IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Human Journals
Research Article
November 2021 Vol.:22, Issue:4

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

IJPPR
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ISSN 2349-7203
HUMAN

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Submitted: 20 October 2021
Accepted: 25 October 2021
Published: 30 November 2021

HUMAN JOURNALS
www.ijppr.humanjournals.com

Keywords: *Cordyceps militaris*, Transfersomes, Solid Lipid Nanoparticles, UV development, and method validation.

ABSTRACT

Cordyceps militaris, an effective antioxidant along with antihyperglycemic, antitumor, and immunomodulatory effects, belongs to the family Cordycipitaceae. The medicinal mushroom *Cordyceps militaris* has an active constituent cordycepin which possesses various pharmacological effects. The UV method development and validation of *Cordyceps militaris* extract were carried out. The extract was scanned over a UV-visible range for its wavelength of maximum absorbance. Various calibration standards were prepared, and absorbance was recorded at a 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 the hot homogenization technique. The formulations were subjected to various evaluation parameters and the results suggested that the formulations were stable 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 the 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 24 hours which indicates that the formulations showed a 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.

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 focusing 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 antioxidative, antihyperglycemic, antitumor, and immunomodulatory effects. In addition, our group has demonstrated that cultivating *C. militaris* can induce apoptosis and autophagy in human glioblastoma cells.[3]

1.1 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 fig. 1.[5]

1.2 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. Sometimes an anamorphic state, which is *Isaria*, is found. Masses of white mycelia form around the parasitized insect; however, these may not be of the same species.[6]

Figure No. 1: *Cordycep Militaris*

Chemical constituents:

The constituents of medicinal mushroom *Cordyceps militaris*, especially the anti-cancer agent cordycepin (3′-deoxyadenosine), are expected to play evolutionary roles in the pharmacognosy sector in the future as fig.2.[7]

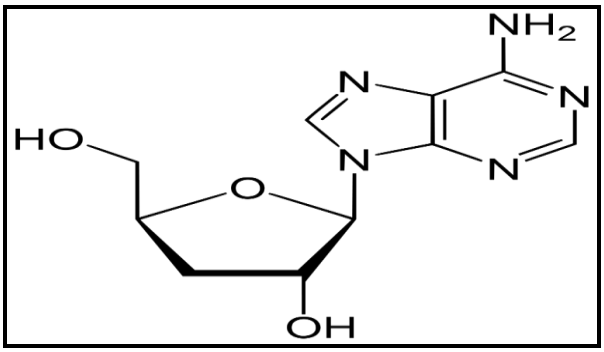

Figure No. 2: Chemical structure of Cordycepin

Experimental Instruments and reagents

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. *Cordyceps militaris* extract was obtained as a 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 *Cordyceps militaris* extract (10mg) was correctly gauged and moved into the 10ml Volumetric Flask and broke down appropriately in 1 ml acetone: DMSO and weakened sufficiently with phosphate support pH 6.8 to accomplish the last convergence of 1000 µg/ml (Stock-1). Stock-1 was appropriately weakened using the versatile stage to accomplish a 100 µg/ml (Stock-2) arrangement.

Determination of wavelength of maximum absorbance (λmax)

The Stock-2 was filtered using full output mode with medium checking speed for a whole scope of UV/VIS Spectrophotometer, reaching out from 800-200 nm with a co-dissolvable structure as a clear. After getting the reach, λmax was perceived. The over technique was reiterated threefold.

Preparation of calibration curve

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

Method Validation

Created UV methodology for the assessment of *Cordyceps 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 an 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 a basic figure for setting up the 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 the standard extension of the analyte of captivated. Three assorted plans of *Cordyceps militaris* extract were masterminded three-fold at a level of 80%, 100%, and 120% of its predefined focus (8, 10, 12 µg/ml). In predefined fixations, a particular amount of *Cordyceps militaris* extract was incorporated (standard

extension technique) and exactness was chosen dependent on percent recovery. For computing the percent recuperation, the 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

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

Robustness

The robustness of the created UV methodology was gotten using particular frequencies. The frequency was purposefully changed to 237nm and 246nm. *Cordyceps militaris* extract (6 µg/ml) was arranged freely, (n=5) and the test was broken down at λmax 237nm and 246nm for *Cordyceps militaris* extract content. The outcome was chosen as far as % RSD.[12]

Ruggedness

Ruggedness, the UV/VIS procedure was completed by dissecting a three-fold trial of *Cordyceps 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.

$$LOQ = 10xSD/S$$

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- Transfersomes and Solid Lipid Nanoparticles:**

Preparation of Transfersomes of *Cordyceps militaris* extract

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

Preparation of Solid Lipid Nanoparticles (SLNs) of *Cordyceps militaris* extract

The hot homogenization method was used for the preparation of solid lipid nanoparticles. The lipid phase and aqueous phase were prepared separately. The lipid phase contained an accurately weighed 10mg of *Cordyceps militaries* extract which was solubilized in about 10ml of solvent DMSO and acetone in a 1:1 ratio along with Glyceryl monostearate (GMS). The aqueous phase consisted of tween 80 solubilized in about 25ml of distilled water. Once both phases reached the same temperature (40^{o}C), the lipid phase was added to the aqueous phase dropwise with continuous stirring. The volume was made up to 50ml. The resultant mixture was homogenized for 1 hour at 9000rpm. The prepared SLNs were cooled to room temperature.

Table No. 1: Formula for transfersomes and SLNs of *Cordyceps militaris* extract

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

➢ **Post Formulation evaluation and characterization of Transfersomes and Solid Lipid Nanoparticles of *Cordyceps militaris* extract**

Encapsulation efficiency

By calculating the amount of free cordyceps in the dispersion medium from the below equation, the entrapment efficiency of cordycepin SLNs as well as in transfersomes was determined.

$$\% \text{ Encapsulation Efficiency} = \frac{\text{Total amount of drug} - \text{Amount of unbound drug}}{\text{The total amount of drug}} \times 100$$

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

Percentage drug content

1ml of SLN dispersion and liposomal dispersion were taken in different test tubes and diluted with 10ml of pH 6.8phosphate 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 262nm using a UV-Vis spectrophotometer.

$$\% \text{ Drug loading} = \frac{\text{Amount of entrapped drug in dispersion}}{\text{Total weight of the dispersion}} \times 100$$

Particle size and polydispersity index:

The mean particle size and polydispersity index of SLNs and transfersomes were measured by photon correlation spectroscopy using the 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 sack of semi-permeable membranes (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 37ºC 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 was carried out at predetermined intervals of time up to 24h, 4ml aliquots were withdrawn and replaced by the same volume of fresh buffer. The concentration of cordyceps 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 10nm gold-palladium thickness. The surface morphology was studied for the two formulations.

➢ RESULTS AND DISCUSSION

Figure No. 3: Formulation of Liposome and SLNs

UV Method Validation and Calibration Curve

The full sweep was read using the UV program and the λmax was perceived. It was discovered to be 262 nm for *Cordyceps militaris* extract.

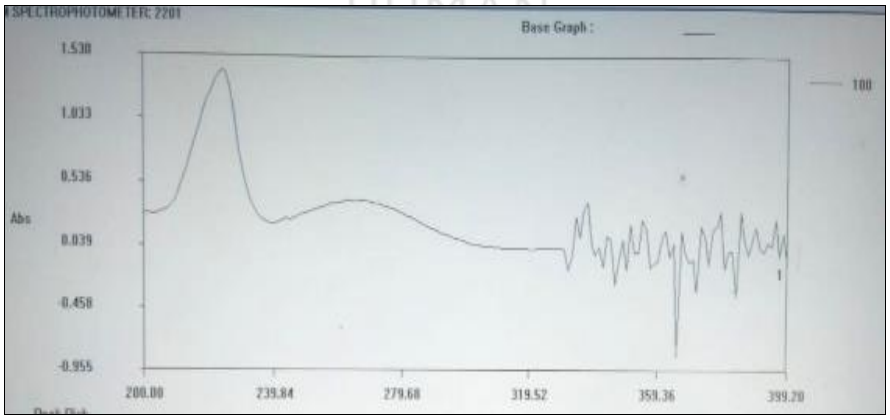

Figure No. 4: Absorbance maxima graph for *Cordyceps militaris* extract

Calibration plot for *Cordyceps militaris* extract

The readings were taken in triplicate and the graph was plotted between absorbance on the y-axis and concentration in µg/ml on the 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 λmax of 262 nm.

Table No. 2: Absorbance readings of *Cordycep militaris* extract on UV spectrophotometer

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

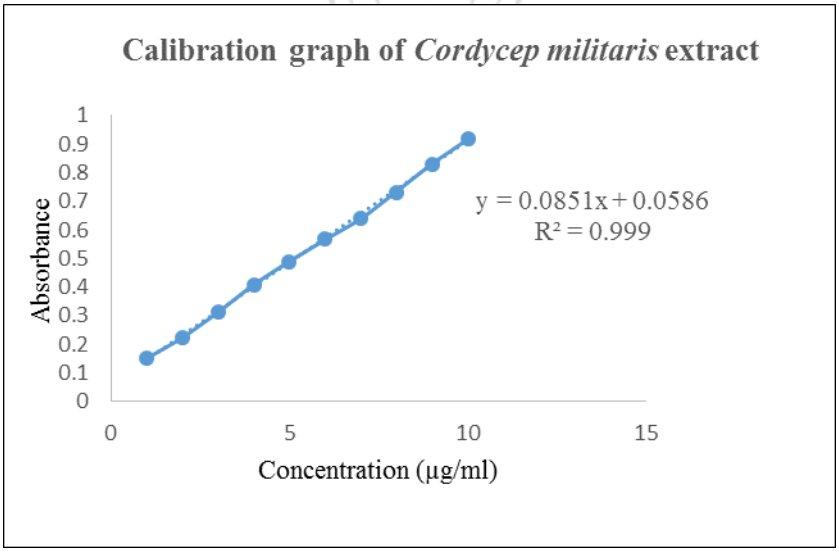

Figure No. 5: Calibration plot of *Cordyceps militaris* extract

Accuracy

At 80 % standard development, the mean recuperation of *Cordyceps 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. % RSD was discovered to be under 2 for the *Cordyceps*

militaris extract recuperation contemplates as shown in Table. From the after-effects of exactness examines, it was seen that made UV system is significantly precise as the percent recovery was in the middle of 99.49 to 100.39%, and the % RSD was well under 2%.

Table No. 3: Accuracy data of UV method for *Cordyceps militaris* extract

S. No.	Concentration (%)	Original level (µg/mL)	Amount added (µg/mL)	% Recovery	Mean % Recovery	% RSD
1	80	10	8	99.18	99.87	1.66
2	80	10	8	98.65		
3	80	10	8	101.77		
4	100	10	10	99.97	100.39	0.41
5	100	10	10	100.40		
6	100	10	10	100.81		
7	120	10	12	100.30	99.49	1.05
8	120	10	12	98.86		
9	120	10	12	98.29		

Precision

% RSD estimations of intra-day exactness considered were discovered to be in the middle of 0.21 and 1.62 however those of between-day accuracy consider were 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.

Table No. 4: Intra-day precision data of UV method of *Cordyceps militaris* extract

S No.	Conc. µg/mL	Morning			Afternoon			Evening		
		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	0.408±0.0024	98.12	1.62	0.409±0.0057	97.90	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	99.60	0.27	0.566±0.0032	99.65	0.25	0.569±0.0013	99.97	0.91

Table No. 5: Inter-day precision data of UV method of *Cordyceps 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	1.60
2	5	0.489±0.0045	100.32	0.49	0.488±0.0076	100.05	0.38	0.481±0.0069	100.12	0.50
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

Robustness

% RSD esteems were discovered to be in the middle of 0.26 and 0.43.

Table No. 6: Robustness data of UV method for *Cordyceps militaris* extract

S. 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

Ruggedness

Test examination and data planning achieved into % RSD esteems somewhere in the range of 0.33 and 0.50.

Table No. 7: Ruggedness data of UV method for *Cordyceps militaris* extract

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	0.50
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

LOD and LOQ

LOD and LOQ of the proposed UV system were discovered to be 8.635and 26.169µg/ml independently. Lower LOQ regard shown that the proposed strategy would be sensible for investigating the examples containing little measures of *Cordyceps militaris* extract.

Table No. 8: LOD & LOQ data for UV method for *Cordyceps militaris* extract

1	LOD	8.635 µg/ml
2	LOQ	26.169 µg/ml

Post Formulation evaluation and characterization of Transfersomes and Solid Lipid Nanoparticles of *Cordyceps militaris* extract

The prepared Transfersomes and Solid Lipid Nanoparticles of *Cordyceps militaris* extract were evaluated for various parameters which are shown in table 9. 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 the two formulations. The particle size and PDI for the two formulations are within range.

Table No. 9: Characterization of Transfersomes and Solid Lipid Nanoparticles of *Cordyceps militaris*

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

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

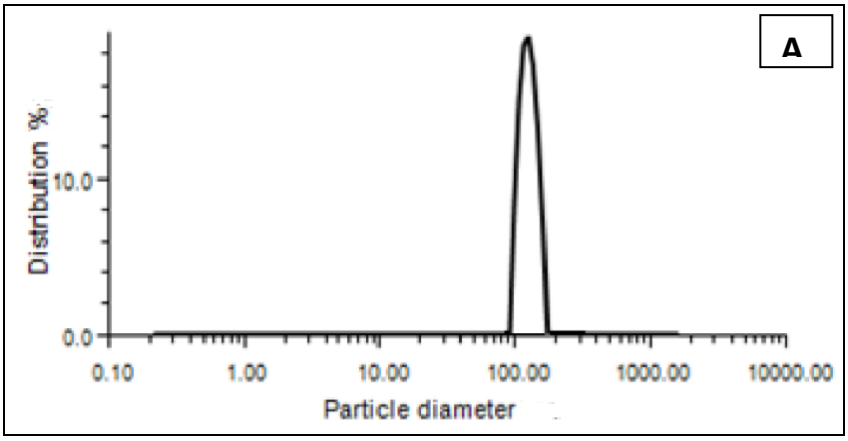

Figure No. 6: Particle size graph for transfersomes

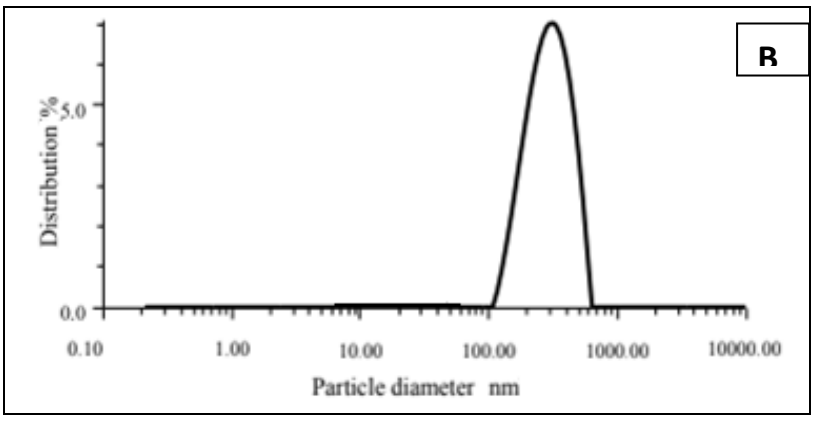

Figure No. 7: Particle size graph for SLNs

In-vitro drug release studies

The concentration of cordyceps was determined spectrophotometrically in each sample of liposomal and SLN formulation. The results of the *in-vitro* drug release study for 24 hours are shown in table 10. 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 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 for 24 hours was in a controlled manner.

Table No. 10: *In-vitro* drug release studies for transfersomes and SLNs

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

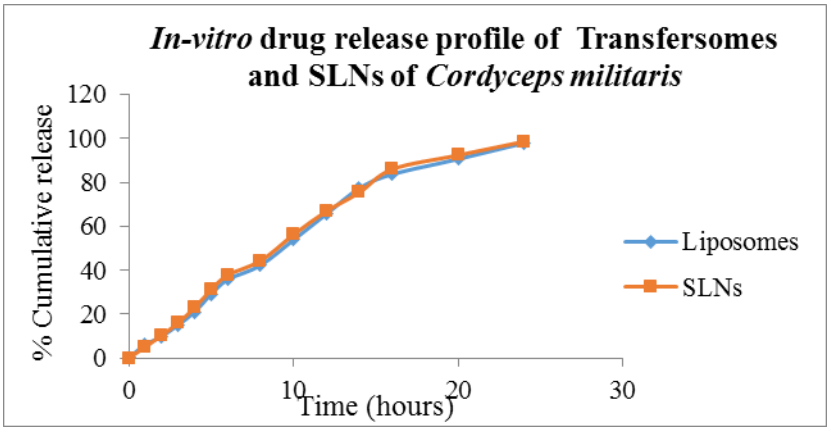

Figure No. 8: *In-vitro* drug release profile of transfersomes and SLNs of *Cordyceps militaris*

SEM analysis

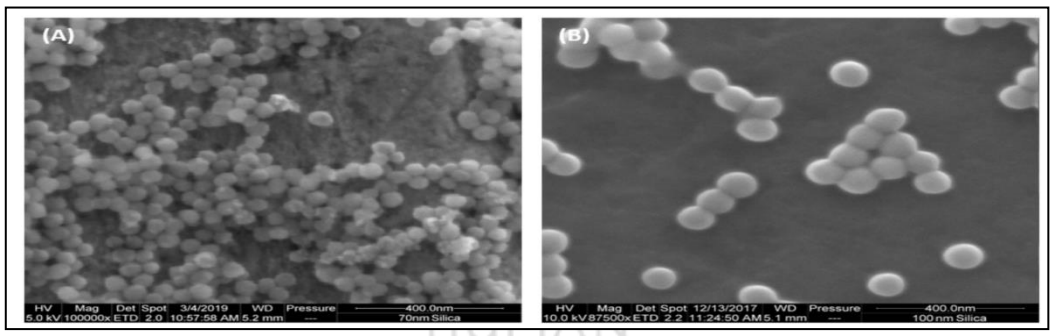

Figure No. 9: SEM analysis of transfersomes (A) and SLNs (B) of *Cordyceps militaris*

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 8.

CONCLUSION

The proposed spectrophotometric method was found to be, simple, sensitive, accurate, and precise for the 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 the concentration range of 1-10 µg/ml. The transfersomes and SLNs of *Cordyceps militaris* were prepared successfully and various characterization 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.

ACKNOWLEDGEMENTS

The author is very grateful to Honorable Chairman S. Gurvinder Singh Bahra, Rayat Bahra Group, Punjab, India for their constant encouragement and support in preparing this article. The authors hereby declare no “conflict of Interest”.

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