Chapter-5

BIOACTIVITY EVALUATION OF

2,3,6-trimethyloct-6-enal
5. BIOACTIVITY EVALUATION OF 2,3,6-trimethyloct-6-enal

5.1. Methodology followed to analyze the hepatoprotective and antioxidant activity of 2,3,6-trimethyloct-6-enal

5.1.1. CCl₄ induced hepatic damage:

The hepatic cell injury was induced by using carbon tetrachloride (CCl₄) as suggested by Ozbek et al, 2004 with slight modifications. Mice were divided into six groups (n=6), Group I received a single dose of water 1mL/kg b.w. daily for 5 days and received liquid paraffin (1mL/kg) once daily for 5 days. Group II received CCl₄ (0.5 mL/kg body weight): liquid paraffin (1:1) once daily for 5 days. Group III received standard drug silymarin (50 mg/kg b.w.) once daily for 5 days. Test groups animals (Groups IV–VI) were administered at a dose of 100, 200 and 300 mg/kg body weight per orally of the isolated compound respectively, in the form of suspension with water. Groups III–VI animals were administered simultaneously CCl₄: liquid paraffin (1:1, 2 mL/kg body weight) once daily for 5 days after 30 min of administration of the doses. Animals were sacrificed 24 h after the last treatment.

5.1.2. Analysis of hepatoprotective activity by using serum biochemical parameters:

Blood serum was used for biochemical analysis, such as the serum glutamic pyruvates transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT), serum alkaline phosphatase (SALP) and total bilirubin. The SGOT and SGPT level were measured as per
the method suggested by Reitman and Frankle (1957). The total bilirubin content was measured as per the method of Grof (1938) and the SALP level was measured as per the method of Sood (1999).

Method for the measurement of SGPT (Serum glutamic pyruvic transaminase) in blood serum samples

Assay Procedure:

1.0 mL of working reagent (2mL of R₂ with 8mL of R₁) was added to 100 µl of serum / plasma, mixed well, incubated for 1 min and the change in optical density was observed every 60 seconds for 180 seconds against distilled water at 340nm.

Calculations:

Average change was observed in absorbance per minute (ΔAbs/min) and release of GPT was expressed in U/L.

At 340 nm in U/L = ΔAbs⁻¹ x 1746

At 334 nm in U/L = ΔAbs⁻¹ x 1780

At 365 nm in U/L = ΔAbs⁻¹ x 3235

where, ΔAbs= change in absorbance; 1746, 1780, 3235= dilution factor.

Method for the measurement of SCOT (Serum glutamic oxaloacetic transaminase) in blood serum samples:
Assay Procedure:

1.0 mL of working reagent (2mL of R₂ with 8mL of R₁) was added to 100 µl of serum / plasma, mixed well, incubated for 1 min and the change in optical density was observed every 60 seconds for 180 seconds against distilled water at 340nm.

Calculations:

Average change was observed in absorbance per minute (ΔAbs/min) and release of GOT in U/L.

At 340 nm in U/L = ΔAbs⁻¹ x 1746

At 334 nm in U/L = ΔAbs⁻¹ x 1780

At 365 nm in U/L = ΔAbs⁻¹ x 3235

where, ΔAbs= change in absorbance; 1746, 1780, 3235= dilution factor.

Method for the measurement of SALP (Serum alkaline phosphatase) in blood serum samples:

Assay procedure:

Four clean dry test tubes were taken and labelled as - Blank (B), Standard (S), Control (C) and Test (T). 1.05 mL of distilled water in (B) and 1.0 mL of distilled water each in (S), (C) and (T) was pipetted out. Then 1 mL of Buffer Reagent (L₁) and 0.1 mL of Substrate Reagent (L₂) was added in all the four test tubes, mixed well and was allowed to stand for 3 min at 37°C. 0.05 mL of sample was added in (T), 0.05 mL of Phenol Standard (S) was
added on (S), mixed well and was allowed to stand for 15 min at 37°C. Then 1 mL of Colour Reagent (L3) was added in all the four test tubes, 0.05 mL of sample was added in (C), mixed well and absorbance was measured against distilled water at 510 nm.

Calculation:

\[
\text{Total ALP activity in K.A. units} = \frac{\text{Abs.T} - \text{Abs.C}}{\text{Abs.S} - \text{Abs.B}} \times 10
\]

Method for the measurement of Total bilirubin (TB) in blood serum samples:

Assay procedure:

Two clean dry test tubes were taken and labelled as \( T_1 \) and \( T_2 \). 0.5 mL of Reagent Diazo A in \( T_1 \) and \( T_2 \) and 0.05 mL of Diazo B in \( T_1 \) was added and mixed thoroughly. Then 0.5 mL of Activator, 1.0 mL of distilled water, 0.1 mL of serum sample was added in \( T_1 \) and \( T_2 \), mixed well, kept at room temperature in the dark for 5 min and absorbance was read at 540 nm.

Calculation

Total bilirubin mg/dl A= of \( T_1 \) - \( T_2 \) x 26.31

where, 26.31= dilution factor.
5.1.3. Preparation of liver homogenate:

Liver samples from the sacrificed mice was quickly removed and pursued with ice-cold saline. A portion of the liver was homogenized in chilled sodium phosphate buffer (0.1M, pH 7.4) using a Potter Eleven homogenizer. The homogenate obtained was centrifuged in a cooling centrifuge at 12,000xg for 30 min at 4°C to separate the nuclear debris. The supernatant was collected and used for the subsequent assays (Mohandas et al., 1984).

5.1.4. Analysis of antioxidant activity by measuring antioxidant enzymatic and non enzymatic levels:

Lipid Peroxidation (LPO) assay:

Determination of Hepatic TBARS which signifies the rate of lipid peroxidation was carried out by following the standard method (Uchiyama and Mihara, 1978; Lee and Lim, 2008). Briefly the tissue was homogenized in chilled 0.1M potassium chloride solution. The assay mixture contained 0.5 mL of liver homogenate, 3mL of 1% H₃PO₄ and 1 mL of 0.6% TBA. The mixture was heated at 100°C for 45 min, the reaction mixture was then allowed to cool at room temperature and 3 mL of n-butanol was added to it and shaken vigorously so as to separate the butanolic phase, it was then subjected to centrifugation at 4000xg for 10 min and absorbance was determined at 535 nm.

Reduced Glutathione (GSH) content:

Reduced Glutathione content was determined by the method of Ellman et al., (1958). 0.2 mL of tissue homogenate was mixed with 1.8 mL of EDTA solution. To this 3.0 mL of
precipitating reagent (1.67g of metaphosphoric acid, 0.2g of EDTA disodium salt, 30g sodium chloride in 1 L of distilled water) was added mixed thoroughly and kept for 5 min before centrifugation. 2.0 mL of the filtrate, 4.0 mL of 0.3 M disodium hydrogen phosphate solution and 1.0 mL of DTNB (5,5-dithiobis-2-nitrobenzoic acid) reagent were added and absorbance was read at 412 nm.

Catalase (CAT) assay:

CAT assay was performed following the method based on the disappearance of H$_2$O$_2$ at 25°C (Pedraza-chaverri et al., 2005). 5µl of dilute homogenate (1:40) was mixed with 720 µl of 30 mM H$_2$O$_2$ in 10 mM potassium phosphate solution and the reaction was followed at 240 nm. Decomposition of H$_2$O$_2$ by CAT contained follows a first order kinetics as $K = \frac{2.3}{t} \log \frac{A_0}{A}$ where $K$ is the first order rate constant, $t$ is the time for which the decrease of H$_2$O$_2$ due to CAT activity was measured for 15s and $A_0/A$ is the optical density at time 0 and at 15s respectively.

Superoxide dismutase (SOD) activity:

Superoxide dismutase activity was measured by the method of Dhindsa et al., (1981) The reaction mixture consisted of 1.5 mL phosphate buffer (0.1 M, pH 7.4) 0.1 mL NBT (2.25 mM, 0.1mL tissue homogenate, 0.1mL sodium carbonate (1.5 M), 0.2 mL methionine (200 mm), 0.1 mL EDTA (3 mM) 1mL distilled water and 0.1 riboflavin (60mM) in the total volume of 3 mL. It was incubated in light for 60 min at room temperature. The rate of reaction was measured by recording changes in absorbance at 560 nm due to formation of formazone, a reaction product of NBT.
Glutathione Peroxidase (GPx) assay:

100 μl of the tissue homogenate diluted in 1:100 was added to 800 μl of reaction mixture containing 50 mM potassium phosphate solution pH 7.0, 1mM EDTA, 1mM sodium azide, 0.2mM NADPH, 1Unit/mL of GR and 1mM GSH. The reaction mixture was incubated at room temperature for 5 min before addition of 32 μl of 2.5 mM H$_2$O$_2$ solution for the initiation of the reaction and finally absorbance at 340 nm was recorded for 3 min and the activity was calculated focusing on millimolar absorption co-efficient for NADPH being 6.22. In case of blank reactions homogenates were replaced by distilled water which was subtracted from each assay (Pedraza-Chaverri et al., 2005).

5.1.5. Histopathological observation:

The mice liver tissues were fixed in 10% formalin for 24 hours and dehydrated with a solution of ethanol and embedded in paraffin. The serial sections were cut 5μm thick and stained with Haematoxylin-Eosin dye and observed under microscope (BX41, OLYMPUS) to note the changes in the liver tissue (Jothy et al., 2011).

5.1.6. Statistical analysis:

The data presented here were expressed as mean ± SE. The results were analysed by one way ANOVA with Tukey- Kramer multiple comparisons Test. The level of significance was accepted at P<0.001, P<0.01 and P<0.05.
5.2. Protective effect of 2,3,6-trimethyloct-6-enal on serum biochemical parameters against CCl₄ induced hepatotoxicity in Swiss albino mice:

Mice treated with a single dose of CCl₄ developed significant (P<0.001, control vs. toxic group) hepatic damage as observed from elevated levels of SGOT (78.29 ± 2.19 IU/L compared to control 26.61 ± 1.55 IU/L), SGPT (94.54± 1.51 IU/L compared to control 18.85 ± 2.05 IU/L), SALP (127.25±2.19 IU/L compared to control 41.67 ± 2.11 IU/L) and serum bilirubin (3.75±0.02 IU/L compared to control 0.31 ± 0.01 IU/L). Treatment with 100, 200, 300 mg/kg b.w. p.o. dose of 2,3,6-trimethyloct-6-enal exhibited significant (P<0.001 compared to toxic group and P<0.001, P<0.01 and P<0.05 compared to control group) protective activity in serum enzyme and bilirubin levels of swiss albino mice (Table 5.2.1). 100 mg/kg b.w. p.o. dose of 2,3,6-trimethyloct-6-enal exhibited maximum level of (significant at P<0.001 compared to toxic group) protective effect in serum biochemical parameters (Table 5.2.2). The protective efficacy of 100 mg/kg dose of 2,3,6-trimethyloct-6-enal was similar to that of protective efficacy exhibited by standard drug Silymarin (fig.5.2). The results were also supported by histopathological observations (fig. 5.4).
Table: 5.2.1. Protective effect of 2,3,6-trimethyloct-6-enal on serum biochemical parameters against CCl₄ induced hepatotoxicity in Swiss albino mice:

<table>
<thead>
<tr>
<th>Doses</th>
<th>SGOT (IU/L)</th>
<th>SGPT (IU/L)</th>
<th>SALP (IU/L)</th>
<th>Bilirubin (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>26.61 ± 1.55</td>
<td>18.85 ± 2.05</td>
<td>41.67 ± 2.11</td>
<td>0.31 ± 0.01</td>
</tr>
<tr>
<td>Group II</td>
<td>78.29 ± 2.19</td>
<td>94.54 ± 1.51</td>
<td>127.25 ± 2.19</td>
<td>3.75 ± 0.02</td>
</tr>
<tr>
<td>Group III</td>
<td>35.11 ± 1.62</td>
<td>23.08 ± 1.11</td>
<td>58.79 ± 0.85</td>
<td>0.74 ± 0.01</td>
</tr>
<tr>
<td>Group IV</td>
<td>32.20 ± 0.74</td>
<td>23.44 ± 1.13</td>
<td>60.24 ± 0.93</td>
<td>0.72 ± 0.01</td>
</tr>
<tr>
<td>Group V</td>
<td>39.76 ± 3.04</td>
<td>27.16 ± 1.65</td>
<td>69.15 ± 2.11</td>
<td>0.81 ± 0.03</td>
</tr>
<tr>
<td>Group VI</td>
<td>47.25 ± 2.19</td>
<td>34.89 ± 2.95</td>
<td>89.83 ± 1.88</td>
<td>0.98 ± 0.03</td>
</tr>
</tbody>
</table>

n=6 animal, values are given as mean±SE, values are statistically significant at P<0.001, P<0.01 and P<0.05 level of significance.

a- compared with control P<0.001,
b- compared with control P<0.01,
c- compared with control P<0.05,
d- compared with standard P<0.001,
e- compared with toxic P<0.001.

Group I-control, GroupII- toxic (CCl₄), Group III- standard (Silymarin), Group IV- 100 mg/kg b.w.p.o. dose of 2,3,6-trimethyloct-6- enal; Group V-200 mg/kg b.w.p.o. dose of 2,3,6-trimethyloct-6- enal; GroupVI-300 mg/kg b.w.p.o. dose of 2,3,6-trimethyloct-6- enal.
Table 5.2.2. % of Hepatoprotection offered by 2,3,6-trimethyloct-6-enal at different dose concentration manner on serum biochemical parameters against CCl₄ induced hepatotoxicity in Swiss albino mice:

<table>
<thead>
<tr>
<th>Doses</th>
<th>SGOT (%)</th>
<th>SGPT (%)</th>
<th>SALP (%)</th>
<th>Bilirubin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group III</td>
<td>83 approx</td>
<td>94 approx</td>
<td>79 approx</td>
<td>87 approx</td>
</tr>
<tr>
<td>Group IV</td>
<td>89 approx</td>
<td>93 approx</td>
<td>78 approx</td>
<td>88 approx</td>
</tr>
<tr>
<td>Group V</td>
<td>74 approx</td>
<td>89 approx</td>
<td>67 approx</td>
<td>85 approx</td>
</tr>
<tr>
<td>Group VI</td>
<td>60 approx</td>
<td>78 approx</td>
<td>43 approx</td>
<td>80 approx</td>
</tr>
</tbody>
</table>

Group III- standard (Silymarin), Group IV- 100 mg/kg b.w.p.o. dose of 2,3,6-trimethyloct-6-enal, Group V-200 mg/kg b.w.p.o. dose of 2,3,6-trimethyloct-6-enal, Group VI- 300 mg/kg b.w.p.o. dose of 2,3,6-trimethyloct-6-enal.
Fig: 5.2. Protective efficacy offered by 2,3,6-trimethyloct-6-enal at different dose concentration manner on serum biochemical parameters against CCl₄ induced hepatotoxicity in Swiss albino mice. (A) changes in SGOT level, (B) changes in SGPT level, (C) changes in SALP level, (D) changes in Bilirubin level.

- a- compared with control P<0.001,
- b- compared with control P<0.01,
- c- compared with control P<0.05,
- d- compared with standard P<0.001,
- e- compared with toxic P<0.001.

Group I- control, GroupII- toxic (CCl₄), Group III- standard (Silymarin), Group IV- 100 mg/kg b.w.p.o. dose of 2,3,6-trimethyloct-6-enal; Group V- 200 mg/kg b.w.p.o. dose of 2,3,6-trimethyloct-6-enal; GroupVI- 300 mg/kg b.w.p.o. dose of 2,3,6-trimethyloct-6-enal.
5.3. Antioxidant efficacy of 2,3,6-trimethyloct-6-enal on tissue enzymatic and non-enzymatic levels of Swiss albino mice against CCl₄ induced hepatic damage:

Mice treated with a single dose of CCl₄ developed significant hepatic damage as observed from elevated levels of LPO and decreased levels of SOD, CAT, GPx, and GSH in hepatic cells. Pretreatment with 2,3,6-trimethyloct-6-enal at dose concentration of 100, 200, 300 mg/kg b.w. p.o. conferred significant (P<0.001 and P<0.01 compared to control, P<0.001 compared to toxic and P<0.01 compared to standard) protective activity in antioxidant enzymatic and non-enzymatic levels of Swiss albino mice by lowering the elevated levels of LPO and by increasing the decreased levels of SOD, CAT, GPx, and GSH (Table 5.3.1). 100 mg/kg b.w. p.o. dose of 2,3,6-trimethyloct-6-enal exhibited a maximum level (P<0.001 compared to toxic) of antioxidant activity and 300 mg/kg b.w.p.o. dose exhibited least level (P<0.001 compared to toxic, standard and control respectively) of antioxidant activity (Fig.5.3). The protective efficacy of 100 mg/kg dose of 2,3,6-trimethyloct-6-enal was similar to that of protective efficacy exhibited by standard drug Silymarin (50 mg/kg b.w.p.o.). The result was also supported by histopathological observations (fig.5.4)
Table: 5.3.1. Antioxidant efficacy of 2,3,6-trimethyloct-6-enal at different dose concentration manner on tissue enzymatic and non enzymatic levels of Swiss albino mice against CCl₄ induced hepatic damage:

<table>
<thead>
<tr>
<th>Doses</th>
<th>LPO (nmoles TBARS/mg protein)</th>
<th>GSH (unit/mg protein)</th>
<th>CAT (unit/mg protein)</th>
<th>SOD (unit/mg protein)</th>
<th>GPx (unit/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>0.56 ± 0.01</td>
<td>54.67 ± 1.31</td>
<td>0.58 ± 0.02</td>
<td>1.12 ± 0.00</td>
<td>67.36 ± 1.13</td>
</tr>
<tr>
<td>Group II</td>
<td>3.97 ± 0.04^a</td>
<td>11.84 ± 0.51^a</td>
<td>0.14 ± 0.01^a</td>
<td>0.32 ± 0.01^a</td>
<td>14.98 ± 1.01^a</td>
</tr>
<tr>
<td>Group III</td>
<td>0.83 ± 0.03^a,e</td>
<td>43.03 ± 1.41^b,e</td>
<td>0.46 ± 0.01^e</td>
<td>0.86 ± 0.01^a,e</td>
<td>48.30 ± 1.71^a,c</td>
</tr>
<tr>
<td>Group IV</td>
<td>0.81 ± 0.02^a,e</td>
<td>42.72 ± 1.46^b,e</td>
<td>0.49 ± 0.02^e</td>
<td>0.82 ± 0.01^a,e</td>
<td>52.95 ± 2.70^b,e</td>
</tr>
<tr>
<td>Group V</td>
<td>0.92 ± 0.01^a,c</td>
<td>37.60 ± 1.32^a,c</td>
<td>0.42 ± 0.01^b,e</td>
<td>0.79 ± 0.01^a,c</td>
<td>46.38 ± 0.90^a,c</td>
</tr>
<tr>
<td>Group VI</td>
<td>1.12±0.02^a,d,e</td>
<td>31.76±0.90^a,d,e</td>
<td>0.35±0.01^a,c</td>
<td>0.72±0.01^a,c,f</td>
<td>35.67±1.98^a,c,g</td>
</tr>
</tbody>
</table>

n=6 animal, values are given as mean±SE, values are statistically significant at P<0.001, P<0.01 and P<0.05 level of significance.

a- compared with control P<0.001, b- compared with control P<0.01,c- compared with control P<0.05, d- compared with standard P<0.001, e- compared with toxic P<0.001,f- compared with standard P<0.01, g- compared with standard P<0.001

Group I-control, Group II- toxic (CCl₄), Group III- standard (Silymarin), Group IV- 100 mg/kg b.w.p.o. dose of 2,3,6-trimethyloct-6-enal; Group V-200 mg/kg b.w.p.o. dose of 2,3,6-trimethyloct-6-enal; Group VI-300 mg/kg b.w.p.o. dose of 2,3,6-trimethyloct-6-enal.
Fig: 5.3. Antioxidant efficacy offered by 2,3,6-trimethyloct-6-enal at different dose concentration manner on tissue enzymatic and non enzymatic levels of Swiss albino mice against CCl₄ induced hepatic damage. (A) changes in LPO level, (B) changes in GSH level, (C) changes in CAT level, (D) changes in SOD level, (E) changes in GPx level

a- compared with control P<0.001, b- compared with control P<0.01, c- compared with control P<0.05, d- compared with standard P<0.001, e- compared with toxic P<0.001, f- compared with standard P<0.01, g- compared with standard P<0.001

Group I-control, GroupII- toxic (CCl₄), Group III- standard (Silymarin), Group IV- 100 mg/kg b.w.p.o. dose of 2,3,6-trimethyloct-6-enal; Group V-200 mg/kg b.w.p.o. dose of 2,3,6-trimethyloct-6-enal; GroupVI-300 mg/kg b.w.p.o. dose of 2,3,6-trimethyloct-6-enal.
5.4. Histopathology of treated mice livers after treatment with 2,3,6-trimethyloct-6-enal at different dose concentration manner:

The protective efficacy of isolated compound 2,3,6-trimethyloct-6-enal (100, 200, 300 mg/kg b.w.p.o.) isolated from bark of *Pajanelia longifolia* (Willd.) K. Schuman were further analysed by histopathological study where it was found that 100 mg/kg b.w.p.o. dose of the 2,3,6-trimethyloct-6-enal exhibited a maximum level of healing of necrosis (Fig 5.4D) which was nearly similar to the control group where normal hepatocytes (Fig 5.4A) were found. Whereas, the liver sections of CCl₄ intoxicated mice showed cellular degradation and necrosis (Fig 5.4B). On the other hand healing of necrosis was found to be similar in the case of 100 mg/kg b.w.p.o dose of 2,3,6-trimethyloct-6-enal and 50 mg/kg b.w.p.o. dose of standard drug Silymarin (Fig 5.4D and 5.4C respectively).
Fig: 5.4. Histopathological studies of sections of mice liver on 6th day after treatment. (A) Control, (B) CCl₄ (0.5 ml/kg b.w.i.p.), (C) Silymarin (50 mg/kg b.w.p.o.), (D) 100 mg/kg b.w.p.o. dose of 2,3,6-trimethyloct-6-enal, (E) 200 mg/kg b.w.p.o. dose of 2,3,6-trimethyloct-6-enal, (F) 300 mg/kg b.w.p.o. dose of 2,3,6-trimethyloct-6-enal

(arrow mark in (B) indicates : necrotic lesions); (C, D, E, F respectively indicates: healing of necrosis)