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PAPER

Self-nanoemulsifying drug delivery system (SNEDDS) for oral delivery of arteether: pharmacokinetics, toxicity and antimalarial activity in mice

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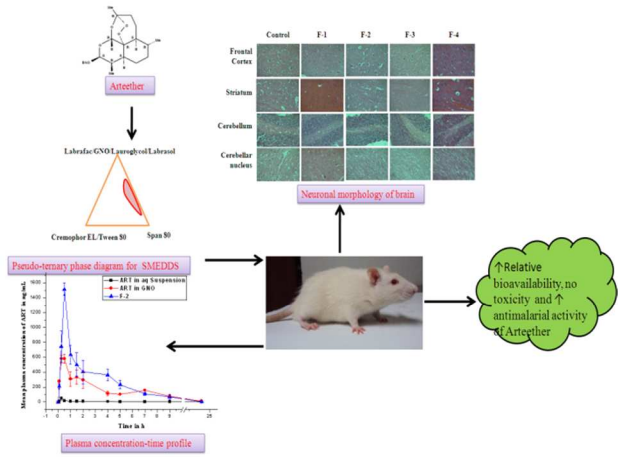
Abstract: The aim of the study was to develop oral arteether (AE) nano formulations and to investigate its effects in rats; for complete and effective treatment of *Plasmodium yoelii nigeriensis* infected mice at reduced dose by increasing relative bioavailability. Nano-formulations of arteether have been developed. The relative bioavailability (RB%) was assessed by calculating individual AUC0–t, AUC0-∞ and Cmax values. Haematological, biochemical parameters were estimated in rats and sections of brain and peripheral organs were analyzed for histopathological changes. The formulations were tested for antimalarial efficacy and safety in Plasmodium yoelii nigeriensis infected swiss mice. The AUC in case of lipid formulations (AUC0-t 4.98±0.79 h. µg/ml) and AUC0-∞ (5.02±0.80 h. µg/ml) were significantly higher ($p < 0.05$) than AE in ground nut oil (GNO) and AE aqueous suspension. The Cmax was also significantly higher for all the formulations. The RB% has been found to be significantly high (257%) in formulations with respect to AE in GNO. No considerable changes have been monitored in the serum biochemical parameters in rats. These formulations have been found to be highly effective against Plasmodium yoelii nigeriensis infected swiss mice even at the lower dose of 12.5 mg/kg x5 days. Overall the developed formulations are safe and provide a non-toxic platform for further clinical studies and can be used in artemisinin-based combination therapies (ACTs).

Graphical abstract:



Representing nontoxic effect of arteether SNEDDS, having improved bioavailability and toxicity

1. Introduction

Malaria is a major health problem in tropical and subtropical countries and almost 106 countries has a major effect of it and is associated with morbidity and mortality even in the twenty first century. According to WHO world malaria report 2013; more than six hundred thousand people die every year due to malaria which includes the maximum number of children, that means almost 1300 lives are lost per day due to malaria, although the figure of mortality has been reduced from the last few years but still it's a huge number[1]. Malaria is caused by *Plasmodium* species in which most life threatening is *Plasmodium falciparum.* Malaria prevalence is on high risk due to the rise in the development of resistant parasites, a poor rate of discovery in antiparasitic segments and high cost of antimalarial drugs[2]. There is a requirement of new antimalarial drugs or to fully exploit the use of existing drugs. Mass-drug-administration has been recently proposed as an option in the malaria chemotherapy as it has rolled back the burden of horrific parasitic diseases (e.g. river blindness, lymphatic filariasis, trachoma)[3]. In 2012, the WHO recommended seasonal malaria chemoprevention with a combination of sulfadoxine-pyrimethamine and amodiaquine (SP+AQ) for children aged between 3 and 59 months in areas of high seasonal malaria transmission across the Sahel sub-region. Artemisinin is a sesquiterpene lactone which is isolated from the plant Artemesia annua L. The derivatives of this artemisinin are now widely used to cure complex malaria. Artemisinin derivatives including dihydroartemisinin, artesunate, artemether and AE are the keystones of the treatment for *Plasmodium falciparum* malaria due to their high potency and rapid action. AE is an ethyl ether derivative of dihydroartemisinin, which is dihydro derivative of artemisinine and is one of the most promising candidates for the cure of malaria. We have chosen AE as our candidate drug as it is developed by our institute (Central Drug Research Institute, India), and is a potential alternate for quinine because of drug resistance and safety issues seen with quinine. It may also be more effective than other artemisinin derivatives because of its oil solubility, longer half life, and increased chemical stability[4]. AE contain stable endoperoxide bridge, this bridge is proposed to be responsible for its antimalarial activity. AE also possess gametocytocidal properties by inhibiting parasite transmission[5-7]. It is particularly effective against *Plasmodium falciparum* malaria parasites that are resistant to conventional antimalarial drugs[7-9]. Artemisinin partial resistance has been documented in *Plasmodium falciparum* malaria in the region of Southeast Asia i.e in Cambodia, Thailand, Myanmar, and Vietnam [10-13]. To overcome this resistance, the treatments of artemisinin compounds are usually

associated with other conventional drugs[14]. Artemisinin-based combination therapies (ACTs) are recommended by WHO as the first-line treatment for uncomplicated *Plasmodium falciparum* malaria as it is believed and has been proven that drugs with different mode of action when combined together can reduce the risk of resistant parasite, but still their widespread use for treating patients with *Plasmodium falciparum* malaria raises the question of emerging drug resistance. In combination, the role of the artemisinin compound is to reduce the main parasite load during the first three days of treatment, while the role of the partner drug is to eliminate the remaining parasites[15]. Self nanoemulsifying drug delivery system (SNEDDS) are isotropic mixtures of lipid/oil, surfactant and drug substance that rapidly form a fine oil-in-water micro emulsions, providing large surface area for increased absorption, when exposed to aqueous media under conditions of gentle agitation or digestive motility that would be encountered in the gastrointestinal tract[16, 17]. SNEDDS are considered as a captivating approach because of high drug solubilizing capacity and enhancement in both rate and extent of absorption by the lymphatic uptake[18-21]. Moreover, it is possible to form blends that are composed of several excipients: like pure triglyceride (TG) oils or blends of different TG, diglyceride (DG) and monoglyceride (MG) oils, or blends of different TG, DG and MG. In addition different types of surfactants (lipophilic and hydrophilic) can be added as co-solvents[22]. The oral administration of lipophilic drugs presents a main challenge because of the low aqueous solubility. Orally administrated SNEDDS widens accessibility of lipidic excipients with particular characteristics to offer flexibility of function with respect to improving bioavailability of poorly water-soluble drugs by manipulating their release profiles and protecting them from enzymatic and/or chemical hydrolysis while facilitating their passage in the gastrointestinal tract until their intestinal absorption[23]. It can be simply manufactured using hot or cold mixing, at low cost, which is of special interest to developing countries. Moreover these liquid SNEDDS can be converted into solid SNEDDS, there are several reports in which the liquid SNEDDS have been successfully formulated to solid SNEDDS by using Aerosil 200[24], Mannitol and sucrose monopalmitate [25], calcium silicate, magnesium aluminum silicate and silicon dioxide[26] by spray drying method and can be formulated into tablets, capsules or pellets which are easy to dispense.

AE is associated with several shortcomings, poor aqueous solubility, and low oral bioavailability[4]. To overcome the problems associated with artemisinins; there were several formulations proposed such as conventional and long circulating liposomes, alone and in combinations with curcumin which were found to be effective against malaria infected mice[27, 28]. Solid lipid nanoparticles of AE have also been formulated which results in improved oral bioavailability[29]. SNEDDS[30], can also be one of the approach to develop an oral formulation of AE which can overcome its limiting steps. The combination therapy with sub-therapeutic dose of β-arteether and curcumin has been formulated as lipid based drug delivery system as the promising approach for the treatment of malaria[31].

In the present investigation, we have developed SNEDDS of AE which has been found to increase the bioavailability and were found effective against malaria infected mice at low dose of AE and with improved bio-availability. In the future aspects, these SNEDDS can be converted into solid SNEDDS and can also be combined with other conventional antimalarial drugs for effective combination therapy in malaria treatment.

2. Results

2.1. Solubility study and its compatibility

The SNEDDS consisted of oil, surfactants and AE should be a clear and monophasic liquid at ambient temperature when introduced to aqueous phase and should have good solvent properties to allow presentation of the AE in solution. All the vehicles showed good solubility of AE (table I). Among the tested vehicles in this study, Labrafac, Lauroglycol and GNO were selected because of maximum solubility of AE and GNO was also reported for its enhanced absorption of AE. Moreover, these vehicles have great miscibility with surfactant mixture and form the spontaneous emulsion when come in contact with aqueous phase with smaller-average diameter of globules and formed clear solution. Labrasol was excluded for the preparation of SNEDDS as it was found to be poorly miscible with other surfactants. Thus, labrafac, lauroglycol and GNO was selected as an oily vehicle due to AE good solubility and good emulsion-forming ability for preparing an optimal SNEDDS formulation resulting in the improvement of AE loading and in the formation of spontaneous fine emulsion.

The vehicles and the excipients have been evaluated for their compatibility with AE, which were found to be acceptable (Table II).

2.2. Construction of pseudo ternary phase diagrams.

A series of SNEDDS were prepared and their self-emulsifying properties were observed visually when it comes into contact with aqueous phase were observed visually. It has been reported that the drug incorporated in the SNEDDS might have some effect on the self-emulsifying performance. Thus, pseudo-ternary phase diagrams were constructed in the presence of AE to identify the self-emulsifying regions with maximum drug loading and to optimize the concentration of oil and surfactant in the SNEDDS. Labrafac and Lauroglycol showed significant high amount of AE incorporation due to high solubility of AE in it. The phase diagram of the system containing Labrafac/Lauroglycol/GNO as an oil and Cremophor EL and Span 80 as a surfactant with AE has been shown in Fig. I. It was observed that within the self-emulsifying region there was increased spontaneity of the self-emulsification process. The efficiency of emulsification was good when the surfactant concentration was more than 60% v/v of SNEDDS formulation. It was observed that the spontaneous emulsion formation was not efficient with less than 30% v/v of surfactant in SNEDDS.

2.3. Development of SNEDDS

We aimed to design AE loaded SNEDDS that could self-emulsify spontaneously when in contact with physiological medium. AE has been reported to have poor absorption when given as aqueous solution with a base line in mind that when AE is given with the fat rich diet, it has an improved absorption. Hence, the lipid-based SNEDDS were designed based on the literature to enhance the solubility and thus bioavailability of AE and to obtain self-emulsifying properties with the selected excipients. SNEDDS were prepared using the phase diagram method and four preparations of SNEDDS were finalized, which gave a clear emulsion on dilution with aqueous phase and were subjected for further studies.

2.4. Characterization of the SNEDDS

2.4.1. Effect of dilution of SNEDDS

F-1, F-2, F-3 and F-4 have been characterized by diluting them with the aqueous phase in a volumetric flask containing TDW and agitated to form a fine emulsion. The visual parameters revealed the formation of spontaneous clear emulsion and the globule size in a range of 55-160nm. All the SNEDDS were diluted with TDW to an appropriate concentration before determining the zeta potential. Each sample was analyzed thrice (Table IV).

The F-1, F-2, F-3 and F-4 were evaluated for its self emulsification in simulated gastrointestinal fluid. The size of the globules after dilution and AE solubility were assessed after keeping them in room temperature for 2 h in simulated gastric fluid and up to 8 h in simulated intestinal fluid. The optimized SNEDDS which were chosen for further studies had a size of approximately 80 nm and a polydispersity index (PDI) less than 0.2, indicating homogeneous distribution of size (Table V).

In contrast, SNEDDS with an upper PDI of 0.5 and the globule size larger than 450 nm was rejected. The globule size of the emulsions is an important parameter for self-emulsifying systems. Indeed, it influences the speed and the quantity of released and absorbed compounds.

2.5. Cytotoxicity study

The cytotoxicity of all the SNEDDS without AE was found in acceptable limit when tested against Caco-2 cell lines by MTT assay. The cell viability remained >90% for all the SNEDDS against Caco-2 cell lines indicating safety of excipients used. At equivalent higher concentration of AE in F-1, F-2, F-3 and F-4 (10µg/ml) less than 45% of cell viability was observed where as at lower equivalent concentration of AE in F-1, F-2, F-3 and F-4 (5µg/ml) the cell viability was more than 60%. The data has been represented in Fig. II.

2.6. *In Vivo* Studies

2.6.1. Repeated dose oral toxicity study

SNEDDS were evaluated for their toxicological effects. The dose was selected as twice the effective dose predicted in murine malaria models. Mortalities in rats have not been observed in any of the treatment or in control groups throughout the experiment. The animals feed were normal throughout the experiment with normal behavior. The increase in body weight of animals in all treatment groups was comparable to that of the control group. No adverse effects in terms of general health of animals have been observed upon oral administration of SNEDDS. The various hematological parameters of treatment groups did not vary significantly from control group (Fig. III). These results also affirmed well with the serum biochemistry profiles of animals. The serum biochemical parameters (Fig. IV) and serum hepatic markers (Fig. V) of the treatment groups did not vary significantly from that of the control group. The serum ALT levels of treatment groups did not vary significantly from that of control group, in animals of either sex. It was in line with earlier reports that AE is not associated with hematological and renal adverse effects[32]. The AE has been previously reported to be neurotoxic when given in oily solution in comparison to AE given in aqueous form for a long period of time, so the SNEDDS were evaluated for neurotoxicity by histological examination of various neuronal region of the brain in control and SNEDDS, where no significant difference was observed from normal histology (Fig VI). Histological examinations of liver and other organs such as kidney, spleen and stomach showed no evidence of hepatotoxicity and were indistinguishable from controls (Fig VII).

2.6.2. Pharmacokinetic studies

The data was subjected to non-compartmental analysis. The plasma concentration-time profile of F-1, F-2, F-3 and F-4, AE in GNO and AE aqueous suspension has been shown in figure VIII. As shown in table VI, the C_{max} and $AUC_{0-\infty}$ for all the SNEDDS is significantly higher than AE aqueous suspension. However, compared to the AE in GNO, a significant difference for these parameters was found for F-1 and F-3 only. The area under curve (AUC) in case of F-1(AUC_{0-t} 4.98±0.79 h. µg/ml) and $AUC_{0-\infty}$ (5.02±0.80 h. µg/ml) was significantly higher ($p < 0.05$) than AE in GNO (AUC_{0-t} 2.43±.6 h. µg/ml) and $AUC_{0-\infty}$ (2.47±0.64 h. µg/ml) and AE aqueous suspension (AUC_{0-t} 0.046±0.008 h. µg/ml) and $AUC_{0-\infty}$ (0.046±0.007 h. µg/ml), whereas AUC of F-2, F-3 and F-4 were AUC_{0-t} 3.55±0.75 h. µg/ml and $AUC_{0-\infty}$ 3.57±0.74 h. µg/ml, AUC_{0-t} 6.33±1.21 h. µg/ml and $AUC_{0-\infty}$ 6.36±1.21 h. µg/ml, and AUC_{0-t} 3.30±0.51 h. µg/ml and $AUC_{0-\infty}$ 3.32±0.53 h. µg/ml respectively. $t_{1/2}$ of F-1 was found to be 0.39±0.14 h whereas AE in GNO has $t_{1/2}$ of 2.48±0.57 h and AE aqueous suspension has $t_{1/2}$ of 0.78±0.23 h. The C_{max} of F-1 (1.35±0.73µg/ml) was also significantly higher ($p < 0.05$) than AE in GNO (0.58±0.055 µg/ml) and AE aqueous suspension (0.048±0.003 µg/ml) whereas T_{max} of F-1, F-2, F-3, F-4 and AE in GNO was 1.5±0.7 h, 1.51±0.08 h, 2.08±0.38 h, 2.53±0.17 h and 0.37±0.17 h respectively. The RB% of F-1, F-2, F-3 and F-4 has been found to be 203.29, 144.53, 257.49 and 133.74% high respectively with respect to AE in GNO and when compared to AE in aqueous suspension F-1, F-2, F-3 and F-4 were 10913.04, 7760.87, 13826 and 7217% respectively.

2.6.3. Efficacy of SNEDDS against *Plasmodium yoelii nigeriensis* mice

The efficacy of F-1, F-2, F-3 and F-4 were tested against *Plasmodium yoelii nigeriensis*. infected mice and were compared with the equivalent oral dose of AE given in the GNO as an oily solution and with the control group which was kept without the treatment. The SNEDDS used in this study were well tolerated by the experimental mice and there was no abnormality seen in the behavior, food/water consumption and general activity of the animals throughout the treatment and post treatment period[33]. The anti-malarial profile of these formulations is given in Table VII.

F-1, F-2, F-3 and F-4 when evaluated for their antimalarial efficacy, they demonstrated almost similar results. The percent cure rate at dose 40mg/kg x5 days and 25mg/kg x5 days was found to be 100%. Mean survival time of this group was >28 days showing no parasitaemia up to 28 days. At the dose of 12.5mg/kg x5 days the cure rate for F-1 was observed to be 100% with mean survival time of >28 days showing no parasitaemia, where as for F-2 no mortality has been observed till day 28 but the parasitaemia was present from day 18 of the treatment with 40% cure rate, where as for F-3 and F-4 the % cure rate was 80 %. This indicates that 12.5mg/kg x 5days is curative dose for the F-1, whereas for F-2, F-3 and F-4 the curative dose has been found to be 25mg/kg x 5days. The activity of these SNEDDS was confirmed by the repeated experiments. The AE in GNO was also given in which the curative dose was found to be 40mg/kg x5 days with the mean survival time of >28 days but at the dose of 25mg/kg x 5 days the cure rate was observed as 80% with mean survival time 19.6±7.66 days showing parasitaemia on day 21 of the experiment and at the lower dose that is at the dose of 12.5mg/kg x5 days the cure rate was observed to be only 30% with mean survival time 17.3±7.65 days showing parasitaemia on day 7 of the experiment. Both blank SNEDDS and oil without AE showed the parasitaemia on the fourth day of the experiment and all the mice died within the day 7 of the treatment. The control group which was untreated has the parasitaemia more than 50% on the fourth day of the experiment with all the mice died within the day 7 of the treatment. This might be due to the formation of lipophilic protective layer over the AE molecules and its slow release from SNEDDS resulting in enhanced activity and reduced curative dose. The survival graph has been represented in figure IX.

These results imply that oral formulation of AE can be developed using SNEDDS for potential application in malaria. This important lead would be very useful for the development of solid oral dosage form of AE, if liquid SNEDDS are converted to solid SNEDDS[24, 25]. It has been reported previously that when oral AE was given in GNO, there was an increased neurotoxicity and

mortality as compared to aqueous suspension[34], but in the present study SNEDDS of AE has not been found to be associated with neurotoxicity. These SNEDDS can also be combined with other conventional antimalarial drugs (such as fansidar, lumefantrine). These combinations can be expected to possess positive impact on the effective treatment of complicated malaria[31].

3. Discussion

SNEDDS, lipid based formulations which offer the potential for enhancing the absorption of water insoluble drugs was prepared for oral delivery of AE. SNEDDS was our choice of formulation as it has been reported that bioavailability of lipophilic drugs can be improved in the presence of fatty acids. SNEDDS self-emulsify themselves when they come in contact with aqueous phase. These SNEDDS also provide the stability to the drug due to the absence of the aqueous phase related degradation and also protects the drug from enzymatic and chemical hydrolysis in the gastrointestinal tract until their intestinal absorption which increases bioavailability of lipophilic drugs. SNEDDS of some of the active pharmaceutical ingredients are also available commercially which include cyclosporine, ritonavir saquinavir and amprenavir [35]. The liquid SNEDDS can easily be formulated into solid SNEDDS, these solid SNEDDS are easy to dispense and can be converted to tablet, capsules and even to pallets. We have developed SNEDDS with the blend of drug, oil and surfactants. These surfactants were known to increase the permeability by disturbing the cell membrane and thus by enhancing the absorption of poorly soluble drugs[36, 37]. Further, AE is highly lipophilic compound and has good solubility in oils, these factors, inspired us to develop SNEDDS which can solve the problem associated with the AE of oral bioavailability and contribute towards absorption via lymphatic route[31].

The SNEDDS formulations were found to be robust when tested for the effect of dilution. The developed optimized formulations spontaneously formed self-emulsion having very small globule size (55-160nm), which is a significant parameter as it influences the absorption of AE. This may be attributed to cremophor and lauroglycol which increased the solubilization capacity of AE and high kinetic stability of SNEDDS on dilution.

The cytotoxicity data reveals that the excipients used are safe and can be used further for the preparation of SNEDDS based formulations which can be used in humans. The cell viability remained >90% for all the SNEDDS against Caco-2 cell lines indicating safety of excipients used. The Caco-2 cell was used for the study since they originate from enterocytes and their viability data can share several biological and biochemical properties of both the resident and activated peritoneal macrophages.

Repeated dose oral toxicity study reveals no toxicity in terms of mortality, serum biochemical parameters and serum hepatic markers of the treatment groups. There was neither sign of toxicity nor significant change in water and food consumption and body weights of mice in all groups during the 14 days observation period or they are comparable to control. Changes in serum hepatic markers of treatment groups were insignificant compared to control group. These results are in conformity with our cytotoxicity data which indicates that the excipients used are well digested by the animals.

AE has been reported to be associated with the neurotoxicity, although due to the significant number of cases reported of *Plasmodium falciparum* malaria and artemisinins the best answer to them; not much importance has been given to their high dose neurotoxicity[38], so the histological examination of various neuronal brain regions after the administration of SNEDDS have been carried out where no sign of toxicity was found and the results were parallel to the control. Liver is the major site of detoxification in the body for all drugs/toxins. Therefore it is an important organ in any toxicological study. Histological examinations of liver showed no evidence of hepatotoxicity and were indistinguishable from controls. Kidneys are the main organs in the body susceptible to the toxic effects of drugs. Histological sections of the kidney derived from rats treated showed normal appearance of the renal capsules and tubules. Macroscopic and histological evaluation of other target organs such as spleen and stomach tissues showed no evidence of inflammation, cell lysis, or lesions; the natural architecture of the organs remained unaffected. Thus, repeated dose toxicity study illustrated the safety of developed SNEDDS on oral administration in the context of malaria infection.

We have used sensitive and selective LC-MS technique to study pharmacokinetic profile of AE. The liquid–liquid extraction method gave high and consistent recoveries for AE and I.S. and provided clean extracts. This analytical method was applied to estimate the levels of AE in rat plasma following an oral dose of 25mg/kg in SNEDDS AUC reflects the extent of drug absorption and C_{max} and T_{max} are important features of the plasma level profile, these parameters are characteristics of the drug formulation and all important for comparative bioavailability (bioequivalence) studies. The significantly high AUC was achieved in comparison to the both AE in GNO and AE in aqueous suspension. The superior performance of SNEDDS may be accredited to the formation of the fine emulsion droplets and subsequent lipolysis and formation of mixed micelles providing larger surface area for the absorption of AE. Cremophor EL and Tween 80, which inhibits P-glycoprotein activity (which serves to protect the body from xenotoxins) resulted in enhanced intestinal permeability of AE. Oleic acid present in Tween 80 also increases chylomicron secretion which consecutively improves the lymphatic transport of AE. However, poor oral bioavailability of AE in GNO and AE in aqueous suspension might include instability in the gastrointestinal fluids and limited aqueous solubility and dissolution of AE. The results of pharmacokinetics clearly indicate the significant enhancement in the bioavailability of AE in F-1, F-2, F-3 and F-4. The pharmacokinetic data was also supported by the antimalarial activity of the SNEDDS. . High solubility of drug in the long chain oil present in SNEDDS and due to increased mucosal permeability caused by the presence of surfactants, is likely to improve lymphatic absorption of the drug and thus enhancing the bioavailability of AE. Rapid clearance of parasitaemia in mice was observed which might be due to fast-acting schizontocide activity of AE. F-1, F-2, F-3 and F-4 completely cured *Plasmodium yoelii* infected mice by the oral route at low dose compared to AE in GNO. The dose of 12.5mg/kg for 5 days was a curative dose in our F-1 formulation without symptom of parasitaemia and mortality even after the completion of our experiment which might be attributed to the fact that AE was not degraded and remains stable for prolonged period providing higher concentration of AE for activity. The increased permeability of intestinal membrane and increased absorption from the site might also be the reason. The curative dose of ART in GNO was 40 mg/kg for 5 days. Whereas the mean survival time of the untreated mice or mice treated with GNO only was less than 6 days. GNO has been taken as a control in our study as it is rich in mono- and polyunsaturated fatty acids, such as linolenic acid, linoleic acid and oleic acid, lack antimalarial activity. The results clearly demonstrate that F-1, F-2, F-3 and F-4 were highly active against *P. yoelii* infected mice.

These formulations if combined with other conventional drugs, as in ACTs, can possible reduce mortality among *Plasmodium*

falciparum cases particularly in children, pregnant women who are at maximum risk and can overcome the problems associated with malaria infections.

4. Materials and methods

4.1. Materials

Arteether (AE) was kindly supplied by Themis Medicare, Mumbai, India. Labrafac, Labrasol and Lauroglycol were supplied by Gattefosse, Saint Priest cedex, France as a free gift. Tween 80, Span 80, and cremophor EL was purchased from Sigma Aldrich (USA, St. Louis), Ground nut oil (GNO) was purchased from the local market as Premio refined oil. Acetonitrile used was of spectroscopic grade and purchased from Merck (India). All other reagents and chemicals were of analytical grade. All materials were used without further purification. The water used in all experiments was prepared in a three-stage Millipore Milli-Q plus 185 purification system (Bedford, MA, US).

4.2. Pre-formulation Studies

4.2.1. Solubility studies

Excess amount of AE was placed in 1 ml of vehicle (GNO, Labrafac PG, Labrasol, Lauroglycol 90, Soyabean oil, Seasome oil). Then, the mixture was vortexed and kept for 48h at ambient temperature in a shaking water bath to facilitate the solubilization. The samples were centrifuged at 5000g for 20 min to remove the un-dissolved AE. The supernatant was taken and diluted with methanol for quantification of AE by HPLC. The HPLC system was equipped with 10 ATVP binary gradient pumps (Shimadzu), a Rheodyne (Cotati, CA, USA) model 7125 injector with a 20 µl loop and SPD-M10 AVP U V detector (Shimadzu). HPLC was carried out on a C18 column (250mm, 4mm, and 5µm) (Merck). The injection volume was 20µl and the column effluent was monitored at 215 nm. Data was acquired and processed using Class VP software. The mobile phase consisted of a mixture of Acetonitrile: water (70: 30 v/v) [39]. Chromatography was performed at a flow rate of 1.0 ml/minute. Solubility of AE in various excipients has been described in table I.

4.2.2. Drug-Excipients Compatibility Studies

In this dosage form the AE remains in close contact with one or more excipients; thus, the latter could affect the stability of the AE. The AE was kept with the excipients in the closed container for the particular period of time and then the properties of AE were monitored at different time points up to three months and the amount of AE was estimated using HPLC method described above. Knowledge of AE-excipients interactions is useful in selecting appropriate excipients. The compatibility of excipients with AE was tested before preparing the SNEDDS (Table II).

4.3. Construction of ternary phase diagram

The ternary phase diagrams of systems containing oil and blend of surfactants was made to identify the existence of self-emulsifying oil formulation fields and to optimize the concentration of oil that could self-emulsify under dilution and gentle agitation in presence of aqueous medium. Since the free energy required to form an emulsion is very low, the formation is thermodynamically spontaneous. The oily mixture of AE and surfactant was prepared and varied in different percentage of the total preparation, each of them, representing an apex of the triangle. A series of self-emulsifying systems were prepared in the formula with varying concentrations of vehicle and surfactants including AE. For any mixture, the total of surfactant and oil concentrations always added to 100%. Compositions were evaluated for nanoemulsions formation by diluting 1ml of each of the 64 mixtures to 100 ml with triple distilled water. Dispersions, having globule size 200 nm or below were considered desirable. The area of nanoemulsions formation was identified for the respective system in which nanoemulsions with desired globule size were obtained. A formulation (0.2 ml) was introduced into 300 ml of triple distilled water (TDW) in a glass beaker at 37°C and the contents were mixed gently on a vortex. The tendency to emulsify spontaneously by forming a fine milky emulsion and also the progress of emulsion droplets were observed by visual examination. All studies were repeated thrice, with similar observations being made between repeats. The series of SNEDDS were prepared and their self-emulsifying properties were observed visually.

4.4. Development of SNEDDS

A series of SNEDDS were prepared by dissolving AE in the oil followed by mixture of surfactant at ambient temperature. The AE-SNEDDS, which were used for further study, were F-1, F-2, F-3 and F-4, the composition of these formulations has been depicted in Table III. The final mixture was vortexed vigorously for 20min to achieve complete mixing until a clear solution was obtained. The SNEDDS were examined for any signs of turbidity or phase separation prior to self-emulsification and globule size studies. These SNEDDS were equilibrated to ambient temperature for 24 h and then stored at room temperature.

4.5. Characterization of the SNEDDS

4.5.1. Self-emulsification of SNEDDS in simulated gastro-intestinal fluids

F-1, F-2, F-3 and F-4 were evaluated for their self-emulsification as they will come in contact with the different physiological fluid after the oral administration. The size and solubility of SNEDDS were measured in TDW, in simulated gastric fluid (prepared by dissolving 2.0 g of sodium chloride and 3.2 g of purified pepsin, in 7.0 ml of hydrochloric acid and water up to 1000 ml) and in simulated intestinal fluid (prepared by dissolving 6.8 g of monobasic potassium phosphate in 250 ml of water and then adding 77 ml of 0.2 N sodium hydroxide and 500 ml of water, 10.0 g of pancreatin was added and the resulting solution was adjusted with 0.2 N sodium hydroxide or 0.2 N hydrochloric acid to a pH of 6.8 ± 0.1 and finally diluted to 1000 ml) [40]. The size of the lipid droplets was determined at 25°C by photon correlation spectroscopy using Zetasizer Nano ZS (Malvern Instrument Ltd., UK).

4.5.2. Globule size and zeta potential

The globule size of F-1, F-2, F-3 and F-4 was been determined by Zetasizer Nano ZS model (Malvern Instruments) and the zeta potential was determined by laser Doppler anemometry using a Malvern Zetasizer. All the SNEDDS has been diluted with TDW to an appropriate concentration before determining the zeta potential. The measurements were carried out in the fully automatic mode. Each sample was analyzed thrice.

4.5.3. *In vitro* studies Cytotoxicity studies

Cytotoxicity studies were carried out using MTT assay on Caco-2 cell line to assess the safety of excipients used in the preparation of SNEDDS. Caco-2 cell lines were grown to a density of 2-2.5 million cells in 25 cm^2 flat bottom tissue culture flasks[41]. F-1, F-2, F-3 and F-4 with and without AE were examined after dilution in DMEM for its *in-vitro* cytotoxicity according to the methods reported earlier[42]. The optical density of the treated cells were measured using a multiwell scanning spectrophotometer (MRX Microplate Reader, Dynatech Laboratories Inc., Chantilly, VA, US) at a wavelength of 570 nm.

4.6. In Vivo studies

4.6.1. Repeated dose oral toxicity study

4.6.1.1. Animal handling

Animal studies were executed to analyze the toxicological effect of SNEDDS on the repeated dose oral toxicity, in terms of serum, liver enzymes levels and biomarkers of hepatotoxicity. The toxicity in brain and peripheral organs were also evaluated.

Wistar rats of about 150-200 g were obtained from National laboratory animal center CDRI. The animals were maintained at the controlled temperature of 23±1ºC, humidity of 55±5%, in a 14 h light/10 h dark cycle. Throughout the study, the animals were provided with soy-free and filtered drinking water.

The toxicity of SNEDDS after oral administration of multiple doses has been evaluated. Neurotoxicity in animal models (mice, rats, dogs and rhesus monkeys) after the multiple im doses of AE has been reported previously[43, 44]. Some studies on neurotoxicity of AE in rats after multiple (7 days) injections of AE in sesame oil (AESO) resulted in 7.5 fold high level of AE accumulation in the blood due to very slow and prolonged absorption of AE from the injection site, which were associated with neurotoxicity in brain, besides causing anorexia and gastrointestinal toxicity[45]. Formulation of AE in GNO has also been reported for having no neurotoxicity in animals by IM/oral/rectal routes [19] as well as in clinical trials in which 3 doses through IM injection were administered[46-48]. Although due to widespread in the malaria and developing resistant strains of *Plasmodium*, allowance has been given to the neurotoxicity effect of artemisinins[38].

4.6.1.2. Study protocol and drug treatment

SNEDDS were evaluated at dose equivalent to 50 mg/kg for toxicity studies; this dose has been selected as it is twice the curative dose for malaria in AE in GNO. The SNEDDS were diluted suitably with water. The Wistar rats of either sex, were assigned to five test groups consisting four animals each for histopathological hematological and serum biomarkers; Group I (F-I), Group II (F-II), Group III (F-III), Group IV (F-IV), Group V (control). Animals in each test group were administered SNEDDS at the dose of 50mg/kg body weight for 14 days, once daily, by oral gavages.

4.6.1.3. Hematological Parameters

Blood was withdrawn from retro orbital plexus of rats in tubes containing anticoagulant EDTA; the tubes were tabbed to mix the blood with anticoagulant properly to prevent the blood-coagulation. Blood haematological analysis was done by using automated hematoanalyser (SYSMEX XT-2000). Different haematological parameters viz. RBC, WBC, Hgb, MCV, MCHC, MCH, and differential population of leukocytes was estimated.

4.6.1.4. Biochemical Parameters

Blood was withdrawn from retro orbital plexus of rats of different experimental groups and allowed to stand undisturbed for 30 min. Serum was separated by centrifugation and levels of urea, alanine transaminase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), total bilirubin (T-BIL) and creatinine (CRT) etc. were estimated using a fully automated biochemical analyzer (Merck-selectra junior).

4.6.1.5. Histological Analysis

To evaluate the morphological alterations Haematoxylin and Eosin (HE) staining was performed in sections of brain and peripheral organs. Animals were perfused intracardially with ice-cold 0.1M phosphate-buffered saline (PBS) followed by cold para-formaldehyde (4% wt/vol) in 0.1 M PBS. Selected brain sections were cut based, two blocks were taken from each brain; one encompassing the midbrain, the other encompassing the caudal pons and rostral medulla (as well as the cerebellum). The blocks were embedded in paraffin and sectioned. Animals decapitated other peripheral organs like stomach, liver, spleen and kidney were also removed and processed for paraffin embedded sectioning. 4-5 µm thick lateral sections were cut on a Microtome (Leica, USA) and were collected on poly L-lysine coated slides and processed for Hematoxylin and eosin staining. Images were captured on upright microscope at 40x magnification.

4.7. Pharmacokinetic Studies by LC-MS:

The pharmacokinetic study to evaluate the oral absorption of F-1, F-2, F-3 and F-4 has been carried out using LC-MS technique[49, 50].

HPLC system consisting of Series 200 pumps and auto sampler with temperature controlled Peltier-tray (Perkin-Elmer instruments, Norwalk, USA) was used to inject 10µL aliquots of the processed samples on a X-bridge C18 column (4.6 mm × 50 mm, 5.0 µm). The system was run in isocratic mode with mobile phase consisting of methanol and 0.01 M ammonium acetate (pH 5.0) in the ratio of 95:5 (v/v). Mobile phase was duly filtered through 0.22 µm millipore filter (Billerica, USA) and degassed ultrasonically for 15 min and delivered at a flow rate of 0.8 ml/min for chromatographic separation.

Mass spectrometric detection was performed on a QTRAP 4000 mass spectrometer (Applied Biosystems, MDS Sciex Toronto, and Canada) equipped with an API electrospray ionization (ESI) source. The ion spray voltage was set at 5500 V. The instrument parameters viz., nebulizer gas, curtain gas, auxillary gas and collision gas were set at 50, 35, 45 and 12, respectively. Compounds parameters viz., de-clustering potential (DP), collision energy (CE), entrance potential (EP) and collision exit potential (CXP) were 35, 13, 10, 15 V and 35, 16, 10, 10 V for AE and internal standard, artemisinin, respectively. Zero air was used as source gas while nitrogen was used as both curtain and collision gas. The mass spectrometer was operated at ESI positive ion mode and detection of the ions was performed in the multiple reaction monitoring (MRM) mode, monitoring the transition of m/z 330.0 precursor ion $[M+H]^+$ to the m/z 267.0 product ion for AE and m/z 300.4 precursor ion $[M+H]^+$ to the m/z 209.4product ion for IS. Quadrupoles Q1 and Q3 were set on unit resolution. Data acquisition and quantitation were performed using analyst software version 1.6 (Applied Biosystems, MDS SciexToronto, Canada).

4.7.1. *In vivo* Pharmacokinetic study:

Young, adult male Sprague-Dawley (SD) rats, weighing 200±20 g, were procured from the National Laboratory Animal Center, CSIR-CDRI (Lucknow, India). Rats were housed in well ventilated cages at room temperature (24±2°C) and 40-60 % relative humidity while on a regular 12 h light-dark cycle. The animals were acclimatized for a minimum period of 3 days prior to the experiment. Approval from the Local Animal Ethics Committee was sought and the study protocols were approved before the commencement of the studies. To evaluate the enhancement in the bioavailability of developed formulations (F-1, F-2, F-3 and F-4), it was compared with the AE aqueous suspension (AE suspended in water) and AE in GNO (known amount of AE dissolved in GNO by warming at 50°C). SNEDDS, AE aqueous suspension and AE in GNO were administered orally at an equivalent dose of 25 mg/kg. Blood samples were collected from the retro orbital plexus of rats under light ether anesthesia into microfuge tubes containing heparin as an anti-coagulant at 0.08, 0.25, 0.5, 1.0, 1.5, 2.0, 4.0, 5.0, 7.0, 9.0 and 24.0 h post-dosing. Plasma samples were harvested by centrifuging the blood at 13000 rpm for 10 min and stored frozen at −70 ± 10◦C until analysis. Plasma (100 µl) samples were spiked with internal standard (IS), and processed as describe.

4.7.2. Sample preparation:

A simple liquid–liquid extraction method was followed for extraction of AE and IS from rat plasma. To 100 µl of plasma aliquot, IS solution (10 µl of 40ng/ml working stock) equivalent to 4.0 ng was added and mixed for 15s on a cyclomixer (Spinix Tarsons, Kolkata, India), followed by extraction with 2.0 ml of hexane: ethyl acetate, 1:1 (v/v), mixture. The mixture was vortexed for 3 min, followed by centrifugation for 5 min at 2000 × g on Sigma 3-16K (Frankfurt, Germany). An aliquot of 1.6 ml

of organic layer was separated and evaporated to dryness under vacuum in speed vac concentrator (Savant Instrument, Farmingdale, USA). The residue was reconstituted in 200 µl of the mobile phase and 10 µl was injected onto analytical column. The recovery of AE and IS, through liquid–liquid extraction procedure, have been determined by comparing the responses of the analytes extracted from replicate quality control (QC) samples (n = 6) with the response of analytes from post-extracted plasma standard sample at equivalent concentrations. Recoveries of AE were determined at lower limit of quantitation, QC low and QC high concentrations viz., 5, 15 and 800 ng/ml, whereas the recovery of IS was determined at a single concentration of 40.0 ng/ml [51].

4.7.3. Pharmacokinetic analysis:

The observed maximum plasma concentration (C_{max}) and the time to reach the maximum plasma concentration (T_{max}) were obtained by visual inspection of the experimental data. The data was subjected to non-compartmental pharmacokinetics analysis using WinNonlin (version 5.1, Pharsight Corporation, Mountain View, USA). Area under curve (AUC) from 0 to 24hr (AUC_{0-24}) was calculated using linear trapezoidal rule. AUC from 0 to infinity ($AUC_{0-\infty}$) was calculated as the sum of AUC_{0-t} and C_{last}/k_{el}, where, C_{last} represents the last quantifiable concentration and k_{el} represents the elimination rate constant.

4.7.4. Relative bioavailability (RB %):

The RB % of AE in F-1, F-2, F-3 and F-4 formulations has been calculated in order to determine the percentage enhancement in bioavailability.

The RB % was calculated as follows:

$$\text{RB \%} = \frac{\text{AUC of formulation}}{\text{AUC of control}} \times 100$$

4.8. *In vivo* antimalarial efficacy of SNEDDS

Swiss mice (20±2g) of either sex, infected with *Plasmodium yoelii nigeriensis* were used in the study. *Plasmodium yoelii nigeriensis* has been reported to be resistant to chloroquine 128mg/kg x 4, mefloquine 128 mg/kg x 4 and quinine (300mg/kg x 4)[52-54]. All the experiments were conducted with the approval of Institutional Animal Ethics Committee. Mice were kept under a controlled climate conditions (23±2ºC; RH=60%) and photoperiod (12h light-dark cycles) in the animal house. Animals were fed on a standard mouse diet and provided with clean drinking water *ad libitum*. Mice were inoculated with $1x10^6$ inoculum of *Plasmodium yoelii nigeriensis* infected RBC by i/p route and after 4-5 hrs of giving the infection, the treatment was started from the same day. All the SNEDDS of AE were administered through oral route only and standard AE in GNO was also given at all the doses.

4.8.1. Administration of the drug

F-1, F-2, F-3 and F-4 were administered orally to the *Plasmodium yoelii nigeriensis* infected mice. Initially 40 mg/kg dose for 5 days was used for the treatment and the formulations, which cured the mice at a particular dose, were further tested with a lower dose of 25 mg/kg and 12.5 mg/kg.

4.8.2. Antimalarial activity assessment

Blood schizontocidal activity of F-1, F-2, F-3 and F-4 was assessed according to method reported earlier with some modifications [55]. F-1, F-2, F-3 and F-4 were administered once daily by oral route, starting from the day of infection (day 0) and continuing once daily administration for a total of 5 days. Thin blood smears were prepared from tail vein of each animal on day 4, 7, 10, 14, 21, 24 and day 28 in order to check the parasitaemia. Smears were fixed with methanol and stained with Giemsa's stain. These smears were examined for % parasitaemia and their ±S.D. was also calculated. If animals remained negative without parasitaemia till day 28, formulation was considered to be 100% curative at that particular dose.

5. Statistical analysis

All results have been expressed as means ±SD (n=3-4). Differences were compared using one-way analysis of variance (ANOVA) followed by the Turkey-Kramer multiple comparison test, using Graph Pad Instat software (Graph Pad Software Inc. CA, USA). $p<0.05$ denotes significance in all cases.

6. Conclusions

In conclusion, the present investigation illustrated the potential use of SNEDDS for improving the pharmacokinetics of AE by the oral route. The results indicate the significant improvement in the relative bioavailability in rats without causing any toxicity. SNEDDS of AE can be the promising delivery system and if combined with other recommended conventional drugs of malaria, it can possibly employ for the artemisinin based combination therapy in resistant malaria.

6. Ethical Statement:

Animal studies were conducted in accordance with current legislation of institute on animal experiments, and protocol was approved by the 'Institutional Animal Ethical Committee' of CSIR-Central Drug Research Institute, India. They were housed in plastic cages in climatically controlled rooms and fed with standard rodent food pellet (Lipton India Ltd, Bombay) and water *ad libitum*. The study was carried out with the guidelines of Council for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

7. Acknowledgments

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8. Conflict of Interest statement

The authors declare they have no competing financial interest.

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Figure captions:

Figure I: Pseudo-ternary phase diagram: The doted section represents isotropic regions for formulations with various concentrations forming spontaneous emulsions when exposed to aqueous medium

Figure II: % Cell Viability of SNEDDS at different concentration.

Figure III: Hematological parameters in Wistar rats.

Figure IV: Serum biochemistry parameters in Wistar rats.

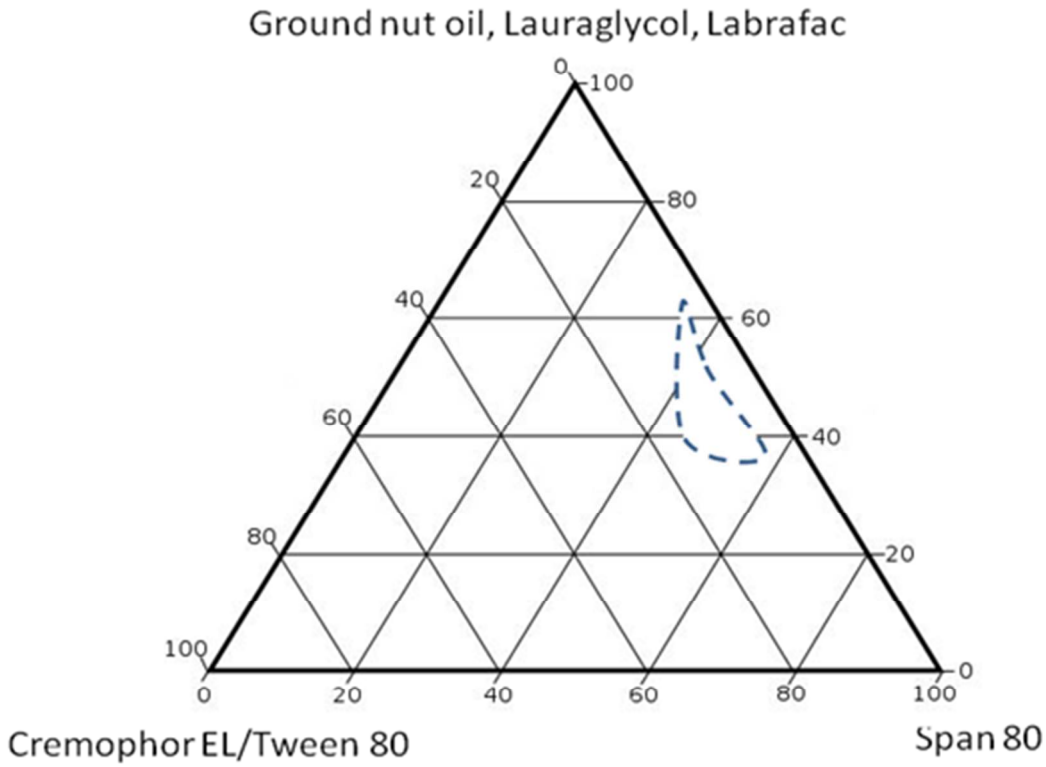
Figure V: Serum hepatic markers in Wistar rats.

Figure VI: Representing histological photomicrographs of various neuronal morphology in different brain regions in control and SNEDDS.

Figure VII: Representing histological photomicrographs of various organs in control and SNEDDS. The various organ sections were stained with haematoxylin and eosin.

Figure VIII (A, B, C and D): Plasma concentration-time profile of SNEDDS (F-1, F-2, F-3 and F-4) and AE in GNO and AE aqueous suspension upon oral administration. Data represented as mean ± S.D. (n=4)

Figure IX: Survival of infected mice treated with AE-SNEDDS at 25 and 12.5 mg/kg×5days (A) F-1 (B) F-2 (C) F-3 and (D) F-4



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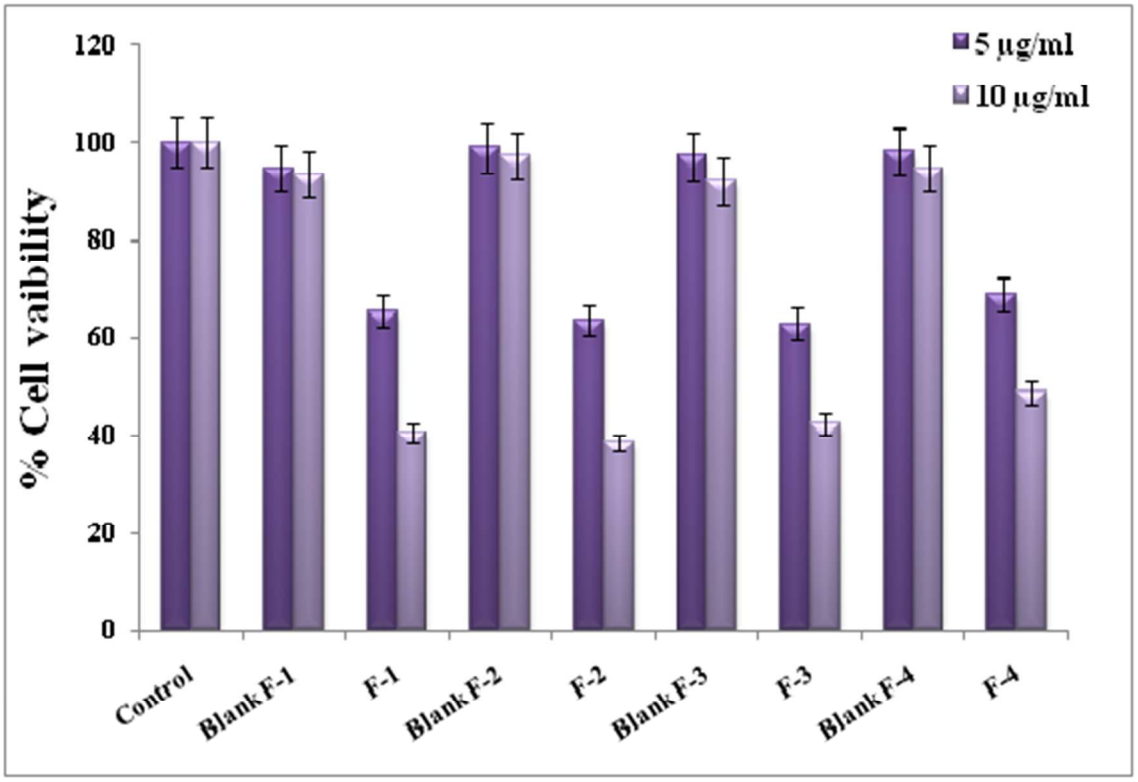

Figure II: % Cell Viability of SNEDDS at different concentration.

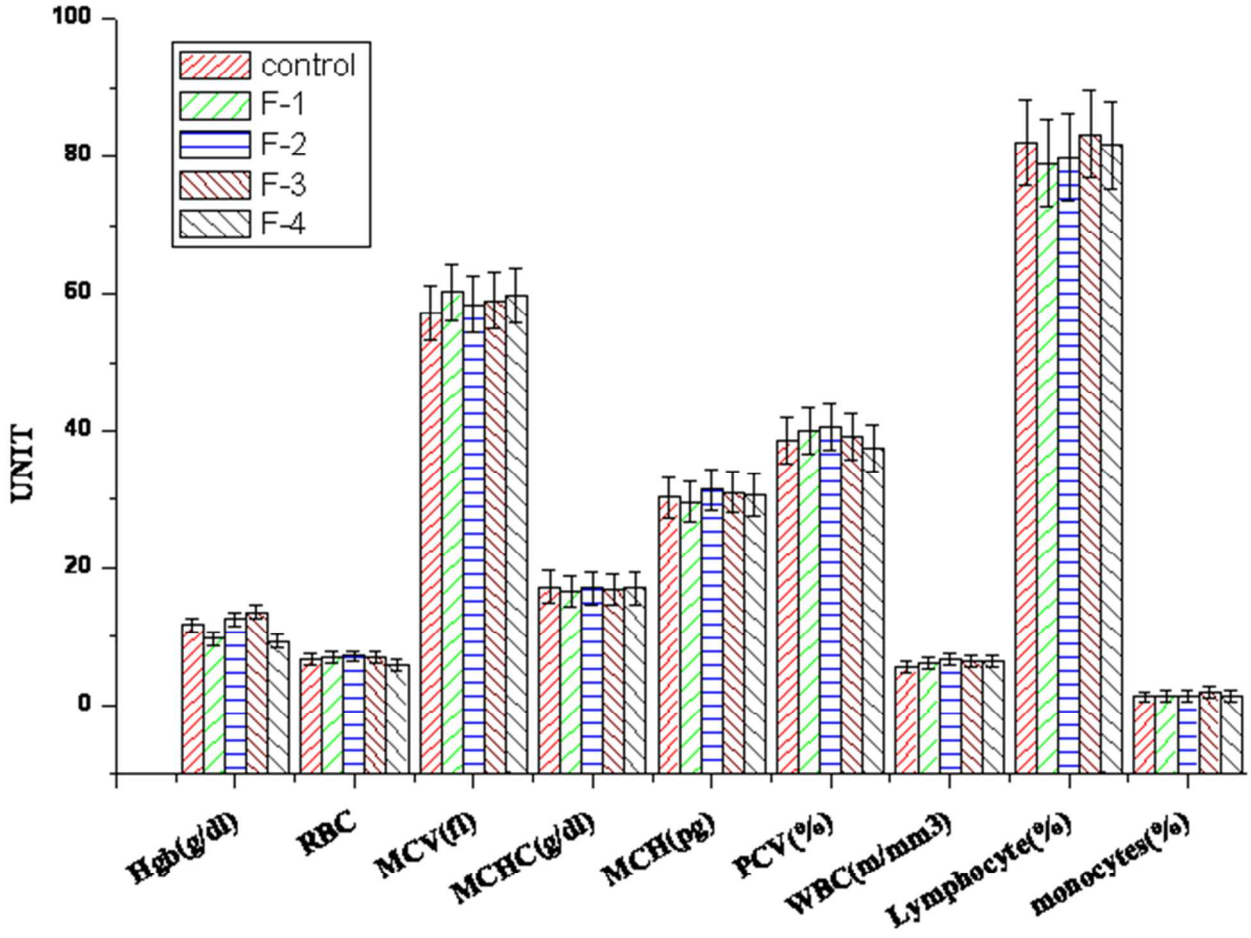

Figure III: Hematological parameters in Wistar rats.

Hgb: haemoglobin; RBC: Red blood cells; MCV: Mean corpuscular volume; MCHC: Mean corpuscular haemoglobin concentration; MCH: Mean corpuscular hemoglobin; PCV: Packed cell volume; WBC: White blood cells

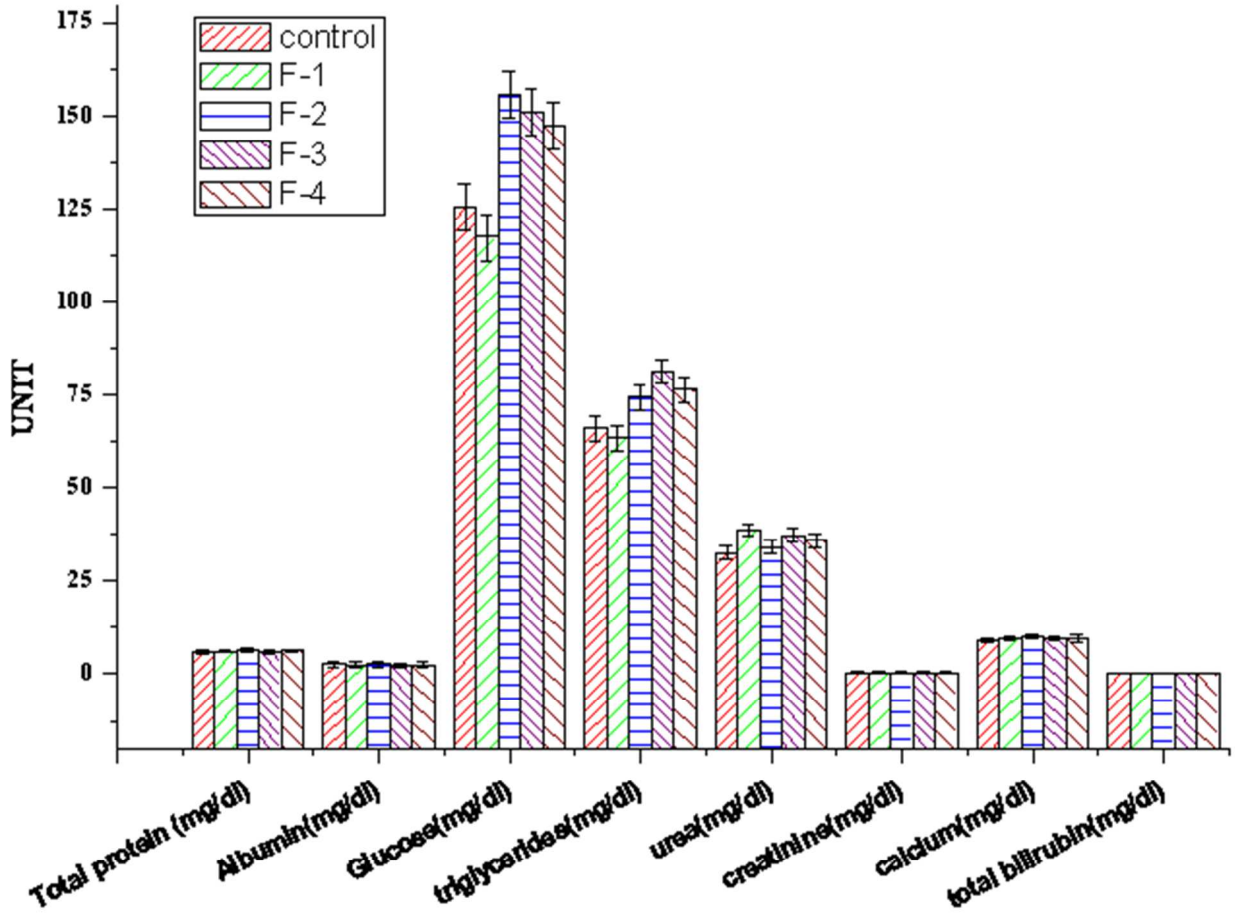

Figure IV: Serum biochemistry parameters in Wistar rats.

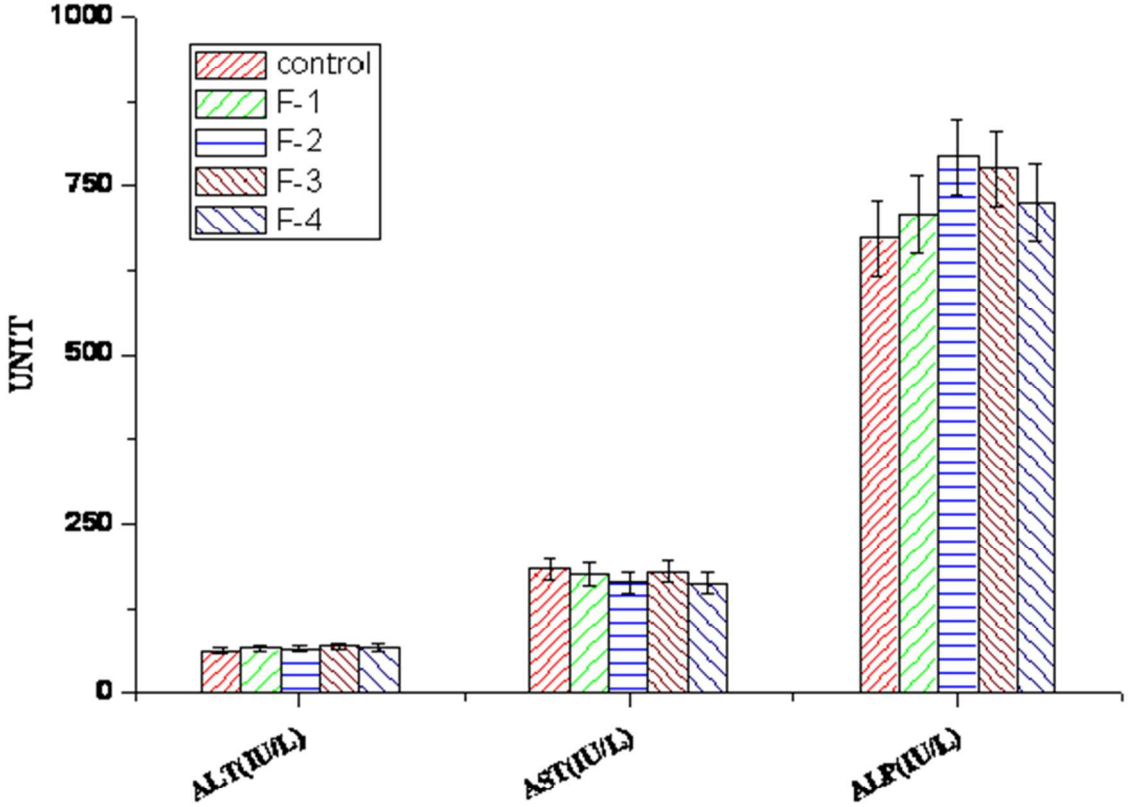

Figure V: Serum hepatic markers in Wistar rats.

ALT: Alanine amino transferase; AST: Aspartate amino transferase; ALP: Alkaline phosphatase

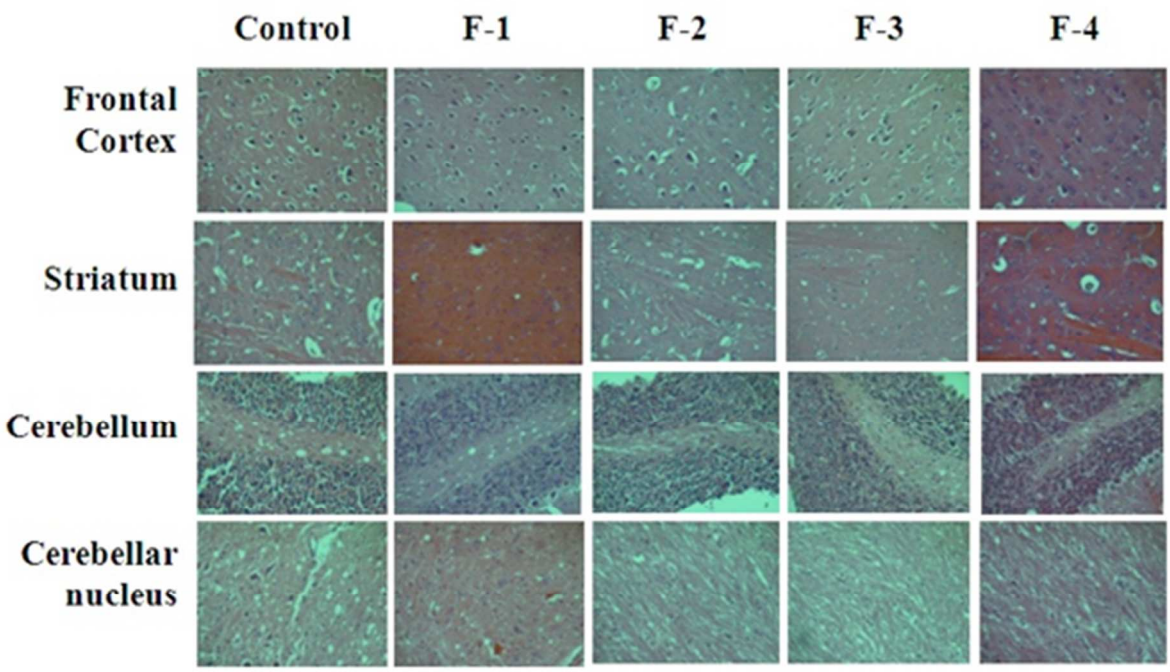

Figure VI: Representing histological photomicrographs of various neuronal morphology in different brain regions in control and SNEDDS.

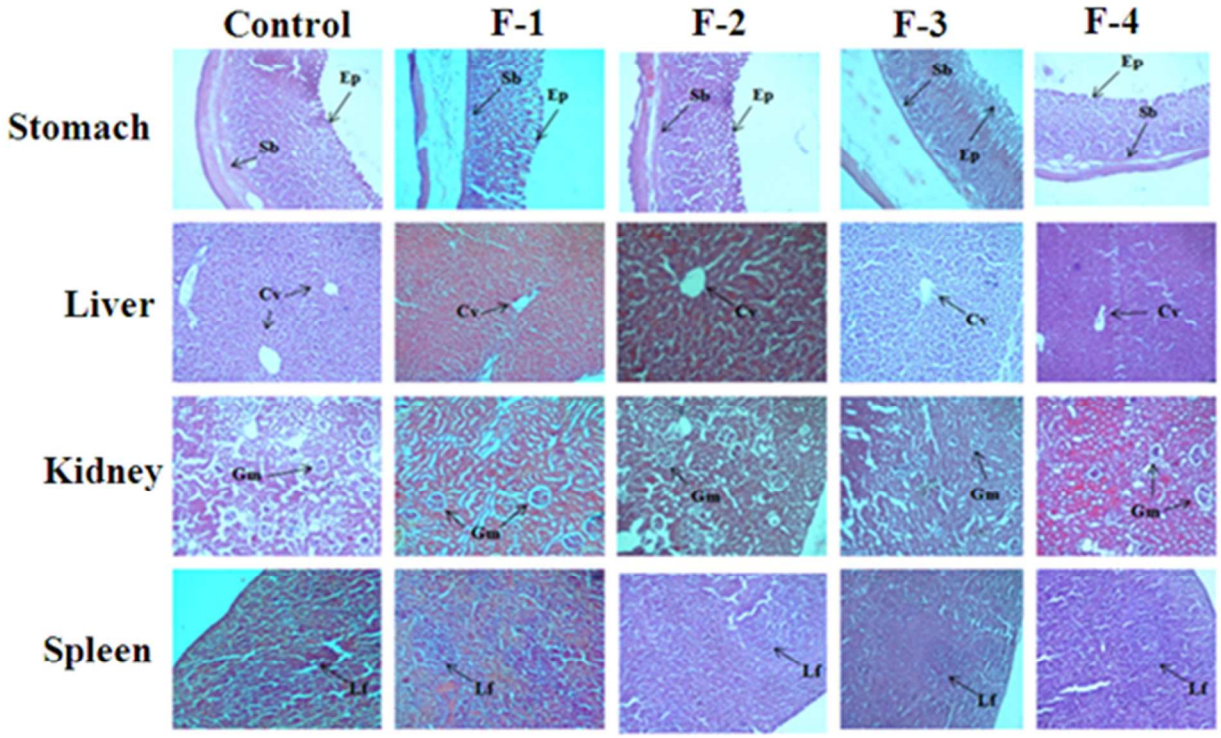

Figure VII: Representing histological photomicrographs of various peripheral organs in control and SNEDDS. The various organ sections were stained with haematoxylin and eosin. (Sb: submucosa, Ep: epithelium, Cv: Central Vein, Lf: Lymphoid follicle, Gm: Glomerulus.)

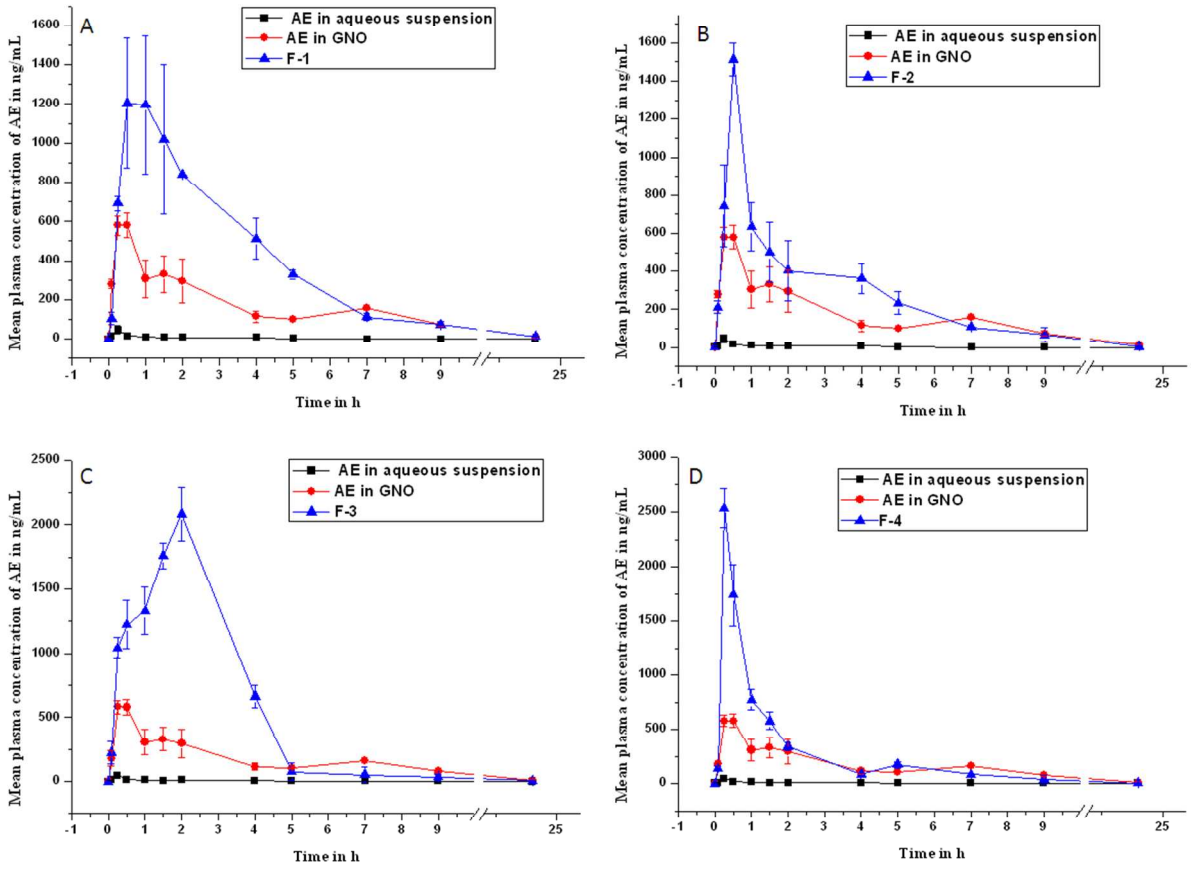

Figure VIII (A, B, C and D): Plasma concentration-time profile of SNEDDS (F-1, F-2, F-3 and F-4) and AE in GNO and AE aqueous suspension upon oral administration. Data represented as mean ± S.D. (n=4)

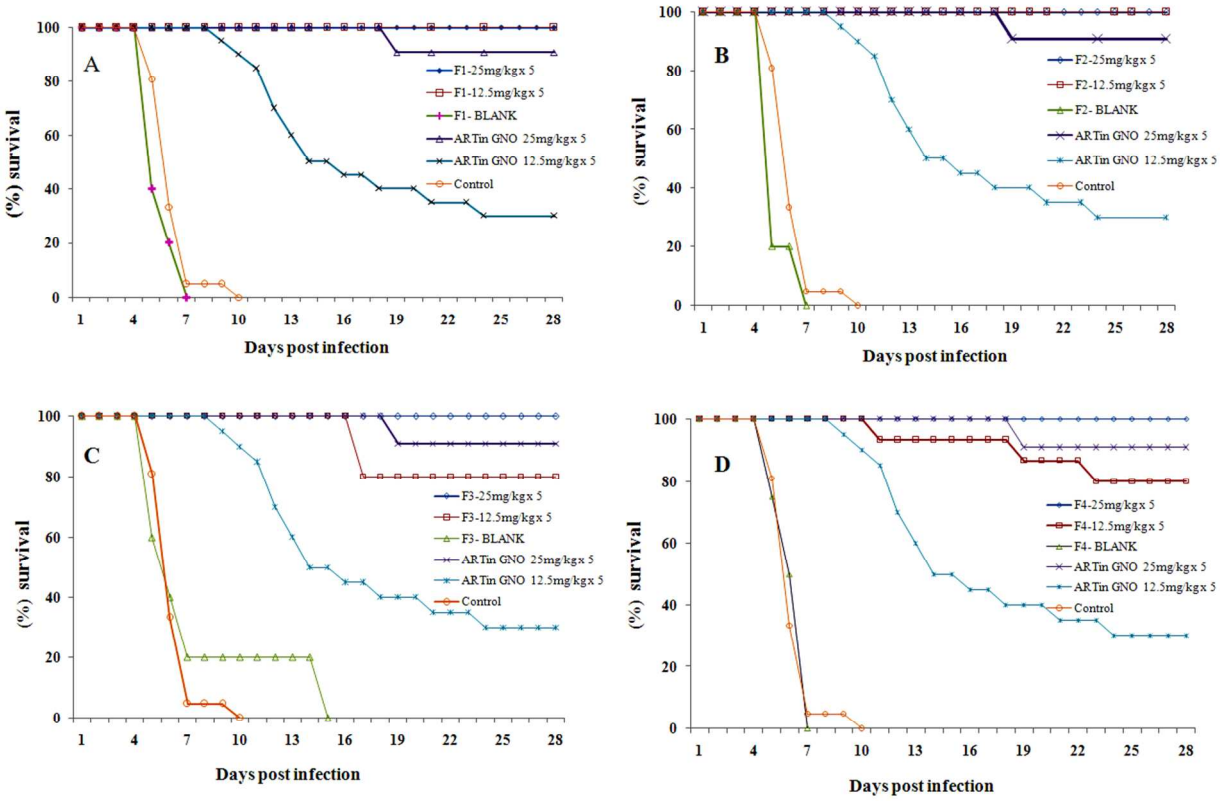

Figure IX: Survival of infected mice treated with AE-SNEDDS at 25 and 12.5 mg/kg×5days (A) F-1 (B) F-2 (C) F-3 and (D) F-4

Table I: Solubility of AE in various excipients

Vehicle	**Composition**	**Solubility of AE (mg/ml) mean**
Lauroglycol 90	Propylene glycol laurate	438±43.8
Labrafac PG	Propylene glycol Dicaprylate/ Dicaprate	412±34
Labrasol	Caprylocaproyl polyoxylate glycerides	225±28
GNO	Triglycerides of long chain fatty acids	100±12.6
Soyabean oil	Triglycerides of long chain fatty acids	80±17
Seasome oil	Poly unsaturated fatty acids	75±22

Table II: Drug-Excipients Compatibility Study

Drug + Excipients	**Parameters**	**Condition**	**Comments**
		RT40°C±2°C/75% ±5%RH	
AE + Lauroglycol 90	Proper miscibility	No change in AE	Compatible
AE + Labrafac PG	Proper miscibility	No change in AE	Compatible
AE + GNO	Proper miscibility	No change in AE	Compatible
AE + Cremephore EL	Proper miscibility	No change in AE	Compatible
AE + Tween 80	Proper miscibility	No change in AE	Compatible
AE + Span 80	Proper miscibility	No change in AE	Compatible

Table III:-Description of composition of various SNEDDS

Composition	F-1	F-2	F-3	F-4
AE (mg)	250	250	250	250
Ground nut oil (mg)	-	-	300	-
Labrafac (mg)		-	-	300
Lauroglycol (mg)	300	300	-	-
Cremophor EL (mg)	500	-	-	500
Tween 80 (mg)	-	500	500	-
Span 80 (mg)	200	200	200	200

Table IV: Physicochemical characterization of SNEDDS

Formulation	Zeta potential (mv)	Average globule size (nm)	Poly Dispersity
F-I	-12.0±1.6	125±15.4	0.234±0.05
F-II	-25.3±1.5	178±12.6	0.189±0.06
F-III	-28.2±2.8	224±16.7	0.197±0.08
F-IV	-25.2±2.6	268±09.6	0.161±0.04

*Values are expressed **mean ±SD**; (n=3)*

Table V: Self emulsification of SNEDDS in simulated gastro-intestinal fluids

Formulation	Gastric pH 1.2 buffer		Intestinal pH 7.5 buffer		Triple distilled water	
	Globule size (nm)	PDI	Globule Size(nm)	PDI	Globule size (nm)	PDI
F-1	124.12±9.6	0.11	117.23±7.2	0.16	118.25±6.1	0.18
F-2	126.22±8.4	0.09	126.73±8.1	0.29	139.42±4.4	0.26
F-3	128.19±7.9	0.18	112.28±7.9	0.14	115.44±9.3	0.17
F-4	129.13±8.5	0.13	118.41±4.6	0.11	116.25±5.2	0.15

Table VI: Pharmacokinetic parameters of AE in GNO, AE aqueous suspension and SNEDDS upon oral administration. Data represented as mean ± S.D. (n=4)

Parameter	AE aqueous suspension, 25 mg/kg	AE in GNO, 25mg/kg	F-1, 25mg/kg	F-2, 25mg/Kg	F-3, 25mg/Kg	F-4, 25mg/Kg
$t_{1/2}$ (h)	0.78±0.23	2.48±0.57	0.39±0.14*#	2.17±0.92*	2.13±1.56*	3.11±0.43*
AUC_{0-t} (h.µg/mL)	0.046±0.008	2.43±.65	4.98±0.79*#	3.55±0.75*	6.33±1.21*#	3.30±0.51*
$AUC_{0-\infty}$ (h. µg/mL)	0.046±0.007	2.47±0.64	5.02±0.80*#	3.57±0.74*	6.36±1.21*#	3.32±0.53*
C_{max} (µg/mL)	0.048±0.003	0.58±0.055	1.35±0.73*	1.51±0.08*#	2.08±0.38*#	2.53±0.17*#
T_{max} (h)	0.25±0.00	0.37±0.17	1.5±0.7*	0.5±0.0*	2.0±0.0	0.25±0.0

**Significantly different compared to AE aqueous suspension (P<0.05)*

 # Significantly different compared to AE in GNO (P<0.05)

Table VII: Antimalarial efficacy of oral formulations of AE against MDR P. y. nig in swiss mice.

Formulations	Dose (mg/kg) x5 days	% Mean Parasitaemia ± S.D.								Mean Survival Time (MST)	%Cure Rate
		Day 4	Day 7	Day 10	Day 14	Day 18	Day 21	Day 24	Day 28		
F-1	40	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	>28	100
	25	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	>28	100
	12.5	0.0 (5)	0.0 (5)	0.0 (5)	0.0 (5)	0.0 (5)	0.0 (5)	0.0 (5)	0.0 (5)	>28	100
	Blank	64.2±11 (5)	dead	-	-	-	-	-	-	5.2±1.16	0
F-2	40	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	>28	100
	25	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	>28	100
	12.5	0.0 (5)	0.0 (5)	0.0 (5)	0.0 (5)	0.008±.016 (5)	2.7±3.48 (5)	8.4±10.36 (5)	10.46±20.77 (5)	>28	40
	Blank	56±16.9 (5)	dead	-	-	-	-	-	-	5±1.1	0
F-3	40	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	>28	100
	25	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	>28	100
	12.5	0.0 (5)	0.0 (5)	0.0 (5)	0.02±0.04 (5)	0.0 (4)	0.0 (4)	0.0 (4)	0.0 (4)	25.8±4.4	80
	Blank	54±15.4 (5)	35.4±0.0 (1)	23±0.0 (1)	39.07±0.0 (1)	dead	-	-	-	7.4±3.92	0
F-4	40	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	>28	100
	25	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	0.0 (15)	>28	100
	12.5	0.0 (15)	0.0 (15)	2.16±8.10 (15)	0.05±0.20 (14)	0.0 (13)	1.95±6.76 (13)	0.0 (12)	0.0 (12)	25.86±4.80	80
	Blank	40.5±14.4 (4)	dead			-	-	-	-	6.25±0.83	0
AE in GNO	40	0.0 (5)	0.0 (5)	0.0 (5)	0.0 (5)	0.0 (5)	0.0 (5)	0.0 (5)	0.0 (5)	>28	100
	25	0.0 (11)	0.0 (11)	0.0 (11)	0.11±0.34 (11)	0.0 (10)	0.0 (10)	0.0 (10)	0.0 (10)	27.09±2.87	91
	12.5	0.0 (20)	0.0061±0.017 (20)	13.78±26.26 (18)	3.19±7.42 (10)	2.87±7.60 (8)	5.52±12.93 (8)	0.0 (6)	0.0 (6)	18.29±7.08	30
	Blank	43.8±28.8 (5)	dead	-	-	-	-	-	-	6.5±1.16	0
Control	-	40.25±19.49 (21)	41±0 (1)	dead	-	-	-	-	-	6.23±0.81	0

**Pooled data of 2-3 experiments.*
No. of surviving mice are given in parentheses.