Protective effects of propolis on inorganic mercury induced oxidative stress in mice

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Protective potential of propolis was evaluated against mercury induced oxidative stress and antioxidant enzymatic alterations in mice liver. Exposure to mercuric chloride (HgCl₂; 5 mg/kg; ip) induced oxidative stress by increasing lipid peroxidation and oxidized glutathione level along with concomitant decrease in glutathione and various antioxidant enzymes. Mercury intoxication deviated the activity of liver marker enzymes in serum. Conjoint treatment of propolis (200 mg/kg; po) inhibited lipid peroxidation and oxidized glutathione level, whereas increased glutathione level. Activities of antioxidants enzymes i.e., superoxide dismutase, catalase, glutathione-S-transferase and glucose-6-phosphate dehydrogenase were also restored concomitantly towards control after propolis administration. Release of serum transaminases, alkaline phosphatase, lactate dehydrogenase and γ-glutamyl transpeptidase were significantly restored towards control after propolis treatment. Results suggest that propolis augments the antioxidants defense against mercury induced toxicity and provides evidence that it has therapeutic potential as hepatoprotective agent.

Keywords: Hepatoprotection, Lipid peroxidation, Liver, Mercuric chloride, Propolis

Propolis is a resinous material, collected by honey bees from plant exudates that is used for construction and repair of honey comb. It has pleasant aromatic odor and yellow-green to dark brown color depending on its source and age¹. In recent years, it has attracted much attention to researchers to explore its usefulness in the field of medicine and cosmetics² with the added advantage of lesser side effects³ as it true for many natural prescriptions⁴. It has a broad spectrum of biological activities against hepatitis⁵, arthritis⁶ and as hepatoprotective agent against galactosamine⁷, econazole⁸, tert-butyl hydroperoxide⁹, paracetamol¹⁰, ethanol¹¹ and carbon tetrachloride¹² induced toxicity. Synergism between propolis and antibiotics¹³, antibacterial agents¹⁴ and with chelators against light metal¹⁵ and heavy metal intoxication¹⁶ has also been proposed.

Mercury (Hg) is one of the highly toxic metals that are present in environment as pollutants. Its exposure occurs primarily via the food chain due to its accumulation in food stuffs¹⁷. Both inorganic and organic forms of mercury exert toxic effects on central nervous system, kidney and liver¹⁸ due to its interaction with many sulfur containing proteins¹⁹ and induction of oxidative stress²⁰. Endogenous antioxidant enzymes such as super oxide dismutase (SOD) and catalase (CAT) are involved in the protection against oxidative stress²¹,²². Induction of these antioxidant enzymes indicate an adaptive onset of the redox defense system, whereas inhibition is thought to contribute to oxidative stress following mercury intoxication²³. In the present study, an attempt has been made to evaluate protective potential of propolis against mercury induced hepatotoxicity by studying liver function tests, status of endogenous antioxidant defense system and hepatic histopathology to improve health status.

Materials and Methods

Animals and chemicals — Female Swiss albino mice (25±5 g body weight) were inbred and maintained in the institutional animal facility under standard husbandry conditions of light (14 hr) and dark (10 hr) at 25°C ± 2°C and 60-70% RH. Animals were fed dry pellets of standard animal diet (provided by the animal facility) and given drinking water ad libitum. The experimental protocols were approved

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and carried out according to the guidelines set by the institutional animal ethics committee.

Mercuric chloride (HgCl$_2$) and silymarin were purchased from Sigma-Aldrich company. Crude propolis used in this investigation was collected from the hive of *Apis mellifera* at Gwalior, MP, India. All the other chemicals used in the study were of highest purity and analytical reagents grade quality.

**Preparation and administration of doses** — Mercuric chloride was dissolved in triple distilled water (5 mg/kg) and administered to the animals intraperitoneally (ip) once daily for three days. Aqueous suspension of propolis (200 mg/kg) and silymarin (50 mg/kg) were prepared in 1% gum acacia suspension (GAS) and administered orally (po) for three days. The selection of the respective doses of propolis and toxicant was made on the basis of our previous studies\(^{24-27}\) and other published reports\(^{28}\). Silymarin was given as positive control in respect to propolis.

**Experimental design** — Animals were divided into 5 groups of 6 each and experiment was conducted according to Scheme 1.

**Assessment of blood biochemistry** — Blood was drawn from the animals by puncturing retro-orbital venous sinus, serum was isolated and used to determine the leakage of aspartate aminotransferase (AST)\(^{29}\), alanine aminotransferase (ALT)\(^{29}\), lactate dehydrogenase (LDH)\(^{30}\) and serum alkaline phosphatase (SALP)\(^{31}\). The γ-glutamyl transpeptidase (γ-GT) was measured using Merck’s kit as per the manufacturer’s instructions.

**Assessment of oxidative stress and antioxidant status** — Lipid peroxidation (LPO) was measured by estimation of thiobarbituric acid reactive substances (TBARS) as described by Sharma and Krishna Murti\(^{32}\). Hepatic reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured by the method of Roberts and Francetic\(^{33}\). Total protein was estimated by the method of Lowry *et al.*\(^{34}\) using bovine serum albumin as standard.

**Assessment of antioxidant enzymes** — Total glutathione-S-transferase (GST) activity was measured according to the method of Habig *et al.*\(^{35}\). Total SOD activity was estimated by measuring inhibition of autooxidation of epinephrine\(^{36}\) and CAT activity was estimated as per Aebi\(^{37}\). Activity of glucose-6-phosphate dehydrogenase (G6PDH) was measured by the method of Askar *et al.*\(^{38}\).

**Statistical analysis** — Results were expressed as mean ± SE of 6 animals. The data were subjected to statistical analysis through one-way analysis of variance (ANOVA) and statistical significance was set a priori at \( P \leq 0.05 \). Next, the data were also subjected to a Student’s *t*-test\(^{39}\) with a statistical significance set a priori at \( P \leq 0.01 \) and \( P \leq 0.05 \).

**Results**

**Assessment of blood biochemistry** — Table 1 represents the effect of propolis extract on mercury

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatments</th>
<th>Day 1-3 (0700 hrs)</th>
<th>Day 1-3 (1000 hrs)</th>
<th>Day 4 (1000 hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Control</td>
<td>Saline (5 ml/kg; ip)</td>
<td>GAS (5 ml/kg; po)</td>
<td>Euthanized</td>
</tr>
<tr>
<td>Group 2</td>
<td>Propolis <em>per se</em></td>
<td>Saline (5 ml/kg; ip)</td>
<td>Propolis (200 mg/kg; po)</td>
<td>Euthanized</td>
</tr>
<tr>
<td>Group 3</td>
<td>Exp. control</td>
<td>HgCl$_2$(5 mg/kg; ip)</td>
<td>GAS (5 ml/kg; po)</td>
<td>Euthanized</td>
</tr>
<tr>
<td>Group 4</td>
<td>Propolis</td>
<td>HgCl$_2$(5 mg/kg; ip)</td>
<td>Propolis (200 mg/kg; po)</td>
<td>Euthanized</td>
</tr>
<tr>
<td>Group 5</td>
<td>Silymarin</td>
<td>HgCl$_2$(5 mg/kg; ip)</td>
<td>Silymarin (50 mg/kg; po)</td>
<td>Euthanized</td>
</tr>
</tbody>
</table>

Animals of all the groups were euthanized after 24 hr of the last treatment.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>AST (IU/L)</th>
<th>ALT (IU/L)</th>
<th>SALP (mg Pi/100ml/hr)</th>
<th>LDH (μ mole py/min/L)</th>
<th>γ-GT (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>68±3.78</td>
<td>48.1±2.65</td>
<td>210±11.6</td>
<td>40.4±2.23</td>
<td>1.10±0.06</td>
</tr>
<tr>
<td>Propolis <em>per se</em></td>
<td>66±3.65</td>
<td>46.7±2.58</td>
<td>212±11.7</td>
<td>39.9±2.20</td>
<td>1.20±0.06</td>
</tr>
<tr>
<td>Mercury</td>
<td>188±10.3*</td>
<td>121±6.58*</td>
<td>920±50.8*</td>
<td>335±18.5*</td>
<td>9.40±0.51*</td>
</tr>
<tr>
<td>Mercury+propolis</td>
<td>109±6.02**</td>
<td>76.6±4.22**</td>
<td>383±21.1**</td>
<td>119±5.77**</td>
<td>4.70±0.25**</td>
</tr>
<tr>
<td>Mercury+silymarin</td>
<td>105±5.80**</td>
<td>71.8±3.96**</td>
<td>371±20.5**</td>
<td>112±6.19**</td>
<td>4.50±0.24**</td>
</tr>
<tr>
<td>F variance at 5 % level</td>
<td>70.9γ</td>
<td>59.1γ</td>
<td>137.2γ</td>
<td>202.3γ</td>
<td>169.2γ</td>
</tr>
</tbody>
</table>

*Significant Hg vs control at \( P \leq 0.01 \); **Significant treatments vs Hg at \( P \leq 0.01 \) for student’s *t*-test.

*Significant for ANOVA at \( P \leq 0.05 \).
induced serum biochemical alterations. Leakage of AST, ALT, SALP, γ-GT and LDH was significantly increased in circulation after mercury intoxication ($P \leq 0.01$). Conjoint treatment of propolis significantly reduced the leakage of all these enzymes and maintained their level towards control ($P \leq 0.01$). More than 70% protection was noticed in all the parameters except γ-GT, which showed more than 60% protection in its release.

Assessment of oxidative stress and antioxidant status — Protective effect of propolis on mercury induced oxidative stress and antioxidant status are shown in Fig. 1 (a-d). Hepatic LPO was significantly enhanced after mercury administration ($P \leq 0.01$). Propolis and silymarin therapy significantly inhibited LPO ($P \leq 0.01$) thus, reduced hepatic peroxidative stress more than 80%. Acute administration to mercury caused significant decrease in hepatic GSH contents and increased GSSG contents ($P \leq 0.01$). Conjoint therapy of propolis improved GSH level towards control and hampered GSSG production ($P \leq 0.01$)

Assessment of antioxidant enzymes — Mercury administration diminished hepatic antioxidant enzymes by decreasing the activities of GST, G6PDH, SOD and CAT in a significant manner ($P \leq 0.01$) (Fig. 2, e-h). Treatment of propolis and silymarin reversed the activities of all these enzymes towards control ($P \leq 0.01$). More than 50% protection was found in SOD, whereas over 60% improvement was noticed in the activities of CAT, GST and G6PDH with propolis treatment.

Discussion

Increase in LPO and GSSG with concomitant decrease in GSH level and antioxidant enzymes was a clear indication of oxidative stress produced by acute exposure to mercury. In fact, therapy with propolis extract and silymarin seemed to afford protection against this noxious stimulus. Because of high affinity of mercury to thiol groups, it affects living organisms by damaging thiol proteins and enzymes. The LPO is an autocatalytic free-radical process whereby polyunsaturated fatty acids in cell membranes undergo degradation by a chain reaction to yield lipid hydroperoxides, which subsequently decompose to form a variety of toxic products, including malondialdehyde.

Induction of LPO by mercury suggests that cell membrane permeability may be affected by this process$^{40,41}$. Administration of propolis protected the liver markedly against mercury induced toxicity by diminishing LPO. Flavonoids and phenolics provide protection as good antioxidants against LPO induced pathogenesis$^{42}$ and also act as effective chelators for several toxic metal ions$^{43}$. Antioxidants have a protective effect against tissue injuries in the pathogenesis of which LPO may be involved. Quercetin, a major component of propolis, is well known to inhibit LPO by scavenging free radicals and/or transition metal ions$^{44}$.
Mercury administration depleted GSH content and increased GSSG in liver that made hepatocytes more susceptible to oxidative damage, especially during increased free radical production. GSH is an important intracellular antioxidant that spontaneously neutralizes several electrophiles and reactive oxygen species\(^{45}\), whereas GSH/GSSG ratio maintains the redox status of the cell\(^{46}\). Concomitant restoration of GSH pool after propolis treatment is consistent with other reports\(^{15}\). GSH is a substrate of enzyme GST, a phase II enzyme that plays a key role in cellular detoxification of xenobiotics, electrophiles and reactive oxygen species through their conjugation to GSH\(^{47}\). In the present study, reduction in GST activity after mercury exposure may hamper the conjugation reaction that consequently produced toxic effects. GSSG is reduced back to GSH by NADPH dependent reaction. G6PDH, the first enzyme of pentose phosphate pathway, is an important site for NADPH production. Reduction in the activity of G6PDH enzyme after mercury intoxication may affect the rate of generation of NADPH and thus, affect the redox status of the cell. Propolis therapy maintained the normal status of GST and G6PDH activities, therefore, improved the antioxidant status of the hepatocytes in terms of increased GSH and decreased GSSG contents. Propolis prevents hepatic disorders induced by APAP\(^{10}\) and CCl\(_4\)\(^{12,24}\) as well as toxic metal induced-lipid peroxidation in liver\(^{15}\) due to the presence of flavonoids and phenolics present in it.

Mercury ion perturbs mitochondrial inner membrane function that results in depletion of mitochondrial reduced glutathione content and increased formation of H\(_2\)O\(_2\) by the mitochondrial electron transport chain\(^{48}\). Increased H\(_2\)O\(_2\) formation is accompanied by increased peroxidation of mitochondrial lipids, consistent with an oxidative stress-like condition\(^{23}\). Cells have a number of mechanisms to protect themselves from the toxic effect of reactive oxygen species. The SOD removes super oxides (O\(_2^\cdot\)) by converting it into H\(_2\)O\(_2\), which is rapidly converted to water by CAT. Therefore, any alteration in the activity of these two enzymes may result in a number of deleterious effects due to accumulation of superoxide radicals and hydrogen peroxide. In the present study, propolis extract significantly restored the SOD and CAT activity towards normal, which indicated that propolis could scavenge reactive free radicals that eventually lessen the oxidative tissue damage and subsequently improved the activities of these antioxidant enzymes. It can be proposed that the beneficial effects of pharmacological dose of propolis may be due to its antioxidant properties that could revive cellular endogenous antioxidant defense system.

Mercury intoxication produced significant hepatic damage as evidenced by substantial increase in the leakage of AST, ALT, SALP, LDH and γ-GT into the circulation due to hepatocellular necrosis or alteration in permeability of cell membrane\(^{49}\). Reduction of these endpoints in serum after propolis administration was a clear indication of stabilization of plasma membranes as well as repair of hepatic tissue damage caused by mercury. This effect is in agreement with the commonly accepted view that serum level of transaminases would return to normal after the healing of hepatic parenchyma and regeneration of hepatocytes.

So far, therapeutic potential of propolis has been evaluated against several environmental toxicants\(^{10,12,15,16}\). Presence of more than 300 compounds, particularly flavonoids, phenolics and their esters in particular are responsible for several biological activities of propolis\(^{50}\). It can be postulated that several bioactive compounds present in it may protect oxidative damage by directly neutralizing reactive oxidants, increase the capacity of endogenous antioxidant defense and modulate the cellular redox state\(^{51}\). This may be due to the favorable capacity of propolis to pass through the membrane and to accumulate in both hydrophilic and hydrophobic environments for protecting cells against oxidative stress and scavenging free radicals\(^{52}\). Thus, it can be concluded that propolis has a strong potential to provide protection against mercury induced oxidative stress in liver.

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