A preliminary pharmacokinetic and toxicity study of nigerloxin

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A rapid RP-HPLC method was developed for the detection and quantitation of nigerloxin, a fungal metabolite active against diabetic complications, in rat plasma. The method was validated and employed to analyze samples obtained from a pharmacokinetic study after oral administration of the nigerloxin at 100 mM/kg body weight to wistar rats. The assay exhibited a dose-dependent response in a linear range from 2-500 µg/mL in rat plasma. The Cmax, AUC, and t1/2 were found to be 10.68±2.05, 49.01±8.23, 6.5±1.4, respectively. In another study, nigerloxin was fed to rats over a period of 14, 28, and 90 days at 100 mM/kg body weight. The results showed that nigerloxin fed rats were not adversely affected, based on the evaluation of various toxicological parameters. Thus, the results of the present study and the preliminary pharmacokinetic profile of the metabolite indicated the absence of any toxicity and promised its possible future application as a molecule against diabetic complications without any side effects.

Keywords: Aldose reductase, Nigerloxin, Pharmacokinetic study, Toxicological study

Diabetes control and complications trials have identified hyperglycemia as a major risk factor for the development of diabetic complications¹. Long-term vascular complications still represent the primary cause of morbidity and mortality in diabetic patients². Hence, the development of new agents with safety and effectiveness in reducing diabetic complications is an urgent need. There are some newer strategies tested in clinical trials for diabetic complications by targeting different pathways from both synthetic and natural sources. During the screening for enzyme inhibitors from microbial sources, a novel inhibitor of lipoxygenase and aldose reductase was isolated from Aspergillus niger and designated as nigerloxin (Fig. 1)³-⁴. This spore metabolite is specially produced in solid state fermentation⁵ and exhibited potent antioxidant activity by in vitro methods⁶ and reducing the oxidative stress in in vivo streptozotocin-induced diabetic rats². Along with its antioxidant potential during eye lens abnormalities in galactose-fed rats, it also helped in the reduction of the cataract⁸ and provided beneficial influence against diabetic nephropathy⁹, as determined in experimental animals. The safety and preliminary pharmacokinetic studies of nigerloxin have also been reported in this manuscript.

Materials and Methods

Chemicals

HPLC-grade methanol and acetic acid were purchased from Merck (Germany); Heparin was purchased from Sigma (USA); Trisodium citrate, ethyl acetate, chloroform, and ethanol were obtained from SRL (India). All other chemicals used were of analytical grade.

Experimental animals

Male wistar rats (CFT strain) weighing 180±10 g were obtained from Animal House Facility of the institutional (Institute Animal Ethics Committee (IAEC) protocol approval no. 334/14). Animals were maintained in polypropylene cages at room temperature with 12 h light and dark cycles. Necessary permission from the institute ethical committee was obtained, and all the experiments were conducted by the guidelines prescribed by Committee for Control and Supervision on Experiments on Animals (CPCSEA). Rat blank plasma was prepared

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Fig. 1 — Structure of nigerloxin
from control rats housed in Animal House Facility of the Institute.

Production of nigerloxin

Nigerloxin was produced through solid state fermentation of A. niger w105. Briefly, 20 g of wheat bran in 500 mL Erlenmeyer flask was moistened with 30 mL of distilled water containing 5% (w/v) trisodium citrate and sterilized for 1 h. This medium was inoculated with three days old inoculum, and the flasks were incubated in a slanting position at 30°C for 4 days. At the end of the fermentation, 200 mL ethyl acetate was added to each flask and agitated on a rotary shaker at 150 rpm at 30°C for 90 min. The mixture was filtered using cheesecloth and the ethyl acetate layer was filtered through Whatman No. 1 filter paper, followed by the addition of anhydrous sodium sulphate. The extract was concentrated to dryness by distillation under vacuum and re-suspended in 100 mL chloroform and centrifuged at 2000 rpm for 20 min. The residue was dried and dissolved in warm ethanol, treated with activated charcoal and then filtered through Whatman No.1 filter paper. The filtrate was concentrated under vacuum to obtain pure nigerloxin powder extract. The purity of nigerloxin was confirmed by HPLC.

Instrumentation and conditions

Nigerloxin samples were analyzed on HPLC system, which consisted of chromatographic pumps (LC-20AT; Shimadzu), and a photodiode array detector (SPD-M20A; Shimadzu). All analytical samples were separated using a C18 column (150 mm × 4.6 mm i.d.; particle size= 5 µm, Phenomenex Luna, Torrance, CA, USA). The mobile phase of HPLC analysis consisted of a gradient solvent system comprising of methanol-water containing 5% acetic acid at a flow rate of 1.0 ml/min. The concentration of the methanol in the mobile phase was changed from 10 to 90% in 15 min, and 100% methanol was eluted for 5 min. The samples were injected in a fixed volume of 10 µL and the detection was carried out in photodiode array detector at 292 nm.

Calibration standards and quality control samples

The stock solution of nigerloxin (5.0 mg/mL) was prepared in ethanol-water (50:50, v/v). Similarly, 1 mg/mL working standard solution of the internal standard (IS) (Kojic Acid) was prepared by the dilution of IS stock solution with methanol, and working solutions for calibration and controls were prepared from stock solutions in the same manner. All the solutions were stored at 4°C. Nigerloxin calibration standards were prepared by spiking blank plasma with appropriate amounts of the working standard solutions. Calibration plots were built in the range of 2–500 µg/mL for nigerloxin in rat plasma. About 10 µL of the appropriate working solution was added to 100 µL of blank plasma followed by vortex mixing. Quality control (QC) samples were prepared in the same manner as the calibration standards in three different plasma concentrations (50, 100 and 400 µg/mL). The analytical standards and QC samples were stored at −20°C until further use. The calibration standards and QC samples were pretreated by liquid-liquid extraction with methanol acetic acid before HPLC analysis.

Sample preparation

In a 2.0 mL centrifuge tube, an aliquot of 10 µL of the standard solution (2 µg/mL) was added to 100 µL plasma sample, followed by addition of 1.0 mL methanol: Acetic Acid (1:10 v/v). The sample was vortex-mixed for 1.0 min. After centrifugation at 5000×g for 10 min, the supernatant was transferred into a 2 mL glass tube and dried under nitrogen stream. The dried residue was reconstituted in 100 µL of methanol, and 10 µL aliquot of the reconstituted solution was injected into HPLC for further analysis.

Method validation

In order to validate the proposed method, selectivity, linearity, accuracy, precision, and recovery were investigated for the method, according to the guidelines of the Food and Drug Administration (USFDA)10 and European Medicines Agency (EMA)11. Validation runs were conducted on three consecutive days. Each validation run consisted of one set of calibration standards and three replicates of QC plasma samples.

Calibration curves were obtained by analyzing spiked samples on three separate days. Peak area ratios of nigerloxin were plotted against nigerloxin concentrations. Standard curves were well fitted to the equations by linear regression, with a weighting factor of the reciprocal of the concentration (1/x) in the concentration range of 2–500 µg/mL. The lower limit of quantitation (LLOQ) was defined as the lowest concentration obtained from the calibration curves.

To evaluate the matrix effect, blank rat plasma was extracted and then spiked with the analyte at 400 and 100 µg/mL (n=6). The corresponding peak areas were
then compared with those of neat standard solutions at equivalent concentrations. This peak area ratio is defined as the matrix effect. The matrix effect of the standard was evaluated at a concentration of 100 µg/mL in a similar manner. Accuracy and precision were evaluated by the determination of QC samples at three concentration levels (50, 100, and 400 µg/mL) in six replicates over three validation. The precision is expressed by (RSD).

The recovery of nigerloxin was evaluated by comparing the peak area of extracted samples with those of reference solutions reconstituted in blank plasma extracts (n=6). The recovery of the standard was determined as described. The carry-over value was assessed by following injection of a blank plasma sample immediately after 3 repeats of the upper limit of quantification (ULOQ), after which the response was checked for accuracy.

Pharmacokinetic studies
All the experiments were conducted strictly by the approved guidelines of the IACE and handling and care of animals was carried out strictly according to the standard guidelines laid by the IAEC. Thirty six rats were randomly distributed into 12 groups (n=3) and maintained under a basal diet for 48 h. Animals were given a single dose of nigerloxin (100 mM/kg body weight) through gavage administration. Blood from the eyes of animals of each group was collected by ocular bleeding using a heparinised capillary tube for every 1 h for 48 h and transferred into heparinised tubes. Centrifugation was carried out at 1500 g for 10 min at 4°C to obtain plasma, which was used to estimate nigerloxin by HPLC method. Plasma nigerloxin concentration vs time data for each rat was analyzed.

Safety assessment of nigerloxin
Twenty-four animals were randomly distributed into two groups (n=6). Animals were maintained for 4 weeks on basal pellet diet. For acute toxicological studies, animals were gavaged with a single dose of 100 mM/kg body weight nigerloxin and for subacute toxicity studies, animals were gavaged daily for 28 and 90 days, while control animals received water. All the animals were observed for 24 h at every 4 h duration and then daily for the onset of any signs of toxicity till the end of the experiment. The body weight of the animals was recorded every week. At the end of the experiment, animals were fasted overnight and were sacrificed under light ether anesthesia, and liver, lungs, kidney, spleen, brain, adrenals, and heart from each rat were excised and weighed. The organ:body weight ratio was calculated before histopathological analyses. Tissues were fixed in Bouin fixative and processed to obtain sections of 5 mm and stained using hematoxylin and eosin.

Hematological studies
Blood samples were collected by heart puncture from each group for hematological profile, including hemoglobin concentration (Hb), packed cell volume (PCV), erythrocyte and leucocyte count, differential counts, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelets (PLT) were analysed according to the method described by Breton-Gorius, (1986).

Clinical chemistry
Blood samples were collected in heparinised tubes and centrifuged at 1500 rpm for 10 min to obtain plasma. Activities of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) were determined in plasma along with the estimation of total urea, uric acid, creatinine, total cholesterol and total triglycerides using commercial biochemical kits (Aggappe Diagnostics Ltd. India). Before use, the kits were calibrated according to manufacturer’s instructions.

Results
Extraction efficiency of nigerloxin with methanol:acetic acid (9:1) showed an acceptable recovery (around 85% and 95%), with lesser matrix effect and appearance of symmetric peak shapes. Thus, the above combination of methanol and acetic acid was used for extraction and analysis of nigerloxin in further experiments (Fig. 2).

Method validation
The calibration curve was expressed by the equation, Y= 0.1166X + 0.7554, r= 0.990, where y represents the nigerloxin peak area and X represents the plasma concentration of nigerloxin. The LLOQ of nigerloxin in plasma was determined to be 2.5 µg/mL, and the precision and accuracy at LLOQ were 7.5% and 82.2%, respectively. Mean recoveries of nigerloxin were higher than 78.6%. The recovery of IS (50 µg/mL) was 88.7%.

The precision of the method was determined by calculating RSD for control samples at three
concentration levels over three days of validation tests. The intra-day, inter-day precision and accuracy of nigerloxin are summarized in Table 1.

Pharmacokinetic studies

The described method was applied to quantify nigerloxin in the rat plasma, following oral dosage of 100 mM/kg bodyweight. The concentration-time profile was shown in Figure 3, the area under curve (AUC) was calculated by the linear trapezoidal method. The main pharmacokinetic parameters including AUC, elimination half-life ($t_{1/2}$), elimination rate constant ($k_{elim}$), clearance rate (CL), the volume of distribution (Vd) were determined according to standard equations. Following oral administration of nigerloxin, a gradual increase in its concentration was

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Precision RSD (%)</th>
<th>Accuracy %</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra Day</td>
<td>Inter Day</td>
<td>Intra Day</td>
</tr>
<tr>
<td>50</td>
<td>8.4±0.65</td>
<td>7.6±0.82</td>
<td>86.2±4.1</td>
</tr>
<tr>
<td>100</td>
<td>4.2±0.37</td>
<td>3.7±0.37</td>
<td>87.2±3.4</td>
</tr>
<tr>
<td>400</td>
<td>2.1±0.22</td>
<td>4.6±0.61</td>
<td>93.7±3.1</td>
</tr>
</tbody>
</table>

Fig. 2 — HPLC Chromatogram of pure nigerloxin (A); blank rat plasma spiked with nigerloxin and internal standard (IS) (B); and rat plasma sample at 4 h after oral administration of nigerloxin (C)
observed in plasma up to 6 h, followed by a gradual reduction until 24 h (Fig. 3). The $C_{\text{max}}$ was 10.68 mM/mL in the plasma and $T_{\text{max}}$ was 5±0.86 h. This level reduced gradually to 0.03 mM/mL at the end of 24 h. The AUC, elimination rate, elimination half-life, the volume of distribution and oral clearance values are described in Table 2.

No abnormality observed between the control and nigerloxin fed rats was further evidenced by the absence of any histological changes in various tissues/organs examined (Fig. 4).

**Safety assessment of nigerloxin**

Relative weights of various vital organs in the nigerloxin treated rats as compared to control rats were described in Table 3 & 4. The data on various hematological parameters are presented in Table 5.

Analysis of various marker enzymes, such as SGOT, SGPT, ALP, and LDH of 4 weeks feeding of nigerloxin are presented in Table 6 and the effect of repeated doses of nigerloxin for 90 days on marker enzymes are represented in Table 7.

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**Table 2 — Single dose (100 mM/Kg bodyweight) oral pharmacokinetic parameters of rat plasma levels of nigerloxin**

<table>
<thead>
<tr>
<th>Group</th>
<th>$C_{\text{max}}$ (mMoles/mL)</th>
<th>$T_{\text{max}}$ (h)</th>
<th>AUC (mMoles/mL/h)</th>
<th>$t_{1/2}$ (h)</th>
<th>$K_{\text{elim}}$ (L/h)</th>
<th>CL (L/h)</th>
<th>$V_d$ (L/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nigerloxin</td>
<td>10.68 ±2.05</td>
<td>5 ±0.85</td>
<td>49.01 ±8.23</td>
<td>7 ±1.4</td>
<td>0.107±1.4</td>
<td>2.04 ±1.4</td>
<td>18.9±1.4</td>
</tr>
</tbody>
</table>

[$C_{\text{max}}$, maximum plasma concentration; $T_{\text{max}}$, time to reach $C_{\text{max}}$; AUC, area under the plasma concentration time curve; $t_{1/2}$, elimination half-life; $K_{\text{elim}}$, elimination rate constant; CL, clearance rate; $V_d$, volume of distribution. Values are mean±standard error of three animals]

**Table 3 — Relative organ weights of female and male rats fed with 100 mM/Kg bodyweight nigerloxin for 4 weeks**

<table>
<thead>
<tr>
<th>Group</th>
<th>Kidney</th>
<th>Liver</th>
<th>Lungs</th>
<th>Spleen</th>
<th>Heart</th>
<th>Adrenal</th>
</tr>
</thead>
<tbody>
<tr>
<td>FC</td>
<td>0.569±0.056a</td>
<td>3.06±0.06a</td>
<td>0.48±0.06a</td>
<td>0.256±0.03a</td>
<td>0.275±0.01a</td>
<td>0.025±0.008a</td>
</tr>
<tr>
<td>FT</td>
<td>0.588±0.011a</td>
<td>2.98±0.05a</td>
<td>0.53±0.07a</td>
<td>0.270±0.01a</td>
<td>0.284±0.01a</td>
<td>0.019±0.004a</td>
</tr>
<tr>
<td>MC</td>
<td>0.562±0.016a</td>
<td>2.87±0.30a</td>
<td>0.549±0.01a</td>
<td>0.274±0.03a</td>
<td>0.301±0.01a</td>
<td>0.017±0.001a</td>
</tr>
<tr>
<td>MT</td>
<td>0.568±0.018a</td>
<td>3.33±0.16a</td>
<td>0.525±0.01a</td>
<td>0.242±0.04a</td>
<td>0.296±0.02a</td>
<td>0.019±0.004a</td>
</tr>
</tbody>
</table>

[FC, Female control; FT, Female Test (nigerloxin 100 mM/kg body weight); MC, Male Control; MT, Male Test (nigerloxin 100 mM/kg body weight). Values are mean±standard error of three animals. Means with the same letter (a) within the same column do not differ significantly ($P <0.05$)]

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Fig. 3 — Mean plasma concentration-time profile of nigerloxin after 100 mM/kg oral administration to male wistar rats (n=3)

Fig. 4 — Histopathology images of control liver, kidney and lungs (A, C, and E respectively), nigerloxin treated liver, kidney and lungs (B, D, and F respectively)
Table 4 — Relative organ weights of female and male rats fed with 100 mM/Kg body weight nigerloxin for 12 weeks

<table>
<thead>
<tr>
<th>Group</th>
<th>Liver</th>
<th>Lungs</th>
<th>Kidney</th>
<th>Heart</th>
<th>Adrenal</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>FC</td>
<td>3.017±0.26a</td>
<td>0.54±0.03a</td>
<td>0.53±0.03a</td>
<td>0.29±0.03a</td>
<td>0.02±0.001a</td>
<td>0.52±0.1a</td>
</tr>
<tr>
<td>FT</td>
<td>3.12±0.16a</td>
<td>0.58±0.05a</td>
<td>0.48±0.02a</td>
<td>0.28±0.01a</td>
<td>0.02±0.003a</td>
<td>0.51±0.2a</td>
</tr>
<tr>
<td>MC</td>
<td>3.16±0.26a</td>
<td>0.55±0.06a</td>
<td>0.53±0.04a</td>
<td>0.31±0.08a</td>
<td>0.03±0.002a</td>
<td>0.56±0.13a</td>
</tr>
<tr>
<td>MT</td>
<td>3.33±0.05a</td>
<td>0.57±0.03a</td>
<td>0.54±0.01a</td>
<td>0.29±0.01a</td>
<td>0.02±0.001a</td>
<td>0.58±0.2a</td>
</tr>
</tbody>
</table>

[FC, Female control; FT, Female Test (nigerloxin 100 mM/kg body weight); MC, Male Control; MT, Male Test (nigerloxin 100mM/kg body weight). Values are mean±standard error of three animals. Means with the same letter (a) within the same column do not differ significantly (P <0.05)]

Table 5 — Haematological Profiles of Female and male rats fed with 100 mM/Kg Bodyweight nigerloxin for 12 weeks

<table>
<thead>
<tr>
<th>Group</th>
<th>Hb (g/dL)</th>
<th>RBC</th>
<th>WBC</th>
<th>HCT%</th>
<th>MCH</th>
<th>MCHC (g/dL)</th>
<th>MCV (fL)</th>
<th>PLT</th>
<th>LYM%</th>
</tr>
</thead>
<tbody>
<tr>
<td>FC</td>
<td>15.97±0.33a</td>
<td>8.72±0.20a</td>
<td>12.60±1.7a</td>
<td>46.87±0.6a</td>
<td>18.20±0.30a</td>
<td>34.07±0.83a</td>
<td>53.53±0.82a</td>
<td>672.67±110a</td>
<td>76.33±1.9a</td>
</tr>
<tr>
<td>FT</td>
<td>18.10±1.02a</td>
<td>9.18±0.07a</td>
<td>10.73±1.5a</td>
<td>49.77±1.7a</td>
<td>19.47±0.73a</td>
<td>36.10±1.02a</td>
<td>53.83±0.74a</td>
<td>780.33±154a</td>
<td>69.80±2.0a</td>
</tr>
<tr>
<td>MC</td>
<td>16.93±2.00a</td>
<td>8.90±0.87a</td>
<td>10.00±2.2a</td>
<td>46.77±5.1a</td>
<td>18.67±0.55a</td>
<td>35.87±0.78a</td>
<td>52.13±1.37a</td>
<td>370.00±65.7a</td>
<td>75.77±3.6a</td>
</tr>
<tr>
<td>MT</td>
<td>16.40±1.80a</td>
<td>9.00±0.86a</td>
<td>10.70±2.1a</td>
<td>47.50±4.3a</td>
<td>18.13±0.64a</td>
<td>34.43±0.86a</td>
<td>53.63±1.46a</td>
<td>400.00±90.1a</td>
<td>73.23±5.2a</td>
</tr>
</tbody>
</table>

[FC, Female control; FT, Female Test (nigerloxin 100 mM/kg body weight); MC, Male Control; MT, Male Test (nigerloxin 100 mM/Kg body weight); Hb, haemoglobin; RBC, red blood cells; WBC, white blood cells; HCT, haematocrit; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; PLT, platelet; LYM, lymphocytes. Values are mean±standard error of three animals. Means with the same letter (a) within the same row do not differ significantly (P <0.05)]

Table 6 — Effects of nigerloxin (100 mM/Kg bodyweight) on serum enzymes and metabolites of the rats fed for 4 weeks

<table>
<thead>
<tr>
<th>Group</th>
<th>SGOT</th>
<th>SGPT</th>
<th>ALP</th>
<th>LDH</th>
<th>Creatine</th>
<th>Creatine kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>FC</td>
<td>113.8±2.81a</td>
<td>38.67±2.12a</td>
<td>219.3±6.06a</td>
<td>950±21.8a</td>
<td>0.65±0.02a</td>
<td>556.9±18.08a</td>
</tr>
<tr>
<td>FT</td>
<td>102.7±3.27a</td>
<td>44.96±1.42a</td>
<td>223.21±1.84a</td>
<td>953±22.99a</td>
<td>0.58±0.03a</td>
<td>519.9±8.02a</td>
</tr>
<tr>
<td>MC</td>
<td>104.1±3.91a</td>
<td>40.62±3.38a</td>
<td>211.81±9.29a</td>
<td>932.23±22.9a</td>
<td>0.57±0.02a</td>
<td>543.2±13.81a</td>
</tr>
<tr>
<td>MT</td>
<td>94.99±4.12a</td>
<td>35.26±2.67a</td>
<td>206.22±8.45a</td>
<td>899.64±24.0a</td>
<td>0.51±0.02a</td>
<td>514.8±5.71a</td>
</tr>
</tbody>
</table>

[FC, Female control; FT, Female Test (nigerloxin 100 mM/kg body weight); MC, Male Control; MT, Male Test (nigerloxin 100 mM/Kg body weight); SGOT, serum glutamate oxaloacetate transaminase (U/L); SGPT, serum glutamate pyruvate transaminase (U/L); ALP, alkaline phosphatase (U/L); LDH, lactate dehydrogenase (U/L). Values are mean±standard error of three animals. Means with the letter (a) within the same row do not differ significantly (P <0.05)]

Table 7 — Effects of nigerloxin (100 mM/Kg bodyweight) on serum enzymes of the rats fed for 12 weeks

<table>
<thead>
<tr>
<th>Group</th>
<th>AST</th>
<th>ALT</th>
<th>ALP</th>
<th>LDH</th>
<th>Creatine</th>
<th>Creatine kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>FC</td>
<td>163.6±6.9a</td>
<td>73±5.4a</td>
<td>164.2±1.7a</td>
<td>943.6±22.12a</td>
<td>0.56±0.04a</td>
<td>759.8±23.23a</td>
</tr>
<tr>
<td>FT</td>
<td>152.8±5.6a</td>
<td>68.6±1.6a</td>
<td>154±4.1a</td>
<td>909.2±23.14a</td>
<td>0.50±0.04a</td>
<td>726.6±20.21a</td>
</tr>
<tr>
<td>MC</td>
<td>156.9±6.7a</td>
<td>60.4±3.6a</td>
<td>157.8±5.7a</td>
<td>804.1±17.68a</td>
<td>0.56±0.05a</td>
<td>773.6±15.86a</td>
</tr>
<tr>
<td>MT</td>
<td>151.1±5.8a</td>
<td>66.1±3.6a</td>
<td>144.2±1.6a</td>
<td>810.7±46.74a</td>
<td>0.57±0.01a</td>
<td>777.1±18.19a</td>
</tr>
</tbody>
</table>

[FC, Female control; FT, Female Test (nigerloxin 100 mM/kg body weight); MC, Male Control; MT, Male Test (nigerloxin 100 mM/kg body weight); SGOT, serum glutamate oxaloacetate transaminase (U/L); SGPT, serum glutamate pyruvate transaminase (U/L); ALP, alkaline phosphatase (U/L); LDH, lactate dehydrogenase (U/L). Values are mean±standard error of three animals. Means with the same letter (a) within the same row do not differ significantly (P <0.05)]

Discussion

The beneficial influence of nigerloxin in diabetic complications, such as cataract, nephropathy, and oxidative stress is previously described in our earlier report7,8 suggested its possible application as a drug, and prompted us to study its preliminary pharmacokinetics and the safety aspects of its application as a potential drug for treatment of diabetic complications.

The inclusion of methanol in the mobile phase (methanol-water) facilitated the better separation of nigerloxin with a sharp peak17. For HPLC separation, it is important to remove the proteins and other potential interference from the biological samples.
prior to analysis\textsuperscript{18,19}. In the present studies, methanol: acetic acid precipitated protein in the biological sample and minimised the matrix effect in the detection of nigerloxin.

Evaluation of the methods used for the detection of nigerloxin was carried out to ensure that the results produced are suitable and proper for their further use. Validation is an important requirement for any new drug discovered before clinical trial applications. It provides assurance that developed method and instrument utilized in the method would yield consistent results that accurately reflect the quality characteristics of the drug tested\textsuperscript{11}. Method for the detection of nigerloxin the calibration curve was linear in the range of 2–500 μg/mL with a correlation coefficient of 0.99. Linear regressions of the peak area ratios versus concentrations were fitted well over the range of nigerloxin in rat plasma. The minimum concentration of nigerloxin detected was found to be 2.5 μg/mL, and precision and accuracy were found to be within the acceptable limits.

The precision of the method was determined by calculating Relative Standard Deviation (RSD) for control samples at different concentration levels over three days of validation tests. The accuracies for all tested concentrations were within nominal range both precisions (within and between runs) were acceptable. All the results of the tested samples were within the acceptable criteria (RSD%: 15%). These results substantiate the precision and accuracy of the developed method for detection of nigerloxin and it follows the guideline as described\textsuperscript{10}.

Sample carryover effect is the major factor which influences the accuracy and precision of the validated method in HPLC and Mass Spectroscopy analysis. None of the analytes showed any significant peak (≥20% of the LLOQ) in blank samples injected after the ULOQ samples. Extending extra 2 min at the end of the elution in gradient solvent could effectively clear the system, thereby eliminating carry-over between samples.

The dose level of 100 mM/kg body weight was found to be beneficial in controlling diabetic complications\textsuperscript{7}. Hence, the same dose was selected to study the preliminary pharmacokinetic properties of the compound. Nigerloxin appeared within 4-6 h in the blood, and maximum concentration of nigerloxin in plasma reached after 5 h. Although there was no previous pharmacokinetic study on nigerloxin, the $T_{\text{max}}$ of nigerloxin was found to be within the range of 4-6 h, which is acceptable, as reported for some of the natural product extracts\textsuperscript{19}. The half-life of nigerloxin was found to be 6.5 h and the elimination rate was found to be 0.1 L/h (Table 2).

Safety of any drug on its use over a period of time at a particular concentration is important in drug discovery. The effective dose of 100 mM/kg body weight was administered to check the safety level of nigerloxin over a period of 28 and 90 days repeated toxicity study; nigerloxin at 100 mM/kg body weight did not result in any treatment related deaths. Also, the rats fed with nigerloxin did not develop any clinical signs of toxicity either immediately or during the span of its administration.

There were no significant differences ($P <0.05$) with respect to feed intake and gain in body weight of rats fed with nigerloxin when compared with control. Similarly, there were no significant differences ($P <0.05$) in the relative weights of various vital organs in the nigerloxin treated rats as compared to control. No significant changes were observed in any of the hematological parameters between control and nigerloxin fed rats.

The adverse effect of any drug on the physical and physiological conditions would be preliminarily observed by monitoring the levels of marker enzymes in the liver, such as SGOT, SGPT, and ALP and any alteration in the levels of the marker enzyme reflect the effect of the drug on the liver and other organs. Administration of nigerloxin at 100 mM/kg body weight over a period of 28 and 90 days did not result in any significant changes in SGOT and SGPT activities. Elevated levels of LDH were found in myocardial infarction, liver diseases, hemolytic anemias, pernicious anemia and pulmonary diseases\textsuperscript{30} and a common marker released extensively during tissue damage or injury. Administration of nigerloxin did not result in any significant changes in the activity of LDH.

Absence of any histological changes on nigerloxin treatment in various tissues/organs examined shows that it had no adverse effect on the cellular structure of these organs. Gross examination of vital organs during autopsy did not reveal any abnormalities that could be attributed to the feeding of nigerloxin.

**Conclusion**

Nigerloxin is a fungal metabolite from \textit{A. niger}, which was shown to be beneficial against diabetic complication and oxidative stress. The results of the
present studies indicate the absence of any toxicity of nigerloxin, which promises its possible future application as a drug against diabetic complications without any side effects. The observations of this study open up the scope of its various clinical trials to substantiate our claim of its application as an agent to ameliorate the various complications of diabetes.

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References