Preparation and Investigation of Antioxidant, Antibacterial and Antityrosinase Potentials of O/W Nanoemulsions Containing Extract of *Tamarindus indica* L. Fruit Pulp

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ABSTRACT

Tamarindus indica L. or tamarind has been used for medical purposes since ancient time. In addition, interesting biological activities of tamarind make it appropriate for cosmetic or hygienic applications. In the present work, tamarind fruit pulp extract was selected as an active ingredient, aiming for cosmetic uses. The main objectives of the current study were to formulate o/w nanoemulsions as a carrier vehicle for tamarind fruit pulp extract, to investigate the stability of tamarind fruit pulp extract loaded nanoemulsions and to assess the antioxidant, antityrosinase and antibacterial potentials of the tamarind fruit pulp extract loaded nanoemulsions. In the beginning, four samples of tamarind fruit pulp extracts (both sour and sweet types) were investigated for their bioactivities which were antioxidant, antityrosinase, and antibacterial properties against two gram positive bacteria which were *Staphylococcus aureus* and *Staphylococcus epidermidis*. Two strains of bacteria commonly cause malodor in axilla areas. Owing to its outstanding bioactivities, the sweet tamarind fruit pulp extract from Guangzhou Phytochem Sciences Inc., China was chosen. Two concentrations of the sweet tamarind fruit pulp extract, 3.3%w/w and 6.6%w/w, which was 50-times and 100-times of EC_{50} of antioxidant activity, were employed. For the formulation process, the blank nanoemulsions were firstly prepared and the optimum formulation would be selected as a vehicle for the sweet tamarind fruit pulp extract. The blank nanoemulsions consisted of 5%w/w surfactant mixture (Tween 80 and Span 80, as an emulsifier), synthetic oil (caprylic/capric triglyceride, as an emollient), water (as a vehicle), glycerine (as a humectant). Apart from the basic ingredients of nanoemulsions (oil phase and water phase), the hydrocolloids with different charge types: xanthan gum (anionic), guar gum (nonionic) and cationic guar

gum (as thickeners) were also incorporated into the formulations and the influence of the charge types on the physicochemical properties of the nanoemulsions were also examined. The concentrations of the hydrocolloids used were 0.10 and 0.25%w/w. Seven formulations (F1-F7) of blank nanoemulsions were prepared using a high pressure homogenization process. The prepared blank nanoemulsions were examined for types, physicochemical properties (pH, viscosity, droplet size, zeta potential, polydispersity index (PDI)) and physical stability under stress conditions: 4 freeze-thaw cycles and storage for 1 month at three various temperatures, 4 ± 2°C, room temperature (30 ± 2°C) and 45 ± 2°C. It was found that all blank formulations were indicated as o/w using dye solubility and conductivity tests. The pH values of the formulations were between 6 to 7. The formulations with and without hydrocolloids displayed low viscosity. The droplet sizes of the blank nanoemulsions were in the range of 100-800 nm. The types of hydrocolloids affected the droplet sizes and the zeta potential of blank nanoemulsions. In general, the increased droplet sizes were observed in the formulations containing hydrocolloids. The freshly prepared nanoemulsions containing cationic guar gum displayed net positively charged zeta potential whereas the rest showed negative charge values. However, the surface charge of the formulations containing cationic guar gum changed to negative after 1 month stability test. The formulation without any hydrocolloids (F1) had the highest zeta potential (-63 mV) and low PDI, <0.2 with droplet sizes less than 200 nm. Therefore, the blank nanoemulsion without hydrocolloids (F1) was chosen to produce tamarind fruit pulp extract loaded nanoemulsions (lotions). The tamarind fruit pulp extract loaded nanoemulsions were checked for types, physicochemical properties, physical and chemical stabilities for 2 months as well as bioactivity properties as previously mentioned. The tamarind extract loaded formulations remained o/w type-nanoemulsions. For both concentrations (3.3 and 6.6%w/w), there were significant differences in physicochemical properties, in particular, conductivity values, pH and viscosity, between the blank nanoemulsions and tamarind extract loaded nanoemulsions ($p < 0.05$). Incorporation of tamarind fruit pulp extract into the blank nanoemulsions (F1) resulted in a decreased pH and a markedly increased viscosity and conductivity of the formulations ($p < 0.05$). Overall, the tamarind extract loaded nanoemulsions had droplet sizes approximately 130 nm, zeta potential about -38 mV

and PDI values less than < 0.2. The formulation which contained 3.3% tamarind fruit pulp extract (F1-3.3TE) exhibited better physicochemical properties as well as physical and chemical stabilities when compared with the formulation containing 6.6% tamarind fruit pulp extract (F1-6.6TE). The formulation which contained 6.6%w/w tamarind extract was not stable (phase separation) after storage at three temperatures for 2 months. According to high performance liquid chromatography (HPLC) analysis, tartaric acid, as a marker in tamarind fruit pulp extract, remained greater than 90% under stress conditions in the formulation F1-3.3TE. Using synthetic membrane and diffusion cells (in-house construction), similar to modified Franz diffusion cells, the *in vitro* release results revealed that the release profiles of tartaric acid (tamarind fruit pulp extract) from o/w nanoemulsions were fitted best to Higuchi model with the highest r^2 of 0.97. In the case of bioactivity investigation, both tamarind fruit pulp extract loaded nanoemulsions were found to have antioxidant activity (2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay), antityrosinase property (Dopachrome technique) and antibacterial activity (agar well diffusion technique) against *Staphylococcus aureus* and *Staphylococcus epidermidis*. The most promising biological property of the tamarind fruit pulp extract loaded nanoemulsion was antibacterial activity since the antioxidant and antityrosinase activities of the formulations were declined under the stress temperature conditions. In conclusion, the current results showed the potential of developed tamarind fruit pulp extract loaded nanoemulsions for cosmetic application such as antiaging, skin lightening preparations and deodorant products.

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LIST OF ABBREVIATIONS AND SYMBOLS

Abs	=	absorbance
^{o}C	=	degree Celsius
CGG	=	cationic guar gum
Conc.	=	concentration
DPPH	=	2,2-diphenyl-1-picrylhydrazyl
EC_{50}	=	half maximal effective concentration
et al.	=	and others
etc.	=	and other things
F1-3.3TE	=	blank nanoemulsion with 3.3% tamarind fruit pulp extract
F1-6.6TE	=	blank nanoemulsion with 6.6% tamarind fruit pulp extract
g	=	gram
GG	=	guar gum
>	=	greater than
<	=	lower than
h	=	hour
IC_{50}	=	half maximal inhibitory concentration
ICH	=	International Conference on Harmonization
M	=	molar

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

mM	=	millimolar
mg	=	milligram
min	=	minute
MBC	=	minimum bactericidal concentration
MIC	=	minimum inhibitory concentration
mL	=	milliliter
MW	=	molecular weight
µg	=	microgram
µg/mL	=	microgram per milliliter
µL	=	microliter
µS	=	microsiemens
n	=	number of sample
nm	=	nanometer
p	=	probability
PDI	=	polydispersity index
pH	=	power of hydrogen
q.s.	=	a sufficient quantity
rpm	=	revolutions per minute
r^2	=	coefficient of determination
RSD	=	relative standard deviation

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

SD	=	standard deviation
USP	=	United States Pharmacopeia
w/v	=	weight by volume
w/w	=	weight by weight
XG	=	xanthan gum

CHAPTER I
INTRODUCTION

1.1. Background and rationale

Plant extracts and natural compounds have been introduced as active ingredients in a variety of topical cosmetic products such as antiaging products, moisturizers, deodorants, and decorative products. For topical application, botanical additives are considered safe by the United States Food and Drug Administration (Draelos, 2005). *Tamarindus indica* L. named as tamarind is one of the plants commonly used in cosmetic products. It is a plant with multiple medical purposes, such as antimicrobial, antioxidant, antityrosinase, and analgesic (Menezes *et al.*, 2016). Various parts of tamarind including seeds, barks, roots, leaves, and fruits have been used for culinary, chemicals, textiles, pharmaceutical, and cosmetics (Menezes *et al.*, 2016; Saideswara and Mary, 2012). Among these parts, the fruit pulp is drawing attention due to several reported bioactive activities. The crude extract of tamarind fruit pulps contains several organic acids which are tartaric acid, acetic acid, formic acid, and malic acid. These organic acids possess antioxidant properties with improving the efficacy of superoxide dismutase, catalase and glutathione peroxide in animals (Maenthaisong *et al.*, 2009; Menezes *et al.*, 2016). According to Gupta *et al.* (2015), tamarind fruit pulp increased collagen content in rats. Other studies from Maenthaisong *et al.* (2009) and Vardhan and Pandey (2014) indicated that tamarind fruit pulp extract had potential skin lightening. This was possibly due to certain compounds, such as polyphenols or aromatic acids in tamarind fruit pulp extract, could interfere with melanin synthesis. In addition, Abukakar *et al.* (2008) found that the aqueous tamarind fruit pulp extract had antibacterial activities which inhibited the growth of the test microorganism: *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*.

Application of certain bioactive properties of tamarind fruit pulp extract for cosmetic purpose is an interesting approach. The antioxidant and antityrosinase activities are appropriate for antiaging and skin lightening products. Skin aging can be classified into two types, which are intrinsic aging (natural aging process) and extrinsic aging. A number of characteristic symptoms of intrinsic aging are fine

wrinkles of the skin, seborrheic keratosis, and cherry hemangiomas. Extrinsic aging involves external, environment factors. Long exposure to the sunlight, especially, ultraviolet ray, can cause photoaging or photodamage. The significant signs of extrinsic aging include hyperpigmentation, age spots and coarsely wrinkled skin (Rahvar, 2014). To some extent, these skin conditions, especially, intrinsic aging and photoaging are associated with oxidative stress (Saliou *et al.*, 2014). The use of antioxidant will alleviate or prevent these signs of aging. Skin color of human is greatly affected by melanin pigment. Melanogenesis, a process of melanin production, involves enzyme. The most important one is tyrosinase, which converts L-tyrosine to several intermediate substances and eventually to melanin pigment (D'Mello *et al.*, 2016). Accordingly, tyrosinase inhibitors in skin lightening products will help to obtain skin lighter complexion. In the case of antioxidant property, such activity of the tamarind fruit pulp extract can be useful for personal care products (e.g. deodorants and antibacterial soaps). Skin microflora, especially coryneform bacteria and micrococcaceae e.g. *Staphylococcus epidermidis*, is responsible for malodor of underarm. Sweat, mainly secreted by eccrine gland and secretion of apocrine gland such as isovaleric acid and androstenone, plays a role in underarm odor (Schreiber, 2014). Antibacterial agents in deodorants will reduce underarm odor by destroying or suppressing the growth of such bacteria. The use of natural active ingredients like tamarind fruit pulp extract will decrease the undesirable side effects which are normally caused by synthetic chemicals. For example, it can reduce the risk of resistance to common antibiotics by replacing the normal antibacterial agents like triclosan in deodorant products.

The crucial factors that can affect the activities of pharmaceutical/cosmetic products are the vehicles (carries) and technologies used on the delivery process of active substances into/through the skin. The skin layer which is known as a formidable barrier is the stratum corneum. Nanotechnology could be an effective tool to allow active ingredients to exert their action. Nowadays, nanotechnology has been widely employed in topical and transdermal formulations such as nanoparticles and nanoemulsions. Nanoemulsions are kinetically stable dispersions of two different liquids (oil and water) stabilized by an emulsifier (generally surfactant or surfactant system) (Sharma *et al.*, 2010). In recent years, the use of nanoemulsions increase

dramatically as potential vehicles for delivering of cosmetics and for optimizing liquidation of active ingredients in the receptor (Sharma and Sarangdevot, 2012). Nanoemulsions are relatively stable physically in comparison with conventional emulsions due to their small droplet size (20-500 nm) (Gupta *et al.*, 2016). Other benefits of nanoemulsions include aesthetic appearance, active ingredients easily penetrate into/through the skin and ease of application (Gupta *et al.*, 2016; Sharma *et al.*, 2010). To date, very few studies have been carried out to formulate nanoemulsions as vehicles for tamarind fruit pulp extract. Previously, some researchers prepared matrix patch and cleansing lotion (emulsion) as carriers for tamarind pulp extract (Maenthaisong *et al.*, 2009; Viyoch *et al.*, 2003). Based on the aforementioned reasons, nanoemulsions had been selected as a carrier for the extract of tamarind fruit pulp.

In the present study, topical oil-in-water (o/w) nanoemulsions (lotions) were prepared using high pressure homogenization. This method was selected to produce the blank nanoemulsions since it was the most used technique when the plant extracts were involved (Zorzi *et al.*, 2015). The ordinary emulsions were firstly prepared using cold process emulsifiers (e.g. Simulgel FL) or conventional liquid emulsifiers such as nonionic surfactants (e.g. Tween 80 and Span 80) to avoid heating and to reduce the time. Nonionic surfactants are less toxic and irritant than ionic surfactants (Eccleston, 2013). Synthetic oils such as caprylic/capric triglyceride were used as an emollient in the oil phase. Several advantages of synthetic oil have been mentioned, for example, clear, colorless, odorless, good spreading on the skin, no tackiness and good stability (Severino *et al.*, 2011). Owing to heat generated during the high pressure homogenization process, the tamarind fruit pulp extract was later incorporated into the blank nanoemulsions. The concentrations of tamarind fruit pulp extract used were based on the bioactivity studies (here 3.3 and 6.6%w/w). The tamarind fruit pulp extract was in the external phase, water, so that it would be easily released from the vehicles. Other valuable properties of tamarind fruit pulp extract loaded nanoemulsions (o/w) were non-tacky, non-greasy, and cooling effects from the water evaporation.

1.2. The objectives of the research

1.2.1 To formulate and study physicochemical properties of nanoemulsions containing extract of *Tamarindus indica* L. fruit pulp

1.2.2. To investigate the stability of nanoemulsions containing extract of *Tamarindus indica* L. fruit pulp

1.2.3. To evaluate antioxidant, antibacterial, and antityrosinase activities of nanoemulsions containing extract of *Tamarindus indica* L. fruit pulp

1.3. The expected outcomes of the research

1.3.1. To obtain stable nanoemulsions containing extract of *Tamarindus indica* L. fruit pulp with effective bioactive properties.

1.3.2. To obtain nanoemulsions containing extract of *Tamarindus indica* L. fruit pulp with appropriate physicochemical properties as follows: the viscosity in range of lotions (typically below 50,000 centipoise), the range of skin pH (4-6), good appearance and spreadability as lotion.

CHAPTER 2
LILERATURE REVIEWS

2.1. Description of *Tamarindus indica* L.

Naturally, plants have been used as dependence sources of medicines from generation to generation (Nwodo *et al.*, 2011). Plants contain a lot of valuable substances which make great contribution in treating sickness and preventing symptoms. One of them is *Tamarindus indica* (Linn) or tamarind **(Figure 2.1)**. *Tamarindus indica* L. is belonged to Leguminosea (Fabaceae) family. It is native to tropical Africa such as Sudan and Ethiopia. Now, it is widely distributed to other tropical climate areas such as Thailand and Indonesia (Wyk, 2015). There are two different types of tamarind which are acidic and sweet fruit. The sweet fruit contains more glucose than acidic fruit (Azad, 2018).

All parts of tamarind are almost useful. Tamarind seed comprises the seed coat (20-30 per cent) and the kernel or endosperm (70-75 per cent). The seed in tamarind is around 40 per cent of the total fruit (Bagul *et al.*, 2015). Tamarind seed was reported to possess antioxidant (Lourith *et al.*, 2009), anti-inflammatory and analgesic properties (Hivrale *et al.*, 2013). The stem bark and leaves were used as decoction mixed with potash for treating stomach disorder (Komutarin *et al.*, 2004). According to Mulyani *et al.* (2017), tamarind leaves extract loaded cream could inhibit collagenase with IC_{50}: 1,792.75 μg/mL.

Nowadays, tamarind fruit is economically important and most valuable, especially the pod. Pods have length and width around 7.5-20 cm and 2.5 cm, respectively. Pods are scurfy and slightly constricted between the brown-ash seeds. In endocarp, there are around 3-12 seeds covered by brown-red, sweetish-acidic, and consumed pulp (Bhusari *et al.*, 2014). The pulp comprises organic acids (e.g. tartaric acid, acetic acid, citric acid, malic acid and succinic acid), amino acids, invert sugar (25-30%), and some thiazoles (2-ethylthiazole, 2-methylthiazole) as fragrances (Bhadoriya *et al.*, 2011).

Using high performance liquid chromatography (HLPC) technique, various substances have been detected in the extract of tamarind fruit pulp powder (Tril *et al.*, 2014) **(see Table 2.1)**.

Table 2.1. Percentage of sugars and organic acids contained in tamarind fruit pulp extract (Tril *et al.*, 2014).

Constituents	Amount (mg/g)
Glucose	155.65 ± 2.93
Fructose	113.51 ± 2.38
Malic acid	21.64 ± 1.65
Tartaric acid	40.97 ± 1.70
Fumaric acid	0.07 ± 0.01

Different organic acids are found in tamarind fruit pulp such as tartaric acid (8-23.8%), lactic acid (2%), citric acid, and malic acid (Toungos, 2019). Chemical structures of some organic acids are shown in **Figure 2.2**. The organic acid is an uncommon plant acid, which is conducted from the primary carbohydrate products of photosynthesis, and once formed, it cannot be found in the other plants because of the specific enzymes. The tartaric acid is synthesized in tamarind leaves under the UV light and transferred to the flowers and fruits (El-Siddig, 2006).

Figure 2.1. *Tamarindus indica* L.
(https://en.wikipedia.org/wiki/File:Tamarind_clara.jpg [Accessed: March 29, 2019])

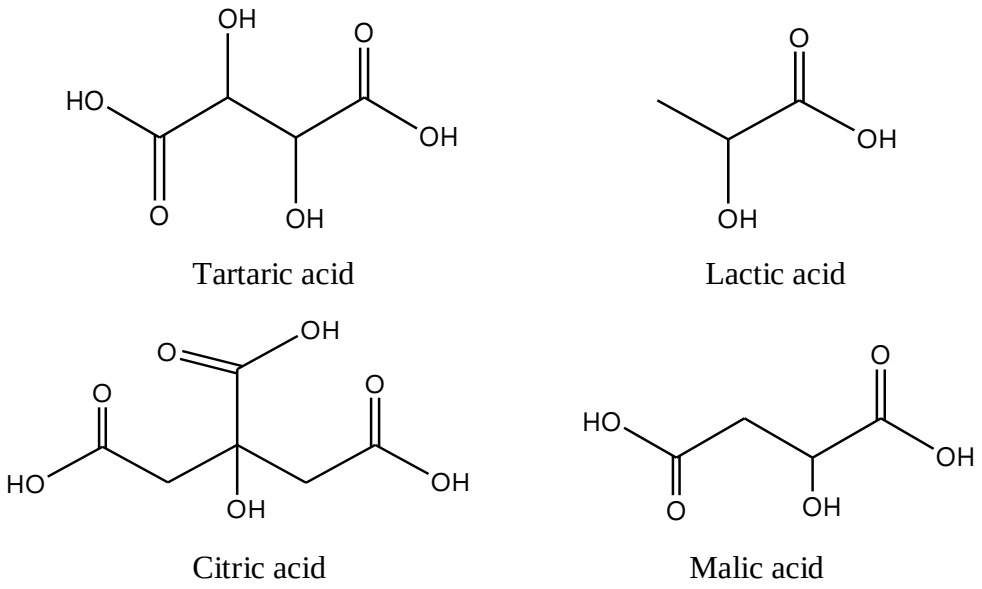


Figure 2.2. Chemical structures of some organic acids contained in tamarind fruit pulp (El-Siddig, 2006).

The highest content of tartaric acid is found in new leaves and the content reduces with time and seasonal variations. The content of tartaric acid in leaves decreases from 28 to 12% because it transfers to the fruit during ripening. In that time, the acidity does not disappear and induces sugar levels. Therefore, tamarind is well-known as the most acidic and sweetest fruit (El-Siddig, 2006).

2.2. Antioxidant properties of *Tamarindus indica* L.

Antioxidant activity of plant is usually connected to phenolic compounds. Phenolic compounds have common structures which can reduce radical with giving hydrogen atom and singlet oxygen scavengers between other reaction mechanisms. Stabilizing or destroying free radical is one of the antioxidant mechanism at the cellular level to prevent damaging of cell structures (Ali and Shah, 2010). The study conducted by Bhadoriya *et al.* (2011) identified that several parts of tamarind such as seeds, fruits, and leaves were organic sources of antioxidants which can be alternative to replacing synthetic antioxidants. However, plan extracts contain very complex substances that can show antioxidant and pro-oxidant properties (Kähkönen *et al.*, 2001).

Radical scavenging activity can be performed using the radical 1,1-diphenyl-2-picryl hydrazyl (DPPH) (Wyk, 2015). Antioxidant substances in samples will react with DPPH and convert to 2,2-diphenyl-1-picryl hydrazine. The level of colorless of DPPH in solution indicates the scavenging activity of antioxidant substances in samples. The strongest radical has the lowest the half maximal inhibitory concentration (IC_{50}) value. The results are usually performed as total antioxidant capacity (TTA) with minimal modification. To investigate the scavenging activity, the absorbance of sample and control are measured using spectrophotometer and is calculated as follows:

$$\text{Scavenging activity (\%)} = \left[1 - \left(\frac{\text{absorbance of sample}}{\text{absorbance of control}}\right)\right] \times 100 \quad \textbf{Eq. 2.1}$$

According to Ugwuona and Onweluzo (2013), aqueous extract of tamarind fruit pulp had lower IC_{50} value than that of the ethanol extract, which were 7.32% and 38.17%, respectively. From the results, it could be seen that the scavenging activity of aqueous extract was higher than that of the ethanol extract. Another research conducted by Atawodi *et al.* (2014) revealed that the *in vitro* antioxidant activity of tamarind fruit pulp was 143 ± 3.5 µg TE/g (Trolox as standard), which was higher than the other parts of tamarind plant.

Amir *et al.* (2016) reported that *Tamarindus indica* L. fruit extract reduced liver toxicity from anti-tubercular drug by antioxidant activity. The presence of polyphenols and flavonoids have been recognized as an antioxidant agent, acting as radical terminators with therapeutic and physiological function (Atawodi *et al.*, 2007; Ullah and Khan, 2008). It was revealed that the polyphenols and flavonoids had variation contents in different parts of tamarind and the highest level was found in fruit pulp of the plant (Atawodi *et al.*, 2014).

2.3. Antibacterial properties of *Tamarindus indica* L.

Resistance of antibiotic drugs is one of the reasons why human beings explore new isolated compounds from plant extracts as antimicrobial herbal medicines. The ability of plants in synthesizing beneficial chemicals can be useful for human to prevent bacterial infection (Abukakar *et al.*, 2008). However, sometimes these

compounds have a lot of functions in human which can be overlapping action, such as antioxidant effect, decreasing of enzyme production, and reduction of inflammation.

Tamarind was reported as a potential antibacterial agent against the clinical human pathogens and could be beneficial in the field of therapeutics (Srinivas and Raja, 2017). Srinivasan *et al.* (2001) found that water extract of tamarind fruit pulp was effective against gram-negative and gram-positive bacteria and fungi.

Gram-negative bacteria such as *Escherichia coli* and *Enterobacter aerogenes* have thick lipid content in cell walls while gram-positive bacteria such as *Staphylococcus epidermidis* and *Bacillus subtilis* contains teichoic acid in peptidoglycan layer. This makes it a formidable barrier for the penetration of several antibiotic molecules. In addition, bacterial enzymes are capable of breaking down foreign molecules in the periplasmic space thus increasing antibiotic drug resistance (Shan *et al.*, 2007). According to Gupta *et al.* (2016), the aqueous-ethanolic extract of tamarind has better antibacterial activity against gram-positive bacteria than gram-negative bacteria. The diameters of inhibition zone of *Staphylococcus aureus* and *Staphylococcus epidermidis*, gram-positive bacteria, were 19.0 mm and 18.0 mm, respectively whereas the inhibition zone of *Pseudomonas aeruginosa*, gram-negative bacteria, was 16.0 mm. These inhibition zones were larger than that of sodium propionate, a positive control. The same results were observed by Nwodo *et al.* (2011); the cold water extract of tamarind fruit pulp showed antibacterial activity against all of the nondiarrhea-genic bacterial strains. The pH of fruit pulp is useful for controlling and developing the spora growth of all microbes including bacteria (Srinivasan *et al.*, 2001).

2.4. Antityrosinase properties of *Tamarindus indica* L.

Melanin is a pigment that can give color for eyes, hair, and skin in humans. Melanocytes cells are the place for producing melanin which is later distributed to stratum basale through a physiological process called melanogenesis (Claus and Decker, 2006). There are two types of melanin pigment, which are eumelanin and pheomelanin. Eumelanin is black or brown pigment while pheomelanin is red or yellow pigment **(Figure 2.3)** (Commo *et al.*, 2004). The essential function of melanin is to absorb UV sunlight and to remove reactive oxygen species for damaging UV

light. Increasing melanin production can cause hyperpigmentation in epidermis and dermis (Briganti *et al.*, 2003). Hyperpigmentation depends on both the activity of tyrosinase enzyme and increased numbers of melanocytes. In enzymatic conversion of L-tyrosine, the melanocyte will be responsible for biosynthesis of melanin. However, the production of melanin is not always the same in every part of the skin, especially in skin face. Nowadays, hyperpigmentation is the phenomenon which makes cosmetic industries develop the products to reduce it. A number of natural ingredients are used in cosmetic formulations based on their properties as antioxidant and antityrosinase activities (Azad, 2018).

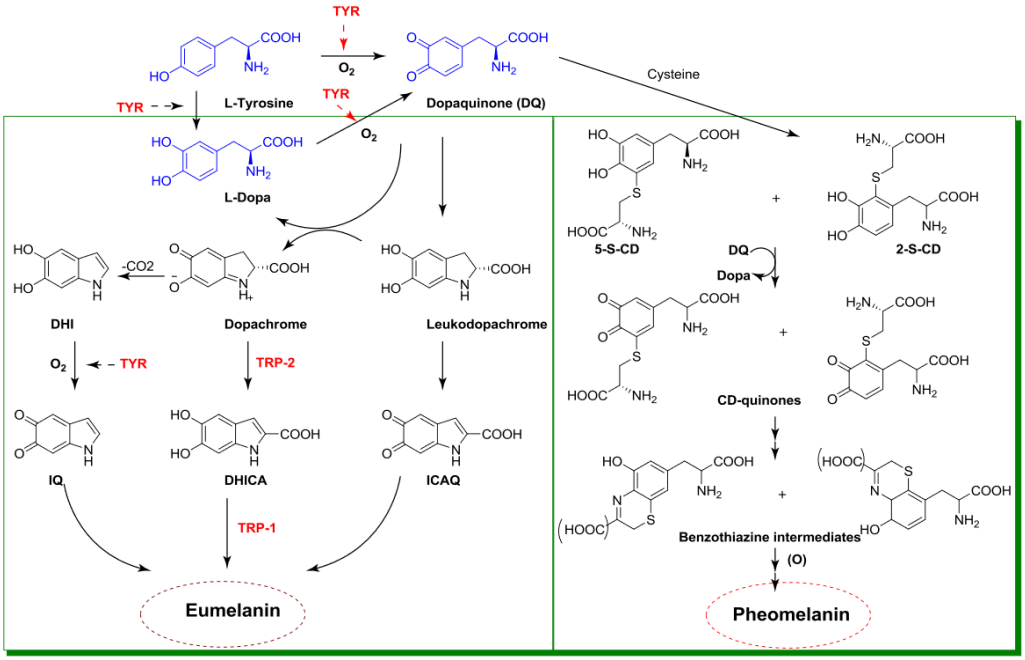


Figure 2.3. Melanogenesis pathway (Pillaiyar *et al.*, 2017). *Abbreviations:* TYR, tyrosinase; DQ, dopaquinone; L-Dopa, L-3,4-dihydroxyphenylalanine; DHICA, 5,6-dihydroxyindole-2 carboxylic acid; DHI, 5,6-dihydroxyindole; ICAQ, indole-2-carboxylic acid-5,6-quinone; IQ, indole-5,6-quinone; HBTA, 5-hydroxy-1,4-benzothiazinylalanin.

Tyrosinase inhibitors are substances which can decrease enzymatic reaction, such as melanisation of human skin. The inhibition of tyrosinase capability is depending on hydroxyl groups of phenolic compounds that can bond to active site of the enzyme to reduce enzymatic activity (Baek *et al.*, 2008). The study conducted by

Thongmuang and Sudjaroen (2013) revealed that % tyrosinase inhibition of methanolic extract of tamarind fruit was 52.13 ± 0.42 while gallic acid as a positive control had % inhibition 51.02 ± 0.45. The rate of tyrosinase activity of tamarind extract was the same as gallic acid. Another research reported by Vardhan and Pandey (2014) found that 20% fruit pulp extract of tamarind had % tyrosinase inhibition 100.99% compared to 0.05% kojic acid which had % inhibition 87.16%.

Antityrosinase activity can be determined with dopachrome method with L-DOPA (L-3,4-dihydroxyphenylalanine) as a substrate with little modification (Liu *et al.*, 2009). Dopachrome, which is intermediate between tyrosine and melanin, is a cyclization product of L-DOPA (Wang *et al.*, 2018). The absorbance of extracts or substances and a positive control is generally measured at 475 nm as dopachrome (red) was converted to dihydroxyindole (colorless). The percentage of tyrosinase inhibition (I%) is calculated as follow:

$$I\% = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100 \qquad \textbf{Eq. 2.2}$$

where $A_{control}$ is the absorbance of the control reaction. Mostly, kojic acid is used as a positive control because its well-documented as a tyrosinase inhibitor. A_{sample} is absorbance of extracts or references that will be determined as antityrosinase (Salleh *et al.*, 2014).

2.5. Related studies of tamarind fruit pulp extract in dermatological formulations

In some research, tamarind fruit pulp extract has been employed in dermatological formulations. Viyoch *et al.* (2013) reported that o/w emulsion loaded tamarind fruit pulp extract for facial skin cleansing had the suitable physical characteristics and stability and also in the range of skin pH. In this study, the percentage of tartaric acid, the highest component in the pulp extract, was found to be 28.49 ± 0.43 %w/w.

Maenthaisong *et al.* (2009) prepared 8% w/w of the aqueous extract of tamarind fruit pulp (2% w/w of tartaric acid) loaded oil-in-water emulsion as cleansing lotions. They tested the products with female volunteers, who applied the test product and placebo (emulsion without extract) on each side of the face. The test product containing tamarind extract exhibited lower significant melanin value than the

placebo did ($p<0.005$). Volunteers' satisfaction scores (Asian skin type) of the cleansing effect and lightening effect were better than those of the placebo ($p<0.05$).

In another study, tamarind fruit pulp extract (lyophilized powder) was incorporated into bio-adhesive hydrogel patches using crosslinked chitosan–starch as polymeric matrix. The patches were developed in order to control the release of alpha hydroxyl acids (e.g. tartaric acid) in tamarind extract. Polymer type, polymer ratio and degree of crosslinking were varied. The amount of tartaric acid released from the patches was found to be fitting with Higuchi's model (Viyoch *et al.,* 2005).

2.6. Emulsions and nanoemulsions

Emulsions are a system with two type different liquids, which are water and oil. These two liquids can be a dispersion by the presence of a third compound: the emulsifier. There are two types of emulsion which are oil-in water emulsion (o/w) and water-in-oil emulsion (w/o). Emulsion (w/o) contains water droplets dispersed in oil phase, while emulsion (o/w) contains oil droplets dispersed in water phase (Singh *et al.*, 2017).

In general, the mean droplet diameter of emulsions is around 1 µm, however microemulsions and nanoemulsions can be formed with droplet sizes in the 100-500 nm range. For cosmetic products, nanoemulsions not only can penetrate the active compounds easily into the skin because of small droplet sizes but also have good spreadability and more kinetically stable compared to macroemulsions (Gupta *et al.*, 2016; Rocha-Filho *et al.*, 2017). The droplet diameter is usually around <500 nm. The very small droplet can decrease problems of inherent sedimentation, creaming, coalescence, or flocculation during storage because of the potency of Brownian motion to decrease the gravity force (Kabri *et al.*, 2011). Certain properties of conventional emulsions (macroemulsions) and nanoemulsions are summarized in **Figure 2.4**.

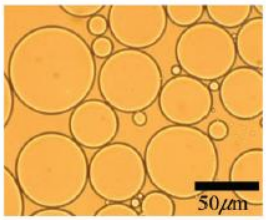
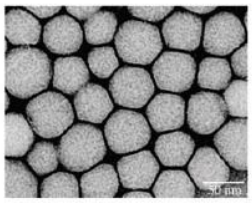
	macroemulsions	nanoemulsions
		
size	1-100 μm	20-500 nm
shape	spherical	spherical
stability	thermodynamically unstable, weakly kinetically stable	thermodynamically unstable, kinetically stable
method of preparation	high & low energy methods	high & low energy methods
polydispersity	often high (>40%)	typically low (<10-20%)

Figure 2.4. Comparison of macroemulsions and nanoemulsions (size, shape, stability, method of preparation and polydispersity) (Gupta *et al*., 2016).

Like emulsions, the formulation of nanoemulsions includes active ingredient(s), additive(s) and emulsifier(s). The physical stabilities of nanoemulsions depend on the preparation method. There are primarily two techniques to prepare nanoemulsions, which are high energy methods such as high pressure homogenization and low energy methods such as emulsion inversion point, see **Figure 2.5** (Abbas *et al.*, 2014). In high energy methods, the input energy density (ε) is around 10^8-10^{10} W/kg while the input energy density (ε) for low energy methods is significantly less and is around 10^3-10^5 W/kg (Gupta *et al.*, 2016). Preparing o/w nanoemulsions using high energy method is usually converted macroemulsions in a homogenizer. A high pressure can push the macroemulsions and reduce the droplet size to the nano range until the size become constant. In high energy methods, it begins with w/o macroemulsions to get w/o nanoemulsions. However, in low energy methods, w/o macroemulsions is transformed into o/w nanoemulsions following changes either in temperature or composition (Gupta *et al.*, 2016).

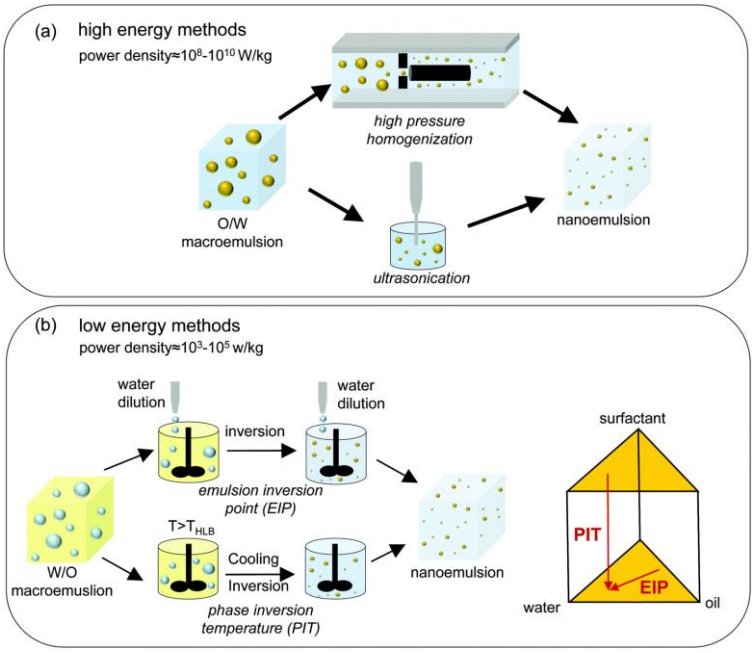


Figure 2.5. The methods in the preparations of nanoemulsions (Gupta *et al.*, 2016).

Nanoemulsions have interesting properties such as nano droplet size, rheological stability, and using small amount of surfactant compared with microemulsions (Sharma *et al.*, 2010). These properties make nanoemulsions as promising carriers for delivering a variety of active agents to the site of action (Peshkovsky *et al.*, 2013). As a result, nanoemulsions have been widely applied in cosmetic, pharmaceutical and medicinal fields.

In cosmetic systems, nanoemulsions are beneficial because small droplet size can easily penetrate into/through the stratum corneum (SC) of the skin. In addition, the small size of nanoemulsions can increase the amount of active ingredients reaching the target sites. This is owing to a closure contact with the stratum corneum (Morganti, 2010). Nanoemulsion are also more useful for lipophilic compounds to be transferred to the active sites of the skin (Sharma and Sarangdevot, 2012).

According Mahdi *et al.* (2011), *Phyllanthus urinaria*-loaded palm kernel oil esters nanoemulsions were formulated successfully by low energy method. In the *in vitro* release study, it was demonstrated that 51.30% and 51.02% of extract-loaded formulations were passed through cellulose acetate membrane with scavenging 30.05% and 29.89% of DPPH activity, indicating as a skin antiaging agent. In another research (Liang *et al.*, 2012), the stable peppermint oil (PO) nanoemulsions were produced using high energy method by incorporating PO with medium-chain triacylglycerol. The minimum inhibitory concentrations (Micillo *et al.*) of two strains

of microorganism, *Listeria monocytogenes* and *Staphylococcus aureus* were almost the same for both bulk PO and PO nanoemulsions.

Owing to thermodynamic instability of nanoemulsion systems, physicochemical characterization after formulation preparations should be performed (Sharma *et al.*, 2010). The size of colloidal structures can be determined by several techniques such as transmission electron spectroscopy, laser diffraction, and small range X-ray (Zorzi *et al.*, 2015). However, both hydrodynamic particle size and particle size of formulated nanoemulsions are generally performed by Dynamic Light Scattering (DLS) method. This method measures the Brownian motion of the particles (Tang *et al.*, 2012). Besides particle size, polydispersity index (PDI) is also one of the important characteristics of formulated nanoemulsions. It indicates the similarity of droplet size in nanoemulsion systems. The near zero PDI value is indicating a monodisperse droplet population, while a large PDI value (near one) is indicating a huge range of droplet size (Ribeiro *et al.*, 2015). PDI value can interfere with flocculation phenomena (Gianeti *et al.*, 2012). Nanoemulsions containing extract of *Opuntia ficus-indica* (L.) Mill showed good stability after observed time with droplet size 148.8 ± 1.6 nm and PDI 0.26 ± 0.01 (Ribeiro *et al*., 2015).

The stability of nanoemulsion systems can also be assessed by zeta potential value. The values between |-25 mV| and |-30 mV| for zeta potential is considered enough to prevent coalescence by creating the energy barrier (Shanmugam and Ashokkumar, 2014). Nanoemulsions are the formulation with two different types of liquids that can be stabilized using surfactant or surfactant system (emulsifier(s)). Selection of emulsifier is the crucial part to obtain nanoemulsions with good physical stability. The emulsifiers are holding the disperse droplets in surrounding medium then decreasing interfacial tension or increasing droplet-droplet repulsion (Solans *et al.*, 2005; Tadros *et al.*, 2004).

Type of nanoemulsions after preparation can be determined using the same technique as that of emulsions, which can be done by dye solubility test, and conductivity test (Rungseevijitprapa *et al.*, 2009). In the dye solubility test, the type of nanoemulsions can be revealed with oil or water-soluble colours. For o/w nanoemulsions, when water-soluble dye is added, the uniformity of colour is achieved and vice versa for w/o nanoemulsions. For conductivity test, the conductivity

increases strongly for o/w nanoemulsions and decreases in the case of w/o nanoemulsions (Ribeiro *et al.*, 2015).

The rheological characteristics of emulsified system are crucial parameters for product applications, including pumping, manufacturing, filling as well as aesthetic proposes in the market-ready product (Soriano *et al.*, 2001). As a rule, the rheological study is the evaluation of thixotropic properties, showing the proper picture of the physical properties and structural stability of formulation (Keunings, 2000).

The other influential factor is determining the amount of the active ingredient(s) loaded in nanoemulsions **(Table 2.2)**, especially when using the extract as the active ingredients. Since most extract contains unspecific substances and are unclearly identified. The recommended way to proceed is measuring the dry residue and the percentage of marker (s) presented in dry residue (Zorzi *et al.*, 2015).

Table 2.2. Extract percentage (dry weight) usually associated in nanotechnology-based system (Zorzi *et al*., 2015).

System	Amount of extract (%)
Nanoemulsions	0.5-20
Nanoparticles	0.5-20
Liposomes	0.1-2.0

In the current study, o/w nanoemulsions were prepared using cold-process emulsifier, Simulgel FL or conventional liquid emulsifier (Tween 80 and Span 80), synthetic oils such as caprylic/capric triglyceride (as emollient) and glycerine (as humectant). In addition, certain natural hydrocolloids (as thickeners) with different charge types were also added into the blank nanoemusions in order to examine the effect of charge type on the physicochemical property of the prepared nanoemulsions. Natural and semi-natural gums: xanthan gum (anionic charge due to carboxylic groups), guar gum (no charge) and cationic guar gum (cationic charge due to ammonium groups) were selected. The physicochemical properties of the ingredients used to formulate the nanoemulsions are summarized in **Tables 2.3-2.9**.

Table 2.3. Properties of tamarind fruit pulp extract (data from sweet tamarind fruit pulp from Guangzhou Phytochem Sciences Inc).

***Tamarindus indica* L. fruit pulp extract**	
Country of Origin	Guangdong province, P.R. China
Production	The tamarind fruit pulp extract powder was prepared by liquid-solid extraction and the ratio of proportion extraction was 4:1 (tamarind: solvent (water)). Spray drying process was used to produce the dry powder.
Colour and appearance	Fine brown yellow powder
Odour	Sweet fruity
Moisture content	≤5% (4.32%)
Ash	≤5% (3.52%)
pH value (1% in water)	3.86

Table 2.4. Properties of tartaric acid, a major organic acid, contained in tamarind fruit pulp extract (Gawronski and Gawronska, 1999).

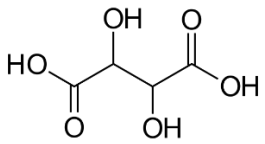
Tartaric acid	
Molecular Structure	 (https://en.wikipedia.org/wiki/Tartaric_acid)
Chemical Name	2,3-Dihydroxybutanedioic acid
Formula	$C_4H_6O_6$
Molecular Weight (g/mol)	150.087
Solubility	1.33 kg/L (L or D-tartaric)
Appearance	White powder
Use	Marker (main component in tamarind fruit pulp extract)

Table 2.5. Properties of Tween 80 as surfactant (conventional emulsifiers) used in the current study (Sigma-Aldrich product detail) (http://www.sigmaaldrich.com) (Remington, 2006).

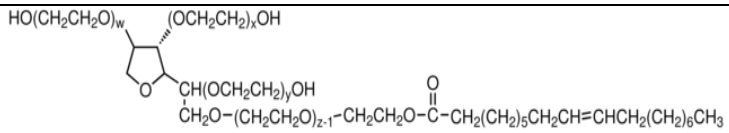
Tween 80	
Molecular Structure	
Chemical Name	Polyoxyethylene (20) sorbitan monooleate
Formula	$C_{64}H_{124}O_{26}$ (approximate)
Molecular Weight (g/mol)	Average 1310
HLB	15.0
Appearance	Viscous liquid
Use	Nonionic surfactant

Table 2.6. Properties of Span 80 as surfactant (conventional emulsifiers) used in the current study (Sigma-Aldrich product detail) (http://www.sigmaaldrich.com) (Remington, 2006).

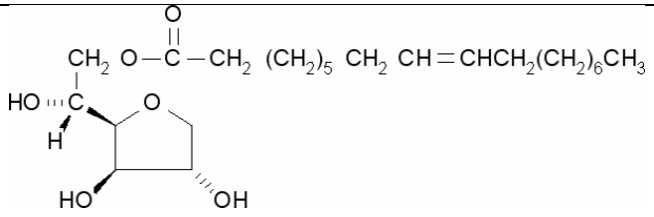
Span 80	
Molecular Structure	
Chemical Name	Sorbitan monooleate
Formula	$C_{24}H_{44}O_6$
Molecular Weight (g/mol)	428.61
HLB	4.3
Appearance	Viscous liquid
Use	Nonionic surfactant

Table 2.7. Properties of cold-process emulsifier used in the current study (https://pubchem.ncbi.nlm.nih.gov/compound/2hydroxyethyl_acrylate#section=CAS).

Simulgel FL	
Chemical Name	Hydroxyethyl Acrylate/Sodium Acryloyldimethyl Taurate Copolymer; Isohexadecane; Polysorbate 60
Appearance	Viscous liquid
Use	Nonionic emulsifier and thickener

Table 2.8. Properties of synthetic oils used in the current study (https://pubchem.ncbi.nlm.nih.gov/compound/121596031#section=Top).

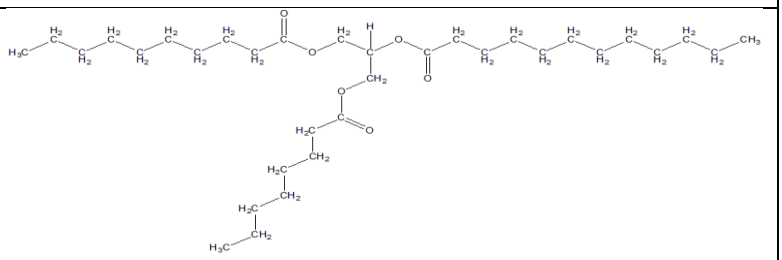
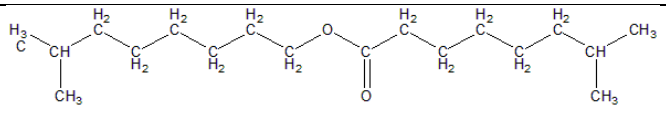
Caprylic/capric triglyceride	
Molecular Structure	
Chemical Name	Caprylic/capric/lauric triglyceride
Formula	$C_{33}H_{62}O_6$
Molecular Weight (g/mol)	554.853
Required HLB	11
Appearance	Viscous liquid
Use	Emollient
Isononyl isononanoate	
Molecular Structure	
Chemical Name	3,5,5-Trimethylhexyl 3,5,5-trimethylhexanoate
Formula	$C_{18}H_{18}O_2$
Molecular Weight (g/mol)	284.484
Appearance	Viscous liquid
Use	Emollient

Table 2.9. Properties of thickeners (natural gum) used in the current study (Holzwarth, 1978; Kadajji and Betageri, 2011; Katzbauer, 1998; Mansa and Detellier, 2013; Rowe *et al.*, 2009; Sathya and Prabaharan, 2015).

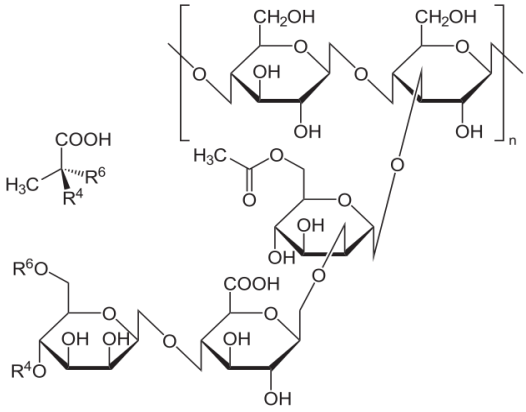
Xanthan gum	
Molecular Structure	 (By NEUROtiker - Own work, Public Domain, https://commons.wikimedia.org/w/index.php?curid=3744710)
Chemical Name	Polysaccharide xanthan
Synonyms	Glucomannan; Glucomannan mayo; Galactomannane; Rhodopol 23; Xanthan; Xantempo
Formula	$C_{35}H_{49}O_{29}$ (monomer)
Molecular Weight (g/mol)	Approximate 2 x 10^6
Ionic charge	Anionic polysaccharide
Solubility	Soluble in both hot and cold water
Origin	Bacterial fermentation
Appearance	Free flowing powder
Use	Thickener, viscosity-inducing agent

Table 2.9. Properties of thickeners (natural gum) used in the current study (Holzwarth, 1978; Kadajji and Betageri, 2011; Katzbauer, 1998; Mansa and Detellier, 2013; Rowe *et al.*, 2009; Sathya and Prabaharan, 2015) (continued).

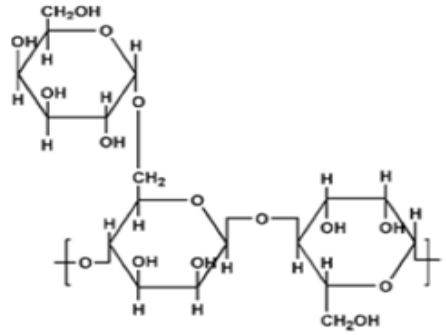
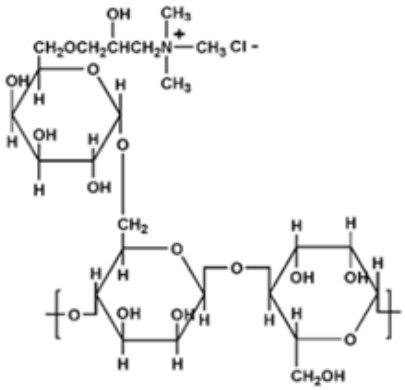
Guar gum	
Molecular Structure	
Chemical Name	Galactomannan polysaccharide
Synonyms	E412, guar flour, jaguar gum
Formula	$(C_6H_{12}O_6)_n$
Molecular Weight (g/mol)	Approximate 220,000
Ionic charge	Nonionic polysaccharide
Solubility	Soluble in cold water
Origin	Derived from the endosperm of the seed of *Cyamopsis tetragonoloba* (guar bean or Indian cluster bean)
Appearance	Free flowing powder
Use	Thickener, viscosity-inducing agent

Table 2.9. Properties of thickeners (natural gum) used in the current study (Holzwarth, 1978; Kadajji and Betageri, 2011; Katzbauer, 1998; Mansa and Detellier, 2013; Rowe *et al.*, 2009; Sathya and Prabaharan, 2015) (continued).

Cationic Guar gum	
Molecular Structure	
Chemical Name	Guar hydroxypropyltrimonium chloride
Synonyms	Guar hydroxypropyl trimethyl ammonium chloride
Formula	$(C_6H_{12}O_6)_n$
Molecular Weight (g/mol)	Approximate 220,000
Ionic charge	Cationic polysaccharide
Origin	Quaternary ammonium derivative of guar gum
Solubility	Soluble in cold water
Appearance	Free flowing powder
Use	Thickeners, viscosity-inducing agent

CHAPTER 3
MATERIALS AND METHODS

3.1. Materials and equipment

3.1.1 Materials

The materials used in the current study, as follows:

1. 3,4–dihydroxy-L-phenylalanine (L-DOPA), analytical grade (Sigma Chemical Co.,MO, USA)
2. Cellulose acetate membranes (Spectra/Por®3 Dialysis Membrane, MWCO 3,500 Dalton) (Spectrum Laboratories, Inc., CA, USA)
3. Dihydrogen orthophosphate dihydrate, Analytical grades (Univar, Australia)
4. Disodium hydrogen orthophosphate, Analytical grades (Univar, Australia)
5. Guar gum (CA0706), pharmaceutical grade (Chemipan Corporation Co., Ltd, Bangkok, Thailand)
6. Isononyl isononanoate, pharmaceutical grade (MySkinRecipes, Bangkok, Thailand)
7. Jaguar C-13S (cationic guar gum), pharmaceutical grade (JKK Chemical Limited, Bangkok, Thailand).
8. Kojic acid, analytical grade (Sigma Chemical Co.,MO, USA)
9. L-ascorbic acid, analytical grade (S.M. Chemical Supplies Co., Ltd., Bangkok, Thailand)
10. Microtubes 2 mL, MCT-200-C (Axygen Scientific, USA)
11. Mueller Hinton Agar, Difco BBL, USA
12. Mueller Hinton Broth, Difco BBL, USA
13. Myristol (Caprylic/capric triglyceride), pharmaceutical grade (Chemipan Corporation Co., Ltd, Bangkok, Thailand)
14. Orthophosporic acid, Analytical grades (Univar, Australia)
15. Pipet tips 0.5-10 µL, Pipetman T300 (Axygen Scientific, USA)
16. Pipet tips 1-200 µL, Pipetman T-200-Y (Axygen Scientific, USA)
17. Pipet tips 1-1000 µL, Pipetman T-1000-B (Axygen Scientific, USA)

18. Ponceau 4R (cochineal red A or brilliant scarlet 4R), pharmaceutical grade (P.C. Drug Center Co., Ltd., Bangkok, Thailand)
19. Refined glycerine USP, pharmaceutical grade (P.C. Drug Center, Bangkok, Thailand)
20. Simulgel FL, pharmaceutical grade (SEPPIC S.A.,Puteaux, France)
21. Sodium chloride, Analytical grades (Univar, Australia)
22. Span 80, pharmaceutical grade (P.C. Drug Center, Bangkok, Thailand)
23. *Staphylococcus aureus* ATCC® 29213™ and *Staphylococcus epidermidis* ATCC® 12228™
24. Syringe Filter 0.2 micron, 13 mm, FTS-NL13020-010 (Filtrex, China)
25. Tamarind fruit pulp extract (Fuyang Bestop Implex Ltd., Anhui, China)
26. Tamarind fruit pulp extract (Guanzhou Phytochem Sciences Inc., Guanzhou, China)
27. Tamarind fruit pulp by a local market in Songkhla Province, Thailand
28. Tartaric acid, analytical grade (racemic form, analytical Grade, 99.7% purity)
29. Tyrosinase, analytical grade (S.M. Chemical Supplies Co., Ltd., Bangkok, Thailand)
30. Tween 80, pharmaceutical grade (P.C. Drug Center, Bangkok, Thailand)
31. Xanthan gum, pharmaceutical grade (P.C. Drug Center, Bangkok, Thailand)

3.1.2. Equipment

The equipment used in the current study is listed in **Table 3.1.**

Table 3.1. Instrumental equipment used in the current study.

Instrument	Model	Company
2-decimal electrical balance	Sartorius Basic	Scientific Promotion Co., Ltd
4-decimal electrical balance	Mettler AE 200	Mettler-Toledo GmbH, Greifensee, Switzerland
96-well plate	167008 Nuclon^{TM} Delta Surface	Thermo Fisher Scientific, Suzhou, Jiangsu
Bob-cup rheometer	LVDV-III Ultra	Brookfield Engineering, Laboratories, Inc., MA.

Table 3.1. Instrumental equipment used in the current study (continued).

Instrument	Model	Company
Circulating water bath (for the rheometer)	Model 6 (T Lauda)	Lauda Dr. R. Wobser GmbH & Co., Lauda-Königshofen, Germany
Centrifuge	Z 323 K	Hermle LaborTechnik GmbH, Wehingen, Germany
Conductivity meter	Mettler Toledo S230	Mettler –Toledo Inc., OH, U.S.A.
Diffusion cells (in-house construction) similar to Modified Franz diffusion cells (Hanson model 57-6M, Hanson Research Corporation, U.S.A.)	-	NK Supply Laboratory Equipment for Retail, Bangkok, Thailand
- Magnetic stirrer	Variomag Telemodul 40S	H+P Labortechnik GmbH, Munich, Germany
- Circulating water bath	57-951-004	Hanson Research, Chatsworth, USA
High pressure homogenizer	Microfluidics M-110P	PCL Holding Co., Ltd
High speed homogenizer	Mettler AE 200	Scientific Industries, Inc., U.S.A.
HPLC system		
- pump	LC-20AD	Shimadzu, Japan
- diode array detector	SPD-M20A	Shimadzu, Japan
- degasser	DGU-20A5	Shimadzu, Japan
- auto sampler	SIL-20A	Shimadzu, Japan
Microplate reader	NS-100 Nano scan	Hercuvan Lab Systems, UK
Multiple channel micropipette 20-200 µL	Acura 885, 66071021	Socorex, Swiss
Micropipette 100 µL	Pipetman, W51451B	Gilson, France

Table 3.1. Instrumental equipment used in the current study (continued).

Instrument	Model	Company
Micropipette 200 µL	Acura 825, 18073477	Socorex, Swiss
Micropipette 1000 µL	Acura 885, 18091304	Socorex, Swiss
pH meter	Seven easy S20	Mettler Toledo Inc., USA
Reverse-phase HPLC Column	Appollo C18 (5µm, 4.6 x 250 mm)	Alltech, IL, U.S.A.
Ultrasonic bath	Crest 950 T	A Crest Group Inc. Company, Penang, Malaysia
Vortex mixer	Vortex Genie	Scientific Industries, Inc., U.S.A.
Zeta potential analyzer	ZetaPALsa	Brookhaven Instruments Corp. U.S.A.

3.2. Methods

3.2.1. Screening of tamarind fruit pulp extracts

In the preliminary study, four samples of tamarind fruit pulp extracts were investigated for their bioactivities. The samples were 1) sample No. 1, sweet tamarind fruit pulp extract (powder) purchased from Guangzhou Phytochem Sciences Inc, China 2); sample No.2, tamarind fruit pulp extract (powder) purchased from Fuyang Bestop Import and Export Ltd, Anhui, China; 3) sample No. 3, tamarind fruit pulp extract, using water as a solvent; and 4) sample No. 4, tamarind fruit pulp extract, using 50% ethanol as a solvent. Samples No. 2-4 were sour tamarind. Tamarind samples No. 3 and 4 were prepared based on the method described by Viyoch *et al.* (2003) and Gupta *et al.* (2014). The fresh tamarind fruit pulps (sour type) were supplied by a local market in Na Mom District, Songkhla Province, Thailand. Briefly, 1 kg of the fruit pulps were homogenized using a blender (Panasonic MX-GX1462) with a certain solvent (tamarind: solvent = 1:5), macerated for 24-hours, filtered and

subjected to lyophilization process to produce tamarind fruit pulp extract powder. All tamarind extract powders were obtained from tamarind fruit pulps without seeds. These tamarind fruit pulp extract samples were tested for tartaric acid content, antioxidant, antityrosinase and antibacterial activities. The sample which had the best bioactivity would be selected as the active ingredient incorporated into the blank nanoemulsions.

3.2.1.1. Antioxidant activity

Antioxidant property of tamarind fruit pulp extracts was determined using scavenging activity of 2, 2-Diphenyl-1-picrylhydrazyl (DPPH). The analysis was according to Martinello *et al.* (2006) and Yuan *et al.* (2012) with slight modification. Tamarind fruit pulp extracts at different concentrations **(see Table 3.2)** were prepared with distilled water. Positive control, which was ascorbic acid (0.1-50 µg/mL in distilled water) and test samples of tamarind fruit pulp extract were added to DPPH solution (6×10^{-5} M in 80% ethanol). This method was tested in a 96-well plate. The composition of reaction mixtures is summarized in **Table 3.3**. The mixture was kept in the dark at 25°C for 30 min. The absorbance of the resulting solution was then measured spectrophotometrically at 517 nm using a microplate reader. All determinations were performed in quadruplicates. The EC_{50} (effective concentration of sample required to scavenging DPPH radical by 50%) was obtained by plotting between % inhibition and concentration of samples.

Table 3.2. Concentrations of each samples for antioxidant test.

Sample	Concentration	Note
Ascorbic acid	0.1-50 µg/mL	Dissolved in distilled water, clear solution (sonicate around 5 min)
Sample 1, sweet tamarind fruit pulp extract (Guangzhou Phytochem Sciences Inc)	0.029-15 mg/mL	Dissolved in distilled water, sonicate 10 min, the stock solution was filtered before dilution
Sample 2, Organic water soluble tamarind fruit pulp extract (Fuyang Bestop Import and Export Ltd)	0.059-30 mg/mL	Dissolved in distilled water, sonicate 10 min, the stock solution was filtered before dilution

Table 3.2. Concentrations of each samples for antioxidant test (continued).

Sample	Concentration	Note
Sample 3, tamarind fruit pulp extract (water)	0.78-40 mg/mL	Dissolved in distilled water, sonicate 10 min, the stock solution was filtered before dilution
Sample 4, tamarind fruit pulp extract (50% ethanol)	0.78-40 mg/mL	Dissolved in distilled water, sonicate 10 min, the stock solution was filtered before dilution

The reduction in the absorbance of the DPPH solution indicates the free radical scavenging activities of the test samples. The % antioxidant activity was calculated according to the following **Eq. 3.1**:

$$\text{Scavenging activity (\%)} = \left[1 - \left(\frac{\text{absorbance of sample}}{\text{absorbance of control}}\right)\right] \times 100 \qquad \textbf{Eq. 3.1}$$

Table 3.3. Compositions of mixtures in 96-well plate.

	Sample solution (µL)	Ascorbic acid solution (µL)	Absolute ethanol (µL)	Distilled water (µL)	DPPH solution (µL)	Total (µL)
Control	-	-	-	100	100	200
Control blank	-	-	100	100	-	200
Sample blank	100	-	100	-	-	200
Sample	100	-	-	-	100	200
Positive control	-	100	-	-	100	200

3.2.1.2. Antityrosinase activity

The inhibitory effect of tamarind fruit pulp extracts on tyrosinase activity was determined using Dopachrome technique. The assay was carried out based on the methods of Dej-adisai *et al.* (2014), Vardhan and Pandey (2014) and Pintus *et al.* (2015) using a 96-well plate and a microplate reader. L-DOPA (0.85 mM in 0.1 M phosphate buffer pH 6.8) was used as a substrate and kojic acid solution was employed as a positive control (0.1-1 mg/mL in distilled water) (Vardhan and Pandey, 2014). Mushroom tyrosinase solution (203.3 unit/mg) was prepared with the same buffer, 0.1 M phosphate buffer pH 6.8. Tamarind fruit pulp extracts at suitable concentrations **(see Table 3.4)** were prepared in distilled water.

Table 3.4. Concentrations of each samples for antityrosinase test.

Sample	Concentration	Note
Kojic acid	0.1-1 mg/mL	Clear solution
Sample 1, sweet tamarind fruit pulp extract (Guangzhou Phytochem Sciences Inc)	0.468-15 mg/mL	Dissolved in distilled water, sonicate 10 min, the stock solution was filtered before dilution
Sample 2, Organic water soluble tamarind fruit pulp extract (Fuyang Bestop Import and Export Ltd)	0.938-30 mg/mL	Dissolved in distilled water, sonicate 10 min, the stock solution was filtered before dilution
Sample 3, tamarind fruit pulp extract (water)	1.25-40mg/mL	Dissolved in distilled water, sonicate 10 min, the stock solution was filtered before dilution
Sample 4, tamarind fruit pulp extract (50% ethanol)	1.25-40 mg/mL	Dissolved in distilled water, sonicate 10 min, the stock solution was filtered before dilution
L-DOPA solution	0.85 mM	Dissolved in 0.1 M phosphate buffer pH 6.8
Mushroom tyrosinase solution	203.3 unit/mg	Dissolve in 0.1 M phosphate buffer pH 6.8

The content of each well, as shown in **Table 3.5**, except L-DOPA, was mixed and pre-incubated at 25-30 °C for 10 minutes before determining the absorbance at 492 nm. Later, 0.85 mM L-DOPA (20 µL) was added in every well and the optical density was determined again at 492 nm using a microplate reader. Then the samples were incubated at 25-30 °C for 30 min and the optical density was determined again with a microplate reader. The inhibition of tyrosinase activity was calculated with the following, **Eq. 3.2** (Dej-adisai *et al.*, 2014). The EC_{50} (effective concentration of

sample required to tyrosinase activity by 50%) was obtained by plotting between % inhibition and concentration of samples.

$$\%\text{Tyrosinase inhibitory} = \frac{(A-B)-(C-D)}{A-B} \times 100 \quad \textbf{Eq. 3.2}$$

Where,

A = the difference of optical density before and after incubation at 492 nm without test sample

B = the difference of optical density before and after incubation at 492 nm without test sample and enzyme

C = the difference of optical density before and after incubation at 492 nm with test sample

D = the difference of optical density before and after incubation at 492 nm with test sample, but without enzyme

Table 3.5. Compositions of mixtures in 96-well plate.

	Sample solution (µL)	**Mushroom tyrosinase solution (µL)**	**Kojic acid solution (µL)**	**Phosphate buffer pH 6.8 (µL)**	**0.85 mM L-DOPA solution (µL)**	**Distilled water (µL)**	**Total (µL)**
A	-	20	-	140	20	20	200
B	-	-	-	160	20	20	200
C	20	-	-	160	20	-	200
D	20	20	-	140	20	-	200
E	-	20	20	140	20	-	200

A=control, B=control blank, C=sample blank, D=sample, E=positive control

3.2.1.3 Antibacterial activity

Normal microflora found in the skin, especially in axillary, includes gram-positive bacteria such as *Staphylococcus aureus* and *Staphylococcus epidermidis*. Minimum inhibitory concentration (Micillo *et al.*) and minimum bactericidal concentration (MBC) of tamarind fruit pulp extract were determined by broth microdilution using twofold serial dilutions in a Mueller–Hinton broth medium (Akinyemi *et al.*, 2006; Gunes *et al.*, 2016). Two bacterial strains, *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* TISTR 517, were provided by

Thailand Institute of Scientific and Technological Research and Department of Medical Sciences Center. Khlong Luang, Pathum Thani.

The MIC of tamarind fruit pulp extract was determined by diluting the various concentrations ranging from 0.02-8 mg/mL. A standard drug positive control was gentamicin sulfate (0.01-4 µg/mL). The stock solution of tamarind fruit pulp extracts for microbial assay was prepared by dissolving with sterile water to give final concentration of 32 mg/mL. The solution was further two-fold serially diluted using Mueller-Hinton broth (MHB) as the solvent for determining MIC and MBC (Sungkharak *et al.*, 2016). The experimental procedures began with dispensing 50 µL of tamarind pulp extract at different concentrations into a 96-well microtiter plate. Then, 50 µL of a standardized suspension (fresh cultures of standard strains were prepared and adjusted the density of turbidity by determining the absorbance reading of 0.08 – 0.1 at 625 nm and further diluted 1:100 in MHB) of the test organisms were added to each well. A positive control was the well containing 50 µL of medium and 50 µL of the inoculums whereas a negative control was the well containing 100 µL of medium. The plates were sealed and then incubated at 37 ± 2°C for 20 h. After reaching the incubation time, 5 µL of a 0.2% blue colored resazurin solution were added in each well and then incubated for 5 h. The plates were inspected for a color change from blue to pink. The lowest concentration showing a blue color was taken as the MIC value based on the lack of reduction of the blue resazurin to the pink resorufin by the microbial dehydrogenase enzyme. The MBC was determined as for the MIC test and the lowest concentrations near to those with no color change were selected. A loop from each of those wells was inoculated on Mueller-Hinton Agar. The complete absence of growth was considered to represent the MBC.

3.2.2. Determination of amounts of tartaric acid in tamarind fruit pulp extracts

The quantity of a certain organic acid, tartaric acid, in tamarind fruit pulp extracts was determined by high performance liquid chromatography (HPLC). The standard solutions of tartaric acid ranging from 5 to 120 µg/mL (7 concentrations) were prepared using HPLC mobile phase (0.006 M phosphoric acid (H_3PO_4)) as solvent. A tamarind fruit pulp extract stock solution was prepared with the mobile phase, diluted to a suitable concentration (20 mg/mL) with the same solvent, sonicated, filtered and injected into the HPLC column. The amount of tartaric acid

was calculated using standard curve. Four replications were performed. The amount of tartaric acid in sweet tamarind fruit pulp extract and sour tamarind fruit pulp extract supplied by China were found to be 0.3240 ± 0.0631 and 0.6395 ± 0.0194 mg% (w/w)**,** respectively. A much higher tartaric acid contents in tamarind fruit pulp water extract and 50% ethanol extract were obtained, which were 23.0050 ± 3.6648 mg% (w/w) and 24.8569 ± 1.0059 mg% (w/w), respectively.

3.2.2.1. High performance liquid chromatography (HPLC) analysis

The HPLC conditions were based on the method of Kordis-Krapez *et al.* (2001) with some modification. A reverse-phase Appollo C18 (5 µm, 4.6 x 250 mm, Alltech, IL, USA) with guard column was used to determine the amount of tartaric acid. The HPLC system consisted of a Shimadzu pump (model LC-20AD, Shimadzu, Japan), a Shimadzu diode array detector (model SPD-M20A, Shimadzu, Japan) a Shimadzu degasser (model DGU-20A5, Shimadzu, Japan) and a Shimadzu autosampler (model SIL-20A, Shimadzu, Japan). The mobile phase was 0.006 M phosphoric acid (pH = 2.1) at a flow rate of 0.8 mL/minute. The UV detection wavelength was 210 nm. The injection volume was 20 µL. The column temperature was 25°C. The system was operated by the LC Solution Software. The validation of HPLC method was performed based on the guidelines of the International Conference on Harmonization of Technical Requirement for the Registration of Pharmaceuticals for Human Use (ICH, 2005). To determine the amount of tartaric acid in the samples, the standard stock solution of tartaric acid was prepared by dissolving 10 mg of standard tartaric acid (accurate weight) in the mobile phase and adjusted to volume in 50 mL volumetric flask. The seven standard solutions (5, 10, 20, 40, 80, 100 and 120 µg/mL) of tartaric acid were prepared by further diluting the stock solution with the mobile phase. The standard curve was constructed by plotting the peak areas of tartaric acid standard solutions against the concentrations. Using linear regression analysis, the linearity range was between 5 - 120 µg/mL with a coefficient of determination (r^2) of 0.9998. The percentage of relative standard deviation (%RSD) of intra-day and inter-day was less than 2%, indicating the precision of the method. The accuracy (% recovery) was in the range of 101.39-103.74% (acceptable limit 90-115%) with RSD less than 2%. The limit of detection (LOD) and limit of quantification (LOQ) values were 0.05 µg/mL and 0.17 µg/mL, respectively. The specificity of the analytical

procedure was also assessed in order to check interferences that may be caused by other ingredients in nanoemulsion formulations. It was found that interference peaks did not interfere with tartaric acid peak. For *the in vitro* release study of tamarind fruit pulp extract loaded nanoemulsions, the stock solution (10 mg in 50 mL) of standard tartaric acid was prepared in the receptor fluid, 0.9% w/v sodium chloride solution. The standard curve was constructed between concentrations of standard tartaric acid solutions (1, 5, 10, 30, 60, 80 and 100 µg/mL) against peak area. Coefficient of determination from linear regression analysis was 0.9997. The accuracy was between 99.67-111.61% and % RSD of precision was less than 2%. The LOD and LOQ were 0.13 and 0.43 µg/mL, respectively (see Appendix A for more details).

3.2.3. Preparation of blank nanoemulsions

In a preliminary study, the nanoemulsion vehicles were prepared using a cold-process emulsifier, Simulgel FL (hydroxyethyl acrylate/sodium acryloyldimethyl taurate copolymer (and) isohexadecane (and) polysorbate 60), at 0.5, 1 and 1.5%w/w. Synthetic oils used as emollients were isononyl isononanoate (low polar oil) and caprylic/capric triglyceride (Myritol 318) (high polar oil) at 10-20%w/w. The other ingredients of the formulations were glycerine (10%w/w) as a humectant and distilled water as a vehicle. The emulsions were firstly prepared using a high speed homogenizer. Then, the obtained emulsions were passed through the high pressure homogenizer to prepare nanoemulsions. After a few hours, there was phase separation (creaming) for all formulations prepared with Simulgel FL. A slower rate of creaming happened with the high polar oil. It was probably because the polymer chains of Simulgel FL were damaged by the high shear (force) and high heat (about 60°C) generated during the high pressure homogenization process. Therefore, the Simulgel FL was replaced by the conventional liquid emulsifier, Tween and Span. The use of conventional emulsifier, Tween 80 and Span 80, was based on the study of Songkro *et al.* (2011), who formulated the nanoemulsions of plaunoi extract with these two nonionic surfactants. Here, the concentrations of the emulsifier (a surfactant mixture: Tween 80 plus Span 80) were 5 and 10%w/w. The other ingredients were the same as the method of cold-process emulsifier. The nanoemulsions were successfully prepared. However, at 10 % surfactant mixture, and 10% caprylic/capric triglyceride,

almost transparent nanoemulsions were obtained. As a result, the 5% of surfactant mixture and 20% of synthetic oil were selected since they could provide acceptable milky white appearance. Nevertheless, the viscosity of the formulations was quite low. Thus, natural hydrocolloids (thickeners) were incorporated into the formulations. This included xanthan gum (negative charge), guar gum (no charge) and cationic guar gum at suitable concentrations, 0.10 and 0.25%w/w. In general, a concentration range for such natural hydrocolloids is up to 1%. Based on the preliminary, 0.5% w/w concentration produced very high viscous preparation, resulting in clogging in a homogenizer. Based on the preliminary study, the modified formulations of blank nanoemulsions are summarized in **Table 3.6**. The amount of Tween 80 and Span 80, the surfactant mixture, was theoretically calculated based on the required Hydrophilic Lipophilic Balance (HLB) of the oil phase, (here caprylic/capric triglyceride with required HLB = 11). Each formulation was prepared in triplicate (n=3).

Table 3.6. Compositions of blank nanoemulsions.

Ingredient	**%w/w**						
	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**
Tween 80 (emulsifier)	3.13	3.13	3.13	3.13	3.13	3.13	3.13
Span 80 (emulsifier)	1.87	1.87	1.87	1.87	1.87	1.87	1.87
Caprylic/capric triglyceride (emollient)	20	20	20	20	20	20	20
Glycerine (humectant)	10	10	10	10	10	10	10
Xanthan gum (thickener)	-	0.10	0.25	-	-	-	-
Guar gum (thickener)	-	-	-	0.10	0.25	-	-
Cationic guar gum (thickener)	-	-	-	-	-	0.10	0.25
Distilled water (vehicle)	65	64.75	64.5	64.75	64.5	64.75	64.5

The blank nanoemulsions were prepared by emulsifying the surfactant mixture, water, glycerine, hydrocolloid (fully hydrated in water portion) and synthetic oil **(see Table 3.6)** with the aid of a high speed homogenizer (series X10/25, model Ystral, D-79282, Germany; shaft number 10G) at 16,000 rpm for about 5 min to

obtain conventional emulsions. Afterwards, the emulsions were passed through a high pressure homogenizer (model M-110 P, Microfluidics Corp., United States) at 20,000 psi for three cycles to produce the nanoemulsions. Further increasing number of cycles showed no effect on droplet sizes according to preliminary study.

The blank nanoemulsions were firstly prepared with a high pressure homogenizer. After that, the optimized blank nanoemulsions (cool state) were incorporated with the suitable amount of selected tamarind fruit pulp extract based on the bioactivity study (here the sweet tamarind fruit pulp extract purchased from Guangzhou Phytochem Sciences Inc, China).

3.2.4. Preparation of tamarind fruit pulp extract loaded o/w nanoemulsions

In the current study, two concentrations of the sweet tamarind fruit pulp extract, 6.6%w/w and 3.3%w/w, which were 100-times and 50-times of EC_{50} (antioxidant activity, **see Table 4.1**), respectively were used. To inhibit mold (fungi) growth, appropriate preservatives: 1%w/w ethylhexylglycerin and 0.2%w/w phenoxyethanol were added into the formulations. Tamarind fruit pulp loaded nanoemulsions were prepared by mixing the selected blank nanoemulsions with sweet tamarind fruit pulp extract powder using a magnetic stirrer (speed 300 rpm) for overnight. The blank formulations had the same ingredients as the tamarind fruit pulp extract loaded nanoemulsions except for the sweet tamarind fruit pulp extract.

3.2.5. Investigation of Physicochemical Properties

The physicochemical properties of freshly prepared formulations (1 day-old preparations) and the formulations under stability investigation were determined as follows:

3.2.5.1. Characterization of nanoemulsion type

The type of the prepared nanoemulsions was determined by using dye solubility test and conductivity measurement. The dye solubility test was performed with Ponceau 4 R aqueous solution. The o/w nanoemulsions were expected to be miscible with the aqueous solution whereas the w/o nanoemulsions exhibited immiscibility. To confirm the dye test, the conductivity of nanoemulsions was carried out using a conductivity meter (model CM-115, Kyoto Electronics Manufacturing Co., Ltd., Kyoto, Japan). All measurements were performed in triplicate at 25 °C.

3.2.5.2. pH measurement

The pH of nanoemulsions were measured in triplicate using a digital pH meter (model Seven easy S20, Mettler -Toledo Inc., OH, USA). The measurements were performed at 25 ℃.

3.2.5.3. Viscosity and flow measurement

The viscosity and flow properties of the formulations were determined in triplicate using bob-cup Brookfield rheometer (model LVDV-III Ultra, Brookfield Engineering Laboratories Inc., MA, USA) with a spindle number SC4-31 and a small sample adapter. Brookfield software (version V 3.1-1) was employed to operate the measurement. Five different shear rates (rpm) were conducted in order to construct rheograms. The measurements were performed at 32 ℃, the skin surface temperature.

3.2.5.4. Zeta potential, droplet size, and polydispersity index (PDI) analysis

The zeta potential, droplet size and PDI of the nanoemulsions were determined with light-scattering technique using Zeta potential analyzer (model ZetaPALS, Brookhaven Instruments Corporation, NY, USA). All measurements were performed at 25 ℃. The measurements were carried out at a fixed angle of 90°C. Nanoemulsions were appropriately diluted with deionized water before the measurement; 15 µL of test formulation was mixed with 20 mL of deionized water. No phase separation occurred after the dilution. All results represent mean ± SD (standard deviation) of ten measurements on the sample.

3.2.6. Stability of blank nanoemulsions

The physical stability of blank nanoemulsions was evaluated by 1) centrifugation (Rocha-Filho et al. 2017); 2) heating-cooling cycles (4 cycles) (Thai Community Product Standard No. 550/2553); and 3) accelerated stability study (international conference on harmonization (ICH) guidelines). The centrifugation test was performed on freshly prepared nanoemulsions at 3,000 rpm for 30 min. The appearance and homogeneity were evaluated by visual observation. For heating-cooling cycle test, each cycle consisted of keeping the samples at cooling temperature of 4°C for 24 h and then at high temperature of 45°C for 24 h. The accelerated stability study on nanoemulsions was performed by keeping the samples (n = 3 in each formulation) at low temperature (4 ± 2°C), room temperature (30 ± 2°C) and

high temperature (45 ± 2°C) for the period of 1 month. All samples were evaluated for the pH, viscosity, and physical changes (e.g. phase separation, precipitation, colour, and clarity) by visual observation after a month. The droplet size, PDI and zeta potential were determined at the end of storage and compared with those of a freshly prepared preparation.

3.2.7. Stability of tamarind fruit pulp extract loaded nanoemulsions

The physical stability of the nanoemulsions which contain tamarind fruit pulp extract was performed similar to that of blank nanoemulsions with the longer periods of accelerated stability studies (2 months). The chemical changes of the stable tamarind fruit pulp extract loaded formulations were investigated by measuring the amount of non-degraded tartaric acid at the end of the experimental period using HPLC technique. The tamarind fruit pulp extract loaded nanoemulsions were accurately weighed (2 g) into a centrifuge tube, added with 5 mL of a mixture of absolute ethanol and distilled water (1:1 by volume) vortexed and sonicated for 30 min. Then, a mixture was centrifuged at 6,000 rpm (model Z 323 K, Hermle LaborTechnik GmbH, Wehingen, Germany) for 10 minutes at 25 °C. The resultant solution was filtered through a membrane pore size of 0.2 µm if necessary. An aliquot of clear solution was further diluted with appropriate amount of HPLC mobile phase and was assessed for the amount of tartaric acid remained in the samples. The technique was modified from the study of Özer *et al.* (2007) who investigated the antityrosinase activity of creams containing plant extract. Ellagic acid was the main constituent of the extract. In a preliminary study, distilled water was also used as an extraction solvent, however, a mixture of absolute ethanol and distilled water (1:1 by volume) provided higher % recovery of tartaric acid than the distilled water did.

3.2.8. In vitro release study of tamarind fruit pulp extract loaded nanoemulsions

The release of tartaric acid, one of the organic acids of tamarind fruit pulp extract, was determined in order to check whether the active ingredient (tamarind fruit pulp extract) could be released from the nanoemulsion vehicles or not. In the current study, only freshly prepared tamarind fruit pulp extract loaded nanoemulsions were investigated. The *in vitro* release tests were performed by using synthetic cellulose acetate membrane (Spectra/Por®3, Spectrum Laboratories, Inc., CA, USA) and

diffusion cells (in-house construction), similar to modified Franz diffusion cells (Hanson model 57-6 M, Hanson Research Corporation, CA, USA). Each amber glass diffusion cell was composed of two parts: donor compartment for sample application and receptor compartment for receptor fluid. The diffusional area of the cell was 1.767 cm^2 and the receptor compartment had a capacity of 12 mL (magnetic bar and spring included). The hydrated membranes, which was previously cut into suitable size, were immersed in receptor medium (0.9% w/v sodium chloride solution) for 15 min before starting the experiment. Then, the membrane was placed on the top of the receptor compartment filled with degassed receptor medium. Importantly, air bubbles in receptor fluid must be removed before the membrane was positioned. The two parts of diffusion cells were hold together with a cell clamp. After equilibration for 30 min, the sample (1 g accurately weight) of each formulation was carefully applied into the donor compartment. Parafilm, a glass slide, and aluminum foil were placed on top of the donor chamber in order to avoid evaporation. The circulating water bath was maintained at 37 ± 1°C. The receptor fluid was stirred continuously at 300 rpm using a magnetic bar and a magnetic stirrer (Variomag Telemodul 40S, H+P Labortechnik, Munich, Germany). At suitable time intervals (30 min to 24 h), 500 µL aliquot part was collected. After sampling, the volume collected was replaced immediately with fresh receptor medium. The amount of tartaric acid released into the receptor medium was assayed by the HPLC method. Three to four replications were performed for each formulation. The cumulative amount of tartaric acid released (Q_t) through the synthetic membrane into the receptor medium was determined by following equation:

$$Q_t = V_r C_t + \sum_{i=0}^{t-1} V_s C_i \quad \textbf{Eq. 3.3}$$

where

V_r = volume of receptor fluid (12 mL)

C_t = tartaric acid concentration of the receptor fluid at each sampling time

V_s = volume of sampling solution (0.5 mL)

C_i = tartaric acid concentration of the i^{th} sample

The release profiles were achieved by plotting cumulative amount of tartaric acid released in the receptor fluid per unit area against time (t). The release kinetics of tartaric acid were analyzed by three kinetic models, namely zero order, first order and Higuchi's model as shown in **Table 3.7**.

Table 3.7. Kinetics of drug release.

Model	Equation
Zero order	$Q_t = Q_0 + K_0t$
First order	$\ln (Q_t) = lnQ_0 + K_1t$
Higuchi	$Q_t = K_H^{1/2}$

where Q_t, is the cumulative amounts of tartaric acid released in time t; Q_0 is initial amount of tartaric acid in dissolution media (here receptor fluid); K_0, K_1, K_H are rate constants of zero order, first order and Higuchi, respectively (Costa *et al.*, 2013).

3.2.9. Bioactivity of tamarind fruit pulp extract loaded nanoemulsions

The tamarind fruit pulp extract loaded nanoemulsions were further tested for the bioactivity properties. Samples tested were freshly prepared formulations and the formulations under stress conditions: heating-cooling cycles and storage at three different temperatures, 4 ± 2°C, 30 ± 2°C and 45 ± 2°C for 2 months. Blank formulations were also employed to check the effect of blank nanoemulsions on the bioactivity properties.

3.2.9.1. Antioxidant activity

The antioxidant activity of the formulations was determined as previously described. However, for tamarind fruit pulp extract loaded nanoemulsions EC_{50} was not calculated. The results were expressed in % scavenging activity (SCV). Tartaric acid in the tamarind fruit pulp extract loaded nanoemulsions was extracted by suitable solvent (here, a mixture of absolute ethanol and distilled water (1:1 by volume)). The procedure was the same as the determination of non-degraded tartaric acid in nanoemulsions with the exception of the dilution with HPLC mobile phase. The clear solution was further tested for bioactivity property. The concentrations were 25µL/mL; 50 µL/mL; 100 µL/mL of tamarind fruit pulp extract equals to F1-3.3TE

(0.825 mg/mL); F1-3.3TE (1.65 mg/mL); F1-3.3TE (3.3 mg/mL) and F1-6.6TE (1.65 mg/mL); F1-6.6TE (3.3 mg/mL); F1-6.6TE (6.6 mg/mL).

3.2.9.2. Antityrosinase activity

The antityrosinase activity of the formulations was determined as previously described. However, for tamarind fruit pulp extract loaded nanoemulsions EC50 was not calculated. The results were expressed in %inhibition. Tartaric acid in the tamarind fruit pulp extract loaded nanoemulsions was extracted by suitable solvent (here, a mixture of absolute ethanol and distilled water (1:1 by volume)). The procedure was the same as the determination of non-degraded tartaric acid in nanoemulsions with the exception of the dilution with HPLC mobile phase. The clear solution was further tested for bioactivity property. The clear solution was further tested for bioactivity property. The concentrations were 25µL/mL; 50 µL/mL; 100 µL/mL equals to 0.825-3.3 mg/mL for F1-3.3TE and 1.65-6.6 mg/mL for F1-6.6TE.

3.2.9.3. Antibacterial activity

The antimicrobial property of the formulations containing tamarind fruit pulp extract were determined using agar well diffusion technique (Balouiri *et al.*, 2016; Gupta *et al.*, 2014). Two bacterial strains, which were *Staphylococcus aureus* and *Staphylococcus epidermidis* were incubated in Mueller-Hinton agar (MHA) for 24 h at 35-37°C and adjusted to obtain turbidity 25% transmittance at 540 nm. A 21-mL of MHA was poured into sterile 100 mm × 20 mm petri dishes and allowed to solidify. The following procedures were performed by streaking microbial suspension on MHA plate using a sterile cotton swab, boring the agar wells in the agar plate using sterile corn borers (6.0 mm diameter) and introducing a 50 µL volume of the samples into the wells of the agar plates. The plates were incubated at 35-37ºC for 16-18 h. The zone of inhibition was recorded to the nearest size in mm (Chien *et al.*, 2016). Gentamicin (0.1% w/w) was prepared in nanoemulsion as a positive control, while blank nanoemulsions were used as a negative control for *Staphylococcus aureus* and *Staphylococcus epidermidis*. All the experiments were performed in triplicate.

3.2.10. Statistical analysis

Data were expressed as means ± standard deviation (SD). Statistical comparisons were made using paired t test and Student's t test or One-way analysis of variance (ANOVA). Differences at $p < 0.05$ were considered to be significant. The analysis was performed by using SPSS statistics version 20.

CHAPTER 4
RESULTS AND DISCUSSION

4.1. Screening of tamarind fruit pulp extracts

In order to obtain the most suitable tamarind fruit pulp extract used for nanoemulsion formulations, the screening of tamarind fruit pulp extract samples was performed by determining antioxidant, antityrosinase and antibacterial properties of the extracts.

4.1.1. Antioxidant activity of tamarind fruit pulp extracts

The antioxidant activities of tamarind fruit pulp extracts obtained from different sources are summarized in **Table 4.1**. The antioxidant activity of ascorbic acid, a well-known antioxidant agent, was measured to prove the method accuracy. According to the results, the sweet tamarind fruit pulp extract possessed relatively higher antioxidant property among the samples. Accordingly, it was selected as an active ingredient in the further study. It was noted that the higher antioxidant activity of the sweet tamarind fruit pulp extract resulted from the combined organic acids even though it contained the lowest amount of tartaric acid (0.3240 ± 0.0631 mg% (w/w)).

Table 4.1. Effective concentration of positive control and tamarind fruit pulp extract samples required to scavenging DPPH radical by 50%.

Samples	Test concentrations	EC_{50}
Ascorbic acid (positive control)	0.1-50 µg/mL	2.8698 µg/mL
Sample 1, sweet tamarind fruit pulp extract (Guangzhou Phytochem Sciences Inc)	0.029-15 mg/mL	0.6681 mg/mL
Sample 2, Organic water soluble tamarind fruit pulp extract (Fuyang Bestop Import and Export Ltd)	0.059-30 mg/mL	13.9115 mg/mL
Sample 3, tamarind fruit pulp extract (water)	0.78-40 mg/mL	11.3154 mg/mL
Sample 4, tamarind fruit pulp extract (50% ethanol)	0.78-40 mg/mL	4.0025 mg/mL

4.1.2. Antityrosinase activity of tamarind fruit pulp extracts

The antityrosinase activities of tamarind fruit pulp extracts obtained from different sources are shown in **Table 4.2**. The antityrosinase activity of kojic acid, a well-known antityrosinase agent, was measured to prove the method accuracy. According to the results, the sweet tamarind fruit pulp extract possessed relatively higher antityrosinase property (with low EC_{50} value) among the samples. Accordingly, it was selected as an active ingredient in the further study.

Table 4.2. Effective concentration of positive control and tamarind fruit pulp extract samples required to inhibit tyrosinase enzyme by 50%.

Samples	**Test concentrations**	**EC_{50}**
Kojic acid (positive control)	0.1-1 mg/mL	0.519 mg/mL
Sample 1, sweet tamarind fruit pulp extract (Guangzhou Phytochem Sciences Inc)	0.47-15 mg/mL	15.983 mg/mL
Sample 2, Organic water soluble tamarind fruit pulp extract (Fuyang Bestop Import and Export Ltd)	0.94-30 mg/mL	37.211 mg/mL
Sample 3, tamarind fruit pulp extract (water)	1.25-40 mg/mL	43.057 mg/mL
Sample 4, tamarind fruit pulp extract (50% ethanol)	1.25-40 mg/mL	26.600 mg/mL

4.1.3. Antibacterial activity of tamarind fruit pulp extracts

The antibacterial activity of tamarind fruit pulp extracts was evaluated by broth macrodilution and the results are displayed in **Table 4.3**. The lowest concentration of tamarind fruit pulp extracts inhibiting the bacterial growth was considered the MIC. The extracts were found to be effective against *Staphylococcus epidermidis* and *Staphylococcus aureus*. Among the test extracts, the lowest MIC value was obtained from Sample 1, sweet tamarind fruit pulp extract **(Table 4.3)**. The sweet tamarind fruit pulp extract showed highest activity against these gram positive bacterial species which cause malodor in underarm areas. For MBC, the lowest

concentration which kill bacteria, was usually higher than MIC. In the current study, MBC could not be determined because all tamarind fruit pulp extract samples turned pink (resorufin). Thus, all test tamarind fruit pulp extract samples had MBC values greater than 8 mg/mL. Antibacterial activities of tamarind fruit pulp extract against gram positive bacteria have been reported (Gupta *et al.*, 2014).

Table 4.3. MIC of positive control and tamarind fruit pulp extract samples against *Staphylococcus epidermidis* and *Staphylococcus aureus*.

Samples	**Test concentrations**	**MIC**	
		Staphylococcus epidermidis	***Staphylococcus aureus***
0.1%w/w Gentamicin sulfate	0.01-4 µg/mL	0.25 µg/mL	0.25 µg/mL
Sample 1, sweet tamarind fruit pulp extract (Guangzhou Phytochem Sciences Inc)	0.02-8 mg/mL	8 mg/mL	8 mg/mL
Sample 2, Organic water soluble tamarind fruit pulp extract (Fuyang Bestop Import and Export Ltd)	0.02-8 mg/mL	>8 mg/mL	>8 mg/mL
Sample 3, tamarind fruit pulp extract (water)	0.02-8 mg/mL	>8 mg/mL	>8 mg/mL
Sample 4, tamarind fruit pulp extract (50% ethanol)	0.02-8 mg/mL	>8 mg/mL	>8 mg/mL

Based on the three bioactivities, sweet tamarind fruit pulp extract from China was a promising candidate to be used as the active ingredient for nanoemulsion formulations.

4.2. Blank nanoemulsions

After high pressure homogenization process, all prepared blank nanoemulsions (F1-F7) showed creamy white appearances with no phase separation.

4.2.1. Characteristics of nanoemulsion types

In the current investigation, both electrical conductivity and dye solubility test were employed to determine the type of blank nanoemulsions. For o/w nanoemulsions with water as external or continuous phase, water could conduct an electrical current, resulting in rather high conductivity values of the o/w formulations (Khan *et al.*, 2011) as shown in **Table 4.4**. It was observed that the formulation F1 with no thickener exhibited the lowest conductivity values in comparison with the other formulations (F2-F7) which contained natural and semi-natural gums: xanthan gum, guar gum or cationic guar gum. These thickeners dissolved in the water phase and could produce charged electrical particles. The amount of the thickener added into the formulations also affected the conductivity behavior of the formulations. In the case of dye solubility test, it was found that Ponceau 4 R solution, a water soluble dye, dissolved uniformly throughout the system, meaning the formulations were o/w nanoemulsions.

Table 4.4. Conductivity and dye solubility tests of various blank nanoemulsions.

Formulation	**Conductivity value (µs/cm) (mean ± SD, n=3, where n is number of samples)**	**Dye solubility**
F1 (no thickener)	83.70 ± 1.49	miscible
F2 (0.1% XG)	129.87 ± 4.84	miscible
F3 (0.25% XG)	204.07 ± 1.12	miscible
F4 (0.10% GG)	160.13 ± 6.99	miscible
F5 (0.25% GG)	187.13 ± 5.56	miscible
F6 (0.10% CGG)	167.40 ± 0.46	miscible
F7 (0.25% CGG)	296.07 ± 1.21	miscible

XG=xanthan gum, GG = guar gum, CGG = cationic guar gum

4.2.2. Physicochemical property and stability

Physicochemical properties of blank nanoemulsions at three conditions: freshly prepared (24-h), freeze-thaw cycles, and storage for one month at three temperatures were evaluated. The results are summarized in **Tables 4.5-4.7**.

The pH of skin is one of the factor that the formulators need to consider when designing the skin care products and cosmetics. This is due to improper pH of the dermatological products can cause skin irritation (Mahdi *et al.*, 2011). In general, skin pH ranging from 4 to 6 (slightly acidic) has been reported (Baumann and Castanedo-Tardan, 2009). As seen from **Table 4.5**, the pH values of nanoemulsions were close to the range of skin pH. Under the stability conditions, the pH values of all formulations appeared to change slightly but were still in the acceptable range of the skin pH. In the case of viscosity, in general the viscosity of the prepared formulations was quite low, less than 30 cps, when determined at skin surface temperature of 32°C. As seen from **Table 4.6**, under the freeze-thaw and 1 month of storage at three different temperatures 4°C, 30°C and 45°C, there were drastic changes in viscosity values of the formulations F3 through F7, yet the viscosity was still relatively low. Slightly changes of viscosity values were observed for the formulations F1 and F2. The representative rheograms of some formulations, F1, F2 and F3, are shown in **Figure 4.1**. The nanoemulsions F1 through F3 tended to exhibit shear-thinning behavior (pseudoplastic flows). Under the experimental stress conditions, the flow properties of formulations F1 (no thickener) and F2 (0.1% xanthan gum) did not considerably change, which was different from the formulation F3 (0.25% xanthan gum). The concentrations of xanthan gum affected the flow behavior of the formulations. This was clearly observed when the concentration of xanthan gum was sufficiently increased. The polymer chains of the thickener (here 0.25% xanthan gum) were probably under ordered-disordered conformational transition during the stress conditions, resulting in the intense changes of viscosity. According to Garcıa-Ochoa *et al.* (2000), the viscosity of xanthan solution can be changed depending on temperature; between 10-80ºC the viscosity of xanthan solution was decreased and this behavior was fully irreversible. However, this phenomenon can be easily happened at high concentration of xanthan gum because of the interaction between polymer molecules (Wyatt *et al.*, 2011). It was found that the viscosity of the formulation F1 which did not contain any thickeners was very low. The addition of hydrocolloid, xanthan gum, did not effectively increase the viscosity of the formulation as expected (F3, 0.25% xanthan gum). The same results were also obtained in the case of the other two thickeners, guar gum and cationic guar gum. The

polymer chains of the thickeners were probably damaged under the process of high pressure homogenization (high pressure and rather high heat about 60°C). Therefore, the viscosity of the nanoemulsions containing thickeners was low. Such range of hydrocolloid concentrations could enormously increase the viscosity of the o/w emulsions having the same ingredients (data not show).

Table 4.5. pH of blank nanoemulsions.

Formulation	pH (mean ± SD, n=3, where n is number of samples)				
	Freshly prepared	After Freeze-thaw (4 cycles)	After 1 month		
			4 ± 2°C	30 ± 2°C	45 ± 2°C
F1 (no thickener)	6.65 ± 0.02	6.26 ± 0.06	6.34 ± 0.06	6.32 ± 0.03	5.96 ± 0.04
F2 (0.1% XG)	6.54 ± 0.02	6.08 ± 0.01	6.74 ± 0.03	6.34 ± 0.02	6.19 ± 0.02
F3 (0.25% XG)	6.48 ± 0.04	6.13 ± 0.01	6.60 ± 0.01	6.21 ± 0.02	5.53 ± 0.03
F4 (0.10% GG)	6.56 ± 0.03	6.19 ± 0.02	6.64 ± 0.04	6.31 ± 0.02	6.16 ± 0.05
F5 (0.25% GG)	6.86 ± 0.05	6.60 ± 0.01	7.18 ± 0.06	6.64 ± 0.02	6.32 ± 0.01
F6 (0.10% CGG)	6.82 ± 0.05	6.50 ± 0.01	6.88 ± 0.01	6.68 ± 0.01	6.34 ± 0.03
F7 (0.25% CGG)	6.72 ± 0.01	6.40 ± 0.01	6.67 ± 0.04	6.64 ± 0.03	6.29 ± 0.02

XG=xanthan gum, GG = guar gum, CGG = cationic guar gum

Table 4.6. Viscosity of blank nanoemulsions.

Formulation	Viscosity (cps) at 30 rpm, 32°C (mean ± SD, n=3, where n is number of samples)				
	Freshly prepared	After Freeze-thaw (4 cycles)	After 1 month		
			4 ± 2°C	30 ± 2°C	45 ± 2°C
F1 (no thickener)	6.39 ± 0.35	8.00 ± 0.00	6.34 ± 0.35	10.28 ± 0.25	6.22 ± 0.19
F2 (0.1% XG)	7.77 ± 0.39	8.13 ± 0.23	8.11 ± 0.19	8.30 ± 0.26	7.28 ± 0.25
F3 (0.25% XG)	12.27 ± 0.46	17.44 ± 0.10	9.11 ± 0.19	21.98 ± 0.04	9.39 ± 0.10
F4 (0.10% GG)	5.17 ± 0.28	8.00 ± 0.00	28.00 ± 0.00	10.11 ± 0.19	30.28 ± 0.25
F5 (0.25% GG)	12.13 ± 0.23	3.11 ± 0.19	7.99 ± 0.02	11.00 ± 0.00	7.11 ± 0.19
F6 (0.10% CGG)	6.11 ± 0.19	4.49 ± 0.16	27.28 ± 0.12	8.30 ± 0.26	29.22 ± 0.19
F7 (0.25% CGG)	7.78 ± 0.38	7.11 ± 0.19	13.11 ± 0.19	12.33 ± 0.00	20.41 ± 0.09

XG=xanthan gum, GG = guar gum, CGG = cationic guar gum

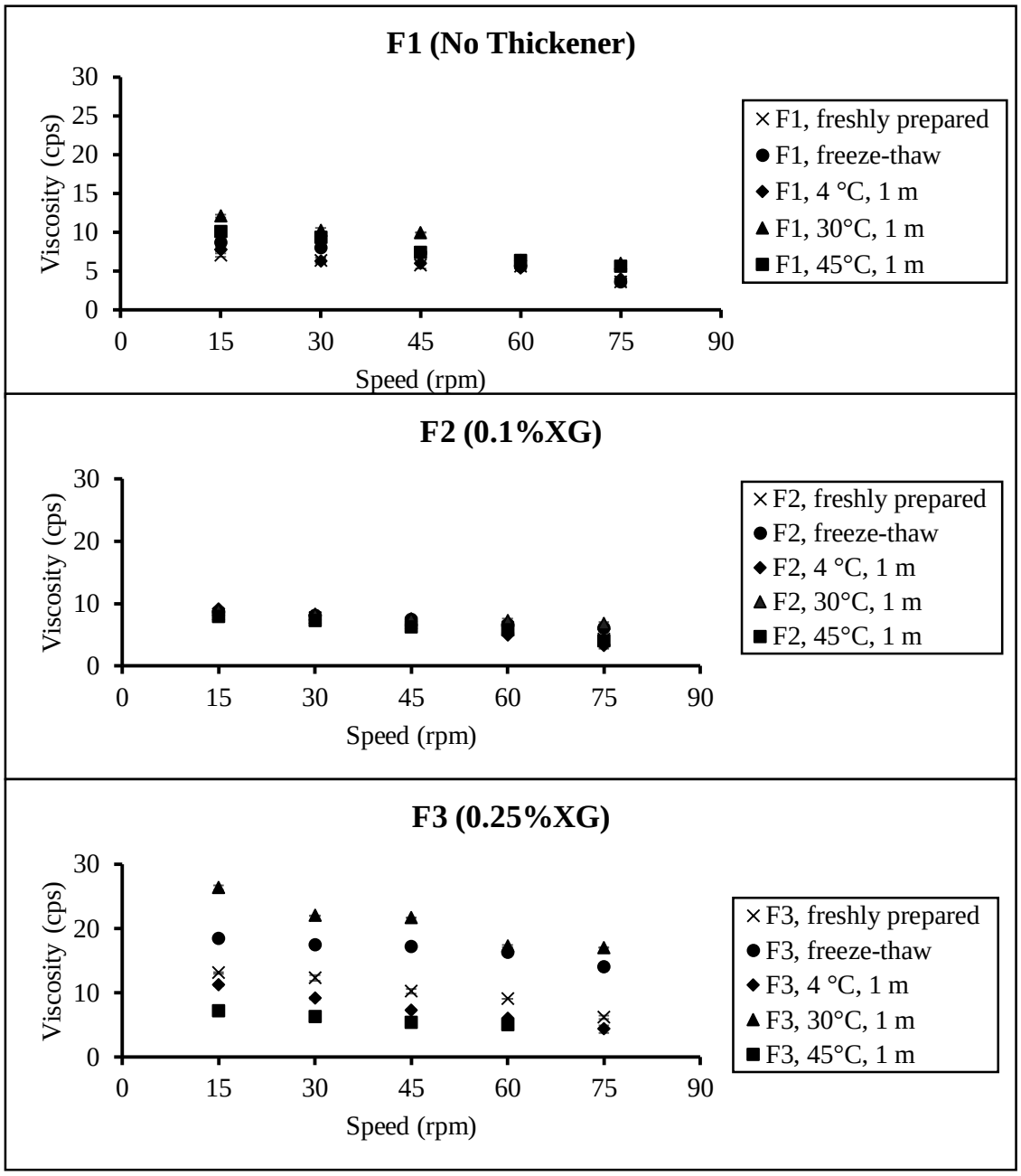


Figure 4.1. Representative rheograms of nanoemulsions, F1, F2 and F3 at different conditions determined at 32°C. XG=Xanthan gum. Each point represents mean ± SD, n =3; where n = number of samples.

Initially, the centrifugation method was used to check the physical stability of all 1-day old. There was no phase separation of blank nanoemulsions under the studied centrifugation condition (3,000 rpm for 30 min), indicating the physical stability of the formulations. In addition, the stability of nanoemulsion systems can be evaluated by determining several important parameters such as droplets sizes, PDI

and zeta potential (surface charge of droplet). Therefore, in the current study, these factors were investigated after 24 h preparation, freeze-thaw and over 1 month at three different temperatures: 4°C, room temperature (about 30°C) and 45°C. The results of these parameters are summarized in **Table 4.7**. It is generally recognized that the change in droplet sizes reflects the stability of the systems (Ribeiro *et al*., 2015). The droplet sizes of nanoemulsion formulations were approximately 100-800 nm, which were considered in the range of nanoemulsions (< 1 µm). It was observed that the type of hydrocolloids used affected the droplet sizes of nanoemulsions. For freshly prepared formulations, the largest sizes were found in the formulation F7 which contained 0.25% cationic guar gum. These hydrocolloids were probably adsorbed on the droplet surfaces, resulting in the increment of the sizes. Some magnitudes of changes in the droplet sizes were observed when the nanoemulsions were subjected to freeze-thaw treatment and stored for 1 month at three temperature conditions. The droplet sizes of the formulation F1 remained constant for all experimental conditions, whereas there were significant changes of droplet sizes in the formulations F5 through F7 which contained guar gum or cationic guar gum. The PDI indicates the uniformity of droplet sizes in nanoemulsion systems. PDI value closer to zero indicates homogeneity of droplets (Ribeiro *et al.*, 2015). It was found that the freshly prepared nanoemulsions possessed low PDI values (between 0.137-0.362). The formulations F1through F4 had PDI values less than the formulations F5 through F7 did, indicating more homogeneous of droplet sizes in these nanoemulsion formulations. There were certain changes of PDI values for freeze-thaw and all three temperature conditions. The highest PDI values obtained was 0.440 in the formulations F5 and F6. The formulations F5, F6 and F7 with PDI values higher than 0.3 would produce unstable nanoemulsions owing to Ostwald ripening phenomena. It was noted that the formulations F1 through F4 which had smaller droplet sizes also had smaller values of PDI when compared with the formulations F5 through F7. The more stable nanoemulsions would be achieved with the formulations F1 through F4. Zeta potential is related to surface charge of droplets. Although all nanoemulsion formulations contained non ionizable surfactants (nonionic), they showed positive or negative zeta potential values. As seen from **Table 4.7**, the zeta potential of the prepared formulations was between -63.02 to +12.28 mV for 1-day old preparation. Most

nanoemulsion formulations displayed negatively charged zeta potential values, except for the formulations containing cationic guar gum (F6 and F7). The negative charge of the o/w nanoemulsions F1-F5 was possibly owing to the spontaneous adsorption of hydroxyl ions from water at the oil-water interface (Mahdi *et al.*, 2011; Marinova *et al.*, 1996). In addition, the negative zeta potential values were involved with the polyoxyethylene chains in the nonionic surfactants (a mixture of Tween 80 and Span 80) which was used as the emulsifier (Maruno *et al.*, 2009). Interestingly, the nanoemulsions containing cationic guar gum (F6 and F7) showed net positive zeta potential values for freshly prepared formulations and after freeze-thaw cycles. The cationic molecules of the hydrocolloid were possibly adsorbed on the surface of droplets and thus providing positive charge. However, after keeping in three different temperatures for 1 month, the zeta potential values became negative. The zeta potential of these formulations shifted from positive charge to slightly negative, suggesting the change in the electrostatic interactions. Desorption of cationic molecules on the surface of nanoemulsion droplets may occur after a certain period of time. This may be caused by the affinity of water (hydroxyl ions) exceeding the affinity of quaternary ammonium groups of cationic guar gum. The stability of the colloidal systems can be presumed based on the magnitude of zeta potential. In general, sufficient high values of zeta potential reflect low/no incidence of droplet flocculation (aggregation of droplets), repulsive forces exceeding attractive forces (van der Waals) (Abdulkarim *et al.*, 2010). Zeta potential between ±30 and ±60 mV provides moderate stable systems and the values between ±60 and ±100 mV produce good to excellent stable systems (Nash, 1996). Based on the zeta potential values, the suitable blank nanoemulsions were the formulation F1 (no thickener) and the formulations F2 (0.10% xanthan gum) and F3 (0.25% xanthan gum). The increased zeta potential values were observed in the formulations F4 and F5 which contained guar gum after 1 month of storage at three different temperatures.

Under environmental stress conditions; freeze-thaw cycles, 4°C and 45°C for 1 month, all blank nanoemulsion formulations remained homogeneous and creamy white similar to the freshly prepared formulations although changes in certain physicochemical properties were found in some formulations.

Table 4.7. Droplet sizes, PDI and zeta potential of blank nanoemulsions.

Formulation	Droplet size (diameter) (nm) (mean ± SD, n=10, where n is number of measurements on the sample)				
	Freshly prepared	After Freeze-thaw (4 cycles)	After 1 month		
			4 ± 2°C	30 ± 2°C	45 ± 2°C
F1 (no thickener)	139.69 ± 2.16	139.86 ± 2.95	140.00 ± 5.22	140.46 ± 1.57	138.95 ± 3.88
F2 (0.1% XG)	117.59 ± 3.38	113.96 ± 2.37	114.14 ± 2.07	118.85 ± 2.53	114.49 ± 2.55
F3 (0.25% XG)	124.24 ± 2.79	115.59 ± 2.67	128.47 ± 4.52	121.78 ± 2.66	130.34 ± 3.53
F4 (0.10% GG)	104.67 ± 1.49	116.22 ± 2.48	105.96 ± 2.35	110.46 ± 3.54	111.00 ± 2.93
F5 (0.25% GG)	448.32 ± 3.60	484.26 ± 3.19	664.99 ± 3.15	565.69 ± 2.67	714.76 ± 3.34
F6 (0.10% CGG)	425.23 ± 2.86	623.37 ± 2.78	524.84 ± 3.39	445.61 ± 3.92	344.31 ± 3.24
F7 (0.25% CGG)	823.78 ± 3.60	516.56 ± 2.38	675.09 ± 3.02	628.37 ± 5.17	575.15 ± 3.29
Formulation	PDI values (mean ± SD, n=10, where n is number of measurements on the sample)				
	Freshly prepared	After Freeze-thaw (4 cycles)	After 1 month		
			4 ± 2°C	30 ± 2°C	45 ± 2°C
F1 (no thickener)	0.137 ± 0.039	0.170 ± 0.034	0.158 ± 0.060	0.184 ± 0.031	0.141 ± 0.041
F2 (0.1% XG)	0.145 ± 0.039	0.173 ± 0.039	0.167 ± 0.051	0.160 ± 0.040	0.147 ± 0.052
F3 (0.25% XG)	0.174 ± 0.039	0.171 ± 0.059	0.179 ± 0.079	0.181 ± 0.024	0.151 ± 0.056
F4 (0.10% GG)	0.135 ± 0.044	0.135 ± 0.039	0.165 ± 0.039	0.150 ± 0.040	0.195 ± 0.045
F5 (0.25% GG)	0.226 ± 0.070	0.397 ± 0.014	0.440 ± 0.042	0.344 ± 0.054	0.313 ± 0.063
F6 (0.10% CGG)	0.264 ± 0.020	0.341 ± 0.056	0.291 ± 0.038	0.440 ± 0.042	0.288 ± 0.051
F7 (0.25% CGG)	0.362 ± 0.006	0.310 ± 0.076	0.362 ± 0.047	0.351 ± 0.073	0.368 ± 0.040

XG=xanthan gum, GG = guar gum, CGG = cationic guar gum

Table 4.7. Droplet sizes, PDI and zeta potential of blank nanoemulsions (continued).

Formulation	Zeta potential (mV) (mean ± SD, n=10, where n is number of measurements on the sample)				
	Freshly prepared	After Freeze-thaw (4 cycles)	After 1 month		
			4 ± 2°C	30 ± 2°C	45 ± 2°C
F1 (no thickener)	-63.02 ± 2.81	-22.34 ± 4.77	-38.50 ± 0.42	-38.52 ± 0.46	-38.34 ± 0.24
F2 (0.1% XG)	-37.42 ± 2.02	-22.69 ± 6.15	-38.53 ± 0.75	-38.21 ± 0.37	-38.72 ± 0.50
F3 (0.25% XG)	-32.94 ± 2.55	-31.59 ± 4.77	-38.50 ± 0.57	-38.08 ± 0.72	-38.28 ± 0.47
F4 (0.10% GG)	-18.09 ± 2.09	-19.12 ± 7.44	-38.45 ± 0.74	-38.15 ± 0.65	-38.26 ± 0.49
F5 (0.25% GG)	-19.06 ± 1.99	-27.22 ± 3.49	-38.29 ± 0.41	-38.7 ± 0.30	-38.43 ± 0.66
F6 (0.10% CGG)	6.24 ± 1.73	5.52 ± 1.43	-2.90 ± 0.04	-3.02 ± 0.05	-3.00 ± 0.03
F7 (0.25% CGG)	12.28 ± 1.30	10.28 ± 2.11	-3.01 ± 0.04	-3.00 ± 0.04	-2.97 ± 0.05

XG=xanthan gum, GG = guar gum, CGG = cationic guar gum

4.3. Tamarind fruit pulp extract loaded nanoemulsions

According to the above results, the most promising blank nanoemulsion candidate to be incorporated with the sweet tamarind fruit pulp extract (powder) would be the formulation F1. For comparison purpose, the formulations F3 (0.25% xanthan gum), F5 (0.25% guar gum) and F7 (0.25% cationic guar gum) were also selected. Based on the antioxidant activity study, the concentrations of the sweet tamarind fruit pulp extract used in the current study were 100-times and 50-times of the EC_{50} which were 6.6 and 3.3%w/w, respectively. After mixing the ingredients (blank nanoemulsion and tamarind powder) together using a magnetic stirrer overnight, it was found that there was phase separation in the formulations which contained xanthan gum, guar gum or cationic guar gum. The incompatibility between tamarind fruit pulp extract powder and these hydrocolloids may occur. The stable tamarind fruit pulp extract loaded nanoemulsion was achieved only in the formulation F1 which did not contain any hydrocolloids. However, after a few days, mold growth was observed. Thus, suitable preservatives, 1%w/w ethylhexylglycerin and 0.2%w/w phenoxyethanol (paraben free) were incorporated into the formulation.

The o/w nanoemulsions (F1) containing sweet tamarind fruit pulp extract were further investigated for physicochemical properties, stability and bioactivities.

4.3.1. Characteristics of tamarind fruit pulp extract loaded nanoemulsions

The formulations which contained 3.3%w/w or 6.6%w/w sweet tamarind fruit pulp extract were called as F1-3.3TE and F1-6.6TE, respectively. The freshly prepared formulations had brown color, homogeneity and no precipitation **(Figure 4.2)**. There was no phase separation under the centrifugation test. Using dye solubility test, the two tamarind fruit pulp extract loaded nanoemulsions showed miscibility. The average conductivity values of the freshly prepared tamarind fruit pulp extract loaded nanoemulsion, F1-3.3TE, were 1,170.67 ± 12.10 µs/cm, which were significant higher than those of the blank nanoemulsion F1 (83.70 ± 1.49 µs/cm) ($p < 0.05$). Similar result was also observed in the case of the nanoemulsion F1-6.6TE (2,071.33 ± 9.61 µs/cm) when compared with the blank F1 ($p < 0.05$). It was obvious that the incorporated tamarind fruit pulp extract markedly affected the conductivity of the nanoemulsion system. Based on both tests, the type of tamarind fruit pulp extract loaded nanoemulsions remained o/w system. The remarkably high conductivity of plant extracts may be associated with the loss of membrane integrity of the extracts during measuring process and the increased ionic content in the formulations (Anjali *et al.*, 2010; Lovelock *et al.*, 2006)

4.3.2. Physicochemical property and physical stability of tamarind fruit pulp extract loaded nanoemulsions

The pH and viscosity of the tamarind fruit pulp extract loaded formulations in comparison with the blank formulations are summarized in **Tables 4.8-4.9**. For the blank formulations, the stability was determined for 1 month. The longer time of 2 months was employed for the tamarind fruit pulp extract loaded formulations. The pH measurement is one of the important indicators that can convey the quality of the products. Highly acidic or alkaline products can cause skin irritation. Adding tamarind fruit pulp extract into the blank nanoemulsion (F1) significant decreased the pH of the preparations ($p <0.05$) for all experimental conditions because of the organic acids in the tamarind fruit pulp extract. The freshly prepared tamarind fruit pulp extract loaded nanoemulsions had weak acidic pH around 5. Different degrees of

pH changes were observed for the formulations kept for 1 month or 2 months at three various temperatures. As seen from **Table 4.8**, the temperature of 4 °C (refrigerator) appeared to be the most appropriate condition to keep the tamarind products since the formulations F1-3.3TE and F1-6.6TE had pH values around 5 similar to the freshly prepared formulations. These pH values were considered safe for dermatological products since they were still in the range of the skin pH (4-6). Nevertheless, for safety reasons, the irritation potential of the preparations should be investigated in the future work. In the case of viscosity, the viscosity of tamarind fruit pulp extract loaded nanoemulsions was significantly higher than that of the blank nanoemulsion, F1 ($p < 0.05$). The viscosity of the freshly prepared formulations was in the range of 200-300 cps. The increased viscosity was probably attributed to constituents such as carbohydrates, fibers and invert sugars in the sweet tamarind fruit pulp. Under the stress condition, the viscosity of the tamarind fruit pulp extract loaded nanoemulsions kept at 4°C did not differ statistically in comparison with the freshly prepared nanoemulsions. This result also supported that the suitable storage condition for tamarind fruit pulp extract loaded nanoemulsions was cold place, around 4°C. Notably, the formulation F1-6.6TE was not stable (phase separation) at the end of 2 months. After 2 months, the pH values of the formulation F1-6.6TE were similar to those of the formulation F1-3.3TE, yet the formulation F1-3.3TE remained stable (no phase separation). Thus, it was postulated that high amount of tamarind fruit pulp extract caused the instability of the nanoemulsion system. Certain constituents in the tamarind fruit pulp extract was possibly incompatibly with the emulsifier, a mixture of Tween 80 and Span 80. The viscosity measurement at the end of 2 months of this formulation, F1-6.6TE, was not performed.

Figure 4.2. Appearance of tamarind fruit pulp extract loaded nanoemulsion (F1-3.3TE).

Table 4.8. pH of tamarind fruit pulp extract loaded nanoemulsions.

Formulation	pH (mean ± SD, n=3, where n is number of samples)							
	Freshly prepared	After Freeze-thaw (4 cycles)	After 1 month			After 2 months		
			4 ± 2°C	30 ± 2°C	45 ± 2°C	4 ± 2°C	30 ± 2°C	45 ± 2°C
F1 (blank)	6.65 ± 0.02	6.26 ± 0.06	6.34 ± 0.06	6.32 ± 0.03	5.96 ± 0.04	n.d.	n.d.	n.d.
F1-3.3TE	5.28 ± 0.06	5.01 ± 0.02	5.43 ± 0.03	4.76 ± 0.05	4.75 ± 0.03	5.15 ± 0.03	4.45 ± 0.03	4.72 ± 0.04
F1-6.6TE	5.15 ± 0.01	4.79 ± 0.02	5.23 ± 0.02	4.14 ± 0.03	4.84 ±0.03	5.24 ± 0.05	4.10 ± 0.09	4.79 ± 0.02

F1-3.3TE =Formulation F1 (no thickener) containing 3.3%w/w tamarind fruit pulp extract; F1-6.6TE = Formulation F1 (no thickener) containing 6.6%w/w tamarind fruit pulp extract; * Phase separation

Table 4.9. Viscosity of tamarind fruit pulp extract loaded nanoemulsions.

Formulation	Viscosity (cps) at 30 rpm, 32°C (mean ± SD, n=3, where n is number of samples)							
	Freshly prepared	After Freeze-thaw (4 cycles)	After 1 month			After 2 months		
			4 ± 2°C	30 ± 2°C	45 ± 2°C	4 ± 2°C	30 ± 2°C	45 ± 2°C
F1 (blank)	6.39 ± 0.35	8.00 ± 0.00	6.34 ± 0.35	10.28 ± 0.25	9.39 ± 0.10	n.d.	n.d.	n.d.
F1-3.3TE	259.94 ± 34.63	239.08 ± 1.44	249.74 ± 1.90	370.52 ± 3.45	282.74 ± 6.52	248.52 ± 3.09	368.83 ± 3.11	186.37 ± 1.03
F1-6.6TE	306.70 ± 46.09	302.44 ± 0.50	286.24 ± 2.40	413.28 ± 5.56	390.06 ± 8.43	n.d.*	n.d.*	n.d.*

F1-3.3TE =Formulation F1 (no thickener) containing 3.3%w/w tamarind fruit pulp extract; F1-6.6TE = Formulation F1 (no thickener) containing 6.6%w/w tamarind fruit pulp extract; * Phase separation

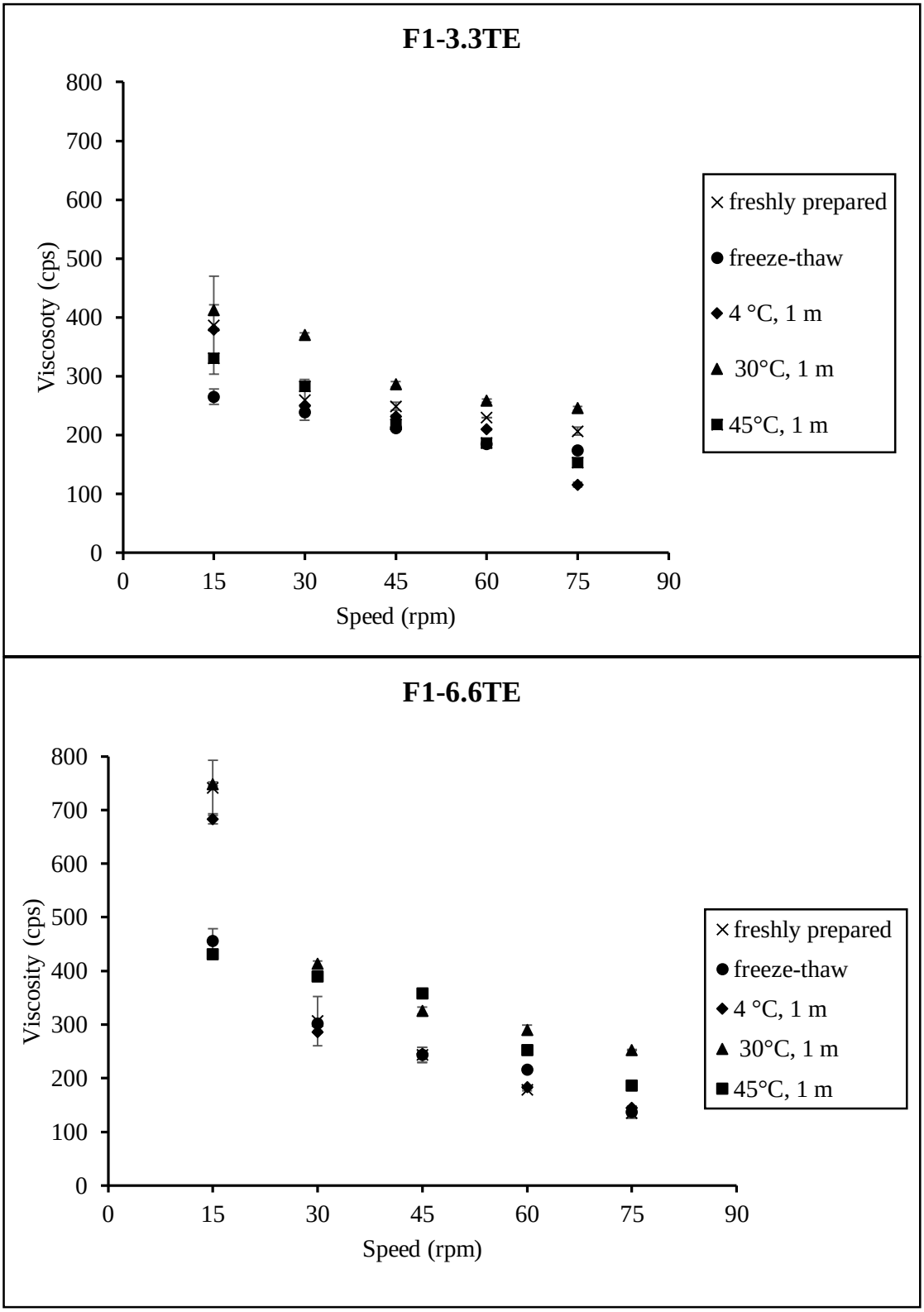


Figure 4.3. Rheograms of tamarind fruit pulp extract loaded nanoemulsions, F1-3.3TE and F1-6.6TE stored at different conditions for 1 month, determined at 32ºC. Each point represents mean ± SD, n =3; where n = number of samples.

The flow curves of the two tamarind fruit pulp extract loaded nanoemulsions under stress environment are exhibited in **Figure 4.3**. The shear thinning behavior was observed for both formulations. There were certain changes in the viscosity of the tamarind fruit pulp extract loaded formulations under freeze-thaw and after storing at three different temperatures when compared with the freshly prepared nanoemulsions. The formulation F1-6.6TE displayed greater changes in viscosity values than the formulation F1-3.3TE did. It is generally recognized that viscosity represents the characteristic properties of fluids. Viscosity measurements can provide useful information concerning natures/structures of nanoemulsions, phase separation, and also the release of active ingredients from the formulations (Santos *et al.*, 2008). Based on the current study, it was likely that the tamarind fruit pulp extract somewhat altered the microstructures of the nanoemulsion systems.

The droplet size, PDI and zeta potential of freshly prepared tamarind fruit pulp extract loaded nanoemulsions and the loaded formulations under environmental stress conditions are summarized in **Table 4.10**. As previously mentions, these three parameters can be used as indicators for the stability of the formulations. As seen from **Table 4.10**, there was significant difference in the droplet sizes between the unloaded and the freshly prepared tamarind fruit pulp extract loaded nanoemulsions ($p < 0.05$). The results clearly showed the influence of tamarind fruit pulp extract on the droplet sizes of the nanoemulsion system. A significant increase in droplet sizes of the formulation F1-3.3TE was observed at the end of 2 months (from 123.47 ± 5.32 nm to 136.39 ± 3.47 nm, $p < 0.05$). This may be owing to the ionized forms of organic acids in the tamarind fruit pulp extract being adsorbed on the surface of droplets. Nevertheless, they were still in an acceptable nanoemulsion range. The uniformity of the droplets was indicated by the PDI. For all experimental conditions, the tamarind fruit pulp extract loaded nanoemulsions displayed the PDI values less than 0.2, indicating high homogeneity of the nanoemulsion droplets (low polydispersity). Overall, the formulation F1-3.3TE had lesser PDI values than the formulation F1-6.6TE did. Another indicator for formulation stability was zeta potential measurement. Incorporation of tamarind fruit pulp extract into the blank nanoemulsions did not affect the surface charge of nanoemulsion droplets. All formulations displayed negative zeta potential. In the case of freshly prepared

preparations, the zeta potential of extracted loaded formulations (about -38 mV) was markedly lower than that of the unloaded nanoemulsion (about -63.02 mV) ($p < 0.05$). However, the zeta potential values of the extract loaded formulations were greater than 30 mV, indicating adequate stable systems. As seen from **Table 4.10**, the zeta potential of the formulation F1-3.3TE maintained in the range of -38 mV throughout 2 months for all three storage temperatures. The formulation F1-6.6TE was not stable (phase separation) after 2 months so these parameters were not measured. High amount of tamarind fruit pulp extract may affect the emulsifying ability of the nonionic surfactants (Tween 80 and Span 80), resulting in the phase separation of nanoemulsion system.

Table 4.10. Droplet sizes, PDI and zeta potential of tamarind fruit pulp extract loaded nanoemulsions.

Formulation	Droplet size (diameter) (nm) (mean ± SD, n=10, where n is number of measurements on the sample)							
	Freshly prepared	After Freeze-thaw (4 cycles)	After 1 month			After 2 months		
			4 ± 2°C	30 ± 2°C	45 ± 2°C	4 ± 2°C	30 ± 2°C	45 ± 2°C
F1 (blank)	139.69 ± 2.16	139.86 ± 2.95	140.00 ± 5.22	140.46 ± 1.57	138.95 ± 3.88	n.d.	n.d.	n.d.
F1-3.3TE	123.47 ± 5.32	127.90 ± 3.05	127.04 ± 2.36	168.33 ± 4.22	130.37 ± 2.20	133.06 ± 2.97	133.07 ± 1.92	136.39 ±3.47
F1-6.6TE	128.42 ± 2.91	130.11 ± 2.96	128.60 ± 2.59	275.65 ± 7.21	130.64 ± 3.42	n.d.	n.d.	n.d.
Formulation	**PDI values (mean ± SD, n=10, where n is number of measurements on the sample)**							
	Freshly prepared	After Freeze-thaw (4 cycles)	After 1 month			After 2 months		
			4 ± 2°C	30 ± 2°C	45 ± 2°C	4 ± 2°C	30 ± 2°C	45 ± 2°C
F1 (blank)	0.137 ± 0.039	0.170 ± 0.034	0.158 ± 0.060	0.184 ± 0.031	0.141 ± 0.041	n.d.	n.d.	n.d.
F1-3.3TE	0.105 ± 0.051	0.120 ± 0.058	0.126 ± 0.044	0.138 ± 0.043	0.149 ± 0.038	0.141 ± 0.040	0.171 ± 0.046	0.197 ± 0.043
F1-6.6TE	0.137 ± 0.060	0.146 ± 0.059	0.150 ± 0.047	0.150 ± 0.046	0.146 ± 0.043	n.d.	n.d.	n.d.

F1-3.3TE =Formulation F1 (no thickener) containing 3.3%w/w tamarind fruit pulp extract; F1-6.6TE = Formulation F1 (no thickener) containing 6.6%w/w tamarind fruit pulp extract; * Phase separation

Table 4.10. Droplet sizes, PDI and zeta potential of tamarind fruit pulp extract loaded nanoemulsions (continued).

Formulation	Zeta potential (mV) (mean ± SD, n=10, where n is number of measurements on the sample)							
	Freshly prepared	After Freeze-thaw (4 cycles)	After 1 month			After 2 months		
			4 ± 2°C	30 ± 2°C	45 ± 2°C	4 ± 2°C	30 ± 2°C	45 ± 2°C
F1 (blank)	-63.02 ± 2.81	-22.34 ± 4.77	-38.50 ± 0.42	-38.52 ± 0.46	-38.34 ± 0.24	n.d.	n.d.	n.d.
F1-3.3TE	-38.52 ± 0.46	-38.52 ± 0.46	-38.37 ± 0.35	-38.36 ± 0.30	-38.55 ± 0.29	-38.65 ± 0.36	-38.36 ± 0.30	-38.12 ± 0.38
F1-6.6TE	-38.15 ± 0.65	-38.15 ± 0.65	-38.51 ± 0.41	-38.12 ± 0.38	-38.42 ± 0.25	n.d.*	n.d.*	n.d.*

F1-3.3TE =Formulation F1 (no thickener) containing 3.3%w/w tamarind fruit pulp extract; F1-6.6TE = Formulation F1 (no thickener) containing 6.6%w/w tamarind fruit pulp extract; * Phase separation.

4.3.3. Chemical stability of tamarind fruit pulp extract loaded nanoemulsions

The initial percentage of tartaric acid remaining from nanoemulsions containing tamarind fruit pulp extract in freshly prepared, after freeze-thaw, after storage at 4 ± 2ºC, 30 ± 2ºC, and 45 ± 2ºC for 2 months are shown in **Figure 4.4**. The stability was defined as the remaining concentration no less than 90% of original concentration of active substances (Bajaj *et al.*, 2012). It was found that the amounts of tartaric acid (tamarind fruit pulp extract) remained in the formulation F1-3.3TE were greater than 90% w/w (96.5-106.5%) when compared with its original concentrations (100%w/w). This results indicated that the estimated shelf life of the formulation F1-3.3TE was at least 2 months. In contrast, at the end in the stability test period, F1-6.6TE had precipitation for all storage temperatures. As a result, the initial percentage of tartaric acid remained in the formulation F1-6.6TE were not further analyzed. As shown in **Figure 4.4**, the initial percentage of tartaric acid of the formulation were lower than 90% after freeze-thaw and 2-month stability. Therefore, the formulation F1-6.6TE was not considered stable. A phase separation was probably due to coagulation of the extract.

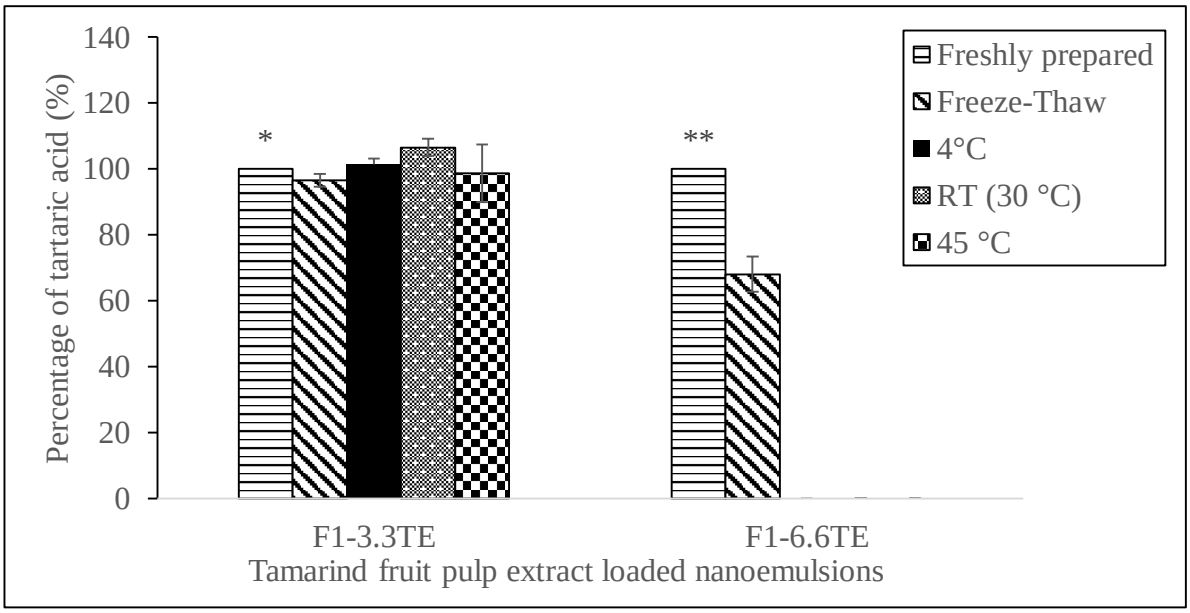


Figure 4.4. Percentage initial tartaric acid remained in tamarind fruit pulp extract loaded nanoemulsions in different conditions. Each point represents mean ± SD, n=3, where n is number of samples. *Actual initial tartaric acid concentration = 0.5968 mg% (w/w) (equivalent to 100%) ** Actual initial tartaric acid concentration = 0.8786 mg% (w/w) (equivalent to 100%).

4.4. *In vitro* release of tamarind fruit pulp extract loaded nanoemulsions

The *in vitro* release study was carried out for the freshly prepared tamarind fruit pulp extract loaded nanoemulsions. The amount of tartaric acid (tamarind fruit pulp extract) released across a synthetic membrane against time is depicted in **Figure 4.5**. At several time points, the formulation F1-6.6TE displayed significantly higher amount of tartaric acid released than the formulation F1-3.3TE did ($p < 0.05$). It could be concluded that the amount of tartaric acid released was directly related to the concentration of tamarind fruit pulp extract incorporated into the formulations. For both formulations, the drastic increase was observed in the first 6 h. However, from 8 h to 24 h, the formulation F1-6.6TE showed slight increase of tartaric acid released. At this period, there was no significant increase of tartaric acid in the case of the formulation F1-3.3TE. It reached a plateau state after 10 hours of experimental period.

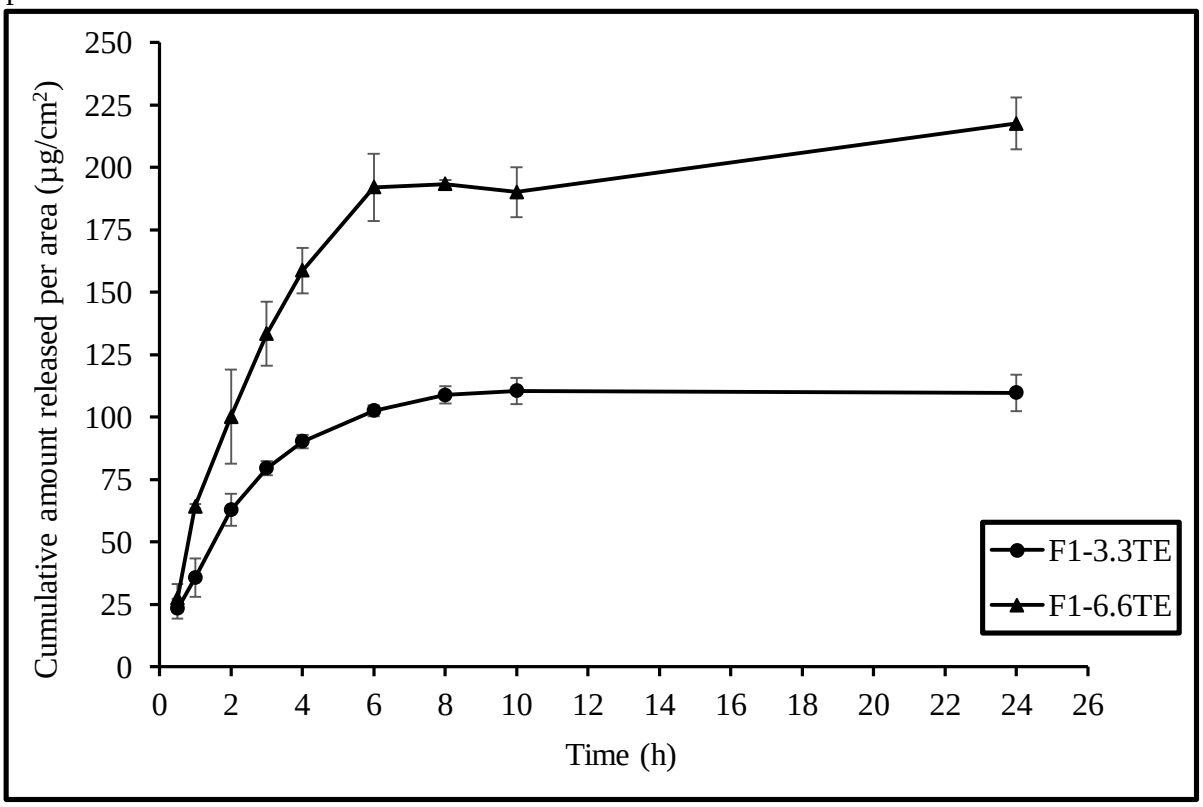


Figure 4.5. *In vitro* release of tartaric acid from tamarind fruit pulp extract loaded nanoemulsions across cellulose acetate membrane. Each point represents mean ± SD, n=3-4, where n is number of replications.

The release profiles were further evaluated for the best fit using different mathematical kinetic models: zero order, first order and Higuchi model. The best fit was based on the values of the coefficient of determination (r^2). For all three models, the release parameters of both formulations were assessed from 0.5 h to 6 h of the release profiles using linear regression analysis. The release rate constants (slopes of curves) and r^2 of each formulation are summarized in **Table 4.11**. The best fit was found to be Higuchi model for both formulations with r^2 of 0.97. This suggests diffusion release mechanism of tartaric acid (tamarind fruit pulp extract) from o/w nanoemulsions. The current results are in agreement with the other investigation. In a study conducted by Sakulku *et al.* (2009), the release of citronella oil from citronella oil nanoemulsions also followed Higuchi model with r^2 ranging from 0.8726 to 0.9618. Another study from Sood *et al.* (2014) revealed that the release of curcumin from nanoemulsions was only 74.34 ± 1.3% at the end of 24 hours. The release data of nanoemulsion formulations was fitted into zero order, first order and Higuchi's equations. However, the r^2 value for Higuchi's model was much higher compared to zero and first order models.

The rate constant or release rate of the formulation F1-6.6TE was statistically higher than that of the formulation F1-3.3TE ($p<0.05$). This was because of higher amount of tamarind fruit pulp extract.

Table 4.11. Release parameters of tamarind fruit pulp extract loaded nanoemulsions (mean ±SD, n=3-4, where n is number of replications).

Formulation	Zero order		First order		Higuchi	
	r^2	K_0 (µg/cm^2/h)	r^2	K_1 (1/h)	r^2	K_H (µg/cm^2/h$^{1/2}$)
F1-3.3TE	0.9070	14.555 ± 1.013	0.7969	0.258 ± 0.039	0.9744	47.831 ± 2.956
F1-6.6TE	0.9017	26.572 ± 4.719	0.7627	0.277 ± 0.061	0.9672	87.190 ± 14.401

K_0, K_1, K_H are rate constants of zero order, first order and Higuchi, respectively (calculated from 0.5 h to 6 h)

4.5. Bioactivity study of tamarind fruit pulp extract loaded nanoemulsions

4.5.1. Antioxidant activity

The antioxidant property (% scavenging activity) of tamarind fruit pulp extract loaded nanoemulsions, F1-3.3TE and F1-6.6TE were investigated and the results of freshly prepared formulations and under stress conditions are summarized in **Table 4.12**. The initial amounts of these two formulations were varied into three different concentrations by mixing with a mixture of absolute ethanol and distilled water (1:1 by volume). This was in order to study the effects of the amounts of tamarind fruit pulp extract (TE) on the antioxidant properties. The results revealed that % scavenging activity (antioxidant activity) was directly related to the concentrations of tamarind fruit pulp extract. The higher the amount of tamarind fruit pulp extract, the higher the % scavenging activity was obtained. It was noticed that even though the concentration of tamarind fruit pulp extract in certain samples was the same e.g. F1-3.3TE (3.3 mg/mL) and F1-6.6TE (3.3 mg/mL), the antioxidant activities were markedly different. For the same concentration, the formulation F1-3.3TE had better scavenging activity than the formulation F1-6.6TE did. This was probably due to the poor chemical stability of the formulation F1-6.6TE. The antioxidant properties of the formulations were caused by several organic acids present in the tamarind fruit pulp extract. It was reported that high % scavenging activity of pomegranate juice was associated with high level of organic acids in the juice (Tezcan *et al.*, 2009).

Under the freeze-thaw condition, the antioxidant activities of all samples decreased. The stress condition could affect the degradation of some chemical structures that attributed to the antioxidant activity (Lim and Murtijaya, 2007). For one month stability, the storage condition at 4°C provided the highest antioxidant activity similarly to that of the freshly prepared nanoemulsions. The decreased antioxidant activity was clearly detected when the storage temperature rising from 4 to 45°C. It was found that the formulation F1-6.6TE showed more decrease in antioxidant activity than the formulation F1-3.3TE did. After two months, the antioxidant activity of the formulation F1-3.3TE (all concentrations tested) significantly reduced when compared with the freshly prepared formulation ($p < 0.05$). Among the three storage temperatures, the formulation stored in the lowest

temperature of 4°C exhibited the highest antioxidant activity, whereas the formulations kept in 45ºC showed the lowest antioxidant activity. The increasing of temperature also decreased the antioxidant activity of ginger extract in sun flower oil (Salariya and Habib, 2003).

As previously mentioned, the non-degrade tartaric acid remained in the formulations was greater than 90% w/w when compared with its original concentration; yet the antioxidant activity of the formulation after 2 months was very low. It was likely that the antioxidant property of tamarind fruit pulp extract loaded nanoemulsion was largely caused by other organic acids present in the tamarind fruit pulp extract and tartaric acid played only minor role in this activity. It was revealed in the screening of tamarind fruit pulp extract section that the sweet tamarind fruit pulp extract had the lowest tartaric acid content but displayed the highest EC_{50}. In this case, the antioxidant activity was definitely attributed to other organic acids.

To maintain the antioxidant activity of tamarind fruit pulp extract loaded nanoemulsions, suitable extra antioxidant agents should be added into the formulations.

Table 4.12. Percent scavenging activity of tamarind fruit pulp extract loaded nanoemulsions determined by DPPH assay.

Formulation	Scavenging activity (%) (mean ± SD, n=5, where n is number of replications)							
	Freshly prepared	After Freeze-thaw (4 cycles)	After 1 month			After 2 months		
			4 ± 2°C	30 ± 2°C	45 ± 2°C	4 ± 2°C	30 ± 2°C	45 ± 2°C
F1-3.3TE (0.825 mg/mL*)	51.80 ± 0.37	13.68 ± 0.37	50.61 ± 0.78	44.30 ± 1.92	32.84 ± 1.42	18.99 ± 2.06	10.76 ± 0.78	10.26 ± 0.42
F1-3.3TE (1.65 mg/mL*)	67.24 ± 0.79	28.31 ± 1.03	65.30 ± 0.76	64.44 ± 1.28	54.47 ± 0.97	36.67 ± 2.33	24.58 ± 1.55	18.18 ± 1.99
F1-3.3TE (3.3 mg/mL*)	81.66 ± 0.77	48.56 ± 1.54	81.41 ± 0.41	78.24 ± 1.12	70.60 ± 1.39	43.42 ± 0.90	40.94 ± 1.99	34.13 ± 0.71
F1-6.6TE (1.65 mg/mL*)	49.36 ± 0.86	35.21 ± 0.91	32.48 ± 0.98	29.19 ± 1.19	14.17 ± 0.81	n.d.**	n.d.**	n.d.**
F1-6.6TE (3.3 mg/mL*)	63.80 ± 0.79	54.72 ± 0.96	52.84 ± 0.44	50.48 ± 1.57	35.08 ± 0.47	n.d.**	n.d.**	n.d.**
F1-6.6TE (6.6 mg/mL*)	84.16 ± 0.89	71.09 ± 1.12	79.38 ± 0.85	78.00 ± 1.30	57.91 ± 1.84	n.d.**	n.d.**	n.d.**

Scavenging activity of ascorbic acid, positive control (3.125 µg/mL) was 50.52 ± 1.84 %
Scavenging activity of blank nanoemulsion (F1) was 0%, *concentration of tamarind fruit pulp extract, ** Phase separation

4.5.2. Antityrosinase activity

The antityrosinase activity (% inhibition) of tamarind fruit pulp extract loaded nanoemulsions, F1-3.3TE and F1-6.6TE were investigated and the results of freshly prepared formulations and under stress conditions are displayed in **Table 4.13**. The initial amounts of these two formulations were varied into three different concentrations by mixing with a mixture of absolute ethanol and distilled water (1:1 by volume). Similar to the antioxidant activity, the effects of the amounts of tamarind fruit pulp extract on the antityrosinase properties were evaluated. As seen from **Table 4.13**, there was direct relationship between % inhibition of tyrosinase and the concentrations of tamarind fruit pulp extract present in the samples. In each formulation, high amount of tamarind fruit pulp extract produced greater percent tyrosinase inhibition. The differences in antityrosinase activities were still observed for the same concentrations of tamarind fruit pulp extract. The antityrosinase properties of the formulations were attributed to the presence of certain compounds such as aromatic aldehydes, aromatic acids or polyphenols in the tamarind fruit pulp extract (Vardhan and Pandey, 2014).

It was found that the antityrosinase activities of the test samples significantly decreased after freeze-thaw treatment ($p < 0.05$) except for the samples F1-6.6TE (3.3mg/mL) and F1-6.6TE (6.6 mg/mL). This phenomenon may be related to high percentage of tamarind fruit pulp extract in the freshly prepared formulation (F1-6.6TE) that can give more active compound(s) for tyrosinase inhibition (Lim *et al.*, 2009). For one month stability, the antityrosinase activity of the formulations reduced in different degrees when the storage temperature increased from 4 to 45°C. At 4 °C, the formulation F1-3.3TE at three different concentrations exhibited percent tyrosinase inhibition similar to those of the freshly prepared formulations unlike the formulation F1-6.6TE at three concentrations. Immobilization of tyrosinase enzyme in low temperature have been reported by Liu *et al.* (2000). Thus, the antityrosinase activity of the formulation F1-3.3TE kept in low temperature was similar to that at freshly prepared condition.

After two months, overall, the antityrosinase activity of the formulation F1-3.3TE tended to reduce when compared with the freshly prepared formulations. In general, the formulation stored in the lowest temperature of 4°C exhibited the highest antityrosinase activity when compared with the other two temperatures.

Table 4.13. Percent tyrosinase inhibition of tamarind fruit pulp extract loaded nanoemulsions determined by tyrosinase assay.

Formulation	Inhibition (%) (mean ± SD, n=5, where n is number of replications)							
	Freshly prepared	After Freeze-thaw (4 cycles)	After 1 month			After 2 months		
			4 ± 2°C	30 ± 2°C	45 ± 2°C	4 ± 2°C	30 ± 2°C	45 ± 2°C
F1-3.3TE (0.825 mg/mL*)	16.213 ± 2.060	7.958 ± 0.941	15.884 ± 1.965	15.336 ± 1.344	9.056 ± 0.712	16.706 ± 0.712	6.479 ± 0.599	7.737 ± 0.712
F1-3.3TE (1.65 mg/mL*)	37.246 ± 1.196	28.143 ± 2.236	35.815 ± 0.477	26.507 ± 0.689	15.056 ± 1.684	27.273 ± 0.583	12.401 ± 0.403	13.745 ± 0.477
F1-3.3TE (3.3 mg/mL*)	52.801 ± 0.941	42.753 ± 2.093	51.203 ± 0.909	49.189 ± 1.011	41.167 ± 0.712	45.105 ± 0.308	35.796 ± 0.599	51.203 ± 0.909
F1-6.6TE (1.65 mg/mL*)	26.952 ± 0.489	20.301 ± 2.654	18.217 ± 1.744	14.586 ± 0.936	10.004 ± 0.810	n.d.**	n.d.**	n.d.**
F1-6.6TE (3.3 mg/mL*)	34.888 ± 2.406	34.476 ± 1.480	30.394 ± 1.455	25.697 ± 0.557	21.609 ± 0.381	n.d.**	n.d.**	n.d.**
F1-6.6TE (6.6 mg/mL*)	54.389 ± 1.151	54.289 ± 1.151	51.970 ± 0.488	43.938 ± 1.032	42.938 ± 0.551	n.d.**	n.d.**	n.d.**

Antityrosinase activity of kojic acid, positive control (0.548 mg/mL) was 51.99 ± 3.017%
Antityrosinase activity of blank nanoemulsion (F1) was 0%, *concentration of tamarind fruit pulp extract, ** Phase separation

4.5.3. Antibacterial activity

The antibacterial property of tamarind fruit pulp extract loaded nanoemulsions against *Staphylococcus aureus* and *Staphylococcus epidermidis* were carried out using agar well diffusion technique. In addition, gentamicin sulfate (0.1% w/w) prepared in nanoemulsion and blank nanoemulsion were employed as a positive control and a negative control, respectively. The zone of inhibition of each sample was determined as shown in **Figures 4.6-4.7**. The results of inhibition zones of freshly prepared formulations and the formulations under stress conditions are summarized in **Tables 4.14 and 4.15**. There was no inhibition zone of blank nanoemulsion F1 against two bacterial strains, therefore the antibacterial effects of the products against *Staphylococcus aureus* and *Staphylococcus epidermidis* were derived from tamarind fruit pulp extract in the formulation only. For both strains, the inhibition zones of the two formulations were different. The freshly prepared formulation F1-6.6TE showed significantly higher zone of inhibition than that of the formulation F1-3.3TE ($p < 0.05$). However, at the end of 1 month, the zone of inhibitions for both strains were similar ($p > 0.05$). Even though the amount of tamarind fruit pulp extract in the formulation F1-6.6TE was 2-times of the formulation F1-3.3TE. This phenomenon may be related to the decreased penetration of formulation F1-6.6TE into the media because of the high viscosity of the formulation **(Table 4.9)**. As seen from **Tables 4.14 and 4.15**, the two tamarind fruit pulp extract loaded formulations were more effective against *Staphylococcus epidermidis*. It was reported that some antibiotics such as cefotaxime and amikacin easily penetrated through *Staphylococcus epidermidis* biofilms than *Staphylococcus aureus* biofilms (Singh *et al.*, 2010).

Under stress experimental condition, no decrease of inhibition zone was observed for both formulations. These results indicated that tamarind fruit pulp extract at 3.3%w/w was sufficient for antibacterial property. In addition, at the end of 2 months, there was the instability of the formulation F1-6.6TE. The antibacterial activity of tamarind fruit pulp extract loaded nanoemulsions was probably associated to the presence of phytochemicals in the extract, such as alkaloids, anthraquinones, saponins and glycosides (Abukakar *et al.*, 2008). The antibacterial activity of tamarind fruit pulp extract against *Staphylococcus aureus* and *Staphylococcus epidermidis* was previously reported (Gupta *et al.*, 2014).

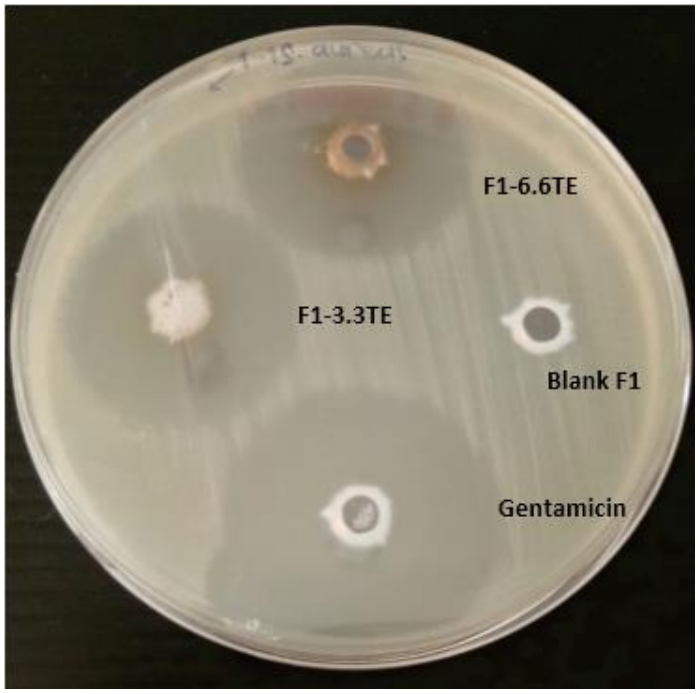


Figure 4.6. Zone of inhibition (mm) of *Staphylococcus aureus* from samples, negative and positive controls.

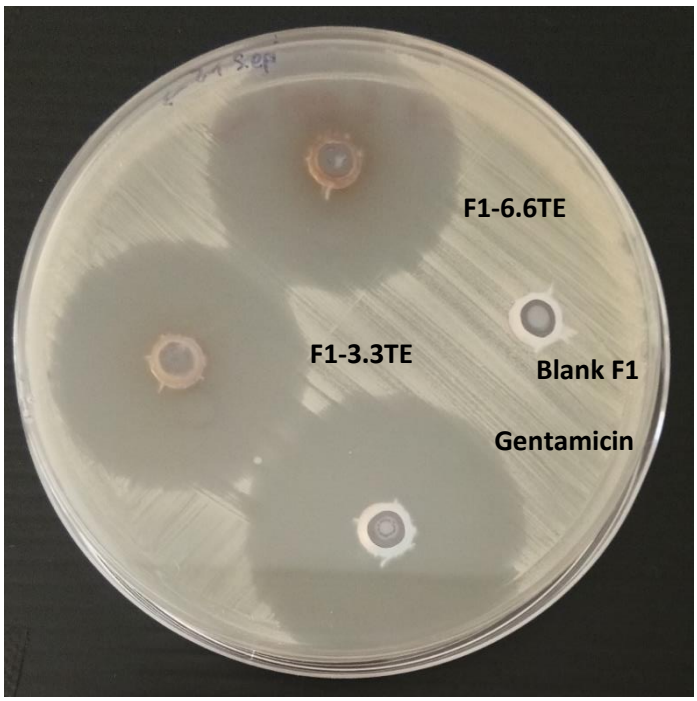


Figure 4.7. Zone of inhibition (mm) of *Staphylococcus epidermidis* from samples, negative and positive controls.

Table 4.14. Antibacterial activity of tamarind fruit pulp extract loaded nanoemulsions against *Staphylococcus aureus* determined by agar well diffusion technique.

Formulation	*Staphylococcus aureus*							
	Zone of inhibition (mm) (mean ± SD, n=3, where n is number of replications)							
	Freshly prepared	**After Freeze-thaw (4 cycles)**	**After 1 month**			**After 2 months**		
			4 ± 2°C	**30 ± 2°C**	**45 ± 2°C**	**4 ± 2°C**	**30 ± 2°C**	**45 ± 2°C**
F1-3.3TE	24.00 ± 0.50	25.33 ± 0.58	28.67 ± 1.15	28.67 ± 0.58	30.00 ± 0.00	26.00 ± 0.00	27.33 ± 1.53	26.00 ± 0.00
F1-6.6TE	26.00 ± 1.00	27.67 ± 0.58	30.00 ± 0.00	30.00 ± 0.00	29.33 ± 0.58	n.d.*	n.d.*	n.d.*
0.1%w/w gentamicin sulfate nanoemulsion	28.00 ± 0.00	28.00 ± 0.00	31.00 ± 1.00			30.67 ± 0.58		

Zone of inhibition of blank nanoemulsion was 0 mm (no inhibition zone)
* Phase separation

Table 4.15. Antibacterial activity of tamarind fruit pulp extract loaded nanoemulsions against *Staphylococcus epidermidis* determined by agar well diffusion technique.

Formulation	*Staphylococcus epidermidis*							
	Zone of inhibition (mm) (mean ± SD, n=3, where n is number of replications)							
	Freshly prepared	**After Freeze-thaw (4 cycles)**	**After 1 month**			**After 2 months**		
			4 ± 2°C	**30 ± 2°C**	**45 ± 2°C**	**4 ± 2°C**	**30 ± 2°C**	**45 ± 2°C**
F1-3.3TE	27.33 ± 0.58	27.67 ± 0.58	31.00 ± 0.00	31.00 ± 0.00	31.00 ± 0.00	29.33 ± 0.58	28.33 ± 0.58	27.00 ± 0.00
F1-6.6TE	27.33 ± 0.58	27.67 ± 0.58	32.00 ± 0.00	31.33 ± 0.58	32.33 ± 0.58	n.d.	n.d.	n.d.
0.1%w/w gentamicin sulfate nanoemulsion	29.67 ± 0.58	29.67 ± 0.58	33.00 ± 1.00			32.33 ± 0.58		

Zone of inhibition of blank nanoemulsion was 0 mm (no inhibition zone)
*Phase separation

CHAPTER 5
CONCLUSIONS

Owing to growing of cosmetic industry, a variety of natural active compounds derived from plants have been recognized. Tamarind fruit pulp which possess interesting bioactivity was chosen as the active ingredient in the current study. Certain bioactivities of tamarind fruit pulp could be applied for various cosmetic products including antiaging, skin lightening and hygienic preparations. In order to find the most suitable tamarind fruit pulp extract, four samples of tamarind fruit pulp extracts were investigated for their bioactivities which were antioxidant, antityrosinase and antibacterial activities. The 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay was used for antioxidant activity and dopachrome technique was employed for antityrosinase activity. The microdilution test was used for investigating the antibacterial property of tamarind fruit pulp extract against two bacteria strains *Staphylococcus aureus* and *Staphylococcus epidermidis*, which causing malodor of underarms. After testing with certain methods, the most suitable tamarind fruit pulp extract was found to be the sweet tamarind fruit pulp extract purchased from Guangzhou Phytochem Sciences Inc, China. It had the best bioactivity among the four samples.

In the current study, several blank o/w nanoemulsion formulations (lotions) were formulated in order to be used as the carrier for tamarind fruit pulp extract. The basic ingredients of the formulation were composed of 5%w/w surfactant mixture (Tween 80 and Span 80, as an emulsifier), synthetic oil (caprylic/capric triglyceride, as an emollient), water (as a vehicle), and glycerine (as a humectant). In addition, hydrocolloids (as thickeners) at different charge types, 0.1 and 0.25%w/w, were also incorporated into the formulations in an attempt to investigate the effect of charge types on the physicochemical property of blank nanoemulsions. The three hydrocolloid polymers used were xanthan gum (anionic charge), guar gum (no charge) and cationic guar gum (cationic charge). The preparation procedures comprised 2 steps. The first one involved the preparation of conventional emulsions by emulsifying the previously mentioned components and hydrocolloids (fully hydrated in water portion) with a high speed homogenizer. The second process started

with passing the prepared emulsions through a high pressure homogenizer in order to reduce the droplet sizes to be nanoscale. Afterwards, determination of nanoemulsion type and investigation of physicochemical properties (appearance, pH, viscosity, zeta potential, droplet size, PDI) of the prepared formulations were carried out. By using dye solubility test and conductivity measurement, all prepared formulations were o/w nanoemulsion system. It was observed that charge types of hydrocolloids somewhat affected the physicochemical properties of the nanoemulsions, especially cationic guar gum. For example, all freshly prepared formulations had negative zeta potential except for the formulations containing cationic guar gum which displayed positive zeta potential values. Based on the physical stability and physicochemical parameters, the optimum formulation was found to be the nanoemulsion without hydrocolloids (F1). The zeta potential (> -30 mV), PDI (< 0.2) and droplet size (about 140 nm) of the selected formulation were satisfactory and in the acceptable range of the nanoemulsion system. The pH of the selected blank nanoemulsion was weakly acidic and the viscosity was quite low.

Two concentrations of tamarind fruit pulp extract based on the bioactivity study were employed: 3.3%w/w and 6.6%w/w. The incorporation of the sweet tamarind fruit pulp extract and the blank nanoemulsion, F1 (without hydrocolloids) did not show any phase separation unlike the other formulations which were composed of hydrocolloids. The tamarind fruit pulp extract loaded nanoemulsions remained o/w nanoemulsions with significantly higher conductivity values than that of its corresponding blank formulation. After incorporation of tamarind fruit pulp extract into the suitable blank nanoemulsion, the viscosity of the formulations markedly increased. Similar to the unloaded formulation, the pseudoplastic flows were observed for tamarind fruit pulp extract loaded nanoemulsions. More acidic pH values of the formulations were obtained. Nevertheless, they were still in acceptable range of skin pH (pH 4-6). When compared with another formulation (6.6% w/w tamarind fruit pulp extract), the nanoemulsion formulation which contained tamarind fruit pulp extract at 3.3%w/w exhibited better physicochemical properties and stability under stress conditions: 4 freeze-thaw cycles and storage at 3 different temperatures, 4°C, 30°C and 45°C for 2 months. There was no phase separation at the

end of 2 months in the case of the formulation containing 3.3% w/w tamarind fruit pulp extract. By HPLC analysis, the percent tartaric acid remained in the formulation which was composed of 3.3%w/w tamarind fruit pulp extract was greater than 90% under the stress experimental condition. *In vitro* release study revealed that the release characteristics of tartaric acid from the tamarind fruit pulp extract loaded nanoemulsions followed Higuchi model (r^2 = 0.97). For the freshly prepared formulations, markedly higher release rates of the nanoemulsions containing 6.6%w/w tamarind fruit pulp extract were obtained.

Bioactivity properties of tamarind fruit pulp extract loaded nanoemulsions were investigated using the same methods as previously mentioned except for the antibacterial activity. The agar well diffusion technique was used in the case of the latter test. Unlike antibacterial property, both antioxidant and antityrosinase activities of the two formulations gradually decreased under stress experimental conditions. Remarkably, the nanoemulsion which contained 3.3%w/w tamarind fruit pulp extract had relatively better antioxidant activity, antityrosinase activity and similar antibacterial activity when compared with the formulation containing 6.6%w/w tamarind fruit pulp extract. These results indicated that amount of tamarind fruit pulp extract loaded in the nanoemulsion system obviously affected the physicochemical properties and bioactivities of the preparations.

According to the current work, the most satisfactory property of the tamarind fruit pulp extract loaded nanoemulsion was the antibacterial activity since it was able to maintain the antibacterial property against *Staphylococcus aureus* and *Staphylococcus epidermidis* throughout the period of study. The suitable concentration of tamarind fruit pulp extract loaded into the nanoemulsion system was 3.3%w/w.

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APPENDIX

APPENDIX A

VALIDATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) METHOD

The HPLC method was chosen to determine the amounts of tartaric acid, one of the organic acids, in the following samples:

1) Tamarind fruit pulp extracts
2) Tamarind fruit pulp extract loaded nanoemulsions
3) Tamarind fruit pulp extract (tartaric acid) released in the receptor fluid (0.9% sodium chloride solution) for the *in vitro* release study

A. Chromatographic conditions and instrumental setting

Chromatographic experiment was performed using HPLC system consisting of a reverse-phase Appollo C18 (5 µm, 4.6 x 250 mm, Alltech, IL, USA) with guard column. The HPLC system consists of a Shimadzu pump (model LC-20AD, Shimadzu, Japan), a Shimadzu diode array detector (model SPD-M20A, Shimadzu, Japan) a Shimadzu degasser (model DGU-20A5, Shimadzu, Japan) and a Shimadzu autosampler (model SIL-20A, Shimadzu, Japan). The HPLC analysis were based on the method of Kordis-Krapez *et al.* (2001). The mobile phase was 0.006 M phosphoric acid (pH = 2.1) at a flow rate of 0.8 mL/minute. The UV detection wavelength was 210 nm. The injection volume was 20µL. The column temperature was 25 °C. LC Solution Software was used to operate the system.

B. Determination of tartaric acid

1) Determination of tartaric acid in tamarind fruit pulp extracts and tamarind fruit pulp extract loaded nanoemulsions

Quantitative determination of tartaric acid using HPLC

The standard solutions of tartaric acid ranging from 5 to 120 µg/mL (7 concentrations) were prepared using HPLC mobile phase (0.006 M phosphoric acid (H_3PO_4)) as solvent. The typical chromatogram of standard tartaric acid solution in mobile phase, 0.006 M phosphoric acid (pH 2.1) is shown in **Figure A.1**. The retention time for tartaric acid in mobile phase was 4.4 minutes.

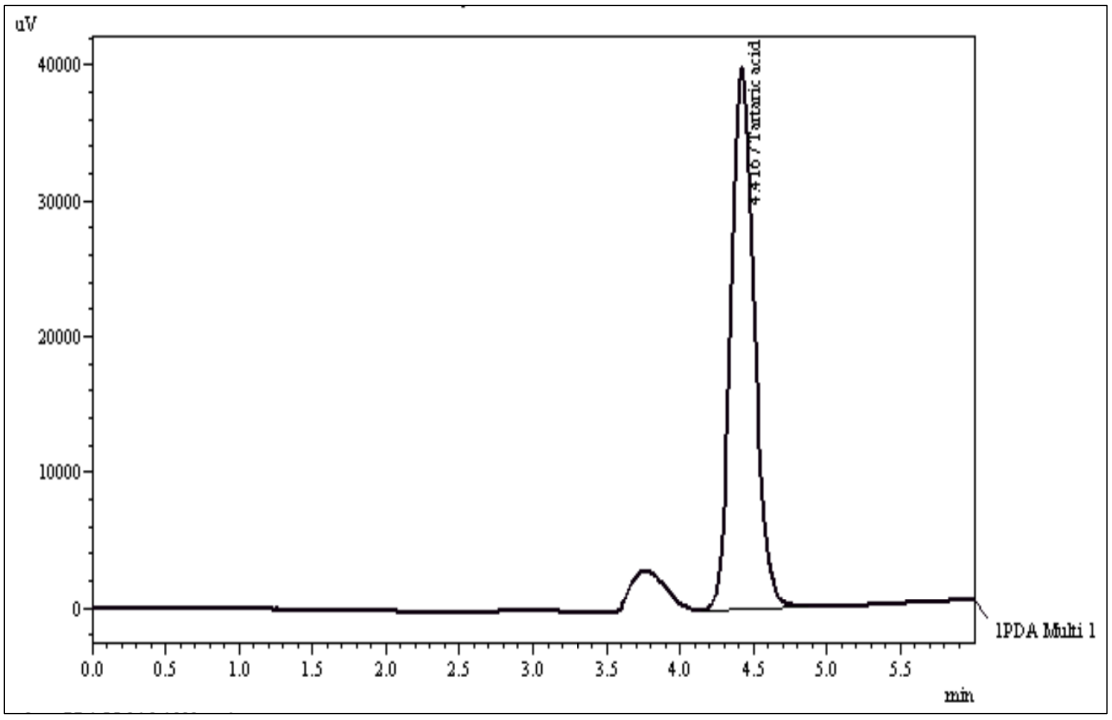


Figure A.1. Chromatogram of standard solution of tartaric acid (60 µg/mL) in mobile phase, 0.006 M phosphoric acid (pH 2.1).

Validation of HPLC method

For validation of analytical method, the guidelines of the International Conference on Harmonization of Technical Requirement for the Registration of Pharmaceuticals for Human Use were followed (ICH, 2005). The validation parameters consisted of specificity, linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ).

Specificity

The analytical method was performed in order to examine the specificity of tartaric acid determination in tamarind fruit pulp extracts and tamarind fruit pulp extract loaded nanoemulsion formulations. Under the chromatographic conditions selected, the peak of tartaric acid (active ingredient) must separate from other organic acids or other constituents in the tamarind fruit pulp extracts. A tamarind fruit pulp extract stock solution was prepared with the mobile phase, diluted to a suitable concentration (20 mg/mL) with the same solvent, sonicated, filtered and injected into the HPLC

column. The chromatogram of tartaric acid in the sweet tamarind fruit pulp extract is displayed in **Figure A.2**.

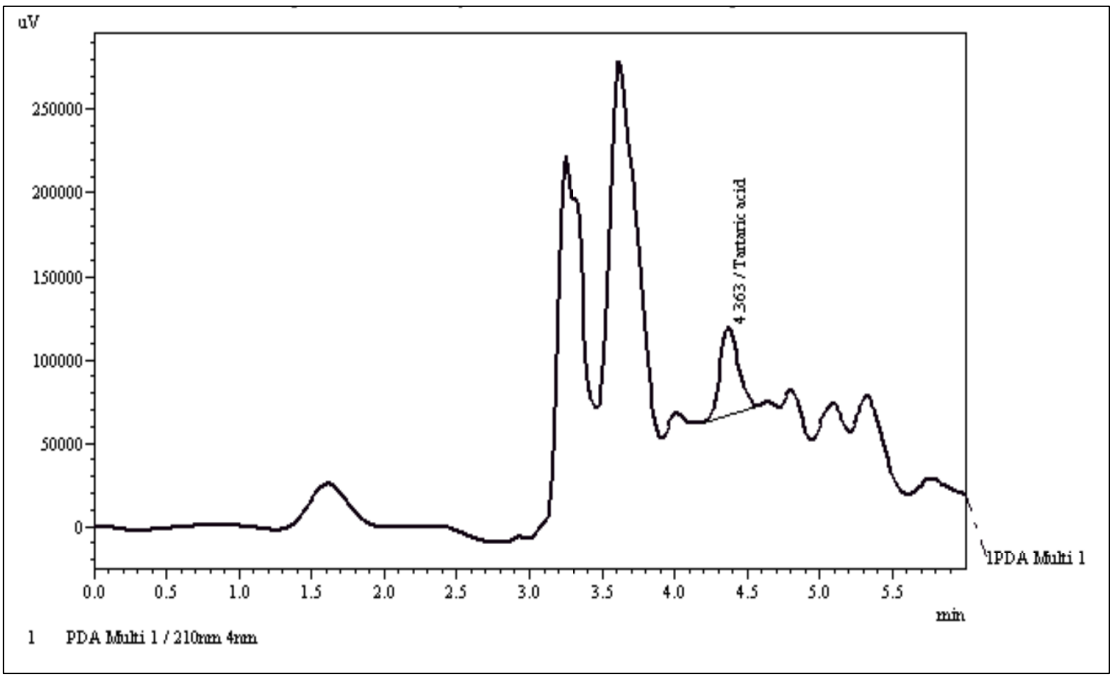


Figure A.2. Chromatogram of tartaric acid in tamarind fruit pulp extract.

In order to ensure the tartaric acid peak, a known concentration of standard tartaric acid was spiking into the same amount of tamarind fruit pulp extract sample. In the current analysis, three concentrations of standard tartaric acid, which were 0.1, 0.3 and 0.5 µg/mL, were used. The % recovery was checked by comparing the total amount recovered by proposed method and the actual amount. It was found that the percentage of recovery were 94.65; 94.06 and 99.70, respectively, which were in the acceptable limit range (90-115%) (ICH, 2005). Thus, the peak position of tartaric acid in the sweet tamarind fruit pulp extract was confirmed using retention time comparison with the standard tartaric acid **(Figure A.1)** and standard addition method.

In the case of nanoemulsions, the peak of tartaric acid must not interfere with the peaks of other compositions in the nanoemulsions (vehicles). To analyze the specificity, chromatogram of tartaric acid spiked in the blank nanoemulsion was compared with chromatogram of the blank nanoemulsions. Determination of non-degraded tartaric acid in nanoemulsions was extracted by suitable solvent (here, a

mixture of absolute ethanol and distilled water (1:1 by volume)). Therefore, the same solvent was used to extract tartaric acid in the blank nanoemulsion and standard tartaric acid loaded nanoemulsion. **Figure A.3** shows the assay of blank nanoemulsion, while **Figure A.4** shows the assay of standard tartaric acid (60 and 80 µg/mL) spiked in blank nanoemulsion. The tartaric acid peak was separated from the blank nanoemulsion. Therefore, the results of specificity studies demonstrated that the peak of tartaric acid in the blank nanoemulsion was not interfered by the peaks of any components in nanoemulsion vehicles.

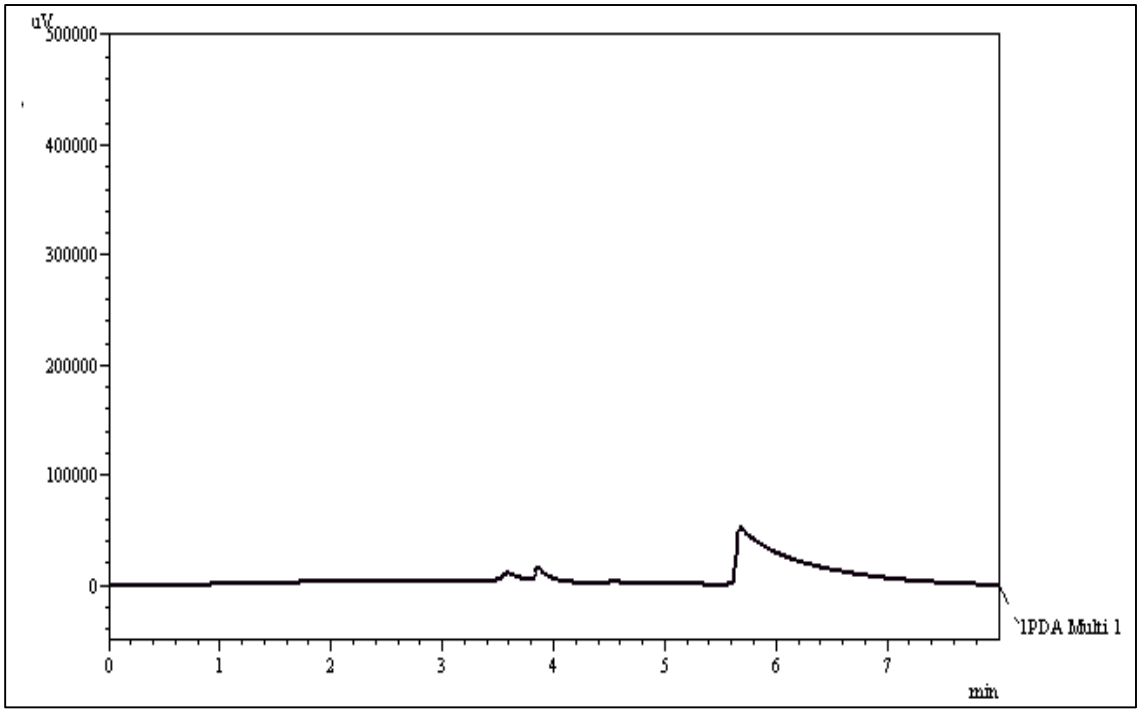


Figure A.3. Chromatogram of blank nanoemulsion (F1).

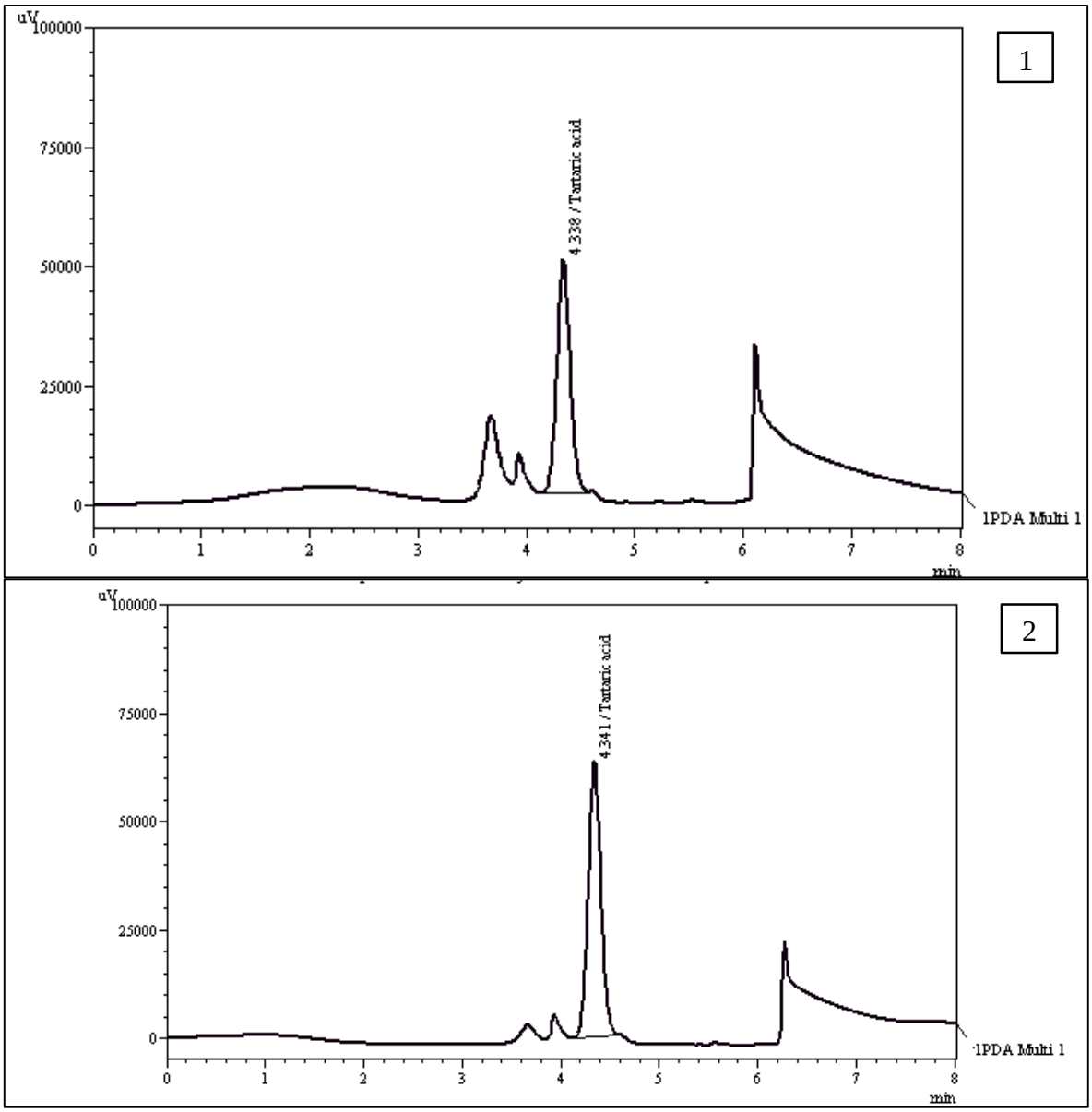


Figure A.4. Chromatogram of blank nanoemulsion spiked with standard tartaric acid (1) Concentration of tartaric acid = 60 µg/mL (2) Concentration of tartaric acid = 80 µg/mL.

Linearity

A stock solution of tartaric acid at the concentration of 500 µg/mL was prepared by dissolving standard tartaric acid in the HPLC mobile phase. This solution was further diluted with the same solvent to provide a series of the standard concentrations: 5, 10, 20, 40, 80, 100 and 120 µg/mL. Each concentration was prepared in sets of three and each one was injected three times. The standard curve was constructed by plotting the

average peak areas against concentrations of standard tartaric acid solution. Linear regression analysis of the means peak areas versus their concentrations was performed. Linearity acceptability was judged by coefficient of determination (r^2) equals or more than 0.999 (Komutarin *et al.*, 2004). The standard calibration curve of tartaric acid solution is demonstrated in **Figure A.5**. The response of standard tartaric acid was found to be linear with increasing tartaric acid concentration. The linear regression equation was y=6177.527x-31.521 with the coefficient of determination of 0.9998 (desired coefficient of determination: ≥0.999).

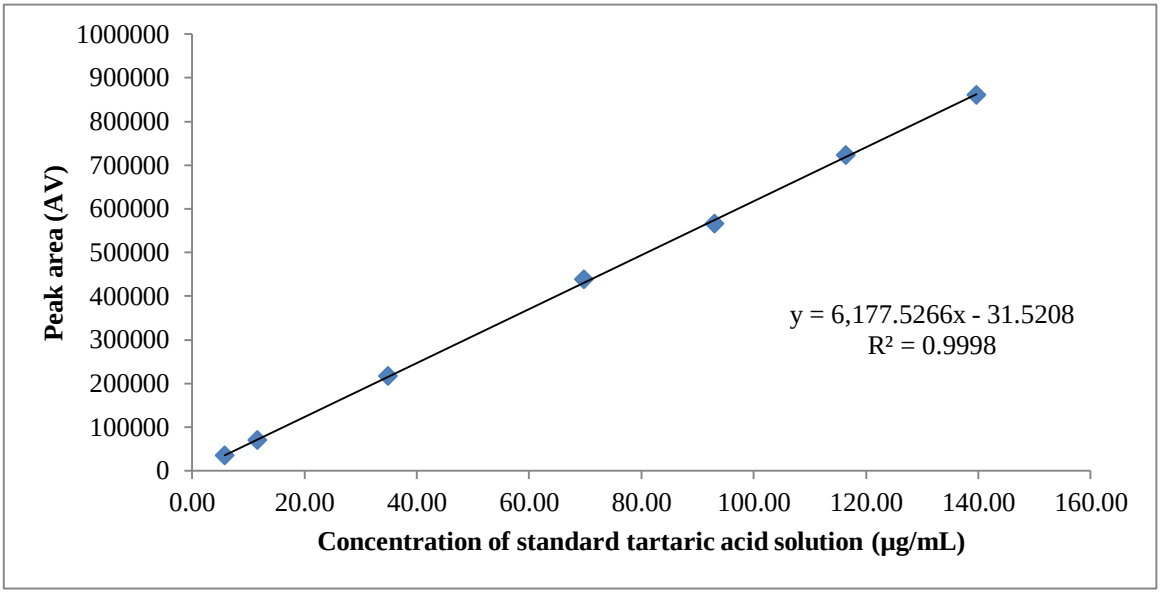


Figure A.5. Calibration curve of tartaric acid in mobile phase, 0.006 M phosphoric acid (pH 2.1). The plotted data are mean ± SD (n=3).

Precision

The precision of the procedure was evaluated through repeatability and expressed by percentage of relative standard deviation (%RSD) using the following equation (Epshtein, 2004). Both intra-day and inter-day were assessed:

$$\%RSD = \frac{SD \times 100}{mean} \quad \textbf{Eq. A.1}$$

The intra-day precision

Three sets of three standard solutions (16, 50, and 100 µg/mL) of tartaric acid were prepared and analyzed by HPLC within one day (ICH, 2005). Each concentration was determined in triplicates. The standard deviation and percent relative standard deviation of peak area were calculated. The %RSD should not more than 2.0%.

The inter-day precision

Three sets of three concentrations (16, 50, and 100 µg/mL) of tartaric acid were prepared and analyzed by HPLC on different days for three consecutive days (ICH, 2005). Each concentration was determined in triplicates. The data was used to calculate standard deviation and percent relative standard deviation of peak area. The %RSD should not more than 2.0%.

Accuracy

The accuracy was evaluated by comparing the amounts of tartaric acid found and amount of tartaric acid added. The data obtained from the precision study was used to calculate % recovery with the acceptable limit of 90 to 115% (ICH, 2005).

In the current analytical method, intra-day and inter-day variability was employed to evaluate the analytical accuracy and precision of the assay. The accuracy of the measured concentration was reported in term of % recovery. It was found that percentages of average recovery of tartaric acid standard solution in intra-day variability at the concentrations of 16, 50 and 100 µg/mL were 101.39 ± 0.63%; 103.05 ± 0.46%; 103.41 ± 0.82%, respectively **(see Table A.1)**. These values indicated high degree of accuracy. For the accuracy (% recovery) of tartaric acid standard solution in inter-day variability, it was in the range of 101.13-103.99% (acceptable limit 90-115%) as shown in **Table A.2**. The intra-day %RSD and inter-day precision of the method exhibited %RSD values between 0.444 to 0.797 and 0.678 to 1.097, respectively **(Table A.3)**. The %RSD of these experiments was less than 2%, indicating the method was adequately precise.

Table A.1. Accuracy and intra-day variability of tartaric acid analysis.

Standard Tartaric acid solution added (µg/mL)	Set	Measured Concentration (µg/mL)	Mean Measured Concentration (µg/mL) ± SD	Mean % Accuracy ± SD
16.160	1	16.432	16.386 ± 0.101	101.39 ± 0.63
	2	16.270		
	3	16.456		
53.868	1	55.661	55.511 ± 0.246	103.05 ± 0.46
	2	55.645		
	3	55.226		
107.736	1	110.867	111.414 ± 0.888	103.41 ± 0.82
	2	110.937		
	3	112.439		

Table A.2. Accuracy and inter-day variability of tartaric acid analysis.

0-day				
Standard Tartaric acid solution added (µg/mL)	**Set**	**Measured Concentration (µg/mL)**	**Mean Measured Concentration (µg/mL) ± SD**	**Mean % Accuracy ± SD**
16.160	1	16.432	16.386 ± 0.101	101.39 ± 0.63
	2	16.270		
	3	16.456		
53.868	1	55.661	55.511 ± 0.246	103.05 ± 0.46
	2	55.645		
	3	55.226		
107.736	1	110.867	111.414 ± 0.888	103.41 ± 0.82
	2	110.937		
	3	112.439		
1-day				
Standard Tartaric acid solution added (µg/mL)	**Set**	**Measured Concentration (µg/mL)**	**Mean Measured Concentration (µg/mL) ± SD**	**Mean % Accuracy ± SD**
16.160	1	16.272	16.343 ± 0.064	101.13 ± 0.39
	2	16.362		
	3	16.395		
53.868	1	55.811	56.016 ± 0.277	103.99 ± 0.51
	2	55.908		
	3	56.331		
107.736	1	112.796	111.767 ± 0.946	103.74 ± 0.88
	2	111.571		
	3	110.934		

Table A.2. Accuracy and inter-day variability of tartaric acid analysis (continued).

2-day				
Standard Tartaric acid solution added (µg/mL)	**Set**	**Measured Concentration (µg/mL)**	**Mean Measured Concentration (µg/mL) ± SD**	**Mean % Accuracy ± SD**
16.160	1	16.778	16.696 ± 0.071	103.31 ± 0.44
	2	16.658		
	3	16.653		
53.868	1	54.685	55.075 ± 0.412	102.24 ± 0.76
	2	55.035		
	3	55.505		
107.736	1	111.031	110.799 ± 0.559	102.84 ± 0.52
	2	111.205		
	3	110.162		

Table A.3. Percentage of relative standard deviation (%RSD) both intra-day and inter-day variability.

Intra-day			
Day	**Concentration (µg/mL)**		
	15	**50**	**100**
0	16.432	55.661	110.867
	16.269	55.645	110.937
	16.456	55.226	112.439
Mean	16.386	55.511	111.414
SD	0.102	0.247	0.888
%RSD	0.621	0.444	0.797
Inter-day			
Day	**Concentration (µg/mL)**		
	15	**15**	**15**
0	16.432	55.661	110.867
	16.269	55.645	110.937
	16.456	55.226	112.439
1	16.272	55.811	112.796
	16.362	55.908	111.571
	16.395	56.331	110.934
2	16.778	54.685	111.031
	16.658	55.035	111.205
	16.653	55.505	110.162
Mean	16.475	55.534	111.327
SD	0.181	0.493	0.747
%RSD	1.097	0.888	0.678

The limit of detection (LOD) and the limit of quantification (LOQ)

Based on the linearity study, calibration curve was constructed. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated by the standard deviation and the slope of the calibration curve based on the following equations.

$$LOD = \frac{3\sigma}{S} \qquad \textbf{Eq. A.2}$$

$$LOQ = \frac{10\sigma}{S} \qquad \textbf{Eq. A.3}$$

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

The LOD and LOQ values for analysis of tartaric acid in tamarind fruit pulp extract and in the tamarind fruit pulp extract loaded nanoemulsions, calculated based on **Equations A.2 and A.3**, were 0.05 µg/mL and 0.17 µg/mL, respectively.

2) Determination of tartaric acid in the receptor fluid (0.9% sodium chloride solution) for the *in vitro* release study

Quantitative determination of tartaric acid using HPLC

The standard solutions of tartaric acid prepared in receptor fluid (0.9% sodium chloride solution) were injected into the HPLC column. The chromatogram of standard solution of tartaric acid is exhibited in **Figure A.6**. The retention time for tartaric acid in receptor fluid was 4.392 minutes, which was similar to that of tartaric acid in the mobile phase.

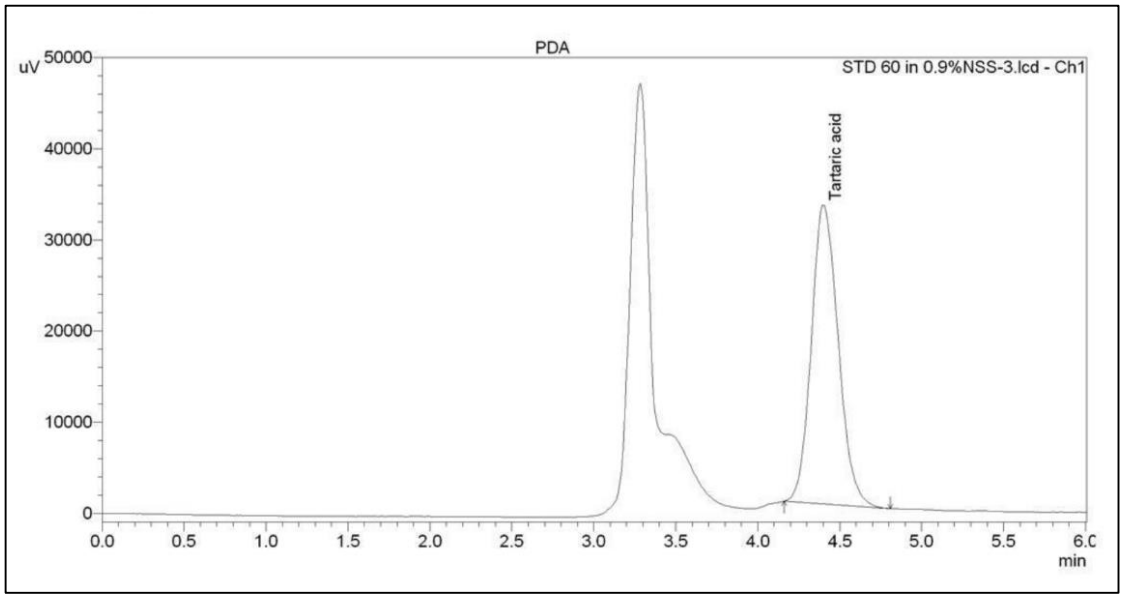


Figure A.6. Chromatogram of standard solution of tartaric acid (60 µg/mL) in the receptor fluid, 0.9% w/v sodium chloride solution.

Validation of HPLC method

Specificity

This parameter was determined in order to examine the specific determination of tartaric acid under the *in vitro* release study. The peak of tartaric acid must separate from the peaks of the nanoemulsion carriers, the receptor fluid and interference peaks from synthetic membrane. The chromatogram of the standard solution of tartaric acid was compared with the chromatogram of the blank nanoemulsion as well as the chromatogram of receptor fluid. **Figure A.7** shows the assay of receptor fluid (0.9% w/v sodium chloride solution), while **Figure A.8** shows blank nanoemulsion (F1) across the synthetic cellulose acetate membrane into the receptor fluid (0.9% w/v sodium chloride solution). It was found that the peak of standard tartaric acid **(see Figure A.7**) was separated from all interferences caused by receptor fluid, synthetic membrane or blank nanoemulsions.

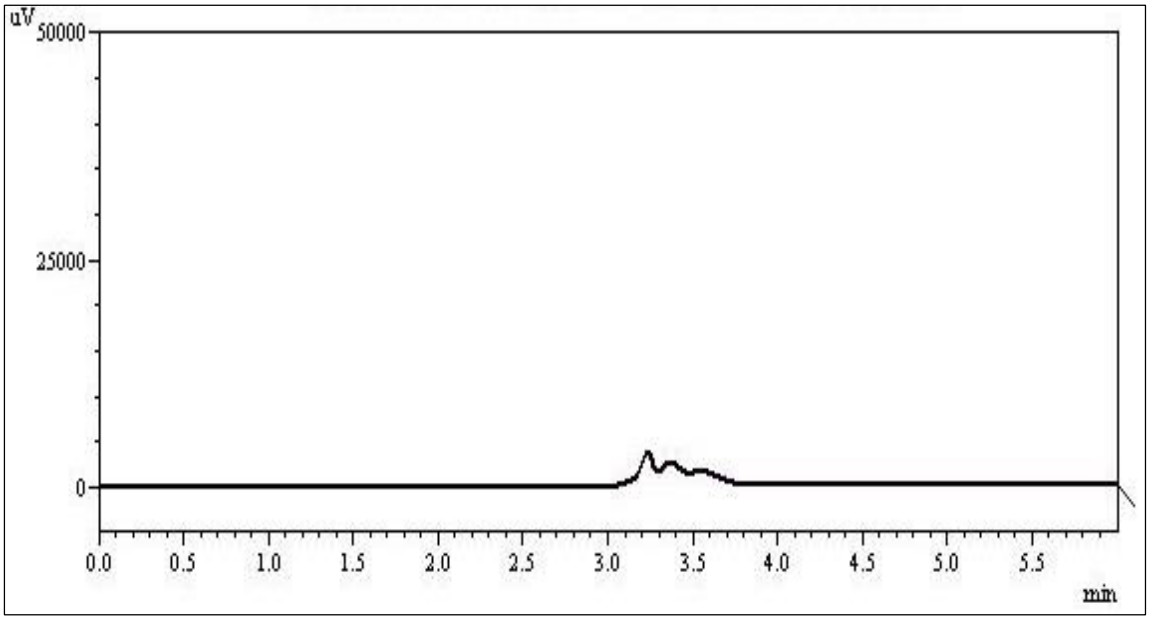


Figure A.7. Chromatogram of receptor fluid, 0.9% w/v sodium chloride solution.

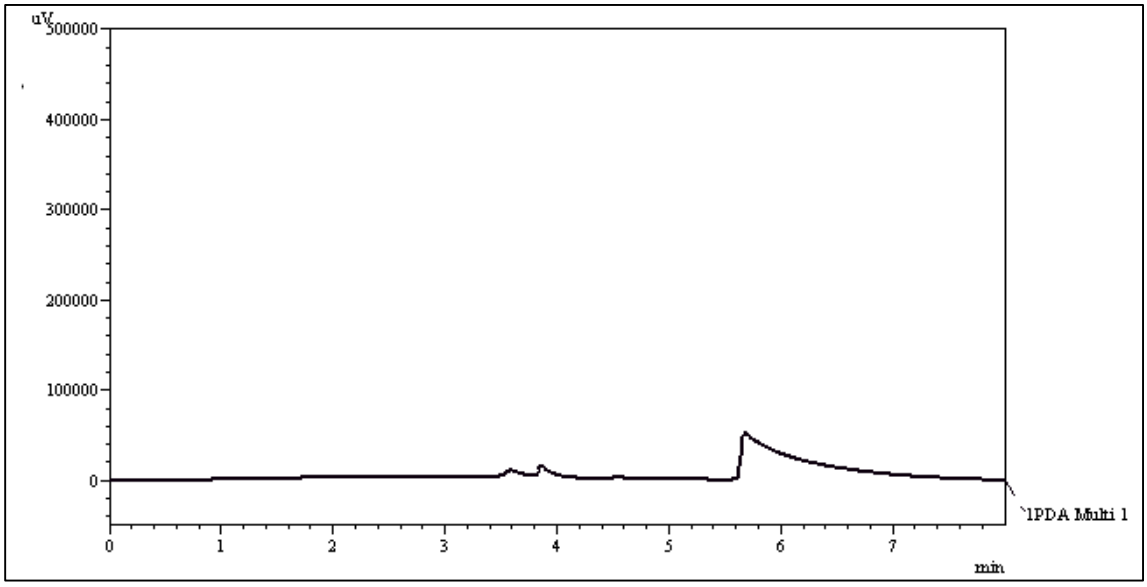


Figure A.8. Chromatogram of blank nanoemulsion (F1) released across the synthetic cellulose acetate membrane into the receptor fluid, 0.9% w/v sodium chloride solution.

Linearity

The stock solution of tartaric acid at the concentration of 500 µg/mL was prepared by dissolving tartaric acid in a receptor fluid, 0.9 %w/v sodium chloride solution (normal saline solution). In the preliminary study, isotonic phosphate buffer pH 7.4 was also tested as a receptor medium. However, the peaks of solvent front caused by the buffer could not completely separate from the peak of tartaric acid. Thus, 0.9 % normal saline solution was used. This stock solution was further diluted with the same solvent to desired concentrations of 1, 5, 10, 30, 60, 80, 100 µg/mL. Each concentration was prepared in sets of three and each one was injected three times. The standard curve was constructed by plotting the peak areas of tartaric acid against their concentrations. Linear regression analysis of the mean peak areas versus their concentrations was performed. According to, the linearity was studied by methods of regression analysis in terms of linear equation: Y= a + bC, where Y is the peak height or area and C is the amount of concentration. The coefficient of determination (r^2) should at least at the level of $r^2 > 0.999$ (Epshtein, 2004; Kazusaki *et al.*, 2012).

The calibration curve of tartaric acid in the receptor fluid was constructed with concentrations ranging from 1 to 100 µg/mL as shown in **Figure A.9**. A coefficient of determination from linear regression (y = 6,122.0752x - 1,593.6167) analysis was 0.9997.

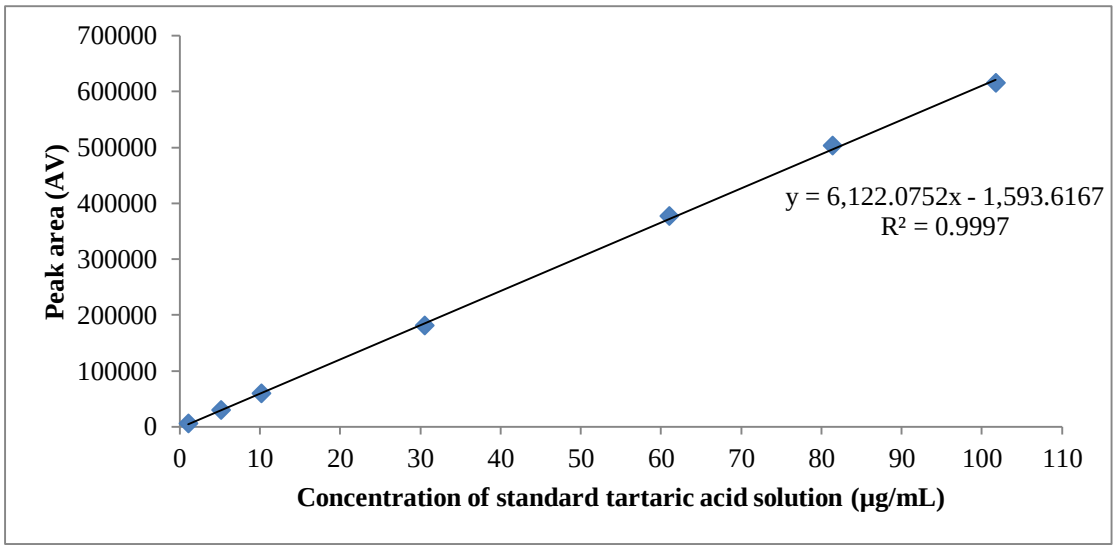


Figure A.9. Calibration curve of tartaric acid in the receptor fluid, 0.9% w/v sodium chloride solution. The plotted data are mean ± SD (n=3).

Precision

The precision of the procedure was evaluated through repeatability. The results were expressed by %RSD using **Equation A.1**. Both intra-day and inter-day were assessed.

The intra-day precision

Three sets of three standard solutions (2, 20 and 50 µg/mL) of tartaric acid were prepared and analyzed by HPLC within one day. Based on the ICH guideline three suitable concentration (low, medium, and high) on the linear range can be used. Each concentration was determined in triplicates. The standard deviation and percent relative standard deviation of peak area were calculated. The %RSD should not more than 2.0%.

The inter-day precision

Three sets of three different concentrations (2, 20 and 50 µg/mL) of tartaric acid were prepared and analyzed by HPLC on different days for three consecutive days. Each concentration was determined in triplicates. The data was used to calculate standard deviation and percent relative standard deviation of peak area. The %RSD should not more than 2.0%.

Accuracy

The accuracy was evaluated by comparing amounts of tartaric found and amount of tartaric added. The data obtained from the precision study was used to calculate % recovery with the acceptable limit of 90 to 115% (ICH, 2005). For precision and accuracy validation, percent of average recovery of tartaric acid standard solution in intra-day variability **(Table A.4)** at the concentration of 2, 20 and 50 µg/mL were 111.61 ± 1.41%; 102.31 ± 0.52%; 99.67 ± 0.24%, respectively. These values indicated high degree of accuracy. The accuracy (% recovery) of tartaric acid standard solution in inter-day variability **(Table A.5)** was in the range of 99.67-111.34%. The %RSD of these experiments was less than 2%, which were between 0.239 to 1.259 for intra-day and between 0.755 to 1.817 for inter-day variability as shown in **Table A.6**. Both % recovery and % RSD were in acceptable limits of ICH guideline, suggesting the suitability of the method for analyzing the amount of tartaric acid released in the receptor fluid (0.9% sodium chloride solution).

Table A.4. Accuracy and intra-day variability of tartaric acid analysis.

Standard Tartaric acid solution added (µg/mL)	Set	Measured Concentration (µg/mL)	Mean Measured Concentration (µg/mL) ± SD	Mean % Accuracy ± SD
2.104	1	2.318	2.348 ± 0.030	111.61 ± 1.41
	2	2.377		
	3	2.348		
21.037	1	21.549	21.524 ± 0.109	102.31 ± 0.52
	2	21.618		
	3	21.404		
52.592	1	52.277	52.419 ± 0.125	99.67 ± 0.24
	2	52.466		
	3	52.514		

Table A.5. Accuracy and inter-day variability of tartaric acid analysis

0-day				
Standard Tartaric acid solution added (µg/mL)	**Set**	**Measured Concentration (µg/mL)**	**Mean Measured Concentration (µg/mL) ± SD**	**Mean % Accuracy ± SD**
2.104	1	2.318	2.348 ± 0.030	111.61 ± 1.41
	2	2.377		
	3	2.348		
21.037	1	21.549	21.524 ± 0.109	102.31 ± 0.52
	2	21.618		
	3	21.404		
52.592	1	52.277	52.419 ± 0.125	99.67 ± 0.24
	2	52.466		
	3	52.514		
1-day				
Standard Tartaric acid solution added (µg/mL)	**Set**	**Measured Concentration (µg/mL)**	**Mean Measured Concentration (µg/mL) ± SD**	**Mean % Accuracy ± SD**
2.104	1	2.338	2.342 ± 0.013	111.34 ± 0.61
	2	2.357		
	3	2.332		
21.037	1	21.777	21.538 ± 0.434	102.38 ± 2.06
	2	21.800		
	3	21.038		
52.592	1	52.541	52.602 ± 0.150	100.02 ± 0.28
	2	52.492		
	3	52.772		

Table A.5. Accuracy and inter-day variability of tartaric acid analysis (continued)

2-day				
Standard Tartaric acid solution added (µg/mL)	**Set**	**Measured Concentration (µg/mL)**	**Mean Measured Concentration (µg/mL) ± SD**	**Mean % Accuracy ± SD**
2.104	1	2.346	2.340 ± 0.013	111.25 ± 0.61
	2	2.350		
	3	2.326		
21.037	1	21.783	21.428 ± 0.632	101.86 ± 3.00
	2	21.802		
	3	20.698		
52.592	1	51.810	52.798 ± 0.891	100.39 ± 1.69
	2	53.540		
	3	53.004		

Table A.6. Percentage of relative standard deviation (%RSD) both intra-day and inter-day variability.

Intra-day			
Day	**Concentration (µg/mL)**		
	2	**20**	**50**
0	2.318	21.549	52.277
	2.377	21.618	52.466
	2.348	21.404	52.514
Mean	2.348	21.524	52.419
SD	0.030	0.109	0.125
%RSD	1.259	0.507	0.239
Inter-day			
Day	**Concentration (µg/mL)**		
	15	**15**	**15**
0	2.318	21.549	52.277
	2.377	21.618	52.466
	2.348	21.404	52.514
1	2.338	21.777	52.541
	2.357	21.799	52.492
	2.332	21.038	52.772
2	2.346	21.783	51.809
	2.350	21.802	53.539
	2.326	20.698	53.044
Mean	2.344	21.497	52.606
SD	0.018	0.390	0.487
%RSD	0.755	1.817	0.919

The limit of detection (LOD) and the limit of quantification (LOQ)

The LOD and the LOQ of the analysis were 0.13 and 0.43 µg/mL, respectively.

In conclusions, these results suggested that the established HPLC method used in the current study was specific, accurate, reproducible and sensitive for the analysis of tartaric acid in tamarind fruit pulp extracts, in tamarind fruit pulp extract loaded nanoemulsions, and tartaric acid released in the receptor fluid (*in vitro* release study).

APPENDIX B
THE DEVELOPMENT OF NANOEMULSION FORMULATIONS

Blank nanoemulsions (unloaded nanoemulsions)

Table B.1. Pictures of blank nanoemulsions after freshly prepared, freeze-thaw, 1 month storage and centrifugation test.

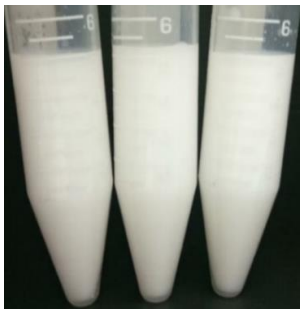
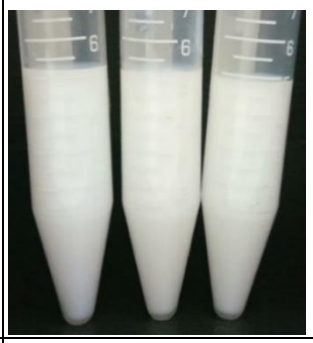
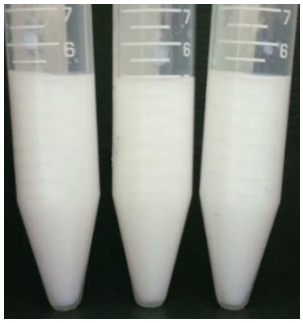
Freshly Prepared			
Formulation		**Centrifugation test**	**Appearances**
F1			Homogeneous, milky opaque, no precipitation after centifuge
F2			Homogeneous, milky opaque, no precipitation after centifuge
F3			Homogeneous, milky opaque, no precipitation after centifuge
F4			Homogeneous, milky opaque, no precipitation after centifuge

Table B.1. Pictures of blank nanoemulsions after freshly prepared, freeze-thaw, 1 month storage and centrifugation test (continued).

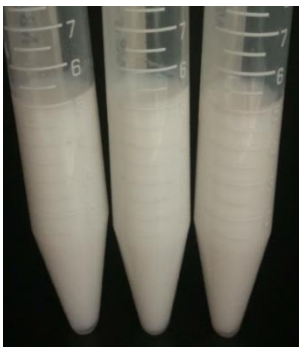
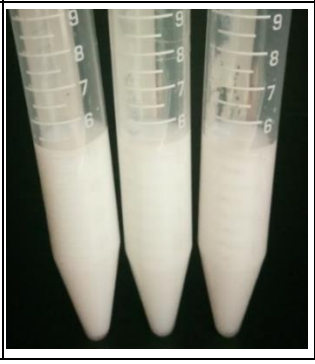
Freshly Prepared			
Formulation		**Centrifugation test**	**Appearances**
F5			Homogeneous, milky opaque, no precipitation after centifuge
F6			Homogeneous, milky opaque, no precipitation after centifuge
F7			Homogeneous, milky opaque, no precipitation after centifuge

Table B.1. Pictures of blank nanoemulsions after freshly prepared, freeze-thaw, 1 month storage and centrifugation test (continued).

Freeze-thaw		
Formulation		**Appearances**
F1		Homogeneous, milky opaque, no precipitation
F2		Homogeneous, milky opaque, no precipitation
F3		Homogeneous, milky opaque, no precipitation
F4		Homogeneous, milky opaque, no precipitation

Table B.1. Pictures of blank nanoemulsions after freshly prepared, freeze-thaw, 1 month storage and centrifugation test (continued).

Freeze-thaw		
Formulation		**Appearances**
F5		Homogeneous, milky opaque, no precipitation
F6		Homogeneous, milky opaque, no precipitation
F7		Homogeneous, milky opaque, no precipitation

Table B.1. Pictures of blank nanoemulsion after freshly prepared, freeze-thaw, 1 month storage and centrifugation test (continued).

1-month (4 ± 2°C)		
Formulation		**Appearances**
F1		Homogeneous, milky opaque, no precipitation
F2		Homogeneous, milky opaque, no precipitation
F3		Homogeneous, milky opaque, no precipitation
F4		Homogeneous, milky opaque, no precipitation

Table B.1. Pictures of blank nanoemulsions after freshly prepared, freeze-thaw, 1 month storage and centrifugation test (continued).

1-month (4 ± 2°C)		
Formulation		**Appearances**
F5		Homogeneous, milky opaque, no precipitation
F6		Homogeneous, milky opaque, no precipitation
F7		Homogeneous, milky opaque, no precipitation

Table B.1. Pictures of blank nanoemulsions after freshly prepared, freeze-thaw, 1 month storage and centrifugation test (continued).

1-month (30 ± 2°C)		
Formulation		**Appearances**
F1		Homogeneous, milky opaque, no precipitation
F2		Homogeneous, milky opaque, no precipitation
F3		Homogeneous, milky opaque, no precipitation
F4		Homogeneous, milky opaque, no precipitation

Table B.1. Pictures of blank nanoemulsions after freshly prepared, freeze-thaw, 1 month storage and centrifugation test (continued).

1-month (30 ± 2°C)		
Formulation		**Appearances**
F5		Homogeneous, milky opaque, no precipitation
F6		Homogeneous, milky opaque, no precipitation
F7		Homogeneous, milky opaque, no precipitation

Table B.1. Pictures of blank nanoemulsions after freshly prepared, freeze-thaw, 1 month storage and centrifugation test (continued).

1-month (45 ± 2°C)		
Formulation		**Appearances**
F1		Homogeneous, milky opaque, no precipitation
F2		Homogeneous, milky opaque, no precipitation
F3		Homogeneous, milky opaque, no precipitation
F4		Homogeneous, milky opaque, no precipitation

Table B.1. Pictures of blank nanoemulsions after freshly prepared, freeze-thaw, 1 month storage and centrifugation test (continued).

1-month (45 ± 2°C)		
Formulation		**Appearances**
F5		Homogeneous, milky opaque, no precipitation
F6		Homogeneous, milky opaque, no precipitation
F7		Homogeneous, milky opaque, no precipitation

Tamarind fruit pulp extract loaded nanoemulsions

Table B.2. Pictures of tamarind fruit pulp extract loaded nanoemulsions after freshly prepared, freeze-thaw, 1-2 months storage and centrifugation test.

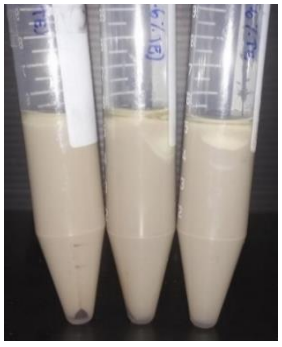
<table>
<tr><th colspan="4">Freshly Prepared</th></tr>
<tr><th colspan="2">Formulation</th><th>Centrifugation test</th><th>Appearances</th></tr>
<tr><td>F1-3.3TE</td><td>  </td><td>  </td><td>Homogeneous, milky opaque, no precipitation after centifuge</td></tr>
<tr><td>F1-6.6TE</td><td>  </td><td>  </td><td>Homogeneous, milky opaque, no precipitation after centifuge</td></tr>
<tr><th colspan="4">Freeze-thaw</th></tr>
<tr><th colspan="2">Formulation</th><th colspan="2">Appearances</th></tr>
<tr><td>F1-3.3TE</td><td colspan="2">  </td><td>Homogeneous, milky opaque, no precipitation</td></tr>
<tr><td>F1-6.6TE</td><td colspan="2">  </td><td>Homogeneous, milky opaque, no precipitation</td></tr>
</table>

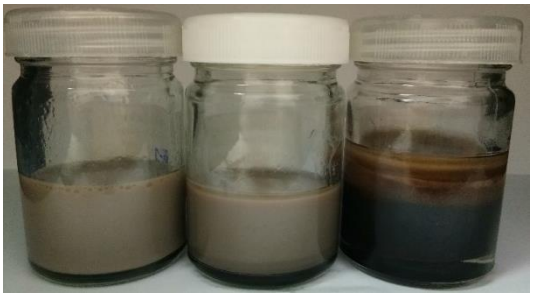
Table B.2. Pictures of tamarind fruit pulp extract loaded nanoemulsions after freshly prepared, freeze-thaw, 1-2 months storage and centrifugation test (continued).

1-month (4 ± 2°C)		
Formulation		**Appearances**
F1-3.3TE		Homogeneous, milky opaque, no precipitation
F1-6.6TE		Homogeneous, milky opaque, no precipitation
1-month (30 ± 2°C)		
Formulation		**Appearances**
F1-3.3TE		Homogeneous, milky opaque, no precipitation
F1-6.6TE		Homogeneous, milky opaque, no precipitation

Table B.2. Pictures of tamarind fruit pulp extract loaded nanoemulsions after freshly prepared freeze-thaw, 1-2 months storage and centrifugation test (continued).

1-month (45 ± 2°C)		
Formulation		**Appearances**
F1-3.3TE		Homogeneous, milky opaque, no precipitation
F1-6.6TE		Homogeneous, milky opaque, no precipitation
2-month (4 ± 2°C)		
Formulation		**Appearances**
F1-3.3TE		Homogeneous, milky opaque, no precipitation
F1-6.6TE		Non homogenous, phase separation, precipitation

Table B.2. Pictures of tamarind fruit pulp extract loaded nanoemulsions after freshly prepared, freeze-thaw, 1-2 months storage and centrifugation test (continued).

2-month (30 ± 2°C)		
Formulation		**Appearances**
F1-3.3TE		Homogeneous, milky opaque, no precipitation
F1-6.6TE		Non homogenous, phase separation, precipitation
2-month (45 ± 2°C)		
Formulation		**Appearances**
F1-3.3TE		Homogeneous, milky opaque, no precipitation
F1-6.6TE		Non homogenous, phase separation, precipitation

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Higher Education Research Promotion and The Thailand's Education Hub for Southern Region ASEAN Countries Project Office of The Higher Education Comission (TEH-AC 022/2017).

List of Publication and Proceeding

Isnaini, Nadia., Sadli, Sari, Irma. Formulasi dan Uji Aktivitas Deodoran *Spray* Ekstrak Etanol 70% Herba Patikan Kebo (*Euphorbia hirta* L.) terhadap Pertumbuhan Bakteri *Staphylococcus epidermidis* secara *In Vitro*. National Seminar with theme "Peranan Riset dan Inovasi untuk Mewujudkan Pengembangan Industri Nasional yang Berdaya Saing dan Bernilai Tambah Tinggi Sera Berwawasan Lingkungan", Banda Aceh, 3rd November 2016 (Oral presentation).