Hepatoprotective effect of trimethylgallic acid esters against carbon tetrachloride-induced liver injury in rats

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Gallic acid and its derivatives are potential therapeutic agents for treating various oxidative stress mediated disorders. In the present study, we investigated the hepatoprotective effects of newly synthesized conjugated trimethylgallic acid (TMGA) esters against carbon tetrachloride (CCl₄)-induced hepatotoxicity in rats. Animals were pre-treated with TMGA esters at their respective doses for 7 days against CCl₄-induced hepatotoxicity. The histopathological changes were evaluated to find out degenerative fatty changes including vacuole formation, inflammation and tissue necrosis. Various biomarkers of oxidative stress (lipid peroxidation, glutathione levels, and endogenous antioxidant enzyme activities), liver enzymes (AST and ALT), triacylglycerol and cholesterol were evaluated. Pre-treatment with TMGA esters (MRG, MGG, MSG, and MUG at the dose of 28.71, 30.03, 31.35, 33.62 mg/kg/day), respectively reversed the CCl₄-induced liver injury scores (reduced vacuole formation, inflammation and necrosis), biochemical parameters of plasma (increased AST, ALT, TG, and cholesterol), antioxidant enzymes (increased lipid peroxidation and nitrite levels; decreased glutathione levels, superoxide dismutase and catalase activities) in liver tissues and inflammatory surge (serum TNF-α) significantly. The study revealed that TMGA esters exerted hepatoprotective effects in CCl₄-induced rats, specifically by modulating oxidative-nitrosative stress and inflammation.

Keywords: CCl₄, Cytokines, Gallic acid, Inflammation, Lipid peroxidation, Liver, Necrosis, TMGA, TNF-α

Liver is a pivotal organ principally involved in metabolism, storage and excretion of metabolites. Hepatotoxins such as ethanol, paracetamol, carbon tetrachloride, etc., are known to cause damage to liver¹⁵. The animal model of liver injuries induced by various hepatotoxins showed similar trend but with slight variations such as increased membrane permeability, lipid peroxidation and cell death comparable to chronic hepatic disease in humans. Upon stimulation from various hepatotoxins, kupffer cells release proinflammatory mediators such as tumor necrosis factor-alpha (TNF-α) and nitrous oxide (NO) and eventually result in accumulation of reactive nitrogen species (ROS). The ROS cause lipid peroxidation and membrane degradation that leads to liver damage and inflammatory response²,⁵,⁶.

Various epidemiological studies consistently indicate a positive association between the consumption of plant food or natural origin and prevention of diseases. Some of the beneficial effect is attributed to the presence of phenolic phytochemicals that are considered to play an important role as dietary antioxidants for the prevention of the oxidative damage caused by reactive oxygen species in living systems⁷,⁸. Gallic acid (GA) and its derivatives are widely distributed in the plant kingdom and represent a large family of plant secondary polyphenolic metabolites. They are present in the form of either methylated GAs (syringic acid) or galloyl conjugates of catechin derivatives, i.e., flavan-3-ols, or polygalloyl esters of glucose, quinic acid or glycerol⁹. GA and its natural derivatives have been widely reported for various biological and pharmacological activities including anti-inflammatory and hepatoprotective effects¹⁰,¹¹. These activities are possibly linked with their antioxidant potential due to their ability to prevent damage from free radicals or to prevent generation of these free radicals²,⁵,⁷,¹².

Based on these observations and previous studies reported from our laboratory¹³-¹⁵, it is suggested that there are potential advantages in giving naturally occurring phenolic/alcoholic agents with complementary pharmacological activities in the form of a single chemical/drug entity. Such agents are designed with improved physicochemical properties such as bioavailability and release of the parent drugs at the
site of action\textsuperscript{14,16,17}. Hence, in our recent studies, we reported the synthesis of some new gallic acid (GA) esters and evaluation of these esters against chronic stress-induced anxiety-like behavior and oxidative stress in mice\textsuperscript{18,19}. In the present study, we investigated the effect of these ester derivatives against \textit{CCl}_4-induced hepatotoxicity in rats.

**Materials and Methods**

Experimental animals— Twelve week old male Wistar rats (250-280 g) were procured from Central Animal House, Panjab University, Chandigarh, India. They were housed in individual cages under standard (25±2°C, 60–70% humidity) laboratory conditions, maintained on a 12 h natural day–night cycle, with free access to standard food pellets and water. Animals were acclimatized to laboratory conditions before the experimentation. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC/98-112/UIPS) and conducted strictly according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

Test compounds and drug treatment— The test compounds 3′-Hydroxyphenyl-3,4,5-trimethylgallate (MRG), 2′-Methoxyphenyl-3,4,5-trimethylgallate (MGG), 3′,4′-(Methylenedioxy)phenyl-3,4,5-trime-thylgallate (MSG) and 2′-Oxo-2′\textit{H}-chromene-7′-yl-3,4,5-trimethylgallate (MUG) were synthesized and characterized in our laboratory. Silymarin has been widely reported as a hepatoprotective drug in the literature. The key mechanism that ensures hepatoprotection appears to be free radical scavenging. Silymarin is able to neutralize the hepatotoxicity of several agents including ethanol, paracetamol (acetaminophen) and carbon tetrachloride in animal models\textsuperscript{20}. Silymarin was used as a positive control in this study. The drug was procured from Ind-Swift Laboratories, Chandigarh, India. All other chemicals used for biochemical and molecular estimations were of analytical grade. The doses of the test compounds were selected according based on previous published study\textsuperscript{15}. The animals were randomly divided into 7 experimental groups, containing 6 animals in each group: Group I, naive animals and received olive oil; Group II, control animals and received \textit{CCl}_4 (1.5 mg/kg) in olive oil in a ratio of 1:1 v/v; Group III, standard group, \textit{CCl}_4-treated animals with pre-treatment of silymarin (50 mg/kg; p.o. for 7 days); and Groups IV–VII, \textit{CCl}_4-treated animals with pre-treatment of test compounds MRG, MGG, MSG, and MUG at the dose of 28.71, 30.03, 31.35, 33.62 mg/kg/day, p.o. for 7 days, respectively. \textit{CCl}_4 was dissolved in olive oil in ratio of 1:1 (v/v) and was given intraperitoneally at a dose of 1.5 ml/kg on 6\textsuperscript{th} and 7\textsuperscript{th} day of treatment.

On day 8, 24 h after \textit{CCl}_4 treatment, rats were sacrificed by cervical dislocation. Blood sample was obtained via cardiac puncture into tubes, and serum was separated by centrifugation at 2000×g for 10 min at 4°C. Liver was excised immediately, blotted off blood, rinsed in pH 7.4 PBS and stored at −80°C until analyzed. The weight of liver was recorded and expressed as a relative organ weight\textsuperscript{21}.

Liver histopathological evaluation— Liver samples were processed separately for histological observations. Initially, the material was fixed in 10% buffered neutral formalin for 48 h. The sections were cut in 5-mm thickness, stained with hematoxylin and eosin, and examined microscopically for histopathological changes\textsuperscript{22} including injury scores calculated as fatty changes or vacuole formation, inflammation and tissue necrosis. The histopathological scores were calculated by a pathologist as described by Tang et al.\textsuperscript{23}.

Homogenization of liver— The liver was homogenized (10%, w/v) in 0.1 mol/L phosphate buffer (pH 7.4). Homogenates were centrifuged for 20 min at 15000×g. The supernatant was collected for estimation of lipid peroxidation and reduced glutathione levels. The post nuclear fractions for catalase assay were obtained by centrifugation of the homogenate at 1000 g for 20 min at 4°C. For other enzyme assays, the homogenate was centrifuged at 12000×g for 60 min at 4°C.

Estimation of biochemical parameters of plasma— The enzymatic activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), triacylglycerol (TG), and total cholesterol (TC) in the plasma were measured colorimetrically with an automatic analyzer (Hitachi 902 Automatic Analyzer).

Estimation of antioxidant enzymes in liver tissues

Estimation of malondialdehyde (MDA)— The extent of lipid peroxidation was determined quantitatively by performing the method as described by Wills\textsuperscript{24}. The amount of MDA was measured by reaction with thiobarbituric acid at 532 nm using Perkin Elmer Lambda 20 spectrophotometer (Norwalk, CT, USA). The values were calculated using the molar extinction coefficient of chromophore (1.56 × 10 M\textsuperscript{-1} cm\textsuperscript{-1}).

Nitrite estimation— The accumulation of nitrite in the supernatant, an indicator of the production of nitric
oxide was determined by a colorimetric assay with Greiss reagent (0.1% N-(1-naphthyl) ethylene diamine dihydrochloride, 1% sulphanilamide and 5% phosphoric acid)\textsuperscript{25}. Equal volumes of the supernatant and the Greiss reagent were mixed and the mixture was incubated for 10 min at room temperature (24°C) in the dark. The absorbance was measured at 540 nm using Perkin Elmer Lambda 20 spectrophotometer (Norwalk, CT, USA). The concentration of nitrite in the supernatant was determined from sodium nitrite standard curve.

**Estimation of reduced glutathione**— Reduced glutathione in the liver was estimated according to the method of Ellman\textsuperscript{26}. Homogenate (1 mL) was precipitated with 1.0 mL of 4% sulfosalicylic acid and the samples were immediately centrifuged at 1200xg for 15 min at 4°C. The assay mixture contained 0.1 mL of supernatant, 2.7 mL of phosphate buffer of pH 8.0 and 0.2 mL of 0.01 M dithiobistnitrobenzoic acid (DTNB). The yellow colour developed was read immediately at 412 nm using Perkin Elmer lambda 20 spectrophotometer (Norwalk, CT, USA). The results were expressed as micromoles of reduced glutathione per milligram of protein.

**Estimation of superoxide dismutase activity**— Superoxide dismutase (SOD) activity was assayed by the method of Kono\textsuperscript{27}. The assay system consists of EDTA 0.1 mM, sodium carbonate 50 mM, and 96 mM of nitro blue tetrazolium (NBT). In the cuvette, 2 mL of the above mixture, 0.05 mL of hydroxylamine, and 0.05 mL of the supernatant was added and autoxidation of hydroxylamine was measured for 2 min at 30 s interval by measuring absorbance at 560 nm using Perkin Elmer Lambda 20 spectrophotometer.

**Estimation of catalase**— Catalase activity was determined by the method of Luck\textsuperscript{28}, wherein breakdown of hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) is measured at 240 nm. Briefly, the assay mixture consisted of 3 mL of H\textsubscript{2}O\textsubscript{2}, phosphate buffer and 0.05 mL of supernatant of tissue homogenate (10%), and the change in absorbance was recorded at 240 nm using Perkin Elmer lambda 20 spectrophotometer (Norwalk, CT, USA). The results were expressed as micromoles of H\textsubscript{2}O\textsubscript{2} decomposed per milligram of protein/min.

**Estimation of TNF-α**— TNF-α activates several intracellular pathways to regulate inflammation, cell death, and proliferation. In the liver, TNF-α is not only a mediator of hepatotoxicity but also contributes to the restoration of functional liver mass by driving hepatocyte proliferation and liver regeneration\textsuperscript{29}. TNF-α was estimated using TNF-a kit (R&D systems). It is a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) using a microtiter plate reader at 450 nm. Concentrations (picogram per milliliter) of TNF-α were calculated from plotted standard curve. TNF-α levels were expressed as mean ± SEM.

**Statistical analyses**— One specific group of rats was assigned to one specific drug treatment condition and each group comprised six rats (n=6). All the values were expressed as means ± SEM. The data were analyzed by One Way ANOVA followed by Tukey’s test. \(P \leq 0.05\) was considered as statistically significant.

**Results**

**Histopathological evaluation**— Histopathology assessment of liver was performed for all groups. As shown in Fig. 1, CCl\textsubscript{4}-induced liver injury caused fatty degeneration of hepatic cells and vacuole formation in

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Fig. 1— Effect of test compounds on CCl\textsubscript{4}-induced hepatotoxicity in rats. (A) Naïve group; (B) Control group (animals treated with CCl\textsubscript{4} 1.5 ml/kg showed severe fatty degeneration and vacuole formation); (C) Silymarin group; and (D–G) CCl\textsubscript{4}-induced animals treated with test compounds MRG, MGG, MSG and MUG, respectively.
the central vein. Table 1 summarizes the data of liver damage induced by CCl₄ in histopathology. The level of vacuole formation and inflammation were significant after acute CCl₄ treatment as compared to naïve group. However, pretreatment with test compounds MRG (28.71 mg/kg), MGG (30.03 mg/kg), MSG (31.35 mg/kg) and MUG (33.62 mg/kg) significantly attenuated injury scores of vacuole formation and inflammation as compared to control.

**Effect of test compounds on CCl₄-induced changes in ALT and AST**—Serum aminotransferase activities have long been considered effective indicators of hepatic injury. ALT and AST are two biochemical markers generally used for early stage assessment of liver injury. Table 2 shows that CCl₄ had significantly raised serum ALT and AST level in rats’ liver as compared to naïve group indicating the incident of liver injury. However, pretreatment with test compounds MRG (28.71 mg/kg), MGG (30.03 mg/kg), MSG (31.35 mg/kg) and MUG (33.62 mg/kg) significantly reduced both ALT and AST levels compared to naïve group also confirming the CCl₄-induced liver injury (Table 2). Pretreatment with MGG (30.03 mg/kg), MSG (31.35 mg/kg) and MUG (33.62 mg/kg) significantly reduced the rise in TG and cholesterol as compared to control group (CCl₄ treated). The protective effects of the compounds on TG and cholesterol were comparable to that of the standard drug (silymarin50 mg/kg) in CCl₄ treated group.

**Effect of test compounds on CCl₄-induced changes in lipid peroxidation and nitrite levels**—Thiobarbituric acid-reactive substance (TBARS) and nitrite levels were increased significantly in the liver homogenate of CCl₄ treated rats as compared with naïve group. However, pretreatment with MRG (28.71 mg/kg), MGG (30.03 mg/kg), MSG (31.35 mg/kg) and MUG (33.62 mg/kg) significantly reduced both TBARS and nitrite levels as compared to control group (CCl₄ treated). The protective effects of the compounds on TBARS and nitrite levels were comparable to that of the standard drug (silymarin50 mg/kg) in CCl₄ treated group.

**Effect of test compounds on CCl₄-induced changes in the antioxidant profile**—Reduced glutathione (GSH) levels and enzymatic activities of superoxide dismutase (SOD) and catalase were decreased significantly in CCl₄ treated group. The protective effects of the compounds on TBARS and nitrite levels were comparable to that of the standard drug (silymarin50 mg/kg) in CCl₄ treated group.

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Fatty change (vacuole formation)</th>
<th>Inflammation</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve (Olive oil)</td>
<td>0.00±0.00b</td>
<td>0.00±0.00</td>
<td>-</td>
</tr>
<tr>
<td>Control (CCl₄ treated)</td>
<td>2.01±0.06</td>
<td>0.89±0.06</td>
<td>0.88±0.06</td>
</tr>
<tr>
<td>Silymarin (50)</td>
<td>0.06±0.06</td>
<td>0.14±0.06</td>
<td>0.08±0.08</td>
</tr>
<tr>
<td>MRG (28.71)</td>
<td>0.80±0.00</td>
<td>0.22±0.00</td>
<td>0.12±0.11</td>
</tr>
<tr>
<td>MGG (30.03)</td>
<td>1.08±0.00</td>
<td>0.35±0.00</td>
<td>0.29±0.00</td>
</tr>
<tr>
<td>MSG (31.35)</td>
<td>0.24±0.00</td>
<td>0.10±0.00</td>
<td>0.10±0.00</td>
</tr>
<tr>
<td>MUG (33.62)</td>
<td>1.12±0.00</td>
<td>0.38±0.00</td>
<td>0.32±0.00</td>
</tr>
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</table>

Values are expressed as mean ± SEM (n = 6)

<table>
<thead>
<tr>
<th>Treatment(mg/kg)</th>
<th>ALT (U/L) (%)</th>
<th>AST (U/L) (%)</th>
<th>TG (mmol/L) (%)</th>
<th>Cholesterol (mmol/L) (%)</th>
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</thead>
<tbody>
<tr>
<td>Naïve (Olive oil)</td>
<td>73 ± 1.8(100)</td>
<td>64.14 ± 2.0(100)</td>
<td>12.4 ± 1.5(100)</td>
<td>16.56 ± 1.3(100)</td>
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<tr>
<td>Control (CCl₄ treated)</td>
<td>240 ± 18.6e(328.8)</td>
<td>278.31 ± 22.8e(434)</td>
<td>34.31 ± 2.2e(276.7)</td>
<td>31.4 ± 1.4e(189.6)</td>
</tr>
<tr>
<td>Silymarin (50)</td>
<td>89 ± 11.9d(121.9)</td>
<td>98.43 ± 18.2d(153.5)</td>
<td>16.21 ± 1.4d(130.7)</td>
<td>18.32 ± 1.9d(110.6)</td>
</tr>
<tr>
<td>MRG (28.71)</td>
<td>137 ± 12.6b(187.7)</td>
<td>121.6 ± 15.7b(189.6)</td>
<td>23.19 ± 2.1b(187.0)</td>
<td>22.5 ± 1.6b(135.9)</td>
</tr>
<tr>
<td>MGG (30.03)</td>
<td>126 ± 11.3a(172.6)</td>
<td>112.1 ± 18.3a(174.8)</td>
<td>20.2 ± 1.9a(162.9)</td>
<td>20.8 ± 1.4a(125.6)</td>
</tr>
<tr>
<td>MSG (31.35)</td>
<td>96 ± 10.2a(131.6)</td>
<td>102.64 ± 16.9a(160)</td>
<td>17.6 ± 1.8a(141.9)</td>
<td>19.28 ± 1.6a(116.4)</td>
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<tr>
<td>MUG (33.62)</td>
<td>131 ± 14.3a(179.5)</td>
<td>118.3 ± 17.52a(184.4)</td>
<td>19.8 ± 1.9a(159.7)</td>
<td>21.2 ± 1.2a(128)</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM (n = 6)

*P < 0.05 as compared to naïve group; **P < 0.05 as compared to control group; and ***P < 0.05 as compared to silymarin group.
metabolites, namely trichloromethyl free radicals by hepatic microsomal CYP and produce toxic produce hepatic injury that involves biotransformation.

Discussion

Carbon tetrachloride (CCL₄) has been reported to produce hepatic injury that involves biotransformation by hepatic microsomal CYP and produce toxic metabolites, namely trichloromethyl free radicals. CYP2E1 is the major isoenzyme involved in bioactivation of CCL₄ and subsequent production of free radicals. Trichloromethyl free radicals initiate lipid peroxidation of the membrane of the endoplasmic reticulum and cause a chain reaction. In addition, injury to liver tissues alters their transport function and membrane permeability, leading to leakage of enzymes from the cells. Therefore, the marked release of serum markers such as AST, ALT, TG, and cholesterol into the circulation indicates severe damage to hepatic tissue membranes due to CCL₄ intoxication. It is further marked by an increase the level of TNF-α, a proinflammatory cytokine, which in turn can trigger other inflammatory chemokine, explicitly, NO.

In the present study, we used CCL₄-induced hepatotoxic model to determine whether administration of newly synthesized TMGA ester compounds can alleviate or reverse the CCL₄-induced hepatotoxicity in rats. In this study, rats that were exposed to CCL₄ exhibited significant fatty degeneration of hepatic cells and vacuole formation in the central vein as compared to control animals. Treatment with test compounds (MRG, MGG, MSG and MUG) and the standard drug silymarin significantly reduced the injury score of vacuole formation and inflammation as revealed by histopathological examination. CCL₄-induced raised ALT and AST levels were significantly restored by test compounds pretreatment. Further, these test compounds demonstrated a significant amelioration in elevated serum TG and cholesterol levels.

Oxidative stress plays an important role in hepatic damage. In aerobic cells, mitochondria are the major source of free radicals and these are also a sensitive target for free radical-mediated damage. An increase in free radical generation causes oxidative stress and is responsible for the hepatic injury. Therefore, inhibition of free radical damage with antioxidant supplementation has become an attractive therapeutic strategy for reducing the risk of liver disease. Gallic acid and other phenolic compounds have been reported to possess considerable antioxidant activity. To find out whether the hepatoprotective effects of conjugated esters is
mediated through its antioxidant action, CCl₄-induced mitochondrial oxidative stress was measured in liver cells in both the presence and absence of these test compounds. Treatment with CCl₄ increased peroxidation of mitochondrial lipids and depleted the mitochondrial total glutathione, superoxide dismutase and catalase contents (Table 3). Pretreatment with test compounds (MRG, MGG, MSG and MUG) at their experimental doses significantly prevented mitochondrial lipid peroxidation, depletion of total glutathione, superoxide dismutase and catalase contents which could be attributed to the radical scavenging antioxidant properties of the parent moieties of constituent esters. Moreover, the multipledoses of pretreatment of esters could have significantly boosted the antioxidant enzyme activities in CCl₄-induced hepatotoxic rats.

The results of the test compound MSG were comparable with silymarin and showed excellent protection against CCl₄-induced liver damage. However, except for lipid peroxidation and reduced glutathione activities in liver tissues, there was no significant difference between MSG and silymarin at their experimental doses. The results suggested that the inhibition of CCl₄-induced mitochondrial oxidative stress by test compounds could be due to the scavenging of free radicals, the formation of which is augmented by CCl₄-induced hepatic damage. Furthermore, several studies have also reported the role of antioxidant in protection against oxidative liver injury⁷,⁸,³³. 

TNF-α is one of the pro-inflammatory cytokines which are early mediators of tissue damage and repair. The test compounds significantly inhibited the increase in TNF-α production from Kupffer cells following CCl₄ injection, due to its anti-inflammatory activity. Nitric oxide (NO) is an inflammatory mediator and highly reactive oxidant produced by iNOS, which is released by Kupffer cells upon exposure to CCl₄²⁹,³³. In all test compounds treated groups, NO level was reverted to normal level. MSG was the most effective compound to revert the elevation of NO level after induction with CCl₄ followed by test compounds. Thus, by suppressing NO production in liver, compounds depicted their potential properties as hepatoprotective agent.

In conclusion, newly synthesized TMGA esters showed significant hepatoprotective effects against CCl₄-induced hepatotoxicity in rats and these hepatoprotective effects might be possibly linked with their antioxidant potential.

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