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Original Article

PREPARATION AND CHARACTERIZATION OF *NIGELLA SATIVA* MICROEMULSIONS

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ABSTRACT

The aims of this study were to develop and characterize an oil-in-water (o/w) *Nigella sativa (N. sativa)* microemulsion. The microemulsions were prepared by drop-wise titration of *N. sativa* oil into mixtures of surfactant blends (Span 20, Span 80, Tween 20, Tween 80, Tween 85) and water. All transparent ternary mixtures were characterized for their viscosity and droplet size. The stability of the microemulsion was evaluated by subjecting them to stressful conditions, namely centrifugation (2000 g for 20 minutes) and heating in a drying oven (60 °C to 105 °C for 5 hours) and the droplet size was determined following one month storage at room temperature (25 °C) thereafter. Based on the results, a phase diagram was constructed from corresponding volumes of those 3 components. *N. sativa* mixtures (ranging from 7.4% to 10.7%) prepared at HLB 16 of surfactant blends (Tween 20: Tween 80; 6:4) with water (ranging from 17.9% to 18.5%) yielded transparent liquids. The constructed phase diagram displayed regions of a few types of microemulsion and emulsion. Interestingly, droplet size of freshly prepared mixtures was wider in range (5 to 15.6 nm) than the size following stressful condition (11.3 to 12.4 nm). It was concluded that *N. sativa* oil could be formulated into microemulsion at specific HLB value of surfactant blends. Such system was envisaged to enable routine rapid *in vitro* test on neuron cell lines loaded with *N. sativa* oil or possibly other lipophilic materials whenever viewing of neurite extension is required.

Keywords: *Nigella sativa*, Microemulsions, Sorbitan-based surfactants, Phase diagram

INTRODUCTION

Nigella sativa L. *(N. sativa)* from Ranunculaceae family is a plant that is usually found in the Mediterranean area, the desert of the Middle East, the South Asia, and the Far East. The oil has been used to treat human illnesses since Pharaoh's era and is often mentioned in some of the oldest religious and medical manuscripts. *N. sativa* was selected as the model lipophilic agent in this study to evaluate its potential as neuro-regenerative agent. It was reported earlier that *N. sativa* carries numerous therapeutic effects including as antioxidant, anti-diabetic and anti-inflammatory [1]. Active constituents that thought to be responsible for its multiple therapeutic properties include several fatty acids, nigellicine, arvacrol, thymoquinone, tannins, essential amino acids, and oxy-coumarin [2]. Studies also had been done on the main active constituent of *N. sativa,* thymoquinone and it showed potential pharmacological effect on pathologies implicating neuro-degeneration such as cerebral ischemia [2]. In a study done by Kanter [3] on sciatic nerves in experimental diabetic neuropathy, it showed that there were fewer morphologic alterations had occurred to the drug-induced diabetic rats after treatment with thymoquinone and especially *N. sativa*. Myelin breakdown had significantly reduced and ultra structural features of axon had remarkably improved. In another separate study, it was also found that thymoquinone was effective in preventing hippocampal neuro-degenerative in rats exposed to toluene [4].

However, it is not feasible to directly test the *N. sativa* oil on cell line due to its immiscibility with the aqueous media. This makes rapid screening for therapeutic activity impossible. Although an emulsion system of the oily agent of interest can be formulated to improve its miscibility with the aqueous media, its high turbidity makes viewing under the microscope difficult. Formulation of a clear and transparent liquid form of *N. sativa* is required particularly in the study of its ability to induce neurite extension on the neuron cell line. A microemulsion was proposed to be the best form of the oily agent for the purpose.

Microemulsions have been extensively used to improve solubility of poorly water-soluble materials [5]. It is defined as the dispersion of oil and water that requires stabilization by surfactants to impart a thermodynamically stable system [6]. The microemulsions can be spontaneously formed, but at a narrow range of concentration of the three components. The size of microemulsion must be below 100 nm, which is significantly smaller than emulsions and must be accompanied by a transparent characteristic [7]. Microemulsion can be further categorized into three types; Type I (oil-in-water microemulsion); Type II (water-in-oil microemulsion) and Type III (bicontinuous microemulsion) [8]. All these can be distinguished based on the nature of the dispersed phase and the dispersion medium of the microemulsion. A co-surfactant free of oil-in-water (o/w) microemulsion could be employed using a mixture of nonionic surfactants [9], in which the hydrophilic surfactants (Tween 80, 60, 40 and 20) were combined to the hydrophobic surfactants (Span 80, 60, 40 and 20). In this study, sorbitan-based surfactants with different combination were tested in the preparation of sorbitan-based *N. sativa* microemulsions.

MATERIALS AND METHODS

N. sativa oil, imported from Karachi, Pakistan was supplied by Al-Mustafa Enterprises (Brand Name: Hemani). Surfactants namely Span 20 (HLB = 8.6), Span 80 (HLB = 4.3), Tween 20 (HLB = 16.7), Tween 80 (HLB = 15), Tween 85 (HLB = 11) were purchased from Merck (Germany). Water of deionized grade was used.

Preparation of *N. sativa* microemulsions

Briefly, surfactants were prepared by blending Span 80, Tween 80, Span 20, Tween 85 and Tween 20 at pre-determined HLB values (Figure 1). Spontaneous emulsification was then applied by mixing deionized water with appropriate surfactant blends at different ratios (Table 1) using magnetic stirrer for 10 minutes at 640 rpm. After that, the *N. sativa* oil was titrated drop-wise until a transparent solution was formed. Only formulation that appeared transparent was further characterized and subjected to stress-testing.

Determination of Droplet Size

The size analysis was conducted for fresh microemulsion and following storage at either stressful or at normal condition (25°C).

The droplet size of the microemulsions was analyzed by Zetasizer Nano series ((Malvern Instrument Ltd., United Kingdom)) by briefly loading 1.5 x 10^{-3} L of individual microemulsion into a polystyrene cuvette. The reading was obtained at 173° detection angle. The droplet size was expressed as the volume mean diameter of triplicates.

Stress-testing and Characterization of the Microemulsions

Method previously employed by Cho et al. [9] was adopted. Briefly, the microemulsions were subjected to extreme conditions by centrifuging them at 2000g for 20 minutes and heated at 60°C to 105°C for 5 hours in a drying oven (Memmert, Germany). The microemulsions were observed by naked eyes for any phase separation following the stressful conditions. Apart from particle sizing, the zeta potential of the microemulsion was also measured. The measurement of the zeta potential was done using Malvern ZetaSizer Nanoseries Z (Malvern Instrument Ltd., United Kingdom), in which 1.5 x 10^{-3} L microemulsions were loaded into a clear disposable zeta cell and the reading was reported as mean ± SD.

Construction of Phase Diagram

The ternary diagram was plotted for evaluation by Triangular Diagram plotting Spreadsheet (TRI-PLOT, United Kingdom).

Statistical Analysis

Statistical analysis was performed where all data were represented as the mean value of triplicate samples. One-Way ANOVA (Minitab 16, USA) was used to determine the significant difference ($p < 0.05$) between the mean values.

RESULTS AND DISCUSSION

Formulation of *N. sativa* Microemulsions

Microemulsion needs a proper HLB value because it will indicate how the surfactant will behave in the solution. Hence, to manipulate the HLB value, combination of two surfactants is used. In order to ensure the presence of adequate number of surfactant molecules to stabilize the nanodroplets in the system, an optimum concentration of surfactants are used [10]. This is important to promote the formation of microemulsions within the range of 100 nm. Also, microemulsions will form under the influence of two factors; interfacial layer and interfacial tension [11].

Additionally, in the free dispersion energy, the droplet size of microemulsion systems is usually minimally equilibrated at 100 – 1000Å of change [7]. For this reason, an optimum surfactant concentration is required in accommodating and in consideration of these interfacial areas. The most important outcome in this study is in the production of flexible layer and a very low tension at the interface of water/oil of the microemulsions.

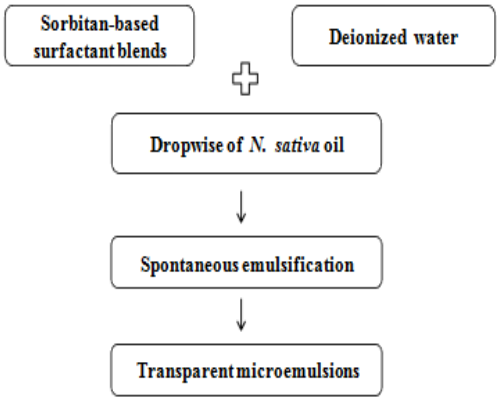

Fig. 1: A flowchart to describe preparation of sorbitan-based *N. sativa* microemulsions

Table 1: Preparations of 16 formulations made up of various surfactant types, blends and different HLB. Abbreviation used: F = Formulation; W = water; O = oil, S = surfactant; S80 = Span80; S20 = Span20; T20 = Tween20; T80 = Tween80; T85 = Tween85

F	Percentage (%)					Surfactant Blends	HLB
	W: S	(W: S): O	W	S	O		
1	-	-	66.7		33.3	-	-
2	-	-	33.3		66.7	-	-
3	-	-	8.3	16.7	75.0	83% S80 / 17% T85	5.5
4	-	-	16.1	64.5	19.4	68% S80 / 15% S20 / 17% T20	7
5	18.2:81.8	25:75	-	-	-	68% S80 / 15% S20 / 17% T20	7
6	16.7:83.3	25:75	-	-	-	28% S80 / 72% T85	9
7	-	-	14.3	71.4	14.3	28% S80 / 72% T85	9
8	-	-	4.8	23.8	71.4	60% T20 / 40% T85	14
9	-	-	27.8	55.5	16.7	60% T20 / 40% T85	14
10	-	-	12.7	75.9	11.4	60% T20 / 40% T85	14
11	-	-	25.0	60.0	15.0	60% T20 / 40% T85	14
12	-	-	17.2	69.0	13.8	60% T20 / 40% T80	16
13	-	-	22.0	65.0	13.0	60% T20 / 40% T80	16
14	-	-	17.9	71.4	10.7	60% T20 / 40% T80	16
15	-	-	18.2	72.7	9.1	60% T20 / 40% T80	16
16	-	-	18.5	74.1	7.4	60% T20 / 40% T80	16

Referring to (Table 2), formulation 1 and 2 were used as the controls in this experiment. This was because; to form a microemulsion, a sufficient amount of surfactants were needed in the formulation. As formulation 1 and 2 lack in the surfactant, no microemulsions should form in the formulae.

Type II microemulsions were prepared using surfactant blends with HLB values of 5.5 and 7, resulted in formulation 3 and 4. However, these formulations failed to produce microemulsions as they appeared turbid. This implied that *N. sativa* oil was incompatible with the surfactant blends' chain length [12]. The results suggested that fatty acid compositions in *N. sativa* oil interacted differently in a ternary phase constituted of surfactants, water and oil.

Formulation 5 (HLB 7) and 6 (HLB 9), a modified formulation adopted from a study by Rukmini et al. [13] were also fabricated. They were of Type II and Type I microemulsion systems, respectively. However, these also did not produce microemulsions, probably because the oil type and surfactant blends used in this study was different than that of Rukhmini's.

As the theoretical HLB values of 8 to 16 from the pseudoternary graph were indicative of a suitable oil-in-water microemulsion system, hence the formulations 6 to 16 were prepared. Type I of microemulsion system was prioritized in this project, as the aqueous phase must be created in order to give a whole homogenized system when loading into the cell lines. These medium HLB values also

indicated that a more stable oil-in-water microemulsion might be formed [14]. Therefore, at the very first place in this experiment, formulation 12 using combined surfactants of HLB 16 (60% Tween 20 and 40% Tween 80) successfully produced transparent solution. Following this result, the formulation 13 (HLB 16), 14 (HLB 16), 15 (HLB 16) and 16 (HLB 16) were then generated with little modification in terms of the oil concentrations and the volume of surfactant blends. However, of all the transparent solutions produced, only formulation 14, 15 and 16 exhibited nano range size (Figure 2).

Table 2: HLB values of sorbitan-based *N. sativa* micro emulsions. Abbreviation used: F = Formulation; W = water; O = oil, S = surfactant; S80 = Span80; S20 = Span20; T20 = Tween20; T80 = Tween80; T85 = Tween85

F	Surfactant Blends	HLB Value	Observation
1	-	-	Cloudy
2	-	-	Cloudy
3	83% S80 / 17% T85	5.5	Cloudy
4	68% S80 / 15% S20 / 17% T20	7	Cloudy
5	68% S80 / 15% S20 / 17% T20	7	Slightly cloudy
6	28% S80 / 72% T85	9	Cloudy
7	28% S80 / 72% T85	9	Cloudy
8	60% T20 / 40% T85	14	Cloudy
9	60% T20 / 40% T85	14	Slightly cloudy
10	60% T20 / 40% T85	14	Slightly cloudy
11	60% T20 / 40% T85	14	Translucent
12	60% T20 / 40% T80	16	Transparent
13	60% T20 / 40% T80	16	Transparent
14	60% T20 / 40% T80	16	Transparent
15	60% T20 / 40% T80	16	Transparent
16	60% T20 / 40% T80	16	Transparent

Formulation 14 Formulation 15 Formulation 16

Fig. 2: Physical appearance of successful sorbitan-based *N. sativa* microemulsions

In this experiment, different concentrations of the *N. sativa* oil have been formulated using different HLB value of the surfactant blends. It was shown that at HLB 16, a high concentration of the *N. sativa* oil would produce a cloudy solution. Hence, the microemulsion systems were achieved when oil concentrations were reduced in corresponding to the decrement in the surfactant blends concentration to the lowest concentration possible.

Droplet Size of *N. sativa* Microemulsions

In measuring the particle size of the microemulsions, measurement was done only to trial preparations with transparent appearance as this characteristic gave a high possibility of the microemulsion production. Therefore, only formulations 11 to 16 were tested. The result showed that only formulation 14, 15 and 16 could be defined as microemulsion (Table 3) as they exhibited less than 100 nm range of particle size. The result One-Way ANOVA posthoc Tukey's test multiple comparison also showed that these three formulations were significantly different ($p < 0.05$) having 97.50% of individual confidence level.

Table 3: Physical characteristic of sorbitan-based *N. sativa* microemulsions

Formulation	Percentage (%)			Mean Particle Size before Storage ± SD (nm)	Mean Particle Size after Storage ± SD (nm)
	Water	Surfactant	Oil		
14	17.9	71.4	10.7	4.99 ± 0.89	12.39 ± 0.17
15	18.2	72.7	9.1	8.06 ± 1.84	11.43 ± 0.19
16	18.5	74.1	7.4	15.62 ± 2.3	11.31 ± 0.08

Abbreviation used: F = Formulation; SD = Standard deviation

These formulations remained transparent with no turbidity after being subjected to vortex mixer. In contrast, formulations 11, 12 and 13 were not termed microemulsions despite their translucent/ transparent appearance by naked eyes observations as their droplets size was not in the nanodiameter region.

Stress-testing and Characterization of the Microemulsions

Some of the characteristics of a stable microemulsion are (a) it does not give phase separation, (b) it forms spontaneously at room temperature and (c) it requires very little energy as compared to conventional emulsions. Hence, to check for the thermodynamic stability of *N. sativa* formulations, the following stress-testings were conducted (Table 4).

For stability study of the microemulsion systems, the zeta potential measurements were taken, in which for formulation 14, the reading obtained was -35.4 ± 1.20 mV (Figure 3), followed by formulation 15 having -32.27 ± 1.20 mV (Figure 4) and formulation 16 with -32.03 ± 2.00 mV (Figure 5). The high value of these readings indicated that microemulsions have excellent dispersion stability. Also, this showed that the systems remained stable upon storage, which was later confirmed by turbidity observation following storage for a month at room temperature.

Table 4: The stress-testing observation for phase separation of sorbitan-based *N. sativa* microemulsions

Formulation	Centrifugation	Heating
14	No separation	No separation
15	No separation	No separation
16	No separation	No separation

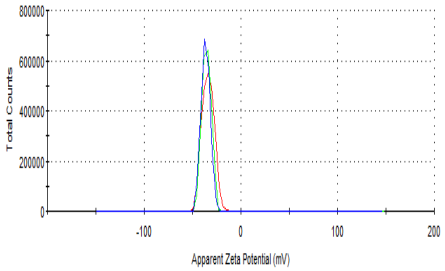

Fig. 3: The triplicate measurement of the zeta potential of formulation 14

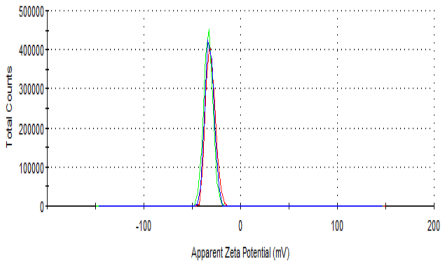

Fig. 4: The triplicate measurement of the zeta potential of formulation 15

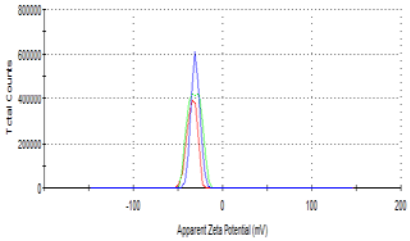

Fig. 5: The triplicate measurement of the zeta potential of formulation 16

The stability of the microemulsions can be evaluated via the turbidity changes [2]. Turbidity is parallel to the mean diameter of the particles, thus giving it the most common method in measuring microemulsions stability. The turbidity of formulation 14, 15 and 16 was negative, indicating that the microemulsions remained stable following exposure to stressful conditions. The microemulsions were also monitored for one month storage at room temperature.

There was no significant change in the appearance of the microemulsion, and turbidity result was negative, even after the stored microemulsions were heated and centrifuged (Table 5). This might happen due to the behaviour of the polysorbate chain [10], in which any change in the turbidity of the microemulsion system was probably affected by the changes in the size of the head-group of the surfactant systems [15].

Table 5: The stability study of sorbitan-based *N. sativa* microemulsions

Formulation	Storage	Centrifugation after Storage	Heating after Storage
14	Clear	Clear / No separation	Clear / No separation
15	Clear	Clear / No separation	Clear / No separation
16	Clear	Clear / No separation	Clear / No separation

Phase behavior of *N. sativa* microemulsions

The components of microemulsion structure can be explained through a pseudoternary phase diagram. In high concentration of oil, the water molecules solubilized-reverse micelles can be produced by the surfactant in its hydrophilic portion [10]. Lack of water will result in the change from a clear to turbid emulsions. In contrast, continuous addition of water produces water-in-oil microemulsions in which the interfacial layer of the combined surfactants will contain stable water droplets. More addition of water will result in a translucent region in which the water is shoved between double layers of surfactants. However, if this continues, the lamellar structure will break down, thus forming oil-in-water microemulsions, where surfactants stabilized-oil droplets become the continuous phase in the system.

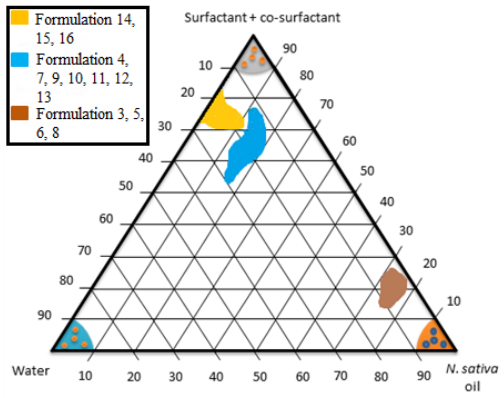

Fig. 6: Pseudoternary diagram of sorbitan-based *N. sativa* microemulsions

In order to determine the phase behaviour of the microemulsions, Triangular Diagram plotting Spreadsheet (TRI-PLOT, United Kingdom) was used to plot pseudoternary diagram using formulation 3 to 16. Formation of these microemulsion systems were explained in a pseudoternary phase diagram and their regions were observed (Figure 6). Formulation 3, 5, 6 and 8 were located at the lower right of the diagram, indicated that they were of the macroemulsion systems.

On the other hand, formulation 4, 7, 9, 10, 11, 12 and 13 were scattered at the upper middle of the diagram, suggesting the formation of the liquid crystal percolate or the bicontinuous structure of the solution systems [7]. In addition, formulation 14, 15 and 16 were closely spotted near to the upper left of the phase region, indicating that these formulations were oil-in-water microemulsions and corresponding to the hypothetical phase pseudoternary diagram [7].

In order to retain the good solubilizing effect of microemulsion, it must therefore be formulated accordingly. The most important one is the prudent selection of surfactants to obtain an ultralow interfacial tension (< 10^{-3}mN/m) at the oil/water interface [7]. By monitoring parameters like concentration, structure and temperature, the surfactant could act as the building block to form aggregates of molecules. The chemical structure of surfactants must be considered as the microemulsion formation was highly influenced by the compatibility of the oil and surfactant chain length [12, 16]. Commonly, microemulsions contain high quantity of surfactants and hence have the propensity to be high in toxicity. As far as the safety usage of the surfactants is concerned, polysorbate is known to be safe for the human usage, as these surfactants are of the nonionic type. According to U. S. Food and Drug Administration (FDA); 10mg/kg body weight per day was considered as the acceptable daily intake (ADI) for 4 known surfactants Tweens; Tween 20, Tween 60, Tween 65 and Tween 80. For this reason, these surfactants are applied widely in the pharmaceutical industry,

food as well as in cosmetics. They are inexpensive and most importantly, minimally toxic to living organisms [17]. In addition, there have been no occurrences of genotoxicity and carcinogenicity as reported by Japan Food Additives Commission [18]. Study also showed that taking 2g of Tween 20 for 3 times per day in one week gave no adverse effect of the human body system [18]. The highest amount of surfactant used in this study only 2.4g in total, fell within allowable safe limit and therefore, these microemulsions could be concluded as safe and non-toxic for human purposes. In this study, the method of using several surfactants in combination was employed, as combined use of surfactants has a few advantages. Among others, this can increase the stability properties, improve the loading of the bioactive compound and allow use of smaller size particle with good self-emulsifying properties [19]. Some co-surfactants such as phenol and ethanol are not used due to their toxicity and irritating potential, caused by the short and medium length chains of the alcohols [15]. They are also avoided as they can result in the partition in the continuous phase [20] and hence disrupt microemulsion formation.

CONCLUSION

Stable sorbitan-based *N. Sativa* oil-in-water microemulsions had been successfully produced by appropriate blending of surfactant types with *N. sativa* oil and water. The findings also suggested that range of HLB ratio for similar surfactant groups could be utilized as guideline to find correct ratio between the ternary components. The correct formulation of *N. sativa* microemulsion is very important to allow use *in vitro* study where it may influence the direction of studies involving *N. sativa.*

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