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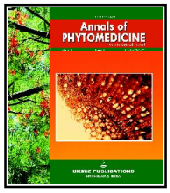

Original Article : Open Access

# Formulation, characterization and evaluation of *in vitro* antioxidant potential of melatonin and quercetin loaded liposomes

**Veerendra C.Yeligar◆, Manjiri A. Rajmane**, Yasmin H. Momin* and Rajendra C. Doijad***

*KIMDTU's Krishna Institute of Pharmacy, Karad-415110, Maharashtra State, India*

**Department of Pharmaceutical Chemistry, Annasaheb Dange College of B.Pharmacy, Ashta-416301, Maharashtra State, India*

***Ashokrao Mane College of Pharmacy, Peth Vadgaon-416112, Kolhapur, Maharashtra State, India*

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**Abstract**

The aim of the work was to use the thin film hydration process under vacuum condition to synthesize liposome loaded with melatonin and quercetin, as well as to improve the bioavailability of melatonin and quercetin from liposome. Phosphatidyl choline, cholesterol ratio, pH of hydration medium, rotating speed, and volume of solvent system were investigated as parameters impacting the entrapment of melatonin and quercetin liposome for formulation optimization. The result revealed that the $\lambda_{max}$ of the melatonin and quercetin was at 276 and 372 nm, respectively. The size of optimized liposome formulation was 43.25 nm by FESEM having a zeta potential of –26.4 mV. Average particle sizes were found to be 1109 nm and PDI of 0.624. The drug entrapment efficiency was found to be 58.56% and 95.03% for melatonin and quercet, in respectively. The percentage drug release respectively for melatonin and quercetin was 68.1% and 79.5% in 24 h, respectively. The $IC_{50}$ value of melatonin and quercetin for DPPH inhibition was found to be 9.315µg/ml and 6.261 µg/ml, respectively and for synthesized liposome 5.64 µg/ml. Finally, the results concluded that the prepared liposome could be used as controlled drug delivery system that provided the better bioavailability and antioxidant activity as compared with the standard ascorbic acid, quercetin and melatonin.

## 1. Introduction

Novel drug delivery is considered as a best well-known technique for targeted drug delivery system by which drugs' bioavailability can be improved and also controlling the rate of drug delivery for the better therapeutic efficacy. Preparation and use of liposome is such an advanced methodology in drug delivery system which improves therapeutic efficacy. Liposome is spherical natural phospholipids vesicles consisting of one or several lipid bilayers which delineate an aqueous cavity (Lorin *et al*., 2004). The main structural components of liposome are lipids such as egg or soybean phosphatidyl choline, di-palmitoylphosphatidylcholine (DPPC), similar to those found in biological membranes (Mufamadi *et al*., 2011; Suntres, 2011). Cholesterol, a sterol, most commonly used in thereafter, in liposome formulations the empty spaces between the phospholipids molecules are filled with cholesterol which strengthens the liposomal membrane and provides structural stability (Sirisha *et al*., 2012). In the last decades, liposome's have received considerable attention due to their particular characteristics such as high biocompatibility, low toxicity, ability to encapsulate both hydrophilic and hydrophobic compounds (Malam, *et al*., 2009; Chen *et al*., 2010). As drug delivery systems, liposomes have been proposed for transporting different therapeutic agents for cancer treatment, vaccine immunization, gene therapy, radiopharmaceuticals and cosmetic formulations (Torchilin, 2005; Irache *et al*., 2011).

**Corresponding author: Dr. Veerendra C.Yeligar**

*Professor, KIMDTU's Krishna Institute of Pharmacy, Karad-415110, Maharashtra State, India*

**E-mail: yveerendra27@gmail.com**

**Tel.: +91-9975019279**



Melatonin (N-acetyl-5-methoxytryptamine, MT) is a synchronizer of biological rhythms that is found in animals and humans. Melatonin is a neurohormone, secreted primarily by the pineal gland in the hours of darkness. The organs like retina, lens, ovary, testis and bone marrow in a circadian rhythm also produce MT. Melatonin plays an important role as a regulator of sleep and can also mediate several cellular, neuroendocrine and physiological processes. Moreover, the free radical scavenging activity of MT established it as an endogenous antioxidant (Tan *et al*., 2000). For such relevant biological properties associated with extremely low toxicities, MT can be utilized for its therapeutic potential, especially in patients suffering from delayed sleep phase syndrome, sleep disturbances in blind people. It also facilitates adaptation to jet lag in travelers, cosmonauts and shift workers (Srinivasan *et al*., 2008; Roach and Sargent, 2019). Further, several clinical and preclinical studies are in progress in order to justify the therapeutic efficacy of this neurohormone especially in the fields of cancer treatment and topical protectant (Najafi *et al*., 2017). When just a modest proportion of the free drug reaches the cancer, systemic administration of the drug is the most typical clinical failure of chemotherapy in anticancer therapy as active pharmaceutical ingredients in chemotherapy are particularly cytotoxic to both malignant and non-cancerous cells. As a result, tumors treatment must concentrate on the tumor's blood vessels. Anticancer drugs incorporated in the liposomal system provide safe platforms for drug delivery system. Targeted administration of liposomal anticancer drugs may be advantageous in reducing the harmful effects of free anticancer drugs on normal tissues.

Quercetin (3,3',4',5,7-pentahydroxyflavone, a natural flavonoid, ubiquitously present in the plant kingdom, is considered to be one

of the best antioxidant flavonoids due to the high number and position of hydroxyl groups, and conjugated π-orbital (Gibellini *et al*., 2011). It has been shown that QU can prevent or protect against damage caused by free radicals in various ways. One way is by directly scavenging free radicals, leading to more stable and less reactive radicals (Nijveld *et al*., 2001; Das *et al*., 2021). Other mechanisms include modulation of gene expression, interactions with different biological enzyme systems and inhibition of lipid peroxidation (Molina *et al*., 2003; Baghel *et al*., 2012; Imad Uddin *et al*., 2020). Also, quercetin is particularly known for its ability to chelates transition metal ions such as iron ions (Maiti *et al*., 2005). Scientific literatures have revealed that quercetin exhibits different biological effects, including anti-inflammatory, antiallergic, antimicrobial (Baghel *et al*., 2012; Yadav *et al*., 2021), Effect of piperine and quercetin pretreatment on safety profile of marbofloxacin after repeated oral administration in broiler chickens (Patel *et al*., 2020). The effect of opuntiae latior fruit juice and quercetin on biochemical parameters and histopathological changes in diabetic rats (Kotadiya *et al*., 2018). Notwithstanding these biological potential in the management and treatment of human diseases, the use of quercetin is restrained by its low aqueous solubility, high rate of metabolism, rapid clearance from plasma and cells, and poor bioavailability. These problems can be overcome by developing a liposomal delivery system to solubilized QU, improve its pharmacokinetic and pharmacological properties (Landi-Librandi *et al*., 2012; Mignet *et al*., 2013) as liposome facilitate intracellular delivery and prolong the retention time of encapsulated drugs inside cells. The use of liposomes has been shown to maintain the desirable, synergistic drug ratios as well as to coordinate the release of the encapsulated drug combinations to achieve increased antioxidant activity. In view of these advantages, our approach is to deliver a combination regimen with synergistic drug ratios through liposome's to develop potentially effective treatment options. The drug combination to be co-encapsulated in liposomes is melatonin and quercetin. This is further supported by our previous findings that the melatonin/quercetin combination, given at molar ratios of 1:1, 1:2, 4:1, 1:2 and 1:4 out of this with the molar ratio of 1:4 which is more than the expected additive effect (Kirakosyan *et al*., 2010). One goal in the development of drug delivery systems, including liposome's, is to incorporate a sufficient amount of drug in order to assure an optimum concentration at the site of action, and thus therapeutic effectiveness. To achieve optimum encapsulation of a drug into liposome's, parameters influencing both the carrier and the drug such as liposome size, surface charge, lipid composition, bilayers rigidity, need to be considered during the early stages of development (Porfire *et al*., 2013).

The main objective of the research was to develop new melatonin and quercetin-loaded liposomes utilizing a simple thin film hydration process as an antioxidant formulation. Hence, melatonin and quercetin-loaded liposomes were produced and characterized in this study, and the percent inhibition of DPPH as an antioxidant activity was also assessed.

## 2. Materials and Methods

### 2.1 Material

Quercetin and melatonin (99%) were purchased from Swapnaroop Pharmaceuticals, Aurangabad, India. Phosphatidyl choline was purchased from Research lab, Mumbai. Cholesterol, chloroform, and methanol were purchased from Molychem, Mumbai.1, 1-diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sisco Research Laboratories, Taloja. All other materials and solvents used were of analytical grade.

### 2.2 Method

#### 2.2.1 Preparation of melatonin and quercetin loaded liposome

Multi-lamellar vesicles (MLV) liposome consisting of mixtures of phosphatidyl choline and cholesterol in different molar ratios (Table. 1) as lipid phase was obtained by thin film hydration technique. Briefly, the lipid mixture and melatonin and quercetin (1:4) was dissolved in 3:2 v/v of chloroform: methanol which was then removed under vacuum at 45°C, thus obtained a thin film of dry lipid on the flask wall using a rotary flash evaporator until film was formed. After the dry residue appeared, completely removed all the traces of solvent. The film was then hydrated by adding phosphate buffer (pH 5.4) under vigorous mechanical shaking with a vortex mixer until vesicle formation. The liposome was further reduced in size using Sonicator to form small unilamellar vesicles. The liposomal suspension was characterized for physical appearance, entrapment efficiency, SEM, FTIR, DSC, *in vitro* drug release (Satyavathi, *et al*., 2015).

**Table1: Composition of liposome formulations**

| Formulation code | PC:CH | Phosphatidyl choline (mg) | Cholesterol (mg) |
|---|---|---|---|
| B1 | 1:0 | 100 | 0 |
| B2 | 1:0.1 | 94.33 | 5.67 |
| B3 | 1:0.2 | 89.27 | 10.73 |
| B4 | 1:0.3 | 84.73 | 15.27 |
| B5 | 1:0.4 | 80.63 | 19.73 |
| B6 | 1:0.5 | 76.90 | 23.10 |
| B7 | 1:0.6 | 73.51 | 26.49 |
| B8 | 1:0.7 | 70.40 | 29.60 |
| B9 | 1:0.8 | 67.54 | 32.46 |
| B10 | 1:0.9 | 64.91 | 35.09 |
| B11 | 1:1 | 62.48 | 37.52 |
| B12 | 1:1.1 | 60.23 | 39.77 |
| B13 | 1:1.2 | 58.11 | 41.89 |
| B14 | 1:1.3 | 56.15 | 43.84 |
| B15 | 1:1.4 | 54.32 | 45.67 |
| B16 | 1:1.5 | 52.61 | 47.38 |
| B17 | 1:1.6 | 50.99 | 49.00 |
| B18 | 1:1.7 | 49.48 | 50.51 |
| B19 | 1:1.8 | 48.05 | 51.99 |

### 2.3 Optimization of liposome preparation

#### 2.3.1 Speed of the rotary evaporator

The final output of liposomal preparation was governed by the formation of a thin and uniform film. The average speed of rotation was 100 rpm during film formation as well as during hydration.

### 2.3.2 The ratio and volume of solvent system

The chloroform and methanol solvent system was optimized by taking various combinations of these organic solvents. The ratios 3:1, 3:2 and 3:3 were tried and the film was evaluated in terms of its uniformity.

### 2.3.3 pH of the hydrating media

The effect of pH of the phosphate buffer was studied on the formulation. Entrapment efficiency was calculated when the pH of the hydration buffer was changed to levels closer to the drug's pka. Distilled water and phosphate buffer pH 4.0, 5.4, 6.4 and 7.0 were used as hydrating media and formulations were studied for entrapment the efficiency.

## 2.4 Characterization of liposome

### 2.4.1 Determination of vesicle entrapment efficiency

The drug loaded liposome was separated from the aqueous phase by centrifugation at 15000 rpm using microspin centrifuge for 30-45 min until a clear supernatant was obtained. The amount of drug loaded was estimated by both direct and indirect methods.

In direct method, the supernatant was decanted after centrifugation. The formed pellet was washed with phosphate buffer and vortexes, in order to remove the free drug adsorbed on the surface of liposome. This step was repeated thrice to completely separate the entrapped drug. The resulting pellet was then dissolved in methanol and sonicated for 20 min. The resulting methanolic solution was then analyzed by UV spectrophotometer at 372 nm and 276 nm using the developed analytical method.

% Entrapment efficiency (E.E.),

$$= \frac{(\text{Amount of drug quantified in the liposome})}{\text{Total amount of drug added}} \times 100$$

### 2.4.2 Determination of vesicle size and size distribution

Vesicle size was determined by dynamic light scattering (DLS) using a computerized inspection system (Malvern particle size analyzer). Freshly prepared batches of vesicles were diluted ten-fold with filtered distill water and then analyzed at 25°C using ten runs at each scan. Optimization time was fixed at 120s. The measurements were done in triplicate.

### 2.4.3 Zeta potential measurement

Liposomal suspension was diluted with filtered distilled water and zeta potential was measured in triplicate with the help of Malvern zetasizer (Aparajita and Padmini, 2015).

### 2.4.4 Field emission scanning electron microscopy (FESEM)

A drop of liposome (liquid form) was spread on conducting carbon tape which was already pasted on aluminum stub (base) of FESEM (TESCAN, Model: Mira3) chamber. The stub was kept in oven at 100°C for 10 h to ensure total moisture removal. The image was captured using electron detector which detects the secondary electron emitted from sample when high energy electron beam with high voltage (kV) strikes on sample surface.

### 2.4.5 Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra are used to detect the interaction of drug with other excipients. FTIR spectrum of drug and optimized liposome batch was obtained on FTIR (Agilent Cary 630) instrument. The spectrum of optimized batch of liposome was taken. Sample of liposome was kept onto sample holder surface for analysis. The spectrum was scanned over the range of 4000-400 $cm^{-1}$.

### 2.4.6 Differential scanning calorimeter analysis

Thermal properties of pure drug and optimized batch of liposome was analyzed by DSC (TA Instruments, USA Model: SDT 2960). Indium standard was used to calibrate the DSC temperature and enthalpy scale. Nitrogen was used as the purge gas through DSC cell at flow rate of 50 ml per min and 100 ml per min through the cooling unit. The sample (1 drop) was heated in a hermetically sealed aluminum pans. Heat runs for each sample were set from 25 to 5000 at a heating rate of 100°C/min.

### 2.4.7 Drug release studies

Drug release studies were carried out according using dialysis method. The experiments were performed using 200 ml of the medium which was prepared by using a magnetic needle. Dialysis membrane was used as a barrier to isolate the donor and the receptor phase. Liposomal dispersion was placed in a dialysis bag of cellulose acetate, which was immersed in the medium and magnetically stirred. The stirring was carried out at 100 rpm at 37°C ± 0.5°C. The simulation of GI transit condition was achieved by altering the pH of the dissolution medium at different time intervals. For the initial 2 h, the pH of the dissolution medium was adjusted to 1.2 using 0.1 NHCl. Subsequently, the pH of the dissolution medium was adjusted to 7.4 with 0.1 N NaOH and buffer salts, and maintained up to 20 h. Samples taken from the receiver solution at predetermined times were replenished with equal volumes of fresh buffer and spectrometrically assayed for drug content by UV-visible spectroscopy (Ahmed *et al*., 2013).

### 2.4.8 *In vitro* antioxidant activity

The free radical scavenging activity of liposome suspension was evaluated by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) inhibition assay. 0.1 mM solution of DPPH in methanol was prepared, and 1 ml of this solution was added to 3 ml of the solution of all liposome suspension at different concentrations (2, 4, 6, 8 and 10 µg/ml). The mixtures were shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm using UV-visible spectrophotometer. Ascorbic acid was used as the reference standard. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability to scavenging the DPPH radical was calculated by using the following formula.

% Inhibition (DPPH scavenging effect) = $\{(A_0 - A_1)/A_0) \times 100\}$.

where, $A_0$ is the absorbance of the control reaction, and $A_1$ is the absorbance in presence of all of the liposome suspension samples and reference standard (Shen *et al*., 2010).

# 3. Results

## 3.1 Effect of process parameters on liposome preparation

### 3.1.1 Speed of rotation of rotary evaporator

During hydration of lipid film sufficient energy and vigorous shaking is required for the dried film to get hydrated and form liposome, thus 100-150 rpm was found adequate to form uniform liposomal dispersion. Effect of speed of rotation of evaporator on the film formation was studied.

### 3.1.2 The ratio and volume of solvent blend

The choice of solvent is usually chloroform owing to its low boiling point (61.15°C) and easy removal under vacuum leading to thin film deposition on the walls of the flask. Chloroform: methanol 3:2 ratio was selected to give an even and uniform film of liposome ranging from 0.1 to 1 µg.

### 3.1.3 pH of the hydrating media

Maximum ionization (~90%) would hence occur at pH values ± one unit of the pka value. Phosphate buffer pH 5.4 was selected as it yields uniform suspension with minimal sedimentation and maximum entrapment efficiency. Sedimentation was observed when pH of the buffer was raised beyond 7 whereas phase separation was observed towards acidic pH.

**Table 2: Entrapment efficiency of liposome formulations**

| Formulation | Entrapment efficiency (%) | |
|---|---|---|
| | Melatonin | Quercetin |
| B1 | 41.18 ± 0.015 | 72.60 ± 0.008 |
| B2 | 35.89 ± 0.012 | 56.33 ± 0.004 |
| B3 | 34.93 ± 0.013 | 66.95 ± 0.006 |
| B4 | 31.41 ± 0.010 | 58.55 ± 0.010 |
| B5 | 20.67 ± 0.013 | 46.23 ± 0.011 |
| B6 | 35.89 ± 0.016 | 64.55 ± 0.013 |
| B7 | 48.07 ± 0.014 | 84.76 ± 0.014 |
| B8 | 35.41 ± 0.010 | 48.45 ± 0.016 |
| B9 | 58.65 ± 0.011 | 95.03 ± 0.017 |
| B10 | 63.71 ± 0.017 | 76.19 ± 0.014 |
| B11 | 35.09 ± 0.016 | 73.97 ± 0.012 |
| B12 | 40.08 ± 0.014 | 70.79 ± 0.018 |
| B13 | 31.57 ± 0.018 | 68.83 ± 0.017 |
| B14 | 53.23 ± 0.012 | 77.05 ± 0.018 |
| B15 | 58.97 ± 0.019 | 65.92 ± 0.013 |
| B16 | 53.34 ± 0.016 | 75.34 ± 0.011 |
| B17 | 49.03 ± 0.015 | 72.43 ± 0.019 |
| B18 | 51.12 ± 0.010 | 70.19 ± 0.009 |
| B19 | 48.00 ± 0.009 | 67.19 ± 0.015 |

## 3.2 Characterization of liposome

### 3.2.1 Drug entrapment efficiency

Table 2 shows that drug entrapment efficiency increased with increasing the amount of polymer. The entrapment efficiency of melatonin and quercetin was found to be in the range from 20.67 % to 73.68 % and 46.23 % to 95.03 %, respectively. It was observed that the entrapment efficiency depends on the concentration of cholesterol and phosphatidyl choline.

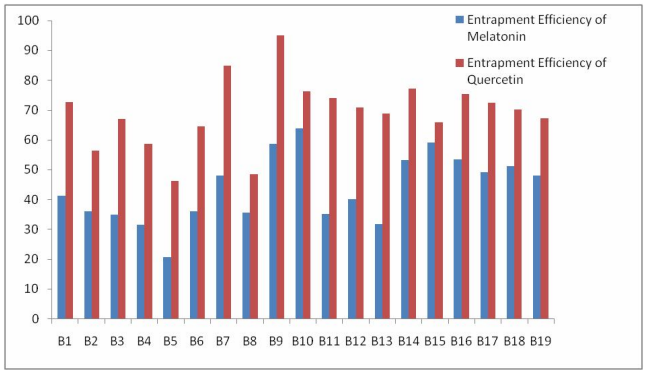


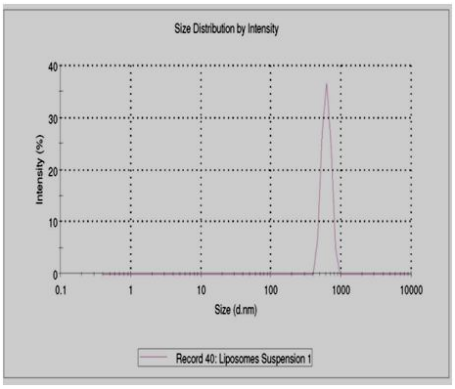

**Figure 1: Drug entrapment efficiency of liposome formulation.**

### 3.2.2 Determination of vesicle size and size distribution

Characterization of mean particle size distribution is one of the most important critical parameter in the manufacture of many products. The particle size distribution has a direct influence on material properties. Determination of mean average particle size of liposome suspension was carried out by using Malvern instrument ver. 6.12. The average particle size of preparation was found to be 110.9 nm and the polydispersity index (PDI) was 0.624 (Figure 2A).

### 3.2.3 Zeta potential measurement

Zeta potential is a measure of the magnitude of the electrostatic charge repulsion or attraction between particles, and is one of the fundamental parameters known to affect stability in terms of dispersion, aggregation or flocculation, and can be applied to improve the stability of formulations of dispersed systems. Determination of average zeta potential of liposome suspension was carried out by using Malvern zetasizer ver. 6.12. Zeta potential of prepared optimized liposome was found to –26.4 mV which indicate sufficient stability (Figure 2B).



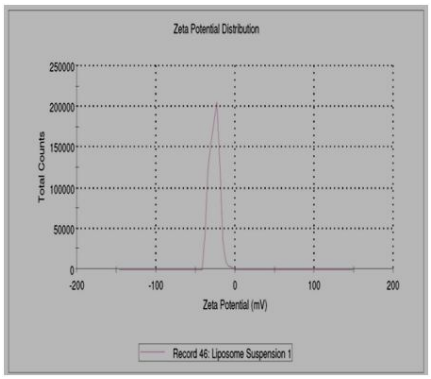



**Figure 2: (A) Size distribution report, and (B) Zeta potential report of optimized formulation B9 liposome.**

### 3.2.4 Field emission scanning electron microscopy (FESEM)

The particle size for B9 optimized formulation of liposome was found to be 43.25 nm.

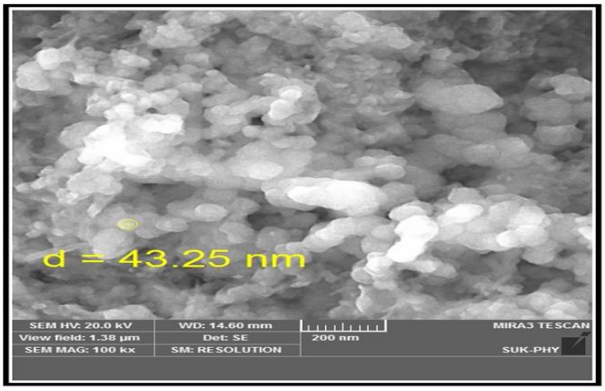



**Figure 3: Field Emission Scanning Electron Microscopy of optimized formulation B9 liposome.**

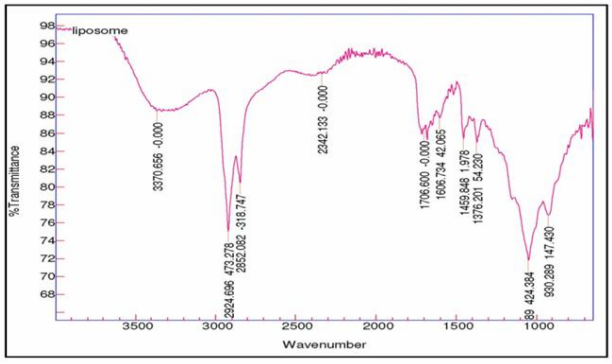



**Figure 4: FTIR spectrum of optimized formulation of liposome B9.**

### 3.2.5 Fourier transform infrared spectroscopy

To study the interaction between the drugs and other excipients, FTIR spectroscopy was performed. Figure 4 shows IR spectra of liposome formulation (B9). The characteristic peaks of melatonin (N-H stretching – 3264 $cm^{-1}$, C-O $^{-1}$100 $cm^{-1}$, C-H stretching – 2922 $cm^{-1}$, C-N – 1367 $cm^{-1}$) and quercetin (C=O – 1656 $cm^{-1}$, O-H – 3227 $cm^{-1}$, C-O – 1162 $cm^{-1}$ , C=C – 1602 $cm^{-1}$) appeared in liposome formulation (N-H stretching – 3370 $cm^{-1}$ , C-O – 1054 $cm^{-1}$ , C-H stretching - 2924 $cm^{-1}$ , C-N – 1376 $cm^{-1}$ , C=O – 1706 $cm^{-1}$ , C-O – 1054 $cm^{-1}$ , C=C – 1606 $cm^{-1}$ ) with slight shift in peak position. These insignificant peak shifts indicate the absence of any interaction between the drugs and excipients used in the formulation.

### 3.2.6 Differential scanning calorimeter (DSC)

Thermal behavior of melatonin, quercetin, phosphatidyl choline, cholesterol and the physical mixture were studied using DSC. A DSC thermogram of melatonin and quercetin showed an endotherm at 115$^0$C and 310.36$^0$C. Broad peaks were observed for phosphatidyl choline from ~50 to 220$^0$C. For cholesterol, the melting process took place with maximum peak at 147.10$^0$C. The thermogram of the physical mixture was almost the overlap of each individual component, except for some slight differences. DSC thermogram of the physical mixture showed the peak of Melatonin at ~ 115$^0$C and cholesterol at ~147$^0$C and integrated peak of quercetin at ~310$^0$C. The area under the curve for the quercetin thermogram was lesser as compared to that of quercetin alone. This may be due to the melting of the lipid components and their interactions with quercetin.

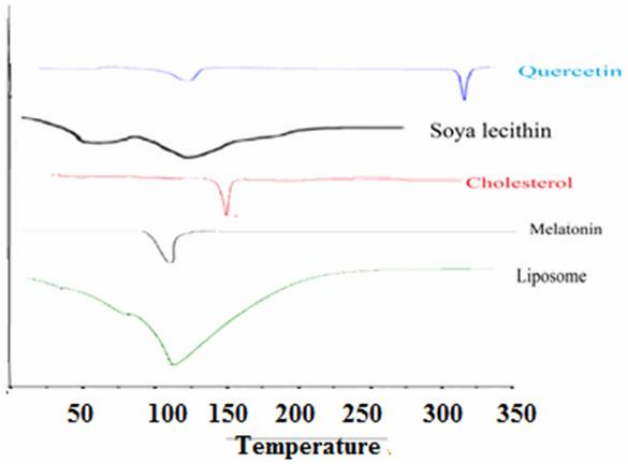



**Figure 5: DSC thermogram of optimized liposome formulation B9.**

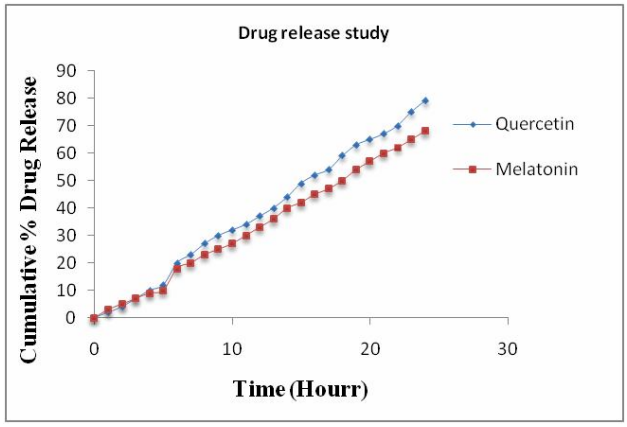



**Figure 6: Drug release study of optimized formulation B9.**

### 3.2.7 Drug release

The *in vitro* drug release curve of optimize formulation (B9) liposome suspension in phosphate buffer of pH 7.4 is shown in Figure 6. The drug release (%) for melatonin and quercetin was 68.1 % and 79.5 % in 24 h, respectively. An initial burst release was observed during first 5 h, followed by a continuous and slower release.

### 3.2.8 *In vitro* antioxidant activity

The *in vitro* antioxidant activity of liposome suspension was very good and found close to the standard ascorbic acid. The $IC_{50}$ value of melatonin, quercetin, ascorbic acid and liposome suspension were found to be 9.315 µg/ml, 6.261 µg/ml, 5.076 µg/ml and 5.645 µg/ml, respectively. Absorbance of control reaction = 1.009

**Table 3: Observed value of DPPH assay**

| Concentration | % Inhibition of DPPH by quercetin | % Inhibition of DPPH by melatonin | % Inhibition of DPPH by liposome | % Inhibition of DPPH by ascorbic acid |
|---|---|---|---|---|
| 2 | 88.01 | 86.03 | 86.03 | 86.03 |
| 4 | 90.98 | 89.99 | 91.98 | 92.97 |
| 6 | 97.92 | 95.94 | 94.95 | 93.96 |
| 8 | 98.91 | 96.93 | 98.91 | 97.92 |
| 10 | 99.9 | 99.9 | 99.9 | 99.9 |
| IC 50 Value | 6.261 | 9.315 | 5.645 | 5.076 |

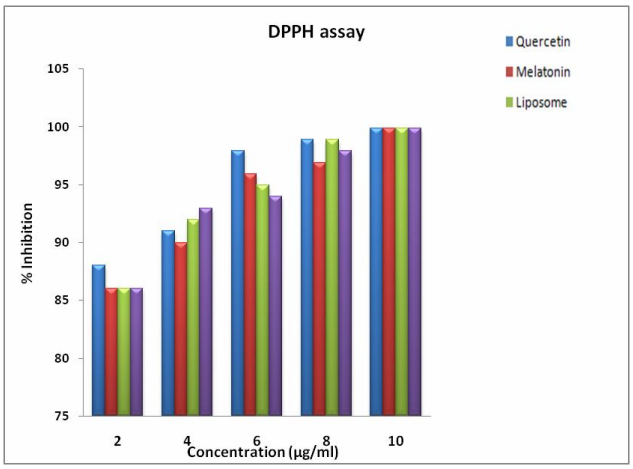



**Figure 7: Histogram of *in vitro* antioxidant activity of quercetin, melatonin and liposome.**

## 4. Discussion

Various parameters such as drug entrapment efficiency, vesicle size and size distribution, zeta potential measurement, field emission scanning electron microscopy (SEM), fourier transform infrared spectroscopy (FTIR), and differential scanning calorimetry (DSC) were used to characterize the liposomes in this study, revealing that the synthesis of liposomes was successfully achieved. Phospholipids (PL) and cholesterol (CH), with good biocompatibility and biodegradability, are used as liposome wall materials, and are able to enhance the bioactivity by improving drug solubility and bioavailability, *in vitro* and *in vivo* sustainability, as well as inhibits influence with the external environment. Uneven and bumpy film formed at higher speed of rotation (150 rpm), has been reported (Jessy and Maria, 2014) for the insufficient time of contact and improper distribution of heat that may have not allowed the liquid to gel transitions of the lipid. In contrary, lower speeds increases the time of contact of the film with the hot water in the water bath. Based upon these observations, 80 rpm seemed to be optimum for formation of uniform film.

The extent of ionization is influenced by the pka of any drug. Pka is the one of the significant factor that influences drug entrapment. The pka of melatonin and quercetin are cited as 15.8 and 6.4, respectively in the literature. Hence, further batches were prepared at pH value of 5.4 (Aparajita and Padmini, 2015).

For drug release, as expected, encapsulation of melatonin and quercetin into liposome led to controlled release rate due to the well-known reservoir effect of liposome. The burst release occurred due to the presence of the free melatonin and quercetin in the external phase and on the surface of the liposome (Haider *et al.*, 2020). The present study was also followed the same trend.

The fate of intravenously injected liposome is determined by a number of properties and among those, the particle size and zeta potential are most important. Moreover, also adsorbed enzyme to the liposome may possibly affect its zeta potential. Remarkable value of zeta potential (>20 mV), should stabilize the liposome and protect against their aggregation. Therefore, the zeta potential changes might be a valuable source of information shedding an additional light on liposome properties, including the hydrolysis reaction (Chibowski and Szczeœ, 2016). The increased size of the liposome is probably due to their aggregation, possibly caused also by the enzyme adhesion and/or bridging mechanism by the hydrolysis products which also showed in the present study and confirmed with FTIR study and DSC.

The DSC spectrum of the complex reveals the characteristic absence of the melting peak of quercetin at $310^0$C. The complete absence of the melting endotherm of quercetin suggested significant interaction of quercetin with the bilayers structure. This phenomenon can be assumed as proof of interactions between the components of the respective binary systems (Bhalerao and Harshal, 2003; Leyva-Porras *et al.*, 2020). This can be considered as indicative of drug amorphization and/or inclusion complex formation.

Furthermore, previous reports revealed antioxidant activity of the liposome. According to the histogram (Figure 7) the DPPH radical scavenging activity of liposome showed an increased percent inhibition of DPPH with concentration, indicating a clear relationship

between DPPH inhibition and liposome concentration. This investigation also revealed that the prepared liposome had the same percentage of DPPH inhibition.

## 5. Conclusion

Multiple batches of liposomes were prepared using the thin film hydration method, which is a simplistic method that can be employed in the laboratory production of liposomes, to evaluate the effect of the rotary evaporator's rotation speed. 3:2 v/v of chloroform: methanol mixture was used for uniform dispersion of drugs in lipid phase. The FTIR and DSC studies revealed absence of any physicochemical interaction components of formulation. The liposomes have major role in medical treatment and research which provide a stabilized therapeutic drug or drug combination in order to overcome difficulties in tissue and cellular uptake and provide better biodistruibution as well in site when used *in vivo* as the combination containing melatonin and quercetin provide antioxidant properties may have positive effects in the treatment of pulmonary diseases characterized by especially edema, inflammation, and fibrosis. The prepared liposome could be used as controlled drug delivery system which provides the better bioavailability and antioxidant activities comparable to the standard drug ascorbic acid.

## Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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