Preparation of microemulsion containing *Lycopersicon esculentum* extract: *In vitro* characterization and stability studies

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Abstract: Lycopene, the active component of *Lycopersicon esculentum* species, has been reported for the protecting capabilities against ultra-violet induced skin pigmentation, antioxygen and antityrosinase activities. In the present study, extract of tomato fruit was obtained from the *Lycopersicon esculentum* plant using solvent system comprised of hexane-ethanol-acetone. The phyto-chemical active constituent "lycopene" was then identified by spectrophotometric technique at 470nm. Micro emulsions were developed containing different ratio of water, isopropyl myristate (oil), tween 80 and propylene glycol as surfactant and co-surfactant respectively via pseudoternary phase diagram. Various physico-chemical tests were performed including globular size, conductivity, viscosity, scanning electron microscopy (SEM), refractive index (RI) and pH measurement for the formulation characterization. Results of physical and chemical stability studies showed that the micro emulsion with proportion of surfactant: co-surfactant of 2:1(S_{mix}) was found to be optimized formulation and with enhanced stability. Therefore, concluded that the stability of the micro emulsion was dependent on the proportions of surfactant/co-surfactant, water and oil in the preparation.

Keywords: *Lycopersicon esculentum*, antioxidant, micro emulsion, stability testing.

INTRODUCTION

Tomatoes are one of the richest sources of lycopene, providing red colour of fruits (Burton-Freeman and Sesso, 2014). Scientifically tomato is "*Lycopersicon esculentum*", containing "lycopene" to be a major constituent offering antioxidant properties (Choksi and Joshi, 2007). Other sources may also include watermelon, papaya, apricots and guava (Rozzi *et al*., 2002). Tomatoes and tomato derived products have been reported to prevent cardiovascular disease, certain types of the cancer (mainly prostate cancer) and erythema due to exposure of ultraviolet (UV) light (Stahl *et al*., 2001; Salehi *et al*., 2019). The measurement and extraction of lycopene from tomatoes is essential for determining the potential health benefits (Periago *et al*., 2004). The major fraction of lycopene (72-92%) is observed in tomato fruit skin and its water insoluble portion (Shi and Le Maguer, 2019). Structure of lycopene possess thirteen double bonds containing an acyclic carotenoid responsible for reactive oxygen species (ROS) scavenging, usually formed under conditions of (photo) oxidative stress (Zhu *et al*., 2019). Lycopene is a C-40 carotenoid, documented to be 10-folds more capable to bind with nascent oxygen than α-tocopherol/β-carotene (Martí *et al*., 2019). Many studies support the protective action of lycopene against photo damage because of inhibition of UVB-prompted ornithine myeloperoxidase and decarboxylase, this minimizes the inflammatory effects (Groten *et al*., 2019; Bungau *et al*., 2019). Recent investigation further confirms that the lycopene inversely affects the skin roughness, formation of furrows and wrinkles hence reducing skin aging (Lopes *et al*., 2010).

Microemulsions are referred to be the thermodynamically stable dispersions of aqueous and non-aqueous. Surfactants and co-surfactant alone or in combination are employed in these preparations for the stabilization of product. Microemulsions offer more advantages as a drug delivery system than the conventional (macro) emulsions including ease of formulation without high-energy input, enhanced stability and nano-scale systems with a higher solubilization capacity (Dantas *et al*., 2010).

Nowadays treatment with natural sources has been practiced worldwide owing to excellent therapeutic response with least toxic and adverse effects (Glynn and Bhikha, 2019). The current study was intended to develop and optimize the topical formulation of *Lycopersicon esculentum* pulp extract using different proportion of surfactant and co-surfactant. Different physico-chemical tests were carried out to evaluate and compare the quality attributes of blank microemulsion with microemulsion containing drug.

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MATERIALS AND METHOD

Plant material and chemicals

Pulp along with skin of *Lycopersicon esculentum, (*variety Saahil; tomatoes) was utilized as plant material. *Lycopersicon esculentum* voucher no. (419-6-1934) was obtained from Herbarium of department of Botany, Faisalabad University). Lycopene pure (for UV standardization) was purchased from Sigma-Aldrich, tween 80 (Franken Chemical, Germany), isopropyl myristate (Merck, Germany), co-surfactant propylene glycol (Merck, Germany), n-hexane (BDH Chemicals, England) was purchased from the market.

Instruments

Electrical top loading balance (ThermoFisher Scientific), hot plate with magnetic stirrer (VelpScienifca, Germany), zetasizer (Malvern, ZEN3600, UK), Abbe refractometer (HEDAO, China), scanning electron microscope SEM (19800 Hitachi S-3400N VP-SEM, Japan), X-ray diffractometer (Bruker D8 Discover, Germany), Brookfield viscometer (DV-III+Pro Brookfield., USA), Conductometer (WTW, Cond 197i, Germany), pH meter (WTW pH-197i, Germany).

Extraction and standardization process

Extraction of *Lycopersicon esculentum* pulp was carried out by shaking it thoroughly with a solvent system composed of ethanol: hexane: acetone (25:50:25). For the separation of drug into polar and non-polar layers, 15 mL distil water was subsequently added into drug solution. Lycopene being non-polar was isolated from the upper lipophilic layer; however pigments of polar nature were contained in the lower polar phase (Souza *et al.,* 2008). Sodium sulfate anhydride (1.0 g) was added to the hexane phase and then stirred for 30 sec and finally filtered to remove aqueous residue. All hexane carotenoid extract content was taken to a flat bottom balloons (250 mL). At 40ºC, mixture was concentrated through vacuum rotary evaporator for around half an hour. Samples obtained from hexane layer were shifted to amber colour volumetric balloons (light protection) and stored in refrigerator (Lopes and Reed, 2010). A UV-visible spectrophotometer was utilized for identification of drug content at wavelength of 470 nm using n-hexane as blank (Bunghez *et al*., 2011).

Preparation and Optimization of microemulsion

Microemulsions (MEs) were formulated utilizing pseudoternary phase diagram. Tween 80 and propylene glycol were used as surfactant and co-surfactant respectively for the emulsification of aqueous and non-aqueous phase (isopropyl myristate). The surfactant and co-surfactant were mixed in different ratios (2:1 and 4:1) as shown in fig. 1. Oil, water and surfactants were blended on hot plate at 25^{o}C till complete emulsification then mixture was retained untouched for one day. The microemulsion regions were finally determined through the pseudoternary phase diagram. The microemulsion with 2:1 ratio indicated higher regions of microemulsion on the phase diagram, hence such formulation (optimized) was chosen for characterization.

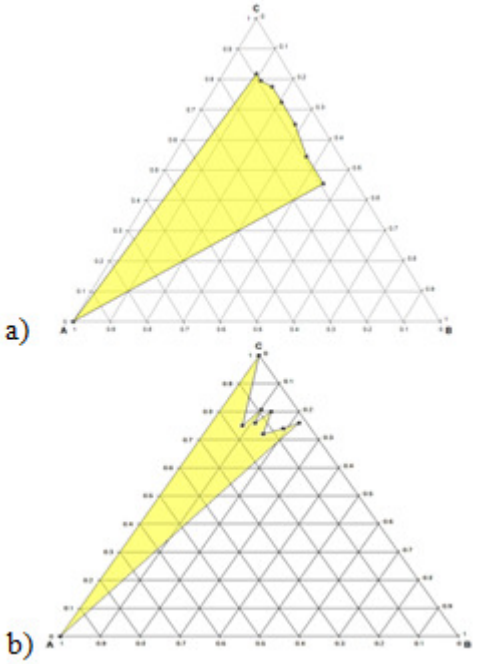

Fig. 1: The pseudoternary phase diagram of surfactant mixture Oil (correspondent to a), S_{mix} (correspondent to b) and Water (correspondent to C). S_{mix}2:1and b) S_{mix}4:1.

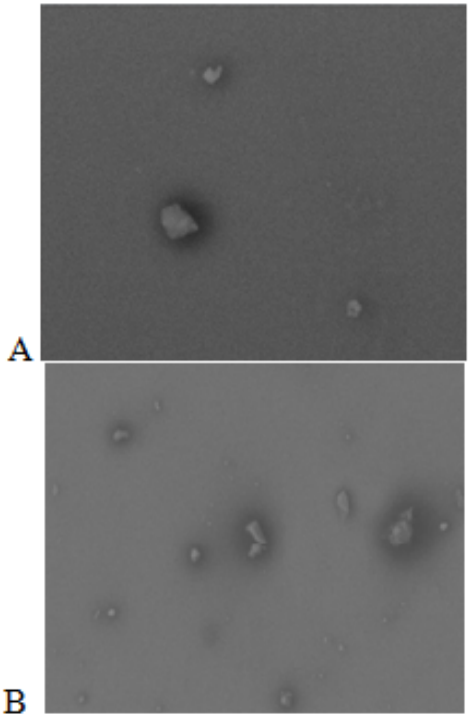

Fig. 2: A) SEM of Blank Microemulsion B) SEM of Microemulsion containing drug.

Table 1: Physico-chemical Properties of blank and microemulsion containing *Lycopersicon esculentum* extract (n=3±SD)

Characterization	Viscosity (cP)	Particle size(nm)	R I	PDI	Spreadability (cm)	Conductivity (µS/cm)	pH
Blank Microemulsion	4.76±0.12	24.95±0.4	1.443	1.000	5.5	11.5	5.93
Microemulsion containing extract	3.91±0.05	32.50±0.2	1.437	0.941	5.40	9.3	5.87

Table 2: Physical stability of Blank (B) and Formulation (F) at different temperatures

Parameters		Fresh		24 hrs		72 hrs		7 days		30 days		60 days		90 days	
		B	F	B	F	B	F	B	F	B	F	B	F	B	F
Colour	4°C	W	R	W	R	W	R	W	R	W	R	W	R	W	R
	25°C	W	R	W	R	W	R	W	R	W	R	W	R	W	R
	40°C	W	R	W	R	W	R	W	R	W	R	W	R	W	R
	50°C	W	R	W	R	W	R	W	R	W	R	W	R	W	R
Turbidity & loss of Clarity	4 °C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	25°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	40 °C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	50°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Phase separation	4°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	25°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	40 °C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	50°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Centrifugation stability (6000 rpm, 30 min)	4°C	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	25°C	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	40 °C	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	50°C	S	S	S	S	S	S	S	S	S	S	S	S	S	S

* S = Stable, W = White; R= Red

Table 3: Chemical Stability: mean values of pH and conductivity of Blank (B) and Formulation(F) microemulsions at various temperatures

Parameters		Fresh		24 hrs		72 hrs		7 days		30 days		60 days		90days	
		B	F	B	F	B	F	B	F	B	F	B	F	B	F
pH	4°C	5.93	5.87	5.83	5.77	5.69	5.62	5.86	5.67	5.82	5.64	5.74	5.46	5.73	5.27
	25°C	5.93	5.87	5.79	5.73	5.73	5.67	5.71	5.61	5.89	5.67	5.77	5.45	5.81	5.43
	40°C	5.93	5.87	5.84	5.76	5.74	5.58	5.78	5.51	5.81	5.66	5.72	5.37	5.88	5.34
	50°C	5.93	5.87	5.89	5.78	5.81	5.60	5.74	5.56	5.87	5.53	5.76	5.56	5.89	5.47
Conductivity	4°C	11.5	9.3	12.3	9.6	12.1	8.1	13.3	16.8	15.7	11.4	14.1	18.7	17.3	12.1
	25°C	11.5	9.3	14.7	12.3	18.3	13.7-	21.8	10.3	25.7	7.9	15.7	11.7	14.3	18.3
	40°C	11.5	9.3	11.2	7.1	24.5	13.7	16.5	15.2	24.7	13.4	11.7	13.3	20.3	13.6
	50°C	11.5	9.3	16.4	12.9	17.6	16.2	13.1	16.8	23.7	18.8	15.7	12.8	16.3	16.3

Physical stability studies

The stability of microemulsion (2:1) was evaluated by varying the storage temperature and centrifugation (6000 rpm for 30 minutes at 25°C). Blank and microemulsion containing the *Lycopersicon esculentum* extract were kept at different storage conditions of 4^0C, 25^0C, 40^0C and 50^0C in incubators. Physical stability (macroscopic appearance, phase separation, flocculation or precipitation, loss of clarity or turbidity) and chemical parameters (pH and conductivity) were analysed for 90 days period at defined time intervals. Stored sample were further characterized for zeta size, antioxidant activity, refractive index (RI), X-ray diffraction, scanning electron microscopy, thermal analysis and spreadibility.

Zeta potential, particle size, refractive index measurements

Globular size of the phases was determined at 25ºC by zetasizer. Test samples were kept in transparent disposable zeta cells and then the results were recorded. The RI of samples was measured digitally by refractometer.

Polydispersity index (PDI) and homogeneity

Microemulsion of drug extract was assessed for appearance, presence of aggregates and homogeneity by visual inspection at 25ºC. Polydispersity indexes of formulations were determined by zetasizer instrument.

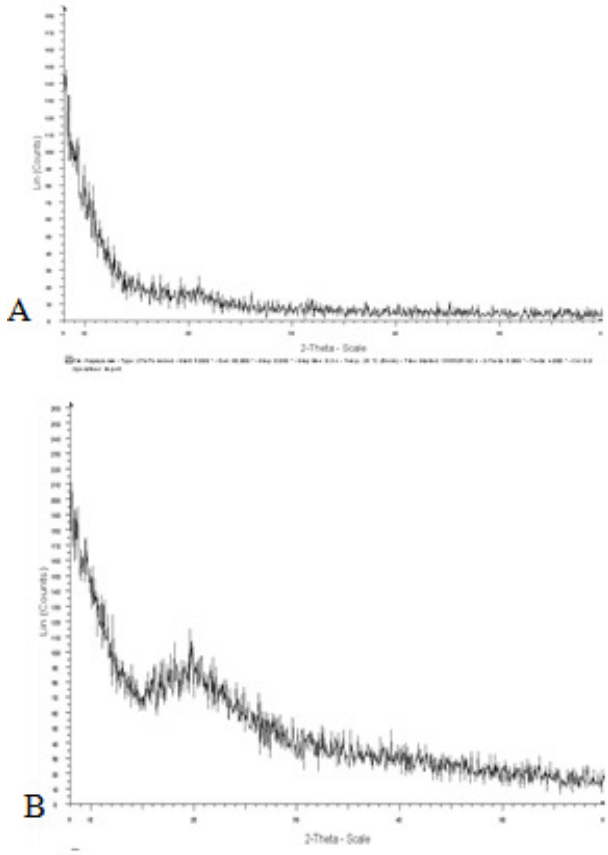

Fig. 3: A) X-Ray Diffractogram of Blank microemulsion B) X-Ray Diffractogram of microemulsion containing drug extract

Scanning electron microscopy (SEM) and XRD (X-ray diffraction)

It was utilized to evaluate the microstructure of blank and drug containing microemulsions at variable pressure. Crystalline components of microemulsion containing drug extract were identified through X-ray diffractometer with Ni-filtered CuK alpha radiation source. The tube voltage of 35KV, current of 35 mA and scanning rate of 5°min^{-1}, over a range of 8°-60° diffraction angle (2θ) range was employed.

Thermogravimetric analysis (TGA) and Differential Scanning Calorimetry (DSC)

In aluminium pans, small quantities of blank (2-4 mL) and drug containing microemulsion were taken and then sealed prior to conduction. Evaluation was made at a rate of 10 C/min, with nitrogen flow of 25 mL/min, over temperature range of 0^0C to 500^0C. Thermograms of blank and extract containing microemulsion was observed separately.

Spreadability, viscosity, conductivity and pH measurements

Spreadability of blank and test formulations was measured by placing a fixed amount of trial and blank (500 mg) on the lower slide while the other slide containing 10g weight was rubbed over it. Circular region of preparation was appeared owing to compression, and were subsequently measured (Desai, 2004). Sample's viscosity was analyzed using viscometer with spindle no. 41 at shear rate of 200 rpm. Electrical conductivity of MEs was determined using conductivity meter. The pH of microemulsions was evaluated by electrode pH meter at temperature 25 ºC.

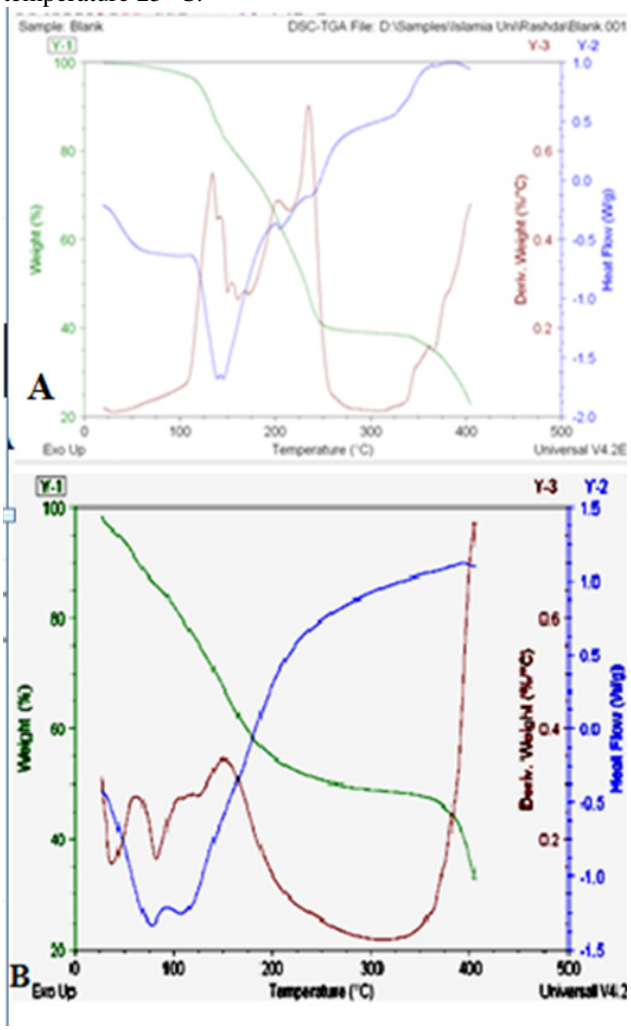

Fig. 4: A) Thermogram of Blank microemulsion B) Thermogram of Drug containing microemulsion *(TGA correspondent to blue line and DSC correspondent to black line)

STATISTICAL ANALYSIS

All the runs were repeated thrice and the results were presented as mean ± SD. Data was examined statistically through one-way analysis of variance (ANOVA). $P<0.05$ was taken to be significant with 95% confidence interval.

RESULTS

Pseudo-ternary phase diagrams of the quaternary system containing water/ IPM, Tween 80/propylene glycol along with different proportions of surfactant-co-surfactant are shown in fig. 1a and 1b. The globules size of ME containing *Lycopersicon esculentum* extract was found to be 37.5±0.2 nm with a PDI of 0.941 (table 1). Microemulsion globules' shape and size were confirmed through SEM technology (fig. 2A and 2B). Drug containing formulation possessed the lowest average particle size of 7.03±0.2nm with PDI of 0.362±0.032. Consistency of formulation was found to be more or less similar showing no significant difference between the viscosities of the trial formulation and the blank MEs (P>0.05). Higher conductivity values confirmed that the O/W type of emulsion. Viscosity, refractive index, pH and spreadability were found to be appropriate for drug loaded emulsions. Physical and chemical stability test confirmed no phase inversion and separation at various temperatures during storage period.

DISCUSSION

Microemulsions are preferred systems than conventional topical emulsions due to higher stability and enhanced life storage time. These preparations are documented to be transparent, monophasic having an ultra-low interfacial tension and viscosity (Gadhave and Waghmare, 2014). In the present investigation, pseudo-ternary phase diagram was used to evaluate the quaternary system of water/ IPM, tween 80/ propylene glycol with different surfactant-co-surfactant proportions. Non-ionic surfactants are biocompatible and non-toxic hence has been commonly utilized in the formulation of many cosmetic and pharmaceutical products. The globules size of drug loaded microemulsion was found to be appropriate (37.5±0.2 nm) with fine polydispersity index of 0.941 (table 1). PDI is a determination of globules homogeneousness that ranges from 0-1. PDI value closer to zero reflects absolute homogeneity of the globules (Moghimipour *et al*., 2013). Thus, PDI indicates the uniformity with distribution size range (micro to nano globules) of ME formulations. Correlation among mean particle size, PDI and globule of blank and formulation were evaluated by analysis of variance and were found to be non-significant (p>0.05). The size distribution is a major testing parameter of ME as it directly affects the stability of the emulsion. Confirmation of microemulsion's globules size and shape of blank and trial was done by SEM (fig. 2). Nemich and Laxman also used SEM to study microstructures of nebivolol micro emulsion formulation (Nemichand and Laxman, 2016). The particle sizes of MEs with and without drug loading were determined and no significant difference was noticed in average size after drug incorporation. The minimum mean particle size of formulation was found to be 7.03±0.2nm with polydispersity index of 0.362±0.032. Recently a study was conducted by Soradech *et al*., to develop the cosmetic microemulsion of *Tiliacora triandra* using pseudoternary phase diagram. Authors have noticed that the drug incorporation did not affect the droplet size and PDI of blank microemulsion trial. However; the pH of the ME may be influenced upon mixing of active plant ingredient and the intensity of fluctuation has mainly dependent on the nature of the constituents present in extract (Soradech *et al*., 2018).

Viscosity is one of the significant parameter for characterization of microemulsions. It has documented that the viscosity of the macroemulsions are apparently higher than ME. Low viscosity may result in decrease contact time of drug with the skin leading to inefficient therapeutic activity. However, this could be reduced through addition of viscosity builders or gelling agents and now a days microemulsion based gels are commercially available worldwide (Kansagra and Mallick, 2016; Gadhave and Waghmare, 2014). The mean viscosity of the blank ME and ME containing extract was measured as 4.76±0.12cP and 3.91±0.05 cP respectively with p>0.05 reflecting insignificant difference between the viscosities of the two formulations. Moghimipour and co-workers formulated ME systems of naproxen, with viscosity range of 253.73 to 802.63 cP, found to be highly consistent than present ME systems (Moghimipour *et al*., 2013). Thicker MEs were actually prepared using higher proportion of surfactant and co-surfactant (4:1 and 6:1) in S_{mix}. Spreadability is basically an ability of formulation being used for easy and smooth application on a surface. If a topical drug formulation is spread on a constant layer, a standard dose will be applied, and consequently the efficacy would be increased. Suitable spreadability also contributes to an appropriate extrudability from the package, easy topical application, and most important, patient preference and compliance (Garg *et al*., 2002). Therefore, spreadability is one of the significant parameters used for characterization of emulsions, gels, and other topical application (Ashara *et al*., 2014). Presently, the blank and drug loaded MEs have shown the good spreadability values ranged between 5.5 to 5.40 with statistically significant difference.

The refractive index of the blank and microemulsion having *Lycopersicon esculentum* extract was observed to be 1.443 and 1.437, correspondingly, illustrating the transparency of the emulsion. Various other researchers have also utilized RI in the analysis of microemulsion and its values correspond to our study. It is reported that the transparent samples usually possess refractive indices of 1 to 2 when viewed under visible light (Kumar *et al*., 2016; Moghimipour *et al*., 2012).

A correlation was noticed between particular structure of ME and its electrical conductivity. The blank ME and ME

containing extract had an average conductivity of 11.5 and 9.3 µS/cm, correspondingly. O/W emulsions generally illustrate the similar conductivity pattern as that of aqueous water phase alone. While the W/O have shown 100-1000 times less conduction than water due to immobility of ions in presence of oil insulations (Prieto and Calvo, 2013). Modi and co fellows also prepared an aceclofenac nanoemulsion gel formulation for topical administration of drug. The conductivity of the trial formulations was assessed to be 0.0867 µS/cm to 0.149 µS/cm therefore, confirming the presence of water in oil (W/O) system (Modi and Patel, 2011). In our MEs higher values of conductivity was observed as it was an O/W system. Skin pH is variable as described in literature from pH 4.0 to 7.0, depending on the skin region (Lambers *et al*., 2006). The blank ME and drug loaded ME had appropriate pH values of 5.93 and 5.87, respectively. Moreover; incorporation of *Lycopersicon esculentum* extract didn't considerably affect/alter the pH of the system. Hence, in this study viscosity, refractive index, pH and spreadability were found to be appropriate for drug loaded emulsions.

XRD graph (fig. 3) of ME containing extract showed the amorphous nature of the formulation, further verified with DSC and TGA thermograms (figs. 4A & 4B). There is no sharp peak in these thermograms, indicating the absence of melting point of any crystalline substance.

Stability testing of microemulsion formulations

Kansagra and Mallickhad developed the antifungal microemuslsion of luliconazole to treat skin infections. In that previous study optimized drug loaded luliconazole ME and ME based gel was selected among various trials on the basis physico-chemical characterization and stability studies. During stability testing researchers mainly target the globular size and the PDI (Kansagra and Mallick, 2016). Presently, results of the physical stability of the prepared ME are presented in table 2. The heterogeneous system physical stability was evaluated via centrifugation (phase separation test). Although ME presented lower viscosity level with higher PDI, but there were no signs of phase separation, alteration, precipitation or flocculation upon macroscopic examination, still found to be stable when exposed to stressed conditions (centrifugation). Therefore, the centrifugation testing corroborates superior physical adherence of trials (blank and drug containing ME) kept at different temperatures.

Chemical stability studies were conducted at various pH values for all ME samples at different storage conditions (4^0C, 25^0C, 40^0C and 50^0C). The effect of temperature was studied by Prieto and Calvo during the formulation optimization of microemulsion using tween 80. Authors have reported the dehydration of surfactant, mainly of head region when exposed to moderate to higher temperatures therefore becoming more hydrophobic. Viscosity of certain preparations was also observed to be decreased however, could be enhanced if temperature was further increased from 30˚C to 40˚C owing to micellization. This investigation clearly reflects the temperature dependent activity of the surfactant tween 80 (Prieto and Calvo, 2013). Negligible variations upon time passage were observed however; a slight decrease in pH was observed in ME but still found to be statistically insignificant by ANOVA (P>0.05) (table 3). Conductivity values of all samples of both blank and active formulations showed no increase during 90 days of storage time period. Therefore, the designed MEs were found stable with no percolation of water. Thus, physical and chemical stability test confirmed no phase inversion and separation at various temperatures during storage period.

CONCLUSION

On the basis of results, it is concluded that blank and *Lycopersicon esculentum* extract containing microemulsion are highly stable in 2:1 ratio of surfactant and co-surfactant, IPM and water system. Physical characterization shows that newly formulated drug microemulsion trial has an appropriate viscosity with optimum spreadability and polydispersibility index. No phase separation, turbidity, inversion and cracking was seen in drug ME trials during 90 days of stability period. Moreover; globular size of the drug preparation was maintained with thermodynamic stability at various storage conditions, provided in laboratory. Through present technique, plant extracts could be successfully loaded directly in microemulsion systems that were thought to be unstable in such environment.

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