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Research Article

STUDIES ON FORMULATION AND EVALUATION OF LIPOSOME FOR CUTANEOUS LEISHMANIASIS

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ABSTRACT
Aim of present study was to design liposome for allopurinol delivery against cutaneous leishmaniasis (CL). Leishmaniasis is disease caused by pathogenic protozoan parasites of the genus *Leishmania*. The disease is complex and untreated cases of leishmaniasis may result to chronic form such as mucosal leishmaniasis. There are several therapeutics available to treat CL, however, in recent time allopurinol has been used to tackle the chronic cases. The conventional formulations of allopurinol remain associated with several limitations both pharmacokinetic and pharmacodynamic. In this study, nano-design liposome has been used to enhance pharmacokinetic and pharmacodynamic parameters. The liposomes were prepared by dried thin film hydration technique using a rotary evaporator with drug and carrier (soybean lecithin). The prepared liposomes formulations were evaluated for physical and chemical characteristics like average vesicle size, shape, zeta potential, % free drug, assay and stability studies. This developed liposomal drug delivery system was also evaluated for *in-vitro* drug release studies by pH 7.4 phosphate buffer using membrane diffusion method. The cumulative percentage of drug release was reported highest in the LF4 i.e. 98.08±1.54. Additionally, *ex vivo* studies have shown that allopurinol loaded into liposome has higher permeation (2.68µg/cm^2) compared to allopurinol alone. These finding demonstrate liposome based allopurinol based drug delivery is effective compared to conventional formations.

Keywords: Cutaneous Leishmaniasis, Allopurinol, Liposomes, Formulations, Characterization.

1. INTRODUCTION
Diseases caused by harmful protozoan parasites of the genus Leishmania are known as leishmaniasis. Infections begin when a sand fly vector injects Leishmania parasites into a mammalian host's skin. *Leishmania* causes a wide variety of inflammatory cutaneous diseases. The kind of dermatological illness is determined by the invading *Leishmania* species, but treatment benefit is also influenced by a combination of inflammatory and anti-inflammatory host immune response characteristics [1]. Several publications look at the numerous cutaneous syndromes that have been observed in individuals, as well as detailed observations of the systemic inflammation connected to diverse cutaneous pathologic exposure to different *Leishmania* species. However, parasite persistence following therapeutic bacteraemia remission is an interesting element of *Leishmania* species infestations, a property that may potentially result in downstream cutaneous symptoms and may also be important for establishing immunological resistance against re-infection [2].

Cutaneous leishmaniasis (CL) is the most common clinical form of leishmaniasis globally, with just seven countries accounting for 90% of all CL cases: Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria. *Leishmania* parasites are divided into Old World species like *L. major*, *L. infantum*, and *L. tropica* (found in the Mediterranean basin, the Middle East, the Horn of Africa and the Indian subcontinent) and New World species like *L. amazonensis*, *L. chagasi*, *L. mexicana*, *L. naiffi*, *L. braziliensis*, and *L. guyanensis* (found in Middle and South America) [3]. Most Old World species induce self-limiting ulcers, while New World species create a disease known as American tegumentary leishmaniasis, which includes CL as well as MCL and the much rarer diffuse and disseminated cutaneous leishmaniasis (DCL) [4].

In this study, allopurinol, a xanthine oxidase enzyme inhibitor (approved by the Food and Drug Administration in 1966 for therapeutic use) was used. In humans, allopurinol is a purine analogue that is used to treat gout.

It is mainly used to reduce blood urate levels as a xanthine oxidase inhibitor. Allopurinol's anti-leishmanial activity was first described by Pfaller and Marr in 1974, and it is assumed to be related to inhibition of the leishmanial enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) [5]. As part of the parasite's purine salvage pathway, HGPRT converts dephosphorylated purines to nucleoside monophosphates. Allopurinol that has been phosphorylated is more likely to be incorporated into nucleic acids, inhibiting protein translation and selecting parasite death. To successfully treat Cutaneous or Visceral Leishmaniasis, allopurinol was seldom used in conjunction with amphotericin B or pentavalent antimonials [6].

The methodologies, formulations, technologies and systems for conveying a pharmaceutical substance throughout the body as required to safely accomplish its intended therapeutic effects are referred to as novel drug delivery systems (NDDS). NDDS is a drug delivery method that differs from other drug administration systems. NDDS is a blend of cutting-edge technology and a dosage form that is considerably superior to traditional dose forms. The goal of NDDS is to deliver a therapeutic quantity of medicine to the right location in the body quickly and then maintain the proper drug concentration [7]. Micellar solutions, vesicle and liquid crystal dispersions, microspheres and nanoparticles are colloidal drug carrier systems made up of microscopic particles with diameters ranging from 10 nm to 400 nm. They have a lot of potential as medication delivery methods. The goal of these formulations is to create systems with optimal drug loading and release qualities, as well as a long shelf life and minimal toxicity [8].

Liposomes were first introduced in 1980 for external pharmaceutical delivery and since then, they've aroused a lot of conjecture regarding their potential utility as a drug transporter and storage for controlled drug release within various epidermal surfaces. In a variety of clinical studies, liposomal pharmaceutical compositions have been demonstrated to be superior to standard drug delivery systems. Psoriasis, mycoses, idiopathic hirsutism, and epidermal infections, among other dermatological diseases and disorders, have been demonstrated to benefit from liposomal formulations [9]. Liposomes are small (unilamellar or multilamellar) vesicles formed by phospholipid self-assembly in an aqueous environment, resulting in closed bilayer forms that have been explored as drug carriers to improve therapeutic agent delivery. Liposomes have long been regarded as one of the most outstanding, diverse, and adaptable carrier structures, capable of transporting a wide range of materials and applications [10].

The primary goal of this study was to develop and analyse allopurinol liposomes. The influence of different stabilisers on drug entrapment efficiency will be used to target the site of action, and non-pegylated liposomes will be used to decrease adverse effects.

2. MATERIAL AND METHODS

2.1. Chemicals

Allopurinol was obtained as a gift sample from Hema Pharmaceuticals Pvt. Ltd., India. Himedia Ltd., India provided soya phosphatidylcholine, dicetyl phosphate, ethyl acetate, and butyl acetate.

2.2. Instrumentations

Electronic Weighing Balance (A&D Company HR 200, Japan), Mechanical Stirrer (Remi Motors, India), Magnetic Stirrer (MC Dalal & Co., India), UV Visible Spectrophotometer (Shimadzu, Japan), Particle size analyser (CILAS-1604L, France), Scanning Electron Microscopy (Hitachi, Japan), Ultracentrifuge (Eppendorf centrifuge, Germany), Wrist action shaker (Yarco, New Delhi, India), and Fourier Transform Infrared spectroscopy (ATR-FTIR Prestige 21, Shimadzu Corp., Japan) were used in this study.

2.3. Preparation of Liposomes

Liposome was prepared by dried thin film hydration technique using a rotary evaporator method reported by Riaz *et al* (1996) with some modifications [11]. Phosphatidylcholine, cholesterol and allopurinol were dissolved in a chloroform/methanol (2:1 v/v) combination and then placed to a pear-shaped flask attached to a Rotavap or (Büchi-type) apparatus. The thin film was created by slowly removing the solvents at 40°C while the speed was sustained at 150r/min and vacuum was provided. The lipid film was vacuum-sealed for 1-2 hours in a desiccator to eliminate solvent residues, and then hydrated at 40°C with a Saline Phosphate Buffer solution of pH 7.4 under continual flask rotation until a dispersal system was produced (about 1 hr). The resultant suspension of multilamellar vesicles was vortexed during two 5-minute intervals before being maintained for 30 min.

2.4. Drug-interaction studies

The possibility of any such interaction between the drug and the utilized excipients; soya lecithin and cholesterol were monitored by Fourier transformed infrared (FT-IR)

spectroscopy to observe the compatibility and inherent stability of the formulation. Any change in the physical mixture of drug and excipients was reported, which may be interpreted as an interaction.

2.5. Physical characterization of liposomes

Determination of average vesicle size of allopurinol liposomes with carriers was a very important characteristic.

2.5.1. Entrapment efficiency

To extract the free medication from the liposome suspension, an ultracentrifuge was used at 5000 rpm for 1 hr. Liposomes were suspended in the supernatant, and unbound drug was found on the centrifugation tube's wall. The supernatant was collected and centrifuged for 30 minutes at 5000 rpm. A clear supernatant solution and liposome pellets were produced. Only liposomes were present in the pellet, therefore it was resuspended in distilled water until next treatment. The liposomes were immersed in 10 mL of methanol and afterwards sonicated for 10 mins to remove any non-entrapped medicines. The drug was released once the vesicles were ruptured and the drug content was calculated. The drug's absorbance was taken at 393.7 nm [12]. The entrapment efficiency was then calculated using following equation.

Amount of drug entrapped = Amount of drug present in supernatant – total amount of drug added

% Entrapment efficiency = (Entrapped drug/Total drug added) × 100

2.5.2. Polydispersity index

Polydispersity was determined according to the equation [13]:

Polydispersity = D (0.9) - D (0.1) / D (0.5)

D (0.9) corresponds to particle size immediately above 90% of the sample, D (0.5) corresponds to particle size immediately above 50% of the sample, D (0.1) corresponds to particle size immediately above 10% of the sample.

2.5.3. Scanning electron microscopy

Scanning electron microscopy (SEM) was used to determine the surface morphology (roundness, smoothness, and aggregation formation) of allopurinol liposomes with carrier. SEM samples were placed on metal studs and amplified to a magnification of 2000x [14].

2.5.4. Zeta potential analysis

The zeta potential's intensity reveals the colloidal system's inherent resilience. If all of the suspended particles have a significant negative or positive zeta potential, these will reject one another and have had no inclination to join together [15]. There would be no force to keep the particles from colliding and flocculating if the zeta predicted values of something like the particles are low. The magnitude of the zeta potential is connected to the stability of colloidal dispersions, which is why it is so important. Colloidal particles with a high zeta potential (negative or positive) are electrically stable, while those with a low zeta potential coagulate.

2.5.5. In-vitro drug release studies

In-vitro release study of allopurinol-loaded liposomal was carried out by using phosphate (pH 7.4) buffer as the recipient media at 37±0.5°C using dialysis membrane packet in dissolution apparatus. At pre-determined intervals, aliquots (1 mL) were removed (2 hr, 4 hr, 6 hr, 8 hr, 10 hr, and 24 hr) and after withdrawal of aliquots, the recipient medium was replenished with the same volume of buffer solution (1mL). The concentration of drug was determined by UV-Visible spectrophotometry [16].

2.5.6. Short term stability studies

The capacity of a certain formulation in a specific container may be characterised as the consistency of a pharmaceutical delivery system. The short-term stability study was carried out to test the physical and chemical stability of the liquid state of allopurinol liposomal preparations for up to 3 months at 40°C and room temperature [17]. The storage period was used to estimate the stability factor, such as Assay.

2.5.7. Ex-vivo skin permeation of allopurinol

Allopurinol skin permeation was tested using pig skin, supplied from a nearby butcher. The tests were carried out in a non-occluding environment. The donor and receptor compartments of vertical Franz-type diffusion cells with an effective permeation area of 1.5 cm^2 were installed between the donor and receptor segments of skin with a thickness of 450 μm in the abdomen region. 3 mL of pH 7.5 phosphate buffer was used in the receptor solution, which was continually agitated with a magnetic bar. A 250 μL liposome containing allopurinol was administered to the donor compartment after the skin surface was set at room temperature, *i.e.* 37°C. Furthermore, 400μL of receptor fluid was removed every 24 hours and replaced with an equal amount of fresh solution, which was then evaluated for drug

concentration. The drug accumulation concentration of the skin was also determined by immersing it in 25 mL methanol overnight, centrifuged, and concentrated. Permeation tests with an allopurinol conjugated in liposome were also done in the same way [18].

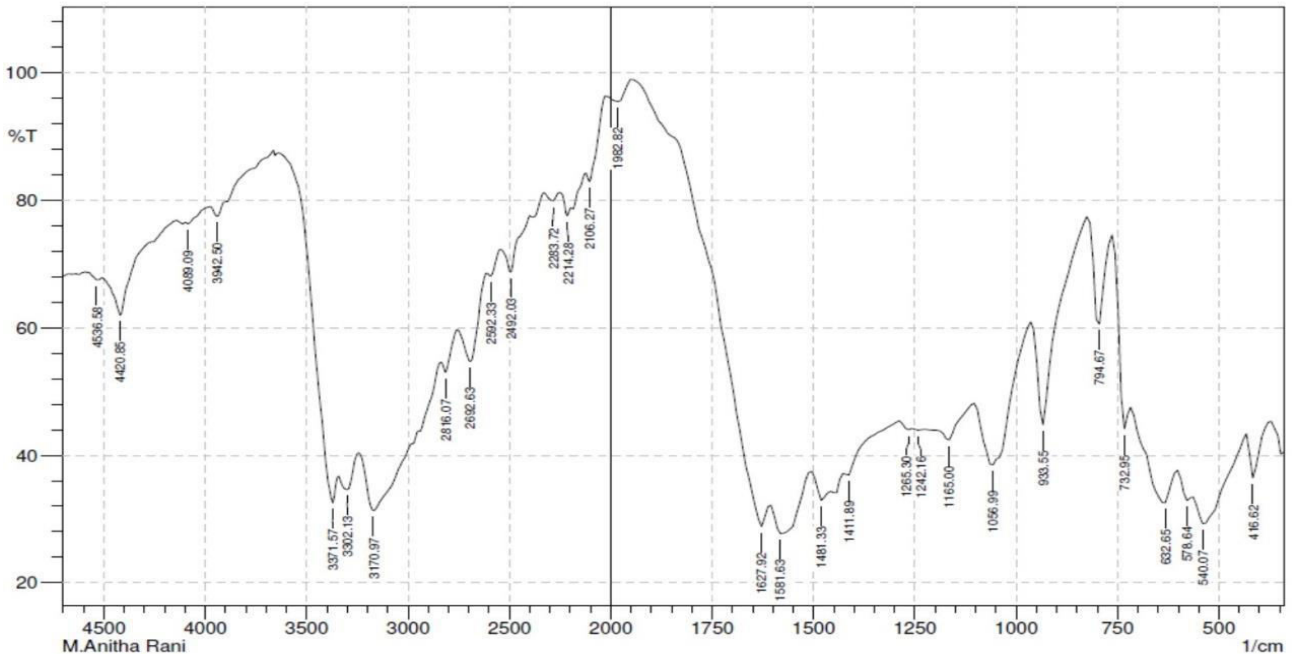
2.5.8. Stability study

The optimized batch (LF4) was subjected to 4°C temperature as well as studied under accelerated stability conditions (40°C±2°C and 75%±5% RH) for the duration of 90 days. The product was packed in an aluminium foil and kept inside a PVC bottle. After the completion of the study, the drug content was determined using assay procedure.

3. RESULTS

3.1. Drug-interaction study

The FTIR spectra for the drug, excipients (soya lecithin and cholesterol), and drug-excipients blends were recorded to determine the possible interactions. Predominantly, the drug displayed characteristic peaks (Fig, 1). In the physical mixture with the excipients, no such overlapping peaks were noticed, which confirmed no such drug-excipient interactions.



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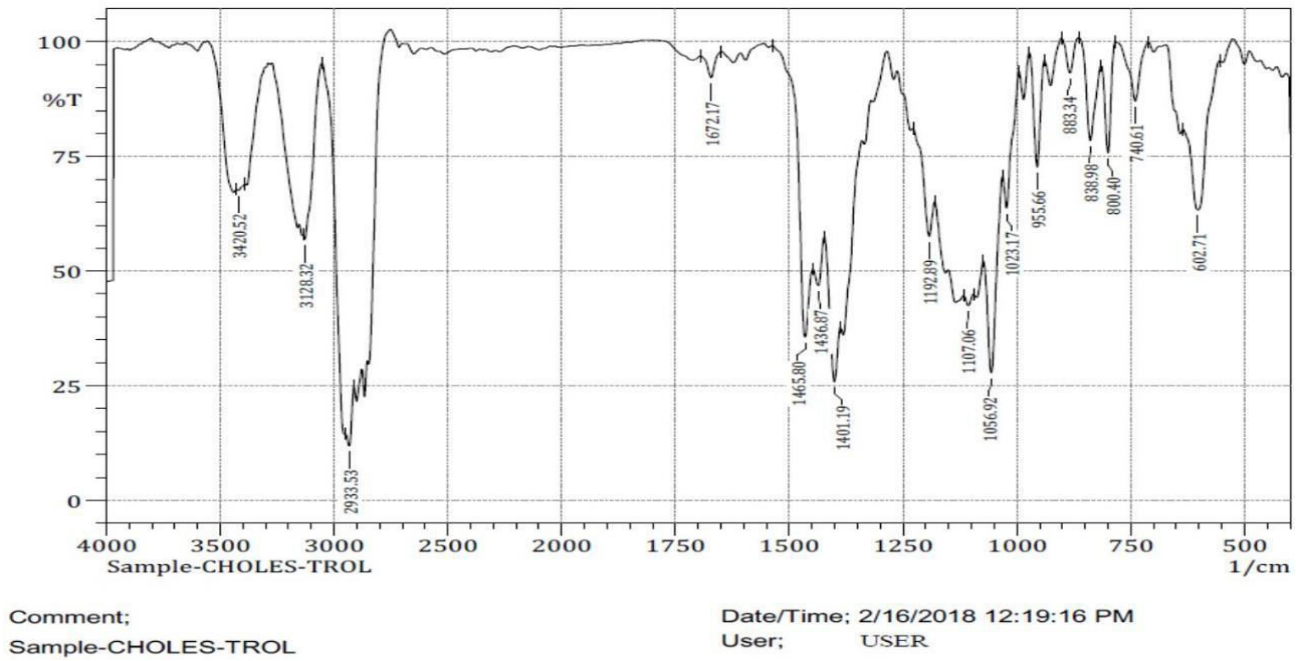

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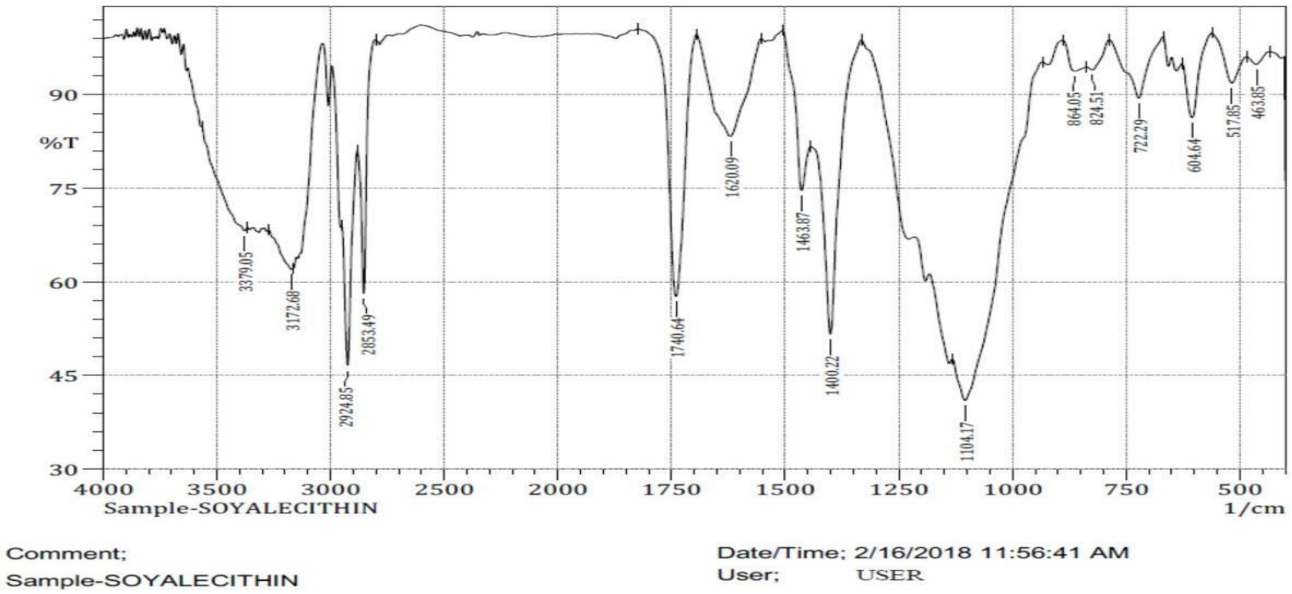

C

Fig. 1: FT-IR spectra of (A) Allopurinol (B) Cholesterol (C) Soya lecithin

3.2. Drug entrapment

The average percent drug entrapment efficiency of the six formulations ranges from 43.62% to 85.26% (Table 1). The formulation LF5 demonstrated the highest drug entrapment efficacy of 85.26% (Fig. 2C).

3.3. Particle size

The particle size of drug-loaded liposomes was found to be 18.091 µm -28.6 µm (Fig. 2B). The formulation LF6 showed the smallest particle size of 18.091 µm.

3.4. Zeta potential

All liposomes were negatively charged and zeta-potential values varied between 16.37 mV - 44.64 mV (Fig. 2A), which is considered as an optimal potential for assuring particle stability. It was observed that £ potential of prepared liposomes has sufficient charge to inhibit aggregation of vesicles. The formulation LF2 expressed the most optimized zeta potential of -29.25 mV (Fig. 3). The range of -30 mV to +30 mV is considered the most optimized for ensuring the particle stability [19].

3.5. Particle morphology

Liposomes obtained were spherical shaped, which could have an impact on drug-release (Fig. 4). No drug crystals were visible in SEM-images, regardless of the preparation technique or the loaded drug [20].

3.6. Drug release profile

The drug release from liposome formulations demonstrated variable release in the range of 86.87% to 98.08% (Table 2). The highest release was observed in LF4 group with cumulative % drug release of 98.09% (Fig. 5A).

3.7. *Ex vivo* skin permeation of allopurinol

Cumulative amount of allopurinol permeated through the skin *ex vivo* from liposome. As shown in the result, a lag time is required for formulation to be retained on skin surface before penetration into skin marked as donor compartment. The time after retention shown drug release and penetration increased with time and reported maximum at 18 hr (2.68µg/cm^2) (Table 3). Compared to the allopurinol as conventional formulation drug permeation pattern reported a decline over the time (Fig. 5B). The permeation of allopurinol remain largely constant after reaching a higher value demonstrated drug applied to the skin retained and act as local reservoir enable slow and steady state permeation in case of liposome based formulation [21].

3.8. Release profile

Fitting the *in vitro* drug release data into zero-order, first-order and Higuchi model helped in determining the probable drug release mechanism from the liposome formulations. Table 4 explains the values of release exponent (n) of the formulations (F2, F4, and F6) with the probable release mechanism(s). The release exponent (n) was found to be more than 0.5 with good linearity which indicated that drug release involved non-Fickian diffusion which closely overlaps with the swelling erosion study. It was also observed from the

release exponent (n) that drug release was governed by multiple processes and can be defined as an anomalous diffusion, where both diffusion and erosion mechanism works (Fig. 6).

Table 1: Evaluation of allopurinol-loaded liposomal formulation

Formulation Code	Average Particle (µm)	Zeta potential (mV)	Drug Entrapment (%)
LF1	28.6±0.13	44.64±1.29	43.62±0.77
LF2	21.023±0.17	29.25±1.15	58.79±0.59
LF3	19.092±0.13	16.37±2.55	49.03±1.38
LF4	26.134±0.33	43.03±1.25	59.29±2.02
LF5	22.091±0.19	39.55±2.41	85.26±1.34
LF6	18.091±0.28	26.77±1.91	73.97±1.27

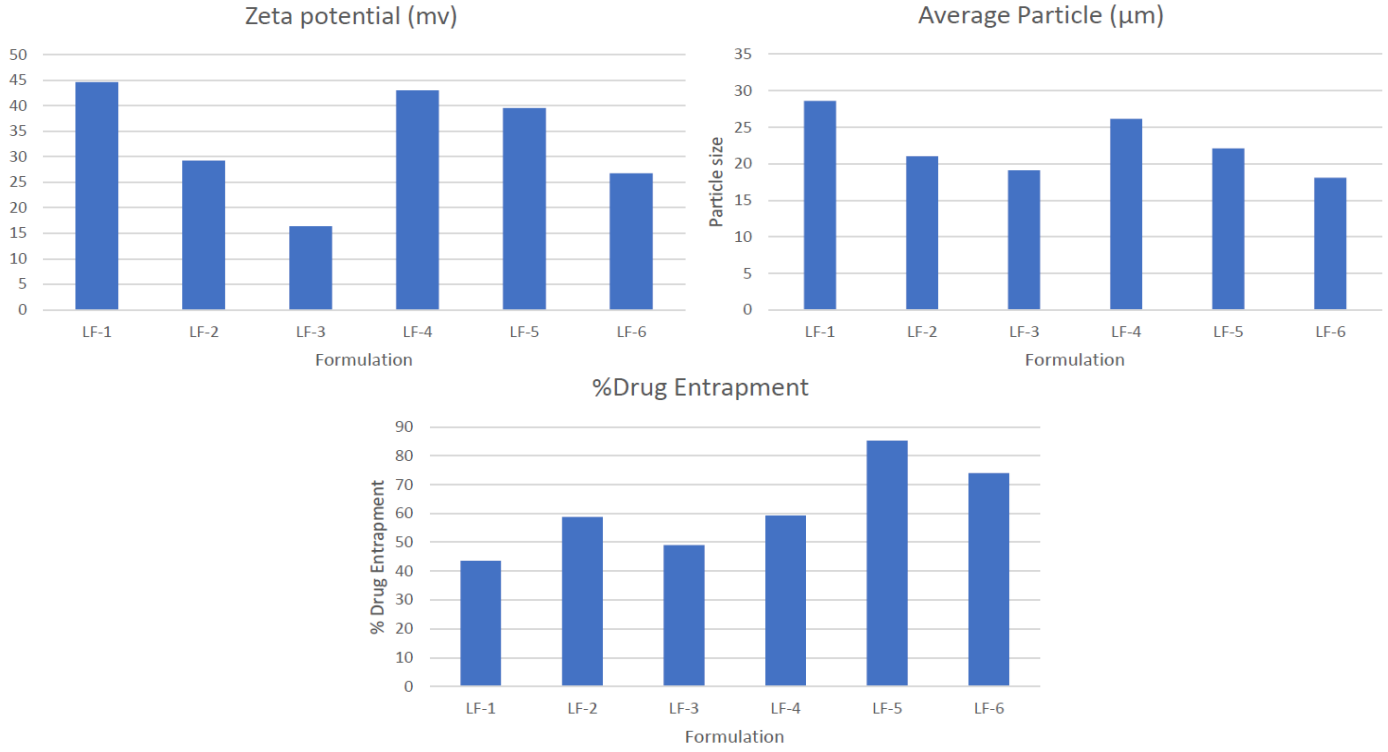

Fig. 2: Zeta potential (A), Mean particle size (B), and % Drug entrapment (C) of allopurinol-loaded liposomal formulation

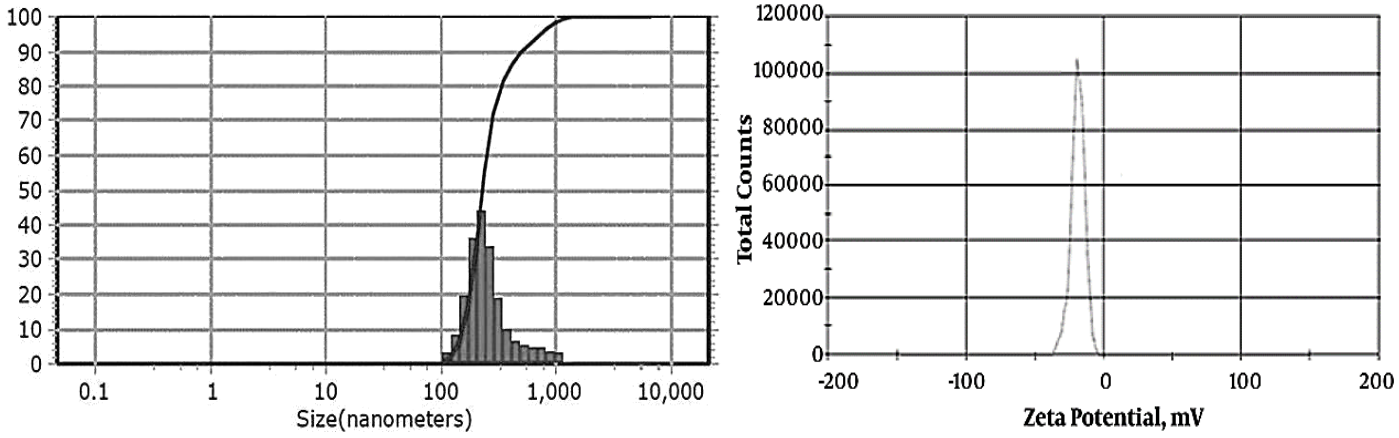

Fig. 3: (A) Particle size distribution of allopurinol-loaded liposomal formulation. (B) Zeta potential of allopurinol-loaded liposomal formulation

Table 2: Cumulative % drug release of allopurinol-loaded liposomal formulation

F. Code/Time	Cumulative % drug release					
	LF1	LF2	LF3	LF4	LF5	LF6
1	13.11	13.99	14.99	15.21	14.39	14.01
2	14.51	15.87	16.23	19.23	17.63	18.91
3	17.14	18.54	19.37	23.21	21.33	22.78
4	22.33	23.15	23.55	27.89	27.98	28.13
6	25.78	28.97	29.78	37.55	31.54	32.74
8	36.11	37.21	37.89	49.51	50.31	42.19
10	41.27	43.19	49.99	53.79	59.19	49.17
12	49.33	47.99	69.88	59.77	63.01	53.18
16	57.89	59.79	70.01	63.79	69.18	57.99
20	71.23	84.11	87.89	89.01	85.15	86.19
24	89.11	91.99	94.99	98.08	86.87	93.79

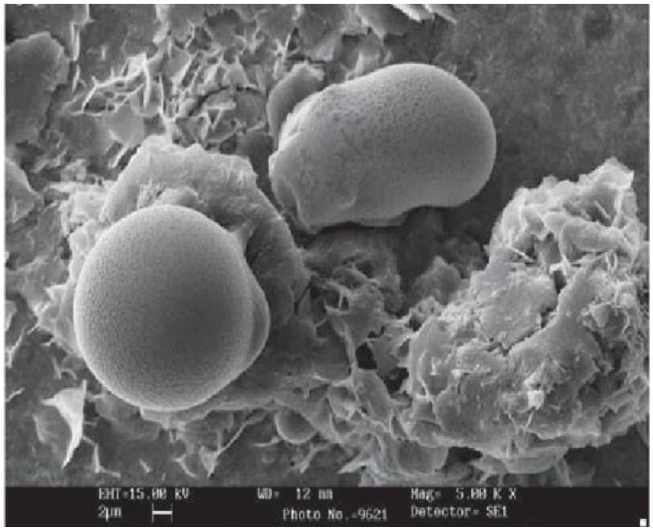

Fig. 4: SEM Microphotograph of allopurinol-loaded liposomal formulation (LF4).

Table 3: Drug permeation profile for cutaneous leishmaniasis and treated with allopurinol with and without liposome mediated

Time (hrs)	Amount of drug permeated from different formulations (µg/cm^2)	
	Allopurinol in conventional formulation	Allopurinol loaded liposome
0	00	00
10	0.324	0.689
12	0.851	1.14
14	1.20	1.68
16	1.57	2.10
18	2.06	2.68
20	1.85	2.53
22	1.74	2.48
24	1.67	2.42
26	1.64	2.38

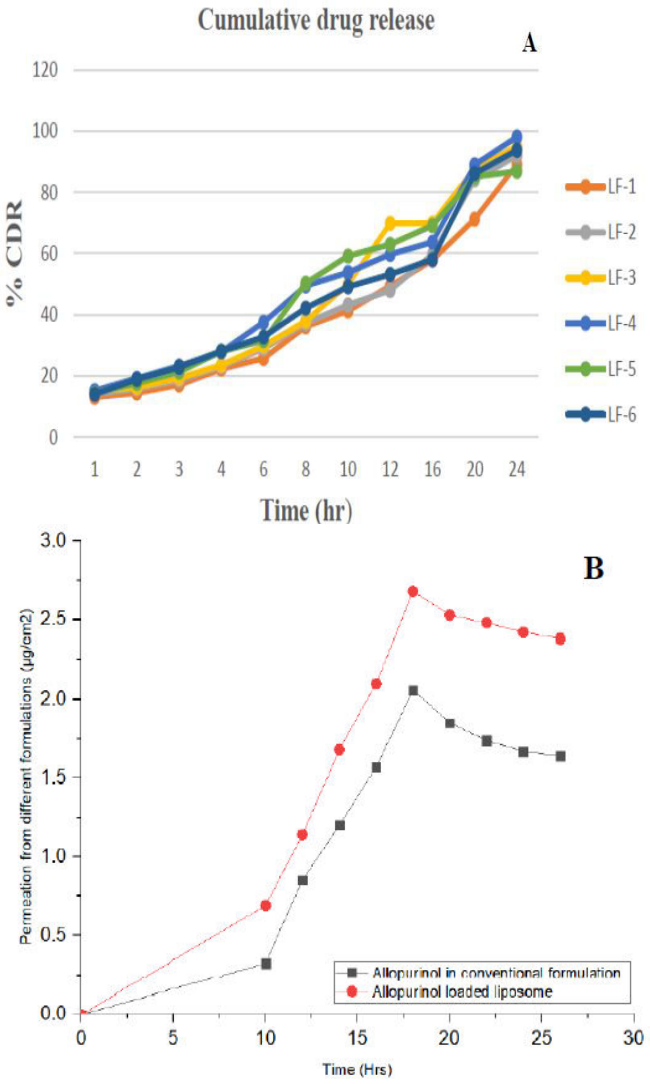

Fig. 5: (A) Cumulative % of drug release of allopurinol-loaded liposomal formulation. (B) Drug permeation profile for cutaneous leishmaniasis and treated with allopurinol with and without liposome mediated

3.9. Stability studies

The stability study under both 4°C temperature and accelerated conditions of temperature and humidity (40°C±2°C and 75%±5% RH) expressed no as such noticeable alteration in terms of drug content of the optimized formulation (F4). In all conditions, the change in drug content (0.5%) was detected after the duration of 40 days (at 4°C temperature) and 60 days (at 40°C temperature) that can be considered within the prescribed limits (Fig. 7). It may be concluded that the prepared tablet was stable under accelerated conditions. Table 5 depicts the assay results.

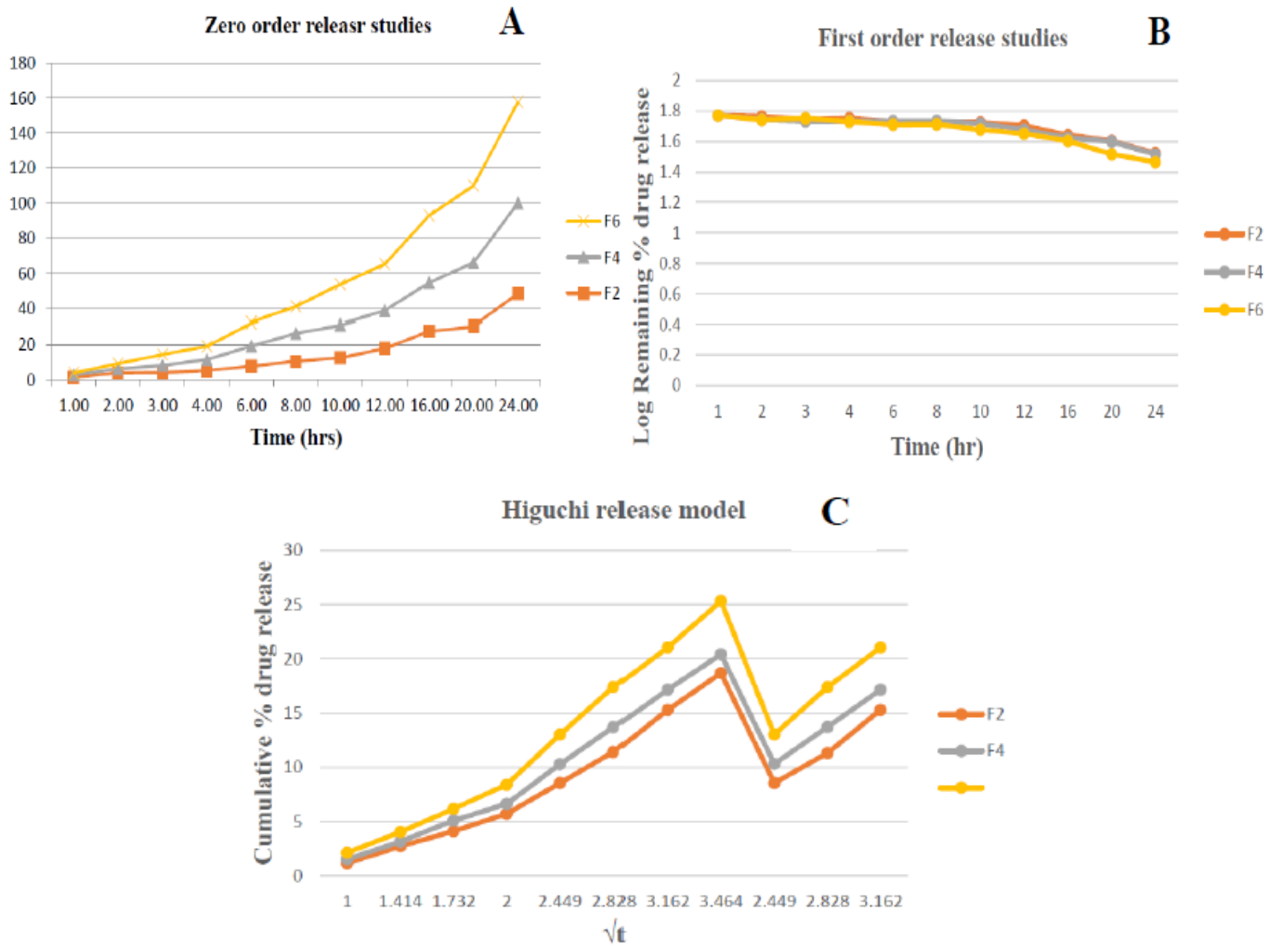

Fig. 5: *In vitro* drug release data: (A) Zero-order (B) First-order (C) Higuchi model

Table 5: Effect of temperature on assay of Allopurinol

Code ↓	Effect of stability on assay (%) at 4°C				
Days →	**0**	**15**	**30**	**40**	**60**
LF2	98.9±0.47	96.7±0.57	98.9±0.82	97.7±1.79	97.8±0.58
LF4	94.3±0.76	96.9±0.56	94.6±1.14	96.8±0.55	97.5±0.98
LF6	99±1.5	98.3±1.5	98.7±1.7	98.4±0.85	97.3±1.5
	Effect of stability on assay (%) at 40°C±2°C and 75%±5% RH				
LF2	99.9±0.67	98.6±0.46	98.6±0.54	95.78±0.34	-
LF4	96.4±0.96	97.4±0.79	96.8±0.68	94.78±0.62	-
LF6	98.5±1.7	99.6±0.63	97.7±0.67	94.82±0.76	-

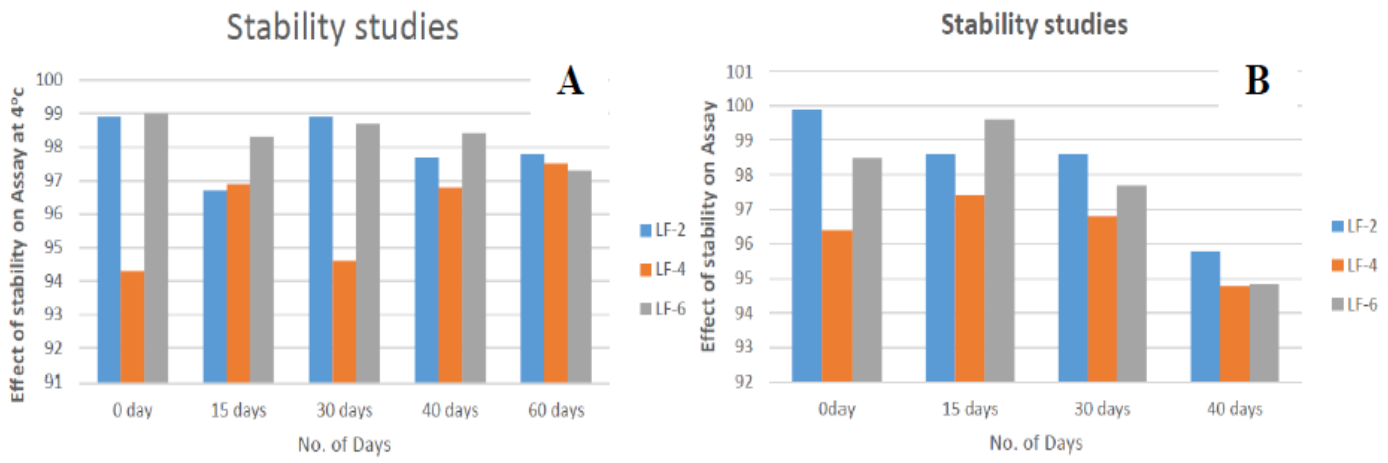

Fig. 7: Drug content observed under (A) Stability study at 4°C temperature (B) Stability study at 40°C± 2°C and 75%±5% RH.

4. DISCUSSION

For improved therapeutic effectiveness, liposomes might be used as a drug cargo in a variety of therapies [22]. To treat cutaneous leishmaniasis, researchers placed allopurinol onto liposomes. With medicine and a carrier (soybean lecithin), liposomes were created using a rotary evaporator and a dried thin film hydration technique [23, 24].

Allopurinol formulations were made using several stabilisers such as dicetylphosphate and stearylamine, with all other parameters such as temperature, vacuum and RPM being constant [25, 26]. Using a wet approach, the particle size distribution of allopurinol liposomes formulations LF2, LF4 and LF6 was examined. The particle diameter in LF6 Formulation was ideal when compared to LF2 and LF4 [27]. Liposome shape and surface texture were evaluated using SEM. The flat surfaces of the particles in the LF2 and LF6 formulations were visible in SEM microphotographs. The zeta potential reports for LF2, LF4 and LF6 formulations are 29.25 mV, 43.03 mV and 26.77 mV, respectively, which are close to the arbitrary value. The research demonstrates that the prepared liposomal solution has an excellent stability value [28, 29]. The *in vitro* drug release of all formulations was studied using a pH 7.4 phosphate buffer and the membrane diffusion method. The distribution of medicine from the F6 formulation was proven to be maintained to a degree when compared to the LF1, LF2, LF3, LF4 and LF5 formulations. The stability of the allopurinol liposomes was tested after 60 days of storage at 40°C and room temperature [30]. The sample assays were established solely based on the storage time. Liposomes were shown to be stable after 60 days at 40°C [31].

El-Badry *et al.*, (2014) investigated the *in vitro* penetration of ketotifen produced in deformable liposomes over rabbit pinna skin using Tween 80 as the edge activator and found comparable results [32]. According to the study, drug encapsulation of hydrophilic drugs may result in fast dispersion on human skin, where phospholipids might form a lipid barrier. Only liposomes containing Allopurinol were employed for penetration in this case, and once the formulation was dried, a lipid film formed on the skin, inhibiting drug release. Cevc *et al*. (2008) observed that drying a ketoprofen-containing carrier formulation on the skin surface enhances drug-carrier interaction, reducing skin penetration [33]. Honeywell-Nguyen *et al.*, (2004) observed that elastic vesicles formed by phosphatidylcholine and sodium cholate improve calcein penetration in a non-encapsulated form [34]. The test conditions, in which vesicles are kept at 32°C under non-occlusive conditions for an extended period of time, publicising fusion and inordinate dehydration [35] and beginning to form a film on the skin's surface, establishing a barrier to stop drug permeation, could be one reason for the poor drug accrual in the skin after implementation of elastic liposomes.

This might be due to the misconception that liposomes are hollow spheres surrounded by a lipid bilayer layer. Once applied to the skin surface, they remain in the top layer of the stratum corneum, acting as a drug reservoir, while liposomes may rapidly change their shape and pass the skin barrier [36]. The difficulty in loading hydrophobic medicines into the bilayer without

affecting the vesicles' deformability and elastic characteristics is, however, a severe restriction. When compared to other vesicles, liposomes have an irregular spherical form and greater skin permeability values [37]. In the instance of drug penetration through the skin, the reaction rose dramatically when the concentration was raised [38]. The main explanation for this is allopurinol's permeation-enhancing potential in liposomes, which was further boosted by the combined impact of phospholipid [39].

5. CONCLUSION

The purpose of this study was to manufacture and test allopurinol liposomes. The liposomes were made utilising a rotary evaporator and a dehydrated thin film hydration process with medication, carrier, ammonium sulphate and stabilisers. Temperature, vacuum and RPM were all kept within acceptable limits. The liposomes were prepared and kept in a freeze-dried state before being delivered for further testing. Physical and chemical properties such as average vesicle size, shape and zeta potential were assessed in liposomes of the LF1, LF2, LF3, LF4, LF5 and LF6 formulations. The percent free drug and assay of the produced liposomes of LF1 to LF6 were assessed. When contrasted to certain other preparations of LF1, F2, F3, F4 and F5, the percent free drug was highest in F6. When contrasted with different formulations of LF1, LF3, LF4, LF5 and LF6, the Assay performed best in LF2 (Neutral). This new liposomal drug delivery system was further tested for dissolution utilising the membrane diffusion technique using a pH 7.4 phosphate buffer. When compared to LF1, LF2, LF3, LF4 and LF5 formulations, the drug release from LF6 (negative) formulation was observed to be maintained to a degree. After 60 days of storage at 4°C and room temperature, the allopurinol Liposome's stability was assessed. The samples' assay was measured as a function of time spent in storage at various intervals. Liposomes kept at 4°C were shown to remain stable for three months. Physical characterisation, *in-vitro* assessment, release kinetics, and stability tests revealed that charged Liposomes carrying allopurinol could be utilised to treat fungal illnesses in the same way that regular medication and neutral liposomes could.

6. ACKNOWLEDGEMENT

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Conflict of Interest

Author declares no conflict of interest.

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