Consolidated report for R&D Project

Project Title

"In-vivo hypocholesterolemic effect of bioconjugates of starch nanoparticles with gamma oryzanol and tocotrienols extracted from rice bran"

DBT Sanction Order No. & Date: No.BT/PR16804/NER/95/294/2015, Date: 10th Nov, 2016

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Section-A: Project Details

A1. Project Title: In-vivo hypocholesterolemic effect of bioconjugates of starch nanoparticles with gamma oryzanol and tocotrienols extracted from rice bran.

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A4. Institute:

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A6. Total Cost: 79.60 lakhs

A7. Duration: Three years+ 6 months extension

A8. Approved Objectives of the Project:

1. To extract two hypocholesterol compounds (γ-oryzanol and tocotrienols) from rice bran
2. To synthesize starch nanoparticle from broken rice
3. To study the interaction of γ-oryzanol and tocotrienols with starch nanoparticle (preparation of bionanoconjugate)
4. To determine anticholesterol effect of γ-oryzanol, tocotrienols and their bionanocaonjugates in vivo

A9. Specific Recommendations made by the Task Force (if any):

Recommendation of the First Meeting of the Technical Expert Committee on Knowledge Generation & Discovery Research, New Tools & Technologies held on 1st May, 2019 at NER-BPMC, New Delhi was "Very Good".

Section-B: Scientific and Technical Progress

B1.Progress made against the Approved Objectives, Targets & Timelines during the Reporting Period (1000-1500 words for interim reports; 2500-3500 words for final report; data must be included in the form of up to 3 figures and/or tables for interim reports; up to 7 figures and/or tables for final reports):

Introduction

A number of natural products contain valuable compounds having counter effect on hypercholesterolemia. Rice bran (RB), a by-product of rice processing, is a good source of vitamin E having γ-oryzanol and tocols (tocopherol+tocotrienols) components in it that exhibit hypocholesterolemic effect. The major problem with these compounds is their poor bioavailability. Primary concern with their effectiveness is whether these compounds when taken orally in the form of rice bran oil reach the target organ or not. We have hypothesized that bioconjugate of starch with tocotrienols and γ-oryzanol will have enhanced bioavailability than free form of tocotrienols and γ-oryzanol when tested in an animal model. Starch being biodegradable and nontoxic compound was chosen to act as a nano carrier. Therefore, the aim of our present study was to investigate the efficacy of prepared bioconjugate in animal model for anti-cholesterol effect.

Objectives

1. To extract two hypocholesterol compounds (γ-oryzanol and tocotrienols) from bran of rice varieties of Assam
2. To synthesize starch nanoparticle and starch tocotrienol bioconjugate
3. To study the interaction of γ-oryzanol and tocotrienols with starch nanoparticle (preparation of bionanoconjugate)
4. To determine anti-cholesterol effect of γ-oryzanol, tocotrienols and their bio-nano-conjugates in vivo.

Objective 1: To extract two hypocholesterol compounds (γ-oryzanol and tocotrienols) from bran of rice varieties of Assam

1.1 Rice bran characterization

Paddy was milled to 6% degree of polish and the bran (RB) that was obtained was heat-treated at 80 ^{o}C for 15 min to inactivate endogenous lipase in a hot air oven (JSGW 1210D/10, India), cooled, and kept at -20 ^{o}C in vacuum sealed low-density polyethylene (LDPE) pouch until

further use. RB was analysed for proximate principles, peroxide value, free fatty acid, and unsaponifiable matter analysis as per the method of AOAC. Proximate analysis (data not given) along with quality ensuring parameters like peroxide value, free fatty acid and unsaponifiable matters were evaluated. The peroxide value, free fatty acid and unsaponifiable matter of RB were 1.5±0.42 meq O_2/kg, 12.86±1.58 % and 3.2±0.42 %, respectively.

1.2 Selection of suitable solvent and HPLC detection

The solvent suitability for maximizing the recovery of tocotrienols (T3) from RB sample was done using ethanol, isopropanol, hexane and methanol in absolute concentration by conventional extraction method (**Fig. 1)**. Extract concentrate (RBE) was further subjected to double dispersive liquid liquid microextraction (DLLME) for the estimation of T3. The α-, δ- and γ-T3 fractions in RBE were quantified using HPLC (Ultimate 3000, Thermo Scientific). The calibration curves were obtained by varying the concentration ranging from 100-1000 μg mL^{-1} for γ-T3 and 100-1000 ng mL^{-1} for α- and δ-T3. Limit of detection (LOD) and the limit of quantification (LOQ) were calculated from the standard curve using the equations LOD = 3.3 SD/slope and LOQ = 10 SD/slope.

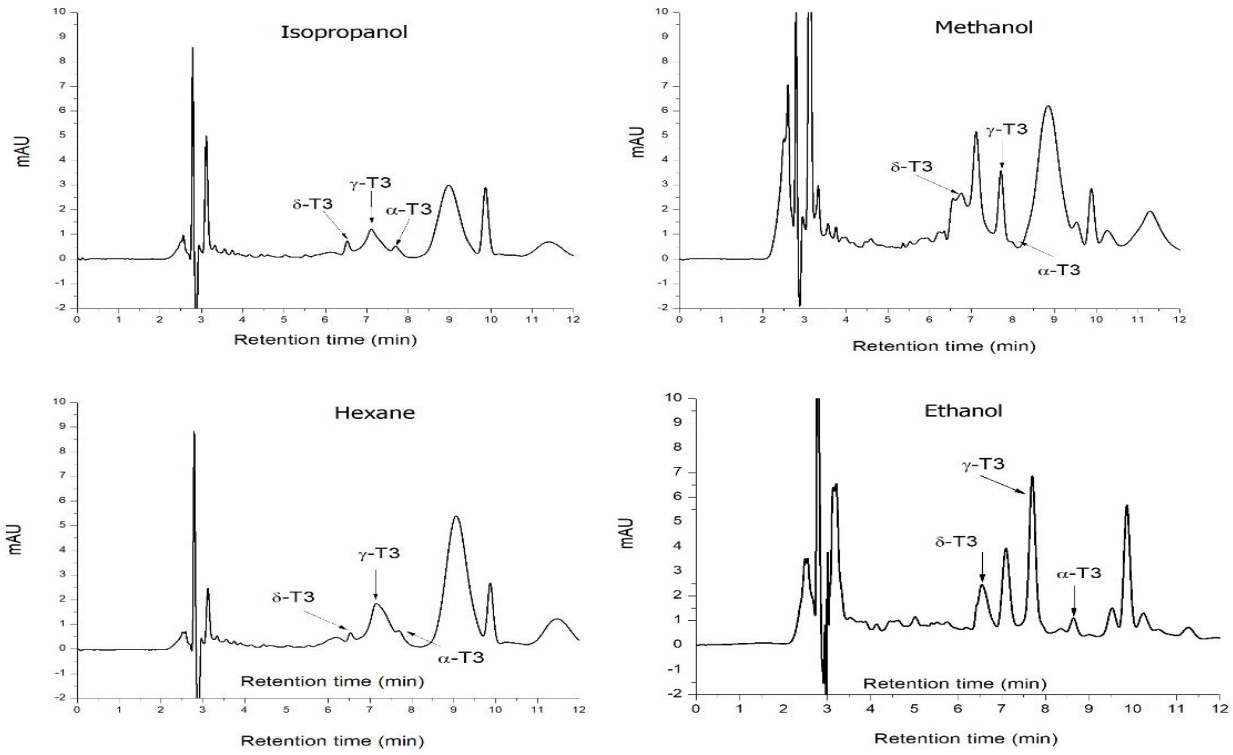

Fig. 1. HPLC chromatograms of tocotrienols extracted in different solvents.

1.3 Optimization of UAE based parameters for the extraction of T3 enriched RBE using RSM

RSM was employed for the optimization of the extraction process of RBE with maximum T3 content by UAE using a probe sonicator (U500, Takashi) with 13 mm probe diameter immersed 2 cm in the solute-solvent mix from the top. Three independent variables selected during ultrasonication were X_1= power (W), X_2= time (min) and X_3= solvent volume (mL) with the respective levels of **+1** (X_1=400 W, X_2=30 min, & X_3=8 mL), **0** (X_1=300 W, X_2=20 min & X_3=7 mL) and **-1** (X_1=200 W, X_2=10 min & X_3=6 mL) using Box Behnken experimental design with Design-Expert version 7.0.0 (Stat-Ease Inc., Minneapolis, MN, USA).

A total of seventeen experiments (**Table 1)** were conducted varying ultrasonication power time and solute:solvent concentration to optimize specific energy (J mL^{-1}) and T3 content (µg mL^{-1}). The least-square technique was used to calculate the coefficient of regression of the linear, intercept, and quadratic terms of the model. A second-order polynomial model was rendered by multiple linear regression analysis of the experimental data for the best fit model. The quadratic mathematical model expressions against various responses deduced from BBD showing the relationship between the independent and dependent variables are given below:

Specific energy (J mL^{-1}) = + 62.60 + 6.62* Power + 2.90* Time – 1.55* Solvent + 3.22* Power* Time + 0.04* Power* Solvent + 0.74* Time* Solvent + 1.68* $Power^2$ – 3.54* $Time^2$ - 0.55* $Solvent^2$ (1)

T3 (µg mL^{-1}) = +171.63 – 24.77* Power – 4.25* Time – 0.43* Solvent – 6.70* Power*Time – 4.61* Power* Solvent + 1.16* Time* Solvent – 6.60* $Power^2$ + 5.50* $Time^2$ – 1.58* $Solvent^2$ (2)

The 3D-response surface plots showing the interaction effects of three independent variables on the whole model for different responses were obtained (**data not presented**). The significance of the model as well as for linear, interaction and quadratic terms of the response variables were deduced from values of *p*, F and R^2 (**data not presented**). Probability values (*p*-value) revealed that the respective models had significant ($p<0.05$) effects on the responses. The lack of fit was non-significant in each case, which is desirable.

1.4 Optimum reaction conditions and model verification

At the optimal condition of 200 W power, 30 min exposure time, and 7.2 mL solvent volume, the experimental responses were 52.38±0.14 J mL^{-1} and 199.34±0.63 µg mL^{-1} for specific energy and T3 concentrations, respectively. HPLC chromatograms **(Fig.2**) revealed that the concentration of α-, δ- and γ-T3 in the RBE obtained after the optimized conditions was 40.43±1.52, 33.09±1.87, and 125.82±0.83 µg mL^{-1}, respectively. Predicted values are in agreement with the experimental values and no significant difference was observed ($p>0.05$). From the experiment, ultrasonication can be considered as a promising technique for the extraction of T3 enriched RBE at low power intensity.

Table 1. Box-Behnken design with the experimental responses for specific energy and T3 concentration in RBE.

Run	A	B	C	Power (W)	Time (min)	Solvent (mL)	Specific energy (J mL^{-1})	T3 concentration in RBE (µg mL^{-1})			
								α-T3	δ-T3	γ-T3	Total
1	-1	-1	0	200	10	7	54.86	52.45	42.95	94.83	190.24
2	1	-1	0	400	10	7	61.33	32.18	35.14	88.29	155.62
3	-1	1	0	200	30	7	53.72	49.67	40.60	108.60	198.87
4	1	1	0	400	30	7	73.07	27.83	30.06	79.54	137.43
5	-1	0	-1	200	20	6	57.91	47.78	36.98	98.48	183.24
6	1	0	-1	400	20	6	71.40	28.62	31.54	81.27	141.42
7	-1	0	1	200	20	8	55.99	48.72	39.92	106.09	194.72
8	1	0	1	400	20	8	69.64	31.40	25.55	77.51	134.46
9	0	-1	-1	300	10	6	58.30	46.70	38.35	99.75	184.81
10	0	1	-1	300	30	6	63.11	43.18	32.51	94.58	170.27
11	0	-1	1	300	10	8	52.44	46.01	40.03	92.48	178.53
12	0	1	1	300	30	8	60.22	35.82	38.49	94.31	168.62
13	0	0	0	300	20	7	63.99	35.54	32.95	101.77	170.26
14	0	0	0	300	20	7	61.00	37.68	32.07	99.70	169.45
15	0	0	0	300	20	7	61.61	35.48	34.39	97.82	167.69
16	0	0	0	300	20	7	64.38	38.04	39.74	98.87	176.65
17	0	0	0	300	20	7	62.02	36.04	38.24	99.84	174.12

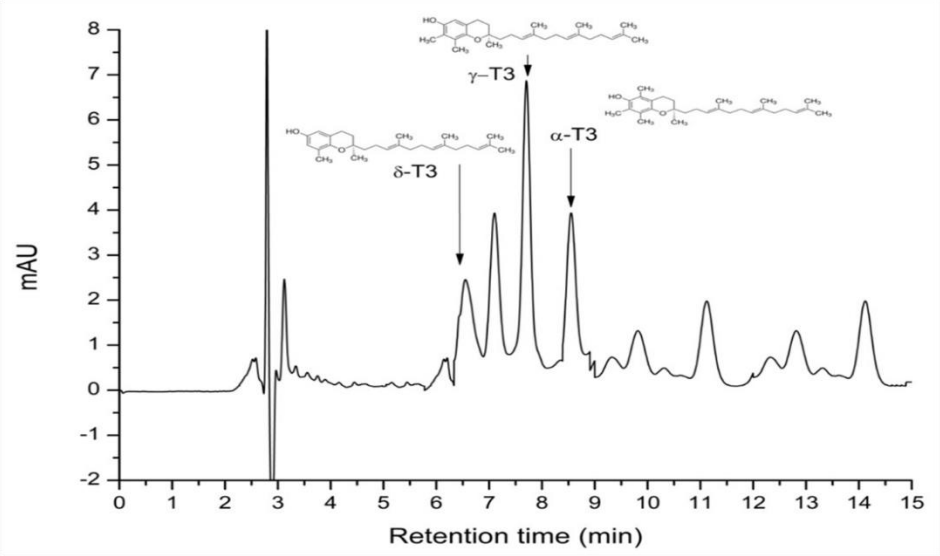

Fig.2. HPLC chromatogram of optimal extract of tocotrienols

1.5 Extraction, separation and identification of gamma-oryzanol from rice bran

The extraction process was done using direct solvent extraction method in room temperature. Ethyl acetate was used as the extracting solvent because of its low toxicity and easy solubility of γ-oryzanol in ethyl acetate as compared to ethanol. After extraction, separation and identification of γ-oryzanol was done using high performance thin layer chromatography **(Fig.3).**

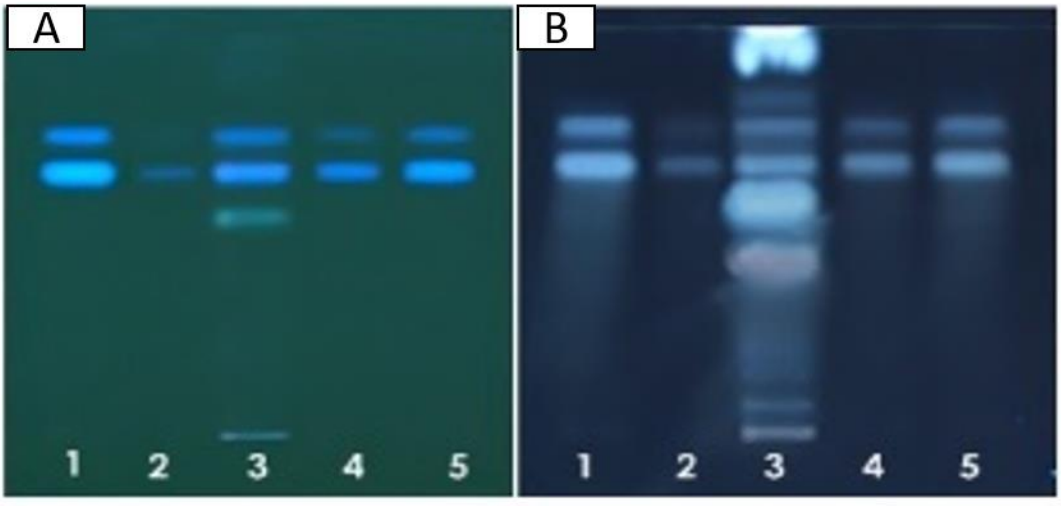

Fig.3. HPTLC profiles of extracted rice bran (lane 3 – conc. not yet determined) and std. gamma-oryzanol (lane – 1,2,4, and 5 with conc. of 1 µg, 0.12 µg, 0.25 µg and 0.5 µg respectively) (A) Visualization under UV 366; (B) Visualization under UV 366 after derivatization with marquis reagent.

1.6 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) inhibition and kinetic study against RBE

RBE showed 60.42±2.04 % of inhibition against HMGR (at 100 μg mL^{-1} of total α-, δ-, γ-T3 present in the extract). Pure α-, δ-, γ-T3 showed 36.96±1.63, 52.51±3.54, 70.71±2.80 % inhibition at 100 μg mL^{-1} of HMGR, respectively, which indicated that all the isomeric forms of T3 are effective in controlling HMGR activity along with RBE. However, γ-T3 has higher potentiality in comparison to the other forms. The inhibitory effect of T3 can be correlated with the formation of hydrogen and hydrophobic interaction with the active amino acid sites of HMGR. γ-T3 recorded lowest IC_{50} of 11.33 μg mL^{-1} followed by 19.17μg mL^{-1} for δ-T3. The α-T3 exhibited the highest IC_{50} among the isomers i.e., 26.73 μg mL^{-1}. RBE showed IC_{50} of 20.4 μg mL^{-1}.

Detailed kinetics of enzyme inhibition of pure α-, δ- and γ-T3 along with RBE against HMGR was performed. The kinetic parameters namely, maximum reaction velocity/rate (V_{max}) and MM constant (K_m, relates the substrate-binding affinity) were calculated. The inhibition activities of the target inhibitors were determined spectrophotometrically using HMG-CoA as a substrate (concentrations from 0.0012–0.0180 μMol). Study showed that the enzyme reaction rate was affected by increase in inhibitor concentration. The V_{max} value decreased with increasing concentration of α-, δ-, γ-T3 and RBE indicating their concentration dependent inhibitory activity. The mode of inhibition determined by the LB plot showed that both the vertical axis and slope of the tested inhibitors do not intercept with each other. Thus, an uncompetitive mode of inhibition was observed against α- and γ-Tocotrienol along with RBE, where both the K_m and V_{max} values were decreased. On the other hand, δ-T3 showed a mixed type of inhibition where K_m values were higher than the V_{max} values (**Figures not shown**).

Objective 2. To synthesize starch nanoparticle and starch tocotrienol bioconjugate

Biodegradable starch nanoparticle was synthesized to act as a nano-carrier of γ-tocotrienol.

2.1.1 Particle size distribution, surface charge, structural properties (NMR) and fluorescent microscopy

Particle size distribution (PSD), polydispersity index (PDI), and zeta potential (ZP) were measured by a Nano Zetasizer Measurement (Nano ZS, Malven, UK) at 25^{o}C with a fixed scattering angle of 90^{o}. Particle size of starch is 1-2 μm. It was found that the particle size of

hydrophobic starches ranged between 50 and 200 nm **(Fig. 4).** Zeta potential ranged between -5 and -10 mV and polydispersity index ranged from 0.1-0.6.

The starch nanoparticles (StNP) were conjugated with linoleic acid (LA) to attach hydrophobic group and the conjugate was coded as StNP-LA. The Proton NMR (^{1}H) spectra of samples were run on a JEOL 400 MHz spectrometer with 32 scan times in a concentration of 25 mg mL^{-1} at 25°C. The spectra were obtained at 60 °C with a pulse angle of 30°, a delay time of 10 s and an acquisition time of 2 s. Proton NMR suggests the presence of LA in starch molecules **(Fig.5)**.

Fluorescence microscopy were characterized using (Olympus model IX83, Japan) two stains, namely, Nile Red and Nile Blue having excitation emission at 488 and 633 nm argon for staining of LA and polar group in starch nanoparticle, respectively. It showed the conjugation of LA with starch **(Fig.6)**.

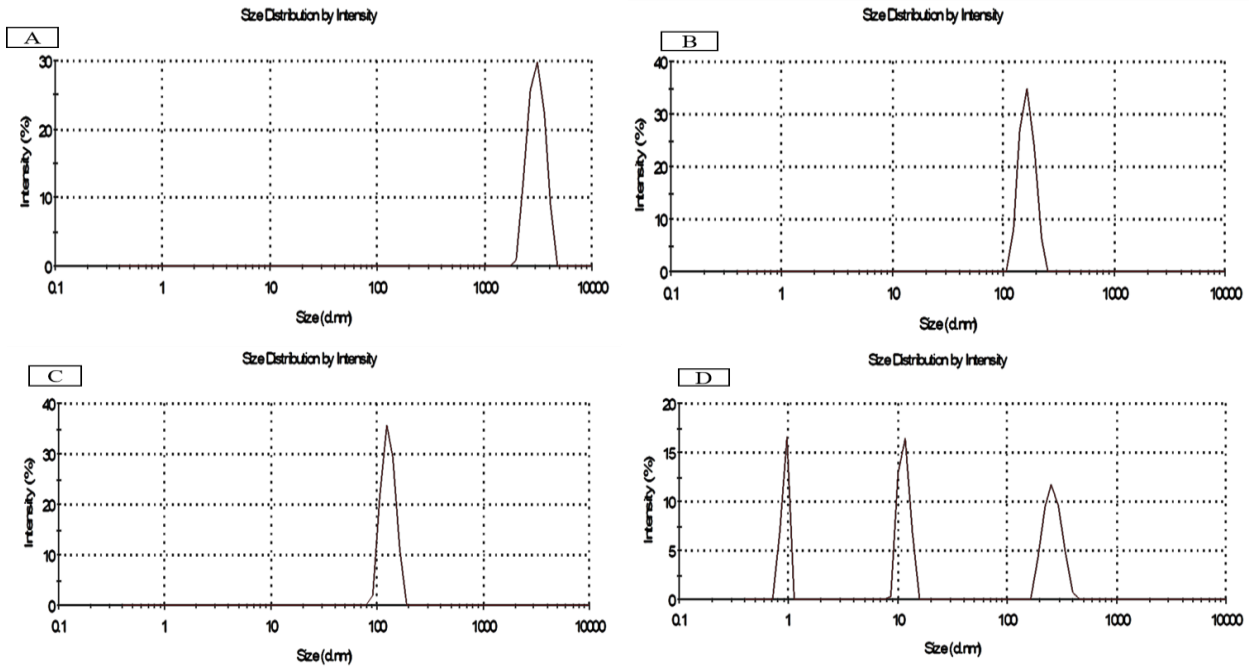

Fig.4. Particle size analysis of samples: A) Native starch; B) StNP-LAU15; C) StNP-LAU30; and D) StNP-LAU45.

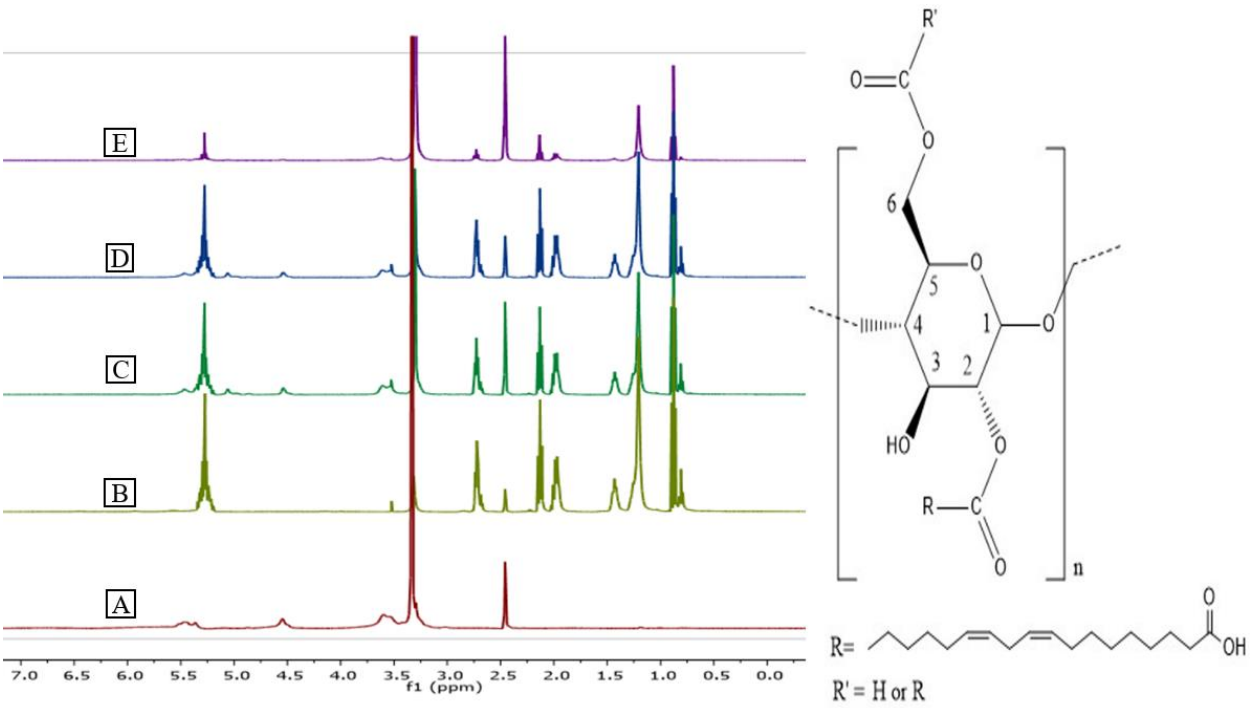

Fig.5. Proton NMR of starch nanoparticles conjugated with linoleic acid: A) Native starch; B) StNP-LAU15; C) StNP-LAU30; and D) StNP-LAU45.

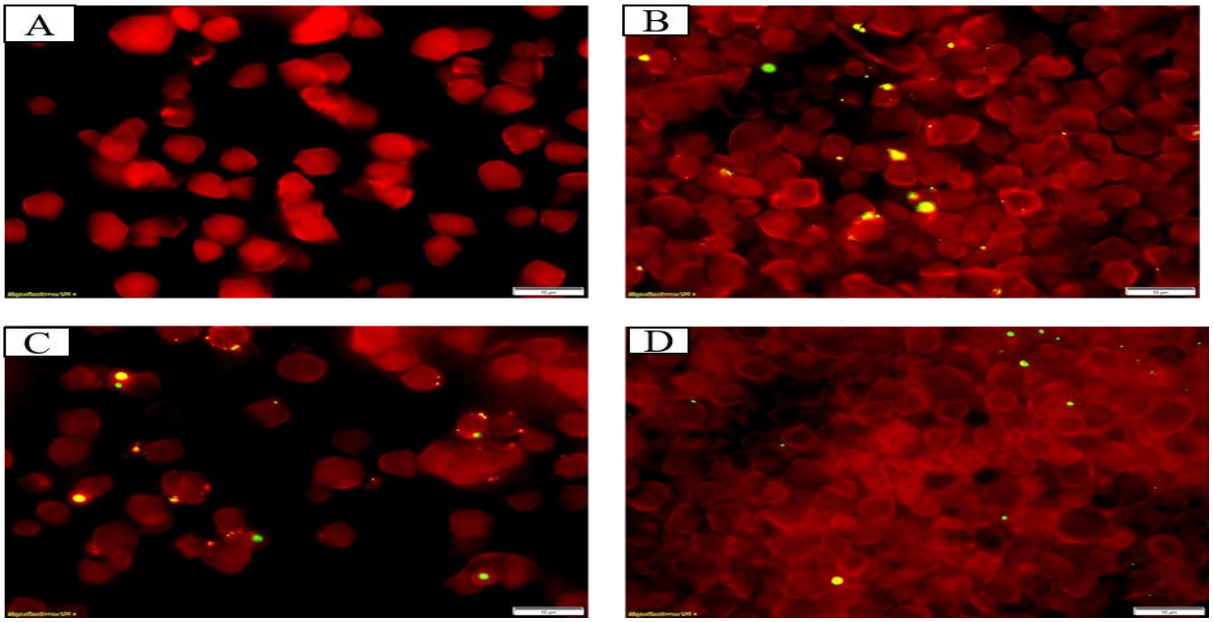

Fig.6. Fluorescent microscopy of starch nanoparticles conjugated with linoleic acid: A) Native starch; B) StNP-LAU15; C) StNP-LAU30; and D) StNP-LAU45

2.1.2 Protein adsorption

The protein resistance properties of native starch and StNP subjected to different ultrasonic treatment time to incorporate LA (StNP-LAU15, StNP-LAU30, and StNP-LAU45) were compared. Bovine serum albumin (BSA, mg mL^{-1} in phosphate buffer) solution was prepared at different pH levels (pH 6.5 and 7.4). Protein resistance was determined for two different time intervals (2 and 4h). To determine the bound protein, sodium dodecyl sulphate (SDS) was added and sonicated for 30 min and measured in a UV–Vis spectrometer at 280 nm to evaluate the adsorption amount of BSA anchored to the samples with the help of standard curve of BSA. With increase in ultrasonication treatment time, LA incorporation was enhanced which significantly improved protein resistance **(Fig. 7)**.

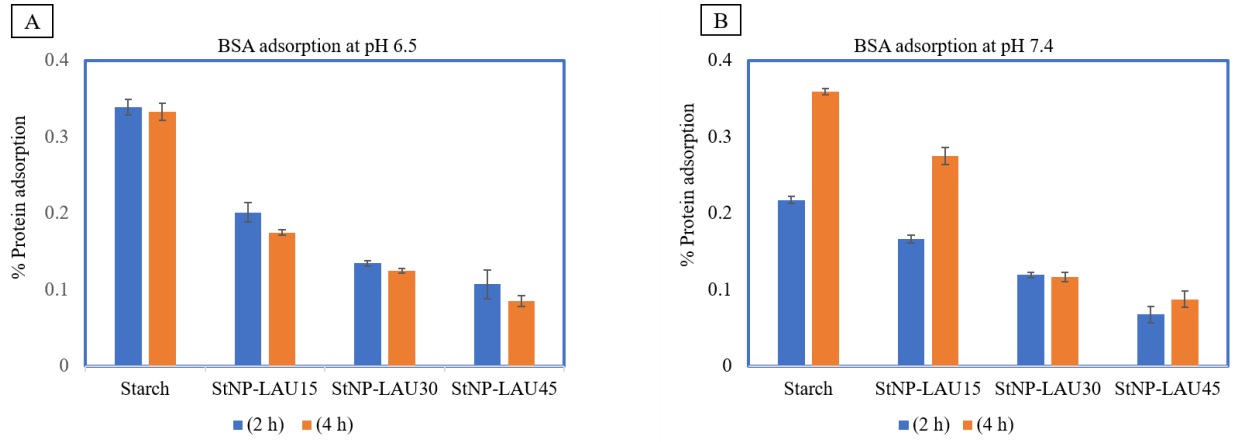

Fig.7. Protein resistance of starch conjugates: A) BSA adsorption at pH 6.5 and B) pH 7.4

2.1.3 In vitro GI hydrolysis

For in vitro starch digestibility tests, sequential digestion was carried out through mouth-stomach-pancreas and the aliquots were centrifuged at 7500 rpm for 20 min and supernatants were filtered through 0.22 membrane filter. In vitro starch digestibility was monitored using 3,5-dinitro salicylic acid (DNS) method in UV-spectrophotometer. Concentration of hydrolysed starch (glucose released) content was analysed from calibration curve. Digestibility of LA incorporated samples had improved which is an important factor for any drug carrier material. Intestinal hydrolysis of starch had decreased in StNP-LAU30 and StNP-LAU45 in comparison to the native starch (**Fig. 8)**.

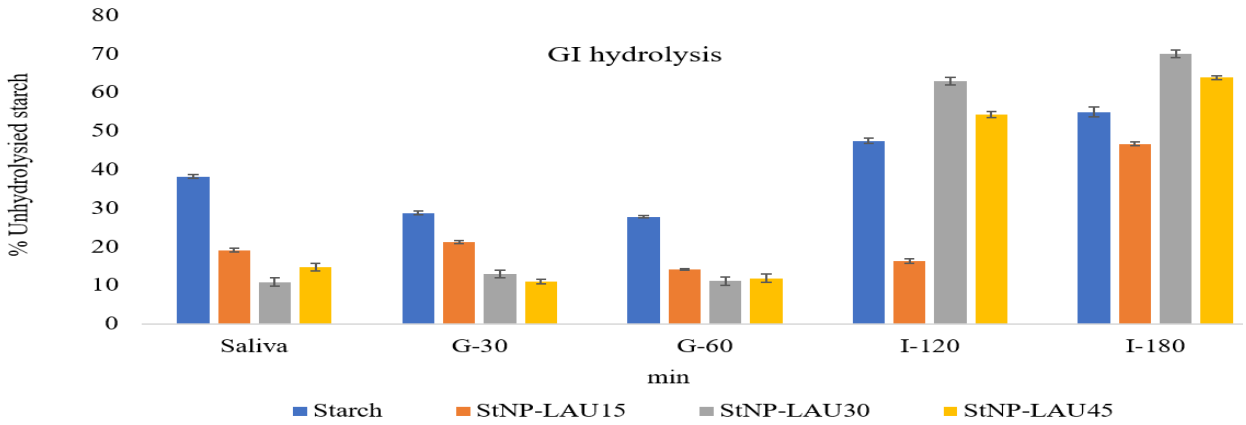

Fig.8. In vitro gastro-intestinal study of native starch and LA incorporated starches

2.2 Conjugation of TRF and γ-oryzanol with St-la using ultrasonic energy

One gram of StNP-LA was dissolved in water in different beakers and stirred in a magnetic stirrer at 100 rpm and separately, 150 mg TRF was dissolved in 20 mL ethanol. TRF solution was added to the StNP-LA solution drop-wise. The stirring speed was increased from 100 to 500 rpm at 5 min interval and kept for 30 min further. Solution was freeze dried and kept at 4ºC till further analysis. Similarly, γ-oryzanol was conjugated with StNP-LA with ultrasonic treatments for 15, 30 and 45 min and coded as St-LA-ORZ1, St-LA-ORZ2 and St-LA-ORZ3.

2.2.1 Particle size, surface charge analysis

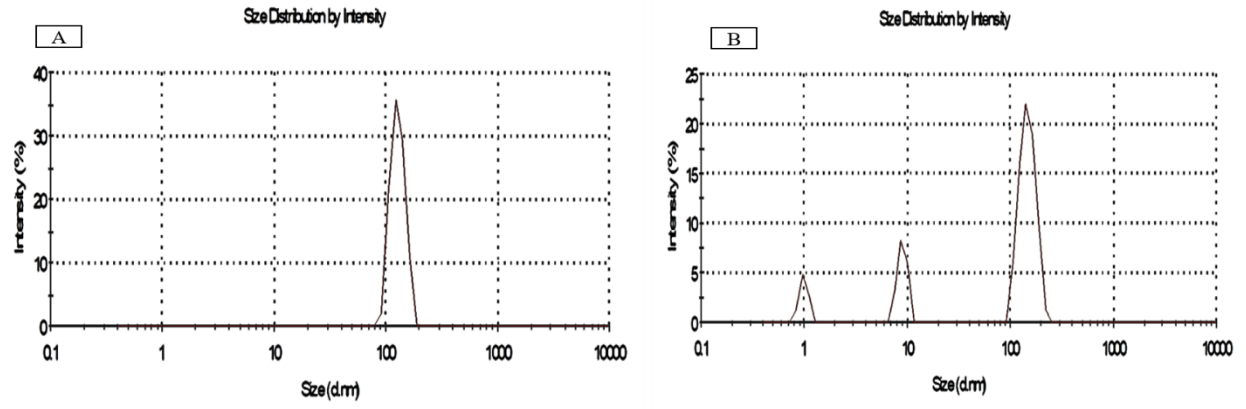

Fig.9.Particle size analysis of StNP-LA (U30) and StNP-LA-TRF conjugate

The particle size of developed LA incorporated starch conjugated with TRF and oryzanol are depicted in **Fig. 9 and Fig. 10,** respectively.

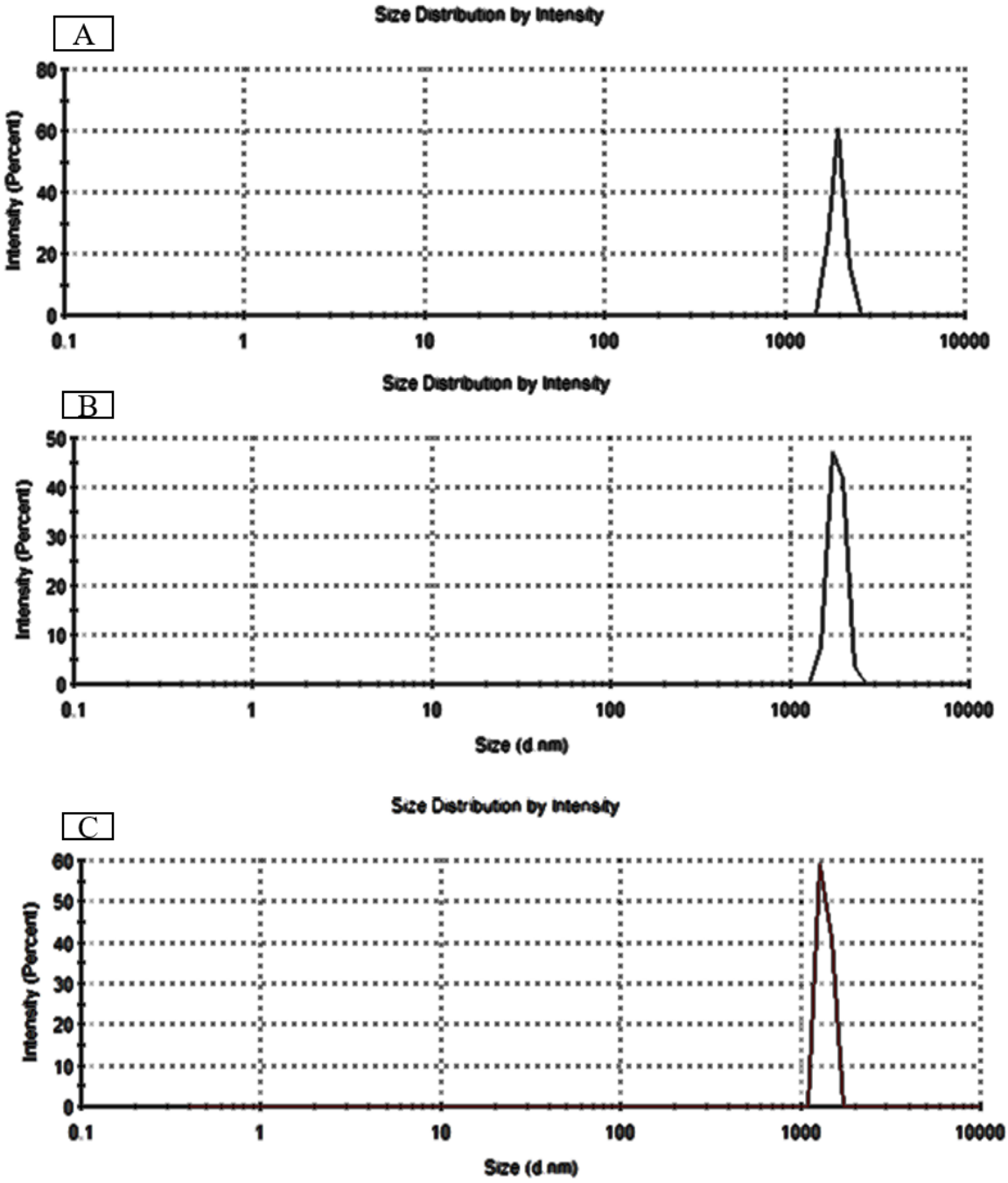

Fig.10. Particle size of starch oryzanol conjugates. A. St-LA-ORZ1, B. St-LA-ORZ2 and C. St-LA-ORZ3

2.2.2 Atomic Force Microscopy (AFM) analysis

Particle morphology was further confirmed using an atomic force microscope (NTEGRA Prima, NT-MDT Technology) in semi-contact mode using silicon cantilever having spring constant 0.03 N/m. Scans were performed over several portions of the films for different scan areas.

Sample solutions (5 mg/mL) were prepared and adsorbed using drop casting method onto silicon substrate (1 cm^2). Substrate cleaning was done before using RCA standard methods. For processing images SPIP software (Image Metrology, Copenhagen, Denmark was used. Topography and 3D images are presented in order to show a better definition of particles. From AFM data particles size of StNP-LA and StNP-LA-TRF conjugate was found to be 30 and 60 nm, respectively.

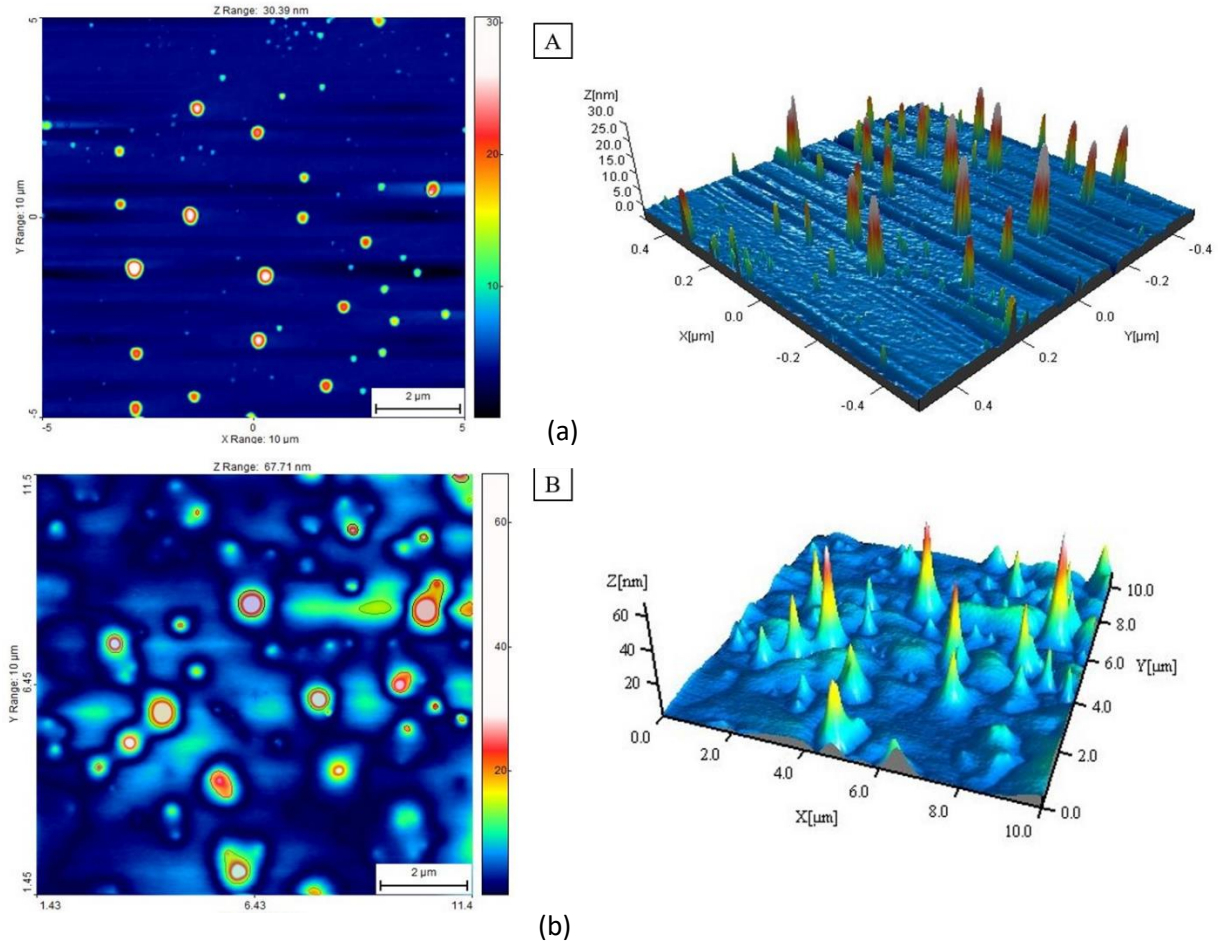

Fig. 11. AFM analysis of (A) StNP-LA, and (B) StNP-LA-TRF

2.2.3 Confocal laser scanning microscopy

Confocal laser scanning microscopy was done to confirm that gamma-oryzanol had incorporated inside the starch granules. StNP-LA-TRF and StNP-LA-ORZ were taken under normal light, and excitation and emission spectra of 488 nm and 640-750 nm, respectively (Figure not shown).

2.2.4 Encapsulation efficiency and release percentage

As per procedure followed, % encapsulation of tocotrienol was 74% (110 mg) and encapsulation of oryzanol was 60%.

2.2.5 Release study

Gastro-intestinal release of tocotrienol was found to be 21mg/ml (Figure not given). Release of γ-oryzanol from samples of StNP-LA-ORZ differing in ultrasonic treatment was also found to improve with starch conjugation to 30-40% in gastric stage and 80-90% in intestinal stage (**Fig.12**).

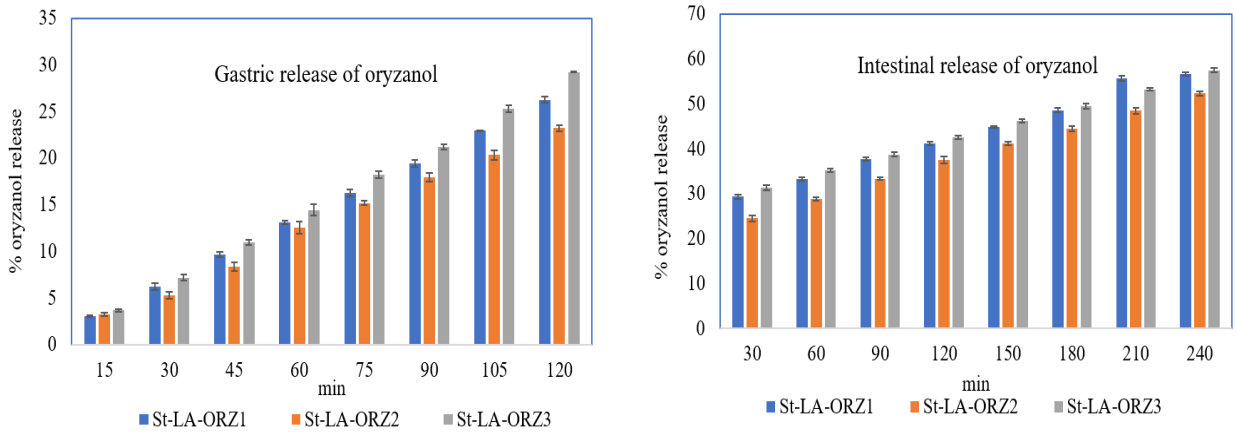

Fig. 12. In vitro release of γ-oryzanol from St-LA complex with respect to time.

2.2.6 Cell line toxicity

The human hepatocellular carcinoma cell line (HepG2) was purchased from National Centre for Cell Science (NCCS, Pune, India) and was grown in Dulbecco's Modified Eagles Medium (DMEM) containing 10% Fetal bovine serum (FBS) and 1% antibiotics (100 U mL^{-1}penicillin, 100 µg mL^{-1} streptomycin). Approximately, $5x10^3$cells were seeded in each well of 96-well plates in triplicate and kept for 24 h at 37 ºC, 100% RH, 5% CO_2 and 95% air. Cells were then treated with different concentrations of samples ranging from 20 to 500 µgmL^{-1} for 24 h. To determine the viability of the cells, 50 µL of 5 mg mL^{-1} MTT 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide was added in each well and incubated for 4 h at 37 ºC. After incubation, the MTT solution was removed by aspiration and the formazan crystals were solubilized by 100 µL of dimethyl sulfoxide followed by measuring absorbance at 595 nm using ELISA reader (Thermo Scientific, USA).

All the samples were tested in different concentration for its effect on cell viability. The results suggested that with the exposure of TRF from 20.0 to 500.0 µg/mL concentrations, cell deaths were in the range of 15.27±2.16 % to 45.22±2.08 % after 24 h (**Fig. 13A**) with an IC_{50} value of 4.66±0.20 µg/mL (R^2=0.950). Oil Red O stain solution was use to visualize the intra-cellular perforation and localization of TRF and conjugates in the cell (HepG2) (**Fig. 13 B-E)**. Cell permeation had enhanced the permeability of conjugated form.

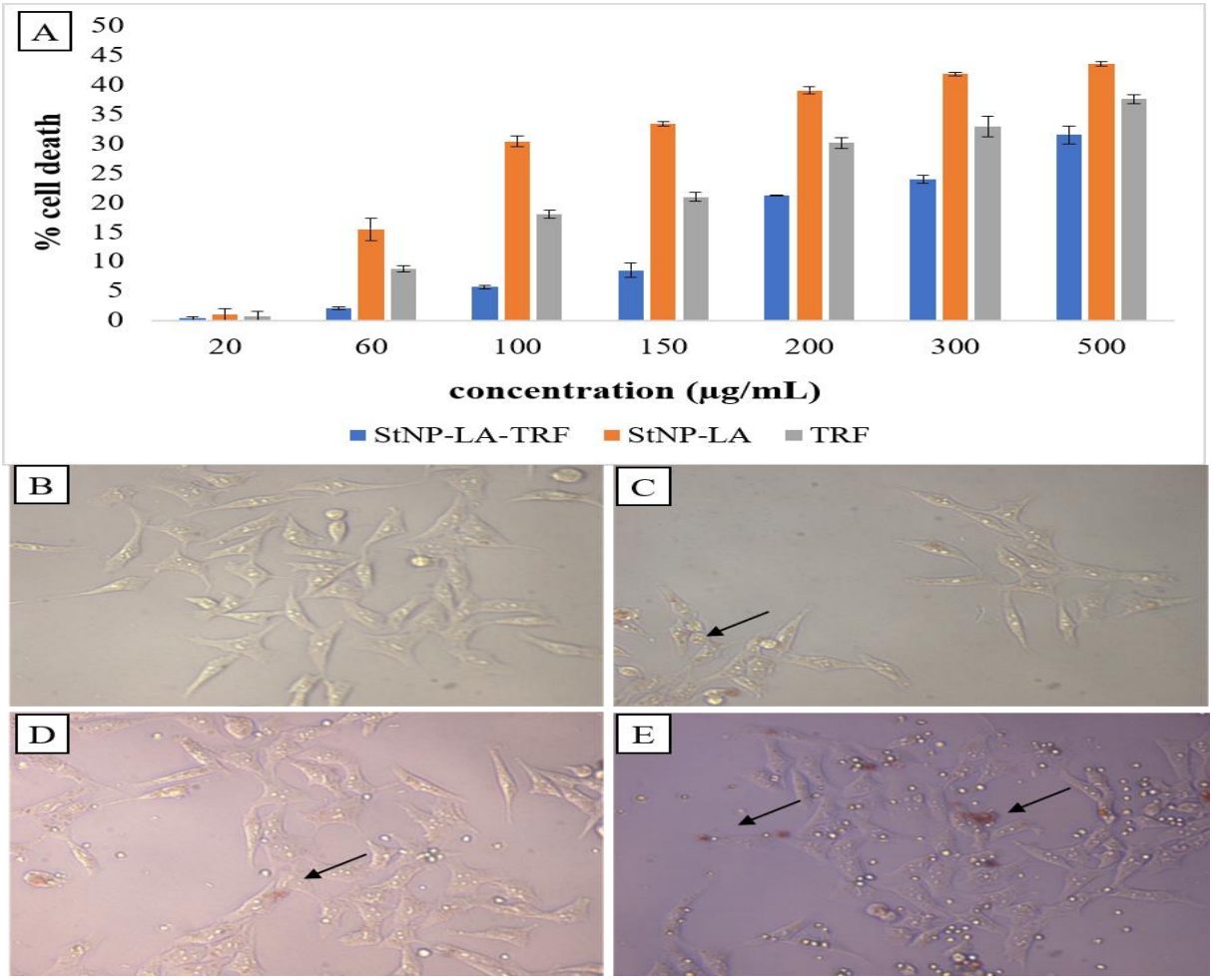

Fig. 13. A. Cell line toxicity and B-E. cell permeability. Arrow marks show the penetration of TRF into HepG2 cell.

Objective 3: To study the interaction of tocotrienols with HMGR

Computational simulations provide a powerful tool for gaining insights on the underlying phenomena of interaction between nanoparticles and biomolecules and facilitate the establishment of quantitative relationships between the structure of these particles and their

interactive activities. Computational modelling in general allows researchers to systematically experiment with different factors and its outcome in the nano-bio interaction. Through this process, researchers can actually visualize which interactions and effects are important for deciding the behaviour of a particular system, and which are not. In Molecular Dynamics inter-atomic interactions can be defined a priori, and with given initial and boundary conditions, the time-dependent, dynamic changes of the system can be computed and visualized.

Molecular docking studies were done to investigate the possible interaction of α-, δ- and γ-T3 as ligands with the receptor sites of HMGR using Autodock vina (AutoDock Tool 1.5.6). The Grid boxes of centre_x=10.461, centre_y=-15.084, centre_z=33.264; size_x=32, size_y=32 size_z=32 with a spacing of 0.375 Å between the grid points were established relative to native ligands. Protein-ligand docking score, ligand properties, 2D and 3D structures were retrieved. For hydrophobic surface structure, UCSF Chimera software

All the structures *viz*. α-T3 (CID: 5282347; Mol wt. 424.7 g mol^{-1}), δ-T3 (CID: 5282350; Mol wt. 396.6 g mol^{-1}) and γ- T3 (CID: 5282349; Mol wt. 410.6 g mol^{-1}) were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and energy minimization (Force field MMFF94) was performed using Avogadro saved as PDB files. Preliminary structure preparation like deletion of the water molecule and addition of hydrogen was done using the tool Discovery Studio R2 Client (Version 2.1). The 3-D structure of HMGR complexed with HMG and CoA was obtained from Protein Data Bank, PDB (source: RCSB database, https://www.rcsb.org/pdb) having PDB id 1 DQA. Before subjecting to docking, protein energy minimization was conducted in the AMBER14 software package. In brief, the protein molecule was kept constant using harmonic constraints, and 1000 steps of the steepest descent algorithm followed by 1000 steps of the conjugate gradient were used. The subsequent minimization was carried out without any constraints for 2000 cycles (1000 cycle of steepest descent and 1000 cycle of conjugate gradient. The energy minimized structure of HMGR protein was considered for the docking process.

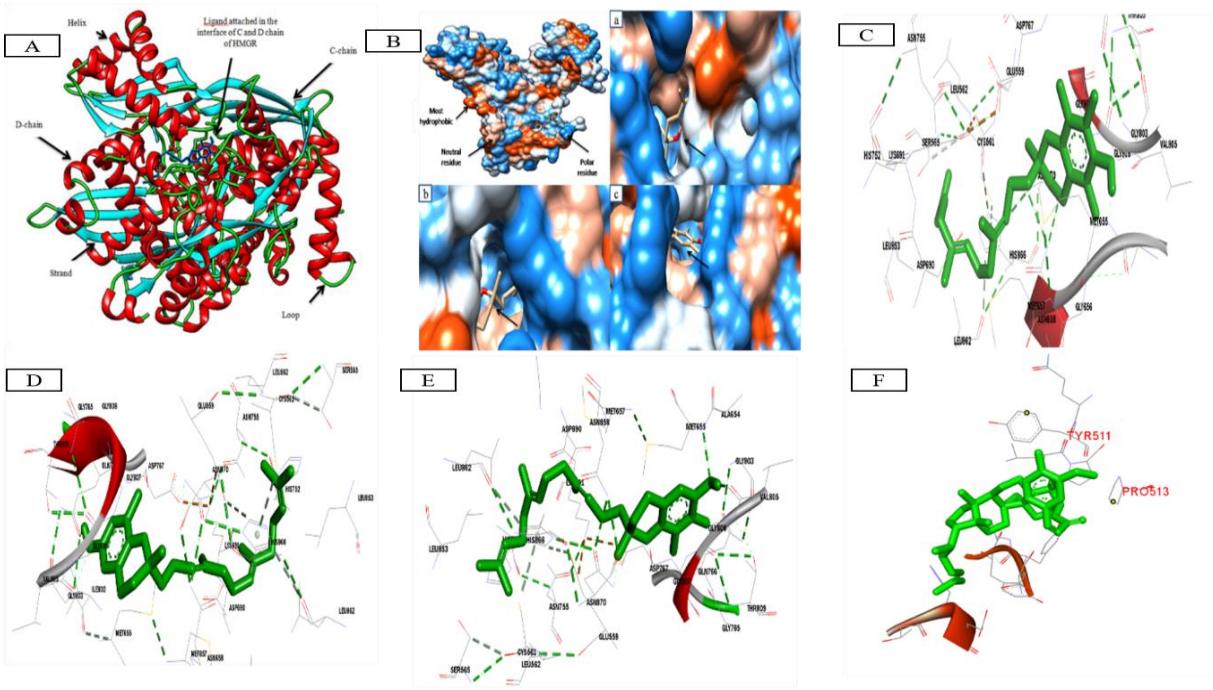

Fig. 14. Molecular interaction study: A & B) Ribbon and surfcae HMGR-tocotrienol complex; C) 2D complex of HMGR with α- T3; D) 2D complex of HMGR with δ- T3; E) 2D complex of HMGR with γ-T3; and F) 2D complex of HMGR with γ-oryzanol**.**

Molecular docking studies were performed to explore the binding of α-, δ- and γ-T3 (ligands) at the active sites of HMGR (receptor) using Auto Dock vina and visualized using Discovery Studio. Docking results showed significant hydrogen bond interactions between the HMGR amino acid residues with T3 hydroxyl moiety along with the pi alkyl interactions of methyl groups present in the isoprenoid chain (**Fig.14**). Other force of interactions such as van der Waals attraction (hydrophobic interaction), alkyl, pi sigma and pi-pi stacking are also involved stabilizing the T3-HMGR structure (**Table 2**). It was evident from this study that, the major interaction of T3 existed, where most of the NADPH catalytic site amino acids were involved. It was also observed that T3 successfully incorporated inside the active site (**Fig. 14)**. However, due to the presence of rigid bonds in the chroman ring it cannot completely occupy the HMG-CoA binding site and thereby may not act as a competitive inhibitor. Enzyme kinetic studies also suggested the uncompetitive mode of inhibition for α- and γ-T3 and mixed mode inhibition for δ-T3. Thus, from this study it can be concluded that the possible interaction of T3 with HMGR

takes place majorly at the NADPH binding site after the formation of HMGR-HMG CoA [ES] complex to restrict the pathway of cholesterol biosynthesis by inhibiting the HMGR activity.

Table 2. Interaction/binding site and binding affinity of α-, δ- and γ-tocotrienols

Ligand	CID	Molecular weight (g/mol)	Protein-ligand interaction/binding site				Binding affinity (kcal/mol)
			van der Waals	Pi alkyl	Pi-Pi stacked	Hydrogen/distances (Å)	
α- T3	5282347	424.7	Asn870, Arg590, Lys691, Gly656, Val805,Asn658, Asp690, Glu559, Asn870, Gly656	His866, Leu862, Met657	Gly806	Met655 (2.80), Val805(2.39)	-9.4
δ- T3	5282350	396.6	Leu562, Ala856, Ser852, Lys691, Asn755, Lys722, Arg590, Ser865, Lys691, Ser565	His752, Leu853, Cys561	Tyr579	Gln766 (2.55)	-8.7
γ- T3	5282349	410.6	Arg590,Gln766 Asp767, Gly807 Glu559, Leu562, Ser565, Asn755,Ala654, Thr809	Met657 Leu862, His866	Gly806	Met655 (2.82), Val805(2.41)	-9.1

Objective 4: To determine anti-cholesterol effect of tocotrienols and their bio-nano-conjugates in vivo

4.1. Animal study with high fat diet to develop hyperlipidaemia and fatty liver

We fed high fat diet to SD male rats for four weeks along with a single intraperitoneal dose of streptozotocin (STZ, 40mg/kg) after two weeks to develop hyperlipidemia and hepatic fat accumulation.

Groups studied:

1.Control: Fed with control diet

2.HFD+STZ: High fat diet and STZ injection (Hyperlipidemia group)

3.HFD+STZ+StNP: Group 2 fed with starch nanoparticle

(referred to as StNP-LA in objectives 1-3)

4.HFD+STZ+T3: Group 2 fed with tocotrienol

5. HFD+STZ+BioC: Group 2 fed with tocotrienol bioconjugate starch nanoparticle

(referred to as StNP-LA-TRF in objectives 1-3)

4.2 Body weight change

We have measured the change in body weight in all five groups. There was a body weight loss in HFD+STZ group. The improvement of body weight loss was improved only in HFD+STZ+T3 group **(Fig.15a)**.

4.3 Liver weight and Body weight ratio

The liver weight and body weight ratio represent the presence of hepatomegaly and liver disorder in high fat and STZ treated rats. As expected we found significant increase in liver weight and body weight ratio in HFD+STZ group when compared to control group. However, this increase ratio was decreased in all three treated group **(Fig.15b)**.

4.4 Fasting blood glucose levels

There was a significant increase in fasting blood glucose levels in HFD group after 72 hours of STZ injection. Although we have not found drastic reduction of fasting blood glucose levels in any treated group, slight reduction of blood glucose levels was observed in both HFD+T3 and HFD+BioC groups **(Fig.15c)**.

4.5 Serum triglyceride levels

There was a significant increase in serum triglyceride levels in HFD group at the end of four weeks. Significant reduction of serum triglyceride levels was observed in both HFD+StNP, and HFD+T3 groups but not in HFD+BioC group **(Fig.15d)**.

4.6 Serum AST levels

There was no change in serum AST levels in any of the groups evaluated in the present study **(Fig.15e)**.

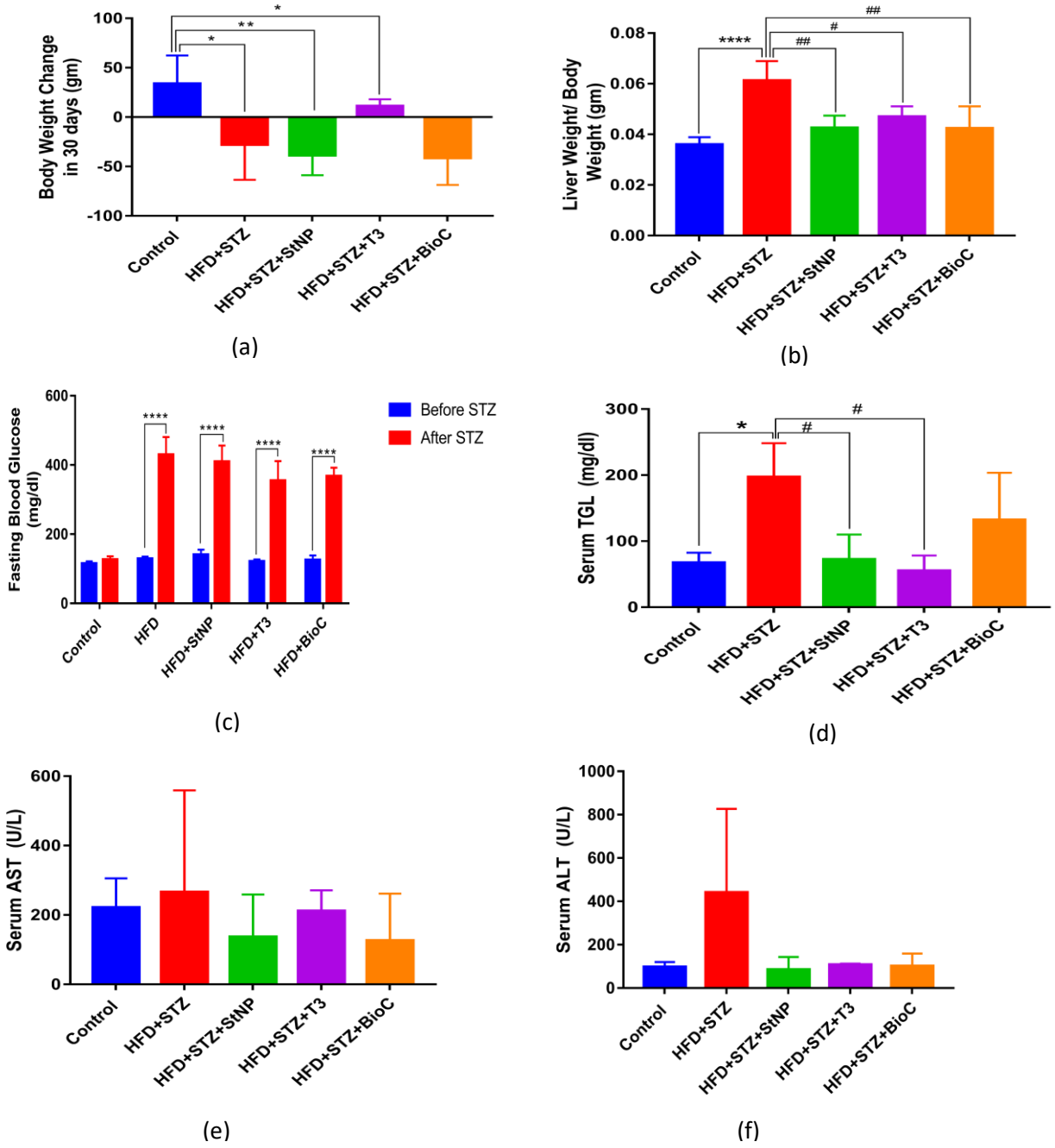

Fig. 15. (a)Body weight change; (b) Liver weight change; (c) Blood glucose level; (d) Serum TGL level , (e) Serum AST level; and (f) Serum ALT level

4.7 Serum ALT levels

The increased serum ALT levels represent the presence of hepatic injury in rats. We have found increased serum ALT levels in high fat and STZ treated rats. However, normalization of serum ALT levels was observed in all three treated groups HFD+StNP, HFD+T3 and HFD+BioC groups **(Fig.15f)**.

4.8 Fat accumulation in liver

To find the fat accumulation in liver, we did Oil Red O staining in the frozen liver sections. Liver sections from three different magnifications (10X, 20X, and 40X) showed the presence of fat in the liver of HFD+STZ group when compared to control **(Fig.16)**. The fat accumulation was decreased in both HFD+T3 and HFD+BioC groups. However, marked improvement was observed only in HFD+BioC group.

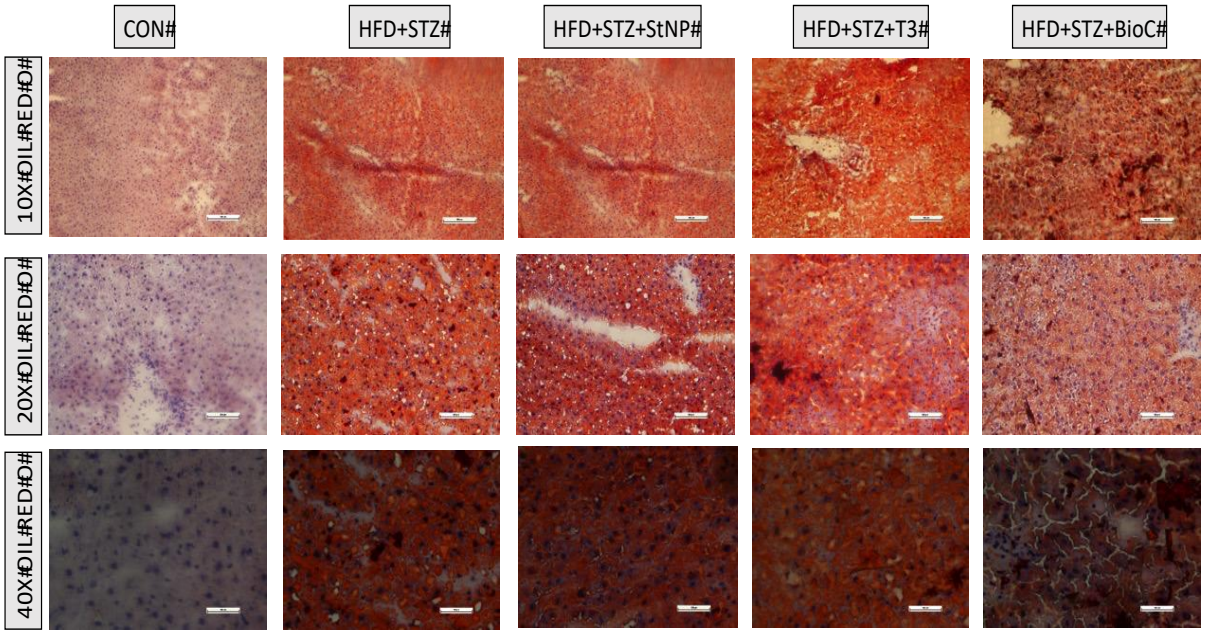

Fig. 16. Microscopic view for liver fat accumulation

4.9 Fibrosis in liver

To find the presence of fibrosis in liver, we did Masson's trichrome staining in the formalin fixed paraffin liver sections. Liver sections from three different magnifications (10X, 20X, and 40X) showed the presence of fibrosis (blue colour) in the liver of HFD+STZ group when compared to control **(Fig.17)**. The fibrosis was shown maximum in HFD+T3 groups. However, fibrosis was normalised in HFD+BioC group.

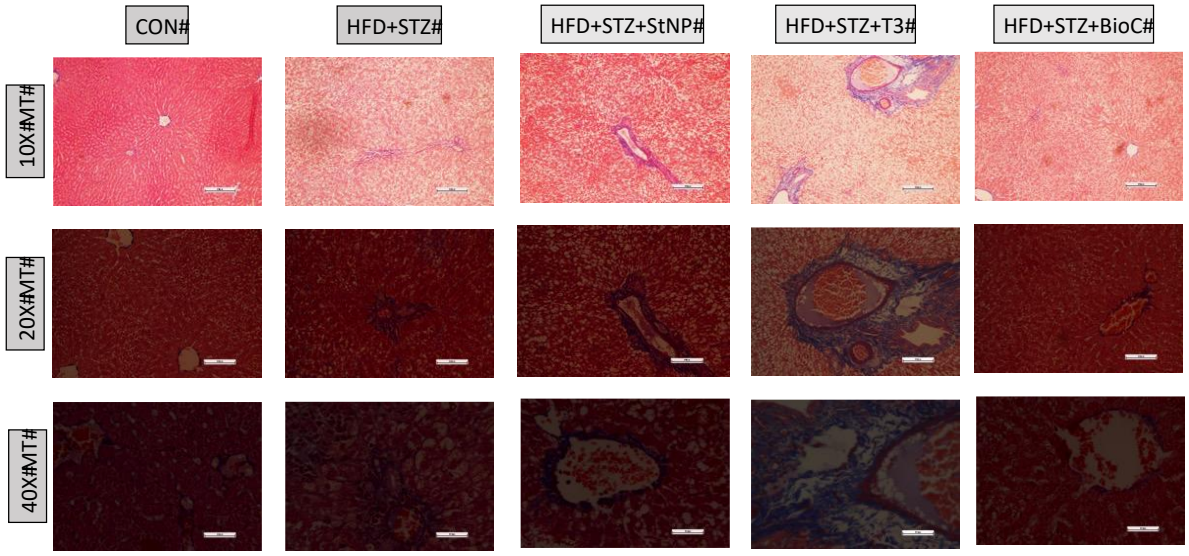

Fig. 17. Microscopic view for liver fibrosis

4.10 Hematoxylin and eosin staining in liver

To find the presence of lipid droplets and hepatocytes injury, we did Hematoxylin and eosin staining in the formalin fixed paraffin liver sections. Liver sections from three different magnifications (10X, 20X, and 40X) showed the presence of lipid droplets (white microbubbles) in the liver of HFD+STZ group when compared to control **(Fig.18)**. However, the lipid droplets number and sizes were decreased in both HFD+STZ+T3 and HFD+BioC group.

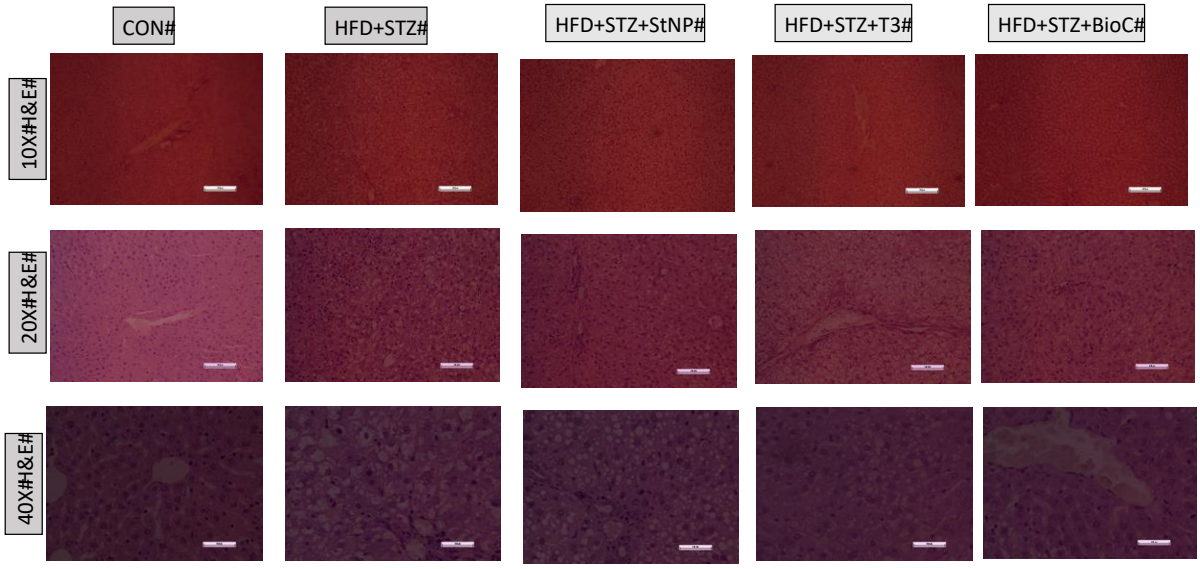

Fig.18. Microscopic view for liver lipid droplets

B2. Summary and Conclusions of the Progress made so far:

Present study demonstrated that the ultrasonic assisted extraction of TRF (α-, δ-, γ-T3) from rice bran inhibited HMGR. The fraction showed concentration dependent inhibitory effect and affinity towards HMGR at low concentration. The α-T3 and γ-T3 along with RBE followed uncompetitive mode inhibition while mixed type was seen in δ-T3. The hydrophobic starch nanoparticles developed by incorporating linolenic acid proved to be a good drug carrier molecule having both protein and gastro intestinal resistance properties. The conjugate of starch nanoparticle with oryzanol exhibited improved gastro-intestinal stability.

Further, StNP-LA-TRF conjugates were tested on HepG2 cell line and animal model. Cell line study showed the improved cell permeability of bioconjugates. In-vivo animal study showed that high fat diet along with streptozotocin injection decreased body weight and increased liver weight/body weight ratio. Further, we have found increase in fasting blood glucose levels, serum triglyceride and serum ALT levels in the diseased rats. More interestingly, our histopathology study confirmed the presence of lipid accumulation and fibrosis in the liver of high fat diet rats. Our intervention study clearly indicated that both tocotrienol and tocotrienol conjugated in starch nanoparticle (StNP-LA-TRF) reduced fasting blood glucose levels, serum triglyceride and ALT levels. But liver fat accumulation and liver fibrosis were only attenuated by tocotrienol conjugated in starch nanoparticle (StNP-LA-TRF) dosage form. The study indicates that starch nanoparticles conjugated with tocotrienol (StNP-LA-TRF) may be more efficient to penetrate in the liver tissue and thus able to reduce the fat accumulation and fibrosis in liver. One interesting finding is that only starch nanoparticle (ST-LA) was also found to be effective in reducing few serum parameters. Further studies are required to ascertain the reason for such effect.

B3. Details of New Leads Obtained, if any:

1. Ultrasonication effectively incorporated linoleic acid into starch to give a hydrophobic conjugate having good gastrointestinal stability, low serum levels of TGL, AST and ALT.
2. Conjugation of tocotrienol with starch nanoparticle-linoleic acid conjugate was effective in reducing liver fat accumulation and liver fibrosis besides lowering serum parameters.

B4. Details of Publications & Patents, if any:

1. Gautam, G., Duary, R .K., Gupta, K., and Mahanta C.L. (2020). Inhibition mechanism of 3-hydroxy, 3-methyl glutaryl CoA reductase by tocotrienol-rich rice bran fraction optimally extracted with ultrasonic energy. *International Journal of Biological Macromolecules,* 164, 1328-1341.https://authors.elsevier.com/sd/article/S0141-8130(20)33955-6.

Section-C: Details of Grant Utilization#

For Tezpur University

C1. Equipment Acquired or Placed Order with Actual Cost:

Equipment purchased by Tezpur University

1. Sonicator: Rs. 2,99,250 .00

2. HPLC: Rs. 24,37,638.00

C2. Manpower Staffing and Expenditure Details:

Manpower engaged	Stipend (Rs.)	Duration	Expenditure (Rs.)	Amount due (Rs.)
JRF (one position)	Rs. 25,000/-	04/01/2017 to 01/03/2017	72,580.00	NIL
		24/07/2017 to 28/02/2019	4,80,645.00	NIL
SRF (one position)	28,000/-	24/04/2019 to 31/12/2020	2,30,533.00	84,000.00
		01/01/2021 to 06/05/2021	NIL	33,600.00
		Total (Rs.)	7,83,758.00	1,17,600.00

C3. Details of Recurring Expenditure:

Tezpur University

i. Contingency: Rs. 1,58,228.00
ii. Consumables: Rs. 6,54,634.00
iii. Travel: Rs. 38,656.00

C4. Financial Requirements for the Next Year with Justifications:

Project closed on 06/05/2020

Sanjay Banerjee

(Dr. Sanjay K Banerjee)

PI

Charu Lata Mahanta

(Dr. Charu Lata Mahanta)

PI

UTILISATION CERTIFICATE

(for the financial year ending 1st April 2020- 6th May 2020)

(Rs. in lakhs)

1.	Title of the project/scheme :	In vivo hypocholesterolemic effect of bioconjugates of starch nanoparticles with gamma oryzanol and tocotrienols extracted from rice bran
2.	Name of the Organisation:	Tezpur University
3.	Principal Investigator :	Dr. Charu Lata Mahanta
4.	Deptt. of Biotechnology sanction order No. &date of sanctioning the project :	File No. BT/PR16804/NER/95/294/2015
5.	Amount brought forward from the previous financial year quoting DBT letter No. & date in which the authority to carry forward the said amount was given :	Rs. 0. 08722 brought forward
6.	Amount received from DBT during the financial year	NIL
7.	Other receipts/interest earned, if any, on the DBT grants :	Rs. 0.00218 (as interest)
8.	Total amount that was available for expenditure during the financial year (Sl. nos. 5, 6 and 7) :	Rs. 0.0894 (including interest)
9.	Actual expenditure (excluding commitments) incurred during the financial year(statement of expenditure is enclosed) :	Rs. NIL
10.	Unspent balance refunded, if any (Please give details of cheque No. etc.)	NIL
11.	Balance amount available at the end of the financial year :	Rs. 0.0894
12.	Amount allowed to be carried forward to the next financial year vide letter No. & date :	Rs. 0.0894 to be carried forward

P.T.O

Certified that the amount of Rs. NIL mentioned against col. 9 has been utilised on the project / scheme for the purpose for which it was sanctioned and that the balance of Rs. 0.0894 remaining unutilized at the end of the year ~~has been surrendered to Govt. (Vide No. .....dated .....)~~ / will be adjusted towards the grants-in-aid payable in the project.

Certified that I have satisfied myself that the conditions on which the grants-in-aid was sanctioned have been duly fulfilled / are being fulfilled and that I have exercised the following checks to see that the money was actually utilised for the purpose for which it was sanctioned.

Kinds of checks exercised:

4. Accounts audited by qualified Chartered Accountant appointed by the University as Internal Auditor
5. The AG (Audit), Guwahati has already audited the account.
6. All equipment, glassware, chemicals, raw materials etc., purchased from the grant are entered in the stock book.

Charu Lata Mahanta

(PROJECT INVESTIGATOR)

(Signed and stamped)

Principal Investigator
Dept. of Food Engg. & Tech.
Tezpur University
Tezpur -784 028, Assam

(FINANCE OFFICER)

(Signed and stamped)

Finance Officer
Tezpur University

(HEAD OF THE INSTITUTE)

(Signed and stamped)

Registrar
Tezpur University

FINALCONSOLIDATED STATEMENT OF EXPENDITURE
(FOR FINAL SETTLEMENT OF ACCOUNTS)

1. **Title of the Project** : **In vivo hypocholesterolemic effect of bioconjugates of starch nanoparticles with gamma oryzanol and tocotrienols extracted from rice bran**

2. **Sanctioned Project Cost** :**Rs 79.60 lakhs (48.70 to Tezpur University)**
3. **Revised cost, if any** : **NIL**
4. **Duration of the project** : **3 years**
5. **Sanction Order No. & Date** : **File No. BT/PR16804/NER/95/294/2015, Dated: 07/11/2016**
6. **Date of commencement of Project** : **07/11/2016**
7. **Extension, if any** : **6 months**
8. **Date of completion of project** : **06/05/2020**

Details of grant, expenditure and balance

S. No.	Heads	Sanct-ioned Cost	Year-wise Releases made						Year-wise Expenditure incurred						
			1st yr	2nd yr	3rd Yr	4th yr	5th yr	Total	1st yr	2nd yr	3rd yr	4th yr	5th yr	Total	Balance
A.	Non-recurring pageno.-														
	Equipments	28.91	28.91	NIL	NIL	NIL	NIL	28.91	-	2.99250	24.37638	-	-	27.36888	1.54112
B.	Recurring														
1.	Manpower	10.29	3.30	2.04	NIL	NIL	NIL	5.34	0.7258	2.05645	2.75000	2.30533	NIL	7.83758	(-)2.49758
2.	Consumables	6.00	1.50	2.50	NIL	NIL	NIL	4.00	1.49705	NIL	1.42896	1.11738	NIL	4.04339	(-)0.04339
3.	Travel	1.00	0.25	0.30	NIL	NIL	NIL	0.55	NIL	NIL	0.38656	NIL	NIL	0.38656	0.16344
4.	Contingency	1.50	0.50	0.16	NIL	NIL	NIL	0.66	NIL	0.03967	0.57948	NIL	NIL	0.61915	0.04085
5.	Overhead	1.00	0.25	0.30	NIL	NIL	NIL	0.55	0.15625	NIL	0.20629	NIL	NIL	0.36254	0.18746
	Total	19.79	5.80	5.30	NIL	NIL	NIL	11.1	2.3791	2.09612	5.35129	3.42271	NIL	13.24922	(-)2.14922
	Grand Total (A+B)	48.70	34.71	5.30	NIL	NIL	NIL	40.01	2.3791	5.08862	29.72767	3.42271	NIL	40.6181	(-)0.6081
	Interest earned	-	0.34280	0.25526	0.09472	0.00254	0.00218	0.6975	N/A	N/A	N/A	N/A	N/A	N/A	0.6975
	All total	48.70	35.0528	5.55526	0.09472	0.00254	0.00218	40.7075	2.3791	5.08862	29.72767	3.42271		40.6181	0.0894

Charu Lata Mahanta
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