Chapter I

Acute and sub-chronic toxicity evaluation of Eugenia jambolana seed extract.
Acute and sub-chronic toxicity evaluation of *Eugenia jambolana* seed extract.

INTRODUCTION

India is considered to be the largest producer of medicinal herbs and is rightly called the botanical garden of the world (Dubey et al., 2004). Use of herbal medicines is an age old practice in India and recent studies have documented a rise in usage of alternative therapies not only in the developing countries like India but also in other developed countries worldwide (Singh and Prakash, 2008). In USA, 12-19% of the total use of complementary and alternative medicines comprises of herbal drugs (Tindle et al.2005). However, there are questions raised on the efficacy and safety of these drugs as majority of the traditional herbal preparations lack of scientific data pertaining to its toxic dose and clinical use. A prevailing myth associated with drugs of herbal origin is that they are always 'safe' with no 'side effects' but, studies have reported hepatotoxicity (Larrey and Pageaux, 1995), nephrotoxicity (Saxena and Panhotra, 2003; Singh and Prakash, 2008) and associated side effects. According to United States Food and Drug Administration act (USFDA), herbal drugs do not fall in the category of medicine and hence there are no rigorous safety evaluations. This necessitates determinations of toxicity dosage of any herbal preparation through preclinical acute and sub chronic toxicity evaluations (Jadeja et al., 2011).

There is no dearth of literature available on beneficial and medicinal uses of EJSE but, the available data lacks a systematic safety/toxicity evaluation of the same. Hence, it
was thought pertinent to evaluate the same in acute and chronic toxicity model as per the Organisation for Economic Co-operation and Development (OECD) guidelines.

MATERIALS AND METHODS

Preparation of extract

Fruits of *Eugenia jambolana* were handpicked from fruit shop. The pulp was removed and the seeds were washed several times with Milli-Q water and shade dried at room temperature. Kernel of the seeds was separated from the seed coat, powdered in an electrical grinder. Hundred gm of kernel powder was suspended in 1000 ml of Milli-Q water overnight and then sieved through several layers of sterile muslin cloth. The resultant residual paste was extracted with 70% ethanol at room temperature and later, was evaporated in a rotatory evaporator at 40–50 °C (Ravi et al., 2003, 2004, 2005; Sharma et al., 2003). The yield of ethanolic extract was 5 g/100 g of dried seed powder.

Experimental animals

*Swiss albino* mice of either sex (Obtained from Zydus research centre, Ahmedabad, India) were housed and maintained in clean polypropylene cages and fed with laboratory chow (M/S Pranav agro, Ltd Baroda, India) and water *ad libitum*. The experimental protocol was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) India and, approved by the animal ethical committee of The Department of Zoology, The M.S. University of Baroda, Vadodara (Approval No.827/ac/04/CPCSEA).
Acute oral toxicity in mice

The acute oral toxicity study was conducted using the limit test procedure as per OECD test guidelines on acute oral toxicity test 401 (OECD, 2001). Thirty two *Swiss* albino mice of either sex were divided into four groups (n=8) and were orally administered with a single dose of 1000, 2000, 3000, 4000 or 5000 mg/kg BW of EJSE. Animals were observed for possible behavioural changes such as tremors, convulsions, sleep, altered feeding, salivation, altered somato-motor activities and diarrhoea till 72 hr post treatment.

Sub chronic oral toxicity in mice

The sub chronic oral toxicity study was conducted according to OECD Test 407 (OECD, 1995). Thirty two *Swiss* albino mice of either sex were divided into four groups (n=8) and maintained for 28 days for this experiment. Group I was orally fed with carboxy methyl cellulose (CMC; 0.5 %) that served as control whereas, Groups 2, 3 and 4 were orally administered with 1000, 2000 or 3000 mg/kg of EJSE respectively. After 28 days of treatment, blood was collected from overnight fasted mice via retro-orbital sinus under mild anaesthesia and plasma was separated for further biochemical analysis. Thereafter, the animals were sacrificed by cervical dislocation under mild ether anaesthesia, and brain, heart, lungs, liver, spleen, kidney and adrenal were excised and weighed.

Cage side observations

After treatment with EJSE, the mice were observed daily for possible changes in appearance of skin, fur and eyes. Also, the animals were closely observed for
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somatomotor activity, respiratory and behaviour changes, tremors, convulsions, salivation, diarrhoea etc.

**Body Weight and Food and Water Intake**

Body weight of each experimental animal was recorded using a weighing balance (CITIZEN Model 1621K, Taiwan) at the end of every week during the entire study. Food and water intake in all the experimental groups were monitored daily at 09:00 hrs. Known quantity of food was given to the respective experimental groups and the leftover food was weighed and the quantity was subtracted from the total to record food intake per mice. Also, mice were provided with known volume of reverse osmosis grade water in a 500 ml measuring cylinder and leftover volume of water was measured every 24 hr interval to calculate water intake.

**Plasma biochemical analysis**

Plasma sodium, potassium (Tulip Diagnostics, Pvt. Ltd), calcium (Lab Care Diagnostics India Pvt. Ltd), Creatine Kinase-MB (CK-MB), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), urea, creatinine, cholesterol, HDL (Reckon diagnostics, Vadodara, India), triglyceride (Beckon Diagnostics, Vadodara, India) and fasting blood glucose (Bayer diagnostics, India Ltd) were assayed using commercially available kits as per the instruction of the manufacturer.
Estimation of Plasma Sodium

**Principle:** Sodium is precipitated as a triple salt with magnesium and uranyl acetate. The excess of the uranyl ion are reacted with ferrocyanide in an acidic medium to develop a brownish colour. The intensity of the colour produced is inversely proportional to the concentration of sodium in the sample.

\[
\text{Uranyl ion + Mg ions + Na}^+ = \text{Uranyl Mg-Na precipitate}
\]

\[
\text{Free Uranyl ions + K}_4\text{Fe(CN)}_6 = \text{Brown colour complex}
\]

**Procedure:** Precipitating reagent 1ml L1 is mixed well with 20μl of serum and kept at room temperature for five minutes and then centrifuged. Clear supernatant is then separated out and it is then mixed with Acid reagent L2 (20 μl) against blank (distilled water) within 15 minutes.

Readings is taken at 530nm/Green filter.

Estimation of Plasma Potassium

**Principle:** Potassium reacts with sodium tetraphenyl boron in a specially prepared buffer to form a colloidal suspension. The amount of the turbidity produced is directly proportional to the concentration of potassium in the sample.

\[
\text{Tetraphenyl Boron + K}^+ \rightarrow \text{White turbidity}
\]

**Procedure:** Pipette into clean dry test tubes labelled as Blank, standard and test. Potassium reagent 1ml is mixed with 20 microns of serum, and in standard 20 micron of K+ standard is taken. Absorbance are taken against blank with plain K+ reagent (L1)

Readings is taken at 630nm/Red filter.
**Estimation of Plasma Calcium**

**Principle:** At a neutral pH, the Ca$^{2+}$ forms with arsenazo III a complex, the colour intensity of which is directly proportional to the concentration of calcium in the sample.

**Procedure:** Serum (25 micron) is incubated at room temperature for 5 minutes. The final absorbance of the sample and standard against the blank is read at red filter. Readings is taken at 650nm filter.

**Estimation of Creatine Kinase-MB**

**Principle:** Enzopak CK-MB is based on the principle of specific immunoinhibition by a blend of monoclonal antibody which completely inhibit CK-MM activity and 50% of CK-MB activity. While not affecting the B subunit activity of CK-MB and CK-BB, the CK-BB activity is measured. The CK-MB activity is obtained by multiplying the CK-BB activity by two. Increased levels may be found due to severe exercise and by large multiple intramuscular injections. While other symptoms and suggestive history, serum CK estimation is an important parameter of choice for myocardial infraction and follows up.

Creatine Phosphate + ADP $\rightarrow$ Creatine + ADP

ATP + Glucose $\rightarrow$ G-6 PO$_4$ + ADP

G-6 PO$_4$ + NADP $\rightarrow$ 6-Phosphogluconate + NADPH + H

**Procedure:** Working reagent 1ml is incubated at 37 degree for 5 minutes and then mixed with 50 microns of serum. The first absorbance of the test is taken at 300 seconds and
thereafter at 30, 60, 90, and 120 seconds at 340 nm. Determining the mean change in absorbance per min.

The reaction is monitored by measuring the increase in absorbance at 340 nm.

Estimation of Lactate dehydrogenase

**Principle:** Potassium reacts with sodium tetraphenyl boron in a specially prepared buffer to form a colloidal suspension. The amount of the turbidity produced is directly proportional to the concentration of potassium in the sample.

Tetraphenyl Boron + K+ \rightarrow White turbidity

**Procedure:** Pipette into clean dry test tubes labelled as Blank, standard and test. Potassium reagent 1ml is mixed with 20 microns of serum, and in standard 20 micron of K+ standard is taken. Absorbance are taken against blank with plain K+ reagent (L1) Readings is taken at 630nm/Red filter.

Estimation of Alanine aminotransferase

**Principle:** L-Alanine and alpha-ketoglutarate reacts in the presence of GPT in the sample to yield pyruvate and L-glutamate.

\[
\text{L-Alanine + alpha ketoglutarate} \rightarrow \text{Pyruvate + L-Glutamate}
\]

\[
\text{Pyruvate + NADH} \rightarrow \text{Lactate + NAD}
\]
**Procedure:** Type of reaction is kinetic and the factor taken is 3376. 1ml reagent is taken and 50 microns of serum is added. The absorbance is measured at an interval of 30 seconds for 2 minutes at 340nm.

_Estimation of Alkaline phosphatase_

**Principle:** Alkaline Phosphatase in a sample hydrolyses paranitrophenyl phosphate into paranitrophenol and phosphate, in the presence of magnesium ions. The rate of increase in absorbance of the reaction mixture at 405nm due to liberation of paranitrophenol is proportional to the alkaline phosphatase activity.

\[
p\text{-Nitrophenyl Phosphate} + H_2O \rightarrow p\text{-Nitrophenol} + \text{Phosphate}
\]

**Procedure:** Type of reaction is kinetic and the factor taken is 2713. Four readings are taken at an interval of 30 seconds. Buffersubstrate reagent 1ml is mixed with 20 microns of serum sample. The readings of the test are taken immediately as it is a kinetic reaction. The reaction is read at 30, 60, 90, and 120 seconds at 405 nm. The mean change in absorbance per minute are calculated as test result.

_Estimation of Urea_

**Principle:** Urease breaks down urea into ammonia and carbon dioxide. In alkaline medium, ammonia reacts with hypochlorite and salicylate to form dicarboxyindophenol, a coloured compound. The reaction is catalysed by sodium nitroprusside. The intensity of colour produced is measured photometrically at 570nm.

\[
\text{Urea} + H_2O \rightarrow \text{Ammonia} + CO_2
\]
Ammonia + Salicylate $\rightarrow$ 2-2 Dicarboxy Indophenol

**Procedure:** Type of reaction is End Point reaction. 5 microns of sample serum is incubated at room temperature with working enzyme reagent at 37 degrees for five minutes. After that 1ml working colour reagent (c) is mixed well and incubated for ten minutes at room temperature. The absorbance of test and standard against reagent blank is read at 570nm with red filter.

**Estimation of Creatinine**

**Principle:** Creatinine present in the serum reacts with alkaline picrate to form a coloured complex. The rate of formation of coloured complex is directly proportional to creatinine concentration. This rate of reaction (intensity of colour produced) is measured photometrically at 510nm and is compared with that of the standard.

$$\text{Creatinine + Alkaline Pictrate} \rightarrow \text{Creatinine Picrate Complex}.$$ 

**Procedure:** Type of reaction is fixed time and readings are taken at 510nm. Flow cell temperature is maintained at 30 degree throughout the test procedure. 1ml of reagent is mixed with 100 microns of sample serum. Mix and aspirated. Absorbance is recorded at 20 second and again at 80 second against distilled water. The absorance of test and standard against reagent blank is read at 510nm.
Estimation of Cholesterol

**Principle:** The cholesterol esters are hydrolysed to free cholesterol esterase. The free cholesterol is then oxidised by cholesterol oxidase to cholesten4en30ne with the simultaneous production of hydrogen peroxide. The hydrogen peroxide reacts with 4-aminoantipyrine and phenolic compound in the presence of peroxidase to yield a coloured complex which is read at 505 nm.

\[
\text{Cholesterol ester} \rightarrow \text{Cholesterol} + \text{Fattyacid}
\]

\[
\text{Cholesterol} + \text{O}_2 \rightarrow \text{Cholesterol-4en-3one} + \text{H}_2\text{O}_2
\]

**Procedure:** It is an end point reaction. Flow cell temperature is maintained at 30 degree. 1ml cholesterol reagent is mixed with 10 microns of sample serum. It is incubated at 37 degrees for 10 minutes and the absorbance is read. The absorbance of test and standard against reagent blank is read at 505nm.

Estimation of Triglyceride

**Principle:** Triglycerides incubated with lipoprotein lipase are hydrolysed to free fatty acid and glycerol. Glycerol kinase catalyzes the conversion of glycerol and ATP to glycerol-3-phosphate and ADP. The glycerol-3-phosphate gets oxidised to dihydroxy acetone phosphate by glycerol phosphate oxidase. Hydrogen peroxide formed in this reaction with the help of peroxidase, reacts with chromogens. 4-aminoantipyrine/ ESPT to gives a purple coloured complex which is read at 546nm filter.

\[
\text{Tryglyceride} \rightarrow \text{Glycerol} + \text{Free Fatty Acids}
\]

\[
\text{Glycerol} + \text{ATP} \rightarrow \text{Glycerol-3-p} + \text{ADP}
\]
Glycerol-3-p + O₂ → DHAP + H₂O₂
H₂O₂ + 4-aminoantipyrine + ESPT → Purple Quinonimine

**Procedure:** Enzyme reagent 1ml and 10microns serum is mixed well and incubated for 10 minutes at 37 degree. After 10 minutes the absorbance of standard and sample against blank is taken. The absorbance of test and standard against reagent blank is read at 546nm green filter.

*Estimation of HDL*

**Principle:** High density lipoproteins(HDL) are separated from other lipoprotein fraction by treating serum with phosphotungstic acid and magnesium chloride. HDL remains in solution while all other lipoprotein fractions are precipitated; cholesterol content of which is estimated by enzymatic method.

Serum + PTA Reagent → Supematant(HDL) + Precipitates

**Procedure:** Fresh clear serum under fasting condition with no haemolysis is the specimen of choice. Sample serum 20microns is added to 20 micron of 3-HDL-cholesterol and it is mixed well. After 5 minutes it is centrifuged at 3500 rpm for 10 minutes. This supernatant 50 microns is incubated with 1ml cholesterol reagent for 10 minutes. The reading is taken in green filter.
Estimation of Blood glucose

Principle: Glucose oxidase oxidises the specific substrate, beta-D-glucose, to gluconic acid and hydrogen peroxide is generated. Hydrogen peroxide thus produced is acted upon by peroxidase and oxygen is liberated. The liberated oxygen is transferred to chromogen system consisting of 4-aminoantipyrine and phenolic compound to produce red quinoneimine dye. The intensity of colour is directly proportional to the concentration of glucose and is measured photometrically at 505 nm green filter.

\[
\text{Glucose} + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{Gluconate} + \text{H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + \text{Phenolic Compound} \rightarrow \text{Coloured Complex} + \text{H}_2\text{O}_2
\]

Procedure: Fresh clear serum under fasting condition with no hemolysis is the specimen of choice. The test is performed as early as possible to prevent glycolysis. Sample serum 10 microns is added to 1 ml of working reagent. It is mixed well and incubated for 5 min at 37°C. The absorbance is then measured with Green filter.

Hematological analysis

At the end of experimental protocol blood was collected in K3-EDTA coated tubes and haemoglobin content, total red and white blood cell counts (RBC and WBC), hematocrit (HCT) value, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution of width-coefficient of variance (RD-WCV), red cell distribution width standard deviation (RD-WSD), platelets count (PLT), mean platelet volume (MPV), red cell distribution width (RDW) and platelet crit (PCT) of blood samples were measured using BC-2300 Haematology Analyzer (Shenzhen Mindray Bio-Medical Electronics Co., Ltd).
**Histopathological Examination**

At the end of experimental period, all animals were subjected to a detailed gross necropsy viz. examination of external surface of the body, all orifices and, the content in cranial, thoracic and abdominal cavities. Autopsy of brain, heart, lungs, liver, kidney and spleen was done and their wet weights were recorded immediately.

Heart, liver and kidney of experimental mice were fixed in 4% buffered paraformaldehyde, dehydrated in graded alcohol series and embedded in paraffin wax using automated tissue processor and 5μm thick sections were cut and stained with hematoxyline and eosin and examined under Leica DMRB microscope. Photographs were taken with Canon power shot S70 digital Camera at 100 X magnification.

**Statistical analysis**

Statistical evaluation of the data was done by one way ANOVA followed by Bonferroni’s multiple comparison test. The results were expressed as mean ± S.E.M using Graph Pad Prism version 3.0 for Windows, Graph Pad Software, San Diego California USA.

**RESULTS**

**Acute oral toxicity**

There were no noticeable behavioural changes recorded in rats subjected to acute toxicity. There was no mortality recorded and hence, LD₅₀ could not be determined. As per an arithmetic calculation, it can be concluded that LD₅₀ of the EJSE is > 5000mg/kg bodyweight (Table.1).
Sub-chronic oral toxicity

Plasma metabolites and electrolytes

Effect of EJSE administration on plasma metabolites and electrolytes were assessed. EJSE caused significant decrement in plasma TC, TG and LDL levels without any alteration in plasma HDL and VLDL levels. The plasma \( \text{Na}^+ \), \( \text{K}^+ \) and \( \text{Ca}^{2+} \) levels recorded non significant alterations in the experimental groups (Table 2, Figure 1 & 2).

Plasma markers of cardiac, hepatic and renal damage

EJSE administration did not alter plasma markers of cardiac damage (LDH and CK-MB). However, plasma urea, creatinine and ALP levels were significantly increased after administration of high dose of EJSE (3000mg/kg BW) while, plasma AST, ALT, and billirubin levels were unaltered (Table 3 and Figure 3 & 4).

Hematological analysis

Haematological parameters such as haemoglobin, WBC, RBC, haematocrite, MCV, MCH, MCHC and platelets were assessed in control and treated animals. Sub chronic administration of EJSE did not alter any of the haematological parameters (Table 4, Figure 5 & 6).

Body weight gain, food and fluid intake and, organ weight

A dose dependent decrement in the body weight and food intake however, non significant alteration recorded in water intake of experimental groups (Table 5 and Figure 7). Organ
weights and their necropsy analysis revealed no major changes in experimental groups administered three doses of EJSE (Table 6, Figure 8).

**Histopathological evaluations**

There were no gross aberrations in the structural integrity of heart, liver and kidney after administration of EJSE (1000, 2000 or 3000mg/kg) (Figure 9). The cells appeared normal with intact nuclei. There was no evidence of cellular condensation observed in any of the tissues. Also, there were no visual evidences of apoptotic or necrotic changes.
Table 1: Arithmetic calculation of 50% lethal dose (LD₅₀) after acute administration of EJSE to Swiss albino mice.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Dose (mg/kg)</th>
<th>Dose difference (DD)</th>
<th>No of mice (N)</th>
<th>No of Dead</th>
<th>Death (%)</th>
<th>Mean dead (Md)</th>
<th>DD x Md</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>00</td>
<td>00</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EJSE1000</td>
<td>1000</td>
<td>1000</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EJSE2000</td>
<td>2000</td>
<td>1000</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EJSE3000</td>
<td>3000</td>
<td>1000</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EJSE4000</td>
<td>4000</td>
<td>1000</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EJSE5000</td>
<td>5000</td>
<td>1000</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>5000</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

LD₅₀ was calculated from the above table as follows:–

\[
LD_{50} = LD_{100} - DD \times MD
\]

Where, \( LD_{50} \) = 50% lethal dose, \( LD_{100} \) = 100% lethal, \( N \) = No. of animals/group, \( MD \) = Mean death for a group, \( DD \) = Dose deference (between two consecutive doses).

Therefore, \( LD_{50} \) of EJSE = \((> 5000) - 1000 \times 0\)

\( > 5000 - 0 \)

\( > 5000 \text{ mg/kg bodyweight} \)
Tables 2: Effect of chronic (28 days) administration of EJSE on plasma metabolic indices in Swiss albino mice.

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>EJSE1000</th>
<th>EJSE2000</th>
<th>EJSE3000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>45.33±2.55</td>
<td>40.00±4.51</td>
<td>41.00±3.05</td>
<td>33.33±4.80</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>46.95±3.05</td>
<td>36.22±2.42</td>
<td>36.59±1.79</td>
<td>34.43±1.29*</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>24.30±0.35</td>
<td>23.83±0.36</td>
<td>24.00±0.56</td>
<td>23.78±0.46</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>32.00±1.33</td>
<td>30.50±2.36</td>
<td>26.56±2.04</td>
<td>12.81±0.93*</td>
</tr>
<tr>
<td>VLDL (mg/dl)</td>
<td>8.98±0.48</td>
<td>7.41±0.23</td>
<td>7.81±0.56</td>
<td>7.02±0.32</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>93.83±7.52</td>
<td>86.28±1.92</td>
<td>76.76±4.69</td>
<td>73.50±3.03</td>
</tr>
<tr>
<td>Sodium (mmol/l)</td>
<td>135.6±1.47</td>
<td>135.4±1.63</td>
<td>137.4±1.50</td>
<td>137.2±2.77</td>
</tr>
<tr>
<td>Potassium (mmol/l)</td>
<td>4.22±0.06</td>
<td>4.26±0.07</td>
<td>4.44±0.09</td>
<td>4.44±0.11</td>
</tr>
<tr>
<td>Calcium (mmol/l)</td>
<td>9.38±0.18</td>
<td>9.73±0.08</td>
<td>9.33±0.12</td>
<td>9.51±0.14</td>
</tr>
</tbody>
</table>

Results are expressed as Mean ± S.E.M for n=8. *p<0.05 compared to CON.
Figure 1: Effect of chronic (28 days) administration of EJSE on plasma metabolic indices in Swiss albino mice.

Results are expressed as Mean ± S.E.M for n=8. *p<0.05 compared to CON.
Figure 2: Effect of chronic (28 days) administration of EJSE on plasma metabolic indices in Swiss albino mice.

Results are expressed as Mean ± S.E.M for n=8. *p<0.05 compared to CON.
Tables 3: Effect of chronic (28 days) administration of EJSE on plasma markers of cardiac, hepatic and renal function in Swiss albino mice.

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>EJSE1000</th>
<th>EJSE2000</th>
<th>EJSE3000</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cardiac injury markers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDH (U/l)</td>
<td>113.6±15.41</td>
<td>123.2±7.44</td>
<td>117.0±8.86</td>
<td>143.9±17.45</td>
</tr>
<tr>
<td>CK-MB (U/l)</td>
<td>53.00±7.21</td>
<td>55.60±8.30</td>
<td>50.60±3.58</td>
<td></td>
</tr>
<tr>
<td><strong>Hepatic injury markers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>34.00±3.31</td>
<td>40.60±3.47</td>
<td>43.00±1.51</td>
<td></td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>24.83±2.40</td>
<td>27.00±1.53</td>
<td>26.67±1.66</td>
<td></td>
</tr>
<tr>
<td>ALP (U/l)</td>
<td>11.34±1.21</td>
<td>14.53±1.67</td>
<td>15.32±1.99</td>
<td></td>
</tr>
<tr>
<td>Billirubin (mg/dl)</td>
<td>0.40±0.027</td>
<td>0.47±0.046</td>
<td>0.49±0.011*</td>
<td></td>
</tr>
<tr>
<td><strong>Renal injury markers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea (mg/dl)</td>
<td>54.72±3.28</td>
<td>60.66±2.87</td>
<td>64.72±4.97</td>
<td>74.13±2.78**</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.43±0.015</td>
<td>0.43±0.015</td>
<td>0.52±0.013*</td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed as Mean ± S.E.M for n=8. *p<0.05 and **p<0.05 compared to CON.
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**Figure 3:** Effect of chronic (28 days) administration of EJSE on plasma markers of cardiac and renal function in *Swiss* albino mice.

**Markers of Cardiac Damage**

<table>
<thead>
<tr>
<th>Lactate dehydrogenase</th>
<th>Creatine Kinase-MB</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Graph of Lactate dehydrogenase" /></td>
<td><img src="image2" alt="Graph of Creatine Kinase-MB" /></td>
</tr>
</tbody>
</table>

**Markers of Renal Damage**

<table>
<thead>
<tr>
<th>Urea</th>
<th>Creatinine</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image3" alt="Graph of Urea" /></td>
<td><img src="image4" alt="Graph of Creatinine" /></td>
</tr>
</tbody>
</table>

Results are expressed as Mean ± S.E.M for n=8. *p<0.05 and **p<0.05 compared to CON.
Figure 4: Effect of chronic (28 days) administration of EJSE on plasma markers of cardiac, hepatic and renal function in Swiss albino mice.

Results are expressed as Mean ± S.E.M for n=8. *p<0.05 and **p<0.05 compared to CON.
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Tables 4: Effect of chronic (28 days) administration of EJSE haematological parameters of Swiss albino mice.

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>EJSE1000</th>
<th>EJSE2000</th>
<th>EJSE3000</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (10^3/μl)</td>
<td>6.76±1.26</td>
<td>5.57±1.67</td>
<td>4.16±0.88</td>
<td>4.20±0.85</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>14.30±0.25</td>
<td>15.17±0.16</td>
<td>14.07±0.29</td>
<td>14.30±0.11</td>
</tr>
<tr>
<td>RBC (10^{12}/l)</td>
<td>8.20±0.17</td>
<td>8.73±0.03</td>
<td>8.06±0.16</td>
<td>8.35±0.04</td>
</tr>
<tr>
<td>Hematocrite (%)</td>
<td>35.80±0.49</td>
<td>36.83±0.46</td>
<td>34.60±0.60</td>
<td>35.03±0.1</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>43.70±0.37</td>
<td>42.20±0.45</td>
<td>43.00±0.49</td>
<td>42.03±0.40</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>17.40±0.05</td>
<td>17.34±0.20</td>
<td>17.40±0.10</td>
<td>17.07±0.12</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>39.90±0.26</td>
<td>41.13±0.20</td>
<td>40.60±0.25</td>
<td>40.77±0.38</td>
</tr>
<tr>
<td>RCD-WCV (%)</td>
<td>16.10±1.66</td>
<td>16.60±1.84</td>
<td>16.60±1.72</td>
<td>16.60±1.72</td>
</tr>
<tr>
<td>RCD-WSD (fl)</td>
<td>19.87±0.23</td>
<td>19.40±0.09</td>
<td>19.87±0.46</td>
<td>19.17±0.23</td>
</tr>
<tr>
<td>Platelets (10^3/μl)</td>
<td>5.88±0.09</td>
<td>5.22±0.26</td>
<td>5.26±0.38</td>
<td>5.44±0.12</td>
</tr>
<tr>
<td>MPV (fl)</td>
<td>10.17±0.03</td>
<td>9.70±0.05</td>
<td>9.80±0.15</td>
<td>9.90±0.05</td>
</tr>
<tr>
<td>RCDW</td>
<td>14.80±0.05</td>
<td>14.50±0.11</td>
<td>14.63±0.06</td>
<td>14.60±0.05</td>
</tr>
<tr>
<td>Plateletcrit (%)</td>
<td>0.59±0.008</td>
<td>0.50±0.02</td>
<td>0.51±0.04</td>
<td>0.53±0.01</td>
</tr>
</tbody>
</table>

Results are expressed as Mean ± S.E.M for n=8. *p<0.05 compared to CON.
Figure 5: Effect of chronic (28 days) administration of EJSE haematological parameters of Swiss albino mice.

Results are expressed as Mean ± S.E.M for n=8. *p<0.05 compared to CON.
Figure 6: Effect of chronic (28 days) administration of EJSE haematological parameters of Swiss albino mice.

Results are expressed as Mean ± S.E.M for n=8. *p<0.05 compared to CON.
Tables 5: Effect of chronic (28 days) administration of EJSE on bodyweight, food and fluid intake of *Swiss* albino mice.

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>EJSE1000</th>
<th>EJSE2000</th>
<th>EJSE3000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight&lt;sup&gt;g&lt;/sup&gt;</td>
<td>Initial (g)</td>
<td>22.45±1.33</td>
<td>23.57±1.12</td>
<td>24.02±0.51</td>
</tr>
<tr>
<td></td>
<td>Final (g)</td>
<td>26.85±0.59</td>
<td>25.40±0.41</td>
<td>25.67±0.66</td>
</tr>
<tr>
<td>Weight gain</td>
<td>4.40±0.02</td>
<td>1.82±0.04*</td>
<td>1.65±0.05**</td>
<td>1.05±0.03***</td>
</tr>
<tr>
<td>Food intake</td>
<td>4.33±0.13</td>
<td>4.03±0.11</td>
<td>3.87±0.24</td>
<td>3.55±0.31</td>
</tr>
<tr>
<td>(g/day)</td>
<td>Fluid intake</td>
<td>8.99±0.63</td>
<td>10.01±0.50</td>
<td>9.08±0.39</td>
</tr>
<tr>
<td>(ml/day)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed as Mean ± S.E.M for n=8. *p<0.05, **p<0.01 and ***p<0.001 compared to CON.
Figure 7: Effect of chronic (28 days) administration of EJSE on bodyweight, food and fluid intake of Swiss albino mice.

Results are expressed as Mean ± S.E.M for n=8. *p<0.05, **p<0.01 and ***p<0.001 compared to CON.
Tables 6: Effect of chronic (28 days) administration of EJSE organ weights of Swiss albino mice.

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>EJSE1000</th>
<th>EJSE2000</th>
<th>EJSE3000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain (g)</td>
<td>0.40±0.01</td>
<td>0.39±0.01</td>
<td>0.41±0.02</td>
<td>0.42±0.03</td>
</tr>
<tr>
<td>Heart (g)</td>
<td>0.12±0.004</td>
<td>0.12±0.005</td>
<td>0.12±0.003</td>
<td>0.11±0.005</td>
</tr>
<tr>
<td>Lungs (g)</td>
<td>0.16±0.014</td>
<td>0.18±0.008</td>
<td>0.18±0.01</td>
<td>0.17±0.009</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>0.98±0.02</td>
<td>0.96±0.04</td>
<td>1.00±0.02</td>
<td>0.98±0.02</td>
</tr>
<tr>
<td>Spleen (g)</td>
<td>0.12±0.010</td>
<td>0.12±0.009</td>
<td>0.13±0.012</td>
<td>0.26±0.014</td>
</tr>
<tr>
<td>Adrenal (g)</td>
<td>0.03±0.001</td>
<td>0.03±0.001</td>
<td>0.04±0.002</td>
<td>0.037±0.002</td>
</tr>
<tr>
<td>Kidney (g)</td>
<td>0.28±0.006</td>
<td>0.27±0.008</td>
<td>0.27±0.005</td>
<td>0.26±0.008</td>
</tr>
</tbody>
</table>

Results are expressed as Mean ± S.E.M for n=8. *p<0.05 compared to CON
Figure 8: Effect of chronic (28 days) administration of EJSE organ weights of *Swiss* albino mice.

Results are expressed as Mean ± S.E.M for n=8. *p<0.05 compared to CON
Figure 9: Photomicrographs of Heart of control (1) and 1000, 2000 and 3000mg/kg EJSE treated mice (2, 3, 4), Liver of control (5) and 1000, 2000 and 3000mg/kg EJSE treated mice (6, 7, 8) and kidney of control (9) and 1000, 2000 and 3000mg/kg EJSE treated mice (10, 11, 12) stained with hematoxyline and eosin (100X).
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DISCUSSION

No mortality was recorded following a single acute dose of EJSE (5000 mg/kg bw), LD₅₀ was arithmetically calculated to be > 5000 mg/kg bw (Dede and Dogara, 2004; Saidu et al., 2007). There were no observable symptoms of any behavioural alterations post 72 hours, indicating that EJSE has no major side effects even at high dose (5000 mg/kg bw).

Alterations in plasma lipid content and fluctuations in electrolytes following administration of an herbal extract are imperative to be assessed. It is also mandatory to monitor the same following low as well as higher doses of EJSE. Observed decrement in plasma TC, TG, LDL and VLDL and unchanged HDL levels are in accordance with one observed hypolipidemic potential of EJSE (Chapter 3). However, non significant alterations were recorded following administration of low dose of EJSE (1000 or 2000 mg/kg BW) suggesting that, hypolipidemic effect of EJSE in normolipidemic mice manifests only at a higher dose. No significant alterations were observed in plasma glucose and electrolyte levels suggesting that EJSE does not manifest any major changes in the glycaemic status or electrolyte balance even at higher doses (1000, 2000 or 3000 mg/kg BW).

Plasma levels of Ck-MB and LDH have been used as a marker of cardiac damage under various toxic manifestations (Jadeja et al., 2010; Thounaojam et al., 2010). Our study recorded non significant alterations in the level of plasma CK-MB and LDH after sub chronic administration of EJSE indicating no damage to cardiomyocytes. These observations get further substantiated with the microscopic evaluation of cardiac tissue wherein, no observable alterations in their cellular integrity could be noted after administration of different doses of EJSE.
Some herbal extracts can be heptotoxic and their oral administration leads to elevated levels of plasma AST and ALT (Mukinda et al., 2010; Udem et al., 2010). Oral administration of EJSE has been reported to reduce streptozotocin and carbon tetrachloride induced elevation in plasma AST and ALT at a dose range of 100 to 500 mg/kg bw (Jasmine and Daisy, 2007; Sisodia and Bhatnagar, 2009; Sundaram et al., 2009). In our study the plasma AST and ALT levels in EJSE administered mice were comparable with those of control animals and the same was further corroborated with comparable histoarchitectural details of liver in control and EJSE administered groups. It is inferable from these observations that, EJSE is non toxic to hepatic tissue in the dosage employed herein. EJSE can therefore be considered as safe and non toxic to liver thus, adding further validity to its already reported hepatoprotective potential.

Studies have shown that, use of traditional herbal medicines in treating renal diseases is limited by their adverse effects on renal functions (Saxena and Panhotra, 2003; Singh and Prakash, 2008). No significant alterations in plasma markers of renal damage (urea and creatinine) could be after sub chronic administration of EJSE in low doses (1000 and 2000 mg/ kg bw). However, significant elevation in plasma levels of these markers could be were observed after administration of high dose of EJSE (3000 mg/kg bw). However, no histoarchitecture destruction was observable in the renal tissue of mice administered lower as well as higher doses of EJSE. It is inferable from these observations that, EJSE may manifest moderate levels of renal damage at very high dose (3000 mg/kg bw) but, is never the less safe at therapeutic dosage (100 - 500 mg/kg bw).
A haemogram reflects upon the haemolytic property of any herbal extract in question (Mukinda et al., 2007, 2010) and the same was assessed in our study. No significant alterations were seen in the haematological profile of any of the experimental groups studied herein. Also, food and water intake and absolute weight of vital organs were comparable in control and EJSE treated groups. However, a dose dependent decrement in body weight gain was recorded at the end of 28 days. Significant decrement in plasma lipid profile and body weight gain observed after EJSE administration is attributable to reduced fat absorption through intestine and effective elimination of lipids through faeces as observed in EJSE treated atherogenic rats (Chapter 3).

It can be concluded from the study that, EJSE is non toxic to cardiac and hepatic tissue and moderately nephrotoxic at high dose (3000 mg/kg BW). Hence, the Lowest Observable Adverse Effect Level (LOAEL) for EJSE is 3000 mg/kg BW while, the No Observable Adverse Effect Level (NOAEL) is up to 2000 mg/kg BW (Rhiouani et al., 2008). According to World Health Organization toxicity guidelines for herbal extracts, it can be concluded that the calculated Acceptable Daily Intake (ADI) is equal to NOAEL (2000mg/kg BW) ÷ 100; wherein, 100 is safety factor (Rosidaha et al., 2009). Thus, ADI for EJSE for mice is 20 mg/kg BW. An extrapolation of these results to an average adult human (70kg) would be 1.4 g of EJSE or 28g of dried EJ powder (based on final percentage yield (5% w/w) after extraction).
Summary

The aim of the present study was to investigate safety evaluation of ethanolic seed extract of *Eugenia jambolana* (EJSE) using acute and sub-chronic toxicity assays in Swiss albino mice as per OECD guidelines. Mice administered a single dose (1000, 2000, 3000, 4000 or 5000mg/kg BW) of EJSE and changes in patterns of behavior and mortality were observed. Also, plasma levels of metabolites, hepatic, cardiac and renal function markers, electrolytes, blood count and histopathology of major organs were monitored in mice chronically treated with EJSE (1000, 2000 or 3000 mg/kg BW) for 28 days. Since no mortality was recorded in the acute toxicity evaluation up to a dose of 5000mg/kg bodyweight of EJSE, LD50 was assumed to be >5000mg/kg BW. In the sub-chronic toxicity evaluation, no adverse observations were recorded in mice administered with 2000mg/kg EJSE; however at 3000 mg/kg dose, moderate increase in the plasma levels of urea and creatinine was observed. Hence, LOAEL for EJSE was found to be 3000 mg/kg BW and NOAEL was adjudged as 2000 mg/kg BW.