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IN-VITRO ANTILEISHMANIAL EVALUATION OF AMPHOTERICIN B-MILTEFOSINE NANOVESICLES

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Keywords:

Antileishmanial activity; AmB-MTF nanovesicles; Macrophages; targeted delivery.

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ABSTRACT: Leishmaniasis is a neglected tropical infectious disease affecting poor people, especially in the developing countries of the world. It is a systemic disease caused by a protozoan parasite leishmania donovani and is fatal if left untreated. The aim of the present study is to develop and evaluate the *in-vitro* antileishmanial activity of the combination of amphotericin B and mitefosine nano vesicles for targeted delivery to macrophages in the treatment of visceral leishmaniasis to increase the efficacy, decrease emergency of resistance and increase overall patient compliance. Drug-loaded nanovesicles were prepared by ethanol injection method. Physicochemical characterization and cytotoxicity, and antileishmanial activity evaluations were assessed. Nanovesicles of optimal AmB-MTF formulation was prepared from phosphatidylcholine-cholesterol-stearic acid (20:4:1 w/w) lipids, ethanol-water (1:4 v/v) dispersion medium, and AmB-MTF (1:1 w/w) drugs at drug loading of 1:8 and stirring rate of 1000 rpm. AmB-MTF 1:1 nanovesicles exhibited a mean particle size of 145.6 nm, polydispersity index (PDI) 0.19, zeta potential -27.3 mV and drug entrapment efficiency 87%. The AmB-MTF combination nanovesicles demonstrated low CC50 against J774 A.1 murine macrophage cells (18.12 µM) compared to the plain AmB (3.71µM), AmB nanovesicles (13.61µM) or AmB-MTF physical mixture (13.64 µM). *In-vitro* antileishmanial activity against promastigotes study indicated that IC_{50} of AmB-MTF1:1 nanovesicles (0.046) was significantly decreased ($p<0.05$) compared to plain AmB-MTF mixture (0.063) or AmB nanovesicles (0.055). To conclude, AmB-MTF nanovesicles could be a safe and reliable therapeutic option over conventional AmB-MTF combination therapy with due consideration *in-vivo* evaluations to be investigated.

INTRODUCTION: Leishmaniasis is a neglected tropical infectious disease affecting poor people, especially the developing countries of the globe [1]. Leishmaniasis has traditionally been classified in three major forms based on clinical symptoms;

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visceral leishmaniasis, cutaneous leishmaniasis, and mucocutaneous leishmaniasis. Visceral leishmaniasis is a systemic disease typically caused by *L. donovani* complex, which includes three species: L. *donovani donovani, L d. infantum*, *L. d. chagasi*. *L. donovani* is the causative for visceral leishmaniasis in the Indian subcontinent and East Africa [2].

Visceral lesihmaniasis is estimated to cause 500,000 new cases and 50,000 deaths annually. In 2015, 90% of worldwide visceral leishmaniasis

cases were reported from seven countries: Brazil, Ethiopia, India, Kenya, Somalia, South Sudan, and Sudan [3]. Visceral leishmaniasis is one in which leishmania parasites migrate and reside in macrophages where the parasite multiplies exponentially until the macrophage is ruptured, releasing the amastigotes into the blood.

The localization of the parasite intracellularly in macrophages presents challenges to drug delivery, as the drug must achieve antiparasitic concentration to the parasite within macrophages [4]. Nanoparticulate intravenous drug delivery system to macrophages presents a novel approach for effective and efficient treatment of visceral leishmaniasis. The beneficial feature of novel drug delivery systems of leishmaniasis treatment has been clearly demonstrated by the higher efficacy and lower toxicity of lipid formulations of amphotericin B compared to amphotericin B solutions. The most frequently used nanoparticles in macrophage targeting through IV route are microscopic lipid-based nanovesicular systems [5]. Mononuclear phagocyte cells engulf leishmania parasites and remove drug particles from the body's circulation. The natural capability of engulfing foreign particles of macrophages makes drug-loaded nanovesicles and leishmania parasites to be internalized that enhances the efficacy of the drugs against the parasites at higher concentrations in the macrophage cells [6].

A very high therapeutic index, short treatment courses and the absence of side effects make lipid formulations of amphotericin B (AmBisome) the most attractive existing treatment for visceral leishmaniasis. However, cases of resistance development from AmB isomerate reported and became a future challenge on administering this formulation alone [7]. In combating the risk of resistance and increasing the efficacy of the existing drugs, WHO recommended using a combination of drugs in different parts of the world. WHO recommended the use of liposomal amphotericin B (5 mg/kg by infusion, single dose) and miltefosine (daily for 7 days 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 days of miltefosine and 6-10 days of liposomal amphotericin B to only 7 days combination therapy at reduced total exposure of each drug to patients [3, 8]. The aim of the present study was to develop a combination of mitefosine and amphotericin B nanovesicles for targeted delivery to macrophages to treat visceral leishmaniasis.

MATERIALS AND METHODS: Amphotericin B (SIGMA St Louis, USA), miltefosine (Cayman chemicals, USA), phosphatidylcholine (Spectrum Chemicals, USA), cholesterol (MB Biomedicals, USA), and stearic acid (Fisher chemicals; 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 (Fisher Chemicals, USA), and all other reagents were used AS received. All chemicals used were analytical grade.

Preparation of Nanovesicles: Drug loaded nanovesicles were prepared using ethanol injection technique according to the method by Tanga *et al.* [9]. 30 mg phosphatidylcholine, 6.5 mg cholesterol, 1.5 mg stearic acid, and miltefosine (5 mg or 15 mg for AmB-MTF 1:1 or 1:3 nanovesicle formulations, respectively) were accurately weighed and dissolved in ethanol at 75 °C. 25 mg/mL AmB solution was prepared in DMSO and a 5 mg AmB equivalent of AmB solution was preheated to 75°C and added to the lipid solution. The lipid solution was sonicated in a bath sonicator (Intertek, WB10, China) for 10 min at 75 °C to dissolve the entire content in ethanol with the endpoint as a clear yellowish solution. 8 ml of phosphate buffer saline (pH 7.4) was heated in a round bottom flask to 75 °C in a water bath on a hot plate magnetic stirrer (Heidolph magnetic stirrers 0416, Germany).

The clear yellowish drug sonicated solution (at the same temperature) was added through a syringe phosphate buffer saline (PBS) (pH 7.4) medium in the round bottom flask under stirring at 1000 to rpm. The solution was stirred for 5 min, and later it was probe sonicated (Microson TM, XL2000, USA) for 1 min at 20 KHz. The hot water bath was removed, and stirring was further continued for about 15 min to bring the system to room temperature. Ethanol was removed from the nanovesicles suspension by using rotavapor (Heidolph Bushi rotavapor, R-114, Germany).

Finally, the nanovesicle suspension was stored in an airtight amber glass container at 2-8 °C.

Particle Size and Zeta Potential Determination: The average particle size, polydispersity index (PDI), and zeta potential measurements were evaluated by the dynamic light scattering technique. All analyses were carried out using Zetasizer (Zitasizer, USA) instrument. The measurements of particle size, polydispersity index, and zeta potential for each sample were carried out in triplicates and reported as mean values [10].

Drug Entrapment Efficiency: Drug entrapment efficiency (DEE) was estimated according to Lankallapalli *et al.* and Bose *et a1* [1, 12]. Nanovesicles suspension containing an equivalent of 1 mg AmB was diluted 1 to 5 with DMSO-distilled water (1:25 v/v) and centrifuged (Eppendorf AG 5404, Germany) at 20,000 g for 30 min at 4 °C. The supernatant was carefully separated and kept in a separate glass tube. For entrapped drugs, the precipitate was dissolved in 5 ml DMSO-methanol (1:5 v/v). 1 ml of this solution was diluted appropriately to make 20 ml solution of drug in DMSO-methanol-water (1:4:5 v/v) solvent, and AmB was determined directly from this solution at 408 nm using UV-Vis spectroscopy (Hitachi-2910, Japan). For the un entrapped drug, 1 ml of the clear supernatant solution collected previously was diluted appropriately to form 5 ml solution of drug 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 the sum of drug content in the supernatant and in the precipitate. The AmB entrapment efficiency was calculated by using the following formula. Miltefosine entrapment efficiency is considered to be 100%; for that, it is the structural component of the nanovesicles due to its surfactant-like action [13].

$$\text{DEE (\%)} = (\text{entraped drug content}) / (\text{Total drug content}) \times 100$$

In-vitro Drug Release Studies: *In-vitro* release of AmB from nanovesicles was evaluated by the dialysis bag diffusion technique [12, 14]. 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 cut-off 14,000 Da, Ward's Science, USA), which has been priorly soaked for 15 min in distilled water. The dialysis tubing containing the 5 ml solution 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 the release medium. 2 ml of samples were withdrawn at regular time intervals (2, 4, 6, 12, 24, 48, and 72 h and the same volume was replaced by the fresh medium at the same temperature. The sample solutions were diluted appropriately to make a solution of AmB in DMSO-methanol- water (1:4:5) from which the samples were analyzed by UV-Vis spectroscopy at 408 nm. All the experiments were performed in triplicate, and the average values were taken. Kinetic analysis of the release data was performed by fitting to kinetic release models -zero order and first order, and release mechanism models Higuchi, Hixson-Crowell, and Peppas- Korsmeyer to characterize the release behaviour [15, 16, 17]. The release profile data was also analyzed using the model-independent dissolution efficiency approach to compare release profiles [18].

Characterization of Amb Formulations Aggregation: AmB aggregation level in nanovesicles formulations was evaluated according to previous reports [19, 20]. AmB containing nanovesicle formulations were dispersed in PBS (pH7.4) to yield a 10 µg/ml suspension. UV-visible optical spectra were obtained using a Hitachi spectrophotometer (Hitachi U-2910, Japan)in double-beam mode against plain lipid nanovesicles solution as blank. All spectra were obtained at room temperature (25 °C) in the wavelength range of 300-450 nm. The spectrum of each AmB containing nanovesicular formulation was compared with a spectrum of a 10 µg/mL solution of pure AmB in PBS (pH 7.4), and in a mixture of DMSO-methanol-water (1:4:5), solvent scanned under the same condition.

Lyophilization: 2 ml of the optimal AmB-MTF nanovesicle suspensions were kept in a deep freezer (-80 °C) overnight and were freeze-dried under vacuum (1.25 Bar) at -52 °C (Labconco Freezone 6 Lyophilizer, USA) [10].

The dried samples were kept in a desiccator at 2-8 °C for further use.

Fourier Transform Infrared Spectroscopy: The Fourier transform infrared (FTIR) spectra of AmB and miltefosine plain drugs and freeze-dried AmB-MTF nanovesicles were analyzed using an FTIR spectrophotometer (Thermo Nicholet 6700, Portugal) [10]. Samples were diluted with KBr powder in a pellet die and the mixture was pressed at a high-pressure gauge to form a thin transparent disc. The FTIR measurements were performed at wavenumbers ranging from 4000 to 450 cm−1 at a constant rate of 10 °C/min under an argon purge.

Stability Evaluations: The optimized nanovesicles were stored over a period of four months at 2-8 °C. The storage stability was evaluated by measuring the changes in particle size, zeta potential, and drug entrapment efficiency [10, 21].

Cytotoxicity Assay: This assay was performed using MTT based in vitro toxicity assay method according to studies previously reported [21, 22]. Different concentrations of test solutions of AmB and MTF plain drugs and nanovesicle formulations were added to wells of 96 well microtiter plates. Suspension of 1×10^5 J774 A.1 macrophage cells in RPMI-1640 macrophages medium was added to each well, and the plate was incubated (at 37 °C, 5% CO_2) for 72 h. MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; thiazolyl blue) solution was added to each well at concentration of 5 mg/mL and incubated for 3 hours. Sodium dodecyl sulphate (SDS) solution in 50% aqueous dimethylformamide was added, and the plate was last incubated for 3 h. The absorbance of solutions in each well of the microplate was determined at 570 nm using a molecular device plate reader (Spectra max M5, USA). Negative control (without drug) and positive control (with reference drugs) were used for comparison and treated like the test solution wells.

Promastigote Assay: Antileishmanial activity of AmB and AmB-MTF nanovesicles was evaluated on log-phase promastigotes of L. donovani. AmB and AmB-MTF nanovesicle solutions at different concentrations were added in a 96-well flat-bottom microtiter plate containing an M199 growth medium. For miltefosine and amphotericin B interaction promastigote assay, mixture solutions of the drugs at required concentrations were prepared and plated similarly. Stationary phase parasites were seeded at 2 × 106 parasites/mL per well to each test solution wells and the plates were incubated at 26 °C for 72 h. MTS-PMS (20:1) was added and further incubated at 26 °C for 5 h. 10% SDS in water solution was added, and the plates were last incubated at 37 °C for 1 h.

The relative amount of purple formazan produced by viable cells per well was measured photometrically at 490 nm using a molecular device plate reader (Spectra max M5, USA). Negative and positive controls were used for comparison and were treated like the test solution wells. The IC_{50} (concentration of compound needed for 50% inhibition of promastigotes growth) value of test substances was determined from the graph representing different concentrations of the compounds plotted against % of promastigote cell viability [22, 23].

RESULTS AND DISCUSSION: A study was conducted to identify an optimal composition of ingredients and conditions for AmB-MTF nanovesicles formulations that best provide nanovesicles with suitable physicochemical and formulation properties for the intended visceral leishmaniasis treatment through intravenous administration. Hence, phosphatidylcholine-cholesterol-stearic acid 20:4:1, drug-lipid proportion 1:8, ethanol-water composition 1:4, stirring rate 1000 rpm, and AmB-MTF combination 1:1 were identified as optimal compositions for optimized AmB-MTF nanovesicles formulation (data not shown here).

AmB-MTF nanovesicles were prepared at the optimized composition, and AmB nanovesicles and AmB-MTF 1:3 nanovesicles were prepared in the same condition as AmB-MTF 1:1 combination for comparison purposes. AmB-MTF 1:3 nanovesicle wasn't considered in the physicochemical characterization for that it was found to have less antileishmanial activity than AmB-MTF 1:1 combination. In general, AmB-MTF nanovesicles physicochemical characterization and antileishmanial activity evaluation studies are presented in this section.

Particle size Distribution, Zeta Potential and Entrapment Efficiency: Particle size, PDI, zeta potential and drug entrapment efficiency evaluations of nanovesicles are presented in **Table 1.** The mean vesicle size of AmB and AmB-MTF 1:1 nanovesicles were found to be 219.0 and 145.6 nm, respectively. Zeta potential results of AmB nanovesicles and AmB-MTF 1:1 nanovesicles were -30.2 and -27.3 mV, respectively. AmB drug entrapment efficiency in AmB and AmB-MTF 1:1 nanovesicles was high and comparable (89.3 and 87.0%).The polydispersity index (PDI) for AmB-MTF 1:1 nanovesicles (0.19) was significantly lower than ($P<0.05$) that of the AmB nanovesicles (0.45). Generally, AmB-MTF 1:1 and AmB nanovesicles didn't show significant differences ($P>0.05$) in most parameters except for PDI. According to International Organization for Standardization (ISO), PDI values bigger than 0.7 indicate a sample has a very broad particle size distribution. In contrast, PDI of 0.3 and below indicates a homogenous population of vesicles [Danaei *et al.*, 2018. This shows the size distribution of AmB-MTF 1:1 nanovesicles (PDI =0.19) has a more homogenous population of vesicles where AS AmB nanovesicles indicated a wide particle size distribution. The lower PDI of AmB-MTF 1:1 nanovesicles may be due to miltefosine's intrinsic surfactant action property to form small and uniform size nanovesicles [24]. Lipid nanocarriers larger than 100-150 nm can be taken up by phagocytes or remain in liver tissues for an extended time once extravasated from blood vessels [25]. AmB and AmB-MTF nanovesicles of this study are in line with this size range that might help the formulations to have longer retention in the liver for the best purpose of combating the parasite therein.

TABLE 1: PARTICLE SIZE, PDI, ZETA POTENTIAL, AND DEE EVALUATIONS OF NANOVESICLES (MEAN ± S. D., N=3)

Formulation		Particle Size (nm)	PDI	Zeta Pot. (mV)	DEE (%)
Am BNV	(Initial)	219.0±55.6	0.45±0.08	-30.2±6.7	89.2±3.4
	(After 4 months)	232.0±50.1	0.42±0.08	-26.6±6.7	86.3±1.5
AmB-MTF 1:1 NV	(Initial)	145.6±8.4	0.19±0.04	-27.6±2.3	86.3±1.4
	(After 4 months)	145.8±4.9	0.23±0.02	-25.8±0.3	83.7±4.8

FIG. 1: PHOTOGRAPHS FOR AMB NANOVESICLE AND AMB-MTF 1:1 NANOVESICLE

Degree of Amb Aggregation: The UV-visible spectra of polyenes are very sensitive to conformational changes induced by different molecular interactions, including aggregation. Hence, UV-Vis spectral characterization method was used to monitor the presence of aggregation species of AmB in AmB containing nanovesicles in PBS (pH 7.4). A UV-Vis spectrum of AmB in organic solvents wherein the drug exists in monomeric form shows four absorption bands with decreasing intensities as we go to the lower wavelengths [19]. In this study, the results are presented in **Fig. 2.** Indicated the spectra of monomeric (in DMSO-methanol-water) and aggregated (in PBS) forms of free solutions of 10 µg/ml concentration. The aggregation level of AmB in AmB nanovesicles was evaluated at the same 10 µg/ml concentration in PBS. Although the positions and intensities of the band may vary, the ratio of the intensities of absorption at the lowest (around 346): the highest (around 408) wavelength may be taken as a measure of the level of aggregation [28]. The results indicated the AmB aggregation level for free AmB, AmB nanovesicles and AmB-MTF 1:1 nanovesicles was 1.7, 4.92 and 4.11, respectively, in PBS. The results also indicated a blue shift in the short wavelength bands from 346.5 nm (in DMSO-methanol-water solvent) to 338.5, 325.5 and 326.5 nm of free AmB, AmB nanovesicles, and AmB-MTF 1:1 nanovesicles, respectively in PBS pH 7.4. This may be due to the formation of strongly coupled super aggregates of AmB molecules in the lipid structure, beyond the weak dimer interactions in the free AmB [29].

According to the general conviction, the aggregated structures of AmB are composed of dimers, which may justify the week aggregated structures of free AmB in PBS at 338.5 nm. In addition to that, the formation of super aggregation may also be due to the higher heating temperature of about 75 °C used during preparation [29]. Clinically, "super-aggregation" of AmB is beneficial for a decreased risk of toxicity from AmB. The weakly-coupled aggregates of AmB seem to be associated with a high risk of toxic side effects than the super-aggregate structures, because such weak-coupled dimer structures may directly assemble into the porous structures that are able to affect the physiological transmembrane ion transport [29]. Similar studies reported heating of Fungizone® induced the formation of monomers and super-aggregates that results in the heated Fungizone® being less toxic than Fungizone® [30].

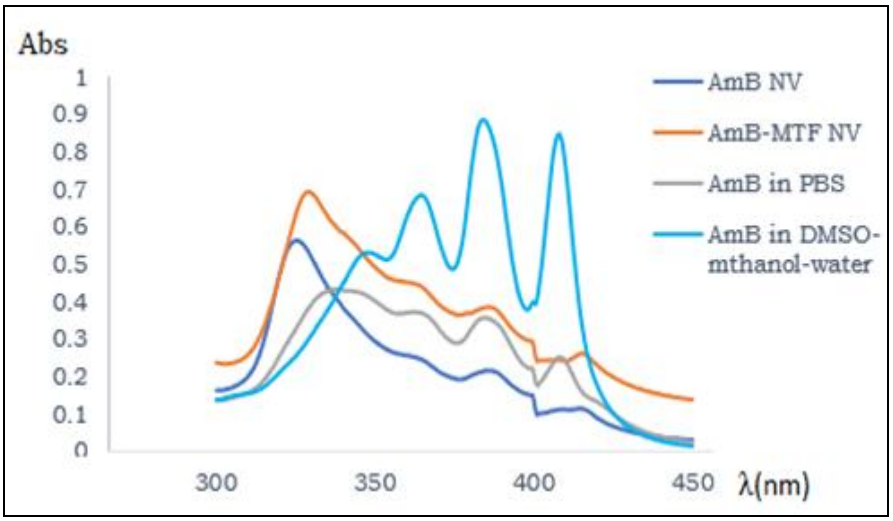

FIG. 2: UV-VIS SPECTA OF AMB FROMAMB AND AMB-MTF 1:1 NANOVESICLES

The degree of aggregation of Ambisome is also high (>5) with aggregates of a mixture of supra-aggregates and monomers with an absorption maximum at about 324 nm; vis it is a relatively non-toxic form of AmB on use [30].

Drug-excipients Interaction Study: Unintended physicochemical interaction of an excipient with a drug substance in a dosage form can result in the complexation or binding of the drug, resulting in slow and/or incomplete drug release in a dissolution medium.

In this study, drug-excipients interaction was studied using Fourier transform infrared (FTIR) spectroscopy **Fig. 3.**

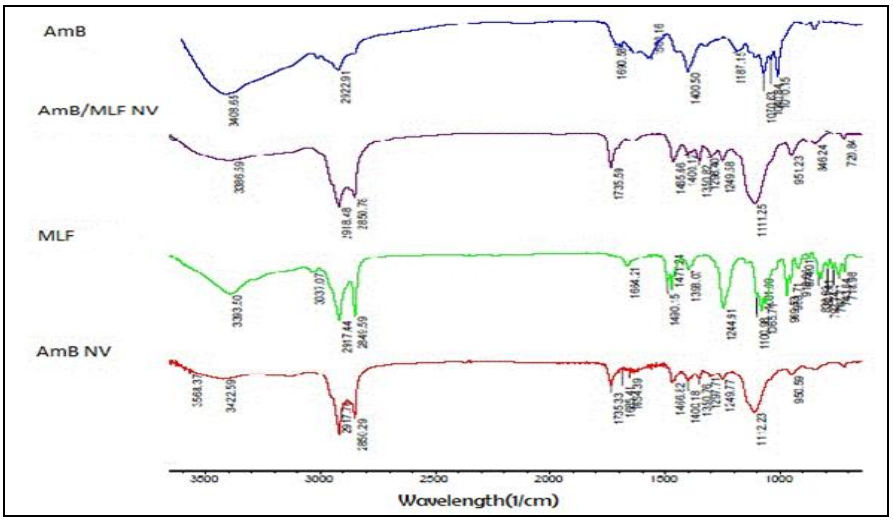

FIG. 3: FOURIER TRANSFORM INFRARED SPECTROSCOPIC SPECTRA OF NANOVESICLES

The characteristic peaks of free AmB were observed at wavenumbers 2922 and 3408 cm^{-131}. Such peaks for free miltefosine were shown at wavenumbers 2917 and 1490 cm^{-1} in the functional group region of the respective pure drugs spectrum. These characteristic peaks in the spectra of AmB correspond to 2923 cm^{-1} CH_2 and CH_3 stretching and 3400 cm^{-1} OH stretching vibrations [25]. On the other hand, in the spectra of miltefosine samples, the 2900 cm^{-1} and 1500 cm^{-1} in the functional regions correspond to CH^2 stretching and CH_2 bending vibrations, respectively [32]. Those characteristic peaks also appeared in the spectra of AmB nanovesicles and/ or AmB - MTF 1:1 nanovesicles at about the same wavenumbers, indicating no interaction between the drug and formulation excipients.

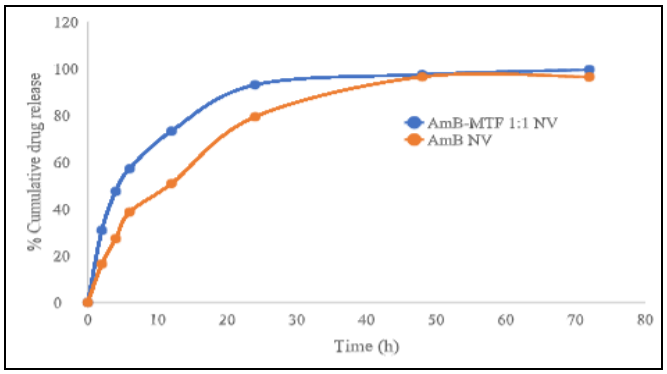

FIG. 4: *IN-VITRO* RELEASE PROFILES OF NANOVESICLES IN PBS (pH 7.4)

***In-vitro* Drug Release Study:** *In-vitro* drug release studies for AmB and AmB-MTF 1:1 nanovesicles are shown in **Fig. 4.** The results of the in vitro drug release study of the AmB and AmB-MTF 1:1 nanovesicles showed drug release of 96.38% and 99.51%, respectively, in 72 h. The results also showed that more than 50% of AmB was released in the first 12 h and more than 80% was released in the first 24 h while the remaining undergo a more extended-release up to a period of 72 h in both AmB and AmB-MTF 1:1 nanovesicles.

Comparison of release profiles of the two preparations indicated AmB-MTF 1:1 nanovesicles apparently showed relatively a higher mean dissolution efficiency (DE) (87.46%) compared to AmB nanovesicles (81.1%) **Table 2.** This may be due to the inclusion of a surfactant-like nature of the drug miltefosine [26].

However, the ANOVA analysis of the dissolution efficiency showed the difference in release profiles of AmB and AmB-MTF 1:1 nanovesicles wasn't statistically significant (p= 0.102).

TABLE 2: DISSOLUTION EFFICIENCY OF NANO-VESICLES (MEAN ± S.D., N=3)

Nanovesicles	DE value (%)
AmB nanovesicles	81.1±6.9
AmB-MTF 1:1 nanovesicles	87.46±3.74

TABLE 3: RESULTS OF DRUG RELEASE MODEL FITTING FOR NANOVESICLES

Model		AmB nanovesicles	AMB-MTF (1:1) nanovesicles
Zero order	r	0.785	0.889
	k_0 (% hr^{-1})	0.107	0.125
First order	r	0.767	0.819
	k_1 (hr^{-1})	0.013	0.021
Higuchi	r	0.921	0.974
Hixon-Crowell	r	0.561	0.678
Korsmeyer–Peppas	r	0.958	0.980
	n	0.318	0.5

For spherical particles, the release rate exponent 'n' value that fits well within the 0.43 and 0.85 range indicates an anomalous or non-Fickian diffusion.

The limits $n \leq 0.43$ indicates Fickian diffusion, and n = 0.85 shows case II release transport (non-Fickian, zero-order release) [27]. Thus, the release rate exponent 'n' value of 0.5 for AmB-MTF 1:1 nanovesicles shows that the release mechanism from those nanovesicles was non-Fickian diffusion where drug release is mediated by both through diffusion in the matrices or by the dissolution of nanovesicles. However, the 'n' value for AmB only nanovesicles was 0.318, indicating it follows classical Fickian diffusion.

Stability Study: The stability of nanovesicles of AmB and AmB-MTF 1:1 was determined after a storage period of four months by assessing the changes in vesicle size, PDI, zeta potential and DEE **Table 1**. In all the parameters evaluated, there wasn't any significant change (P>0.05) in

properties of AmB nanovesicles and AmB-MTF 1:1 nanovesicles over the four months of storage period. The phospholipid and cholesterol components of nanovesicles may contribute to the integrity and stability of the vesicles [10].

Cytotoxicity Study: The results of the cytotoxicity study showed nanovesicles of AmB and AmB-MTF 1:3 exhibited CC_{50} values of 13.61 and 18.12 µM, respectively. The CC_{50} measures for plain AmB and AmB-MTF 1:3 physical mixture were 3.71 and 13.64 µM, respectively **Table 4.** The results indicated AmB and AmB-MTF nanovesicles exhibited lesser toxicity to cells compared to the plain drug forms.

The cytotoxicity test of blank lipid nanovesicles showed no substantial growth inhibition of macrophages at concentrations of about 100 µg/mL of total lipid used in the preparations. Similarly, lipid formulations of AmB are reported to decrease the toxicity of the drug to macrophage cells as compared to the plain drug or its conventional dosage forms [33]. The reasons that lipid formulations influence the toxicity Maybe by rendering a slow release of AmB as monomers or super aggregates, and due to higher temperature 75 °C of nanovesicles preparation [29, 34].

TABLE 4: CC50 OF DRUGS AND NANOVESICLES ON J774 A.1 CELLS

Drug / Formulation	CC_{50} (µM) (mean ± s. d., n=3)
AmB	3.71±2.1
AmB NV	13.61±4.4
AmB-MTF 1:3 mixture	13.64±3.05
AmB-MTF 1:3 NV	18.12±3.4

Amphotericin B and Miltefosine Interaction On l. Donovani Promastigotes: The IC_{50} of AmB and miltefosine against *L. donovani* promastigotes were 0.076 and 11.13 µM, respectively 72 h post-treatment.

Combinatorial promastigote assay performed on varying AmB concentrations at fixed and sub-optimal concentrations of miltefosine (0.1875, 0.375, 0.75 or 1.5 µM) showed a decrease in the IC_{50} of AmB significantly from 0.076 µM (AmB alone) to about 0.061 µM (Amb-MLF) ($p<0.05$). Normalized parasite viability with comparison to untreated controls also showed increased antileishmanial activity of AmB-MTF combinations at those concentrations studied. The result also indicated a significant decrease in parasite viability from 93% to 59% upon adding 0.1875, 0.375, 0.75, or 1.5 µM miltefosine at 0.063 µM concentration of AmB **Fig 5.** This finding is in line with studies reporting the presence of a mild synergistic effect of AmB and miltefosine combination against *L. donovani in-vitro* at a lower concentration of miltefosine [35]. Similarly, a separate report indicated that antileishmanial activity of miltefosine-paromomycin was enhanced upon a combination of a lower dose of miltefosine on the top dose of paromomycin *in-vivo*. These are also supported by reports that different interactions were observed upon combining antimalarial drugs at various dose levels [35, 36]. Hence, it can be inferred from these results that it could be possible to obtain an increased efficacy and reduced risk of toxicity using AmB and miltefosine combination formulation.

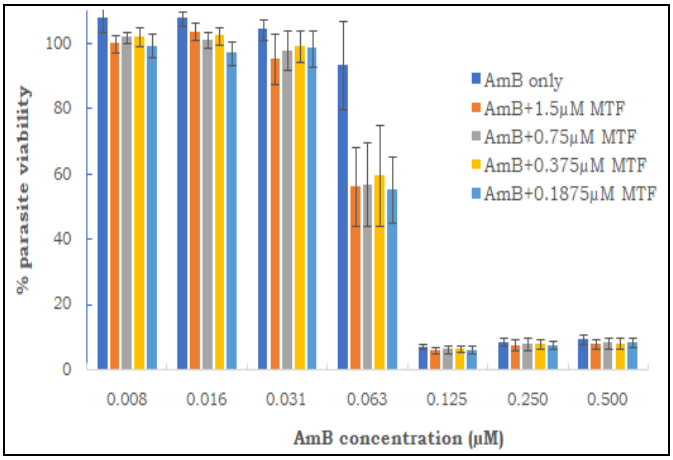

FIG. 5: PERCENTAGE PARASITE VIABILITY BY AMB AND MILTEFOSINE

***In-vitro* Antileishmanial Activity of Nano-vesicles:** Antileishmanial activity of nanovesicles of AmB, AmB-MTF 1:1, and AmB-MTF 1:3 against promastigotes 72 h post-treatment are presented in **Table 5.** The results showed IC^{50} of nanovesicles of AmB, AmB-MTF 1:1 and AmB-MTF 1:3 were 0.055 ± 0.032, 0.046 ± 0.027 and 0.046 ± 0.039, respectively.

This indicates AmB and AmB-MTF nanovesicles showed a significantly decreased ($p<0.05$) IC^{50} compared to IC^{50} of plain AmB (0.081 ± 0.044). This may be due to the mild synergetic effect of the combination of AmB and miltefosine at its lower concentrations [35].

TABLE 5: IC_{50} OF PLAIN DRUGS AND NANOVESICLES AGAINST *L. DONOVANI* PROMASTIGOTES (MEAN ± D., N=3)

Drug/formulation	IC_{50} (µM)
AmB	0.081±0.044
AmB NV	0.055±0.032
AmB-MTF 1:1 NV	0.046±0.027
AmB-MTF 1:3 NV	0.046±0.039

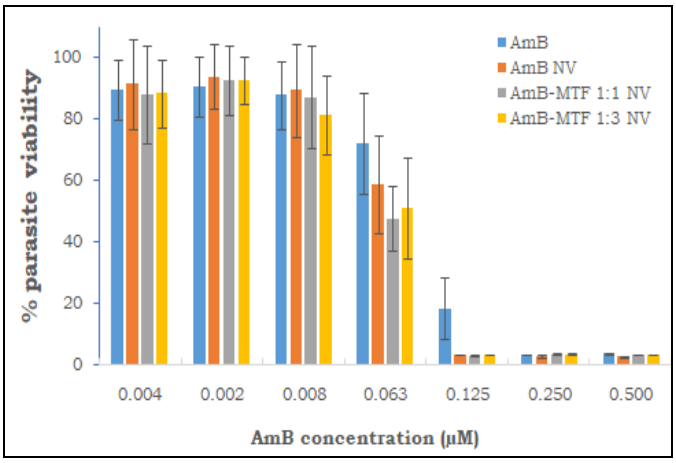

FIG. 6: PERCENTAGE OF PARASITES VIABILITY BY NANOVESICLES

The parasites' growth inhibition plot for antileishmanial activity evaluation of nanovesicle formulations is presented in **Fig. 6**. A significant variation ($p<0.05$) of parasite viability of 72%, 58%, 26% and 50% was found for plain AmB, AmB nanovesicle, AmB-MTF 1:1 nanovesicle and AmB-MTF 1:3 nanovesicle, respectively, at 0.063 µM AmB concentrations post 72 h treatment. The results also showed combination nanovesicles of AmB-MTF 1:1 and AmB-MTF 1:3 exhibited higher growth inhibition compared to plain AmB and AMB nanovesicles.

This may be due to an additive effect of the drugs AmB and miltefosine against the parasite through the different mechanisms of the individual drugs [35]. However, the antileishmanial activity for AmB-MTF 1:3 nanovesicles exhibited a less parasitic inhibition effect compared to AmB-MTF 1:1 nanovesicles at the same concentration of 0.063 µM AmB. This may be due to that miltefosine stably associated closer to 100% with lipid bilayers at its lower molar fractions [37].

Miltefosine insert in between molecules of cholesterol and phospholipids in the lipid bilayers in erect form like the AmB molecules do. This is due to the high affinity of AmB and miltefosine for cholesterol in lipid bilayers [37, 38, 39].

CONCLUSION: AmB and AmB-MTF nanovesicles were prepared by the ethanol injection method. Physicochemical evaluation of nanovesicles of AmB and AmB-MTF 1:1 showed that the formulations exhibited very good compatibility, extended drug release, efficient storage stability, and high drug entrapment efficiency. The drug-loaded nano vesicular formulations' cytotoxic activity was less toxic than plain drugs against murine J774. A macrophage cell. The nanovesicles of AmB-MTF 1:1combination showed remarkable efficacy in the invitro antileishmanial activity in comparison to plain AmB and AmB nanovesicles against *L. donovani* promastigotes. To conclude, AmB-MTF 1:1 nanovesicles could be a safe and reliable therapeutic option over a conventional AmB-MTF combination therapy. Further, the potential of the AmB-MTF 1:1combination nanovesicles formulation needs to be investigated *in-vivo* for the formulation to be considered as a better option over the conventional delivery in the treatment of visceral leishmaniasis.

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CONFLICTS OF INTERESTS: Nil

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