Original article

THAI BULLETIN OF PHARMACEUTICAL SCIENCES
ISSN 2586-8659 (online)

OPTIMIZATION AND EVALUATION OF MICROEMULSIONS CONTAINING GRAPE SEED OIL

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ABSTRACT

Grape seed oil, obtained from grape seeds (*Vitis Vinifera* L.), is used in cosmetic products. It demonstrates antifungal, antioxidant, anti-inflammation, and antibacterial activities. Microemulsions are a type of drug delivery system that has thermodynamic stability. Grape seed oil-loaded microemulsions were prepared by mixing water, oil, surfactant, and co-surfactant in an appropriate ratio. The objectives of this study were to develop grape seed oil-loaded microemulsions and to investigate the effects of surfactants (polysorbate 80 and polysorbate 20) and co-surfactants (ethanol, isopropyl alcohol, Cetiol® HE, and glycerin) on the physicochemical properties of the microemulsions. The stability was studied for 30 days at room temperature and protected from light. In addition, the antioxidant activity of grape seed oil and microemulsion formulations was analyzed. From the pseudo-ternary phase diagram, polysorbate 80, and Cetiol® HE at a ratio of 1:2 showed the largest microemulsion area. The amount of the loaded-grape seed oil and surfactant mixtures were 5-15% and 75-85%, respectively. All formulations were transparent light-yellow emulsions showing good thermodynamic stability. Moreover, surface charges, conductivity, pH, and viscosity were desirable. After the 30 day-stability tests, all microemulsion formulations presented good thermodynamic stability with no apparent changes observed. The antioxidant activity increased as grape seed oil was added in greater quantities. Despite the fact that the size of the particle was greater than 300 nm and the PDI value was high, the findings indicated that a microemulsion system containing grape seed oil, polysorbate 80, and Cetiol® HE could be utilized with good antioxidant activity.

Keywords: grape seed oil, microemulsion, polysorbate 80, Cetiol® HE, antioxidant activity

Received: 29 October 2022; Revised: 1 December 2022; Accepted: 2 December 2022

Introduction

Grapes (*Vitis vinifera* L.) are one of the most important crop fruits in the world. Grape seeds have 8-20% oil content, which is rich in essential fatty acids. In fact, mono and polyunsaturated fatty acids account for approximately 90% of the total amount of grape seed oil. The major component of the oil includes 3-15% oleic acid, 58-78% linoleic acid, and other saturated fatty acids. Vitamins and phytosterols are the second most abundant compounds of lipophilic molecules in grape seeds. Phytosterols' biological significance originated from their antioxidant activity and impacts on cholesterol metabolism. Also, grapes contain phenols, a flavonoid with antioxidant activities, particularly in the grape seeds. Furthermore, grape seed oil contains phenolic compounds such as gallic acid, catechin, epicatechin, procyanidins, and pro-anthocyanidins, or condensed tannins, which are known for their antioxidant activity.[1] Similarly, it has gained interest for its antimicrobial properties against various viral, fungus, and microorganism strains.[2] Consequently, topical grape seed oil was found to hasten wound healing in mice, and this was thought to be because it controlled the redox-sensitive processes that promote the growth of new epithelial tissue. Interestingly, grape seed oil is a delightful skincare component that influences skin hydration. It is extremely light; therefore, it is comfortably absorbed into the skin and tends to leave no oil residue.[3]

Microemulsion is a transparent colloidal system composed of oil, surfactant, co-surfactant, and water. The oil-water interfacial tension is drastically decreased by the surfactant, which is observable at the interface between the two phases. The dispersions spontaneously emulsify and are thermodynamically stable.[4,5] In addition, it is anticipated to be used in topical drug delivery due to its sufficient solubility, low skin irritation, and high permeability.[6] The skin is an important route to deliver active substances, especially those that aim to act locally on the applied tissues. Otherwise, it can be used to deliver active molecules for systemic actions. The most crucial and necessary step in the creation of a microemulsion formulation is the ternary phase diagram. With the assistance of these diagrams, researchers can determine the composition that gives a microemulsion system and examine the effect of the weight ratio of surfactant and co-surfactant on the area where a stable microemulsion can be obtained.[7]

Previous studies have shown that microemulsions of oils, such as tea tree oil[8] and clove oil[9], have been developed with antioxidant and antimicrobial activities, whereas grape seed oil has been formed as a nanoemulsion system.[10] However, the disadvantages of a nanoemulsion are being a thermodynamically unstable colloidal dispersion[11] requiring high energy in the preparation process.[12] Herein, grape seed oil was used as the active ingredient and oil phase in the preparation of microemulsion with antioxidant activity. The effect of surfactants (polysorbate 80 and polysorbate 20) and co-surfactants (ethanol, isopropyl alcohol, Cetiol® HE, and glycerin) on the physicochemical characteristics of the microemulsion was studied. A 30-day physicochemical stability test was also carried out. Additionally, the antioxidant activity of the formulated microemulsion was assessed.

Materials and Methods

Materials

Grape seed oil was acquired from Thai-China Flavours and Fragrances Industry Co., Ltd (Phra Nakhon Si Ayutthaya, Thailand). Cetiol® HE was received from P.C. Drug Center Co., Ltd (Bangkok, Thailand). Polysorbate 80, polysorbate 20, ethanol, and isopropyl alcohol were purchased from QRëC™ (New Zealand). Glycerol was bought from Namsiang (Ho Chi Minh City, Vietnam). The reagents used in the experiment were used without further purification and were analytical grade.

Construction of pseudo-ternary phase diagrams and preparation of microemulsion

The water titration method was used to create pseudo-ternary phase diagrams at an ambient temperature. As shown in Table 1, 24 systems containing various surfactant and co-surfactant mixtures (S_{mix}) were studied using grape seed oil as the oil phase. The S_{mix} is composed of polysorbate 80 and polysorbate 20 as the surfactants, and ethanol, glycerol, isopropyl alcohol, and Cetiol® HE as co-surfactants. The ratio of S_{mix} (surfactant:co-surfactant) was varied into 1:1, 1:2, and 2:1. Oil and S_{mix} were mixed in various weight ratios of 0:10, 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1, and 10:0. These mixtures were thoroughly mixed on magnetic stirrers before being titrated with deionized water in a drop-wise manner and shaken vigorously. The water titration ceased when the clear solution changed to a turbid mixture. The amount of water added was recorded and calculated in percentages of the component in the total formulation content. The percentages of oil, S_{mix}, and water were used to construct the phase diagram of each system. From the phase diagram, formulations of grape seed oil-loaded microemulsions were selected from the microemulsion occurring area for further examinations.

Characterizations of grape seed oil-loaded microemulsion

Particle size, polydispersity index (PDI), and zeta potential (ZP) evaluation

The particle size, PDI, and ZP of the microemulsion formulations were analyzed using a Zetasizer Nano-ZS (Malvern, Worcestershire, UK) at room temperature. The sample was filled in a zetasizer cell and placed in the particle size analyzer for the measurements. The samples were measured without dilution.

Conductivity evaluation

The electrical conductivity of the microemulsion was determined using a conductivity meter (ECTestr11+, USA) at room temperature.

pH evaluation

The pH value of the formulations was measured at room temperature using a pH meter (LAQUA twin, Horiba, Japan).

Viscosity evaluation

Viscosity measurements were carried out with an LV SC4-18 spindle on a viscometer (DV2T, AMETEK Brookfield, USA) at 25°C for 60 sec. The spindle speed was fixed at 4-8 rpm for the measurements.

Thermodynamic stability test

Centrifugation (Optima L-80, Beckman Instruments, USA) at 10,000 rpm for 30 minutes at 25°C was used to assess the thermodynamic stability of the microemulsion.

Physicochemical stability test

The physicochemical stability test was performed at room temperature and protected from light for 30 days. The particle size, PDI, surface charge, conductivity, pH, viscosity, and thermodynamic stability of microemulsions were evaluated.

Antioxidant activity test

The ability of grape seed oil-loaded microemulsion to scavenge free radicals was tested using the DPPH radical scavenging assay.[9] The potential of oil to donate hydrogen atoms was determined by decolorizing a methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). In the presence of antioxidants, DPPH produces a purple color in methanol solution and falls away to colors of yellow.

Briefly, 500 µL of each sample (grape seed oil and grape seed oil-loaded microemulsion) was mixed with 500 µL of methanol to give a stock solution. The solution was then vortexed for 5 min and centrifuged at 10,000 rpm for 15 min to remove any other existing bubbles that occurred in the dissolving process. To prepare test solutions, the stock solutions were further diluted at the ratio of 1:1 and 1:10 for grape seed oil and 1:1 for the microemulsion. A solution of 80 µg/mL DPPH in methanol was prepared, and 100 µL of DPPH solution was mixed with 100 µL of the test solution. The standard curve using Trolox as the standard substance was prepared in the range of 1–25 µg/mL with a correlation coefficient of 0.999. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 515 nm. The percentage of DPPH radical scavenging activity was calculated using Equation 1:

$$\%DPPH\ radical\ scavenging\ activity = \frac{A_{control} - (A_{sample} - A_{blank})}{A_{control}} \times 100 \quad ----(1)$$

where $A_{control}$ is the absorbance of the control (DPPH with all components except grape seed oil), A_{sample} is the absorbance of the sample (sample with DPPH), and A_{blank} is the absorbance of the blank sample (sample without DPPH).

Statistical analysis

All tests were done in three replications. Data are shown as mean ± standard deviation (SD). The data were analyzed with independent T-tests and F-tests at a 95% confidence interval using Microsoft® Excel 2019 (p-value < 0.05).

Results and Discussion

Construction of pseudo-ternary phase diagrams and preparation of microemulsion

The systems constructed to create pseudo-ternary phase diagrams of grape seed oil-loaded microemulsion formulations are shown in Table 1. A microemulsion area was observed when the system was composed of polysorbate 80 as the surfactant and Cetiol® HE or glycerol as the co-surfactant at the ratios of 1:1 and 1:2.

The pseudo-ternary phase diagrams with various surfactant weight ratios are shown in Figure 1. The shaded area represents the region that provides translucent microemulsion, while the other areas give turbid mixtures representing no microemulsion formation. The system composed of polysorbate 80 and Cetiol® HE at the ratios of 1:1 and 1:2 exhibited the largest microemulsion region. Unfortunately, the 2:1 ratios did not exhibit microemulsion since there may have been insufficient amounts of co-surfactant to maintain a thermodynamically stable system. Polysorbate 80 and Cetiol® HE are nonionic surfactants which were used as an emulsifier and co-emulsifier, respectively. Microemulsions stabilized by these non-ionic compounds present a strong barrier between the internal and external phases. The character of the adsorbed surface film created by the emulsifiers is regarded as being of primary importance in determining emulsion stability. Moreover, Cetiol® HE as a co-emulsifying agent accumulates substantially at the interface layer, increasing the fluidity of the interfacial film by penetrating into the surfactant layer. The presence of co-surfactants contributes to the interfacial film with sufficient flexibility to take up different curvatures required to form microemulsions over a wide range of composition.[13]

Table 1 Systems for construction of pseudo-ternary phase diagram

System	Surfactant	Co-surfactant	Ratio of surfactant: co-surfactant
1-3	Polysorbate 80	Ethanol	1:1 1:2 2:1
4-6	Polysorbate 80	Isopropyl alcohol	1:1 1:2 2:1
7-9	Polysorbate 80	Cetiol® HE	1:1 *1:2 *2:1
10-12	Polysorbate 80	Glycerol	1:1 *1:2 *2:1
13-15	Polysorbate 20	Ethanol	1:1 1:2 2:1
16-18	Polysorbate 20	Isopropyl alcohol	1:1 1:2 2:1
19-21	Polysorbate 20	Cetiol® HE	1:1 1:2 2:1
22-24	Polysorbate 20	Glycerol	1:1 1:2 2:1

* Microemulsion area was found.

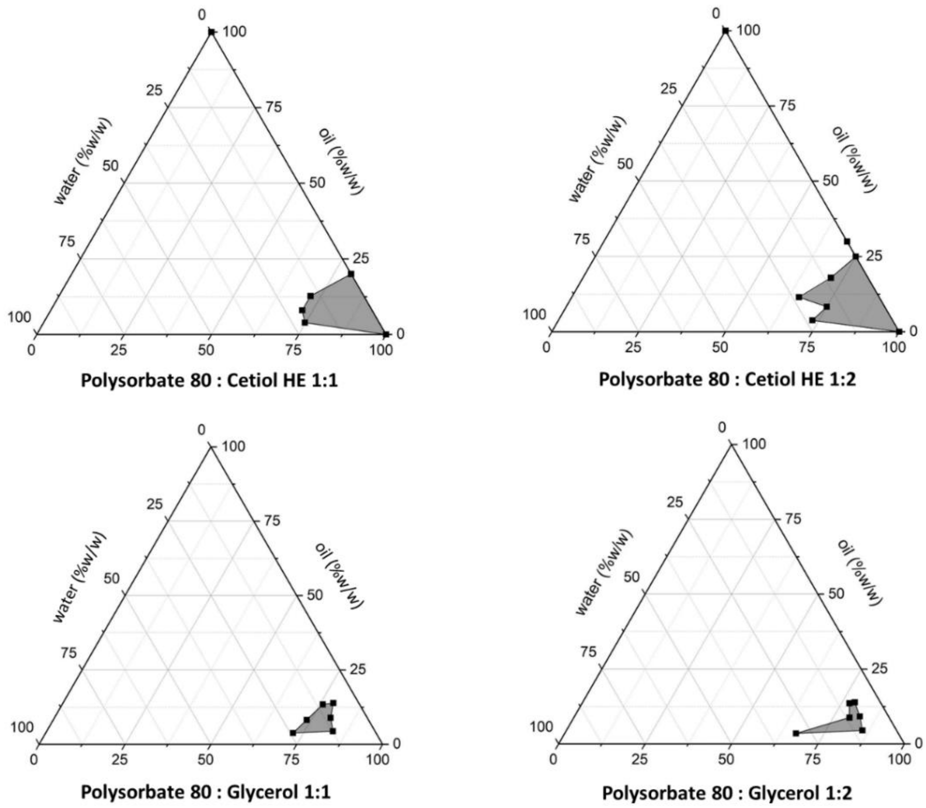

Figure 1 Pseudo-ternary phase diagrams of systems component of grape seed oil, surfactant mixtures and water

Physicochemical characteristics of grape seed oil-loaded microemulsion

Grape seed oil can be loaded into the microemulsion up to 15% while using 75–85% of S_{mix}. Grape seed oil-loaded microemulsion formulations using Cetiol® HE and glycerol as co-surfactants were prepared (Table 2). All formulations were transparent and homogeneous with pH values of 7.51 to 8.04. After centrifugation at 25°C, no phase separation was seen, indicating thermodynamic stability. The particle size ranged from 451.80 to 7,573 nm. The particle size increased because the amount of oil and S_{mix} were increased; however, the formulations were still transparent with no phase separation. This is probably due to the surfactant concentration being high enough to initially obtain full coverage of the internal phase droplets as well as the presence of free surfactant molecules.[14] Figure S1 (supplementary information) showed the size distribution pattern of Rx 6, which was a representative pattern of all formulations. In spite of the large particle size and size distribution, the major particle size was still in the nano-scale range which may be the reason that the microemulsion thermodynamic stability was achieved. Furthermore, when attractive steric forces were prominent, raising the oil content led to fewer oil molecules piercing the interfacial film, resulting in larger particles.[15] Ideally, the microemulsion is thermodynamically stable, transparent, and isotropic dispersion with particle sizes ranging from 5 to 100 nm.[16] However, the product's bioavailability was not dependent on the particle size.[17] According to a previous study, the prepared phytosterol-loaded microemulsion had particles that were larger than 300 nm.[18] PDI was more than 0.7, which means board size distribution. The zeta potential values (-0.023 to 0.054 mV) indicated that Cetiol® HE is a nonionic surfactant. The conductivity values varied between 0.60 and 6.17 µS/cm. The higher water content of the microemulsion increased its electrical conductivity. It has been previously reported that the conductivity of w/o microemulsion is likely to be lower than 10 µS/cm, and the o/w microemulsion has relatively high conductivity as compared with w/o microemulsion (about 10-100 µS/cm).[19,20] The viscosity ranged from 267.17 to 405.67 cP. The increase in the amount of oil and altered S_{mix} from 1:1 to 1:2 decreased viscosity. Due to their large particle sizes of Rx 5 and Rx 10 in Table 2, they were not chosen for the following experiment. The antioxidant activity test was performed on Rx 1-4 and Rx 6-9.

Physicochemical stability study

After 30-day storage at room temperature, the particle size, PDI, ZP, conductivity, pH, viscosity, and thermodynamic stability of the microemulsion were investigated. It was found that there were no substantial changes to any parameter after the storage. The appearance of microemulsion formulations was clear, homogeneous, and showed no sign of phase separation. Though there may be some changes in the particle size which was obvious because the data showed rather large PDI values. This may be due to the component of the grape seed oil that contains various oils and fatty acids which could form different sizes of internal phase droplets with the surfactant and co-surfactant used. The heterogeneous droplets in the formulation, however, could provide a thermodynamically stable system. This is because the content of total emulsifier and the type of emulsifier used created a strong adsorption film which acts as a barrier to maintain the stability of the microemulsions. The results of physicochemical characteristics after the 30-day stability study are shown in Table S1 (supplementary information).

Table 2 The microemulsion formulations in various concentrations of grape seed oil and surfactant mixture and their physicochemical characteristics

Rx No.	Amount (%w/w)			Physicochemical characteristics (mean ± SD), n = 3				
	Oil	S_{mix}	Water	Size (nm)	PDI	Zeta potential (mV)	Conductivity (µs/cm)	Viscosity (cP)
System: Polysorbate 80:Cetiol® HE 1:1								
1	5	80	15	646.43 ± 13.71	1.00	-0.023 ± 0.024	4.40 ± 1.87	405.67 ± 6.51
2	10	80	10	1407.67 ± 59.36	1.00	0.054 ± 0.042	2.77 ± 0.40	384.93 ± 1.60
3	15	80	5	4262.00 ± 1923.03	0.91 ± 0.16	-0.011 ± 0.049	0.60 ± 0.10	371.50 ± 3.77
4	10	75	15	756.53 ± 6.24	0.99 ± 0.02	0.000 ± 0.017	5.10 ± 0.89	388.17 ± 7.25
5	10	85	5	7321.67 ± 2946.38	0.78 ± 0.22	-0.007 ± 0.013	0.70 ± 0.97	350.47 ± 14.16
System: Polysorbate 80:Cetiol® HE 1:2								
6	5	80	15	451.80 ± 5.63	0.93 ± 0.03	0.012 ± 0.034	5.73 ± 1.29	316.03 ± 5.45
7	10	80	10	1750.33 ± 99.79	1.00	0.016 ± 0.018	2.60 ± 0.72	315.00 ± 7.50
8	15	80	5	2200.00 ± 196.64	1.00	0.023 ± 0.051	0.80 ± 0.36	267.17 ± 19.25
9	10	75	15	804.17 ± 17.43	1.00	0.001 ± 0.014	6.17 ± 1.17	297.87 ± 4.50
10	10	85	5	7573.00 ± 745.91	0.74 ± 0.08	-0.011 ± 0.031	1.00 ± 0.20	294.50 ± 4.27

Antioxidant activity test

The amount of grape seed oil in the formulation determines its antioxidant activity. This high antioxidant capacity is associated with the high content of gallic acid, catechin, epicatechin, procyanidins, and pro-anthocyanidins in grape seed and seed oil, and might be the result of the synergistic combination of these phenolic compounds.[21] The amount of S_{mix} and the ratio of surfactant and co-surfactant did not affect antioxidant activity (Figure. 2). When compared to 10 µg/mL Trolox, Rx 4, and Rx 9 showed no significant difference in %inhibition, while Rx 3 and Rx 8 showed superior antioxidant activity. The percent DPPH inhibition of grape seed oil after 10-fold dilutions did not differ substantially from 10 µg/mL Trolox. The control that contained all components in the formulations except grape seed oil exhibited slight antioxidant activity (% DPPH inhibition: 10.23 ± 0.77 to 10.45 ± 0.90) compared to the preparations shown in Figure 2. After considering both physicochemical properties and antioxidant activity data, four appropriate formulations (Rx 3, 4, 8, and 9) were recommended. The highest antioxidant activity was found in Rx 3 and Rx 8 due to the highest amount of grape seed oil in the formulations. Rx 4 and Rx 9 had lower antioxidant activity than Rx 3 and 8, but their particle size was smaller. Following the stability test, Rx 9 was determined to be the best formulation for further development.

Conclusion

Grape seed oil with antioxidant activity was formulated into microemulsion formulations. Polysorbate 80 and Cetiol® HE were selected as surfactant and co-surfactant, respectively. The appearance of grape seed oil-loaded microemulsion was a clear pale yellow homogeneous solution. The particle size depended on the amount of composition. The conductivity varied with the water content in the formulation. After the 30-day stability test, the formulation was found to be thermodynamically

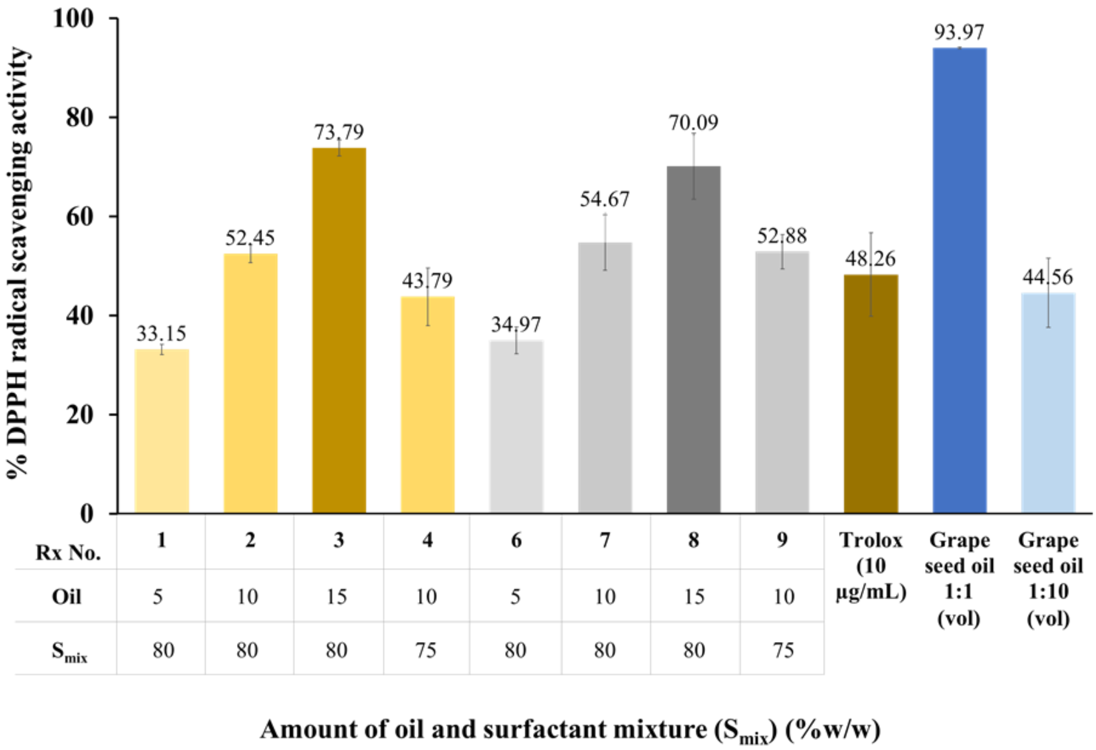

Figure 2 Antioxidant activity of grape seed oil-loaded microemulsion, grape seed oil and Trolox

stable, with no phase separation or other instability characteristics observed. The antioxidant activity of formulations depended on the amount of grape seed oil. Appropriate formulations were considered based on the physicochemical characteristics, stability, and antioxidant activity. In order to achieve a larger microemulsion area, smaller particle size, and higher loading amount of grape seed oil, more research utilizing other surfactant and co-surfactant types is required.

Acknowledgment

We would like to express our gratitude to the Faculty of Pharmacy, Silpakorn University for supporting our work.

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