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Preparation and characterization of isoniazid-loaded crude soybean lecithin liposomes

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ABSTRACT

Tuberculosis (TB) is a poverty related infectious disease that is rapidly giving rise to public health concerns. Lengthy drug administration and frequent adverse side-effects associated with TB treatment make anti-tubercular drugs (ATDs) good candidates for drug delivery studies. This work aimed to formulate and prepare liposomes as a cost-effective option for ATD delivery. Liposomes were prepared by film hydration using crude soybean lecithin (CL) and not pure phospholipids as in the normal practice. Cholesterol was also used (up to 25% mass ratio), and isoniazid (INH) was encapsulated as model drug using a freeze-thaw loading technique. Purified soybean lecithin (PL) was also used for comparative purposes, under the same conditions. INH-loaded liposomes were characterized for particle size, Zeta Potential (ZP), encapsulation efficiency (EE) and drug release. Physicochemical properties were investigated using thermogravimetric analysis, differential scanning calorimetry, X-ray diffraction and Fourier transform infrared. INH-loaded CL-based liposomes showed high EE ($79 \pm 2.45\%$). The average particle size (813.00 ± 9.21 nm) and ZP (-42.80 ± 4.31 mV) of this formulation are promising for the treatment of TB by pulmonary delivery. These findings suggest the possibility of encapsulating ATDs in liposomes made of crude soybean lecithin that is cheap and readily available.

1. Introduction

Tuberculosis (TB) is one of the top two most devastating infectious diseases worldwide, second only to the human immunodeficiency virus (HIV) infection. In 2015, the world health organization (WHO) reported 10.4 million of new active TB-cases and 1.8 million deaths (World Health Organization, 2016). The current anti-TB regimen offers high cure rates – up to 95% – in the case of drug susceptible TB (Nuermberger et al., 2010). The pharmacological concerns in this field include the poor bioavailability of some anti-TB drugs, rapid first pass metabolism and non-selective biodistribution. These result in extended TB treatment of 6 to 24 months, using multiple drugs. This lengthy therapeutic regimen is associated with several adverse effects and low patient compliance that promotes both the development of drug resistance and therapeutic failure (Sosnik et al., 2010). The firt line anti-TB drugs are isoniazid, rifampicin, pyrazinamide and ethambutol (Kadare et al., 2014). Due to various side effects related to their clinical use, all these antimicrobial agents are good candidates for drug delivery in order to control their pharmacokinetics beneficially.

Liposomes are lipid-based vesicular devices that are widely used as drug delivery systems. Among all the current delivery systems, liposomes represent the most clinically established drug carriers (Guptha, 2015). Liposomes are known to be taken up by the cells of mononuclear phagocytic system, especially macrophages. This

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feature is known as a longstanding inconvenience in terms of circulation half-life, when the site of interest is beyond macrophages (Immordino et al., 2006). However, the causative agent of TB, *Mycobacterium tuberculosis,* resides in macrophages, making treatment difficult (Tăbăran and Catoi, 2014). The biological fate of liposomes therefore enhances their appropriateness as a vehicle for anti-TB drug delivery.

Liposomes are already being extensively studied for the delivery of anti-tubercular drugs. Chimote and Banerjee (2010) encapsulated isoniazid in liposomes using dipalmitoyl phosphatidylcholine, DPPC. These INH-loaded DPPC-liposomes demonstrated a dual therapeutic benefit for the management of TB – pulmonary medication and lung surfactant mimic action. Vyas et al. (2004) developed ligand-anchored and negatively charged liposomal aerosols that enhanced and sustained the release of rifampicin (RIF) in macrophages. This formulation showed higher clearance rate of *Mycobacterium smegmatis* residing in rat alveolar macrophages than non-decorated and neutral RIF-loaded liposomes. Recently, Patil et al. (2015) prepared and characterized freeze-dried RIF-loaded liposomes that demonstrated controlled and sustained release profile by intra-tracheal instillation to Wister rat model. In addition, dry powder formulations based on INH-loaded pro-liposomes have demonstrated attractive flow ability, powder performance and high antimicrobial activity in infected macrophages without any inflammatory or cytotoxic effects (Rojanarat et al., 2011). Other groups have reported similar successful encapsulations of anti-TB drugs in liposomal systems (Singh et al., 2015; Patil-Gadhe et al., 2014; Manca et al., 2012; Pandey et al., 2004).

However, the costly formulation status of liposomes is often due to the use of expensive synthetic or purified natural phospholipids (Li et al., 2015; Yokota et al., 2012), which might preclude their use in the management of poverty related diseases like TB, where many patients cannot afford the long regimen. A recent review by Machado et al. demonstrated the importance of using cost effective naturally occurring phospholipids to make liposomes for food ingredient delivery (Machado et al., 2014). The feasibility of using crude soybean lecithin for food-liposome preparation was established by Yokota et al. This group has successfully encapsulated casein hydrolysate in order to mask its bitter taste and odour (Yokota et al., 2012). The aim of our study was to develop anti-TB drug-loaded liposomes using this FDA-approved naturally occurring phospholipids mixture, crude soybean lecithin (CL), which is cheap and readily available, by studying the properties of CL-based liposomes with anti-TB drugs.

2. Materials and methods

2.1. Materials

Soybean lecithin granules used in this study were sourced from Health Connection Wholefoods (USA). According to the manufacturer, these granules (100 g) contained mainly phosphatidylcholine (23 g), phosphatidylinositol (14 g), polyunsaturated fat (35 g), saturated fat (13 g), glycaemic carbohydrates (8 g), sodium (0.11 g) and total energy up to 2940 kJ. Cholesterol used was from Carlo Erba/Divisione Chimica (Italy). Isoniazid, mono- and dibasic sodium phosphate were purchased from Sigma Aldrich (Germany). Ethyl acetate and acetone used were from Protea Chemicals (South Africa) while chloroform was from BM Scientific/Parow Industria (South Africa). Methanol from Merck (Germany) and acetonitrile from Ranbaxy Fine Chemicals Ltd (India) were of analytical high performance liquid chromatography (HPLC) grade. Unless indicated, all materials were used without further purification.

2.2. Methods

2.2.1. Purification of soybean lecithin

Crude soybean lecithin was purified according to the procedure described by Mertins et al. (2008) with a slight modification. Briefly, the crude soybean lecithin (10 g) was dissolved in ethyl acetate (100 ml), after which HPLC grade water (4 ml) was added while stirring slowly in order to prevent the formation of two phases. After allowing the liquid mixture to stand, the supernatant was decanted and the bottom phase, which looked like a gel, was dispersed in acetone (60 ml). Any agglomerates that formed were disrupted using a glass rod. Thereafter, the acetone was decanted and a further portion of acetone (60 ml) added, repeating the crushing process. Finally, the precipitate was filtered under vacuum and dried in a desiccator connected to a vacuum pump for 48 h. The purified lecithin (7.99 g) was collected and kept at room temperature. Further purification using liquid chromatography did not improve the quality of the lipid mixture significantly and was not considered in further studies.

2.2.2. Validation studies

An HPLC method for the quantification of isoniazid (INH) was validated for linearity, accuracy, repeatability (intraday precision) and intermediate precision (inter-day precision). The chromatographic system consisted of an Agilent HP1100 LC–MSD and equipped with a quaternary pump, in-line degasser, DAD detector, 1100 MSD and ChemStation for collection and analysis of data. A ZORBAX Elipse Plus C18 4.6 i.d.x 150 mm × 5 μm column was used for reversed-phase HPLC analysis. The mixture of aqueous solution of monobasic sodium phosphate 0.01 M and acetonitrile (90:10) was used as mobile phase on isocratic elution mode. From a 1000 μg/ml stock solution of INH in HPLC grade water, five different concentrations (5–500 μg/ml) were prepared and filtered using 0.45 μm Millipore filters before their injection.

Over six days of experiments, three daily injections of 20 μl for each INH fresh solution were performed the HPLC instrument. The flow rate of the mobile phase was 1 ml/min and the UV detection was set at 254 nm. The duration of each elution was 5 min and the retention time for INH was found at 1.2 min. The average peak areas obtained were plotted against respective concentrations to construct the calibration curve in order to determine the linearity range, regression equation and correlation coefficient. The percentage recovery and relative standard deviations (RSD) were calculated in order to evaluate accuracy and precision respectively. The limits of detection (LOD) and quantification (LOQ) were established as the ratio of the standard deviation for the lower concentration in the calibration curve to the slope of the regression equation times 3 for LOD and 10 for LOQ (ICH Experts, 2005; CLSI, 2004).

2.2.3. Preparation of liposomes

The crude soybean lecithin (CL) or the purified version (PL) was used in combination with cholesterol at the mass ratio of 3:1. Basically, 0.10 g of lipid components was dissolved in 1 ml of chloroform in an ultra clean 25 ml round bottom flask. The obtained solution was dried at 50 °C under vacuum using a rotary evaporator (Büchi Rotavapor R-205, Switzerland) at 200 rpm for 5 min. The round bottom flask was removed and stored in a vacuum desiccator at room temperature overnight. Phosphate buffer (3 ml, pH 7.0) in HPLC grade water was added to the thin lipid film. The mixture was heated for an hour at 60 °C under stirring at 400 rpm to hydrate the lipids. The medium was then homogenized in a bath sonicator (Digital Ultrasonic Cleaner, Spellbound 909) at 60 °C for 20 min to produce a suspension of CL-based liposomes or PL-based liposomes. On the other hand, 3 ml of

phosphate buffer (pH 7.0) was treated in the same conditions to produce a control solution free from lipids.

2.2.4. Encapsulation of isoniazid

The suspension of empty liposomes obtained above was directly added to the aqueous solution of Isoniazid (INH), 0.20 g in 1 ml of pH 7.0 phosphate buffer, contained in a Beckman centrifuge tube. Without allowing the mixture to cool, it was gently shaken for 30 s and subjected to 2 cycles of freeze-thawing. For each freeze-thaw cycle, the centrifuge tube containing the preparation was immersed in liquid nitrogen (−196 °C) for 5 min and thereafter in an oil bath set at 65 °C for 10 min, according to a procedure by Costa et al. (2014).

Thereafter, the mixture was allowed to cool at room temperature for 30 min and 20 ml of HPLC grade water were added to produce a volume required for centrifugation. The lipid-free solution previously prepared was treated in the same conditions to produce INH control solution. Empty liposomes were freeze-thawed following the procedure described above, apart from the addition of 1 ml of phosphate buffer (pH 7.0) instead of INH solution.

2.2.5. Determination of encapsulation efficiency

The prepared liposomal mixture was centrifuged at 20,000 rpm for 20 min using Beckman Coulter Allegra 64R centrifuge at 25 °C. The supernatant was decanted off and the remaining liposomes were rinsed five times with 20 ml of HPLC grade water to remove the free INH molecules. The liposomes were freeze-dried and kept for further characterizations. INH in the supernatant and in the control solution was quantified using the validated HPLC method. Dilutions (1/100) were done and all the solutions were filtered using 0.45 μm Millipore filters. A ZORBAX Elipse Plus C18 4.6 i.d.x 150 mm × 5 μm column was used with the mixture of aqueous solution of monobasic sodium phosphate 0.01 M and acetonitrile (90:10 v/v) as mobile phase on isocratic elution mode. For each sample, three injections of 20 μl were performed into the HPLC instrument for the quantitative analysis using a mobile phase flow rate of 1 ml/min and UV detection set at 254 nm. The percentage encapsulation efficiency (% EE) was calculated according to the following equation (Costa et al., 2014; Singh et al., 2011):

$$\frac{(Cc - Cs)}{Cc} \times 100$$

In this equation, C_S and C_C represent respectively the concentrations of INH in the supernatant and in the control solution used to estimate the theoretical concentration of INH.

2.2.6. Particles size and Zeta Potential determination

Freeze-dried liposomes were analyzed for particles size, size distribution and zeta potential using Zetasizer nano (Malvern Instruments). The freeze-dried preparations were gently dispersed in HPLC grade water at room temperature to produce a yellow suspension. The measurements were performed at the scattering angle of 173° using either an ordinary cuvette or a capillary one according to the determination, particles size or Zeta Potential respectively.

2.2.7. Particle shape analysis

The shape of freeze-dried liposomes was investigated by transmission electron microscopy (Zeiss Libra 120KV TEM instrument). The aqueous liposomal suspensions from particle size and zeta potential analyses were used for TEM experiments. For each sample, a drop was deposited on the copper grid and the solvent in excess was absorbed using a piece of filter paper. The prepared TEM samples were allowed to dry over 48 h at room temperature.

2.2.8. Physicochemical characterizations

2.2.8.1. Thermogravimetric analysis (TGA). TGA was used as a tool to study the thermal behavior of freeze-dried CL- and PL-based liposomes and investigate any physicochemical interactions between INH and lipid components. The samples were heated in nitrogen saturated atmosphere using PerkinElmer TGA-4000 instrument. The heat range was 30 to 800 °C at a flow rate of 10 °C/min. The inert nitrogen gas flow was set at 20 ml/min. TGA thermograms with respective mass loss curves were recorded and data were analyzed using the software TGA Pyris series.

2.2.8.2. Differential scanning calorimetry (DSC). Isoniazid encapsulated in freeze-dried CL- and PL-based liposomes was characterized in terms of thermal events using PerkinElmer DSC-6000 instrument that gives upward endothermic peaks. The samples were heated from 30 to 450 °C at a flow rate of 10 °C/min in a nitrogen saturated atmosphere. The inert nitrogen gas flow was set at 20 ml/min. The reference was an empty aluminum-based pan. DSC thermograms with respective heat flow curves were recorded and data were processed using the software DSC Pyris series.

2.2.8.3. X-Ray diffraction (XRD). XRD patterns were evaluated in order to assess the crystalline state of INH in freeze-dried liposomes. The experiments were conducted using Cu-Kα radiation set at 1.5404 Å with a nickel filter, on Bruker D8 Discover instrument equipped with a Lynx Eye detector. The freeze-dried samples were placed on a glass sample holder and the diffraction patterns were recorded in the range of 2θ from 10 to 60°with a step size of 1°/min and a slit width of 6.0 mm.

2.2.8.4. Fourier transform infrared spectroscopy. The presence of INH in liposomes was investigated by Fourier transform infrared (FT-IR) spectroscopy. The IR spectra were recorded by the attenuated total reflection method using PerkinElmer Spectrum 100 FT-IR Spectrometer. For each experiment, 8 scans were done in the frequency range of 4000–650 cm^{-1} and data were processed using the software FT-IR Spectrum.

2.2.9. INH release study

INH-loaded liposomes were firstly evaluated for INH content using the procedure described by Rojanarat et al. (2011) with a slight variation regarding aliquot size. The freeze-dried sample (5 mg) was placed in a 25 ml volumetric flask containing methanol (10 ml) to destroy liposomal structure. The obtained dispersion was copped to volume using HPLC grade water. The volumetric flask was placed in a bath sonicator (Digital Ultrasonic Cleaner, Spellbound 909) at 35 °C for 30 min to promote particle dissolution and the obtained solution was analyzed using the validated HPLC method.

The release profile of the freeze-dried INH-loaded liposomes was determined according to the procedure used by Chimote and Banerjee (2010), modified in terms of the composition of release medium and volume of liposomal suspension. In brief, an amount of freeze-dried liposomes corresponding to 5 mg of INH was gently dispersed in 5 ml of pH 7.4 buffer in 10 ml volumetric flask at room temperature. The volume was copped to 10 ml using the same dispersing medium. One ml of the prepared suspension was transferred into a cellulose dialysis tubing membrane (Membra-Cel MD 10–14 × 100 CLR, Sigma Aldrich). The dialysis bag was placed in a glass vial containing 20 ml of pH 7.4 phosphate buffer. The content of the vial was then maintained at 37 °C under a stirring condition of 100 rpm. An aliquot of the draining medium (5 ml) was withdrawn after 0.5; 1; 1.5; 2; 3; 4; 5; 7; 9 and 12 h, for INH quantification using the validated HPLC method. After every

sampling, an equivalent volume of fresh buffer was added to the release medium for its renewal.

3. Results and discussion

3.1. Validation studies

Good linearity (Fig. 1), intraday- and interday-repeatability (Table 1) were established for different concentrations of INH. The calibration line indicates a good linear correlation in the range of 5–500 μg/ml, the correlation coefficient ($R^2 = 0.9996$) being acceptable as compared to the standard value ($R^2 = 0.998$) (Shabir, 2004). The LOD and LOQ calculated were 0.013 μg/ml and 0.042 μg/ml, respectively. The RSD values for intraday analysis show repeatability of less than 2% (Ayyappa et al., 2011). Furthermore, the intermediate precision evaluated over six days of experiments show RSD values (Table 1) less than 5% (Bartolomeo and Maisano, 2006; Milán-Segovia et al., 2007). The obtained percentage recoveries being within a range of 95.39–102.42%, the accuracy of the method is quite valid as the acceptable percentage recovery range is 90–110% (Bartolomeo et al., 2006).

3.2. Encapsulation efficiency (%EE)

A preliminary study using principal component analysis (not shown), indicated that liposome formation was best achieved at a lecithin/cholesterol mass ratio of 3:1, using two or 3 freeze-thaw loading cycles. Contrary to expectation, further cycles steadily decreased the% EE of INH-loaded CL-based liposomes and PL-based liposomes. Under these conditions the best%EE were found to be 79 ± 2.45% and 21 ± 2.10%, respectively. This higher% EE of CL-based liposome may be related to the presence of hydrophilic compounds in the crude soybean lecithin, especially carbohydrates present that can stabilize INH. Also, as INH is not ionized at pH 7.0, some of its molecules may be located either in lipid membranes or inside of the liposomal vesicles. A considerable attention must be given to the percentage (%) EE of CL-based liposomes obtained. In fact, limited encapsulation efficiency of hydrophilic drugs in liposomes, made by thin film hydration method, is common in the literature (Pattni et al., 2015). To quote a few, Rojanarat et al. (2011) and Chimote and Banerjee (2010) reported respectively 18–30% and 36.7 ± 1.8% of encapsulation efficiency of INH as a single drug in liposomes. The cost effectiveness of CL-based liposomes prepared can be praised here as the entrapment efficiency has an impact on the economical status of drug carriers (Kadare et al., 2014). The outstanding% EE, particularly, of CL-based liposomes was the main reason of their selection for further analysis using various physicochemical techniques.

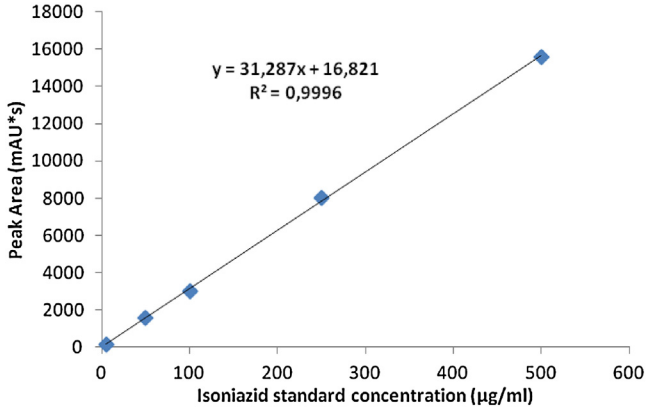

Fig. 1. Standard plot of Isoniazid over the concentration range of 5–500 μg/ml.

3.3. Particles size and zeta potential

The results of particle size, polydispersity index (PDI) and Zeta Potential (ZP) are presented in Table 2. All the liposomal formulations showed average particle sizes in the range of 800–1100 nm. This range of size is acceptable for both the deep lung deposition (Paranjpe and Müller-Goymann, 2014) and macrophage uptake (Chuan et al., 2013). The zeta potential values obtained (from −42.80 ± 4.31 to −69.10 ± 2.36 for liposomes prepared) are valuable for the liposomes stability during the storage and cell internalization as macrophage scavenger receptors recognize negatively charged particles (Kelly et al., 2011). The encapsulation of INH did not have a large impact on the size or surface charge of liposomes, although charges were slightly more negative for the INH-loaded liposomes (p-value > 0.05). PL-based liposomes, however, were more negatively charged than CL-based liposomes. This was expected due to the fact that the purification process of crude soybean lecithin increases the proportion of phospholipids (Mertins et al., 2008), which influences the charge on the liposomes.

3.4. Particle shape analysis

All the formulations prepared exhibited particles with roughly spherical shapes (using TEM data). The presence of spherical morphology was indicative of liposomal vesicles. No liposomal particles aggregation was found during the samples microscopic observation. This is due to the negative charges on the surface of particles, allowing particles to repel each other. Some typical TEM micrographs of empty and INH-loaded liposomes are presented in Fig. 2a and b.

3.5. Thermogravimetric analysis

TGA allowed the investigation of liposome thermal stability. The thermal behavior of INH-loaded liposomes looks different to either the empty liposomes or pure INH (Fig. 3), as expected, and shows a behavior intermediate to these two extremes. The mass loss curve of INH-loaded CL-based liposomes presents two stages of thermal decomposition. The first decomposition occurred from 134 to 180 °C, where a much smaller second one started and finished around 420 °C. Since only one thermal decomposition of pure INH occurred (from 170 to 305 °C), this additional decomposition seen in the liposome formulations must be related to the additional components present in the lecithin. The earlier thermal decomposition of INH-loaded CL-based liposomes in comparison to the empty liposomes might be due to the disruption of the tight packing of lipids to accommodate the INH in the liposomes, and again may indicate that at least some of the INH is present in the lipid bilayers rather than inside the aqueous cavity. This cannot be confirmed currently, but further studies are underway.

3.6. Differential scanning calorimetry

DSC analysis of INH shows the main endothermic peak around 173 °C (as expected where the melting point of INH = 171 °C). This shifted to about 130 °C in DSC curves of INH-loaded CL-based liposomes (Fig. 4). This lowering of "melting point" is common in impure mixtures and may reflect a distribution of INH in the crude soybean lecithin as well as the loss of some crystalline structure. However, the presence of the characteristic endothermic peak of INH indicates there is no major physicochemical interaction in the formulation (Patil et al., 2015).

Table 1
RSD and recovery values over 6 days of experiments.

Concentration (μg/ml)	Intraday precision (%RSD)	Interday precision (% RSD)	Recovery (%, accuracy)
5	0.85	3.09	95.39
50	0.33	1.39	101.67
100	0.87	0.53	95.56
250	0.13	0.45	102.42
500	0.15	0.45	99.56

Table 2
Average particle size, polydispersity index (PDI), Zeta Potential (ZP) and% EE.

Formulations	Average size ± SD (nm)	PDI ± SD	ZP ± SD (mV)	EE ± SD (%)
INH-CL-Liposomes	813.00 ± 9.21	0.29 ± 0.06	−42.80 ± 4.31	78.78 ± 2.45
Empty CL-Liposomes	874.80 ± 5.15	0.48 ± 0.07	−47.20 ± 1.61	–
INH-PL-Liposomes	1081.00 ± 20.43	0.43 ± 0.07	−66.00 ± 0.65	20.90 ± 2.10
Empty PL-Liposomes	1053.00 ± 3.02	0.39 ± 0.10	−69.10 ± 2.36	–

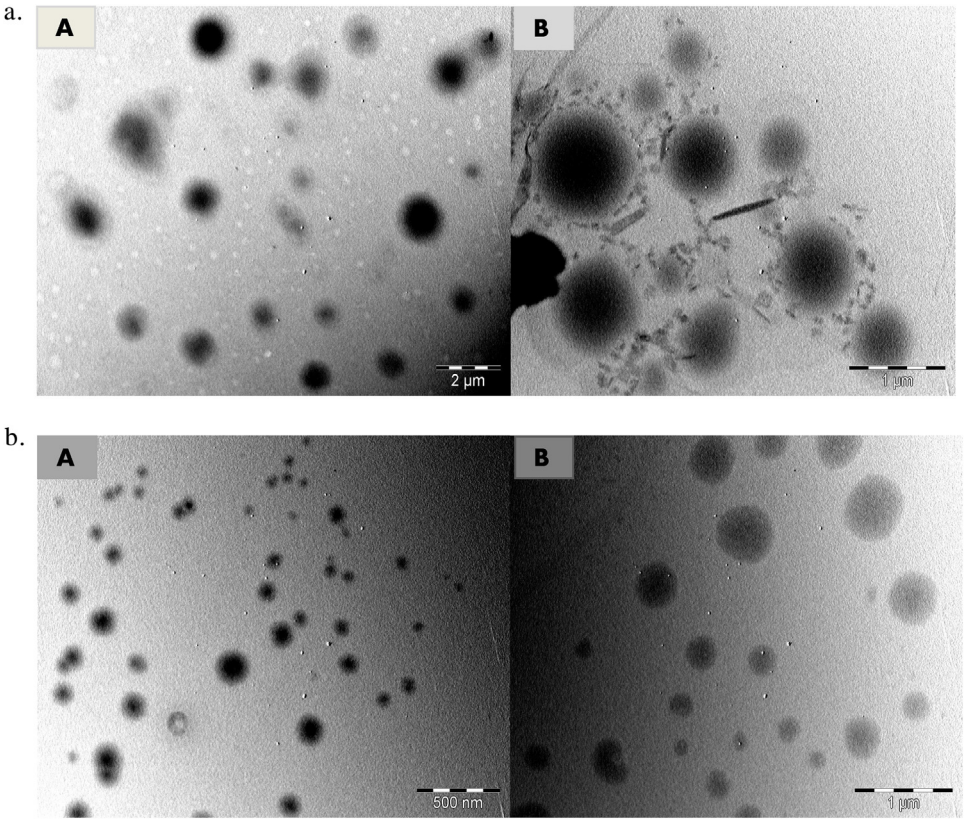

Fig. 2. a: TEM micrographs of empty CL-based liposomes (A) and INH-loaded ones (B). b: TEM micrographs of empty PC-based liposomes (A) and INH-loaded ones (B).

3.7. X-Ray diffraction

The XRD patterns of the pure INH, INH-loaded CL-based liposomes and empty liposomes are presented in Fig. 5. While empty CL-loaded liposomes show a broad amorphous profile, some crystalline peaks can be observed in the pattern of INH-loaded CL-loaded liposomes. The characteristic peaks of INH at 12°, 16°, 17°, 20° and in the range of 25°–30° are found in the INH-loaded liposomes pattern, confirming the presence of INH in liposomes. These XRD data supports earlier DSC and TGA results concerning the change in the physical structure of INH in liposomes, possibly in a more amorphous state (lower intensity and broadened nature of peaks).

3.8. Fourier-transform infrared spectroscopy

FT-IR spectra were collected to confirm the presence of INH in CL-based liposomes. A comparison in Fig. 6 of the FT-IR spectra for pure INH, INH-loaded CL-based liposomes and empty liposomes shows the presence of INH in the liposomes. The characteristic FT-IR bands of INH at 3107 to 3301 cm^{-1} are assigned to primary and secondary N—H stretching vibrations of hydrazide group along

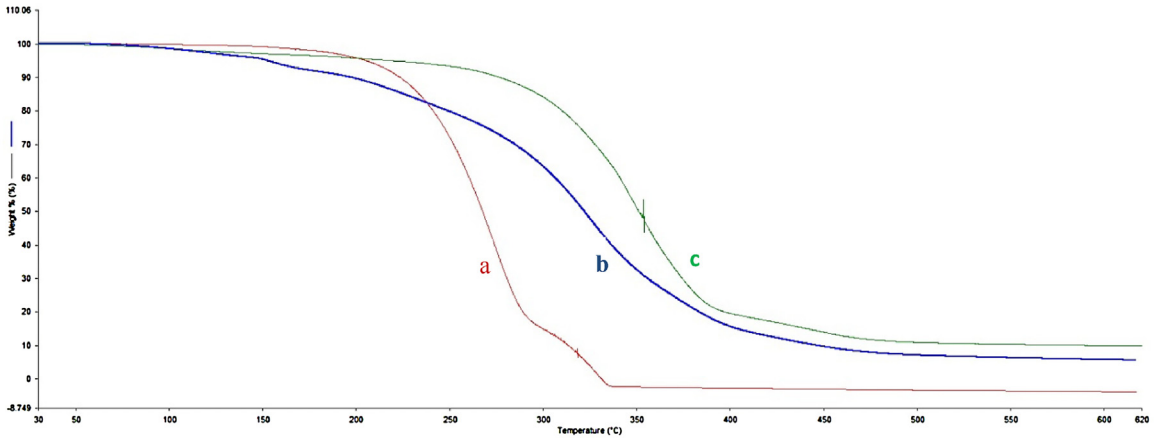

Fig. 3. TGA mass loss curves of pure INH (a), INH-loaded CL-based liposomes (b) and Empty CL-based liposomes (c).

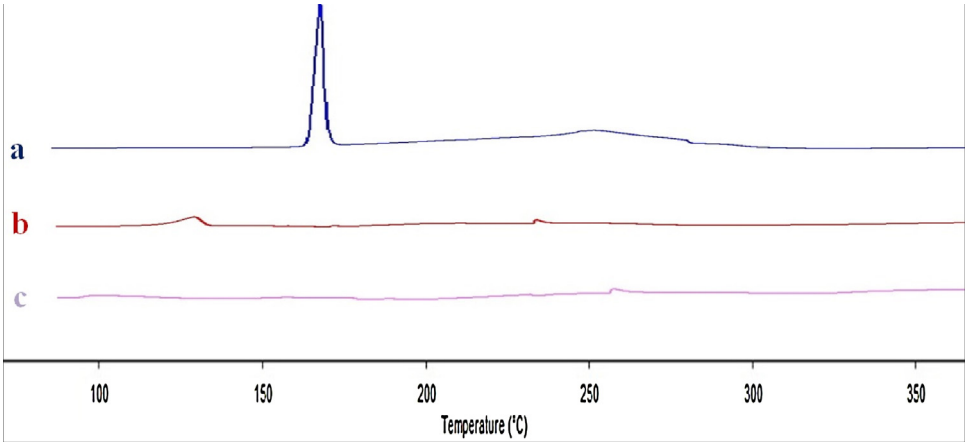

Fig. 4. DSC Curves of pure INH (a), INH-loaded CL-based liposomes (b) and Empty CL-based liposomes (c).

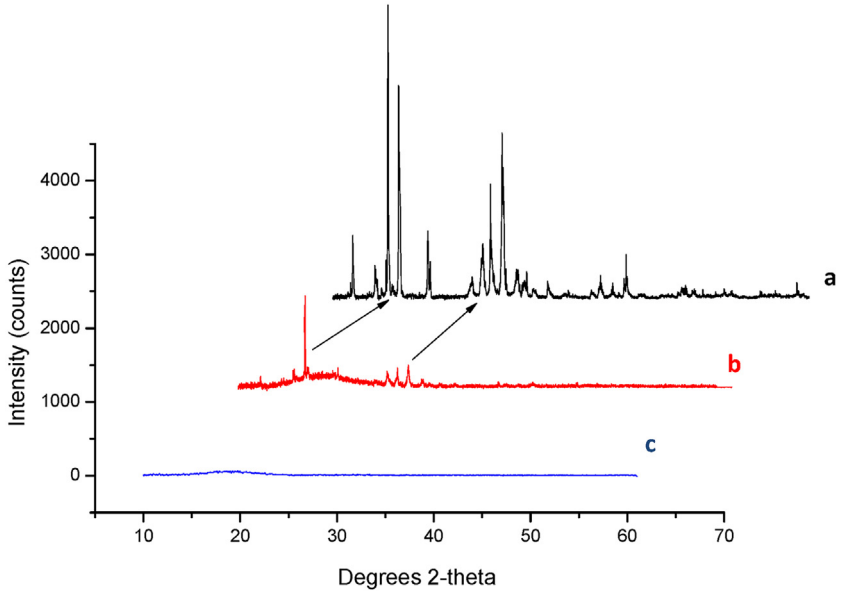

Fig. 5. XRD patterns of pure INH (a), INH-loaded CL-based liposomes (b) and Empty CL-based liposomes (c).

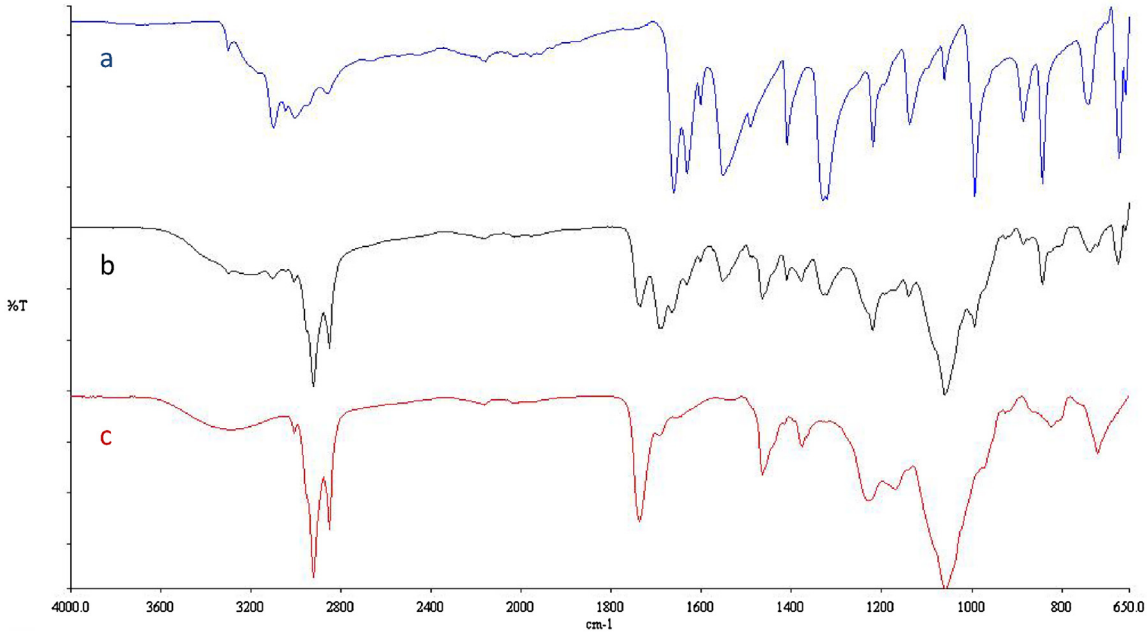

Fig. 6. FT-IR Spectra of pure INH (a), INH-loaded CL-based liposomes (b), and Empty CL-based liposomes (c).

with the hydrazide C=O, aromatic N=C, C=C and pyridine ring stretches at 1662, 1632 1602 and 1553 cm^{-1}, respectively.

3.9. INH release study

The results of zero order kinetic release study are presents in Table 3. A burst drug release was observed after 30 min for all the preparations studied. This initial burst release of 35% INH from CL-based liposomes is lower than PL-based liposomes and free INH, which showed burst release values of 45 and 58%, respectively. The low value for free INH is indicative of less than ideal diffusion through the dialysis membrane, and therefore the% release for INH from the liposomes is likely to be higher than recorded.

Over 1.5 h, all of the free INH was released into the bulk solution (100% within 5 h), while both the PL and CL-liposomes took much longer. After 12 h, PL-based liposomes released up to 68% whereas the percentage release of CL-based liposomes was only 50% under the same conditions. Hence, the release kinetics of CL-based liposomes was more controlled and sustained than that of PL-based liposomes (Fig. 7). Overall, CL-based liposomes showed an initial rapid release of a third of the encapsulated INH followed by a slower release for the remainder of the experiment. This release profile suggests rapid release and dissolution of INH molecules from liposomal membranes at 37 °C, followed by a slow diffusion of INH molecules from the internal cavities of the carrier. Studies to confirm the kinetics of release are ongoing in our labs. This release profile may be beneficial in addition to the deep lung deposition and high macrophage uptake of liposomes, over free INH for TB therapy.

Table 3
Data from the *in vitro* release study.

Time (hour)	%INH-CL-Liposomes	%INH-PL-Liposomes	% Free INH
0	0	0	0
0,5	35	45	58
1	40	56	70
1,5	41	61	95
2	43	62	97
3	44	63	99
4	46	64	99
5	47	65	100
7	48	66	–
9	49	67	–
12	50	68	–

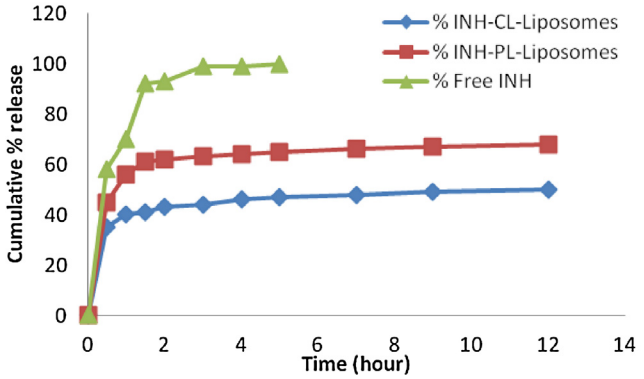

Fig. 7. *In vitro* release kinetics of free INH (green), INH-loaded PL-based (red) and CL-based liposomes (blue) in pH 7.4 buffer over 12 h at 37 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Conclusion

The present study was focused on the encapsulation of INH in liposomes using crude soybean lecithin as an alternative to expensive synthetic or purified lipids. The feasibility of successfully encapsulating INH in the liposomes at high efficiencies (>78%) make the use of this natural phospholipid mixture attractive for further development. This is the first report on the development of INH-loaded CL-based liposomes by freeze-thaw loading technique, and we are pleased that the high encapsulation efficiency (78.78 ± 2.45) of INH was achieved in just two freeze-thaw cycles. To the best of our knowledge this has not yet been reported in the literature. The physicochemical properties of INH-loaded CL-based liposomes revealed attractive particle size (813 ± 9.21 nm) and zeta potential (-42.80 ± 4.31 mV), which are promising for

pulmonary delivery and macrophage targeting. These features could be useful for improving the bioavailability of anti-TB drugs at the site of interest, while reducing their side effects and dosing frequency (through targeted sustained and controlled release). The present findings suggest the possibility of encapsulating drugs efficiently in liposomes using crude soybean lecithin. The use of this naturally occurring phospholipid mixture for liposomal drug delivery would revolutionize the need for expensive synthetic or purified phospholipids in this field. Further investigations on the formulation developed for cost effective treatments of poverty related diseases like TB is currently ongoing in our labs.

Conflict of interest

The authors declare no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work.

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