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Research Article

Formulation and evaluation of Rifampicin and Ofloxacin niosomes for Drug-resistant TB on Logarithmic-phase cultures of *Mycobacterium tuberculosis*

Paramita Chowdhury* and Uma Shankar MS

Department of Pharmaceutics, Krupanidhi College of Pharmacy, Sarjapura Main Road, Carmelaram Post, Bangalore-560 035, Karnataka, India

ABSTRACT

Nanotechnology has offered enormous improvement in field of therapeutics by means of designing of nanocarriers that can cross biological barriers and are able to target cellular reservoirs of *Mycobacterium tuberculosis* (*M.tuberculosis*). Niosomes are mainly consisting of non-ionic surfactants that have been found to form vesicles, capable of entrapping hydrophilic and hydrophobic molecules. Niosomes of rifampicin and ofloxacin were prepared by ether injection method. A procedure is described for producing a dry product, proniosomes that may be hydrated immediately before use to yield aqueous niosome dispersions, which minimize problems of aggregation, fusion and leaking, and provide additional convenience in transportation, distribution, storage and dosing. The prepared rifampicin and ofloxacin niosomes and proniosomes showed a vesicle size in the range of 100-300nm, the entrapment efficiency were 81.76% and 92.06% of niosomes and proniosomes respectively. The in vitro release study suggested that the action extended till 15days and all formulations followed non-Fickian anomalous diffusion that plays an important role in controlling the drug release. The bactericidal activities of the formulations were studied by BACTEC radiometric method using the resistant strains (RF 8554) and sensitive strains (H37RV) of *Mycobacterium tuberculosis* that showed greater inhibition and reduced growth index.

Keywords: BACTEC; in vitro; *Mycobacterium tuberculosis*; Niosomes; Ofloxacin; Proniosomes; Rifampicin

INTRODUCTION

Tuberculosis is an important public health problem; about two billion people (one third of the world's population) were infected with TB (Arachi A, 1991). Nearly half a million cases of MDR-TB emerge every year as a result of under-investment in basic activities to control TB, poor management of the supply and quality of anti tuberculosis drugs, improper treatment of TB patients and transmission of the disease in congregate settings (Global Report, 2011). Rifampicin used as first-line drug for treatment of tuberculosis, showed lesser resistance as compared to isoniazid. It is a semisynthetic compound with a molecular weight of 822.94. The chemical name is 3-[[[4-Methyl-1-piperazinyl] imino] methyl] rifamycin or 5,6,9,17,19,21 - hexahydroxy - 23 - methoxy - 2,4,12,16,20,22 - heptamethyl - 8 - [N - (4 - methyl - 1 - piper - azinyl) formimidoyl] - 2,7 - (epoxypentadeca - [1,11,13] trienimino) naphtho [2,1 - b]furan - 1,11(2H) - dione 21-acetate. Ofloxacin is a synthetic second-generation chemotherapeutic antibiotic, active against both typical and atypical bacterial

respiratory pathogen. The molecular weight is 361.37 and the chemical name is 7-fluoro-2-methyl-6-(4-methylpiperazin-1-yl)-10-oxo-4-oxa-1-azatricyclo [7.3.1.0^{5,13}]trideca-5(13),6,8,11-tetraene-11-carboxylic acid. Niosomes reside in lungs due to alveolar and effect of alveolar phagocytic cells with small sized vesicles, which can pass through capillaries (Li VHK *et al.*, 1987). Designed targeted drug delivery system (niosomes) were taken up by liver and breaks down to release the free drug that eventually re enter the circulation and maintain plasma drug levels (Khandari JN *et al.*, 2008). Rifampicin and Ofloxacin showed extended release of drug, which suffices to lesser days of treatment, decreased drug dose and increased patient compliance. The duration of treatment under DOTS strategy may also be reduced to the greater extent.

MATERIALS & METHODS

Reagents and chemicals

Rifampicin and Ofloxacin were obtained as a gift sample from Karnataka Antibiotics Pharmaceuticals Ltd. Potassium bromide was obtained from Merck specialties Pvt. Ltd. Cholesterol (99% purity) was obtained from Sigma Aldrich, Japan. Disodium hydrogen phosphate and Potassium dihydrogen phosphate were obtained from Spectrum reagent and chemicals Pvt. Ltd. The other chemicals were of analytical grade and were obtained from S.D. Fine Chemical Ltd.

* Corresponding Author

Email: chowdhury.paramita88@gmail.com

Contact: +91-9932079310

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Preparation of niosomes

Different niosomal formulations were prepared by the ether injection technique introduced by Deamer and Bangham in 1976. Drug, non ionic surfactant span (20,60 and span 80) and cholesterol were weighed accurately in the ratio of 1:2:1 and dissolved in diethyl ether and was mixed to the solution of drug dissolved in methanol (Yoshioka T *et al.*, 1994). The resulting solution was injected through 20-gauge needle at a rate of 1ml/min into warm aqueous solution of phosphate buffer saline. Evaporation of methanol leads to formulation of single layered vesicles. The suspension was cooled in ice bath and was sonicated using probe sonicator to obtain unilamellar niosomes (Tourtout E *et al.*, 1994).

Characterization of Niosomes

1. Particle Size Measurement

The morphology of prepared niosomes was done by optical microscopy and scanning electron microscopy. The particle size analysis was carried out using an optical microscope with a calibrated eyepiece micrometer. About 200 niosomes and proniosomes were measured individually, average was taken and their size distribution range and mean diameter was calculated (Sinico C *et al.*, 2003).

2. Drug Entrapment Efficiency

The prepared niosomes were filled into dialysis bag and was dialyzed for 24 hours using 100ml of phosphate buffer saline (PBS, pH 7.4) (Udupa N *et al.*, 1993). The dialyzed niosomes were lysed with 0.1% v/v Triton X-100 and the drug content was estimated by UV spectrophotometer for ofloxacin and rifampicin at 294 nm and 332 nm respectively (Jain CP *et al.*, 1995).

3. Compatibility study

The stability of a formulation depends on the compatibility of the drug with the excipients. Drug and each excipient were separately passed through #20. Different drug excipient mixtures were introduced in glass vials covered with aluminium foil having small holes and were kept under two different conditions, one at 2-8°C (refrigerator) and other at 40°C±2°C and 75%±5% RH (stability chamber) (Karkai R *et al.*, 2008). The FT-IR studies were carried out using Shimadzu model 8033. The thermo gravimetric and differential scanning calorimetry curve was obtained using SDT Q600 V20.9 BUILD20.

STABILITY STUDIES

Freshly prepared niosomal formulations were selected for stability testing at three different temperature condition i.e., refrigeration temperature (4-8°C), room

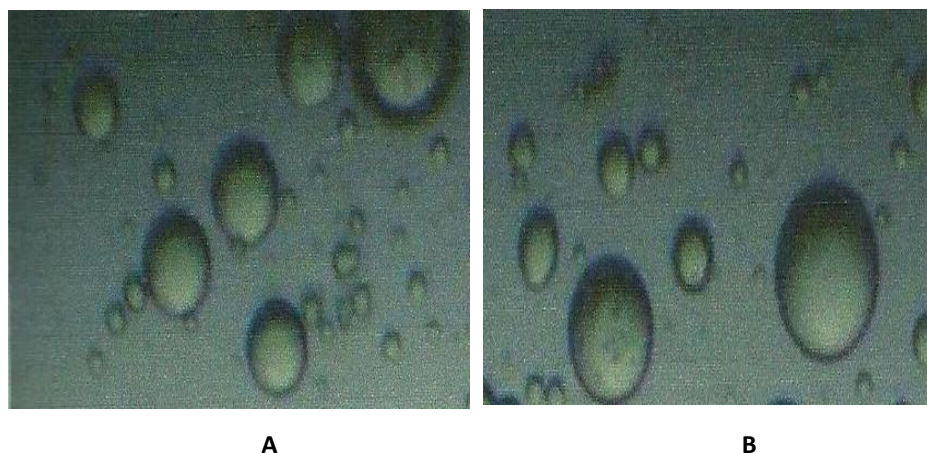


Figure 1: Optical Photomicrograph of a) Ofloxacin and b) Rifampicin niosomes at 100X

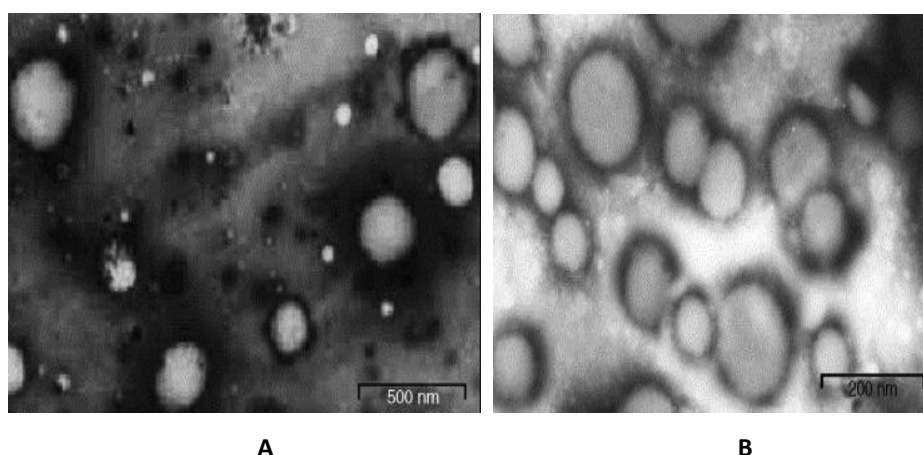


Figure 2: Scanning electron microscope of a) Ofloxacin and b) Rifampicin niosomes at 100X

temperature ($25\pm 2^\circ\text{C}$) and oven temperature ($45\pm 2^\circ\text{C}$). Formulation was kept under specific storage conditions in vials. Samples were withdrawn and observed periodically under microscope for any change in integrity, shape, size of niosomes or presence of crystalline structure or drug crystal. The amount of drug retained at each time interval was checked by determining the entrapment procedure. Zeta potential determination was determined by Malvern Zetasizer Nano Z instrument (Prabagar Balakrishnan *et al.*, 2009).

IN VITRO STUDIES

1. Diffusion studies

The niosomal preparation of ofloxacin and rifampicin were filled in separate dialysis bag, which acts a donor compartment. Dialysis bag was placed in a beaker containing 250ml phosphate buffer saline of pH 7.4 that acts as a receptor compartment. The temperature of the receptor medium is maintained at $37\pm 1^\circ\text{C}$ and the medium was agitated at a moderate speed using magnetic stirrer. Aliquots of sample were withdrawn periodically and after each withdrawal same volume medium was replaced. The collected samples were analyzed by UV spectroscopy at 294nm and 332 nm respectively (Ruckmani K *et al.*, 2003).

BACTERIOLOGICAL STUDY

1. BACTEC radiometric method

BATEC radiometric method was used where 0.1 ml of broth culture of the strains (H37Rv & RF 8554) from 12B medium Growth Index (GI) 300-500 in all drug containing 12B medium was inoculated. The same broth was diluted 1/100 times with diluting fluid, and 0.1 ml of this diluted sample was inoculated in the drug free medium and incubated at 37°C . Drugs were added to 12B medium to give final concentration as single drug with or without niosome. The readings were taken for all the vials daily for 5-7 days. When the reading of the control vial reached more than a GI of 30, then the ΔGI of the control reading was compared with the ΔGI of the test medium. ΔGI is the difference between two consecutive days readings (Global report, 2011).

RESULTS AND DISCUSSIONS

Niosomes of Ofloxacin and Rifampicin were prepared by ether injection method. The niosomes were subjected at 100X to optical microscopy and scanning electron microscopy for characterizing size shape of niosomes. Vesicles were found to be spherical in shape and size was found to be in range of 5-25 μm are given in Fig 1 & 2.

The percentage entrapment efficiency of ofloxacin niosomes (OFLO-S60) and rifampicin niosomes (RIF-S60) were 81.53% and 81.30% as shown in Fig 3.

The stability studies of niosomal formulations were carried out at refrigeration temperature ($4-8^\circ\text{C}$), room temperature ($25\pm 2^\circ\text{C}$) and oven temperature ($45\pm 2^\circ\text{C}$)

and 75% RH. Leakage of the drug from the prepared niosomes was analyzed in terms of percentage retained. Storage at refrigerated temperature showed a promising result of 94.45% of drug retained after 5 weeks. At room temperature the % retained after five weeks was 75.13%. The percentage of drug retained at 45°C and 75% RH was 44.35%. The results showed that the niosomal formulations were quite stable at refrigeration and room temperature as there was higher percentage of drug retained in niosomes. Percentage drug retained at 45°C had increased remarkably which may be due to melting of surfactant and lipid. The Percentage of drug retained at refrigeration is the highest this exhibiting stable formulation.

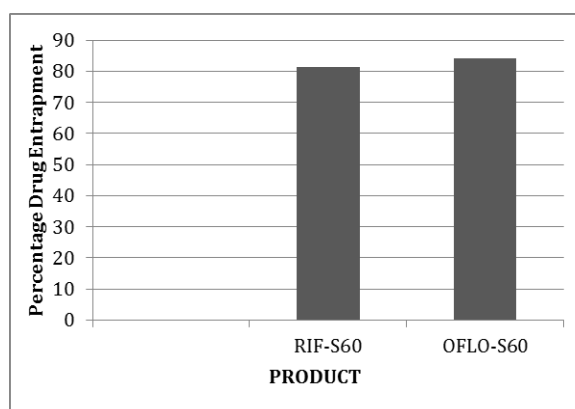


Figure 3: Drug Entrapment Efficiency

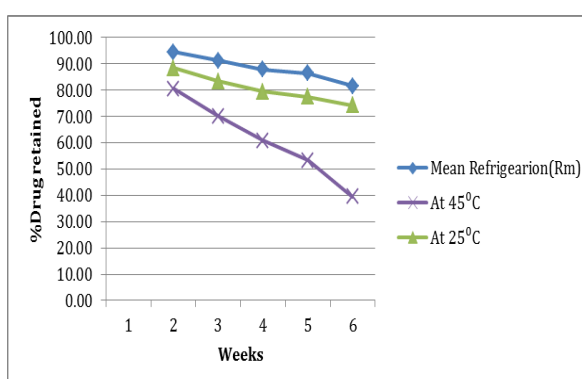


Figure 4: Stability studies

The DSC thermogram of rifampicin, ofloxacin and 1:1 drug-excipients physical mixtures were obtained and compared. In this study DSC revealed the compatibility of rifampicin with span 60 and cholesterol, ofloxacin with span 60 and cholesterol were compatible with drug. The *in vitro* release study was carried by diffusion method using sigma dialysis membrane as a barrier. To ascertain the drug release mechanism and release rate, data of prepared formulations were fitted with various release models. The models selected were Zero order, First order, Higuchi, Korsmeyer-Peppas. R^2 value in case of Higuchi release was found to be higher than zero order and first order in all the formulations suggesting that the drugs released from all the formulations by diffusion process. The 'n' value in case of Korsmeyer-Peppas model suggest whether the diffusion was fickian and non-fickian. The results suggested

that drugs released from all the formulations followed non-fickian anomalous diffusion, which plays important role in controlling the drug release. It is shown in figure 5-12.

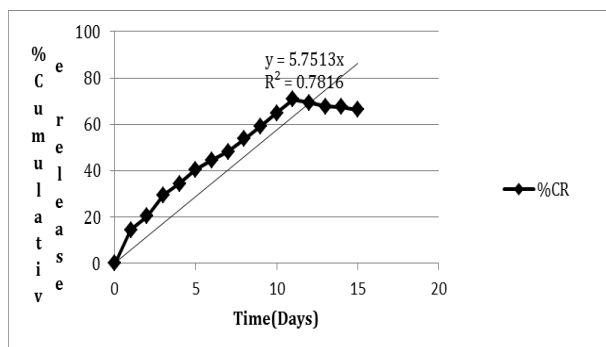


Figure 5: Zero-order drug release graph of OFLO-S60

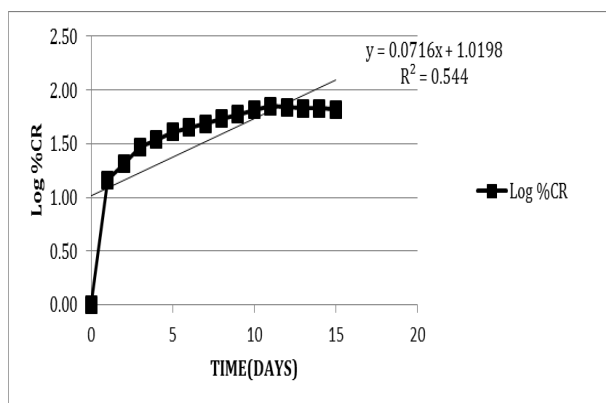


Figure 6: First-order drug release graph of OFLO-S60

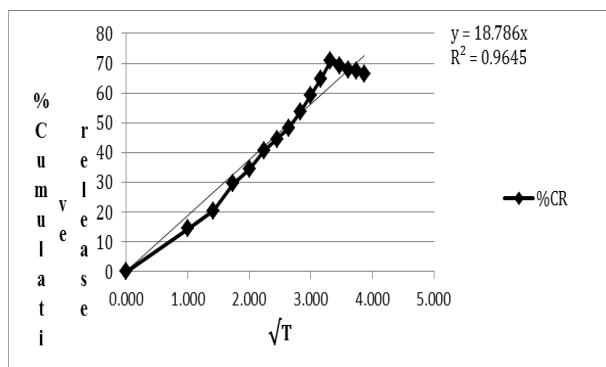


Figure 7: Higuchi release graph of OFLO-S60

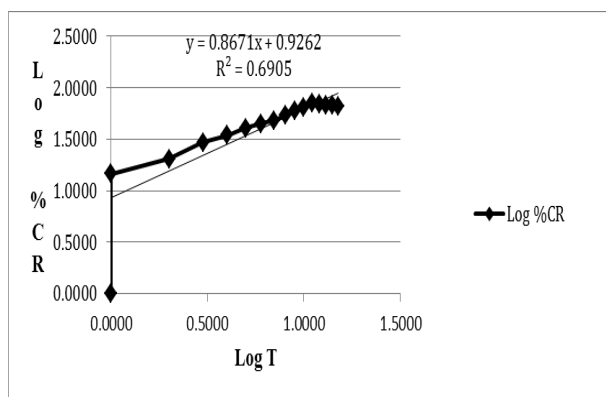


Figure 8: Korsmeyer-Peppas release graph of OFLO-S60

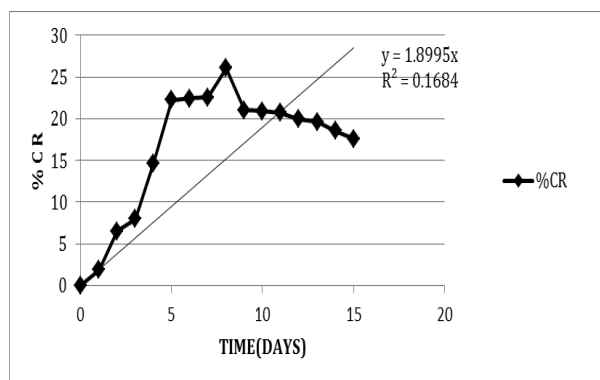


Figure 9: Zero-order drug release graph of RIF-S60

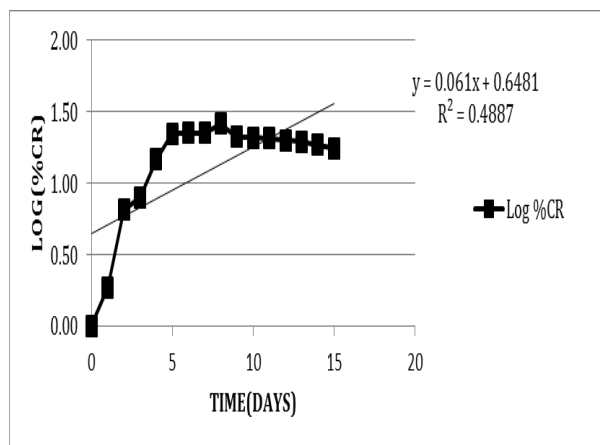


Figure 10: First-order drug release graph of RIF-S60

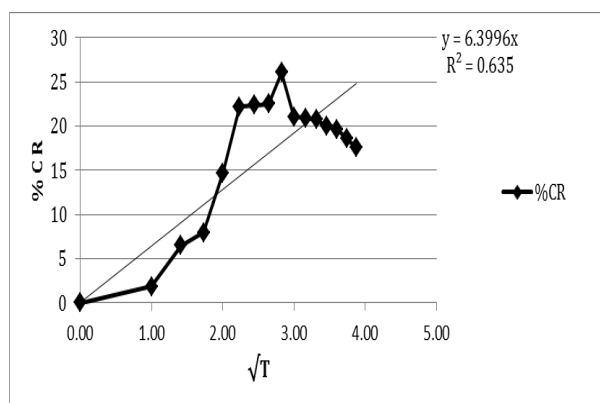


Figure 11: Higuchi release graph of RIF-S60

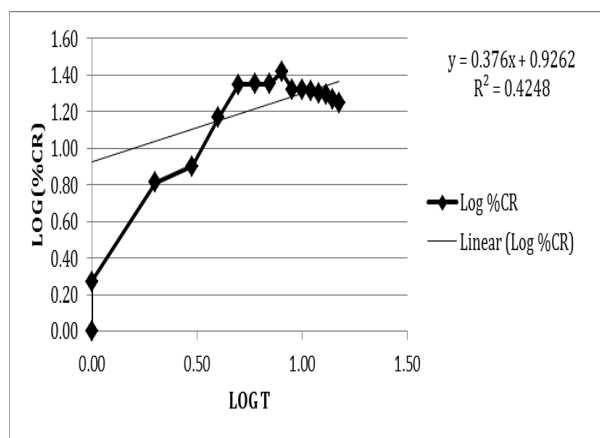


Figure 12: Korsmeyer-Peppas release graph of RIF-S60

The bactericidal activity of the drugs (Rifampicin and Ofloxacin) and in niosomal formulations (RIF-S60 and OFLO-S60) were studied by BACTEC radiometric method using the resistant strains (RF8554) and sensitive strains (H37Rv) of *Mycobacterium tuberculosis*.

Rifampicin drug 5mg/ml solutions were prepared in 0.5ml of DMSO and 4.5ml of sterile distilled water. From the stock solution 80µg/ml and 40µg/ml is prepared, so that 0.1ml (8 µg/ml) correspond to 2 µg/ml and 1 µg/ml in 4ml of BACTEC medium.

Ofloxacin drug 10mg/10ml solution was prepared in sterile 0.1N NaOH. Subsequent dilutions were made with sterile distilled water and aseptically added to the BACTEC media to give the desired final concentrations as 0.125 µg/ml and 0.25 µg/ml. Equivalent dilutions were made for rifampicin niosomes and ofloxacin niosomes separately. The results of Daily Growth Index (GI) readings were taken for 7 days. The results of Daily Growth Index (GI) readings were taken for 7 days. The control reading of resistant strain (FR8554) of *M.tuberculosis* showed a marked multiplication from the 4th day onwards. Rifampicin (1 µg & 2 µg) and ofloxacin (0.125 µg & 0.250 µg) drug showed that the strain is resistant to drug reduced action is increased Growth Index and attained a maximum of 999 in 6th day.

Rifampicin (1 µg & 2 µg) and Ofloxacin (0.125 µg & 0.25 µg) drug showed that the strain is resistant to the drug and the reduced the activity by the increased Growth index and attained a maximum of 999 on 6th day. Rifampicin niosomes (RIF-S60) (1 µg & 2 µg) and Ofloxacin niosomes (OFLO-S60) (0.125 µg and 0.25 µg) showed that the Growth index were increasing uniformly for 5 days and it reached a maximum of 999 indicating the resistance, but greater activity for niosomes were shown relatively less Growth index and reached a maximum of 999 on the 6th day, which was equal to drug alone (Fig 13 &14).

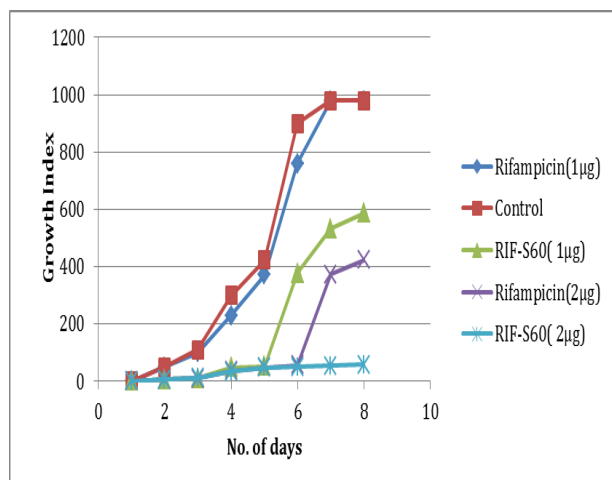


Figure 13: Comparative bactericidal activity of Rifampicin (1µg & 2 µg) and RIF-S60 (1 µg & 2 µg) using resistant strain (RF 8554) of *M.tuberculosis*

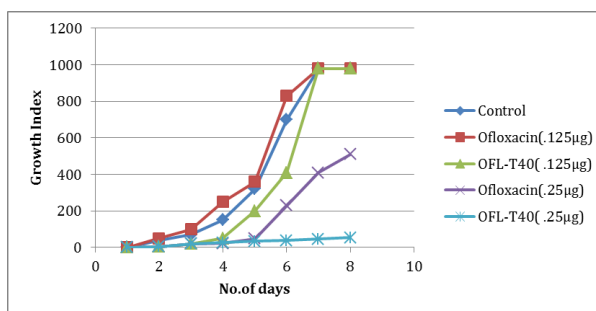


Figure 14: Comparative bactericidal activity of Ofloxacin (0.125µg & 0.25 µg) and OFLO-S60 (0.125µg & 0.25 µg) using resistant strain (RF 8554) of *M.tuberculosis*

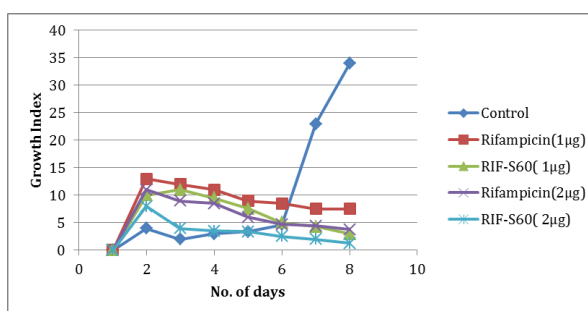


Figure 15: Comparative bactericidal activity of Rifampicin (1µg & 2 µg) and RIF-S60 (1 µg & 2 µg) using sensitive strain (H37Rv) of *M.tuberculosis*

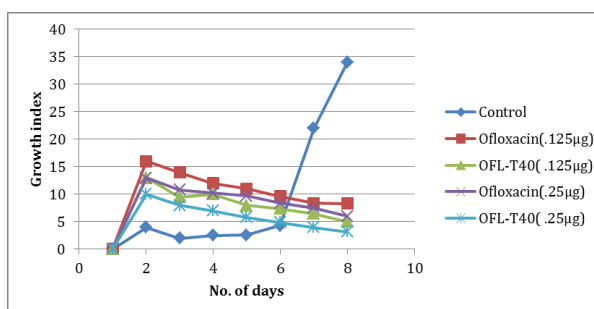


Figure 16: Comparative bactericidal activity of Ofloxacin (0.125µg & 0.25 µg) and OFLO-S60 (0.125µg & 0.25 µg) using sensitive strain (H37Rv) of *M.tuberculosis*

For sensitive strain H37Rv same concentrations of pure drug and niosomes were used in BACTEC medium (Figures 5.70 to 5.73). It also showed that the formulations of niosome having greater inhibition and reduced Growth index than the drug alone for sensitive strain (Fig 15 &16).

CONCLUSION

It can be concluded that the formulated niosomes of Rifampicin and Ofloxacin using widely accepted and physiologically safe excipients was capable of exhibiting prolonged release properties. They are thus may be reducing the dose intake, avoid hepatic first pass metabolism, dose related adverse effects and ultimately improve the patient compliance and drug efficiency. The treatment under DOTS strategy may also be re-

duced to a greater extent. The release of rifampicin and ofloxacin from niosomes and proniosomes was zero order diffusion controlled as indicated by higher R^2 values in zero order and Higuchi model. The 'n' values obtained from the Korsmeyer-Peppas model showed that the release mechanism was non-Fickian. The bactericidal study showed that drugs loaded in niosome vesicles showed improved bactericidal effect against the tuberculi bacilli.

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