PROTECTIVE EFFECT OF ETHANOLIC EXTRACT OF PROPOLIS ON ISONIAZID INDUCED HEPATOTOXICITY IN MALE ALBINO MICE

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ABSTRACT

Background: Different herbal extracts and drugs had been tried to combat the isoniazid induced hepatotoxicity; there is no study before using propolis for abating isoniazid induced liver toxicity.

Objective: The purpose of this study was to assess protective effect of ethanolic extract of propolis (EEP) in isoniazid induced hepatotoxicity in male albino mice.

Methods: Forty five male albino mice were divided into five groups, i.e. group A served as control, groups B, C, D and E were experimental. Group B was treated with isoniazid 100 mg/kg for 30 days. Groups C, D and E were pretreated with EEP 150 mg/kg for 10, 20 and 30 days respectively, followed by treatment with isoniazid for another 30 days. Blood samples were obtained on 30th day in groups A and B and on 40th, 50th and 60th days respectively in groups C, D and E for assessing liver enzymes and total bilirubin. Serial sections of liver 4µm thick were stained with H & E, PAS and PASD for microscopic examination.

Results: Group B showed an increase in serum ALT, AST, ALP and total bilirubin levels; the general architecture of liver was deranged; necrosis, apoptosis, pyknosis, vacuoles and periportal inflammation were also observed. A decrease in the level of liver enzymes was observed in mice of groups C, D and E. Preparations from groups C and D showed some evidence of recovery, whereas in group E significant recovery was observed.

Conclusion: Pretreatment with EEP (150 mg/kg) showed time related hepatoprotection against INH induced hepatotoxicity in male albino mice.

Key words: Isoniazid, ALT, AST, ALP, Bilirubin, Propolis.

INTRODUCTION

Mycobacterium tuberculosis (TB) is one of the major causes of morbidity and mortality among infectious diseases, which primarily affects lungs and secondarily other organs of the body. Standard treatment of tuberculosis consists of a six to nine months course of antibiotics named isoniazid (INH), pyrazinamide (PZA), rifampicin (RIF), and ethambutol or streptomycin. These antitubercular drugs are also hepatotoxic; the rate of their hepatotoxicity is much higher in developing countries such as India and China (8 – 28%), compared to that in advanced countries (2 - 3%), even when the same dose schedule is used. Other common side effects of anti-tubercular drugs are skin allergic reactions, gastrointestinal and neurological disorders. Among these side effects, hepatotoxicity is the one regarded as serious and is the focus of the present study; 2 – 3% cases of jaundice, 7 – 8% of other side effects reported in hospitals, and about 30% of sudden and severe liver failure are caused by drugs induced liver injury. Isoniazid is acetylated by the liver enzyme N-acetyltransferase 2 (NAT2) and then hydrolyzed, resulting in formation of isonicotinic acid and acetylated hydrazine; the later compound is activated by cytochrome P-450. The metabolic oxidation of acetylated hydrazine leads to reactive acetylated species diacetylhydrazine. Acetyl hydrazine and diacetylhydrazine bind with liver cell macromolecules, causing liver damage. These toxic metabolites produce many histological changes such as hepatocyte vacuolar degeneration and necrosis. Diffuse micro vesicular fatty change with mild portal triaditis and necrosis was also reported. It was reported in another study that oxidative stress produced by antitubercular drugs caused hepatic injury. Human genetic researches have revealed that cytochrome P450 2E1 (CYP2E1) is involved in isoniazid induced hepatotoxicity. An elevated CYP2E1 activity may lead to a higher production of hepatotoxins. Experimental studies showed that isoniazid and hydrazine stimulate CYP2E1 activity. Experimental studies on animals suggest that isoniazid administration in toxic doses distress hepatocellular membrane integrity, producing an increase in alanine transaminase (ALT), aspartate transaminase...
(AST), alkaline phosphatase (ALP) and total bilirubin in serum values. The regression of isoniazid hepatotoxicity generally takes weeks, after discontinuation of isoniazid.

The word ‘pro-polis’ is derivative of Greek words ‘pro’-, for, and ‘polis’- the city, i.e., protection of the hive. It consists of strongly adhesive sticky gum, collected, converted and used by bees to close up cracks in their hives. Bees (Apis mellifera L.) collect a sequence of gums, resins and balms of viscous consistency from flower buds and tree barks.

Propolis consists of 50 – 70% resins and 10 – 15% essential oils mixed with 30 – 50% wax for proper uniformity and 5 – 10% pollen. Propolis also contains vitamins, folic acid, nicotinic acid and many minerals, including calcium, magnesium, manganese, vanadium, iron, copper, strontium, aluminum and silicon. Propolis is antibacterial, antiviral, antiprotozoan and antifungal in its properties. Propolis possesses curative and preventive effects against inflammation, cardiac disease, diabetes mellitus and cancer. Hippocrates, the well – known Greek general practitioner prescribed propolis externally, to treat sores, wounds, and for resolution of indurations and ulcers.

Although protective effect of different herbs and drugs on hepatotoxicity had been studied, further, it has been reported that treatment with propolis showed a dose dependent protective effect on alcohol, carbon tetrachloride (CCI₄) and paracetamol induced hepatotoxicity, there is hardly any work to show the effect of EEP on isoniazid induced hepatotoxicity. Consequently the present study was undertaken to see the effect of propolis on isoniazid induced hepatotoxicity.

METHODS

It was a randomized control trial study. Simple random sampling technique using lottery method was used. Forty five healthy adult male albino mice, 6-8 weeks old, weighing 30g ± 5g were obtained from the colony raised at Veterinary Research Institute (VRI), Lahore; these were divided into five groups A, B, C, D and E, each having nine mice. Each group was housed individually in an stainless steel cage with wooden shavings at the floor in the experimental research laboratory of University of Health Sciences, Lahore. The animals were kept at controlled room temperature (23 ± 2°C), humidity (50 ± 5%) and, light and dark cycle of 12 hours each. Each group was housed in an individual stainless steel cage with wooden shavings at the floor in the experimental research laboratory of University of Health Sciences, Lahore. The animals were kept at controlled room temperature (23 ± 2°C), humidity (50 ± 5%) and, light and dark cycle of 12 hours each. The animals were fed on standard mouse diet and water ad libitum. The body weight of animals was recorded in the beginning, regularly every week and at the end. The experiment started after a period of 1 week of acclimatization.

Group A served as control; each mouse was given 10 ml/kg of normal saline (0.9%) orally for 30 days; in Group B, mice were given isoniazid 100 mg/kg daily, dissolved in 10 ml distilled water, orally for 30 days; the animals were sacrificed the next day after the experimental period. Mice belonging to Groups C, D and E were given ethanolic extract of propolis150 mg/kg daily dissolved in 10 ml normal saline, orally for first 10, 20 and 30 days respectively and then isoniazid 100 mg/kg dissolved in 10 ml distilled water, orally for next 30 days; these were sacrificed on 40, 50 and 60 days respectively.

Crude Propolis was obtained from the hive of Apis mellifera. I. from the University of Agriculture, Faisalabad. Ethanolic extract of propolis was prepared at PC-SIR (Pakistan Council of Scientific and Industrial Research) laboratory, Lahore, was standardized by keeping it in ultraviolet light for 24 hours and stored in amber colored bottles at 4°C. Ethanolic extract of propolis was given orally daily at a dose of 150 mg/kg body weight dissolved in 10 ml of 0.9% normal saline.

Isoniazid was obtained in powdered form, from Sigma Chemicals Company (St. Louis, MO, USA). Dose of isoniazid was based on previous studies in which isoniazid was given at 100 mg/kg/day, orally for 30 days, dissolved in 10 ml distilled water, using mouse as experimental models, respectively.

The animals were sacrificed under anesthesia and 2 – 3 ml of blood was taken using 3 ml disposable syringe, by cardiac puncture on 30 th day in groups A and B, 40th, 50th and 60th day in groups C, D and E respectively. It was collected in sterile vacuotainers with gel. The blood was allowed to stand for 1 hour, centrifuged at a speed of 3000 rpm for 10 minutes, using bench top centrifuge. The serum was collected with the help of sterilized dropper and stored in sterile appendorf tubes and kept at -20°C until used for the biochemical estimations.

Kits for ALT (Alanine aminotransferase), AST (Aspartate aminotransferase) and total bilirubin were obtained from Randox Laboratories Ltd. United Kingdom and kit for ALP (Alkaline phosphatase) was obtained from Human Company, Germany.

The liver of the mouse was removed soon after sacrificing the animal and 3 – 5 mm thick pieces were excised after gross examinations, fixed in formalin for 48 hours and processed to prepare paraffin blocks. Sections 4µm thick were obtained and stained with Haematoxylin and eosin (H & E), Periodic acid Schiff’s (PAS) and Periodic acid Schiff’s diastase (PASD) stains.

Statistical Analysis

The data of the groups was entered into and analyzed by computer software SPSS (Statistical Package for Social Sciences) version 18.0. Mean (± S.E) was given for quantitative variables like number of cells, body weight, ALT, AST etc. One way ANOVA (Analysis of Variance) was applied to compare the mean body weight, AST, ALT, etc. among study groups. Sphapiro–Wilk test was applied to check normality. Post Hoc Tukey test was applied to observe mean difference
Table 1: Mean values of serum biochemical parameters of liver in U/L/mg/dl among groups.

<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>Group A Mean (± S.E)</th>
<th>Group B Mean (± S.E)</th>
<th>Group C Mean (± S.E)</th>
<th>Group D Mean (± S.E)</th>
<th>Group E Mean (± S.E)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean value of serum ALT in U/L</td>
<td>61.22 ± 3.22</td>
<td>153.00 ± 14.079</td>
<td>96.88 ± 10.842</td>
<td>75.44 ± 4.167</td>
<td>59.88 ± 2.750</td>
<td>0.001*</td>
</tr>
<tr>
<td>Mean value of serum AST in U/L</td>
<td>71.33 ± 4.99</td>
<td>190.22 ± 14.61</td>
<td>82.66 ± 8.42</td>
<td>67.44 ± 5.177</td>
<td>58.22 ± 3.90</td>
<td>0.001*</td>
</tr>
<tr>
<td>Mean value of serum ALP in U/L</td>
<td>97.55 ± 6.664</td>
<td>187.77 ± 19.73</td>
<td>96.22 ± 7.15</td>
<td>95.44 ± 7.02</td>
<td>89.00 ± 3.24</td>
<td>0.001*</td>
</tr>
<tr>
<td>Mean value of serum T. Bil. in mg/dl</td>
<td>0.457 ± 0.074</td>
<td>2.166 ± 0.265</td>
<td>1.11 ± 0.193</td>
<td>0.744 ± 0.182</td>
<td>0.566 ± 0.076</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

*p value ≤ 0.05 is statistically significant.
S.E, Standard error of mean; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; ALP, Alkaline phosphatase; T.Bil, Total bilirubin.

among groups. Fisher’s exact test was applied to observe associations between qualitative variables. P value ≤ 0.05 was considered as statistically significant.

**RESULTS**

Liver functions were assessed biochemically using enzymatic markers ALT, AST, ALP and total serum bilirubin; their values were raised significantly (p value < 0.001) in group B, confirming isoniazid as hepatotoxic drug; the values of the test came down significantly by EEP in groups C, D and E, showing its hepatoprotective effect (Table 1).

In the present study, all animals were active and healthy; however, those of group B showed a slight degree of sluggishness. The difference in the body weight of mice was statistically insignificant at the start of experiment (p value 0.207). The gain in body weights was less in animals of group B when compared to other groups at the end of the experimental period and the difference was statistically significant. A statistically significant reduction in weight and volume of liver was observed in isoniazid treated group B when compared to group A (p value < 0.039 and 0.026 respectively). Upon administration of ethanolic extract of propolis, there was a statistically significant increase in the liver weight and volume in groups C, D and E when compared to group B. There was statistically insignificant difference in liver weight and volume when groups C, D, and E were compared with group A (p value > 0.05) at the end of experimental period.

Group A showed normal liver histology i.e. dark brown color of liver having smooth surface. Microscopically each hepatic lobule had a vein in its centre and portal triads at its periphery. The hepatocytes were arranged in radiating cords proceeding to the periphery from the central vein, having sinusoids in between, lined by discontinuous endothelial cells with flattened nuclei; among them were kupffer cells with rounded prominent nuclei. The hepatic sinusoids were seen to be anastomosing with each other and opening into the lumen of central vein, lined by flattened endothelial cells. Hepatocytes were polyhedral in form, each with a vesicular nucleus and 1 – 2 nucleoli. Some nuclei were pyknotic and binucleated. Dilatation of sinusoids and central veins, filled with RBCs was an evidence of vascular congestion. Portal area comprised a branch each of the portal vein, hepatic artery and bile duct. Branch of portal vein had wide lumen, lined by endothelial cells and contained erythrocytes; that of the hepatic artery had narrow lumen and thick wall as compared to the portal vein; the bile duct had, however, a lining of low cuboidal epithelium. There was no...
Fig. 2: Photomicrograph of liver section from group B, showing totally deranged general architecture with deformed hepatocytes (H) having vacuoles (V) in their cytoplasm. Centrilobular necrosis (Ne) was evident by vacuolar degeneration and fatty metaplasia around central vein (CV), filled with blood (B), indicative of vascular congestion. H & E stain X 400.

Fig. 3: Photomicrograph showing group C with general architecture restored near to normal. Many cells are binucleated (BN). Sinusoids (S), Kupffer cells (K), Blood (B) in the sinusoids. Few pyknotic nuclei (PK) and lymphocytes (L) were present. H&E stain X 400.

Fig. 4: Photomicrograph of liver section from group D, showing hepatocytes (H), rounded to polyhedral in shape having nucleus (N) in their centre. Many binucleated (BN) with their prominent nucleoli (PNu), Kupffer cells (K) in sinusoidal spaces (S). H&E stain X 400.

Fig. 5: Photomicrograph of liver section from group E, showing near to normal structure sinusoidal spaces (S), Kupffer cells (K), Hepatocytes (H), rounded or polyhedral in shape having single nucleus (N), many binucleated (BN) with prominent nucleoli (PNu) are present, indicative of regeneration. H&E stain X 400.

evidence of perportal inflammation. In group B, the histopathological studies of liver showed loss of general architecture of liver, ballooning degeneration of hepatocytes, focal areas of hepatocyte necrosis, pyknosis, apoptosis, vacuolar degeneration, vascular congestion with perportal inflammation (Fig. 2), all supported by biochemical analysis, which showed rise in animals of groups C, D and E which were pretreated with EEP for 10, 20 and 30 days respectively and were subsequently given INH for 30 days each, histological examination of the liver and biochemical parameters gradually improved and near normal values were obtained in group E (Fig. 3, 4 and 5). Group C mice were given EEP (150 mg/kg) for 10 days and INH for next 30 days, showed some evidence of recovery and regeneration in some hepatocytes with some pyknotic nuclei and lymphocytes (Fig. 3). Group D mice were given EEP (150 mg/kg) for 20 days and INH for next 30 days showed evidence of greater regeneration than in group C (Fig. 4). Group E mice were given EEP (150 mg/kg) for 30 days and INH for next 30 days showed marked evidence of regeneration (Fig. 5).
Liver injury by isoniazid and its protection with propolis was assessed by examining liver histologically. Statistical analysis of the findings by ANOVA and Post Hoc Tukey test showed increase in size of hepatocytes, their nuclei, size of central vein and number of kuffer cells in group B, and the difference when compared with group A was statistically significant (p value < 0.001); however, size of hepatocytes in groups C, D and size of their nuclei in C, D and E decreased and was comparable to those of group A. Size of central vein and number of kuffer cells in groups C, D and E decreased but were not comparable to group A. This means that in groups C, D and E, the toxic effects of INH were reduced progressively depending upon the length of treatment with EEP; the enzyme levels and the histological picture of the liver structure came to nearly normal level of the control in group E where EEP was given for 30 days before treating the animals with INH. Evidences of regeneration were observed in the form of multinucleated hepatocytes with prominent nucleoli and increased cytoplasmic eosinophilia of the cells. Statistical analysis by ANOVA and Fisher’s exact test showed highly significant differences between values of the control group and EEP treated groups when compared to isoniazid treated group (p value < 0.001). This is clearly indicative of protective effect of ethanolic extract of propolis in isoniazid induced hepatotoxicity.

DISCUSSION

In the current study, we found that the ethanolic extract of propolis prevented the reduction in body weights in groups C, D and E upon treatment with isoniazid. It had been reported earlier that propolis extract prevented experimentally induced diabetic nephropathy in rats; further, oral administration of propolis extract in doses of 100, 200 and 300 mg/kg/day significantly increased the body weight of rats which was reduced on account of oxidative stress produced by diabetes. Propolis has a strong antioxidant and free radical scavenging effect.

In present investigations, there was a statistically significant reduction in relative weight and volume of liver with isoniazid treatment in mice of group B as compared to the mice in group A Treatment with ethanolic extract of propolis increased the liver weight and volume in groups C and D; the increase was statistically significant when compared to group B (p value < 0.039 and 0.027). Our results were not in accord with previous studies in which supplementation of various antioxidants i.e. garlic and carotenoids were reported to prevent the isoniazid induced hepatotoxicity, and there was no effect on the liver weight of experimental animals.

Hepatic injury is indicated by release of intracellular enzymes into circulation, such as AST, ALT, ALP and LDH. Their estimations are useful quantitative marker for measuring the extent of hepatocellular damage by toxic substances. In the current investigations it was observed that in group B, the serum levels of diagnostic liver enzymes ALT, AST, ALP and total bilirubin, were significantly increased as compared to group A (p < 0.001); this was positively indicative of damage to the plasmalemma of hepatocytes by isoniazid, resulting in leakage of the enzymes. Our observations are in accord with those reported earlier that CCl₄ is hepatotoxic, as was evident by increased levels of hepatic enzymes in rat model of the experimental work.

In the current study, pretreatment with ethanolic extract of propolis is considered to be responsible for keeping the enzymes near to normal values. Similar results were reported earlier where propolis pretreatment was reported to decrease the elevation of serum transaminases produced by paracetamol (acetaminophen), CCl₄ and abuse of alcohol.

In the current study, hepatocytes and their nuclei in group B were observed to increase in size and so was the case with hepatocytes. Similar observation was reported earlier in which isoniazid treatment in rodents produced fat vacuoles and inflammatory changes in liver, further, vascular dilatation was also seen in isoniazid treated group.

In the current study pretreatment of groups C, D and E with EEP maintained the size of hepatocytes and nuclei to near normal and comparable to the control group. Further, the changes in vascular size seen upon isoniazid treatment were nearly comparable to the control group in EEP pretreated groups C, D and E, although the changes were statistically insignificant. Comparable results had been reported earlier when the central vein and portal triad congestion was reduced to normal on administering propolis in the experimental model of rat wherein hepatotoxicity was induced by CCl₄.

Observation in the current investigation that the general architecture of the liver nearly remained normal in groups C, D and E which were pretreated with EEP. These results are comparable to earlier reports in which treatment of rats with CCl₄ caused degeneration and disintegration of liver architecture; in this study hepatic lesions were characterized by massive hepatic necrosis and large vacuolation; it was also reported earlier that pretreatment with propolis extract showed restoration of liver to normal lobular pattern with well form polygonal hepatocytes having conspicuous nuclei in experimental modules, using rats. It was seen in the current investigation that the number of Kupffer cells which increased in group B and these were restored to nearly control level in groups D and E treated with EEP for 20 and 30 days. These results are in accord with previous studies in which it was postulated that depletion of Kupffer cells protect the liver injury from alkyating agent, for example melphelan and the industrial chemical, thioacetamide.
cells activators, however, are reported to markedly enhance hepatotoxicity induced by acetaminophen, alcohol and halobenzenes.21

In the current investigations, INH in group B caused statistically significant degree of necrosis, pyknosis and perportal inflammation when compared with the control (p-value < 0.001); these were indicative of degenerative changes and toxic effects of INH on liver. Pyknosis is the irreversible condensation of chromatin of the nucleus leading onto apoptosis; these nuclei appear as dark and condensed chromatin material.

Evidences of regeneration were present in propolis treated groups. Multinucleated hepatocytes (mostly binucleated), with prominent nuclei and increased cytoplasmic eosinophilia are regarded as the evidence of regeneration. In current study, in groups C, D and E there were many binucleated hepatocytes present which corroborated with the early reported work in which pretreatment with propolis extract for 5 days after CCl₄ administration for three weeks showed binucleated hepatocytes with no evidence of neutrophilic infiltration and fatty changes. These changes are indicative of hepatoprotective effect.

Hydrazine is the toxic metabolite of isoniazid, which is metabolized and subsequently detoxified by cytochrome P-450 system in liver. Hepatotoxicity could be due to any disturbance in cytochrome P-450 system or in detoxification pathway.21,22 It was reported that animals receiving isoniazid showed perportal inflammation, fatty changes, liver cell necrosis, ballooning degeneration, vascular congestion, pyknosis and apoptosis; these histopathological changes were reversed by various drugs and herbs such as cimetidine, Pisonia Aculeate (Nyctaginaceae), Jetepar (Glucometamine, glucodiamine, nicotinamide ascorbate), Embelia Tsjeriam Cottam fruit, Vitex negundo (five leaved chaste tree) leaf extract and aqueous extract of Azadirachta indica (Neem leaves) respectively.5,18,23-26 INH produces oxidative stress and free radicals which are quenched by these antioxidants. Similarly, it is proposed that propolis, being an antioxidant, prevented isoniazid induced hepatic injury.

Further, trials are necessary regarding dose, route of administration, formulation and duration to assess its full benefits.

It is Concluded that the present study is the first in which ethanolic extract of propolis was studied against isoniazid induced hepatotoxicity. The investigations provide an efficient, cheap and affordable hepatoprotective natural protection for INH produce hepatotoxicity.

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