FORMULATION AND EVALUATION OF AMPHOTERICIN B AND MILTEFOSINE COMBINATION NANOVESICLES

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ABSTRACT

Objective: The aim of the present investigation is to formulate and evaluate amphotericin B-miltefosine combination nanovesicles for application in the treatment of visceral leishmaniasis.

Methods: Amphotericin B-miltefosine combination (AmB-MTF) nanovesicles were prepared by ethanol injection method. Formulations of nanovesicles were evaluated at varying conditions of lipids composition, drug-lipid proportion, ethanol-water composition and stirring rate, on drug entrapment efficiency and particle size.

Results: The study showed that entrapment efficiency was significantly affected (p<0.01) by the effects of lipids composition, drug-lipid proportion, ethanol-water composition, and stirring rate. Particle size of nanovesicles was significantly affected (p<0.05) by drug-lipid proportion and stirring rate. An optimized formulation of amphotericin B-miltefosine nanovesicles was prepared at optimal factors composition of: phosphatidylcholine-cholesterol-stearic acid 20:4:1, drug-lipid 1:8, AmB-MTF 1:1; ethanol-water 1:4 ratios, and stirring rate 1000 rpm. The AmB-MTF 1:1 nanovesicles formulation showed particle size of 145.6 nm, poly dispersity index 0.19, zeta potential-27.3 mV and drug entrapment efficiency 87%.

Conclusion: Evaluation of AmB-MTF 1:1 nanovesicles showed development of a successful formulation with very good compatibility, extended drug release, convenient vesicle size and high drug entrapment efficiency. To conclude, AmB-MTF 1:1 nanovesicles formulation could be a safe and reliable therapeutic option over the conventional combination therapy provided further antileishmanial investigations are investigated *in vitro* and *in vivo.*

Keywords: Nanovesicles, AmB-MTF, Ethanol injection method, Leishmaniasis

INTRODUCTION

Visceral leishmaniasis is the most severe form of leishmaniasis by which patients nearly always die if untreated. It causes half a million new cases and 50,000 deaths each year. Visceral leishmaniasis remains to be a challenge for poor people in developing countries. The current treatment for visceral leishmaniasis is with drugs developed 50 y ago. Pharmaceutical companies are not attracted for developing new therapeutic options for neglected infectious diseases due to low incentives in the area [1, 2].

Hence, the best strategy that remains as effective solution in crisis associated with the neglected diseases is using combination of the exciting drugs and developing novel drug delivery systems to improve the efficacy, specificity, tolerability, and emergence of resistance of existing antiparasitic agents [3]. Nanocarriers drug delivery to the liver and spleen, the main infection site of visceral leishmaniasis, have been developed [4]. Ambisome is the most effective and safe amphotericin B lipid liposomal formulation currently available on market. However, ambisome monotherapy unresponsiveness and drug resistance cases have also been reported in some places such as Bahir, India [5].

In combating the risk of resistance and to increase the efficacy of the existing drugs, WHO recommended use of combination of drugs. WHO recommended the use of liposomal AmB (5 mg/kg by infusion, single dose) and miltefosine (daily for 7 d orally) for the treatment of visceral leishmaniasis caused by *L. donovani* in Bangladesh, Bhutan, India and Nepal. This decreases the treatment duration by monotherapy from 28 d of miltefosine and 6-10 d of liposomal AmB to only 7 d combination therapy at reduced total exposure of each drug to patients [1, 6]. However, the two drugs are given in different routes and as separate dosage forms, and miltefosine is given in its conventional delivery that has significant gastrointestinal disturbances and hemolytic effects. Thus, the objective of the present investigation is to formulate and evaluate AmB-MTF combination lipid nanovesicles in a single dosage form and for targeted drug delivery to infection sites in the treatment of visceral leishmaniasis.

MATERIALS AND METHODS

Materials

Amphotericin B (Sigma St Louis, USA), miltefosine (Cayman chemicals, USA), phosphatidylcholine (Spectrum chemicals, USA), cholesterol (MB Biomedicals, USA), oleic acid (Fisher; USA), lecithin (Fisher; USA) and stearic acid (Fisher; USA) were purchased. Distilled water, tween 80 (Fisher chemicals; USA), methanol (Fisher chemicals; USA), ethyl alcohol (Dacon Laboratories, USA), dimethyl sulfoxide (Fisher chemicals, USA), phosphate buffered saline tablets (Fisher chemicals, USA), and all other reagents were all used as received. All chemicals used were analytical grade.

Methods

Preparation of nanovesicles

Drug loaded nanovesicles were prepared using ethanol injection technique based on Tanga *et al.* [7]. Accurately weighed AmB was dissolved in dimethyl sulfoxide (DMSO) to form 25 mg/ml solution. Specified amounts of lipids and 5 mg of miltefosine were accurately measured and dissolved in ethanol and heated to 75 °C as per the compositions given in table 1. A required amount of AmB solution was preheated to 75 °C and added to the lipid solution. The ethanolic solution was kept in sonicator bath for about 10 min to dissolve the entire content in ethanol with the end point as a clear yellowish solution. This solution was heated to 75 °C in the hot plate water bath (Heidolph magnetic stirrers 0416, Germany), injected into phosphate buffer saline (PBS) (pH 7.4) aqueous medium preheated at 75 °C in a round bottom flask, stirred continuously at a required rate for 5 min and probe sonicated (Microson TM, XL2000, USA) for 1 minute at a frequency of 20 kHz. The hot water bath was removed and the magnetic stirring was further continued at the same speed for about 15 min to bring the system to room temperature. Ethanol

was removed from the preparation by using rotavapor (Heidolph Bushi Rotavapor, R-114, Germany). Finally, the vesicular dispersion was stored in airtight amber glass container at 2-8 °C.

Drug entrapment efficiency

Drug entrapment efficiency (DEE) was estimated according to Lankallapalli *et al.,* and Bose *et al.,* [8,9]. Nanovesicles suspension containing an equivalent of 1 mg AmB was diluted 1 to 5 with DMSO-distilled water (1:25 v/v) and centrifuged (Eppendruf AG 5404, Germany) at 20,000g for 30 min at 4 °C. The supernatant was carefully separated and kept in a separate glass tube. For entrapped drug, the precipitate was dissolved in 5 ml DMSO-methanol (1:5 v/v). 1 ml of this solution was diluted appropriately to make a solution of 20 ml in DMSO-methanol-water (1:4:5 v/v) and AmB was determined directly from this solution at 408 nm using UV-Vis spectroscopy (Hitachi-2910, Japan). For the unentrapped drug, 1 ml of the clear supernatant solution collected previously was diluted appropriately to form 5 ml solution in DMSO-methanol-water (1:4:5) and AmB was determined directly from this solution at 408 nm. The total drug content was obtained as sum of drug content in the supernatant and in the precipitate. The AmB entrapment efficiency was calculated by using the following formula. In the case of miltefosine, entrapment efficiency is considered to be 100%, because it is the structural part of the nanovesicles due to its surfactant like action [10].

$$DEE\ (\%) = \frac{\text{Entraped drug content}}{\text{Total drug content}} \times 100$$

Particle size and zeta potential determination

The average particle size, polydispersity index (PDI) and zeta potential measurements were evaluated by dynamic light scattering technique. All analyses were carried out using Zeta sizer (Zita sizer, USA) for nanoparticles. The measurements of particle size, polydispersity index and zeta potential for each sample were carried out in triplicates and reported as mean values [11].

In vitro drug release studies

In vitro release of AmB from nanovesicles was evaluated by the dialysis bag diffusion technique [9, 12]. Sample of nanovesicles suspension equivalent to 2 mg AmB was diluted to 5 ml in PBS (pH 7.4). The resulting 5 ml solution was transferred to dialysis tubing (MW cutt-off 14,000 Da, Ward's science, USA) which has been priorly soaked for 15 min in distilled water. The dialysis tubing with the 5 ml sample in was sealed at both ends, and immersed into a receptor compartment containing 95 ml dissolution medium of PBS (pH 7.4) and 1% tween 80. The receptor compartment was stirred at 100 rpm and maintained at 37±0.5 °C. The receptor compartment was covered to prevent the evaporation of release medium. 2 ml of samples were withdrawn at time intervals (0, 2, 4, 6, 12, 24, 48 and 72 h), and the same volume was replaced by fresh medium at same temperature. The sample solutions in DMSO-methanol-water (1:4:5) were prepared using appropriate dilution and were analyzed by UV-Vis spectroscopy at 408 nm. All the experiments were performed in triplicate, and the average values were taken.

Fourier transform infrared spectroscopy

The Fourier transform infrared (FTIR) spectra of AmB and miltefosine plain drugs, and freeze-dried AmB-MTF nanovesicle formulations were analyzed using a FTIR spectrophotometer (Thermo Nicholet 6700, Portugal) [11, 12]. Samples were diluted with KBr powder in a pellet die, and the mixture was pressed at high pressure gauge to form a thin transparent disc. The FTIR measurements were performed at wave numbers ranging from 4000 to 450 cm^{-1} at constant rate of 10 °C/minute under an argon purge.

RESULTS AND DISCUSSION

AmB-MTF nanovesicles were prepared by ethanol injection method. Nanovesicles were evaluated at different conditions of lipids composition, drug-lipid proportion, ethanol-water composition and stirring rate on drug entrapment efficiency and particle size. In the preparation of nanovesicles, ethanol soluble components; phosphatidylcholine (PC), cholesterol, stearic acid and miltefosine were first dissolved in ethanol at 75 °C to assure a homogeneous mixture of lipids for spontaneous formation of small unilamellar vesicles (SUVs) upon injection [12]. AmB was dissolved in DMSO (25 mg/ml) to enhance its entrapment in nanovesicles. This is in accord with previous findings that the amount of poorly water soluble drugs encapsulated into vesicles is usually quite high with solvent aid [14]. Due to limited drug solubility in the external aqueous phase, the solubilized AmB in organic solvents tended to have a higher affinity to the phospholipid membrane components of the nanovesicles, thereby resulting in enhanced entrapment efficiency [14]. The concentration of lipids in ethanol solution was fixed at around 25 mg/ml to insure formation of SUVs in the vesicle formation. Studies showed that decreasing phospholipid concentrations in ethanol from 50 mg/ml to about 10 mg/ml produced SUVs of a significant lower particle size, although entrapment efficiency may be decreased as the lipid concentration decreased [15]. The effects of ingredients and process variables on nanovesicles formulations is presented in table 1.

Table 1: Effects of different factors in preparation of AmB-MTF nanovesicles

Factors		Particle size (nm)	DEE (%)
Stirring rate (rpm)	*8 mg cholesterol, 30 mg PC, 1:10 AmB: lipid and 1:4 Ethanol: PBS.*		
	500	196.3±6.9	81.45±2.26
	1000	193.6±6.3	79.05±5.41
	1200	167.6±14.6	67.97±2.96
Lipid (mg)	*1000 rpm,1:10 AmB: lipid, and 1:4 Ethanol:* PBS, total lipid (40 mg)		
	Stearic acid, Oleic acid, Tween 80 (10:1:1)	171.5±10.7	37.41±4.8
	Stearic acid,Lecithin, Cholesterol (0.5:7:2)	184.0±11.7	45.43±5.77
	PC, Cholesterol (7:2)	192.5±9.0	77.63±2.73
	Stearic acid, PC, Cholesterol (0.5:7:2)	193.3±6.9	93.07±3.19
Drug: lipid (mg)	*1000 rpm, 8 mg cholesterol, 30 mg PC, and 1:4 Ethanol: PBS.*		
	1:5	202.6±1.7	61.26±4.39
	1:10	192.5±6.3	79.43±5.49
	1:20	169.7±17.4	88.54±3.31
Ethanol-PBS(pH 7.4)	*1000 rpm, 8 mg cholesterol, 30 mg PC, 1:10 AmB: lipid*		
	2:1	---	---
	1:1	184.6±3.3	38.34±4.09
	1:4	193.6±6.3	79.43±5.49

Values represent mean±SD (n=3), weight of miltefosine in all formulations was 5 mg, total ethanol-water volume was 10 ml.

Effect of stirring rate

The effect of stirring rate on vesicle size and entrapment efficiency was assessed and the results are presented in table 1. In order to investigate the effect of stirring rate, formulations were prepared at 500, 1000 and 1200 rpm while all other factors put at specified values in the table. The results showed particle size decreased from 193.6 to 167.6 nm when the stirring rate was increased from 1000 to 1200 rpm. The particle size also decreased from 196.3 to 193.6 nm by increasing stirring rate from 500 to 1000 rpm. Overall, the results

showed increasing stirring rate from 500 to 1200 rpm significantly decreased the size of nanovesicles from 196.3 to 167.6 (p<0.05). This may be due to the increased shearing action that has decreased aggregation of small vesicles leading to formation of smaller particle size vesicles [16]. The change in nanovesicles size due to stirring rate can also be explained by the intensification of micro mixing between the two phases, organic and aqueous during preparation that produces small droplets and hence small size vesicles [17].

Increasing stirring rate from 500 to 1200 rpm showed a decrease of entrapment efficiency from 81.45 to 67.97% (p<0.05). This decrease entrapment efficiency upon increasing the stirring rate may be due to the increased surface area of the nanovesicles that caused the drug molecules exposed to the surface leading more drug escape to the medium [18].

Effect of lipids

Different lipids were evaluated for entrapment efficiency and particle size properties in the preparation of drug loaded nanovesicles (table 1). Inclusion of PC and cholesterol showed an increased in entrapment efficiency up to 93.07%. Nanoparticles prepared from lecithin, stearic acid and cholesterol exhibited lower entrapment (45.43%) as compared to nanovesicles containing cholesterol, stearic acid and PC (93.07%). Nanoparticles prepared from lipids of stearic acid and oleic acid in the presence of tween 80 exhibited the lowest entrapment efficiency of 37.41%. It was evident from this that the inclusion of different types of lipids produced a significant effect on the entrapment efficiency (p<0.001). Entrapment of AmB increased from 77.63 to 93.07% upon adding stearic acid (p<0.05). A similar study showed liposomes prepared with stearic acid had better entrapment efficiency, and stearic acid acted like cholesterol in stabilizing liposomal structure [19]. The increase in entrapment of AmB could also be due to the amine group of the drug interacted with the carboxylic headgroup of stearic acid through establishment of electrostatic interactions [20]. However, the average particle sizes of vesicles with and without stearic acid was 192.5 and 193.3 nm, respectively, showing that stearic acid didn't cause a significant change in particle size of the vesicles. The effect of use of different lipids didn't generally exhibit statistically significant change on particles size distribution of nanovesicles produced (p = 0.081).

Effect of drug-lipid proportion

The effect of drug-lipid proportion in nanovesicles preparation was assessed and presented in table 1. The results indicated increasing the proportion of AmB significantly affected the particle size and entrapment efficiency. The particle size is decreased from 202.6 to 169.7 nm (p<0.05) with a decrease in the drug's proportional from 1:5 to 1:20. The decrease in particle size upon decreased initial loading of AmB could be due to the decrease in the viscosity of the organic phase with resultant higher diffusion of the lipid solution as small droplets into aqueous medium leading in lower particle size of nanovesicles. A similar study showed increasing initial loading of AmB increased the particle size with widened size distribution [21]. Decreasing the proportion of drug from 1:5 to 1:20 resulted in a significant increase in DEE from 61.26 to 88.54% (p<0.001). This shows decreasing the drug-to-lipid ratio significantly enhanced the retention of the drug in the lipid. The increase in percent entrapment might be due to the presence of more internal phase in the lipid bilayer for dissolving the drug when lipid content is high [22]. The study also indicated the entrapment of AmB and stability of vesicles was found to be higher when the preparation temperature was about 75 °C as compared to lower temperatures such as 60 °C.

Effect of ethanol-water composition

The effect of different proportion of ethanol and water (PBS solution) in the process of vesicular preparations was investigated and the result is presented in table 1. At 2:1 ratio (66 % ethanol v/v in PBS), unstable preparation with sedimentation of drug at the bottom of the container was observed within hours, and those

preparations were not further characterized. 1:1 (50%) and 1:4 (20% ethanol v/v) ratios showed changes in entrapment efficiency of the drug in nanovesicles. An increase in ethanol percentage from 20 to 50% caused the entrapment significantly decreased from 79.43 to 38.4% (p<0.01). However, the effect of an increase of ethanol percentage from 20 to 50% on nanovesicles diameter wasn't statistically significant (p = 0.09).

In general, factors evaluation study was conducted to evaluate the effects of factors; lipids composition, drug lipid proportion, ethanol-water composition and stirring rate on particle size and entrapment efficiency. The study demonstrated that, although particle size of vesicles were significantly affected by some of the factors, the vesicle sizes were still within the recommended rang of nanoparticles size from 100-250 nm for systemic drug delivery under all used ranges of the factors. However, drug entrapment efficiency was affected highly that ranges from 37.42 to 93.07% at varying compositions of the factors. All the factors also showed significant effects on drug entrapment efficiency (p<0.05), and the entrapment efficiency in some of the nanovesicles was very low. Thus, an optimal composition of factors was selected based on that to produce adequate entrapment efficiency. Hence, PC-cholesterol-stearic acid 20:4:1, drug-lipid 1:8, ethanol-aqueous medium 1:4 and stirring rate 1000 rpm were selected as optimal factors composition for optimal AmB-MTF nanovesicles formulation development in the optimized formulation study.

AmB-MTF 1:1 nanovesicles formulation

An optimal formulation of AmB-MTF nanovesicles was prepared at the chosen values: PC-cholesterol-stearic acid 20:4:1, drug-lipid 1:8, AmB-MTF 1:1, ethanol-water 1:4, and stirring rate 1000 rpm. This optimal factors composition was chosen based on the factors evaluation study. The AmB and miltefosine proportion of AmB: MTF 1:1 (w/w) was choosen based on their therapeutic doses to patients, and preliminary AmB and miltefosine interaction study on promastigotes (data not shown here).

AmB-MTF 1:1 nanovesicles showed vesicle size 145.6 nm, poly dispersity index (PDI) 0.19, zeta potential-27.3 mV and DEE 87% (table 2). PDI is used to describe the degree of non-uniformity of a size distribution of particles. According to international organization for standards (ISO), PDI values closer to 0 are seen with highly monodisperse standards, whereas PDI values 0.7-1.0 indicate that the sample has a very broad particle size distribution. In drug delivery applications using lipid-based carriers, a PDI of 0.3 and below is considered to be acceptable [23]. This may indicate the size distribution of AmB-MTF 1:1 nanovesicles (PDI = 0.19) is within the acceptable range. The relative smaller particle size and PDI values in AmB-MTF 1:1 nanovesicles can be due to the surfactant nature of miltefosine that could produce smaller and uniform sized droplets and hence smaller nanovesicles during preparation [24]. Zeta potential results of AmB-MTF nanovesicles was-27.3 mV, and AmB entrapment was 87.0%. Particle size and zeta potential distributions of AmB-MTF 1:1 nanovesicles are presented in fig. 1.

***In vitro* drug release study**

The results of *in vitro* drug release study of the AmB-MTF 1:1 nanovesicles formulation showed drug release of 99.51% in 72 h. It is also evident from the result that about 50% of AmB was released in the first 6 h, and about 93% of AmB was released in the first 24 h while the remaining undergo a more extended release up to a period of 72 h (fig. 2). The higher drug release rate of the poorly-water soluble AmB in the first 12 h may be due to the surfactant effect of miltefosine [25]. *In vitro* drug release study is performed in order to assess the potential of nanovesicles to control the release of drugs incorporated for prolonging the action [26].

Table 2: Particle size, PDI, Zeta potential and DEE evaluations of AmB-MTF nanovesicle

Formulation	Particle size (nm)	PDI	Zeta potential (mV)	DEE (%)
AmB-MTF 1:1 nanovesicles	145.6±8.4	0.19±0.04	-27.3±2.8	87.0±4.2

Values represent mean±SD (n =3)

AmB/MTF 1:1 nanovesicle   Zeta potential distribution   Particle size distribution
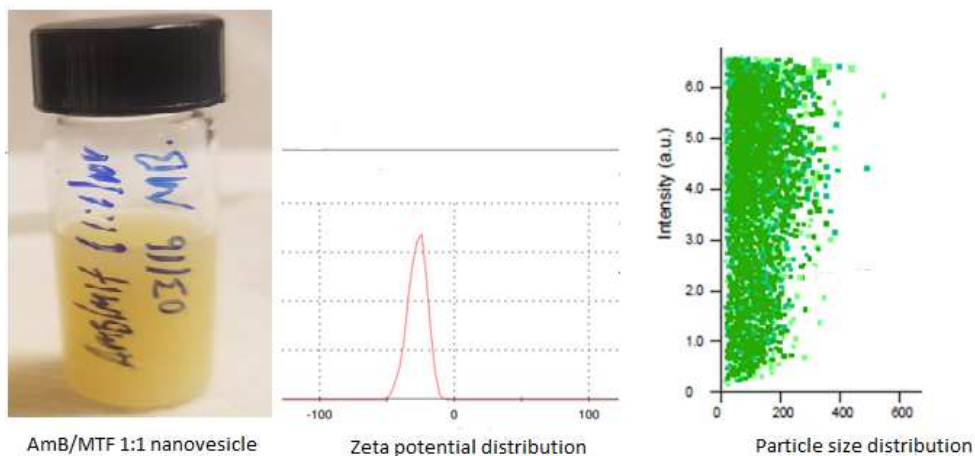
Fig. 1: Particle size (nm) and zeta potential distributions (mV) of the AmB-MTF 1:1 nanovesicles
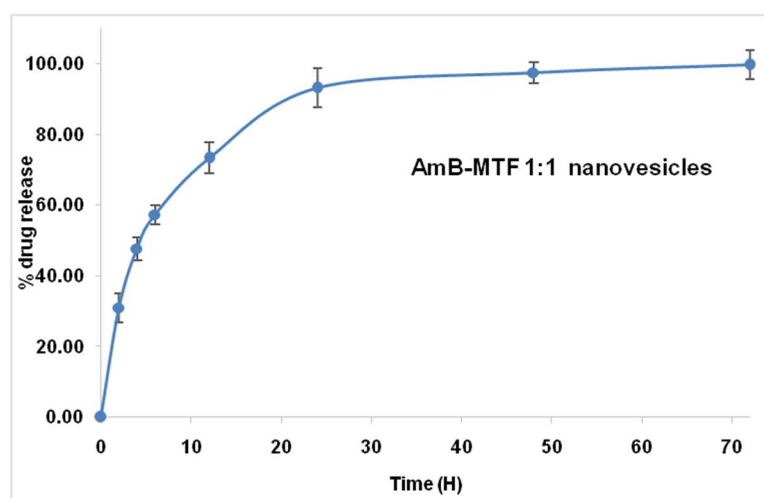

Fig. 2: *In vitro* drug release profile of AmB-MTF 1:1 nanovesicles in PBS (pH 7.4)

Drug-excipients interaction study

Drug-excipients interaction was studied using Fourier transform infrared (FTIR) spectroscopy, and the results are presented in fig. 3. AmB demonstrates characteristic peaks in FTIR at 2920 cm⁻¹ (due to its CH_2 and CH_3 stretching) and 3400 cm⁻¹ (due to OH stretching) [27]. In this study, both plain AmB and AmB-MTF 1:1 nanovesicles showed these characteristic peaks of AmB at about the same wavelengths. Similarly, miltefosine exhibits its characteristic FTIR peaks at 2900 cm⁻¹ (CH_2 stretching) and 1500 cm⁻¹ (CH_2 bending) [28]. The results of this study also illustrated plain AmB and miltefosine, and AmB-MTF 1:1 nanovesicles showed these characteristic peaks at about the same wavelength. According to this study, there was no apparent interaction between the drugs and formulation excipients [29, 30].
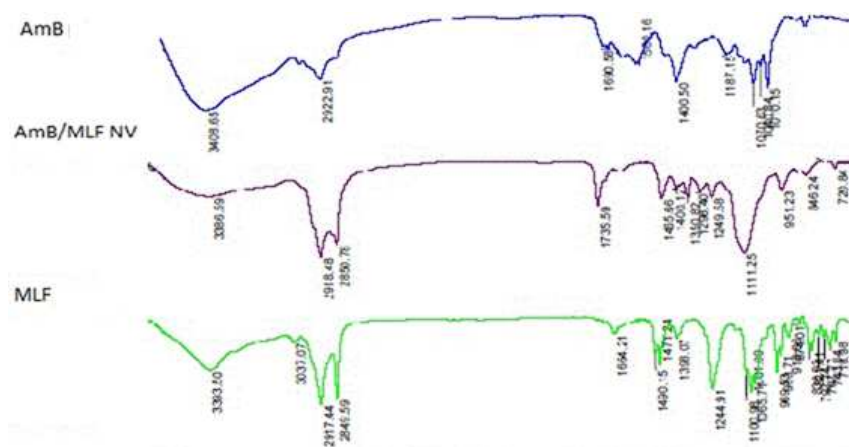

Fig. 3: FTIR spectra of AmB, miltefosine and AmB-MTF nanovesicles

CONCLUSION

AmB-MTF nanovesicles were prepared by the ethanol injection method. The study showed that entrapment efficiency was significantly affected ($p<0.01$) by the effects of lipids, drug-lipid proportion, ethanol-water composition and stirring rate. Particle size of the nanovesicles were significantly affected ($p<0.05$) by drug-lipid proportion and stirring rate. The study also demonstrated that although both entrapment efficiency and particle size showed significant changes, drug entrapment efficiency was the most important response variable. Hence, an optimal composition of factors: PC-cholesterol-stearic acid 20:4:1, drug-lipid 1:8, ethanol-aqueous medium 1:4 and stirring rate 1000 rpm was chosen based on that to produce adequate entrapment efficiency for optimal AmB-MTF nanovesicles preparation. Hence, optimized AmB-MTF1:1 nanovesicles was prepared at the chosen optimal factors composition, and its evaluation showed development of a successful formulation with very good compatibility, extended drug release, convenient particle size and high drug entrapment efficiency. This study concludes AmB-MTF 1:1 nanovesicles could be a safe and reliable therapeutic option over conventional AmB-MTF combination therapy. However, for the AmB-MTF 1:1 nanovesicles formulation to be used as better option of the conventional combinations in the treatment of visceral leishmaniasis, further antileishmanial investigations need to be conducted *in vitro* and *in vivo.*

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AUTHORS CONTRIBUTIONS

All the authors listed in this manuscript have contributed equally.

CONFLICT OF INTERESTS

The author declares that there is no conflict of interest related to this report

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