Screening for the protective efficacy of two different extracts of Pithecellobium dulce in CCl₄ and Isoniazid induced hepatic injury in wistar rats.

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Abstract: Hepatoprotective effect of ethanolic and hexane:benzene:acetone (1:1:1) extracts of Pithecellobium dulce (EEPD and HBAPD respectively) in carbon tetra chloride (CCl₄) and Isoniazid (INH) induced toxicity in wistar rats was evaluated. Two parallel sets of experiments were performed. Hepatotoxicity was induced by either single dose of CCl₄ or regular doses of Isoniazid (100 mg/kg b.w., i.p.). In both experiments standard drug (100 mg/kg, p.o.) and test extracts (500 mg/kg, p.o. for both) were given for ten days. Serum liver function enzymes were estimated spectrophotometrically. Both CCl₄ and INH treated animals showed significant increased values of serum alkaline phosphatase, serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase and Tbil (P<0.001 for all). However, these were found to be restored in one or both test extracts. The decreased values of these parameters were seemed to be less or more equivalent to standard drug. Present findings suggest that the PD extracts can ameliorate hepatic injury. However, different extracts showed similar protective activities against parameter used, in both experiments. Further investigations are needed to reveal the mode of action of the same.

Keywords: CCl₄, hepatoprotective, Silymarin, Pithecellobium dulce, total bilirubin, Isoniazid

I. Introduction

Liver diseases are caused by toxins, drugs, microbial infections, foreign particulars etc. Hepatic tissues are major site of that participate in almost all the biochemical pathways of growth, defence, nutrient supply and storage, energy provision, detoxification and drug metabolism [2,3]. Therefore, abnormality in its functioning directly affects the overall health [4]. Because hepatic cells are core site of detoxification and drug metabolism, it routinely exposed by certain toxin/drug/ foreign agents [3-5].

Most of the hepatotoxins cause tissue damage via generation of excessive free radicals [4]. Carbon tetrachloride (CCl₄) is one of the known pro-oxidative compounds which transformed into a trichloromethyl peroxyl radical (+OOCCl₃) in tissues [8] and causes harms to tissue lipids and proteins [9]. On the other hand, Isoniazid (INH) is one of the most commonly used antibiotic has been found to be associated with severe hepatotoxicity. Both above mentioned toxins have been found to be associated with increased production of free radicals. Moreover, treatments with these pro-oxidatives have been reported to cause membrane lipid and protein oxidation and exert deleterious effects on enzymatic system of the cells.

Antioxidants are well known protective agents which either inhibit generation of free radicals or by enhance their removal [7,9]. A number of herbs have been known to have antioxidative property [6,10,11,12]. Pithecellobium dulce (PD) is reported to be used as drug in dysentery, dermatitis, inflammation, leprosy, peptic ulcer, toothache, and venereal disease etc. in addition to this, evidence of free radical scavenging potential of the same have also been reported. Though, some reports on hepato-protective efficacy of PD have been documented, but available data also showed disparity in the protective potencies of herbs in different extraction medium. Since, different extraction medium contain different active constituents so medicinal properties of the same herb vary with extraction system. In present study, ethanolic and hexane:benzene:acetone (1:1:1) leave extracts of Pithecellobium dulce (EEPD and HBAPD respectively) were investigated for their relative efficacies against CCl₄ and INH induced systems. For this, Enzymatic activities such as serum alkaline phosphatase (ALP), serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) and total bilirubin (TBil) were taken as test parameters. In both experiments, silymarin was used as standard drug. Tissue histology was performed to validate the results.

II. Materials and methods

1.1 Chemicals

All chemicals were of the highest commercially available purity. The bark of Syzygium cumini were collected from D-1 University campus, Dewas road, Ujjain, M.P., India. The identification of plant was done in Dept of Botany, Safia College of science, Bhopal. (M.P.) and the voucher specimen 436/ Bot /saf/13, was deposited in the Safia College of Science Bhopal (M.P.).
2.2 Preparation of extracts

The leaves of PD were air dried and ground to a coarse powder. The powdered leaves were exhaustively extracted in soxhlet extractor with ethanol. Removal of solvent under reduced pressure afforded solid mass. The yield of ethanolic extract was stored for further research. To prepare HBAPD, the powdered leaves were exhaustively extracted in soxhlet extractor with hexane: benzene: acetone, (1:1:1). Removal of solvent under reduced pressure afforded solid mass. Thus obtained extracts was stored for further research.

2.3 Animals

Healthy in-bred albino wistar rats of either sex (2-2.5 months old) were housed in polypropylene cages under constant temperature (27±2°C) and photo-schedule (14 h light and 10 h dark). They were provided rodent feed (Golden Feeds, New Delhi, India) ad libitum and had free access to boiled drinking water. The approval of departmental ethical committee for handling and maintenance for experimental animals was also obtained before starting the experiments.

2.4 Induction of hepatotoxicity

Hepatic injury was induced by single oral administration of CCl₄ mixed with olive oil as vehicle in 1:1 ratio (3 ml/kg of rat body weight). While in other experiment regular doses of INH (100 mg/kg b.w., i.p.) were given to healthy animals for successive ten days.

2.5 Acute oral toxicity study

Acute oral toxicity study was carried out in young healthy female mice using the ‘Limit dose test of up and down procedure’ (UDP) according to Organization for Economic Corporation and Development guidelines 425. Dose up to 2000 mg/kg body weight (bw) was given in an increasing dose order and animals were checked for general behavioural, physical and autonomic changes.

Experimental design- the overall research work was divided into two experiments.

In the experiment 1st, a total of 30 rats were used, which were divided into 5 groups having 6 animals in each group as follows: Group I: Normal control rats received 1ml/100gm of 0.5% CMC using an intragastric tube. Group II: Negative control rats received CCl₄ 3 ml/kg, p.o. only once at day 1. Group III: Rats received CCl₄ 3 ml/kg, p.o. and Silymarin (100mg/kg, p.o.), designated as STAND group. Group IV: Rats received CCl₄ 3 ml/kg, p.o. and ethanolic extract of Pithecellobium dulce, (500mg/kg p.o.), desinated as EEPD Group. Group V: Rats received CCl₄ 3 ml/kg, p.o. and hexane:benzene:acetone (1:1:1) extract of Pithecellobium dulce, (500mg/kg p.o.), desinated as HBAPD Group.

In the experiment 2nd, a total of 30 rats were used, which were divided into 5 groups having 6 animals in each group as follows: Group I: Normal control rats received 1ml/100gm of 0.5% CMC using an intragastric tube. Group II: Negative control group given isoniazid (100mg/kg, i.p.), designated as induced group. Group III: Rats received isoniazid 100mg/kg, i.p. and silymarin (100mg/kg, p.o.), designated as stand group. Group IV: Rats received isoniazid 100mg/kg, i.p. and ethanolic extract of Pithecellobium dulce, (500mg/kg p.o.), desinated as EEPD Group. Group V: Rats received isoniazid 100mg/kg, i.p. and hexane:benzene:acetone (1:1:1) extract of Pithecellobium dulce, (500mg/kg p.o.), desinated as HBAPD Group.

At the end of the experiments on 10th day, animals were kept on overnight fasting and blood was collected by orbital puncture method. This blood was then allowed to clot for 30 minutes at room temperature. The serum was separated by centrifugation at 3000 rpm at 30°C for 15 minutes.

2.6 Biochemical estimation

The serum samples were analyzed spectro-photometrically for ALP, SGOT, SGPT and total bilirubin (TBil) levels using standard kits (Span diagnostics Ltd). Activity of SGOT and SGPT were measured at 505 nm and is expressed as IU/ L of serum. In ALP assay the blue color developed which was read at 510 nm against blank and the activity is expressed as IU/L of serum. To estimate the TBil readings were taken at 540 nm, the level of total bilirubin was expressed as mg/ dL of serum [20].

2.7 Histological study

Histopathological study was done using protocol of Deepa and Varlakshmi [21]. Tissues were fixed in 10% formaldehyde and then dehydrated in descending grades of isopropanol, cleared in xylene, and then embedded in molten paraffin wax. These fixed tissues were subsequently cut in to 5 µm thick sections using microtome. The ribbons were then stained with hematoxylin and eosin and viewed under light microscope to study histopathological changes.
2.8 Statistical analysis

Data are expressed as mean ± SE. Statistical analysis was done by using one-way ANOVA followed by unpaired student’s t-test and p-values of 5% and less were considered as significant.

III. Results and discussion

In acute toxicity test, both extracts were found to be safe up to 2000 mg/kg body weight. While, in first experiment, as compared to normal control animals CCl₄ induced group showed significant increased values of serum ALP, SGOT, SGPT and TBil (P<0.001, for all). As already established elsewhere, here also treatment with standard drug significantly reduced the values of all above mentioned parameters (P<0.001 for all). In case of EEPD and HBAPD drug therapies significant protection were observed (at least P<0.01 for all) than CCl₄ induced animals, but maximum reduction of serum ALP and TBil was seen in EEPD, while, HBAPD administered animals exhibited maximum protection for SGOT and SGPT (P<0.05 than EEPD group).

In second experiment, Treatment of INH exhibited considerable increase in serum values of all test parameters (P<0.001) while parallel drug therapy of silymarin or herbal extracts showed drastic decrease in almost all of the used parameters (at least P<0.05 for all). Here, animals kept on EEPD therapy showed maximum restoration in ALP, SGOT (P<0.01 for both) and SGPT (P<0.01) than HBAPD group. However, for TBil no significant differences were seen among different extracts treated groups in both experiments.

### Table – 1: Acute toxicity study of AEBM, EEBM and BAEBM bark.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Extract used</th>
<th>100</th>
<th>200</th>
<th>500</th>
<th>1000</th>
<th>2000</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EEPD</td>
<td>All animal survived</td>
<td>All animal survived</td>
<td>All animal survived</td>
<td>All animal survived</td>
<td>All animal survived</td>
</tr>
<tr>
<td>2</td>
<td>HBAPD</td>
<td>All animal survived</td>
<td>All animal survived</td>
<td>All animal survived</td>
<td>All animal survived</td>
<td>One died and rest survived</td>
</tr>
</tbody>
</table>

Where, (no. of animals in each group=3) drug doses in mg/kg body weight. EEPD (ethanolic extracts of *Pithecellobium dulce*) and HBAPD (hexane:benzene:acetone (1:1:1) extract of *Pithecellobium dulce*).

**Figure 1:** Effects on different parameters. Cont (Normal control), Induced (CCl₄ treated), STAND (CCl₄ + Silymarin), EEPD (CCl₄ + ethanolic extract of PD) and HBAPD (CCl₄ + hexane:benzene:acetone (1:1:1) extract of PD). Each bar represents the mean±SE (n=6), *P<0.001, as compared to normal control, *P<0.05, *P<0.01 and *P<0.001 as compared to CCl₄ treated group, *P<0.05, *P<0.01 and *P<0.001, as compared to standard drug while *P<0.05 increased than other herbal extract treated group.
Screening for the protective efficacy of two different extracts of *Pithecellobium dulce* in *CCl₄* and

**Figure 2:** Effects on different parameters. Cont (Normal control), Induced (INH treated), STAND (INH + Silymarin), EEPD (INH+ ethanolic extract of PD) and HBAPD (INH+ hexane:benzene:acetone (1:1:1) extract of PD). Each bar represents the mean±SE (n=6), *P<0.001, as compared to normal control, *P<0.05, *P<0.01 and *P<0.001 as compared to INH treated group, *P<0.05, *P<0.01 and *P<0.001, as compared to standard drug while *P<0.05 increased than other herbal extract treated group.

**Figure 3:** Histology of different experimental animals. Where, Cont (Normal control), Induced (CCl₄ treated), STAND (CCl₄ + Silymarin), EEPD (CCl₄+ ethanolic extract of PD) and HBAPD (CCl₄+ hexane:benzene:acetone (1:1:1) extract of PD).

**Figure 4:** Histology of different experimental animals. Where, Cont (Normal control), Induced (INH treated), STAND (INH + Silymarin), EEPD (INH+ ethanolic extract of PD) and HBAPD (INH+ Hexane:Benzen:Acetone (1:1:1) extract of PD).
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IV. Conclusion

The leaves has been known to contain high amount of protein, crude fibre, cyclitol, dulcitol, Octacosanol, β-D-glucoside of α-spinasterol, α-spinasterol and kaempferol-3-rhamnoside and numbers of polyphenolic and flavonoids. these chemicals are known to exert anti-oxidative, anti-diabetic, antibacterial and anti-cancer activities. The present work was aimed to reveal the better protection of PD extracts against CCl₄ and INH induced hepato-toxicity in rats. Finding revalidate the safe and non-toxic effects of the test extracts with acute toxicity assay, as also observed earlier.

Induction of oxidative injury with the CCl₄ treatment has already been reported earlier [6,8,9]. This result in excessively generation of free radicals, these may cause oxidative damages to cellular lipids, proteins and DNA. Free radicals also lead the leakage of cytosolic proteins into the blood stream [18,21]. This may be uses as reliable markers of hepatocellular damage. In this investigation also in CCl₄ induced rats the increased levels of SGOT, SGPT, ALP and TBil indicated severity of hepatocellular damage [8, 22]. The INH used also found to provoke oxidative injury particularly in liver cells […] also resembles with earlier investigations […]

Clinically, enhanced level of TBil indicated hepatobiliary disease and/or severe disordered hepatocellular functions [25]. The extracts mediated control of the increased bilirubin level suggests the possibility of the extracts being able to stabilize biliary dysfunction. Parallally, ALP is a hydrolase enzyme chiefly found in the cells lining the biliary ducts of the liver. Increased level of ALP indicates large bile duct obstruction, intrahepatic cholestasis, or infiltrative diseases of the liver [13,17]. In addition, SGOT and SGPT both are transaminase enzymes and play central role in amino acid metabolism. Both of these are found in the different body’s organs such as liver, heart, skeletal muscle, kidneys, brain, and red blood cells. Serum SGOT and SGPT level, and their ratio (SGOT:SGPT ratio) are frequently measured clinically as biomarkers for liver health. Their increased level has been linked with abnormal liver functions, though these are not very specific to liver disease. Too the toxin treated animals showed increased levels of these enzymes. Conversely, extract therapies attenuated the increased level of these enzymes in serum. Recovery towards the normalization suggests that these extracts caused parenchymal cell regeneration in liver, thus protecting membrane fragility and thereby decreasing enzyme leakage [27].

Histopathological examination of liver sections of the normal control group demonstrated normal cellular architecture with separate hepatic cells and a central vein. While, both induced groups exhibited disarrangement of hepatic cells with intense necrosis. The liver sections of the rat treated with different drug therapies showed more or less recovery of the tissue damage than induced group.

Here, all three extracts were observed to be significantly effective against both toxins. Earlier studies showed that PD leaf posses benenficial polyphenolic compound, flavonoids, tennins, and other protective bioactive chemicals. Thus the presence of these active ingredients could be considered responsible for the hepatoprotective activity of leave’s extracts. Further, comparison of with silymarin indicated that in both experiments, both extracts were nearly equal effective as standard drug used. In addition, both EEPD and HBAPD were found to effective against different serum parameters used. These findings revealed that different extracts possess different active compounds which served via different mechanism. Therefore, to get maximum benefits, either additional alteration in the composition of extracts or mixed extracts therapy could be applied. In conclusion, treatment CCl₄ and INH both responsible for drastic elevated values of liver stress markers, indicated severe hepato-toxic events, which were observed to be completely recovered in silymarin drug therapy. Though, the three extracts posses appreciably effective protection against both toxins but for most of the parameters, these were comparatively less effective than standard drug. In addition, EEPD was seemed to be more effective in some test parameters than HBAPD. Thus, assay dependent protective potencies of the test extracts possibly indicated the presence of diverse group of active compounds in them which served via different pathways. Further investigations are needed to confirm the findings I addition to this altered extraction method or mixed extracts therapy could be analysed for their possible better amelioration.

Acknowledge

Help of the co-scholars of School of studies in chemistry and biochemistry department, Vikram University, Ujjain (M.P.) are gratefully acknowledged.

References

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