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Original Article

FORMULATION AND EVALUATION OF ETHAMBUTOL POLYMERIC NANOPARTICLES

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ABSTRACT

Objective: Tuberculosis (TB) is an infectious bacterial disease caused by *Mycobacterium tuberculosis* which most commonly affects the lungs. TB has the highest mortality rate than any other infectious disease occurs worldwide. The main objective of the present investigation was to develop polymeric nanoparticles based drug delivery system to sustain the ethambutol (ETB) release by reducing the dose frequency.

Methods: The Preformulation studies of drug ETB were done by physical characterization, melting point determination, and UV spectrophotometric analysis. The ETB loaded nanoparticles were prepared by double-emulsion (W/O/W) solvent evaporation/diffusion technique. The prepared polymeric nanoparticles were evaluated for particle size, polydispersity index, zeta potential, drug entrapment efficiency, drug loading, drug-polymer compatibility study, surface morphology, *in vitro* drug release, and release kinetics.

Results: Based on the result obtained from the prepared formulations, F11 showed the best result and was selected as the optimized formulation. Optimized batch (F11) showed better entrapment efficiency (73.3%), good drug loading capacity (13.21%), optimum particle size (136.1 nm), and zeta potential (25.2 mV) with % cumulative drug release of 79.08% at the end of 24 h.

Conclusion: These results attributed that developed polymeric nanoparticles could be effective in sustaining the ETB release over 24 h. Moreover, the developed nanoparticles could be an alternate method for ETB delivery with a prolonged drug release profile and a better therapeutic effect can be achieved for the treatment of tuberculosis.

Keywords: *Mycobacterium tuberculosis*, Ethambutol, Eudragit, Polymeric nanoparticle

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INTRODUCTION

Tuberculosis (TB) is a highly contagious persistent infection caused by *Mycobacterium tuberculosis* and *Mycobacterium Bovis* and has the highest mortality rate than any other infectious disease. TB is the world's second most common cause of death after HIV/AIDS [1]. Treatment of TB involves the administration of a combination of two or more first-line anti-TB drugs namely, Rifampicin, Isoniazid, and Ethambutol in a fixed proportion in a single dosage form for the initial two months followed by Rifampicin and Isoniazid for four months, described as RHZE2/RH4 [2, 3].

Nanoparticle-based drug delivery systems form the crux of nanomedicine. They are suitable for targeting chronic diseases such as tuberculosis [4]. Experimental data support the possibility of intermittent chemotherapy with key first-line as well as second-line anti-tuberculosis drugs by employing synthetic or natural carriers, chiefly polymers [5]. Besides the sustained release of drugs in plasma and organs, other potential advantages of this system include the possibility of selecting various routes of chemotherapy, reduction in drug dosage, adverse effects, drug interactions, and targeting drug-resistant and latent bacteria [6-8].

Nanoparticles are defined as particulate dispersions or solid particles with a size in the range of 10-1000 nm. The major goals in designing nanoparticles as a delivery system are to control particle size, surface properties [9]. Nanoparticles are prepared majorly by these methods namely solvent evaporation, nanoprecipitation/solvent displacement, ionic gelation or coacervation of hydrophilic polymers, emulsification/solvent diffusion, double emulsification solvent evaporation, supercritical fluid technology, polymerization of monomer dialysis and salting-out method [10-13]. For hydrophilic compounds; encapsulation, double emulsion solvent evaporation is the most popular technique among other methods of preparation. It is hypothesized that by combining the double emulsion evaporation and diffusion technique at the same time could result in a better encapsulation efficiency of hydrophilic molecules in nanoparticles [14-16]. The ideal nanoparticles should be biodegradable, stable, non-immunogenic, non-thrombogenic, non-toxic, easy to fabricate, cost-effective, and able to release their payloads only at the target site [17]. The nanoparticles are generally characterized by their size, morphology, and surface charge, using such advanced microscopic techniques as dynamic light scattering (DLS), scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM) zeta sizer [18, 19].

In the present study, an attempt was made to develop a novel nanoscopic drug delivery portal, polymeric nanoparticles bearing ETB, and evaluated its anti-tuberculosis efficacy by in vitro methods. The prepared nanoparticles were characterized for their size, zeta potential, entrapment efficiency, drug loading, surface morphology, and in vitro drug release profile for monitoring the efficient release of ETB.

MATERIALS AND METHODS

Materials

Ethambutol (ETB) was purchased from Sigma Aldrich, St. Louis, MO, USA. Eudragit RS-100 was purchased from Evonik Industries, Essen, Germany. Span 80, PVA, and Methanol were purchased from SD Chemicals, Maharashtra India. Dichloro methane, sodium hydroxide and sodium chloride from Qualigens Fine Chemicals Pvt. Ltd., Mumbai, India. Potassium dihydrogen phosphate and Potassium chloride were purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. All the other reagents and chemicals used were of analytical grade.

Preformulation studies

Preformulation is a phase of the research and development process to develop stable, safe, and effective dosage forms. In this study, ETB was

selected as a model anti-tuberculosis drug. The selected drug ETB was identified by various methods like physical characterization, melting point determination, UV-spectrophotometric study, and infrared (IR) spectroscopy [20].

Physical characterization of drug

ETB was physically characterized based on appearance, color, odor, and taste [20].

Melting point determination

The capillary melting point apparatus was used to determine the melting point of the drug. The melting point of a drug can be determined by introducing a tiny amount of drug into a one-sided closed small capillary tube. Thermometer attached in a heating bath, the bath was heated slowly and temperatures were observed at which melting begins and is completed [21].

UV spectrophotometric study

UV spectrophotometric study was carried out to determine the λmax of ETB in a phosphate buffer solution of pH 6.8, hydrochloric acid buffer pH 1.2, and distilled water as per Indian Pharmacopoeia 2010. A standard stock solution of ETB was prepared by dissolving 100 mg of drug in a 100 ml volumetric flask and the volume was made up to 100 ml by using phosphate buffer solution of pH 6.8 to get the concentration 1000 µg/ml of standard ETB. From the standard stock solution, 1 ml of sample was pipetted out into a 10 ml volumetric flask and the volume was made up to 10 ml with phosphate buffer solution pH 6.8 to get the concentration (100 µg/ml). Again from this, 1 ml is taken and diluted to 10 ml of phosphate buffer solution 6.8 to get the desired concentration (10 µg/ml) and scanned in the wavelength region between 200 nm to 400 nm by using UV-VIS spectrophotometer (*Elico UV-SL210, India*). The same procedure was repeated with hydrochloric acid buffer pH 1.2 and distilled water [22].

Calibration curve of ETB in phosphate buffer pH 6.8/hydrochloric acid buffer pH 1.2/distilled water

A standard stock solution of ETB (1000 µg/ml) was prepared by taking 100 mg of ETB in 100 ml of phosphate buffer pH 6.8. From the standard stock solution, 1 ml of the sample was further diluted to 10 ml with phosphate buffer pH 6.8 into a 10 ml volumetric flask and diluted up to the mark. Aliquots of 2, 4, 6, 8, and 10 ml of stock solution were pipetted out into 10 ml volumetric flasks. The volume was made up to the mark with phosphate buffer pH 6.8. These dilutions give 2, 4, 6, 8, and 10 µg/ml concentration of ETB respectively. The absorbance was measured in the UV-Visible spectrophotometer at 267 nm using phosphate buffer pH 6.8 as blank and the graph was plotted (concentration versus absorbance). The same procedure was followed for the preparation of the calibration curve of ETB in 0.1N HCl and distilled water respectively [22].

Preparation of ETB loaded polymeric nanoparticles

The ETB loaded nanoparticles were prepared using a double-emulsion (W/O/W) solvent evaporation/diffusion technique. Briefly, the specified amount of Eudragit RS-100 was dissolved in 20 ml of an organic mixture of dichloromethane containing Span 80 (2%, v/v) as an emulsifier. 100 mg ETB was dissolved in 5 ml of distilled water and then emulsified in the polymer solution through magnetic stirring at 1000-1200 rpm for 15 min. The primary W/O emulsion was further added to 25 ml of external water containing poloxamer or PVA as a secondary surfactant with magnetic stirring for 10 min to achieve the stable double emulsion (W/O/W). The nanoparticles suspending in the emulsion were collected by ultracentrifugation at 11000 rpm for 40 min and washed with distilled water three times. Finally, the products were dried by lyophilization and stored at-4 °C for further evaluation [23-26].

Evaluation of ETB loaded polymeric nanoparticles

Particle size, polydispersity index, and zeta potential

Particle size, polydispersity index of polymeric nanoparticles was measured by Photon Correlation Spectroscopy using Zetasizer (*Beckman Coulter Counter, USA*). The zeta potential of the polymeric nanoparticles was measured by the same instruments at 25 °C [27].

Entrapment efficiency and drug loading

The entrapment efficiency of ETB was determined by indirect method i.e. by measuring the concentration of the free drug in the aqueous phase of Nano suspension. The amount of free drug was analyzed by a UV-Visible spectrophotometer at a wavelength of 267 nm. The drug entrapment efficiency (EE) and drug loading (DL) was calculated using the following equation [28]:

$$\text{Entrapment efficiency (\%)} = \frac{\text{Amount of total drug} - \text{Amount of free drug}}{\text{Amount of total drug}} \times 100$$

$$\text{Drug loading (\%)} = \frac{\text{Amount of total drug} - \text{Amount of free drug}}{\text{Amount of dry nanoparticles}} \times 100$$

Drug-polymer compatibility study

The study of the compatibility between the drug and the excipients is an important process in the development of a stable solid dosage form. Incompatibility between drug and excipient can alter the stability and bioavailability of drugs, affecting its safety and efficacy. To determine any type of interaction between the drug and excipients, Fourier-transform infrared (FTIR) spectroscopy and X-ray diffraction (XRD) analysis were done for the drug, polymer, physical mixture and formulation [29].

FT-IR spectroscopy

The FT-IR spectra of ETB, Eudragit RS-100, physical mixture of drug and polymer (1:1), and formulation were observed on the FT-IR spectrophotometer (*FTIR-4100, Jasco, Tokyo, Japan*) by using KBr method. The sample was grounded gently with anhydrous KBr and compressed to form a pellet. The scanning range was 400 and 4000 cm^{-1} [30].

XRD analysis

The X-ray diffractograms of ETB, Eudragit RS-100 physical mixture and formulation were procured on an X-ray diffractometer (*Rigaku MiniFlex II, Tokyo, Japan*) for examining the physical state of ETB and its interaction with other ingredients in the formulation. The source of X-ray was Copper Kα (λ=1.5405 °A) monochromatic radiation, operated at 30 kV and 15 mA. The samples were scanned between 2 theta ranges of 10°-80° [30].

Morphological characterization

The morphological characteristics of prepared nanoparticles were observed by Environmental Scanning Electron Microscope (*Quanta 200, FEI Company, Eindhoven, The Netherlands*). The samples for SEM were prepared by sprinkling the nanoparticle powder on a double adhesive tape that sticks to an aluminum stub. They were then vacuum-coated with platinum for 45s. The samples were then randomly scanned and photographs were taken randomly [31].

***In vitro* drug release studies**

The *in vitro* drug release of ETB from the polymeric nanoparticles was performed by the dialysis bag diffusion technique. The drug release studies of the ETB solution and ETB loaded Eudragit RS-100 Nano-suspension carried out in 250 ml of phosphate buffer saline pH 6.8 maintained at 37±2 °C with a magnetic stirrer with constant heating equipment. A sample of 5 ml of Nano-suspension was filled in a dialysis pouch with the two ends tied by a thread. The pH value was selected to simulate the physiological pH of 6.8. Aliquot samples of 5 ml were withdrawn at the regular interval. The same volume of fresh media was replaced to maintain the sink condition. The aliquots were diluted with fresh media. The amount of drug released was measured by using a UV-Visible spectrophotometer at the wavelength of 267 nm against phosphate buffer pH 6.8 as a blank [32-34].

Kinetic analysis of drug release data

To know the mechanism and kinetics of drug release from nanoparticles, *in vitro* drug release data were fitted to various kinetic models like zero-order model ($Q_t = k_0 t$), first-order model ($\log Q_0 - \log Q_t = k_1 t/2.303$), Higuchi model ($Q_t = k_H \sqrt{t}$) and Korsmeyer-Peppas model ($Q_t = k_{KP} t^n$).

Where t is the time, Q_t is the amount of drug released at time t, Q_0 is the initial amount of the drug in the nanoparticles, k_0 is the zero-

order rate constant, k_1 is the first-order rate constant, k_H is the Higuchi constant reflecting the design variables of the system and k_{KP} is the rate constant in Korsmeyer-Peppas model equation and n is the release exponent [35, 36].

Stability studies

Stability study of the optimized batch of nanoparticles was performed under accelerated stability conditions (40 °C±2 °C/75±5% RH) by keeping in stability testing chamber for three months according to ICH guidelines for stability testing of new products. The samples were withdrawn at a different interval (0, 1, and 3 mo) and evaluated in terms of particle size, zeta potential, and entrapment efficiency [37-39].

Data analysis

The experimental data were processed using Microsoft Excel 2007 software and results were expressed as mean±SD.

RESULTS AND DISCUSSION

Physical characterization of drug

ETB was evaluated for its physical properties and it was observed that ETB is a white, crystalline powder, almost odorless and bitter in taste with the solubility in chloroform, methylene chloride, and sparingly soluble in water. The physical properties of the ETB were found similar to those reported in Indian Pharmacopoeia 1996 [40].

Melting point determination

The melting point of ETB was found to be 87.2 °C, which corresponds to the literature value of 87.5 °C to 88.8 °C which signifies the identity and purity of the drug [41].

UV spectrophotometric study

UV Spectrophotometric study was carried out to determine the λmax of ETB in phosphate buffer pH 6.8, hydrochloric acid buffer pH 1.2, and distilled water. Scanned λmax for ETB was found at 267 nm in all the Medias [42].

Calibration curve of ETB in phosphate buffer pH 6.8/hydrochloric acid buffer pH 1.2/distilled water

The calibration curve of ETB was prepared in phosphate buffer pH 6.8, hydrochloric acid buffer pH 1.2, and distilled water. The R-square (R^2) value of the calibration curve in each media was found at almost 0.999 which signifies a statistically linear and straight calibration curve [43]. The λmax of the drug was found to be at 267 nm [44] and no shift in the λmax of the drug was observed in different tested Media. The calibration curve of ETB in phosphate buffer pH 6.8, hydrochloric acid buffer pH 1.2 and distilled water were shown in fig. 1, fig. 2 and fig. 3.

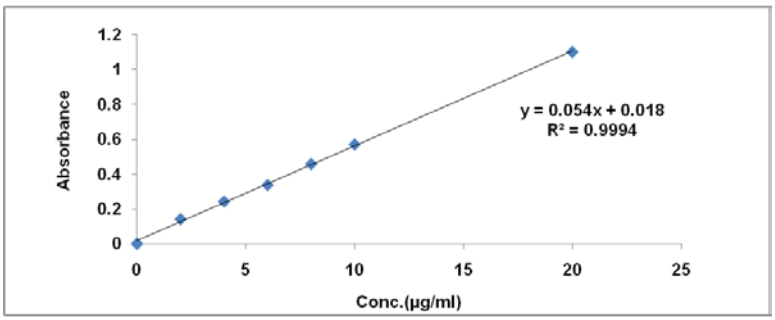

Fig. 1: Calibration curve of ETB in phosphate buffer pH 6.8

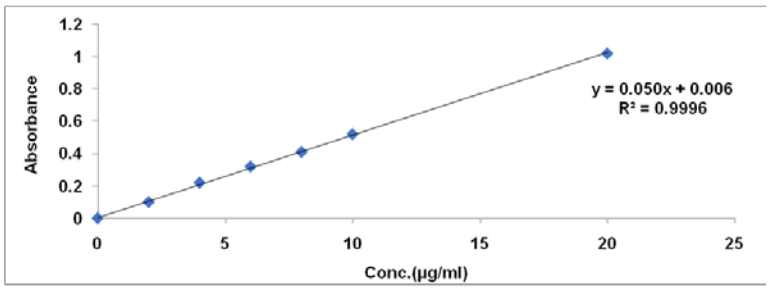

Fig. 2: Calibration curve of ETB in hydrochloric acid buffer pH 1.2

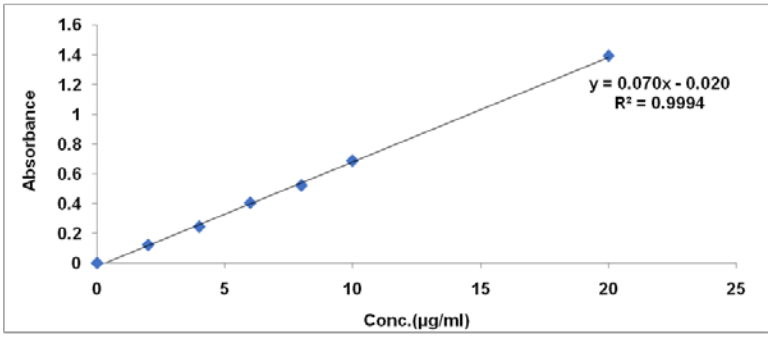

Fig. 3: Calibration curve of ETB in distilled water

Preparation and evaluation of ETB loaded polymeric nanoparticles

Effect of various process variables

The polymeric nanoparticles were prepared by Double-emulsion (W/O/W) solvent evaporation/diffusion technique. The compositions of different formulations were shown in table 1. The effects of different process variables like different surfactants with varying concentration and steering time on particle size, PDI, zeta potential, % entrapment efficiency, and % drug loading were analyzed. The nanoparticles were further optimized in terms of particle size and entrapment efficiency. The *in vitro* release and stability of polymeric nanoparticles were also studied. The morphological character of ETB loaded polymeric nanoparticles was studied by using scanning electron microscopy.

Effect of stirring time

The duration of steering has a great impact on the emulsification process and the size of the particle formed [45]. The primary and secondary stirring time was employed during the W/O/W emulsification process. At low stirring time 10:5 min (Primary stirring time: secondary stirring time); 15:5 min (primary stirring time: secondary stirring time) the emulsion was not formed properly. But at high stirring time 20:10 min (primary stirring time: secondary stirring time) the emulsification was found to be optimum for the formation of stable W/O/W emulsion.

Effect of secondary surfactant

The influence of different types of surfactants was also investigated. The type and concentration of surfactant also impact on the stability of emulsion and size of particles as well [46, 47]. The average particle size, polydispersity index (PDI) of the ETB loaded Eudragit RS-100 nanoparticles are illustrated in table 2. The particle size and PDI were significantly affected by the surfactant. A small particle size (45.5 nm) with low PDI (0.237) was obtained when the poloxamer solution was used as an aqueous surfactant compared to the PVA batch where the particle size and PDI were 81.80 nm and 0.248 respectively. These findings suggest that poloxamer 188 is more efficient in stabilizing the emulsion with smaller particles as compared to PVA.

Table 1: Composition of different formulations

Batch	Drug	Polymer	Surfactant	Ratio
F1	ETB	EUDRAGIT RS-100	PVA(45000)	1:0.5
F2	ETB	EUDRAGIT RS-100	PVA(45000)	1:1
F3	ETB	EUDRAGIT RS-100	PVA(45000)	1:2
F4	ETB	EUDRAGIT RS-100	PVA(45000)	1:3
F5	ETB	EUDRAGIT RS-100	PVA(125000)	1:0.5
F6	ETB	EUDRAGIT RS-100	PVA(125000)	1:1
F7	ETB	EUDRAGIT RS-100	PVA(125000)	1:2
F8	ETB	EUDRAGIT RS-100	PVA(125000)	1:3
F9	ETB	EUDRAGIT RS-100	POLOXAMER 188	1:0.5
F10	ETB	EUDRAGIT RS-100	POLOXAMER 188	1:1
F11	ETB	EUDRAGIT RS-100	POLOXAMER 188	1:2
F12	ETB	EUDRAGIT RS-100	POLOXAMER 188	1:3
F13	ETB	EUDRAGIT RS-100	POLOXAMER 407	1:0.5
F14	ETB	EUDRAGIT RS-100	POLOXAMER 407	1:1
F15	ETB	EUDRAGIT RS-100	POLOXAMER 407	1:2
F16	ETB	EUDRAGIT RS-100	POLOXAMER 407	1:3

Particle size

The particle size of prepared nanoparticles was observed in the range of 45.51 nm to 300.4 nm. The amount of polymer used in the formulation has a great impact on the size of the particles formed [48]. In this study also the amount of EUDRAGIT RS-100 has shown a significant effect on particle size. Increasing EUDRAGIT RS-100 concentration led to an increase in viscosity of the organic phase, hence reducing the net shear stress and promoting the formation of a droplet with the larger size. Also, an increase in the surfactant concentration i.e. PVA or poloxamer significantly decreases the particle size. Nanoparticles smaller than 10 nm can be rapidly cleared by the kidneys or through extravasation, while larger nanoparticles may have a higher tendency to be cleared by cells of the mononuclear phagocyte system [49]. It was observed that nanoparticles<100 nm have a higher potential to circulate in the blood for long periods and experience reduced hepatic filtration [50].

Table 2: Particle size, polydispersity index, and zeta potential of prepared formulation

Batch	Particle size (nm)	PDI	Zeta potential (mV)
F1	136.0	0.299	3.23
F2	277.8	0.248	5.03
F3	81.8	0.448	3.53
F4	117.8	0.325	11.10
F5	248.4	0.823	6.24
F6	131.6	0.369	13.24
F7	300.4	0.286	9.24
F8	247.5	0.274	8.98
F9	106.0	0.672	3.64
F10	247.5	0.274	13.70
F11	136.0	0.299	25.20
F12	133.2	0.481	13.20
F13	139.3	0.287	4.23
F14	87.6	0.314	1.16
F15	66.1	0.237	2.80
F16	45.5	0.364	4.09

The polydispersity index (PDI)

PDI of nanoparticles was observed in the range of 0.237 to 0.672 with a low coefficient of variation value of 0.11. Generally, PDI ranges from Zero to One. Results suggest that a high surfactant concentration (1%, w/v or higher) leads to smaller particles with a satisfactory PDI and this may be attributed to the fact that higher surfactant concentration ensures a good emulsification process and therefore leads to the formation of particles of small size and with uniform size distribution [51].

Zeta potential

Zeta potential of the optimized batch was found to be+25.2 mV (Nanoparticles with a zeta potential between-10 and+10 mV are considered relatively neutral, while nanoparticles with zeta potentials of greater than+25 mV or less than-25 mV are considered strongly cationic and strongly anionic, respectively and stable [52]. The positive charge of nanoparticles is due to the carboxyl group of EUDRAGIT RS-100.

Entrapment efficiency (EE)

The % entrapment efficiency was found to be high for the hydrophilic nature of drugs lying between 61.4 to 80.9 % and results were shown in table 3. The difference in entrapment efficiency mainly depends upon the amount of Eudragit RS-100 and the concentration of surfactant [53]. The amount of Eudragit RS-100 shows a significant effect on entrapment efficiency, since increasing Eudragit RS-100 concentration led to an increase in viscosity of the organic phase. Increasing viscosity could increase the drug resistance diffusion into the aqueous phase and thus enhance the drug entrapment efficiency. With the increase of poloxamer concentration, particle size decreases which leads to low entrapment of drugs.

Drug loading (DL)

The % drug loading was found to be in the range of 13.21% to 42.7% and results were shown in table 3. The amount of Eudragit RS-100 showed a significant effect on % DL. With the increase of EUDRAGIT RS-100 concentration the % DL decreased.

Table 3: Entrapment efficiency (% EE), drug loading (% DL) and cumulative drug release (% CDR) of prepared batches of formulation

Batch	% EE	% DL	% CDR
F1	76.98±0.06	42.54±0.02	82.25±0.31
F2	74.10±0.03	25.12±0.01	80.26±0.24
F3	69.50±0.21	17.12±0.02	79.82±0.56
F4	65.12±0.30	10.23±0.02	73.25±0.25
F5	66.27±0.16	38.70±0.12	79.82±.024
F6	67.12±.01	26.73±0.32	74.59±0.29
F7	72.24±0.02	20.12±0.21	79.57±0.65
F8	75.04±0.045	13.23±0.34	76.23±0.24
F9	61.40±0.02	39.97±0.25	77.11±0.75
F10	63.20±0.012	27.37±0.31	73.90±0.65
F11	**73.30±0.14**	**13.21±0.65**	**79.08±0.42**
F12	75.20±0.24	10.24±0.02	78.23±0.02
F13	69.22±0.03	40.20±0.21	80.73±0.04
F14	66.01±0.01	27.96±0.31	80.79±0.216
F15	72.25±0.014	19.60±0.21	76.88±0.24
F16	75.23±0.02	15.20±0.24	75.90±0.036

Results are presented as mean±SD (n=3).

FT-IR spectroscopy

The FT-IR spectra of Eudragit RS-100 appeared at 1727.21 cm^{-1} for the carbonyl peak (C=O stretching) which corresponds to the FTIR spectra of Eudragit RS-100 found in the literature [54]. FT-IR spectra of ETB show the broad peak at 3414-3200 cm^{-1} (NH_2 stretching), at 3000-2850 cm^{-1} (-CH stretching) and at 1713.72 cm^{-1} for the carbonyl peak (C=O stretching) which were similar with the standard ETB [55]. In the formulation of Eudragit RS-100 and ETB, all the characteristic peaks of polymer and the drug are retained showing no significant interaction between them. The FT-IR spectra of ETB, Eudragit RS-100, and optimized formulations were shown in fig. 4, fig. 5 and fig. 6.

XRD analysis

The diffraction pattern of pure ETB shows that the drug is crystalline in nature, with many characteristic peaks observed between 11-45 (2θ value). The XRD pattern of the ETB loaded polymeric nanoparticles shows that most of the characteristic peaks of ETB were retained which suggests that no significant incompatibility between drug and other excipients within polymeric nanoparticles. The XRD of ETB shows sharp picks which implies the crystalline nature of the formulation [56]. The XRD spectra of ETB, Eudragit RS-100, ETB-Eudragit RS-100 physical mixture, and optimized formulation were shown in fig. 7.

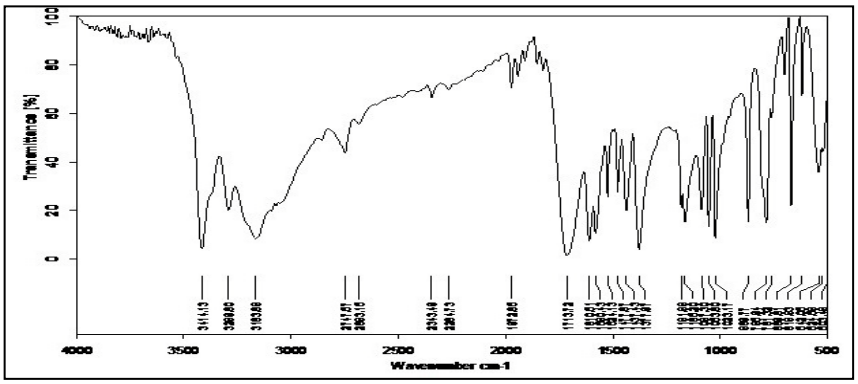

Fig. 4: FTIR spectra of ethambutol

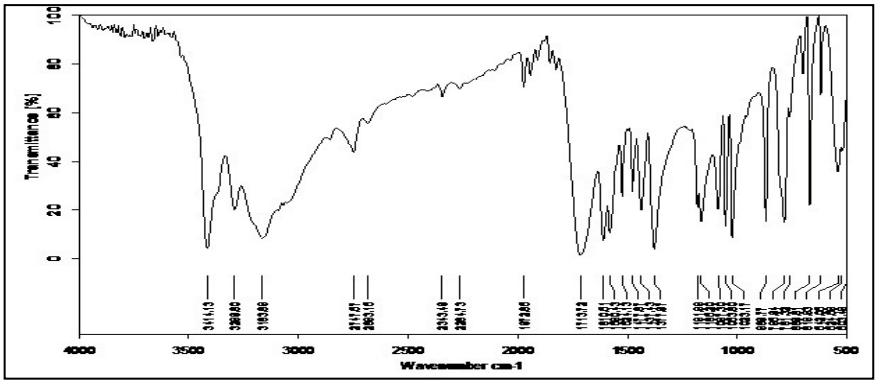

Fig. 5: FTIR spectra of eudragit RS-100

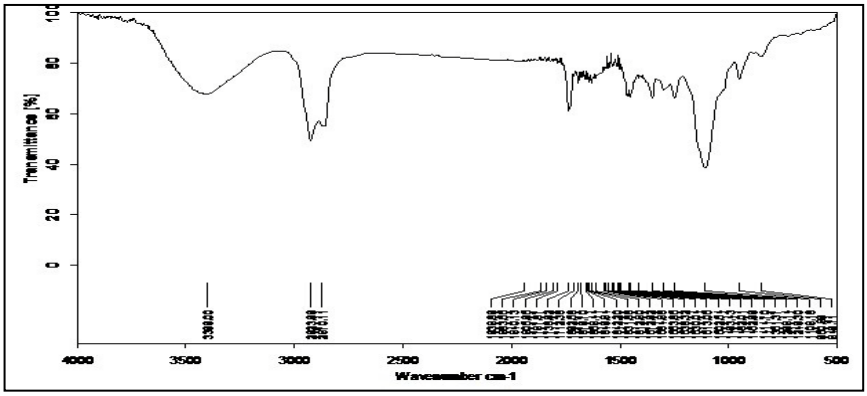

Fig. 6: FTIR spectra of optimized formulations

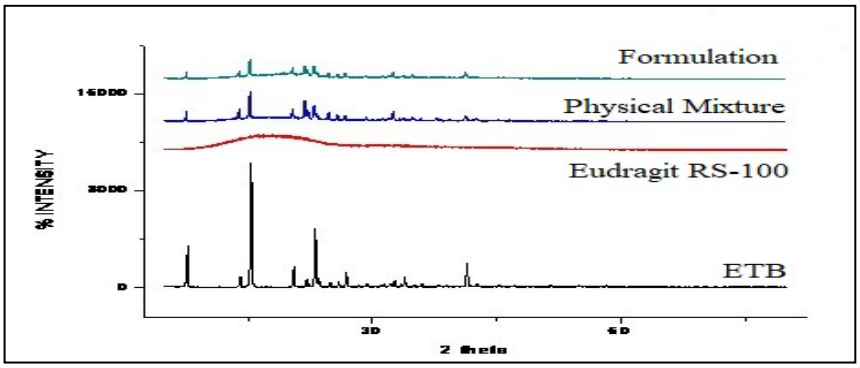

Fig. 7: X-ray diffractograms of ETB, Eudragit RS-100, physical mixture, and optimized formulation

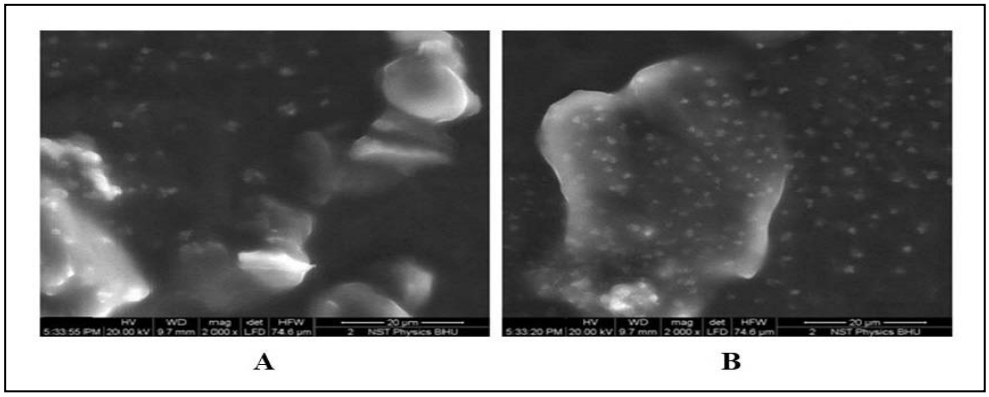

Fig. 8: SEM photographs

Morphological characterization

The external morphology of the nanoparticles was studied using SEM revealed that all nanoparticles are somewhat spherical in shape and are of Nano-size range but with substantial agglomeration. The degree of nanoparticle fusion was notable in fig. 8 (A and B). A reason for this behavior was that during the lyophilization process solvent was removed from nanoformulation. This affected the droplet equilibrium resulting in coalescence and agglomeration during the early step of lyophilization [57]. The surface of nanoparticles was smooth with few very small pores which seem to be associated with evaporation of solvent from the surface.

***In vitro* drug release studies**

The *in vitro* release study was performed in phosphate buffer pH 6.8 and the study was continued up to 24 h. The results of *in vitro* dissolution studies on the polymeric nanoparticles, F1 to F16 were shown in table 4 to table 7. The results allow for the following observations and inferences.

When ETB pure drug was studied for its dissolution, it was seen that a high percent (%) of ETB was dissolved within 1 h. Almost all the drug (90%) dissolved within 6 h and later there was no further release.

Formulations F1 to F16: Formulations F1 to F4 prepared using PVA (45000) sustained the drug release as the time prolonged. These formulations showed a biphasic release profile; an initial sustained release phase up to 11 h followed by a controlled release phase up to 24 h. The initial burst release of the drug is due to the presence of the drug on the surface of particles. The sustained release pattern of the drug may be attributed to the alteration in the stability and swelling profile of the polymeric matrix system. The release was gradual and controlled; and by the end of 24 h, 82 % of the drug was released from formulation F1.

Formulations F5 to F8 prepared using PVA (125000) sustained the drug release as the time prolonged. These formulations showed a biphasic release profile. The release was gradual and controlled and by the end of 24 h, 82% of the drug was released from formulation F5. In this study the % drug release from the NPs was found around 80% when PVA was used as a stabilizer which is similar to the study carried out by Dustin *et al.* [58].

Formulations F13 to F16 prepared using poloxamer 407 sustained the drug release as the time prolonged. These formulations showed a biphasic release profile. This type of sustained-release pattern of the drug is more pronounced in the presence of poloxamer as a stabilizing agent, due to the alteration in the stability of the polymeric matrix system. The release was gradual and controlled and by the end of 24 h, 81% of the drug was released from formulation F14.

Table 4: *In vitro* drug release studies of ETB and prepared formulations using PVA (45000 da)

Time (h)	ETB Aq. Sol	F1	F2	F3	F4
0	0	0	0	0	0
0.25	12.25±0.02	6.37±0.23	8.57±0.31	6.37±0.36	6.37±0.36
0.5	18.98±0.02	11.26±0.23	11.26±0.03	11.26±0.45	11.26±0.45
0.75	26.69±0.10	12.98±0.24	12.98±0.04	12.98±0.56	12.98±0.56
1	29.95±0.23	21.06±0.02	18.36±0.03	21.06±0.36	21.06±0.36
1.5	35.60±0.12	24.24±0.0.21	21.79±0.05	24.24±0.46	24.24±0.46
2	46.20±0.13	29.38±0.24	29.38±0.04	29.38±0.25	29.38±0.25
3	53.20±0.13	32.25±0.014	30.25±0.26	33.25±0.45	33.25±0.45
4	68.97±0.21	35.26±0.14	37.46±0.36	35.26±0.62	35.26±0.62
5	86.24±0.56	42.85±0.14	45.54±0.46	42.85±0.45	42.85±0.45
6	96.24±0.47	47.25±0.23	47.25±0.64	45.53±0.15	47.25±0.23
7	98.25±0.55	51.41±0.21	48.97±0.23	46.53±0.01	48.97±0.12
8		54.60±0.24	54.60±0.54	51.00±0.23	54.60±0.02
9		58.51±0.14	59.98±0.35	56.00±0.14	59.98±0.04
10		67.33±0.56	64.88±0.36	63.00±0.23	64.88±0.03
11		70.76±0.65	78.35±0.45	72.23±0.01	68.25±0.32
12		75.41±0.32	79.25±0.46	76.20±0.02	69.26±0.04
24		82.25±0.75	80.26±0.36	79.82±0.13	73.25±0.02

Results are presented as mean±SD (n=3).

Table 5: *In vitro* drug release profile of prepared formulations using PVA (125000 da)

Time (h)	F5	F6	F7	F8
0	0	0	0	0
0.25	8.57±0.03	6.37±0.26	5.39±0.31	4.65±0.31
0.5	11.26±0.01	11.26±0.23	6.37±0.12	6.37±0.01
0.75	12.98±0.32	12.98±0.36	11.26±0.21	9.55±0.02
1	18.36±0.36	21.06±0.24	12.98±0.14	12.98±0.31
1.5	21.79±0.25	24.24±0.26	21.06±0.25	21.06±0.21
2	25.00±0.24	29.38±0.56	24.24±0.12	24.24±0.42
3	34.03±0.36	32.20±0.24	33.45±0.01	36.45±0.24
4	37.46±0.45	35.26±0.36	35.26±0.02	38.93±0.36
5	45.54±0.36	42.85±0.45	42.85±0.35	42.85±0.24
6	47.25±0.45	47.25±0.78	47.25±0.21	47.25±0.25
7	48.97±0.23	51.41±0.62	51.41±0.25	51.41±0.26
8	54.60±0.01	54.60±0.35	54.60±0.21	54.60±0.24
9	59.98±0.25	58.51±0.21	58.51±0.45	58.51±0.36
10	64.88±0.15	67.33±0.36	67.33±0.42	67.33±0.25
11	78.35±0.25	70.76±0.14	74.48±0.25	70.76±0.26
12	81.28±0.36	75.41±0.02	75.08±0.21	75.90±0.36
24	81.82±0.25	79.82±0.21	75.59±0.12	79.57±0.45

Results are presented as mean±SD (n=3).

Table 6: *In vitro* drug release studies prepared formulations using poloxamer 188

Time (h)	F9	F10	F11	F12
0	0	0	0	0
0.25	1.38±0.21	8.32±0.24	7.34±0.02	5.63±0.21
0.5	2.75±0.31	15.91±0.32	11.75±0.01	8.57±0.23
0.75	4.13±0.45	19.10±0.25	16.89±0.01	14.45±0.24
1	5.50±0.02	23.26±0.24	21.79±0.04	21.79±0.23
1.5	8.25±0.01	29.38±0.23	27.91±0.05	27.42±0.25
2	11.00±0.03	35.50±0.65	30.85±0.05	33.30±0.25
3	16.50±0.05	41.22±0.25	35.50±0.32	35.75±0.26
4	22.00±0.32	45.54±0.25	41.13±0.01	40.40±0.02
5	27.50±0.01	48.72±0.12	45.54±0.05	43.58±0.03
6	33.00±0.03	51.90±0.21	48.72±0.02	47.99±0.01
7	38.50±0.05	57.78±0.31	51.90±0.06	53.86±0.04
8	44.00±0.32	59.98±0.01	55.33±0.14	56.31±0.05
9	49.50±0.42	61.94±0.02	71.40±0.25	63.41±0.06
10	55.01±0.12	68.06±0.02	70.35±0.36	64.64±0.05
11	60.51±0.32	73.20±0.03	70.35±0.45	71.98±0.12
12	66.01±0.10	75.90±0.04	69.58±0.36	76.39±0.04
24	76.23±0.32	77.12±0.02	73.93±0.12	79.08±0.05

Results are presented as mean±SD (n=3).

Table 7: *In vitro* drug release studies prepared formulations using poloxamer 407

Time (h)	F13	F14	F15	F16
0	0	0	0	0
0.25	1.38±0.02	9.55±0.05	7.34±0.24	8.32±0.02
0.5	2.75±0.03	13.71±0.03	13.71±0.23	11.75±0.04
0.75	4.13±0.04	16.89±0.02	19.10±0.15	13.71±0.05
1	5.50±0.25	23.50±0.05	24.24±0.26	16.89±0.02
1.5	8.25±0.15	25.22±0.03	29.38±0.13	24.24±0.25
2	11.00±0.45	30.85±0.05	33.30±0.15	30.36±0.15
3	16.50±0.04	36.23±0.04	38.19±0.14	32.02±0.24
4	22.00±0.03	45.54±0.06	45.54±0.13	35.50±0.26
5	27.50±0.35	48.23±0.05	48.72±0.17	40.15±0.21
6	33.00±0.42	51.41±0.36	51.41±0.10	46.27±0.02
7	38.50±0.24	57.78±0.24	55.33±0.05	49.21±0.01
8	44.00±0.23	59.98±0.26	57.54±0.09	56.31±0.21
9	49.50±0.15	62.92±0.24	64.88±0.08	60.23±0.02
10	55.01±0.01	65.37±0.15	70.76±0.08	60.47±0.01
11	60.51±0.02	70.76±0.12	71.98±0.01	70.27±0.02
12	66.01±0.03	78.35±0.15	73.45±0.05	72.47±0.02
24	78.23±0.05	80.79±0.23	76.88±0.15	75.90±0.15

Results are presented as mean±SD (n=3).

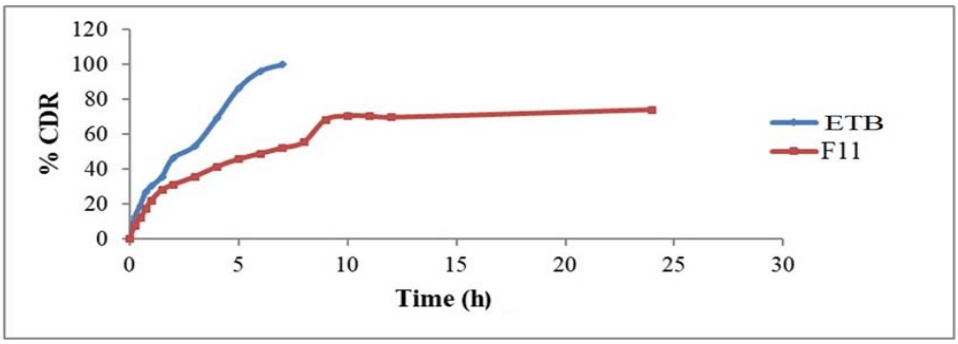

Fig. 9: *In vitro* drug release profile of pure drug ETB and formulation F11 in Phosphate buffer pH 6.8

Kinetic analysis of drug release data

The drug release mechanism and kinetics from the NPs were investigated by fitting the drug release data in various kinetic models. After the model fitting the correlational coefficient (R^2) of the various kinetic models (table 8) was compared and it has been seen that the Korsmeyer-Peppas (K-P) model was found to be best fitted for optimized formulation F11. The n value (0.43) indicates the release pattern of the drug from NPs was maintained by the Fickian diffusion mechanism [59].

Table 8: Correlational coefficient (R^2) of the various kinetic models

Batch	Zero order model		First order model		Higuchi model	K-P model	
	K_0	R^2	K_1	R^2	R^2	R^2	n
F1	3.45	0.895	0.086	0.798	0.958	0.967	0.478
F2	3.54	0.937	0.073	0.782	0.939	0.956	0.523
F3	3.25	0.824	0.071	0.783	0.937	0.949	0.489
F4	3.28	0.826	0.073	0.782	0.937	0.95	0.487
F5	3.25	0.826	0.075	0.786	0.94	0.959	0.535
F6	3.45	0.895	0.081	0.795	0.958	0.967	0.478
F7	3.36	0.876	0.075	0.786	0.765	0.722	0.562
F8	3.65	0.889	0.073	0.793	0.954	0.961	0.596
F9	3.54	0.92	0.072	0.881	0.967	0.968	0.51
F10	3.38	0.832	0.066	0.725	0.938	0.964	0.408
F11	3.4	0.79	0.065	0.728	0.939	0.947	0.435
F12	3.61	0.872	0.073	0.766	0.949	0.967	0.439
F13	3.33	0.876	0.083	0.752	0.948	0.964	0.45
F14	3.56	0.963	0.076	0.761	0.954	0.968	0.439
F15	3.25	0.835	0.068	0.729	0.938	0.967	0.347
F16	3.45	0.871	0.073	0.788	0.954	0.954	0.495

Selection and optimization of prototype formula

The optimization was aimed at maximizing % entrapment efficiency and % drug loading of ETB in the formulation while minimizing particle size for the new formulation. Sixteen formulations were prepared and optimized by changing the drug: polymer ratio, type of surfactant. Particle size and drug entrapment efficiency were in the range of 45.51 nm to 300.4 nm and 61.4% to 80.9% respectively. Polydispersity index was in the range of 0.237 to 0.672 with a low coefficient of variation value of 0.11. Among the entire batches prepared, the F11 batch was considered as optimized formulation showing the best results in terms of desired particle size, PDI, Zeta Potential, and good entrapment efficiency and % drug loading. Based on the result of these six batches, optimized batch (F11) was prepared using EUDRAGIT RS-100, poloxamer188 (1% w/v) and the optimized has shown better entrapment efficiency (73.3%), drug loading (13.21%) and optimum particle size (136 nm).

Stability studies

The stability of optimized, ETB loaded polymeric nanoparticles developed in the present study was evaluated as per International Conference on Harmonization (ICH) guidelines. The storage conditions recommended by ICH for stability testing are summarized in table 9 and table 10.

The storage conditions for accelerated testing are 40 °C±2 °C, 75±5% RH for 6 mo as per ICH and WHO guidelines. If the product is unstable in the above conditions, intermediate conditions (30 °C±2 °C, 65±5% RH) are recommended. WHO has prescribed testing at 0, 1, 2, 3, and 6 mo during storage. ICH has not given testing time-frequency [60]. In the present study, as the formulations developed comes under the category of solid oral dosage forms/solids for reconstitution/dry and lyophilized powders in vials, a storage condition of 40±2 °C, 75±5% RH for 6 mo was used as per accelerated stability studies.

Table 9: Effect of storage condition (40±2 °C/75±5% RH) on characterization parameters of optimized formulation (F11)

Parameters	0 D	30 D	60 D	90 D
Particle Size (nm)	133.2±1.48	160.2±3.20	182.36±5.10	213.51±6.92
Zeta Potential (mV)	25.2±0.03	13.7±0.24	9.24±0.37	3.53±0.23
% EE	69.13±0.59	63.78±0.87	55.17±0.74	49.86±1.23

Results are presented as mean±SD (n=3).

Table 10: ETB release profiles from optimized formulation (F11) during the stability studies (before and after storage) at (40±2 °C/75±5% RH)

Time (h)	% CDR		
	Before storage	After storage	
		3 Mo	6 Mo
0	0	0	0
0.25	8.32±0.01	10.45±0.04	10.24±0.23
0.5	15.91±0.23	17.12±0.05	16.25±0.01
0.75	19.10±0.14	21.23±0.42	22.35±0.25
1	23.26±0.25	25.21±0.03	23.25±0.56
1.5	29.38±0.24	29.56±0.23	31.38±0.45
2	35.50±0.26	37.56±0.25	34.50±0.78
3	39.23±0.25	41.20±0.24	36.23±0.69
4	45.54±0.24	47.28±0.02	42.64±0.45
5	48.72±0.63	52.14±0.36	49.72±0.36
6	51.90±0.85	54.96±0.56	53.90±0.24
7	57.78±0.24	59.54±0.45	57.78±0.26
8	59.98±0.12	61.24±0.36	59.98±0.27
9	61.94±0.23	64.25±0.25	66.94±0.36
10	68.06±0.23	66.64±0.24	71.06±0.45
11	73.20±0.24	69.47±0.36	73.20±0.78
12	75.90±0.26	73.52±0.54	76.90±0.64
24	77.12±0.36	75.70±0.68	78.12±0.32

Results are presented as mean±SD (n=3)

The optimized batch of polymeric nanoparticles of ETB was charged on accelerated stability and monitored for particle size, zeta potential, entrapment efficiency, and *in vitro* dissolution profile studies at 40±2 °C/75±5% RH for 6 mo (table 9 and table 10). Characteristic parameters of Polymeric nanoparticles like particle size, zeta potential and entrapment efficiency conducted for 6 mo of storage had shown that there were no significant changes during the storage. The stability was evaluated based on the measurement of particle size, zeta potential, and entrapment efficiency at an interval of 30 d for 3 mo. It was found that the formulation was stable for two months as no significant change in particle size and entrapment efficiency was observed. However, the particle size increased from 133 nm to 213 nm at the end of 3 mo.

CONCLUSION

The Ethambutol loaded polymeric nanoparticles were formulated by the double-emulsion (W/O/W) solvent evaporation/diffusion technique and found to be compatible between drug and other excipients within polymeric nanoparticles. Among the 16 formulations, F11 which was prepared using ETB (100 mg), Eudragit RS-100 (200 mg), poloxamer (1% w/v) showed the smallest particle size (136.1 nm), better entrapment efficiency (73.3%), good drug loading capacity (13.21%) and zeta potential (25.2 mV) with % cumulative drug release of 79.08% at the end of 24 h was selected as the optimized formulation. The optimized batch was found to be stable for 90 d. Thus, a once-a-day polymeric nanoparticle-based controlled drug delivery system of Ethambutol can be developed for a better therapeutic effect in the treatment of tuberculosis.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

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