DEVELOPMENT OF HERBAL NANOPARTICULATE FORMULATION FOR THE TREATMENT OF SOLID TUMOR IN MICE

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Abstract:

The relevance of nanotechnology to drug delivery systems for cancer therapy has progressively received great attention. Castalin has been initiate to be very efficacious against many different types of cancer cells. Our present work investigated the efficiency of encapsulation of castalin in poly (lactic-co- glycolic acid) (PLGA) nanoparticles using solid/oil/water emulsion solvent evaporation method. to conclude, the in vivo anti-tumor activity of Castalin-loaded PLGA NPs was evaluated in tumor-bearing mice. The NPs obtained had smooth surfaces with particle sizes ranged from 161 ± 3.05 to 164 ± 8.7 nm with slightly positive surface charge ranged from 1.09 ± 0.15 to 3.71 ± 0.44 mV. Entrapment of Castalin ranged between 85.5 ± 1.8% and 87 ± 1.3% with yields not less than 102.32%. Total protein adsorbed was less than 25.5 µg total protein/1 mg NP. In vitro drug liberate was less than 89.1% at 12 h. to finish, significant reductions in tumor growth rate and mortality rate were observed for PLGA NP formulations compared to both Castalin solution and naked NPs.

Keywords: *Nanoparticles; PLGA; Castalin; cancer; sustained release; in vivo rat model.*

Introduction

The most outstanding approach is targeted drug delivery (TDD), in which the drug is included into a nanocarrier such as a liposome, niosome, nanoemulsion, or nanoparticles. Cancer is a widespread disease in which cells grow and divide abnormally and out of control, resulting in a mass known as a tumor. There is considerable interest in new technologies that can differentiate between normal and cancer cells and specifically target the tumor. TDD increases both drug efficacy and reduces drug toxicity, and it could overcome a wide range of obstacles such as drug solubility and instability, as well as facilitate drug delivery to the target cell. Different drug targeting strategies exist, namely passive and active targeting *(Mohamed et al., 2019).* Passive targeting depends on a unique phenomenon of most solid tumors known as the enhanced permeability and retention (EPR) effect, in which molecules of certain sizes are preferentially taken up by and accumulate in the tumors *(Danaei et al., 2018).*

A large number of NP delivery systems have been developed, in which the drug to be delivered is dissolved, entrapped, and encapsulated within the matrix *(Lövestam et al., 2010).* NPs conjugated with biodegradable polymers such as PLGA have pulled considerable attention as a result of their ability for active and passive tumor-targeting *(Xiaowei et al., 2015).* NPs are considered to be a drug delivery system that enables unique approaches for cancer treatment, and to be one of the most important means utilized in nanomedicine *(Jiang et al., 2007).* The external dimensions of NPs range from a few nanometers up to 1000 nm. It is well known that NPs coated with PEG can accumulate in different types of solid tumors due to the EPR effect; they are considered suitable vehicles for hydrophobic drugs, able to attain efficient tumor targeting with the fewest adverse reactions *(Venkatasubbu et al., 2013; Siqi et al., 2019*)

Preparation of Castalin NPs

Castalin loaded PLGA nanoparticles have been organized through nanoprecipitation approach. In short, by dissolving 100 mg of PLGA changed into dissolved in 1 mL of acetone and then introduced drop clever to 10 mL of TPGS solution (0.3 % w/v) with continuous stirring. Castalin 10 mg turned into dissolved 10 ml of water. Organic section obtained with the addition of PLGA and TPGS solution in acetone changed into introduced drop clever to aqueous segment of Castalin solution upon stirring. Acetone turned into allowed evaporating through continuous magnetic stirring for 4 h. Castalin loaded PLGA nanoparticles were then recovered from the nano-dispersion with the aid of centrifugation at 15,000g force for 35 min at 4°C. Then, brought about precipitated PLGA nanoparticles have been suspended in mannitol solution (5% w/v) and lyophilized for 72 h.

Nitric Oxide Radical scavenging assay

Sodium nitroprusside in aqueous media at physiological pH spontaneously generates nitric oxide which interacts with oxygen to provide nitrite ions that can be measured the use of Griess reagent at 546 nm spectro photometrically.

Nitric oxide scavenging assay is carried out as in keeping with the approach of *Sreejayan and Rao et al*.In short, 200 µl of 10nM sodium nitroprusside and 200 µl of

check solution/reference trendy of numerous concentrations are incubated at room temperature for 150 minutes. Upload 500 µl Griess reagent and incubated for 10 mints at room temperature measure the absorbance at 546 nm spectro photometrically. Check substances are changed though buffer media for a manage.

The results (**Table 1 and Figure 1**) of NO scavenging activity of the standard drug cisplatin, Castalin and Castalin nanoparticles were shown as percentage of Nitric oxide or reactive nitrogen species, formed during their reaction with oxygen or with superoxides, such as N02, N2O4, N3O4, NO3- and NO2 found to be very reactive. These ionic compounds are responsible for altering the structural and functional behavior of many cellular components. The Sodium nitroprusside solution is incubated in phosphate buffer saline at 25° C for 2 hours resulted in linear time-dependent nitrite production, which was reduced by the tested Castalin and Castalin nanoparticles. This may be due to the antioxidant principles in the Castalin, which compete with oxygen to react with nitric oxide thereby inhibiting the generation of nitrite. Castalin and Castalin nanoparticles shows approximately equal when compare with standard cisplatin. The maximum NO inhibition percentage of standard cisplatin and Castalin and Castalin nanoparticles was found to be 91.98%, 65.86% and 75.22 respectively. IC_{50} values of standard cisplatin and Castalin and Castalin nanoparticles were found to be 23.66, 21.66 and20.88 respectively.

Table 1: Nitric Oxide Radical scavenging assay results are represented follows

Drug	**Concentration (µg/ml)**	**Absorbance 546 nm**	**% Inhibition**	**IC_{50}**
Control	0.0	0.5301	0.0	25.66
Standard (Cisplatin)	5	0.4892	7.22	
	10	0.4268	20.92	
	20	0.3078	41.56	
	40	0.1576	66.82	
	80	0.1148	75.94	
	160	0.0260	92.98	
Castalin	5	0.5132	4.88	22.26
	10	0.4802	10.12	
	20	0.3622	29.98	
	40	0.2798	46.59	
	80	0.2382	55.18	
	160	0.2104	67.86	
Castalin nanoparticle	5	0.4994	5.98	23.88
	10	0.4522	14.12	
	20	0.3368	34.34	
	40	0.2128	51.42	
	80	0.1726	64.26	
	160	0.1098	78.22	

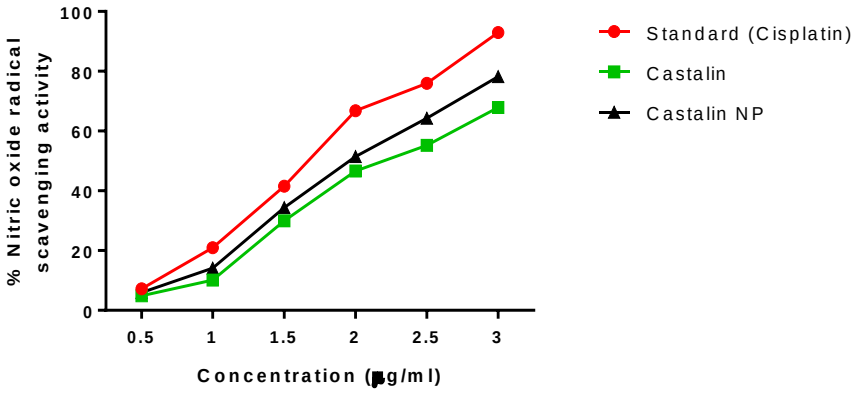

Figure 1. Nitric oxide scavenging assay of standard drug cisplatin, Castalin and Castalin nanoparticles

Cytotoxicity research the usage of HepG2 mobile line by way of MTT assay

HepG2 cell line changed into obtained from Amla cancer studies Institute, India. 50,000 cells / nicely were taken and seeded in 96 nicely plates and incubated for twenty-four hrs at 37^0 C, 5% CO2 incubator. Castalin and Castalin nanoparticles to be tested are added from zero-320µg/ml attention in RPMI with out FBS & are incubated for 24 hr. 100µl/properly of the MTT (five mg/10ml of MTT in 1X PBS) was introduced to incubate the Castalin and Castalin nanoparticles samples to the respective wells and incubated for 3to 4 hours.MTT reagent turned into discarded through pipetting without stressful cells and one hundred µl of DMSO turned into introduced rapidly to solubilize the formazan. Absorbance turned into measured at 590 nm. Inhibition calculation is made using the formulation.

% Inhibition=one hundred-((OD of pattern)/(OD of manipulate))×a hundred

Castalin and Castalin nanoparticles showed significant dose-dependent inhibition of growth of HepG2 cells at IC50 values of 115.2µg/ml **(Table 2 and Figure 2)**. It was found that there were cytotoxic effects with increasing concentration on HepG2 cell line from 10µg to 320µg concentration. Compared to the untreated Hep G2 cells, only 20% growth and cell proliferation is maintained even in the treated cells.

Table 2: Cytotoxicity studies using HepG2 cell line by MTT assay results are tabulated follows

Drug	**Concentration (µg/ml)**	**OD at 590 nm**	**% Inhibition**	**IC_{50}**
Control		0.8156	0.00	46.12
Standard (Cisplatin)	10	0.7012	10.89	
	20	0.6102	28.12	
	40	0.4789	49.34	
	80	0.2846	67.24	

	160	0.2159	75.36	
	320	0.1196	91.26	
Castalin	10	0.7580	7.98	42.24
	20	0.6623	19.92	
	40	0.5190	38.23	
	80	0.3265	57.12	
	160	0.2684	64.68	
	320	0.1524	79.98	
Castalin nanoparticle	10	0.7336	9.89	44.38
	20	0.6324	22.46	
	40	0.4912	41.12	
	80	0.3068	60.29	
	160	0.2348	69.12	
	320	0.1322	85.48	

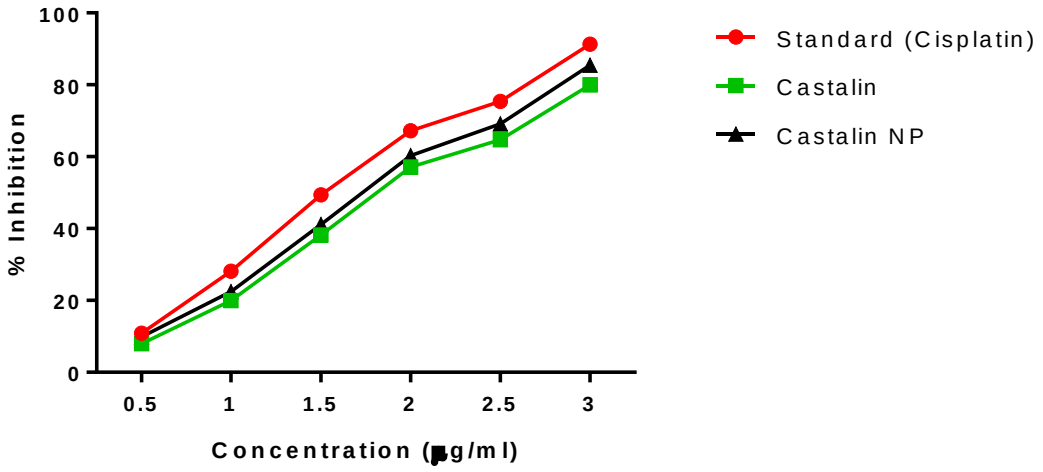

Figure 2. Cytotoxic study of Castalin and Castalin nanoparticles

Acute oral toxicity study

Acute toxicity studies of castalin nanoparticles was carried out in female rats by using Organisation for Economic Co-operation and Development (OECD) guideline 423 as per that the dose intensity used as 5, 50, 300, 2000, 4000 mg/kg. Before oral administration of a single dose of the test samples, the rats were deprived of food for 3 h. Doses of the test samples were given using oral gavage to the rats. After treatment for first four critical hrs then over period of 24 hr thereafter daily for 14 days general behavioral changes symptoms of toxicity and mortality were observed in all the rats. Oral management of the castalin nanoparticles at 300 mg/kg did not produces any clinical signs of toxicity or deaths of the rats. As there were no mortality and clinical signs of toxicity in the tested dose, LD50 value of castalin nanoparticles was found to be greater than 300 mg/kg.

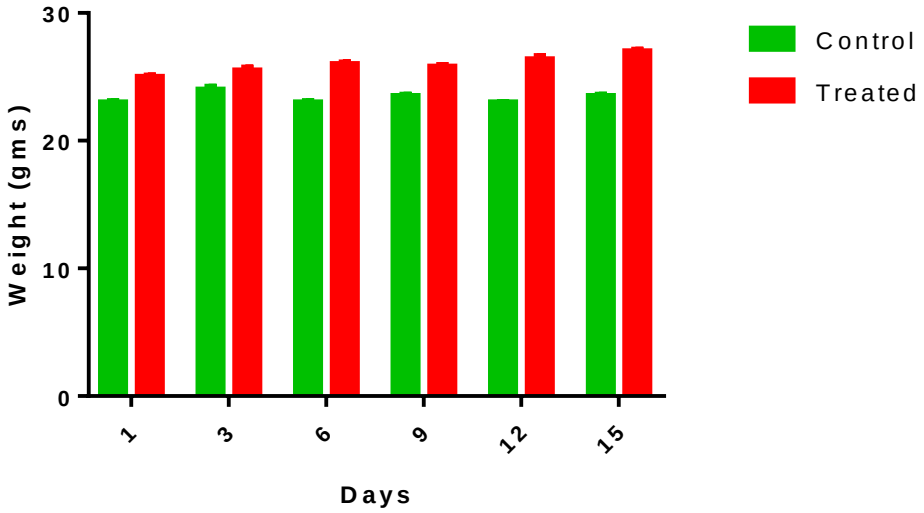

Figure 3: Bodyweight changes of rats followed by the administration of castalin nanoparticles, *p<0.05

Pharmacokinetic analysis

Two groups of six male Wistar albino rats 200-250 gms were kept under a twelve-hour light/dark cycle on standard lab chow. Animals were fasted overnight and received Castalin solution and its nanoformulation at a dose of 30mg /kg body weight by oral gavage. At 30, 60, 90 and 120 min, animals were subjected to retro orbital puncture while they were under diethyl-ether anaesthesia to collect 1 ml blood in to heparinized tubes, centrifuged immediately at 7000 × g for 15 min, plasma was then decanted and stored at −80˚C until analysis. The organs (liver, heart, spleen, lung, kidney and brain) were removed and transferred into 50 ml tubes. Whole blood was collected by cardiac puncture into heparinized tubes.

Quantification of castalin using high-performance liquid chromatography (HPLC) method using C18 Luna column5-µm particle size, 25 cm×3.00 mm I.D. (Phenomenex, Torrance, CA, USA) was used as mobile phase composed by water–formic acid (99.5:0.5, v/v) (solvent A) and acetonitrile (solvent B) was used. The flow rate was 0.5ml/min. The injection volume was 10 µL and the wavelength was set at 246 nm.

Pharmacokinetic calculations were performed on each individual set of data using the WinNonlin Standard Edition Version 2.1 by non-compartmental method. Pharmacokinetic results are represented as mean ± SEM. Statistical analysis was performed by t test (SPSS version10.0) to compare different groups. The level of significance was set at $p < 0.05$.

Castalin and its nanoformulation were preferred for the pharmacokinetics studies. Figure shows the mean plasma castalin concentration versus time profiles before and after oral administration of Castalin nanoparticles at a dose of 30 mg/kg bw. The peak concentration (Cmax) and time of peak concentration (Tmax) were obtained directly from the individual plasma castalin concentration versus time profiles. The area under the concentration time curve from 0 to 120 min ($AUC_{0\text{-}120}$) was calculated using the trapezoidal method. The AUC determines the bioavailability of the drug for a specified dose of the formulation. These oral pharmacokinetic parameters are listed in Table. As shown in Figure, plasma castalin

concentration was significantly higher in rats administrated with castalin nanoparticles than the castalin solution. The Cmax value of castalin in the castalin nanoparticles group (511±68.2µg/L) was much higher than that obtained with the castalin solution (102.1±12.8 µg/L). The AUC_{0-120} value of castalin after oral administration of castalin nanoparticles was 33582.6 µg min/L, which was 4 fold greater than that after castalin solution administration. The data revealed that smaller the particle size greater the effect on enhanced castalin absorption by oral administration. The HPLC chromatogram of castalin in the rat plasma obtained at 30 min is presented in the figure below.

Table 3. Pharmacokinetic parameters of castalin in rats following oral administration of castalin solution and nanoparticles by a dose of 30 mg/kg bw.

Sample	**Cmax (µg/L)**	**Tmax (Min)**	**AUC_{0-120}(µg min/L)**
Castalin solution	102.1±12.8	108	8102.2
Castalin nanoparticles	511±68.2	30	33582.6

AUC, part under the plasma concentraton vs time curves; Cmax, maximum concentration and Tmax, time to reach the maximum concentration.

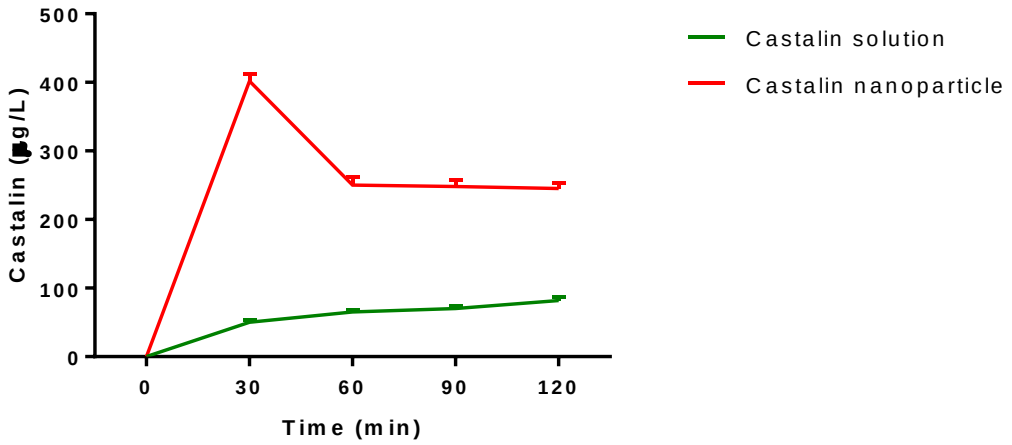

Figure 4. Represents Mean plasma concentration-time curve oral administration of castalin solution and nanoparticles to rats at a dose of 30 mg/kg body weight (n=6)

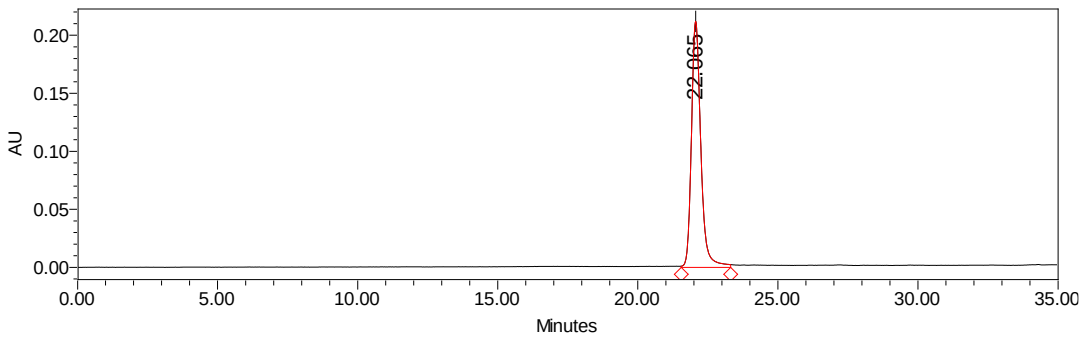

Figure 5.HPLC chromatogram of castalin in rat plasma collected at 30 min

Evaluation of Anticancer Activity

Induction of cancer using DAL Cells

Cell line-induced cancer in Swiss albino mice were used to evaluate the anticancer activity of castalin nanoparticles. DAL cells were supplied by Amala Cancer Research Centre, Trissur, Kerala, India. Upon intraperitoneal transplantation; the DAL cells were maintained in vivo in the study animals. The cell counts were done, and further dilutions were made so that the total cell should be 1×10^6cells. The tumor was let to grow in the mice for minimum of 7 days before starting treatments.

Treatment Schedule

Swiss albino mice (20–25 g) were divided into four groups of 6 animals each. All the animals in each groups except group I received DAL cells (1×10^6 cells/mouse, ip). This was taken as day "0."

Group I – Normal control (saline 1ml/kg body weight)

Group II- DAL control.

Group III – Castalin solution (past 24 hof DAL transplantation, animals during group III received castalin solution by a dose of 30 mg/kg bw orally for 14 days)

Group IV - Castalin nanoparticles (once 24 h of DAL transplantation, animals in group IV received castalin nanoparticles at a dose of 30 mg/kg bw orally for 14 days)

After administration of the last dose, three mice from each group were kept fasting for 18 h and blood was collected by retro-orbital plexus for the estimation of biochemical and hematological parameters. For the study of antitumor activity animals are sacrificed. The rest of the animals in each group were kept alive with food and water ad libitum to check percentage increase in life span of the tumor host to determine the mean survival time (MST).Antitumor activity of castalin nanoparticles was assessed by observation of changes with respect to the following parameters, viz, tumor volume and weight. by taking the weight of mice before and after ascitic fluid collected from the peritoneal cavity tumor weight was measured by taking it in a graduated centrifuge tube and expressed in milliliter. The tumor weight was measured by taking the weight of the mice before and after the collection of the ascitic fluid from peritoneal cavity and expressed in gram.

Percentage Enlarge Life Span

The effect of castalin nanoparticles on tumor growth was monitored by recording the mortality of the experimental mice. Percentage enlarge in life span (% ILS) was calculated by the subsequent formula:

$$ILS\ (\%) = \left[\frac{Mean\ survival\ time\ of\ the\ treated\ group}{Mean\ survival\ time\ of\ the\ conrol\ group}\right] - 1 \times 100$$

$$MST = \frac{Day\ of\ the\ first\ death + Day\ of\ the\ last\ death}{2}$$

Tumor Cell (Viable/Nonviable) Count

The ascitic fluid was taken in a white blood cell (WBC) pipette and diluted 20 times. Then, a drop of the diluted cell suspension was cited on the Neubauer's counting chamber and the number of cells in 64 small squares was counted. The viability and nonviability of the cell were checked by trypanblue assay. The cells were stained with trypan blue (0.4 % in normal saline) dye. The cells that did not take up the dye were viable, and those that took the dye were nonviable. These viable and nonviable cells were counted.

$$Cell\ count = \frac{Number\ of\ cells\ \times Dilution\ factor}{Area\ \times Thickness\ of\ liquid\ film}$$

Haematological Parameters

Collected blood was used for the assessment of hemoglobin (Hb) content, red blood cell (RBC) count, and WBC count by standard procedures.

Biochemical Parameters

The blood samples be allowed to clot, and the serum was separated by centrifugation at 5,000 rpm for 10 min. Serum was wear to estimate the biochemical parameters like total cholesterol, triglycerides, aspartate aminotransferase (AST),alanine aminotransferase (ALT), and alkaline phosphatise (ALP). All the analysis be performed by using commercially available kits from Span Diagnostics Ltd., Surat, India. Animals were then sacrificed by ether anesthesia, and the liver was dissected out, washed, and transferred to an ice-cold saline solution. The organ was weighed, and portion of the organ was fixed in 10 % formalin for histopathological examinations.

The data with antitumor activity study were expressed as the mean±SEM and statistically analyzed by one-way analysis of variance (ANOVA) followed by Dunnett'spost hoc test by Graph Pad Prism V7. $P<0.05$ was considered as significant and $P<0.01$ as highly significant.

Antitumor Activity

When compared with treated animals the tumor weight and tumor volume and viable cell count were found to be significantly increased and non viable cell count was significantly decreased in DAL control animals. Administration of castalin nanoparticles at the dose of30 mg/kg bw significantly decreased the tumor volume and viable cell count. Nonviable cell count was significantly higher in castalin nanoparticles treated animals when compared with DAL control animals. Further, the median survival time was increased on administration of castalin nanoparticles.

Table 4. Effect of castalin nanoparticles on tumor volume, tumor weight, total cell count, viable and nonviable cell count, and median survival time (MST) and percentage increase life span (% ILS) in DAL-bearing mice.

Study parameters	**DAL control**	**DAL + castalin solution**	**DAL+castalin nanoparticles**
Tumor volume (ml)	3.1±0.02	0.74±0.02*	0.87±0.04*
Tumor weight (g)	2.38±0.02	0.82±0.10*	0.89±0.02*

Total cell ($x10^7$ cell/ml)	9.64±0.28	5.02±0.14*	4.98±0.12*
Viable cell ($x10^7$ cell/ml)	8.18±0.16	0.59±0.88*	1.02±0.02*
Nonviable cell ($x10^7$ cell/ml)	0.12±0.02	2.36±0.08*	2.44±0.04*
MST (days)	15±1	32±0.5	38±1.4
ILS (%)	0	74.24	77.82

Values are represented as mean±SD from six observations in each group. *P<0.05 (one-way ANOVA between DAL control group vs treated groups followed byDunnett's test)

Haematological Parameter

There was increased level of WBC and decreased level of hemoglobin (Hb) and RBC in DAL control group as compared to normal control group. After treatment with castalin nanoparticles by the dose of 30 mg/kg in DAL-bearing mice significantly increased the RBC count, Hb content, and significantly reduced the WBC count as compared with the DAL control group.

Table 5. Effect of castalin nanoparticles on hematological parameters in DAL-bearing mice.

Study parameters	**Control**	**DAL control**	**DAL+castalin solution**	**DAL+castalin nanoparticle**
RBC (cellx106/µl)	4.79±0.28	$2.48±0.12^{a*}$	$4.02±98^{b*}$	$4.38±0.24^{c*}$
WBC (cellx106/µl)	4.18±0.22	$6.37±0.12^{a*}$	$4.06±0.19^{b*}$	$4.69±0.28^{c*}$
Hb (g/dl)	10.08±0.12	$5.02±0.21^{a*}$	$8.16±0.67^{b*}$	$9.08±0.21^{c*}$

Values be represented as mean±SD from six observations in each group. *P<0.05 (one-way ANOVA between DAL control group and the treated groups followed by Dunnett's test[a]DAL control group vs normal group, [b]castalin solution treated group vs DAL control group, [c]castalin nanoparticles treated group vs DAL manage group

Table 6. Effect of castalin nanoparticles on serum enzyme and lipid protein in DAL-bearing mice.

Study parameter	**Control**	**DAL control**	**DAL+castalin solution**	**DAL+castalin nanoparticles**
Cholesterol (mg/dl)	97.12±1.89	$152.24±1.2^{a*}$	$120.09±2.4^{b*}$	$100.12±1.2^{c*}$
TGL (mg/dl)	115.26±2.2	$224.12±2.5^{a*}$	$131.34±1.8^{b*}$	$125.98±2.4^{c*}$
AST (U/L)	32.19±1.2	$91.02±1.8^{a*}$	$46.26±2.5^{b*}$	$35.97±1.6^{c*}$
ALT (U/L)	30.28±1.34	$81.04±3.1^{a*}$	$41.49±3.2^{b*}$	$30.18±0.8^{c*}$
ALP (U/L)	108.98±1.68	$220.08±1.8^{a*}$	$120.89±1.5^{b*}$	$100.23±1.4^{c*}$

Results are represented as mean±SD from six observations in each group. *P<0.05; **P<0.01 (one-way ANOVA between DAL manage group and the treated groups followed by Dunnett's test)[a]DAL control group vs normal group, [b]castalin solution treated group vs DAL control group, [c]castalin nanoparticles treated group vs DAL manage group

Serum Enzyme and Lipid Protein

Biochemical parameters like AST, ALT, ALP, cholesterol and triglyceride in the DAL control group were significantly increased as compared to the normal control group. Treatment with castalin nanoparticles in DAL-bearing mice significantly decreased cholesterol, triglyceride, and the biochemical parameters.

Histopathological Examination

Control animals for liver section of, toxic control animals, Castalin treated and castalin nanoparticles treated are given in figure below.

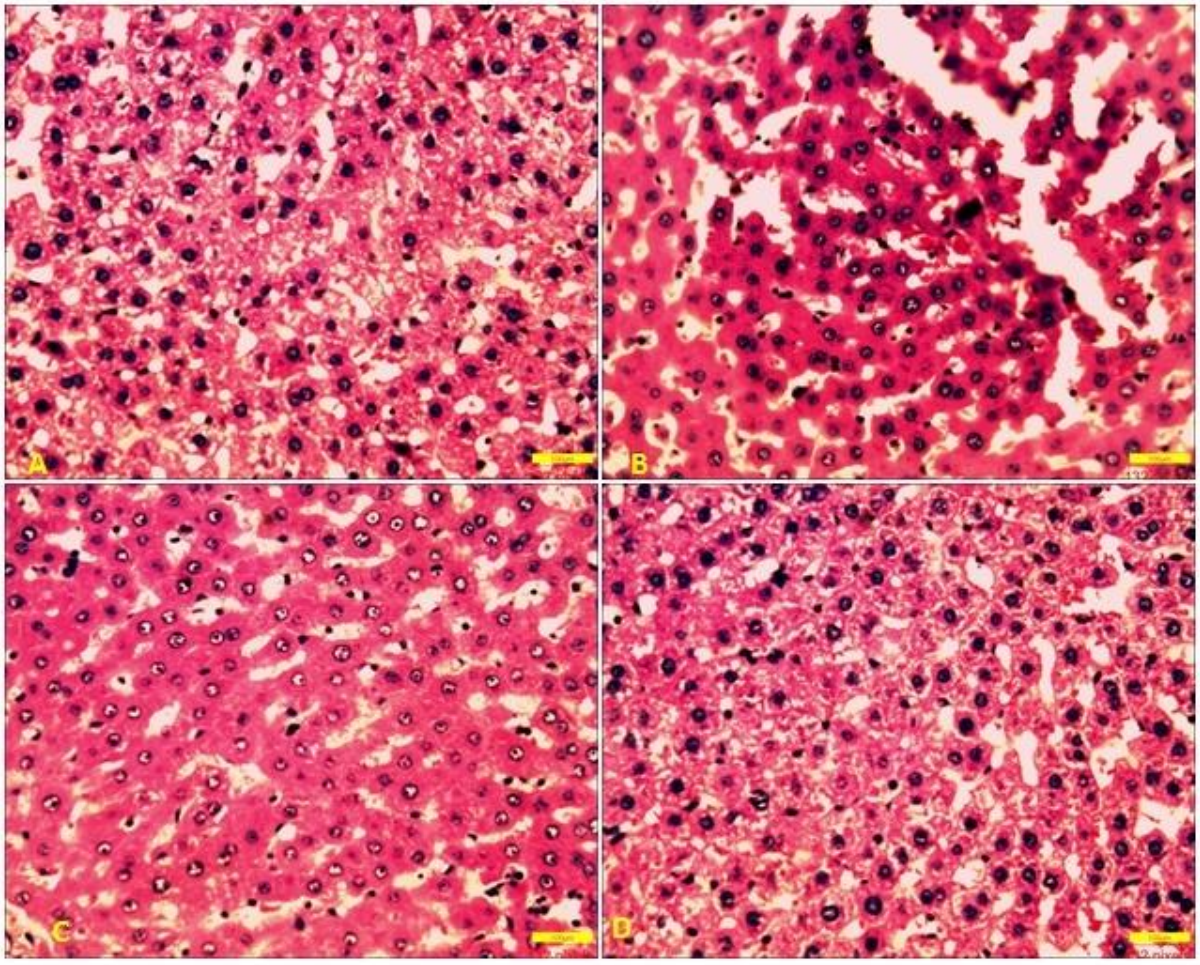

Figure 6. (A) Liver section of normal control shows structure of liver with sheets of hepatocytes separated by sinusoids cartial vein and portal tract appears normal. (B) Liver section of toxic control shows structure of the liver presenting hepatic congestion at sinusoids and the portal vessel, pericenter globular microsteatosis, Kupffer cell proliferation,hepatocyte diffuse necrosis, and mononuclear infiltrate. (C) castalin solution for liver section was treated

shows the liver structure presenting mild hepatic congestion at sinusoids and the portal vessel,pericenter globular microsteatosis, no Kupffer cell proliferation, mild hepatocyte diffuse necrosis, and mononuclear infiltrate. (D) Liver section of castalin nanoparticles treated shows structure of the liver presenting no hepatic congestion at sinusoids and the portal vessel, pericenter globular microsteatosis, no Kupffer cell proliferation, no hepatocyte diffuse necrosis, and mo

Invitro drug release study

100 ml of phosphate buffer, pH adjusted to7.4, was poured into a well-closed glass vessel as the dissolution medium for the in vitro release test. Castalin nanoparticles (5 ml) was transferred to a dialysis bag (molecular weight cut off 5000–10,000), and then the dialysis bag was placed in the glass vessel. The vessels were placed in an incubator shaker and shaken horizontally at 37 °C and 100 strokes per min. The sample (1 ml) was withdrawn from the system at predetermined time intervals and filtered through a 0.45-µmhydrophilic filter membrane. The drug content was measured by the HPLC technique was calculated by the drug content. The diffusion profile of pure drug suspension through a dialysis bag was examined as control. The pure drug suspension was prepared by dispersing 1 ml Castalin solution (5 mg/ml) in 4 ml of double distilled water. Each experiment was performed in triplicate.

The in vitro release behavior of Castalin from the solution and nanoparticles were studied for 24h as shown in the figure. Biphasic release pattern was observed. Initially a burst release followed by a sustained release. All most all the 100% drug in the solution get released in 2h. There was again an initial burst release observed It was found that there was an initial burst release of Castalin from the nanoparticles, and this may be due to Castalin adsorption on the surface of the nanoparticles. A sustained drug release of about 60 % was observed for the nanoparticles over 24 h.

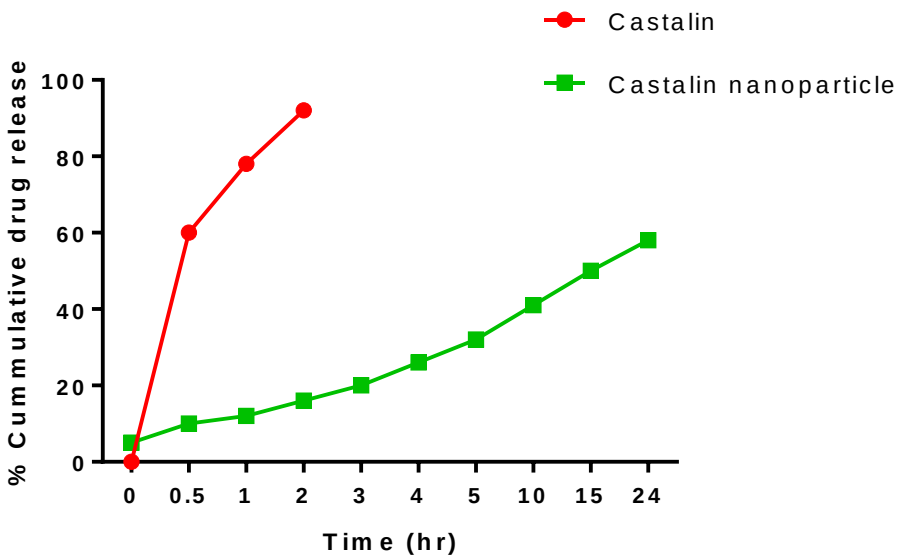

Figure 7.Cumulative Percentage drug release of pure drug Castalin and Castalin nanoparticles within Phosphate buffer solution (pH 7.4)

Stability study

To explore the impact of storage temperature on the stability of castalin nanoparticles, the nanoparticles stored at 4±2°C the same as 25±2°C in the dark over duration of 360 days. A mount in particle size and also reduction in zeta potential as same as entrapment efficiency were observed with storage time at both the storage conditions. just before examine any changes in the drug release profile during storage, drug release studies were done and also compared to the preliminary formulations. A minor difference in release rate was practical from both the formulations stored at special conditions. A sustained drug release was noticed with both the formulations stored at different storage conditions.

Table 7. Stability study data of castalin nanoparticles stored at two different temperatures

Samples	**4°C±2°C**	**25°C±2°C, 60%±5%RH**
	Particle size (nm)	
0	264	264
6	301	311
12	303	318
	Entrapment efficiency (%)	
0	49	49
6	48	45
12	46	44
	Zeta potential (-mV)	
0	-25	-25
6	-25	-24
12	-24	-24

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- *Treatment. The column used was a phenomenex ® Luna C18, 5 µm, 250 mm × 4.6 mm (Phenomenex, Torrance, CA, USA) with a phenomenex ® guard column, 4 mm length × 3 mm id (Phenomenex, Torrance, CA, USA)*
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