ANTI-TYROSINASE AND ANTIOXIDANT ACTIVITIES OF *MORINDA CITRIFOLIA* FRUIT EXTRACT AND DEVELOPMENT OF NANOEMULSIONS

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นางสาวพนิดา บริสุทธิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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By	Miss Panida Borisut
Field of Study	Pharmaceutical Technology
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พนิดา บริสุทธิ์: ฤทธิ์ต้านไทโรซิเนสและฤทธิ์ต้านออกซิเดชันของสารสกัดผลยอและการพัฒนานาโนอิมัลชัน. (ANTI-TYROSINASE AND ANTIOXIDANT ACTIVITIES OF *MORINDA CITRIFOLIA* FRUIT EXTRACT AND DEVELOPMENT OF NANOEMULSIONS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ดร.อัญญพร ตันศิริคงคล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.ดร. อุบลทิพย์ นิมมานนิตย์, 173 หน้า.

งานวิจัยนี้ มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ยับยั้งเอนไซม์ไทโรซิเนสและฤทธิ์ยับยั้งการเกิดออกซิเดชันสารสกัดจากผลยอที่ปลูกในประเทศไทยและนำมาพัฒนาเพื่อใช้ในทางเครื่องสำอาง น้ำคั้นจากผลยอถูกทำให้เป็นผลแห้งโดยวิธี Freeze-dried ส่วนกากที่แยกได้จะนำไปสกัดด้วยตัวทำละลายเอธานอล, อะซิโตน, และ เอธิลอะซิเตต จากนั้นทดสอบฤทธิ์ต้านไทโรซิเนสและฤทธิ์ต้านออกซิเดชันของสารสกัดที่ได้ โดยสารสกัดที่มีฤทธิ์ดีที่สุดถูกนำไปสกัดแยกส่วนด้วยตัวทำละลายเฮกเซนและเอธิลอะซิเตต เพื่อให้ได้สารสกัดที่มีฤทธิ์ดีที่สุดและนำไปศึกษาความเป็นพิษต่อเซลล์เคราติโนไซต์ (HaCat cells) ทดสอบด้วยวิธี MTT assay สารสกัดจะถูกเก็บที่อุณหภูมิ 4, 30, และ 40 องศาเซลเซียส เป็นเวลา 6 เดือน เพื่อศึกษาความคงตัวทางเคมีโดยการวิเคราะห์ปริมาณสารสำคัญ scopoletin ในสารสกัดเทียบกับเวลาเริ่มต้นด้วยเทคนิค HPLC ที่ผ่านการตรวจสอบความถูกต้องของวิธีวิเคราะห์แล้ว ส่วนความคงตัวทางชีวภาพจะศึกษาจากฤทธิ์ต้านอนุมูลอิสระ DPPH นอกจากนี้สารสกัดส่วนดังกล่าวจะนำไปใช้ในการพัฒนานาโนอิมัลชันชนิดน้ำมันในน้ำ โดยประเมินคุณสมบัติทางกายภาพและความคงตัวเบื้องต้นของสูตรตำรับเพื่อคัดเลือกไปทดสอบความคงตัวและทดสอบการซึมผ่านผิวหนังแบบนอกกาย ผลการทดลองพบว่าสารสกัดจากกากของผลยอด้วยตัวทำละลายเอธานอลมีฤทธิ์ต้านออกซิเดชันดีที่สุด (IC_{50} = 0.234 มก./มล.) ซึ่งมีฤทธิ์ฤทธิ์ยับยั้งเอนไซม์ไทโรซิเนสใกล้เคียงกับสารสกัดจากกากด้วยตัวทำละลายอื่น สารสกัดส่วนดังกล่าวจึงถูกเลือกเพื่อนำไปสกัดแยกส่วน โดยพบว่าสารสกัดส่วนที่ละลายในตัวทำละลายเอธิลอะซิเตตที่ได้จากการสกัดแยกส่วนมีฤทธิ์ต้านออกซิเดชันและฤทธิ์ยับยั้งเอนไซม์ไทโรซิเนสดีที่สุด มีค่า IC_{50} = 1.201 มก./มล.และ 0.014 มก./มล.ตามลำดับ และไม่พบความเป็นพิษต่อเซลล์ของสารสกัดที่ความเข้มข้น 1.2 มก./มล. ความคงตัวของสารสกัดไม่พบการเปลี่ยนแปลงของสารสำคัญ scopoletin เช่นเดียวกับฤทธิ์ต้านอนุมูลอิสระ DPPH ของสารสกัดเมื่อเก็บที่อุณหภูมิ 4 และ 30 องศาเซลเซียสเป็นเวลา 6 เดือน แต่พบการลดลงเล็กน้อยของสาร scopoletin และฤทธิ์ต้านอนุมูลอิสระเมื่อเก็บที่ 40 องศาเซลเซียส สูตรตำรับนาโนอิมัลชันที่เลือกประกอบด้วยส่วนน้ำมันคือ caprylic/capric triglyceride, สารก่ออิมัลชันคือ TWEEN®80-SPAN®20 และสารช่วยละลาย Cremophor® RH-40 โดยพบว่าสูตรตำรับที่เตรียมได้ มีความคงตัวดีต่อสภาวะเร่งอุณหภูมิ และที่เก็บในอุณหภูมิ 4 และ 30 องศาเซลเซียส ความคงตัวทางเคมีของสาร scopoletin ในสูตรตำรับไม่พบการเปลี่ยนแปลง การปลดปล่อย scopoletin ออกจากนาโนอิมัลชันไม่แตกต่างจากสารละลายสารสกัด และพบปริมาณสาร scopoletin ซึมผ่านผิวหนังของลูกสุกรของนาโนอิมัลชันมากกว่าสูตรตำรับอิมัลชันธรรมดาอย่างมีนัยสำคัญ

ภาควิชาวิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม ลายมือชื่อนิสิต.....

สาขาวิชา….....เทคโนโลยีเภสัชกรรม.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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PANIDA BORISUT: ANTI-TYROSINASE AND ANTIOXIDANT ACTIVITIES OF *MORINDA CITRIFOLIA* FRUIT EXTRACT AND DEVELOPMENT OF NANOEMULSIONS. ADVISOR: ANYARPORN TANSIRIKONGKOL, Ph.D., CO-ADVISOR: ASSOC. PROF. UBONTHIP NIMMANNIT, Ph.D., 173 pp.

The present study focused on anti-tyrosinase and antioxidant activities of Thai *Morinda citrifolia* fruit extracts as potential ingredient used for nanoemulsions formulation in cosmetic applications. The fruit juice was freeze-dried. The residue was further extracted by ethanol, acetone and ethyl acetate. All of the extracts were determined for their inhibitory activities against tyrosinase enzyme and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. The extract which showed highest activity was further partitioned by hexane and ethyl acetate. The IC_{50} of partitioning fractions was determined for their activities. The extract with highest activities was selected for further investigations. The cytotoxicity evaluation was determined by MTT assay using keratinocyte (HaCaT cells). The selected extract was incubated at 4°C, 30°C and 40°C for 6 months. The chemical constituents were determined by validated HPLC condition with scopoletin as a marker. The o/w nanoemulsions with small droplet size was produced and investigated for its stability and *in vitro* permeation profile. As the results of extraction, the ethanol extraction showed the highest antioxidant activity (IC_{50} =0.234 mg/ml) with comparable anti-tyrosinase activity to other solvent extracts. After partition, ethyl acetate soluble fraction exhibited highest activities with IC_{50} value of anti-tyrosinase and DPPH assay were 1.201 mg/ml and 0.014 mg/ml, respectively. The extract at concentration of 1.2 mg/ml had no effect on cell viability. The stability determination of the extract showed insignificant reduction of the scopoletin concentration as well as its antioxidant activity against DPPH radicals kept at 4°C and 30°C for 6 months. However, it showed slightly reduction after storage at 40°C for 4 months. This reduction was in an acceptable range. Caprylic/capric triglyceride was chosen as the oil phase, TWEEN®80-SPAN®20 as emulsifiers, and cremophor® RH-40 as solubilizing enhancer for nanoemulsions formulation. The prepared nanoemulsions showed good physical stability under heat-cool stress conditions and kept at 4°C and 30°C over 3 months. The chemical compound, scopoletin, incorporated in nanoemulsions exhibited good stability. The release profile of extract-loaded nanoemulsions was not different compared to the solution of extract. The extract-loaded nanoemulsions showed significantly better permeation through porcine membrane than conventional o/w emulsion.

Department : Pharmaceutics and Industrial Pharmacy Student's Signature .....

Field of Study : Pharmaceutical Technology Advisor's Signature .....

Academic Year : 2011 Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

%	percentage
°C	degree Celsius (centigrade)
µg	microgram (s)
µM	micromolar
ANOVA	analysis of ariance
EDTA	ethylene diamine tetraacetic acid
et al.	et alii, and other
g	gram
h	hour
HCl	hydrochloric acid
mg	milligram (s)
min	minute (s)
mM	millimolar
PBS	phosphate buffer saline
pH	the negative logarithm of hydrogen ion concentration
PdI	poly disperse index
SD	standard deviation

CHAPTER I

INTRODUCTION

Cosmetic industry has gained more interest due to its constant increase in demand. New compounds have been investigated based on their activities, such as antioxidant, anti-tyrosinase, to provide active ingredients used in the cosmetic products. Traditional herbal medicines provide a major source for development of potential ingredients used in skin care cosmetics since many of them contain compounds with whitening and anti-oxidant benefits. Extracts from herbs have, therefore, drawn more attention worldwide.

Morinda citrifolia L., commonly known as noni, is typically found in Southeast Asia and other tropical areas. It has been long used as traditional medicine for prevention and improvement of various diseases, such as arthritis, infections, colds, cancers, diabetes, etc. (Wang, et al., 2002). In the past decade, commercial noni fruit products supplied as a dietary supplement have grown tremendously in the global market. It is widely consumed for preventing several diseases such as diabetes, high blood pressure and arteriosclerosis. This wide consumption has promoted an increase in research on the phytochemical constituents and biological activities of noni. A relatively large number of scientific publications on noni have been published in recent years. Many studies have revealed its anti-inflammatory (Akihisa, et al., 2007; Deng, et al., 2007), antibacterial (Pawlus, et al., 2005), antioxidant (Zin, et al., 2002), and anti-melanogenesis (Matsuda, et al., 2009) activities. Furthermore, over 100 compounds have been identified in noni fruit. Coumarins and flavonoids, which are predominant in noni fruit, have been indicated to possess a variety of biological activities and may play an important role in antioxidant activity of noni fruit (West, et al., 2010). Lignans (americanol A, americanin A, morindolin, and isoprincepin) isolated from noni fruit exhibited remarkably strong inhibitory activity against copper-induced oxidation of low-density lipoprotein particles *in vitro* (Kamiya*, et al.,* 2004). The neolignan, americanin A from a noni fruit extract was found to be a potent antioxidant against 1,1-diphenyl-2-picrylhydrazyl (DPPH) and peroxy nitrite radicals (Su*,* et al., 2005). The iridoid glycosides, hemiterpene glycosides, and saccharide fatty acid esters from the methanol extract of noni fruit may be valuable as potential skin

whitening agents due to the remarkably reduction of melanin contents tested on the α-MSH-stimulated B16 melanoma cells (Akihisa, et al., 2010).

Scientific researches showing abundant potential activities of noni as a good source of anti-melanogenesis and antioxidant agents support the possibility to develop noni extract as the promising whitening and antioxidant agent used in the skin care cosmetic. Therefore, study on such activities of noni cultivated in Thailand for cosmetic purpose is of interest. However, the extraction method must be optimized to obtain the extract with highest activities. The quantitative analysis of the phytochemical compositions of the extract should be developed. Importantly, cytotoxicity should be screened in order to assess any harmful effects for applying to skin care products.

Skin, an organ comprising complex organization and chemical structure, is the main barrier for transportation of substances into the body. The outermost region, stratum corneum, plays an important role for this function. The special structure of stratum corneum comprises columns of tightly packed corneocytes, which merged together with desmosomes and sealed tightly with special inter-cellular lipids. As a consequence, the delivery of topically applied drugs and cosmetics is governed by many factors including the molecular size, the lipophilicity of the components, type of formulation, presence of penetration enhancers and physical state of the stratum corneum. These limitations may explain the negative response of some compounds that possess high ability profiles *in vitro* but have no or less effects *in vivo* (Cevc and Vierl, 2010).

In the past few decades, the development of novel drug delivery system has gained considerable attention. These systems may enhance the delivery of some drug and cosmetic components through the skin by maximizing drug partition into the skin. These novel formulations are also reported to have remarkable advantages over conventional formulations, including improvement in the efficacy of formulations, enhancement in solubility and stability of the active compounds, and protection from physical and chemical degradation (Saraf, 2010). Thus the novel drug delivery systems might be able to incorporate herbal extract to enhance its skin penetration to active sites or overcome problems associated with plant extracts.

Nanoemulsions are referred to mini-emulsion or emulsion systems with an average drop size range of 20–200 nm. Nanoemulsions are, therefore, transparent or translucent and kinetically stable. The attraction of nanoemulsions used in personal care and cosmetic applications is due to the very small droplet size. The small-sized droplet gives the large reduction in the gravity force and the Brownian motion, which inhibits creaming or sedimentation during storage. Moreover, due to their small size, nanoemulsions enhance the skin penetration and are, therefore, suitable for delivery of active ingredients through the skin surface. In addition, the transparent nature of the system and the reasonable surfactant concentration may give them a pleasant skin feel and aesthetic character that may approach customer perception and acceptability (Tadros, et al. 2004).

Taken together, the present study hypothesized that i) *Morinda citrifolia* fruit extract has anti-tyrosinase and antioxidant effects that could be used as whitening agent in the skin care cosmetics ii) Nanotechnology used in the formulation improves the stability and enhance the skin penetration of the extract. Therefore, this research will develop the optimized extracting method of *Morinda citrifolia* fruit cultivated in Thailand and will evaluate their biological activities, cytotoxicity and stability of the extract. Finally, nanoemulsions containing noni extract will be formulated and its performance and stability will also be evaluated.

This investigation will provide the information on the anti-tyrosinase and antioxidant activities of *Morinda citrifolia* extract. The information can be used to support the development of the effective and safe novel skin whitening agents from Thai herb. The effective nano-sized formulation for cosmetic applications will also be obtained.

CHAPTER II

LITERATURE REVIEWS

1. Botanical, Chemical, and Pharmaceutical Aspects of *Morinda citrifolia*

Morinda citrifolia L. is commonly known as Noni, Indian Mulberry, Ba Ji Tian, Nono or Nonu, Cheese Fruit, and Nhau. It is found throughout the world especially in South and Southeast Asia, East Africa, the Hawaii Islands and the Caribbean. *Morinda citrifolia* is small green plant with large bright green and elliptical leaves, and white tubular flowers. The fruit is 12 cm in length with a lumpy surface covered by polygonal-shaped sections. The seed has reddish brown color with triangular shaped and air sac attached at one end. The mature fruit has a foul taste and odor (Wang et al, 2002). The picture of *Morinda citrifolia* is shown in Figure 1.

Figure 1 The picture of *Morinda citrifolia*

Morinda citrifolia plant has been used in various combinations for herbal remedies. The fruit has been reported for the treatment of oral sore, mouth ulcer, tooth decay, gingivitis, bacterial-, viral-, and fungal-infection, anti-inflammation, haemorrhiods, seizure, diabetes, and hypertension (Pawlus and Kinghorn, 2007).

Several studies have focused on isolation of active compound from *Morinda citrifolia*. The roots contain a group of anthraquinones, particularly, rubiadin,

damnacanthal, and alizarin-1-methyl ether, naphthoquinone derivatives and sterols (Pawlus and Kinghorn, 2007, Kamiya et al, 2009). While, iridoids, flavonoids, triterpenoids, and anthraquinones were found from leaves (Sang et al., 2001(a), Sang et al., 2001(b), Pawlus et al., 2005, Takashima, et al., 2007, Deng, West, and Jensen, 2008, Deng et al., 2009). Main compound found in fruits are iridoids such as asperuloside, asperulosidic acid, deacetylasperuloside, deacetylasperulosicdic acid, flavonoids compounds such as quercetin, and kaempferol, and also coumarins compound such as scopoletin (Su et al., 2005; Akihisa et al., 2010; Wang et al., 1999; Deng, West, and Jensen, 2010; Potterat et al., 2007; Kamiya et al., 2004; Saludes, Garson, and Franzblau, 2002). Moreover, some flavonoids, lignans, and triterpenoids isolated from seeds have been reported (Masuda et al., 2009). List of compounds isolated from various parts of *Morinda citrifolia* are presented in Table 1 and chemical structures of some compounds are shown in Figure 2.

Table 1 The compounds isolated from various parts of *Morinda citrifolia*

Chemical group	**Phytochemical compound**	**Part**	**Ref.**
Anthraquinones	Damnacanthol and Damnacanthol glycosides	Roots	Pawlus and Kinghorn, 2007; Kamiya et al., 2009;
	Morindone-6-O-b -D-primeveroside	Roots	Kamiya et al., 2009
	Rubiadin	Roots	Pawlus and Kinghorn, 2007
Iridoids	Asperuloside	Leaves	Sang et al., 2001(a)
	Asperulosidic acid	Leaves	Sang et al., 2001(a)
	Citrifolinoside A	Leaves	Sang et al., 2001(a)
Flavonoids	Kaempferol	Leaves	Deng et al., 2008
	Quercetin	Leaves	Deng et al., 2008
	Rutin	Leaves	Sang et al., 2001(b); Deng et al., 2008
Triterpenoids	β-Sitosterol	Leaves	Pawlus et al., 2005

Chemical group	Phytochemical compound	Part	Ref.
and sterols	Ursolic acid	Leaves	Takashima et al., 2007
Anthraquinones	Alizarin	Leaves	Deng et al., 2009
	Lucidin	Leaves	Deng et al., 2009
	Rubiadin	Leaves	Deng et al., 2009
Iridoids	Asperuloside	Fruits	Su et al., 2005; Akihisa et al., 2010
	Asperulosidic acid	Fruits	Wang et al., 1999; Kamiya et al., 2005; Su et al., 2005; Samoylenko et al., 2006; Potterat et al., 2007
	Citrifolinin B	Fruits	Su et al., 2005
	Deacetylasperuloside	Fruits	Su et al., 2005
	Deacetylasperulosicdic acid	Fruits	Kamiya et al., 2005; Samoylenko et al., 2006; Akihisa et al., 2010; Potterat et al., 2007
Flavonoids	Kaempferol	Fruits	Deng et al., 2007
	Narcissoside	Fruits	Su et al., 2005
	Nicotifloroside	Fruits	Sang et al., 2001; Su et al., 2005
	Quercetin	Fruits	Deng et al., 2007; Deng et al., 2010
	Rutin	Fruits	Wang et al., 1999; Deng et al., 2010; Potterat et al., 2007
Lignans	Americanin A	Fruits	Kamiya et al., 2004; Su et al., 2005
	Americanoic acid	Fruits	Kamiya et al., 2004

Chemical group	Phytochemical compound	Part	Ref.
	Americanol A	Fruits	Kamiya et al., 2004
	3,3'-Bisdemethylpinoresinol	Fruits	Kamiya et al., 2004; Deng et al., 2007
	Isoprincepin	Fruits	Kamiya et al., 2004
	Morindolin	Fruits	Kamiya et al., 2004
Anthraquinones	Alizarin	Fruits	Deng et al., 2009; Potterat et al., 2007
	5,15-Dimethylmorindol	Fruits	Kamiya et al., 2005 Deng et al., 2009; Deng et al., 2010
	Lucidin	Fruits	Deng et al., 2009
	Rubiadin	Fruits	Deng et al., 2009
Coumarin	Isoscopoletin	Fruits	Deng et al., 2007
	Scopoletin	Fruits	Saludes et al., 2002; Samoylenko et al., 2006; Deng et al., 2007; Deng et al., 2010; Potterat et al., 2007
Flavonoids	Quercetin	Seeds	Masuda et al., 2009
Lignans	Americanin A	Seeds	Masuda et al., 2009
	3,3'-Bisdemethyltanegool	Seeds	Masuda et al., 2009
Triterpenoids and sterols	Ursolic acid	Seeds	Masuda et al., 2009

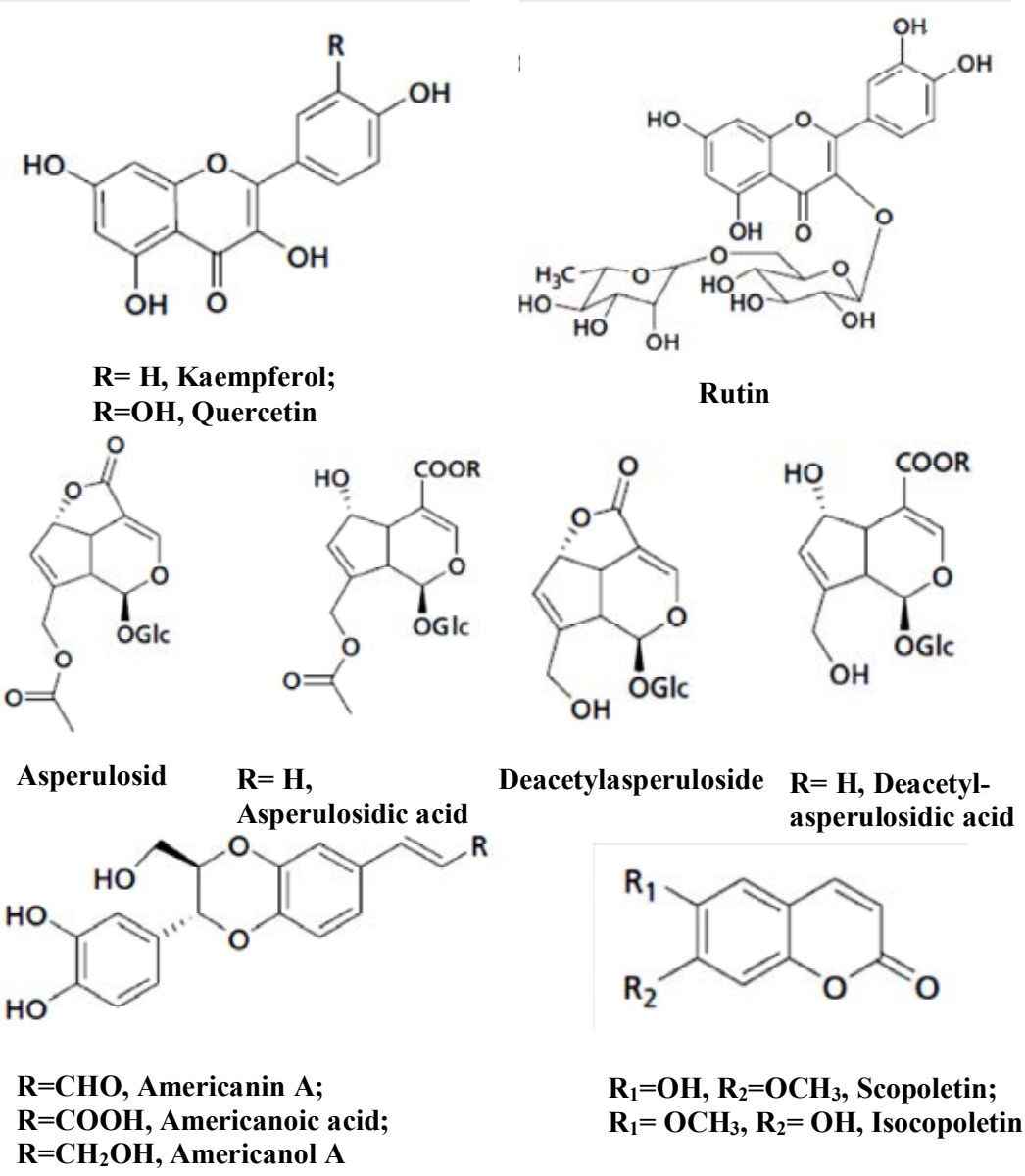


Figure 2 Chemical structures of some isolated compounds from *Morinda citrifolia*

A number of in vitro studies have been performed on both crude extracts and isolated active constituents of *Morinda citrifolia*. The antioxidant, anti-tyrosinase, anti-melanogenesis, and anti-inflammatory activities have been reported.

Chang-hong et al., 2007 suggested that several compounds such as isoscopoletin, aesculetin, quercetin isolated from the successive extraction of ferment *Morinda citrifolia* juice with ethyl acetate contributed a scavenging activity toward hydroxyl radicals. Masuda et al., 2009, have reported the anti-elastase, anti-tyrosinase, and

DPPH scavenging activity of ethyl acetate fraction partitioned from ethanol extract of dried seeds. These activities were contributed from ursolic acid, 3,3'-bisdemethylpinoresinol, americanin A, and quercetin which were active constituents isolated from the assay fraction. Zin, Hamid, and Osman, 2002 reported that the methanol and ethyl acetate extract of *Morinda citrifolia* roots and the ethyl acetate extract from leave and fruits possessed significant antioxidant activity. The further study isolated and identified the anti-oxidative compound from *Morinda citrifolia* fruits extract as catechin and epicatechin. Su et al., 2005 reported neolignan and americanin A isolated from butanol-soluble fraction of methanol extract as a potent antioxidants. Deng et al., 2007 also reported that quercetin and kaempferol isolated from ethyl acetate partition of *Morinda citrifolia* fruits found to exhibit COX-2 enzyme and to be a potent 5-LO (5-lipooxygenase) inhibitor. Recently, Akihisa et al., 2010 reported the butanol- and ethyl acetate- soluble fraction partitioned from methanol extract of *Morinda citrifolia* fruits possessed a potent anti-melanogenesis in the α-MSH-stimulated B16 melanoma cells. Furthermore, the investigation from Nitteranon, 2010 on the anti-inflammatory and cancer protective effects of ethyl acetate extract of *Morinda citrifolia* fruit purees also reported that the isolated scopoletin and quercetin were able to inhibit the production of nitric oxide (NO) in lipopolysaccharide-induced RAW 264.7 macrophage cells and exhibited quinine reductase (QR) induction in cultured Hepa 1C1C7 cells. They suggested that these isolated compounds might be efficient to suppress inflammatory and carcinogenic process related to nitric oxide and COX-2 gene over expression.

2. Nanoemulsions

Nanoemulsions are the emulsions with the extremely small droplet size ranging from 20-200 nm (Solan, 2005; Sadurni, 2005). Owing to the small size range, nanoemulsions are transparent or translucent with low viscosity and high kinetic stability. Moreover, this small size range provides a large surface area that is suitable for delivery of active ingredients through the skin. These reasons make nanoemulsions become a potential drug delivery system for pharmaceutical and cosmetic application. It also possesses low skin irritation potential compared to microemulsions which contains high amount of surfactant. In comparison to

liposomes, nanoemulsions contains lipophilic core which enhances the drug loading capacity of active compound. It is, therefore, more favorable to deliver lipophilic compound and improves the chemical stability (Shah, Bhalodia, and Shalat, 2010; Zhou et al., 2010; Tadros et al., 2004). Nanoemulsions are, hence, suitable for various applications in oral, parenteral, ophthalmal, and topical formulations (Sandip, and Mansoor, 2007).

2.1 Compositions and preparations of nanoemulsions

Nanoemulsions consist of oil phase, water phase, surfactants and other ingredients similar to that of conventional emulsions. The oil phase are usually natural oil such as purified soybean, corn, cotton seed, sunflower, and peanut oil, or the synthetic lipids, fatty acids such as medium or long chain triglycerides and perfluorochemicals. Since nanoemulsions mostly contain two liquids phases that are likely to separate from each other, it is necessary to add a surfactant to decrease the interfacial tension between those two phases in appropriate amount to facilitate the droplet formation. Nevertheless, due to the ultrafine droplets dispersion, nanoemulsions require sufficient mechanical energy during preparation process and the appropriate composition to make a stable system (Sadip and Mansoor, 2007).

There are a large number of factors to be controlled in the formation of nanoemulsions. Mason et al., 2006 reviewed some recommendation to prepare the stable nanoemulsions as following. The disperse phase should be insoluble in the continuous phase to avoid Oswald ripening instability. The surfactants used in the formulation should not form liquid crystalline phase as occurring in microemulsions, this structure could be formed in the system containing short chain alkanes, alcohols, water and surfactants. The surfactant concentration should be optimized to complete surface coverage which is above the critical micelle concentration (CMC). Moreover, the surfactants should present in the bulk continuous phase as well as on the interfaces in the way that able to provide the excess amount of surfactant for rapidly coating the new surface area of nano-scale droplets during emulsification. Additionally, the extreme shear should be applied to rapture the emulsions droplets into nanoscale-size. As mention above, nanoemulsions are a dispersion of very small droplets which the

energy input from mechanical device or from chemical potential of the ingredients is required to prepare a stable system.

2.1.1 The high-energy emulsification method

The high-energy emulsification method can be used to produce nanoscale droplets. The process requires shear stirring, high pressure homogenization, and/or ultra-sonication for energy supply. By introduce the high shear, the interfacial area is created and it is simultaneously coated by the excess surfactant in the continuous phase to yield core emulsions. They are then further homogenized by forcing the premixed emulsions through the rigid micro-channel of high pressure homogenizer or microfluidizer to rapture the droplets into much smaller size. The process needs repeatation to produce a more uniform nanoemlsions. By this concerning, the size of the obtained nanoemulsions can be controlled by appropriate adjustment of pressure setting and number of cycles run. Nanoemulsions can also be prepared by direct ultra-sonication in which the final droplet size depends on the intensity, mode, and duration of sonication (Solan et al., 2005; Sadurni at al., 2005; Sandip and Mansoor, 2007; Mason et al., 2006; Wooster, Golding, and Sanguansri, 2008, and Meleson, Graves, and Mason. 2004).

Recently, Zhou et al., 2010, has successfully developed small droplet size nanoemulsions by high energy emulsification method using lecithin as the emulsifier. The optimized formulation was prepared by emulsification condition at 50 °C with 1200 rpm for 2 minutes and by high pressure homogenizer at a pressure of 1,000 bar for 4 cycles. The optimized formulation showed very small droplet size ranging from 58 to 92 nm. Bespinar, Cornelia, and Borchert, 2010, also found that the small size (ranging from 120- 240 nm) with narrow size distribution and highly stable nanoemulsions can be prepared by low homogenization pressure but higher numbers of homogenization cycles (eg. 300 bar at 10 cycles). These results have proved that the stable emulsions with desirable size can be prepared by the efficient high pressure homogenization method. Similar result was reported in the study of Floury et al., 2003 who reviewed the influent factors on the preparation of nanoemulsions stabilized by methyl cellulose. The high temperature during emulsification facilitated droplet size break-up into small size due to the decrease in viscosity of both the oil and water

phase. Homogenization pressure below 200 mPa was found to reduce in size of nanoemulsions. This result in agreement with Desrumaux, Loisel, and Marcand, 2000 who suggested, the droplet coalescence occurs immediately after passing through the slit of the valve. The too high homogenization pressure would not allow the surfactant to adsorb and emulsify the newly formed droplet. In such that high pressure and high turbulent intensity, the droplets collision was promoted, and therefore, increase the coalescence rate.

2.1.2 The low-energy emulsification method

Nanoemulsions can be formed spontaneously by the use of phase transitions that taking place during emulsification process in the case of low-energy emulsification method. The phase inversion temperature (PIT) method is based on the changes in solubility of non-ionic surfactants with temperature. For oil in water emulsification system, the polyoxyethylene chain of non-ionic surfactant becomes lipophilic or dehydrated at high temperature. It increases affinity toward the oil phase resulting in the formation of W/O vesicle. At low temperature the surfactant tends to have the higher affinity to water and turns the curvature forming O/W droplet. At the intermediate temperature, around 55-65 °C, the spontaneous curvature becomes close to zero. The surfactant has an equal affinity for both oil and water phase, resulting in a bicontinuous phase formation. The extremely reduce in surface tension facilitates the emulsification. Nanoemulsions with small internal droplet can be formed with low energy input (Rao and McClements, 2011).

2.2 Characterization of nanoemulsions

2.2.1 Particle size

The size of droplet dispersion of nanoemulsions plays an important role on their behavior such as physical appearance, efficacy as well as their stability. The particle size of nanoemulsions can be measured by several methods, for example, light scattering spectroscopic method, electron microscopy, and analytical ultracentrifugation (Sandip and Mansoor, 2007).

2.2.2 Particle size distribution

The size distribution is another major factor to determine the stability of nanoemulsions. A large distribution of the particle size accelerates the aggregation and coalescence of the droplet that may lead to the instability of the formulation. The droplet size distribution of nanoemulsions can also be measured by Zetasizer (Malvern instruments, UK) (Sandip and Mansoor, 2007).

2.2.3 Surface characterization

Similar to the size, surface charge has a significant effect on stability of formulation. Nanoemulsions droplets are form by the surface active agents as a result of the interfacial phenomenon. The stability of the system is a balance between the attractive Van Der Waal's forces and the electrostatic repulsion force. In anionic surfactant system, the net surface charge around the droplet, contributing the repulsion force, is commonly expressed in term of zeta potential. Conventionally, the zeta potential indicates the degree of repulsion. If the zeta potential is high (more than 30 mV either in positive and negative term), a stable system can be maintained. In contrast, if the zeta potential falls below this level, the emulsions droplets would aggregate which leads to the instability of the system. The zeta potential is usually measured by a Zetasizer (Malvern instruments, UK) or the ZetaPlus instrument (Brookhaven instruments corporation, Holtsville, USA) (Sandip and Mansoor, 2007 and Bali, Ali, and Ali, 2010).

2.3 Stability of nanoemulsions

Nanoemulsions are kinetically stable against creaming and sedimentation owing to the small droplet size. However, there are two main mechanisms on destabilization of nanoemulsions. First, coalescence destabilization commonly occurs when the two droplets come into contact and tends to fuse together resulting in the formation of larger droplets with smaller surface area. This type of instability is usually overcome by choosing the surfactants that provide the electrostatic repulsion force as ionic surfactants or the surfactants with highly steric stabilization effect as synthetic nonionic surfactants. By this attempt, the charged head groups or long hydrophilic tail will shield the droplets repelling from each other and keep them apart as a consequence the instability can be reduced. Yilmaz and Borchert, 2005 reported

that the highly stable nanoemulsions formed in the presence of a hydrophilic (TWEEN®80) and a lipophilic surfactant (LE80), supported by the positive charge lipophilic cosurfactant phytosphingosine. They suggested that when increased the hydrophilic surfactant (TWEEN®80) concentration, the steric stabilization of surfactant was increased. This experiment confirmed the assumption that the formation of the very small particle size with high stability was obtained from the presence of the excess amount of surfactants in the bulk (Baspinar, et al., 2010).

The second mechanism of Oswald ripening arises from the polydispersion and difference in solubility between the small and large droplets. The larger droplets get increase in their size by expense of the smaller droplets. This phenomenon usually occurs if the disperse phase has a relatively high solubility in the continuous phase. This process is driven by the Kelvin effect where the smaller droplets which have higher Laprace pressure will diffusive migrate through the continuous phase to the larger droplets that have less Laprace pressure. This destabilization mechanism can be eliminated by selecting or adding the molecules of the disperse phase that have the very low solubility in the continuous phase. Wooster et al., 2008, reported that nanoemulsions made with high viscosity oils, such as long chain triglycerides (LCT), had considerably larger droplet size (120 nm) than that of nanoemulsions prepared with low viscosity oils such as hexadecane (80 nm). They suggested that the Ostwald ripening might occur according to the highly soluble in the continuous phase of the low viscosity oil. This problem could be prevented by adding large molar volume of long chain triglyceride oils, which makes them insoluble in water thus providing a kinetic barrier to Ostwald ripening. They proposed that these entropy gain associated with oil de-mixing provided a thermodynamic barrier to Ostwald ripening. Not only nanoemulsions created in this work, but also some of thermodynamically stable to Ostwald ripening of the small droplet nanoemulsions was reported when at least 50% of the oil phase is an insoluble triglyceride.

2.4 Topical application of nanoemulsions

Currently, various nanoemulsions have been used as the topical delivery systems. According to the very small droplet sizes, nanoemulsions provide a large surface area and the uniformly deposition on the skin which enhance the occlusion

effect resulting in the reduction of trans-epidermal water loss and also facilitate the skin penetration of the biological active ingredients. Its transparent nature, the fluidity, as well as absence of any thickeners provide an easily to apply and pleasant skin feel. Additionally, nanoemulsions can incorporated both hydrophilic and lipophilc compounds which make them suitable for delivery a large variation of active ingredients with non-toxic and non-irritant properties. By all these reasons, nanoemulsions gain a lot of attention for topical formulation development (Sonneville-Aubrun, Simonnet, and L'Alloret., 2004, Solan, 2005).

Zhou H et al., 2010, found that the lecithin nanoemulsions incorporated in an O/W cream was significantly improved the skin hydration capacity with about 2.5-fold over the blank cream. Moreover, they also reported the permeation improvement of this formulation on the *in vivo* test using rat abdominal skin. Friedman et al., 1995 demonstrated the improvement of transdermal delivery of steroidal and nonsteroidal anti-inflammatory drugs (NSAID) such as betamethasone valerate and dipropionate, indomethacin, diclofenac, piroxicam, and naproxen of a submicron emulsions formulation with average size ~100 nm over the standard creams measured by the carageenan-induced paw edema rat model. In the same manner, Schwarz et al., 1995, also reported the very effective transdermal delivery of diazepam-loaded submicron emulsion produced by medium chain triglyceride studied by protective effect against Pentamethylenetetrazole induced convulsive in mice model. The results suggested that the anticonvulsive activity of Diazepam-loaded submicron emulsion was superior to emulsion cream due to the small droplet size (100-300 nm). The systemic activity of this submicron emulsion also showed significantly greater than that of the emulsion and ointment which may reach the same range as that of parenteral delivery.

Yilmaz and Borchert, 2006, evaluated the skin hydration, elasticity and erythema induction effect in healthy volunteers of positively charged oil/water nanoemulsions. Its effectively enhancement of the skin hydration and viscoelasticity leaded to the conclusion to use as the suitable carrier for low solubility compounds such as ceramides with remarkably improvement in clinical effect. Recently, Puglia et al., 2010, investigated the permeation profile of glycyrrhetic acid nanoemulsions with mean droplet size about 210 nm. The *in vitro* skin permeation studied on subcutaneous-epidermal membrane using franz diffusion model, while, the *in vivo*

experiment was performed on the healthy volunteers to examine the anti-inflammatory effect of the formulation on the UV-induced erythema. Both of these studies showed the significantly increase in permeation of nanoemulsions over the conventional O/W emulsion.

CHAPTER III

MATERIALS AND METHODS

Plant materials

1. Fruits of *Morinda citrifolia* were collected from Nakhorn Ratchasima province, Thailand and purchased from Talad-Thai market, Pathum Thani province, Thailand.

Cell culture

1. Human keratinocyte cell line HaCaT cells (Cell Line Service, Germany)

Materials

1. Absolute ethanol AR grade (Carloerbra, France)
2. Acetone AR grade (Fisher scientific, UK)
3. Acetonitrile Plus for HPLC gradient (Carloerbra, France)
4. Antibiotic Antimycotic Solution 100X (1,000 units Penicillin, 10 mg Streptomycin and 25 µg Amphotericin B per ml) (Sigma-Aldrich, Inc., USA)
5. Caprylic/capric triglyceride (Crodamol GTCC™) (Croda, England)
6. Cremophor RH40 (Sigma-Aldrich, Inc., Germany)
7. 2-Deoxy-D-ribose (Sigma-Aldrich, Inc., USA)
8. Dimethylsulfoxide (DMSO) Analytical grade (Riedel-de Haën, Germany)
9. 1,1-Diphylnyl-2-picryl-hydrazine stable radical (DPPH) (Sigma-Aldrich, Inc., USA)
10. Disodium ethylene diamine tetra-acetic acid (EDTA) (Sigma-Aldrich, Inc., USA)
11. Disodium hydrogen phosphate anhydrous (Carloerbra, France)
12. Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, USA)
13. Ethyl acetate AR grade (Fisher scientific, UK)
14. Fetal bovine serum (FBS) (Invitrogen, USA)
15. Formic acid AR grade (VWR international Ltd., England)
16. Hexane AR grade (Carloerbra, France)
17. Hydrogen peroxide solution (30% w/v) laboratory reagent grade (Fisher scientific, UK)

18. 6-Hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid, 97% (Trolox) (Sigma-Aldrich, Inc., USA)
19. Iron (III) chloride-6-hydrate (Sigma-Aldrich, Inc., USA)
20. Jojoba oil (Inca Jojoba LITE) (PC intertrade, Thailand)
21. Kojic acid (Sigma-Aldrich, Inc., USA)
22. L-ascorbic acid (Sigma-Aldrich, Inc., USA)
23. L-3,4-dihydroxyphenylalanine (L-DOPA) (Sigma-Aldrich, Inc., USA)
24. L-glutamine (GlutaMAX) (Invitrogen, USA)
25. Methyl paraben USP grade (S.Tong chemicals co., Ltd, Thailand)
26. Mineral oil (Liquid paraffin LW) (S.Tong chemicals co., Ltd, Thailand)
27. Mushroom tyrosinase (Sigma-Aldrich, Inc., USA)
28. Phosphate buffered saline (PBS) 10X pH7.4 (Invitrogen, USA)
29. Polyol soluble licorice extract P-T(40) (Namsiang international Ltd., Thailand)
30. Polysorbate 20 (TWEEN® 20-LQ-(SG)) (Croda, England)
31. Polysorbate 80 (TWEEN® 80-LQ-(SG)) (Croda, England)
32. Propylene glycol, U.S.P. grade (Srichand United Dispensary Co., Ltd., Thailand)
33. Sodium dihydrogen phosphate (Carloerbra, France)
34. Sorbitan laurate (SPAN® 20-LQ-(SG)) (Croda, England)
35. Sorbitan oleate (SPAN® 80) (The East Asiatic (Thailand) PCL., Thailand
36. Thiazolyl blue tetrazolium bromide (MTT) (Sigma-Aldrich, Inc., USA)
37. Thiobarbituric acid (Sigma-Aldrich, Inc., USA)
38. Trichloroacetic acid (Sigma-Aldrich, Inc., USA)
39. Trypan blue stain 0.4% (Invitrogen, USA)
40. 0.25% Trysin-EDTA (Invitrogen, USA)
41. Propyl paraben (S.Tong chemicals co., Ltd, Thailand)

Apparatuses

1. Centrifuge (MX-305 High speed refrigerated micro centrifuge, Tomy kogyo, Japan)
2. CO_2 incubator (Forma series II water jacket, Thermo scientific, USA)

3. Column (C-18 column type MGII, 4.6 x 250 mm, 5 µm, Column No. A4AD 03125, Shishedo, Japan)
4. De-ionized water (DI water) system (ELGAStat Option 3B, ELGA, England)
5. Electronic balance (BSA423S-CW, Sartorius Basic, Scientific Promation, co., Ltd., Thailand)
6. Electronic balance (Precision Plus TP 2000, Mettler-toledo, Switzerland)
7. Freeze dryer (Christ-102162, Martin Christ GmbH, Germany)
8. High Performance Liquid Chromatography (HPLC) (Waters 2695 separations module coupled with Waters 2998 photodiode array (PDA) detector, Waters Corporation, USA)
9. High speed homogenizer (T25 digital, Ultra Turrax, IKA, Germany)
10. Incubator (Contherm scientific, New Zealand)
11. Juice separator (SEV-3559, Severin, Germany)
12. Laminar hood biosafety cabinet class II (Mars Safety Class 2, SCANLAF, LaboGene, Denmark)
13. Micro-pipettes (SL-20 (2-20 µL), SL-200 (20-200 µL) and SL-1000 (100-1,000 µL, Brand, Germany)
14. Microplate reader (Power wave XS2, BioTek instruments, USA)
15. Modified Franz diffusion cell (Crown Glass Company, USA)
16. Multi-channel pipettes 20-200 µL (Brand, Germany)
17. Particle size analyzer (Zetasizer NanoZS, Malvern, UK)
18. Pre-freezer (Christ-101840, Martin Christ GmbH, Germany)
19. Rotary vacuum evaporator,
    a. Rotavapor (Hei –VAP, Heidolph, Germany)
    b. Vacuum pump (Rotavac valve control, Heidolph, Germany)
    c. Water bath (Laborata 4011, Heidolph, Germany)
20. Ultra-purifier water system (Maxima UF, England)
21. Ultrasonic bath (Elma sonic S300H, Elma, Germany)
22. Vortex mixer (Vortex ginie2, Scientific industries inc., USA)
23. Water bath (OVE 14, Memmert, Germany)

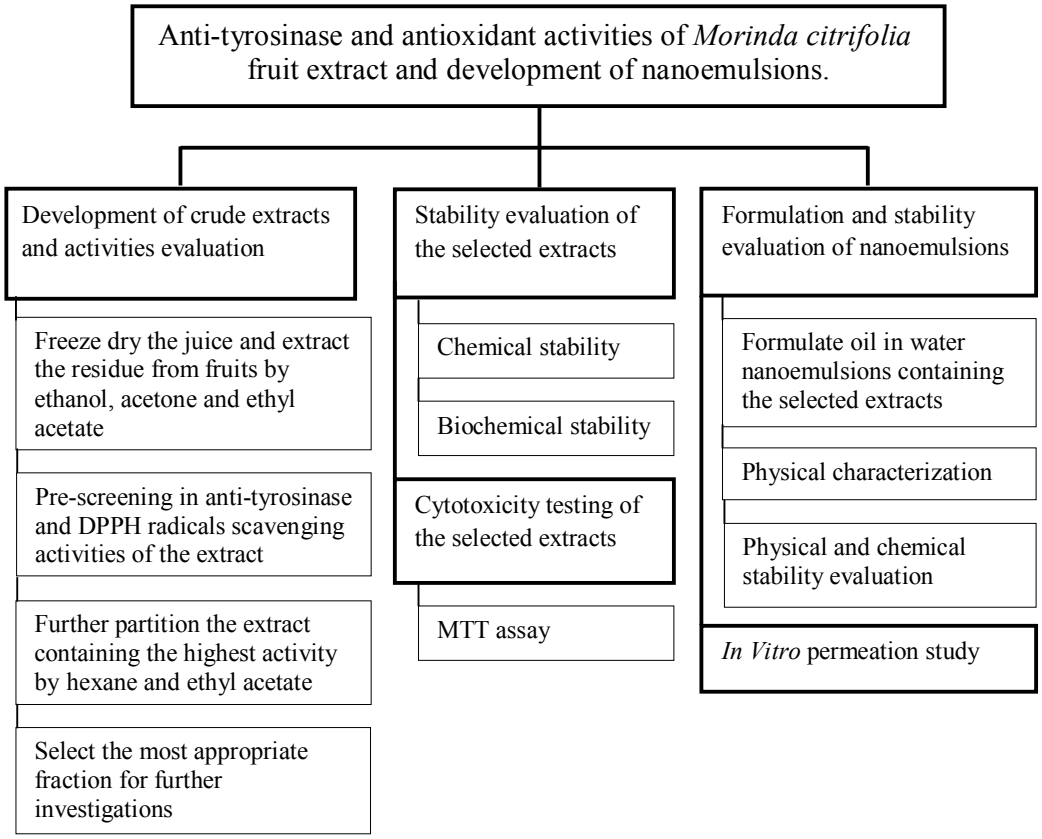
Accessories

1. 125 cm^2 cell culture flask, canted neck with 0.2 μm vent cap (Corning, Inc., USA)
2. Centrifuge tube 15 ml, plug seal cap (Corning, Inc., USA)
3. Centrifuge tube 50 ml, plug seal cap (Corning, Inc., USA)
4. Cryogenic vial (Corning, Inc., USA)
5. Disposable sterile pipette 1 ml (Corning, Inc., USA)
6. Disposable sterile pipette 5 ml (Corning, Inc., USA)
7. Disposable sterile pipette 10 ml (Corning, Inc., USA)
8. Disposable sterile pipette 25 ml (Corning, Inc., USA)
9. 0.45 nylon syringe filters (National scientific, USA)
10. Pipette tips, 1-10 μl universal fit pipette tips (Corning, Inc., USA)
11. Pipette tips, 20-200 μl universal fit pipette tips (Corning, Inc., USA)
12. Pipette tips, 100-1000 μl universal fit pipette tips (Corning, Inc., USA)
13. Syringe needle gauge No.23 (Nipro Corporation, Thailand)
14. Syringe, 3 ml (Nipro Corporation, Thailand)
15. 96-well plates (Costar ® 3912, Corning, Inc., USA)\

Methods

The experiments were divided into five parts:

1. Development of extraction method and activity testing of crude extracts from *Morinda citrifolia* fruit locally grown in Thailand
2. Stability evaluation of *Morinda citrifolia* extract
3. Cytotoxicity evaluation of *Morinda citrifolia* extract
4. Formulation of oil in water nanoemulsions containing *Morinda citrifolia* extract and its stability evaluation
5. *In vitro* permeation study of nanoemulsions containing *Morinda citrifolia* extract



Part 1: Development of extraction method and activity testing of crude extracts from *Morinda citrifolia* locally grown in Thailand

This part of study focused on extraction method providing extract that have the most appropriate biological activity for further studies.

1.1 Extraction

The fresh fruits of *Morinda citrifolia* were squeezed and juice was separated, filtered and freeze-dried. The remaining residue was divided into three parts. Each part was macerated by three kinds of solvent; ethanol, acetone and ethyl acetate, for three days at room temperature. This maceration was repeated for three extraction times. The solvent from each extract was collected and evaporated by the use of rotary evaporator under reduced pressure at 40°C. This process gave four kinds of extract which were i) freeze-dried juice, ii) ethanol extract, iii) acetone extract and iv) ethyl acetate extract. The extraction process was performed according to the diagram shown in Figure 3. The percent yield was calculated.

Freeze-dried juice and extracts from each solvent were screened for their anti-tyrosinase and antioxidant activities by the DOPA chrome enzymatic assay and DPPH radical scavenging activity assay, respectively. The extract that showed highest activity was used for further partition.

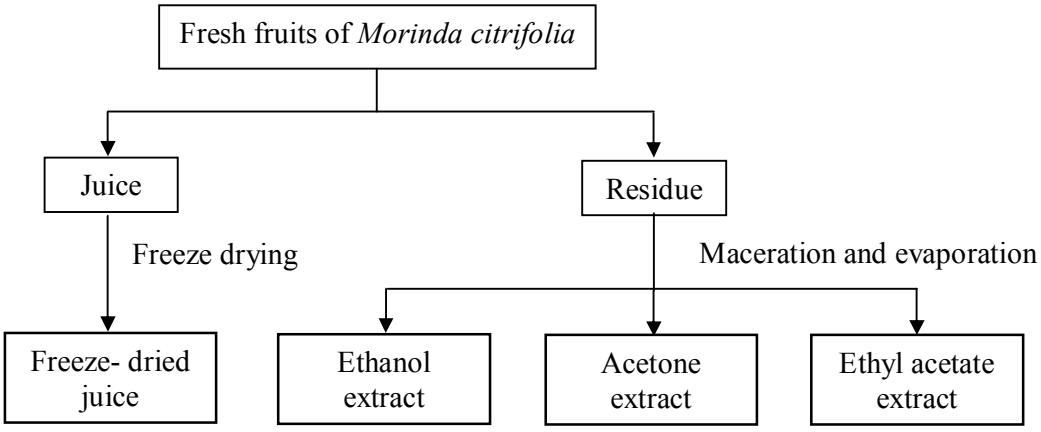


Figure 3 Diagram of the solvent extraction process of *Morinda citrifolia*

1.2 Pre-screening activity of *Morinda citrifolia* extracts

1.2.1 Anti-tyrosinase activity

The tyrosinase inhibitory activity was examined by the DOPAchrome enzymetic method using L-DOPA as a substrate. DOPAchrome, one of the intermediate substances in melanin synthesis pathway, can be detected in visible light with red in color. The potential tyrosinase inhibitor resulted in a decrease in DOPAchrome absorption. In this experiment, the method was modified from the method of Sritularak, 2002 and Jithavech, 2005.

1.2.1.1 Preparation of phosphate buffer (pH 6.8)

Solution A: 20 mM $NaH_2PO_4.H_2O$ (MW. = 137.99) was prepared by dissolving $NaH_2PO_4.H_2O$ 276 mg in 100 mL of DI water.

Solution B: 20 mM Na_2HPO_4 (MW. = 141.96) was prepared by dissolving Na_2HPO_4 284 mg in 100 mL of DI water.

Then, the solution a (70 mL) and solution B (30 mL) were mixed together to provide phosphate buffer pH 6.8.

1.2.1.2 Preparation of 480 units/ml of mushroom tyrosinase

Mushroom tyrosinase enzyme (labeled activity 3933 U/mg) 0.61 mg was dissolved in 5 mL of phosphate buffer pH 6.8.

1.2.1.3 Preparation of 0.85 mM L-DOPA

L-DOPA 0.80 mg was dissolved in 5 mL of phosphate buffer pH 6.8.

1.2.1.4 Preparation of test sample

The test sample was prepared by dissolving the extract with 90% propylene glycol in water for 30 minutes. The stock solution was then diluted with 90% propylene glycol in water until a suitable range of concentration (µg/mL) was obtained.

1.2.1.5 Activity determination

The reaction mixture contained 40 µL of 480 units/ml of mushroom tyrosinase in phosphate buffer, 80 µL of 20 mM phosphate buffer (pH 6.8), and 40 µL of the sample solution. The mixtures were mixed in 96-well plate and pre-incubated for 10 minutes. 40 µL of 0.85 mM L-DOPA in phosphate buffer was added and incubated at ambient temperature for another 10 minutes. The absorbance

of each well was measured at 492 nm by the use of microplate reader. Licorice extract and kojic acid were used as references. Each sample was performed and measured in triplicate.

The anti-tyrosinase activity of the extracts was present in % tyrosinase inhibition. The IC_{50} (concentration at 50% tyrosinase inhibition) of each extract was calculated from the graph plotted between sample concentration versus % tyrosinase inhibition which was calculated as follows:

$$\% \text{ tyrosinase inhibition} = \left(\frac{(A-B)-(C-D)}{(A-B)} \right) x100$$

A: The absorbance after incubation at 492 nm without test sample

B: The absorbance after incubation at 492 nm without test sample and enzyme

C: The absorbance after incubation at 492 nm with test sample

D: The absorbance after incubation at 492 nm with test sample, but without enzyme

The mean IC_{50} values were calculated. Statistical comparison of the IC_{50} among different extracts and reference was made using one-way ANOVA and Tukey or Dunnett T3 test (α= 0.5). The lower IC_{50} value, the greater anti-tyrosinase activity of test sample.

1.2.2 Hydrogen-donating activity (DPPH radical scavenging activity)

Hydrogen-donating activity of the extracts was determined based on their ability to donate hydrogen to DPPH radical. In this study, the method is slightly modified from that of Jithavech (2005).

1.2.2.1 Preparation of 0.1 mM DPPH radical solution

DPPH was accurately weighed 3.943 mg and dissolved in 100 mL of absolute ethanol.

1.2.2.2 Preparation of test sample

The test sample was prepared as stock solution with initial concentration of 1-2 mg/mL. The stock solution was then diluted with absolute ethanol until a suitable range of concentration (µg/mL) was obtained.

1.2.2.3 Activity determination

100 µL of 0.1 mM DPPH radical solution and 100 µL of test sample were mixed in 96-well microplate. The solution was incubated at 37 °C for 30 minutes and the absorbance of the mixture was measured at 517 nm using a microplate reader. Water soluble form of vitamin E (Trolox ®), BHA, and BHT were used as antioxidant references.

The antioxidant activity was calculated as percent inhibition by the following equation:

$$\% \text{ inhibition} = \left(\frac{(\text{Abs. control} - \text{Abs. sample})}{\text{Abs. control}} \right) \text{x } 100$$

Abs. control: The absorbance of mixture containing 100 µL of 0.1 mM DPPH radical solution and 100 µL of absolute ethanol

Abs. sample: The absorbance of mixture containing 100 µL of 0.1 mM DPPH radical solution and 100 µL of test sample

Graph was plotted between concentrations of test sample versus % inhibition. Concentration at which 50% inhibition obtained from the graph was 50 % inhibited concentration (IC_{50} value). The mean ± SD of IC_{50} values were obtained from triplicate experiments in all test samples. Statistical comparison of the IC_{50} among different extracts and reference antioxidants was made using one-way ANOVA and Tukey and Dunnett T3 test (α= 0.5). The lower IC_{50} value, the greater antioxidant activity of test sample.

1.3 Partition of selected crude extract

The selected extract was re-suspended in distilled water and ethanol (9:1) and partitioned with hexane followed by ethyl acetate. The solvent-soluble layers were then collected and evaporated under reduced pressure at 40°C using rotary evaporator to give hexane-soluble fraction and ethyl acetate-soluble fraction. The residual solvent in the aqueous layer was removed under reduced pressure at 40°C

using rotary evaporator. The remaining fraction was concentrated by placing in the evaporating disc on the water bath at 60°C for 72 hours to give a water soluble fraction. The partition process was performed according to the diagram shown in Figure 4. The obtained fraction were packed in a well-closed container, protected from light and kept in a refrigerator (4°C) before further testing.

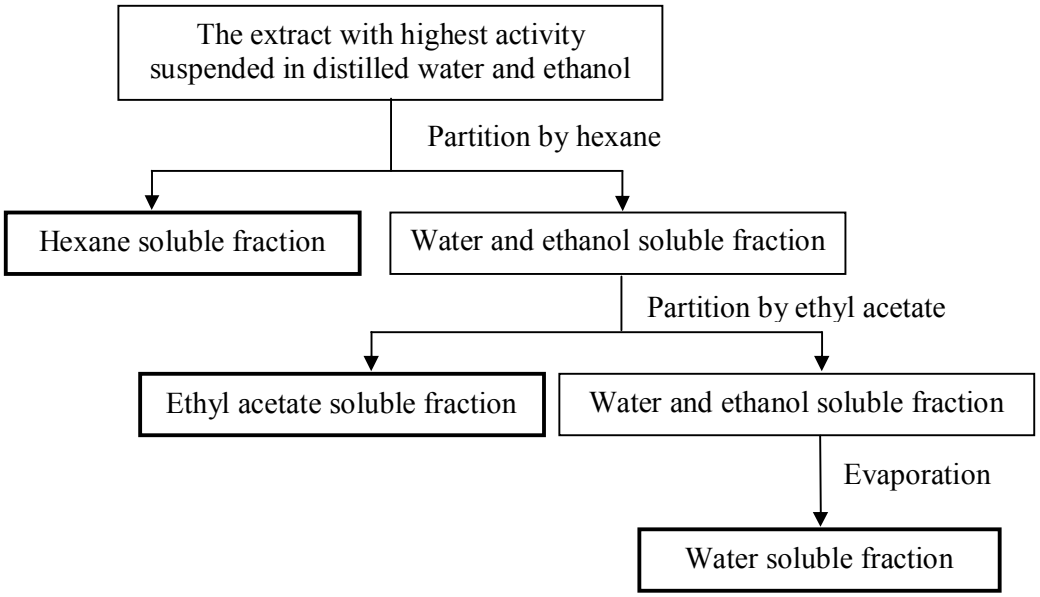


Figure 4 Diagram of the partition process of *Morinda citrifolia* extracts

1.4 Activity testing of partitioned *Morinda citrifolia* extracts

The anti-tyrosinase and antioxidant activities of each fraction were evaluated by the DOPA chrome enzymatic assay and DPPH radical scavenging activity assay, respectively, as mention in section 1.2. The fraction showing highest activities from both tests was selected for further testing in stability, cytotoxicity and formulation.

Part 2: Stability evaluation of *Morinda citrifolia* extract

The purpose of this part was to monitor chemical and biochemical stabilities of selected extract. Chemical stability was evaluated through quantification of biological active compound remaining in the extract using HPLC method.

Biochemical stability was evaluated in term of its remaining antioxidant activity using DPPH radical scavenging assay.

2.1 HPLC condition

2.1.1 HPLC condition development

To determine the biological active compounds in the extract, the HPLC (High Performance Liquid Chromatography) method were developed and modified from the method reported by Deng et al., 2010 to achieve the condition as following:

Column	: C_{18} column (5 µm, 4.6 x 250 mm)
Mobile phase	: (A) = Acetonitrile,
	(B) = 0.1% Formic acid in H_2O (v/v)
	(Isocratic; 30% A: 70%B)
Detection	: UV-Visible spectroscopy at 354 nm
Injection volume	: 10 µL
Column temperature	: 25°C
Flow rate	: 0.5 mL/minute

The biological active compounds in the extract was analyzed using the developed HPLC conditions and compared with the reference standard selecting from the compounds found in *Morinda citrifolia* fruits previously reported. 10 mg of the extract or 1 mg of standard was prepared in absolute ethanol adjusted to the volume of 10.0 ml in volumetric flask. The sample solutions were analyzed and the retention time of the extract and that of the standards such as scopoletin, quercetin, or kaempferol were compared and determined.

2.1.2 Preparation of standard solution for calibration curve

For quantification of biological active compound in the extract, a standard solution containing 100 µg/mL of standard compound in absolute ethanol was prepared. Predetermined volumes of the standard solution were diluted with absolute ethanol to have 0.05, 0.1, 0.5, 1, 2, 10, 20 and 40 µg/mL of standard compound. The samples were filtered through 0.45 membrane filter and then analyzed by HPLC. Calibration curve was obtained by plotting the area under the peak versus concentrations of reference standard. A linear equation from the calibration curve was

generated for quantification of active compound found in the samples. The results were expressed as μg/mL.

2.1.3 Method validation

The developed analytical method was validated according to ICH guideline on following parameter; accuracy, precision, selectivity, linearity and range.

2.1.3.1 Accuracy

Accuracy of an analytical method is the degree of closeness between the actual value of analytes in the sample and the value determined obtained by that method. Accuracy can be determined by analyzing samples with known concentrations and comparing the measured value with the actual value.

Three concentrations (5, 15, and 30 μg/mL) of standard solution in absolute ethanol were measured five replicates each. The accuracy was reported as percent recovery obtained from the mean of measured concentrations (calculated from standard curve) divided by the mean of actual concentrations and multiplied by 100.

2.1.3.2 Precision

The precision of an analytical procedure is the degree of agreement among test results when multiple samplings of homogeneous sample were measured by that method. Intra-day precision was obtained in term of repeatability when the analysis was performed by an analyst using the same equipment at the same day. The inter-day precision or intermediate precision expressed the variation of different-days operation within the same laboratory conditions.

Three concentrations (5, 15, and 30 μg/mL) of each standard solution in absolute ethanol were measured five replicates each in the same days (intra-day precision). Each sample solution was injected into the HPLC system for three days. The percent recoveries of each of sample solution in three days were calculated as intermediate precision (inter-day precision). The precision was determined in term of percent coefficient of variation (%CV) or relative standard deviation (%RSD) of series of measurements.

2.1.3.3 Selectivity

The selectivity of the biological active compound in the sample was determined by comparison of chromatograms among standard solution, sample solution and blank samples. The well resolving from the other peaks and the symmetry of the peaks should be obtained.

2.1.3.4 Linearity and range

The linearity was determined to ensure that testing condition provided test results proportional to the concentration of analyte. A series of sample concentrations was measured. A graph between the response of the analyte and their concentrations was plotted and coefficient of determination (r^2) was calculated. The linearity was determined by a series of three injections of five concentrations (1, 2, 10, 20 and 40 µg/mL) of standard compound in absolute ethanol.

The range was determined in term of interval concentration which demonstrated as good accuracy (98-102%), precision (RSD ≤ 2%) and linearity ($r^2 \geq$ 0.999).

2.2 Determination of biological active compounds in *Morinda citrifolia* extract

Briefly, 1.0 mg/mL sample solution in absolute ethanol was prepared, filtered through 0.45µm membrane filter and analyzed by HPLC method developed in section 2.1. Amount of the active compound in the samples was calculated from the calibration curve. The results were expressed as µg/mL.

2.3 Stability evaluation

2.3.1 Chemical stability evaluation

The extract was packed in a well-closed container, protected from light, and kept in a refrigerator (4°C), at room temperature (30°C ± 2°C), and at 40°C for 6 months. The chemical stability data of the extract was investigated at predetermined time interval (0, 2, 4, and 6 months) using HPLC method to quantify the biological active compound in the extract reported from section 2.2. The result was expressed as percentage in the biological active compound remaining in the extract at particular time point compared to that at time zero.

2.3.1 Biochemical stability evaluation

The biochemical stability study of the selected extract was evaluated in term of its remaining antioxidant activity using DPPH radical scavenging assay. The extract was packed in a well-closed container, protected from light and kept in a refrigerator (4°C), at room temperature (30°C ± 2°C), and at 40°C for 6 months. The antioxidant activity of the extract was investigated at predetermined time intervals (0, 2, 4, and 6 months). Changes in antioxidant activity were investigated.

The result was expresses as the % relative antioxidant activity of each test sample calculated using the following equation:

$$\% \text{ relative antioxidant activity} = [A/B] \times 100$$

A: Concentration of the extract providing 50% inhibition (IC_{50}) at particular time interval (2, 4, or 6 month)

B: Concentration of the extract providing 50% inhibition (IC_{50}) at initial time interval (0 month)

Part 3: Cytotoxicity evaluation for *Morinda citrifolia* extracts

3.1 Cell culture

Human epidermal keratinocyte cells (HaCaT cells) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). HaCaT cells were cultured in DMEM medium in a 5% CO_2 environment at 37° C. All media was supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 units/ml of penicillin/streptomycin.

3.2 Cytotoxicity test

The cytotoxicity test of selected extract was investigated by a modified 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Briefly, the HaCaT cells were prepared by seeding 1x 10^5 cell/well to 96-well plates supplied with 200 µL/well of fresh culture medium. The test cells were incubated at 37°C to allow attachment and initiation of cell growth. Following 72 hours of incubation, the growth medium was removed and replaced with the test extract at predetermined concentrations. The test cells were exposed to the test extract

for 12 hours and were then washed with 200 µL of phosphate buffer saline (PBS). Test cells were further incubated with 10 µL of MTT solution (0.5 mg/mL in phosphate buffer) in 100 µL of the medium at 37 ºC for 4 hours. The MTT solution was removed. The formazan crystals were dissolved using 200 µL of dimethyl sulfoxide (DMSO). The optical density (OD) was detected by spectrophotometry at 570 nm and the relative percentage of cell viability was calculated by dividing the absorbance of treated cells

$$\% \text{ Relative cell viability} = \frac{OD_{570} \text{ treatment}}{OD_{570} \text{ control}} \times 100$$

The non-toxic dose determined by the maximum concentration that shown 90-100 % relative cell viability (Teepe et al., 1993).

Part 4: Formulation of nanoemulsions containing *Morinda citrifolia* extract and its stability evaluation

The objective of this part was to develop the stable oil in water nanoemulsions containing *Morinda citrifolia* extract. Selection of oil and emulsifiers used in the formulation were performed. The selected nanoemulsions were characterized for their physicochemical properties and stability.

4.1 Selection of oil

Oil incorporated into the formulation was selected according to its ability in solubilizing the extract. The effect of oil type, the solubility of the extract in different oils were investigated. Mineral oil and caprylic/capric triglyceride were selected to represent hydrocarbons and medium chain triglycerides. Moreover, the natural oil such as jojoba oil was also used for solubility screening. In this study, the solubility of the selected extract at 12 mg/mL in various oils was preliminary determined by visual detection of clear and homogeneous of the extract in oil. The oil that can dissolve the extract at the proposed concentration was selected for further solubility investigation. An excess amount (50mg) of the extract was mixed in 1 mL of the pre-selected oils using a vortex mixer and sonicated for 30 minutes. The mixture was then kept at room temperature (30°C ± 2°C) for 48 hours to reach

equilibrium. The equilibrated samples were centrifuged at 3,000 rpm for 15 minutes. The supernatant was taken and filtered through a 0.45μm membrane filter. The concentration of the extract was determined using a HPLC method reported in section 2. Oil showing maximum solubility was selected for nanoemulsion preparation.

4.2 Selection of emulsifier

Emulsifiers used in the formulation also affected nanoemulsion formation and stability. The non-ionic emulsifier was selected as emulsifying agent. The emulsifier system consisted of polysorbates emulsifier (TWEEN®20, or TWEEN®80) and sorbitan esters emulsifier (SPAN®20, or SPAN®80). In addition, polyoxy- 40-castor oil (Cremophor® RH) was also included in the experiment in order to improve solubility of the extract in oil phase. The amount of each emulsifier was adjusted to closely match the proper HLB values of an oil phase. The mixed HLB values were calculated using following equation:

$$HLB_{mix} = HLB_T(T\%) + HLB_S(S\%)$$

Where HLB_{mix} = the HLB of the mixed surfactants

HLB_T = the HLB of TWEEN®

HLB_S = the HLB of SPAN®

T% = the mass percentage of TWEEN®

S% = the mass percentage of SPAN® (= 100 - T%)

4.3 Preparation of extract-free nanoemulsions

To screen an optimum composition of the formulation, the oil concentration was varied from 2-10%, while the concentrations of mixed surfactants were adjusted from 5, 10, and 15%. The optimized formulation should have the concentration of oil that is able to dissolve the extract and the concentration of surfactant was minimized.

Oil in water nanoemulsions composed of water, non-ionic surfactants, and medium chain triglyceride or natural oil were prepared using high energy homogenization technique. Briefly, the oil phase and water phase were heated separately to reach 55-60°C. Water phase with 60°C was slowly added to oil phase and was being homogenized using a turbo-agitator (ULTRA TURRAX®) at 6000 rpm for 5 minutes. The effects of amount of oil and types and amount of surfactant were

evaluated through phase separation and particle size analysis after the prepared formulation were kept in room temperature (30°C ± 2°C) for 1 and 4 weeks. The stable formulations that showed desirable sizes were selected to prepare nanoemulsions containing the extract for further studies.

4.3.1 Physical characterization

4.3.1.1 Appearance of nanoemulsions

The formulations were examined visually for physical appearance such as color and phase separation.

4.3.1.2 Particle size analysis of nanoemulsions

The particle size and size distribution of nanoemulsions were examined using a Zetasizer. The 20 µL of nanoemulsions was diluted with 200 µL deionized water before measurement at 25 °C. The measurement was taken at least in triplicate and the average result was determined.

4.4 Preparation of *Morinda citrifolia* extract loaded nanoemulsions

The extract free formulations that showed desirable size and good stability were chosen to prepare the extract loaded nanoemulsions. The selected fraction of *Morinda citrifolia* fruits extract which exhibited highest activities was loaded into the formulation at predetermined concentration. The method of preparation was conducted in the same manner as the preparation of extract free nanoemulsions. The physical appearance and particle size of the prepared formulation were investigated by the method mentioned in section 4.3.1. The optimized formulation having smallest droplet size was selected and brought to stability studies.

4.5 Stability evaluation

For stability evaluation of the obtained formulation, physical stability was determined visually and by particle sizes analysis, while the chemical stability was investigated by quantification of biological active compound using HPLC method.

The selected formulations were evaluated for 8 heating–cooling cycles, each cycle was kept at 4 °C for 24 hours and 40 °C for 24 hours. After completion, the formulations were evaluated for the physical appearance. In addition, the optimized nanoemulsion formulation was kept at 4°C, room temperature (30°C ± 2°C) and accelerated temperature (40 °C) at 75±5% RH for three months. The

samples were taken at specified time intervals (0, 1, 2 and 3 months), physical and chemical stabilities were investigated.

4.5.1 Physical stability evaluation

4.5.1.1 Appearance of extract loaded nanoemulsions

The formulations were visually examined for any physical change in color and separation or precipitation.

4.5.1.2 pH of extract loaded nanoemulsions

The pH of the formulation was investigated using pH meter.

4.5.1.3 Particle size analysis of extract loaded nanoemulsions

The particle size and size distribution of the formulation were examined using a Zetasizer. As mention in section 4.3.1.2, the average size from three measurements of each formulation at any time intervals was recorded and compared to the initial average size.

4.5.2 Chemical stability evaluation of extract loaded nanoemulsions

The quantification of biological active compound in the formulation was analyzed using HPLC method as mentioned in section 2. Briefly, the analysis was carried out by dilution of the extract loaded nanoemulsions with absolute ethanol. The sample solutions were filtered through 0.45μm membrane filter and injected to HPLC. The concentration of active compound in the formulations was calculated from the standard curve. The concentration remaining in the formulation at each particular time was compared to the concentration at initial.

Part 5: *In Vitro* permeation study of nanoemulsions containing *Morinda citrifolia* extract

5.1 The analysis of biological active compound in permeation study

The determination of biological active compound in the receptor fluid was performed using HPLC method as reported in section 2.

The receptor fluid was collected at predetermined time interval and was filtered before injecting into HPLC. The amount of the released extract was calculated from the obtained analytical areas of biological active compound in the solution at any time points. The obtained concentrations were calculated for release and permeation profiles.

5.2 *In Vitro* release study of nanoemulsions containing *Morinda citrifolia* extract

The *in vitro* release study was performed using the modified franz diffusion cells, which consisted of donor compartment, receiver compartment and dialysis membrane lined in between the two compartment.

The study condition was as followed:

Condition: Occlusion with parafilm

Receptor fluid: 40% (v/v) propylene glycol in phosphate buffer pH 7.4 (40 % PG in PBS)

Receptor fluid temperature: 37±0.5 °C

Sampling periods: 0-24 hours

Briefly, the membrane was pre-soaked in distilled water for 24 hours, followed by the receptor fluid for an hour before use. The receiver compartment contained approximately 12 mL of receptor fluid maintaining temperature at 37 ± 0.5 °C. One hour after equilibration, 1 mL of the extract nanoemulsions was placed into the donor compartment. The receptor fluid was continuously mixed by magnetic stirrer throughout the time of study. One mL of receptor fluid was withdrawn at 2, 4, 6, 8, 12, 16, 20 and 24 hours and was replaced with fresh receptor solution to maintain the constant volume during experiment. The examinations were performed in 3 replicates. The samples were analyzed for amount of biological active compound released from nanoemulsions using HPLC method mentioned in section 2.

The cumulative amount of the extract releasing at predetermined time interval was calculated and expressed as % cumulative amount.

$$\% \text{ cumulative amount} = (At/A0) \times 100$$

At = cumulative amount of the active compound released at particular time

A0 = initial amount of the active compound

The solution of the extract in 40% PG in PBS at the comparable saturated solubility was used as control.

5.3 Skin permeation study of nanoemulsions containing *Morinda citrifolia* extract

The *in vitro* skin permeation study was performed on modified franz diffusion cell using full thickness abdominal skin of new born pig with the condition as followed:

Condition: Occlusion with parafilm

Receptor fluid: Phosphate buffer pH 7.4

Receptor fluid temperature: 37±0.5 °C

Sampling periods: 0-48 hours

Briefly, the procine skin was soaked in isotonic phosphate buffer pH 7.4 for an hour before use. The receiver compartment contained approximately 12 mL of receptor fluid maintaining temperature at 37 ± 0.5 °C. One hour after equilibration, 1 mL of the extract nanoemulsions was placed into the donor compartment. The receptor fluid was continuously mixed by magnetic stirrer throughout the time of study. One mL of receptor fluid was withdrawn at time interval of 2, 4, 8, 12, 16, 20, 24, 32 and 48 hours and was replaced with fresh receptor solution to maintain the constant volume during experiment. The examinations were performed in 3 replicates. The samples were analyzed for amount of biological active compound released from nanoemulsions using HPLC method mentioned in section 2. At the end permeation study, the remaining nanoemulsions in the donor compartment and on the skin surface was rinsed with absolute ethanol and analyzed for the remaining extract on the donor compartment. The porcine skin was cut into small pieces and soaked in absolute ethanol. The solvent was filtered through 0.45 μm membrane filter and analyzed for the extract accumulating in the skin. The examinations were performed in triplicates.

The cumulative amount of the extract permeating at predetermined time interval was calculated and expressed as % cumulative amount.

$$\% \text{ cumulative amount} = (At/A0) \times 100$$

At = cumulative amount of the active compound permeated at particular time

A0= initial amount of the active compound

The solution of 40% PG in PBS at the comparable saturated solubility was used as control.

The cumulative amount of the permeated extract per diffusion area was also plotted versus time. The observed study flux was calculated from the slope. The percentage of the extract permeated, the percentage of the extract accumulated in the skin and the steady stage flux were determined and compared amongst the formulation system and control.

Part 6: Statistical analysis

The data in this study were analyzed statistically using one-way analysis of variance (ANOVA). When the significant difference ($p<0.05$) was indicated, the data were subjected to multiple comparison by Tukey or Dunnett test to compare the difference. The statistical package for social sciences (SPSS) program version 16.0 was used in this study.

CHAPTER IV

RESULTS AND DISCUSSION

Part 1: Development of extraction method and activity testing of crude extracts from *Morinda citrifolia* locally grown in Thailand

1.1 Extraction

Juice separated from the fresh fruit of *Morinda citrifolia* was filtered and lyophilized. The obtained freeze-dried juice was sticky powder with yellow-brown in color. The percent yield of the extract was calculated from following:

$$\% \text{ Yield} = \frac{\text{Mass of freeze-dried juice (g)}}{\text{Mass of fresh juice (g)}} \times 100$$

The picture of freeze-dried juice is shown in Figure 5.

The maceration of the residue separated from *Morinda citrifolia* fruits with ethanol, acetone and ethyl acetate yielded ethanol, acetone and ethyl acetate extracts. All of the obtained crude extracts were sticky pastes with dark-brown color with percent yields of 2.71, 2.20 and 3.27, respectively. The percent yields of the crude extracts were calculated from:

$$\% \text{ Yield} = \frac{\text{Mass of crude extract (g)}}{\text{Mass of fresh residue (g)}} \times 100$$

The pictures of crude extracts are shown in Figure 6 and percent yields are listed in Table 2.

All extracts were kept in well-closed containers with silica gel at 4 °C for further studies.

Figure 5 Freeeze-dried juice of *Morinda citrifolia*

Figure 6 Ethanol, acetone and ethyl acetate extracts of *Morinda citrifolia*

Table 2 Percent yields of *Morinda citrifolia* extracts

	Freeze-dried juice	**Ethanol extract**	**Acetone extract**	**Ethyl acetate extract**
Fresh juice weight (g)	1,461	-	-	-
Fresh residue weight (g)	-	283	285	304
Crude extract weight (g)	69.42	9.26	6.26	8.24
%Yield	4.75	3.27	2.20	2.71

The freeze-dried juice gave the highest percent yield, while acetone and ethyl acetate extract showed similar results but less than the percent yield of ethanol extract. This may be attributed to the fact that the juice without any purification contained more varieties groups of compounds including fiber and sugar than the semi-polar compounds extracts which they were not solubilized in the solvent

(Potterat and Hamburger, 2007). The ethanol extract possessed slightly more yield might be due to its higher in polarity that might solubilize more compounds than other 2 solvents.

The obtained freeze-dried juice and residues extract using different solvent were further pre-screened for anti-tyrosinase and antioxidant activities by the DOPAchrome enzymatic assay and DPPH radical scavenging activity assay, respectively to select the suitable fraction for further partitioning.

1.2 Pre-screening activity of *Morinda citrifolia* extracts

1.2.1 Anti-tyrosinase activity

The inhibitory effect on tyrosinase activity of *Morinda citrifolia* extracts was examined by the DOPAchrome enzymatic method using L-DOPA as substrate (Sritularak, 2002; Jithavech, 2005). In this experiment, 80 µL of the test sample solution was added to the reaction mixture to provide the total volume of 200 µL. The final concentration was calculated by the equation below:

$$C_1V_1 = C_2V_2$$

C_1 = Initial concentration (mg/mL)

V_1 = Initial volume (mL)

C_2 = Final concentration (mg/mL)

V_2 = Final volume (mL)

The initial and final concentration (mg/mL) of the test samples were shown in Table 3.

Table 3 The initial and final concentration (mg/mL) of the extract for pre-screening activity in anti-tyrosinase assay

Sample		Concentration (mg/mL)						
		1	**2**	**3**	**4**	**5**	**6**	**7**
Freeze-dried juice	Initial	5.0	10.0	12.5	20.0	25.0	-	-
	Final	2.0	4.0	5.0	8.0	10.0	-	-
Ethanol extract	Initial	6.0	8.0	10.0	12.0	14.0	16.0	20.0
	Final	2.4	3.2	4.0	4.8	5.6	6.4	8.0
Acetone extract	Initial	8.0	10.0	12.0	14.0	16.0	20.0	-
	Final	3.2	4.0	4.8	5.6	6.4	8.0	-

Sample		Concentration (mg/mL)						
		1	**2**	**3**	**4**	**5**	**6**	**7**
Ethyl acetate extract	Initial	6.0	8.0	10.0	12.0	14.0	16.0	-
	Final	2.4	3.2	4.0	4.8	5.6	6.4	-

Anti-tyrosinase activity of all test samples at various concentrations was expressed as percent inhibition and show in Table 4. The result revealed an increase in anty-tyrosinase activity with increasing extract concentration. Each extract presented its unique anti-tyrosinase profile. In order to compare the activity of each extract, the IC_{50} was calculated from the equation of linear regression analysis (Figure 7-10). The IC_{50} of all samples were compared and show in Table 5 and Figure 11.

Table 4 The percentages of anti-tyrosinase activity of all test samples at various concentrations

No.	**Conc.**	**%Inhibition**			
	(mg/mL)	**Freeze-dried juice**	**Ethanol extract**	**Acetone extract**	**Ethyl acetate extract**
1	2.0	7.91 ± 2.47	-	-	-
2	2.4	-	7.82 ± 2.60	-	11.75 ± 2.86
3	3.2	-	16.26 ± 2.84	8.53 ± 6.48	27.56 ±3.74
4	4.0	21.78 ± 2.02	26.02 ± 1.95	17.21 ± 4.45	41.21 ± 2.09
5	4.8	-	35.36 ± 2.84	24.50 ± 5.09	56.71 ±3.86
6	5.0	28.86 ± 2.33	-	-	-
7	5.6	-	44.96 ± 0.06	31.01 ± 5.84	70.78 ± 2.80
8	6.4	-	56.80 ± 1.72	40.01 ± 9.03	84.91 ± 3.81
9	8.0	50.25 ± 2.97	81.97 1.24	52.73 ± 5.32	-
10	10.0	65.38 ± 3.75	-	-	-

Note: %Inhibitions were shown as mean ± SD, n = 3.

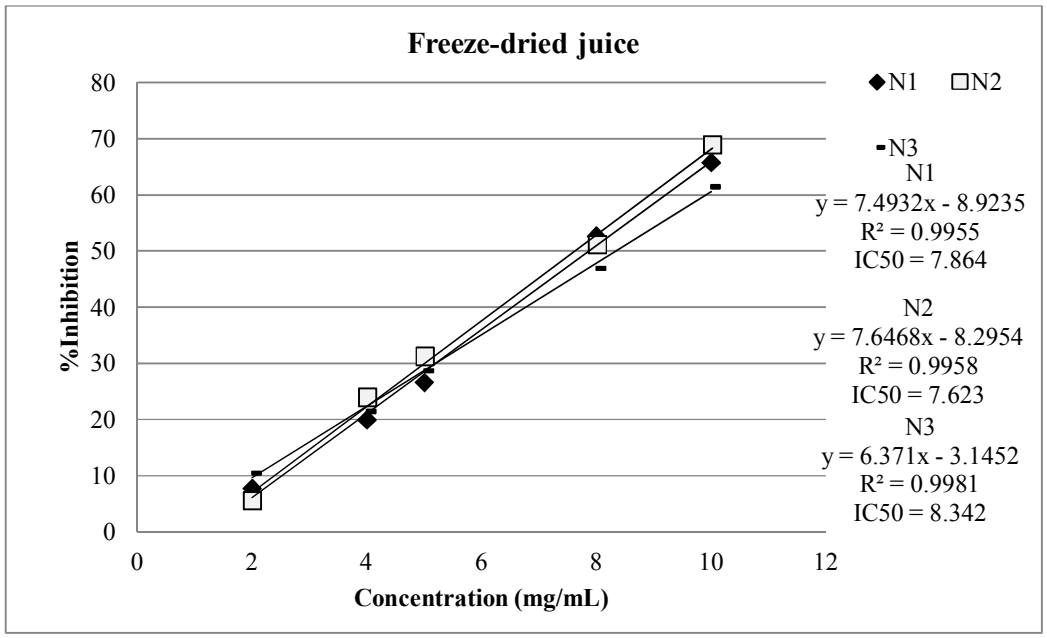


Figure 7 Tyrosinase inhibition of freeze-dried juice

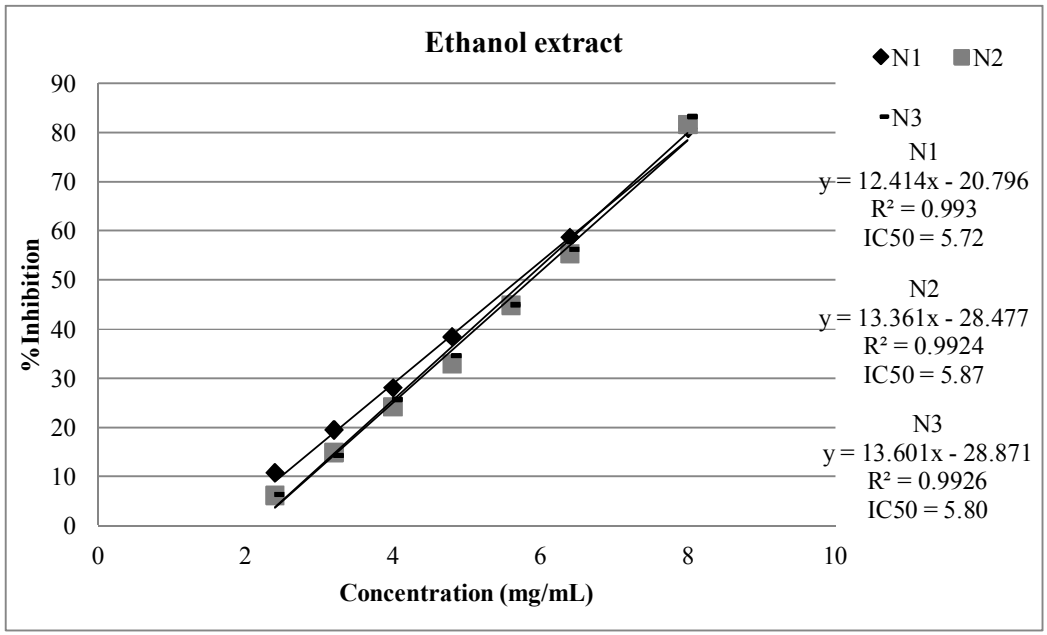


Figure 8 Tyrosinase inhibition of ethanol extract

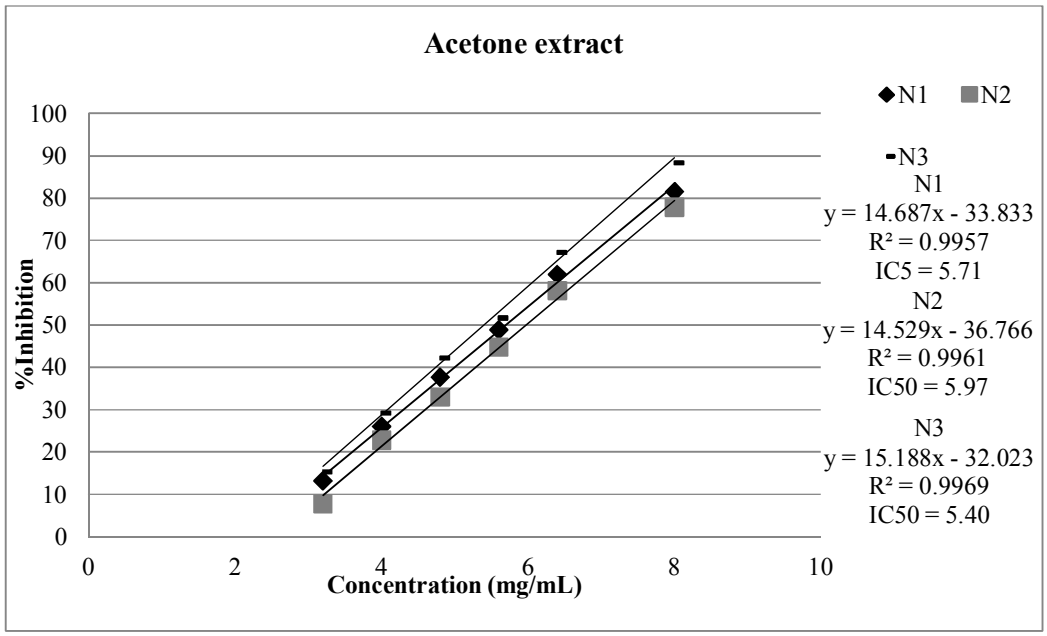


Figure 9 Tyrosinase inhibition of acetone extract

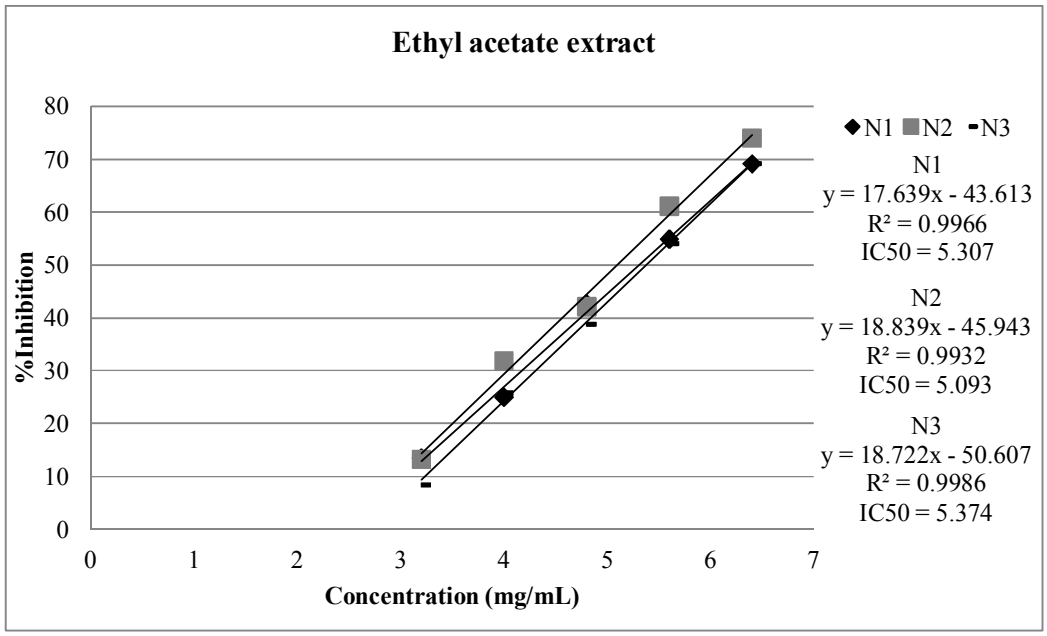


Figure 10 Tyrosinase inhibition of ethyl acetate extract

Table 5 The IC_{50} values calculated from linear equations for anti-tyrosinase activity of each extract and reference standard

sample	IC_{50} (mg/mL)	Mean (mg/mL)	SD
Freeze-dried juice	7.864 7.623 8.342	7.943	0.366
Ethanol extract	5.720 5.870 5.800	5.797	0.075
Acetone extract	5.710 5.400 5.970	5.693	0.285
Ethyl acetate extract	5.307 5.093 5.374	5.258	0.147

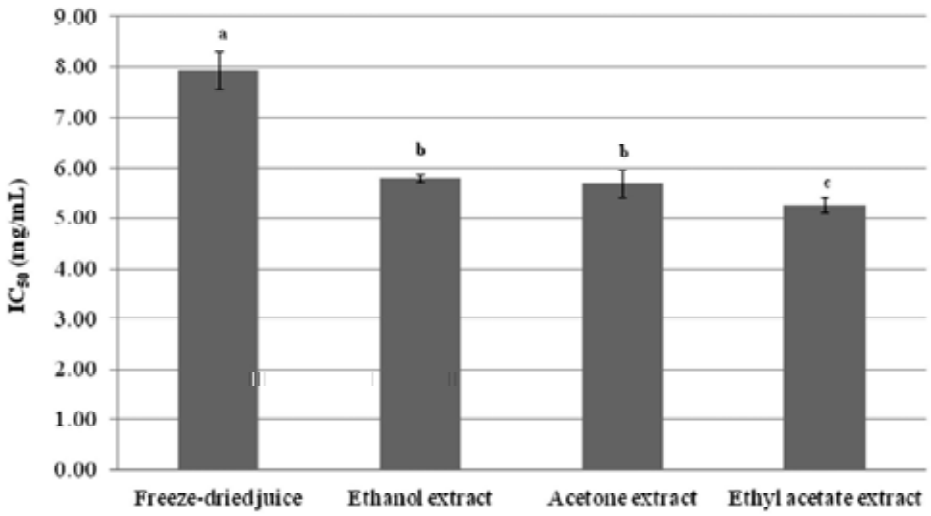


Figure 11 Concentration at 50% tyrosinase inhibition (IC_{50}) of *Morinda citrifolia* extract (significantly different between a and b, P value < 0.05)

The IC_{50} of freeze-dried juice, ethanol extract, acetone extract and ethyl acetate extract on anti-tyrosinase activity were 7.943 ± 0.366, 5.797 ± 0.075, 5.693 ± 0.285, and 5.258 ± 0.147 mg/mL, respectively. By comparing the IC_{50} value, each solvent extract exhibited comparable activities with superior result over freeze-dried juice.

1.2.2 Hydrogen-donating activity (DPPH radical scavenging activity)

The antioxidant activity of *Morinda citrifolia* extracts was examined in term of their activities to donate hydrogen atom to DPPH radicals. The potential antioxidant extracts show a decrease in absorption at 517 nm of the purple DPPH radical to the yellow scavenged one (Jithavech, 2005).

In this experiment, 100 µL of the test sample solution was added to the reaction mixture to provide the total volume of 200 µL. the final concentration was calculated by the equation below:

$$C_1V_1 = C_2V_2$$

C_1 = Initial concentration (mg/mL)
V_1 = Initial volume (mL)
C_2 = Final concentration (mg/mL)
V_2 = Final volume (mL)

The initial and final concentration (mg/mL) of the test samples were shown in table 6.

Table 6 The initial and final concentration (mg/mL) of the extract for pre-screening activity in DPPH radical scavenging assay

Sample		Concentration (mg/mL)						
		1	**2**	**3**	**4**	**5**	**6**	**7**
Freeze-dried juice	Initial	0.40	0.60	0.80	1.00	1.20	1.40	1.60
	Final	0.20	0.30	0.40	0.50	0.60	0.70	0.80
Ethanol extract	Initial	0.20	0.30	0.40	0.50	0.60	-	-
	Final	0.10	0.15	0.20	0.25	0.30	-	-
Acetone extract	Initial	0.30	0.40	0.50	0.60	0.80	1.00	1.20
	Final	0.15	0.20	0.25	0.30	0.40	0.50	0.60
Ethyl acetate extract	Initial	0.40	0.50	0.60	0.80	1.00	-	-
	Final	0.20	0.25	0.30	0.40	0.50	-	-

DPPH radical scavenging activity of all test samples were reported as percent inhibition and shown in Table 7. Each extract resulted in different profile. In

order to compare the radical scavenging activity of each extract, the concentration at 50% antioxidant inhibition (IC_{50}) of each sample was calculated from the equation of linear regression analysis (Figure 12-15). The IC_{50} of all samples were compared and show in Table 8 and Figure 16.

Table 7 The percentages of DPPH scavenging activity of all test samples at various concentrations

No.	Conc. (mg/mL)	%Inhibition			
		Freeze-dried juice	**Ethanol extract**	**Acetone extract**	**Ethyl acetate extract**
1	0.10	-	25.20 ± 2.58	-	-
2	0.15	-	35.78 ± 3.16	23.73 ± 0.80	-
3	0.20	30.13 ± 16.39	44.54 ± 2.95	29.57 ± 2.34	30.52 ± 2.03
4	0.25	-	52.66 ± 3.13	37.10 ± 1.68	36.42 ± 2.10
5	0.30	36.64 ± 19.91	61.23 ± 3.54	42.44 ± 0.60	40.96 ± 1.37
6	0.40	42.24 ± 22.93	-	55.43 ± 1.23	50.57 ± 1.95
7	0.50	46.46 ± 25.20	-	67.45 ± 0.58	60.57 ± 3.21
8	0.60	51.71 ± 28.01	-	82.32 ± 0.55	-
9	0.70	56.90 ± 30.79	-	-	-
10	0.80	63.28 ± 34.23	-	-	-

Note: %Inhibitions were shown as mean ± SD, n = 3.

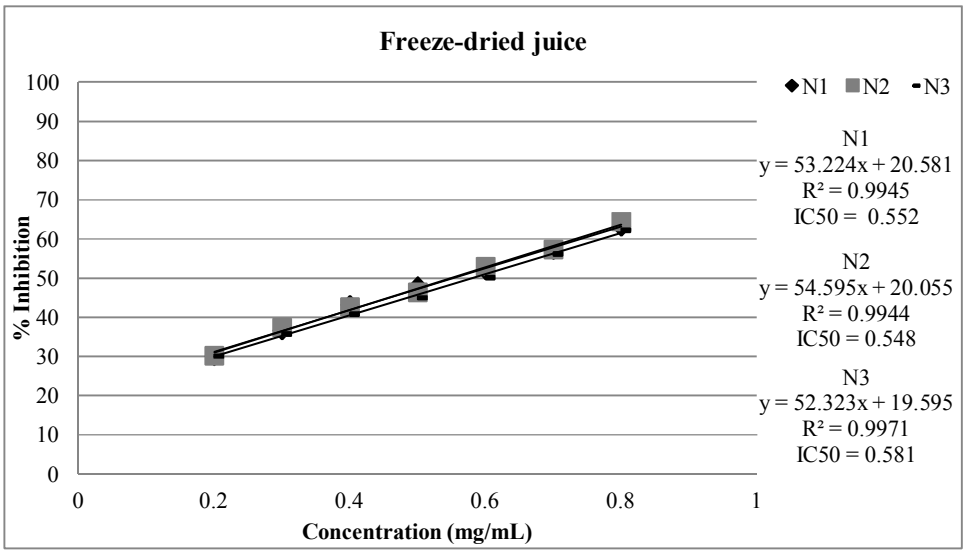


Figure 12 DPPH radical scavenging activity of freeze-dried juice

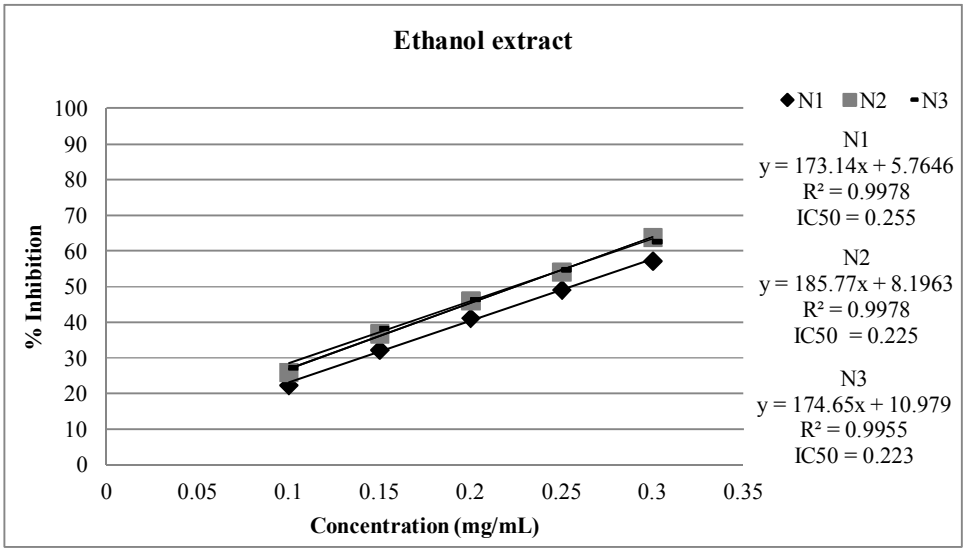


Figure 13 DPPH radical scavenging activity of ethanol extract

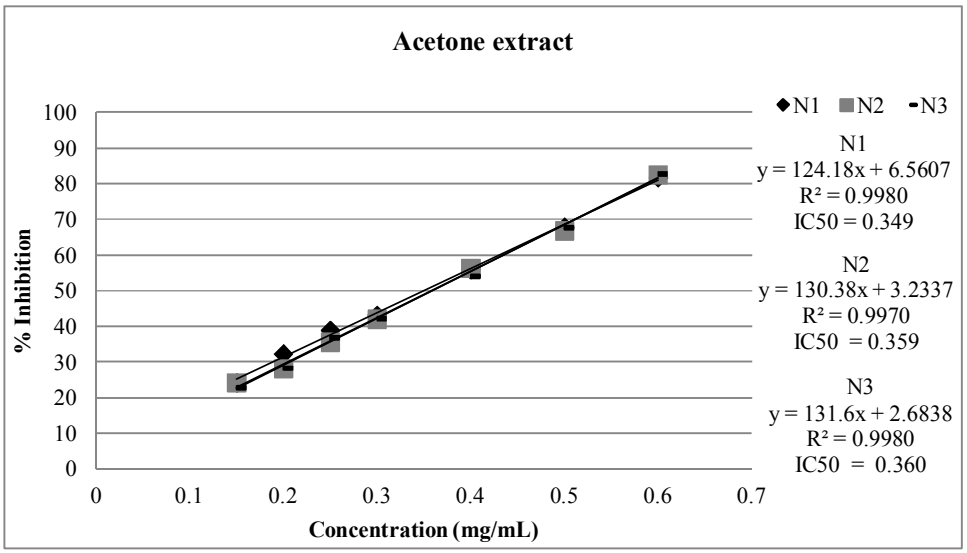


Figure 14 DPPH radical scavenging activity of acetone extract

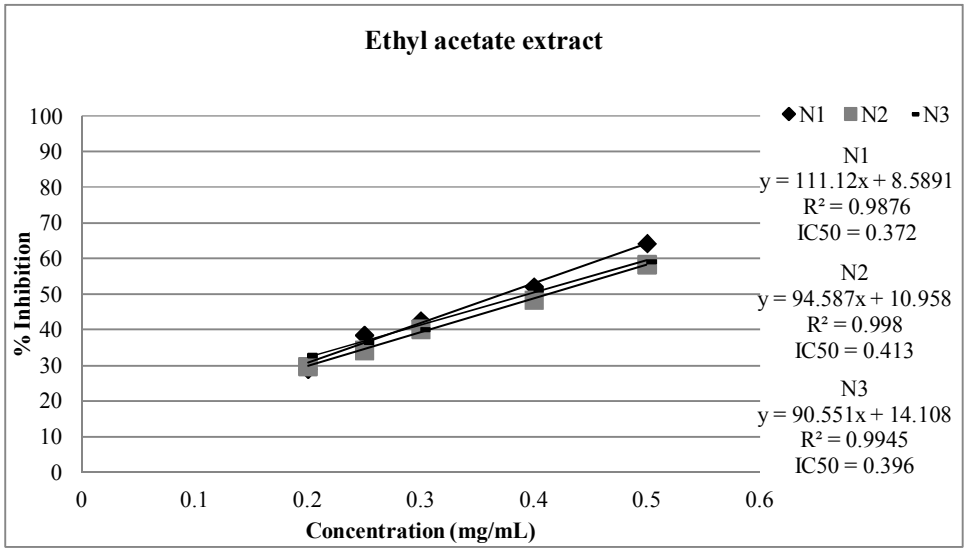


Figure 15 DPPH radical scavenging activity of ethyl acetate extract

Table 8 The IC_{50} values calculated from linear equations for DPPH radical scavenging activity of each extract and reference standard

sample	IC_{50} (mg/mL)	Mean (mg/mL)	SD
Freeze-dried juice	0.552 0.548 0.581	0.560	0.018
Ethanol extract	0.255 0.225 0.223	0.234	0.018

sample	IC_{50} (mg/mL)	Mean (mg/mL)	SD
Acetone extract	0.349	0.356	0.006
	0.359		
	0.360		
Ethyl acetate extract	0.372	0.394	0.021
	0.413		
	0.396		

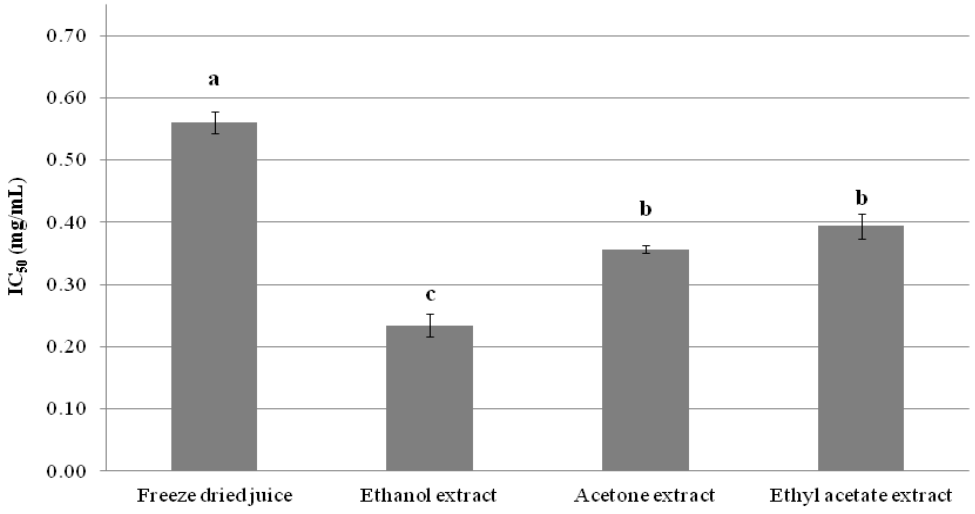


Figure 16 Concentration at 50% inhibition (IC_{50}) of *Morinda citrifolia* extract in DPPH assay (significantly different among a, b, and c, P value < 0.05)

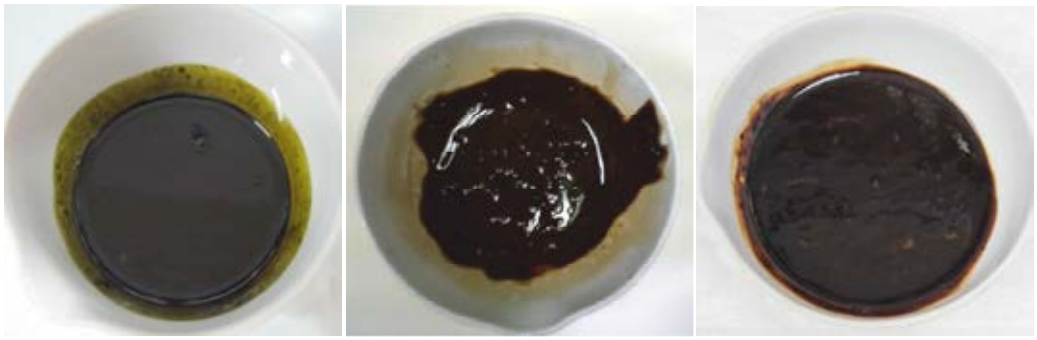
It was found that as the concentration was increased, the %inhibition was increased in all samples. The IC_{50} of freeze-dried juice, ethanol extract, acetone extract and ethyl acetate extract on DPPH radicals scavenging activity were 0.560 ± 0.018, 0.234 ± 0.018, 0.356 ± 0.006, and 0.394 ± 0.021 mg/mL, respectively. By comparing the IC_{50} value, ethanol extract exhibited highest activity, while, acetone and ethyl acetate extract exhibited comparable activities with superior result over freeze-dried juice.

Since the polarity of acetone, ethyl acetate and ethanol are very similar, the same groups of active compounds were extracted in close approximate amount resulting in comparable percent yield and anti-tyrosinase activity profiles. As a result, the solvent extract should be further partitioning with the solvent that have more different in polarity to concentrate and purify the active compounds in the extract

which was expected to show stronger activities. The results from preliminary activity studies of the extracts suggested that ethanol extract was the most appropriate fraction due to its highest antioxidant activity (IC_{50} = 0.242 ± 0.023 mg/mL) with comparable anti-tyrosinase activity (IC_{50} = 5.797 ± 0.075 mg/mL). This result might be because of more active compounds in ethanol extract which is relatively polar than acetone and ethyl acetate. Consequently, the ethanol extract was selected for further partitioning due to its significantly greater in anti-oxidant activity.

1.3 Partition of the selected crude extract

In general, the goal of partitioning is to increase the potency of the botanical extract by concentrating the biological active constituents. Since alcohol is a powerful solvent, alcoholic extract generally contain a wide range of complex constituents, the further purification by different solvent is often performed. In this study, to further purify, the ethanolic extract that showed the highest activity was partitioned by three different solvents. After partitioning, three kinds of extracts which were hexane-, ethyl acetate-, and water-soluble fractions provided 19.53%, 4.29%, and 63.73% yield from its original ethanol extract. Hexane soluble fraction was viscous liquid with dark-green color while ethyl acetate and water soluble fractions were sticky paste with dark-brown color. The pictures of crude extracts after partitioning are shown in Figure 17.



Hexane soluble fraction	Ethyl acetate soluble fraction	Water soluble fraction

Figure 17 Hexane, ethyl acetate and water soluble fraction of ethanolic *Morinda citrifolia* extracts

1.4 Activity testing of partitioned *Morinda citrifolia* extracts

1.4.1 Anti-tyrosinase activity

After partitioning, the anti-tyrosinase activity of each fraction was also investigated. The initial and final concentration (mg/mL) of each fraction and references were shown in table 9.

Table 9 The initial and final concentration (mg/mL) for activity testing of the partitioned extract in anti-tyrosinase assay

Sample		Concentration (mg/mL)					
		1	**2**	**3**	**4**	**5**	**6**
Hexane soluble fraction	Initial	5.0	7.5	10.0	12.5	-	-
	Final	2.0	3.0	4.0	5.0	-	-
Ethyl acetate soluble fraction	Initial	2.0	2.5	2.75	3.0	3.25	3.5
	Final	0.8	1.0	1.1	1.2	1.3	1.4
Aqueous soluble fraction	Initial	5.0	10.0	12.5	20.0	25.0	-
	Final	2.0	4.0	5.0	8.0	10.0	-
Hexane soluble fraction	Initial	0.0080	0.0100	0.0120	0.0160	0.0200	-
	Final	0.0032	0.0040	0.0048	0.0064	0.0080	-
Licorice extract	Initial	0.0100	0.0150	0.0200	0.0300	0.0400	-
	Final	0.0040	0.0060	0.0080	0.0120	0.0160	-
Kojic acid	Initial	5.0	7.5	10.0	12.5	-	-
	Final	2.0	3.0	4.0	5.0	-	-

The IC_{50} values of tyrosinase inhibiting activity of all partitioned fractions were investigated. The relationship between % inhibition and extract concentration are shown in Figure 18-22. The IC_{50} values of tyrosinase inhibiting activity of all partitioned fractions were determined from the linear regression analysis and shown in Table 10 and Figure 23.

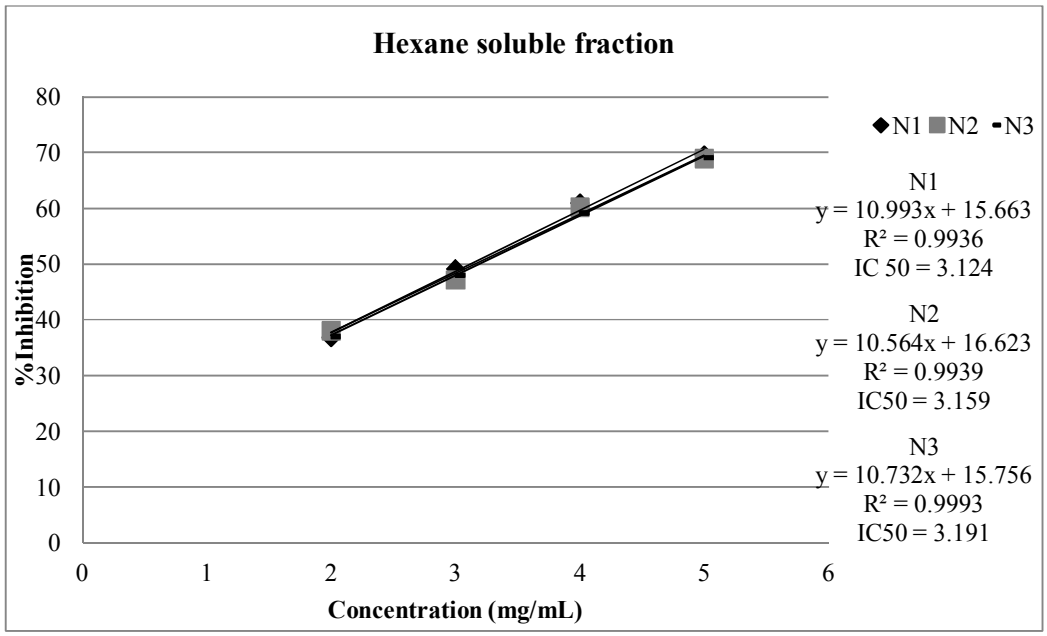


Figure 18 Tyrosinase inhibition of hexane soluble fraction

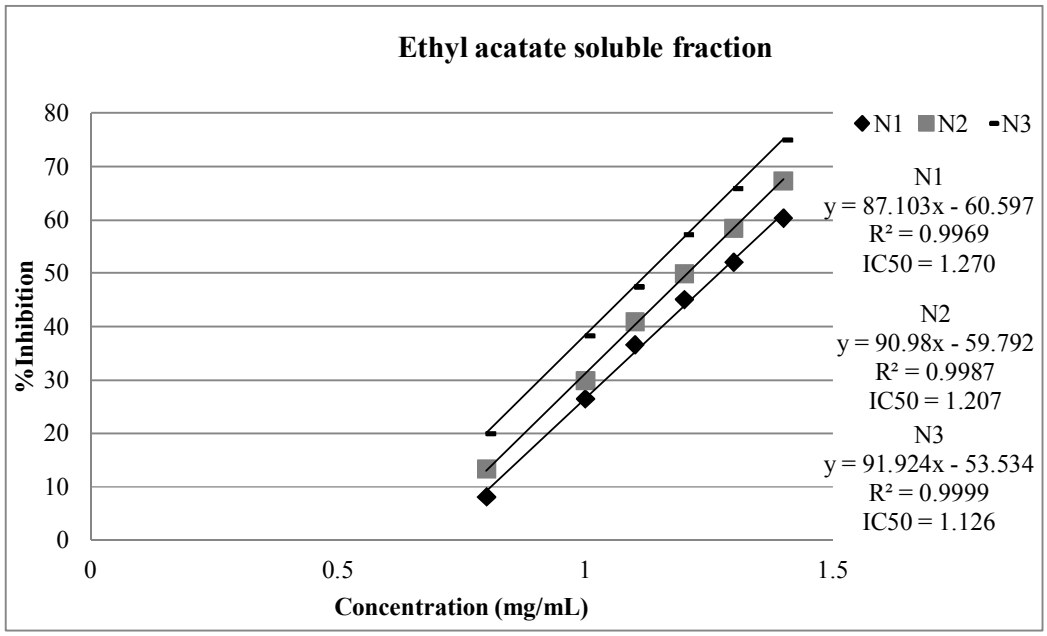


Figure 19 Tyrosinase inhibition of ethyl acetate soluble fraction

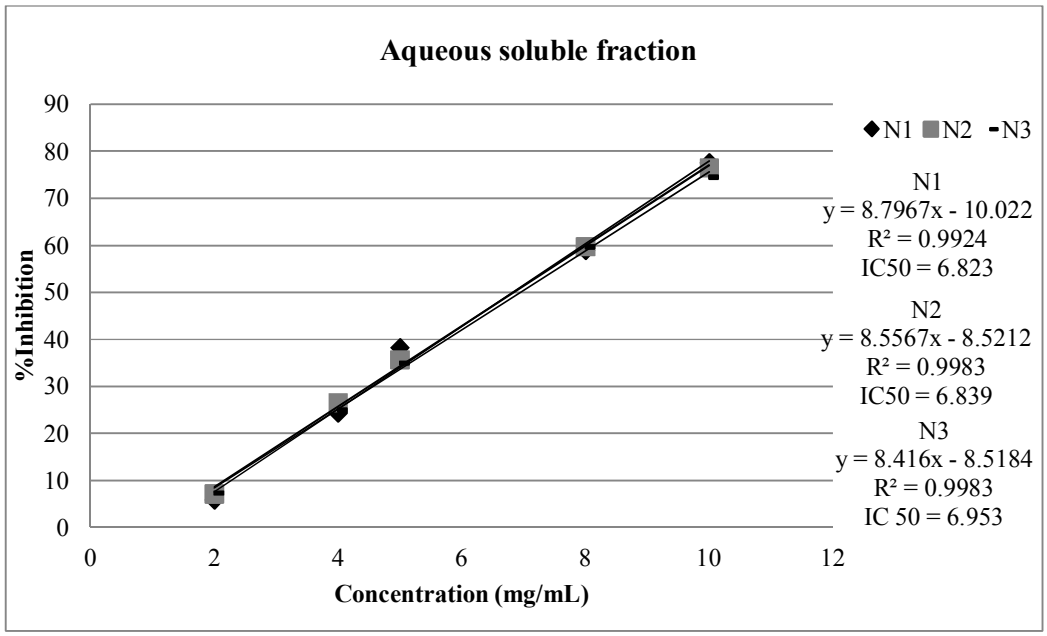


Figure 20 Tyrosinase inhibition of aqueous soluble fraction

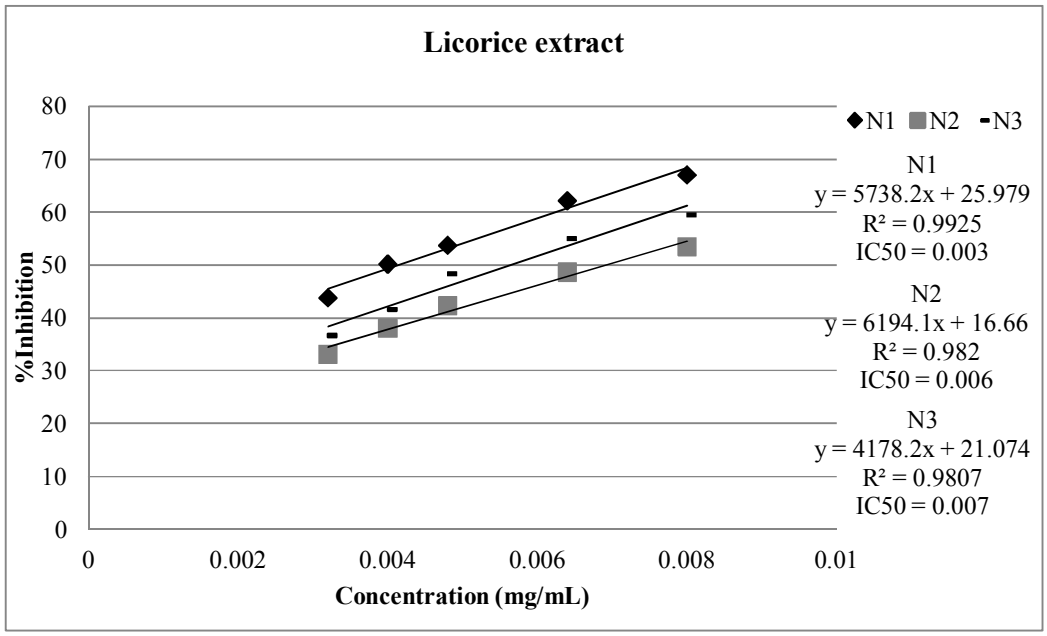


Figure 21 Tyrosinase inhibition of licorice extract

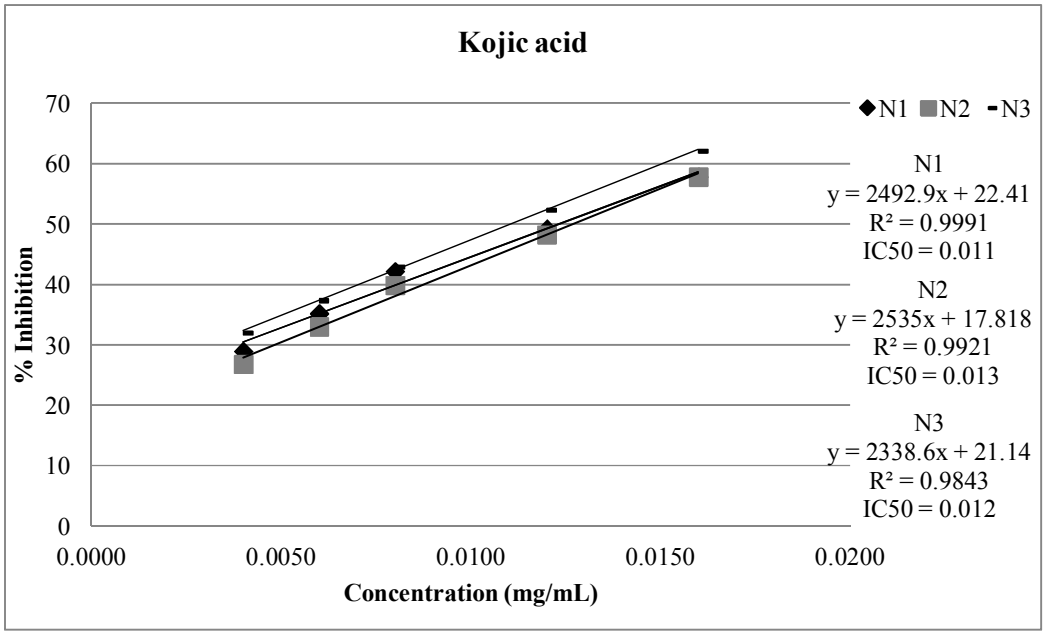


Figure 22 Tyrosinase inhibition of kojic acid

Table 10 The IC_{50} values calculated from linear equations for anti-tyrosinase activity of each extract and reference standard

sample	IC_{50} (mg/mL)	Mean (mg/mL)	SD
Hexane soluble fraction	3.124	3.158	0.034
	3.159		
	3.191		
Ethyl acetate soluble fraction	1.270	1.201	0.072
	1.207		
	1.126		
Aqueous soluble fraction	6.823	6.853	0.089
	6.783		
	6.953		
Licorice extract	0.003	0.005	0.002
	0.006		
	0.007		
Kojic acid	0.011	0.012	0.001
	0.013		
	0.012		

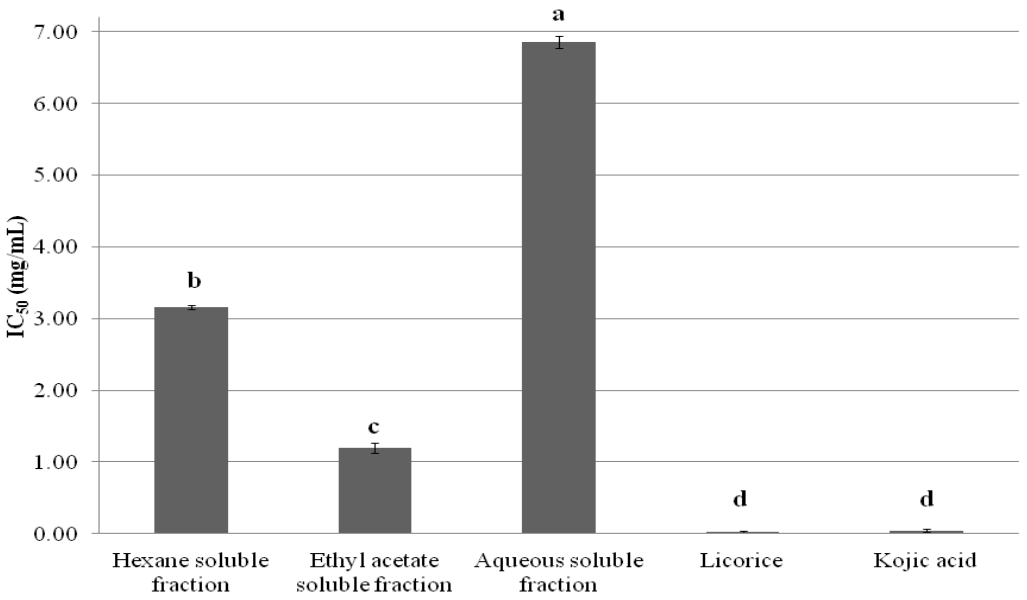


Figure 23 Concentration at 50% tyrosinase inhibition (IC_{50}) of all fractions and references (significantly different among a, b, c, and d, P value < 0.05)

The IC_{50} value of all fractions partitioned from ethanol extract can be ranked from the lowest to the highest, as followed; ethyl acetate soluble fraction (1.201 ± 0.072 mg/mL), hexane soluble fraction (3.158 ± 0.034 mg/mL), and aqueous soluble fraction (6.853 ± 0.089 mg/mL). Since the lower IC_{50} value indicates greater inhibitory potency, it was indicated that ethyl acetate soluble fraction exhibited the highest anti-tyrosinase activity. However, the IC_{50} value of ethyl acetate soluble fraction was 240 and 100 folds higher than the IC_{50} value of licorice extract and kojic acid, respectively.

1.4.2 Hydrogen-donating activity (DPPH radical scavenging activity)

After partitioning, the antioxidant activity of each fraction was also investigated. The initial and final concentration (mg/mL) of each fraction and reference were shown in table 11.

Table 11 The initial and final concentration (mg/mL) for activity testing of the partitioned extract in DPPH radical scavenging assay

Sample		Concentration (mg/mL)						
		1	**2**	**3**	**4**	**5**	**6**	**7**
Hexane soluble fraction	Initial	0.20	0.28	0.32	0.40	0.48	0.52	0.60
	Final	0.10	0.14	0.16	0.20	0.24	0.26	0.30
Ethyl acetate soluble fraction	Initial	0.020	0.024	0.028	0.032	0.036	-	-
	Final	0.010	0.012	0.014	0.016	0.018	-	-
Aqueous soluble fraction	Initial	0.20	0.30	0.40	0.50	0.60	0.70	-
	Final	0.10	0.15	0.20	0.25	0.30	0.35	-
BHA	Initial	0.008	0.010	0.012	0.014	-	-	-
	Final	0.004	0.005	0.006	0.007	-	-	-
BHT	Initial	0.20	0.24	0.28	0.32	0.36	0.40	-
	Final	0.10	0.12	0.14	0.16	0.18	0.20	-
Trolox	Initial	0.0010	0.0020	0.0030	0.0040	0.0050	0.0060	0.0070
	Final	0.0005	0.001	0.0015	0.002	0.0025	0.003	0.0035

The result revealed an increase in radical scavenging activity with increasing extract concentration. The relationship between % inhibition and extract concentration are presented in Figure 24-29. The IC_{50} value of DPPH radical scavenging activity of all partitioned fraction were calculated from the linear regression analysis of each figure. The IC_{50} values of all fractions were summarized and listed in Table 12 and Figure 30.

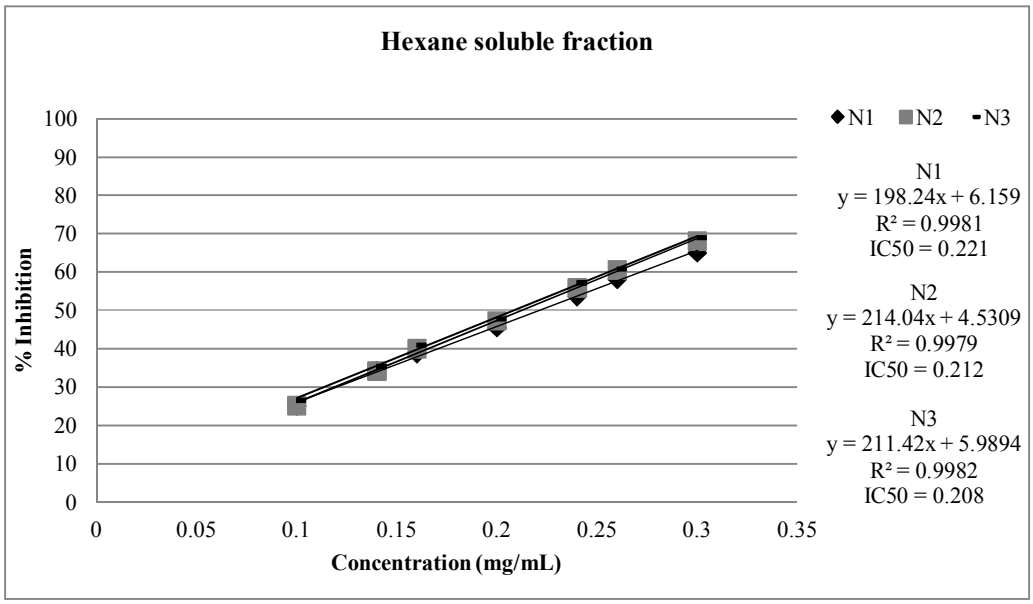


Figure 24 DPPH radical scavenging activity of hexane soluble fraction

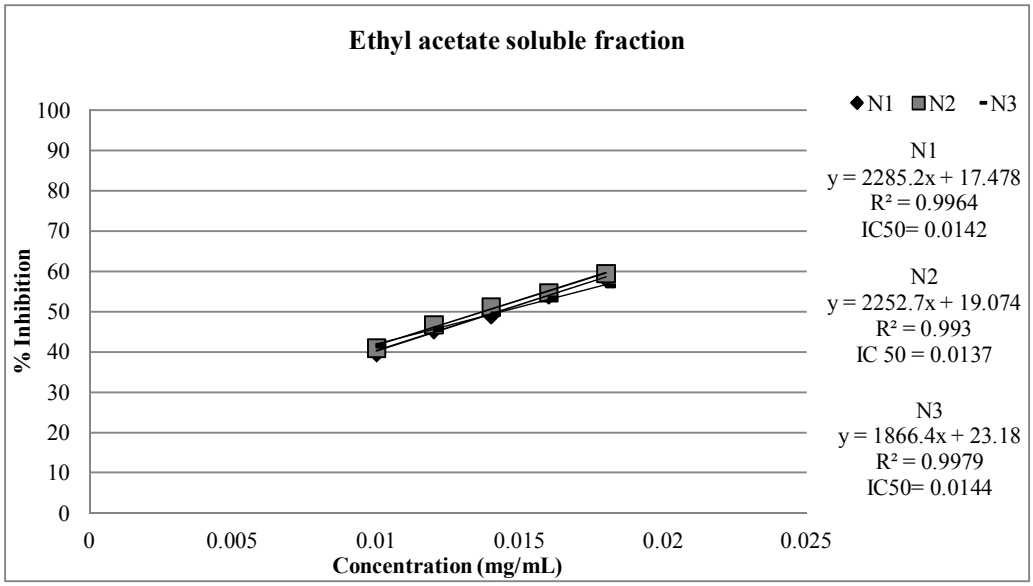


Figure 25 DPPH radical scavenging activity of ethyl acetate soluble fraction

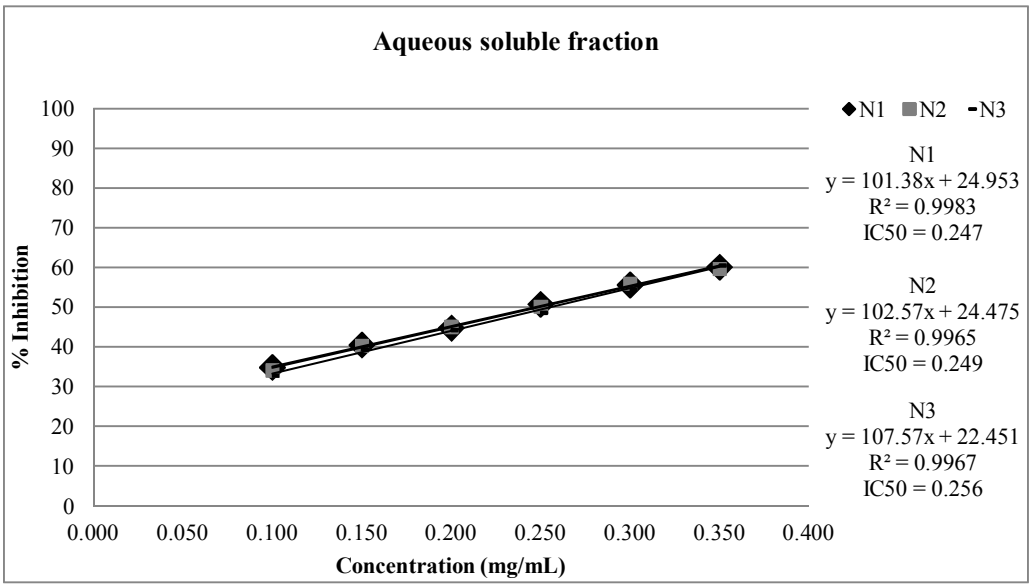


Figure 26 DPPH radical scavenging activity of aqueous soluble fraction

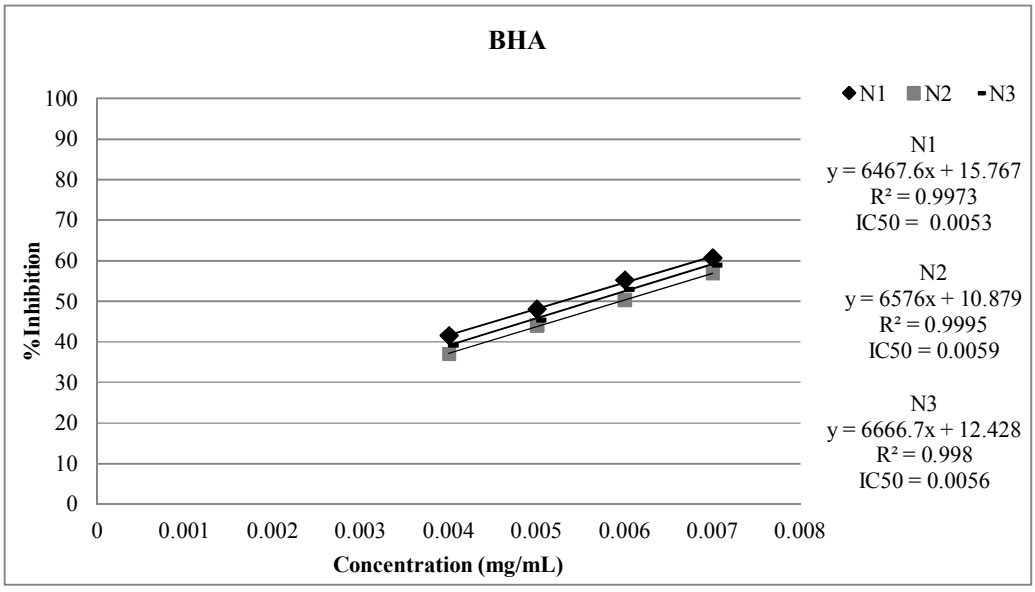


Figure 27 DPPH radical scavenging activity of BHA

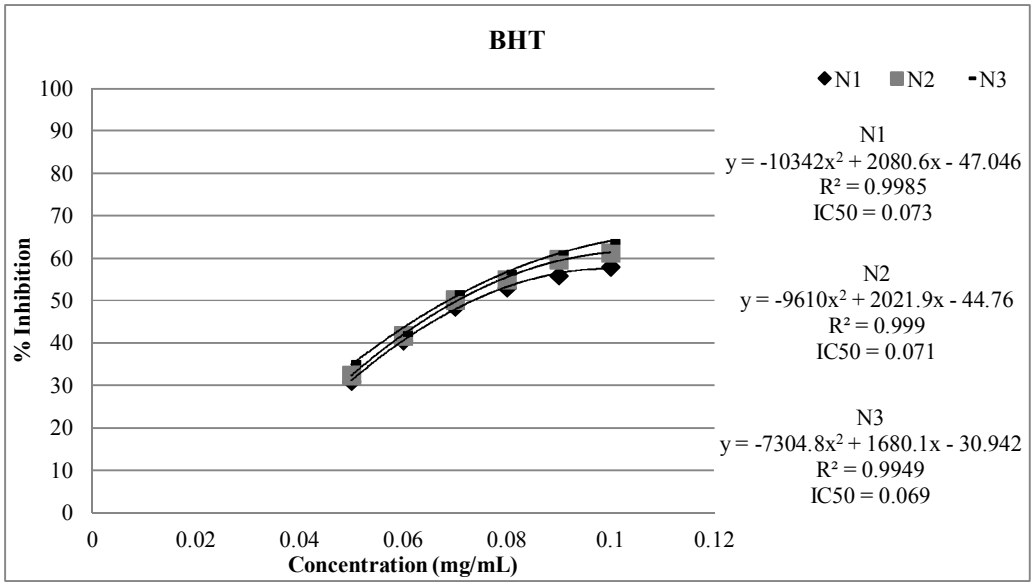


Figure 28 DPPH radical scavenging activity of BHT

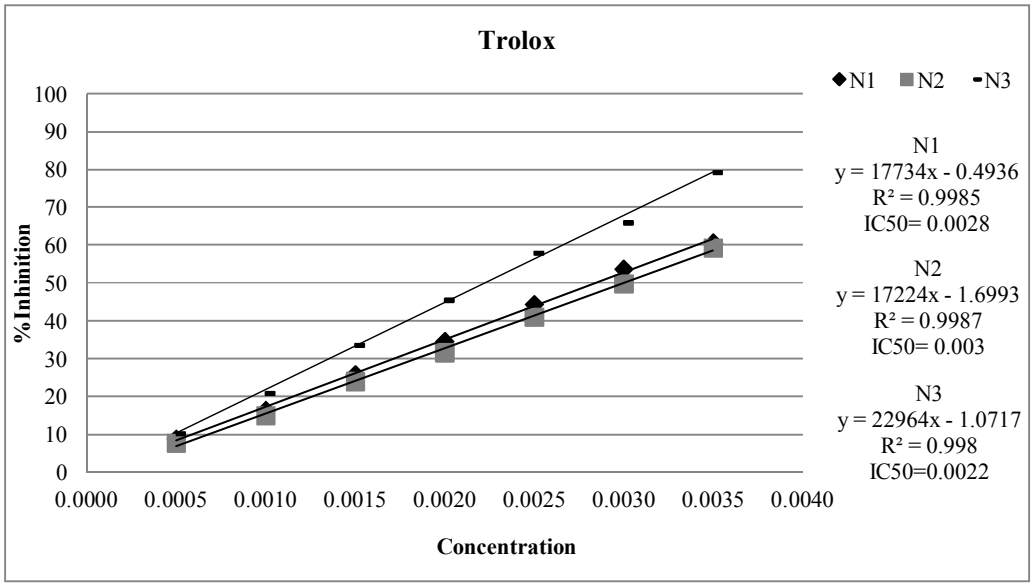


Figure 29 DPPH radical scavenging activity of Trolox

Table 12 The IC_{50} values calculated from linear equations for DPPH radical scavenging activity of each extract and reference standard

sample	IC_{50} (mg/mL)	Mean (mg/mL)	SD
Hexane soluble fraction	0.221 0.212 0.208	0.214	0.007
Ethyl acetate soluble fraction	0.014 0.014 0.014	0.014	0.000
Aqueous soluble fraction	0.247 0.249 0.256	0.251	0.005
BHA	0.0053 0.0059 0.0056	0.006	0.0003
BHT	0.073 0.071 0.069	0.071	0.002
Trolox	0.0028 0.0030 0.0022	0.003	0.0004

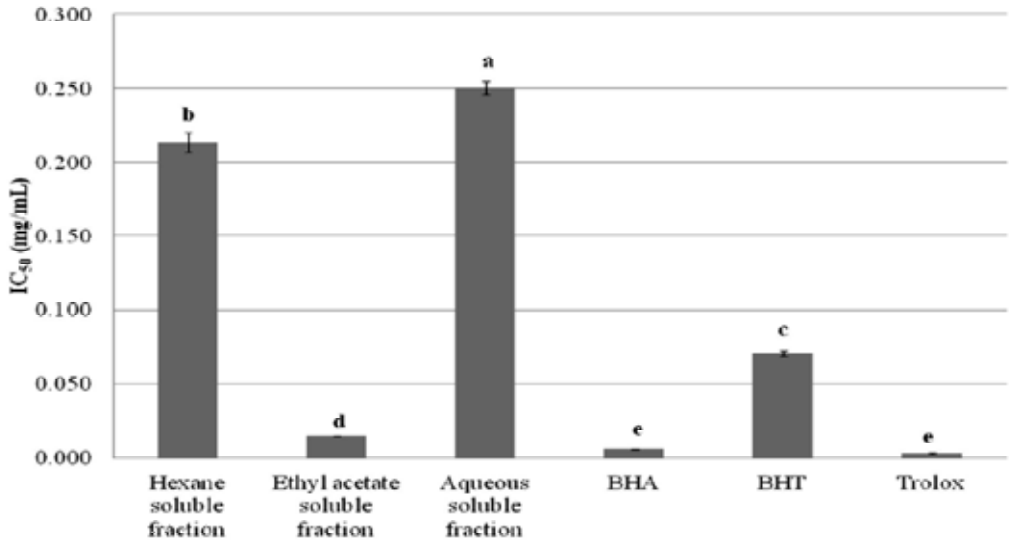


Figure 30 Concentration at 50% inhibition (IC_{50}) of all fractions and references (significantly different among a, b, c, d, and e, P value < 0.05)

The IC_{50} value or DPPH radicals scavenging activity of all fractions partitioned from ethanol extract ranked from the lowest to the highest, as follow; ethyl acetate soluble fraction (0.014 ± 0.000 mg/mL), hexane soluble fraction (0.214 ± 0.007 mg/mL), and aqueous soluble fraction (0.251 ± 0.005 mg/mL). Since the lower IC_{50} value indicates greater inhibitory potency, it indicated that ethyl acetate soluble fraction exhibited the highest DPPH radicals scavenging activity. To compare its activity to the reference, ethyl acetate soluble fraction provided better in antioxidant activity than BHT which is widely used as an antioxidant in pharmaceutical and cosmetics products. However, its activity is inferior than BHA and trolox.

The solvent extraction is a method that extracts the biological active compound from plant using the concept of polarity. The compounds that have the similar polarity to the solvent are solubilized in the solvent and can be concentrated by solvent evaporation. In this study, the solvent extraction exhibited stronger anti-tyrosinase and anti-oxidant activities compared to freeze-dried juice. This might be because the major active compounds extracted by the organic solvent from residue might exhibit more potent activities and might be more concentrated than those in freeze-dried juice. Potterat et al., 2007 reviewed the active compounds from methanol extract from fruit of *Morinda citrifolia* were iridoids compounds such as asperulosidic acid and deacetyl asperulosidic acid, flavonoids compounds such as rutin, kaempferol, quercetin and coumarin such as scopoletin. Those reported compounds contained the phenolic group that is known to exhibit potent anti-melanogenesis and antioxidants activities. Due to its moderate in polarity, those compounds can thus be extracted and concentrated by semi-polar solvent. In contrast the juice which might contain a various compounds with different polarity but in a very small amount of active constituent showed lower in activities compared to solvent extract.

After partition, ethyl acetate soluble fraction exhibited the highest activities with 16.7-fold higher than initial ethanol extract. The result is in accordance to Masuda et al., 2009 who reported that ethyl acetate soluble fraction isolated from methanol extract of *M. citrifolia* seeds exhibited greater inhibitory activity against elastase and tyrosinase, and the DPPH radical scavenging activity. Moreover, the study of Deng et al., 2007 and Su et al., 2005 revealed the compounds in *M. citrifolia* fruits were flavonoids (kaempferol and quercetin), iridoids (asperulosidic acid),

lignans (americanin A, and americanoic acid) and coumarin (scopoletin). It confirmed that the compounds found in *M. citrifolia* fruits had the polarity close to ethyl acetate. Further partitioning with ethyl acetate, therefore, purified and concentrated the active compounds leading to more potent in activities. Consequently, ethyl acetate soluble fraction was used for further cytotoxicity study, stability evaluation and formulation of nanoemulsions.

Part 2: Stability evaluation of *Morinda citrifolia* extract

The chemical stability of the extract was evaluated with respect to quantification of biological active compound remaining in the extract using HPLC method. While, the biochemical stability was evaluated in term of its antioxidant activity using DPPH radical scavenging assay.

2.1 HPLC condition

2.1.1 HPLC condition development

To determine the biological active compounds in the extract, the HPLC (High Performance Liquid Chromatography) method was developed and modified from the method reported by Deng et al., 2010. In short, they expressed the chromatogram of various parts of *Morinda citrifolia* such as fruit juice, dried fruit, leaf, root, seed, and flower. Deacetyl-asperulosidic acid (DAA) and asperulosidic acid (AA) were reported as the active compounds with retention time of 15.94 and 26.08 minutes as detected with UV-spectrophotometry at 235 and 344 nm. Their HPLC condition was as following:

Column	: C_{18} column (5 µm, 4.6 x 250 mm)
Mobile phase	: (A) = Acetonitrile,
	(B) = 0.1% Formic acid in H_2O (v/v)
(Gradient; 0–5 min, 0% A (100% B); and 40-41 min, 30% A (70% B))	
Detection	: UV-Visible spectroscopy at 210 - 400 nm
Injection volume	: 10 µL
Column temperature	: 25°C
Flow rate	: 0.8 mL/min

In this experiment, the ethyl acetate soluble fraction of *Morinda citrifolia* extract in absolute ethanol was analyzed. The obtained chromatogram of the extract was compared with that of the reference standards which were scopoletin, quercetin, and kaempferol. By following the condition from Deng et al., 2010 the HPLC chromatogram of ethyl acetate soluble fraction is shown in Figure 31 and the chromatogram of the standards are shown Figure 32.

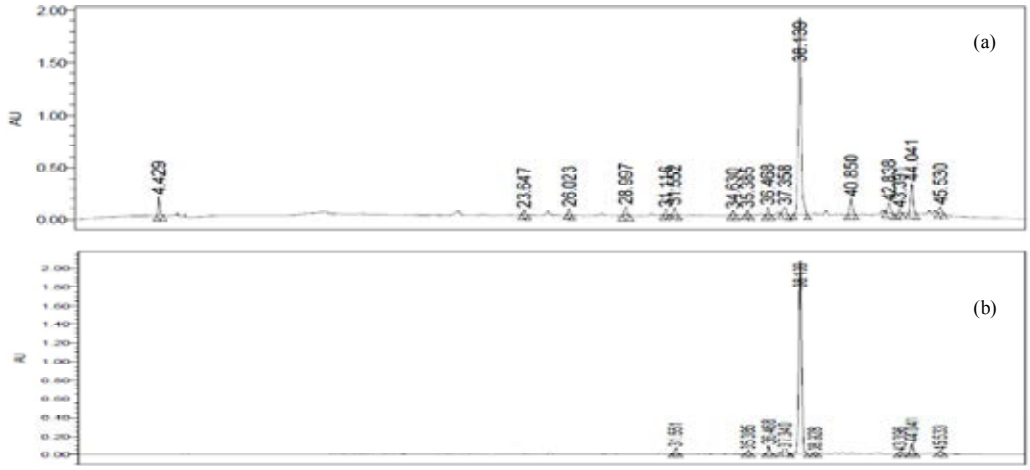


Figure 31 The HPLC chromatogram of ethyl acetate soluble fraction by Deng et al., 2010 condition detected at; (a) 235 nm, and (b) 344 nm

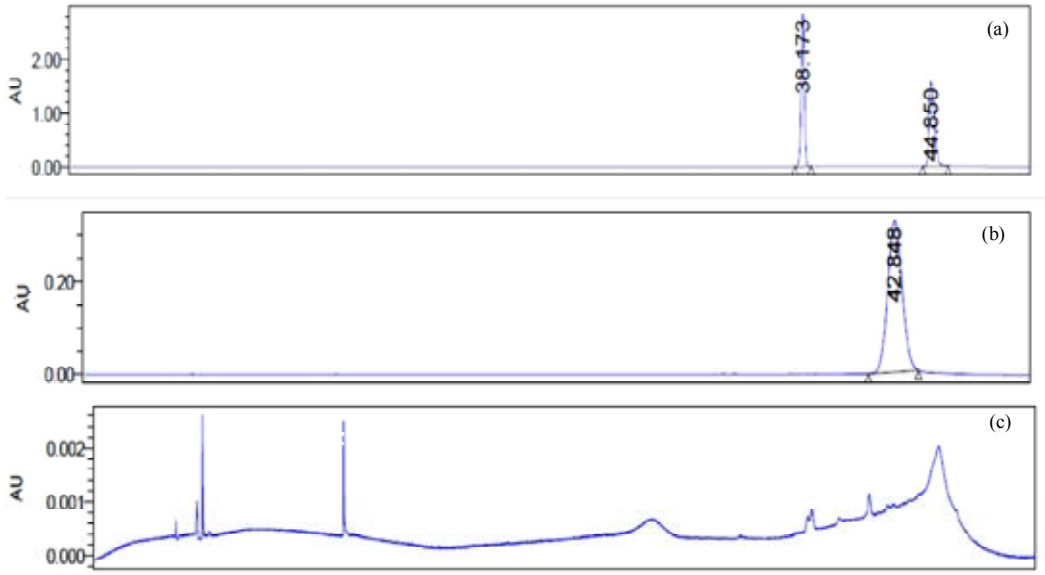


Figure 32 The HPLC chromatogram of standard at 344 nm by Deng et al., 2010 condition; (a) scopoletin, (b) quercetin, and (c) kaempferol

This preliminary condition exhibited the good resolution peaks. The main peak was eluted at 38.139 minute which was the same as the retention time of the standard scopoletin in Figure 32 (a). There was undetected amount of the compound eluted at 15.94 and 26.08 minutes detected at 235 nm which was reported as the retention time of DAA and AA by Deng et al., 2010. Thus, the result suggested that the major active compound in the selected extract was scopoletin. However, to shorten the retention time of the major peak of scopoletin in the extract, the condition was modified by following condition;

Column	: C_{18} column (5 µm, 4.6 x 250 mm)
Mobile phase	: (A) = Acetonitrile,
	(B) = 0.1% Formic acid in H_2O (v/v)
(Gradient; 0–2 min, 0% A (100% B); and 2-20 min, 30% A (70% B))	
Detection	: UV-Visible spectroscopy at 210 - 400 nm
Injection volume	: 10 µL
Column temperature	: 25℃
Flow rate	: 0.5 mL/minute

By this condition, the well resolving chromatogram was obtained with shorter retention time. The chromatograms of the extract and standard scopoletin are shown in Figure 33 and Figure 34, respectively.

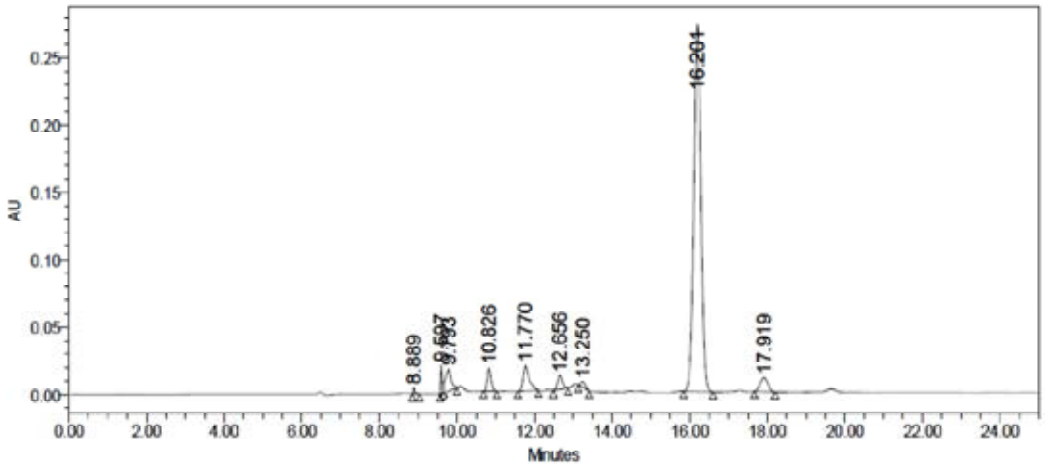


Figure 33 The HPLC chromatogram of ethyl acetate soluble fraction by HPLC gradient condition modified from Deng et al., 2010 detected at 344 nm

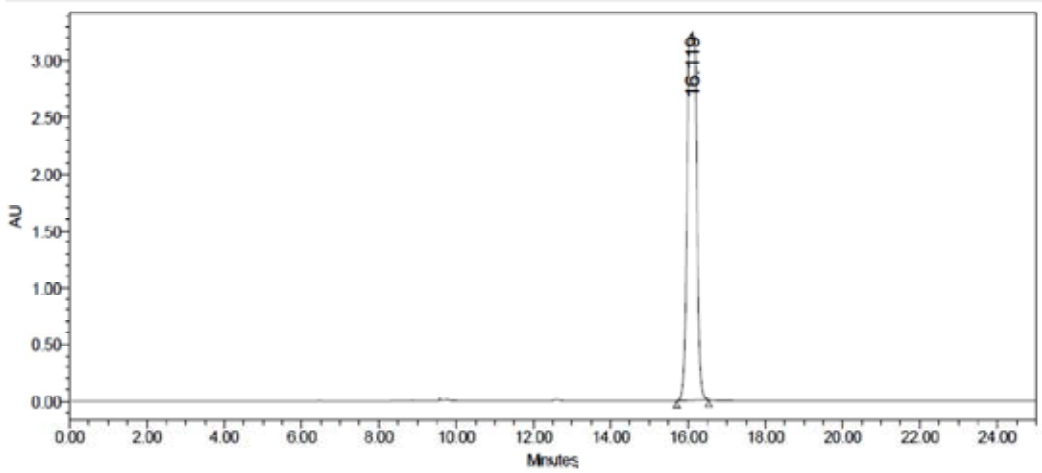


Figure 34 The HPLC chromatogram of standard scopoletin by HPLC gradient condition modified from Deng et al., 2010 detected at 344 nm

The condition was further developed by changing the flow pattern of mobile phase from gradient to isocratic in order to shorten retention time. The developed condition was as following:

Column	: C_{18} column (5 µm, 4.6 x 250 mm)
Mobile phase	: Isocratic Acetonitrile (30%): 0.1% Formic acid in H_2O (70%)
Detection	: UV-Visible spectroscopy at 354 nm
Injection volume	: 10 µL
Column temperature	: 30°C
Flow rate	: 0.5 mL/minute

The chromatogram of extract, scopoletin, quercetin and kaempferol are shown in Figure 35, 36, 37, and 38, respectively.

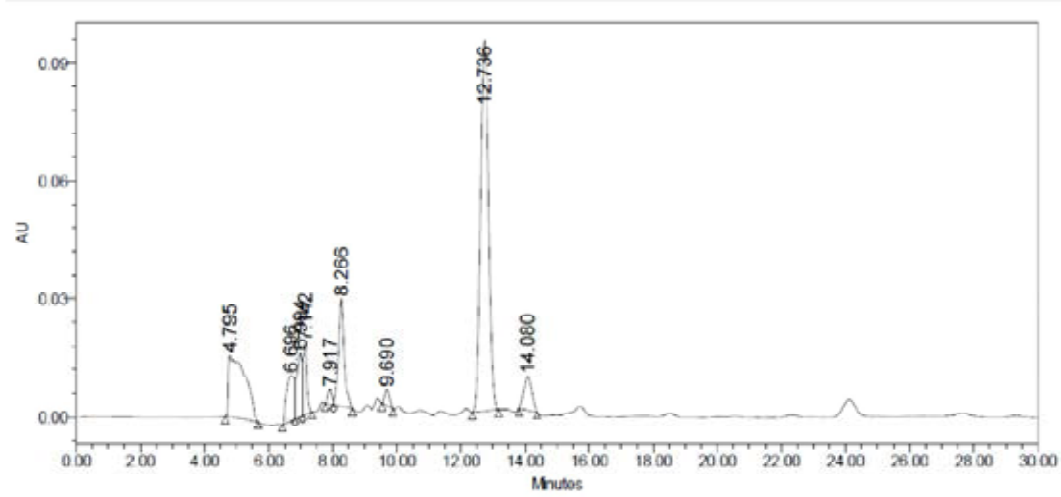


Figure 35 Chromatogram of ethyl acetate soluble fraction of *Morinda citrifolia* extract

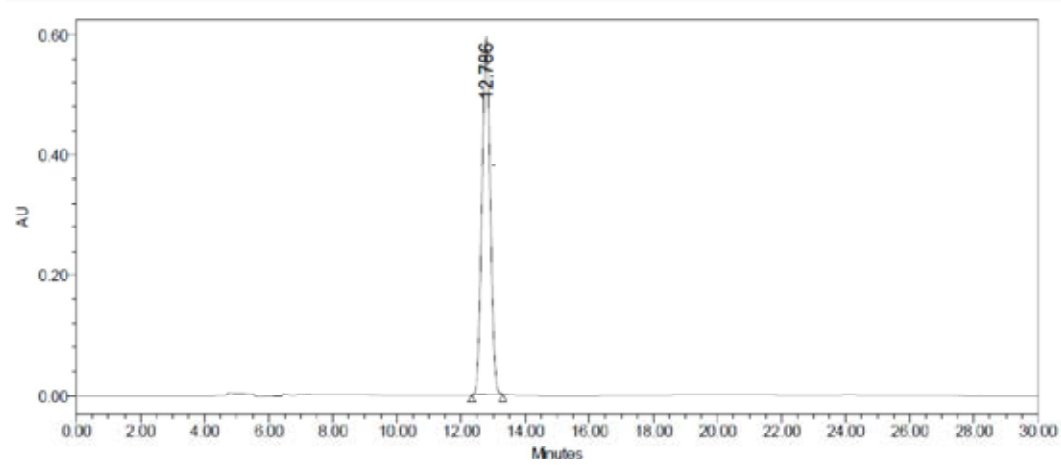


Figure 36 Chromatogram of standard Scopoletin

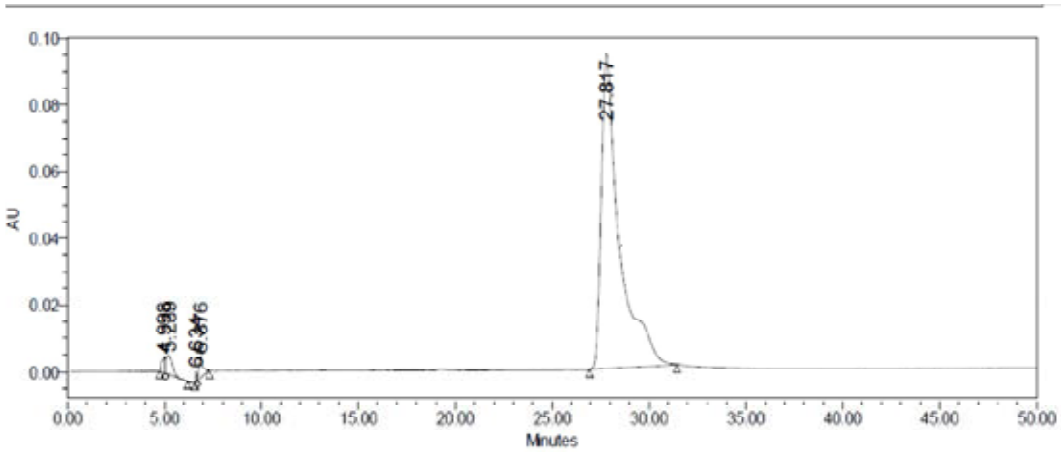


Figure 37 Chromatogram of standard Quercetin

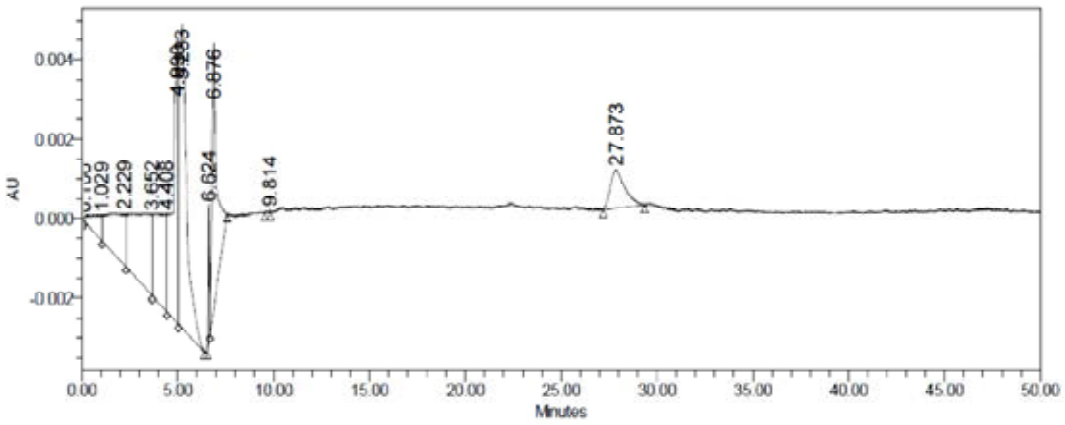


Figure 38 Chromatogram of standard Kaempferol

By following this condition, the main peak was eluted at 12.736 minutes with resolution of 7.30 exhibiting a well separation from other peaks. This main peak was similar to scopoletin reference standard eluted at 12.786 minutes. This result was found to be in accordance with the findings from Deng et al. 2005 who reported scopoletin as one of the compounds isolated from the fruits of *Morinda citrifolia* collected in Tahiti. Moreover, Samoylenko et al. 2007 also revealed the presence of scopoletin in the samples of fresh-squeezed *Morinda citrifolia* fruit juice from Japan. In addition, Potterat et al. 2007 also used scopoleitn as one of the biological markers for HPLC-MS to quantify the active constituent in commercial *Morinda citrifolia* fruits capsules and juice. As a result, in according to the most predominant compound found in the extract in this study, scopoletin was selected to be the active marker for further studies.

2.1.2 Preparation of calibration curve

A calibration curve of scopoletin reference standard was obtained by plotting the peak areas versus concentrations. The calibration data and the calibration curve of scopoletin in absolute ethanol are shown in Table 13 and Figure 39, respectively. A linear equation from the calibration curve was generated and the coefficient of determination (R^2) was calculated as 1.0000. This standard curve was used to calculate the concentration of active scopoletin in the extract.

Table 13 Data of calibration curve of scopoletin in absolute ethanol by HPLC method

Concentration	Peak area				
(µg/mL)	Set 1	Set 2	Set 3	Average	SD
0.05	3317.22	2931.33	3384.17	3210.91	244.42
0.1	7865.11	6728.00	8314.33	7635.81	817.65
0.5	35787.89	35688.33	36357.67	35944.63	361.15
1	75563.89	72955.78	82821.11	77113.59	5111.98
2	150789.22	144269.67	158856.56	151305.15	7307.12
10	758010.00	687293.11	765430.33	736911.15	43130.36
20	1488848.33	1442107.00	1558297.33	1496417.56	58463.82
40	2972736.78	2810218.00	3127761.22	2970238.67	158786.35
R^2	1.0000	0.9998	1.0000	1.0000	-

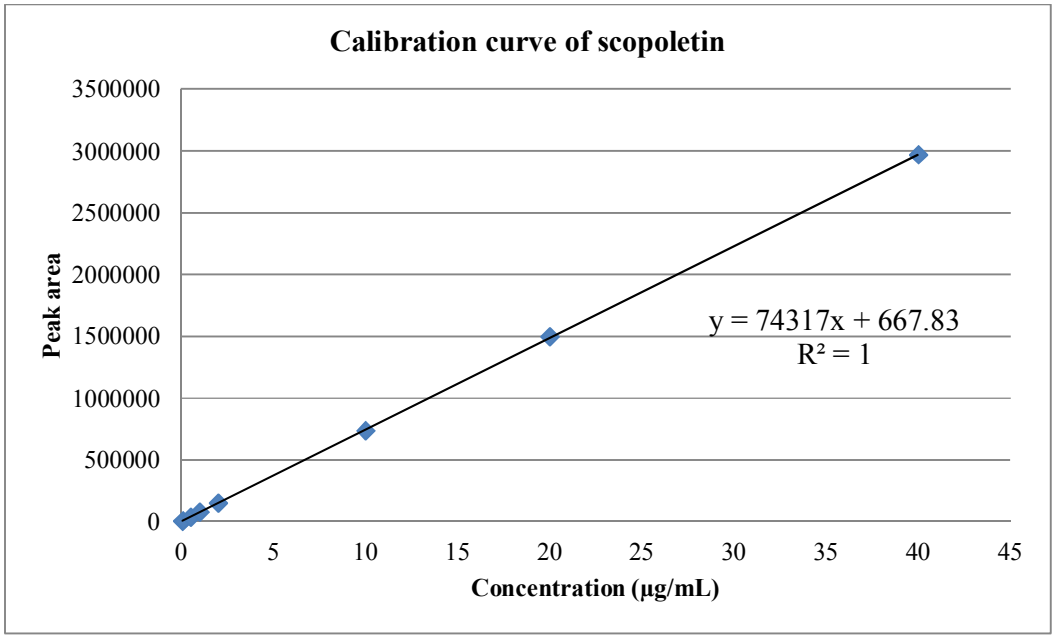


Figure 39 Calibration curve of standard scopoletin in absolute ethanol

2.1.3 Method validation

The developed analytical method was validated according to ICH guideline on following parameter; accuracy, precision, selectivity, linearity and range.

2.1.3.1 Accuracy

Three concentrations (5, 15, and 30 µg/mL) of standard scopoletin solution in absolute ethanol were analyzed in five replicates. The accuracy as percent recovery obtained from the mean of observed concentrations calculated from the calibration curve divided by the mean of actual concentrations and multiplied by 100 was reported in Table 14. The percentages analytical recovery were 99.43 – 100.97 %, which were in the range of 98.00 – 102.00 %. The results indicated the high accuracy of this method. Thus, it would be used for analysis of scopoletin in the sample.

Table 14 Percentage of analytical recovery of scopoletin in absolute ethanol by HPLC method

Actual concentration (µg/mL)	Analytical concentration (µg/mL)	% Recovery	Mean ± SD of % Recovery
5.000	5.0400	100.80	100.97 ± 0.76 (99.72-101.59)
	5.0632	101.26	
	5.0728	101.46	
	4.9858	99.72	
	5.0795	101.59	
15.000	14.8121	98.75	100.12 ± 1.34 (98.75-101.93)
	15.2899	101.93	
	14.9275	99.52	
	15.1653	101.10	
	14.8959	99.31	
30.000	30.0443	100.15	99.43 ± 0.65 (98.85-100.15)
	30.0351	100.12	
	29.6538	98.85	
	29.6718	98.91	
	29.7390	99.13	

2.1.3.2 Precision

The inter-day and intra-day precision of standard scopoletin solution in absolute ethanol analyzed by HPLC method was determined. The result showed that the percentages of relative standard deviation (%RSD) of all

concentrations were in the range of 0.65 – 1.34 % and 0.30 – 0.67 % for intra-day and inter-day precision, respectively. The %RSD of the analytical method was less than 2.00%. The HPLC method was, therefore, precise for quantitative analysis of scopoletin in the sample within the range of study.

Table 15 Data of intra-day precision of scopoletin in absolute ethanol by HPLC method

Actual concentration (µg/mL)	Analytical concentration (µg/mL)	% Recovery	Mean	SD	%RSD
5.000	5.0400	100.80	100.97	0.76	0.75
	5.0632	101.26			
	5.0728	101.46			
	4.9858	99.72			
	5.0795	101.59			
15.000	14.8121	98.75	100.12	1.34	1.34
	15.2899	101.93			
	14.9275	99.52			
	15.1653	101.10			
	14.8959	99.31			
30.000	30.0443	100.15	99.43	0.65	0.65
	30.0351	100.12			
	29.6538	98.85			
	29.6718	98.91			
	29.7390	99.13			

Table 16 Data of inter-day precision of scopoletin in absolute ethanol by HPLC method

Actual concentration (µg/mL)	%Recovery			Mean	SD	%RSD
	Day1	Day2	Day3			
5.000	100.97	100.23	101.16	100.79	0.49	0.49
15.000	100.12	100.52	100.72	100.45	0.31	0.30
30.000	99.43	98.73	100.05	99.42	0.06	0.67

2.1.3.3 Selectivity

The selectivity of the biological active compound in the sample was determined by comparison of chromatograms among standard scopoletin solution, extract solution and blank samples (absolute ethanol) as showed in Figure 40. The main active peak showing the well resolving from the other peaks and the symmetry of the peaks was obtained with average resolution value as 7.8787 and tailing factor as 1.0504. Since, the resolution factor should be more than 1.5 and tailing factor should be less than 2, the obtained chromatogram showed good selectivity and thus would be used to analyze scopoletin in the extract solution.

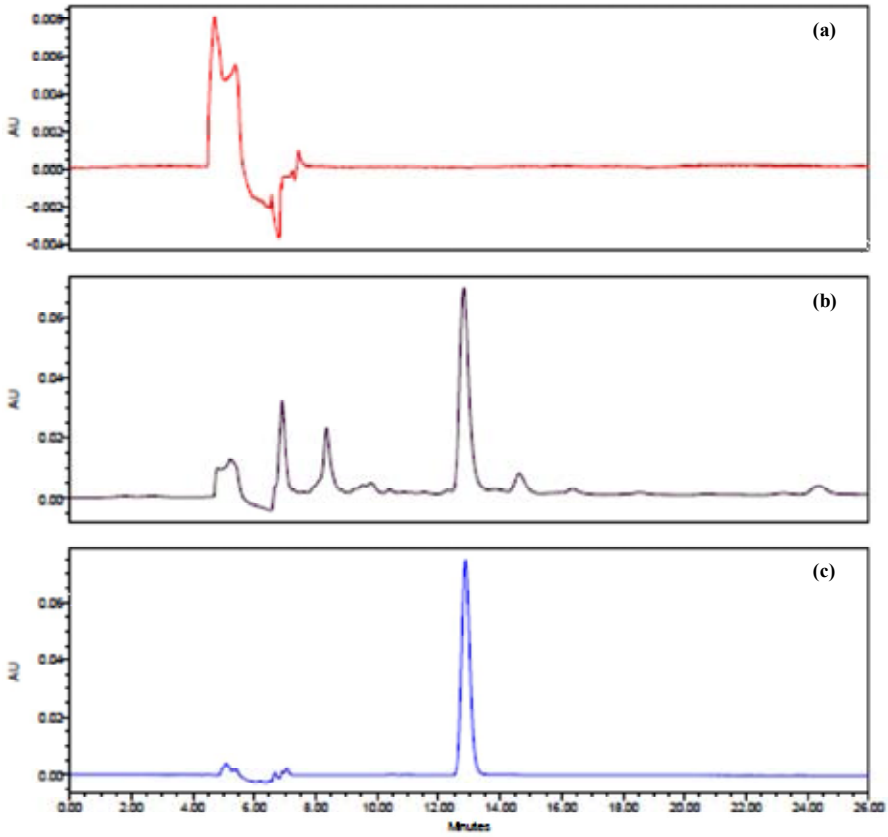


Figure 40 HPLC chromatogram of the sample solution; (a) blank samples (absolute ethanol), (b) extract solution, and (c) standard scopoletin solution

2.1.3.4 Linearity and range

Five concentrations (1, 2, 10, 20 and 40 µg/mL) of standard scopoletin in absolute ethanol were analyzed in triplicates. The analytical amount was

calculated as showed in Table 17. The graph between the analytical concentrations and their actual concentrations were plotted and shown in Figure 41 with the coefficient of determination (R^2) of 1.000. These results indicated that the HPLC method was acceptable for quantitative analysis of scopoletin in the range studied.

Table 17 Data of actual and analytical concentration of scopoletin in absolute ethanol by HPLC method

Concentration (µg/mL)	Analytical concentration (µg/mL)			Average	SD
	Set 1	Set 2	Set 3		
1	0.9318	1.0169	1.0747	1.0078	0.0719
2	2.0608	2.0533	1.9460	2.0200	0.0642
10	10.2682	10.0541	10.2498	10.1907	0.1187
20	19.0566	19.8947	21.1230	20.0248	1.0393
40	40.0885	40.0365	39.8504	39.9918	0.1252

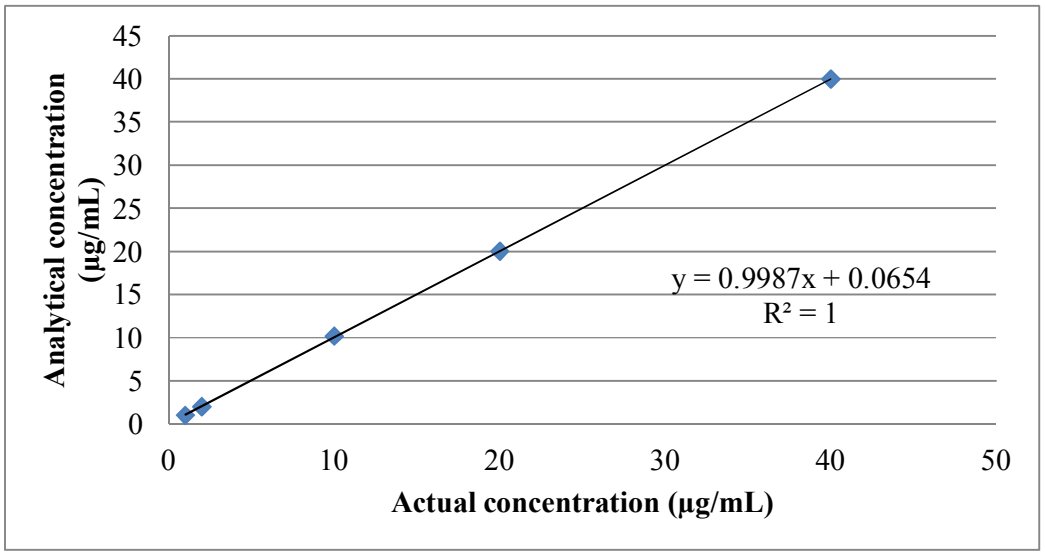


Figure 41 Linearity between the analytical concentration and the actual concentration of standard scopoletin in absolute ethanol

In conclusion, the analysis of scopoletin by HPLC method developed in this study exhibited good accuracy, precision, selectivity, and linearity. As a result, this method was used for determination of the scopoletin content in the extract.

2.2 Determination of scopoletin in the extracts

Freeze-dried juice and the solvent extract were examined for scopoletin. The chromatograms are shown in Figure 42.

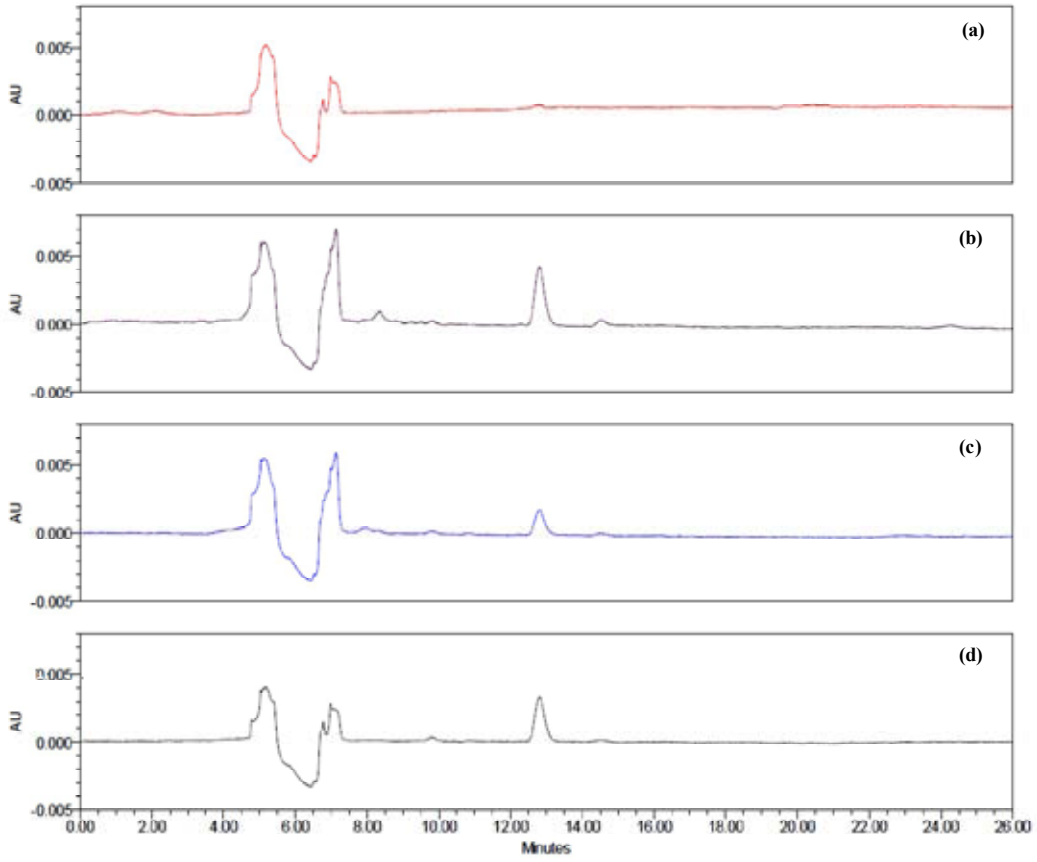


Figure 42 HPLC chromatogram of the sample solution in absolute ethanol; (a) freeze-dried juice, (b) ethanol extract and (c) acetone extract (d) ethyl acetate extract

By comparing the chromatogram of freeze-dried juice and the solvent extracts, it was found that all the solvent extract exhibited comparable in scopoletin content but higher than that was in freeze-dried juice. This result was correlated with their anti-tyrosinase and antioxidant activities that all the solvent extracts possessed higher activity than freeze-dried juice. It could confirm that solvent extraction can extract and concentrate more content of active compound. Moreover, the result revealed that the active compounds in the *Morinda citrifolia* fruits were semi-polar since they were predominantly found in the solvent extraction.

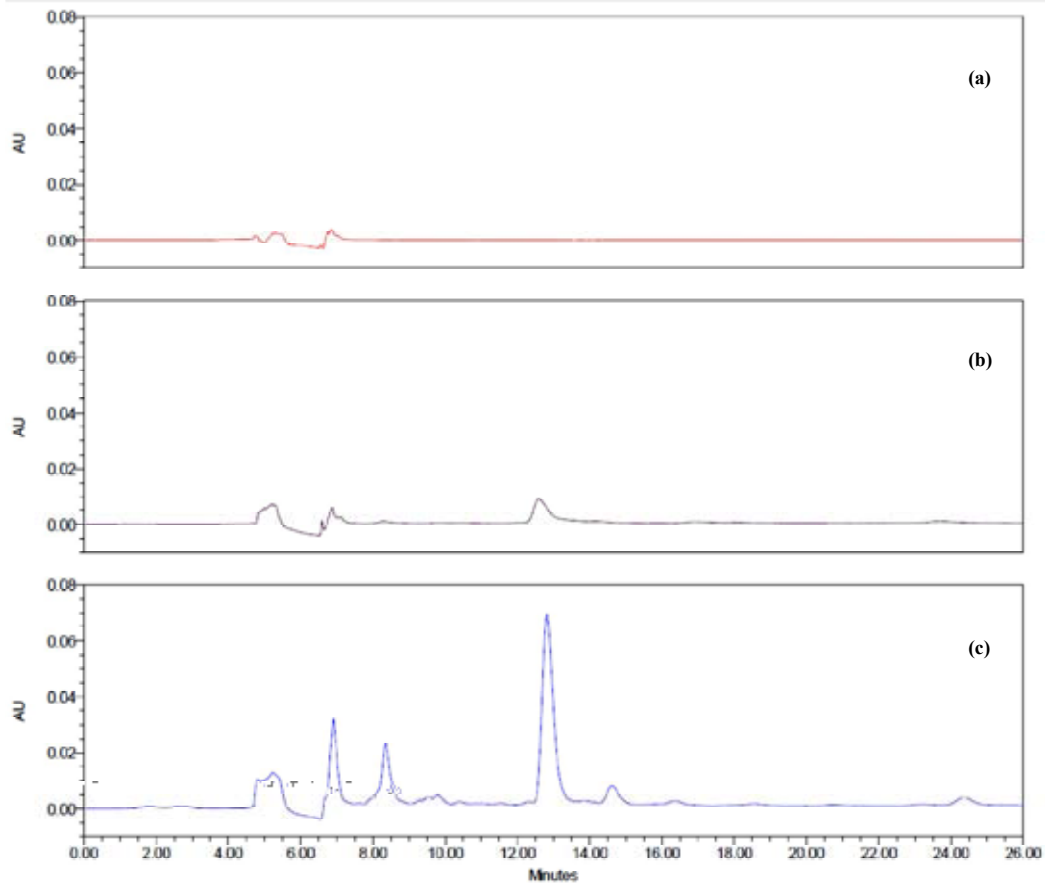


Figure 43 HPLC chromatogram of the sample solution in absolute ethanol; (a) aqueous soluble fraction, (b) hexane soluble fraction and (c) ethyl acetate soluble fraction

After partition, the chromatogram of hexane-, ethyl acetate-, and water soluble fraction showed large different in scopoletin content. Ethyl acetate soluble fraction showing highest activity exhibited highest amount of scopoletin. The higher in scopoletin content, the higher in activity of the extract. It could be then indicated that the main active compound in the extract is scopoletin. Moreover, the chromatogram of ethyl acetate soluble fraction in Figure 43 also showed larger amount of scopoletin than that of the ethanol extract before partitioned which was in accordance to their activity. This may attribute to the fact that the biological active compound in the extract can be concentrated and purified by partitioning process.

Owing to their highest in activity, ethyl acetate soluble fraction was selected for further studies. The quantitative analysis of scopoletin in extract was

performed. The selected extract was analyzed using the HPLC method and the concentration of scopoletin in the extract was calculated from the standard curve obtained in section 2.1.2 (Table 18). The linear regression was obtained from the plot between calculated concentration of scopoletin in the extract and the actual concentration of the extract prepared. The plot showed a linear relationship with R^2 = 0.9992, Figure 44. This linear relationship demonstrated that when the extract concentration was increased, the concentration of scopoletin was also detected increasingly.

Table 18 The actual concentration of extract injected to HPLC and the concentration of scopoletin in the extract calculated from the calibration curve

Actual concentration of extract (mg/mL)	**Peak area**					**Calculated concentration of scopoletin (µg/mL)**
	N1	**N2**	**N3**	**Average**	**SD**	
0.5000	752864	749307	740350	747507.00	6448.26	10.0494
0.8000	1161608	1197814	1187251	1182224.33	18619.05	15.8989
1.0000	1443851	1422800	1451031	1439227.33	14672.46	19.3571
1.2000	1745075	1725739	1759258	1743357.33	16825.39	23.4494
1.4000	2114101	2058202	2001926	2058076.33	56087.61	27.6842
1.6000	2389791	2339336	2343366	2357497.67	28039.34	31.7132

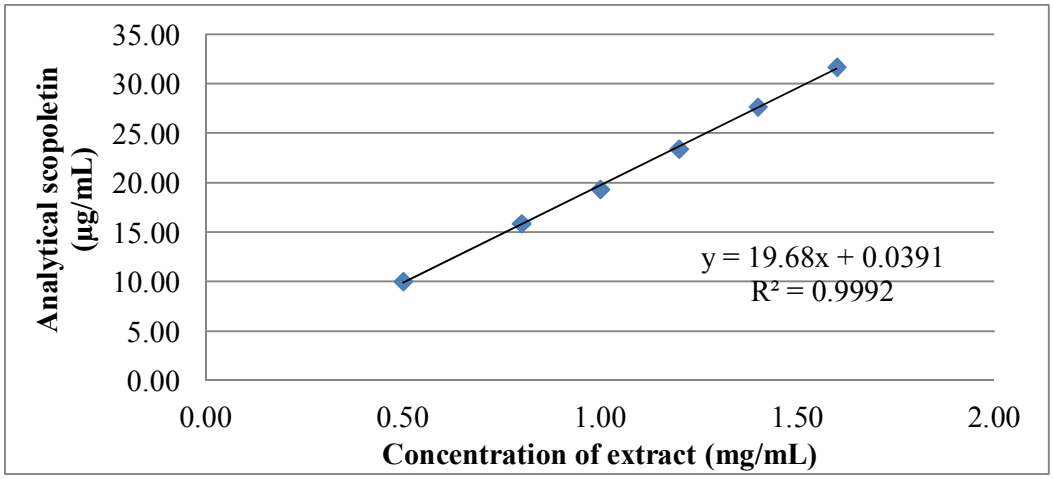


Figure 44 The linear relationship of actual concentration of extract injected to HPLC and the concentration of scopoletin in the extract calculated from the calibration curve

2.3 Stability evaluation

2.3.1 Chemical stability evaluation

To examine stability of the extract, it was packed in a well-closed container, protected from light and kept at 4°C, at room temperature (30°C), and at 40°C for 6 months. The extract was analyzed for the remaining active compound; scopoletin, at various time interval (0, 2, 4, and 6 months). Percentages of scopoletin in the samples were calculated from the standard curve. The data are shown in Table 19, and 20.

Table 19 The peak area of scopoletin in the extract quantified by HPLC

Condition	Time (Month)	Peak area				
		N1	N2	N3	Average	SD
4°C	0	1666167	1673634	1676564	1672122	5360.948
	2	1674940	1678374	1673413	1675576	2540.853
	4	1686070	1675836	1656865	1672924	14818.578
	6	1662370	1647627	1674956	1661651	13678.673
RT	0	1666167	1673634	1676564	1672122	5360.948
	2	1685856	1693074	1661854	1680261	16344.642
	4	1685216	1689315	1649740	1674757	21762.119
	6	1663413	1670096	1652619	1662043	8818.563
40°C	0	1666167	1673634	1676564	1672122	5360.948
	2	1666465	1664864	1664708	1665346	972.286
	4	1667957	1663127	1658509	1663198	4724.396
	6	1659931	1659325	1667921	1662392	4797.544

Table 20 The amount of scopoletin in the extract calculated from calibration curve

Condition	Time (Month)	Analytical scopoletin (µg/mL)				
		N1	N2	N3	Average	SD
4°C	0	22.411	22.511	22.551	22.491	0.072
	2	22.529	22.575	22.508	22.537	0.034
	4	22.679	22.541	22.286	22.502	0.199
	6	22.360	22.161	22.529	22.350	0.184

Condition	Time (Month)	Analytical scopoletin (µg/mL)				
		N1	N2	N3	Average	SD
RT	0	22.411	22.511	22.551	22.491	0.072
	2	22.676	22.773	22.353	22.600	0.220
	4	22.667	22.722	22.190	22.526	0.293
	6	22.374	22.464	22.228	22.355	0.119
40°C	0	22.411	22.511	22.551	22.491	0.072
	2	22.415	22.393	22.391	22.400	0.013
	4	22.435	22.370	22.308	22.371	0.064
	6	22.327	22.319	22.434	22.360	0.065

The percent relative amount of scopoletin remaining in the extract at particular time point compared to the initial amount at time zero was calculated and demonstrated in Table 21 and Figure 45.

Table 21 The relative percentage of scopoletin in extract at particular time point compared to time zero

Condition	Time (Month)	% Relative to time zero				
		N1	N2	N3	Average	SD
4 °C	2	100.17	100.37	100.08	100.21	0.152
	4	100.83	100.22	99.09	100.05	0.887
	6	99.42	98.53	100.17	99.37	0.818
RT	2	100.82	101.25	99.39	100.49	0.978
	4	100.78	101.03	98.66	100.16	1.302
	6	99.48	99.88	98.83	99.40	0.528
40 °C	2	99.66	99.57	99.56	99.59	0.058
	4	99.75	99.46	99.19	99.47	0.283
	6	99.27	99.23	99.75	99.42	0.287

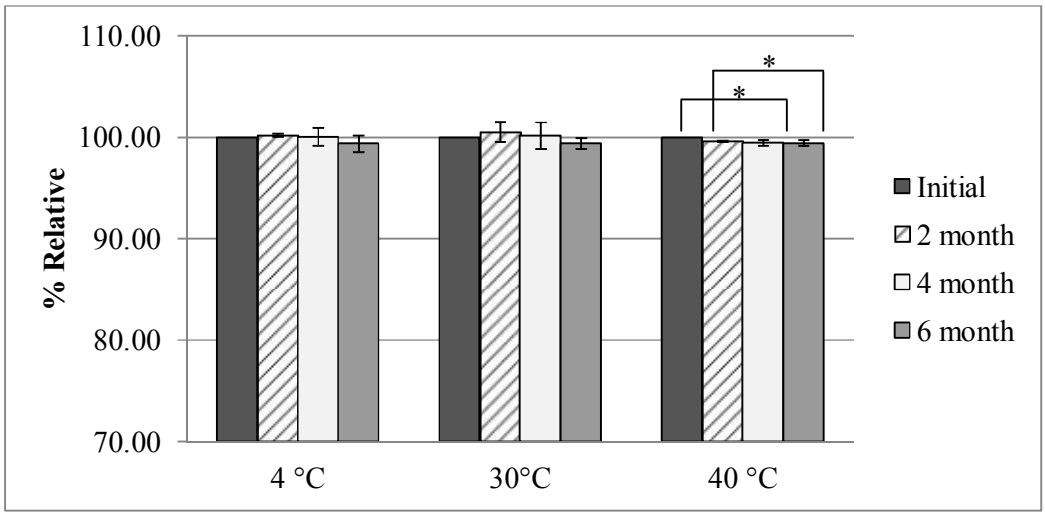


Figure 45 The comparison of relative scopoletin remaining in the extract at particular time point to the extract at time zero. Plots were mean ± SD (n=3), * means significantly different between group (p-value < 0.05)

There was insignificant change in the amount of scopoletin in the extract kept at 4°C and room temperature (30°C). The results indicated the extract was stable at 4°C and at room temperature (30°C). In constant, there was a significantly decrease in the amount of scopoletin in the extract kept at 40 °C after 4 and 6 months of storage. This indicated that scopoletin in the extract might be susceptible to high temperature. Nevertheless, the remaining amount of scopoletin in the extract was still within the acceptable range of 90-110%. The extract was therefore chemically stable at conditions investigated.

2.3.2 Biochemical stability evaluation

The selected extract kept at 4°C, at room temperature (30°C ± 2°C), and at 40°C were analyzed for the remaining antioxidant activity at various time intervals (0, 2, 4, and 6 months). By DPPH radical scavenging assay, the remaining antioxidant activity was determined as IC_{50} show in Table 22. The relative IC_{50} of the extract at particular time point compared to that at time zero was determined and reported in Table 23 and Figure 46.

Table 22 The IC_{50} values for DPPH radical scavenging activity of the extract kept in various conditions at any particular time point and time durations

Storage condition	Time (Month)	IC_{50} (μg/mL)				
		N1	N2	N3	Average	SD
4°C	0	0.0160	0.0140	0.0150	0.0150	0.0010
	2	0.0160	0.0158	0.0158	0.0159	0.0001
	4	0.0166	0.0157	0.0157	0.0160	0.0005
	6	0.0170	0.0176	0.0170	0.0172	0.0003
RT	0	0.0160	0.0140	0.0150	0.0150	0.0010
	2	0.0137	0.0158	0.0161	0.0152	0.0013
	4	0.0176	0.0143	0.0143	0.0154	0.0019
	6	0.0166	0.0172	0.0179	0.0172	0.0007
40°C	0	0.0160	0.0140	0.0150	0.0150	0.0010
	2	0.0158	0.0169	0.0156	0.0161	0.0007
	4	0.0175	0.0177	0.0178	0.0177	0.0001
	6	0.0178	0.0180	0.0182	0.0180	0.0002

Table 23 The relative percentage of IC_{50} values for DPPH radical scavenging activity of the extract kept in various conditions

Storage condition	Time (Month)	% Relative to time zero				
		N1	N2	N3	Average	SD
4 °C	2	106.67	105.33	105.33	105.78	0.7698
	4	110.67	104.67	104.67	106.67	3.4641
	6	113.33	117.33	113.33	114.67	2.3094
RT	2	91.33	105.33	107.33	101.33	8.7178
	4	117.33	95.33	95.33	102.67	12.7017
	6	110.67	114.67	119.33	114.89	4.3376
40 °C	2	105.33	112.67	104.00	107.33	4.6667
	4	116.80	118.09	118.66	117.85	0.9559
	6	118.67	120.00	121.33	120.00	1.3333

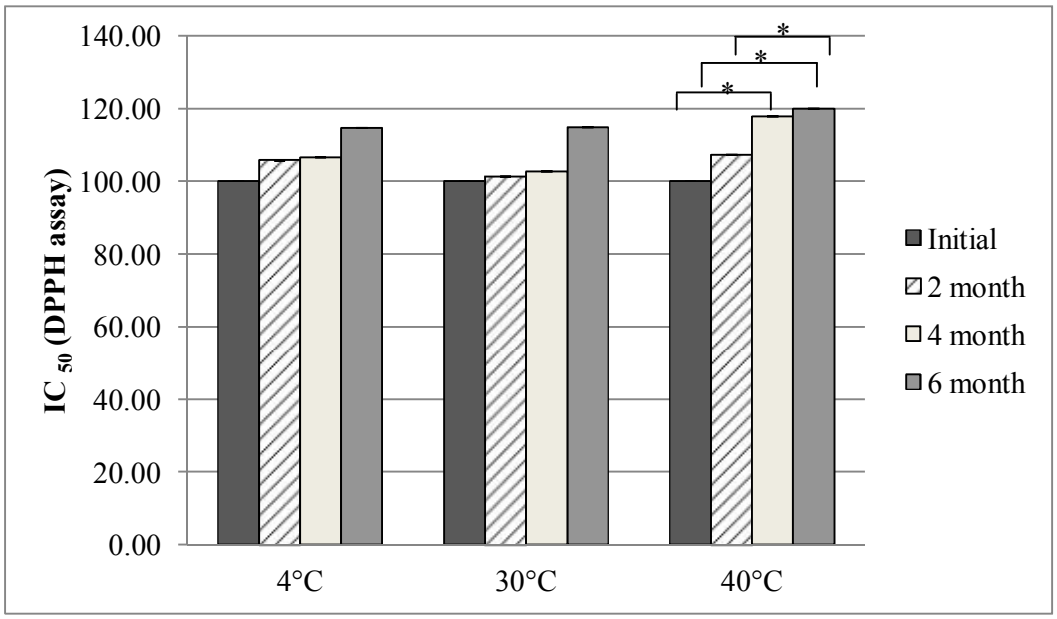


Figure 46 The comparison of the relative percentage of IC_{50} values for DPPH radical scavenging activity of the extract in various conditions. Plots were mean ± SD (n=3), * means significantly different between group (p-value < 0.05)

For the biological stability evaluation of the extract, it was found that there was insignificantly changing in DPPH radical scavenging activity of the extract at 4°C and room temperature (30°C). There was, however, a decrease in antioxidant activity of the extract after 4 and 6 months of storage at 40 °C. The result was in accordance to the chemical stability study of the extract. This finding may indicate that scopoletin in the extract degraded due to high temperature resulting in the lower in antioxidant activity of the extract. This result suggests the storage condition for the extract at room temperature (30°C) or below.

Part 3: Cytotoxicity evaluation for *Morinda citrifolia* extracts

MTT assay is now widely used to quantitate cell proliferation and cytotoxicity of various substance as well as pharmaceutical compounds. The method measures the reduction of the MTT to colored formazan product by the succinate-tetrazolium reductase system, which occurs in the mitochondria of living cells (Phan Toan Thang et al., 2000; Nogueira et al., 2010). The procedures used in this study were based on the method of Mosmann, 1983 with minor modifications by replacing DMSO with 100 µL of 0.04N HCl in isopropanol to dissolve the purple blue formazan crystal.

Human cultured keratinocyte was selected due to its widely use as cell line model for cytotoxicity testing in topical pharmaceutical formulation. Previous studies revealed the good correlation between *in vitro* irritancy test using cultured keratinocyte and the *in vivo* skin irritation model for investigating skin irritancy from surfactant used in topical formulation (Benassi et al., 2003; Osborne and Perkins, 1994; Wilhelm, Bottjer, and Siegers 2001). In order to evaluate the level of harmfulness of the extracts on skin cells, the cytotoxicity effect of the extract was, therefore, determined using the MTT assay in the immortalized keratinocytes (HaCaT) cells.

The cytotoxicity of the extract at different concentrations was evaluated on HaCaT cell for 12 hours which is an average application-time for cosmetic product. The relative percentages of cell viability of the cell treated with extract compared to the untreated control were calculated and shown in Table 24 and Figure 47.

Table 24 The percentage of HaCaT cell viability after to 12 hours incubation with various concentrations of the extract

Concentration	%Relative cell viability				
(μg/mL)	n1	n2	n3	Average	SD
100	103.14	100.61	92.33	98.69	5.66
200	100.66	94.34	96.28	97.09	3.24
400	108.29	99.60	95.27	101.06	6.63
600	107.96	95.29	93.54	98.93	7.87
800	108.28	94.61	84.48	95.79	11.94
1000	113.61	98.37	86.48	99.49	13.60
1200	105.63	100.44	78.68	94.91	14.30
1400	72.59	70.93	66.01	69.84*	3.42
1600	43.57	45.15	58.47	49.06*	8.19
1800	25.31	26.01	38.49	29.94*	7.42
2000	20.52	21.05	36.57	26.05*	9.12

* significant different versus non-treated control ($P < 0.05$).

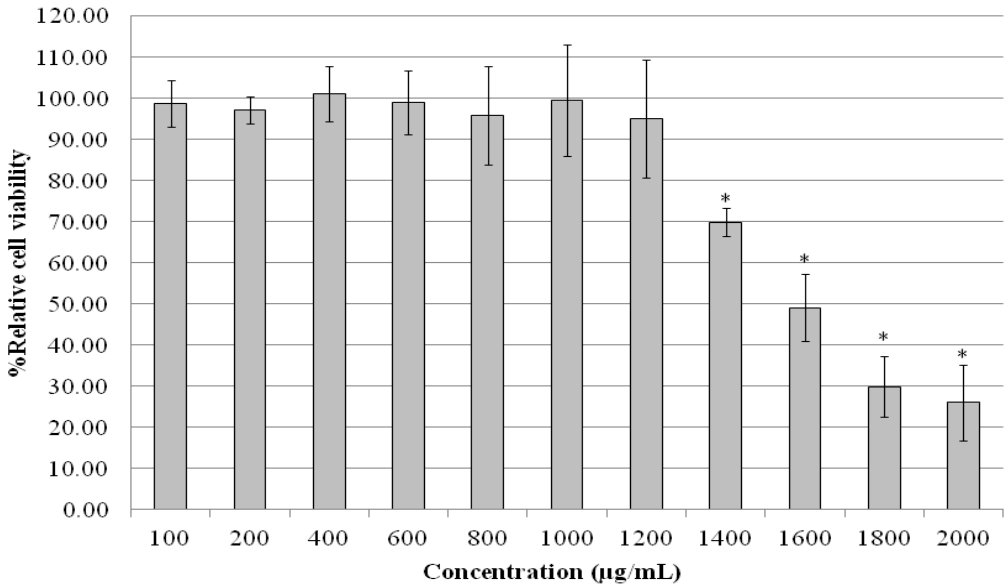


Figure 47 The cytotoxicity effect to HaCaT cells after 12 hours incubation of the extract at different concentrations. Values were represented as relative cell viability compared to control cells and plots were mean ± SD. (n=3). * significant different versus non-treated control ($P < 0.05$).

The results revealed that the remaining HaCaT cell viability were 98.96 ± 5.66%, 97.09 ± 3.24%, 101.06 ± 6.63%, 98.93 ± 7.87%, 95.79 ± 11.94%, 99.49 ± 13.60%, and 94.91 ± 14.30% after 12 hours incubation with the extract at concentration of 100, 200, 400, 600, 800, 1000, and 1200 µg/mL, respectively. The data suggested insignificant alteration in cell viability (Figure 47). However, the percentage of cell viability was significantly decreased at 1400 µg/mL, and reached approximately 50% at 1600 µg/mL (69.84 ± 3.42%, and 49.06 ± 8.19%, respectively). As the non-toxic dose determined by the maximum concentration that shown 90-100 % relative cell viability (Teepe et al., 1993), nontoxic dose after 12 hours incubation was found to be at 1200 µg/ml. By increasing the concentration of the extract, the cell viability was reduced in concentration-dependent manner. These results were in agreement with Akihisa et al., 2010 who reported non-toxic effect of the ethyl acetate soluble fraction from a methanol extract of *Morinda citrifolia* fruits to the B16 murine melanoma cells investigated by MTT assay (92.5 ± 2.33 % of cell count) at 100 µg/mL. It could be concluded that there was no cytotoxicity effect of this extract at low concentration.

In order to achieve the antioxidant and, especially the whitening effect of the extract, its concentration reached the active site should be adequate (Manosroi, et al., 2011). The pre-determined concentration for topical application would be roughly 10 times to the 50% effective concentration (IC_{50}) to account for the loss of active compound during application. The result has shown that the non-toxic dose tested by MTT assay was 1.2 mg/mL. As a result, the extract would be further incorporated in the nanoemulsions at concentration of 12 mg/mL which was 10 times to the non-toxic dose. This concentration was also equal to 10 times of the IC_{50} for anti-tyrosinase assay (1.201 mg/mL) and more than 100 times to the IC_{50} for antioxidant assay (0.014 mg/mL). This result revealed that the extract might exhibit great antioxidant with moderate anti-tyrosinase activities.

Nevertheless, the cytotoxicity testing of this extract should be confirmed on other types of cell such as melanocytes and fibroblast which were also commonly used in toxicity testing for topical formulation. Moreover, the cellular damage could occur in several pathways and can be measured by various aspects such as metabolic activity and plasma membrane integrity. The cytotoxicity of the compound should also be performed by different types of assay in order to support these toxicological studies.

Part 4: Formulation of nanoemulsions containing *Morinda citrifolia* extract and its stability evaluation

4.1 Selection of oil

The preliminary study was screened for the clear and homogeneous solution of 12 mg/mL of extract in various oils (caprylic/capric triglyceride, mineral oil, and jojoba oil). It was found that, no oil in this study could completely dissolve the extract at this concentration. However, when the concentration of the extract was increased to 50 mg/mL, caprylic/capric triglyceride gave less precipitation and provided most intense in color of the solute part. The photograph of the extract at 12 mg/mL and 50 mg/mL in different oil kept at room temperature (30±2°C) for 48 hours were illustrated in Figure 48 and 49, respectively.

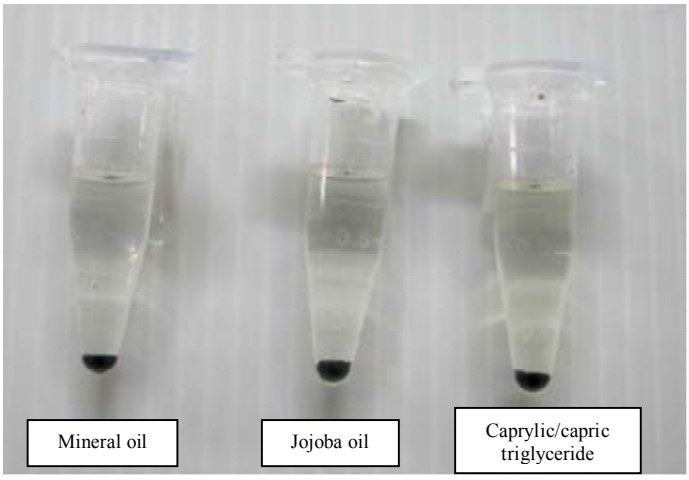


Figure 48 The photograph of the extract at 12 mg/mL in different oil

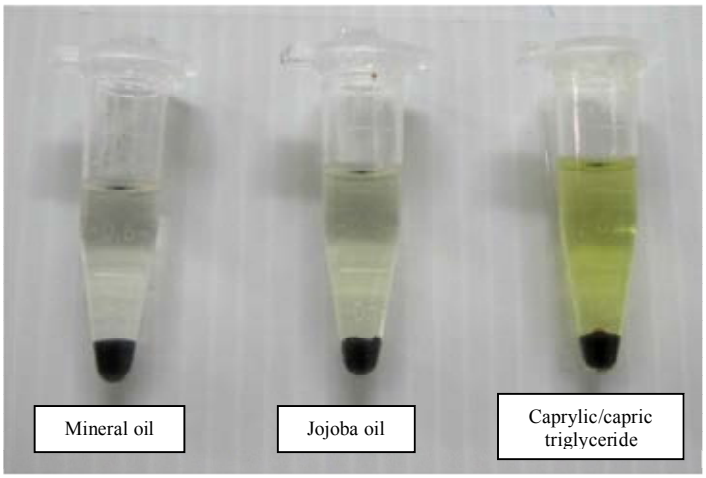


Figure 49 The photograph of the extract at 50 mg/mL in different oil

The screening data indicated that extract seem to be more soluble in caprylic/capric triglyceride than in mineral oil and jojoba oil. This result might be due to the fact that the extract with moderate polarity was likely to dissolve in small-medium chain hydrocarbon with medium molar volume oil such as caprylic/capric triglyceride which is more polar than long-chain hydrocarbon (mineral oil) and ester of long chain natural oil (jojoba oil). Additionally, there were reports from Bouchemal et al., 2004, Sadurni et al., 2005, and Hatanaka et al., 2010 that medium chain triglyceride such as caprylic/capric triglyceride forms the stable nanoemulsions

and possessed biocompatibility and safety for topical use therefore it was selected for the oil in this study.

4.2 Selection of surfactant

Emulsifiers used in the formulation can also impact nanoemulsion formation and stability. In this study, the non-ionic surfactants blending were selected based on their structure and HLB value. The surfactant system should consist of the emulsifying agents having higher and lower HLB than that of the oil used in the formulation. The HLB value of the emulsifier combination matches the HLB of the oil, lead to the formation of stable nanoemulsions (Shinoda et al., 1987).

As important as HLB value, the structure of non-ionic surfactant blend also plays a major role in nanoemulsion formation. Bergstrom and Eriksson, 2000, reported that the synergistic effects in mixed binary non-ionic surfactant system were due to the entropic free energy contributions related to the surfactant head groups. The two non-ionic the head groups with different in sizes showed a large synergistic effect in emulsion formation. For these reasons, the combination of TWEEN® and SPAN® was selected as binary non-ionic surfactants in this study. The emulsifier systems chosen were listed in Table 26.

TWEEN®80 was incorporated as a single surfactant since there were studies successfully produced very small size nanoemulsions using TWEEN®80 (Friedman, Schwarz, and Weisspapir, 1994; Shafiq et al., 2007, Shakeel et al., 2008; Wooster, Golding, and Sanguansri, 2008, and Ziani et al., 2010). TWEEN®80 was selected in this study to see the effect of single surfactant with and without the co emulsifying effect of cremophor® RH-40.

Kong, Chen, and Park, 2011, also studied the optimum composition of TWEEN® and SPAN® surfactant mixture in various combinations. It was suggested that the pair of surfactant mixture that contained the largest difference in hydrocarbon chain length of hydrophobic portion would produce the nanoemulsions with smallest droplet size. In contrast, the larger droplet sizes were obtained in the surfactant systems that contained equal hydrocarbon chain length such as SPAN®20-TWEEN®20, SPAN®60-TWEEN®60, and SPAN®80-TWEEN®80. As a consequence, the combination of SPAN®20 and TWEEN®80, as well as SPAN®80 and TWEEN®20 were used in this study.

Taken together, the TWEEN®80 (T80), a combination of SPAN®20 with TWEEN®80 (S20-T80), and SPAN®80 with TWEEN®20 (S80-T20) were selected as the surfactant system in this experiment. The emulsifier blend was adjusted to satisfy the HLB value equal to 11 which was the required HLB for caprylic/capric triglyceride as the oil phase selected in this study. In addition, cremophor® RH-40 may be added in order to improve solubility of the extract in oil phase. All the HLB values of the surfactants used in this study and the required HLB of the oil were shown in Table 25.

Table 25 The required HLB of oil and the HLB values surfactants at 25°C

Ingredients	**Required HLB**	**HLB**
Caprylic/capric TG	11	
Polysorbate 20 (TWEEN®20)		16.7
Polysorbate 80 (TWEEN®80)		15.0
Sorbitan monolaurate (SPAN®20)		8.6
Sorbitan monooleate (SPAN®80)		4.8
Cremophor® RH-40		12.0– 14.0

4.3 Preparation of extract-free nanoemulsions

In summary, the compositions of nanoemulsions were selected as following;

- Caprylic/capric triglyceride was selected as the oil in the formulation. Cremophor® RH-40 might be included to enhance the solubility of the selected extract. The ratio of the oil and solublizer mixture was varied from 10:0, 9:1, 8:2, 7:3 and 6:4. The concentration of the mixture was adjusted from 5-10%.
- TWEEN®20, TWEEN®80, SPAN®20, and SPAN®80 were used as emulsifying agents with the concentration of mixed surfactant was varied as 5, 10 and 15%.

The percentage of each surfactant and total of the combination are shown in Table 26 and the mass percentages of the ingredients used in the formulation were shown in Table 27, 28, 29, 30, and 31.

The effects of ratio and amount of oil/solubilizer as well as types and amount of surfactant were evaluated by observing for phase separation and particle size analysis of the prepared formulations after keeping at room temperature (30±2°C) for 1 week and 4 weeks. The results are shown in Table 32.

Table 26 The mass percentages of the surfactant combination using in the formulation

Surfactant	Percentage of surfactant used in the formulation						
	1	2	3	4	5	6	7
SPAN®20					3.125	6.250	9.375
SPAN®80			2.300	4.600			
TWEEN®20			2.700	5.400			
TWEEN®80	5.000	10.000			1.875	3.750	5.625
Total	5.000	10.000	5.000	100.000	5.000	10.000	15.000

Table 27 The mass percentages of the ingredients using oil/solubilizer ratio as 10:0 at 5 and 10% with different types and concentrations of surfactant blend

A = oil : solubilizer (10:0)

Ingredients	Formulation (%w/w)									
	A1 T80,05	A2 T80,10	A3 S80T20,05	A4 S80T20,10	A5 S20T80,05	A6 S20T80,10	A7 S20T80,15	A8 S20T80,05	A9 S20T80,10	A10 S20T80,15
Oil phase										
CCTG	5.000	5.000	5.000	5.000	5.000	5.000	5.000	10.000	10.000	10.000
Cremophor RH-40										
SPAN20					3.125	6.250	9.375	3.125	6.250	9.375
SPAN80			2.300	4.600						
Water phase										
TWEEN20			2.700	5.400						
TWEEN80	5.000	10.000			1.875	3.750	5.625	1.875	3.750	5.625
Paraben concentrate	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Water	89.000	84.000	89.000	84.000	89.000	84.000	79.000	84.000	79.000	74.000
Total	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000

S20 = SPAN20, T20 = TWEEN20, S80 = SPAN80, and T80 = TWEEN80; number after comma is the concentration of the surfactant blend

Table 28 The mass percentages of the ingredients using oil/solubilizer ratio as 9:1 at 5 and 10% with different types and concentrations of surfactant blend

B = oil : solubilizer (9:1)

Ingredients	Formulation (%w/w)									
	B1 T80,05	B2 T80,10	B3 S80T20,05	B4 S80T20,10	B5 S20T80,05	B6 S20T80,10	B7 S20T80,15	B8 S20T80,05	B9 S20T80,10	B10 S20T80,15
Oil phase										
CCTG	4.500	4.500	4.500	4.500	4.500	4.500	4.500	9.000	9.000	9.000
Cremophor RH-40	0.500	0.500	0.500	0.500	0.500	0.500	0.500	1.000	1.000	1.000
SPAN20					3.125	6.250	9.375	3.125	6.250	9.375
SPAN80			2.300	4.600						
Water phase										
TWEEN20			2.700	5.400						
TWEEN80	5.000	10.000			1.875	3.750	5.625	1.875	3.750	5.625
Paraben concentrate	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Water	89.000	84.000	89.000	84.000	89.000	84.000	79.000	84.000	79.000	74.000
Total	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000

S20 = SPAN20, T20 = TWEEN20, S80 = SPAN80, and T80 = TWEEN80; number after comma is the concentration of the surfactant blend

Table 29 The mass percentages of the ingredients using oil/solubilizer ratio as 8:2 at 5 and 10% with different types and concentrations of surfactant blend

C = oil : solubilizer (8:2)

Ingredients	Formulation (%w/w)									
	C1 T80,05	C2 T80,10	C3 S80T20,05	C4 S80T20,10	C5 S20T80,05	C6 S20T80,10	C7 S20T80,15	C8 S20T80,05	C9 S20T80,10	C10 S20T80,15
Oil phase										
CCTG	4.000	4.000	4.000	4.000	4.000	4.000	4.000	8.000	8.000	8.000
Cremophor RH-40	1.000	1.000	1.000	1.000	1.000	1.000	1.000	2.000	2.000	2.000
SPAN20					3.125	6.250	9.375	3.125	6.250	9.375
SPAN80			2.300	4.600						
Water phase										
TWEEN20			2.700	5.400						
TWEEN80	5.000	10.000			1.875	3.750	5.625	1.875	3.750	5.625
Paraben concentrate	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Water	89.000	84.000	89.000	84.000	89.000	84.000	79.000	84.000	79.000	74.000
Total	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000

S20 = SPAN20, T20 = TWEEN20, S80 = SPAN80, and T80 = TWEEN80; number after comma is the concentration of the surfactant blend

Table 30 The mass percentages of the ingredients using oil/solubilizer ratio as 7:3 at 5 and 10% with different types and concentrations of surfactant blend

D = oil : solubilizer (7: 3)

Ingredients	Formulation (%w/w)									
	D1 T80,05	D2 T80,10	D3 S80T20,05	D4 S80T20,10	D5 S20T80,05	D6 S20T80,10	D7 S20T80,15	D8 S20T80,05	D9 S20T80,10	D10 S20T80,15
Oil phase										
CCTG	3.500	3.500	3.500	3.500	3.500	3.500	3.500	7.000	7.000	7.000
Cremophor RH-40	1.500	1.500	1.500	1.500	1.500	1.500	1.500	3.000	3.000	3.000
SPAN20					3.125	6.250	9.375	3.125	6.250	9.375
SPAN80			2.300	4.600						
Water phase										
TWEEN20			2.700	5.400						
TWEEN80	5.000	10.000			1.875	3.750	5.625	1.875	3.750	5.625
Paraben concentrate	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Water	89.000	84.000	89.000	84.000	89.000	84.000	79.000	84.000	79.000	74.000
Total	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000

S20 = SPAN20, T20 = TWEEN20, S80 = SPAN80, and T80 = TWEEN80; number after comma is the concentration of the surfactant blend

Table 31 The mass percentages of the ingredients using oil/solubilizer ratio as 6:4 at 5 and 10% with different types and concentrations of surfactant blend

E = oil : solubilizer (6:4)

Ingredients	Formulation (%w/w)									
	E1 T80,05	E2 T80,10	E3 S80T20,05	E4 S80T20,10	E5 S20T80,05	E6 S20T80,10	E7 S20T80,15	E8 S20T80,05	E9 S20T80,10	E10 S20T80,15
Oil phase										
CCTG	3.000	3.000	3.000	3.000	3.000	3.000	3.000	6.000	6.000	6.000
Cremophor RH-40	2.000	2.000	2.000	2.000	2.000	2.000	2.000	4.000	4.000	4.000
SPAN20					3.125	6.250	9.375	3.125	6.250	9.375
SPAN80			2.300	4.600						
Water phase										
TWEEN20			2.700	5.400						
TWEEN80	5.000	10.000			1.875	3.750	5.625	1.875	3.750	5.625
Paraben concentrate	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Water	89.000	84.000	89.000	84.000	89.000	84.000	79.000	84.000	79.000	74.000
Total	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000

S20 = SPAN20, T20 = TWEEN20, S80 = SPAN80, and T80 = TWEEN80; number after comma is the concentration of the surfactant blend

Table 32 The visual observation for phase separation and particle sizes of the pre-screening formulations after 1 and 4 weeks

oil/solubilizer (10:0)	A1 T80,05	A2 T80,10	A3 S80T20,05	A4 S80T20,10	A5 S20T80,05	A6 S20T80,10	A7 S20T80,15	A8 S20T80,05	A9 S20T80,10	A10 S20T80,15
stable emulsion	x	x	x	x	x	x	x	x	x	x
particle size (nm) 1 week	-	-	-	-	-	-	-	-	-	-
particle size (nm) 4 weeks	-	-	-	-	-	-	-	-	-	-
oil/solubilizer (9:1)	B1 T80,05	B2 T80,10	B3 S80T20,05	B4 S80T20,10	B5 S20T80,05	B6 S20T80,10	B7 S20T80,15	B8 S20T80,05	B9 S20T80,10	B10 S20T80,15
stable emulsion	x	x	x	x	x	x	x	✓	✓	✓
Appearance	x	x	x	x	x	x	x	opaque	opaque	opaque
particle size (nm) 1 week	-	-	-	-	-	-	-	170.80	92.65	249.00
particle size (nm) 4 weeks	-	-	-	-	-	-	-	174.90	111.40	258.90
oil/solubilizer (8:2)	C1 T80,05	C2 T80,10	C3 S80T20,05	C4 S80T20,10	C5 S20T80,05	C6 S20T80,10	C7 S20T80,15	C8 S20T80,05	C9 S20T80,10	C10 S20T80,15
stable emulsion	x	x	x	✓	✓	✓	✓	✓	✓	✓
Appearance	x	x	x	opaque	opaque	opaque	opaque	opaque	translucent	opaque
particle size (nm) 1 week	-	-	-	128.80	140.40	111.90	245.60	96.30	28. 85	105. 40
particle size (nm) 4 weeks	-	-	-	151.40	146.60	130.00	269.40	101.00	58.91	132.20
oil/solubilizer (7:3)	D1 T80,05	D2 T80,10	D3 S80T20,05	D4 S80T20,10	D5 S20T80,05	D6 S20T80,10	D7 S20T80,15	D8 S20T80,05	D9 S20T80,10	D10 S20T80,15
stable emulsion	x	x	✓	✓	✓	✓	✓	✓	✓	✓
Appearance	x	x	opaque	opaque	translucent	opaque	opaque	opaque	translucent	opaque
particle size (nm) 1 week	-	-	191.30	208.30	23.17	153.70	288.70	60.38	21.39	117. 10
particle size (nm) 4 weeks	-	-	x	x	25.42	175.60	315.60	63.36	22.25	117.80

Table 32 The visual observation for phase separation and particle sizes of the pre-screening formulations after 1 and 4 weeks (continued)

oil/solubilizer (6:4)	E1 T80,05	E2 T80,10	E3 S80T20,05	E4 S80T20,10	E5 S20T80,05	E6 S20T80,10	E7 S20T80,15	E8 S20T80,05	E9 S20T80,10	E10 S20T80,15
stable emulsion	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Appearance	opaque	opaque	opaque	opaque	translucent	opaque	opaque	translucent	translucent	opaque
particle size (nm) 1 week	161.30	151.20	87.01	173.70	22. 13	98.45	195.60	40.29	18.85	124.40
particle size (nm) 4 week	165.80	159.50	88.54	177.00	22.66	100.90	196.30	42.40	20.43	127.50

S20 = SPAN20, T20 = TWEEN20, S80 = SPAN80, and T80 = TWEEN80; number after comma is the concentration of the surfactant blend

By concerning the effect of cremophor® RH-40, it was found that the phase separation occurred in all systems that contained no cremophor® RH-40 (oil/solubilizer ratio =10:0). When increasing cremophor® RH-40 (oil/solubilizer ratio = 9:1), the nanoemulsions were formed in the system containing 10% S20-T80. The results indicated that S20-T80 surfactant system could facilitate nanoemulsion formation. In comparison to other surfactant systems, nanoemulsions were formed only when increasing cremophor® RH-40 concentration or decreasing the oil concentration. It could be suggested that, in addition to the solubilization enhancer effect, cremophor® RH-40 could be used as co-emulsifying agent to facilitate stable nanoemulsion formation. This may be taken into an account of the structure of cremophor® RH-40 that contained fatty acid ester of glycerol and polyethylene glycol that solubilized and emulsified the oil.

Concerning emulsifier(s) used in the system, it was found that the system that contained only T80 produced unstable emulsions. This result informed that using the hydrophilic surfactant alone without combination with the hydrophobic surfactant might not meet the HLB concept which could not facilitate formation of the stable emulsions. It was in agreement with the finding of Scamehorn, 1986 who suggested that the surfactant mixture provide better performance than a single one. However, nanoemulsions can be formed by using T80 as single surfactant when increasing the concentration of cremophor® RH-40 to the oil/solubilizer ratio of 6:4 (formulation E1 T80,05 and E1 T80,10). This might confirm the co-emulsifying effect of cremophor® RH-40 which facilitate nanoemulsion formation in this study.

The surfactant blend of S20-T80 in the presence of cremophor® RH-40 produced more stable emulsions with smaller droplet size than the system consisted of S80-T20. This result might be explained by the following assumption. For oil in water nanoemulsions, the surfactant molecule would presumable orient at the oil/water interface in such the way that the lipophilic chain of the emulsifier locates into the oil phase and the hydrophilic ring lies on the aqueous side. As a result, the structural similarity between the hydrocarbon moieties of the oil phase and the surfactant is essential for emulsion formation (Gallapalli, and Sheth, 1999). Gallapalli, and Sheth, 1999, also suggested that by keeping the difference in HLB value of the two emulsifiers to a minimum would reduce the droplet size and improve the stability of

nanoemulsions. Combination of S20-T80 used in the study offers smaller value in HLB difference and provides better fit to form more rigid interfacial film. As a result, it produced more stable nanoemulsions with smaller in droplet size. This observation also in consistence with the previous report of Wang et al., 2009 and Kong et al., 2011, who found TWEEN®80 and SPAN®20 produced the smallest droplet size over other surfactant combinations. They suggested that the high difference in hydrocarbon chain length between TWEEN® and SPAN® would facilitate the orientation and the curvature of the surfactant shell to form very small. Moreover, the presence of double bond in the chain of TWEEN®80 which penetrated between the absorbed hydrophobic surfactant would facilitate loosely arrangement of the surfactant at the interface. This would favor the formation of smaller droplet nanoemulsions.

In addition to the required HLB and surfactant composition, the surfactant concentration also plays an important role in nanoemulsions formation. When comparing the concentration of the surfactant used in the formulation for each oil/solubilizer ratio, it was found that the smallest sizes were obtained when the concentration of the oil/solubilizer was equal to the concentration of the surfactant blend. The droplet sizes of the formulation C5(S20T80,05), D5(S20T80,05), and E5(S20T80,05) were 146.60 > 25.42 > 22.66 nm, and the droplet sizes of the formulation C9(S20T80,10), D9 (S20T80, 10), and E9 (S20T80, 10) were 58.91 > 22.25 > 20.43 nm, respectively. These nanoemulsions provided smallest droplet size compared to the formulation with surfactant concentration lower and higher than concentration of oil phase. Similar results have been reported in Henry *et al*, 2009 who investigated the effect of the surfactant on the formation of decane in water nanoemulsions. It was found that the droplet size decreased with increasing the surfactant concentration. Nevertheless, this effect was limited when the surfactant concentration was increased up to 15 mM. They suggested that the continuous increasing in surfactant concentration would reduce the droplet break up. This result was in accordance with the study of Wang et al., 2009, in which the droplet size increased with increasing the emulsifier concentration. They reported that the highly viscous liquid crystalline phase due to high concentration of emulsifying agents provided a high rigidity of the interface which required more energy to produce the

fine emulsion droplets. The insufficient mechanical energy, therefore, resulted in the increase in droplet size.

By focusing only in S20-T80 system which concentration of oil/solubilizer and surfactant were 5%:5% and 10%:10% (w/w), it was found that the droplet sizes of nanoemulsions were decreased when cremophor® RH-40 was increased in the system. This result was then confirmed that cremophor® RH-40 was important for the formulation of nanoemulsions in this study.

In summary, the formulation D5(S20T80,05), D9(S20T80,10), E5(S20T80,05), and E9(S20T80,10) which showed desirable sizes were selected to prepare nanoemulsions containing the extract for further studies. Their pictures are shown in Figure 50.

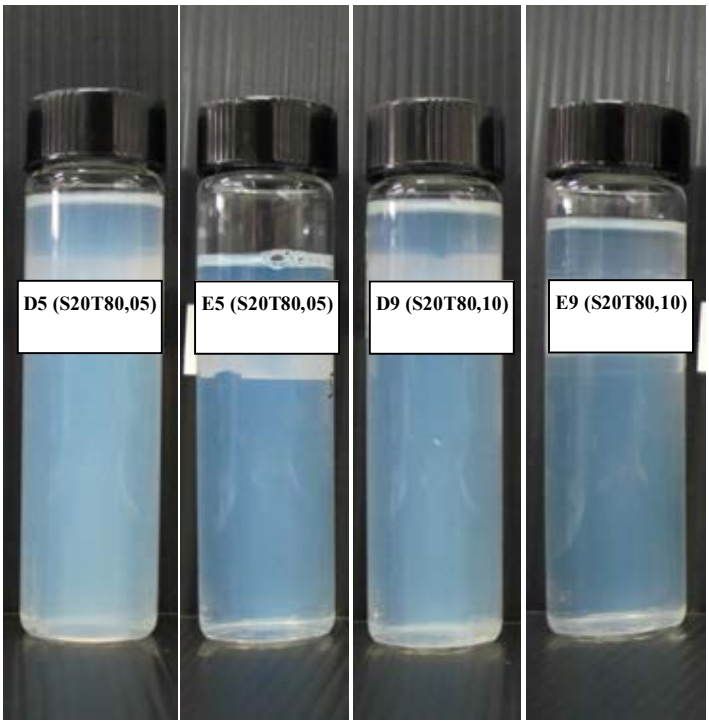


Figure 50 the picture of the extract free nanoemulsions

4.4 Preparation of *Morinda citrifolia* extract loaded nanoemulsions

- Based on the anti-tyrosinase and antioxidant activities in consideration on cytotoxicity mentioned in section 1 and 3. Twelve mg/mL of ethyl acetate soluble fraction of *Morinda citrifolia* extract was selected as the loading dose for development of nanoemulsions.

The 1.2 g of extract was pre-incorporated to the oil/solubilizer phase to prepared 100 mL of nanoemulsions. The preparations were followed according to formulation D5(S20T80,05), D9(S20T80,10), E5(S20T80,05), and E9(S20T80,05). The physical appearance and particle size of the prepared formulation were investigated. The physical stability and chemical stability were also determined.

All of the obtained formulations were homogeneous with brown color. D5(S20T80,05) and E5(S20T80,05) were translucent fluid dispersion while D9(S20T80,10) and E9(S20T80,10) were transparent. Figure 51 demonstrated the picture of the extract loaded nanoemulsions.

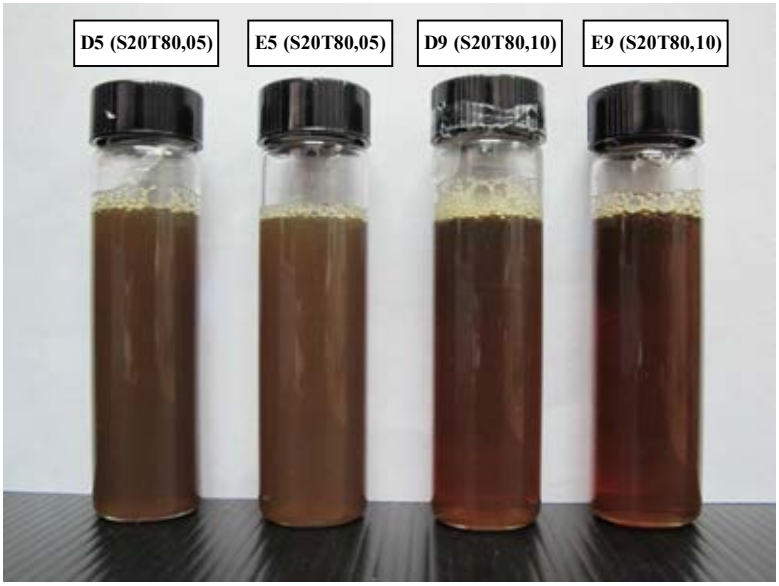


Figure 51 the picture of the extract loaded nanoemulsions.

The pH profiles of the formulations were characterized by pH meter and the droplet size and size distribution were characterized by Zetasizer. The data were shown in Table 33.

Table 33 The pH profile, droplet size, and size distribution of the extract-loaded nanoemulsions

Sample	pH		Size		Polydisperse Index	
	Mean	SD	Mean	SD	Mean	SD
D5(S20T80,05)	3.53	0.0153	29.00	0.101	0.044	0.006
E5(S20T80,05)	3.44	0.0252	33.48	0.180	0.237	0.005
D9(S20T80,10)	3.50	0.0153	32.29	0.035	0.127	0.001
E9(S20T80,10)	3.43	0.0361	25.84	0.311	0.048	0.020

It was found that all formulations possessed similar pH (3.43-3.53) which was insignificantly different. All formulations showed no phase separation and no precipitation of the extract observed after 1 week and 4 weeks of preparation. Nevertheless, formulation E9(S20T80,10) possessed the smallest droplet size (25.84 ± 0.311 nm). As a result, it was selected for further stability evaluation and for *in vitro* release and permeation studies.

4.5 Stability evaluation

4.5.1 Physical stability evaluation

4.5.1.1 Appearance

The extract-loaded nanoemulsions prepared by the formulation E9(S20T80,10) possessed good appearance and stability. No phase separation or change in color were detected during storage at 4°C, at room temperature (30°C), and at 40°C for 3 months and after 8 heating-cooling cycles. The pictures are shown in Figure 52.

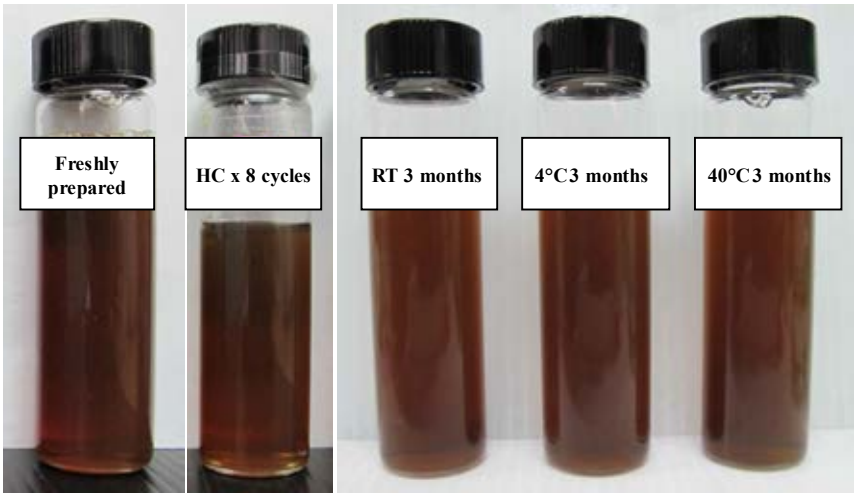


Figure 52 The pictures of the formulation after freshly prepared and after complete the stability test of the extract-loaded nanoemulsion formulation E9(S20T80,10).

4.5.1.2 pH

The pH of the formulation was also investigated using pH meter. The data shown in Table 34 revealed that there were insignificant changes in pH profiles of the extract-loaded nanoemulsions after heating-cooling test and after stability study.

Table 34 The pH profiles of the extract-loaded nanoemulsions after heating-cooling test and stability study

Sample	Time	pH	
		mean	SD
Initial	0	3.50	0.0153
HC condition	8 cycles	3.44	0.0115
4°C	1	3.48	0.0100
	2	3.51	0.0100
	3	3.47	0.0100
RT	1	3.46	0.0493
	2	3.47	0.0451
	3	3.49	0.0265
40°C	1	3.46	0.0153
	2	3.47	0.0173
	3	3.48	0.0173

4.5.1.3 Particle size analysis

The particle size and the polydisperse index are the most representative parameters for the physical stability study of nanoemulsions. The particle sizes and their distributions of the extract-loaded nanoemulsions prepared by formulation E9(S20T80,10) were then determined by a Zetasizer. The mean diameters of the formulation in all condition were in nanometer range and polydispesity index of these particles were less than 0.2, which demonstrated narrow size distribution. Table 35 showed the size analysis of extract-loaded nanoemulsions at initial time point, after 8-heating-cooling cycles, and after storage in various temperatures. Figure 53 illustrated the relationship of the size of extract-loaded formulations in various conditions comparing to initial time.

Table 35 The size analysis of extract-loaded nanoemulsions at time initial, after heating-cooling test and after stability test at any time interval

Sample	Time	Size (nm)		PdI	
		Mean	SD	Mean	SD
Initial	0	25.84	0.311	0.048	0.020
HC condition	8 cycles	25.82	0.325	0.061	0.008
4°C	1	25.43	0.187	0.047	0.013
	2	25.60	0.271	0.035	0.006
	3	25.93	0.313	0.041	0.021
RT	1	25.76	0.126	0.042	0.008
	2	26.27	0.236	0.049	0.006
	3	26.45	0.098	0.037	0.005
40°C	1	26.78*	0.125	0.053	0.004
	2	27.04*	0.370	0.045	0.014
	3	28.57*	0.265	0.073	0.021

*= significant difference from initial ($P < 0.05$)

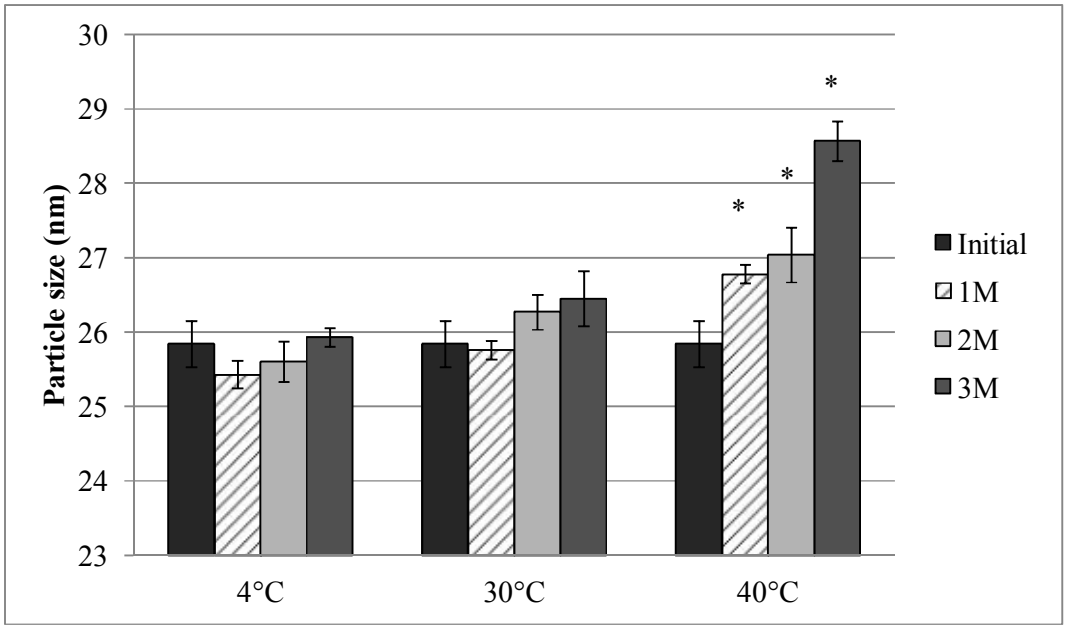


Figure 53 The size after stability test at any time interval comparing to initial, plots were mean ± SD. (n=3). * $P < 0.05$ versus non-treated control.

The data showed insignificant changes in particle size of the formulation kept at 4°C and at room temperature (30°C) compared to time zero. The particle size of the formulation being kept at 40°C was, however, gradually increased. On the other hand, there was no observation of any change in size distribution at any time intervals compared to the average sizes at initial.

This result indicated that the extract-loaded nanoemulsions displayed good stability for at least 3 months at storage temperature of 4°C and room temperature (30°C). It might be due to the optimal compositions of the oil and surfactants that formed the stable system in the formulation. The result also indicated that the extract at the effective concentration can be loaded into the nanoemulsions and did not affect the stability of the formulation. Moreover, the result guided the optimal storage condition for the extract-loaded nanoemulsions in this study. It shall be stored below or equal to 30°C as indicated by the unchanged in mean droplet size and polydisperse index. High temperature that might increase the kinetic energy of the system would let Ostwald ripening and/or coalescence to occur, resulting in the slightly increase in droplet size. However, the particle size was still in the nano-size range and the change in physical appearance could not be observed. It can be

concluded that extract- loaded nanoemulsions prepared by formulation E9(S20T80,10) had physically stable for 3 months of storage at 4°C, 40°C and after complete 8-heating-cooling cycles.

4.5.2 Chemical stability evaluation

The percentage of scopoletin in the formulations after storage in various temperatures at any particular time point were calculated and compared to that was in the formulation at time zero to determine the biochemical stability of the active compound in the formulation.

The quantification of scopoletin in the formulation was analyzed using HPLC method mentions in section 2. To determine the interference from the blank formulation, the chromatogram of the extract-free nanoemulsions was compared to the chromatogram of the extract-loaded nanoemulsions. The results were displayed in Figure 54 (a) and (b).

The chromatogram showed there was no interference from the extract-free nanoemulsions at the retention time of the scopoletin. This HPLC system was used to determine the stability of biological active scopoletin in the formulation.

The extract-loaded nanoemulsions was kept at 4°C, room temperature (30°C) and 40 °C and was determined for the remaining scopoletin in the formulation at 0, 1, 2, and 3 month of storage. Percentages of scopoletin in the formulation were calculated. The data are shown in Table 36. The comparison of the amount of scopoletin remaining in the formulation at any particular time point and to that of time zero was demonstrated in Figure 55.

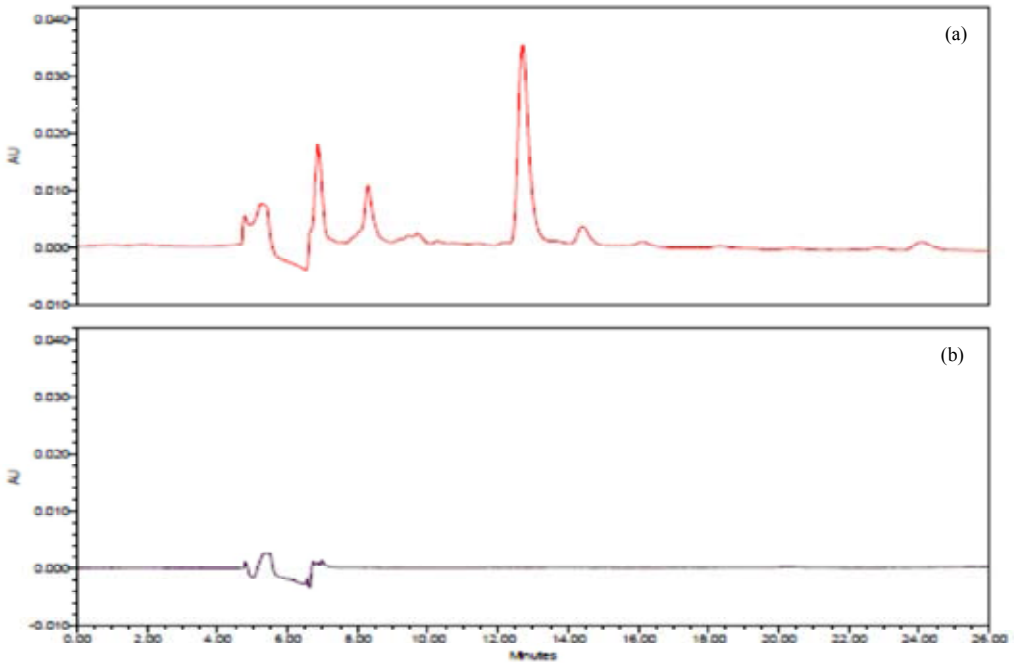


Figure 54 The chromatograms of (a) extract-loaded nanoemulsions and (b) extract-free nanoemulsions

Table 36 The analytical concentration of scopoletin in extract-loaded nanoemulsions calculated from calibration curve

Condition	Time (Month)	Analytical scopoletin (µg/mL)				
		N1	N2	N3	Average	SD
4°C	0	243.634	247.111	250.394	247.046	3.381
	1	248.175	246.062	251.917	248.718	2.965
	2	250.053	246.151	246.069	247.424	2.277
	3	244.932	248.286	243.978	245.732	2.263
RT	0	243.634	247.111	250.394	247.046	3.381
	1	250.448	251.171	246.599	249.406	2.458
	2	247.827	245.554	247.620	247.000	1.257
	3	245.018	246.434	244.002	245.151	1.222
40°C	0	243.634	247.111	250.394	247.046	3.381
	1	245.190	248.824	245.294	246.436	2.069
	2	246.634	246.154	246.462	246.417	0.243
	3	243.651	246.492	244.963	245.035	1.422

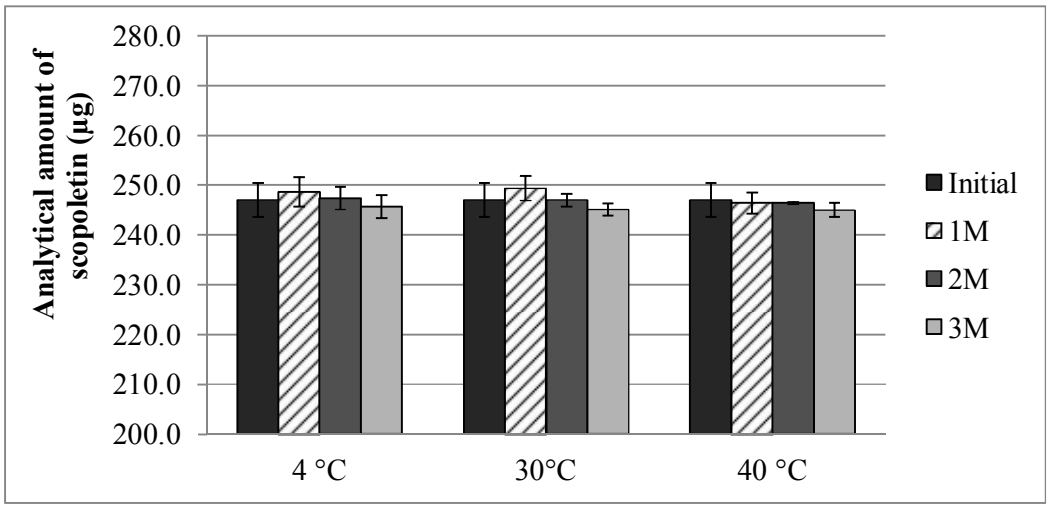


Figure 55 The comparison of scopoletin remaining in the formulation at any particular time point relative to the formulation at time zero. Plots were mean ± SD (n=3)

There was insignificant change in the amount of scopoletin in the extract-loaded nanoemulsions. The results indicated the biological active compound in the obtained nanoemulsions was stable at 4°C, room temperature (30°C) and 40 °C for at least 3 months of storage.

Part 5: *In Vitro* permeation study of nanoemulsions containing *Morinda citrifolia* extract

5.1 The analysis of biological active compound in permeation study

The percentage of scopoletin in the receptor fluid was determined using HPLC condition as reported in section 2. The chromatogram of 40% propylene glycol in phosphate buffer pH 7.4 (40% PG in PBS) and of phosphate buffer pH 7.4 (PBS) used as the receptor fluids in the release and permeation study, respectively, presented no interference peak at the retention time of the scopoletin (Figure 56 (a), (b), (c), and (d)).

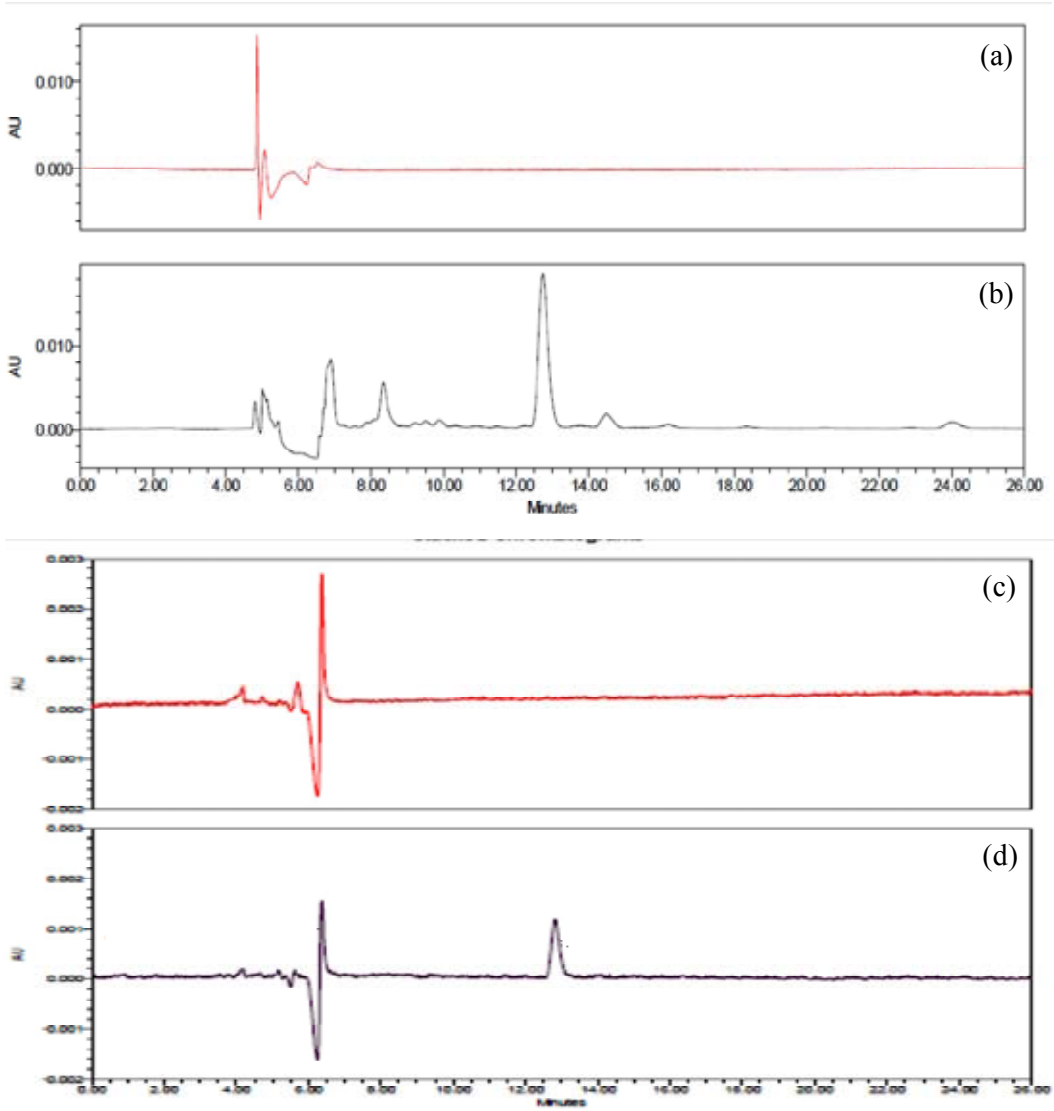


Figure 56 The chromatogram of (a) 40% PG in PBS pH7.4 and (b) extract in 40% PG in PBS pH7.4 (c) PBS pH7.4 and (d) extract in PBS pH7.4

Thus, the developed HPLC condition was used to quantify the biological active scopoletin in the receptor fluids from release and permeation studies.

5.2 *In vitro* release study of nanoemulsions containing *Morinda citrifolia* extract

The extract-loaded formulation E9(S20T80,10) which possessed good appearance and stability was selected for release study. The *in vitro* release study was performed and the amount of released biological active compound was analyzed by

HPLC method. The percentage of the accumulated active compound released at a particular time was calculated comparing to the initial amount. The release profiles of scopoletin from nanoemulsions and solution of extract are shown in Figure 57.

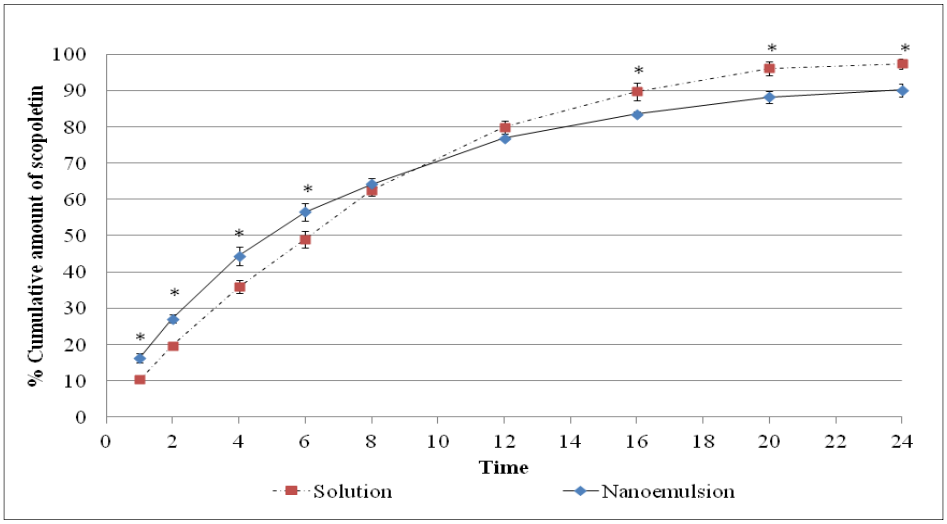


Figure 57 The release profiles of scopoletin from solution and nanoemulsions, the data are present as mean ± SD (n=3), * means significantly different between solution control and nanoemulsions ($P < 0.05$)

Control in the study was the solution of 240 mg/mL extract in 40% PG in PBS. At this concentration, the solution of extract exhibited the comparable saturation compared to 12 mg/mL extract-loaded nanoemulsions.

The amount of scopoletin released from the solution of 40% PG in PBS reached almost 100% within 24 hours. The similar pattern was also observed in the release profile of scopoletin from nanoemulsions in which scopoletin slowly released and reached about 90% within 24 hours. At the first 6 hour, the percent cumulative amount of the released scopoletin from nanoemulsion was found to be higher than that from the solution. The similar percent cumulative amount was observed at 8 and 12 hours, while the amount from nanoemulsion exhibited lower level than that from the solution after 16 hours. Nevertheless, it might be assumed that scopoletin was able to completely release from nanoemulsion without the effect of formulation compared to the 40% PG in PBS solution within 24 hours.

5.3 Skin permeation study of nanoemulsion containing *Morinda citrifolia* extract

The comparison of the skin permeation of scopoletin from nanoemulsion and conventional was examined. The conventional o/w emulsion was prepared using the same type of surfactant blend with the same concentration as nanoemulsion No. A9 (S20T80,10) but cremophor RH-40 was replaced with stearic acid in the conventional o/w emulsion as a stabilizer. The ingredients of nanoemulsions and conventional o/w emulsions were shown in Table 37.

Table 37 The mass percentages of the ingredients of nanoemulsions and conventional formulation used for permeation study and their average droplet sizes.

Ingredients	Nanoemulsions E9 (S20T80,10)	Conventional emulsions
Oil phase		
Capric/cpric triglyceride	6.000	10.000
Cremophor RH-40	4.000	
SPAN®20	6.250	6.250
Stearic acid		0.250
Water phase		
TWEEN®80	3.750	3.750
Paraben concentrate	1.000	1.000
Water	79.000	78.75
Total	100.000	100.000
Droplet sizes (nm)	25.84	353.1

240 mg/ml of extract in 40% PG in PBS solution with the same chemical driving force as nanoemulsions was used as control.

The permeation study was performed using the modified franz diffusion cell. The new born pig skin was chosen as the model membrane as its morphology and permeability are similar to human skin (Klang et al., 2010). The cumulative amounts of scopoletin permeated through the skin was quantified by HPLC and calculated as the amount of scpoletin permeated through the skin at predetermined time (appendix G). The amount of scopoletin detected in the receptor part after 48 hours, the amount of scopoletin detected in the donor part and in the skin and the mass balance are shown in Table 38 and Figure 58.

Table 38 The amount of scopoletin after 48 hours and %mass balance from permeation study of various formulations

Amount of scopoletin (ug) after 48 hours	Formulation		
	Solution	Nanoemulsions	Conventional o/w emulsions
Receptor chamber	46.268 ± 4.896	14.192 ± 0.748	5.966 ± 0.554
Donor part	3487.426 ± 97.565	223.676 ± 6.717	208.017 ± 3.932
Skin	18.509 ± 2.531	1.639 ± 0.252	1.960 ± 0.193
Total	3572.203 ± 135.382	239.507 ± 7.385	215.944 ± 4.372
% Mass balance	97.047 ± 3.352	96.347 ± 1.964	95.323 ± 1.634

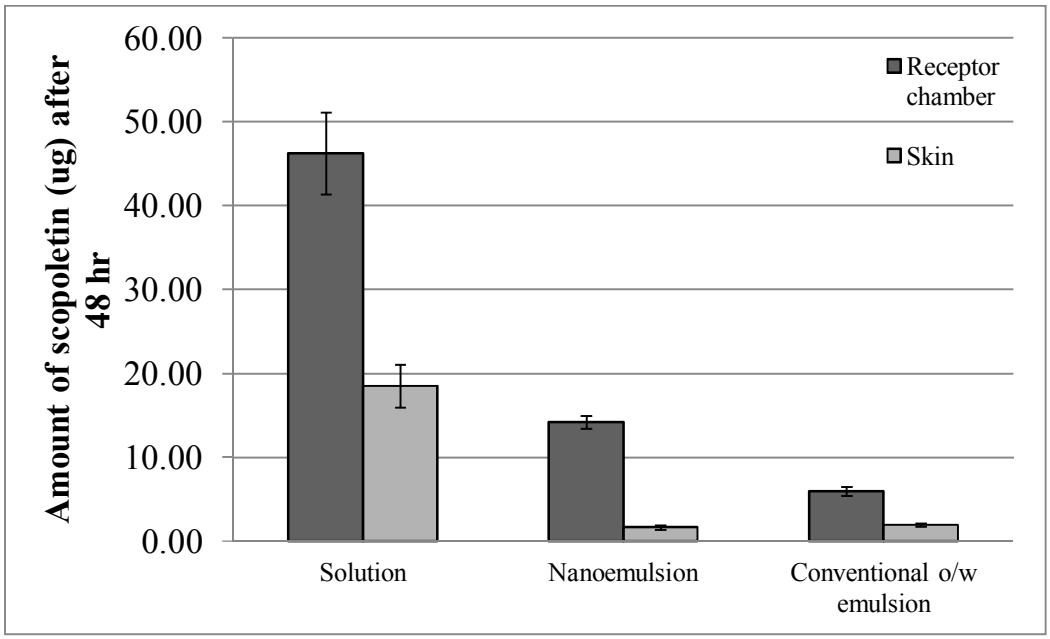


Figure 58 The amount of scopoletin permeated from various formulations after 48 hours detected in the receptor chamber and skin

The data revealed that scopoletin was found to permeate through the skin after 48 hours while a small amount occupied in the skin. The mass balance showed good recovery of scopoletin within the range of 85-115% according to the basic criteria for the *in vitro* assessment of dermal absorption of cosmetic ingredients from the Scientific Committee on Consumer Products, 2006 (Deyan, 2009). The concentration of scopoletin in receptor fluid from solution was found to be highest

followed by that in nanoemulsions and conventional emulsions (46.268 ± 4.896, 14.192 ± 0.748, and 5.966 ± 0.554 ug/ml, respectively).

The concentrations of the extract permeated from nanoemulsions through the skin calculated from the amount of scopoletin found in the receptor chamber were 0.719 mg/mL. It was lower than the non-toxic dose from MTT assay (1.2 mg/ml) with 1.67 fold lower than the IC_{50} for anti-tyrosinase (1.201 mg/ml) but 5.14 fold higher than the IC_{50} for antioxidant (0.014 mg/ml. This result revealed that the extract loaded nanoemulsions might provide the great antioxidant activity with unsatisfied anti-tyrosinase activity. Since the whitening effect of the extract might come from different pathway, the other test observing the anti-melanogenesis activity from the extract should be examined.

The skin absorption depends on various factors including the physicochemical properties of the active compounds such as lipophilicity, partition coefficient, ionization, solubility and molecular weight (Deyan et al., 2009). Scopoletin is a low molecular weight compound (192.17) with logP as 1.3. It was considered to be compatible with both aqueous and lipid layer in skin structure. This led to the detection of scopoletin permeated through to skin into the receptor fluids.

Moreover, the 40% PG in PBS solution which exhibited the same chemical driving force and similar release profile as nanoemulsions exhibited higher amount of permeated scopoletin. This finding might associate with the initial concentration of the extract in the solution that was much higher than that of the two other emulsion formulations.

The percent cumulative amounts of scopoletin permeated through the skin from all formulations at any particular time compared to the initial concentration of scopoletin in the loaded sample was shown in Figure 59.

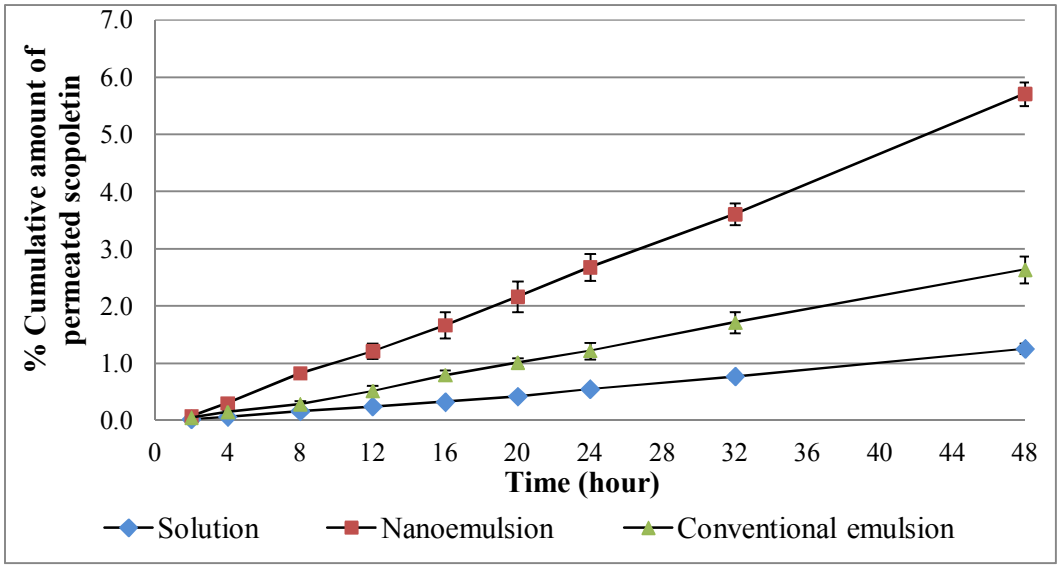


Figure 59 The % cumulative amounts of scopoletin permeated through the skin from solution, nanoemulsions, and conventional emulsions (µg/mL) at any particular time, plots were mean ± SD (n=3)

The data revealed that the percentage of cumulative amounts of scopoletin permeated through the skin from different formulation was increased with time and showed difference in permeation profiles. After 48 hours, the percent cumulative amount of permeated scopoletin from nanoemulsions found to be highest was 5.7073 ± 0.2073 % followed by that from the conventional o/w emulsions and solution at 2.6333 ± 0.2381 and 1.2545 ± 0.0908 %, respectively.

When comparing the % cumulative amount of the extract regardless the initial concentration, the solution showed lowest permeation profile. It might be due to the surfactant effect from formulation that could play a predominant role over the permeation enhancer effect from 40% PG in PBS solution.

The amphiphilic structure of the surface active agents in both nanoemulsions and conventional emulsions that was known to be able to compatible with both aqueous and lipid layer in skin structure led to the delivery of active compounds. In general, the topical formulation applied to the skin will partially absorbed into stratum corneum (SC) and partition into the Brick-and-Mortar structures making this area more hydrophilic. In addition, the surfactant might partition into the hydrophilic protein domains, disrupting their structure that allowing the compound transport through the corneocytes (Nokhodshi et al., 2003; Shin et al.,

2001). The surfactant blend in the formulation was also formed a loose monolayer around the droplet that could facilitate the release of the scopoletin from the lipophilic core of nanoemulsions (Silva et al., 2012).

However, the permeation profile of two similar formulations containing the same percentage of the extract but different in particle size as nanoemulsions and conventional o/w emulsions was compared by plotted the cumulative amounts of scopoletin permeated per unit area as a function of time. The permeation profiles of scopoletin from nanoemulsions and conventional emulsions are shown in Figure 60 and 61, respectively. The permeated rate constant (Flux, J, $\mu g/cm^2h$) of scopoletin at steady state was calculated from the slope of the linear portion of this plot. The cumulative amount per unit area of the extract and Flux values after 48 hours are shown in Table 39.

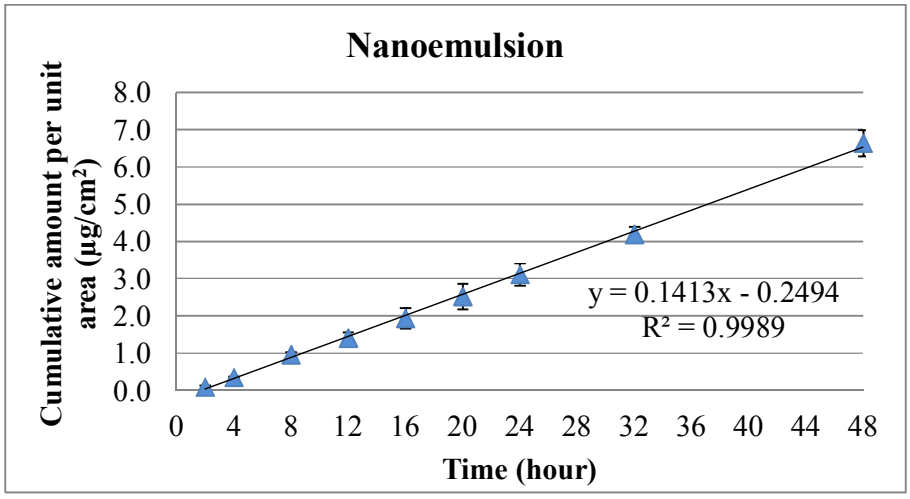


Figure 60 The cumulative amounts per unit area of scopoletin permeated through the skin from nanoemulsions as a function of time, plots were mean ± SD (n=3)

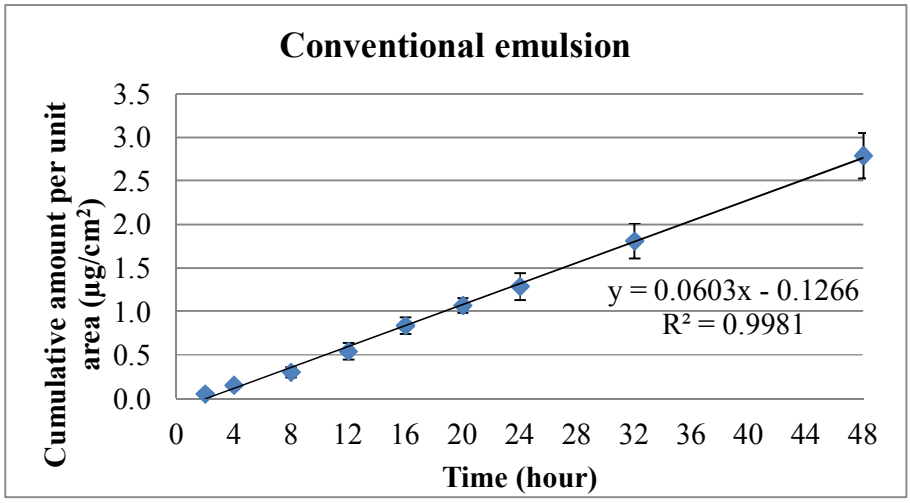


Figure 61 The cumulative amounts per unit area of scopoletin permeated through the skin from conventional o/w emulsions as a function of time, plots were mean ± SD (n=3)

Table 39 The % cumulative amount and Flux values of scopoletin permeated through the skin from various formulations after 48 hours

Formulation	Cumulative amount per unit area after 48 hours ± SD	Flux
	($\mu g/cm^2$)	($\mu g/cm^2h$)
Nanoemulsions	6.6411 ± 0.3498	0.1413
Conventional o/w emulsions	2.7919 ± 0.2593	0.0603

After 48 hours, the permeation of scopoletin from nanoemulsions exhibited significantly higher than the conventional one. The Flux values of nanoemulsions and conventional emulsion were 0.1413, and 0.0603 $\mu g/cm^2h$, respectively.

This finding revealed that the small vesicular sizes of nanoemulsions led to an increase in amount of scopoletin permeated through the skin. Moreover, cremophor RH- 40 might give the permeation enhancer effect that increased the skin permeation of the active compound from nanoemulsions.

The reduction in size is apparently the driving force for permeation enhancement. This data is in accordant with the evidences reported from Shafiq et al., 2007; Kuo et al., 2008; Kotyla et al., 2008 and Yilmaz and Boechet, 2006. Schwarz et al., 1995, observed the penetration of diazepam in two emulsions which identical in

composition and equal drug loading but different only in their droplet sizes. They suggested that the submicron emulsions (100 nm) exhibited significantly better activity than the large droplet cream (1 micron) observed by *in vivo* experiment in mice. This finding was in agreement with the reported from Puglia et al., 2010 who reported the significantly increased in transdermal permeation of glycyrrhetic acid from nanoemulsions in comparison to a conventional o/w emulsions observed both *in vitro* and *in vivo*. They proposed that the permeation enhancement from nanoemulsions could be ascribed to the very small particle size.

From the data, nanoemulsions is a promising system for *Morinda citrifolia* extract due to its ability t-o enhance the skin delivery of active scopoletin. This finding demonstrated the use of *Morinda citrifolia* extract for whitening and/or antiaging applications.

However, as there are many factors involving in skin permeation, *in vivo* investigation on efficacy and also irritancy of extract-loaded nanoemulsions should be further examined.

CHAPTER V

CONCLUSION

The extraction method of *Morinda citrifolia* fruit cultivated in Thailand was developed using different solvent extraction and further purification by partition techniques to obtain more activity. The biological activities in anti-tyrosinase and antioxidant (hydrogen donating) of those extracts were evaluated to obtain the most appropriate solvent and techniques for extraction used in the study. The selected extract was further investigated on its cytotoxicity and stability. Finally, the extract was used as alternative ingredients to formulate into nanoemulsions. The performance and stability of the formulations were also studied. The experiment led to the conclusion as following:

1. At the initial step of extraction, the ethanol extraction showed the highest antioxidant activity (IC_{50} =0.234 mg/ml) with comparable anti-tyrosinase activity to other solvent extracts. It was, then, chosen for further partition.
2. The ethyl acetate soluble fraction exhibited highest activities which IC_{50} value for anti-tyrosinase and DPPH assay were 1.201 mg/ml and 0.014 mg/ml, respectively.
3. The extract at concentration of 1.2 mg/ml had no effect on cell viability.
4. The HPLC condition for quantitative analyzed the compound in the extract was developed and validated. As a result, scopoletin could be determined by this method.
5. The stability testing of the extract was examined at 4 °C and 30 °C during 6 months of storage. They showed good stability by the insignificant reduction of the scopoletin content in the extract as well as the antioxidant activity against DPPH radicals.
6. The nanoemulsions containing caprylic/capric triglyceride as the oil phase, TWEEN®80-SPAN®20 in optimum concentration as emulsifiers, and cremophor RH-40 as solubilizing enhancer for the extract, expressed good physical stability under heat-cool stress conditions and under storage condition at 4°C and 30°C over 3 months.

7. The release profile through cellulose acetate membrane of extract-loaded nanoemulsions and the solution were comparable, while, the permeation profile of the prepared nanoemulsions showed significantly better than conventional o/w emulsions

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APPENDICES

Appendix A

Anti-tyrosinase activity of *Morinda citrifolia* extracts

Table A1. The raw data for anti-tyrosinase inhibition percentage of freeze-dried juice of *Morinda citrifolia* fruits

No.	Conc. (mg/mL)	%Inhibition			Mean	SD
		N1	N2	N3		
1	2.0	7.6923	5.5623	10.4839	7.9128	2.4682
2	4.0	19.9478	23.9420	21.4516	21.7805	2.0173
3	5.0	26.5971	31.2576	28.7100	28.8549	2.3336
4	8.0	52.6728	51.1487	46.9355	50.2523	2.9718
5	10.0	65.7757	68.9238	61.4516	65.3837	3.7515

Table A2. The raw data for anti-tyrosinase inhibition percentage of ethanol extract of *Morinda citrifolia* fruits

No.	Conc. (mg/mL)	%Inhibition			Mean	SD
		N1	N2	N3		
1	2.4	10.8146	6.2038	6.432749	7.8171	2.5985
2	3.2	19.5225	14.9188	14.32749	16.2562	2.8440
3	4.0	28.0899	24.2245	25.73099	26.0151	1.9483
4	4.8	38.4831	32.9394	34.64912	35.3572	2.8389
5	5.6	44.9438	44.9040	45.02924	44.9590	0.0640
6	6.4	58.7079	55.3914	56.28655	56.7953	1.7157
7	8.0	80.8989	81.6839	83.33333	81.9720	1.2425

Table A3. The raw data for anti-tyrosinase inhibition percentage of acetone extract of *Morinda citrifolia* fruits

No.	Conc. (mg/mL)	%Inhibition			Mean	SD
		N1	N2	N3		
1	3.2	13.1650	7.7519	15.3285	8.5291	6.4823
2	4.0	26.0745	22.7907	29.1971	17.2125	4.4544
3	4.8	37.6791	33.0233	42.1898	24.4984	5.0941

No.	Conc. (mg/mL)	%Inhibition			Mean	SD
		N1	N2	N3		
4	5.6	48.8539	44.8062	51.6788	31.3078	5.8441
5	6.4	61.9628	58.1395	67.1533	40.0111	9.0268
6	8.0	81.5186	77.8295	88.3212	52.7338	5.3223

Table A4. The raw data for anti-tyrosinase inhibition percentage of ethyl acetate extract of *Morinda citrifolia* fruits

No.	Conc. (mg/mL)	%Inhibition			Mean	SD
		N1	N2	N3		
1	3.2	13.5135	13.2857	8.4444	11.7479	2.8631
2	4	25.0356	31.8571	25.7778	27.5568	3.7426
3	4.8	42.6743	42.1429	38.8148	41.2106	2.0918
4	5.6	54.9075	61.1429	54.0741	56.7082	3.8631
5	6.4	69.1323	74.0000	69.1852	70.7725	2.7952

Table A5. The raw data for anti-tyrosinase inhibition percentage of hexane soluble fraction

No.	Conc. (mg/mL)	%Inhibition			Mean	SD
		N1	N2	N3		
1	2.0	36.8465	38.0747	37.0170	37.3127	0.6654
2	3.0	49.1720	47.1688	48.0150	48.1186	1.0056
3	4.0	60.9745	60.2011	59.1738	60.1165	0.9034
4	5.0	69.5541	68.9437	69.0717	69.1898	0.3219

Table A6. The raw data for anti-tyrosinase inhibition percentage of ethyl acetate soluble fraction

No.	Conc. (mg/mL)	%Inhibition			Mean	SD
		N1	N2	N3		
1	0.8	8.1081	13.3519	19.9755	13.8118	5.9470
2	1.0	26.4840	29.9026	38.2880	31.5582	6.0736
3	1.1	36.6093	40.9144	47.4952	41.6730	5.4824
4	1.2	45.0860	49.9305	57.2304	50.7489	6.1134
5	1.3	52.0885	58.4812	65.9096	58.8264	6.9170
6	1.4	60.3415	67.3296	74.9828	67.5513	7.3232

Table A7. The raw data for anti-tyrosinase inhibition percentage of aqueous soluble fraction

No.	Conc. (mg/mL)	%Inhibition			Mean	SD
		N1	N2	N3		
1	2.0	5.8465	7.1300	7.3431	6.7732	0.8096
2	4.0	24.3605	26.5428	24.9666	25.2900	1.1265
3	5.0	38.2460	35.6760	34.9800	36.3007	1.7203
4	8.0	58.9525	59.7364	59.6841	59.4577	0.4383
5	10.0	77.5883	76.4539	74.4993	76.1805	1.5625

Table A8. The raw data for anti-tyrosinase inhibition percentage of licorice extract

No.	Conc. (mg/mL)	%Inhibition			Mean	SD
		N1	N2	N3		
1	0.0032	43.8202	33.1226	36.6755	37.8728	5.4484
2	0.0040	50.1873	38.0531	41.6227	43.2877	6.2361
3	0.0048	53.7453	42.3515	48.3509	48.1492	5.6996
4	0.0064	62.1723	48.6726	55.0132	55.2860	6.7540

No.	Conc. (mg/mL)	%Inhibition			Mean	SD
		N1	N2	N3		
5	0.0080	67.0412	53.4766	59.4987	60.0055	6.7965

Table A9. The raw data for anti-tyrosinase inhibition percentage of kojic acid

No.	Conc. (mg/mL)	%Inhibition			Mean	SD
		N1	N2	N3		
1	0.0040	28.9706	26.7895	31.9953	29.2518	2.6142
2	0.0060	35.1719	33.0286	37.3616	35.1874	2.1666
3	0.0080	42.1324	39.8506	42.9398	41.6409	1.6022
4	0.0120	49.2068	48.2380	52.3249	49.9233	2.1356
5	0.0160	57.7941	57.7941	62.1015	59.2299	2.4869

Table A10. One-way analysis of variance of concentration at 50% tyrosinase inhibition (IC_{50}) of *Morinda citrifolia* extract

ANOVA

IC_{50} (mg/mL)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13.025	3	4.342	71.609	0.000
Within Groups	0.485	8	0.061		
Total	13.511	11			

Table A11. Multiple comparison of concentration at 50% tyrosinase inhibition (IC_{50}) of *Morinda citrifolia* extract by Turkey HSD method

Multiple Comparisons

IC_{50} (mg/mL)
Tukey HSD

(I) extract	(J) extract	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Freeze-dried	Ethanol extract	2.14633*	0.20105	0.000	1.5025	2.7902

Multiple Comparisons

IC_{50} (mg/mL)
Tukey HSD

(I) extract	(J) extract	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
juice	Acetone extract	2.24967*	0.20105	0.000	1.6058	2.8935
	Ethyl acetate extract	2.68500*	0.20105	0.000	2.0412	3.3288
Ethanol extract	Freeze-dried juice	-2.14633*	0.20105	0.000	-2.7902	-1.5025
	Acetone extract	0.10333	0.20105	0.953	-0.5405	0.7472
	Ethyl acetate extract	0.53867	0.20105	0.105	-0.1052	1.1825
Acetone extract	Freeze-dried juice	-2.24967*	0.20105	0.000	-2.8935	-1.6058
	Ethanol extract	-0.10333	0.20105	0.953	-0.7472	0.5405
	Ethyl acetate extract	0.43533	0.20105	0.212	-0.2085	1.0792
Ethyl acetate extract	Freeze-dried juice	-2.68500*	0.20105	0.000	-3.3288	-2.0412
	Ethanol extract	-0.53867	0.20105	0.105	-1.1825	0.1052
	Acetone extract	-0.43533	0.20105	0.212	-1.0792	0.2085

Table A12. Homogeneous subset of concentration at 50% tyrosinase inhibition (IC_{50}) of *Morinda citrifolia* extract by Turkey HSD method

Homogeneous Subsets

IC_{50} (mg/mL)
Tukey HSD

extract	N	Subset for alpha = 0.05 1	2
Ethanol extract	3	5.2580	
Acetone extract	3	5.6933	
Ethyl acetate extract	3	5.7967	
Freeze-dried juice	3		7.9430
Sig.		0.105	1.000

Means for groups in homogeneous subsets are displayed.

Table A13. One-way analysis of variance of concentration at 50% tyrosinase inhibition (IC_{50}) of all fractions partitioned from ethanol extract and references

ANOVA

IC_{50} (mg/mL)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	99.359	4	24.840	9.191E3	0.000
Within Groups	0.027	10	0.003		
Total	99.386	14			

Table A14. Multiple comparison of concentration at 50% tyrosinase inhibition (IC_{50}) of all fractions partitioned from ethanol extract and references by Dunnette T3 method

Multiple Comparisons

IC_{50} (mg/mL)
Dunnett T3

(I) extract	(J) extract	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Hexane soluble fraction	Ethyl acetate soluble fraction	1.935000*	0.043236	0.001	1.61770	2.25230
	Aqueous soluble fraction	-3.717000*	0.052590	0.000	-4.12172	-3.31228
	Licorice extract	3.130667*	0.011566	0.000	3.03400	3.22733
	Kojic acid	3.124000*	0.011518	0.000	3.02585	3.22215
Ethyl acetate soluble fraction	Hexane soluble fraction	-1.935000*	0.043236	0.001	-2.25230	-1.61770
	Aqueous soluble fraction	-5.652000*	0.066108	0.000	-5.97800	-5.32600
	Licorice extract	1.195667*	0.041695	0.005	0.83894	1.55240
	Kojic acid	1.189000*	0.041681	0.005	0.83184	1.54616
Aqueous soluble fraction	Hexane soluble fraction	3.717000*	0.052590	0.000	3.31228	4.12172
	Ethyl acetate soluble fraction	5.652000*	0.066108	0.000	5.32600	5.97800
	Licorice extract	6.847667*	0.051330	0.000	6.40820	7.28713
	Kojic acid	6.841000*	0.051319	0.000	6.40119	7.28081
Licorice extract	Hexane soluble fraction	-3.130667*	0.011566	0.000	-3.22733	-3.03400
	Ethyl acetate soluble fraction	-1.195667*	0.041695	0.005	-1.55240	-.83894

Multiple Comparisons

IC_{50} (mg/mL)
Dunnett T3

(I) extract	(J) extract	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Kojic acid	Aqueous soluble fraction	-6.847667*	0.051330	0.000	-7.28713	-6.40820
	Kojic acid	-0.006667	0.001333	0.080	-0.01467	0.00134
	Hexane soluble fraction	-3.124000*	0.011518	0.000	-3.22215	-3.02585
	Ethyl acetate soluble fraction	-1.189000*	0.041681	0.005	-1.54616	-0.83184
	Aqueous soluble fraction	-6.841000*	0.051319	0.000	-7.28081	-6.40119
	Licorice extract	0.006667	0.001333	0.080	-0.00134	0.01467

*. The mean difference is significant at the 0.05 level.

Table A15. Homogeneous subset of concentration at 50% tyrosinase inhibition (IC_{50}) of *Morinda citrifolia* extract by Turkey HSD method

Homogeneous Subsets

IC_{50} (mg/mL)
Tukey HSD[a]

extract	N	Subset for alpha = 0.05			
		1	2	3	4
Licorice extract	3	0.00533			
Kojic acid	3	0.01200			
Ethyl acetate soluble fraction	3		1.20100		
Hexane soluble fraction	3			3.13600	
Aqueous soluble fraction	3				6.85300
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix B

Anti-tyrosinase activity of *Morinda citrifolia* extracts

Table B1. The raw data for DPPH radical scavenging activity (free radical inhibition percentage) of freeze-dried juice of *Morinda citrifolia* fruits

No.	Conc. (mg/mL)	%Inhibition			Mean	SD
		N1	N2	N3		
1	0.20	30.0908	30.1911	30.1075	30.1298	16.3933
2	0.30	36.7056	37.4522	35.7527	36.6368	19.9116
3	0.40	43.1907	42.6752	40.8602	42.2420	22.9342
4	0.50	47.9896	46.3694	45.0269	46.4620	25.1963
5	0.60	52.0104	52.9936	50.1344	51.7128	28.0145
6	0.70	57.1984	57.3248	56.1828	56.9020	30.7863
7	0.80	63.1647	64.4586	62.2312	63.2848	34.2335

Table B2. The raw data for DPPH radical scavenging activity (free radical inhibition percentage) of ethanol extract of *Morinda citrifolia* fruits

No.	Conc. (mg/mL)	%Inhibition			Mean	SD
		N1	N2	N3		
1	0.10	22.3160	26.0123	27.2800	25.2028	2.5791
2	0.15	32.2400	36.8098	38.3003	35.7834	3.1579
3	0.20	41.1500	46.0123	46.4658	44.5427	2.9469
4	0.25	49.0751	54.1104	54.8088	52.6648	3.1283
5	0.30	57.1840	63.8037	62.6883	61.2253	3.5440

Table B3. The raw data for DPPH radical scavenging activity (free radical inhibition percentage) of acetone extract of *Morinda citrifolia* fruits

No.	Conc. (mg/mL)	%Inhibition			Mean	SD
		N1	N2	N3		
1	0.15	24.1718	24.2105	22.8070	23.7298	0.7994
2	0.20	32.2699	28.1287	28.3041	29.5676	2.3420
3	0.25	38.8957	35.5556	36.8421	37.0978	1.6847

No.	Conc. (mg/mL)	%Inhibition			Mean	SD
		N1	N2	N3		
4	0.30	43.1288	42.0360	42.1636	42.4428	0.5975
5	0.40	55.9509	56.3158	54.0351	55.4339	1.2251
6	0.50	67.8528	66.7836	67.7193	67.4519	0.5826
7	0.60	81.6960	82.5146	82.7485	82.3197	0.5527

Table B4. The raw data for DPPH radical scavenging activity (free radical inhibition percentage) of ethyl acetate extract of *Morinda citrifolia* fruits

No.	Conc. (mg/mL)	%Inhibition			Mean	SD
		N1	N2	N3		
1	0.20	28.9854	29.7329	32.8289	30.5157	2.0378
2	0.25	38.4529	34.2625	36.5429	36.4194	2.0979
3	0.30	42.5434	40.1858	40.1392	40.9561	1.3748
4	0.40	52.0740	48.3740	51.2761	50.5747	1.9472
5	0.50	64.2377	58.3043	59.1647	60.5689	3.2062

Table B5. The raw data for DPPH radical scavenging activity (free radical inhibition percentage) of hexane soluble fraction

No.	Conc. (mg/mL)	%Inhibition			Mean	SD
		N1	N2	N3		
1	0.10	25.1208	25.2011	26.5688	25.6302	0.8138
2	0.14	34.2995	34.1823	35.3805	34.6208	0.6606
3	0.16	38.7681	40.0804	40.9880	39.9455	1.1161
4	0.20	45.5314	47.3190	47.7971	46.8825	1.1942
5	0.24	53.6232	55.8981	57.2764	55.5992	1.8448
6	0.26	58.2126	60.5898	60.8812	59.8945	1.4639
7	0.30	65.0966	68.0965	69.0254	67.4062	2.0533

Table B6. The raw data for DPPH radical scavenging activity (free radical inhibition percentage) of ethyl acetate soluble fraction

No.	Conc. (mg/mL)	%Inhibition			Mean	SD
		N1	N2	N3		
1	0.010	39.8675	40.9272	41.5899	40.7949	0.8687
2	0.012	45.5629	46.7550	45.7373	46.0184	0.6438
3	0.014	49.3225	51.2032	49.4240	49.9832	1.0578
4	0.016	54.2005	54.6791	53.3410	54.0736	0.6780
5	0.018	58.4011	59.4920	56.4516	58.1149	1.5403

Table B7. The raw data for DPPH radical scavenging activity (free radical inhibition percentage) of aqueous soluble fraction

No.	Conc. (mg/mL)	%Inhibition			Mean	SD
		N1	N2	N3		
1	0.10	34.8750	34.1270	32.7609	33.9210	1.0720
2	0.15	40.5000	40.3439	39.3659	40.0699	0.6147
3	0.20	44.7500	45.1058	44.2536	44.7032	0.4280
4	0.25	50.7500	50.1323	48.6129	49.8317	1.0998
5	0.30	55.6250	55.9524	54.4254	55.3342	0.8040
6	0.35	60.0830	59.6561	60.5020	60.0804	0.4230

Table B8. The raw data for DPPH radical scavenging activity (free radical inhibition percentage) of BHA

No.	Conc. (mg/mL)	%Inhibition			Mean	SD
		N1	N2	N3		
1	0.004	41.5036	37.0110	39.1906	39.2351	2.2466
2	0.005	47.9918	44.0321	45.3674	45.7971	2.0145
3	0.006	55.2008	50.3018	52.9286	52.8104	2.4516
4	0.007	60.6591	56.8410	58.8924	58.7975	1.9108

Table B9. The raw data for DPPH radical scavenging activity (free radical inhibition percentage) of BHA

No.	Conc. (mg/mL)	%Inhibition			Mean	SD
		N1	N2	N3		
1	0.100	30.9800	32.3529	35.3620	32.8983	2.2413
2	0.120	40.5462	41.7281	42.1826	41.4856	0.8447
3	0.140	48.5011	50.1601	51.8162	50.1591	1.6576
4	0.160	52.9979	54.9626	56.6239	54.8615	1.8152
5	0.180	55.8887	59.6585	61.2179	58.9217	2.7400
6	0.200	57.9229	61.2593	63.8889	61.0237	2.9900

Table B10. The raw data for DPPH radical scavenging activity (free radical inhibition percentage) of BHA

No.	Conc. (mg/mL)	%Inhibition			Mean	SD
		N1	N2	N3		
1	0.0005	8.9431	7.8629	10.3310	9.0457	1.2372
2	0.0010	16.4634	15.1210	20.9629	17.5158	3.0598
3	0.0015	25.9146	24.0927	33.7011	27.9028	5.1034
4	0.0020	34.6545	31.7540	45.5823	37.3303	7.2921
5	0.0025	44.4106	41.1290	58.0321	47.8572	8.9632
6	0.0030	53.7602	49.8992	66.0643	56.5745	8.4420
7	0.0035	60.6707	59.3750	79.3173	66.4543	11.1585

Table B11. One-way analysis of variance of concentration at 50% free radicals inhibition (IC_{50}) of *Morinda citrifolia* extract

ANOVA

IC_{50} (mg/mL)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.163	3	0.054	196.400	0.000
Within Groups	0.002	8	0.000		
Total	0.165	11			

Table B12. Multiple comparison of concentration at 50% free radicals inhibition (IC_{50}) of *Morinda citrifolia* extract by Turkey HSD method

Multiple Comparisons

IC_{50} (mg/mL) Tukey HSD

(I) extract	(J) extract	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Freeze-dried juice	Ethanol extract	0.3260000*	0.0135831	0.000	0.282502	0.369498
	Acetone extract	0.2043333*	0.0135831	0.000	0.160836	0.247831
	Ethyl acetate extract	0.1666667*	0.0135831	0.000	0.123169	0.210164
Ethanol extract	Freeze-dried juice	-0.3260000*	0.0135831	0.000	-0.369498	-0.282502
	Acetone extract	-0.1216667*	0.0135831	0.000	-0.165164	-0.078169
	Ethyl acetate extract	-0.1593333*	0.0135831	0.000	-0.202831	-0.115836
Acetone extract	Freeze-dried juice	-0.2043333*	0.0135831	0.000	-0.247831	-0.160836
	Ethanol extract	0.1216667*	0.0135831	0.000	0.078169	0.165164
	Ethyl acetate extract	-0.0376667	0.0135831	0.092	-0.081164	0.005831
Ethyl acetate extract	Freeze-dried juice	-0.1666667*	0.0135831	0.000	-0.210164	-0.123169
	Ethanol extract	0.1593333*	0.0135831	0.000	0.115836	0.202831
	Acetone extract	0.0376667	0.0135831	0.092	-0.005831	0.081164

*. The mean difference is significant at the 0.05 level.

Table B13. Homogeneous subset of concentration at 50% free radicals inhibition (IC_{50}) of *Morinda citrifolia* extract by Turkey HSD method

Homogeneous Subsets

IC_{50} (mg/mL)

Tukey HSD

extract	N	Subset for alpha = 0.05		
		1	2	3
Ethanol extract	3	0.234333		
Acetone extract	3		0.356000	
Ethyl acetate extract	3		0.393667	
Freeze-dried juice	3			0.560333
Sig.		1.000	0.092	1.000

Means for groups in homogeneous subsets are displayed.

Table B14. One-way analysis of variance of concentration at 50% free radicals inhibition (IC_{50}) of all fractions partitioned from ethanol extract and references

ANOVA

IC_{50} (mg/mL)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.186	5	0.037	3.144E3	0.000
Within Groups	0.000	12	0.000		
Total	0.186	17			

Table B15. Multiple comparison of concentration at 50% free radicals inhibition (IC_{50}) of all fractions partitioned from ethanol extract and references by Dunnette T3 method

Multiple Comparisons

IC_{50} (mg/mL)
Dunnett T3

(I) extract	(J) extract	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Hexane soluble fraction	Ethyl acetate soluble fraction	0.1996667*	0.0038442	0.002	0.163992	0.235341
	Aqueous soluble fraction	-0.0370000*	0.0047140	0.016	-0.062920	-0.011080
	BHA	0.2080667*	0.0038481	0.001	0.172529	0.243604
	BHT	0.1426667*	0.0040139	0.001	0.111591	0.173742
	Trolox	0.2110000*	0.0038517	0.001	0.175588	0.246412
Ethyl acetate soluble fraction	Hexane soluble fraction	-0.1996667*	0.0038442	0.002	-0.235341	-0.163992
	Aqueous soluble fraction	-0.2366667*	0.0027285	0.001	-0.261987	-0.211346
	BHA	0.0084000*	0.0001732	0.002	0.006793	0.010007
	BHT	-0.0570000*	0.0011547	0.002	-0.067716	-0.046284
	Trolox	0.0113333*	0.0002404	0.002	0.009103	0.013564
Aqueous soluble fraction	Hexane soluble fraction	0.0370000*	0.0047140	0.016	0.011080	0.062920
	Ethyl acetate soluble fraction	0.2366667*	0.0027285	0.001	0.211346	0.261987
	BHA	0.2450667*	0.0027339	0.001	0.219938	0.270195
	BHT	0.1796667*	0.0029627	0.000	0.159415	0.199919

Multiple Comparisons

IC_{50} (mg/mL)
Dunnett T3

(I) extract	(J) extract	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
	Trolox	0.2480000*	0.0027390	0.000	0.223045	0.272955
BHA	Hexane soluble fraction	-0.2080667*	0.0038481	0.001	-0.243604	-0.172529
	Ethyl acetate soluble fraction	-0.0084000*	0.0001732	0.002	-0.010007	-0.006793
	Aqueous soluble fraction	-0.2450667*	0.0027339	0.001	-0.270195	-0.219938
	BHT	-0.0654000*	0.0011676	0.001	-0.075690	-0.055110
	Trolox	0.0029333*	0.0002963	0.007	0.001312	0.004554
BHT	Hexane soluble fraction	-0.1426667*	0.0040139	0.001	-0.173742	-0.111591
	Ethyl acetate soluble fraction	0.0570000*	0.0011547	0.002	0.046284	0.067716
	Aqueous soluble fraction	-0.1796667*	0.0029627	0.000	-0.199919	-0.159415
	BHA	0.0654000*	0.0011676	0.001	0.055110	0.075690
	Trolox	0.0683333*	0.0011795	0.001	0.058384	0.078283
Trolox	Hexane soluble fraction	-0.2110000*	0.0038517	0.001	-0.246412	-0.175588
	Ethyl acetate soluble fraction	-0.0113333*	0.0002404	0.002	-0.013564	-0.009103
	Aqueous soluble fraction	-0.2480000*	0.0027390	0.000	-0.272955	-0.223045
	BHA	-0.0029333*	0.0002963	0.007	-0.004554	-0.001312
	BHT	-0.0683333*	0.0011795	0.001	-0.078283	-0.058384

*. The mean difference is significant at the 0.05 level.

Table B16. Homogeneous subset of concentration at 50% free radicals inhibition (IC_{50}) of *Morinda citrifolia* extract by Turkey HSD method

Homogeneous Subsets

IC_{50} (mg/mL)
Tukey HSD[a]

		Subset for alpha = 0.05				
extract	N	1	2	3	4	5
Trolox	3	0.002667				
BHA	3	0.005600				
Ethyl acetate soluble fraction	3		0.014000			
BHT	3			0.071000		
Hexane soluble fraction	3				0.213667	
Aqueous soluble fraction	3					0.250667

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix C

Chemical stability of ethyl acetate soluble fraction

By High Performance Liquid Chromatography method

Table C1. One-way analysis of variance of %relative of the amount scopoletin in the extract at particular time pint compared to time zero when kept at 4°C

ANOVA

%Relative in amount of scopoletin

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.208	3	0.403	1.092	0.407
Within Groups	2.951	8	0.369		
Total	4.159	11			

Table C2. Multiple comparison of %relative of the amount scopoletin in the extract at particular time pint compared to time zero when kept at 4°C by Turkey HSD method

Multiple Comparisons

%Relative in amount of scopoletin

Tukey HSD

(I) sample	(J) sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Time 0	2 month	-0.206667	0.495900	0.974	-1.79471	1.38138
	4 month	-0.046667	0.495900	1.000	-1.63471	1.54138
	6 month	0.626667	0.495900	0.608	-0.96138	2.21471
2 month	Time 0	0.206667	0.495900	0.974	-1.38138	1.79471
	4 month	0.160000	0.495900	0.988	-1.42805	1.74805
	6 month	0.833333	0.495900	0.392	-.75471	2.42138
4 month	Time 0	0.046667	0.495900	1.000	-1.54138	1.63471
	2 month	-0.160000	0.495900	0.988	-1.74805	1.42805
	6 month	0.673333	0.495900	0.556	-.91471	2.26138
6 month	Time 0	-0.626667	0.495900	0.608	-2.21471	0.96138
	2 months	-0.833333	0.495900	0.392	-2.42138	0.75471
	4 months	-0.673333	0.495900	0.556	-2.26138	0.91471

Table C3. Homogeneous subset of %relative of the amount scopoletin in the extract at particular time pint compared to time zero when kept at 4°C by Turkey HSD method

Homogeneous Subsets

Tukey HSD

sample	N	Subset for alpha = 0.05: 1
6 month	3	99.37333

Tukey HSD

sample	N	Subset for alpha = 0.05
		1
Time 0	3	100.00000
4 month	3	100.04667
2 month	3	100.20667
Sig.		0.392

Table C4. One-way analysis of variance of %relative of the amount scopoletin in the extract at particular time pint compared to time zero when kept at room temperature

ANOVA

%Relative in amount of scopoletin

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.875	3	0.625	0.855	0.502
Within Groups	5.849	8	0.731		
Total	7.724	11			

Table C5. Multiple comparison of %relative of the amount scopoletin in the extract at particular time pint compared to time zero when kept at room temperature by Turkey HSD method

Multiple Comparisons

%Relative in amount of scopoletin

Tukey HSD

(I) sample	(J) sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Time 0	2 month	-0.486667	0.698176	0.895	-2.72247	1.74914
	4 month	-0.156667	0.698176	0.996	-2.39247	2.07914
	6 month	0.603333	0.698176	0.823	-1.63247	2.83914
2 month	Time 0	0.486667	0.698176	0.895	-1.74914	2.72247
	4 month	0.330000	0.698176	0.963	-1.90581	2.56581
	6 month	1.090000	0.698176	0.449	-1.14581	3.32581
4 month	Time 0	0.156667	0.698176	0.996	-2.07914	2.39247
	2 month	-0.330000	0.698176	0.963	-2.56581	1.90581
	6 month	0.760000	0.698176	0.706	-1.47581	2.99581
6 month	Time 0	-0.603333	0.698176	0.823	-2.83914	1.63247
	2 months	-1.090000	0.698176	0.449	-3.32581	1.14581
	4 months	-0.760000	0.698176	0.706	-2.99581	1.47581

Table C6. Homogeneous subset of %relative of the amount scopoletin in the extract at particular time pint compared to time zero when kept at room temperature by Turkey HSD method

Homogeneous Subsets

Tukey HSD

		Subset for alpha = 0.05
sample	N	1
6 month	3	99.39667
Time 0	3	100.00000
4 month	3	100.15667
2 month	3	100.48667
Sig.		0.449

Table C7. One-way analysis of variance of %relative of the amount scopoletin in the extract at particular time pint compared to time zero when kept at 40°C

ANOVA

%Relative in amount of scopoletin

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.629	3	0.210	5.080	0.029
Within Groups	0.330	8	0.041		
Total	0.960	11			

Table C8. Multiple comparison of %relative of the amount scopoletin in the extract at particular time pint compared to time zero when kept at 40°C by Turkey HSD method

Multiple Comparisons

%Relative in amount of scopoletin

Tukey HSD

					95% Confidence Interval	
(I) sample	(J) sample	Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Time 0	2 month	0.403333	0.165932	0.148	-0.12804	0.93471
	4 month	0.533333*	0.165932	0.049	0.00196	1.06471
	6 month	0.583333*	0.165932	0.032	0.05196	1.11471
2 month	Time 0	-0.403333	0.165932	0.148	-0.93471	0.12804
	4 month	0.130000	0.165932	0.860	-0.40137	0.66137
	6 month	0.180000	0.165932	0.708	-0.35137	0.71137
4 month	Time 0	-0.533333*	0.165932	0.049	-1.06471	-0.00196

Multiple Comparisons

%Relative in amount of scopoletin

Tukey HSD

(I) sample	(J) sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
	2 month	-0.130000	0.165932	0.860	-0.66137	0.40137
	6 month	0.050000	0.165932	0.990	-0.48137	0.58137
6 month	Time 0	-0.583333*	0.165932	0.032	-1.11471	-0.05196
	2 months	-0.180000	0.165932	0.708	-0.71137	0.35137
	4 months	-0.050000	0.165932	0.990	-0.58137	0.48137

Table C9. Homogeneous subset of %relative of the amount scopoletin in the extract at particular time pint compared to time zero when kept at 40°C by Turkey HSD method

Homogeneous Subsets

Tukey HSD

sample	N	Subset for alpha = 0.05: 1	2
6 month	3	99.41667	
4 month	3	99.46667	
2 month	3	99.59667	99.59667
Time 0	3		1.00000E2
Sig.		0.708	0.148

Appendix D

Biological stability of ethyl acetate soluble fraction

Table D1. One-way analysis of variance of concentration at 50% free radicals inhibition (IC_{50}) of ethyl acetate soluble fraction kept in 4°C at any particular time point

ANOVA

IC_{50} (mg/mL)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.020	3	0.007	3.995	0.052
Within Groups	0.013	8	0.002		
Total	0.034	11			

Table D2. Multiple comparison of concentration at 50% free radicals inhibition (IC_{50}) of ethyl acetate soluble fraction kept in 4°C at any particular time point by Dunnette T3 method

Multiple Comparisons

IC_{50} (mg/mL)
Dunnette T3

		Mean Difference			95% Confidence Interval	
(I) extract	(J) extract	(I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Time 0	2 month	-0.0956667	0.0473369	0.475	-0.457450	0.266117
	4 month	-0.0010000	0.0006506	0.620	-0.004400	0.002400
	6 month	-0.0022000	0.0006110	0.161	-0.005948	0.001548
2 month	Time 0	0.0956667	0.0473369	0.475	-0.266117	0.457450
	2 month	0.0946667	0.0473343	0.482	-0.267189	0.456522
	6 month	0.0934667	0.0473338	0.489	-0.268403	0.455337
4 month	Time 0	0.0010000	0.0006506	0.620	-0.002400	0.004400
	2 month	-0.0946667	0.0473343	0.482	-0.456522	0.267189
	6 month	-0.0012000	0.0003606	0.135	-0.002905	0.000505
6 month	Time 0	0.0022000	0.0006110	0.161	-0.001548	0.005948
	2 month	-0.0934667	0.0473338	0.489	-0.455337	0.268403
	4 month	0.0012000	0.0003606	0.135	-0.000505	0.002905

*. The mean difference is significant at the 0.05 level.

Table D3. Homogeneous subset of concentration at 50% free radicals inhibition (IC_{50}) of ethyl acetate soluble fraction kept in 4°C at any particular time point by Turkey HSD method

Homogeneous Subsets

Tukey HSD

		Subset for alpha = 0.05
extract	N	1
Time 0	3	0.015000
4 month	3	0.016000
6 month	3	0.017200
2 month	3	0.110667
Sig.		0.081

Table D4. One-way analysis of variance of concentration at 50% free radicals inhibition (IC_{50}) of ethyl acetate soluble fraction kept in room temperature at any particular time point

ANOVA

IC_{50} (mg/mL)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.000	3	0.000	1.881	0.211
Within Groups	0.000	8	0.000		
Total	0.000	11			

Table D5. Multiple comparison of concentration at 50% free radicals inhibition (IC_{50}) of ethyl acetate soluble fraction kept in room temperature at any particular time point by Tukey HSD method

Multiple Comparisons

IC_{50} (mg/mL)
Tukey HSD

		Mean Difference			95% Confidence Interval	
(I) extract	(J) extract	(I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Time 0	2 month	-0.0002000	0.0010617	0.997	-0.003600	0.003200
	4 month	-0.0004000	0.0010617	0.981	-0.003800	0.003000
	6 month	-0.0022333	0.0010617	0.231	-0.005633	0.001167
2 month	Time 0	0.0002000	0.0010617	0.997	-0.003200	0.003600
	2 month	-0.0002000	0.0010617	0.997	-0.003600	0.003200
	6 month	-0.0020333	0.0010617	0.294	-0.005433	0.001367

Multiple Comparisons

IC_{50} (mg/mL)
Tukey HSD

(I) extract	(J) extract	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
4 month	Time 0	0.0004000	0.0010617	0.981	-0.003000	0.003800
	2 month	0.0002000	0.0010617	0.997	-0.003200	0.003600
	6 month	-0.0018333	0.0010617	0.371	-0.005233	0.001567
6 month	Time 0	0.0022333	0.0010617	0.231	-0.001167	0.005633
	2 month	0.0020333	0.0010617	0.294	-0.001367	0.005433
	4 month	0.0018333	0.0010617	0.371	-0.001567	0.005233

*. The mean difference is significant at the 0.05 level.

Table D6. Homogeneous subset of concentration at 50% free radicals inhibition (IC_{50}) of ethyl acetate soluble fraction kept in room temperature at any particular time point by Turkey HSD method

Homogeneous Subsets

Tukey HSD

extract	N	Subset for alpha = 0.05
		1
Time 0	3	0.015000
4 month	3	0.015200
6 month	3	0.015400
2 month	3	0.017233
Sig.		0.231

Table D7. One-way analysis of variance of concentration at 50% free radicals inhibition (IC_{50}) of ethyl acetate soluble fraction kept in 40°C at any particular time point

ANOVA

IC_{50} (mg/mL)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.000	3	0.000	15.209	0.001
Within Groups	0.000	8	0.000		
Total	0.000	11			

Table D8. Multiple comparison of concentration at 50% free radicals inhibition (IC_{50}) of ethyl acetate soluble fraction kept in 40°C at any particular time point by Tukey HSD method

Multiple Comparisons

IC_{50} (mg/mL)
Tukey HSD

(I) extract	(J) extract	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Time 0	2 month	-0.0011000	0.0005084	0.213	-0.002728	0.000528
	4 month	-0.0026776*	0.0005084	0.003	-0.004306	-0.001050
	6 month	-0.0030000*	0.0005084	0.002	-0.004628	-0.001372
2 month	Time 0	0.0011000	0.0005084	0.213	-0.000528	0.002728
	2 month	-0.0015776	0.0005084	0.057	-0.003206	0.000050
	6 month	-0.0019000*	0.0005084	0.024	-0.003528	-0.000272
4 month	Time 0	0.0026776*	0.0005084	0.003	0.001050	0.004306
	2 month	0.0015776	0.0005084	0.057	-0.000050	0.003206
	6 month	-0.0003224	0.0005084	0.918	-0.001950	0.001306
6 month	Time 0	0.0030000*	0.0005084	0.002	0.001372	0.004628
	2 month	0.0019000*	0.0005084	0.024	0.000272	0.003528
	4 month	0.0003224	0.0005084	0.918	-0.001306	0.001950

*. The mean difference is significant at the 0.05 level.

Table D9. Homogeneous subset of concentration at 50% free radicals inhibition (IC_{50}) of ethyl acetate soluble fraction kept in room temperature at any particular time point by Turkey HSD method

Homogeneous Subsets

Tukey HSD

		Subset for alpha = 0.05		
extract	N	1	2	3
Time 0	3	0.015000		
2 month	3	0.016100	0.016100	
4 month	3		0.017678	0.017678
6 month	3			0.018000
Sig.		0.213	0.057	0.918

Appendix E

Cytotoxicity of ethyl acetate soluble fraction of *Morinda citrifolia* extract

Table E1. One-way analysis of variance of the %cell viability of HaCaT cells after 12 hours incubation of the extract at different concentrations

ANOVA

%Relative in amount of scopoletin

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	27515.976	11	2501.452	33.452	0.000
Within Groups	1794.634	24	74.776		
Total	29310.610	35			

Table E2. Multiple comparison of the %cell viability of HaCaT cells after 12 hours incubation of the extract at different concentrations by Turkey HSD method

Multiple Comparisons

%Relative in amount of scopoletin

Tukey HSD

(I) sample	(J) Concentration (µg/mL)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Control	100	1.30796	7.06052	1.000	-24.1497	26.7656
	200	2.90645	7.06052	1.000	-22.5512	28.3641
	400	-1.05668	7.06052	1.000	-26.5143	24.4009
	600	1.06959	7.06052	1.000	-24.3880	26.5272
	800	4.21062	7.06052	1.000	-21.2470	29.6682
	1000	0.51229	7.06052	1.000	-24.9453	25.9699
	1200	5.08528	7.06052	1.000	-20.3723	30.5429
	1400	30.15510*	7.06052	0.011	4.6975	55.6127
	1600	50.93547*	7.06052	0.000	25.4779	76.3931
	1800	70.06278*	7.06052	0.000	44.6052	95.5204
	2000	73.95175*	7.06052	0.000	48.4941	99.4094

Table E3. Homogeneous subset of the amount scopoletin in the nanoemulsion kept in various conditions at particular time pint compared to time zero by Turkey HSD method

Homogeneous Subsets

Tukey HSD

		Subset for alpha = 0.05			
sample	N	1	2	3	4
2000	3	26.0482			
1800	3	29.9372			
1600	3	49.0645	49.0645		
1400	3		69.8449	69.8449	
1200	3			94.9147	94.9147
800	3				95.7894
200	3				97.0936
100	3				98.6920
600	3				98.9304
1000	3				99.4877
Control	3				1.0000E2
400	3				1.0106E2
Sig.		0.103	0.187	0.056	0.999

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix F

Physical and chemical stability of extract-loaded nanoemulsions

Table F1. One-way analysis of variance of the particle sizes of the extract-loaded nanoemulsions in various conditions compared to that of initial

ANOVA

Particle size

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	24.159	10	2.416	37.275	0.000
Within Groups	1.426	22	0.065		
Total	25.585	32			

Table F2. Multiple comparison of of the particle sizes of the extract-loaded nanoemulsions in various conditions compared to that of initial by Turkey HSD method

Multiple Comparisons

Particle size

Tukey HSD

(I) sample	(J) sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Initial	HC 8 cycles	0.01667	0.20787	1.000	-0.7264	0.7597
	RT 1M	0.07333	0.20787	1.000	-0.6697	0.8164
	RT 2M	-0.43333	0.20787	0.598	-1.1764	0.3097
	RT 3M	-0.61333	0.20787	0.170	-1.3564	0.1297
	4°C 1M	0.40333	0.20787	0.687	-0.3397	1.1464
	4°C 2M	0.23333	0.20787	0.985	-0.5097	0.9764
	4°C 3M	-0.09667	0.20787	1.000	-0.8397	0.6464
	40°C 1M	-0.94667*	0.20787	0.006	-1.6897	-0.2036
	40°C 2M	-1.20000*	0.20787	0.000	-1.9431	-0.4569
	40°C 3M	-2.73333*	0.20787	0.000	-3.4764	-1.9903
HC 8 cycles	Initial	-0.01667	0.20787	1.000	-0.7597	0.7264
	RT 1M	0.05667	0.20787	1.000	-0.6864	0.7997
	RT 2M	-0.45000	0.20787	0.549	-1.1931	0.2931
	RT 3M	-0.63000	0.20787	0.147	-1.3731	0.1131
	4°C 1M	0.38667	0.20787	0.734	-0.3564	1.1297
	4°C 2M	0.21667	0.20787	0.991	-0.5264	0.9597
	4°C 3M	-0.11333	0.20787	1.000	-0.8564	0.6297
	40°C 1M	-0.96333*	0.20787	0.005	-1.7064	-0.2203
	40°C 2M	-1.21667*	0.20787	0.000	-1.9597	-0.4736

Multiple Comparisons

Particle size

Tukey HSD

(I) sample	(J) sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
	40°C 3M	-2.75000*	0.20787	0.000	-3.4931	-2.0069
RT 1M	Initial	-0.07333	0.20787	1.000	-0.8164	0.6697
	HC 8 cycles	-0.05667	0.20787	1.000	-0.7997	0.6864
	RT 2M	-0.50667	0.20787	0.388	-1.2497	0.2364
	RT 3M	-0.68667	0.20787	0.087	-1.4297	0.0564
	4°C 1M	0.33000	0.20787	0.871	-0.4131	1.0731
	4°C 2M	0.16000	0.20787	0.999	-0.5831	0.9031
	4°C 3M	-0.17000	0.20787	0.999	-0.9131	0.5731
	40°C 1M	-1.02000*	0.20787	0.003	-1.7631	-0.2769
	40°C 2M	-1.27333*	0.20787	0.000	-2.0164	-0.5303
	40°C 3M	-2.80667*	0.20787	0.000	-3.5497	-2.0636
RT 2M	Initial	0.43333	0.20787	0.598	-0.3097	1.1764
	HC 8 cycles	0.45000	0.20787	0.549	-0.2931	1.1931
	RT 1M	0.50667	0.20787	0.388	-0.2364	1.2497
	RT 3M	-0.18000	0.20787	0.998	-0.9231	0.5631
	4°C 1M	0.83667*	0.20787	0.019	0.0936	1.5797
	4°C 2M	0.66667	0.20787	0.105	-0.0764	1.4097
	4°C 3M	0.33667	0.20787	0.858	-0.4064	1.0797
	40°C 1M	-0.51333	0.20787	0.371	-1.2564	0.2297
	40°C 2M	-0.76667*	0.20787	0.039	-1.5097	-0.0236
	40°C 3M	-2.30000*	0.20787	0.000	-3.0431	-1.5569
RT 3M	Initial	.61333	0.20787	0.170	-0.1297	1.3564
	HC 8 cycles	.63000	0.20787	0.147	-0.1131	1.3731
	RT 1M	.68667	0.20787	0.087	-0.0564	1.4297
	RT 2M	.18000	0.20787	0.998	-0.5631	0.9231
	4°C 1M	1.01667*	0.20787	0.003	0.2736	1.7597
	4°C 2M	.84667*	0.20787	0.017	0.1036	1.5897
	4°C 3M	.51667	0.20787	0.363	-0.2264	1.2597
	40°C 1M	-.33333	0.20787	0.865	-1.0764	0.4097

Multiple Comparisons

Particle size

Tukey HSD

(I) sample	(J) sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
	40°C 2M	-.58667	0.20787	0.213	-1.3297	0.1564
	40°C 3M	-2.12000*	0.20787	0.000	-2.8631	-1.3769
4°C 1M	Initial	-0.40333	0.20787	0.687	-1.1464	0.3397
	HC 8 cycles	-0.38667	0.20787	0.734	-1.1297	0.3564
	RT 1M	-0.33000	0.20787	0.871	-1.0731	0.4131
	RT 2M	-0.83667*	0.20787	0.019	-1.5797	-0.0936
	RT 3M	-1.01667*	0.20787	0.003	-1.7597	-0.2736
	4°C 2M	-0.17000	0.20787	0.999	-0.9131	0.5731
	4°C 3M	-0.50000	0.20787	0.406	-1.2431	0.2431
	40°C 1M	-1.35000*	0.20787	0.000	-2.0931	-0.6069
	40°C 2M	-1.60333*	0.20787	0.000	-2.3464	-0.8603
	40°C 3M	-3.13667*	0.20787	0.000	-3.8797	-2.3936
4°C 2M	Initial	-0.23333	0.20787	0.985	-0.9764	0.5097
	HC 8 cycles	-0.21667	0.20787	0.991	-0.9597	0.5264
	RT 1M	-0.16000	0.20787	0.999	-0.9031	0.5831
	RT 2M	-0.66667	0.20787	0.105	-1.4097	0.0764
	RT 3M	-0.84667*	0.20787	0.017	-1.5897	-0.1036
	4°C 1M	0.17000	0.20787	0.999	-0.5731	0.9131
	4°C 3M	-0.33000	0.20787	0.871	-1.0731	0.4131
	40°C 1M	-1.18000*	0.20787	0.000	-1.9231	-0.4369
	40°C 2M	-1.43333*	0.20787	0.000	-2.1764	-0.6903
	40°C 3M	-2.96667*	0.20787	0.000	-3.7097	-2.2236
4°C 3M	Initial	0.09667	0.20787	1.000	-0.6464	0.8397
	HC 8 cycles	0.11333	0.20787	1.000	-0.6297	0.8564
	RT 1M	0.17000	0.20787	0.999	-0.5731	0.9131
	RT 2M	-0.33667	0.20787	0.858	-1.0797	0.4064
	RT 3M	-0.51667	0.20787	0.363	-1.2597	0.2264
	4°C 1M	0.50000	0.20787	0.406	-0.2431	1.2431

Multiple Comparisons

Particle size

Tukey HSD

(I) sample	(J) sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
	4°C 2M	0.33000	0.20787	0.871	-0.4131	1.0731
	40°C 1M	-0.85000*	0.20787	0.016	-1.5931	-0.1069
	40°C 2M	-1.10333*	0.20787	0.001	-1.8464	-0.3603
	40°C 3M	-2.63667*	0.20787	0.000	-3.3797	-1.8936
40°C 1M	Initial	0.94667*	0.20787	0.006	0.2036	1.6897
	HC 8 cycles	0.96333*	0.20787	0.005	0.2203	1.7064
	RT 1M	1.02000*	0.20787	0.003	0.2769	1.7631
	RT 2M	0.51333	0.20787	0.371	-0.2297	1.2564
	RT 3M	0.33333	0.20787	0.865	-0.4097	1.0764
	4°C 1M	1.35000*	0.20787	0.000	0.6069	2.0931
	4°C 2M	1.18000*	0.20787	0.000	0.4369	1.9231
	4°C 3M	0.85000*	0.20787	0.016	0.1069	1.5931
	40°C 2M	-0.25333	0.20787	0.973	-0.9964	0.4897
	40°C 3M	-1.78667*	0.20787	0.000	-2.5297	-1.0436
40°C 2M	Initial	1.20000*	0.20787	0.000	0.4569	1.9431
	HC 8 cycles	1.21667*	0.20787	0.000	0.4736	1.9597
	RT 1M	1.27333*	0.20787	0.000	0.5303	2.0164
	RT 2M	0.76667*	0.20787	0.039	0.0236	1.5097
	RT 3M	0.58667	0.20787	0.213	-0.1564	1.3297
	4°C 1M	1.60333*	0.20787	0.000	0.8603	2.3464
	4°C 2M	1.43333*	0.20787	0.000	0.6903	2.1764
	4°C 3M	1.10333*	0.20787	0.001	0.3603	1.8464
	40°C 1M	0.25333	0.20787	0.973	-0.4897	0.9964
	40°C 3M	-1.53333*	0.20787	0.000	-2.2764	-0.7903
40°C 3M	Initial	2.73333*	0.20787	0.000	1.9903	3.4764
	HC 8 cycles	2.75000*	0.20787	0.000	2.0069	3.4931
	RT 1M	2.80667*	0.20787	0.000	2.0636	3.5497
	RT 2M	2.30000*	0.20787	0.000	1.5569	3.0431
	RT 3M	2.12000*	0.20787	0.000	1.3769	2.8631

Multiple Comparisons

Particle size

Tukey HSD

(I) sample	(J) sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
	4°C 1M	3.13667*	0.20787	0.000	2.3936	3.8797
	4°C 2M	2.96667*	0.20787	0.000	2.2236	3.7097
	4°C 3M	2.63667*	0.20787	0.000	1.8936	3.3797
	40°C 1M	1.78667*	0.20787	0.000	1.0436	2.5297
	40°C 2M	1.53333*	0.20787	0.000	0.7903	2.2764

Table F3. Homogeneous subset of %relative of the particle sizes of the extract-loaded nanoemulsions in various conditions compared to that of initial by Turkey HSD method

Homogeneous Subsets

Tukey HSD

		Subset for alpha = 0.05					
sample	N	1	2	3	4	5	6
4°C 1M	3	25.4333					
4°C 2M	3	25.6033	25.6033				
RT 2M	3	25.7633	25.7633	25.7633			
HC 8 cycles	3	25.8200	25.8200	25.8200			
Initial	3	25.8367	25.8367	25.8367			
4°C 3M	3	25.9333	25.9333	25.9333			
RT 2M	3		26.2700	26.2700	26.2700		
RT 3M	3			26.4500	26.4500	26.4500	
40°C 1M	3				26.7833	26.7833	
40°C 2M	3					27.0367	
40°C 3M	3						28.5700
Sig.	3	0.406	0.105	0.087	0.371	0.213	1.000

Table F4. One-way analysis of variance of the amount scopoletin in the nanoemulsion kept in various conditions at particular time pint compared to time zero

ANOVA

%Relative in amount of scopoletin

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	54.599	9	6.067	1.319	0.288
Within Groups	91.999	20	4.600		
Total	146.598	29			

Table F5. Multiple comparison of the amount scopoletin in the nanoemulsion kept in various conditions at particular time pint compared to time zero by Turkey HSD method

Multiple Comparisons

%Relative in amount of scopoletin Tukey HSD

(I) sample	(J) sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Initial	RT 1M	-2.35955	1.75118	.930	-8.5607	3.8416
	RT 2M	.04618	1.75118	1.000	-6.1550	6.2473
	RT 3M	1.89502	1.75118	.981	-4.3061	8.0961
	4°C 1M	-1.67167	1.75118	.992	-7.8728	4.5295
	4°C 2M	-.37795	1.75118	1.000	-6.5791	5.8232
	4°C 3M	1.31457	1.75118	.999	-4.8866	7.5157
	40°C 1M	.61037	1.75118	1.000	-5.5908	6.8115
	40°C 2M	.62958	1.75118	1.000	-5.5715	6.8307
	40°C 3M	2.01111	1.75118	.972	-4.1900	8.2122
RT 1M	Initial	2.35955	1.75118	.930	-3.8416	8.5607
	RT 2M	2.40572	1.75118	.922	-3.7954	8.6069
	RT 3M	4.25456	1.75118	.360	-1.9466	10.4557
	4°C 1M	.68787	1.75118	1.000	-5.5133	6.8890
	4°C 2M	1.98160	1.75118	.975	-4.2195	8.1827
	4°C 3M	3.67412	1.75118	.548	-2.5270	9.8753
	40°C 1M	2.96991	1.75118	.786	-3.2312	9.1710
	40°C 2M	2.98913	1.75118	.780	-3.2120	9.1903
	40°C 3M	4.37065	1.75118	.327	-1.8305	10.5718
RT 2M	Initial	-.04618	1.75118	1.000	-6.2473	6.1550
	RT 1M	-2.40572	1.75118	.922	-8.6069	3.7954

Multiple Comparisons

%Relative in amount of scopoletin Tukey HSD

(I) sample	(J) sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
	RT 3M	1.84884	1.75118	.984	-4.3523	8.0500
	4°C 1M	-1.71785	1.75118	.990	-7.9190	4.4833
	4°C 2M	-.42413	1.75118	1.000	-6.6253	5.7770
	4°C 3M	1.26840	1.75118	.999	-4.9327	7.4695
	40°C 1M	.56419	1.75118	1.000	-5.6369	6.7653
	40°C 2M	.58340	1.75118	1.000	-5.6177	6.7845
	40°C 3M	1.96493	1.75118	.976	-4.2362	8.1661
RT 3M	Initial	-1.89502	1.75118	.981	-8.0961	4.3061
	RT 1M	-4.25456	1.75118	.360	-10.4557	1.9466
	RT 2M	-1.84884	1.75118	.984	-8.0500	4.3523
	4°C 1M	-3.56669	1.75118	.586	-9.7678	2.6344
	4°C 2M	-2.27297	1.75118	.943	-8.4741	3.9282
	4°C 3M	-.58044	1.75118	1.000	-6.7816	5.6207
	40°C 1M	-1.28465	1.75118	.999	-7.4858	4.9165
	40°C 2M	-1.26544	1.75118	.999	-7.4666	4.9357
	40°C 3M	.11609	1.75118	1.000	-6.0850	6.3172
4°C 1M	Initial	1.67167	1.75118	.992	-4.5295	7.8728
	RT 1M	-.68787	1.75118	1.000	-6.8890	5.5133
	RT 2M	1.71785	1.75118	.990	-4.4833	7.9190
	RT 3M	3.56669	1.75118	.586	-2.6344	9.7678
	4°C 2M	1.29372	1.75118	.999	-4.9074	7.4949
	4°C 3M	2.98625	1.75118	.781	-3.2149	9.1874
	40°C 1M	2.28204	1.75118	.941	-3.9191	8.4832
	40°C 2M	2.30125	1.75118	.939	-3.8999	8.5024
	40°C 3M	3.68278	1.75118	.545	-2.5184	9.8839
4°C 2M	Initial	.37795	1.75118	1.000	-5.8232	6.5791
	RT 1M	-1.98160	1.75118	.975	-8.1827	4.2195
	RT 2M	.42413	1.75118	1.000	-5.7770	6.6253
	RT 3M	2.27297	1.75118	.943	-3.9282	8.4741

Multiple Comparisons

%Relative in amount of scopoletin Tukey HSD

(I) sample	(J) sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
	4°C 1M	-1.29372	1.75118	.999	-7.4949	4.9074
	4°C 3M	1.69252	1.75118	.991	-4.5086	7.8937
	40°C 1M	.98832	1.75118	1.000	-5.2128	7.1894
	40°C 2M	1.00753	1.75118	1.000	-5.1936	7.2087
	40°C 3M	2.38905	1.75118	.925	-3.8121	8.5902
4°C 3M	Initial	-1.31457	1.75118	.999	-7.5157	4.8866
	RT 1M	-3.67412	1.75118	.548	-9.8753	2.5270
	RT 2M	-1.26840	1.75118	.999	-7.4695	4.9327
	RT 3M	.58044	1.75118	1.000	-5.6207	6.7816
	4°C 1M	-2.98625	1.75118	.781	-9.1874	3.2149
	4°C 2M	-1.69252	1.75118	.991	-7.8937	4.5086
	40°C 1M	-.70421	1.75118	1.000	-6.9053	5.4969
	40°C 2M	-.68499	1.75118	1.000	-6.8861	5.5161
	40°C 3M	.69653	1.75118	1.000	-5.5046	6.8977
40°C 1M	Initial	-.61037	1.75118	1.000	-6.8115	5.5908
	RT 1M	-2.96991	1.75118	.786	-9.1710	3.2312
	RT 2M	-.56419	1.75118	1.000	-6.7653	5.6369
	RT 3M	1.28465	1.75118	.999	-4.9165	7.4858
	4°C 1M	-2.28204	1.75118	.941	-8.4832	3.9191
	4°C 2M	-.98832	1.75118	1.000	-7.1894	5.2128
	4°C 3M	.70421	1.75118	1.000	-5.4969	6.9053
	40°C 2M	.01921	1.75118	1.000	-6.1819	6.2203
	40°C 3M	1.40074	1.75118	.998	-4.8004	7.6019
40°C 2M	Initial	-.62958	1.75118	1.000	-6.8307	5.5715
	RT 1M	-2.98913	1.75118	.780	-9.1903	3.2120
	RT 2M	-.58340	1.75118	1.000	-6.7845	5.6177
	RT 3M	1.26544	1.75118	.999	-4.9357	7.4666
	4°C 1M	-2.30125	1.75118	.939	-8.5024	3.8999
	4°C 2M	-1.00753	1.75118	1.000	-7.2087	5.1936

Multiple Comparisons

%Relative in amount of scopoletin Tukey HSD

(I) sample	(J) sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
	4°C 3M	.68499	1.75118	1.000	-5.5161	6.8861
	40°C 1M	-.01921	1.75118	1.000	-6.2203	6.1819
	40°C 3M	1.38152	1.75118	.998	-4.8196	7.5827
40°C 3M	Initial	-2.01111	1.75118	.972	-8.2122	4.1900
	RT 1M	-4.37065	1.75118	.327	-10.5718	1.8305
	RT 2M	-1.96493	1.75118	.976	-8.1661	4.2362
	RT 3M	-.11609	1.75118	1.000	-6.3172	6.0850
	4°C 1M	-3.68278	1.75118	.545	-9.8839	2.5184
	4°C 2M	-2.38905	1.75118	.925	-8.5902	3.8121
	4°C 3M	-.69653	1.75118	1.000	-6.8977	5.5046
	40°C 1M	-1.40074	1.75118	.998	-7.6019	4.8004
	40°C 2M	-1.38152	1.75118	.998	-7.5827	4.8196

Table F6. Homogeneous subset of the amount scopoletin in the nanoemulsion kept in various conditions at particular time pint compared to time zero by Turkey HSD method

Homogeneous Subsets

Tukey HSD

sample	N	Subset for alpha = 0.05: 1
40°C 2M	3	245.0353
RT 1M	3	245.1514
40°C 3M	3	245.7318
40°C 1M	3	246.4168
Initial	3	246.4360
RT 3M	3	247.0002
4°C 2M	3	247.0464
4°C 3M	3	247.4243
RT 2M	3	248.7180
4°C 1M	3	249.4059
Sig.		0.327

Appendix G

In vitro release and permeation studies

Table RRRR The amount of scopoletin from the solution of extract in 40% PG in PBS detected in receptor fluid and in the donor part after 24 hr from release study

Time	Amount of scopoletin (ug/ml)		
	N1	N2	N3
1	374.094	378.537	376.633
2	702.816	698.288	726.024
4	1295.029	1267.839	1333.975
6	1750.217	1737.613	1824.233
8	2356.253	2249.121	2181.617
12	3001.038	2887.472	2777.947
16	3377.407	3264.500	3092.144
20	3580.466	3529.844	3316.404
24	3609.228	3574.184	3382.520
Donor part	49.409	64.422	91.084
Total	3658.637	3638.606	3473.604
% Mass balance	99.95	99.40	98.51

Table RRRR The percentage of cumulative amount of scopoletin from release study of the solution of extract in 40% PG in PBS

Time	% cumulative amount				
	N1	N2	N3	Mean	SD
1	10.22	10.34	10.68	10.41	0.2391
2	19.20	19.08	20.59	19.62	0.8401
4	35.38	34.64	37.83	35.95	1.6717
6	47.81	47.47	51.73	49.01	2.3687
8	64.37	61.44	61.87	62.56	1.5813
12	81.98	78.88	78.78	79.88	1.8214
16	92.27	89.18	87.69	89.71	2.3339
20	97.81	96.43	94.05	96.10	1.9038
24	98.60	97.64	95.93	97.39	1.3552

Table RRRR The amount of scopoletin from extract-loaded nanoemulsion detected in receptor fluid and in the donor part after 24 hr from release study

Time	Amount of scopoletin (ug/ml)		
	N1	N2	N3
1	42.109	36.200	41.193
2	67.233	63.049	68.438
4	112.199	101.191	112.018
6	143.351	131.199	139.525
8	158.387	152.587	159.472
12	187.011	187.620	187.560
16	205.458	204.658	201.473

Time	Amount of scopoletin (ug/ml)		
	N1	N2	N3
20	217.730	217.359	210.311
24	222.602	222.186	214.733
Donor part	14.919	15.598	24.230
Total	237.521	237.784	238.962
% Mass balance	97.06	97.59	98.08

Table RRRR The percentage of cumulative amount of scopoletin from release study of extract-loaded nanoemulsion

Time	% cumulative amount				
	N1	N2	N3	Mean	SD
1	17.21	14.86	16.91	16.32	1.2787
2	27.47	25.88	28.09	27.15	1.1416
4	45.85	41.53	45.97	44.45	2.5299
6	58.58	53.85	57.26	56.56	2.4422
8	64.72	62.63	65.45	64.27	1.4671
12	76.42	77.00	76.98	76.80	0.3306
16	83.96	84.00	82.69	83.55	0.7435
20	88.97	89.21	86.32	88.17	1.6061
24	90.96	91.19	88.13	90.09	1.7042

Table RRRR The amount of scopoletin from the solution of extract in 40% PG in PBS detected in receptor fluid and in the donor part after 48 hr in permeation study

Time	Amount of scopoletin (ug/ml)		
	N1	N2	N3
2	0.902	0.958	0.752
4	2.316	2.303	2.320
8	5.825	6.343	5.629
12	9.358	10.393	7.572
16	13.102	13.133	9.374
20	16.889	15.829	13.723
24	21.332	20.493	18.892
32	30.066	30.447	24.689
48	50.301	47.684	40.820
Donor part	3434.235	3600.027	3428.015
Skin	19.481	20.410	15.636
Total	3504.017	3728.121	3484.471
% Mass balance	93.18	99.14	98.82

Table RRRR The cumulative amount per unit area of scopoletin from the solution of extract in 40% PG in PBS in permeation study

Time	Cumulative amount per unit area (ug/cm^2)				
	N1	N2	N3	Mean	SD
2	0.1055	0.1121	0.0880	0.1018	0.0125
4	0.2709	0.2694	0.2714	0.2706	0.0010
8	0.6814	0.7420	0.6585	0.6939	0.0432
12	1.0947	1.2157	0.8858	1.0654	0.1669
16	1.5326	1.5363	1.0965	1.3885	0.2528
20	1.9756	1.8516	1.6053	1.8109	0.1885
24	2.4954	2.3972	2.2099	2.3675	0.1450
32	3.5170	3.5616	2.8881	3.3222	0.3767
48	5.8841	5.5780	4.7750	5.4124	0.5728

Table RRRR The amount of scopoletin from the extract-loaded nanoemulsion detected in receptor fluid and in the donor part after 48 hr in permeation study

Time	Amount of scopoletin (ug/ml)		
	N1	N2	N3
2	0.177	0.281	0.121
4	0.784	0.758	0.708
8	1.921	2.235	1.995
12	2.686	3.365	2.981
16	3.765	4.832	3.829
20	5.081	6.219	4.849
24	6.238	7.382	6.337
32	8.489	9.336	9.058
48	14.457	14.771	13.348
Donor part	231.077	221.985	217.966
Skin	1.923	1.445	1.548
Total	247.457	238.201	232.862
% Mass balance	98.58	94.89	95.57

Table RRRR The cumulative amount per unit area of scopoletin from extract-loaded nanoemulsion in permeation study

Time	Cumulative amount per unit area (ug/cm^2)				
	N1	N2	N3	Mean	SD
2	0.0207	0.0329	0.0142	0.0226	0.0095
4	0.0917	0.0887	0.0828	0.0877	0.0045
8	0.2247	0.2614	0.2334	0.2398	0.0192
12	0.3142	0.3936	0.3487	0.3522	0.0398
16	0.4404	0.5652	0.4479	0.4845	0.0700

Time	Cumulative amount per unit area (ug/cm^2)				
	N1	N2	N3	Mean	SD
20	0.5944	0.7275	0.5672	0.6297	0.0858
24	0.7297	0.8635	0.7413	0.7782	0.0741
32	0.9930	1.0921	1.0596	1.0482	0.0505
48	1.6911	1.7279	1.5614	1.6601	0.0875

Table RRRR The amount of scopoletin from the extract-loaded conventional emulsion detected in receptor fluid and in the donor part after 48 hr in permeation study

Time	Amount of scopoletin (ug/ml)		
	N1	N2	N3
2	0.092	0.150	0.103
4	0.342	0.411	0.243
8	0.746	0.699	0.502
12	1.021	1.403	1.074
16	1.589	1.996	1.801
20	2.085	2.399	2.393
24	2.400	2.806	3.064
32	3.515	3.746	4.351
48	5.439	5.916	6.544
Donor part	203.479	210.173	210.400
Skin	1.985	2.139	1.756
Total	210.903	218.228	218.700
% Mass balance	93.44	96.16	96.37

Table RRRR The cumulative amount per unit area of scopoletin from extract-loaded conventional emulsion in permeation study

Time	Cumulative amount per unit area (ug/cm^2)				
	N1	N2	N3	Mean	SD
2	0.0108	0.0175	0.0120	0.0135	0.0036
4	0.0400	0.0481	0.0284	0.0388	0.0099
8	0.0873	0.0818	0.0587	0.0759	0.0151
12	0.1194	0.1641	0.1256	0.1364	0.0242
16	0.1859	0.2335	0.2107	0.2100	0.0238
20	0.2439	0.2806	0.2799	0.2682	0.0210
24	0.2807	0.3282	0.3584	0.3225	0.0392
32	0.4112	0.4382	0.5090	0.4528	0.0505
48	0.6362	0.6920	0.7655	0.6979	0.0648

VITA

Miss Panida Borisut was born on October 1983, in Bangkok, Thailand. She graduated the Bachelor degree from the Faculty of Pharmaceutical Sciences, Chulalongkorn University in 2006. She had been worked at S&J International Enterprise, plc., Bangkok, Thailand in department of research and development in cosmetic products for two years before enrolling the Master's Degree of International Pharmaceutical Technology at Chulalongkorn University in Bangkok, Thailand.