CHAPTER – 8

Evaluating the Effect of Different Formulation Approaches on the In-Vitro and In-Vivo Performance of Ezetimibe

ABSTRACT

The aim of this part of study was to compare at the *in-vitro* and *in-vivo* levels, the developed optimized formulations discussed in the previous chapters with each other and to also compare each of the formulation with the commercial tablet product, Ezentia. In the afore discussed chapters, three different formulation approaches were applied and studied to improve the aqueous solubility and dissolution rate of Eze with an aim to improve its bioavailability. One optimized formulation from each of the applied formulation approaches, a pharmaceutical CoC, Eze-ND, a ternary CD complex, E-CD-TPGS and an NC formulation, ESTNC F8, were identified as potential formulations in improving the *in-vitro* and *in-vivo* performance of Eze following oral administration. In this part of research, the *in-vitro* aqueous solubility of all the optimized formulations, Eze-ND, E-CD-TPGS and ESTNC F8 were compared. The *in-vitro* dissolution, *in-vivo* pharmacokinetics and pharmacodynamics were studied for a comparative evaluation among the optimized formulations and against the commercial tablet product, Ezentia. Additionally, the dose reduction efficiencies offered by each of the formulations were studied and reported.

8.1 INTRODUCTION

According to the literature, between 60 and 70 % of the APIs in the pipeline show sufficient membrane permeability but low water solubility and thus belong to BCS class II [Rehder, 2013]. So, the performance of orally delivered BCS class II drugs depends solely on the aqueous solubility and dissolution rate as the drug must dissolve from their dosage form within the GIT in order to be absorbed, first by the tissue of the intestines and ultimately into circulation. Therefore, their bioavailability is solubility and dissolution rate-limited after oral administration. To increase their water solubility and dissolution rate and thus their bioavailability, different formulation approaches may be applied and studied.

Eze is a model BCS class II drug with low water solubility. Pharmacologically, Eze is a hypocholesterolemic that serves as a cholesterol absorption inhibitor unlike other marketed lipid lowering agents that act by inhibiting the synthesis of cholesterol. Eze inhibits cholesterol absorption by small intestine, but, being a P-gp substrate, the *in-vivo* absorption of Eze is lowered by P-gp efflux at the small intestinal brush border. The oral bioavailability of Eze is lowered to as low as 35% due to its low aqueous solubility and P-gp efflux [Bandyopadhyay et al., 2012]. In the present research work, three different formulation approaches were applied and studied to improve the water solubility and dissolution rate and thus the bioavailability of Eze.

In chapter 5, Eze-NA and Eze-ND CoCs were successfully optimized and prepared in the drug:coformer ratio, 1:2 and 1:1, respectively. Both, Eze-NA and Eze-ND CoCs showed marked improvement in solubility and dissolution properties of Eze. Considering the antihypercholesterolemic action of NA, it was conceived that the preparation of NA CoC of Eze would not only enhance the solubility and dissolution

properties of Eze but may also provide an agonistic effect in the treatment of hypercholesterolemia. However, the results of *in-vitro* characterization suggested ND as a more beneficial coformer. Though the difference in antihypercholesterolemic ability of both the CoCs was statistically insignificant, considering the statistically superior solubility, dissolution and pharmacokinetics of Eze-ND in comparison to Eze-NA, the former was noted as the most effective CoC formulation for the oral delivery of Eze. ND, being the higher water soluble coformer, Eze-ND CoCs exhibited superior solubility, dissolution and *in-vivo* behavior of Eze.

The optimization and preparation of ternary CD complexes of Eze was discussed in chapter 6. Both the ternary systems, E-CD-TPGS and E-CD- AA2G served as suitable formulations for Eze not only by improving its aqueous solubility and dissolution but also by providing additive hypolipidemic effect and enhanced bioavailability. However, with the ability of HPBCD to maintain cholesterol homeostasis [Peake and Vance, 2012], and the P-gp inhibitory action of TPGS [Guo et al., 2013], E-CD-TPGS presented its advantage in improving the *in-vivo* absorption of hypocholesterolemic P-gp substrate, Eze, at its site of action, the small intestinal brush border. So, considering the cumulative *in-vitro* and *in-vivo* performances, E-CD-TPGS was noted as the best ternary CD complex formulation to improve the solubility, oral absorption and reduce the bioavailability variations of Eze.

In chapter 7, Eze NCs were successfully optimized and prepared by experimental design approach, by using AA2G (ANCs) and TPGS (TNCs) as stabilizers. Electrostatically stabilized TNCs, the ESTNCs, were identified as the best NC formulations of Eze. AA2G, being purely hydrophilic, could reduce the PS of Eze to nanorange but the saturation solubility and dissolution properties of ANCs were inferior to ESTNCs. Even

the pharmacokinetic and pharmacodynamic performances of ESTNCs were markedly superior to ANCs which may be ascribed to the P-gp efflux inhibitory nature of TPGS. The higher lipid lowering activity of ESTNCs indicated that the inhibition of P-gp efflux transport facilitated higher extent of intestinal absorption and *in-vivo* performance of drug. Oral delivery of Eze as ESTNCs was found to be quite potential in improving the *in-vitro* and *in-vivo* performance of the drug.

The aim of the present chapter was to compare the performance of the optimized formulations, Eze-ND, E-CD-TPGS and ESTNC F8 with each other and to also compare each of the formulation with the commercial product, Ezentia® (10 mg Eze), a marketed Eze tablet from Sun Pharma Pvt. Ltd. The *in-vitro* aqueous solubilities of Eze-ND, E-CD-TPGS and ESTNC F8 were compared. The *in-vitro* dissolution, *in-vivo* pharmacokinetics and pharmacodynamics were studied for the comparative evaluation among the optimized formulations and against the commercial tablet. Finally, the dose reduction efficiencies offered by each of the formulations were studied and reported.

8.2 METHODS

8.2.1 Saturation aqueous solubility studies

The saturation solubility results of Eze-ND, E-CD-TPGS and ESTNC F8 were taken from the previous chapters for a comparison.

8.2.2 Dissolution

The method of dissolution reported for Eze in the "Dissolution Methods" guide of FDA was adapted with modifications. The dissolution studies were carried out by filling pure Eze or each of the formulations equivalent to 10 mg of Eze into hard gelatin capsules.

Ezentia® (10 mg Eze), a marketed Eze tablet from Sun Pharma Pvt. Ltd. was also tested for comparative analysis. Dissolution was studied in 500 mL of three different media - 0.01 N HCl (pH 2) with 0.45% w/v SLS, USP acetate buffer of pH 4.5, containing 0.45% w/v SLS and distilled water (measured pH 6.8) with 0.45% w/v SLS [http://www.accessdata.fda.gov/scripts/cder/dissolution/dsp_SearchResults_Dissolutions.cfm?PrintAll=1; http://www.dissolution.com/ddg/showthread.php?145-USP-change-to-0-01N-HCl; http://www.dissolution.com/ddg/showthread.php?1329-Dissolution-of-ezetimibe-tablets; http://www.drug-dissolution-testing.com/?p=233]. Dissolution was conducted using USP apparatus I (Electrolab, India) at 37±0.5 ˚C and 100 rpm rotation rate. Samples of 5 mL were withdrawn at appropriate time intervals and the dissolution medium volume was made up to 500 mL by replacing the withdrawn samples with fresh prewarmed (37±0.5 ˚C) medium. The collected samples were filtered, appropriately diluted and Eze content was quantified by UV at 232 nm. The cumulative percent drug dissolved at each time point was calculated for all the formulations and the data obtained by six replicate determinations was averaged and recorded. Dissolution was studied for 45 min and the dissolution efficiency (DE) representing the area under the dissolution curve up to 45 min was calculated for each of the systems. $t_{80\%}$ was also noted.

8.2.3 Stability

The optimized formulations, Eze-ND, E-CD-TPGS and ESTNC F8, were subjected to stability studies at 30±2 ˚C/70±5% RH for six months. The basic stability testing details have already been discussed in the formulation specific chapters, 5, 6 and 7. Additionally, after 6 months of storage in stability chamber (Narang Industries, New

Delhi, India) at 30±2 ˚C/70±5% RH, one batch of dry powder formulations, each of Eze-ND, E-CD-TPGS and ESTNC F8, were subjected to FTIR, DSC and XRD. The graphs obtained before and after 6 months were compared.

8.2.4 *In-vivo* preclinical pharmacokinetic study

8.2.4.1 Animals

The pharmacokinetic testing details have already been discussed in the formulation specific chapters, except here, two additional treatment groups were studied, Ezentia tablet and Ezentia tablet suspension. The study protocol was approved and guided by the Central Animal Ethical Committee, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. Male Albino Wister rats (200 – 250 g) were used and the animals were divided into six groups of six animals each. The standard - group I, test - group II, test – group III, test – group IV, test – group V and test – group VI, received pure drug suspension, Ezentia tablet, Ezentia suspension, E-CD-TPGS, Eze-ND and ESTNC F8, respectively. The animals were housed in polypropylene cages and kept at standard laboratory conditions (25±2 ˚C and 55±5% RH). Six animals per cage were accommodated with free access to standard laboratory diet (Lipton feed, Mumbai, India) and water *ad libitum*.

8.2.4.2 Dosing and sampling

All the animals used were fasted overnight for the study and dosed orally using 18-gauge oral feeding needle. A single dose pharmacokinetic study was conducted and the animal groups, I, II, III, IV, V and VI, were respectively, dosed with pure drug, Ezentia tablet, Ezentia suspension, E-CD-TPGS, Eze-ND and ESTNC F8. Pure drug powder, crushed and sieved Ezentia tablet, E-CD-TPGS, Eze-ND and ESTNC F8 were

administered by dispersing in 0.25% w/v NaCMC. All these treatment group animals received 2 mL of 50 mg/kg body weight equivalent dose of Eze, via oral administration. For studying the effect of tablet formulation treatment on test group II, the fraction of tablet corresponding to the weight of the tablet required to give the desired dose was administered to rats by directly loading into the stomach by intragastric gavage. After anaesthetizing the rats with diethyl ether, 500 µL blood samples were collected by retro-orbital puncture at 0 (pre-dose), 0.5, 1, 1.5, 2, 2.5, 4, 12, and 24 h, into heparinized microcentrifuge tubes. Plasma was immediately separated by centrifugation at 5000 rpm for 20 min, spiked with IS and stored at -20 °C until bioanalysis.

8.2.4.3 Drug extraction – same as described under the section 5.3.2.10.3.

8.2.4.4 Plasma drug analysis – same as described under the section 5.3.2.10.4.

8.2.4.5 Pharmacokinetic parameters – same as described under the section 5.3.2.10.5.

8.2.5 *In-vivo* preclinical pharmacodynamic study

The details of antihypercholesterolemic activity**,** the primary pharmacodynamic study, have already been discussed in the formulation specific chapters. A complete pharmacodynamic profile was drawn where the total cholesterol (TC), triglycerides (TG) and high density lipoprotein cholesterol (HDL) were estimated directly and the low density lipoprotein cholesterol (LDL), very low density lipoprotein cholesterol (VLDL), the atherogenic indices (AIs) - Atherogenic Coefficient (AC), Atherogenic Index of Plasma (AIP), CHOLINDEX, Castelli's Risk Index – I (CRI 1) or Cardiac Risk Ratio (CRR) and Castelli's Risk Index – II (CRI 2) were calculated indirectly [Akpmar et al., 2013; Lafta, 2014; Ranjit et al., 2015].

The study protocol was approved and guided by the Central Animal Ethical Committee, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. Male Albino Wister rats (200 – 250 g) were used and the animals were divided into six groups of six animals each. The control - group I, standard - group II, test - group III, test – group IV, test – group V, and test – group VI received cholesterol, pure drug suspension plus cholesterol, Ezentia suspension plus cholesterol, E-CD-TPGS plus cholesterol, Eze-ND plus cholesterol and ESTNC F8 plus cholesterol, respectively. The animals were housed in polypropylene cages and kept at standard laboratory conditions (25±2 ˚C and 55±5% RH). Six animals per cage were accommodated with free access to standard laboratory diet (Lipton feed, Mumbai, India) and water *ad libitum*.

The study was carried out for a total period of 8 weeks wherein the first four weeks, all the animals were fed 200 mg cholesterol in 2 mL coconut oil as high fat diet for inducing hypercholesterolemia. At the end of fourth week, the plasma levels of TC, TG, HDL, LDL, VLDL, AC, AIP, CHOLINDEX, CRI 1 or CRR and CRI 2 were measured for all the groups and the values were considered as baseline values for the following four week study, the actual pharmacodynamic activity study. The first four weeks is a stage 1, hypercholesterolemic induction study and the next four weeks is the stage 2, pharmacodynamic activity study.

The pharmacodynamic study was carried out for 28 days and the animals were fed and dosed orally using 18-gauge oral feeding needle. To carry out the study, all the groups were induced with hypercholesterolemia by administering them with high fat diet (200 mg cholesterol in 2 mL coconut oil). Two hours following the administration of high fat diet, the treatment groups, II, III IV, V and VI were respectively fed with pure drug, Ezentia tablet powder, E-CD-TPGS, Eze-ND and ESTNC F8 dispersed in 0.25% w/v

NaCMC. The daily dose for rats was calculated by considering the rat to human being surface area ratio [Bandyopadhyay et al., 2012; Dixit and Nagarsenker, 2008]. Volume of vehicle (200 µL) and dose levels of 1 mg Eze or equivalent formulation/kg body weight/day were kept constant in each case. Blood samples were collected on day 7, 14, 21 and 28, after anaesthetizing the rats with diethyl ether, by retro-orbital puncture, into anticoagulated microcentrifuge tubes (heparin treated). The plasma was separated by centrifugation at 5000 rpm for 20 mins and stored at 2 ˚C until further use. Percent reduction in the plasma levels of TC, TG and HDL were directly analysed using *in-vitro* Cogent diagnostic kit (Span Diagnostics Ltd., Surat, India). All the remaining parameters were derived as per established equations, from the measured plasma lipid values of TC, TG and HDL. The procedures followed for direct estimation of TC, TG and HDL, were briefed below [Cogent diagnostic kit data].

8.2.5.1 Estimation of TC

The principle involves hydrolyzing the cholesterol esters by cholesterol esterase (CE) to give free cholesterol and fatty acids. In subsequent reaction, cholesterol oxidase (CHOD) oxidizes the 3-OH group of free cholesterol to liberate cholest-4-en-3-one and hydrogen peroxide. In the presence of peroxidase (POD), hydrogen peroxidase couples with 4-aminoantipyrine (4-AAP) and phenol to produce red quinoneimine dye. Absorbance of colored dye is measured at 505 nm which is proportional to amount of TC concentration in the sample.

$$\text{Cholesterol esters} \xrightarrow{\text{CE}} \text{Cholesterol} + \text{Fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{CHOD}} \text{Cholest-4-en-3-one} + H_2O_2$$

$$2\ H_2O_2 + \text{Phenol} + \text{4-AAP} \xrightarrow{\text{POD}} \text{Quinoneimine dye} + H_2O$$

Blank was 1 mL of the cholesterol reagent and standard was prepared by adding 1 mL of the cholesterol reagent into 10 µL of standard cholesterol. For preparing test sample, 1 mL of the cholesterol reagent was added into 10 µL test plasma, all the solutions were mixed properly and incubated at room temperature for 30 min and absorbance were taken at 505 nm against blank. The concentration of TC in plasma was obtained by the following formula:

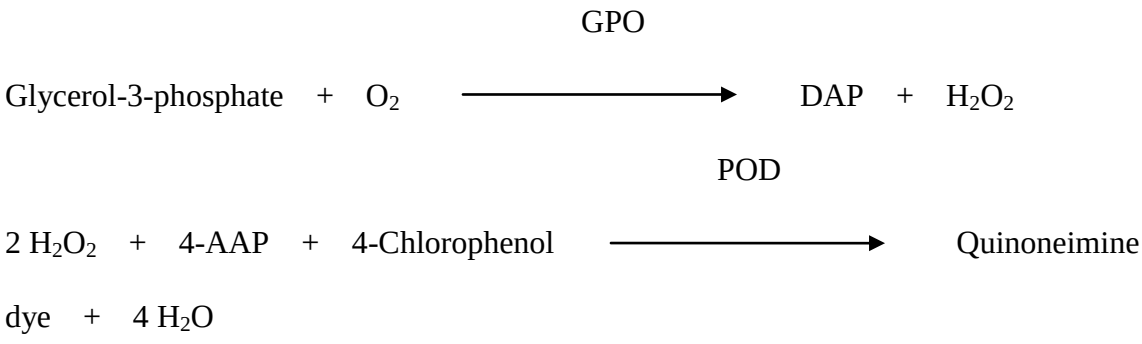
TC (mg/dL) = Absorbance of Test * 200/ Absorbance of Standard

8.2.5.2 Estimation of TG

TGs are hydrolysed by lipoprotein lipase (LPL) to produce glycerol and free fatty acids (FFA). In the presence of glycerol kinase (GK), adenosine triphosphate (ATP) phosphorylates glycerol to produce glycerol-3-phosphate and adenosine diphosphate (ADP). Glycerol-3-phosphate is further oxidized by glycerol-3-phosphate oxidase (GPO) to produce dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2). In presence of peroxidase (POD), hydrogen peroxide couples with 4-aminoantipyrine (4-AAP) and 4-cholorophenol to produce red quinoneimine dye. Absorbance of colored dye was measured at 505 nm which is proportional to TG concentration in the sample.

$$\text{Triglyceride} \xrightarrow{\text{LPL}} \text{Glycerol} + \text{FFA}$$

$$\text{Glycerol} \xrightarrow{\text{GK}} \text{Glycerol-3-phosphate} + \text{ADP}$$



1mL triglyceride mono reagent was added into 10 µL plasma, mixed well and incubated at room temperature for 30 min. The same process was repeated for standard solution (10 µL) and blank (without plasma) and absorbance were taken at 505 nm against blank. The TG concentration was obtained by the following formula:

TG Concentration = Absorbance of Test * 200/ Absorbance of Standard

8.2.5.3 Estimation of HDL

The procedure is the same as for TC estimation, except for HDL determination, the IDL, LDL, VLDL and chylomicron fractions are initially precipitated by addition of polyethylene glycol 6000 (PEG). After centrifugation, the HDL fraction that remains in the supernatant is determined by CHOD-POD method.

Step 1: HDL separation

200 µL precipitating agent was added to 200 µL of plasma, mixed well and kept at room temperature for 10 min. Then it was centrifuged at 2000 rpm for 15 min and clear supernatant was separated.

Step 2: HDL Estimation

Supernatant separated in the first step was processed the same way as in cholesterol estimation. HDL was calculated using following formula:

HDL Concentration = Absorbance of Test * 50 * 2/ Absorbance of Standard.

8.2.5.4 Estimation of other parameters

All the remaining parameters, the LDL, VLDL, the atherogenic indices (AIs) - AC, AIP, CHOLINDEX, CRI 1 or CRR and CRI 2 were calculated by using the formulae as given in Table 8.1.

Table 8.1. Estimation of pharmacodynamic parameters.

Direct assay	**Principle**
Total cholesterol (TC)	CHOD-POD
High Density Lipoprotein Cholesterol (HDL)	PEG-CHOD-POD
Triglycerides (TG)	GPO-POD
Indirect assay	**Formula**
Low Density Lipoprotein Cholesterol (LDL)	TC – HDL – (TG/5)
Very Low Density Lipoprotein Cholesterol (VLDL)	TG/5
Atherogenic coefficient (AC)	(TC-HDL)/HDL
Atherogenic Index of Plasma (AIP)	Log (TG/HDL)
CHOLINDEX	LDL – HDL
Castelli's Risk Index – I (CRI 1) or Cardiac Risk Ratio (CRR)	TC/HDL
Castelli's Risk Index – II (CRI 2)	LDL/HDL

8.2.5.5 Dose reduction efficiency study

The procedure followed was the same as for the pharmacodynamic profile study. All the parameters, TC, TG, HDL, LDL, VLDL, AC, AIP, CHOLINDEX, CRI 1 or CRR and CRI 2 were estimated, except here, the dose of the optimized formulations was reduced for the study. The study protocol was approved and guided by the Central Animal Ethical Committee, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. Male Albino Wister rats (200 – 250 g) were used and the animals were divided into seven groups of six animals each. The control - group I, standard - group II, test -

group III, test – group IV, test – group V, test – group VI, and test – group VII received cholesterol, pure drug suspension plus cholesterol, Ezentia suspension plus cholesterol, E-CD-TPGS plus cholesterol, Eze-ND plus cholesterol, ESTNC $F8^A$ plus cholesterol, and ESTNC $F8^B$ plus cholesterol, respectively. The only difference between ESTNC $F8^A$ and ESTNC $F8^B$ was the dose studied for ESTNC F8. The standard - group II and test - group III received pure drug and Ezentia suspension as 1 mg/kg/day equivalent Eze. The test – group IV, test – group V, and test – group VI received E-CD-TPGS, Eze-ND and ESTNC $F8^A$ as 0.5 mg/kg/day equivalent Eze and test – group VII received ESTNC $F8^B$ as 0.2 mg/kg/day equivalent Eze. The dosing volume of vehicle, 200 µL was kept constant in each case. The animals were housed in polypropylene cages and kept at standard laboratory conditions (25±2 ˚C and 55±5% RH). Six animals per cage were accommodated with free access to standard laboratory diet (Lipton feed, Mumbai, India) and water *ad libitum*.

The study was carried out for a total period of 8 weeks wherein the first four weeks, hypercholesterolemia was induced. At the end of fourth week, the plasma levels of TC, TG, HDL, LDL, VLDL, AC, AIP, CHOLINDEX, CRI 1 or CRR and CRI 2 were measured for all the groups and the values were considered as baseline values for the following four week study, the actual pharmacodynamic activity study. The first four weeks is a stage 1, hypercholesterolemic induction study and the next four weeks is the stage 2, pharmacodynamic activity study.

The pharmacodynamic study was carried out for 28 days and the pharacodynamic parameters were studied on day 7, 14, 21 and 28. Percent reduction in the plasma levels of TC, TG and HDL were directly analysed using *in-vitro* Cogent diagnostic kit (Span

Diagnostics Ltd., Surat, India). All the remaining parameters were derived as per established equations, from the measured plasma lipid values of TC, TG and HDL.

The dose reduction efficiency was reported in two phases. In the first phase report of performance comparison of the treatment groups, plain Eze and Ezentia treatment groups received 1 mg/kg/day equivalent Eze and all the optimized formulations, E-CD-TPGS, Eze-ND and ESTNC-F8^{A} were dosed at 0.5 mg/kg/day equivalent Eze. In the second phase report of performance comparison of the treatment groups, plain Eze and Ezentia treatment groups received 1 mg/kg/day equivalent Eze, the optimized formulations, E-CD-TPGS and Eze-ND received 0.5 mg/kg/day equivalent Eze, and ESTNC-F8^{B} was dosed at 0.2 mg/kg/day equivalent Eze. As already mentioned, the only difference between ESTNC F8^{A} and ESTNC F8^{B} was the dose studied for ESTNC F8.

8.2.6 Statistical analysis

All the results were shown as Mean±SD. The data pertaining to solubility, dissolution and pharmacokinetic investigations were analyzed by one-way ANOVA followed by post hoc Tukey multiple comparison test (p value set 0.05). The pharmacodynamic study results were analyzed by two-way ANOVA followed by post hoc Bonferrroni multiple comparison test.

8.3 RESULTS AND DISCUSSION

8.3.1 Aqueous solubility and dissolution

The solubility of E-CD-TPGS was higher than pure Eze ($p < 0.05$) and the solubility results of Eze-ND and ESTNC were higher than pure Eze as well as E-CD-TPGS.

However, the solubility values of Eze-ND and ESTNC were insignificantly different ($p > 0.05$). The comparison of the solubility values of pure Eze and optimized formulations in distilled water has been provided in Figure 8.1.

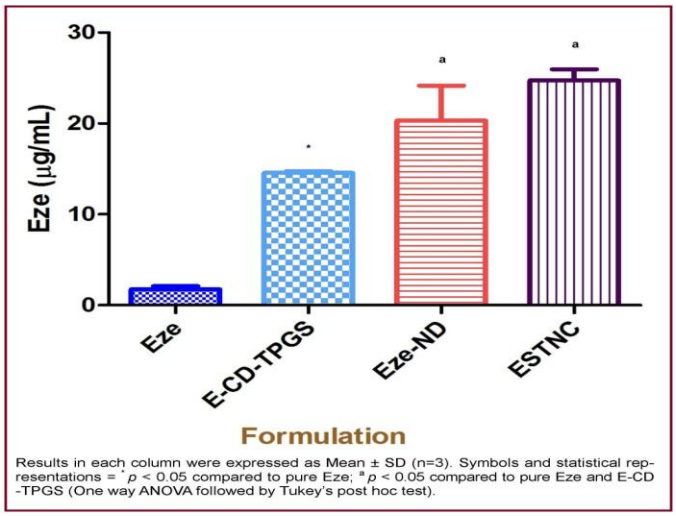


Figure 8.1. Solubility of pure Eze and optimized formulations in distilled water (vertical bars represent SD, n = 3).

Though Eze contains ionisable groups [Figure 2.17 or Figure 6.1], the drug was reported to essentially show a pH independent solubility characteristic across the gastrointestinal pH range. Therefore, pH-based strategies to improve the solubility/dissolution characteristics (e.g. salts, addition of pH modifiers) were never a first-line option [Taupitz et al., 2013]. The same was confirmed by conducting the dissolution of the optimized formulations in three different pH media.

Dissolution was studied at three different pH media containing 0.45% w/v SLS - 0.01 N HCl (pH 2), USP acetate buffer of (pH 4.5), and distilled water (measured pH 6.8). Use of diluted acidic media, 0.01 N HCl (pH 2) more closely represents the stomach pH in the fed state besides having its environmental benefits [http://www.dissolution.com/ddg/showthread.php?145-USP-change-to-0-01N-HCl].

The dissolution medium for Eze as per FDA "Dissolution Methods" guide is USP acetate buffer of pH 4.5 containing 0.45% w/v SLS [http://www.accessdata.fda.gov/scripts/cder/dissolution/dsp_SearchResults_Dissolutions.cfm?PrintAll=1]. The use of distilled water to study the dissolution of Eze CDDS formulations has already been established [Bandyopadhyay et al., 2012; Dixit and Nagarsenker, 2008; Bali et al., 2010 and 2011]. The comparison of $t_{80\%}$ of optimized formulations and Ezentia in USP acetate buffer media (pH 4.5) with 0.45% w/v SLS has been shown in Figure 8.2. The comparison of DE_{45} of pure Eze, optimized formulations and Ezentia in USP acetate buffer media (pH 4.5) with 0.45% w/v SLS has been shown in Figure 8.3.

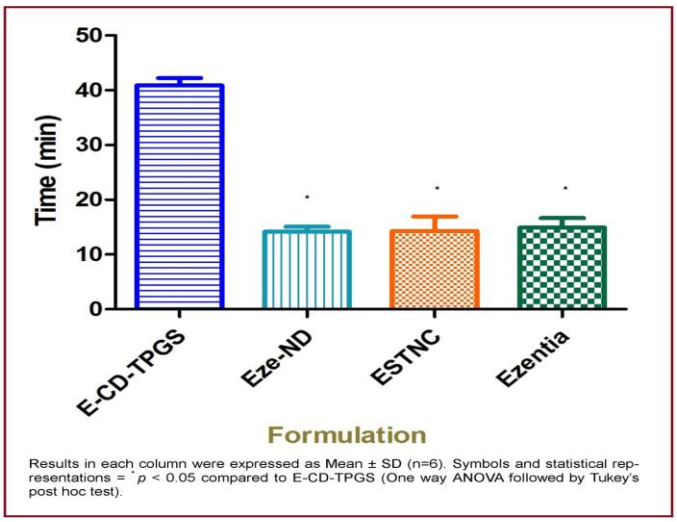


Figure 8.2. Dissolution parameter, $t_{80\%}$ of optimized formulations and Ezentia in USP acetate buffer media (pH 4.5) with 0.45% w/v SLS (vertical bars represent SD, n = 6).

The normal human stomach has a pH which can range from approximately 1-3 but is usually closer to 2. When there is food in the stomach the pH can raise to as high as 4-5. After the food leaves the stomach, bicarbonate ions are secreted to neutralize and

alkalinize the mixture. The pH of the small intestine is approximately in the range 5-8. It is commonly accepted that most of the drug (or food) ingested, gets absorbed from the intestinal part. As absorption depends on dissolution, the majority of the drug should be available in the solution form in this segment of the GI tract. Therefore, for dissolution purposes, these pH environments also appears to be relevant and critical [http://scienceline.ucsb.edu/getkey.php?key=275].

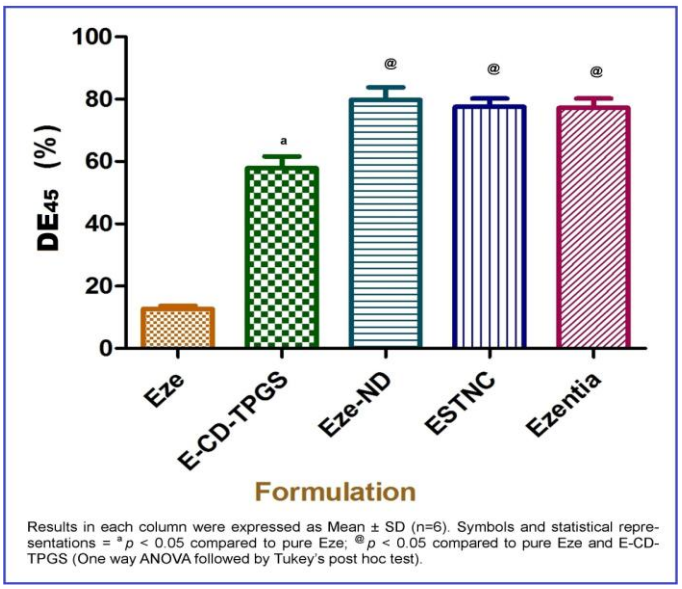


Figure 8.3. DE_{45} of pure Eze, optimized formulations and Ezentia, in USP acetate buffer media (pH 4.5) with 0.45% w/v SLS (vertical bars represent SD, n = 6).

The dissolution graph data and the corresponding profiles drawn using the three different pH media containing 0.45% w/v SLS - 0.01 N HCl (pH 2), USP acetate buffer of (pH 4.5), and distilled water (measured pH 6.8) were respectively shown in Tables, 8.2, 8.3 and 8.4; and Figures, 8.4, 8.5 and 8.6. The aqueous solubility and dissolution parameters in USP acetate buffer of (pH 4.5) containing 0.45% w/v SLS were given in Table 8.5.

Table 8.2. Dissolution graph data of pure Eze, Ezentia, and optimized formulations in 0.01 N HCl (pH 2) containing 0.45% w/v SLS. Results were expressed as Mean±SD (n = 6).

Time (min)	Cumulative % drug dissolved from different batches				
	Pure Eze	**E-CD-TPGS**	**Eze-ND**	**ESTNC - F8**	**Ezentia®**
10	2.8±0.8	41.4±2.8	76.8±3.4	53.4±2.8	71.2±3.6
20	9.6±1.3	55.7±3.4	84.6±4.2	94.2±3.6	82.4±3.7
30	16.2±1.0	72.2±3.86	93.2±3.9	96.4±4.4	91.8±4.4
45	21.8±2.8	85.8±4.98	94.8±4.6	97.8±4.3	95.2±3.9

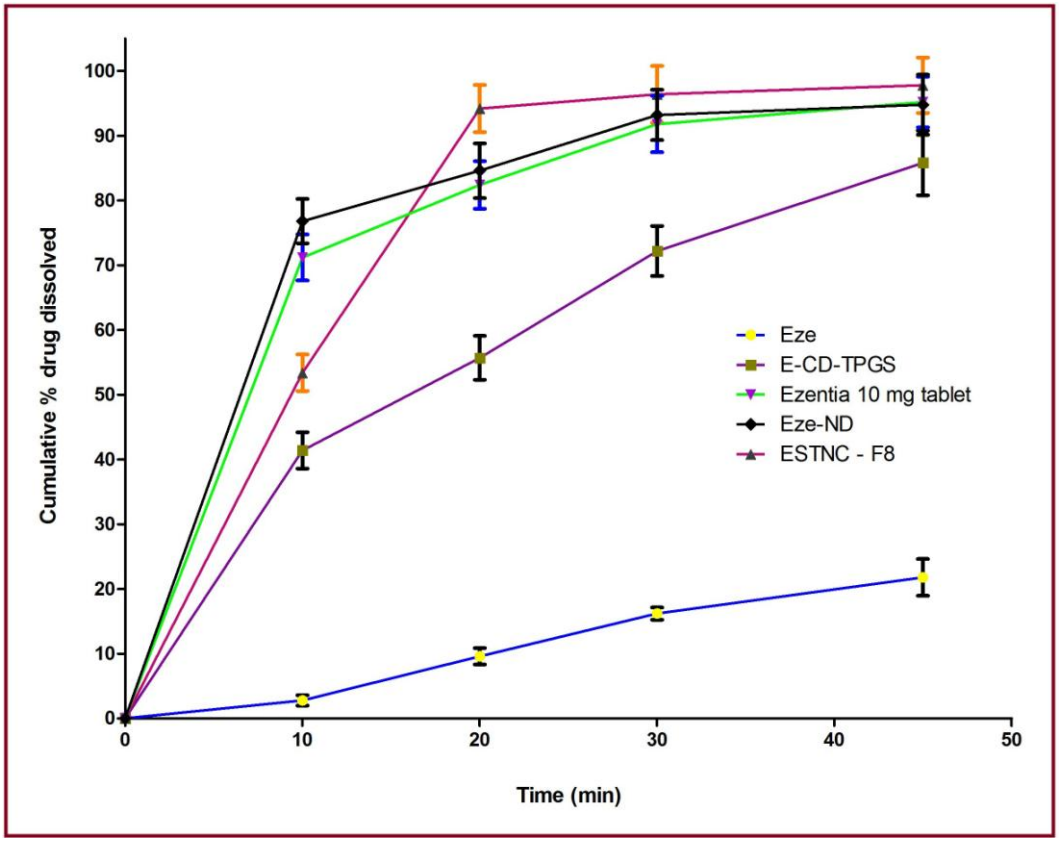


Figure 8.4. Dissolution profiles of pure drug, Ezentia and optimized formulations in 0.01 N HCl (pH 2) containing 0.45% w/v SLS (vertical bars represent SD, n = 6).

Table 8.3. Dissolution graph data of pure Eze, Ezentia, and optimized formulations in USP acetate buffer (pH 4.5) containing 0.45% w/v SLS. Results were expressed as Mean±SD (n = 6).

Time (min)	Cumulative % drug dissolved from different batches				
	Pure Eze	**E-CD-TPGS**	**Eze-ND**	**ESTNC - F8**	**Ezentia®**
10	4.02±2.0	44.04±2.4	79.9±2.4	56.04±2.4	73.02 ±2.2
20	11.2±2.5	58.02±2.2	87.7±2.8	96.02±2.2	85.04 ±2.8
30	18.9±3.0	74.03±3.3	95.5±2.2	98.01±3.3	94.01 ±3.3
45	24.6±1.8	88.01±1.6	96.02±3.2	99.2±1.6	96.04 ±1.9

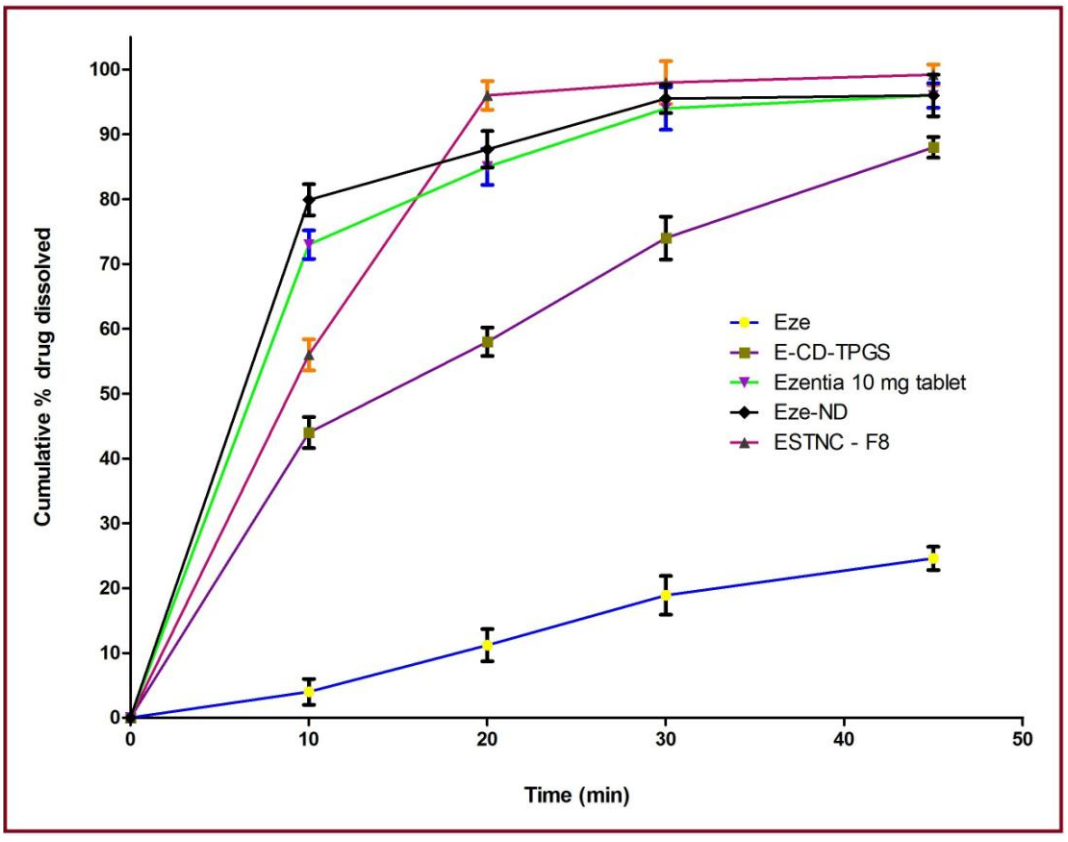


Figure 8.5. Dissolution profiles of pure drug, Ezentia and optimized formulations in USP acetate buffer (pH 4.5) containing 0.45% w/v SLS (vertical bars represent SD, n = 6).

Table 8.4. Dissolution graph data of pure Eze, Ezentia, and optimized formulations in distilled water (measured pH 6.8) containing 0.45% w/v SLS. Results were expressed as Mean±SD (n = 6).

Time (min)	Cumulative % drug dissolved from different batches				
	Pure Eze	**E-CD-TPGS**	**Eze-ND**	**ESTNC - F8**	**Ezentia®**
10	3.6±2.4	42.4±2.0	78.2±2.2	54.4±2.4	71.6±2.4
20	11.0±2.2	57.2±2.5	85.9±2.8	94.4±2.8	83.4±2.2
30	18.2±3.3	73.2±3.0	93.2±3.3	97.2±2.2	92.8±3.3
45	24.2±1.6	86.6±1.8	94.4±1.9	99.0±3.2	94.4±1.6

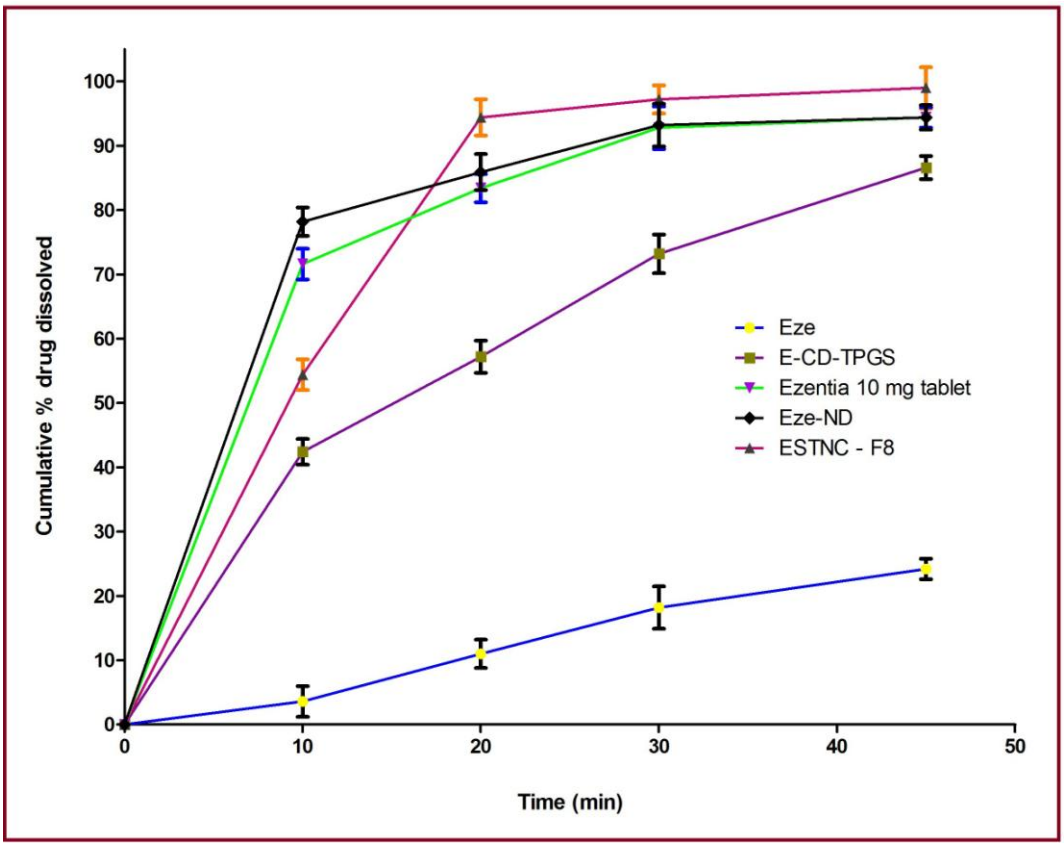


Figure 8.6. Dissolution profiles of pure drug, Ezentia and optimized formulations in distilled water (measured pH 6.8) containing 0.45% w/v SLS (vertical bars represent SD, n = 6).

Table 8.5. Saturation solubility (n = 3) and dissolution (n = 6) parameters of pure drug, optimized formulations and Ezentia (data shown as Mean±SD).

System	Saturation aqueous solubility (µg/mL)	USP acetate buffer (pH 4.5) with 0.45% w/v SLS	
		$t_{80\%}$ (min)	DE_{45} (%)
Pure drug	1.99±0.62	Not Achieved	12.73±0.97
E-CD-TPGS	14.55±0.18^{a***}	40.91±1.33	57.89±3.72^{a***}
Eze-ND	20.32±3.85$^{a***, b*}$	14.13±0.93^{b***}	79.78±3.98$^{@***}$
ESTNC	24.73±1.24$^{a***, b**}$	14.29±2.66^{b***}	77.53±2.64$^{@***}$
Ezentia®	Not applicable	14.9±1.74^{b***}	77.22±2.99$^{@***}$
Symbols and statistical representations = ***p < 0.001, **p < 0.01 and *p < 0.05; a = compared to pure Eze; b = compared to E-CD-TPGS; @ = compared to pure Eze and E-CD-TPGS (One way ANOVA followed by Tukey's post hoc test).			

Table 8.6. Dissolution (n = 6) parameters of pure drug, optimized formulations and Ezentia (data shown as Mean±SD) in other media.

Medium	Distilled water (measured pH 6.8) with 0.45% w/v SLS		0.01 N HCl (pH 2) with 0.45% w/v SLS	
System	$t_{80\%}$ (min)	DE_{45} (%)	$t_{80\%}$ (min)	DE_{45} (%)
Pure drug	Not Achieved	12.33±0.86	Not Achieved	11±0.72
E-CD-TPGS	41.57±1.2	57±2.94^{a***}	42±1.1	56±2.08^{a***}
Eze-ND	14.43±0.88^{b***}	78.09±3.64$^{@***}$	15±0.76^{b***}	78±3.14$^{@***}$
ESTNC	14.71±2.44^{b***}	76.58±2.18$^{@***}$	15±2.22^{b***}	76±2.26$^{@***}$
Ezentia®	15.2±1.52^{b***}	76±2.24$^{@***}$	15.33±1.32^{b***}	76±2.42$^{@***}$
Symbols and statistical representations = ***p < 0.001; a = compared to pure Eze; b = compared to E-CD-TPGS; @ = compared to pure Eze and E-CD-TPGS (One way ANOVA followed by Tukey's post hoc test).				

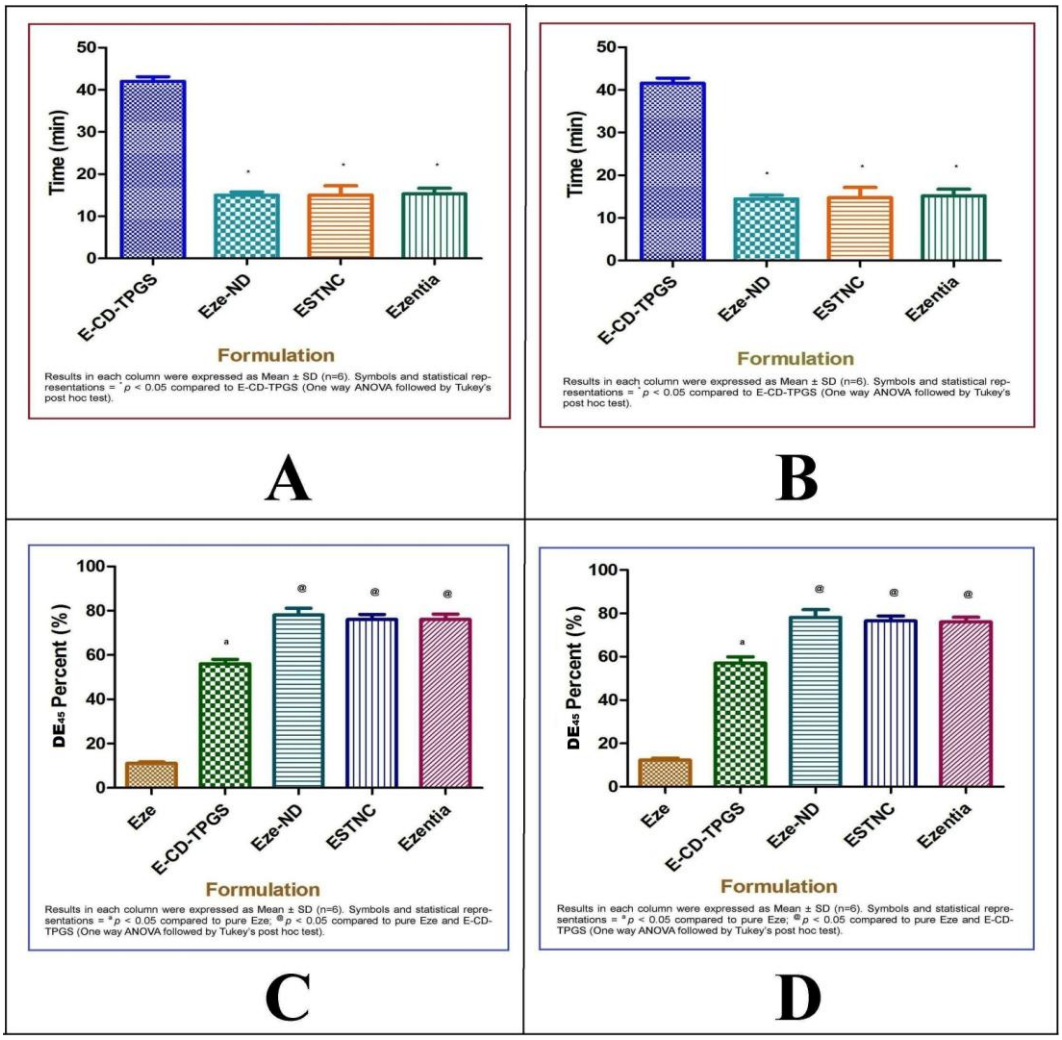


Figure 8.7. Dissolution parameters: $t_{80\%}$ - time (min) of optimized formulations and Ezentia in A. 0.01 N HCl (pH 2) with 0.45% w/v SLS and B. distilled water (measured pH 6.8) with 0.45% w/v SLS; DE_{45} – percent (%) of pure Eze, optimized formulations and Ezentia in C. 0.01 N HCl (pH 2) with 0.45% w/v SLS and D. distilled water (measured pH 6.8) with 0.45% w/v SLS (vertical bars represent SD, n = 6).

The dissolution parameters were measured at three different pH media, 0.01N HCl (pH 2), USP acetate buffer (pH 4.5) and distilled water (measured pH 6.8) and the following observations were made in all the media. The $t_{80\%}$ of Eze-ND, ESTNC and Ezentia were significantly higher than E-CD-TPGS ($p < 0.05$). While the pure drug could not dissolve till 80% during the 45 min dissolution study, the DE_{45} of pure Eze was significantly

lower compared to E-CD-TPGS ($p < 0.05$). The DE_{45} of Eze-ND, ESTNC and Ezentia were significantly higher than E-CD-TPGS ($p < 0.05$). The DE_{45} values of Eze-ND, ESTNC and Ezentia when compared to each other were insignificantly different ($p > 0.05$). The $t_{80\%}$ and DE_{45} parameters derived in media, 0.01 N HCl (pH 2) and distilled water (measured pH 6.8), each with 0.45% w/v SLS, were shown in Table 8.6 and Figure 8.7.

The dissolution in three different pH media was conducted in order to reconfirm the literature information that the solubility and dissolution characteristics of Eze are independent of pH [Taupitz et al., 2013]. Similar performance was observed in all the three media, 0.01N HCl (pH 2), USP acetate buffer (pH 4.5) and distilled water (measured pH 6.8), each with 0.45% w/v SLS. Fresh and degassed distilled water was used and the pH measured before use was 6.8 [https://www.quora.com/What-is-the-pH-of-distilled-water]. The order of the measured parameters did not differ with the change in the pH of dissolution medium. The $t_{80\%}$ and DE_{45} of none of the formulations varied significantly ($p > 0.05$) with the change in the pH of dissolution medium.

8.3.2 Stability

The aim of the stability testing was to study the variation in the quality of the drug product with time under the influence of environmental factors such as temperature and humidity. The purpose of the solid state characterization after stability study was to confirm the retention of solid state properties upon variations in temperature and humidity. The FTIR, DSC and XRD analyses confirmed the retention of solid state characters of the respective formulations. The stability study results in terms of FTIR,

DSC and XRD analyses, comparing the fresh and treated batches of Eze-ND, E-CD-TPGS and ESTNC F8 were shown in Figures, 8.8, 8.9 and 8.10, respectively.

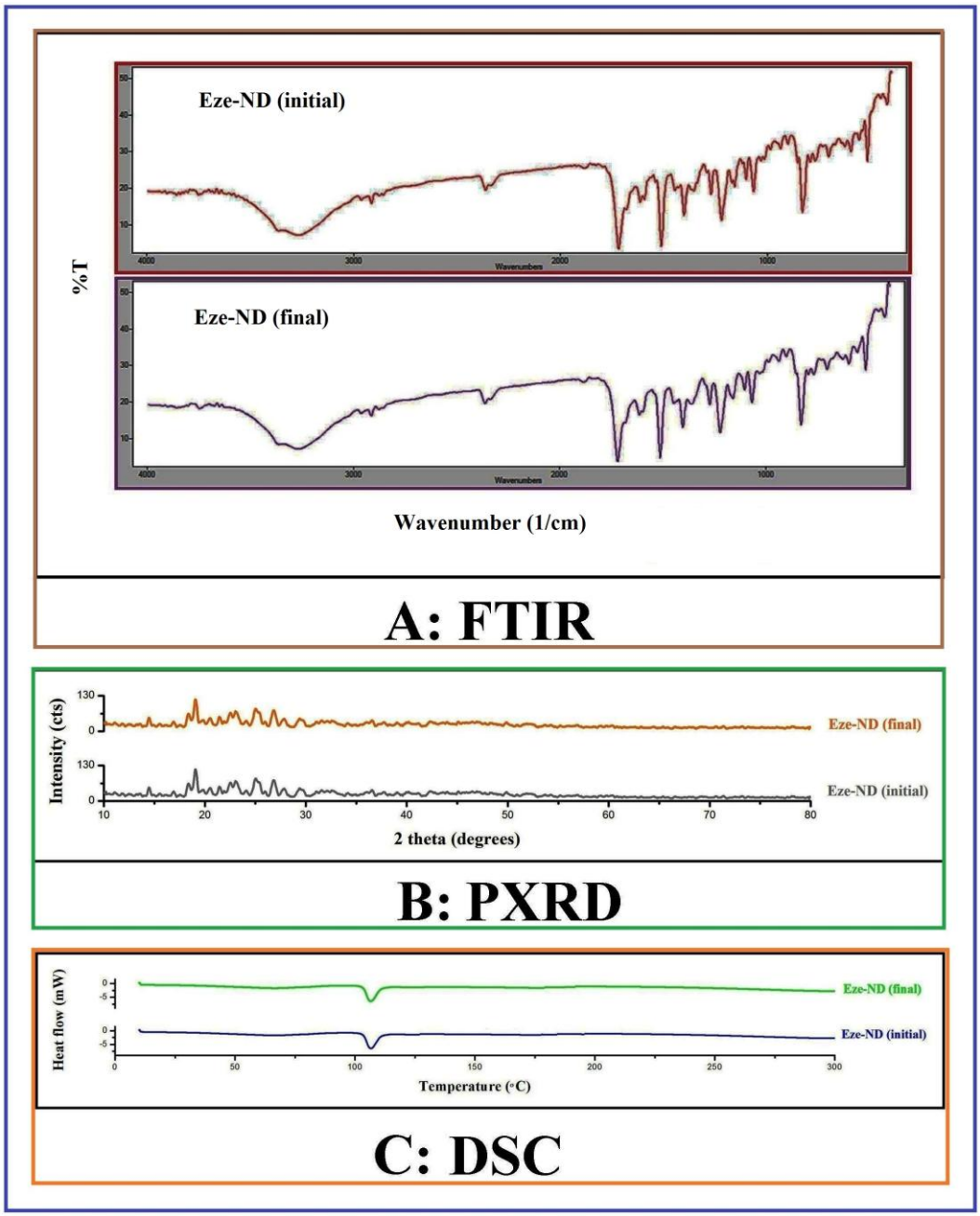


Figure 8.8. FTIR spectra, DSC thermograms and PXRD graphs of a stability study batch of Eze-ND against the respective fresh batch.

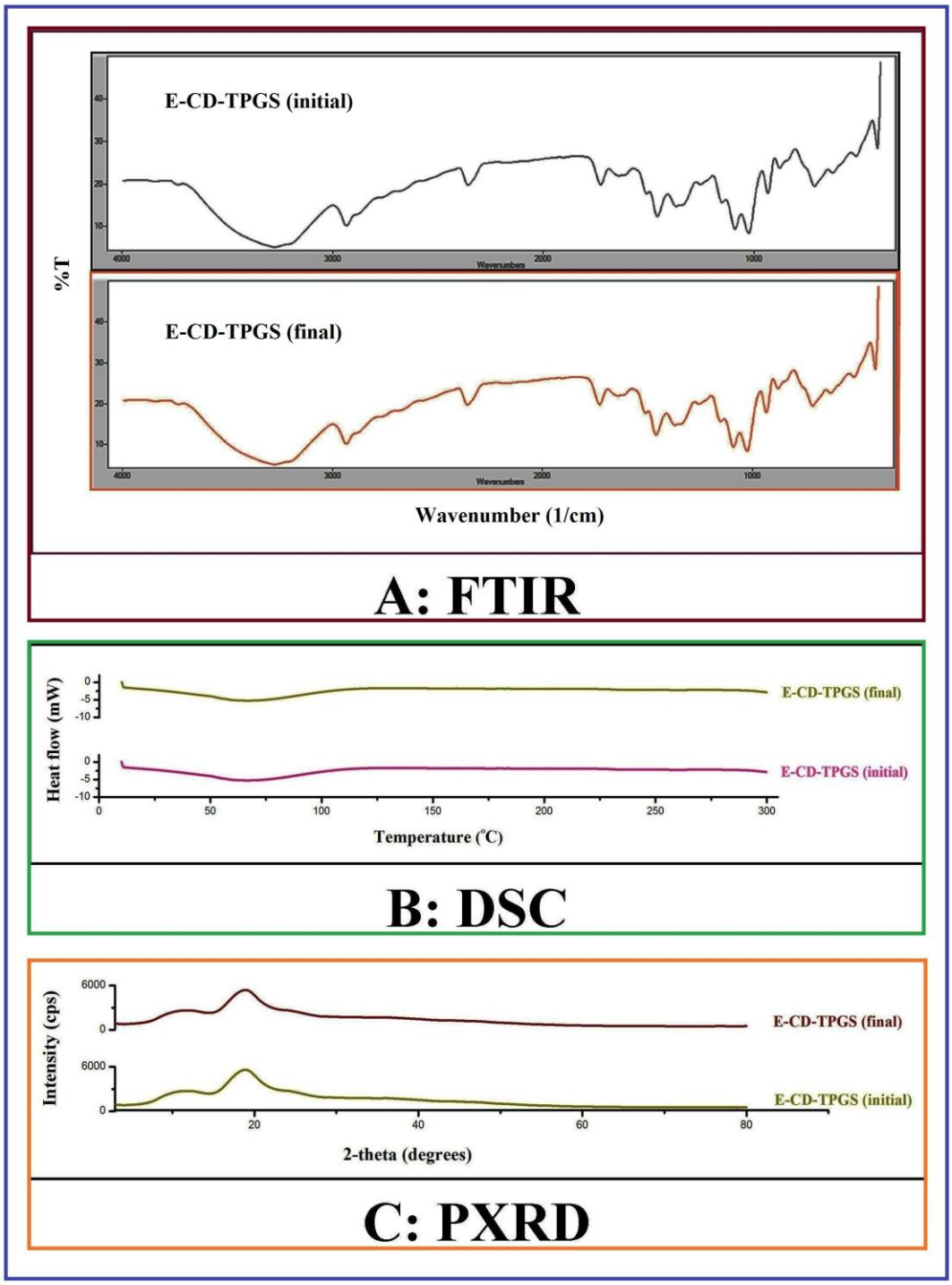


Figure 8.9. FTIR spectra, DSC thermograms and PXRD graphs of a stability study batch of E-CD-TPGS against the respective fresh batch.

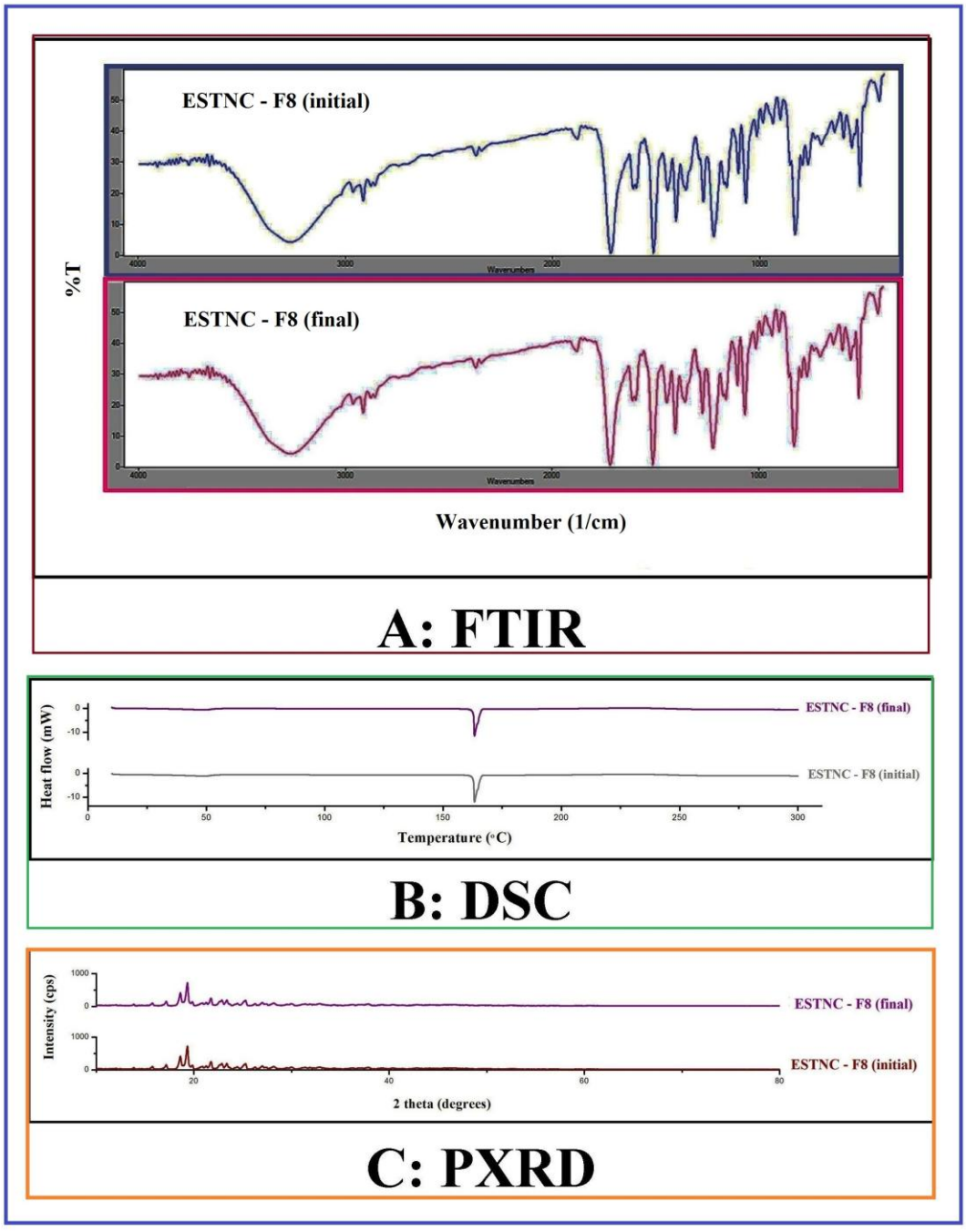


Figure 8.10. FTIR spectra, DSC thermograms and PXRD graphs of a stability study batch of ESTNC –F8 against the respective fresh batch.

On comparing the stored samples with the initial samples, it was observed that the solid state properties of the stored samples were not influenced by the storage conditions. The observations indicated that the formulations were stable and capable of withstanding the environmental fluctuations during storage and handling. All the three optimized formulations, Eze-ND, E-CD-TPGS and ESTNC F8, maintained their stability for the period of 6 months and showed no any noticeable changes in the FTIR, DSC and XRD analyses suggesting that the room temperature storage was acceptable.

8.3.3 *In-vivo* preclinical pharmacokinetic study

8.3.3.1 HPLC-UV plasma drug analysis – method development and validation – same as described under the section 5.4.2.8.1.

8.3.3.2 Pharmacokinetic parameters

The pharmacokinetic parameters were determined using Kinetica 5.0 pharmacokinetic software (Trial version, PK-PD analysis, Thermofischer) and Graphpad Prism software (version 5.03, GraphPad Software, USA). The plasma profiles of total Eze quantified in adult male Albino Wistar rats following single dose oral administration of pure drug suspension, Ezentia suspension, Ezentia tablet, Eze-ND, E-CD-TPGS and ESTNC F8 were reported in Table 8.7. The percent relative bioavailability values (% RB) derived were shown in Table 8.8. The peak plasma concentration (C_{max}) and the time to attain C_{max}, T_{max}, were recorded directly from the plasma concentration – time curve. The area under the plasma concentration – time curve was determined by trapezoidal method.

The pharmacokinetic activity comparison details of each of the optimized formulation against pure drug suspension have already been discussed in the formulation specific chapters.

Table 8.7. Pharmacokinetic parameters compared for pure Eze, Ezentia, Ezentia suspension and optimized formulations (n = 6). Data shown as Mean±SD.

Parameter/ Treatment	T_{max} (h)	C_{max} (ng/mL)	AUC_{0-24h} (ng. h/mL)	$AUC_{0-\infty}$ (ng. h/mL)	$AUMC_{0-24h}$ (ng. h^2/mL)
Pure drug	2±0.00	1912±195.92	17848±1306.54	18664.43±1324.18	163568±3801.65
Ezentia®	2±0.00	1794.5±206.98	16458±1312.48	17068.42±1398.24	150260±3918.96[a]
Ezentia® suspension	2±0.00	2013±245.12	19147±1317.96	20093.25±1384.16	176770±3910.04[b]
E-CD-TPGS	1.5±0.00	3380.5±359.97[@]	24145±1421.89	25215.55±1476.92[@]	206846±4654.93[@]
Eze-ND	1±0.00	4173±396.92[@]	29263±1438.52	31630.63±1482.66[#]	254746±4721.08[#]
ESTNC F8	1±0.00	5146.5±535.94[$]	46704±1856.13	64169.33±1912.32[$]	464738±6821.95[$]

Symbols and statistical representations: significance level set as $p < 0.05$; a = compared to pure Eze; b = compared to pure Eze and Ezentia; @ = compared to pure Eze, Ezentia and Ezentia suspension; # = compared to pure Eze, Ezentia, Ezentia suspension and E-CD-TPGS; $ = compared to pure Eze, Ezentia, Ezentia suspension, E-CD-TPGS and Eze-ND (One way ANOVA followed by Tukey's post hoc test).

In case of tablet formulation, the drug would not be available for absorption until the dosage form undergoes disintegration and further deaggregation into fine particles which can undergo subsequent dissolution. Since in case of pure Eze suspension and tablet suspension, the drug was present in the form of fine particles having large surface area for dissolution, the process of dissolution and subsequent absorption was relatively higher from the tablet suspension compared to tablet. However, the noted T_{max} was 2 h for all the three systems because the tablet formulation offers rapid dissolution due to the presence of favorable surfactant excipients in its composition that nullify the time required for the disintegration and deaggregation steps which are absent for suspensions. It can be seen that the plasma concentration time profile of Eze from all the

optimized formulations presented greater improvement of drug absorption than the marketed tablet formulation and simple drug suspension or tablet suspension.

Table 8.8. Percent relative bioavailability values (n = 6). Data shown as Mean±SD.

% RB = % Relative bioavailability	**Test/ Standard**	**Pure drug**	**Ezentia®**	**Ezentia® suspension**
% $RB_{(0\text{-}24h)}$	**Pure drug**	100±0.00	Not derived	Not derived
	Ezentia®	92.21±1.17	100±0.00	85.95±1.2
	Ezentia® suspension	107.28±1.06	116.34±1.28	100±0.00
	E-CD-TPGS	135.28±2.04	146.71±1.22	126.1±1.11
	Eze-ND	163.96±1.38	177.81±1.71	152.84±1.25
	ESTNC F8	261.67±6.3	283.78±3.19	243.92±2.42
% $RB_{(0\text{-}\infty)}$	**Pure drug**	100±0.00	Not derived	Not derived
	Ezentia®	91.44±1.55	100±0.00	84.94±1.36
	Ezentia® suspension	107.65±1.2	117.73±1.5	100±0.00
	E-CD-TPGS	135.1±2.21	147.74±1.65	125.5±1.03
	Eze-ND	169.47±1.42	185.34±1.5	157.43±1.6
	ESTNC F8	343.82±8.08	376±5.43	319.38±4.57

The 24 h plasma concentration time profiles shown in Figure 8.11 indicated that Eze reached its peak followed by a rapid decline and this pattern repeated leading to the occurrence of multiple peaks in the plasma concentration time graph. The complete plasma drug concentration – time graph data corresponding to Figure 8.11 was given in Table 8.9. These multiple peaks were due to the fact that Eze is subjected to extensive glucuronidation to a phenolic glucuronide at its site of action, the intestine and is then excreted into the bile. It may be possible that Eze experiences enterohepatic recirculation and is repeatedly delivered back to its site of action, the intestinal tract

lumen, after undergoing reabsorption in the ileum. This reabsorption and recirculation processes have the potential to enhance the residence time of Eze in the lumen of the intestinal tract, thereby improving its cholesterol-lowering activity [Bali et al., 2010 and 2011].

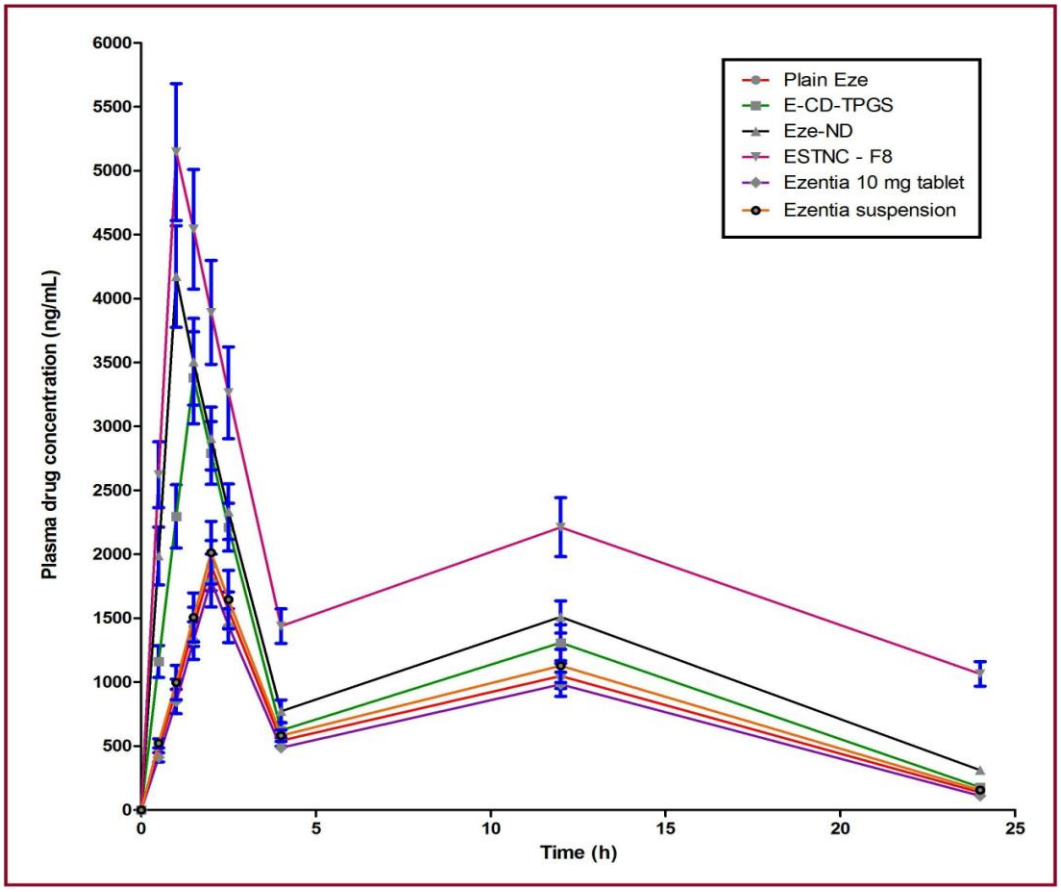


Figure 8.11. Pharmacokinetic profiles of pure Eze, Ezentia, Ezentia suspension and optimized formulations (vertical bars represent SD, n = 6) up to 24 h.

The T_{max} was observed in the order, pure drug = Ezentia = Ezentia suspension > E-CD-TPGS > Eze-ND ≈ ESTNC F8. The T_{max} of pure drug, Ezentia and Ezentia suspension was noted at 2 h, the T_{max} of E-CD-TPGS was observed at 1.5 h and that of Eze-ND and ESTNC F8 was recorded at 1 h. Eze-ND and ESTNC F8 reduced the T_{max} of pure Eze by half. The parameters, C_{max}, $AUC_{0\text{-}24h}$, $AUMC_{0\text{-}24h}$ and $AUC_{0\text{-}\infty}$ were observed in the

order, ESTNC F8 > Eze-ND > E-CD-TPGS > Ezentia suspension > pure drug suspension > Ezentia. The plasma concentration time profiles of pure drug, commercial tablet and optimized formulations, up to 4 h, highlighting the C_{max} were shown in Figure 8.12.

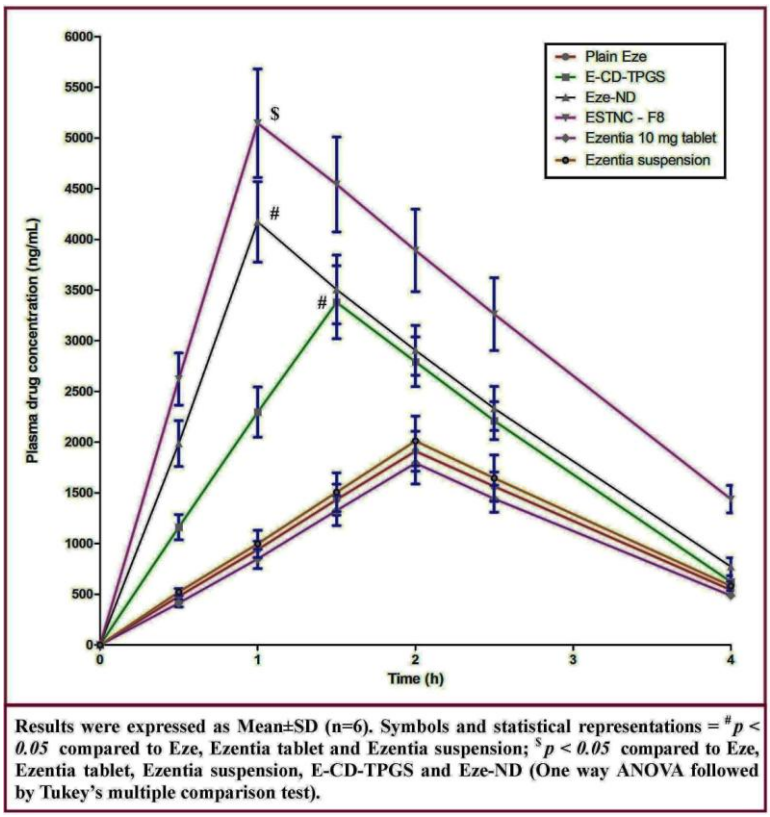


Figure 8.12. Pharmacokinetic profiles of pure Eze, Ezentia, Ezentia suspension and optimized formulations (vertical bars represent SD, n = 6) up to 4 h.

The extent to which each of the formulation approach improved the pharmacokinetic parameters of Eze has already been discussed in the afore described chapters. The MRT values of pure drug, Ezentia and Ezentia suspension were 9.15±1.02, 9.14±1.08 and 9.12±1.10, respectively. Insignificant differences were observed in the MRT values of all the studied drug systems. MRT is an intrinsic property of a drug and there was no change in the intrinsic property of Eze when the drug was formulated into different

formulations [Bali et al., 2010 and 2011]. The percent relative bioavailability values indicated a good possibility of dose reduction for each of the optimized formulation. Therefore, the possible dose reduction efficiencies were evaluated in the following sections of this chapter.

Table 8.9. Plasma drug concentration – time graph data of pure Eze, Ezentia, Ezentia suspension and optimized formulations up to 24 h. Data shown as Mean±SD (n = 6).

Time (h)	Plasma drug concentration (ng/mL) recorded for different treatment groups					
	Plain Eze	**E-CD-TPGS**	**Eze-ND**	**ESTNC - F8**	**Ezentia®**	**Ezentia® suspension**
0.5	480.53±31.42	1162.01±125.49	1987.51±225.96	2623.02±257.21	413.06±35.98	522.08±36.01
1.0	941.04±83.26	2296.04±247.68	4173.02±396.92	5146.51±535.94	849.08±94.96	998.05±133.98
1.5	1434.02±152.78	3380.51±359.97	3507.04±338.87	4543.05±468.21	1326.07±147.62	1507.07±191.74
2.0	1912.04±195.92	2793.03±245.89	2906.03±245.68	3892.04±406.26	1794.52±206.98	2013.09±245.12
2.5	1564.52±141.18	2212.02±186.96	2333.02±217.14	3264.03±359.11	1443.09±132.24	1646.06±228.86
4.0	543.51±44.08	623.04±58.72	772.54±89.12	1438.01±135.22	488.02±29.66	582.05±45.24
12.0	1049.02±96.97	1309.02±140.74	1510.51±125.97	2212.04±230.68	984.04±93.15	1128.07±130.28
24.0	138.04±27.07	178.01±25.26	311.52±30.13	1064.52±95.76	111.06±20.04	156.08±16.98

8.3.4 *In-vivo* preclinical pharmacodynamic study

8.3.4.1 Principle behind performing the plasma cholesterol determination test for pure Eze and formulations – same as described under the section 5.4.2.9.1.

8.3.4.2 Pharmacodynamic potential of pure drug and optimized formulations

Eze is the first drug that blocks the Niemann–Pick C1-Like 1 protein in the enterocytes of the intestine, and inhibits cholesterol absorption. It is a selective cholesterol absorption inhibitor, which potently inhibits the absorption of biliary and dietary cholesterol from the small intestine. Mechanistically, Eze reduces the small intestinal enterocyte uptake and absorption of cholesterol and thus keeps the cholesterol in the

intestinal lumen for excretion. Eze is rapidly absorbed and primarily metabolized in the small intestine and liver to its glucuronide, both of which undergo enterohepatic recycling [Heek et al., 2000; Catapano, 2001]. The pharmacodynamic potential of pure Eze and the optimized formulations was assessed by estimating the lipid profile and atherogenic indices (AIs).

Lipid profile consists of a group of biochemical tests that are often used in predicting, diagnosing and treating hypercholesterolaemia and the most associated lipid related disorder, atherosclerosis. The first step in diagnosis is to define the lipoprotein pattern by chemical analysis of the plasma lipids and lipoproteins. There are accumulated evidences relating the concentrations of lipids (TC and TG) and their associated blood transporting lipoprotein cholesterols (HDL, LDL, VLDL), with the occurrence of cardiovascular diseases (CVDs) in general and atherosclerosis in particular. Epidemiological studies have shown that an elevated concentration of TC in the blood is a powerful risk factor of coronary disease. Increased plasma level of LDL and VLDL is often found in hypertension and diabetes mellitus as a risk factor for CVD. Also high plasma TG level is both an independent and synergistic risk factor for CVD, and is often associated with hypertension, abnormal lipoprotein metabolism, obesity, insulin resistance and diabetes mellitus. On the other hand, decrease in plasma TC, TG and LDL have been considered to reduce risk of CVDs [Lafta, 2014].

Increases in plasma HDL have been considered to reduce risk in CVD. High HDL exerts a protective effect by enhancing reverse cholesterol transport by scavenging excess cholesterol from peripheral tissue, which it esterifies with the aid of lecithin cholesterol acyltransferase and delivers to the liver and steroidogenic organs for subsequent syntheses of bile acids and steroid hormones, and eventual elimination from

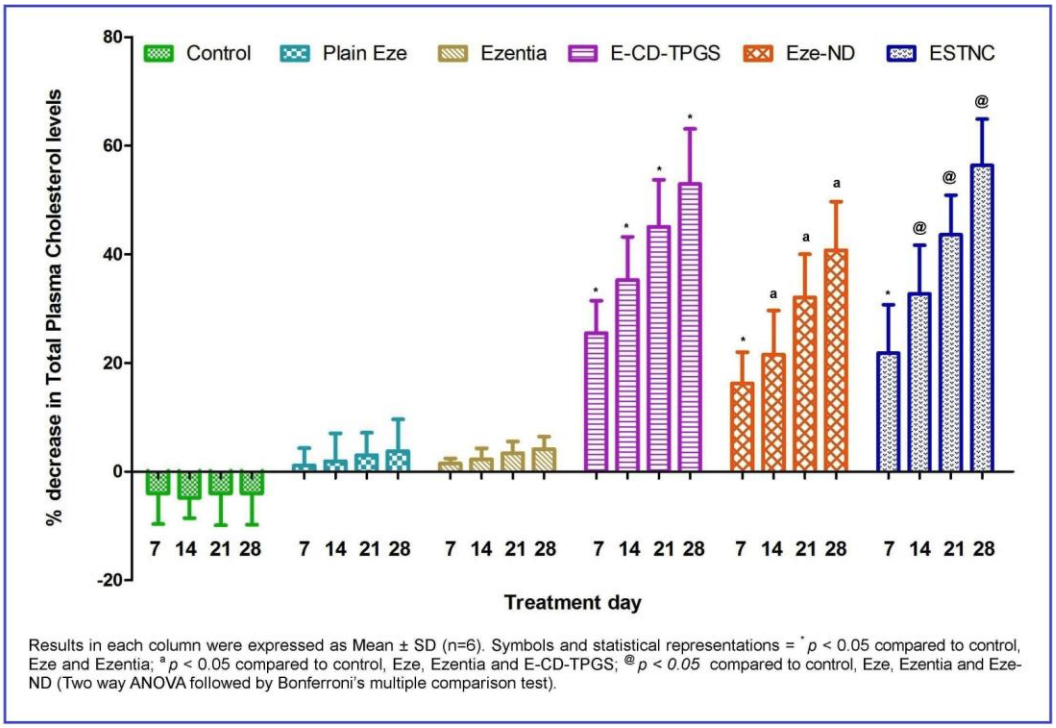
the body, and inhibiting the oxidation of LDL as well as the atherogenic effects of oxidized LDL by virtue of its antioxidant and anti-inflammatory properties [Lafta, 2014].

Additionally, AIs are powerful indicators of the risk assessment of CVD. The AIs like the AC, AIP, CHOLINDEX, CRI 1 or CRR and CRI 2, were calculated. The higher the values, higher are the risks of developing cardiovascular diseases and vice versa. Therefore, the percent decrease in each of the index was calculated and reported [Akpmar et al., 2013; Lafta, 2014; Ranjit et al., 2015].

The study was conducted for a total of eight weeks wherein the first four weeks, all the animal groups were fed with 200 mg cholesterol in 2 mL coconut oil as high fat diet for inducing hypercholesterolemia. The mean percent elevation or decline in plasma lipid levels of all the animals in comparison to day one was noted at the end of four week hypercholesterolemic induction study. These measured values were considered as baseline values for the next stage four week study, the actual pharmacodynamic activity study. The mean elevation levels, calculated for all the animal groups, compared to day one values, were 80-85% for total plasma cholesterol, 75-80% for triglycerides, 200-300% for LDL levels. The mean HDL levels showed a 20-25% decline compared to day one. The percent changes in the plasma levels of TG, TC, HDL, LDL VLDL, AC, AIP, CHOLINDEX, CRI 1 or CRR and CRI 2, for a period of 28 days were shown in the Figure 8.13 to Figure 8.22, wherein the statistically differential performance of each treatment group with respect to the other was indicated. TG, TC and HDL levels were experimentally determined and their values were applied to derive all the remaining parameters. The orders in which the treatment groups performed were presented in Table 8.10.

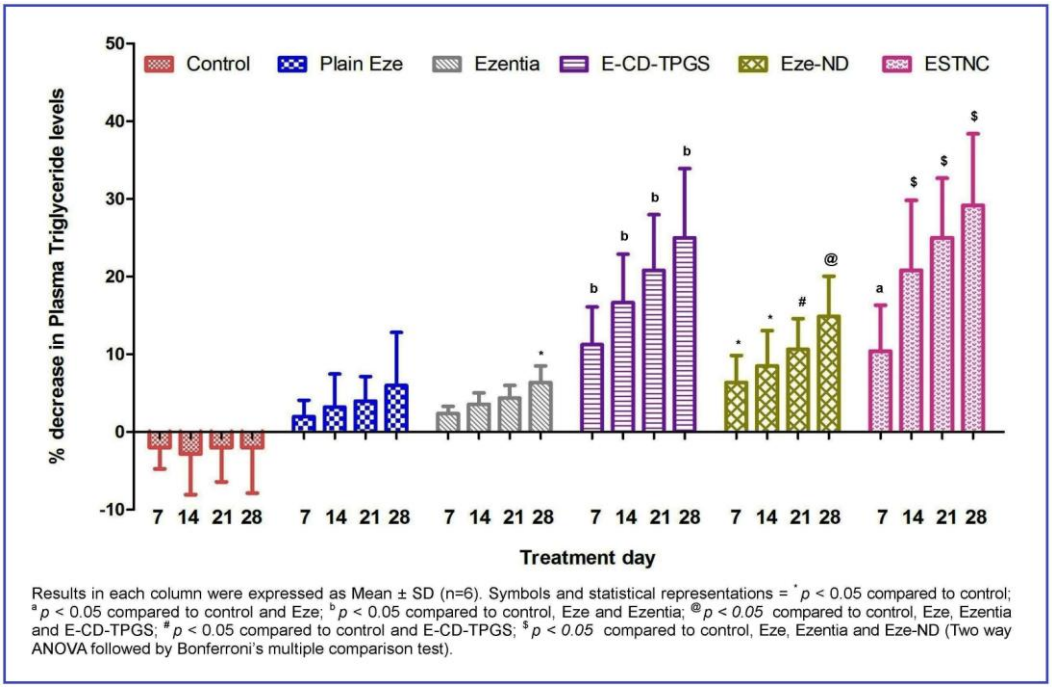
Table 8.10. Order of pharmacodynamic performance of the treatment groups.

Percent reduction in TC:
Day 7, 14, 21: Control < Eze < Ezentia® < Eze-ND < ESTNC < E-CD-TPGS
Day 28: Control < Eze < Ezentia® < Eze-ND < E-CD-TPGS < ESTNC
Percent reduction in TG:
Day 7: Control < Eze < Ezentia® < Eze-ND < ESTNC < E-CD-TPGS
Day 14, 21, 28: Control < Eze < Ezentia® < Eze-ND < E-CD-TPGS < ESTNC
Percent enhancement in HDL:
Day 7: Eze < Control < Eze-ND < Ezentia® < E-CD-TPGS ≈ ESTNC
Day 14, 21, 28: Control < Eze < Ezentia® < Eze-ND < E-CD-TPGS ≈ ESTNC
Percent reduction in LDL:
All days: Control < Eze < Ezentia® < Eze-ND < ESTNC < E-CD-TPGS
Percent reduction in VLDL:
Day 7: Control < Eze < Ezentia® < Eze-ND < ESTNC < E-CD-TPGS
Day 14, 21, 28: Control < Eze < Ezentia® < Eze-ND < E-CD-TPGS < ESTNC
Percent reduction in AC:
Day 7, 14, 21: Control < Eze < Ezentia® < Eze-ND < ESTNC < E-CD-TPGS
Day 28: Control < Eze < Ezentia® < Eze-ND < E-CD-TPGS < ESTNC
Percent reduction in AIP:
Day 7: Control < Eze < Ezentia® < Eze-ND < ESTNC < E-CD-TPGS
Day 14, 21, 28: Control < Eze < Ezentia® < Eze-ND < E-CD-TPGS < ESTNC
Percent reduction in CHOLINDEX:
Day 7: Control < Eze < Ezentia® < Eze-ND < ESTNC < E-CD-TPGS
Day 14, 21, 28: Control < Ezentia® < Eze < Eze-ND < ESTNC < E-CD-TPGS
Percent reduction in CRI 1:
Day 7, 14, 21: Control < Eze < Ezentia® < Eze-ND < ESTNC < E-CD-TPGS
Day 28: Control < Eze < Ezentia® < Eze-ND < E-CD-TPGS < ESTNC
Percent reduction in CRI 2:
All days: Control < Eze < Ezentia® < Eze-ND < ESTNC < E-CD-TPGS



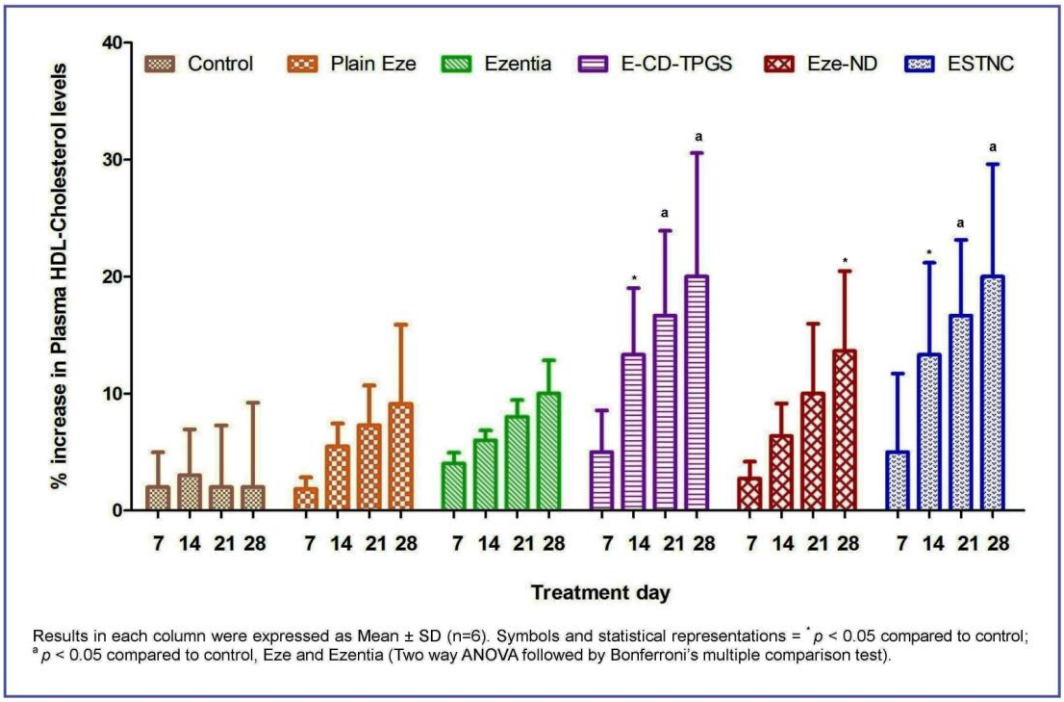
Results in each column were expressed as Mean ± SD (n=6). Symbols and statistical representations = $^{*}p < 0.05$ compared to control, Eze and Ezentia; $^{a}p < 0.05$ compared to control, Eze, Ezentia and E-CD-TPGS; $^{@}p < 0.05$ compared to control, Eze, Ezentia and Eze-ND (Two way ANOVA followed by Bonferroni's multiple comparison test).

Figure 8.13. Percent decrease in total plasma cholesterol levels achieved by various treatment groups.



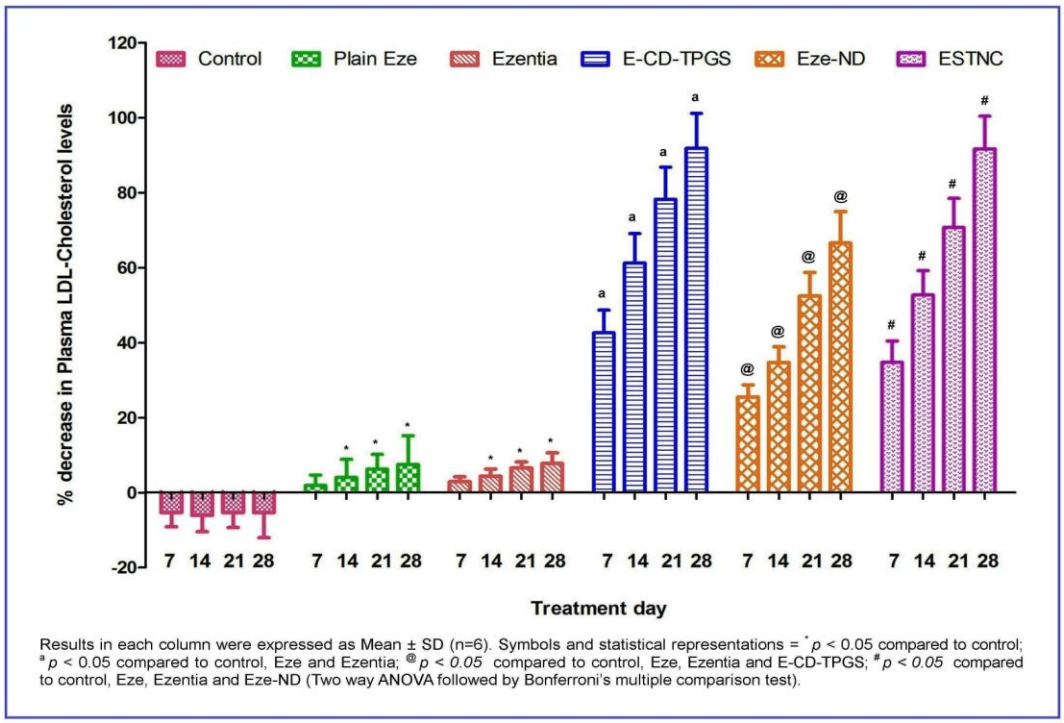
Results in each column were expressed as Mean ± SD (n=6). Symbols and statistical representations = $^{*}p < 0.05$ compared to control; $^{a}p < 0.05$ compared to control and Eze; $^{b}p < 0.05$ compared to control, Eze and Ezentia; $^{@}p < 0.05$ compared to control, Eze, Ezentia and E-CD-TPGS; $^{\#}p < 0.05$ compared to control and E-CD-TPGS; $^{\$}p < 0.05$ compared to control, Eze, Ezentia and Eze-ND (Two way ANOVA followed by Bonferroni's multiple comparison test).

Figure 8.14. Percent decrease in plasma triglyceride levels achieved by various treatment groups.



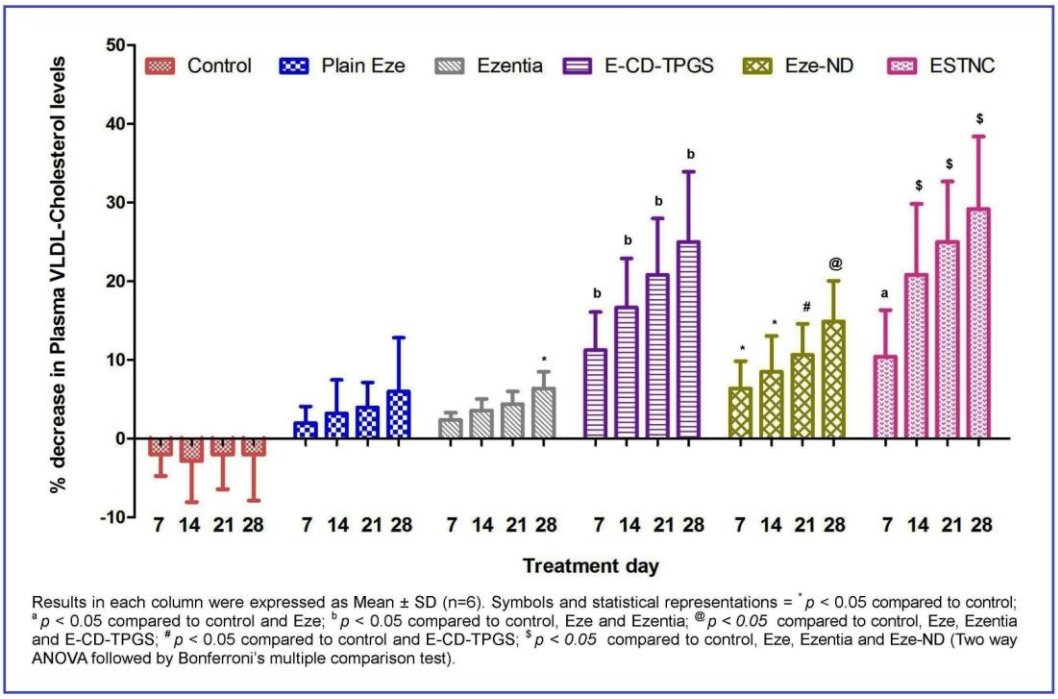
Results in each column were expressed as Mean ± SD (n=6). Symbols and statistical representations = $^{*}p < 0.05$ compared to control; $^{a}p < 0.05$ compared to control, Eze and Ezentia (Two way ANOVA followed by Bonferroni's multiple comparison test).

Figure 8.15. Percent increase in plasma HDL levels achieved by various treatment groups.



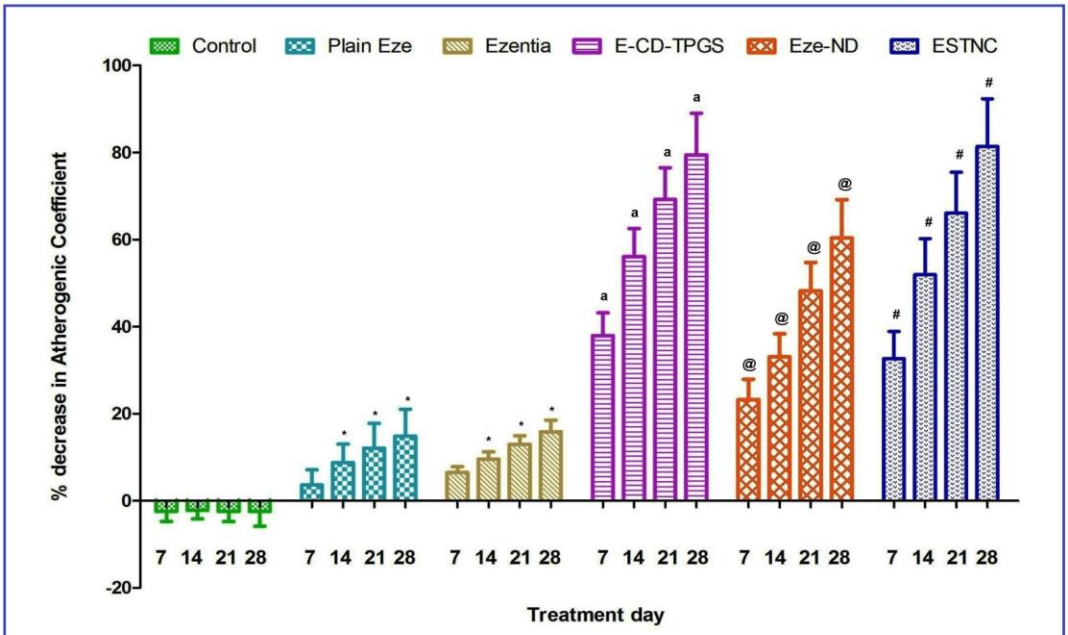
Results in each column were expressed as Mean ± SD (n=6). Symbols and statistical representations = $^{*}p < 0.05$ compared to control; $^{a}p < 0.05$ compared to control, Eze and Ezentia; $^{@}p < 0.05$ compared to control, Eze, Ezentia and E-CD-TPGS; $^{\#}p < 0.05$ compared to control, Eze, Ezentia and Eze-ND (Two way ANOVA followed by Bonferroni's multiple comparison test).

Figure 8.16. Percent decrease in plasma LDL levels achieved by various treatment groups.



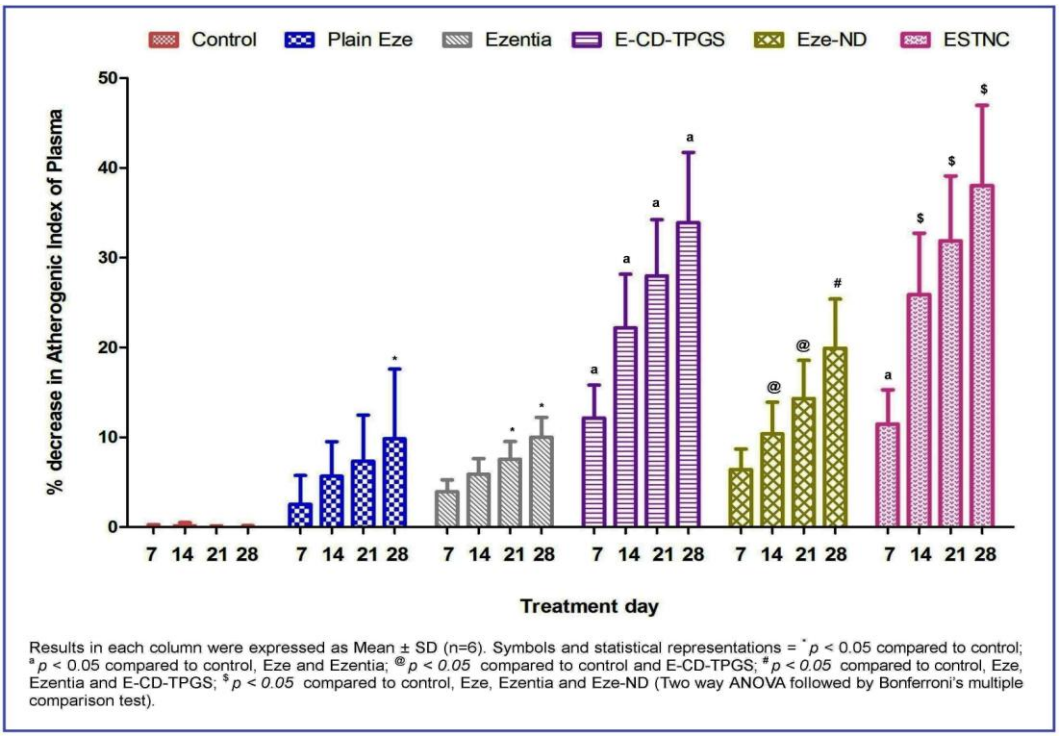
Results in each column were expressed as Mean ± SD (n=6). Symbols and statistical representations = $^{*}p < 0.05$ compared to control; $^{a}p < 0.05$ compared to control and Eze; $^{b}p < 0.05$ compared to control, Eze and Ezentia; $^{@}p < 0.05$ compared to control, Eze, Ezentia and E-CD-TPGS; $^{\#}p < 0.05$ compared to control and E-CD-TPGS; $^{\$}p < 0.05$ compared to control, Eze, Ezentia and Eze-ND (Two way ANOVA followed by Bonferroni's multiple comparison test).

Figure 8.17. Percent decrease in plasma VLDL levels achieved by various treatment groups.



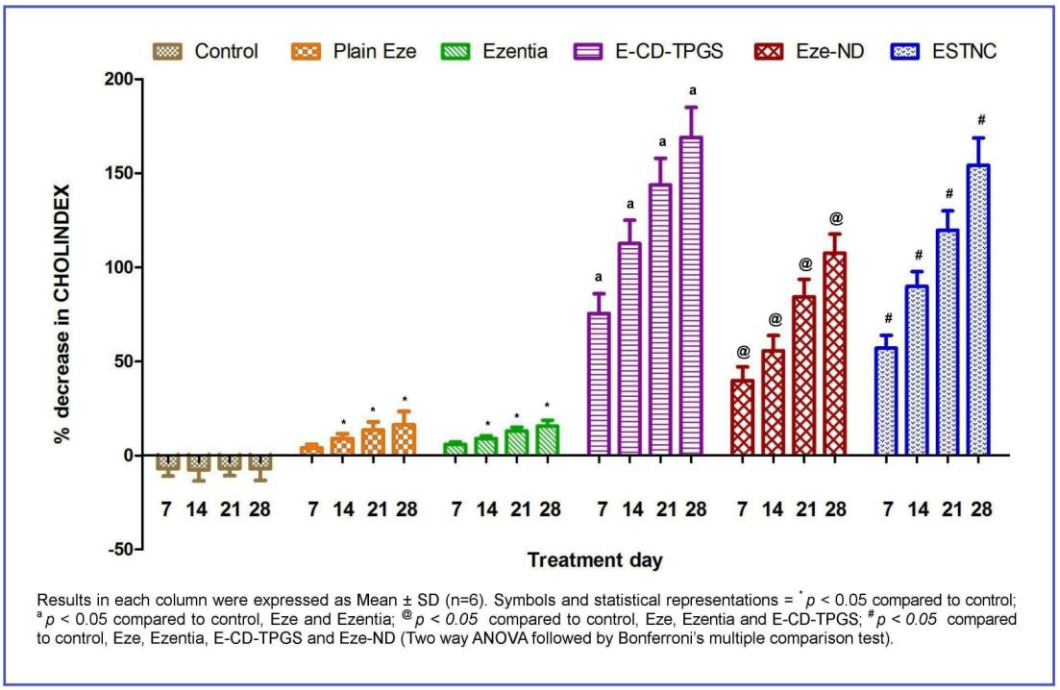
Results in each column were expressed as Mean ± SD (n=6). Symbols and statistical representations = $^{*}p < 0.05$ compared to control; $^{a}p < 0.05$ compared to control, Eze and Ezentia; $^{@}p < 0.05$ compared to control, Eze, Ezentia and E-CD-TPGS; $^{\#}p < 0.05$ compared to control, Eze, Ezentia and Eze-ND (Two way ANOVA followed by Bonferroni's multiple comparison test).

Figure 8.18. Percent decrease in Atherogenic Coefficient achieved by various treatment groups.



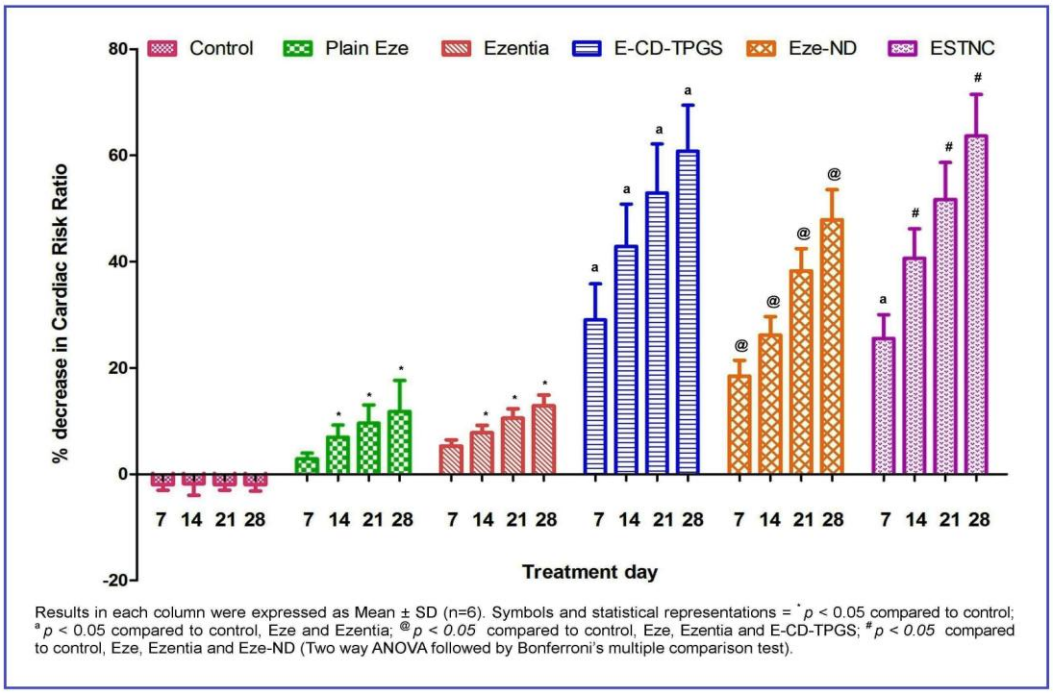
Results in each column were expressed as Mean ± SD (n=6). Symbols and statistical representations = $^{*}p < 0.05$ compared to control; $^{a}p < 0.05$ compared to control, Eze and Ezentia; $^{@}p < 0.05$ compared to control and E-CD-TPGS; $^{\#}p < 0.05$ compared to control, Eze, Ezentia and E-CD-TPGS; $^{\$}p < 0.05$ compared to control, Eze, Ezentia and Eze-ND (Two way ANOVA followed by Bonferroni's multiple comparison test).

Figure 8.19. Percent decrease in Atherogenic Index of Plasma achieved by various treatment groups.



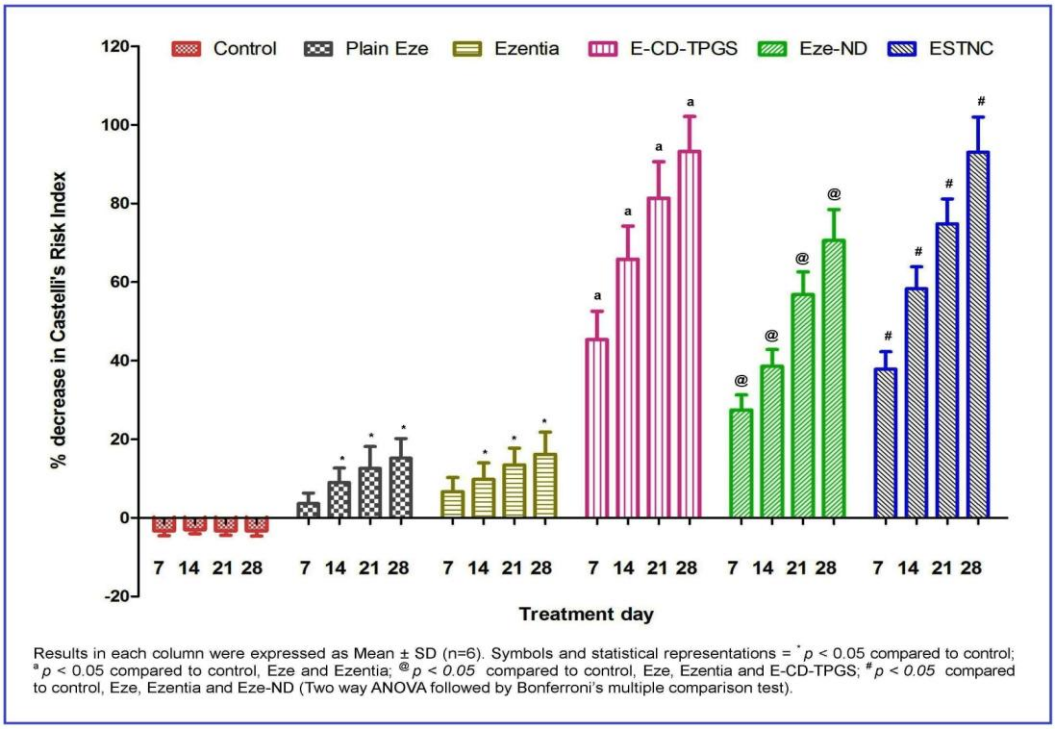
Results in each column were expressed as Mean ± SD (n=6). Symbols and statistical representations = $^{*}p < 0.05$ compared to control; $^{a}p < 0.05$ compared to control, Eze and Ezentia; $^{@}p < 0.05$ compared to control, Eze, Ezentia and E-CD-TPGS; $^{\#}p < 0.05$ compared to control, Eze, Ezentia, E-CD-TPGS and Eze-ND (Two way ANOVA followed by Bonferroni's multiple comparison test).

Figure 8.20. Percent decrease in CHOLINDEX achieved by various treatment groups.



Results in each column were expressed as Mean ± SD (n=6). Symbols and statistical representations = $^{*}p < 0.05$ compared to control; $^{a}p < 0.05$ compared to control, Eze and Ezentia; $^{@}p < 0.05$ compared to control, Eze, Ezentia and E-CD-TPGS; $^{\#}p < 0.05$ compared to control, Eze, Ezentia and Eze-ND (Two way ANOVA followed by Bonferroni's multiple comparison test).

Figure 8.21. Percent decrease in Cardiac Risk Ratio or Castelli's Risk Index I achieved by various treatment groups.



Results in each column were expressed as Mean ± SD (n=6). Symbols and statistical representations = $^{*}p < 0.05$ compared to control; $^{a}p < 0.05$ compared to control, Eze and Ezentia; $^{@}p < 0.05$ compared to control, Eze, Ezentia and E-CD-TPGS; $^{\#}p < 0.05$ compared to control, Eze, Ezentia and Eze-ND (Two way ANOVA followed by Bonferroni's multiple comparison test).

Figure 8.22. Percent decrease in Castelli's Risk Index II achieved by various treatment groups.

The most widely observed order was Control < Eze < Ezentia < Eze-ND < E-CD-TPGS ≈ ESTNC for the various lipid profile parameters and the AIs studied. It may be understood that E-CD-TPGS, because of the presence of HPBCD and TPGS, a cholesterol homeostasis maintaining agent and a P-gp inhibitor, respectively, could have synergistically favored the hypocholesterolemic activity of the P-gp substrate, Eze. In case of ESTNC, the nanosize and the presence of TPGS, a P-gp inhibitor, could have successfully enhanced the hypocholesterolemic activity of the P-gp substrate, Eze, by presenting the drug in a highly solubilized form in blood and aided in its localization at its site of action, respectively.

8.3.4.3 Dose reduction efficiency study

The dose reduction efficiency was studied in two phases. In the first phase, plain Eze and Ezentia treatment groups received 1 mg/kg/day equivalent Eze and all the optimized formulations, E-CD-TPGS, Eze-ND and ESTNC-F8^{A} were dosed at 0.5 mg/kg/day equivalent Eze. In the second phase, plain Eze and Ezentia treatment groups received 1 mg/kg/day equivalent Eze, the optimized formulations, E-CD-TPGS and Eze-ND received 0.5 mg/kg/day equivalent Eze, and ESTNC-F8^{B} was dosed at 0.2 mg/kg/day equivalent Eze. As previously mentioned, the only difference between ESTNC F8^{A} and ESTNC F8^{B} was the dose studied for ESTNC F8.

The lipid profile and AIs management efficacies of pure Eze, Ezentia suspension and the optimized formulations in terms of lipid profile parameters and AIs were summarized in Tables, 8.11 to 8.20 and Figure 8.23 to 8.32. The gist of the order of performances in the dose reduction efficiency studies at the first phase and second phase was presented in Tables 8.21 and 8.22, respectively.

Table 8.11. Percent decrease in total plasma cholesterol levels.

Before dose reduction (all treatment groups other than control received 1 mg/kg/day equivalent Eze) - % decrease in TC						
Day/ Treatment	**Control**	**Eze**	**Ezentia®**	**E-CD-TPGS**	**Eze-ND**	**ESTNC - F8**
Day 7	-4±5.67	1.13±3.21	1.51±2.28	25.49±5.98	16.23±5.76	21.82±8.90
Day 14	-4.8±3.78	1.89±5.18	2.26±4.96	35.29±7.92	21.51±8.18	32.73±8.97
Day 21	-4±5.89	3.02±4.16	3.4±5.28	45.1±8.65	32.08±7.92	43.64±7.26
Day 28	-4±5.75	3.77±5.84	4.15±5.64	52.94±10.15	40.75±8.96	56.36±8.54

After half dose reduction of E-CD-TPGS, Eze-ND and ^ESTNC-F8 - % decrease in TC							
Day/ Treatment	**Control**	**Eze**	**Ezentia®**	**E-CD-TPGS**	**Eze-ND**	**ESTNC - F8^{A}**	**ESTNC - F8^{B}**
Day 7	-4±5.67	1.13±3.21	1.51±2.28	11.76±4.68^{a}	9.43±4.98^{a}	20±3.12$^{\#}$	6.91±3.56
Day 14	-4.8±3.78	1.89±5.18	2.26±4.96	17.65±5.06^{a}	9.43±2.22^{c}	29.09±3.98$^{\#}$	9.09±4.52
Day 21	-4±5.89	3.02±4.16	3.4±5.28	24.31±6.55^{a}	16.98±4.64^{a}	40±5.64$^{\#}$	12.36±4.96
Day 28	-4±5.75	3.77±5.84*	4.15±5.64*	32.16±6.84^{a}	21.51±5.72$^{@}$	52.73±6.88$^{\#}$	15.64±5.22

Results were expressed as Mean±SD (n=6) and only the results after half dose reduction were compared. ^ESTNC-F8^{A} and ESTNC-F8^{B} were dosed at 0.5 and 0.2 mg/kg/day equivalent Eze, respectively. Symbols and statistical representations = $^{*}p < 0.05$ compared to control; $^{a}p < 0.05$ compared to control, Eze and Ezentia; $^{@}p < 0.05$ compared to control, Eze, Ezentia and E-CD-TPGS; $^{c}p < 0.05$ compared to control, Eze and E-CD-TPGS; $^{\#}p < 0.05$ compared to control, Eze, Ezentia, E-CD-TPGS and Eze-ND (Two way ANOVA followed by Bonferroni's multiple comparison test).

Table 8.12. Percent decrease in plasma triglyceride levels.

Before dose reduction (all treatment groups other than control received 1 mg/kg/day equivalent Eze) - % decrease in TG							
Day/ Treatment	**Control**	**Eze**	**Ezentia®**	**E-CD-TPGS**	**Eze-ND**	**ESTNC - F8**	
Day 7	-2.00±2.74	2.00±2.11	2.39±2.26	11.25±4.86	6.38±3.48	10.42±5.92	
Day 14	-2.80±5.26	3.20±4.28	3.59±3.58	16.67±6.24	8.51±4.56	20.83±8.99	
Day 21	-2.00±4.42	4.00±3.14	4.38±3.94	20.83±7.16	10.64±3.96	25.00±7.68	
Day 28	-2.00±5.86	6.00±6.82	6.37±5.26	25.00±8.92	14.89±5.18	29.17±9.21	
After half dose reduction of E-CD-TPGS, Eze-ND and ^ESTNC-F8 - % decrease in TG							
Day/ Treatment	**Control**	**Eze**	**Ezentia®**	**E-CD-TPGS**	**Eze-ND**	**ESTNC - F8^{A}**	**ESTNC - F8^{B}**
Day 7	-2.00±2.74	2.00±2.11	2.39±2.26	6.25±2.82*	2.98±1.08	8.33±2.54^{b}	2.50±1.16
Day 14	-2.80±5.26	3.20±4.28*	3.59±3.58*	9.17±3.74^{a}	3.83±1.28*	18.75±3.16$^{\#}$	5.42±2.28
Day 21	-2.00±4.42	4.00±3.14*	4.38±3.94*	10.83±5.66^{b}	4.68±2.06^{c}	22.5±3.88$^{\#}$	6.25±2.62
Day 28	-2.00±5.86	6.00±6.82*	6.37±5.26*	13.75±6.04^{b}	7.23±2.44^{c}	26.25±5.44$^{\#}$	7.50±3.98

Results were expressed as Mean±SD (n=6) and only the results after half dose reduction were compared. ^ESTNC-F8^{A} and ESTNC-F8^{B} were dosed at 0.5 and 0.2 mg/kg/day equivalent Eze, respectively. Symbols and statistical representations = $^{*}p < 0.05$ compared to control; $^{a}p < 0.05$ compared to control and Eze; $^{b}p < 0.05$ compared to control, Eze and Ezentia; $^{c}p < 0.05$ compared to control and E-CD-TPGS; $^{\#}p < 0.05$ compared to control, Eze, Ezentia, E-CD-TPGS and Eze-ND (Two way ANOVA followed by Bonferroni's multiple comparison test).

Table 8.13. Percent increase in plasma HDL levels.

Before dose reduction (all treatment groups other than control received 1 mg/kg/day equivalent Eze) - % increase in HDL						
Day/ Treatment	**Control**	**Eze**	**Ezentia®**	**E-CD-TPGS**	**Eze-ND**	**ESTNC - F8**
Day 7	2.00±2.97	1.82±1.02	4.00±2.32	5.00±3.56	2.73±1.46	5.00±6.72
Day 14	3.00±3.94	5.45±1.98	6.00±2.08	13.33±5.68	6.36±2.78	13.33±7.86
Day 21	2.00±5.28	7.27±3.42	8.00±3.56	16.67±7.26	10.00±5.97	16.67±6.47
Day 28	2.00±7.22	9.09±6.80	10.00±6.94	20.00±10.57	13.64±6.84	20.00±9.62

After half dose reduction of E-CD-TPGS, Eze-ND and ^ESTNC-F8 - % increase in HDL							
Day/ Treatment	**Control**	**Eze**	**Ezentia®**	**E-CD-TPGS**	**Eze-ND**	**ESTNC - F8^{A}**	**ESTNC - F8^{B}**
Day 7	2.00±2.97	1.82±1.02	4.00±2.32	2.50±0.88	0.91±0.23	3.33±0.76	1.67±0.74
Day 14	3.00±3.94	5.45±1.98	6.00±2.08	5.83±2.22	4.55±1.42	11.67±1.48$^{@}$	3.33±1.26
Day 21	2.00±5.28	7.27±3.42	8.00±3.56*	9.33±4.96*	6.36±2.38	15±3.84$^{\#}$	5.83±2.48
Day 28	2.00±7.22	9.09±6.80*	10.00±6.94*	10.83±5.14*	8.18±3.86*	18.33±5.22$^{\$}$	9.17±4.07

Results were expressed as Mean±SD (n=6) and only the results after half dose reduction were compared. ^ESTNC-F8^{A} and ESTNC-F8^{B} were dosed at 0.5 and 0.2 mg/kg/day equivalent Eze, respectively. Symbols and statistical representations = $^{*}p < 0.05$ compared to control; $^{@}p < 0.05$ compared to control, Eze, E-CD-TPGS and Eze-ND; $^{\#}p < 0.05$ compared to control, Eze, Ezentia and Eze-ND; $^{\$}p < 0.05$ compared to control, Eze, Ezentia, E-CD-TPGS and Eze-ND (Two way ANOVA followed by Bonferroni's multiple comparison test).

Table 8.14. Percent decrease in plasma LDL levels.

Before dose reduction (all treatment groups other than control received 1 mg/kg/day equivalent Eze) - % decrease in LDL						
Day/ Treatment	**Control**	**Eze**	**Ezentia®**	**E-CD-TPGS**	**Eze-ND**	**ESTNC - F8**
Day 7	-5.33±3.79	1.88±2.82	2.91±3.16	42.59±6.07	25.46±3.23	34.73±5.68
Day 14	-6.07±4.38	4.00±4.88	4.37±4.72	61.22±7.82	34.66±4.19	52.69±6.54
Day 21	-5.33±3.98	6.25±3.92	6.55±3.98	78.23±8.57	52.45±6.26	70.66±7.82
Day 28	-5.33±6.72	7.50±7.70	7.77±6.94	91.84±9.28	66.56±8.37	91.62±8.78
After half dose reduction of E-CD-TPGS, Eze-ND and ^ESTNC-F8 - % decrease in LDL						
Day/ Treatment	**Control**	**Eze**	**Ezentia®**	**E-CD-TPGS**	**Eze-ND**	**ESTNC - F8^{A}**	**ESTNC - F8^{B}**
Day 7	-5.33±3.79	1.88±2.82*	2.91±3.16*	19.39±2.87^{a}	14.79±1.68^{a}	31.74±2.62$^{\#}$	11.26±1.26
Day 14	-6.07±4.38	4.00±4.88*	4.37±4.72*	30.00±3.72^{a}	15.77±2.0$^{@}$	46.71±3.18$^{\#}$	14.61±1.80
Day 21	-5.33±3.98	6.25±3.92*	6.55±3.98*	42.45±4.56^{a}	28.40±2.78$^{@}$	64.79±4.24$^{\#}$	20.66±2.14
Day 28	-5.33±6.72	7.50±7.70*	7.77±6.94*	55.71±5.28^{a}	35.64±3.66$^{@}$	85.87±6.88$^{\#}$	26.89±2.44

Results were expressed as Mean±SD (n=6) and only the results after half dose reduction were compared. ^ESTNC-F8^{A} and ESTNC-F8^{B} were dosed at 0.5 and 0.2 mg/kg/day equivalent Eze, respectively. Symbols and statistical representations = $^{*}p < 0.05$ compared to control; $^{a}p < 0.05$ compared to control, Eze and Ezentia; $^{@}p < 0.05$ compared to control, Eze, Ezentia and E-CD-TPGS; $^{\#}p < 0.05$ compared to control, Eze, Ezentia, E-CD-TPGS and Eze-ND (Two way ANOVA followed by Bonferroni's multiple comparison test).

Table 8.15. Percent decrease in plasma VLDL levels.

Before dose reduction (all treatment groups other than control received 1 mg/kg/day equivalent Eze) - % decrease in VLDL						
Day/ Treatment	**Control**	**Eze**	**Ezentia®**	**E-CD-TPGS**	**Eze-ND**	**ESTNC - F8**
Day 7	-2.00±2.74	2.00±2.11	2.39±2.26	11.25±4.86	6.38±3.48	10.42±5.92
Day 14	-2.80±5.26	3.20±4.28	3.59±3.58	16.67±6.24	8.51±4.56	20.83±8.99
Day 21	-2.00±4.42	4.00±3.14	4.38±3.94	20.83±7.16	10.64±3.96	25.00±7.68
Day 28	-2.00±5.86	6.00±6.82	6.37±5.26	25.00±8.92	14.89±5.18	29.17±9.21

After half dose reduction of E-CD-TPGS, Eze-ND and ^ESTNC-F8 - % decrease in VLDL							
Day/ Treatment	**Control**	**Eze**	**Ezentia®**	**E-CD-TPGS**	**Eze-ND**	**ESTNC - F8^{A}**	**ESTNC - F8^{B}**
Day 7	-2.00±2.74	2.00±2.11	2.39±2.26	6.25±2.82*	2.98±1.08	8.33±2.54^{b}	2.50±1.16
Day 14	-2.80±5.26	3.20±4.28*	3.59±3.58*	9.17±3.74^{a}	3.83±1.28*	18.75±3.16$^{\#}$	5.42±2.28
Day 21	-2.00±4.42	4.00±3.14*	4.38±3.94*	10.83±5.66^{b}	4.68±2.06^{c}	22.5±3.88$^{\#}$	6.25±2.62
Day 28	-2.00±5.86	6.00±6.82*	6.37±5.26*	13.75±6.04^{b}	7.23±2.44^{c}	26.25±5.44$^{\#}$	7.50±3.98

Results were expressed as Mean±SD (n=6) and only the results after half dose reduction were compared. ^ESTNC-F8^{A} and ESTNC-F8^{B} were dosed at 0.5 and 0.2 mg/kg/day equivalent Eze, respectively. Symbols and statistical representations = $^{*}p < 0.05$ compared to control; $^{a}p < 0.05$ compared to control and Eze; $^{b}p < 0.05$ compared to control, Eze and Ezentia; $^{c}p < 0.05$ compared to control and E-CD-TPGS; $^{\#}p < 0.05$ compared to control, Eze, Ezentia, E-CD-TPGS and Eze-ND (Two way ANOVA followed by Bonferroni's multiple comparison test).

Table 8.16. Percent decrease in Atherogenic Coefficient.

Before dose reduction (all treatment groups other than control received 1 mg/kg/day equivalent Eze) - % decrease in AC						
Day/ Treatment	**Control**	**Eze**	**Ezentia®**	**E-CD-TPGS**	**Eze-ND**	**ESTNC - F8**
Day 7	-2.45±2.28	3.66±3.48	6.53±3.28	37.97±5.22	23.28±4.66	32.67±6.24
Day 14	-2.18±1.96	8.78±4.26	9.61±3.98	56.11±6.46	33.07±5.28	51.98±8.26
Day 21	-2.45±2.32	12.11±5.68	13.01±4.78	69.23±7.28	48.27±6.54	66.11±9.38
Day 28	-2.45±3.41	14.88±6.16	15.86±6.56	79.49±9.56	60.40±8.79	81.40±10.98

After half dose reduction of E-CD-TPGS, Eze-ND and ^ESTNC-F8 - % decrease in AC							
Day/ Treatment	**Control**	**Eze**	**Ezentia®**	**E-CD-TPGS**	**Eze-ND**	**ESTNC - F8^{A}**	**ESTNC - F8^{B}**
Day 7	-2.45±2.28	3.66±3.48	6.53±3.28	18.2±4.28^{b}	12.93±3.86^{a}	28.88±3.54$^{\#}$	10.79±3.24
Day 14	-2.18±1.96	8.78±4.26*	9.61±3.98*	29.01±2.96^{b}	16.87±4.04$^{@}$	46.69±3.72$^{\#}$	15.38±3.76
Day 21	-2.45±2.32	12.11±5.68*	13.01±4.78*	40.24±5.44^{b}	27.7±4.88$^{@}$	61.17±4.88$^{\#}$	21.99±4.12
Day 28	-2.45±3.41	14.88±6.16*	15.86±6.56*	50.72±6.20^{b}	34.63±5.12$^{@}$	76.81±5.22$^{\#}$	29.06±4.44

Results were expressed as Mean±SD (n=6) and only the results after half dose reduction were compared. ^ESTNC-F8^{A} and ESTNC-F8^{B} were dosed at 0.5 and 0.2 mg/kg/day equivalent Eze, respectively. Symbols and statistical representations = $^{*}p < 0.05$ compared to control; $^{a}p < 0.05$ compared to control and Eze; $^{b}p < 0.05$ compared to control, Eze and Ezentia; $^{@}p < 0.05$ compared to control, Eze, Ezentia and E-CD-TPGS; $^{\#}p < 0.05$ compared to control, Eze, Ezentia, E-CD-TPGS and Eze-ND (Two way ANOVA followed by Bonferroni's multiple comparison test).

Table 8.17. Percent decrease in Atherogenic Index of Plasma.

Before dose reduction (all treatment groups other than control received 1 mg/kg/day equivalent Eze) - % decrease in AIP						
Day/ Treatment	**Control**	**Eze**	**Ezentia®**	**E-CD-TPGS**	**Eze-ND**	**ESTNC - F8**
Day 7	0.00±0.24	2.52±3.22	3.93±3.26	12.13±3.68	6.39±2.28	11.45±3.82
Day 14	0.12±0.36	5.66±3.86	5.87±4.28	22.18±5.98	10.37±3.54	25.88±6.84
Day 21	0.00±0.12	7.33±5.14	7.55±4.84	27.97±6.26	14.31±4.26	31.87±7.22
Day 28	0.00±0.14	9.83±7.76	9.99±5.44	33.90±7.82	19.91±5.48	38.03±8.94

After half dose reduction of E-CD-TPGS, Eze-ND and ^ESTNC-F8 - % decrease in AIP							
Day/ Treatment	**Control**	**Eze**	**Ezentia®**	**E-CD-TPGS**	**Eze-ND**	**ESTNC - F8^{A}**	**ESTNC - F8^{B}**
Day 7	0.00±0.24	2.52±3.22	3.93±3.26	6.44±2.84*	2.71±0.68	8.64±1.24$^{\#}$	3.02±1.28
Day 14	0.12±0.36	5.66±3.86*	5.87±4.28*	11.02±3.16^{a}	5.75±1.96$^{@}$	22.94±1.88$^{\$}$	6.38±2.92
Day 21	0.00±0.12	7.33±5.14*	7.55±4.84*	14.71±3.99^{a}	7.55±2.08$^{@}$	28.47±2.67$^{\$}$	8.75±3.76
Day 28	0.00±0.14	9.83±7.76*	9.99±5.44*	18.09±4.02^{a}	10.59±3.84$^{@}$	34.11±2.92$^{\$}$	11.95±3.98

Results were expressed as Mean±SD (n=6) and only the results after half dose reduction were compared. ^ESTNC-F8^{A} and ESTNC-F8^{B} were dosed at 0.5 and 0.2 mg/kg/day equivalent Eze, respectively. Symbols and statistical representations = $^{*}p < 0.05$ compared to control; $^{a}p < 0.05$ compared to control, Eze and Ezentia; $^{@}p < 0.05$ compared to control and E-CD-TPGS; $^{\#}p < 0.05$ compared to control, Eze and Eze-ND; $^{\$}p < 0.05$ compared to control, Eze, Ezentia, E-CD-TPGS and Eze-ND (Two way ANOVA followed by Bonferroni's multiple comparison test).

Table 8.18. Percent decrease in CHOLINDEX.

Before dose reduction (all treatment groups other than control received 1 mg/kg/day equivalent Eze) - % decrease in CHOLINDEX						
Day/ Treatment	**Control**	**Eze**	**Ezentia®**	**E-CD-TPGS**	**Eze-ND**	**ESTNC - F8**
Day 7	-7.00±4.02	3.81±1.98	5.92±2.84	75.40±10.44	39.81±7.24	57.00±6.74
Day 14	-7.60±5.96	8.95±2.62	8.89±3.48	112.64±12.28	55.56±8.18	89.72±7.92
Day 21	-7.00±3.82	13.33±4.48	12.89±5.26	143.68±14.16	84.26±9.26	119.63±10.28
Day 28	-7.00±6.24	16.19±7.18	15.51±7.78	168.97±15.94	107.41±10.20	154.21±14.44

After half dose reduction of E-CD-TPGS, Eze-ND and ^ESTNC-F8 - % decrease in CHOLINDEX							
Day/ Treatment	**Control**	**Eze**	**Ezentia®**	**E-CD-TPGS**	**Eze-ND**	**ESTNC - F8^{A}**	**ESTNC - F8^{B}**
Day 7	-7.00±4.02	3.81±1.98*	5.92±2.84*	34.48±6.22^{a}	22.78±4.22$^{@}$	51.40±4.54$^{\#}$	18.50±4.06
Day 14	-7.60±5.96	8.95±2.62*	8.89±3.48*	54.71±6.78^{a}	26.11±3.98$^{@}$	79.44±4.92$^{\#}$	24.67±5.22
Day 21	-7.00±3.82	13.33±4.48*	12.89±5.26*	78.16±7.14^{a}	46.11±4.86$^{@}$	109.53±5.26$^{\#}$	35.51±5.77
Day 28	-7.00±6.24	16.19±7.18*	15.51±7.78*	101.61±7.76^{a}	57.96±5.17$^{@}$	144.3±6.22$^{\#}$	47.10±6.06

Results were expressed as Mean±SD (n=6) and only the results after half dose reduction were compared. ^ESTNC-F8^{A} and ESTNC-F8^{B} were dosed at 0.5 and 0.2 mg/kg/day equivalent Eze, respectively. Symbols and statistical representations = $^{*}p < 0.05$ compared to control; $^{a}p < 0.05$ compared to control, Eze and Ezentia; $^{@}p < 0.05$ compared to control, Eze, Ezentia and E-CD-TPGS; $^{\#}p < 0.05$ compared to control, Eze, Ezentia, E-CD-TPGS and Eze-ND (Two way ANOVA followed by Bonferroni's multiple comparison test).

Table 8.19. Percent decrease in Cardiac Risk Ratio or Castelli's Risk Index I.

Before dose reduction (all treatment groups other than control received 1 mg/kg/day equivalent Eze) - % decrease in CRI - I						
Day/ Treatment	**Control**	**Eze**	**Ezentia®**	**E-CD-TPGS**	**Eze-ND**	**ESTNC - F8**
Day 7	-1.96±1.08	2.90±1.06	5.30±2.86	29.04±6.78	18.45±2.98	25.54±4.46
Day 14	-1.75±2.21	6.96±2.28	7.80±3.44	42.91±7.94	26.21±3.46	40.64±5.52
Day 21	-1.96±1.04	9.59±3.42	10.55±4.26	52.94±9.22	38.25±4.22	51.69±6.98
Day 28	-1.96±1.22	11.79±5.86	12.86±4.98	60.78±8.67	47.86±5.67	63.64±7.82

After half dose reduction of E-CD-TPGS, Eze-ND and ^ESTNC-F8 - % decrease in CRI - I							
Day/ Treatment	**Control**	**Eze**	**Ezentia®**	**E-CD-TPGS**	**Eze-ND**	**ESTNC - F8^{A}**	**ESTNC - F8^{B}**
Day 7	-1.96±1.08	2.90±1.06	5.30±2.86	13.92±2.36^{b}	10.25±2.08^{a}	22.58±2.96$^{\#}$	8.44±1.64
Day 14	-1.75±2.21	6.96±2.28*	7.80±3.44*	22.19±3.21^{b}	13.37±2.42$^{@}$	36.5±3.58$^{\#}$	12.02±2.88
Day 21	-1.96±1.04	9.59±3.42*	10.55±4.26*	30.77±3.77^{b}	21.95±3.64$^{@}$	47.83±4.26$^{\#}$	17.19±4.24
Day 28	-1.96±1.22	11.79±5.86*	12.86±4.98*	38.79±4.22^{b}	27.45±3.96$^{@}$	60.05±5.22$^{\#}$	22.72±4.68

Results were expressed as Mean±SD (n=6) and only the results after half dose reduction were compared. ^ESTNC-F8^{A} and ESTNC-F8^{B} were dosed at 0.5 and 0.2 mg/kg/day equivalent Eze, respectively. Symbols and statistical representations = $^{*}p < 0.05$ compared to control; $^{a}p < 0.05$ compared to control and Eze; $^{b}p < 0.05$ compared to control, Eze and Ezentia; $^{@}p < 0.05$ compared to control, Eze, Ezentia and E-CD-TPGS; $^{\#}p < 0.05$ compared to control, Eze, Ezentia, E-CD-TPGS and Eze-ND (Two way ANOVA followed by Bonferroni's multiple comparison test).

Table 8.20. Percent decrease in Castelli's Risk Index II.

Before dose reduction (all treatment groups other than control received 1 mg/kg/day equivalent Eze) - % decrease in CRI - II							
Day/ Treatment	**Control**	**Eze**	**Ezentia®**	**E-CD-TPGS**	**Eze-ND**	**ESTNC - F8**	
Day 7	-3.27±1.26	3.63±2.68	6.65 ± 3.62	45.32±7.24	27.44±3.86	37.84±4.42	
Day 14	-2.98±1.06	8.97±3.72	9.78 ± 4.18	65.79±8.46	38.57±4.24	58.26±5.64	
Day 21	-3.27±1.18	12.61±5.55	13.48 ± 4.26	81.34±9.28	56.78±5.80	74.85±6.28	
Day 28	-3.27±1.34	15.21±4.98	16.15 ± 5.68	93.20±8.92	70.58±7.86	93.01±8.96	
After half dose reduction of E-CD-TPGS, Eze-ND and ^ESTNC-F8 - % decrease in CRI - II							
Day/ Treatment	**Control**	**Eze**	**Ezentia®**	**E-CD-TPGS**	**Eze-ND**	**ESTNC - F8^{A}**	**ESTNC - F8^{B}**
Day 7	-3.27±1.26	3.63±2.68*	6.65±3.62*	21.35±3.32^{a}	15.55±2.70$^{@}$	33.94±2.98$^{\#}$	12.71±2.88
Day 14	-2.98±1.06	8.97±3.72*	9.78±4.18*	33.86±3.87^{a}	19.43±3.02$^{@}$	52.27±3.52$^{\#}$	17.37±3.06
Day 21	-3.27±1.18	12.61±5.55*	13.48±4.26*	47.36±4.22^{a}	32.69±3.34$^{@}$	69.38±4.04$^{\#}$	25.03±3.84
Day 28	-3.27±1.34	15.21±4.98*	16.15±5.68*	60.04±5.64^{a}	40.51±4.16$^{@}$	88.06±6.66$^{\#}$	33.03±4.12

Results were expressed as Mean±SD (n=6) and only the results after half dose reduction were compared. ^ESTNC-F8^{A} and ESTNC-F8^{B} were dosed at 0.5 and 0.2 mg/kg/day equivalent Eze, respectively. Symbols and statistical representations = $^{*}p < 0.05$ compared to control; $^{a}p < 0.05$ compared to control, Eze and Ezentia; $^{@}p < 0.05$ compared to control, Eze, Ezentia and E-CD-TPGS; $^{\#}p < 0.05$ compared to control, Eze, Ezentia, E-CD-TPGS and Eze-ND (Two way ANOVA followed by Bonferroni's multiple comparison test).

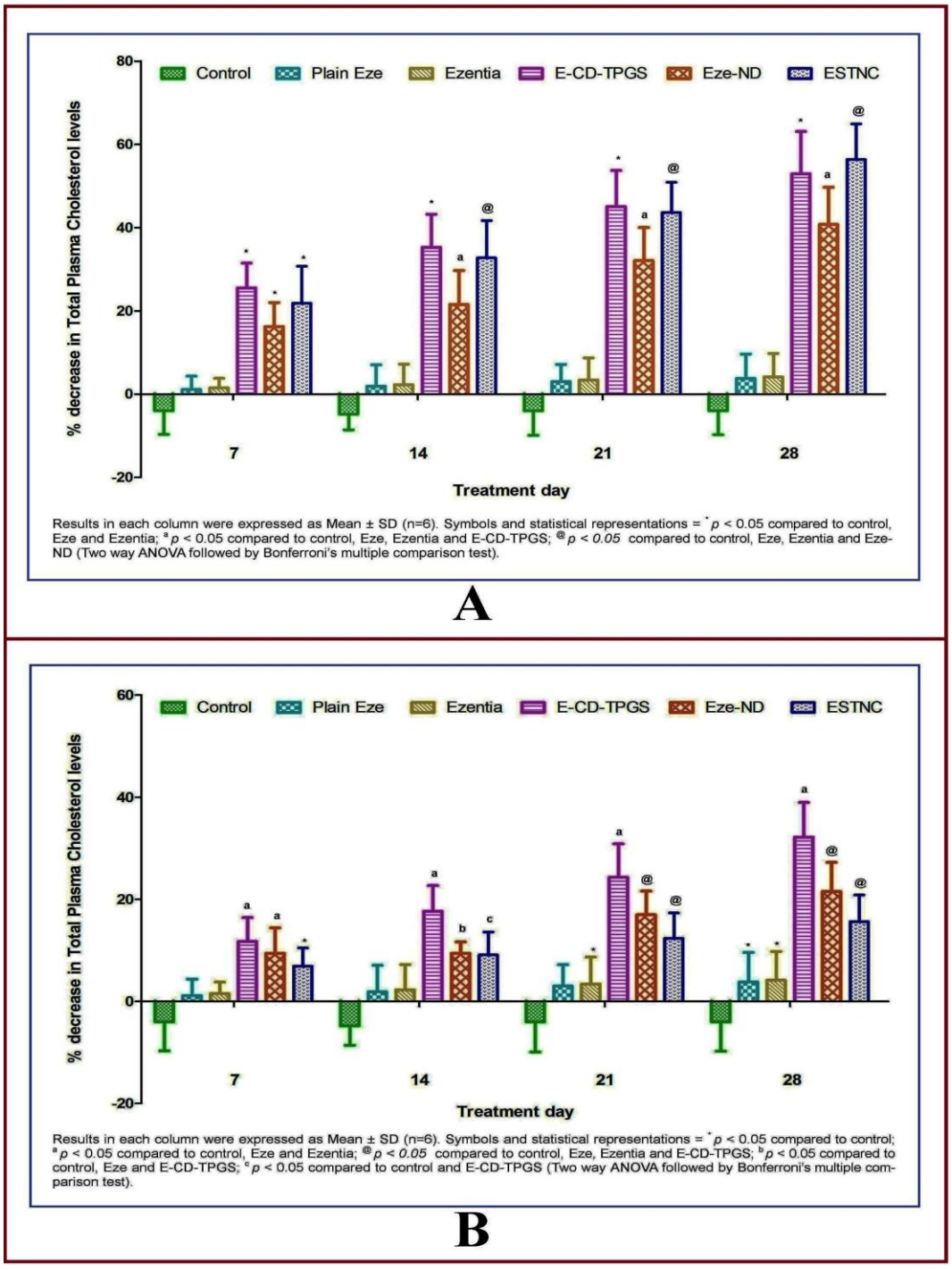


Figure 8.23. Percent decrease in total plasma cholesterol levels – A. Before dose reduction (standard group and test groups received 1 mg/kg/day equivalent Eze) and B. After dose reduction (E-CD-TPGS and Eze-ND were dosed at 0.5 mg/kg/day equivalent Eze; ESTNC-F8 was dosed at 0.2 mg/kg/day equivalent Eze while plain Eze and Ezentia treatment groups received 1 mg/kg/day equivalent Eze).

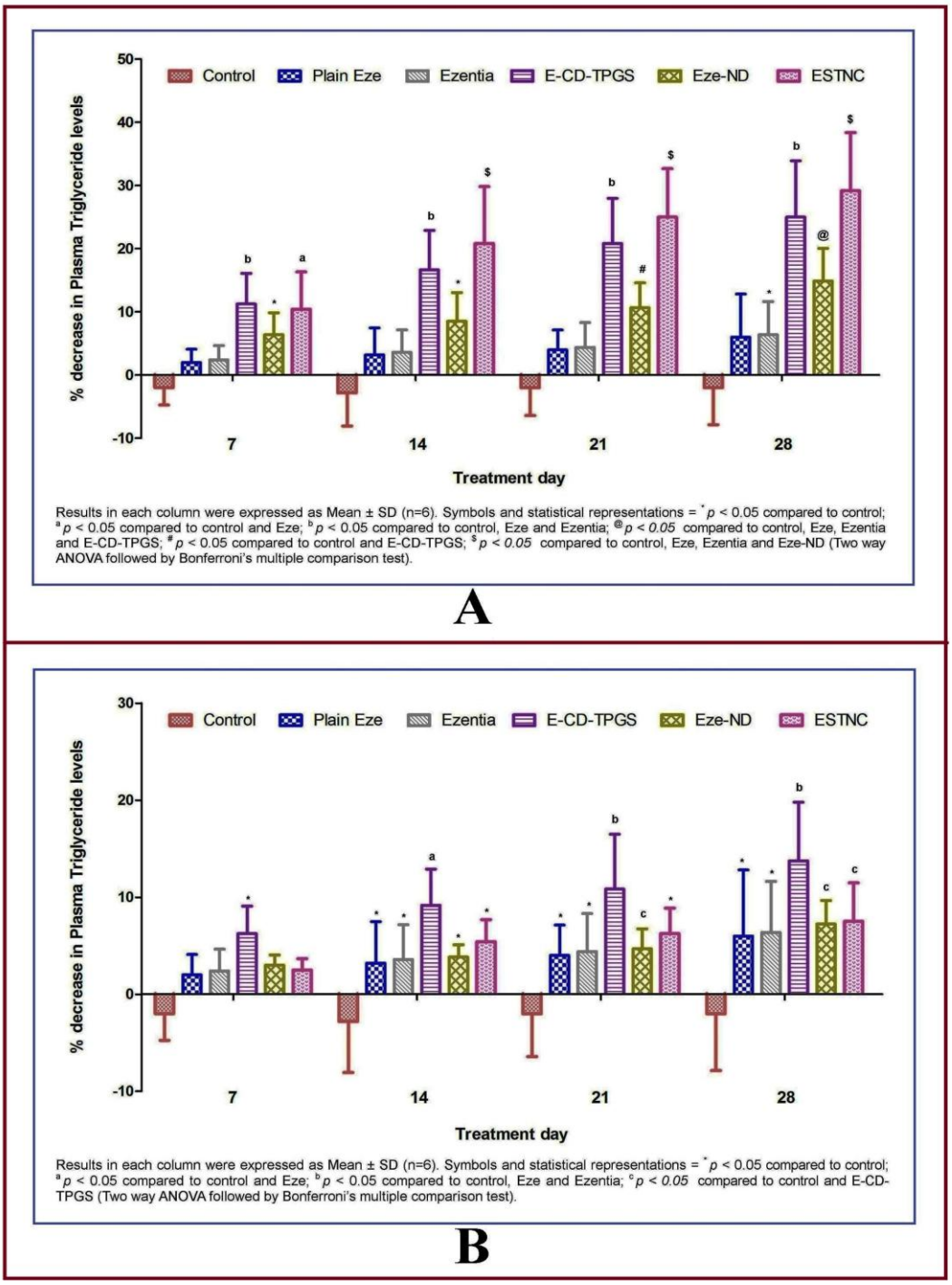


Figure 8.24. Percent decrease in plasma triglyceride levels – A. Before dose reduction (standard group and test groups received 1 mg/kg/day equivalent Eze) and B. After dose reduction (E-CD-TPGS and Eze-ND were dosed at 0.5 mg/kg/day equivalent Eze; ESTNC-F8 was dosed at 0.2 mg/kg/day equivalent Eze while plain Eze and Ezentia treatment groups received 1 mg/kg/day equivalent Eze).

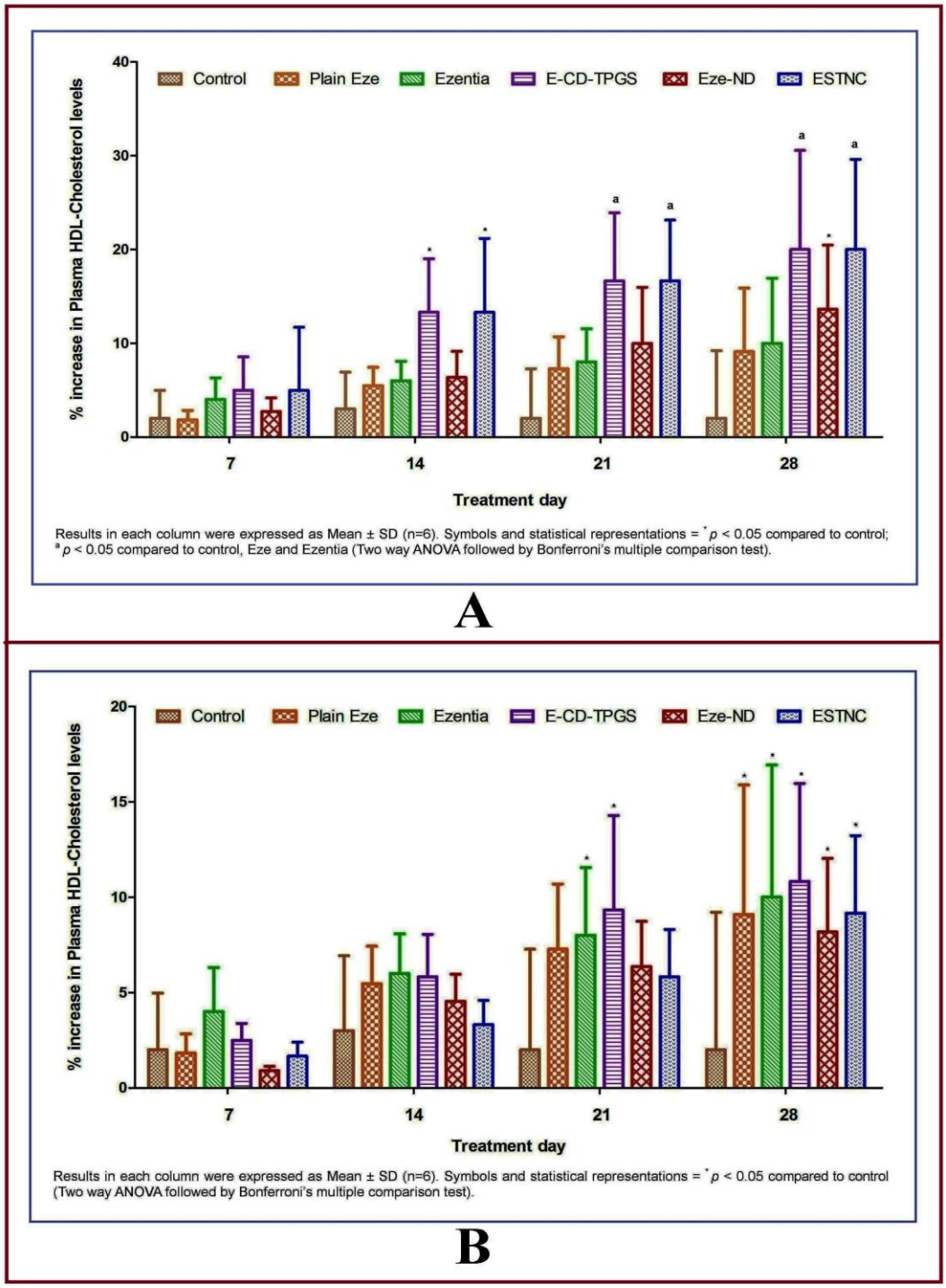


Figure 8.25. Percent increase in plasma HDL levels – A. Before dose reduction (standard group and test groups received 1 mg/kg/day equivalent Eze) and B. After dose reduction (E-CD-TPGS and Eze-ND were dosed at 0.5 mg/kg/day equivalent Eze; ESTNC-F8 was dosed at 0.2 mg/kg/day equivalent Eze while plain Eze and Ezentia treatment groups received 1 mg/kg/day equivalent Eze).

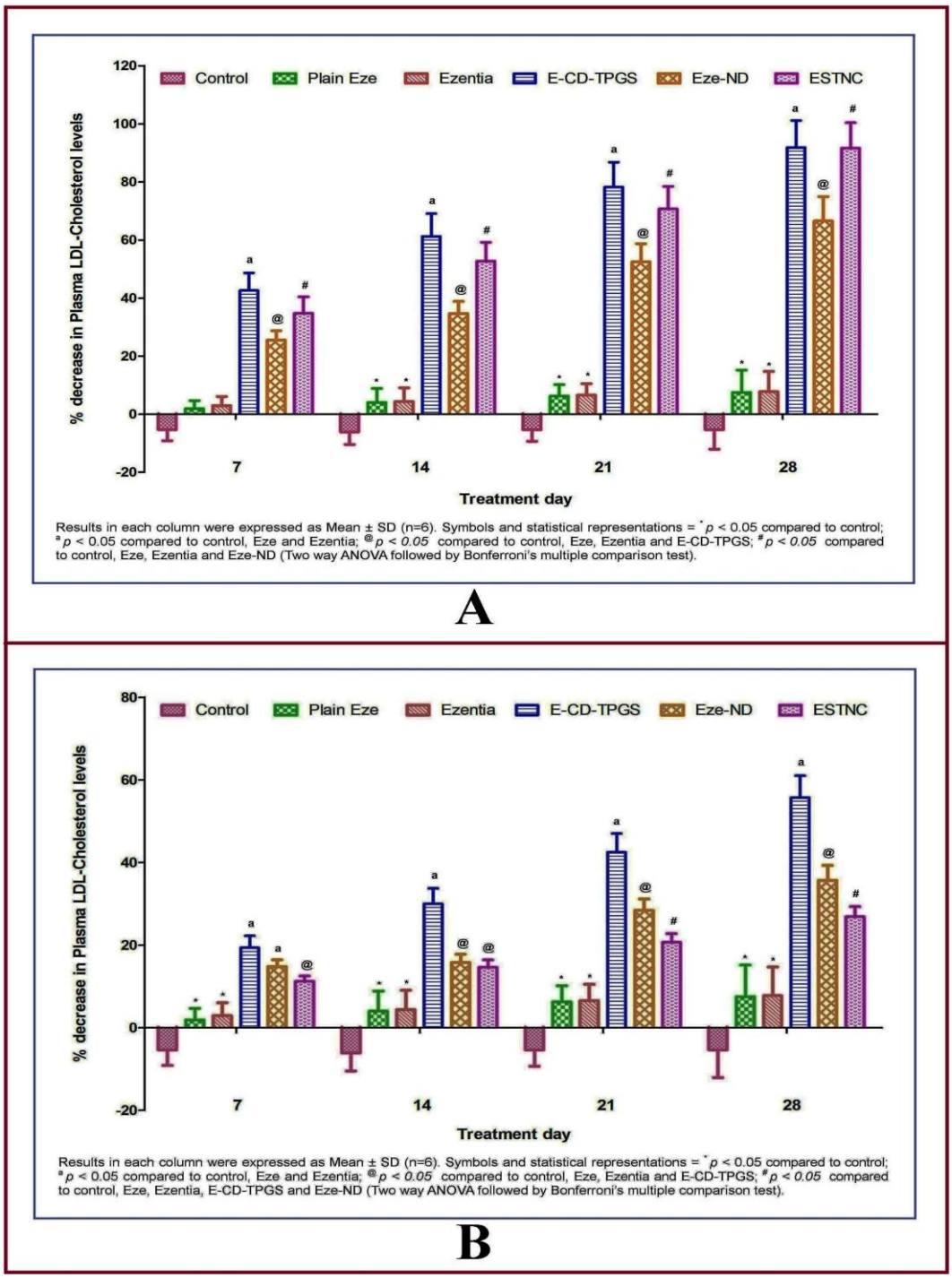


Figure 8.26. Percent decrease in plasma LDL levels – A. Before dose reduction (standard group and test groups received 1 mg/kg/day equivalent Eze) and B. After dose reduction (E-CD-TPGS and Eze-ND were dosed at 0.5 mg/kg/day equivalent Eze; ESTNC-F8 was dosed at 0.2 mg/kg/day equivalent Eze while plain Eze and Ezentia treatment groups received 1 mg/kg/day equivalent Eze).

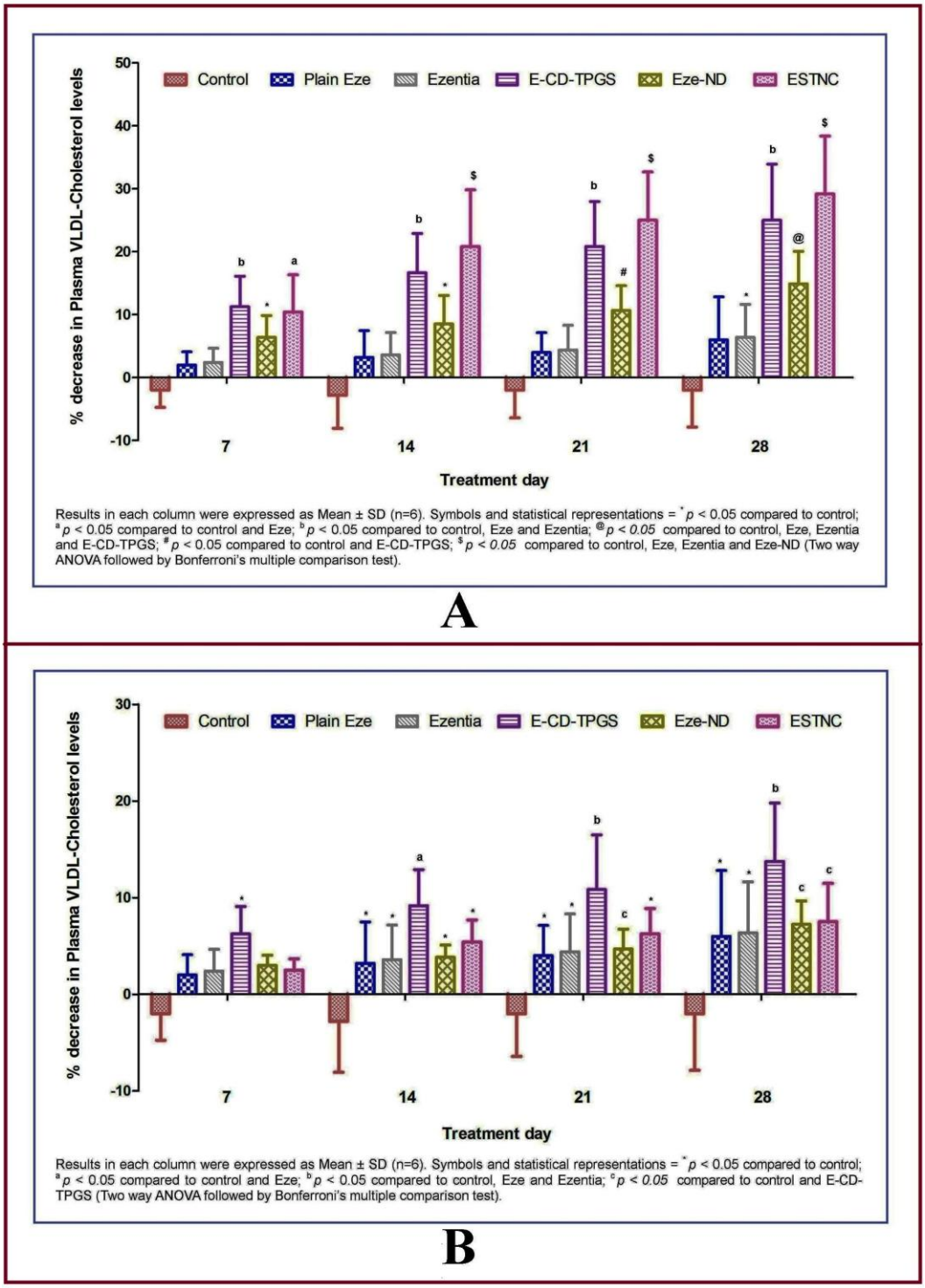


Figure 8.27. Percent decrease in plasma VLDL levels – A. Before dose reduction (standard group and test groups received 1 mg/kg/day equivalent Eze) and B. After dose reduction (E-CD-TPGS and Eze-ND were dosed at 0.5 mg/kg/day equivalent Eze; ESTNC-F8 was dosed at 0.2 mg/kg/day equivalent Eze while plain Eze and Ezentia treatment groups received 1 mg/kg/day equivalent Eze).

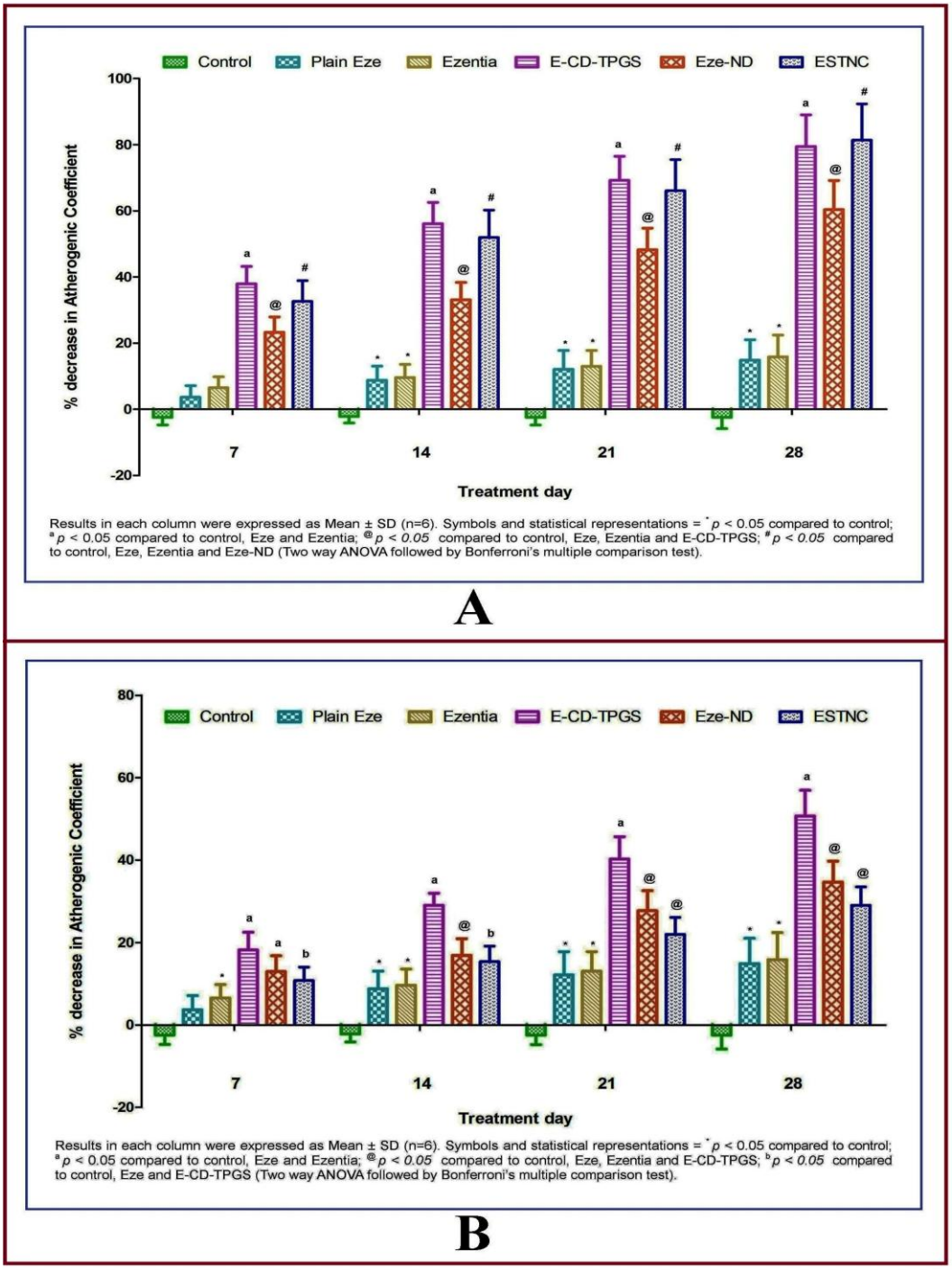


Figure 8.28. Percent decrease in Atherogenic Coefficient – A. Before dose reduction (standard group and test groups received 1 mg/kg/day equivalent Eze) and B. After dose reduction (E-CD-TPGS and Eze-ND were dosed at 0.5 mg/kg/day equivalent Eze; ESTNC-F8 was dosed at 0.2 mg/kg/day equivalent Eze while plain Eze and Ezentia treatment groups received 1 mg/kg/day equivalent Eze).

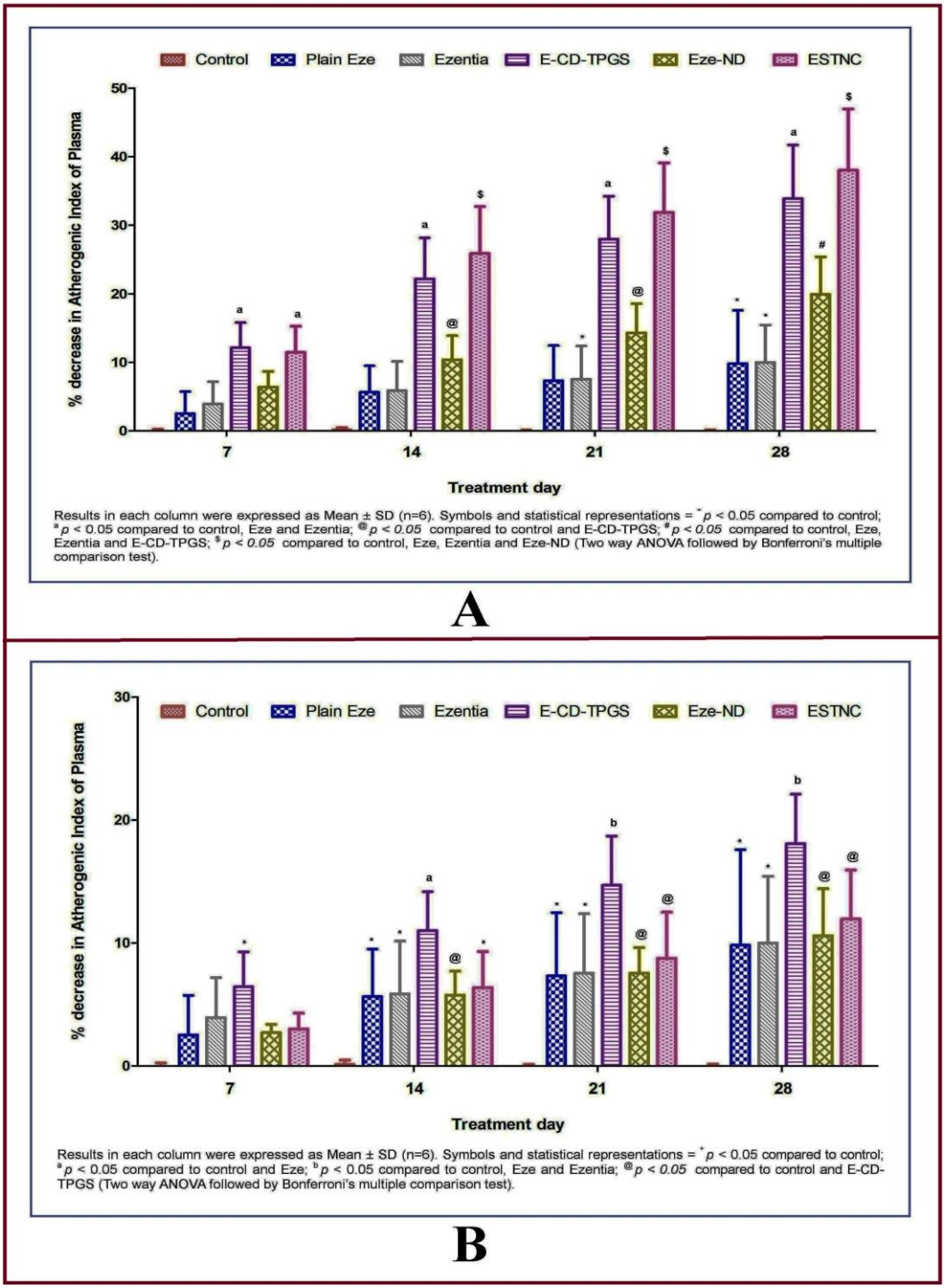


Figure 8.29. Percent decrease in Atherogenic Index of Plasma – A. Before dose reduction (standard group and test groups received 1 mg/kg/day equivalent Eze) and B. After dose reduction (E-CD-TPGS and Eze-ND were dosed at 0.5 mg/kg/day equivalent Eze; ESTNC-F8 was dosed at 0.2 mg/kg/day equivalent Eze while plain Eze and Ezentia treatment groups received 1 mg/kg/day equivalent Eze).

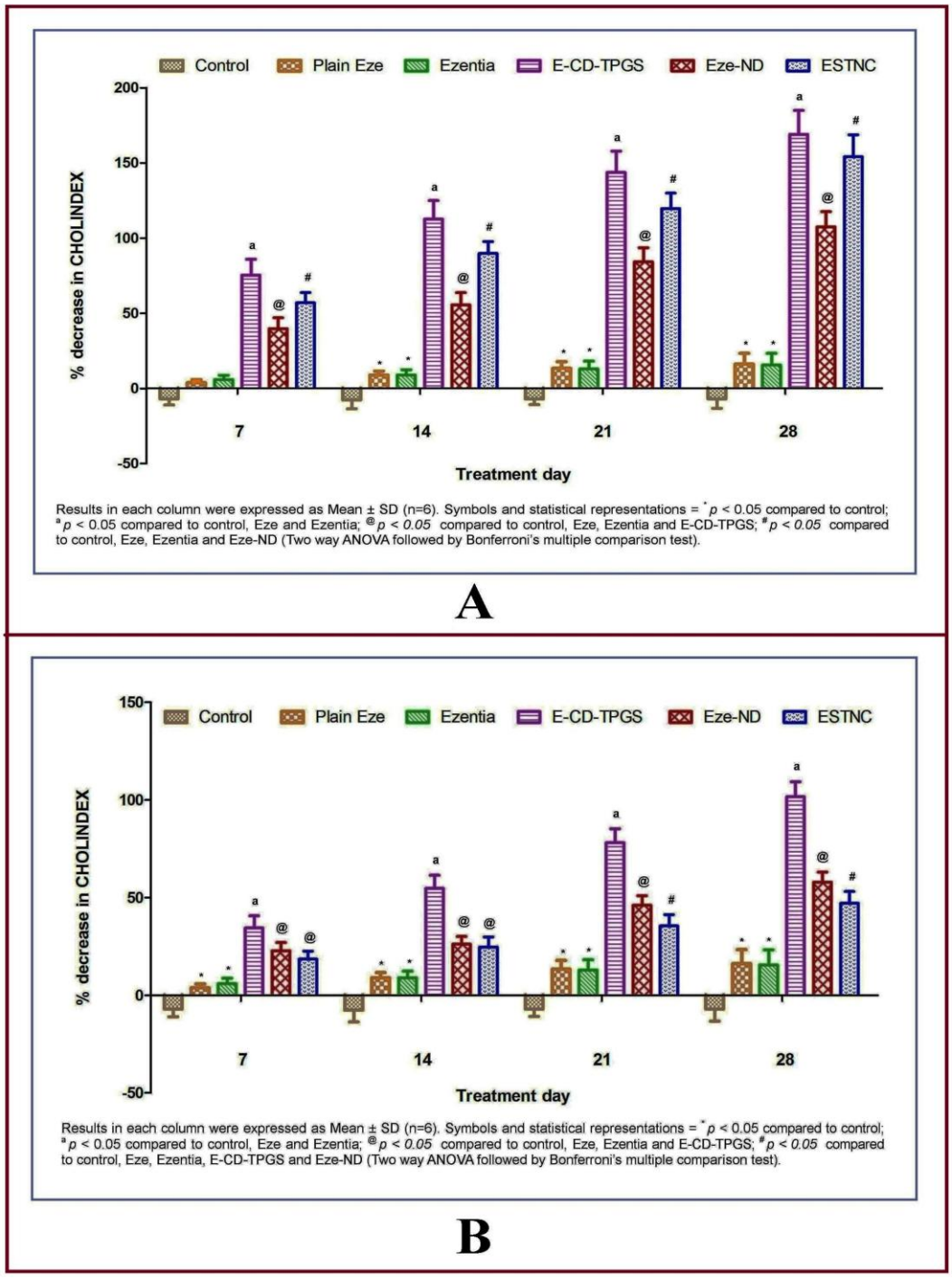


Figure 8.30. Percent decrease in CHOLINDEX – A. Before dose reduction (standard group and test groups received 1 mg/kg/day equivalent Eze) and B. After dose reduction (E-CD-TPGS and Eze-ND were dosed at 0.5 mg/kg/day equivalent Eze; ESTNC-F8 was dosed at 0.2 mg/kg/day equivalent Eze while plain Eze and Ezentia treatment groups received 1 mg/kg/day equivalent Eze).

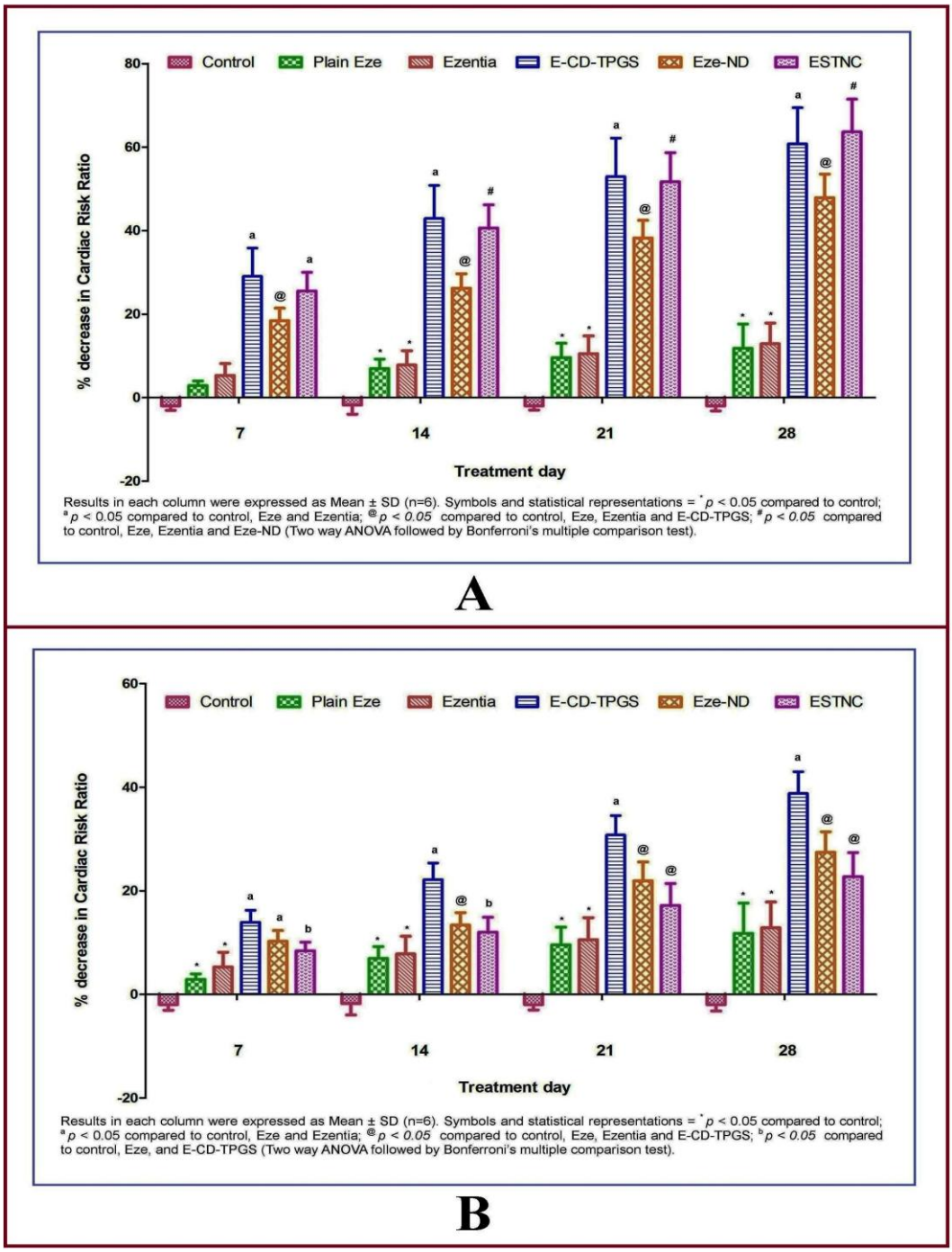


Figure 8.31. Percent decrease in Cardiac Risk Ratio or Castelli's Risk Index I – A. Before dose reduction (standard group and test groups received 1 mg/kg/day equivalent Eze) and B. After dose reduction (E-CD-TPGS and Eze-ND were dosed at 0.5 mg/kg/day equivalent Eze; ESTNC-F8 was dosed at 0.2 mg/kg/day equivalent Eze while plain Eze and Ezentia treatment groups received 1 mg/kg/day equivalent Eze).

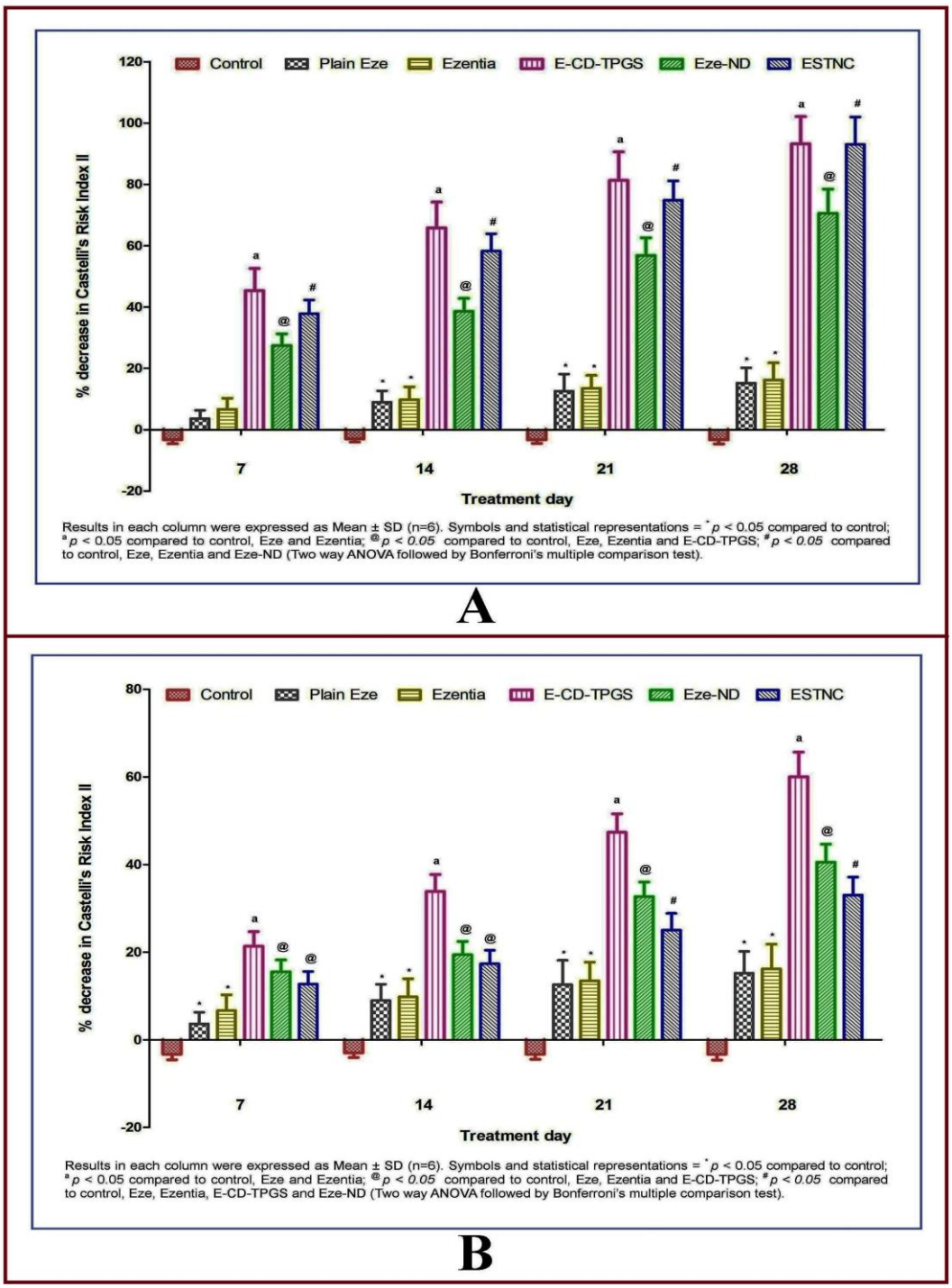


Figure 8.32. Percent decrease in Castelli's Risk Index II – A. Before dose reduction (standard group and test groups received 1 mg/kg/day equivalent Eze) and B. After dose reduction (E-CD-TPGS and Eze-ND were dosed at 0.5 mg/kg/day equivalent Eze; ESTNC-F8 was dosed at 0.2 mg/kg/day equivalent Eze while plain Eze and Ezentia treatment groups received 1 mg/kg/day equivalent Eze).

Table 8.21. First phase dose reduction efficiency studies – order of performance.

Percent reduction in TC:
All days: Control < Eze < Ezentia® < Eze-ND < E-CD-TPGS < ESTNC
Percent reduction in TG:
All days: Control < Eze < Ezentia® < Eze-ND < E-CD-TPGS < ESTNC
Percent enhancement in HDL:
Day 7: Eze-ND < Eze < Control < E-CD-TPGS < ESTNC < Ezentia®
Day 14: Control < Eze-ND < Eze < E-CD-TPGS < Ezentia® < ESTNC
Day 21, 28: Control < Eze-ND < Eze < Ezentia® < E-CD-TPGS < ESTNC
Percent reduction in LDL:
All days: Control < Eze < Ezentia® < Eze-ND < E-CD-TPGS < ESTNC
Percent reduction in VLDL:
All days: Control < Eze < Ezentia® < Eze-ND < E-CD-TPGS < ESTNC
Percent reduction in AC:
All days: Control < Eze < Ezentia® < Eze-ND < E-CD-TPGS < ESTNC
Percent reduction in AIP:
Day 7, 14: Control < Eze < Eze-ND < Ezentia® < E-CD-TPGS < ESTNC
Day 21: Control < Eze < Eze-ND ≈ Ezentia® < E-CD-TPGS < ESTNC
Day 28: Control < Eze < Ezentia® < Eze-ND < E-CD-TPGS < ESTNC
Percent reduction in CHOLINDEX:
Day 7: Control < Eze < Ezentia® < Eze-ND < E-CD-TPGS < ESTNC
Day 14, 21, 28: Control < Ezentia® < Eze < Eze-ND < E-CD-TPGS < ESTNC
Percent reduction in CRI 1:
All days: Control < Eze < Ezentia® < Eze-ND < E-CD-TPGS < ESTNC
Percent reduction in CRI 2:
All days: Control < Eze < Ezentia® < Eze-ND < E-CD-TPGS < ESTNC

Table 8.22. Second phase dose reduction efficiency studies – order of performance.

Percent reduction in TC:
All days: Control < Eze < Ezentia® < ESTNC < Eze-ND < E-CD-TPGS
Percent reduction in TG:
Day 7: Control < Eze < Ezentia® < ESTNC < Eze-ND < E-CD-TPGS
Day 14, 21, 28: Control < Eze < Ezentia® < Eze-ND < ESTNC < E-CD-TPGS
Percent enhancement in HDL:
Day 7: Eze-ND < ESTNC < Eze < Control < E-CD-TPGS < Ezentia®
Day 14: Control < ESTNC < Eze-ND < Eze < E-CD-TPGS < Ezentia®
Day 21: Control < ESTNC < Eze-ND < Eze < Ezentia® < E-CD-TPGS
Day 28: Control < Eze-ND < Eze < ESTNC < Ezentia® < E-CD-TPGS
Percent reduction in LDL:
All days: Control < Eze < Ezentia® < ESTNC < Eze-ND < E-CD-TPGS
Percent reduction in VLDL:
Day 7: Control < Eze < Ezentia® < ESTNC < Eze-ND < E-CD-TPGS
Day 14, 21, 28: Control < Eze < Ezentia® < Eze-ND < ESTNC < E-CD-TPGS
Percent reduction in AC:
All days: Control < Eze < Ezentia® < ESTNC < Eze-ND < E-CD-TPGS
Percent reduction in AIP:
Day 7: Control < Eze < Eze-ND < ESTNC < Ezentia® < E-CD-TPGS
Day 14: Control < Eze < Eze-ND < Ezentia® < ESTNC < E-CD-TPGS
Day 21: Control < Eze < Eze-ND ≈ Ezentia® < ESTNC < E-CD-TPGS
Day 28: Control < Eze < Ezentia® < Eze-ND < ESTNC < E-CD-TPGS
Percent reduction in CHOLINDEX:
Day 7: Control < Eze < Ezentia® < ESTNC < Eze-ND < E-CD-TPGS
Day 14, 21, 28: Control < Ezentia® < Eze < ESTNC < Eze-ND < E-CD-TPGS
Percent reduction in CRI 1:
All days: Control < Eze < Ezentia® < ESTNC < Eze-ND < E-CD-TPGS
Percent reduction in CRI 2:
All days: Control < Eze < Ezentia® < ESTNC < Eze-ND < E-CD-TPGS

The dose reduction studies were conducted with an intention to evaluate the possible dose reduction that may be offered by each of the optimized formulations without compromising on the efficacy levels exhibited by the pure drug or the marketed tablet product, Ezentia. The extent to which the dose was reduced for the optimized formulations was based on the pharmacokinetic performance of the formulations. Briefly, the following observations were drawn from the pharmacokinetic studies. The T_{max} of pure drug, Ezentia and Ezentia suspension was noted at 2 h, the T_{max} of E-CD-TPGS was observed at 1.5 h and that of Eze-ND and ESTNC F8 was recorded at 1 h. Eze-ND and ESTNC F8 reduced the T_{max} of pure Eze by half. Since Eze is already available as a once daily formulation in the market, the decrease in value of T_{max} observed in case of the optimized formulations over pure drug suspension might not serve to change the dosing frequency. However, the enhancement in oral bioavailability of the drug from the optimized formulations in comparison to suspension (as evident from the % RB values) might offer a possibility of considerable dose reduction of the drug when administered in the form of the developed optimized formulations, thus affecting the drug dosing regimen beneficially.

The parameters, C_{max}, $AUC_{0\text{-}24h}$, $AUMC_{0\text{-}24h}$ and $AUC_{0\text{-}\infty}$ were observed in the order, ESTNC F8 > Eze-ND > E-CD-TPGS > Ezentia suspension > pure drug suspension > Ezentia. Eze-ND improved the mean values of, C_{max} of Eze by 2.2 times, $AUC_{0\text{-}24h}$ by 1.6 times and $AUC_{0\text{-}\infty}$ of Eze by 2 times. The mean C_{max} of pure drug was improved by 2 times, the mean $AUC_{0\text{-}24h}$ and $AUC_{0\text{-}\infty}$ of pure drug were improved by 1.4 times by E-CD-TPGS. The mean C_{max} and $AUC_{0\text{-}24h}$ of Eze were improved by 3 times and the mean $AUC_{0\text{-}\infty}$ value of pure drug, was improved by 3.4 times by ESTNC-F8.

Based on the superior pharmacokinetic performance of the optimized formulations compared to the pure drug and the marketed tablet product, Ezentia, the efficacy or the pharmacodynamics of each of the optimized formulations was studied at doses half that of pure drug and Ezentia. Additionally, on account of the markedly superior pharmacokinetic performance exhibited by ESTNC-F8, its efficacy or the pharmacodynamics was studied not only at half dose but also at five times reduced dose compared to pure drug and Ezentia suspension.

The observed order of the studied lipid profile parameters and the AIs, when all the treatment groups received 1 mg/kg/day equivalent Eze, was, Control < Eze < Ezentia < Eze-ND < E-CD-TPGS ≈ ESTNC. At half dose reduction of each the optimized formulations, E-CD-TPGS, Eze-ND and ESTNC-F8 (0.5 mg/kg/day equivalent Eze), the order was Control < Eze < Ezentia < Eze-ND < E-CD-TPGS < ESTNC. During the second phase dose reduction efficiency study, the order was Control < Eze < Ezentia < ESTNC < Eze-ND < E-CD-TPGS which means after five times dose reduction also, ESTNC performed superior to either plain drug or marketed tablet product, Ezentia suspension.

Literature survey from the clinical pharmacology review report on Eze suggested the dose response relation exhibited by Eze as follows. The human dose of Eze is 10 mg and the degree of decrease in the plasma lipid levels were found to be directly related to the increase in dose upto 10 mg [http://www.accessdata.fda.gov/drugsatfda_docs/nda/2002/21445_Zetia_biopharmr_P1.pdf]. It was reported that doses above 10 mg failed to provide further benefit significantly. Human Eze dose of 10 mg/day is approximately equivalent to rat dose of 1 mg/kg/day. When the dose reduction study performances were keenly observed, it

may be noted that the efficacy of the optimized formulations, Eze-ND and E-CD-TPGS decreased more or less proportionally with the decrease in dose from 1 mg/kg/day to 0.5 mg/kg/day. The pharmacodynamic performance of the optimized formulation, ESTNC-F8 was insignificantly different ($p > 0.05$) on comparing its performance at 1 mg/kg/day and 0.5 mg/kg/day dose levels. Only further decrease in dose of ESTNC-F8 to 0.2 mg/kg/day showed significantly different and inferior pharmacodynamic performance ($p < 0.05$) compared to the formulation's own performance at 1 mg/kg/day and 0.5 mg/kg/day dose levels.

So, for ESTNC-F8, by extrapolating the studied rat dose to the human dose, it may be said that the degree of decrease in the plasma lipid levels may be directly related to the increase in dose upto 5 mg and that the doses above 5 mg failed to provide further benefit significantly. Interestingly, ESTNC-F8, even at five times dose reduction, performed superior to either plain drug or marketed tablet product, Ezentia suspension The USFDA considers an NC product as "new drug" because, its markedly superior and unique pharmacokinetic profile is not bioequivalent or comparable to any other solubilized form of the same drug, not even to the drug's own micronized form, administered at the same dosage [Singare et al., 2010]. This consideration was reestablished by the *in-vitro* and *in-vivo* performance of ESTNC F8.

In a nut shell, at reduced dose levels also, all the optimized formulations, Eze-ND, E-CD-TPGS and ESTNC F8 performed superior to both pure Eze and Ezenia suspension. Exceptionally superior dose reduction efficiency was offered by ESTNC F8. Eze-ND and E-CD-TPGS performed better than pure drug and marketed product even after half dose reduction. Outstandingly, ESTNC F8 performed better than pure drug and marketed product even at five times lower dose.

8.4 SUMMARY

From the research work discussed in the previous chapters, a pharmaceutical CoC, Eze-ND, a ternary CD complex, E-CD-TPGS and an NC formulation, ESTNC F8, were identified as potential formulations in improving the *in-vitro* and *in-vivo* performance of Eze and as effective for oral administration of the drug. In the current part of investigation, the *in-vitro* aqueous solubilities of Eze-ND, E-CD-TPGS and ESTNC F8 were compared. Similarly, the *in-vitro* dissolution, *in-vivo* pharmacokinetics and pharmacodynamics were studied for the comparative evaluation among the optimized formulations and against the commercial tablet. Also, the dose reduction efficiencies offered by each of the formulations were studied and reported.

The solubility of E-CD-TPGS was higher than pure Eze ($p < 0.05$) and the solubility results of Eze-ND and ESTNC were higher than pure Eze as well as E-CD-TPGS. However, the solubility values of Eze-ND and ESTNC were insignificantly different ($p < 0.05$).

While the pure drug could not dissolve till 80% during the 45 min dissolution study, the DE_{45} of pure Eze was significantly lower compared to E-CD-TPGS ($p < 0.05$). The DE_{45} of Eze-ND, ESTNC and Ezentia were significantly higher than E-CD-TPGS ($p < 0.05$). The DE_{45} values of Eze-ND, ESTNC and Ezentia when compared to each other were insignificantly different ($p > 0.05$). Similar performance was observed in all the three media which confirmed that the solubility and dissolution characteristics of Eze were independent of pH. The order of all the measured dissolution parameters did not differ with the change in the pH of dissolution medium, the $t_{80\%}$ and DE_{45} of none of the

formulations varied significantly ($p > 0.05$) with the change in the pH of dissolution medium.

The T_{max} was observed in the order, pure drug = Ezentia = Ezentia suspension > E-CD-TPGS > Eze-ND ≈ ESTNC F8. The T_{max} of pure drug, Ezentia and Ezentia suspension was noted at 2 h, the T_{max} of E-CD-TPGS was observed at 1.5 h and that of Eze-ND and ESTNC F8 was recorded at 1 h. Eze-ND and ESTNC F8 reduced the T_{max} of pure Eze by half. The parameters, C_{max}, $AUC_{0\text{-}24h}$, $AUMC_{0\text{-}24h}$ and $AUC_{0\text{-}\infty}$ were observed in the order, ESTNC F8 > Eze-ND > E-CD-TPGS > Ezentia suspension > pure drug suspension > Ezentia.

When all the treatment groups received 1 mg/kg/day equivalent Eze, the observed order of the studied lipid profile parameters and the AIs was Control < Eze < Ezentia < Eze-ND < E-CD-TPGS ≈ ESTNC. At half dose reduction of each the optimized formulations, E-CD-TPGS, Eze-ND and ESTNC-F8, 0.5 mg/kg/day equivalent Eze, the observed order was Control < Eze < Ezentia < Eze-ND < E-CD-TPGS < ESTNC. When the plain Eze and Ezentia treatment groups received 1 mg/kg/day equivalent Eze, the optimized formulations, E-CD-TPGS and Eze-ND received 0.5 mg/kg/day equivalent Eze, and ESTNC-F8 was dosed at 0.2 mg/kg/day equivalent Eze, the observed order was Control < Eze < Ezentia < ESTNC < Eze-ND < E-CD-TPGS. After five times dose reduction also, ESTNC performed superior to either plain drug or marketed tablet product, Ezentia suspension.

The present study successfully compared the *in-vitro* and *in-vivo* performances of the optimized formulations, Eze-ND, E-CD-TPGS and ESTNC F8. The markedly superior performance of ESTNC F8 may be ascribed to the nano size and the P-gp efflux inhibitory nature of the employed NC stabilizer, TPGS. The nanosize and wetting effect

of TPGS offered superior aqueous solubility and dissolution properties at *in-vitro* level. The solubility of ESTNC F8 was higher compared to pure drug as well as the optimized formulations, Eze-ND and E-CD-TPGS. The dissolution properties of ESTNC F8 were higher compared to pure drug and E-CD-TPGS. The pharmacokinetic and lipid lowering activity of ESTNC F8 were higher compared to pure drug, the optimized formulations, Eze-ND and E-CD-TPGS and also compared the marketed tablet product, Ezentia. The dose reduction efficiency studies suggested that Eze-ND and E-CD-TPGS performed better than pure drug and marketed product even at half dose reduction. Outstandingly, ESTNC F8 performed better than pure drug and marketed product even at five times lower dose. At half the dose of ESTNC F8, its pharmacodynamic profile was still significantly ($p < 0.05$) superior to not only pure drug and Ezentia but also compared to the optimized formulations, Eze-ND and E-CD-TPGS. The results indicated that the inhibition of P-gp efflux transport by TPGS facilitated higher extent of intestinal absorption and *in-vivo* performance of drug from ESTNC F8. It may be seen that the oral delivery of Eze as NCs is quite beneficial and ESTNC F8 may be viewed as rational, simplest, economic and most promising formulation in improving the *in-vitro* and *in-vivo* performance of the drug, with highly superior dose reduction efficiency.