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UV Method Development and Validation, Formulation, Development and Characterisation of Transfersomes and SLNs of *Cordycep militaris* Extract

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ABSTRACT

Cordyceps militaris, an effective antioxidant along with anti-hyperglycemic, antitumor, and immune-modulatory effects, belongs to family *Cordycipitaceae*. The medicinal mushroom *Cordyceps militaris* has active constituent cordycepin which possess various pharmacological effects. The UV method development and validation of *Cordyceps militaris* extract was carried out. The extract was scanned over UV-visible range for its wavelength of maximum absorbance. Various calibration standards were prepared, and absorbance was recorded at wavelength of maximum absorbance. Various analytical method validation parameters *viz.* accuracy, precision, LOD, LOQ, robustness and ruggedness were calculated using QC standards. The transfersomes and SLNs of *Cordyceps militaris* were prepared and difference in characteristics was observed. The transfersomes were prepared by method and SLNs were prepared using hot homogenization technique. The formulations were subjected to various evaluation parameters and the results suggested that the formulations were stable as well as the values obtained were within the required range. The encapsulation efficiency for transfersomes and SLNs were reported to be about 80% and 83% respectively. The difference may be due to different types of lipids used in the two formulations. The particle size reported for both the formulations were within the range of 155 nm to 160 nm along with the PDI below 0.3. About 97% to 98% of the active ingredient was released from the formulations over the period of 24 hours which indicates that the formulations showed controlled release drug release pattern. Thus it proved that transfersomes and SLNs are potential drug delivery carriers and can be used to improve the bioavailability as well as controlled release of the active ingredient from the formulation.

Keywords: *Cordyceps militaris*, Transfersomes, Solid lipid nanoparticles, UV development, Method validation

INTRODUCTION

Cordyceps militaris is a species of fungus in the family *Cordycipitaceae*, and the type species of the genus Cordyceps. It was originally described by Carl Linnaeus in 1753 as *Clavaria militaris* [1]. *Cordyceps militaris (C. militaris)*, belonging to the family *Clavicipitaceae*, is a fungus with a long history of widespread use in folk medicine. Although *C. militaris* is found in East Asia, *C. militaris* is difficult to find in the wild due to its rarity. Thus, many investigators have been devoted to focus on the artificial cultivation of *C. militaris* using cutting-edge technology [2]. Many biological and pharmacological functions of this fungus have been identified, such as its anti-oxidative, anti-hyperglycemic, anti-tumor, and immune-modulatory effects. In addition, our group has demonstrated that cultivation of *C. militaris* can induce apoptosis and autophagy in human glioblastoma cells [3].

Macroscopic characteristics

The fungus forms 20-50 mm high, club-shaped and orange/red fruiting bodies, which grow out of dead underground pupae [4]. The club is covered with the stroma, into which the actual fruit bodies, the perithecia, are inserted. The surface appears roughly punctured. The inner fungal tissue is whitish to pale orange as Figure 1 [5].

Figure 1: *Cordycep militaris.*

Microscopic features

The spores are smooth, hyaline, long-filiform, and often septate. They decompose to maturity in 3-7 µm × 1-1.2 µm sub-pores. The asci are long and cylindrical. Sometime an anamorphic state, which is Isaria, is found. Masses of white mycelia form around the parasitised insect; however, these may not be of the same species [6].

Chemical constituents

The constituents of medicinal mushroom *Cordyceps militaris*,

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especially the anti-cancer agent cordycepin (3′-deoxyadenosine), are expected to play evolutionary roles in the pharmacognosy sector in future as Figure 2 [7].

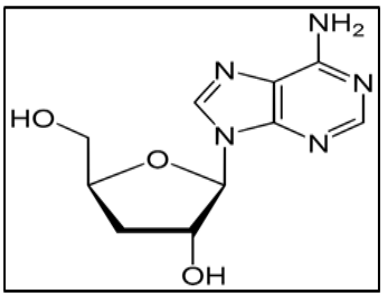

Figure 2: Chemical structure of cordycepin.

MATERIALS AND METHODS

Experimental instruments and reagents

Concurrent a double beam UV-visible spectrometer with spectra manager software was utilized for the UV examination. Quartz cells having 3 cm length with 1 cm way length were utilized for spectral estimation. Weighing balance with inside calibration mode was utilized for the accurate weighing purpose. *Cordycep militaris* extract was obtained as gift sample. Acetone, DMSO, span and chloroform were purchased from CDH, New Delhi. All the chemicals of analytical grade were utilized for the proposed study.

UV method validation and calibration curve

Preparation of working standard drug solution: The standard *Cordycep militaris* extract (10 mg) was correctly gauged and moved into the 10 ml volumetric flask and broke down appropriately in 1 ml acetone : DMSO and weakened sufficient with phosphate support pH 6.8 to accomplish a last convergence of 1000 µg/ml (Stock-1). Stock-1 was appropriately weakened using versatile stage to accomplish a 100 µg/ml (Stock-2) arrangement.

Determination of wavelength of maximum absorbance (λ max): The Stock-2 was filtered using full output mode with medium checking speed for a whole scope of UV/VIS Spectrophotometer, the reaching out from 800-200 nm with a co-dissolvable structure as a clear. Subsequent to getting the reach, λ max was perceived. The over technique was reiterated three-fold.

Preparation of calibration curve: The calibration bend was set up by using Stock-2 to accomplish the ten different adjustments standard addressing 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 µg/ml strength. An absorbance of every adjustment standard was evaluated at λ max 262 nm using fixed frequency assessment mode.

Method validation: Created UV methodology for the assessment of *Cordycep militaris* extract was affirmed as far as boundaries like linearity, expand, precision, strength, unpleasantness, precision, oblige of assessment (LOQ) and compel of area (LOD) using predefined adjustment principles as portrayed under [8].

Linearity and range: Linearity of the proposed UV strategy was set up using ten assorted alignment norms. In light of assessment of adjustment rules, alignment bends regarding absorbance versus focus plots were made and exposed to direct least square relapse investigation. R square regard was viewed as basic figure for setting up linearity of the proposed methodology. The break among upper and lower fixation compel with acceptable linearity was itemized to be the run of the proposed UV strategy [9].

Accuracy: Accuracy is a level of the closeness of the trial incentive to the genuine amount of the substance inside the framework. The exactness of the proposed UV methodology was surveyed using recovery contemplates after standard extension of analyte of captivated. Three assorted plans of *Cordycep militaris* extract were masterminded in three-fold at level of 80%, 100% and 120% of its predefined focus (8, 10, 12 µg/ml). In predefined fixations, particular amount of *Cordycep militaris* extract was incorporated (standard extension technique) and exactness was chosen dependent on percent recovery. For computing the percent recuperation, following condition was used:

$$\% RC = (SPS-S/SP) \times 100$$

Where, % RC=Percent recovery, SPS=Amount found in the spiked sample, SP=Amount added to the sample, S=Amount found in the sample [10].

Intra-day precision and inter-day precision: Precision of the test strategy was reviewed regarding repeatability *via* completing three free examines of *Cordycep militaris* extract test game-plan and the % RSD of assessment (intra-day). Exactness of the methodology was checked by performing same system on three consistent days [11].

Robustness: Robustness the created UV methodology was gotten using particular frequencies. Frequency was purposefully changed to 237 nm and 246 nm. *Cordycep militaris* extract (6 µg/ml) was arranged freely, (n=5) and test was broke down at λ max 237 nm and 246 nm for *Cordycep militaris* extract content. The outcome was chosen as far as % RSD [12].

Ruggedness: Ruggedness, the UV/VIS procedure was completed by dissecting three-fold trial of *Cordycep militaris* extract using two unmistakable specialists. The outcome was outlined as far as % RSD [13].

Limit of Quantification (LOQ): In UV method development LOQ was determined by utilizing the following equation.

$$LOQ = 10 \times SD/S$$

Where, S=slope, SD=Standard deviation of Y-intercepts

Limit of Detection (LOD): In UV method development LOD was determined by utilizing the following equation

$$LOD = 3.3 \times SD/S$$

Where, SD=Standard deviation of Y-intercepts, S=Slope [14].

Formulation development of transfersomes and solid lipid nanoparticles

Preparation of transfersomes of *Cordycep militaris* extract: Soya lecithin and span 60 were weighed and dissolved in methanol and chloroform in ratio 2:1 in round bottom flask. Thin layer was formed on the inner side of round bottom flask by evaporating the solvent under vacuum using rotary evaporator for 10 minutes at 40°C. Further 10 mg *Cordycep militaris* extract was dissolved in acetone and DMSO mixture. Required amount of phosphate buffer pH 6.8 was added to the layer formed to make up the volume up to 50 ml. The mixture was shaken continuously for 1 hr at 40°C to anneal liposome structures. The resulting solution was sonicated for 30 minutes using bath sonicator.

Preparation of Solid Lipid Nanoparticles (SLNs) of *Cordycep militaris* extract: Hot homogenization method was used for the preparation of solid lipid nanoparticles. Lipid phase and aqueous phase were prepared separately. Lipid phase contained an accurately weighed 10 mg of *cordycep millitaris* extract which was solubilized in about 10 ml of solvent DMSO and acetone in 1:1 ratio along with Glyceryl Monostearate (GMS). The aqueous phase consisted of tween 80 solubilized in about 25 ml of distilled water. Once both phase reached the same temperature (40°C), the lipid phase was added to aqueous phase drop wise with continuous stirring. The volume was made up to 50 ml. The resultant mixture was homogenized for 1 hour at 9000 rpm. The prepared SLNs were cooled to room temperature (Table 1).

Chemicals used	Transfersomes	SLNs
Soya Lecithin (mg)	90	-
Span 60 (mg)	10	-
Chloroform (ml)	2	-
Methanol (ml)	4	-
Glyceryl Monostearate (mg)	-	50
DMSO (ml)	-	5
Acetone (ml)	-	5
Tween 80 (ml)	-	0.8
Distilled water (ml)	-	25

Table 1: Formula for transfersomes and SLNs of *Cordycep militaris* extract.

Post formulation evaluation and characterization of transfersomes and solid lipid nanoparticles of *Cordycep militaris* extract

Encapsulation efficiency: By calculating the amount of free *Cordycep* in the dispersion medium from below equation, the entrapment efficiency of *Cordycep* in SLNs as well as in transfersomes was determined.

% Encapsulation efficiency=Total amount of drug-Amount of unbound drug × 100/Total amount of drug

1 ml of SLNs were taken and diluted with 10 ml of pH 6.8 phosphate buffer. The mixture was sonicated in bath sonicator for 20 minutes. It was further centrifuged at 6000 rpm for 30 minutes. The concentration of free C*ordycep* was determined in the supernatant by measuring the UV absorbance at 262 nm on UV absorption spectrophotometer. The entrapment efficiency was determined by difference from the original concentration of drug added. The same procedure was repeated for transfersomes.

Percentage drug content: 1 ml of SLN dispersion and liposomal dispersion were taken in different test tubes and diluted with 10 ml of pH 6.8 phosphate buffer. The solution was centrifuged at 6000 rpm for 40 minutes. Further the solution was diluted to 25 ml with ethanol. Then drug concentration was determined by measuring the absorbance at 262 nm using UV spectrophotometer.

% Drug loading=Amount of entrapped drug in dispersion × 100/Total weight of dispersion

Particle size and polydispersity index: The mean particle size and polydispersity index of SLNs and transfersomes were measured by photon correlation spectroscopy using Malvern Zeta sizer. It was performed at a scattering angle of 90° (at room temperature). The diameter was averaged from three parallel measurements.

***In vitro* drug release studies:** SLNs and Liposomal formulation were accurately weighed and placed in a sac of semi-permeable membrane (Cellophane membrane) separately. The liposome and SLN sacs were transferred into two separate glass beakers containing 50 ml phosphate buffer of pH 6.8. The temperature was maintained at 37°C using the thermostatically controlled heater of the magnetic stirrer and stirred at 140 rpm. The contents of the beakers were closed with aluminum foil to prevent any evaporative losses during the experimental run. Sampling were carried out at predetermined intervals of time up to 24 h, 4 ml aliquots were withdrawn and replaced by same volume of fresh buffer. The concentration of *Cordycep* was determined spectrophotometrically in each sample of liposomal and SLN formulation.

Scanning Electron Microscope (SEM) analysis: The SLNs and liposomal formulation were subjected to SEM analysis. The surface morphology as well as cross section binding of two layers of dried film was examined using a scanning electron microscope (JEOL, JSM 840, Japan). The SLNs and transfersomes were placed separately on a glass disc applied on a metallic stub and subjected to evaporation under a vacuum overnight. The samples were metalized under an argon atmosphere with a 10 nm gold palladium thickness. The surface morphology was studied for the two formulations (Figure 3).

Figure 3: Formulation of liposome and SLNs.

RESULTS AND DISCUSSION

UV method validation and calibration curve

Full sweep was read using UV program and the λ max was perceived. It was discovered to be 262 nm for *Cordycep militaris* extra (Figure 4).

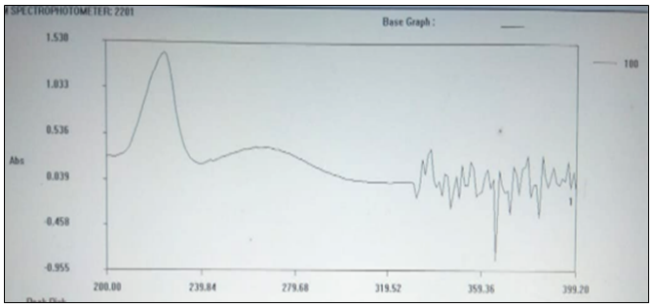

Figure 4: Absorbance maxima graph for *Cordycep militaris* extract.

Calibration plot for *Cordycep militaris* extract: The readings were taken in triplicate and the graph was plotted between absorbance on y-axis and concentration in µg/ml on x-axis. The straight line equation was found to be y=0.0851x+0.0586 with the regression coefficient value of 0.999 with the λ max of 262 nm (Table 2 and Figure 5).

Concentration (µg/ml)	Absorbance mean ± S.D (n=3)
1	0.15 ± 0.0020
2	0.222 ± 0.0015
3	0.311 ± 0.0038
4	0.408 ± 0.0040
5	0.489 ± 0.0017
6	0.568 ± 0.0055
7	0.639 ± 0.0042
8	0.731 ± 0.0011
9	0.830 ± 0.0036
10	0.918 ± 0.0082

Table 2: Absorbance readings of *Cordycep militaris* extract on UV spectrophotometer.

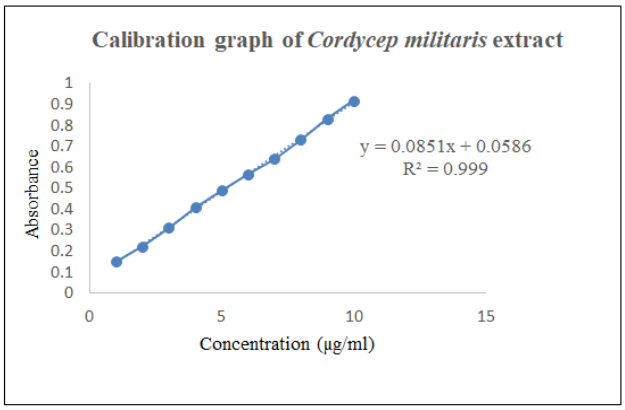

Figure 5: Calibration plot of *Cordycep militaris* extract.

Accuracy: At 80% standard development, mean recuperation of

Cordycep militaris extract was discovered to be 99.87% however at 100% and 120% standard extensions, it was discovered to be 100.39% and 99.49% separately.

Precision: % RSD estimations of intra-day exactness consider were discovered to be in the middle of 0.21 and 1.62 however those of between day accuracy consider was in the middle of 0.21 and 1.60. When all is said in done, % RSD estimations of fewer than 2 seemed the accuracy of made UV procedure (Tables 3-6).

LOD and LOQ: LOD and LOQ of proposed UV system was discovered to be 8.635 and 26.169 µg/ml independently. Lower LOQ regard shown that proposed strategy would be sensible for investigating the examples containing in fact little measures of

Cordycep militaris extract (Table 9).

SI. No.	Concen-tration (%)	Original level (µg/mL)	Amount added (µg/mL)	% Recovery	Mean % Recovery	% RSD
1	80	10	8	99.18	99.9	1.7
2	80	10	8	98.65		
3	80	10	8	101.77		
4	100	10	10	99.97	100	0.4
5	100	10	10	100.4		
6	100	10	10	100.81		
7	120	10	12	100.3	99.5	1.1
8	120	10	12	98.86		
9	120	10	12	98.29		

Table 3: Accuracy data of UV method for *Cordycep militari* extract.

S No.	Conc. µg/ mL	Morning			Afternoon			Evening		
		Absorbance mean ± S.D (n=3)	% Assay	% RSD	Absorbance mean ± S.D	% Assay (n=3)	%RSD	Absorbance mean ± S.D (n=3)	% Assay	% RSD
1	4	0.408 ± 0.0024	98.12	1.62	0.409 ± 0.0057	97.9	1.33	0.401 ± 0.0077	97.03	1.09
2	5	0.489 ± 0.0055	100.27	0.21	0.487 ± 0.0011	100.21	0.31	0.488 ± 0.0016	100.48	1.26
3	6	0.568 ± 0.0047	99.6	0.27	0.566 ± 0.0032	99.65	0.25	0.569 ± 0.0013	99.97	0.91

Table 4: Intra-day precision data of UV method of *Cordycep militaris* extract.

S No.	Conc. µg/ mL	Day 1			Day 2			Day 3		
		Absorbance mean ± S.D (n=3)	% Assay	% RSD	Absorbance mean ± S.D (n=3)	% Assay	% RSD	Absorbance mean ± S.D (n=3)	% Assay	% RSD
1	4	0.401 ± 0.0028	97.68	1.07	0.409 ± 0.0032	96.81	1.44	0.411 ± 0.0019	97	1.6
2	5	0.489 ± 0.0045	100.32	0.49	0.488 ± 0.0076	100.05	0.38	0.481 ± 0.0069	100	0.5
3	6	0.566 ± 0.0015	99.74	0.21	0.560 ± 0.0033	99.58	0.25	0.562 ± 0.0046	100	0.29

Table 5: Inter-day precision data of UV method of *Cordycep militaris* extract.

S No.	Conc. µg/Ml	Day 1			Day 2			Day 3		
		Absorbance mean ± S.D (n=3)	% Assay	% RSD	Absorbance mean ± S.D (n=3)	% Assay	% RSD	Absorbance mean ± S.D (n=3)	% Assay	% RSD
1	4	0.401 ± 0.0028	97.68	1.07	0.409 ± 0.0032	96.81	1.44	0.411 ± 0.0019	97.28	1.6
2	5	0.489 ± 0.0045	100.32	0.49	0.488 ± 0.0076	100.05	0.38	0.481 ± 0.0069	100.12	0.5
3	6	0.566 ± 0.0015	99.74	0.21	0.560 ± 0.0033	99.58	0.25	0.562 ± 0.0046	99.62	0.29

Table 6: Inter-day precision data of UV method of *Cordycep militaris* extract.

Robustness: % RSD esteems were discovered to be in the middle of 0.26 and 0.43 (Table 7).

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SI. No	Concentration (µg/mL)	Wavelength	Absorbance mean ± S.D	% RSD
1	5	262	0.489 ± 0.0012	0.27
2	5	262	0.488 ± 0.0009	0.28
3	5	262	0.489 ± 0.0046	0.26
4	5	246	0.404 ± 0.0055	0.41
5	5	246	0.405 ± 0.0032	0.42
6	5	246	0.401 ± 0.0018	0.43

Table 7: Robustness data of UV method for *Cordycep militaris* extract.

Ruggedness: Test examination and data planning achieved into % RSD esteems somewhere in the range of 0.33 and 0.50 (Table 8).

S. No	Concentration (µg/mL)	Analyst	Absorbance mean ± S.D (n=3)	% RSD
1	5	I	0.413 ± 0.0021	0.48
2	5	II	0.417 ± 0.0008	0.5
3	5	III	0.458 ± 0.0015	0.42
4	5	IV	0.459 ± 0.0030	0.33
5	5	V	0.426 ± 0.0049	0.41
6	5	VI	0.423 ± 0.0077	0.43

Table 8: Ruggedness data of UV method for *Cordycep militaris* extract.

S. No	LOD and LOQ	Value
1	LOD	8.635 µg/ml
2	LOQ	26.169 µg/ml

Table 9: LOD & LOQ data for UV method for *Cordycep militaris* extract.

Post formulation evaluation and characterization of transfersomes and solid lipid nanoparticles of *cordycep militaris* extract

The prepared Transfersomes and Solid Lipid Nanoparticles of *Cordycep militaris* extract were evaluated for various parameters which are shown in Table 10. The encapsulation efficiency for transfersomes and SLNs is 80.16% and 83.25% respectively. the difference may be due to the different lipids used in two formulations. The particle size and PDI for the two formulations is within range (Table 10).

Parameter	Transfersomes	SLNs
Encapsulation Efficiency (%)	80.16	83.25
Drug Content (%)	96.21	95.36
Particle Size (nm)	165	157
PDI	0.31	0.26

Table 10: Characterization of transfersomes and solid lipid nanoparticles of *Cordycep militaris.*

The particle size for transfersomes and SLNs were found to be 165 nm and 157 nm respectively. The particle size analysis graphs for transfersomes and SLNs are shown in Figures 6 and 7.

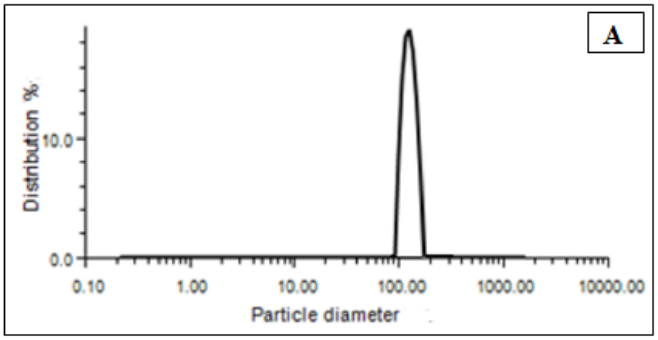

Figure 6: Particle size graph for transfersomes.

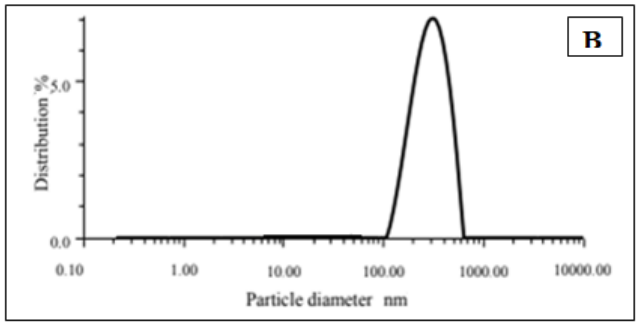

Figure 7: Particle size graph for SLNs.

***In vitro* drug release studies:** The concentration of *Cordycep* was determined spectro-photometrically in each sample of liposomal and SLN formulation. The results of *in vitro* drug release study for 24 hours are shown in the Table 11. At the end of 24 hours, the cumulative drug release for transfersomes was found to be 97.8% and for the SLNs it was found to be 98.5% over the period of 12 hours, approximately 65% of the active ingredient was released from the transfersomes. The release pattern of both the formulations indicated that the drug release over the period of 24 hours was in controlled manner (Figure 8 and Table 11).

Time (hrs)	% Cumulative release	
	Transfersomes	SLNs
0	0.125 ± 0.545	0.029 ± 0.015
1	6.065 ± 0.034	5.023 ± 0.304
2	9.887 ± 0.033	10.337 ± 0.023
3	15.124 ± 0.125	16.054 ± 0.065
4	21.061 ± 0.054	23.001± 0.234
5	29.054 ± 0.055	31.054 ± 0.065
6	35.675 ± 0.549	37.765 ± 0.045
8	42.225 ± 0.071	44.275 ± 0.675
10	53.784 ± 0.024	56.134 ± 0.124
12	65.568 ± 0.275	66.865 ± 0.375
14	77.314 ± 0.006	75.354 ± 0.576
16	83.654 ± 0.176	85.962 ± 0.076
20	90.566 ± 0.343	92.354 ± 0.025
24	97.879 ± 0.036	98.556 ± 0.146

Table 11: *In vitro* drug release studies for transfersomes and SLNs.

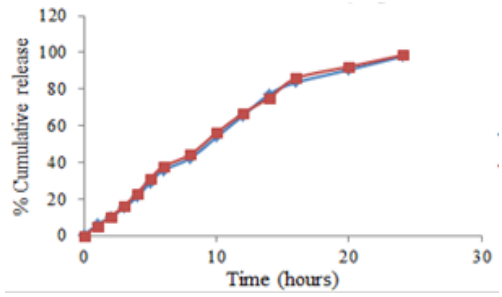

Figure 8: *In vitro* drug release profile of transfersomes and SLNs of *Cordyceps militaris.*

Note: Liposomes SLNs

SEM analysis:

The SEM analysis of transfersomes and SLNs of *Cordyceps militaris* showed that the shapes of transfersomes and SLNs prepared were spherical which is illustrated in Figure 9.

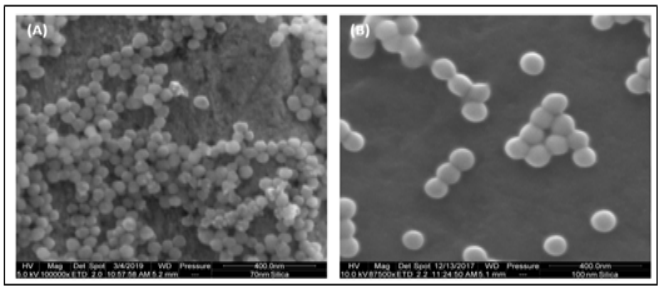

Figure 9: SEM analysis of transfersomes (A) and SLNs (B) of *Cordyceps militaris.*

CONCLUSION

The proposed spectrophotometric method was found to be, simple, sensitive, accurate and precise for determination of *Cordyceps militaris* in its extract. UV spectrum of *Cordyceps militaris* was obtained which exhibits absorption maxima at 262 nm. The calibration curve was linear in concentration range of 1-10 µg/ml. The transfersomes and SLNs of *Cordyceps militaris* were prepared successfully and various characterisation tests showed that the formulations prepared were stable. The % *in vitro* cumulative drug release at the end of 24 hours was found to be 97.879% and 98.556% for transfersomes and SLNs respectively. The results of all the characterization parameters such as % encapsulation efficiency, % drug content, particle size evaluation etc. were found to be satisfied and within the desired range.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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