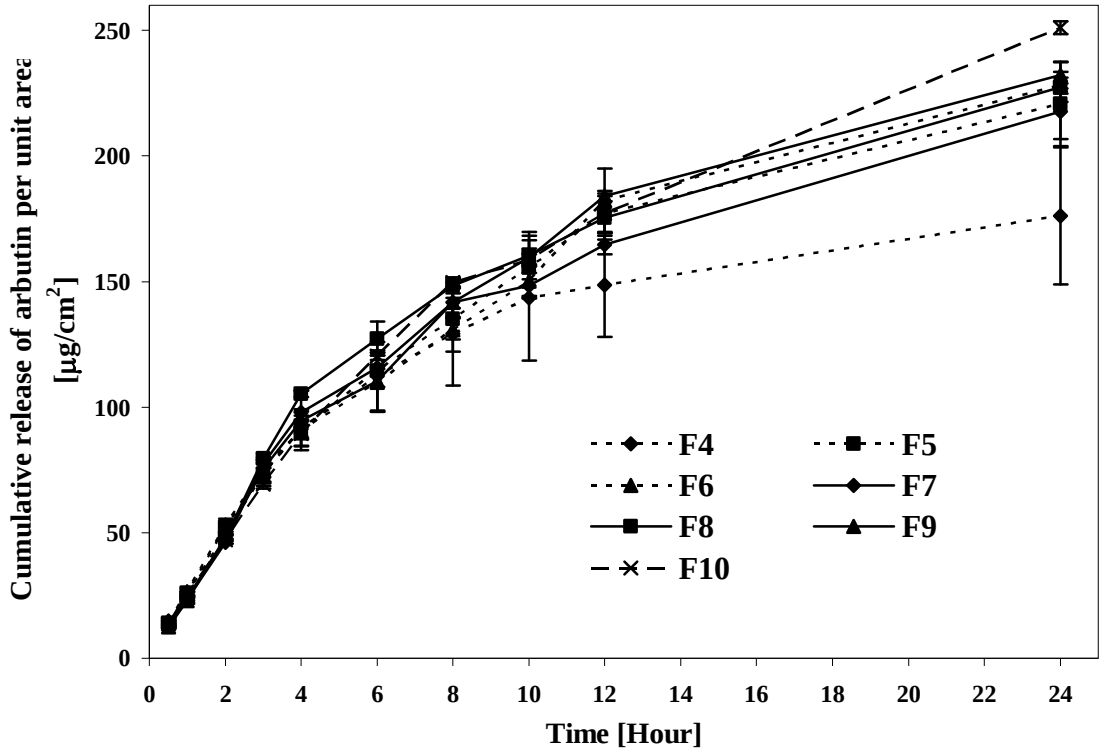


Figure 25 Release profiles of arbutin from w/o type microemulsions containing medium MW chitosan of various concentrations; 0.25% (F4), 0.175% (F5), and 0.1% (F6) and high MW chitosan of various concentrations; 0.25% (F7), 0.175% (F8), and 0.1% (F9) compared with without chitosan (F10) (each point represents mean ± S.E.M.; n=3)

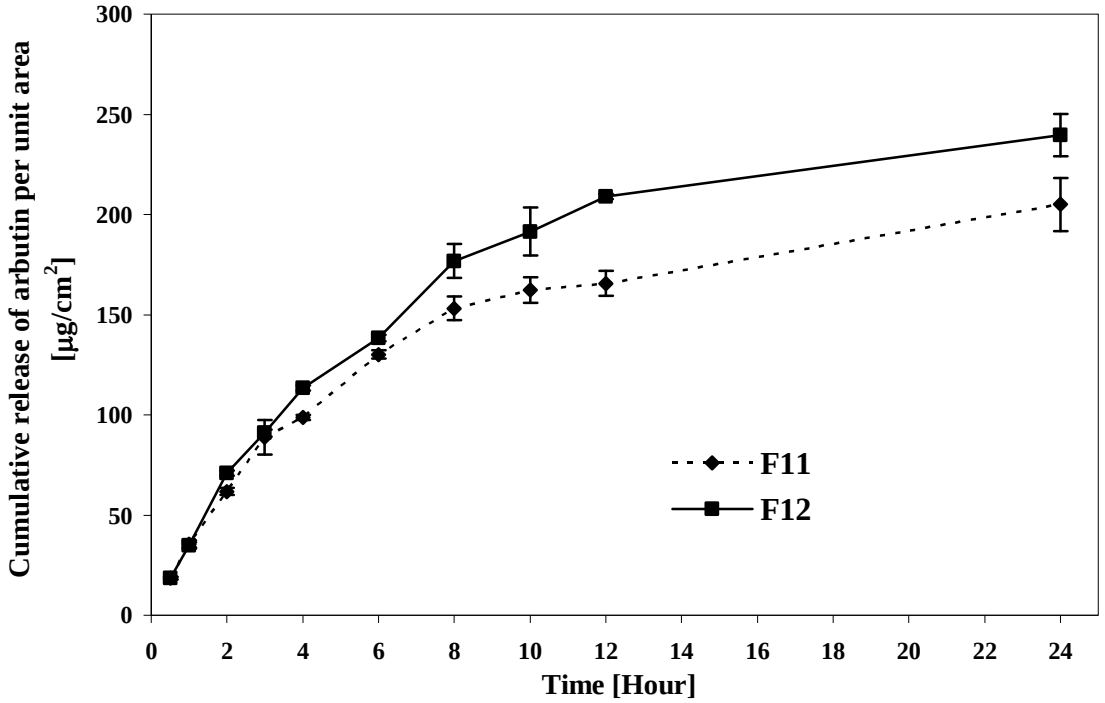


Figure 26 Release profiles of arbutin from bicontinuous type microemulsions with 0.1% medium MW chitosan (F11) and without chitosan (F12) (each point represents mean ± S.E.M.; n=3)

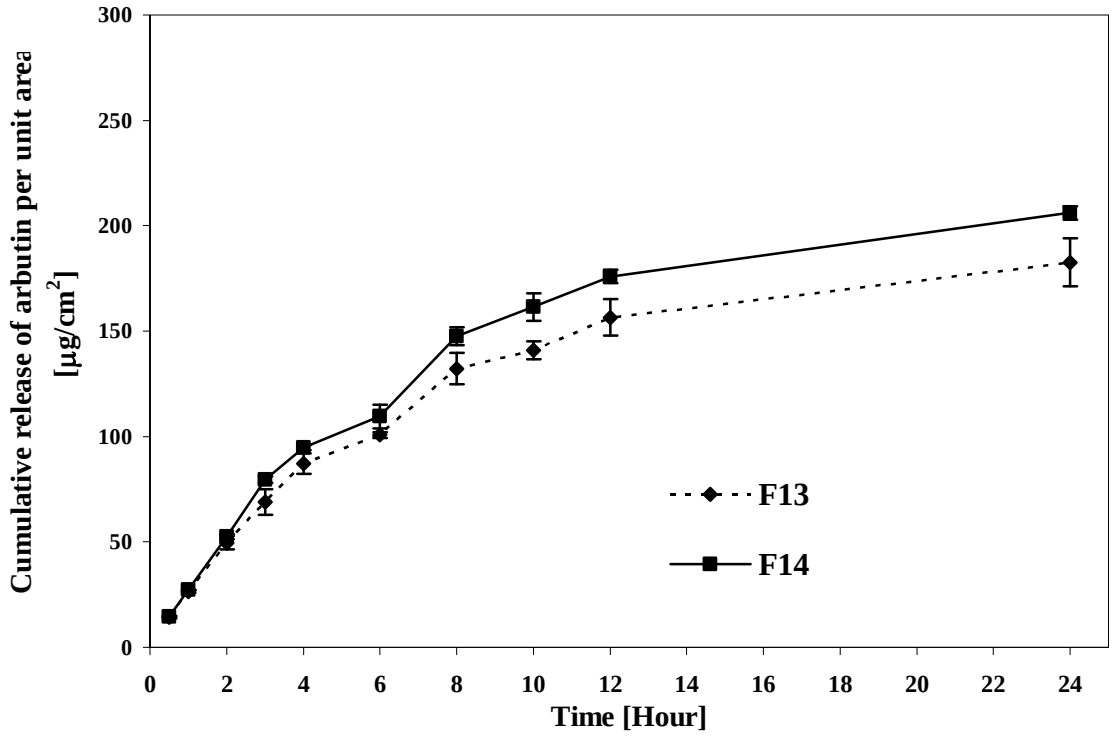


Figure 27 Release profiles of arbutin from w/o/w emulsions with 0.1% medium MW chitosan (F13), and without chitosan (F14) (each point represents mean ± S.E.M.; n=3)

5. Skin permeation of arbutin microemulsions

The *in vitro* permeation was determined using modified Franz diffusion cells. The newborn pig skins were used as a model skin since they are similar to human skin, i.e. the thickness of the stratum corneum and epidermis (Songkro *et al*., 2003a). In addition, their advantages are minimal animal ethical considerations, more readily available, and more easily to be standardized than human skin (Songkro *et al*., 2003a).

In the present experiment, the formulations F1, F2 and F3 (K_m values 2:1, 3:1, and 4:1, respectively) were selected to study the effect of Labrasol® as the skin permeation enhancer. Moreover F4 and F7 were selected to study the influence of molecular weight of WSC to the permeation of arbutin, using F10 as a control (without WSC/TPP). The effect of WSC concentration (F5, F6, F8 and F9) was not studied since the release of arbutin was not significantly different between formulations (One-Way ANOVA, $p>0.05$). In addition, permeation of arbutin from F11 as bicontinuous type microemulsion and F12 was investigated. However, skin permeation from F13 and F14 was not studied since arbutin was limited by its solubility. Moreover, F13 and F14 were bicontinuous structure as continuous phase, similar to F11 and F12 which were represented as bicontinuous type microemulsion.

Table 9 shows the flux and permeation coefficient (K_P) of arbutin from the formulations. The skin permeation of arbutin from F1 to F3 is presented in Fig.28. As shown in Table 9, the flux and K_P of arbutin from F1, F2 and F3 were not different significantly (One-Way ANOVA, $p>0.05$). This result can be explained by the following study (Kreilgaard *et al*., 2000). The influence of structure and composition of microemulsions (Labrasol®/Plurol® isostearique/isostearylic isostearate/water) on

the transdermal delivery of lidocaine (lipophilic model drug) and prilocaine hydrochloride (hydrophilic model drug) was reported (Kreilgaard *et al*., 2000). The steady state flux of such model drugs from microemulsions containing various K_m values (1:1, 2:1 and 3:1) was not different significantly even at the systems with large surfactant content. Moreover, Liu *et al*. (2006) studied the effect of enhancers on the topical delivery of cyclosporine A (CysA). They reported that Labrasol® appeared to be a weak penetration enhancer compared to 40% ethanol and diethylene glycol monoethyl ether (Transcutol®) as shown by low concentrations of CysA in stratum corneum and deeper skin (epidermis and dermis) (Liu *et al*., 2006). In our study, therefore, no influence of Labrasol® on the permeation of arbutin across newborn pig skin was observed. The flux of arbutin from microemulsions containing various K_m values (F1, F2, and F3) was not different significantly (One-Way ANOVA, $p>0.05$).

As shown in Fig.29, the permeated amount of arbutin from the solution was higher than that of all microemulsion formulations. It has been reported that, the hydroxyl groups of the active substances are effective in increasing permeability based on the hydrogen bonding ability of substance with the polar lipids of stratum corneum (Songkro *et al*., 2003b). In our study, the hydroxyl group of phenol ring of arbutin might interact with the polar lipids of stratum corneum, resulting in the increasing permeability of arbutin. In addition, newborn pig skin used in our study was full-thickness skin comprising of epidermis and dermis (Songkro *et al*., 2003a). Since the epidermis layer of such skin was very thin, the presence in the aqueous dermal layer is likely to have a slight effect on the diffusion of substances through the skin (Songkro *et al*., 2003a). From all above reasons, it is possible that free arbutin as dissolved in the solution could contact directly and, then, penetrate through the skin to

provide more amount of obtained arbutin in the receptor fluid than arbutin incorporated in formulation.

Moreover, the influence of molecular weight of WSC (F4 and F7 contained WSC of medium and high MW, respectively) to the permeation of arbutin was studied compared with F10 as controlled. In Table 9, the flux and K_P of arbutin from F7 and F10 was higher than F4 significantly (One-Way ANOVA, $p<0.05$), while no significant difference between F7 and F10 was observed (One-Way ANOVA, $p>0.05$). This phenomenon was correlated to the *in vitro* release of arbutin from such formulations. It is likely that WSC in the inner aqueous phase could form the WSC-TPP matrix and hamper the release of arbutin from the formulations. As reported previously, the particles smaller than 3μm were randomly distributed in the stratum corneum and penetrated the skin, while, the particles larger than 10-20 μm were remained on the stratum corneum and did not penetrate through the skin (Escribano *et al*., 2003). Therefore, nano/microparticles generated from both medium and high MW chitosan (average particle size of 200 nm and 2 μm, respectively) were able to retain in the stratum corneum and penetrate the skin. The important pathway of drug penetration is to across the continuous stratum corneum that is divided in intercellular and paracellular routes. However, most molecules penetrate through skin via intercellular route (Barry, 2001). It is likely that the WSC nano/microparticles containing arbutin were able to penetrate the stratum corneum via intercellular route and, thereafter, retain in the stratum corneum resulting in the release of arbutin in the stratum corneum.

The permeation of arbutin from F11 and F12 is shown in Fig.29. The flux and permeation coefficient of arbutin from F11 was less than that of F12 (Table

9). It is likely that the spherical droplets (F4) could more easily penetrate through the skin than non-spherical droplets (F11) resulting in the slight decrease in flux and permeation coefficient.

The influence of arbutin concentration in F4 on the amount of arbutin permeated across the skin is shown in Fig. 30. The concentrations of arbutin in the microemulsions were 0.5%w/w and 1%w/w which is the maximum concentration of arbutin that could be incorporated into F4. It has been known that the effective method to improve the skin permeation rate and the skin retention of various compounds is the increase in loading dose (Chen *et al*., 2004). In our study, as the arbutin concentration was increased to 1%, the amount of arbutin permeated through skin also increased. The flux of arbutin was increased with increasing the arbutin concentration, while K_P was not increased (Table 9). This phenomenon has been reported previously (Kreilgaard *et al*., 2000). The permeation coefficient of the model drug from the vehicles with high and low drug loading did not diverge significantly, except in the increase in the transdermal flux that was influenced directly by the higher concentration of the drug in the individual vehicle (Kreilgaard *et al*., 2000).

Table 9 Permeation coefficient (K_P) and flux of arbutin from microemulsions through full thickness newborn pig skin (mean ± S.E.M.; n=3)

Formulations	**K_P [x 10^{-3}cm/h]**	**Flux [µg/cm^2/h]**
Arbutin solution	3.66 ± 0.85	18.30 ± 4.25
F1	0.16 ± 0.13	0.79 ± 0.63
F2	0.80 ± 0.43	3.99 ± 2.16
F3	0.12 ± 0.03	0.59 ± 0.14
F4 a (0.5% arbutin)	0.41 ± 0.06	2.07 ± 0.29
F4 b (1% arbutin)	0.30 ± 0.08	2.96 ± 0.79
F7	2.02 ± 0.53	10.10 ± 2.64
F10	2.72 ± 0.53	13.59 ± 2.63
F11	0.07 ± 0.02	0.38 ± 0.13
F12	0.12 ± 0.02	0.61 ± 0.10

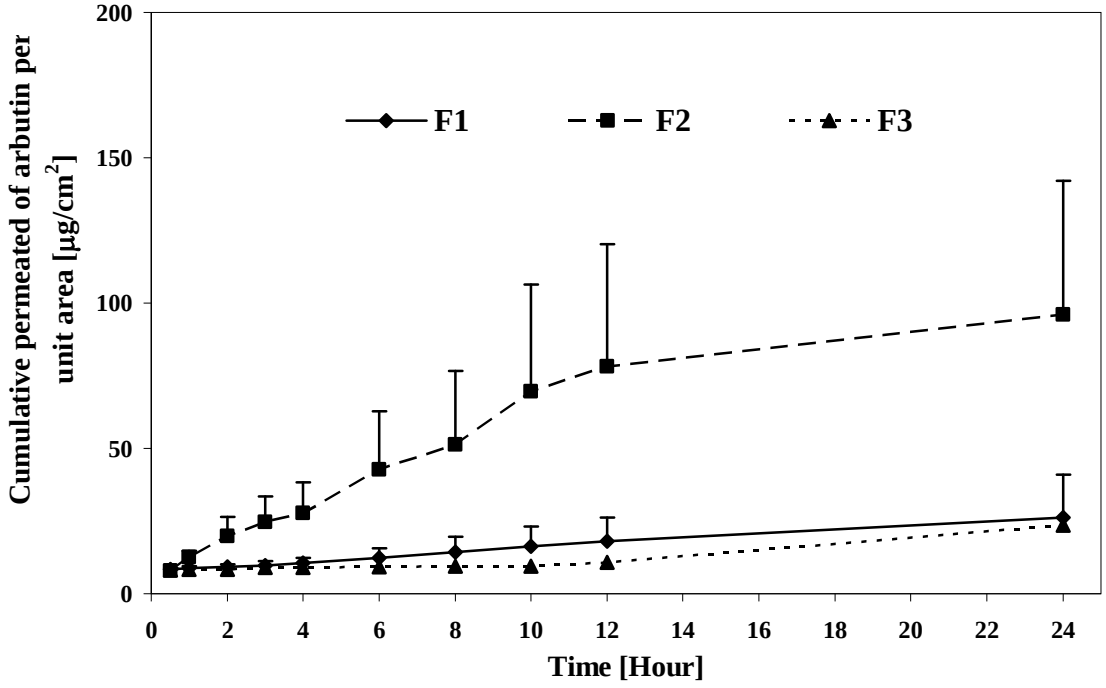


Figure 28 Effects of K_m values of w/o type microemulsions on skin permeation of arbutin (2:1 in F1, 3:1 in F2, and 4:1 in F3) (each point represents mean ± S.E.M.; n=3)

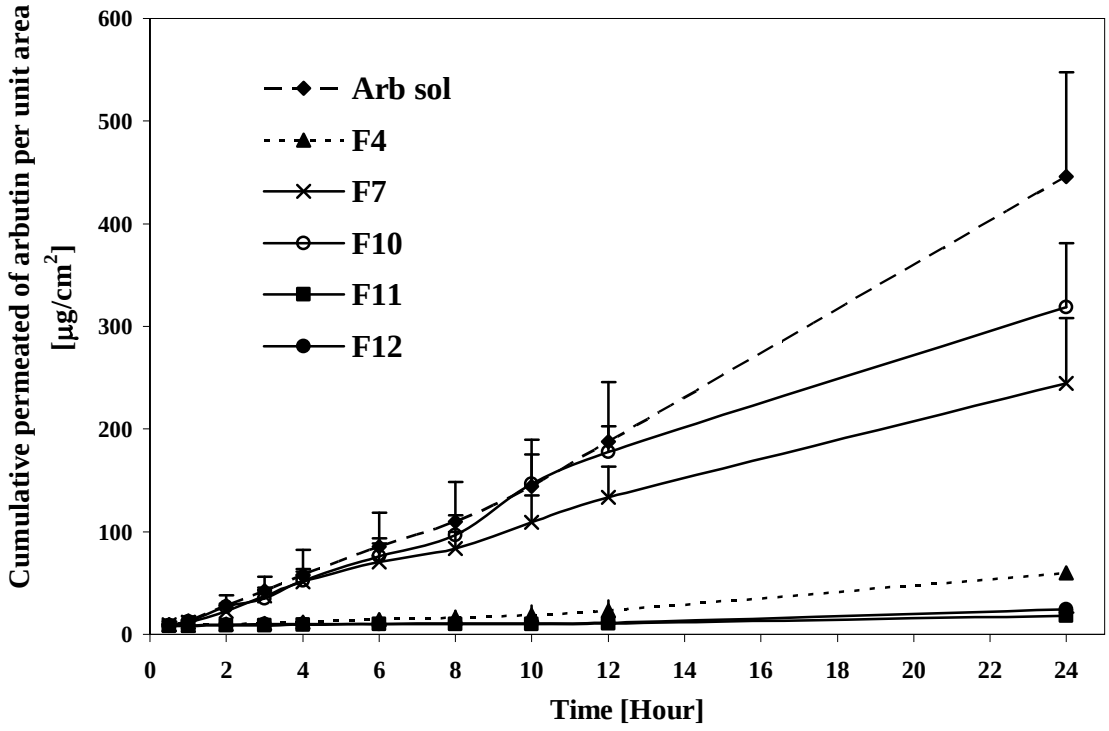


Figure 29 Effects of microemulsion types on skin permeation of arbutin (each point represents mean ± S.E.M.; n=3)

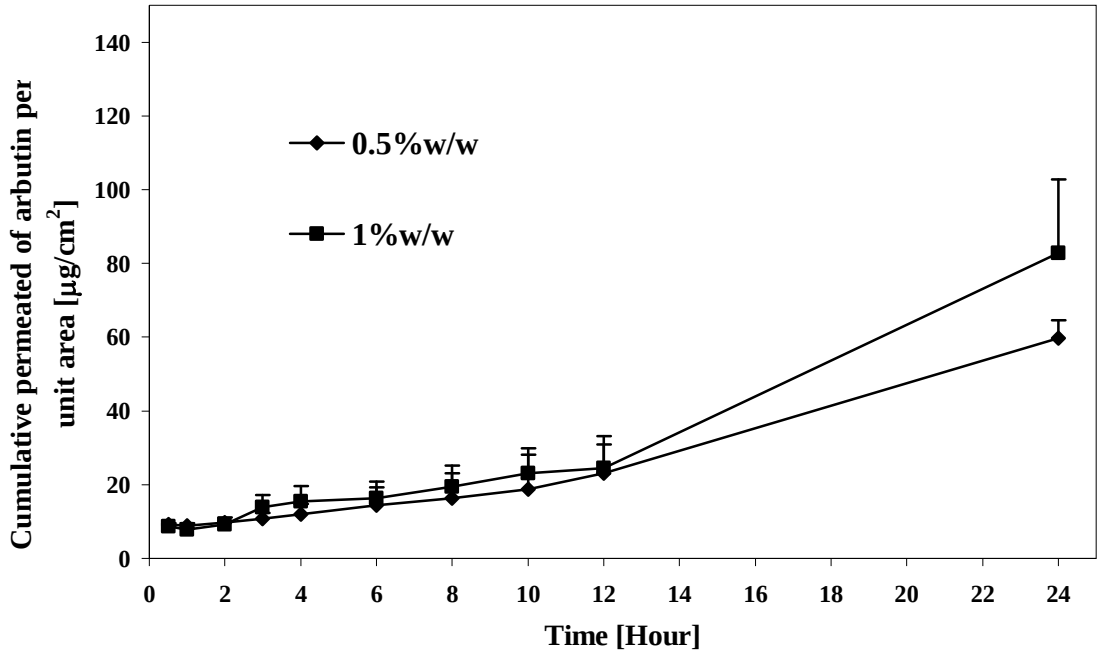


Figure 30 Effects of loading dose of w/o type microemulsions (F4) on skin permeation of arbutin (each point represents mean ± S.E.M.; n=3)

6. Skin retention of arbutin microemulsions

The objective of the topical application of arbutin is to maintain the effective concentration of arbutin near its site of action, melanocytes, which is in the basal layer of epidermis. In the present experiment, after 24 hours of *in vitro* skin permeation experiment, the newborn pig skin was removed from modified Franz diffusion cell and homogenized in distilled water to detect the accumulated amount of arbutin in the skin using HPLC method.

The accumulation amount of arbutin from various formulations is presented in Fig.31. Effects of microemulsions with various K_m values (F1, F2 and F3 as K_m values of 2:1, 3:1 and 4:1, respectively) on the accumulation of arbutin in the skin was not different significantly (one-way ANOVA, $p>0.05$).

Moreover, the influence of different MW of WSC was also studied. The accumulated amount of arbutin in the skin from F4 which contained WSC medium MW, was higher than that from F7 which contained WSC high MW and higher than that from F10 as a control significantly (one-way ANOVA, $p<0.05$). It is likely that F7 seems to lose arbutin permeated through newborn pig skin more than retained arbutin in the skin, whereas, F4 seems to accumulate arbutin in the skin more than penetrate arbutin across the skin. It was correlated to the skin permeation of arbutin from F4 and F7 as shown previously in Fig.29. It is possible that both denser (F4) and looser (F7) matrix could be retained in the skin. The denser matrix seems to keep arbutin in such matrix and accumulate arbutin in the skin, while, the looser matrix seems to lose arbutin from such matrix. Such arbutin is penetrated preferably through the receptor fluid based on the interaction between the hydroxyl group of arbutin and the polar lipids of stratum corneum as mentioned above.

Furthermore, the retention of arbutin in the skin from F11 and F12 as bicontinuous type microemulsion was studied (Fig.31). The accumulated amount of arbutin from F11 was higher than that of F12. It is correlated to the permeation of arbutin from such formulations. F11 contained crosslinked chitosan in the formulation which could retain arbutin in the skin compared with F12 as a control. In addition, the accumulated amount of arbutin in the skin from F4 was as similar as F11. It is likely that both w/o and bicontinuous type of microemulsions containing crosslinked chitosan were able to improve arbutin retained in the skin.

Thereafter, effects of arbutin concentrations in the retained amount of arbutin in the skin were studied as shown in Fig. 32. The amount of arbutin retained in the newborn pig skin of 1% arbutin loading was significantly higher than that of 0.5% loading (approximately 105 and 84 $\mu g/cm^2$, respectively) (One-Sample T-Test, $p<0.05$). Thus, the appropriate concentration of arbutin incorporated in our formulations was likely to be 1%w/w, since it gave the highest amount of arbutin retained in the skin. Moreover, the commercial products contain the arbutin concentration of 1% as the effective concentration for depigmentation (Rendon and Gaviria, 2005).

In conclusion, F4 seems to be the most appropriate formulation, since it provides the highest amount of arbutin retained in the skin.

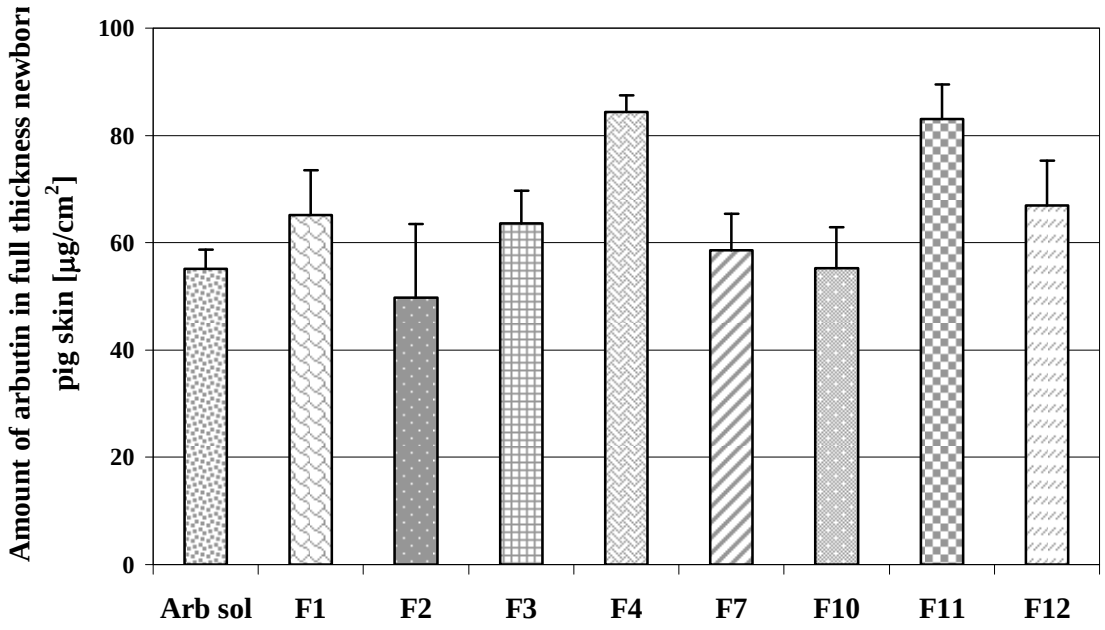


Figure 31 Skin retention of arbutin in various microemulsion formulations compared with 0.5%w/w arbutin solution (mean ± S.E.M.; n=3)

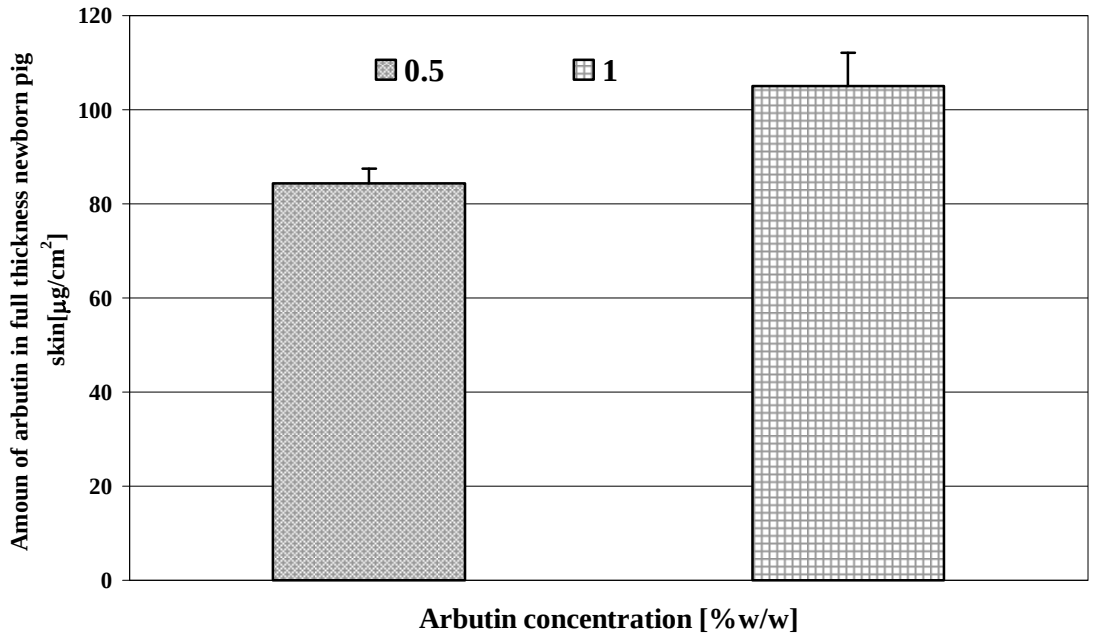


Figure 32 Skin retention of arbutin in w/o type microemulsions (F4) with 0.5%w/w and 1%w/w arbutin loading (mean ± S.E.M.; n=3)

7. Cytotoxicity of microemulsions

Our study presents the new microemulsion systems composed of virgin coconut oil, Labrasol®, Plurol® Oleique, and WSC solution as oil, surfactant, co-surfactant and water phase, respectively. Cytotoxicity of arbutin solutions and microemulsions on melan-a melanocytes was studied using the mitochondrial dehydrogenase (MTT) assay. MTT is a yellow tetrazolium salt that is oxidized by the mitochondrial dehydrogenase enzyme in living cells to give a dark blue formazan product (Sha *et al*., 2005). Firstly, we demonstrated the effect of different diluted microemulsions to the melan-a viability. The concentrations of the microemulsions were varied from 0.001 to 5%v/v to find the optimal concentration of microemulsion for the next experiments as shown in Fig.33. The result revealed that the diluted concentration below 0.1%v/v was too toxic to malan-a melanocytes, in contrast, the diluted concentration over 0.1%v/v showed the viability of over 80%. This result was in agreement with Rozner *et al* (2008). They reported that the optimal diluted concentration using in all experiments was 0.1%v/v microemulsion which showed HaCaT keratinocyte viability of 75% after 18 hours incubation time (Rozner *et al*., 2008). Moreover, all microemulsion formulations showed the optimal diluted concentration of 0.1%v/v used in next experiment as shown in Fig.34. Therefore, the final concentration at 0.1%v/v was the appropriate concentration for further study.

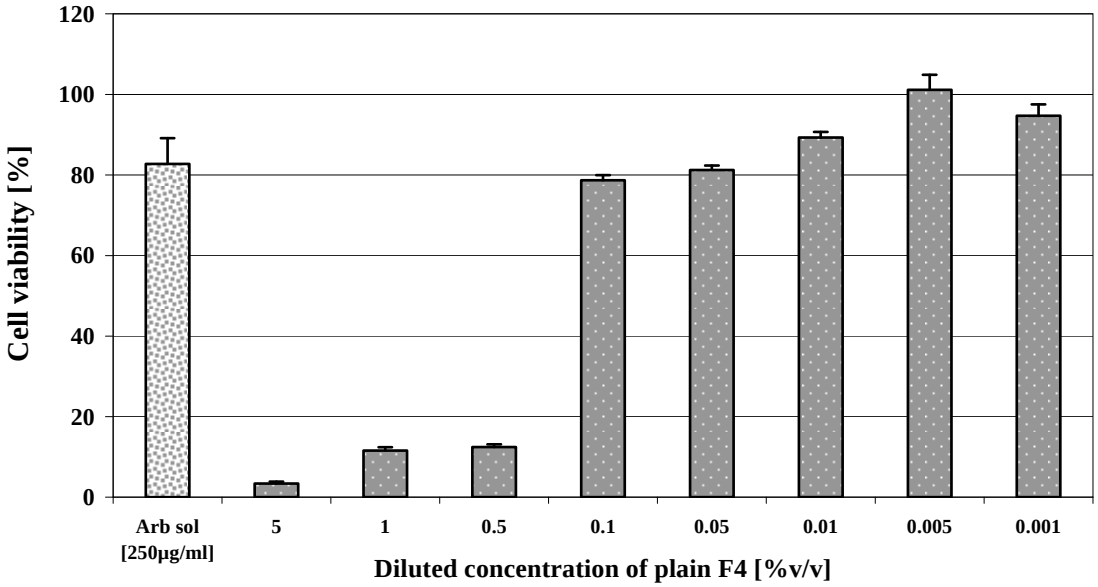


Figure 33 Cytotoxicity of w/o type microemulsion containing medium MW chitosan (F4) (mean ± S.E.M.; n=16)

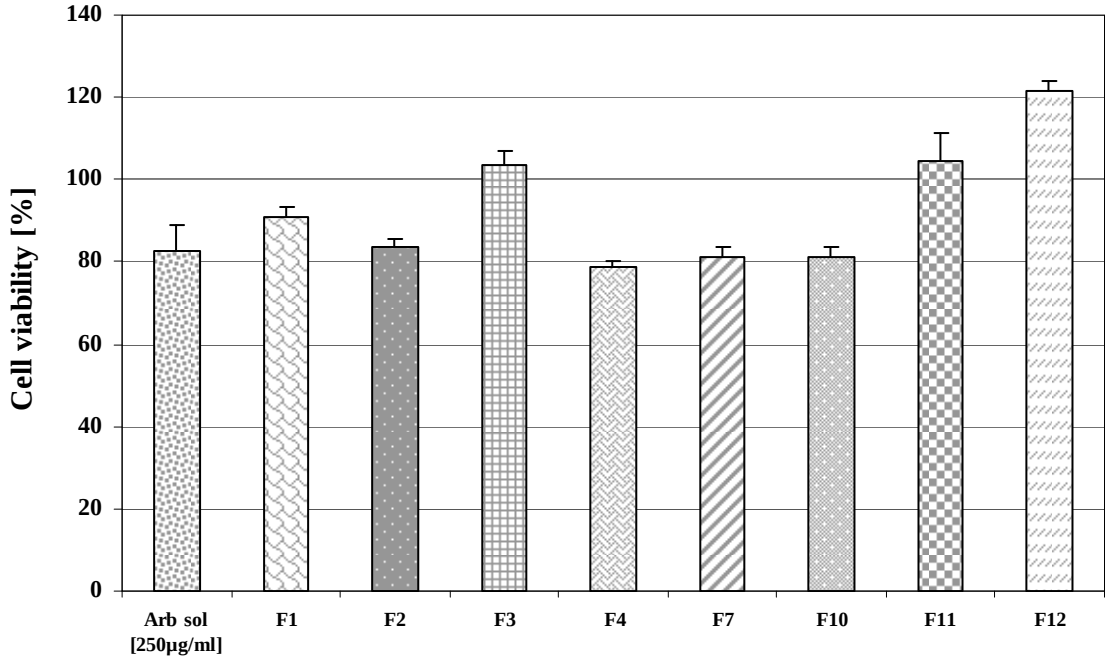


Figure 34 Cytotoxicity of w/o and bicontinuous type microemulsions (mean ± S.E.M; n=16)

8. Whitening activity of microemulsions

The determination of whitening activity of plain microemulsions composed of VCO, Labrasol®, Plurol® Oleique and WSC solution as oil, surfactant, co-surfactant and water phase, respectively, was carried out. VCO contains the potential minor components such as alpha-tocopherol, tocotrienols and polyphenols obtained by wet process (Nevin and Rajamohan, 2004). Moreover, alpha-tocopherol is the most potential form of vitamin E more than other forms. Funasaka *et al*. (1999) reported that alpha-tocopherol is a strong inhibitor of melanin formation among the chemicals tested (arbutin, kojic acid, magnesium L-ascorbyl 2-phosphate and tranexamic acid) in pigmented human melanoma cells (Funasaka *et al*., 1999). For the study of whitening activity of microemulsions, the activity of arbutin in our microemulsions was limited by its solubility. Thus, this present study shows the whitening activity of microemulsion formulation without the presence of arbutin.

8.1 Assay of mushroom tyrosinase activity

Initially, mushroom tyrosinase was preincubated with plain microemulsion at diluted concentration of 0.1%v/v for 10 minutes. Then, L-dopa as substrate was added and the dopachrome formation was measured at 475 nm using microplate reader. The result revealed that F1 to F3 did not differ with each other significantly as shown in Fig.35 (One-Way ANOVA, p>0.05) indicating that the different K_m ratio did not influence to mushroom tyrosinase activity. Moreover, MW of WSC influenced the mushroom tyrosinase activity. As shown in Fig.35, F4 containing WSC nanoparticles generated from medium Mw gave lower mushroom tyrosinase activity than F7 containing WSC generated from high MW and F10 as

controlled. Finally, the bicontinuous type microemulsion was studied. The mushroom tyrosinase of F11 was slightly lower than that of F12 as controlled (One-Sample T Test, $p<0.05$). The result revealed that F11 contained WSC nanoparticles which could lower the mushroom tyrosinase activity compared with F12. Therefore, WSC nanoparticles could be useful to incorporate in our microemulsion system to lower mushroom tyrosinase activity compared with control.

8.2 Melanin assay in melanocytes

Melanin is synthesized in a multi-step biochemical pathway that operates within a specialized intracellular organelle, melanosome (Wang *et al*., 2006). Therefore, melanin formation is the most important determination of skin depigmentation (Wang *et al*., 2006). In present study, malan-a melanocytes were used for determining the effect of various microemulsion formulations on the melanin content. The result revealed that all microemulsion formulation containing WSC nanoparticles could lower melanin formation compared with control. As similar to the mushroom tyrosinase activity, there was no different significantly between F1 to F3 as shown in Fig.36 (One-Way ANOVA, $p>0.05$), indicating that the different K_m ratio did not influence the melanin formation. Thereafter, MW of WSC was studied. The result revealed that F4 containing WSC nanoparticles generated from medium Mw gave significantly lower melanin formation than F7 containing WSC generated from high MW and F10 as controlled (One-Way ANOVA, $p<0.05$). Finally, the bicontinuous type microemulsion was also studied. The melanin formation of F11 was lower than F12 as controlled. Therefore, WSC nanoparticles could be useful to

incorporate in our microemulsion system to lower melanin content compared with control.

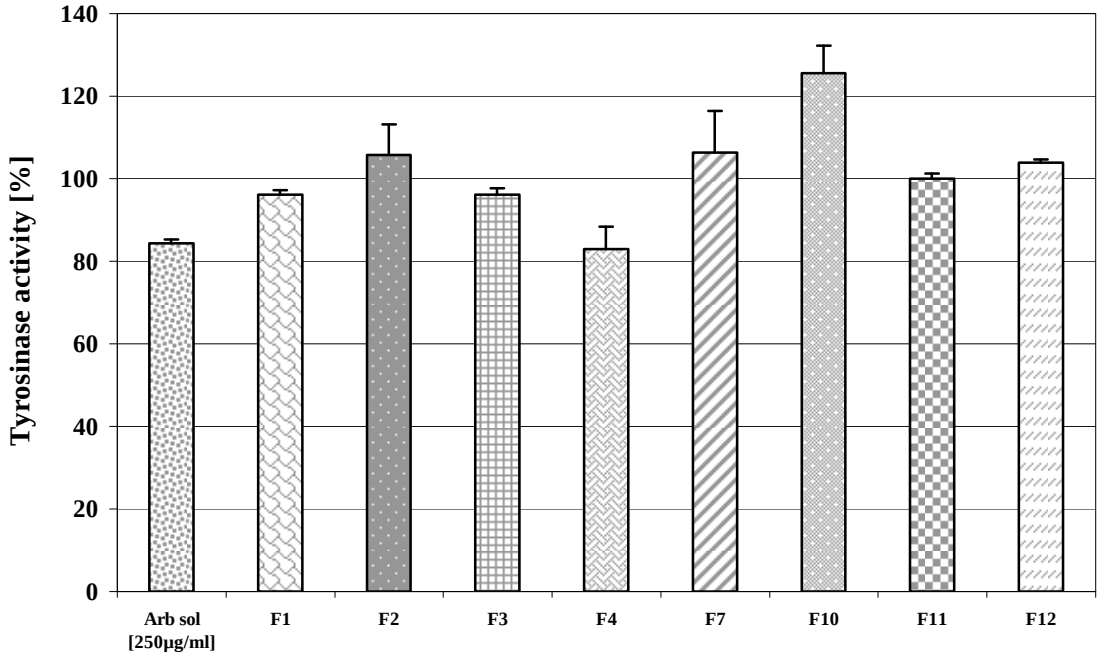


Figure 35 Mushroom tyrosinase activity of w/o and bicontinuous type microemulsions (mean ± S.E.M.; n=3)

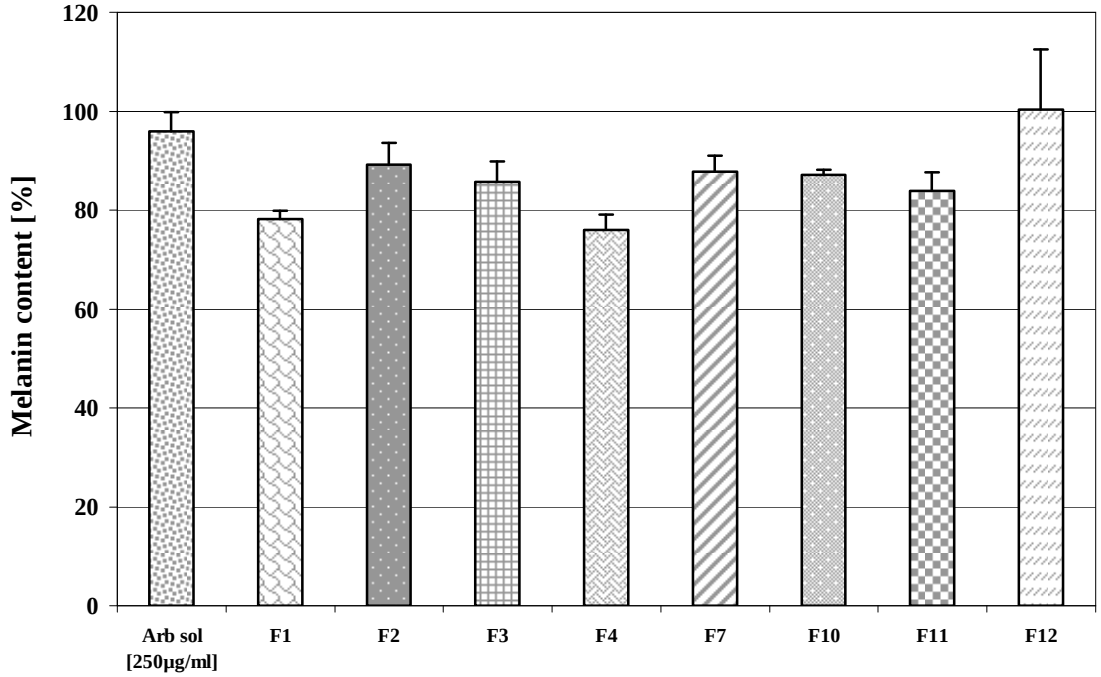


Figure 36 Effects of w/o and bicontinuous type microemulsions on melanin content of melanocytes cultures (mean ± S.E.M.; n=3)

CHAPTER V

CONCLUSION

This study presents the successful microemulsion systems as potential vehicles for topical delivery of arbutin. Our microemulsion systems were composed of virgin coconut oil, Labrasol®, Plurol® Oleique and water phase containing water soluble chitosan (WSC)/TPP. The pseudo-ternary phase diagram showed that the appropriate K_m value for our system was 2:1. The conductivity and DSC determination of the microemulsions indicated the water-in-oil (w/o) type microemulsions. At K_m value of 2:1, DSC study showed the endothermic peak of water phase began to appear above 20% of water phase, indicating the transition from w/o to bicontinuous type microemulsion. The conductance values confirmed the water phase at the concentration of 20% indicating the percolation threshold of such system. Thereafter, the microemulsion formulations containing chitosan nanoparticles were prepared and evaluated as the effective vehicles for arbutin delivery. The results revealed that the most appropriate formulations were the w/o type microemulsions with medium, and high MW WSC/TPP (F4 and F7, respectively). TEM determination indicated the spherical morphology of the WSC/TPP particles with the particle size of 200 nm in F4, and polydispersion of 200 nm to 1-2 μm in F7. The chemical stability of arbutin in microemulsion system containing WSC nanoparticles was investigated. Stability of arbutin in the microemulsions was determined after storage at room temperature, 45°C, and 4°C for 1, 2, and 3 months. F4 was more stable than F7 and F10 (without WSC/TPP) at all storage temperatures (room temperature, 4°C and 45°C). The arbutin content of F4 stored at room temperature and 4°C was 99.02%,

and 100.76%, respectively. At the elevated temperature, improved stability was obvious found in F4 (94.57% arbutin content), while degradation of arbutin was observed in the arbutin solution, F7, and F10 (arbutin content of 80.66%, 90.02%, and 88.46% respectively). Moreover, the results showed the physical stability of F4, and F7 after storage at 45^{o}C for three months.

Furthermore, the *in vitro* release, skin permeation and skin retention of F4 and F7 were investigated compared with F10 as controlled. F4 seems to be the most appropriate formulation based on such experiments, since it gave the controlled release of arbutin, the sustained penetration of arbutin across newborn pig skin, and the highest amount of arbutin retained in such skin.

Finally, the whitening activity of plain microemulsions was studied using mushroom tyrosinase activity and melanin content in melan-a melanocytes as indicators. The non-cytotoxic concentration of plain microemulsions to melan-a melanocytes was not more than 0.1%v/v. Skin whitening activity of the 0.1% dilution of plain F4 was determined. Effects of 250 μg/ml arbutin solution as positive control and plain F4 on tyrosinase activity were similar (84.32% and 82.92%, respectively). The melanin content in melan-a melanocytes of diluted plain F4 was significantly lower than that of the positive control.

Therefore, the results in this study indicated that microemulsion formulation containing medium MW WSC/TPP nanoparticles was a promising arbutin delivery for skin whitening application in cosmetics.

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APPENDIX A

HPLC ASSAY

1. Analytical method validation

1.1 Preparation of standard curve

Standard stock solutions (1000 µg/ml) of arbutin were prepared by dissolving 0.0100 g of arbutin in 10 ml of distilled water or isotonic phosphate buffer pH 7.4. Then, the standard stock solutions of arbutin at the volume of 10, 30, 50, 70 and 100 µl were transferred to 10-ml volume metric flasks. Thereafter, such solutions were adjusted to 10 ml with distilled water or isotonic phosphate buffer pH 7.4 to obtain the concentrations of 1, 3, 5, 7 and 10 µg/ml, respectively. These standard solutions were injected and analyzed using HPLC in triplicate.

1.2 Validation of HPLC analysis

The HPLC validation was investigated by intra-day and inter-day accuracy and precision test. The drug concentrations of 3, 7 and 10 µg/ml were used for the HPLC validation. These solutions were analyzed for the determination of arbutin concentrations using HPLC on the same day and for three consecutive days to evaluate intra-day and inter-day, respectively. The intra-day test was evaluated for three replicates of each concentration on the same day. For the inter-day test, each concentration was quantitated and compared for three consecutive days. The percentage relative standard deviation (%RSD) was calculated to determine the variation within replicates as follows:

$$RSD(\%) = \frac{S.D. \times 100}{\overline{X}}$$

Where S.D. is standard deviation and $\overline{X}$ is a mean of arbutin concentration. In present study, the methods were accepted when the percentage relative standard deviation was less than 10%.

Results of HPLC validation method

1. HPLC validation of arbutin in distilled water

1.1 Linearity

The average peak area was plotted against arbutin concentrations which all data are presented in Table 10. The standard curve of arbutin was linear over the concentration range 1-10 µg/ml (y = 17450x – 2857.4; r^2 = 0.9994) as shown in Fig.37. It was indicated that this standard curve gave high linearity between drug concentrations (µg/ml) and peak area (r^2 > 0.99).

Table 10 Average peak area of HPLC chromatogram of arbutin in distilled water

Arbutin concentration [µg/ml]	Average peak area	R.S.D. [%]
1	15976.78±1266.62	7.93
3	49486.56±763.59	1.54
5	82010.89±1482.75	1.81
7	119117.00±1748.95	1.47
10	172815.89±10958.00	6.34

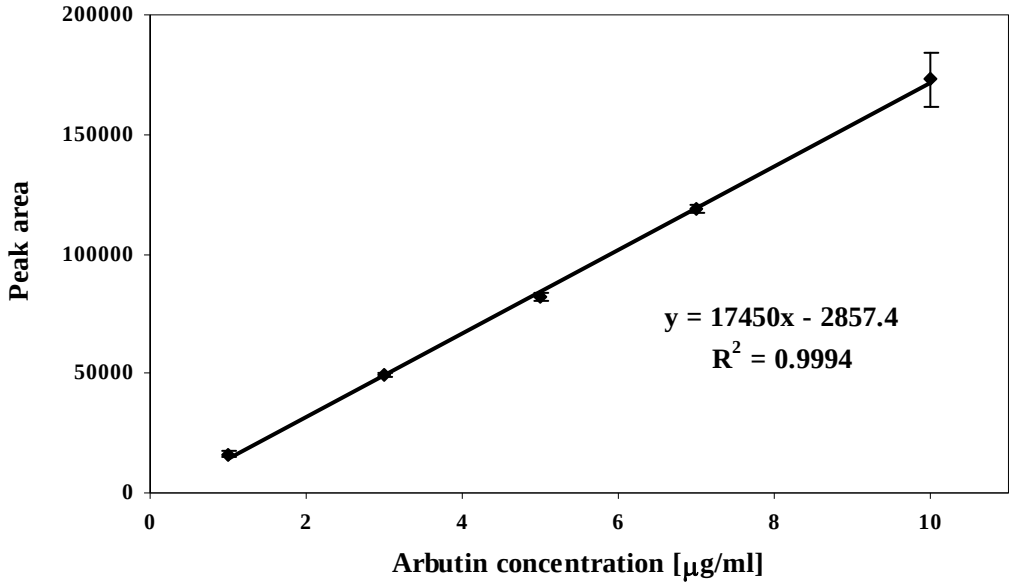

Figure 37 Standard curve of arbutin in distilled water

1.2 Recover/Accuracy

The drug concentrations of 3, 7 and 10 µg/ml were used for the accuracy test in HPLC validation method. All accuracy data were summarized in Table 11. The mean recovery (±S.D.) was 100.74±4.34, 99.71±1.25 and 100.34±3.59 for the arbutin concentration of 3, 7 and 10 µg/ml, respectively. These results showed that the method gave the acceptable coefficients of the percentage relative standard deviation since they were less than 10%.

Table 11 Accuracy of HPLC determination of arbutin in distilled water

Arbutin concentration [µg/ml]	Recovery [%]			Mean±S.D.	R.S.D. [%]
	Day 1	Day 2	Day 3		
3	101.89±5.31	100.33±6.17	100.00±1.53	100.74±4.34	4.30
7	98.57±0.65	100.71±1.68	99.86±1.41	99.71±1.25	1.25
10	100.6±3.48	99.77±0.99	100.67±6.31	100.34±3.59	3.57

1.3 Precision

The drug concentrations of 3, 7 and 10 µg/ml were used for the precision test in HPLC validation method. All precision data were summarized in Table 12. These results showed that the method gave the acceptable coefficients of the percentage relative standard deviation since they were less than 10%.

Table 12 Intra- and inter-day precision of HPLC determination of arbutin in distilled water

Arbutin concentration [µg/ml]	Intra-day		Inter-day	
	Mean±S.D.	R.S.D. [%]	Mean±S.D.	R.S.D. [%]
3	3.00±0.05	1.53	3.02±0.13	4.30
7	6.99±0.10	1.41	6.98±0.09	1.25
10	10.07±0.63	6.26	10.03±0.36	3.57

2. HPLC validation of arbutin in isotonic phosphate buffer, pH 7.4

2.1 Linearity

The average peak area was plotted against arbutin concentrations which all data are presented in Table 13. The standard curve of arbutin was linear over the concentration range 1-10 µg/ml (y = 16176x + 12528; r^2 = 0.9993) as shown in Fig.38. It was indicated that this standard curve gave high linearity between drug concentrateons (µg/ml) and peak area (r^2 > 0.99).

Table 13 Average peak area of HPLC chromatogram of arbutin in isotonic phosphate buffer, pH 7.4

Arbutin concentration [µg/ml]	Average peak area	R.S.D. [%]
1	28703.11±2836.83	9.88
3	62585.67±3403.32	5.44
5	96349.67±621.95	0.64
7	125485.78±2613.82	2.08
10	178501.67±5516.79	3.09

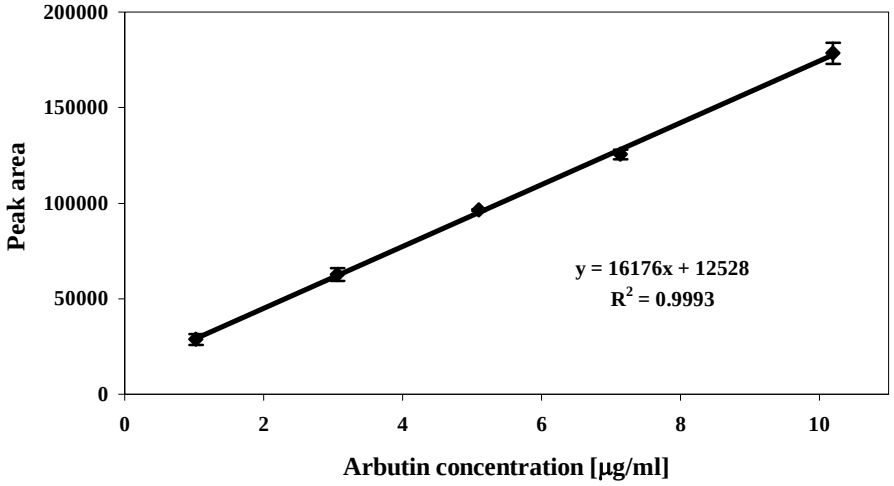

Figure 38 Standard curve of arbutin in isotonic phosphate buffer, pH 7.4

2.2 Recovery/Accuracy

The drug concentrations of 3, 7 and 10 µg/ml were used for the accuracy test in HPLC validation method. All accuracy data were summarized in Table 14. The mean recovery (±S.D.) was 102.00±5.77, 101.13±2.40 and 102.42±2.10 for the arbutin concentration of 3, 7 and 10 µg/ml, respectively. These results showed that the method gave the acceptable coefficients of the percentage relative standard deviation since they were less than 10%.

Table 14 Accuracy of HPLC determination of arbutin in isotonic phosphate buffer, pH 7.4

Arbutin concentration [μg/ml]	Recovery [%]			Mean±S.D.	R.S.D. [%]
	Day 1	Day 2	Day 3		
3	103.11±7.07	103.67±1.85	99.22±8.38	102.00±5.77	5.70
7	99.76±2.32	100.81±2.94	102.81±1.94	101.13±2.40	2.38
10	102.57±3.39	102.97±1.46	101.73±1.44	102.42±2.10	2.05

2.3 Precision

The drug concentrations of 3, 7 and 10 μg/ml were used for the precision test in HPLC validation method. All precision data were summarized in Table 15. These results showed that the method gave the acceptable coefficients of the percentage relative standard deviation since they were less than 10%.

Table 15 Intra- and inter-day precision of HPLC determination of arbutin in isotonic phosphate buffer, pH 7.4

Arbutin concentration [μg/ml]	Intra-day		Inter-day	
	Mean±S.D.	R.S.D. [%]	Mean±S.D.	R.S.D. [%]
3	3.09±0.21	6.86	3.06±0.17	5.70
7	6.98±0.16	2.33	7.08±0.17	2.38
10	10.26±0.34	3.31	10.24±0.21	2.05

APPENDIX B

PREPARATION OF REAGENTS

1. Preparation of isotonic phosphate buffer solution, pH 7.4 (USP XX)

This vehicle required two stock solutions, one containing 8.00 g of monobasic sodium phosphate (NaH_2PO_4) per liter, and the other containing 9.47 g of dibasic sodium phosphate (Na_2HPO_4) per liter, the weights being on the anhydrous basis. For the preparation, the used monobasic sodium phosphate was $NaH_2PO_4 \cdot 2H_2O$. Thus, the vehicle required 10.40 g of $NaH_2PO_4 \cdot 2H_2O$ per liter. The stock solutions used in the proportions are indicated in Table 16.

Table 16 Compositions of isotonic phosphate buffer, pH 7.4

Monobasic sodium phosphate solution [ml]	Dibasic sodium phosphate solution [ml]	Resulting buffer solution, pH	Sodium chloride required for isotonicity [g/100 ml]
20	80	7.4	0.44

Preparation of isotonic phosphate buffer, pH 7.4

1. 10.40 g of monobasic sodium phosphate ($NaH_2PO_4 \cdot 2H_2O$) was dissolved with distilled water in 1000-ml volumetric flask. The monobasic sodium phosphate solution was adjusted to 1000 ml with distilled water.

2. 9.47 g of dibasic sodium phosphate (Na_2HPO_4) was dissolved with distilled water in 1000-ml volumetric flask. The dibasic sodium phosphate solution was adjusted to 1000 ml with distilled water.

3. 200 ml of the monobasic sodium phosphate solution and 800 ml of dibasic sodium phosphate solution were mixed in 1000-ml beaker to make the phosphate buffer, pH 7.4.

4. 4.4 g of sodium chloride was dissolved in the phosphate buffer, pH 7.4 to make isotonic phosphate buffer, pH 7.4.

2. Preparation of 0.05M potassium phosphate solution

The mobile phase used in HPLC method was composed of 0.05M potassium phosphate (KH_2PO_4) and methanol at the ratio of 92:8. The composition for the preparation of 0.05M KH_2PO_4 is shown in Table 17.

Table 17 Compositions of 0.05M potassium phosphate solution

Potassium phosphate [g]	Distilled water [ml]
6.8045	Adjust to 1000 ml

3. Preparation of complete media for melan-a melanocytes

Table 18 Compositions of complete media for melan-a melanocytes

Compositions	Volume [ml]
FBS (10%v/v)	4
Penicillin/Streptomycin solution (1%v/v/)	0.4
Phorbol 12-myristate 13-acetate (PMA) (40μM)	0.2
RPMI1640 with glutamine	35.4

4. Preparation of complete media with phenylthiourea for melan-a melanocytes

Phenylthiourea (PTU) is the tyrosinase inhibitor. Cells are grown with PTU (100-300 μM) for a few days before freezing and also after thawing for improving the recovery rate of melan-a melanocytes. After thawing, pigmentation returns a few days after removing PTU.

Table 19 Compositions of complete media with phenylthiourea for melan-a melanocytes

Compositions	Volume [ml]
FBS (10%v/v)	4
Penicillin/Streptomycin solution (1%v/v/)	0.4
Phorbol 12-myristate 13-acetate (PMA) (40μM)	0.2
Phenylthiourea (PTU) (100mM)	0.08
RPMI1640 with glutamine	35.3

5. Preparation of stock solution of mushroom tyrosinase enzyme

Stock solution (10,000 unit/ml) of mushroom tyrosinase was prepared by dissolving 25 ku of mushroom tyrosinase in 2.5 ml of 50 mM potassium phosphate buffer pH 6.5. This solution was kept at -20°C before used. For used, stock solution of mushroom tyrosinase was diluted by 50 mM potassium phosphate buffer pH 6.8 to obtain mushroom tyrosinase solution at the concentration of 1,000 and 200 unit/ml, respectively.

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Mahasawat, P., Songkro, S. and Oungbho, K. 2008. Whitening activity of chitosan-alginate multiple emulsions containing arbutin. The 6th World Meeting on Pharmaceutics, Biopharmaceutic and Pharmaceutical Technology, (Centre Convencions Internacional Barcelona, Barcelona, Spain, April 7-10, 2008).