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Article

Characterization, Antioxidant and Antibacterial Potentials of *Tamarindus indica* L. Fruit Pulp Extract Loaded O/W Nanoemulsions

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The main purposes of the current study were to formulate o/w nanoemulsions as a carrier for *Tamarindus indica* (tamarind) fruit pulp extract and to study the antioxidant and antibacterial potentials of nanoemulsions containing tamarind extract, focusing on cosmetic/hygiene applications. The o/w nanoemulsions using a mixture of Tween 80 and Span 80 as an emulsifier (5%w/w) were prepared by a high pressure homogenization process. Two concentrations of sweet tamarind extract, 3.3 and 6.6%w/w, based on the bioactivity study**,** were incorporated into the blank nanoemulsions to produce loaded nanoemulsions, F1-3.3TE (3.3%) and F1-6.6TE (6.6%). As compared with the unloaded nanoemulsion, both tamarind extract loaded nanoemulsions showed reduced pH and significantly increased viscosity. Overall, the loaded nanoemulsions had droplet sizes of approximately 130 nm, zeta potential around -38 mV and polydispersity index (PDI) values less than 0.2. The nanoemulsion F1-3.3TE had better stability (e.g. significantly greater % tartaric acid content and lesser PDI value) than the nanoemulsion F1-6.6TE did. The antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl assay revealed that the nanoemulsions F1-3.3TE and F1-6.6TE had scavenging activities of 81.66 ± 0.77% and 63.80 ± 0.79%, respectively. However, antioxidant activity of these two formulations decreased under stress conditions (heating-cooling cycles). Such incidence did not occur for their antibacterial properties investigated by agar well diffusion technique. The two formulations exhibited inhibition zones of approximately 24.0-27.7 mm against *Staphylococcus aureus* and *Staphylococcus epidermidis*, responsible for malodor of underarms. The results suggest the potential of using sweet tamarind pulp extract loaded nanoemulsions as hygiene products.

Keywords: *Tamarindus indica* (tamarind) fruit pulp extract. Nanoemulsions. Antioxidant. Antibacterial.

INTRODUCTION

Plant extracts have been generally recognized as active ingredients in a variety of dermatological products. Tamarind (*Tamarindus indica* L.), a tropical tree native to Africa, has been widely cultivated in several tropical countries including South Asia (e.g. India), Southeast Asia (e.g. Thailand) and Oceania (Wyk, 2015). Two major varieties of tamarind have been recognized; sour (the most common) and sweet. 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, pharmaceuticals and cosmetics (Gaur, Parvez, 2019; Menezes *et al.*, 2016). Among these parts, the fruit pulp has drawn attention due to its several reported bioactive activities, especially antioxidant (Atawodi *et al*., 2014), and antibacterial properties (Bhattacharjee, Bhattacharyya,

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Rai, 2018; Gupta, Prakash, Gupta, 2014; Nwodo *et al*., 2011). The crude extracts of tamarind fruit pulps contain several organic acids, namely tartaric acid, acetic acid, formic acid, and malic acid. These organic acids possess antioxidant properties which improve the efficiency of superoxide dismutase, catalase and glutathione peroxidase in animals (Liu *et al*., 2019; Maenthaisong *et al*., 2009; Menezes *et al*., 2016). The phytochemicals detected which include alkaloids, anthraquinones, saponins and glycosides can provide inhibitory effects for bacteria (Abukakar, Ukwuani, Shehu, 2008; Rana, Sharma, 2018). The antioxidant activity of tamarind pulp extract is suitable for food, pharmaceutical and cosmetic applications, especially anti-aging products. Both intrinsic aging and photoaging are associated with oxidative stress (Saliou *et al*., 2014; Stout, Birch-Machin, 2019). To some extent, the use of antioxidants will alleviate or prevent the signs of aging such as wrinkled skin and age spots. The antibacterial property of the fruit pulp can be applied to both food and personal care products such as deodorant products. Skin microflorae, bacteria in particular, are responsible for malodor of the underarm. This includes gram-positive bacteria, *Staphylococcus spp.* for example, *Staphylococcus aureus* and *Staphylococcus epidermidis*. The activity of bacteria on the secretions of human skin glands (apocrine and eccrine glands) causes body odor (Schreiber, 2014). Antibacterial agents in deodorants reduce underarm odor by destroying or suppressing the growth of such bacteria. The use of natural active ingredients like tamarind extract can decrease the undesirable side effects 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 vehicles (carriers) and technologies used greatly influence the delivery process of active substances into/through the skin. 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 including nanoparticles and nanoemulsions (Borgheti-Cardoso *et al*., 2016; Nastiti *et al*., 2017). Nanoemulsions are kinetically stable dispersions of two immiscible liquids (oil, and water) stabilized by an emulsifier (generally a surfactant or a surfactant system). The crucial functions of emulsifiers include decreasing the interfacial tension between the water and oil phases and stabilizing nanoemulsions through repulsive electrostatic interactions and steric hindrance. In general, droplet sizes of nanoemulsions are around 20-500 nm (Gupta *et al*., 2016). Nanoemulsions can be prepared using either high energy methods (e.g. ultrasonication and high pressure homogenization) or low energy methods (e.g. emulsion inversion point and phase inversion temperature) (Asadinezhad *et al*., 2019; Gupta *et al*., 2016). In recent years, nanoemulsions have become increasingly noteworthy as potential vehicles for the controlled delivery of a variety of active ingredients in particular skin layers. Nanoemulsions are relatively stable physically in comparison with conventional emulsions due to their small droplet sizes. When compared to microemulsions, a lower amount of surfactant is required in nanoemulsion formulations. Other benefits of nanoemulsions include aesthetic appearance, rapid penetration of active ingredients into/through the skin and ease of application (Gupta *et al*., 2016; Van Tran *et al*., 2019). To date, very few studies have been carried out to formulate nanoemulsions as vehicles for tamarind fruit pulp extract. Previously, some researchers prepared matrix patches and cleansing lotions (emulsions) as carriers for tamarind pulp extract (Maenthaisong *et al*., 2009; Viyoch *et al*., 2003). Based on these reasons, nanoemulsions have been selected as a carrier for the extract of tamarind fruit pulp.

In this study, topical oil-in-water (o/w) nanoemulsions (lotions) were first prepared using cold process emulsifiers (e.g. Simulgel FL) or conventional liquid emulsifiers which were nonionic surfactants (e.g. Tween 80 and Span 80) to avoid heating and to reduce time. The high energy method, high pressure homogenization, was selected to produce the nanoemulsion bases. It was the most used technique when the plant extracts were involved (Zorzi *et al*., 2015). 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 and non-greasy; they also displayed cooling effects from the water evaporation.

Therefore, the aims of the current study were to formulate the o/w nanoemulsions as a carrier for tamarind fruit pulp extract and to evaluate the bioactive activities of o/w nanoemulsions containing tamarind fruit pulp extract by determining antioxidant and antibacterial properties.

MATERIAL AND METHODS

Sweet tamarind fruit pulp extract (powder) was purchased from Guangzhou Phytochem Sciences Inc. (China). The origin of tamarind (*Tamarindus indica* L.) was Guangdong province, China. The tamarind extract powder was prepared by liquid-solid extraction and the ratio of proportion extraction was 4:1 (tamarind: solvent). Spray drying process was used to produce the dry powder. Myristol (Caprylic/capric triglyceride) were obtained from Chemipan Corporation Co. Ltd. (Thailand). Tween 80, Span 80, refined glycerine USP, ponceau 4R (cochineal red A or brilliant scarlet 4R), ethylhexylglycerin, phenoxyethanol and light mineral oil were obtained from P.C. Drug Center Co., Ltd. (Thailand). Simulgel FL (hydroxyethyl acrylate/ sodium acryloyldimethyl taurate copolymer and isohexadecane and Polysorbate 60) was purchased from SEPPIC S.A. (France). Orthophosporic acid and sodium chloride were supplied by Univar (Australia). Tartaric acid (racemic form, 99.7% purity), L-ascorbic acid (99% purity) and 2,2 –diphenyl-1-picrylhydrazyl (DDPH) were from S.M. Chemical Supplies Co., Ltd. (Thailand). Absolute ethanol was obtained from VMR International Ltd., Poole, United Kingdom. Methyl alcohol (methanol), HPLC grade, was from J.T. Baker Inc. (USA). Mueller-Hinton agar and Mueller-Hinton broth were supplied by Difco BBL (USA). All chemicals were of analytical grade or pharmaceutical grade except where specified.

Determination of amounts of tartaric acid in tamarind extract

The quantity of tartaric acid, a specific organic acid in the sweet tamarind fruit pulp extract was determined by high performance liquid chromatography (HPLC) (as explained below). The amount of tartaric acid in the sweet tamarind extract was found to be 0.324 ± 0.063 mg% (w/w).

HPLC analysis

The HPLC conditions were based on the method of Kordis-Krapes *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 in our work 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). The linearity, accuracy, and precision of the analytical procedure were assessed and found to be in the acceptable range. The specificity of the HPLC method was also evaluated to check interferences.

Antioxidant activity

Antioxidant property of sweet tamarind fruit pulp extract was determined using scavenging activity of 2, 2-Diphenyl-1-picrylhydrazyl (DPPH). The analysis was performed according to Martinello *et al.* (2006) and Yuan *et al.* (2012) with slight modifications.

Different concentrations of tamarind extract (0.029-15 mg/mL) were prepared using distilled water. The positive control, which was ascorbic acid (0.1-50 µg/mL in distilled water), and test samples of tamarind extract were added to the DPPH solution (6 x 10^{-5} M in 80% ethanol). This method was tested in a 96-well plate. The composition of reaction mixtures is summarized in Table I. The mixture was kept in the dark at 25ºC 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 quadruplicate. The EC_{50} (effective concentration of sample required to scavenging DPPH radical by 50%) was obtained by plotting between % inhibition and concentration of samples.

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. 1:

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

TABLE I - 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

Antibacterial activity (tamarind fruit pulp extract)

Normal microflora found in the skin, especially in axillary, includes gram-positive bacteria such as *S. aureus* and *S. epidermidis*. Minimum inhibitory concentration (MIC) 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, Oluwa, Omomigbehin, 2006; Gunes *et al*., 2016). Two bacterial strains, *S. aureus* ATCC 25923 and *S. 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 extract for microbial assay was prepared by dissolving in sterile water to give a final concentration of 32 mg/mL. The solution was further serially diluted two-fold 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. Fresh cultures of standard strains were prepared and their density of turbidity adjusted by determining the absorbance reading of 0.08 – 0.1 at 625 nm and further diluted 1:100 in MHB. Then, 50 µL of this suspension of the test organisms was added to each well. The wells containing 50 µL of the medium and 50 µL of the inoculums were considered positive controls whereas the wells containing 100 µL of medium were the negative controls. The plates were sealed and then incubated at 37 ± 2ºC for 20 h. After reaching the incubation time, 5 µL of a 0.2% blue colored resazurin solution was 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 considered 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 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.

Preparation of blank nanoemulsions

In the preliminary study, Simulgel FL, a cold process emulsifier and thickener, was first used to prepare the nanoemulsion vehicles. 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. However, the obtained nanoemulsions had phase separation (creaming) after a few hours for all concentrations of Simulgel FL (0.5-1.5%w/w). It was probably because the polymer chains of Simulgel FL (hydroxyethyl acrylate/sodium acryloyldimethyl taurate copolymer [and] isohexadecane [and] polysorbate 60) were damaged by the high shear (force) and high heat (about 60ºC) generated during the high-pressure homogenization process. Therefore, the Simulgel FL was replaced by the conventional liquid emulsifier, Tweens and Spans. The use of conventional emulsifiers, Tween 80 and Span 80, was based on the study of Songkro *et al*. (2011) which formulated the nanoemulsions of plaunoi extract with these two nonionic surfactants. Hydrocolloids such as xanthan gum, guar gum and cationic guar gum were also added into the formulations as thickeners. Although the stable nanoemulsions with suitable physicochemical properties were obtained during 1-month storage, the viscosity of the formulations was considerably low. It is likely that the polymer chains of the hydrocolloids were probably damaged under the process of high-pressure homogenization. In addition, when the nanoemulsions containing hydrocolloids were mixed with tamarind extract powder, there was phase separation within 24 h (data not show). Based on the above results, the final formula of blank nanoemulsion, F1, is as follows:

	%w/w
Tween 80 (emulsifier)	3.13
Span 80 (emulsifier)	1.87
Caprylic/capric triglyceride (emollient)	20
Glycerine (humectant)	10
Distilled water (vehicle)	65

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). The formulation was prepared in triplicate (n=3).

The o/w nanoemulsions were prepared by emulsifying the surfactant mixture, water, glycerine and synthetic oil 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 first prepared with a high-pressure homogenizer. After that, the optimized nanoemulsion bases (cool state) were incorporated with the suitable amount of the tamarind extract based on the bioactivity study.

Preparation of tamarind fruit pulp extract loaded o/w nanoemulsions

In the current study, two concentrations of the sweet tamarind extract, 6.6%w/w and 3.3%w/w, which were 100times and 50times of EC_{50} (antioxidant activity), respectively were used. To inhibit mold (fungi) growth, appropriate preservatives, namely 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 nanoemulsion bases with sweet tamarind fruit pulp extract powder using a magnetic stirrer at 300 rpm overnight.

Investigation of Physicochemical Properties

The physicochemical properties of the prepared formulations were determined as follows:

Characterization of Nanoemulsion Type

The type of the prepared nanoemulsions was determined by dye solubility test (1% Ponceau 4 R aqueous solution) and conductivity measurement using a conductivity meter (model CM-115, Kyoto Electronics Manufacturing Co., Ltd., Kyoto, Japan). All measurements were performed in triplicate at 25°C.

pH Measurement

The pH values of nanoemulsions were measured in triplicate using a digital pH meter (model Seven easy S20, Mettler -Toledo Inc., OH, USA). The pH determination was carried out directly in nanoemulsion formulations without dilution. The measurements were performed at 25 °C.

Viscosity and Flow Measurement

The viscosity and flow properties of the formulations were determined in triplicate using a bob-cup Brookfield rheometer (model LVDV-III Ultra, Brookfield Engineering Laboratories Inc., MA, USA) 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°C, the surface temperature of skin.

Zeta Potential, Droplet size and Polydispersity index (PDI) Analysis

The zeta potential, droplet size and PDI of the nanoemulsions were determined with a light-scattering technique using Zeta potential analyzer (model ZetaPALS, Brookhaven Instruments Corporation, NY, USA). All measurements were carried out at a fixed angle of 90°C at 25°C. Nanoemulsions were appropriately diluted with deionized water before the measurement; 15 µL of the 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.

Stability of nanoemulsions

The physical stability of nanoemulsions was evaluated by 1) centrifugation (Rocha-Filho *et al*., 2017) and 2) heating-cooling cycles (4 cycles) (Thai Community Product Standard No. 550/2553). The centrifugation test was performed on freshly prepared nanoemulsions (24 h) at 3,000 rpm for 30 min. The appearance and homogeneity were evaluated by visual observation. For the heating-cooling cycle test, each cycle consisted of keeping the samples at a cooling temperature of 4ºC for 24 h and then at a high temperature of 45ºC for 24 h. The chemical changes of the formulations were investigated by measuring the amount of non-degraded tartaric acid using the HPLC technique. The tamarind extract loaded nanoemulsions were accurately weighed (2 g) into a centrifuge tube and 5 mL of a mixture of absolute ethanol and distilled water (1:1 by volume) was added. The mixture was vortexed and sonicated for 30 min and centrifuged at 6,000 rpm (model Z 323 K, Hermle LaborTechnik GmbH, Wehingen, Germany) for 10 minutes at 25°C. When necessary, the resultant solution was filtered through a membrane pore size of 0.2 µm. An aliquot of clear solution was further diluted with an appropriate amount of HPLC mobile phase and was assessed for the amount of tartaric acid remaining in the samples. The technique was modified from the study of Özer, Mutlu, Kıvçak (2007) which investigated the antityrosinase activity of creams containing plant extract.

In vitro release study of tamarind extract loaded nanoemulsions

The release of tartaric acid was determined in order to check whether the active ingredient (tamarind fruit pulp extract) could be released from the nanoemulsion vehicle or not. Consequently, the active compound (s) released could exert the antioxidant and antibacterial effects. The release characteristics of the two concentrations of tamarind fruit pulp extracts loaded nanoemulsions, 3.3 and 6.6%w/w, were compared to each other. The *in vitro* release tests were performed by using synthetic cellulose acetate membrane (Spectra/Por 3, Spectrum Laboratories, Inc., CA, USA) and diffusion cells 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: a donor compartment for sample application and a 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 were previously cut into a suitable size, were immersed in a 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. All air bubbles in the receptor fluid were removed before the membrane was positioned. The two parts of diffusion cells were held 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. 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 sink condition was obtained under the experimental conditions. 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 the following equation:

$$Q_t = V_r C_t + \sum_{i=0}^{t-1} V_s C_i \quad \text{Eq. 2}$$

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 II.

TABLE II - Kinetics of drug release

Model	Equation
Zero order	Qt=Q0+K0t
First order	lnQt=lnQ0+K1t
Higuchi	Qt=Q0+KHt1/2

Q_t : cumulative amounts of tartaric acid released in time t; Q0 : initial amount of tartaric acid in receptor fluid; K_0, K_1, K_H : release rate constants of zero order, first order and Higuchi, respectively (Costa *et al*., 2013).

Bioactivity study of tamarind extract loaded nanoemulsions

The tamarind fruit pulp extract loaded nanoemulsions were further tested for their bioactivity properties. Samples tested were freshly prepared formulations and the formulations under stress conditions: heating-cooling cycles. Blank formulations were also employed to check the effect of nanoemulsion bases on the bioactivity properties.

Antioxidant activity

The antioxidant activity of the formulations was determined as previously described. Tartaric acid in the tamarind extract loaded nanoemulsions was extracted by a suitable solvent (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.

Antibacterial activity

The antimicrobial property of the nanoemulsions containing tamarind fruit pulp extract was determined

using the agar well diffusion technique (Balouiri, Sadiki, Ibnsouda, 2016; Gupta, Prakash, Gupta, 2014).

Two bacterial strains, which were *S. aureus* and *S. epidermidis* were incubated in Mueller-Hinton agar (MHA) for 24 h at 35-37°C and adjusted to obtain a turbidity of 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, Yen, Mau, 2016). Gentamicin (0.1% w/w) was prepared in nanoemulsion as a positive control, while blank nanoemulsions were used as a negative control for *S. aureus* and *S. epidermidis*. All experiments were performed in triplicate.

Statistical Analysis

Statistical comparisons were made using Student's t-test, paired t-test or One-way analysis of variance (ANOVA). Differences at $p < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Antioxidant and antibacterial activities of tamarind fruit pulp extract

The antioxidant activity of the sweet tamarind fruit pulp extract is summarized in Table III. The antioxidant activity of ascorbic acid, a well-known antioxidant agent, was measured to prove the method accuracy. It was speculated that antioxidant activity of the sweet tamarind fruit pulp extract was derived from the combined organic acids since it contained low amounts of tartaric acid.

The antibacterial activity of tamarind extracts was evaluated by broth macrodilution and the results are shown in Table III. The lowest concentration of tamarind extract inhibiting the bacterial growth was considered the MIC. The extract was found to be effective against *S. epidermidis* and *S. aureus* which cause malodor in underarm areas. For MBC, the lowest concentration killing bacteria, was usually higher than MIC. In the current study, MBC could not be determined because the tamarind extract sample turned pink (resorufin). Thus, the test tamarind extract had MBC values greater than 8 mg/mL. Antibacterial activities of tamarind extract against gram positive bacteria have been reported (Gupta, Prakash, Gupta, 2014).

Based on the antioxidant and antibacterial activities, sweet tamarind fruit pulp extract from China was a promising candidate to be used as the active ingredient in our study. In the preliminary study, sour tamarind fruit pulp extracts were also investigated for the antioxidant and antibacterial potentials. They exhibited lower scavenging and bacterial activities against two bacterial strains although they contained considerably higher amounts of tartaric acid.

TABLE III - Antioxidant and antibacterial activities of sweet tamarind fruit pulp extract compared with positive controls
A. Effective concentration of positive control and sweet tamarind fruit pulp extract required to scavenging DPPH radical by 50%
B. MIC of positive control and sweet tamarind fruit pulp extract against *S. epidermidis* and *S. aureus*

Samples	Test concentrations	EC_{50}
Ascorbic acid (positive control)	0.1-50 µg/mL	2.8698 µg/mL
Sweet tamarind pulp extract	0.029-15 mg/mL	0.6681 mg/mL

Samples	Test concentrations	MIC	
		S. epidermidis	*S. aureus*
Gentamicin sulfate	0.01-4 µg/mL	0.25 µg/mL	0.25 µg/mL
Sweet tamarind pulp extract	0.02-8 mg/mL	8 mg/mL	8 mg/mL

Blank nanoemulsion and Tamarind extract loaded nanoemulsions

The blank nanoemulsion (F1) showed creamy white appearances with no phase separation. According to electrical conductivity and dye solubility tests, the obtained nanoemulsion was o/w, as shown in Table IV. 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). In addition, Ponceau 4 R solution, a water-soluble dye, dissolved uniformly throughout the system, meaning the formulations were o/w nanoemulsions. Based on the antioxidant activity study (see Table III), the concentrations of the tamarind fruit pulp extract used in the current study were 100times and 50times of the EC_{50} which were 6.6 and 3.3%w/w, respectively. The ingredients (nanoemulsion base and tamarind powder) were mixed together using a magnetic stirrer overnight, and a few days later, 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 containing sweet tamarind fruit pulp extract were further investigated for physicochemical properties, stability and bioactivities.

The tamarind extract loaded nanoemulsions had light brown color, homogeneity and no precipitation. Using a dye solubility test, the two tamarind extract loaded nanoemulsions showed miscibility. The average conductivity values of the freshly prepared tamarind extract loaded nanoemulsions, F1-3.3TE and F1-6.6TE, were significantly higher than those of the blank nanoemulsion F1 ($p < 0.05$). The constituents in the tamarind extract markedly affected the conductivity of the nanoemulsion system. The conductivity value was directly related to the amount of tamarind extract used. Based on both tests, the type of tamarind extract loaded nanoemulsions remained o/w system.

TABLE IV - Conductivity and dye solubility tests of blank nanoemulsion and tamarind extract loaded nanoemulsions

Formulation	Conductivity value (µs/cm) (mean ± SD, n=3, where n is number of samples)	Dye solubility
F1 (blank)	83.70 ± 1.49	miscible
F1-3.3TE	1,170.67 ± 12.10*	miscible
F1-6.6TE	2,071.33 ± 9.61*	miscible

F1-3.3TE = nanoemulsion containing 3.3%w/w tamarind extract; F1-6.6TE = nanoemulsion containing 6.6% w/w tamarind extract, *$p < 0.05$ compared to F1

Physicochemical properties and stability of nanoemulsions

Physicochemical properties of the nanoemulsions are summarized in Table V. The pH of skin is one of the factors that the formulators need to consider when designing the skin care products and cosmetics. This is due to improper pH of the dermatological products which can cause skin irritation (Mahdi *et al*., 2011). In general, skin pH ranging from 4 to 6 (slightly acidic) has been reported (Baumann, Castanedo-Tardan, 2009). As seen from Table V, incorporating tamarind extract into the nanoemulsion base (F1) significantly decreased the pH of the preparations ($p <0.05$). This was because of the organic acids in the tamarind fruit pulp extract. The freshly prepared tamarind fruit pulp extract loaded nanoemulsions, F1-3.3TE and F1-6.6TE, had weak acidic pH, (approximately 5) and significantly lower pH values under stress conditions ($p<0.05$) when compared with their own freshly prepared formulations. The pH of formulation F1-3.3TE statistically differed to that of the formulation F1-6.6TE ($p <0.05$), owing to the different amounts of tamarind extract used. Based on the pH values, two tamarind extract loaded nanoemulsions were considered safe for dermatological products since they were still in the range of the skin pH. Nevertheless, for safety reasons, the irritation potential of the preparations should be investigated in future work. As seen from Table V, the viscosity of the two tamarind

extract loaded nanoemulsions was significantly higher than that of the blank nanoemulsion, F1 ($p < 0.05$). The viscosity of the tamarind extract loaded formulations was in the range of 200-300 cps (at 30 rpm). The increased viscosity was probably attributed to constituents such as carbohydrates, fibers and invert sugars in the sweet tamarind fruit pulp. The flow curves of the two tamarind extract loaded nanoemulsions are exhibited in Figure 1. The shear thinning behavior was observed for both formulations. At the speed of 15 rpm, there was drastic change in the viscosity of the formulation F1-6.6TE under heating-cooling cycles when compared with the formulation F1-3.3TE. Only slight changes in viscosity were observed at the other speeds.

TABLE V - Physicochemical property of tamarind extract loaded nanoemulsions compared with corresponding blank nanoemulsions

Formulation	pH		Viscosity (cps) (at 30 rpm, 32°C)		Droplet size (diameter) (nm)		PDI values		Zeta potential (mV)	
	(mean ± SD, n=3, where n is number of samples)				(mean ± SD, n=10, where n is number of measurements on the sample)					
	Freshly prepared	Heating –cooling	Freshly prepared	Heating –cooling	Freshly prepared	Heating –cooling	Freshly prepared	Heating –cooling	Freshly prepared	Heating –cooling
F1 (blank)	6.65 ± 0.02	6.26 ± 0.06	6.39 ± 0.35	8.00 ± 0.00	139.69 ± 2.16	139.86 ± 2.95	0.137 ± 0.039	0.170 ± 0.034	-63.02 ± 2.81	-22.34 ± 4.77
F1-3.3TE	5.28 ± 0.06*[a]	5.01 ± 0.02*[a]	259.94 ± 34.63*	239.08 ± 1.44*	123.47 ± 5.32*[a]	127.90 ± 3.05*[a]	0.105 ± 0.051	0.120 ± 0.058	-38.52 ± 0.46*	-38.52 ± 0.46*
F1-6.6TE	5.15 ± 0.01*[a]	4.79 ± 0.02*[a]	306.70 ± 46.09*	302.44 ± 0.50*	128.42 ± 2.91*	130.11 ± 2.96*	0.137 ± 0.060	0.146 ± 0.059	-38.15 ± 0.65*	-38.15 ± 0.65*

F1-3.3TE = nanoemulsion containing 3.3%w/w tamarind extract; F1-6.6TE = nanoemulsion containing 6.6% w/w tamarind extract; *$p < 0.05$ compared to F1

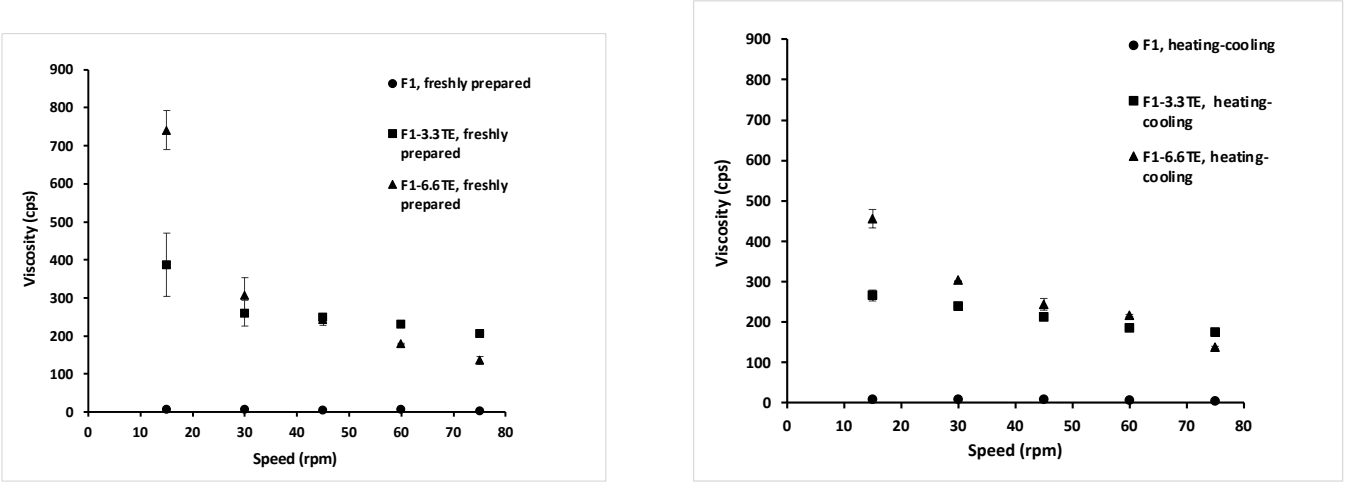

FIGURE 1 - Rheograms of tamarind extract loaded nanoemulsions, F1-3.3TE and F1-6.6TE at freshly prepared (above) and stored at different conditions for 1 month (below), determined at 32ºC. Each point represents mean ± SD, n =3; where n = number of samples.

Initially, the centrifugation method was used to check the physical stability of all 24-h preparations. There was no phase separation of nanoemulsions under the 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 droplet sizes, PDI and zeta potential (surface charge of droplet). It is generally recognized that the change in droplet sizes reflects the stability of the systems (Ribeiro *et al*., 2015). The results of droplet sizes are summarized in Table V. There was significant difference in the droplet sizes between the unloaded and tamarind extract loaded nanoemulsions ($p < 0.05$). The tamarind extract loaded formulations displayed significantly lower droplet sizes, suggesting that tamarind extract was located in the external phase (here, water). The presence of tamarind fruit pulp extract influenced the droplet sizes of the nanoemulsion system. Under heating-cooling conditions, the droplet sizes of the formulation F1-3.3TE were significantly different from those of the freshly prepared formulation ($p<0.05$). However, no significant difference was found in the other formulation (6.6%). The uniformity of the droplets was indicated by the PDI. PDI value closer to zero indicates homogeneity of droplets (Ribeiro *et al*., 2015). For freshly prepared and heating-cooling formulations, the two extract loaded nanoemulsions displayed the PDI values less than 0.2, indicating high homogeneity of the nanoemulsion droplets (low polydispersity). It was found that the formulation F1-3.3TE had lesser PDI values than the formulation F1-6.6TE did. This was possibly due to lesser amount of tamarind extract in the formulation. Another indicator for formulation stability was zeta potential measurement. 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 adequately stable systems. As seen from Table V, the zeta potential of the formulations F1-3.3TE, and F1-6.6TE maintained in the range of -38 mV after the stress condition. The negative charge of the o/w nanoemulsions was possibly due 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 (here, a mixture of Tween 80 and Span 80) which were used as the emulsifiers in the current study (Maruno, Rocha-Filho, 2010).

There was no significant change in the amounts of tartaric acid remaining in the formulation F1-3.3TE after heating-cooling cycles in comparison with the freshly prepared formulation. However, the formulation F1-6.6TE displayed marked reduction of tartaric acid concentrations (about 67%w/w) when compared with its original concentration (100%w/w). Furthermore, the prolonged storage time (2 months) of the formulation F1-6.6TE resulted in a phase separation probably due to coagulation of the extract.

In vitro release of tamarind fruit pulp extract loaded nanoemulsions

The amount of tartaric acid released across a synthetic membrane against time is depicted in Figure 2. At several time points, the formulation F1-6.6TE displayed a significantly higher amount of tartaric acid released than the formulation F1-3.3TE did ($p <0.05$). It could be concluded that the amount released was directly related to the concentration of tamarind extract incorporated into the formulations. For both formulations, drastic increase in the amounts of tartaric acid released was observed in the first 6 h.

The release profiles were further evaluated for the best fit using different mathematical kinetic models: zero order, first order and Higuchi model (Costa *et al*., 2013). 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 VI. The Higuchi model was found to be the best fit for both formulations with r^2 of 0.96. This suggests the diffusion release mechanism of tartaric acid (tamarind fruit pulp extract) from o/w

nanoemulsions. 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 a higher amount of tamarind fruit pulp extract.

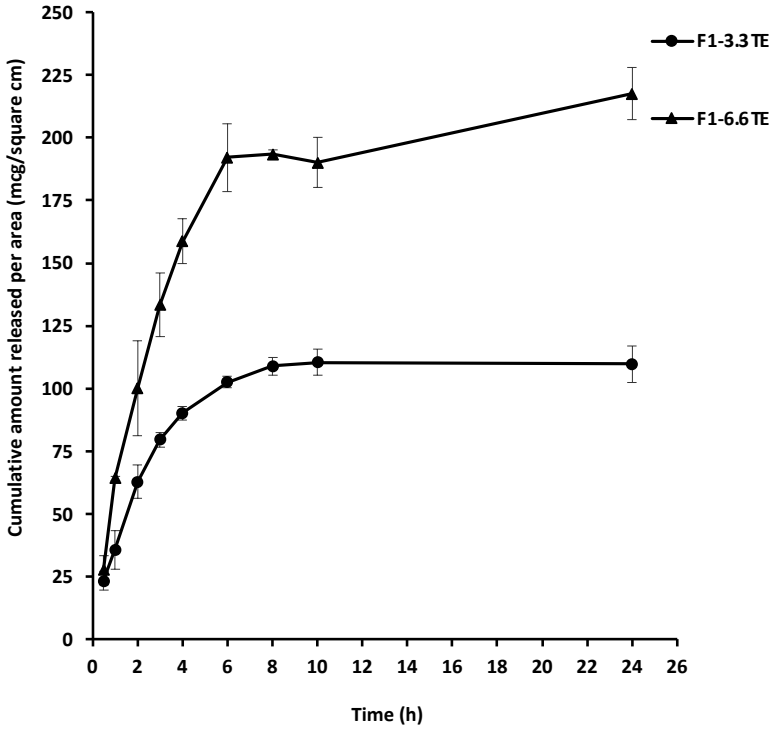

FIGURE 2 - *In vitro* release profiles of tartaric acid from tamarind fruit pulp extract loaded nanoemulsions across cellulose acetate membrane. Each point represents mean ± SD, n=3-4, where n is number of replications.

TABLE VI - Release parameters of tamarind 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/cm²/h)	r^2	K_1 (1/h)	r^2	K_H (µg/cm²/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	0.9671	87.192 ± 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)

Bioactivity study of tamarind fruit pulp extract loaded nanoemulsions

The antioxidant property (% scavenging activity) of tamarind extract loaded nanoemulsions, F1-3.3TE and F1-6.6TE were investigated and the results of freshly prepared formulations and those under stress condition are summarized in Table VII. 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 on the antioxidant properties. The results revealed that % scavenging activity (antioxidant activity) was directly related to the concentrations of tamarind extract. The

higher the amount of tamarind extract, the higher the % scavenging activity was obtained. The antioxidant properties of the formulations were caused by several organic acids in the tamarind extract. It was surprising that the same concentrations of tamarind extract (1.65 and 3.3 mg/mL) of the two nanoemulsions did not give the same range of % scavenging activity. Under the heating-cooling conditions, the antioxidant activities of all samples significantly decreased ($p<0.05$). To maintain the antioxidant activity of tamarind extract loaded nanoemulsions, suitable extra antioxidant agents should be added into the formulations.

TABLE VII - Percent scavenging activity of tamarind extract loaded nanoemulsions determined by DPPH assay.

Formulation	Scavenging activity (%) (mean ± SD, n=5, where n is number of replications)	
	Freshly prepared	Heating-cooling
F1-3.3TE (0.825 mg/mL**)	51.80 ± 0.37[a]	13.68 ± 0.37[a]
F1-3.3TE (1.65 mg/mL**)	67.24 ± 0.79[a]	28.31 ± 1.03a
F1-3.3TE (3.3 mg/mL**)	81.66 ± 0.77[a]	48.56 ± 1.54[a]
F1-6.6TE (1.65 mg/mL**)	49.36 ± 0.86[a]	35.21 ± 0.91[a]
F1-6.6TE (3.3 mg/mL**)	63.80 ± 0.79[a]	54.72 ± 0.96[a]
F1-6.6TE (6.6 mg/mL**)	84.16 ± 0.89[a]	71.09 ± 1.12[a]

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; [a]$p < 0.05$ compared between freshly prepared and heating-cooling

The antibacterial properties of tamarind extract loaded nanoemulsions against *S. aureus* and *S. epidermidis* were investigated using agar well diffusion technique. In addition, gentamicin sulfate (0.1% w/w) prepared in nanoemulsion and the 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 Figure 3. The results of inhibition zones of freshly prepared formulations and the formulations under stress conditions are summarized in Table VIII. There was no inhibition zone of blank nanoemulsion F1 against two bacterial strains, therefore the antibacterial effects of the loaded nanoemulsions were derived from tamarind fruit pulp extract in the formulation only. As seen from Table VIII, the inhibition zones of the formulation F1-6.6 TE for *S. aureus* were significantly greater than those of the formulation F1-3.3TE ($p < 0.05$). Unlike the formulation F1-6.6TE, the formulation F1-3.3TE did not show significant difference in the inhibition zones of *S. aureus* after experiencing heating-cooling conditions. For *S. epidermidis*, the inhibition zones of the two formulations were similar. No significant difference in the inhibition zone was observed ($p < 0.05$) even though the amount of tamarind fruit pulp extract in the formulation F1-6.6TE was two times

that of the formulation F1-3.3TE. In addition, no statistical change in inhibition zones was observed in both formulations under stress conditions. Between the two test bacteria, the formulation F1-3.3TE was more effective against *S. epidermidis* ($p < 0.05$). These results indicated that sweet tamarind fruit pulp extract at lower concentration, 3.3%w/w, was sufficient to be used as an antibacterial agent.

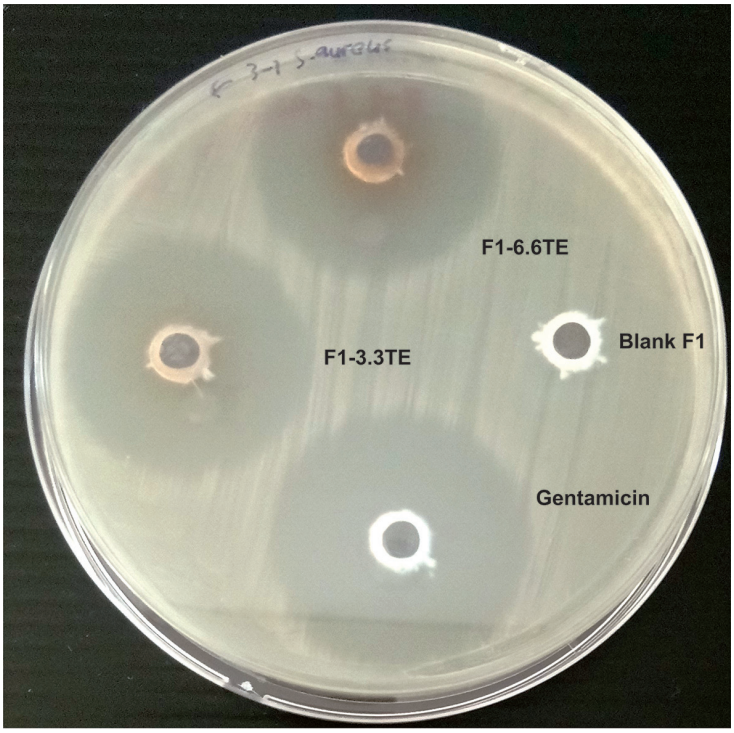

FIGURE 3

FIGURE 3 - Zone of inhibition (mm) of *S. aureus* from samples, negative and positive controls.

TABLE VIII - Antibacterial activity of tamarind extract loaded nanoemulsions against *S. aureus* and *S. epidermidis* determined by agar well diffusion technique

Formulation	Zone of inhibition (mm) (mean ± SD, n=3, where n is number of replications			
	S. aureus		*S. epidermidis*	
	Freshly prepared	**Heating-cooling**	**Freshly prepared**	**Heating-cooling**
F1-3.3TE	24.00 ± 0.50	25.33 ± 0.58	27.33 ± 0.58	27.67 ± 0.58
F1-6.6TE	26.00 ± 1.00[a]	27.67 ± 0.58[a]	27.33 ± 0.58	27.67 ± 0.58
0.1%w/w gentamicin sulfate nanoemulsion	28.00 ± 0.00	28.00 ± 0.00	29.67 ± 0.58	29.67 ± 0.58

Zone of inhibition of blank nanoemulsion was 0 mm (no inhibition zone); [a]$p < 0.05$ compared between freshly prepared and heating-cooling

CONCLUSION

In the current study, sweet tamarind fruit pulp extract loaded o/w nanoemulsions were successfully prepared using a high pressure homogenization process. The preparations were tested for their physicochemical, antioxidant and antibacterial properties in an attempt to be used in cosmetic/hygiene applications such as anti-aging and deodorants. Two concentrations of tamarind extract based on the bioactivity study were employed: 3.3%w/w and 6.6%w/w. Acceptable physicochemical property and relatively better bioactivity of the nanoemulsion containing 3.3%w/w were achieved. The major problem of the prepared tamarind extract loaded nanoemulsions involved the decreased antioxidant activity under stress experimental conditions. Nevertheless, the results suggest that nanoemulsions containing tamarind extract can be considered a promising candidate as a natural deodorant product. In the future, the extra antioxidant agents should be added into the formulations in order to retain the antioxidant property. The long-term stability of the formulations should be performed. In addition, the *in vitro* skin permeation and retention studies of the tamarind fruit pulp extract loaded nanoemulsions should be carried out for better understanding of how nanotechnology influences the delivery of active ingredients (tamarind fruit pulp extract) into/through the skin. For cosmetic products, the permeation of active ingredients through the skin (transdermal delivery) is of concern since it can cause undesirable systemic side effects. The retention/accumulation of cosmetic compounds within the targeted skin layers or at the skin surface (local/topical delivery) are a crucial goal for cosmetic nanocarriers.

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Erratum

In the article "**Characterization, Antioxidant and Antibacterial Potentials of *Tamarindus indica* L. Fruit Pulp Extract Loaded O/W Nanoemulsions**", number doi: 10.1590/s2175-97902022e19373, published in the Brazilian Journal of Pharmaceutical Sciences, vol 58:

Where it was written:

TABLE II - Kinetics of drug release

Model	Equation
Zero order	Qt=Q0+K0t
First order	lnQt=lnQ0+K1t
Higuchi	Qt=Q0+KHt1/2

Q_t : cumulative amounts of tartaric acid released in time t; Q0 : initial amount of tartaric acid in receptor fluid; K_0, K_1, K_H : release rate constants of zero order, first order and Higuchi, respectively (Costa *et al*., 2013).

Should read:

TABLE II - Kinetics of drug release

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

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