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Original Article

FORMULATION AND STABILITY TESTING OF GENTAMICIN-*N. SATIVA* FUSION EMULSIONS FOR OSTEO-HEALING APPLICATION

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ABSTRACT

An alternative osteo-healing formulation with osteo-healing properties was formulated by combining gentamicin and *Nigella sativa* (*N. sativa*) oil in an emulsion to reduce gentamicin toxicity effect over prolonged use in osteo-infection treatment. This work aims to test the aqueous solubility and physicochemical properties of the emulsion. Four emulsions (emulsion A, B, C and D) had been formulated, with final concentration of gentamicin was made constant at 0.1% (w/v) whereas *N. sativa* oil concentration was varied between 32.5% (v/v) to 46.4% (v/v) in all formulations. Then, stability studies of all emulsion were performed by centrifugation at (5000rpm, 5 minutes), at different storage conditions (8°C, 25°C and 50°C), organoleptic characteristics, freeze-thawcycle, pH determination, particle size measurement, zeta-potential analysis, and pH titration analysis. Results showed no phase separation after centrifugation for freshly prepared emulsions. Storage at 8°C, all emulsions also showed no phase separation at all-time points. At 25°C storage condition, three formulations were stable at day 7 but phase separation was formed in all emulsions by day 14showed good stability at day 7 and all emulsions formed phase separation at day 14. No emulsions were stable in storage temperature of 50°C. The particle size of the emulsions increased with an increment of*N. sativa* oil concentration. Zeta-potential analysis showed a range of -32.2 ± 0.15 mV to -48.0 ± 0.45 mV. When pH titration analysis was performed, the zeta potential indicated that the emulsion stability was affected by acidic conditions. We concluded that the use of gentamicin-*N. Sativaemulsions* must take into account the storage condition with preference of low temperature and fresh preparation at higher alkalinity and the lowest possibility of *N. sativa* oil.

Keywords: Gentamicin, *Nigellasativa*, Emulsion, Stability, Osteo-healing.

INTRODUCTION

The usage of gentamicin as a treatment for musculoskeletal infection locally or systemically has become increasingly popular. Local antibiotic delivery system has been used in the treatment of bone and tissue infection, either to supplement or to replace the use of systemic antibiotics [1]. However, antibiotic treatment in patients with poor vascularise infected tissues and osteonecrosis may be inadequate or ineffective. A long-term course of antibiotic therapy is a must but there is the side effect or toxicity over these prolonged therapies [1,2]. Therefore, gentamicin and *Nigella sativa* (*N. sativa*) oil was fused together to lower the toxic effect of gentamicin. *N. sativa* is known as black seed or black cumin and had been used in herbal medicine all over the world for the treatment and prevention of diseases and conditions [3,4,5] such as, decreases DNA damage, prevents initiation of carcinogenesis in colonic tissue [4] and effect against osteoporosis [6]. *N. sativa* has an active compound named thymoquinone, which has antioxidant activity and was derived from the fatty acid constituents present in the seeds. It is believed that the wound healing process due to thymoquinone may be effective in accelerating new bone formation due to their cytoprotective and antioxidant actions, and effect on some mediator of inflammation [7,8,9,10]. In the case of osteoporosis, it was reported that the active compounds of *N. sativa* would improve the well-being of the patients[11].

In pharmaceutical practice, product stability is defined as maintenance of its key features and function through time. Many product property parameters, like its manufacturing process, material bottle packaging, transport and environmental conditions, conditions, and all can influence in its stability[12]. In product development, studies performed to achieve a stable, secure and effective product. Changes can occur in pharmaceutical products can be caused by external factors or related to the nature of the formulation (intrinsic). Time, oxygen, temperature, microorganisms, light, and vibration, and the reactions caused by these factors are categorized as extrinsic factors. On the other hand, intrinsic factors are related to ingredients interactions or to the conditioning material, such as redox reactions, hydrolysis, pH changes, particle size and many more[12,13]. The emulsion stability analysis provides information that indicates the relative product stability in different conditions. However, stability assessments are always relative, because product properties vary with time and depend on various factors. Thus, throughout this study, organoleptic characteristic observation, centrifugation test, freeze-thaw cycle, pH determination, particle size measurement, zeta-potential analysis and pH titration analysis were done to determine stability of emulsions.

MATERIALS AND METHODS

Materials

All the chemicals used in this study were of analytical grade; gentamicin sulphate powder purchased from local pharmacy, *N. sativa* oil purchased from Hemani Trading, Pakistan, sorbitan monolaurate (Span®20) purchased from Sigma (Sigma-Aldrich Co., USA),and PEG-20 sorbitan monolaurate (Tween®20). Distilled water was used for the preparation of emulsions.

Methods

Emulsification process

Gentamicin-*N. sativa* emulsions were formulated (Emulsion A, B, C &D) (Figure 1). Tween®20 and Span®20 were used as surfactant and co-surfactant respectively. Concentration of gentamicin was made constant at 0.1% (w/v) whereas the final concentration of *N. sativa* was varied between 32.5% (v/v) to 46.4% (v/v) in all formulations

(Table 1). During the preparation of the emulsions, the solution was agitated slowly using a magnetic stirrer (Daihan Labtech, India) at a speed of 1500rpm. Gentamicin sulphate powder was diluted in distilled water, followed by adding Tween®20and Span®20, as surfactant and co-surfactant. *N. sativa* oil, then added and stirred thoroughly for 5 minutes (1500rpm). The emulsions were homogenised using T10 basic Ultra-Turrax® (Germany) homogeniser for 5 minutes at 10 000rpm.

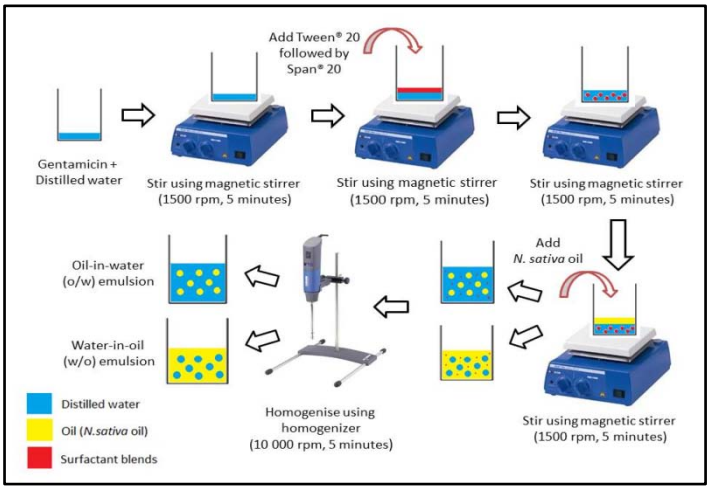

Fig. 1: Schematic diagram of gentamicin-*N. sativa* emulsion formulation (Emulsion A, B, C and D).

Stability tests

The stability tests were performed at different storage conditions for gentamicin-*N. sativa* emulsions. The samples were kept at 8 ± 0.2°C (in refrigerator), 25 ± 0.3°C (in temperature-controlled room) and 50 ± 0.2°C (in oven) under different durations; day 0, 7, 14 and 30 respectively. The samples were then examined for their stability by properties of their organoleptic characteristics, centrifugation tests, particle size measurements, and pH readings.

Organoleptic characteristics

Stable formulation of gentamicin-*N. sativa* emulsions were prepared freshly and investigated organoleptically through the properties of colour, odour, texture and phase separation of the samples. The organoleptic characteristics of the emulsions kept in distinct storage conditions were observed and recorded at various intervals of day 0, 7, 14 and 30.

Centrifugation test

Centrifugation tests were performed for the gentamicin-*N. sativa* emulsions immediately after preparation. The same test was repeated after 7, 14 and 30 days of preparation. Centrifugation conditions were 25°C and 5000 rpm (5 minutes).

Freeze-thaw cycle

Three test samples and a control sample of gentamicin-*N. Sativa* emulsions were prepared in four separate micro-centrifuge tubes. Initial observations were made for all samples. The test samples were placed in a freezer (-20°C) for 24hours and then removed to be allowed to thaw at room temperature for 24 hours. The test samples were then put into an oven with temperature 50°C and left for 24hours.

The test samples were then removed and equilibrated to room temperature for 24 hours. End observations were recorded based on the notability for any signs of phase separation in the test samples. This completes one cycle for the freeze-thaw cycle test. Another two cycles were repeated on the test samples to attain a good degree of confidence in the stability of the emulsions[19].

pH determination

Initial pH values of freshly prepared *gentamicin-N. sativa* emulsions were measured with a calibrated digital pH meter. The pH values of the emulsions kept at different storage conditions were also measured after each interval of day 0, 7, 14 and 30. Electrode of the digital pH meter was immersed directly into the emulsion during measurement. Triplicates were done in all pH measurements and the mean readings were recorded.

Particle size measurement

ZEN1600 Nano Particle Size Analyzer (Malvern Instruments, UK) was used to measure particle size of gentamicin-*N. sativa* emulsions. Ratio 1:1000 of the sample diluted with distilled water. The measurements of the particle size were done in triplicates and the mean results were recorded.

Zeta-potential analysis

The measurement of zeta-potential was measured at 25°C by using Malvern Zetasizer 4 (Malvern Instruments, UK) following 1:1000dilutions in distilled water. The measurements of zeta-potential were done in triplicates and the mean results were recorded.

pH titration analysis

The measurement of pH titration was measured at 25°C by using Malvern Zetasizer 4 (Malvern Instruments, UK) following 1:1000dilutions in distilled water. Measurements of pH titration were done in triplicates and the mean results were recorded.

Table 1: Gentamicin-*N. sativa* emulsion formulations (A, B, C & D denotes different concentration of *N. sativa* oil).

Formulation	Composition (% v/v)				Gentamicin (% w/v)	Observable Phase Separation
	Distilled Water	*N. sativa* Oil	Tween 20	Span 20		
A	38.9	32.5	23.4	5.2	0.1	-
B	37.5	35.0	22.5	5.0	0.1	-
C	34.5	40.2	20.7	4.6	0.1	-
D	30.9	46.4	18.6	4.1	0.1	-

Table 2: Effect of centrifugation of gentamicin-*N. sativa* emulsions (Emulsion A, B, C & D) stored at different storage conditions, observed at day 0, 7, 14 and 30.

	Emulsion A				Emulsion B				Emulsion C				Emulsion D			
Time	Day 0	Day7	Day 14	Day 30	Day 0	Day 7	Day 14	Day 30	Day 0	Day 7	Day 14	Day 30	Day 0	Day 7	Day 14	Day 30
8°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25°C	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+
50°C	-	+	++	++	-	+	++	++	-	+	++	++	-	+	++	++

-= No Change, +=Slight change, ++= More change

Table 3: Organoleptic characteristic observation of gentamicin-*N. sativa* emulsions (Emulsion A, B, C and D) stored at different storage conditions, observed at day 0, 7, 14 and 30.

Duration (Day)	Color												Phase Separation											
	8°C				25°C				50°C				8°C				25°C				50°C			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
Day 0	M	M	M	M	M	M	M	M	M	M	M	M	N	N	N	N	N	N	N	N	N	N	N	N
	W	W	W	W	W	W	W	W	W	W	W	W												
Day 7	M	M	M	M	M	M	M	M	M	M	M	M	N	N	N	N	Y	N	N	N	Y	Y	Y	Y
	W	W	W	W	W	W	W	W	W	W	W	W												
Day 14	M	M	M	M	M	M	M	M	GB	GB	GB	GB	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	Y
	W	W	W	W	W	W	W	W																
Day 30	M	M	M	M	M	M	M	M	GB	GB	GB	GB	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	Y
	W	W	W	W	W	W	W	W																

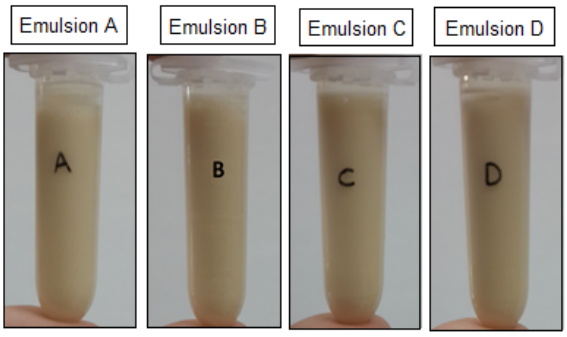

Fig. 2: Gentamicin-*N. sativa* emulsions observed as milky white in colour. Emulsion A, B, C, and D showed ranging in *N. sativa* oil between 32.5 to 46.4% (v/v) while gentamicin concentration is constant at 0.1% (w/v).

RESULTS AND DISCUSSION

Formulation gentamicin-N. sativa emulsions

Gentamicin-*N. sativa* emulsion A, B, C and D were formulated with a constant concentration of gentamicin sulphate (0.1% (w/v)) whereas *N. sativa* oil concentration was ranging from 32.5% to 46.4% (v/v) (Table 1). Further investigations regarding stability of the emulsions were tested in stability tests.

Stability tests

Centrifugation test

Centrifugation test is done to determine the behaviour of an emulsion at the end of storage conditions (Table 2). The result at 8°C, emulsion A, B, C and D showed no phase separation formed at day 0, 7, 14 and 30. At 25°C, all emulsions started to form phase separation at day 14 and onwards. At 50°C, all emulsions formed phase separation at day 7 and onwards. Gravitational force acts in the emulsions generate sample stress and thus increase the particle mobility and started to produce instabilities. Therefore, phase separation was formed after stored in certain storage conditions and durations [14].

Organoleptic characteristics

Organoleptic characteristics are defined as an observation of the appearance of emulsions by its colour, odour, formation of phase separation, precipitation, turbidity and many more[13]. The observation results as in Table 3. The colour of all stable emulsions was milky white (Figure 2). The emulsion smells like *N. sativa*oil and the texture emulsions were sticky. No phase separation was seen at temp 8°C and the colour of emulsions remains milky white. At 25°C phase separations formed at day 7 for emulsion A and at day 14 for emulsion B, C & D. At temperature 50°C, all emulsions changed colour to golden brown and phase separation formed from day 7 onwards. Colour, odour and texture of the emulsion should be unaltered over time [13]. Thus, emulsion must be stored at 8°C to its colour, odour and texture.

Freeze-thaw cycle

Freeze-thaw cycles, evaluate the stability of emulsions by challenging temperature shock that the product could suffer, which cause problems such as phase separation, crystal formation, rheological properties damaged and etc. [14]. Results (Table 4) showed that all emulsions became unstable when challenged with extreme temperature. This may be influenced by polymorphism, degree of lipid crystallinity and phase behaviour of water. When emulsion freeze, lipid droplets become concentrated into freeze-concentratesphase. Thereby coming into close contact with one another in the unfrozen aqueous channel between crystals. The concentration of the lipid droplets in these narrow channels could promote aggregation, flocculation or coalescence during the freeze-thaw process [15,16]. Hence, emulsion must be kept away from extreme conditions and huge fluctuations of temperature.

Table 4: Freeze-thaw cycle results of freshly prepared gentamicin-*N. sativa* emulsions (Emulsion A, B, C & D) for three cycles.

	Phase separation			
Cycle	Emulsion A	Emulsion B	Emulsion C	Emulsion D
Control	-	-	-	-
Cycle 1	+	+	+	+
Cycle 2	++	++	+	+
Cycle 3	++	++	+	+

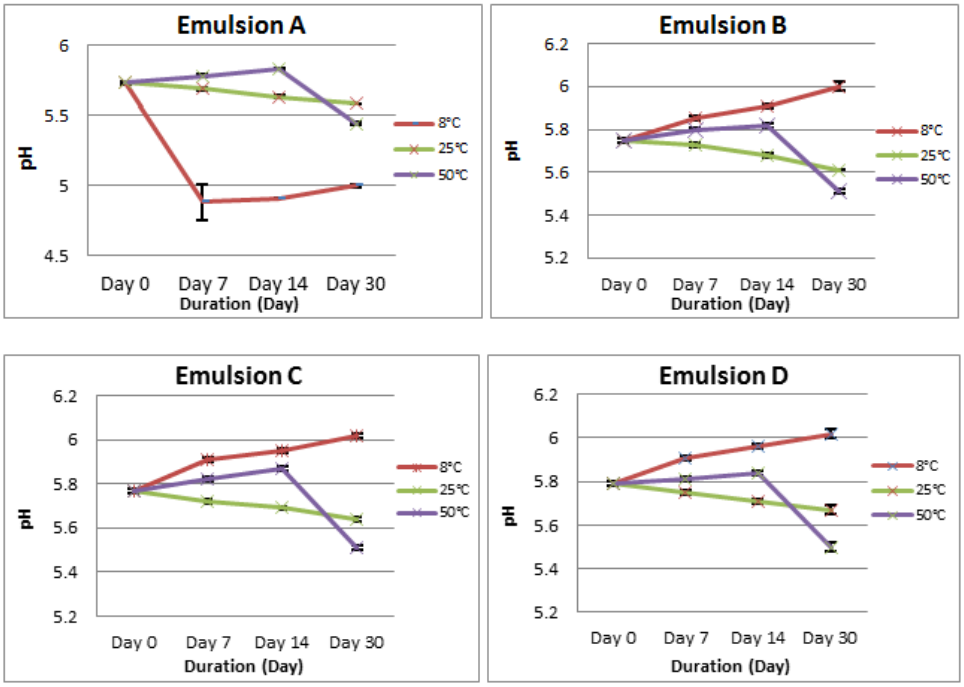

Fig. 3: Measurement of pH-changes in the gentamicin-*N. sativa* emulsions (Emulsion A, B, C & D) stored at different storage conditions, measured at day 0,7, 14 and 30 (n=3).

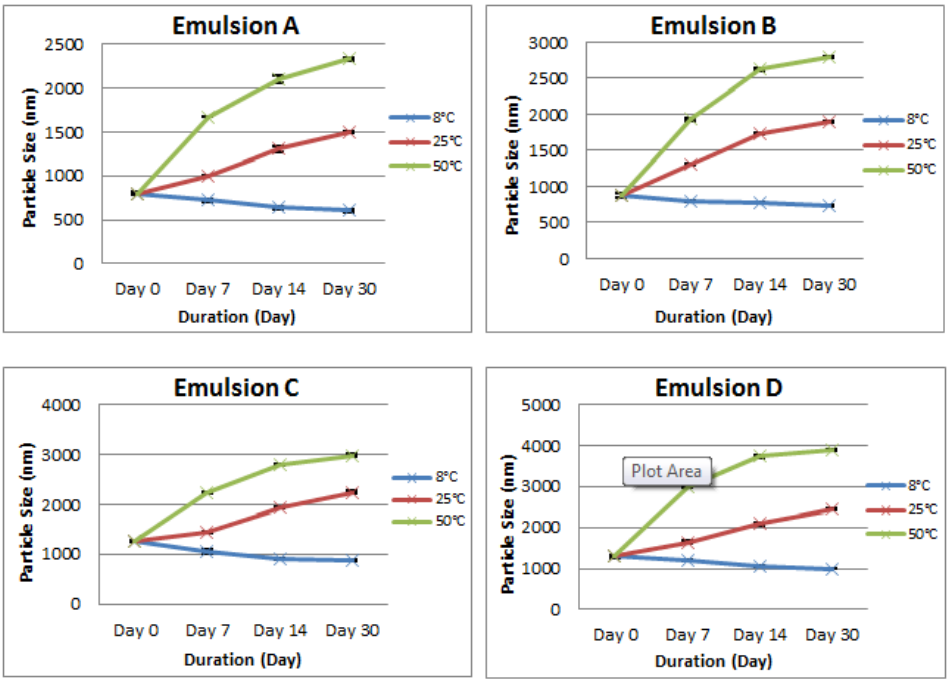

Fig. 4: Measurement of particle sizes of gentamicin-*N. sativa* emulsions (Emulsion A, B, C and D) stored at different storage conditions, measured at day 0, 7,14, and 30 (n=3).

pH determination

pH value for all freshly prepared emulsions were between pH 5.73 to 5.79 (Figure 3). When stored at 8°C, the pH increased in all emulsions while at a25°C storage temperature, pH decreased in all emulsions. Emulsions stored in 50°C showed a fluctuation of pH values. This is because, at high temperature, physicochemical parameters were altered since the temperature elevation possesses direct influence on stability of pharmaceutical dosage forms and the active substance in *N. sativa* oil. Furthermore, low pH emulsions showed poor stability compared to emulsions with higher pH [17]. Increasing of pH could be related to volatile aldehyde oxidation to carboxylic acids or lipid enzymatic hydrolysis that release free fatty acids contains in *N. sativa* oil [18].

Particle size measurement

Particle size of emulsions increased with an increment of *N. sativa* oil concentration (Figure 4). This may be due to increase fatty acid from *N. sativa* oil. Particle sizes of freshly prepared emulsions were ranging from 800 to 1296 nm. In a well-formulated emulsion, the droplet size would be between 1 to 5 micro-meters. At 8°C, particle size of the emulsions became smaller may be due to the attractive forces acting between droplets decreased while inversely, at 25°C and 50°C, the size of particles increased. Sometimes particle size does not affect the stability of the emulsions. Some emulsions with large particles, which were greater than 10 micro-meters showed good long-term stability [19]. But most of the time, attractive forces acting between droplets decrease with smaller particle size and will give better stability against droplet flocculation and coalescence. In addition, the minimum particle size can be achieved by many factors. Previous studies have shown that using a high energy approach (such as homogenizer type, speed, temperature and time), sample composition (such as oil type, surfactant type and relative concentration) and physical properties of component phases (interfacial tension and viscosity) [20]. Thus, homogenizer can be used to achieve minimum particle size so that the stability, appearance, texture and bioavailability of good emulsions can be produced [21,22].

Zeta-potential analysis

The **zeta-potential** of all emulsions were lower than -30mV and became more negative as the *N. sativa* oil increased (Figure 5). The negative charged emulsion was contributed by negative charged surfactants, which are also known as anionic surfactants. The surfactants used were Tween®20 and Span®20 which were categorised as soap surfactants [19]. Additionally, emulsifiers and surfactants can be classified as cationic (positively charged), anionic (negatively charged), amphoteric or zwitter ionic (both positively and negatively charged) and non-ionic (no-charged). Most of emulsifiers and surfactants were amphiphilic molecules which consist of hydrophobic and hydrophilic parts. Polar group of charged substances attached to hydrocarbon chain and exhibit both hydrophobic and hydrophilic characteristics [23]. The influence of the surfactants of the emulsion characteristics was demonstrated that the surface charge might affect physical and chemical stability [24,25]. Thus, it could be considered that all emulsions prepared had good stability and repulsive forces between droplets were larger in the emulsions containing oil concentrations.

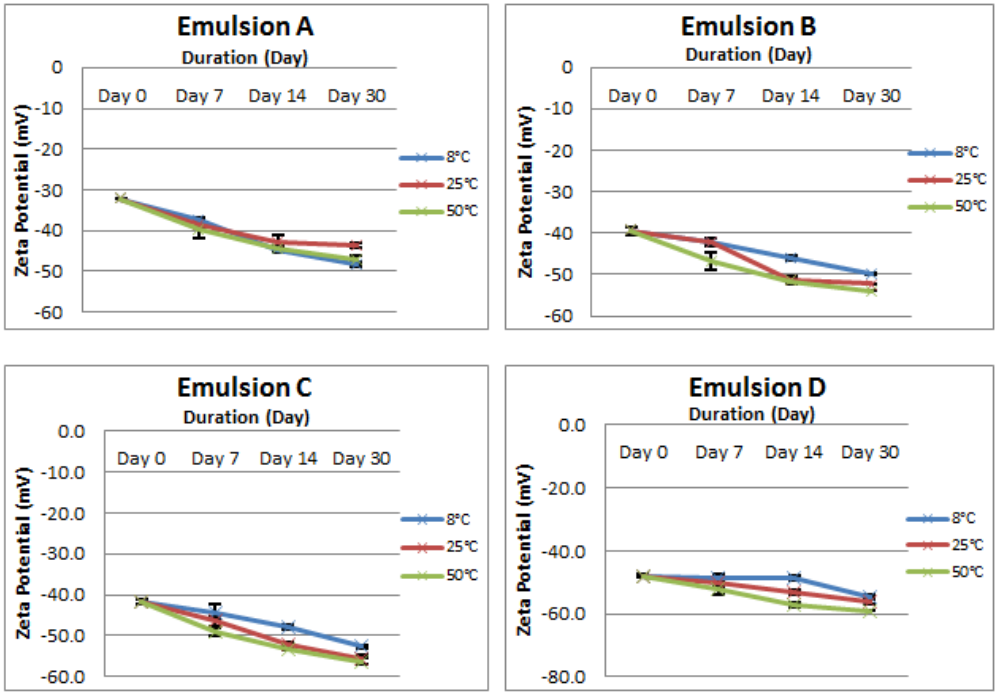

Fig. 5: Measurement of zeta-potential of gentamicin-*N. sativa* emulsions (Emulsion A, B, C and D) stored at different storage conditions, measured at day 0, 7, 14 and 30 (n=3).

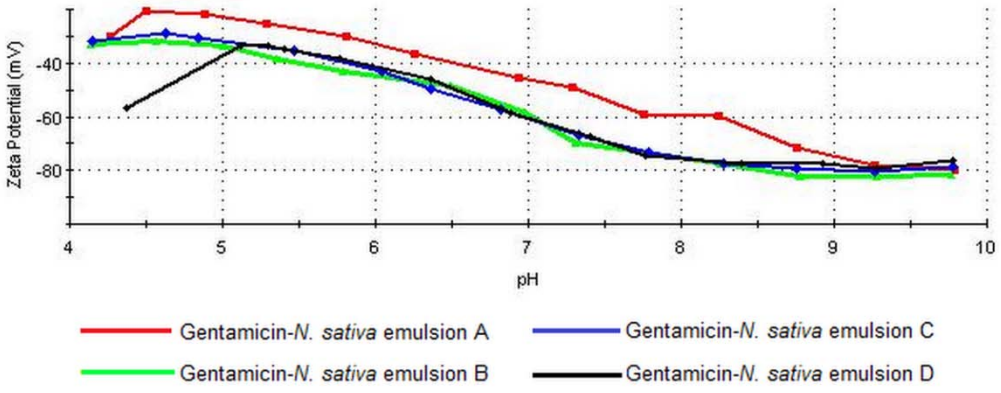

Fig. 6: pH titration range and zeta-potential (mV) of freshly prepared gentamicin-*N. sativa* emulsions (Emulsion A, B, C and D) (n=3).

pH titration analysis

pH titration of freshly prepared emulsions (Figure 6) showed that the emulsion stability was affected by acidic conditions but not by alkaline conditions. Unstable emulsion can be identified by demulsification process, which can be occurred when emulsion prepared at high temperature then decreased progressively. The particles become protonated when adsorb at oil-water interface and detached from the interface in-situ. Thus, the degree of ionisation of emulsion particles was taken into consideration in controlling the coalescence stability of emulsions [26].

CONCLUSION

Although the tools for characterising emulsions are advanced and mechanisms of emulsification understood, it is still difficult to predict the actual results of an emulsification process, because many parameters involved. The emulsions showed that storage temperature does affect the stability of emulsions. Extreme temperature must be avoided during storage of emulsion to maintain the stability. Thus, understanding the role of each parameter can aid in designing the stable gentamicin-N. sativa emulsions.

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CONFLICT OF INTERESTS

Declared None

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