PHARMACOKINETICS AND ENHANCED ORAL BIOAVAILABILITY IN ALBINO RABBITS OF SIMVASTATIN NANOPARTICLES

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ABSTRACT

Aims: The aim of the study was to compare the single dose oral bioavailability of simvastatin nanoparticles (SV), Simvastatin pure drug and marketed tablet formulation in albino rabbits.

Study design: Plasma was analyzed for simvastatin using a sensitive, reproducible, accurate and validated RP-HPLC method. Pharmacokinetic parameters including AUC, Cmax, Tmax, t1/2, MRT and Kel were determined from plasma concentration of the simvastatin nanoparticles, simvastatin pure drug and marketed tablet formulations.

Methodology: Albino rabbits were grouped as standard I (6), Standard II (6) and Test (6). Simvastatin pure drug and simvastatin marketed formulation were administered to standard group. Simvastatin loaded nanoparticles suspension was administered to test group. The pharmacokinetic parameters of SV nanoparticles, SV pure and SV marketed tablets were compared in albino rabbits.

Results: Cmax of SV nanoparticles was found to be 63.35±0.195 ng/ml, whereas Cmax value for the drug suspension and marketed tablet formulation was found to be 34.00 ± 0.100 ng/ml and 46.91± 0.194 ng/ml respectively (p < 0.001). AUC (0–8h) value for the simvastatin nanoparticles, drug suspension and marketed tablet formulation were found to be 235.36 ± 0.101 (ng/ml×h), 152.72±0.20 (ng/ml×h) and 189.22± 0.23 (ng/ml×h) respectively (p<0.001). The results revealed that relative bioavailability (Fr %) was increased as compared to oral control group standard I and II. Conclusion: Simvastatin nanoparticles showed a significant improvement in bioavailability as compared with the pure drug and conventional marketed tablet

Keywords: Bioavailability, Pharmacokinetics, RPHPLC, Simvastatin, Nanoparticles.
INTRODUCTION

Poor solubility is in most cases associated with poor bioavailability. There are two basic approaches to overcome the bioavailability problems of these drugs, increase of saturation solubility and dissolution velocity [1,2]. A much more straightforward way is increasing the dissolution velocity by increasing the surface area of the drug powder. Exhaustive research has been carried out on increasing dissolution and bioavailability of SV such as micronization, molecular dispersion, incorporation of surfactants, inclusion complexation with cyclodextrin, crystal modification, glass formation and co-precipitation; these studies was explored earlier. However, many of the new compounds show such a low solubility that micronisation does not lead to a sufficient increase in bioavailability after oral administration [3,4]. Therefore the next step taken was nanonisation. The drug powder is transferred to drug nanoparticles, typical sizes are around 200-600nm. The main production technologies currently in use top down to produce drug nanoparticles. However, the most convenient dosage form for the patient is a dry product, e.g. tablet or capsule.

Drug delivery approaches aim to develop a carrier system, which can hold the drug molecule effectively and can navigate them towards the right destination without affecting the route and at the same time modify the drug release characteristics. For water insoluble drugs with high permeability, drug absorption by GIT is limited by drug dissolution rate. Solubility/dissolution are good pointer and major contributor to drug bioavailability [5]. Nanoparticles have drawn greater attention because of their solubilisation and transport properties. Simvastatin, which is a potent and effective lipid lowering agent from the family of statins with a good tolerability profile, has systemic bioavailability 5%. Simvastatin is used to control hypercholesterolemia (elevated cholesterol levels) and to prevent cardiovascular disease [6,7]. Simvastatin can decrease low density lipoprotein (LDL) levels by up to 50%. From recent research it has become apparent that simvastatin inhibit the progression of atherosclerosis beyond their effects on LDL [8-11]. Limited analytical methods have been developed for the determination of simvastatin in biological samples along them HPLC methods.

Therefore, the aim of the present investigation was to develop a new, sensitive HPLC method for the estimation of simvastatin in albino rabbits plasma. The outcome of a study depends upon the reliability, reproducibility and sensitivity of the analytical methodology employed [12,13]. Therefore, the bioanalytical method was validated prior to the initiation of the study.
The study was to developed nanoparticles of simvastatin and to assess the bioavailability in comparison with the pure drug and conventional marketed tablets in albino rabbits.

**MATERIAL AND METHODS**

Simvastatin was obtained gift sample from Aurobindo Pharmaceutical Ltd., Hyderabad; Poly (D, L Lactide-co- Glycolide) (PLGA 50:50) was obtained as gift samples from Purac biochem ltd. Netherland; Pluronic F 68 was purchased from sigma chemicals, Mumbai, dialysis bag (cellophane membrane, molecular weight cut off 10000-12000 Da, purchased from Hi-Media, Mumbai, India. All other reagents and chemicals used in this study were of analytical grade.

**COMPATIBILITY STUDIES**

Compatibility of the simvastatin (SV) with PLGA used to formulate nanoparticles (NPs) was established from FTIR spectrum and DSC thermogram analysis. FTIR & DSC spectral analysis of SV and combination of SV and PLGA was carried out to investigate the changes in chemical composition of the drug after combining it with the excipients. Compatibility study was carried out on FTIR (Jasco V-530) and DSC (TA-60, Instruments SDT-2960, USA).

**PREPARATION OF PLGA NANOPARTICLES**

PLGA nanoparticles were prepared by the nanoprecipitation - solvent displacement method [14,15]. Fixed amount of simvastatin was dissolved in acetone. Hydrophilic stabilizer pluronic F-68 of concentration (0.2%, 0.3% & 0.4 % ) was also dissolved in water. PLGA was solubilized in acetone at various concentrations (1:1, 1:2 & 1:3 ). The organic phase was poured into the aqueous solution drop wise, at 1ml/min flow with syringe positioned with the needle directly into stabilizer containing water, which was stirred at 1500 rpm for 2 hrs, thus forming a milky colloidal suspension. The organic solvent was then evaporated by using a Rota evaporator. All experiments were performed in triplicates. Nanoparticles were collected by centrifugation at 15,000 rpm for a period of 1h and supernant phase discarded. The resultant dispersion was dried using a freeze-drying method [16,17].

**CHARACTERISATION OF NANOPARTICLES**

Simvastatin nanoparticles was characterized for particle size, zeta potential and polydispersity index, % entrapment efficiency, % process Yield, % drug content and *in-vitro* drug release studies. The compatibility of drug and excipients were calculated by fourier
transform infrared spectroscopy study. The percentage degree of crystallinity of SV, Physical mixture and various batches of nanoparticles were calculated by differential scanning calorimetry. Detection of the crystallinity of the pure drug and the nanoparticles formulation was performed [18]. The morphology of nanoparticles was observed using scanning electron microscopy. The morphology of nanoparticles was observed by Transmission electron microscopy. The optimized batch of prepared nanoparticles was selected for bioavailability study on albino rabbits.

**DETERMINATION OF SIMVASTATIN IN ALBINO RABBITS BLOOD BY RP-HPLC**

1. **Linearity study of simvastatin**
   Standard working solutions of 1000µg/ml of simvastatin, were prepared using mobile phase as a solvent. Required volume of solution from standard working solution was taken to get final dilutions of required strength for calibration curves and made up the volume with mobile phase. The HPLC analysis of all aliquots was carried out and response factor for each analyte was calculated. [19]

2. **Preparation of Internal Standard Solution**
   Standard stock solution containing lovastatin was prepared by dissolving 50 mg of lovastatin in 20 ml of mobile phase. It was then sonicated for 10 minutes and the final volume of solution was made up to 50 ml with mobile phase to get 1000 µg/ml of lovastatin in a 50 ml volumetric flask.

3. **Recovery Studies**
   Accuracy of analysis was determined by performing recovery studies by spiking different concentrations of pure drug in the pre-analyzed laboratory sample

4. **System Suitability Parameters**
   System suitability parameters were analyzed on freshly prepared standard stock solutions of simvastatin. All these analytes were injected into the chromatographic system under the optimized chromatographic conditions. Parameters that were studied to evaluate the suitability of the system were number of theoretical plates, calibration curve, capacity factor, resolution factor, retention time.

5. **Experimental animals**
   The albino rabbits of either sex having weight 1.5 to 3.0 kg were used for bioavailability
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study of nanoparticles formulation along with free simvastatin. The albino rabbits were kept under standard conditions in animal house of Bharati Vidyapeeth College of Pharmacy, Kolhapur, as per guidelines of CPCSEA; approval letter no. BVCPE/ CPESEA/ IAEC/ 01/17 dated Jan. 12, 2011. All the animals randomly divided into three treatment groups with six animals in each group as follows: standard I (6), Standard II (6) and Test (6). SV suspension and SV marketed formulation were administered to standard group. SV loaded nanoparticles suspension was administered to test group.

Animals had free access to food and water ad libitum. The formulations were provided orally using 23-gauge oral feeding needle. SV pure drug suspension and SV tablet suspension 1mg/kg body weight equivalent dose were orally administered to Reference group (RG) rabbits. SV optimized batch nanoparticles formulation 1 mg/kg body weight equivalent dose was orally administered to Treatment group (TG) rabbits. Rabbits were anaesthetized using ether. Blood samples were withdrawn from marginal ear vein of rabbits at 0.5, 1, 2, 4, 8 and 12 h. The plasma was separated and drug content was estimated using RP-HPLC. In this method acetonitrile and double distilled water (pH 3) (80:20) with 0.1% orthophosphoric acid (OPA) as mobile phase using RP-HPLC (Jasco PU 2080 Pump, UV 2075 Detector).

6. Processing of Blood samples for HPLC analysis

All frozen (below 0°C) plasma samples were thawed at ambient temperature. 200 µl plasma sample was transferred to a 2 ml polypropylene test tube. The tube was vortex and then liquid extraction was carried out with 1 ml of methyl tert-butyl ether. Tube was vortexed for 30 sec. and centrifuged for 15 min at 4°C at 3000 rpm. The supernant was separated and transferred to a clean polypropylene test tube and air dried at 40°C. The residue was reconstituted with 100 µl of methanol and filtered through 0.22 µm syringe filter, then 20 µl volume was injected into RP-HPLC. The flow rate was 1 ml/min and UV detection was performed at 238 nm wavelength. The retention time of simvastatin was determined [20-21].

7. Estimation of Pharmacokinetic Parameters

The pharmacokinetic parameters for simvastatin pure drug suspension, marketed formulation and nanoparticles dispersion following oral administration were determined from plasma concentration data. The total area under the concentration-time curve (AUC) from time zero to infinity was calculated by the trapezoidal rule method. The maximal concentration (Cmax) and the time to maximal concentration (tmax) were obtained directly by observation. The relative bioavailability is determined, when there are no i.v. data, by comparing different
dosage forms. As with calculation of bioavailability, clearance is assumed to be constant. The relative bioavailability was determined from AUC data [22-23]. The pharmacokinetic (PK) parameters were performed by non-compartmental analysis. All values are expressed as the mean ± SD. All the analysis of data was performed using statistical software Graph pad prism 5 version. (GraphPad Software, San Diego, CA, USA), using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. Difference between two parameters were considered stastically significant for $P<.001$. [24]

**RESULTS AND DISCUSSION**

**Characterization of nanoparticles**

FTIR studies showed that the fundamental peaks of SV were retained. Compliance with peak of PLGA and SV, indicate that SV is compatible with the PLGA and pluronic F68. DSC curve of simvastatin(SV)/PLGA physical mixture showed a glass transition peak at 33.83°C corresponding to the PLGA, followed by the endothermal melting peak at 138.98°C indicating its crystalline nature (the endothermic value was 25.73 J/g). Results from FTIR and DSC spectras indicate that there was no chemical interaction between simvastatin (SV) and excipients used in the formulation hence, can be used in the formulation of nanoparticles (NPs). Simvastatin-loaded nanoparticles resulted in maximum supersaturated concentrations from nanoparticles, hence increase in solubility after 48h (81.58 ±1.60µg/ml) in comparison with simvastatin pure drug, i.e increase in solubility approximately 5 fold.

Analysis of results indicates that particle size range was 100- 300 nm. As the concentration of PLGA was increased, particle size also increased but Pluronic F68 surfactant concentration played important role in maintaining particle size in submicron range, which is evident from particle size of batch PS6, which was 122± 1.52 nm with 0.4 % surfactant concentration and 100 mg of PLGA with (1:2) proportion of SV and PLGA. Particle size of nanoparticles was not only dependent on the PLGA amount used in formulation but also dependent on the Pluronic F68 surfactant concentration (i.e increase concentration of surfactant, decrease particle size of nanoparticles). Particle size of optimized batch of PS6 is shown in fig.1. Polydispersity index (PI) of prepared nanoparticles batches were found in the range 0.4508 to 0.9669, which was near to 1 for all nanoparticles batches.
The percentage entrapment efficiency of batches under investigation was in the range of 70.0-85.43%. Batch PS6 showed entrapment efficiency 85.43 ± 0.49%. Prepared nanoparticles batches, PS6 batch zeta potential was (-23.32 ± 0.01), means near to range, which indicates good physical stability of nanoparticles. Cumulative drug release for all formulations batches PS1–PS9 were found to be 77.24± 0.317% to 96.53 ± 0.501% respectively, after 60min. Finally, it can be concluded that the different drug release rates may be attributed to different sizes of the nanoparticles.

The DSC curves of simvastatin nanoparticles no sharp endotherm was seen at 130.19°C (endothermic value was 3.122 J/g), suggesting that SV in nanoparticles was molecularly dispersed as a less crystalline form. This SV was amorphous after being precipitated as nanoparticles; its melting point was decreased indicating reduced crystallinity. The nanoparticles prepared with PLGA of PS6 batch was characterized by less intensity of the diffraction peak, when compared to that of SV. This clearly indicates the significant reduction in the crystallinity of the precipitated SV nanoparticles. Scanning electron microscopy study of prepared SV-loaded PLGA nanoparticles of batch PS6 had a drastic change in the morphology and shape of drug, nearly spherical shape with a relatively uniform size of about 122 nm in diameter and no drug crystals were present in nanoparticles. The morphology of nanoparticles was observed by Transmission electron microscopy (TEM) of optimized batch PS6, spherical shape and uniform size.

**VALIDATION OF METHOD DEVELOPMENT**

The HPLC chromatogram of simvastatin, overlain spectra of HPLC chromatogram of simvastatin and calibration curve of simvastatin by HPLC are given in fig. 2, 3 respectively.
Different parameters of calibration curve such as slope, intercept and coefficient of correlation obtained are given in Table 1.

Table 1: Various constant for calibration curves in HPLC

<table>
<thead>
<tr>
<th>Regression Equation</th>
<th>Simvastatin</th>
<th>Lovastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Data</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y = A + B*C</td>
<td>Simvastatin</td>
<td>Lovastatin</td>
</tr>
<tr>
<td>Slope (B)</td>
<td>2037.28</td>
<td>0.0257</td>
</tr>
<tr>
<td>Intercept (A)</td>
<td>13076.77</td>
<td>0.0200</td>
</tr>
<tr>
<td>Correlation coefficient (R)</td>
<td>0.9910</td>
<td>0.9997</td>
</tr>
</tbody>
</table>

Where C is the concentration in µg/ml and Y is the unit of response factor.

1. Linearity study of simvastatin

The calibration curve for simvastatin was found to be linear in concentration range of
50μg/ml to 250μg/ml. The result of laboratory sample assay is reported in Table 2.

Table 2: Results of Analysis of Laboratory Sample

<table>
<thead>
<tr>
<th>Analyte</th>
<th>% Concentration estimated*(Mean ± S.D.)</th>
<th>% R.S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simvastatin</td>
<td>99.13 ± 1.6517</td>
<td>0.8754</td>
</tr>
<tr>
<td>Lovastatin</td>
<td>100.03±1.9951</td>
<td>1.9945</td>
</tr>
</tbody>
</table>

* Average of six determinations.

2. Recovery Studies

Results of recovery studies indicating that the method is rapid, accurate and reproducible are shown in Table 3.

Table 3: Results of Recovery Studies

<table>
<thead>
<tr>
<th>Analyte</th>
<th>% Recovery estimated*(Mean ± S.D.)</th>
<th>% R.S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simvastatin</td>
<td>99.05 ± 1.13586</td>
<td>0.9632</td>
</tr>
<tr>
<td>Lovastatin</td>
<td>100.59±1.2353</td>
<td>1.2280</td>
</tr>
</tbody>
</table>

* Average of six determinations.

3. System Suitability Parameters

All these analytes were injected into the chromatographic system under the optimized chromatographic conditions shown in Table 4. The limit of detection and limit of quantitation given in Table 5.

Table 4: System Suitability Parameters

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Parameters</th>
<th>Simvastatin</th>
<th>Lovastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Retention Time in minutes (α)</td>
<td>9.073</td>
<td>7.388</td>
</tr>
<tr>
<td>2.</td>
<td>Number of Theoretical plates</td>
<td>9021.26</td>
<td>7642.3</td>
</tr>
<tr>
<td>3.</td>
<td>Asymmetry</td>
<td>2.161</td>
<td>2.015</td>
</tr>
<tr>
<td>4.</td>
<td>Calibration Curve (µg/ml)</td>
<td>10-250</td>
<td>0.2-25.6</td>
</tr>
<tr>
<td>5.</td>
<td>Capacity factor (k')</td>
<td>902.35</td>
<td>738</td>
</tr>
<tr>
<td>6.</td>
<td>Resolution (R_S)</td>
<td>4.52</td>
<td>2.86</td>
</tr>
</tbody>
</table>

Table 5. Limit of Detection and Limit of Quantitation

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Analyte</th>
<th>Simvastatin</th>
<th>Lovastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Limit of Detection (µg/ ml)</td>
<td>0.045</td>
<td>0.031</td>
</tr>
<tr>
<td>2.</td>
<td>Limit of Quantitation ( µg/ ml)</td>
<td>0.035</td>
<td>0.094</td>
</tr>
</tbody>
</table>

4. Estimation of pharmacokinetic parameters

The retention time of HPLC chromatogram of simvastatin and lovastatin were found to be
Pharmacokinetic parameters of SV pure, SV marketed tablets and SV nanoparticles were compared in albino rabbits. Mean plasma simvastatin concentration (in-vivo release profile) was plotted as a function of time. The bioavailability study was performed with objective of estimating simvastatin after oral administration. The plasma concentration (ng/mL) of optimized batch nanoparticles formulation, marketed formulation and pure SV suspension were showed more concentration in plasma (19.11 ± 0.108 ng/mL), 14.31 ± 0.078 and 9.30 ± 0.138 respectively at 0.5 h after oral administration. By comparison of standard and test group it was observed that Cmax of Simvastatin nanoparticles was found to be 63.35 ± 0.195 ng/ml, whereas Cmax value for the drug suspension and marketed tablet formulation was found to be 34.00 ± 0.100 ng/ml and 46.91± 0.194 ng/ml respectively (P<.001) indicating facilitated absorption of simvastatin by nanoparticles. After oral administration, simvastatin nanoparticles were absorbed slower than simvastatin pure and marketed formulation. T max of simvastatin nanoparticles was 2 hrs, whereas it’s value for the drug suspension and marketed tablet formulation was 1 h respectively (P < .001).

AUC (0–8h) value for the simvastatin nanoparticles, drug suspension and marketed tablet formulation were found to be 235.36± 0.101 (ng/ml × h), 152.72 ± 0.20 (ng/ml × h) and 189.22± 0.23 (ng/ml × h) respectively (P<.001). AUC (0–∞ h) value for the simvastatin nanoparticles, drug suspension and marketed tablet formulation were found to be 264.42±0.75 (ng/ml × h), 174.18± 0.88 (ng/ml × h) and 221.17 ±0.89 (ng/ml × h) respectively (P<.001). The elimination rate constant (Ke) value for the simvastatin nanoparticles, drug suspension and marketed tablet formulation were found to be 0.320, 0.269 and 0.256 respectively (P < .001).

The elimination half life (t_{1/2}) for the simvastatin nanoparticles, drug suspension and marketed tablet formulation were found to be 2.70 h, 2.56 h and 2.16 h respectively (P < .001). The mean residence time (MRT) values for the simvastatin nanoparticles, drug suspension and marketed tablet formulation were found to be 3.88 h, 3.68 h and 3.11 h respectively. (P< .001).

The relative bioavailability of oral nanoparticles was determined by taking the area under curve of oral administration of nanoparticles dispersion of optimized batch, pure drug and marketed formulation. The results revealed that relative bioavailability (Fr %) was increased as compared to oral control group standard I & II, it was found that relative bioavailability
was 151.80 % and 119.55 % respectively ($P<.001$). The Pharmacokinetic data for pure drug, marketed formulation & nanoparticles optimized batch formulation in plasma shown in table 6.

The in vivo study results reveals that SV nanoparticles show better bioavailability than drug suspension and marketed formulation. It indicated that significantly enhanced bioavailability of simvastatin has been achieved through formulation of nanoparticles ($P<.001$). The SV nanoparticles have the potential to be used to increase the oral bioavailability of highly lipophilic drugs. Either released drug can be transported across the endothelial cell membrane, therefore achieving enhanced drug absorption. Thus, bioavailability enhancing capacity of nanoparticles formulation of simvastatin could be successfully proven.

**Table 6: Pharmacokinetic data for Pure drug, Marketed formulation & Nanoparticles PS6 batch formulation in plasma.**

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>Pure Drug suspension</th>
<th>Marketed formulation</th>
<th>Nanoparticles PS6 batch***</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ (ng/ml)</td>
<td>34.00 ± 0.100</td>
<td>46.91± 0.194</td>
<td>63.35 ± 0.195</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>AUC (ng/ml × h) (0-8 h)</td>
<td>152.72 ± 0.20</td>
<td>189.22± 0.23</td>
<td>235.36± 0.10</td>
</tr>
<tr>
<td>AUC (ng/ml × h) (0-\infty h)</td>
<td>174.18± 0.88</td>
<td>221.17 ±0.89</td>
<td>264.42±0.75</td>
</tr>
<tr>
<td>$K_e$</td>
<td>0.269</td>
<td>0.320</td>
<td>0.256</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>2.56</td>
<td>2.16</td>
<td>2.70</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>3.68</td>
<td>3.11</td>
<td>3.88</td>
</tr>
<tr>
<td>Fr (%)</td>
<td>--</td>
<td>--</td>
<td>119.55*</td>
</tr>
</tbody>
</table>

* Calculated on AUC (0-\infty h) with simvastatin suspension (Std. I) as reference

** Calculated on AUC (0-8 h) with simvastatin marketed tablet (Std. II) as reference

*** Significant differences: $P < .001$ Data are expressed as mean ± SD, $n=6$.

**CONCLUSION**

In vivo studies on albino rabbits revealed overall increase in bioavailability of the drug upon oral administration of nanoparticles formulation as compared with pure suspension and marketed formulation. The experimental findings collectively support that prepared nanoparticles had the potential to enhance solubility, dissolution rate correlates with faster oral absorption and bioavailability of poorly water soluble drugs. Simvastatin nanoparticles
formulation showed a significant improvement in bioavailability as compared with the conventional tablets. As a result, nanoparticles could be a promising delivery system to enhance the oral bioavailability of highly lipophilic drug of simvastatin.

ACKNOWLEDGEMENTS
Authors wish to acknowledge Aurobindo Pharmaceutical Ltd. (Hyderabad, India), for providing simvastatin as gift sample. We are also grateful to Bharati vidyapeeth, Poona college of Pharmacy, Pune for providing Malvern Mastersizer facility, Shivaji university Kolhapur for SEM, DSC & XRD facilities. We are also grateful to Govt. College of pharmacy, Karad for providing the albino rabbits for study.

COMPETING INTERESTS
The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

AUTHORS CONTRIBUTIONS
Author Anilkumar J Shinde managed the literature searches, designed the study, carried out the experimental work, performed the statistical analysis, and wrote the protocol and first draft of the manuscript. Author Harinath N. More designed the study, wrote the protocol and managed the analyses of the study. All authors read and approved the final manuscript.

REFERENCES


