

Characterization of rice bran wax policosanol and its nanoemulsion formulation

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Abstract: Policosanol, a mixture of long-chain alcohols found in animal and plant waxes, has several biological effects; however, it has a bioavailability of less than 10%. Therefore, there is a need to improve its bioavailability, and one of the ways of doing this is by nanoemulsion formulation. Different droplet size distributions are usually achieved when emulsions are formed, which solely depends on the preparation method used. Mostly, emulsions are intended for better delivery with maintenance of the characteristics and properties of the leading components. In this study, policosanol was extracted from rice bran wax, its composition was determined by gas chromatography mass spectrophotometry, nanoemulsion was made, and the physical stability characteristics were determined. The results showed that policosanol nanoemulsion has a nanosize particle distribution below 100 nm (92.56–94.52 nm), with optimum charge distribution (–55.8 to –45.12 mV), pH (6.79–6.92) and refractive index (1.50); these were monitored and found to be stable for 8 weeks. The stability of policosanol nanoemulsion confers the potential to withstand long storage times.

Keywords: rice bran wax, policosanol, nanoemulsion, characterization

Introduction

Naturally occurring compounds in plants have received tremendous attention for many decades because of their therapeutic and risk-minimizing potential for many diseases, including chronic diseases such as cardiovascular diseases, diabetes, and neurodegenerative diseases.^{1–3} It is not just coincidence that rice, on which most of the world's population depends for survival, contains several health-promoting bioactive compounds.^{4–7} Rice accounts for about 35%–80% of the calorie intake of most people in Asia, including Malaysia, and less than 20% of the calorie intake of a significant number of people among the African and Latin American population.⁸

One of the products of rice milling is rice bran, which contains several compounds that have nutritional and biological potential in its oily extract, rice bran oil (RBO). RBO contains oryzanol, a significant amount of tocopherols, tocotrienols, phytic acid, lecithin, inositol, and wax. In the wax, which was initially considered a waste product, wax esters and aliphatic alcohols (policosanols) are present. Supercritical fluid extraction⁹ is the best extraction method for RBO, but it can be extracted using other methods such as solvent extraction and ohmic heating.^{10,11} From RBO, a eutectic wax substance can be extracted using a number of available methods, such as winterization, and solvent and catalytic methods.^{12–14}

Policosanol is a mixture of naturally occurring high molecular weight aliphatic primary alcohols present in a significant amount in rice bran wax. It was initially

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isolated from sugar cane wax by Cuban researchers and developed by Cuban Dalmer laboratories.¹⁵ Other sources of policosanol include beeswax, wheat, sorghum, maize, rice, broccoli, spinach, alfalfa, and other cereal grains. The major components of policosanol mixture are octacosanol (66%), triacontanol (12%), and hexacosanol (7%). The remaining 15% is made up of other alcohols, namely, tetracosanol, heptacosanol, docosanol, nonacosanol, and tetratriacontanol, which are the minor components.¹⁶ Several reports, including many clinical trials, have shown that policosanols have significant health-promoting activities such as the reduction of lipid levels,^{17–21} platelet aggregation reduction,²² antiviral activity,²³ reduction of prostate enlargement, and relief of intermittent claudication.²⁴ In contrast, there are conflicting findings about the efficacy of policosanol, particularly with regard to lowering lipid levels.^{25–30} However, direct comparisons of the two findings have been limited by differences in regional dietary patterns and by the unknown variability of the composition of the policosanol products that were tested.³¹ Despite the amazing number of reported biological activities of policosanol, its bioavailability was reported to be between 5% and 12%, with absorption after oral administration ranging from 10%–35%.³²

There appears to be little literature on the nanoformulation of policosanol. Only two patents were found that described the formation of policosanol nanoparticles,^{33,34} and one of the patents described formation of policosanol nanoemulsion by continuous stirring and heating.³³ Nanoemulsion is one of the emerging nanotechnologies, that confer the controlled delivery of pharmaceuticals and cosmetics.³⁵ Nanoemulsions are oil-in-water (O/W) or water-in-oil (W/O) transparent liquid dispersions with a diameter of 20–200 nm.^{36,37} It is quite apparent that the smaller the droplet size, the more resistant the nanoemulsion is to destabilization because of gravitational separation, flocculation, and coalescence. To date, no known studies have used high-pressure homogenization to form rice bran wax (RBW) policosanol nanoemulsion. In the present study, policosanol was extracted from RBW, a nanoemulsion was prepared, and its stability characteristics were monitored. The policosanol nanoemulsion formation was performed in two steps: pre-emulsion formation and high-pressure homogenization.

Methodology

Materials

Rice bran was obtained from Bernas milling factory in Kuala Selangor, Selangor, Malaysia. RBO was extracted by supercritical fluid extraction (SFE), using carbon dioxide; RBW was separated from RBO; and policosanol was

extracted from RBW. All the reagents and solvents used were either analytical grade or high-performance liquid chromatography grade.

Extraction of RBO

About 160 g of freshly obtained rice bran, stabilized using an automated microwave oven (2,450 MHz, 550 W, 110°C for 200 seconds), and stored at 4°C was extracted using an SFE machine (Thar 1000 F; Thar Technologies Inc., Pittsburgh, PA, USA) for 3 hours at 60°C, 600 bars pressure, with a carbon dioxide flow rate of 25 g/minute.³⁸ The extracted oil was collected and percentage yield calculated with respect to the rice bran sample weight.

Extraction of RBW from RBO

The RBW in the SFE-extracted RBO sample was separated as acetone insoluble, according to the method proposed by Ramaswamy et al¹³ with slight modification. Acetone chilled to approximately 4°C was added to 5 mL RBO (1:1, volume/volume [v/v]), and the mixture was centrifuged at 4,000 rpm for 25 minutes. Supernatant oil was decanted carefully, and the insoluble portion was washed with 5 mL chilled acetone and centrifuged. The wax obtained was blown with nitrogen, using a nitrogen generator, and then dried in an oven at 60°C. The dried wax was then weighed and stored at 4°C until further analyzed.

Extraction of policosanol

Policosanol was extracted from RBW according to the method described by Cravotto³⁹ with some modification. About 10 g RBW was placed into a 200 mL conical flask and hydrolyzed with 100 mL 0.2 M NaOH by sonication with a Power sonic 505 (Hwashin Technology Co., Seoul, Korea) ultrasonicator 50 Hz, 350 W, at 60°C for 90 minutes. The hydrolyzed mixture was then extracted with an equal volume of petroleum ether, cooled down to 2°C, and then divided into 50 mL centrifuge tubes and centrifuged at 4,000 rpm for 10 minutes. The upper petroleum ether layer and the lower NaOH layer were removed by carefully decanting; the middle yellowish layer (policosanol) was collected and freeze dried.

Determination of policosanol content

Policosanol contained in the RBW was determined by gas chromatography mass spectrophotometry (GC-MS). The GC-MS (TSQ series; Thermo Scientific, Waltham, MA, USA) determination of policosanol was according to the method described by Dunford et al⁴⁰ with some modification. Policosanol standards docosanol, tetracosanol, hexacosanol, and

octacosanol were purchased from Axon Scientific, Selangor, Malaysia. A 5 mM concentration mixture of these standards in chloroform was prepared, 0.5 mL of this mixture was derivatized with 0.2 mL N,O-Bis(trimethylsilyl)trifluoroacetamide by incubating at 60°C for 20 minutes, and then the volume was made up to 1 mL by adding more chloroform after cooling to room temperature. RBW policosanol extracts were derivatized the same way. Policosanol standards and RBW policosanol extracts were first injected into the GC-MS machine.

The GC oven temperature was programmed from 150°C to 300°C with a heating rate of 4°C/minute and maintained at this temperature for 15 minutes. Initial flow rate of the carrier gas, helium, was 1.0 mL/minute. Inlet temperature was 300°C. GC-MS parameters were as follows: the MS transfer line temperature was 280°C, the ion source was kept at 230°C, and the MS quadrupole temperature was kept at 150°C. The ionization energy was 70 eV with 2 scans/second and a mass range of 100–1,000 amu. The standards/samples (2 µL) were injected into GC-MS with a 1:10 split ratio. Data analysis was carried out using the machine's software.

Formation of policosanol nanoemulsion

About 5 g of the extracted policosanol was weighed in a Scott bottle, and 3 g Tween 80 was added; then, 92 g deionized water was added slowly while continuously stirring. The mixture was homogenized at 13,000 rpm for 3 minutes, using an Ultra Turrax® (IKA, Staufen, Germany) to form the pre-emulsion. The pre-emulsion was then passed into a high-pressure homogenizer (Stansted Fluid Power, Ltd., Essex, UK) at 550–600 bars (55–60 MPa), room temperature, for 10 circles, with the emulsion cooled down to 30°C after every circle. The particle size and charge of the formed nanoemulsion was immediately measured using a zeta sizer (Nano-ZS; Malvern Instruments Ltd, Malvern, UK) and was subsequently monitored weekly for 8 weeks. The pH of the nanoemulsion was also monitored using a pH meter (Mettler Toledo, Selangor, Malaysia) throughout the period (8 weeks).

Determination of refractive index

The refractive index of policosanol nanoemulsion was measured using zeta sizer (Nano-ZS) taken at the same time as the particle size and charge measurements.

FTIR

The Fourier transform infrared spectroscopy (FTIR) of the policosanol nanoemulsion was performed according to the method described by Al-Qubaisi et al.⁴¹ The spectra of one drop each of policosanol nanoemulsion, RBO and Tween 80;

RBW and policosanol powder were recorded over the range of 400–4,000 cm⁻¹ on a Thermo Nicolet Nexus, Smart orbit spectrometer, on spectroscopic-grade potassium bromide (KBr) with 10 tons of pressure.

TEM morphology of the policosanol nanoemulsion

Transmission electron microscopy (TEM) was performed by first premixing the sample using a sonicator and then placing a Formvar-coated copper grid on top of a drop of the sample before allowing it to stand for 20 seconds. The copper grid was then removed and placed on top of a drop of 2% uranyl acetate and allowed to stand for 10 seconds; this was then removed with filter paper, allowed to dry on a Petri dish, and after drying, mounted on TEM (CM12; Phillips, Eindhoven, the Netherlands) with an acceleration of 120 kV and a maximum magnification limit of crystal lattice at 660 k times.

Statistical analysis

All analyses were done in triplicate unless otherwise indicated. The data were analyzed using SPSS version 20 statistical package (IBM Corporation, Armonk, NY, USA) by one-way analysis of variance and presented as means and standard deviation (SD). The significance of the difference between comparisons was determined by Duncan's multiple range tests. The significant difference was taken to be a value of $P < 0.05$ at a 95% confidence interval.

Results and discussion

RBW policosanol yield

The yield of RBW policosanol, together with that of RBW and RBO, is as depicted in Table 1. The RBO wax content is variable, depending on many factors such as the rice cultivar, extraction method, and processing conditions.⁴² The highest RBW yield reported ranged from 8.3%–13.7%⁴³ compared with the 1%–3%, 3%–4%, and 5%–7% yields obtained by Reddi et al,⁴⁴ Cousins et al,⁴⁵ and Saunders,⁴⁶ respectively. However, in all of the studies, RBO and RBW were extracted by solvent extraction. In this study, the RBO

Table 1 Yields of RBO, RBW, and policosanol

Sample	Yield, mean (standard deviation)
RBO, g/100 g RB	12.37 (0.083)
RBW, g/100 g RBO	30.17 (0.962)
Policosanol, g/100 g RBW	10.82 (0.396)
Policosanol, (g/100 g RB)	9.81 (0.441)

Abbreviations: RB, rice bran; RBO, rice bran oil; RBW, rice bran wax.

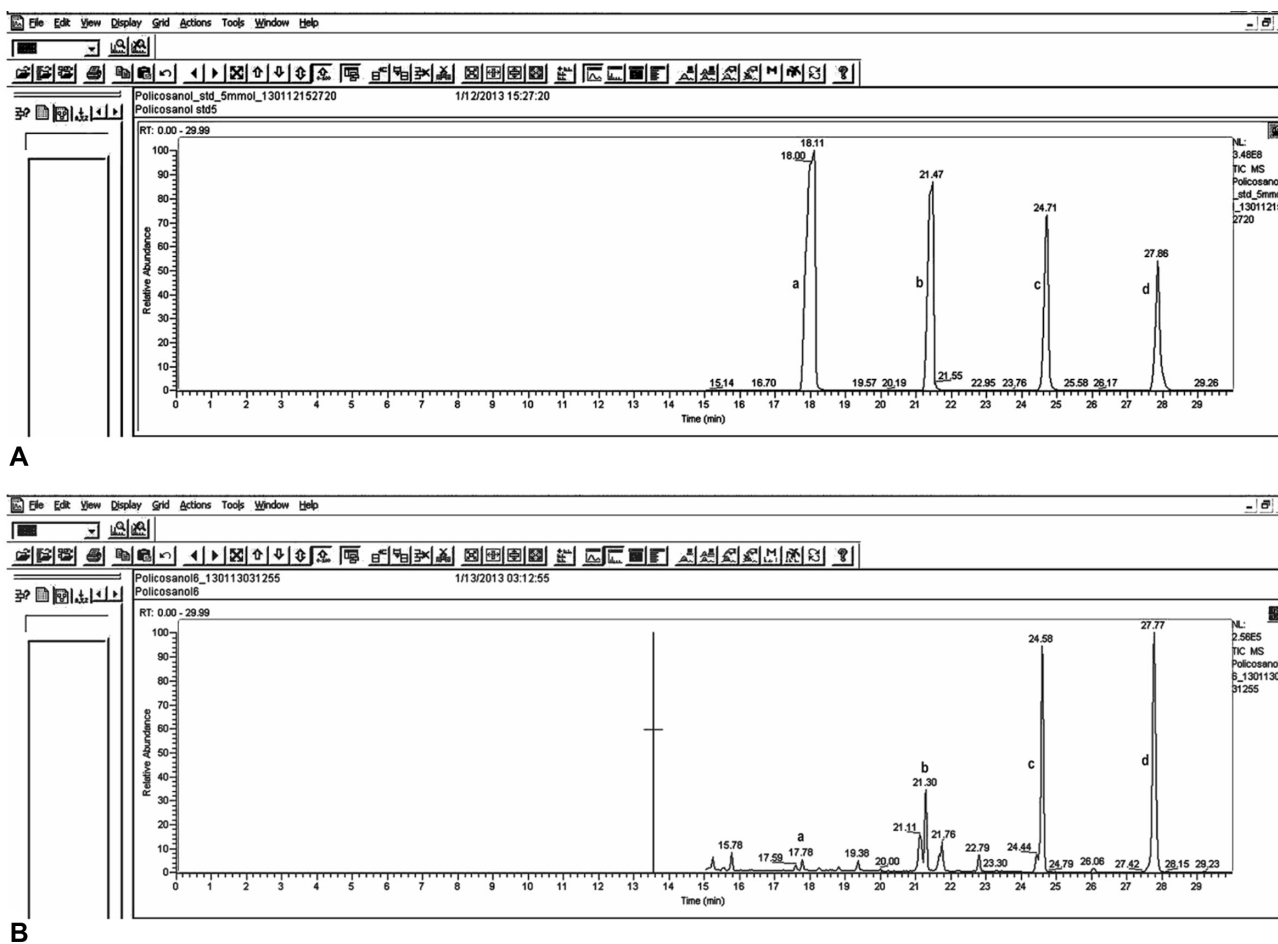


Figure 1 Chromatogram for policosanol standards (**A**), rice bran wax policosanol extract (**B**).
Notes: a, docosanol; b, tetracosanol; c, hexacosanol; d, octacosanol.

was extracted by SFE at 600 bars pressure and a temperature of 60°C, which may account for the high yield in the crude RBW. However, when the RBO was fractionated at 30°C and a pressure of 300 bars, RBW yield fell to 11.60 (0.156) (data not shown).

The yield of policosanol is also variable depending on the extraction method and source. The yield of policosanol from RBW by the high-intensity ultrasound extraction method was 8%.³⁹ In the present study, following the method of Cravotto et al³⁹ with some modification, a higher yield of policosanol was obtained (Table 1). Liu et al⁴⁷ reported that ultrasonic power is more important in the hydrolysis of RBW than ultrasonic frequency. This explains why good policosanol yield was achievable in this study even though low ultrasound frequency was used (50 Hz). However, a study by Wang et al⁴⁸ reported a maximum policosanol yield of up to 28% by saponification of RBW via the water (neutralized) extraction method. In the study, Wang et al compared different methods

of extraction of policosanol, but all the methods appeared cumbersome and time-consuming.

GC-MS policosanol content analysis

The GC-MS chromatograms of standards and extracts were as shown in Figure 1. The policosanol contents concentrations (Table 2) were calculated on the basis of the peak area of the calibration curve of each of the 4 standards used (Figure 2). Each of the components was identified according to recognition

Table 2 Policosanol composition from gas chromatography mass spectrophotometry analysis of RBW and RB extracts

Source	Policosanol content (mg/100 g)			
	Docosanol	Tetracosanol	Hexacosanol	Octacosanol
RBW	60.1 (0.00002)	36.9 (0.00544)	83.2 (0.0076)	102.9 (0.0010)
RB	60.0 (0.00003)	33.8 (0.0003)	80.5 (0.00002)	100.5 (0.00015)

Notes: Data presented as mean (standard deviation).

Abbreviations: RBW, rice bran wax; RB, rice bran.

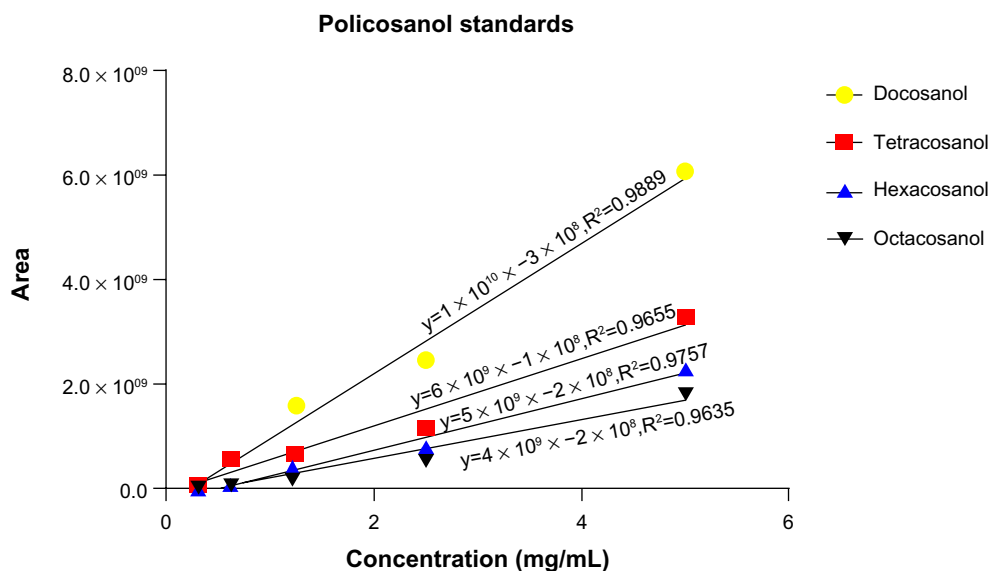


Figure 2 Standard curves for docosanol, tetracosanol, hexacosanol, and octacosanol.

of the mass fragment pattern of its trimethylsilyl derivative as the target ion in *m/z*. The target ions used were *m/z* 383 (qualifier ions, *m/z* 103, 384, 385), *m/z* 411 (qualifier ions, *m/z* 103, 412, 413), *m/z* 439 (qualifier ions, *m/z* 103, 440, 441), and *m/z* 467 (qualifier ions, *m/z* 103, 468, 469) for docosanol, tetracosanol, hexacosanol, and octacosanol, respectively.⁴⁹

The composition of policosanol, based on the chosen standards, showed a significant variation with those obtained in previous studies. For example, octacosanol content was greater than the highest value of 10.53%±0.72% obtained by dry saponification extraction of RBW.⁴⁸ Hexacosanol (29.4%) and tetracosanol (13.0%) were at higher levels than those

reported by Cravotto et al,³⁹ whereas the level of octacosanol was lower (36%) than the 46% reported in their study. All of the policosanol components in this study, docosanol, tetracosanol, hexacosanol, and octacosanol, were higher than those obtained by Asikin et al⁴⁹ for all of the sugarcane cultivars in their study.

Stability study of policosanol nanoemulsion

The particle size distribution profile of policosanol nanoemulsion was monodisperse with a unimodal peak; most of the particles fell below the size of 100 nm (Figure 3).

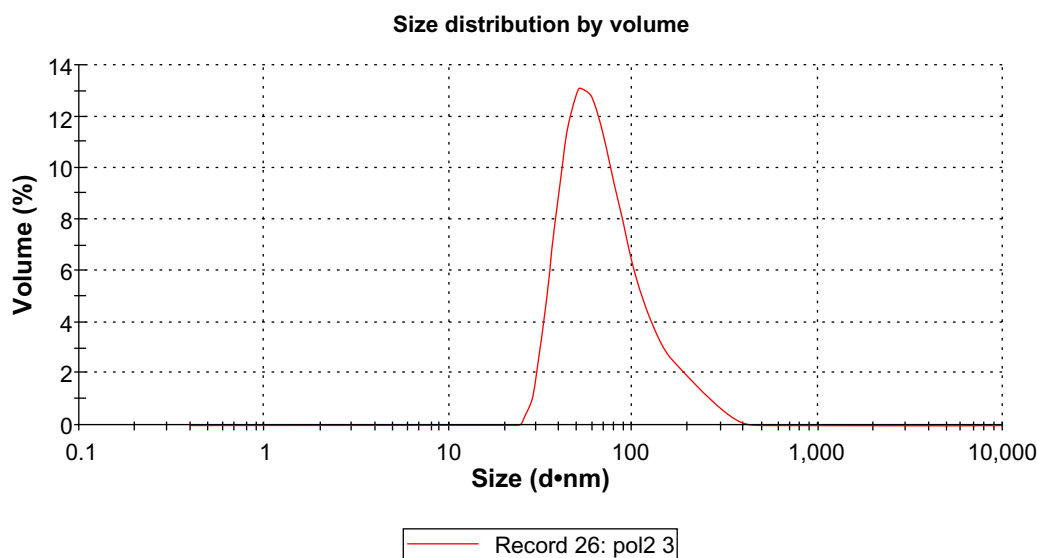


Figure 3 Particle size distribution profile by volume of policosanol nanoemulsion.

Abbreviations: Pol2, policosanol nanoemulsion sample number 2; 3, reading number 3.

Table 3 Policosanol nanoemulsion charge and size distribution over 8 weeks of storage

Storage period (weeks)	0	1	2	3	4	5	6	7	8
Size, nm	92.56 (0.127)	92.69 (0.115)	94.52 (0.189)	93.09 (0.067)	92.76 (0.078)	92.77 (0.059)	92.99 (0.049)	92.99 (0.341)	92.81 (0.055)
Zp, mV	-55.80 (1.609)	-47.1 (0.361)	-45.15 (326)	-45.12 (0.416)	-45.59 (0.639)	-45.42 (0.353)	-45.51 (0.336)	-45.54 (0.192)	-45.81 (0.291)
Polydispersity index	0.270 (0.007)	0.265 (0.003)	0.256 (0.004)	0.264 (0.009)	0.261 (0.012)	0.260 (0.010)	0.259 (0.010)	0.268 (0.025)	0.278 (0.014)
RI	1.59	1.59	1.59	1.59	1.59	1.59	1.59	1.59	1.59
pH	6.81 (0.754)	6.92 (1.04)	6.83 (0.85)	6.89 (0.89)	6.79 (1.17)	6.87 (1.10)	6.80 (0.98)	6.91 (1.07)	6.84 (0.91)

Note: Data are mean (standard deviation).

Abbreviations: Zp, zeta potential; RI, refractive index.

Droplet size is a good indicator of nanoemulsion stability. Hence, changes, especially an increase in particle size, are suggestive of low-stability emulsions. The droplet size of the policosanol nanoemulsion was stable during the 8 week monitoring period (Table 3).

The zeta potential recorded for the nanoemulsion throughout the 8 week period ranged from -55.8 to -45.12 mV (Table 3) and is within the acceptable range of -60 to -30 mV. This is a good sign because nanoemulsions tend to be more stable at large zeta potential, as the charged particles repel one another, preventing aggregate formation.⁵⁰ The initial change in zeta potential after 24 hours (Table 3) could be attributed to the change in pH, as an increase in ambient pH was associated with an increase in zeta potential.⁵¹ However, the zeta potential remained relatively unchanged despite the slight changes in pH that occurred at certain times throughout the monitoring period.

The polydispersity index of the policosanol nanoemulsion was below 0.3 throughout the 8 week period (Table 3). This clearly indicates the high fidelity of the policosanol nanoemulsion and, hence, reflects its overall stability.

The refractive index of an emulsion measures the strength of multiple lights scattered by the emulsion's particles. It gives the idea about the isotropicity of the formulation.^{37,52} Therefore, from the constant value of the refractive index throughout the period of the study (Table 3), it could be concluded that policosanol nanoemulsion was chemically stable in addition to its physical stability.

The pH value of an emulsion is an important determinant of its stability, as changes in pH suggest ongoing chemical reactions that could affect the quality of the system. The policosanol nanoemulsion had a stable pH (6.79-6.92) throughout the period of monitoring, and this pH value falls within the range of pH that is nonirritant to skin and membranes such as mucous membranes.

FTIR of policosanol nanoemulsion

The FTIR spectra of policosanol nanoemulsion, policosanol, RBO, RBW, and Tween 80 are shown in Figure 4. The absorption peak at 3,497 cm^{-1} on Tween 80 spectra is characteristic stretching vibrations of O-H (O-H group) on the Tween 80 molecule,⁵³ whereas the peak at 3,321 cm^{-1} on policosanol nanoemulsion spectra is a characteristic stretching of the O-H of primary aliphatic alcohols and water.⁵⁴⁻⁵⁷ The absorption peaks at 2,916 and 2,846 cm^{-1} on policosanol nanoemulsion, policosanol, RBO, and RBW spectra are attributable to the stretch of C-H aliphatic (group CH_3) on

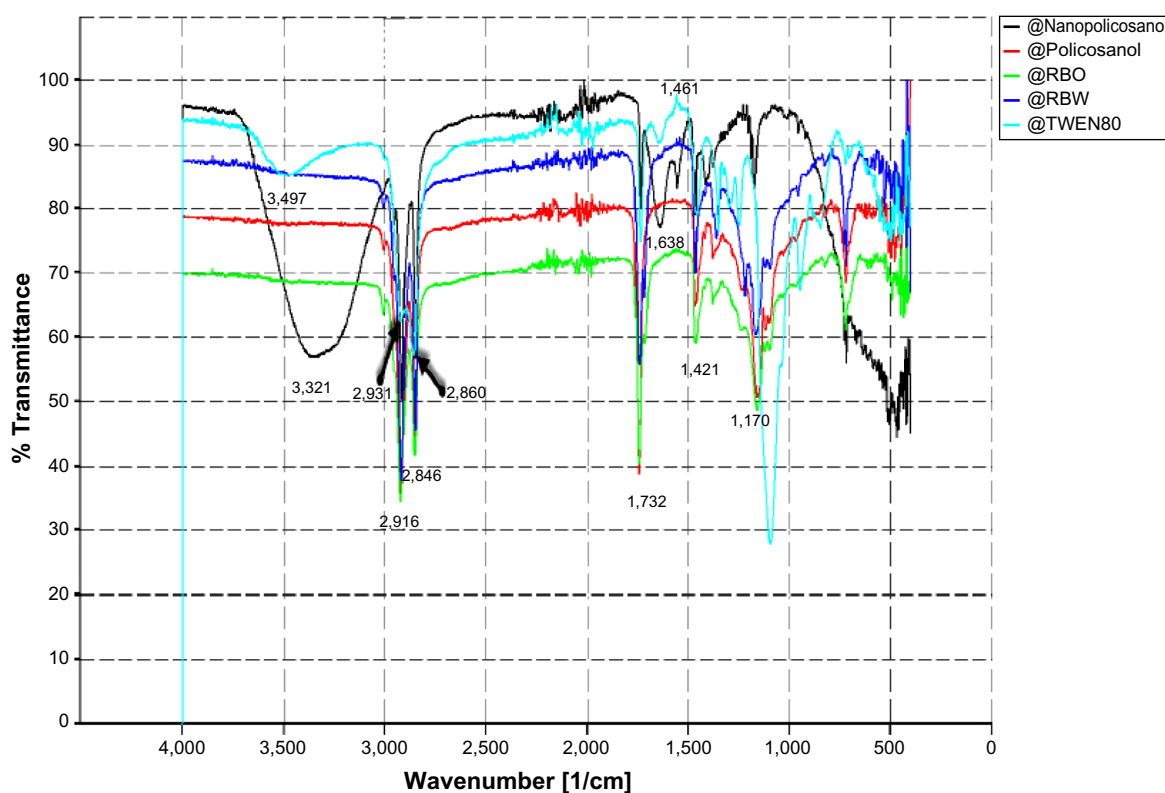


Figure 4 Fourier transform infrared spectra of policosanol nanoemulsion, policosanol, rice bran oil (RBO), rice bran wax (RBW), and Tween 80 (TWEEN80).

the policosanol primary alcohol skeleton.^{54,55} These were different from the peaks at 2,931 and 2,860 on the Tween 80 spectra, which are attributable to asymmetric and symmetric stretching vibrations of methylene ($-\text{CH}_2$).⁵³ The peak at 1,732 cm^{-1} on policosanol nanoemulsion, policosanol, RBO, and RBW spectra is a result of C=O stretching (C=O

of ester group) and appeared at 1,753 cm^{-1} on Tween 80 spectra.^{54,55,58,59} The peaks at 1,638 and 1,461 cm^{-1} appeared only on Tween 80 and policosanol nanoemulsion spectra, and C=C stretching on Tween 80 could be responsible for the 1,638 cm^{-1} peak, whereas the 1,461 cm^{-1} peak could be associated with C-H ($-\text{CH}_2$ group) bending.^{55,59} The peaks at 1,421 and 1,170 cm^{-1} are attributes of H-C-H bending ($-\text{CH}_2$ group rocking) on the policosanol structure.⁵⁹

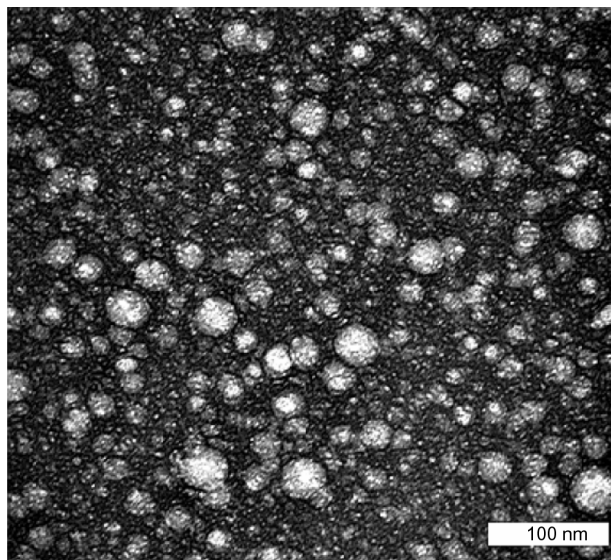


Figure 5 Transmission electron micrograph of policosanol nanoemulsion.

TEM morphology of the policosanol nanoemulsion

The TEM image of policosanol nanoemulsions is shown in Figure 5. It is apparent that the nanoparticles are spherical in shape with diameters smaller than 100 nm, which supported the zeta sizer particle size results. Moreover, most of the particles are solitary, which is suggestive of the repulsion between the particles preventing aggregation, with the exception of a few noticeable agglomerations.

Conclusion

This study confirmed that policosanol can be extracted at low-intensity ultrasound, and high-pressure homogenization is a relatively simple and effective technique for producing

policosanol nanoemulsions. The particle size and size distribution of the nanoemulsion was stable for a period of 8 weeks and remained relatively unchanged afterward in a predetermined monthly check. The policosanol nanoemulsion had good physical stability with no chemically significant changes that occurred during the storage period. Other forms of policosanol nanoparticles are worth exploring; it is possible that formulations with lower particle sizes and higher stability could be achieved. Studies are underway to determine the bioavailability and bioactivity of the formulated policosanol nanoemulsion to ascertain its application and effectiveness in comparison with policosanol in the management of ailments such as hypercholesterolemia.

Disclosure

The authors of this study report no conflicts of interest in the work.

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