International Journal of Applied Pharmaceutics

ISSN- 0975-7058 Vol 14, Issue 3, 2022

Original Article

DEVELOPMENT AND EVALUATION OF SELF EMULSIFIED DRUG DELIVERY SYSTEM OF EMBELIN FOR THE TREATMENT OF AMOEBIASIS

BHARAT LAL[1*], MANOJ GADEWAR[2], ARUN GARG[2]

[1]Department of Pharmaceutics, K. R. Mangalam University, Haryana, India, [2]Department of Pharmacology, K. R. Mangalam University, Haryana, India

*Email: bharatlal049@gmail.com

Received: 24 Jan 2022, Revised and Accepted: 02 Mar 2022

ABSTRACT

Objective: Development and evaluation of self-emulsified drug delivery system of embelin for the treatment of amoebiasis.

Methods: Self-emulsifying drug delivery systems (SEDDS) were prepared by determining the saturation solubility of embelin in different oil, surfactant and co-surfactant. Pseudo-ternary phase diagram was prepared by using chemix software for the selection of surfactant and co-surfactant ratio, and was optimized as 2:1 (Surfactant: Co-surfactant) for SEDDS. The drug was dissolved in surfactant, followed by the addition of co-surfactant and oil in a beaker. The resultant mixtures were stirred continuously by magnetic stirrer and heated at 40 °C to obtain a homogenous mixture. The prepared drug-loaded SEDD formulation was optimized on the basis of emulsification time, size, PDI, zeta potential and precipitation of drugs. Then the anti-amoebic activity of embelin and metronidazole-loaded SEDDS powder was carried out using microplate reader.

Results: The optimum solubility of embelin was calculated in Capmul MCM EP as oil (37 mg/ml), Kolliphor hs 15 as surfactant (150 mg/ml) and PEG 400 as co-surfactant (19 mg/ml). The formulation F7 i.e. 70% Smix and 30% oil was estimated as optimized formulation. The different values of emblein loaded SEDDS was found to be dilution test (no sign of precipitation), centrifugation test (no sign of phase separation), globule size (Dia 217.5 nm) and PDI (108), zeta potential determination (-31.60 mV), viscosity (17.12 cps) and Eosin red confirmed the o/w type of emulsion. Moreover, the angle of repose SEDDS prepared by using Avicel 200 was found to be Θ=27.474, means flow of powder is good with drug loading capacity of 92.7 %. SEM image and *in vitro* drug release indicated the 99.3% drug was released from the SEDDS in 120 min. Cytotoxicity study (MTT assay) shows the Plain Drug Suspension, Embelin loaded SEDDS powder and Metronidazole exhibited 100% viability at the concentration range of 1.56-100 mmol. The anti-amoebic IC_{50} values of embelin, metronidazole and embelin loaded SEDDS powder was found 1.519 µmol, 1.354 µmol, 1.84 µmol and 1.35 µmol, respectively.

Conclusion: Present study showed the stronger amoebicidal action of embelin may associated with some specific interaction of the active embelin with the cell wall of the parasites or with a more effective mechanism of penetration into the parasites through membrane channels.

Keywords: Self-emulsifying powder, Solubility, Ternary phase diagram, Amoebiasis, Embelin

DOI: https://dx.doi.org/10.22159/ijap.2022v14i3.44222. Journal homepage: https://innovareacademics.in/journals/index.php/ijap

INTRODUCTION

Amoebias is a disease caused by a single-celled protozoan, amoeba, which belongs to the class Rhizopoda possessing organoflocomotion known as psuedopodia. Amoebiasis is widespread in its distribution occurring in all parts of the world. The invasive amoebiasis is more prevalent in certain areas of the world including Westand southeast Africa, China, the South East Asia, Mexico and Western portion of South America and in the Indian subcontinent [1]. The symptoms might include diarrhea, cramping, fever and bloody stool. The orally active drugs are found to be more effective in case of amoebic disease or amoebic colitis. The altered physiology of stomach in amoebiasis pose various complications towards the pharmacokinetic profiling of medicine. The orally active medication was found to be very effective in such type of diseases. The dose and bioavailability of the drug may vary with respect to type of formulation administered orally. SEDDS type of formulation was found to be advantageous and compatible on oral administration. SEDDS formulations are isotropic mixtures of oil, surfactants, solvents and co-solvents/surfactants. The principle characteristic of these systems is their ability to form fine oil-in-water (o/w) emulsions or micro-emulsions upon mild agitation following dilution by an aqueous media such as gastrointestinal (GI) fluids. Fine oil droplets would pass rapidly from the stomach and promote wide distribution of the drug throughout the GI tract, thereby minimizing the irritation frequently encountered during extended contact between bulk drug substances and the gut wall. SEDDS have also attracted interest because they can improve the bioavailability of compounds that fall into class II of the biopharmaceutical classification system (BCS). Class II compounds are poorly water-soluble and highly permeable. SEDDS may offer an improvement in both the rate and extent of absorption and reproducibility of plasma concentration profiles [2]. Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone) is a naturally occurring alkyl-substituted hydroxy benzoquinone and a major constituent of Embelia ribes Burm. (Family: Myrsinaceae). The fruit is bitter in taste and has been used to treat fever, inflammatory diseases, and a variety of gastrointestinal ailments for thousands of years [3]. In the Indian system of plant medicine is “Ayurveda”, popularly known as vidanga and it is an ingredient in many Ayurvedic preparations. Embelin is reported to possess antitumor, antibacterial, antioxidant, analgesic, antifertility, wound healing, anticonvulsant, and antidiabetic activities [4]. In the present study, we developed liquid SEDDS with Capmul MCM as oil, Kolliphor hs 15 as surfactant and PEG 400 as co-surfactant. The prepared liquid SEDDS were formulated in to solid SEDDS as a self-emulsifying powder (SEP) by adsorbing on to powder Avicel 200 as carrier. The potential anti-amoebic activity of emebelin and metronidazole-loaded SEDDS was investigated.

MATERIALS AND METHODS

Materials

Capmul MCM (Abitec Co. ltd.), Castor oil (Himedia Laboratories Pvt. Ltd., Mumbai), Cremophor EL (Sigma Aldrich, USA), Embelin(Indofine chemical company), Inc, USA, fetal bovine serum (HiMedia Laboratories Pvt. Ltd., Mumbai), Avicel 200 (Sigma Aldrich, USA), Heparin Sodium (Central Drug House, New Delhi), Kolliphor HS 15 (Sigma Aldrich, USA),n-Octanol (Lobachemie), Oleic acid (Central Drug House, New Delhi) Olive oil (Central Drug House, New Delhi), Pencillin (HiMedia Laboratories Pvt. Ltd., Mumbai), Tween 80 (Himedia Laboratories Pvt. Ltd., Mumbai), MTT Tetrazolium (Central Drug House, New Delhi)were purchased. Entamoebahistolytica strain (HM:

IMSS), Diamond medium was gifted by Jamia Millia Islamia, University, MCF-7 cell line was gifted by NCCS, Pune. All the excipients and reagents were of analytical grade and double distilled water was freshly prepared whenever required throughout the study.

Formulation development: Embelin containing SEDDS

Screening of components (oil, surfactant andco-surfactant)

Selection of components (Oil, Surfactant and Co-surfactant) was done on the basis of saturation solubility of drug in various components as well as USFDA approval of components. The components having higher solubility were selected for the Embelin formulation. Ratio of surfactant and co-surfactant (Smix) was fixed by using pseudoternary phase diagram.

Pseudo ternary phase diagram

Pseudo ternary phase diagram was prepared by using chemix software 5 trial version having triangular format (triangle) which has three coordinates. Each coordinate represents one component of the emulsion system viz. (1) Oil phase (2) SA-Cos ratio phase and (3) aqueous phase. Each coordinate also represents 0 to 100% concentration of each of the phases in the increment of 10%. Water titration method was used to construct pseudoternary phase diagrams because this method is easy and scalable. In this study blank (without drug) SEDDS were prepared to find the area of the particular component system. Surfactant was blended with cosurfactant in fixed weight ratios (1:1, 2:1, 3:1, and 4:1) based on earlier reports [5]. Aliquots of each surfactant and cosurfactant mixture (Smix) were then mixed with oil at ambient temperature. For each phase diagram, the ratio of oil to the Smix was varied as 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8,1:9(v/v). Water was added dropwise to each oil-Smix mixture under vigorous stirring. After equilibrium, the samples were visually checked and determined as being clear emulsions or gels.

Method of preparation of SEDDS formulation

SEDDS were prepared by using the ratio of oil-Smix [Capmul MCM: Kolliphor hs 15: PEG 400(2:1)] and a pseudoternary phase diagram was prepared. Dissolving the drug in surfactant followed by addition of co-surfactant and oil in a beaker. The resultant mixtures were stirred continuously by magnetic stirrer and heated at 40 °C to obtain a homogenous mixture. SEDDS is also known as Emulsion preconcentrate because when it comes in contact with water, it will convert in to Emulsion [5].

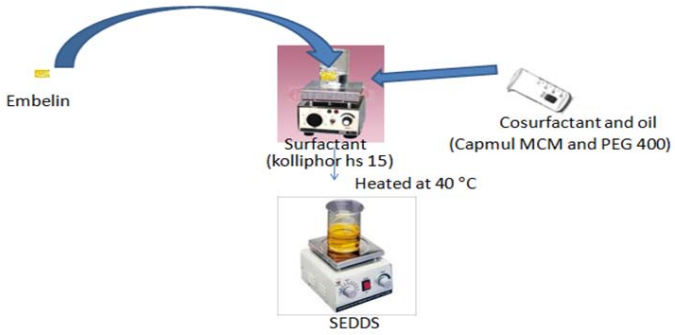


Fig. 1: Self-emulsifying drug delivery system preparation method

Optimization of SEDDS formulation

From above preparedternary phase diagrams, S: CoS (Smix) ratio which gives maximum area was selected and predicted that it should give stable emulsion. Based on the observations in the phase diagram, different batches of SEDDS containing embelin were prepared and optimization was done on the basis of, emulsification time % transmittance, particle size, polydispersity index and zeta potential of the reconstituted emulsions and precipitation of the drug from the respective batches of SEDDS as response parameters.

Characterization of embelin loaded SEDDS

Visual assessment of self-emulsification

Evaluation of the self-emulsifying properties of SEDDS formulations was performed by visual assessment. The USP XXIV type II dissolution apparatus (Electrolab, Mumbai, India) was used to evaluate the efficiency of self-emulsification of different formulations. One gram of each formulation was added drop wise into 500 ml of distilled water maintained at 37 °C with gentle agitation condition provided by rotating paddle at 50 rpm. The time taken for the emulsion (until a clear homogenous system was obtained) formation was assessed visually as reported [5].

Clarity (transparency) test

Transparency of emulsion was determined in terms of percentage transmittance determined using a spectrophotometer at 638.2 nm (UV, 1601, 220X Shimadzu, Japan) wavelength against distilled water blank [6].

Dilution test

Dilution test is conducted to observe whether the dissolved drug precipitates on dilution. The emulsion was diluted with water up to 100 times with stirring. It was then observed for any precipitation by measuring percent transmittance as described above [7].

Centrifugation test

This test is used to specify the stability of the emulsion whether it is monophasic or not. In this, the samples are centrifuged at 7500 rpm for 10 min and then are examined for whether the system is monophasic or biphasic [7].

Globule size and polydispersity index determination

The mean globule size (MGS) and polydispersity index of the emulsion was determined by light scattering based on laser diffraction using the particle size analyzer (Beckman Coulter Counter Delsa Nano C, USA).

Zeta potential determination

Beckman Coulter Counter Delsa Nano C, USA was used to measure the zeta potential of the globules based on the electrophoresis and electrical conductivity of the emulsion samples. The electrophoretic mobility (cm^2/Vs) of the particles was converted to the zeta potential by in-built software based on the Helmholtz-smoluchowski equation.

Viscosity measurement

The viscosity was determined using a rotational viscosity measuring device coupled with concentric cylinders (Brookfield Rheometer, Model-RS3CPS230LS, Middleboro, U. S. A) and the results were recorded. Experiments were performed in triplicate for each sample, and results were presented as average±standard deviation.

Drug release studies

Drug release experiments were conducted using a modified dialysis method [5]. Initially, the dialysis tubing was soaked in the dialysis

medium for 12 h at room temperature which was treated at 40 °C before start of experiment. The diluted SEDDS formulation (equivalent to 10 mg) was placed in dialysis tubing and clamped on both sides. The secured dialysis tube was allowed to rotate freely in the dissolution vessel of USP XXIV type-II dissolution apparatus (Electrolab, Mumbai, India) containing 500 ml of phosphate buffer (pH 7.4) at 37±0.5 °C and stirred at 50 rpm. An aliquot of 5 ml was withdrawn at predetermined time intervals and filtered through 0.45 µm filter. The withdrawn volume was replenished immediately with same volume of fresh medium in order to keep total volume constant and maintain sink conditions. The concentration of embelin in the filtrate was analyzed using UV spectrophotometer at 285 nm. The blank SEDDS without drug was processed similarly and used as a reference to avoid interference from the formulation components, if any. The mean of at least three determinations was used to calculate the drug release [5].

Preparation of solid self-emulsifying powder

Self-emulsifying powder was prepared to overcome the disadvantages associated with liquid SEDDS. Hence to increase the stability and patient compliance optimized formulation F7 was adsorbed onto Avicel 200. The amount of Avicel 200 adsorbed to produce the free-flowing powder was 1g/g (0.5g/g, 1g/g, 1.5 g/g of SEDDS formulation). This low amount of Avicel 200 required to produce free-flowing characteristics may be due to the larger surface area and adsorption capacity of Avicel 200. Thus the Self emulsifying powder containing avicel 200 (1g/g) as adsorbent carrier was selected Aerosil 200 (1 gm) was added slowly and mixed vigorously to get the granular mass. The granular mass were passed through 500 microns mesh (35 mesh) to get a uniform free-flowing granules. The granules were stored over anhydrous calcium chloride in a desiccator [8].

Characterization of embelin loaded self-emulsifying powder

Flow properties

Angle of repose (θ) for embelin loaded solid SEDDS was determined by Carr's method. Briefly, the sample was poured through a funnel with its tip position data fixed height (h) on a horizontal surface until apex of piletouches the tip of the funnel. The angle of repose was calculated using the formula tan θ=h/r where r is radius of the pile of powder. Additionally, the flow rate was determined by measuring the time required for 1.0 g of formulation to flow through funnel with orifice of 1.5 cm diameter. The powder flow property was noted on the basis of time required to pass through orifice as less than 1 s (excellent), less than 5 s (good), less than 10 s (average) and more than 10 s (poor) [8].

Drug content

Embelin loaded SEDDS powder were dissolved in methanol and embelin was extracted by shaking at 37 °C for at least 30 min. Embelin concentration in methanol extract was analyzed using U. V spectrophotometer at 285 nm against blank [9].

Morphological analysis of embelin loaded SEDDS powder

The outer macroscopic structure of the solid self-emulsifying powder(SEP) was investigated by scanning electron microscopy (S-4100, Hitachi, Japan) at 15 keV accelerating voltage.

Drug release studies

Drug release experiments were conducted using a modified dialysis method. Initially, the dialysis tubing was soaked in the dialysis medium for 12 h at room temperature, which was treated at 40 °C before start of the experiment. The diluted SEDDS formulation (equivalent to 10 mg) was placed in dialysis tubing and clamped on both sides. The secured dialysis tube was allowed to rotate freely in the dissolution vessel of USP XXIV type-II dissolution apparatus (Electrolab, Mumbai, India) containing 500 ml of phosphate buffer (pH 7.4) at 37±0.5 °C and stirred at 50 rpm. An aliquot of 5 ml was withdrawn at predetermined time intervals and filtered through 0.45 µm filter. The withdrawn volume was replenished immediately with the same volume of fresh medium in order to keep the total volume constant and maintain sink conditions. The concentration of embelin in the filtrate was analyzed using UV spectrophotometer at 285 nm. The blank SEDDS without drug was processed similarly and used as a reference to avoid interference from the formulation components, if any. The mean of at least three determinations was used to calculate the drug release [5].

***In vitro* study of amoebiasis**

Culture of test organism

E. histolytic a HM1:IMSS was culture in falcon flask by using diamond TPS-1 medium. When the amoebic growth was confluent sub culturing was carried out by chilling the flask on ice to detach the amoebae and diluting the resulting suspension 2 to 3 times with fresh culture medium [10].

Test procedure

DMSO (50 µl) was added to embelin (1 mg) followed by enough culture medium to obtain concentrations of 1 mg/ml and sonicated by bath sonicator. Two-fold serial dilutions were made in the well of 96 well microtiter plates in 170 µl of culture medium. Each plate included metronidazole as a standard amoebicidal drug, control wells (culture medium plus amoebae) and a blank (culture medium only). A suspension of amoebae was prepared from confluent culture by pouring off the medium, adding 2 ml of fresh mdium and chilling the culture on ice to detach the organism from side of the flask. The number of amoeba per milliliter was estimated with a hemacytometer and trypan blue used to confirm viability. Fresh culture medium was added to dillute the suspension to 10^5per ml and 170 µl of this suspension was added to test and control well. An inoculum of $1.7x10^4$ organism was chosen so that confluent growth. Plates were sealed with parafilm and placed in incubating chamber at 37 °C for 72h [10].

Assessment of antiamoebic activity

After incubation, the growth of amoebae checked with a low-power microscope. The culture medium was removed by inverting the plates and shaking them gently. Plates were then immediately washed once sodium chloride solution (0.9%) at 37 °C and dry at room temperature and amoebae were fixed with methanol and, when dry, stained with aqueous eosin (0.5%) for 15 min. Stained plates were washed with distilled water.200 µl portion of 0.1N NaOH solution was added to each well to dissolve the protein and release dye. The optical density of the resulting solution in each well determine at 490 nm with a microplate reader (Bio-red) [10]

***In vitro* cell line studies**

Cytotoxicity studies (MTT assay)

MCF-7 cells were cultured and maintained as a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with10% of fetal calf serum (FCS), antibiotics: 100 IU/ml of penicillin and 100 mg/ml of streptomycin. All cells were cultured in flasks at 37 °C in the 100% humidity atmosphere and 5% of CO2. Only viable cells were used in the assay. Exponentially growing cells were plated at 1.2×10^4 cells per well into 96-well plates and incubated for 48 h before the addition of drugs. Stock solutions of compounds were initially dissolved in 20% (v/v) DMSO and further diluted with a complete fresh medium. The growth-inhibitory effects of the compounds were measured using a standard tetrazolium MTT assay. After 48 h of incubation at 37 °C, the medium was removed and 5 ml of MTT reagent (5 mg/ml) in serum-free medium was added to each well. The plates were incubated at 37 °C for 4 h. At the end of the incubation period, the medium was removed and pure DMSO (100 ml) was added to each well. The metabolized MTT product dissolved in DMSO was quantified by reading the absorbance at 570 nm. All assays were performed in triplicate. Percent viability was defined as the relative absorbance of treated versus untreated control cells. Plates were analyzed in an ELISA plate reader at 570 nm with a reference wavelength of 655 nm [11].

Pharmacokinetics study of embelin

Method described in the previous section after method development by UV was successfully applied to quantify plasma concentration of embelin in pharmacokinetics study.

Animal

Guinea pig of either sex (550-650 gm) was used to study pharmacokinetics of plain drug suspension of embelin (Control) and embelin loaded SEDDS powder formulations (Test) after oral administration. Animals were procured from Animal house of ISF College of Pharmacy, Moga, India and were housed under standard laboratory conditions with free access to food and water. The animals of both groups test and control were fasted overnight (~14 h) and had free access to water throughout the experimental period. Experimental procedure was adopted according to the method reported earlier [12]. Protocol for studies was approved by the Institutional ethical committee at ISF College of Pharmacy, Moga, India. The experiments were conducted with as per CPCSEA (Committee for Prevention, Control and Supervision of Experimental Animals, approval NoISFCP/IAEC/CPCSEA/Meeting No. 7/2012-13/Protocol No.126) guidelines.

Route of administration and withdrawal of blood samples

Plasma drug concentration-time profile and pharmacokinetic parameters were obtained from oral administration of plain drug suspension followed by oral administration of prepared Embelin loaded SEDDS powder formulations. 2 groups of guinea pig were taken for pharmacokinetic studies. Each group had 6 guinea pig. Table 1 represents groups and the dose of embelin administered to guinea pig. The formulation was administered orally with oral feeding cannula.

For oral studies, a group of rats (n = 6) were administered with prepared Embelin loaded SEDDS powder and plain drug suspension at a dose of 10 mg/kg.

Table 1: Experimental layout for pharmacokinetic study

Group	No. of animals	Dose (mg/kg)	Route of administration	Sampling route	Drug
1st	6	10	Oral	Tarsal vein	Embelin Plain Drug Suspension
2nd	6	10	Oral	Tarsal vein	Embelin loaded SEDDS powder

After oral administration of embelin loaded SEDDS powder, 0.5 ml of blood samples were collected from Tarsal vein in previously heparinised [(30 units/10 µl) prepared in 0.9% NaCl solution] and labeled eppendorf's tubes at predose and at 0, 0.5, 1, 2, 4, 6,8, 12,16 and 24 h post-dose. The suspension was prepared by suspending the amount of plain drug powder in 0.3 % of DMSO (dimethyl sulfoxide) in water.

Plasma was harvested from the blood by centrifugation (10000 rpm, 10 min) at zero degree celcius temperature on refrigerated centrifuge apparatus (Ultracentrifuge sigma, U. S. A) and kept at-20 °C until used. Plasma samples collected from guinea pig were analyzed using validated bio-analytical method and drug plasma concentration values were determined from the calibration curve.

Histopathology study

Animals (Guinea pigs) divided into 5 groups (n=3). Embelin loaded self-emulsifying drug delivery systems (SEDDS) were prepared and embelin loaded self-emulsifying drug delivery systems (SEDDS) in powder form were administered orally and compared with the marketed formulation of metronidazole in table 2.

Table 2: Experimental layout for histopathology study

GROUP-1	Disease control
GROUP-2	100 mg/kg/day (5 d), orally for Metronidazole
GROUP-3	10 mg/kg/day (5 d), orally for Embelin powder form
GROUP-4	10 mg/kg/day (5 d), orally for embelin loaded self-emulsifying drug delivery systems (SEDDS).
GROUP-5	10 mg/kg/day (5 d), orally forEmbelin loaded self-emulsifying drug delivery systems(SEDDS) Powder

Inoculation procedure

For inoculation in animals the strain of E. histolytic a was cultured in several tubes of the medium and incubated for 48 h. At the end of incubation, the fluid phase of the cultures pooled, and centrifuged for 10 min. The supernatant was discarded and the deposit was washed three times with phosphate buffer saline (pH 7.2). Finally, the deposit was suspened in small volume of phosphate buffer saline, the count of amoebae was adjusted to $2x10^5$ per ml by adding the required amount of phosphate buffer saline. 0.5 ml of this suspension containing 10^5amoebae was used for infecting each animal [13].

Antiamoebie evaluation in experimental caecal amoebiasis

The animals were maintained on standard pelleted diet and water. The animal was fasted for 36-48 h prior to infection. The inoculum was prepared by pooling a number of 48 h culture flasks and centrifuging at low speed to minimize the bacterial flora and concentrate the culture. The amoebae were counted using a haemocytometer. The animals were carefully anaesthetized with ketamine and a laparotomy was performed to expose the caecum. The amoebae were directly injected into the caecum. The caecum was replaced in the abdomen, the peritoneal cavity closed and the skin sutured. One set of the animals was segregated and kept as the untreated control [13].

Drug administration

All the formulations administered orally to the guinea pig with the aid of a blunt end needle and syringe. Metronidazole was administered similarly. The animals were treated for 5 d, with the treatment commencing 24 h after infection. The animals kept aside as controls were treated with normal saline. The number of animals in each group will be 6. The rats were sacrificed at the end of the sixth day and the caecum was taken out from each animal and the caecal pathology by using stain haematoxylin [13]

RESULTS AND DISCUSSION

Saturation solubility

The extent of the solubility of a substance in a specific solvent is measured as the saturation concentration, where adding more solute does not increase the concentration of the solution and begins to precipitate the excess amount of solute.

Embelin was found to have highest solubility in Capmul MCM (oil), kolliphor hs 15 (surfactant) and PEG 400 (Cosurfactant). Fig. 2 shows the graphical representation of the saturation solubility of embelin in different oils, surfactants and co-surfactants and Data given in mean±SD, n=3.

Formulation development and optimization of embelin loaded SEDDS

Self-emulsifying drug delivery system was prepared according to the procedure mentioned and further, it was optimized on the basis of emulsification time, particle size, PDI, transmittance, precipitation of drug, zeta potential. Process parameters included concentration of oil, surfactant: cosurfactant ratio. A small-sized globule size was the aim of this study because they offered very high surface area for absorption, which leads to quick absorption of the drug and improves oral bioavailability.

Screening of components

One important consideration when formulating a self-emulsifying formulation is avoiding the precipitation of the drug on dilution in the gut lumen *in vivo* (Pouton 2000). Therefore, the components used in the system should have high solubilization capacity for the drug, ensuring the solubilization of the drug in the resultant dispersion. Screening of components was based on saturation solubility of the embelin in different oils, surfactants and co-surfactants. Table 3 shows saturation solubility's of selected components *i.e.* Capmul MCM, Kolliphor hs 15 and PEG 400.

Pseudoternary phase diagram

Self-emulsifying systems form fine oil-water emulsions with only gentle agitation upon their introduction into aqueous media. Surfactant and cosurfactant get preferentially adsorbed at the interface, reducing the interfacial energy as well as providing a mechanical barrier to coalescence. The decrease in the free energy required for the emulsion formation consequently improves the thermodynamic stability of the emulsion formulation [2]. Therefore, the selection of oil and surfactant, and the mixing ratio of oil to S/CoS, plays an important role in the formation of the emulsion. Screening of surfactant: the co-surfactant ratio was done on the basis of pseudoternary phase diagram using the water titration method. Table 4 and fig. 3 shows the screening of different ratios of surfactant: cosurfactant and pseudoternary phase diagrams prepared for different ratios of S: CoS *i.e.* from 1:1, 1:2, 1:3 and 1:4.

Table 3: Selected components on the basis of saturation solubility

Components	Saturation solubility (mg/ml)*
Capmul MCM (Oil)	37.17±.001
Kolliphor hs 15 (Surfactant)	150.42±.006
PEG 400 (Co-surfactant)	150.29±.002

*Data given in mean±SD, n=3

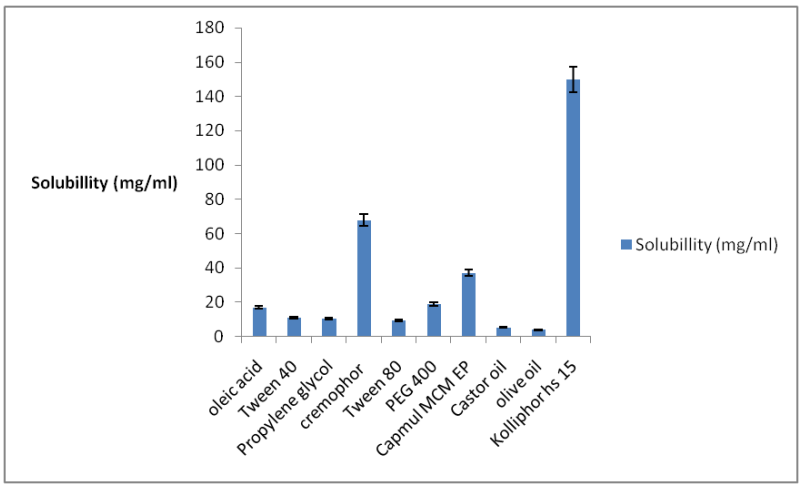


Fig. 2: Saturation solubility of embelin in different excipients

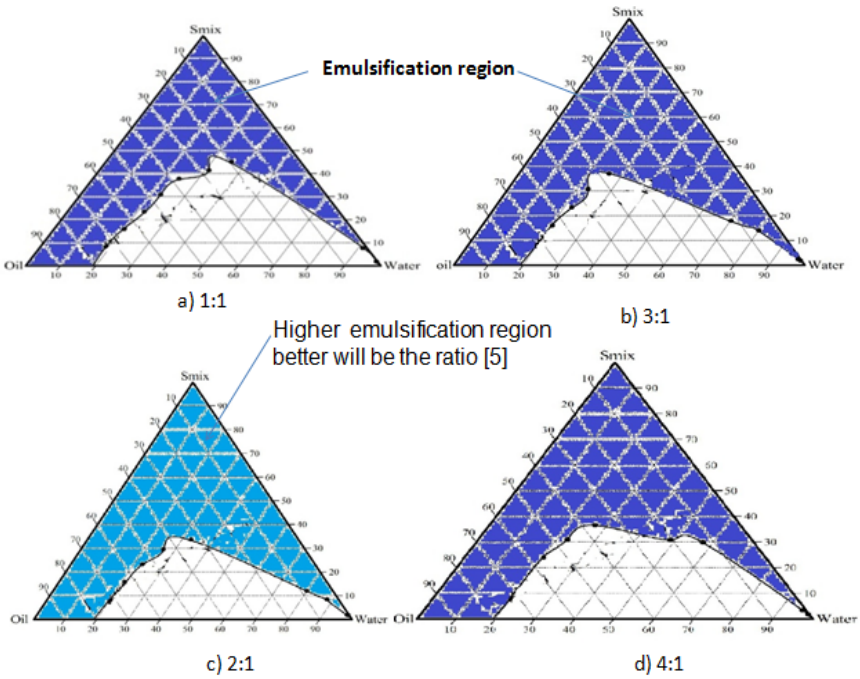


Fig. 3: Pseudoternary phase diagrams of Capmul MCM (oil), Kolliphor hs 15: PEG 400 (SA: Cos) and water system (For Embelin) in Surfactant to co-surfactant ratio of (a) 4:1, (b) 3:1, (c) 2:1 and (d) 1:1

Table 4: Pseudo-ternary phase diagram study for the selection of surfactant and Co-surfactant

Oil (ml) (Capmul MCM)	Smix (ml) (Kolliphor hs 15 and PEG 400)	Dilution with water until emulsion remain clear (ml)			
		4:1(SA: CoS)	3:1(SA: CoS)	2:1(SA: CoS)	1:1(SA: CoS)
0.2	1.8	Infinite	Infinite	**Infinite**	Infinite
0.4	1.6	45	67	**85**	21
0.6	1.4	2.7	8.0	**15**	1.1
0.8	1.2	1.9	4.7	**8**	0.9
1.0	1.0	0.74	0.70	**0.98**	0.65
1.2	0.8	0.59	0.60	**0.71**	0.59
1.4	0.6	0.52	0.57	**0.59**	0.56
1.6	0.4	0.51	0.51	**0.53**	0.50
1.8	0.2	0.50	0.49	**0.51**	0.46

*Data given in mean±SD, n=3

On the basis of this pseudoternary phase diagram study the optimized ratio of surfactant and cosurfactant *i.e.* kolliphor hs 15: PEG 400 was 2:1. Pseudoternary diagrams showed that with the increase in the surfactant concentration there is increase in the emulsifying region. Selection was totally on the basis of region of emulsification *i.e.* more the emulsification region better will be the ratio. Selected ratio *i.e.* 2:1 amongst other ratios provides the rapid emulsification and reduced globule size of the emulsion.

Optimization of the SEDDS formulation

From above ternary phase diagrams, S: CoS (2:1) give maximum area, so it should give a stable emulsion. Based on the observations in the phase diagram, different batches of SEDDS containing Embelin were prepared by increasing the concentration of the oil and diluting in a fixed volume of water. The optimization parameters for the respected batches were emulsification time, % transmittance, particle size, polydispersity index, zeta potential and precipitation of the drug. The dose of the drug and ratio of surfactant: cosurfactant were kept constant *i.e.* 8% and 2:1. With the increase in the oil concentration the S: CoS concentration was decreased simultaneously because of the fixed formulation size. The optimization chart shows an increase in the ratio of the oil phase (Capmul MCM) resulted in a proportional increase in particle size, because of the simultaneous decrease in the S/CoS proportion. Increasing the S/CoS ratio led to a decrease in mean droplet size. This could be attributed to an increased surfactant proportion relative to cosurfactant. It is well known that the addition of surfactants to the emulsion systems causes the interfacial film to stabilize and condense, while the addition of cosurfactant causes the film to expand; thus, the relative proportion of surfactant to cosurfactant has varied effects on the droplet size [2]. Similarly, there is the decrease in % transmittance value with increase in the concentration of the oil due to an increase in globule size of the emulsion formed. Negative zeta potential value of all the respected batches shows the presence of fatty acids in oil phase. For oral emulsion preparations, dilutability is very important parameter to confirm that the drug should not precipitate out on dilution in GIT.). Formulation 7 was the selected one on the basis of emulsification time no precipitation, zeta size, transmittance, PDI and zeta potential.

Table 5: Optimization of self-emulsifying drug delivery system containing embelin, Data expressed as mean±SD, n=3

Formulatⁿ	Drug	Oil	Smix(2:1)	Dilutability with water (200 ml)	Emulsification Time(Sec)	Size (nm)	PDI	Transmittance (%)	Pptⁿof drug	ZetaPotential (mV)
F1	8%w/v	5%	95%	Clear	12	112.65±1.4	0.461±0.003	98.3±0.23	Yes	-15.14±0.11
F2	8%w/v	10%	90%	Clear	15	127.76±0.76	0.351±0.015	96.7±0.15	Yes	-17.29±0.10
F3	8%w/v	15%	85%	Clear	23	140.43±0.44	0.372±0.016	94.8±0.24	Yes	-19.73±0.14
F4	8%w/v	20%	80%	Clear	19	161.11±0.52	0.411±0.007	92.7±0.35	Yes	-22.67±0.42
F5	8%w/v	25%	75%	Clear	25	178.39±1.22	0.345±0.006	91.3±0.29	Yes	-27.78±0.22
F6	8%w/v	28%	72%	Clear	28	201.19±0.98	0.294±0.016	89.8±0.19	Yes	-29.14±0.23
F7	8%w/v	30%	70%	Clear	27	217.50±1.75	0.108±0.003	88.1±0.09	No	-31.68±0.13
F8	8%w/v	32%	68%	Translucent	30	251.0±1.87	0.263±0.012	84.3±0.22	No	-.34.47±0.54
F9	8%w/v	34%	65%	Translucent	33	283.64±2.20	0.239±0.011	79.6±0.13	No	-35.87±0.76
F10	8%w/v	36%	64%	Translucent	37	297.17±3.43	0.221±0.005	71.4±0.23	No	-37.91±0.39
F11	8%w/v	38%	61%	Turbid	36	311.43±3.21	0.311±0.008	67.3±0.17	No	-39.29±0.16
F12	8%w/v	40%	60%	Turbid	40	325.56±2.54	0.285±0.003	53.2±0.12	No	-41.01±0.09

Data expressed as mean±SD, n=3

Characterization of eembelin loaded SEDDS liquid

Clarity (Transparency) test

Transparency of emulsion was determined in terms of percentage transmittance determined using a spectrophotometer at 638.2 nm (UV, 1601, 220X Shimadzu, Japan) wave length against distilled water blank and it was found to be 88.1% as shown in the table 5 [6].

Dilution test

Dilution test is conducted to observe whether the dissolved drug precipitates on dilution. Optimized formulation (F7) was diluted up to 1000 times with water and there was no sign of precipitation as seen visually in the table 5 [7].

Centrifugation test

This test is used to specify the stability of the emulsion, whether it is monophasic or not. In this, the optimized formulation was firstly diluted with 200 ml of water and then centrifuged at 7500 rpm for 10 min. There was no sign of phase separation in the reconstituted emulsion in the table 5 [7].

Globulesize and polydispersity index determination

The average particle size and polydispersity index are important parameters in sedds study as it predicts the stability of formulations systems. Both size and PDI showed that SEDDS is a good formulation. Size and size distribution of optimized reconstituted SEDDS of embelin was determined by photon correlation spectroscopy method using zetasizer (Bechman coulter). Size and size distribution of the optimized formulation is shown in fig. 4 [15].

Zetapotential determination

Zeta potential is a crucial indicator to determine the stability of SEDDS. It is necessary to assess zeta potential of the SEDDS as it can

identify the charge of oil globules in the emulsion. The increase in electrostatic repulsive forces between the globules prevents the coalescence of emulsion. On the contrary, a decrease in electrostatic repulsive forces can cause phase separation. The mean zeta potential of the emulsion obtained by diluting embelin loaded SEDDS with distilled water was −31±0.68 mV and the results are shown in fig. 4. The charge on the oil globules is negative due to free fatty acid present in the oil phase [15].

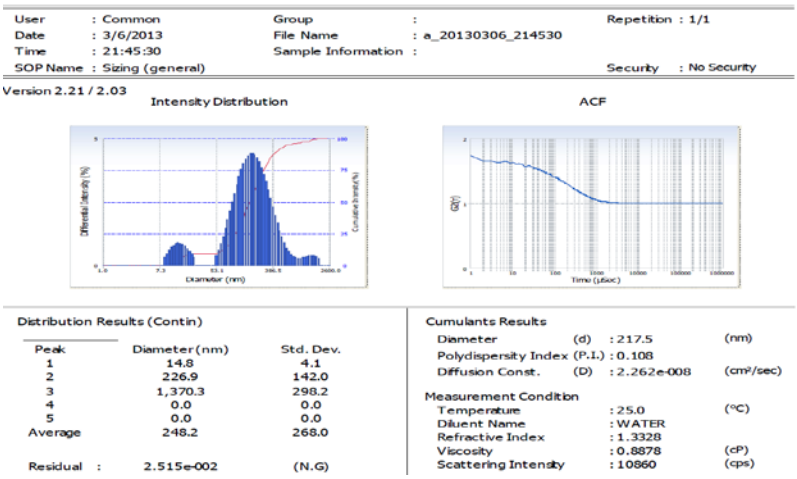


Fig. 4: Size and polydispersity index of optimized embelin loaded SEDDS

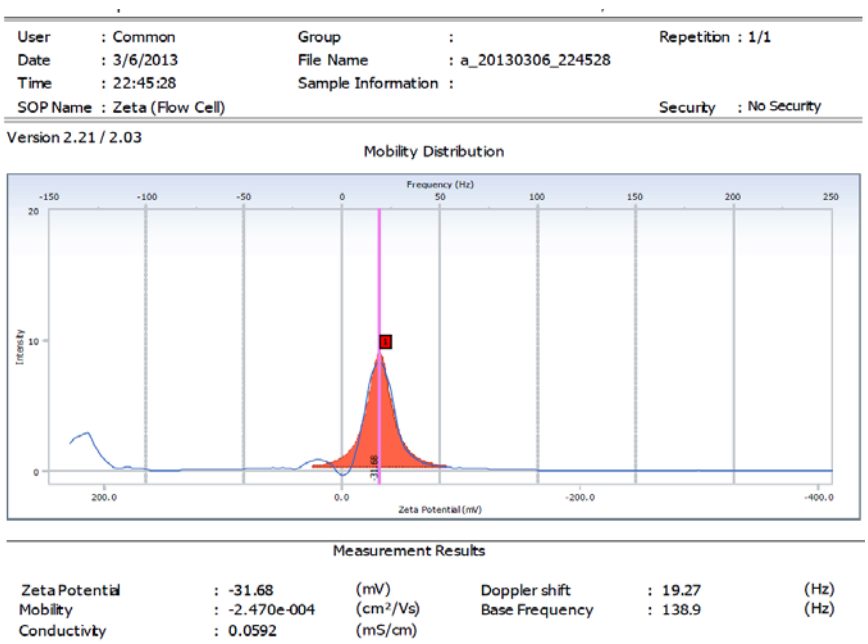


Fig. 5: Zeta potential of optimized embelin loaded SEDDS

Viscosity measurement

The viscosity of the optimized embelin loaded SEDDS(F7) was determined to check its character i.e. o/w or w/o and the viscosity of embelin loaded SEDDS formulation without water was determined to check its ability to be filled in hard or soft gelatin capsules. If the system has very low viscosity, it may enhance the probability of leakage from the capsule and the system with very high viscosity may create problem in pourability [16]. Table 6 shows the viscosity and rheological parameters of optimized Embelin loaded SEDDS in to emulsion and table 4 shows the viscosity and rheological parameters of optimized SEDDS.

Table 6: Viscosity and rheological parameters of optimized Embelin loaded SEDDS

Temp.	Shear rate (Sec^{-1})	% Torque (Nm)	Viscosity (cP)
25 °C	10	6.32±0.43	17.12±0.54
25 °C	20	7.13±0.21	16.37±0.27
25 °C	30	8.72±0.78	16.11±0.32

Data expressed as mean±SD, n=3

The values in the table 6 showed that the type of emulsion was o/w and that value of viscosity was less than 25,000 cps at different shear rates. It implies that the developed SEDDS can be filled in hard gelatin capsules by commercial liquid filling equipments [17].

In vitro drug release graph of embelin loaded SEDDS

In vitro dissolution profile of embelin loaded SEDDS in comparison to plain drug suspension in phosphate buffer pH 7.4 is shown in fig. 6

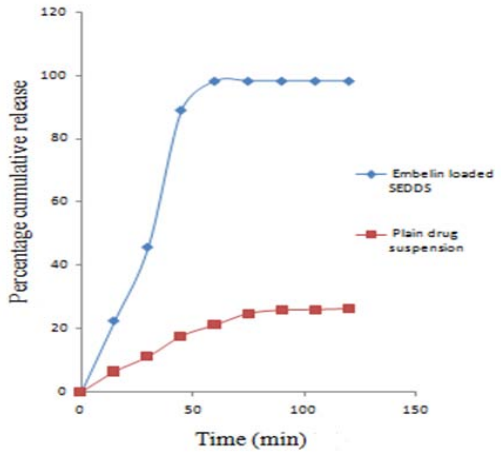


Fig. 6: *In vitro* profiles of Embelin loaded SEDDS and plain drug suspension in phosphate buffer (pH 7.4), data expressed as mean±SD, n=3

Percentage cumulative release was above 95% for embelin loaded SEDDS in phosphate buffer pH 7.4, while it was only 23 % in case of plain drug suspension in phosphate buffer pH-7.4 after 120 h. Significant increase in the rate of release of Embelin from SEDDS compared to plain drug suspension can be attributed to its quick dispersibility and ability to keep drug in the solubilized state.

Characterization of self-emulsifying powder

Self-emulsifying powder was prepared to overcome the disadvantages associated with liquid SEDDS. Hence to increase the stability and patient compliance, optimized formulation F7 was adsorbed onto avicel 200. The amount of avicel 200 adsorbed to produce the free-flowing powder was 1% w/w (0.5% w/w, 1% w/w, 1.5% w/w of SEDDS formulation). This low amount of Avicel 200 required to produce free-flowing characteristics may be due to the larger surface area and adsorption capacity of avicel 200. Thus the Self-emulsifying powder containing avicel 200 (1g/g) as an adsorbent carrier was selected.

Flow properties

Angle of repose

Formula tan θ=H/r= 1.3/2.5=0.52

θ=27.474

The value of angle of repose is greater than 25; this indicates flow of powder is good.

Drug content estimation

Drug content of optimized SEDDS powder formulations was found to be 92.7%.

SEM Image of SEDDS powder

The scanning electron micrographs in fig. 4.17 revealed that the self-emulsifying powder showed smooth granular particles after adsorbing the liquid SEDDS on the surface of avicel 200.

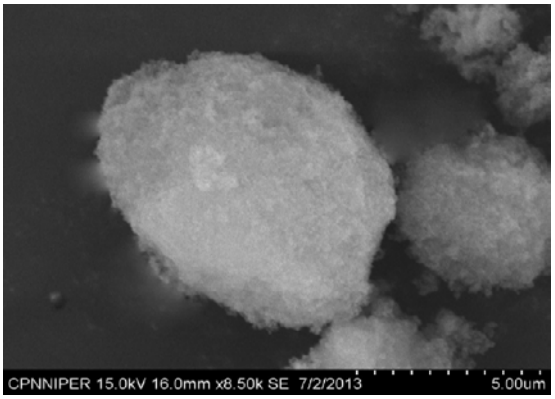


Fig. 7: SEM image of SEDDS powder

Percentage *in vitro* drug release studies

In vitro dissolution profile of embelin loaded SEDDS Powder in comparison to plain drug suspension in buffer pH 7.4 is shown in fig. 4.18

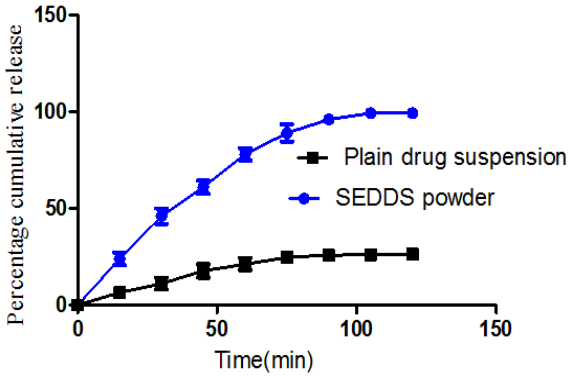
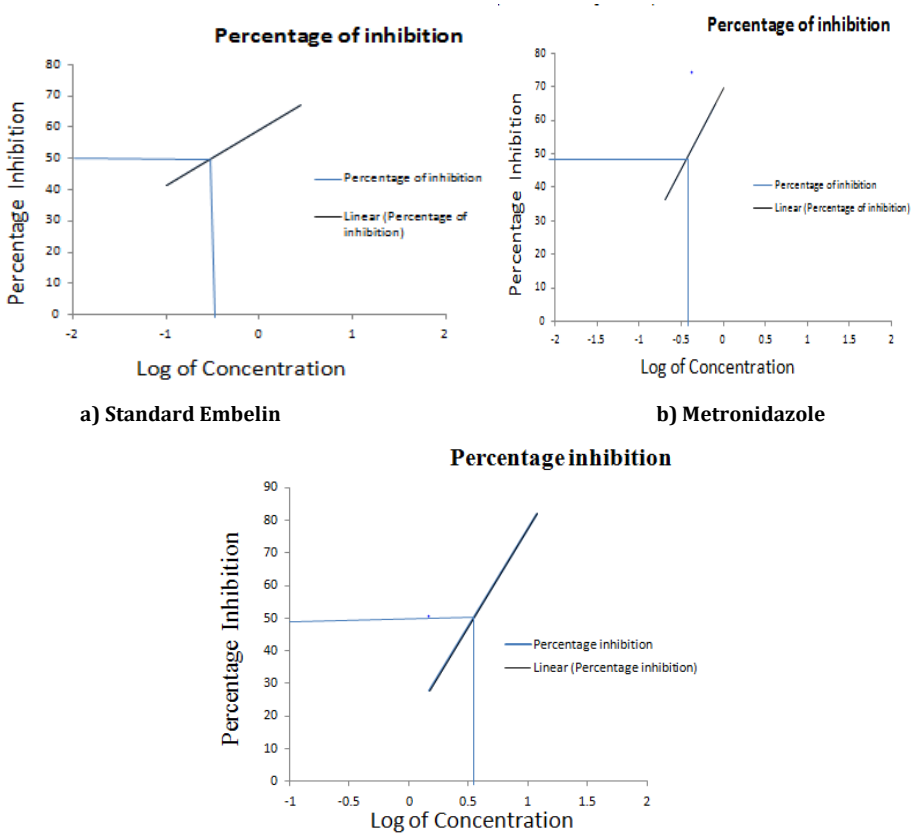


Fig. 8: Dissolution profiles of Embelin loaded SEDDS powder and plain drug suspension in phosphate buffer (pH-7.4), Data expressed as mean±SD, n=3

Percentage cumulative release was above 97% for embelin loaded SEDDS in phosphate buffer pH 7.4 while it was only 23 % in case of plain drug suspension in phosphate buffer pH-7.4 Significant increase in the rate of release of embelin from SEDDS compared to plain drug suspension can be attributed to its quick dispersibility and ability to keep drug in the solubilized state.

In vitro antiamoebic activity

Standard embelin, Isolated embelin amd embelin loaded SEDDS powder were screened *in vitro* for antiamoebic activity against HM1: IMSS strain of *E. histolytica* by the microdilution method. E. histolytica trophozoites were cultured in TYIS-33 growth medium in 96-well microtitre plate. The test compounds (1 mg) were dissolved in DMSO (40 µl). The maximum concentrations of DMSO in the test did not exceed 0.25%, at which level no inhibition of amoebal growth occurred. The stock solutions of the compounds were prepared freshly before use at a concentration of 1 mg/ml. The IC50 values in µM are given in Tables 7. The results were estimated as the percentage of growth inhibition compared with the untreated controls and plotted as probit values as a function of the drug concentration. The IC50 and95% confidence limits were interpolated in the corresponding dose-response curve. Metronidazole was used as the reference drug which had a 50% inhibitory concentration of 1.84 mmol in our experiments.



a) Standard Embelin **b) Metronidazole**

c) Embelin loaded SEDDS powder

Fig. 9: Amoebicidal activity of different sample a) Standard embelin b) Metronidazole c) Embelin loaded SEEDS powder

Table 7: IC_{50} value of different test samples

Test sample	IC_{50} value (µM)
Standard embelin	1.354
Metronidazole	1.84
Embelin loaded SEEDS powder	1.35

*Data expressed as mean±SD, n=3

Cytotoxicity studies

To examine the effect of antiamoebic formulation plain drug suspension, embelin loaded SEDDS powder and metronidazole on cell proliferation; we studied their cytotoxicity on human breast cancer MCF-7 cell line. A subconfluent population of MCF-7 cells was treated with increasing concentrations of compounds and the number of viable cells was measured after 48 h by MTT cell viability assay based on mitochondrial reduction of the MTT tetrazolium dye to a highly coloured blue formazone product. This assay usually shows high correlation with number of living cells and cell proliferation. The concentration range for all the formulations was 1.56-100 mmol. Fig. 4.20depicts the formulation i. e Plain Drug Suspension, embelin loaded SEDDS powder and metronidazole exhibited 100% viability at the concentration range of 1.56-100 mmol These results mean that Plain Drug Suspension, Embelin loaded SEDDS powder and Metronidazole were nontoxic against the human breast cancer MCF-7 cell line.

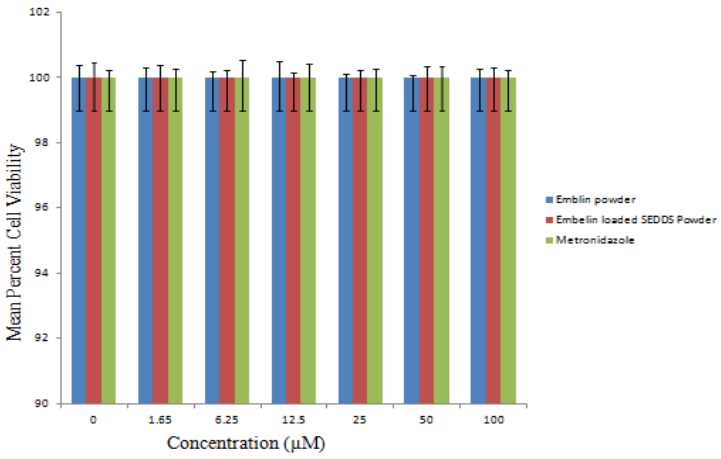


Fig. 10: Percentage of viable cells after 48 h pre-treatment of human breast cancer MCF-7 cells with formulation (Blue color indicate-Embelin powder, Red color indicate-Embelin loaded SEDDS powder and Green Color indicate-Metronidazole), evaluated by MTT assay

Pharmacokinetics study of embelin

In vivo bioavailability and pharmacokinetic studies of Test Groups (Embelin load SEDDS) and Control group (plain drug suspension) were conducted in Two groups of guinea pig containing six animals in each group. Following oral dosage of sample, blood samples were collected at specified time points and embelin concentration was determined in plasma by UV method. Results are tabulated as average plasma concentration of embelin (mcg/ml) for control and test groups along with standard deviation in table 8**.** The values are plotted with plasma concentration Vs time. Fig. 4. [22]. The pharmacokinetic parameters were calculated by using software kinetic.

Software kinetic

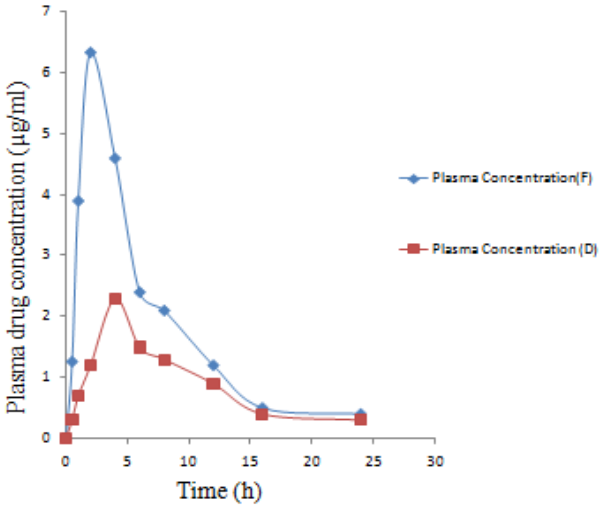


Fig. 11: Plasma concentration time curve following oral administration of Embelin plain drug suspension (D) and Embelin loaded SEDDS formulations (F) groups of guinea pig. Data expressed as mean±SD; * p<0.05 statistically significant (n=6)

Table 8: Pharmacokinetic parameters of control and test sample calculated using plasma concentration time data using software kinetica

Parameters	Fomulation (F7)	Plane drugs (D)
Cmax (µg/ml)	6.33	2.3
Tmax (h)	2	4
AUC(µg-h/ml)	42.062	21.175
$T_{1/2}$ (h)	5.29	6.69

The C_{max} of embelin plain drug suspension was 2.3 µg/ml after 4.0 h, whereas it was 6.33 µg/ml for the embelin loaded SEDDS, This may have been due to the slow diffusion of embelin from the dispersed oil globules to the continuous medium and higher lymphatic uptake may increase the $T_{max.}$ It indicates the release of drug was higher from the embelin loaded SEDDS than from the control. However, plasma half-life of the test formulations (Embelin loaded SEDDS) was less than that obtained with embelin Suspension in guinea pigs. Hence it is evident that the observed bio-availability increase in case of embelin loaded SEDDS formulation is due to increased absorption process with no contribution of the elimination process in it. The relative bioavailability of the embelin loaded SEDDS formulations as a comparison to the plain drug suspension was calculated by using mentioned formula and given in the table 8

$$F = \frac{[AUC]_0^{\infty} D_D}{[AUC]_0^{\infty} D_F} \times 100$$

AUC = Area under curve

F= Optimized Formulation

D=Dose (10 mg/kg)

d= Plain Drug

Relative bioavailability of Embelin loaded SEDDS as a comparison to plain drug is 1.98 fold

Histopathology study

The causative protozoan parasite, *Entamoeba histolytica*, is a potent pathogen. Secreting proteinases that dissolve host tissues, killing host cells on contact, *E histolytica* trophozoites invade the intestinal mucosa, causing amoebic colitis [15] and ulcers [18] which are clearly visible in the disease control histology i. e 1) ulceration 2) colitis by the black spot. Metronidazole, SEDDS, Embelin Loaded SEDDS Powder, Embelin Powder was treated the ulceration and colitis, which are clearly visible that there was no sign of black spot and ulceration at in the histology in fig. 12 Show the *In vivo* treatment of amoebiasis by different formulations, b,c, d, e respectively.

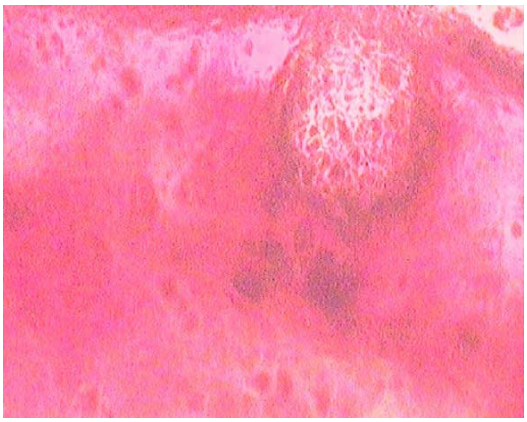


Fig. 12(a): Disease control: show ulceration and colitis by blackspot

Fig. 12(b): Metronidazole treated: show no sign of ulceration and colitis

Fig. 12(c): Embelin loaded SEDDS treated: show no sign of ulceration and colitis

Fig. 12(d): Embelin loaded SEDDS Powder Treated: show no sign of ulceration and colitis

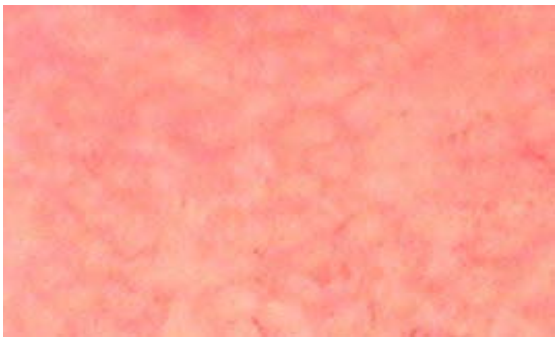


Fig. 12(e): Embelin powder treated: show no sign of ulceration and colitis

CONCLUSION

Present study showed the stronger amoebicidal action of embelin may associated with some specific interaction of the active embelin with the cell wall of the parasites or with a more effective mechanism of penetration into the parasites through the membrane channels. These results of *in vitro* and *in vivo* suggest that embelin is potential therapeutic drug for the treatment of *E. histolytica* infection. Further studies aimed that understanding the molecular mechanism involved in the amoebicidal action of embelin. Development of a self-emulsified drug delivery system leads to an increase the bioavailability of embelin up to 1.98 fold and Cytotoxicity study (MTT assay) shows the Plain Drug Suspension, Embelin loaded SEDDS powder and Metronidazole exhibited 100% viability at the concentration range of 1.56-100 mmol.

FUNDING

Nil

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

REFERENCES

1. Sehgal D, Bhattacharya A, Bhattacharya S. Pathogenesis of infection by *Entamoeba histolytica*. J Biosci. 1996;21(3):423-32. doi: 10.1007/BF02703099.
2. Patel PA, Chaulang GM, Akolkotkar A. Self-emulsifying drug delivery system: a review. Res J Pharm Technol. 2008;1:313-23.
3. Anon, National Medicinal Plants Board. Ministry of Health and Family Welfare press releases; 2008. p. 2-5.
4. Bhandari U, Ansari MN, Islam F, Tripathi CD. The effect of aqueous extract of *Embelia ribes Burm* on serum homocysteine, lipids and oxidative enzymes in methionine-induced hyperhomocysteinemia. Indian J Pharmacol. 2008;40(4):152-7. doi: 10.4103/0253-7613.43161, PMID 20040948.
5. Kallakunta VR, Bandari S, Jukanti Raju, Veerareddy PR. Oral self-emulsifying powder of lercanidipine hydrochloride: formulation and evaluation. Powder Technol. 2012;221:375-82. doi: 10.1016/j.powtec.2012.01.032.
6. Pouton CW. Lipid formulations for oral administration of drugs: non-emulsifying, self-emulsifying and 'self-micro emulsifying drug delivery systems. Eur J Pharm Sci. 2000;11Suppl 2:S93-8. doi: 10.1016/s0928-0987(00)00167-6, PMID 11033431.
7. Elnaggar YSR, El-Massik MA, Abdallah OY. Self-nano emulsifying drug delivery systems of tamoxifen citrate: design and optimization. Int J Pharm. 2009;380(1-2):133-41. doi: 10.1016/j.ijpharm.2009.07.015, PMID 19635537.
8. Dixit RP, Nagarsenker MS. Self-nanoemulsifying granules of ezetimibe: design, optimization and evaluation. Eur J Pharm Sci. 2008;35(3):183-92. doi: 10.1016/j.ejps.2008.06.013, PMID 18652892.
9. Yang SC, Gursoy RN, Lambert G, Benita S. Enhanced oral absorption of paclitaxel in a novel self-microemulsifying drug delivery system with or without concomitant use of P-glycoprotein inhibitors. Pharm Res. 2004;21(2):261-70. doi: 10.1023/b:pham.0000016238.44452.f1, PMID 15032307.
10. Wright CW, O'Neill MJ, Phillipson JD, Warhurst DC. Use of microdilution to assess *in vitro* antiamoebic activities of Brucea javanica fruits, Simarouba amara stem, and a number of quassinoids. Antimicrob Agents Chemother. 1988;32(11):1725-9. doi: 10.1128/AAC.32.11.1725. PMID 2908094.
11. Hayat F, Salahuddin A, Zargan J, Azam A. Synthesis, characterization, antiamoebic activity and cytotoxicity of novel 2-(quinolin-8-yloxy) acetohydrazones and their cyclized products (1,2,3-thiadiazole and 1,2,3-selenadiazole derivatives). Eur J Med Chem. 2010;45(12):6127-34. doi: 10.1016/j.ejmech.2010.09.066, PMID 20961670.
12. Guo F, Zhong H, He J, Xie B, Liu F, Xu H, Liu M, Xu C. Self-micro emulsifying drug delivery system for improved oral bioavailability of dipyridamole: preparation and evaluation. Arch Pharm Res. 2011;34(7):1113-23. doi: 10.1007/s12272-011-0709-8, PMID 21811918.

13. Dineshchandra SK, Dixit PC. Sem and D. Joshi, An experiment study of kutajarishta with special reference to amoebiasis. Anc Sci Life. 1988;VIII:100-2.
14. Guerrant RL, Brush J, Ravdin JI, Sullivan JA, Mandell GL. Interaction between Entamoeba histolytica and human polymorphonuclear neutrophils. J Infect Dis. 1981;143(1):83-93. doi: 10.1093/infdis/143.1.83, PMID 6260869.
15. Gershanik T, Benita S. Self-dispersing lipid formulations for improving oral absorption of lipophilic drugs. Eur J Pharm Biopharm. 2000;50(1):179-88. doi: 10.1016/s0939-6411(00)00089-8, PMID 10840200.
16. Patil P, Patil V, Paradkar A. Formulation of a self-emulsifying system for oral delivery of simvastatin: *in vitro* and *in vivo* evaluation. Acta Pharm. 2007;57(1):111-22. doi: 10.2478/v10007-007-0009-5. PMID 19839411.
17. Bowte WJ. Materials, process and manufacturing considerations for lipid based hard capsules formats. Oral lipid-based formulations-enhancing the bioavailability of poorly water soluble drugs. Hauss DJ. 2007;170:13-39.
18. Pittman FE, Hennigar GR. Sigmoidoscopic and colonic mucosal biopsy findings in amebic colitis. Arch Pathol. 1974;97(3):155-8. PMID 4130018.