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Synergistic protective effect of picrorhiza with honey in acetaminophen induced hepatic injury

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Rhizome of picrorhiza along with honey prevents hepatic damage and cure the acetaminophen (paracetamol) induced hepatotoxicity by modulating the activity of hepatic enzymes. Here, we studied the *in vivo* effects of *Picrorhiza kurroa* and honey on acetaminophen induced hepatotoxicity Balb/c mice model. Hepatic histopathological observations of acetaminophen fed (day-6) group showed more congestion, hemorrhage, necrosis, distorted hepatic architecture and nuclear inclusion. Such damages were recompensed to normal by picrorhiza or honey alone or both in combinations. We observed increased activity of SGPT and SGOT in injured liver tissues, and that too was compensated to normal with picrorhiza or honey alone or both in combinations. We observed 1.27 and 1.23-fold enhanced activity of SGPT in serum and liver lysate, respectively while SGOT showed 1.66 and 1.11 fold enhanced activity. These two enzymes are signature enzymes of liver damage. Thus, our results support that honey may be used with drug picrorhiza due to its synergistic role to enhance hepatoprotective and hepatoregenerative ability along with allopathic drugs to mitigate the hepatotoxic effects.

Keywords: Ayurvedic, Liver damage, Hepatoprotective, Hepatoregenerative, Herbal, Kutki, Picroliv, Picrovin, SGPT, SGOT

Naturally growing medicinal plants are the prime source of many modern drugs[1-5]. Recently, *Aegle marmelos* (Bale) has been reported to protect liver cancer in Balb/c mice model[6]. As per the WHO reports, approximately 80% of world populations are using traditional herbal medicine[7]. An ancient ayurvedic perennial herb *Picrorhiza kurroa* (Fam.: Scrophulariaceae), commonly called kutki, grown in the northwest India on the slopes of the Himalayas between 3000 and 5000 m is used to treat liver diseases, chronic dysentery and bronchial problems[8-10]. Its rhizome contains 26.6 % crystalline glycoside as an active medicinally potential substance[11]. Other ingredients are apocynin, drocinand and cucurbitacin glycoside[9,12]. Apocynin is a potent anti-inflammatory agent[13] while cucurbitacin has antitumour effect[9]. Recently, Sharma *et al.*[14] have demonstrated the anticancer and anti-inflammatory activities of some dietary cucurbits. *P. kurroa* has also antioxidant[15], free radical scavenging activity[16] and immune-modulating activity[17]. Picrovin present in rhizome is known to reverse LDL binding with paracetamol, and thus prevent hepatocyte damage of mice. Picrovin also controls the activity of metabolic enzymes such as glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, ALK phosphatase and lipids of hepatocytes[11]. Since these enzymes are released in serum after tissue injury, hence called marker of hepatic tissue damage.

Honey has been used for its medicinal properties since ancient times and has important role in the recuperation of human health[18,19]. Pure honey contains dextrose, levulose, water and in small proportions of most of the elements present in the human body. The precise composition of honey varies according to the plant species on which the bee forages, but the main constituents are the same in all honeys. Honey (average pH 3.2-4.5) also contains minerals and vitamins (0.02% of its weight) a number of amino acids such as proline, phenylalanine and aspartic acid with a concentration of greater than 200 ppm. The gluconolactone and gluconic acid content of honey helps to maintain its pH range[18-20]. The enzymes present are invertase, glucose oxidase, hydrogen peroxide and amylase (diastase)[18,19]. The H_2O_2 content of honey supports its antimicroabial

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activity[19]. Honey is known to control gastric ulcer when used as a dietary supplement[18,19] and acts as antioxidant[21]. The fructose present in honey is absorbed more slowly from the gastrointestinal tract than glucose and is rapidly taken up by the liver. Hence with honey the blood sugar rises minimally due to fructose ingestion[18,19,22]. It may lower plasma insulin activity, C-reactive protein, and homocysteine in the healthy and diabetic subjects[23].

In the present study, we investigated the effects of honey and traditional medicine *P. kurroa*, alone or in combination to check the level and activity of the hepatic injury marker enzymes including SGPT/ALT (alanine transaminase) and SGOT/AST (aspartate transaminase) in acetaminophen feed mice, if any.

Materials and Methods

Chemicals and Reagents

Acetaminophen, a dispersible Tablet Acton–OR (1000 mg/tab acetaminophen) was obtained from APEX LABORATORY. Enzyme assay kits of SGPT/ALT (Cat No.-K753-100) and SGOT/AST (Cat No.-K753-100) were purchased from BioVision Inc. California 95035, USA via Hysel, New Delhi, India and kept at 4°C till further use. Analytical grade NaCl from SRL, DMEM from Genetix biotech Asia Pvt. Ltd. India, Trypsin from Euroclone were used. Hematoxylin and Eosin were purchased from MERCK, Mumbai, India.

Collection of herbal medicinal plants and honey

Rhizome of *P. kurroa* was purchased from the Ayurvedic Pharmacy, Institute of Medical Sciences, Banaras Hindu University, Varanasi. About 15 g of air-dried rhizome standardized by the company was taken for aqueous extraction. Poly floral honey (as it is not synthetic) was collected from the forest of Mirzapur, UP, India and examined microscopically for any contaminants. In brief, 1:1 preparation was made in laminar hood in aseptic condition. About 15 µL was used for plating on the LB-medium agar-agar plate. No bacterial contamination was observed. Simultaneously, slide of 1:1 solution was prepared and observed under microscope.

Extraction procedure

Fifteen gram of powdered picrorhiza rhizome was taken in 150 mL conical flask and 75 mL of double distilled water (DDW) was added. The mouth of flask was covered with aluminum foil and kept on shaker overnight agitating at 150 rev/min for proper mixing and complete extraction of water soluble materials. Later, extract was filtered first using muslin cloth and then by Whatman no. 1 filter paper. The extract was dried on water bath till complete evaporation of water and powder was recovered, kept at 4ºC for further use.

Cytotoxicity assay

Spleenocyte culture

Balb/c mice were sacrificed according to the guidelines of ethical committee of Banaras Hindu University, UP, India (Oct/06 item no. II (iv) IMS BHU). Spleen were taken out from dissected mice, washed with cold phosphate buffered saline and cell suspension was prepared by mincing tissue in incomplete Dulbecco's modified Eagles medium (DMEM). Erythrocytes lysis buffer (0.15 M NH_4Cl, 1 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.4) were used for lysis of erythrocytes. Then cells were washed twice with incomplete DMEM medium and subjected to centrifuge at 2000 rpm for cell pellet formation. Cells were resuspended in incomplete DMEM medium. Trypan blue were used for counting of cells by haemocytometer, and 2×10^6 cells/mL concentration of cells were cultured in 6-well culture plate and incubated for 2 h at 37°C and 5% CO_2 condition in CO_2 incubator.

Drug treatment and viability assay

After 2 h incubation, cells were treated with different concentration of drug acetaminophen (10, 20, 40, 60 and 80 µg/mL). After dose selection of acetaminophen (60 µg/mL) viability assay was performed with honey (10 µL), picrorhiza (10 µg/mL) and honey (10 µL) + picrorhiza (10 µg/mL) as of mentioned concentration. Treated cells were incubated in CO_2 incubator at 37°C with 5% CO_2 for 1 h. After 1 h, cells were collected by trypsinization from culture plate, pelleted by centrifugation (2000 rpm/4ºC/15 min) and resuspended in the incomplete medium. Viable cells were counted by Trypan blue exclusion assay with haemocytometer.

Animal care, mice group distribution and dose administration

Balb/c male mice, 5-6 wk old, were housed in the Animal house of School of Biotechnology, Banaras Hindu University in air-conditioned with humidity activity 30-50% with 12:12 h light/dark cycles. The animals were allowed to acclimatize for 14 days before any experiment. Standard mice chow and water were available *ad libitum*. Healthy mice, weighed between 25-35 g, were selected for the experimental study. Mice body weights (body wt.) were taken at the regular interval starting from day-0,

followed by day-2, 4, 6 and 8. Mice were given treatment of drugs as mentioned below in details. The drug treated mice & mock mice were sacrificed by cervical dislocation on decided experimental date. The mice suffering with any acute illness, congenital disorder or infection were excluded before group formation. All the mice were kept in hygienic condition. Feeding and care was taken as per the ethical rule and regulation of the concerned committee, BHU, Varanasi, India.

Mice were divided into 4 groups, each containing 5 mice (Table 1). Mock (control) in each group was fed only with double distilled water (DDW), Group I was acetaminophen treated, Group II, acetaminophen +honey, Group III, acetaminophen+picrorhiza, and Group IV, acetaminophen+honey+picrorhiza treated. Sublethal dose of acetaminophen, 625 mg/kg body wt. was given after dispersing in DDW to each mice of respective group. Aqueous extract of picrorhiza 200 mg/kg body wt. of mice was given to respective groups. Honey was diluted in DDW (1:1) and 0.25 mL dose was given orally to respective mice group.

Histopathological slide preparation

Liver samples of all the groups mentioned above were dissected out at the end of the experimental period, washed, blotted dry, fixed in 10% buffered formalin solution and dehydrated with stratified ethanol solutions from 50-100%. The dehydrated liver samples were later used for preparation of paraffin blocks for cryosectioning. Sections of 4 µm in thickness were cut and stained with haematoxylin and counter stained with eosin[24]. Histopathological slides were observed and documented by a light photomicroscope.

Serum and liver lysate preparation

Two mice from each group were sacrificed randomly by cervical dislocation after set time interval to collect blood samples. About 2 mL of blood samples were collected in centrifuge tubes using 2 mL syringe via cardiac puncture and the serum was separated. The liver samples were rinsed with ice cold 0.9% NaCl, weighed and homogenized in ice cold sodium pyrophosphate buffer (pH 8.3) in a ratio of 50 mg/mL. Homogenate was preserved at 4ºC till use.

Enzyme assays

The serum and liver tissue lysate samples were used to assay for hepatic marker enzymes such as Alanine transaminase (SGPT/ALT) and Aspartate transaminase (SGOT/AST). Activities of AST and ALT were assayed according to the 2-4 DNPH method and results were expressed as IU/dL. Procedure was followed as per guide line given in the SGPT/ALT and SGOT/AST kit. In brief, 0.25 mL given reagent-I was incubated at 37ºC for 5 min and then respective samples were added separately into each tube, mixed properly and incubated at 37ºC for 1 h for SGPT and 30 min for SGOT. After incubation, reagent-II was added followed by addition of respective samples and finally reagent-III was added. After 10 min of incubation OD was measured at 505 nm and activity was calculated as per formulae given in the kit protocol.

Results

Cells viability assay with drugs

Acetaminophen at concentration of 60 µg/mL showed a sublethal dose or non-cytotoxic dose as per its cytotoxicity assay showed 78% of viable cells of spleenocytes after 1 h of treatment (Fig. 1a). This non-cytotoxic dose (60 µg/mL) of acetaminophen was used for further experiment with picrorhiza (10 µg/mL), honey (10 µL) and picrorhiza+honey (10 µg/mL +10 µL). Approximately, 22% cell death was recorded with acetaminophen (60 µg/mL) compared to the control (without treatment). We observed enhancement in growth of cells up to 11, 33 and 77% along with honey, picrorhiza and honey+picrorhiza, respectively in comparison to control (without treatment) (Fig. 1b).

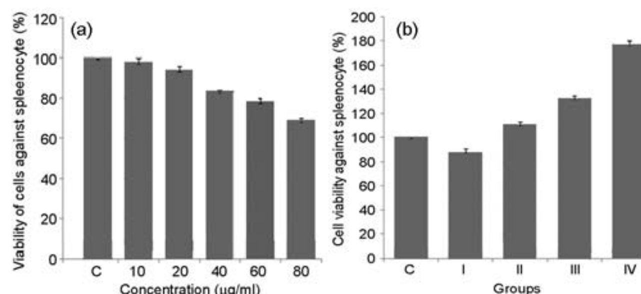

Fig. 1—Cell viability assay with (a) different concentration of acetaminophen (10, 20, 40, 60, and 80 µg/mL); and (b) acetaminophen, and with combinations of honey and picrorhiza upon 1 h of treatment. [C: Control is without any treatment represents 100% viability. Groups I-IV represents A, A+H, A+P, and A+H+P fed mice. A, H, P denotes acetaminophen, honey and picrorhiza, respectively. Concentration of A is 60 µg/mL, P is 10 µg/mL and H, 10 µL (1:1 dilution). Data reported as the mean ± SEM of triplicate experiments performed in similar condition using a Student t-test. *P* <0.05 were considered statistically significant]

Liver histopathological slides symptoms

Hepatic histopathological slides were showing pathological symptoms after treatment with acetaminophen at day-0, 4, 6 and day-8 (Fig. 2). Acetaminophen treated slide at day-0 had normal architecture with distinct bile plugs and without any congestion, haemorrhage, necrosis and normal nuclear characteristics. Day-4 treated slide had distorted hepatic sinusoids while rest of the features was similar to normal or day-0 slide. Day-6 treated slide showed distorted architecture. However, after treatment of honey, picrorhiza and honey+picrorhiza, hepatic histopathological slides at day-6 showed normal architecture as it was in day-0 (Fig. 3). With honey, picrorhiza and honey + picrorhiza there was recovery or more appropriately prevention of distorted hepatic sinusoids and disappearance of congestion, necrosis like normal hepatic architecture (Fig. 3).

Serum SGPT enzyme activity

With only acetaminophen feed, the serum SGPT enzyme activity was increased by 1.48, 2.40, and 4.53 fold, respectively at day-2, 6 and 8 in comparison to control (Fig. 4a), while with acetaminophen + honey feed, the SGPT enzyme activity was increased 1.48, 2.14 and 3.62 fold, respectively at day-2, 6 and 8 in comparison to control (Fig. 4a). With acetaminophen +picrorhiza feed, the SGPT enzyme activity was increased 1.26, 2.02 and 3.69 fold, respectively at day-2, 6 and 8 compared to control (Fig. 4a) whereas with acetaminophen+honey+picrorhiza feed, the SGPT enzyme activity was found increased by 1.25, 2.91 and 2.91 fold at day-2, 6 and 8, respectively compared to the control (Fig. 4a).

Liver lysate SGPT enzyme activity

With only acetaminophen feed, the liver lysate SGPT enzyme activity was increased by 1.22, 1.69 and 3.12 fold at day-2, 6 and 8, respectively when compared to control (Fig. 4b), while with acetaminophen + honey feed, the increase was 1.13, 1.42 and 2.43 fold, respectively (Fig. 4b). With acetaminophen+picrorhiza feed, the liver lysate SGPT enzyme activity was increased by 1.13, 1.34 and 2.19 fold at day-2, 6 and 8, respectively (Fig. 4b) while

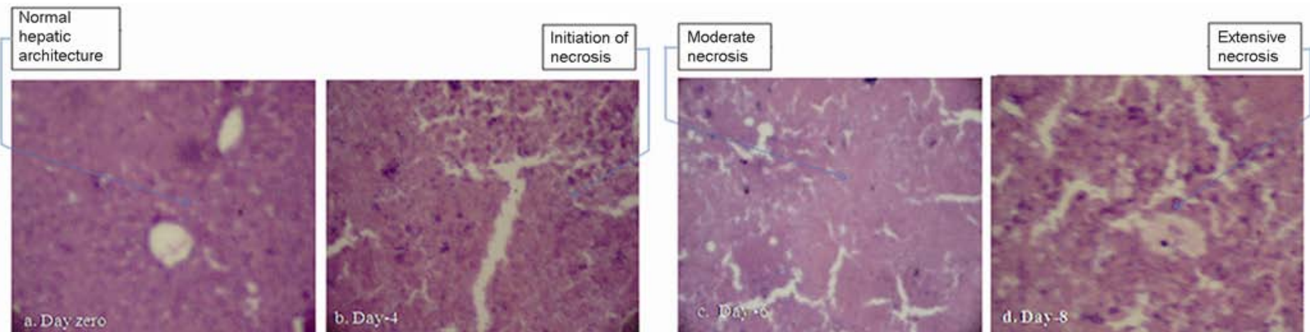

Fig. 2—Histopathological analysis of Liver tissues of only acetaminophen feed mice. [(a) day-0 (control, H (haematoxylin) and E (eosin)x50); (b) day-4 (H and E, x50); (c) day-6 (H and E, x50); and (d) day-8 (H and E, x50)]

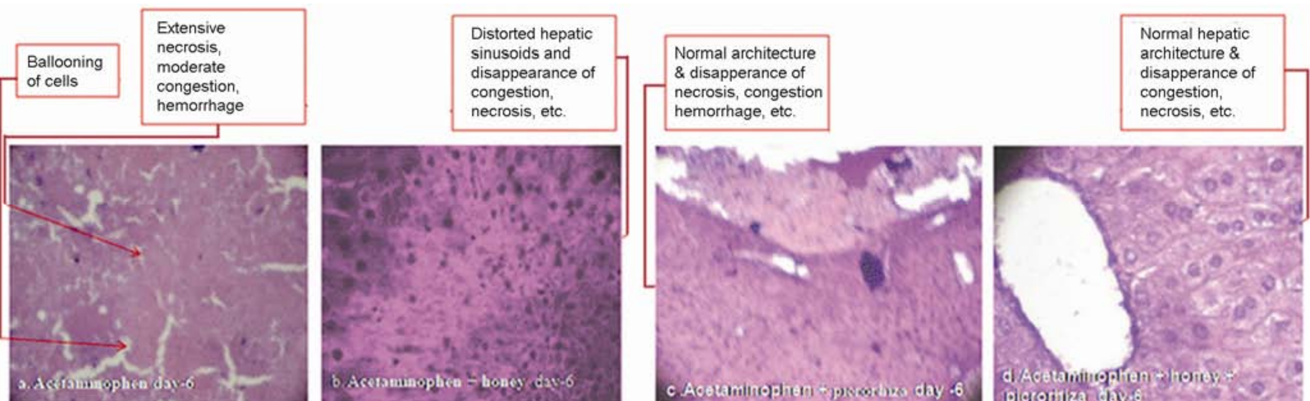

Fig. 3—Histopathological analysis of Liver tissues of mice fed with acetaminophen and with combinations of honey and picrorhiza. [(a) Gr-I at day-6 (H and E, x50); (b) Gr-II at day-6 (H and E, x50); (c) Gr-III at day-6 (H and E, x50); and (d) Gr-IV at day-6 (H and E, x50)]

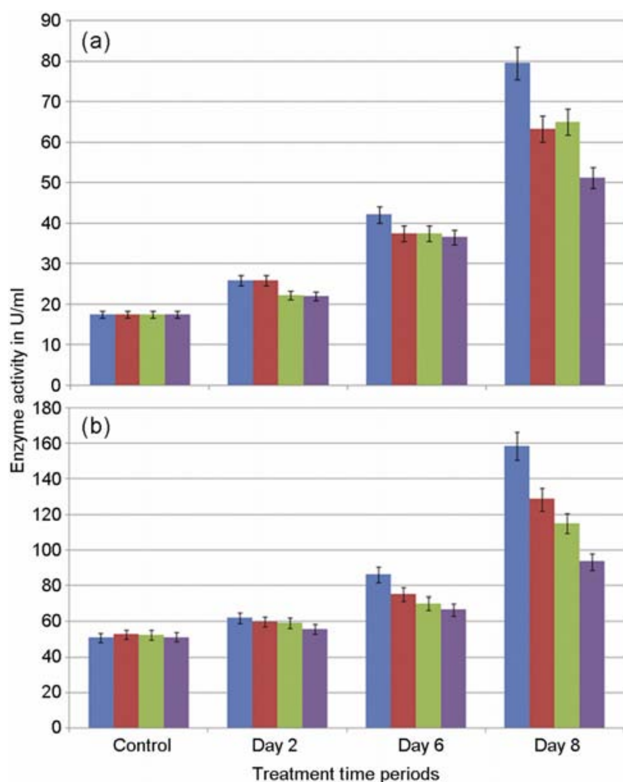

Fig. 4—(a) Serum; and (b) Liver lysate SGPT enzyme activity at day-0 (control), day-2, day-6 and day-8 in mice Groups I-IV fed with A, A+H, A+P, and A+H+P, respectively. . [A, H, P represents acetaminophen, honey and picrorhiza, respectively. Data reported as the mean ± SEM for n=5 per group and compared against PBS control mice by using a Student t-test. *P* <0.05 were considered statistically significant]

with acetaminophen+honey+picrorhiza feed, it was 1.09, 1.30 and 1.83 fold, respectively (Fig. 4b).

Serum SGOT enzyme activity

With only acetaminophen feed, the serum SGOT enzyme activity was increased by 1.03, 2.04 and 3.68 fold at day-2, 6 and 8, respectively compared to control (Fig. 5a) while with acetaminophen + honey feed, the same activity increased by 1.03, 1.49 and 2.68 fold, respectively (Fig. 5a). With acetaminophen + picrorhiza feed, the serum SGOT enzyme activity was non-significant at day-2 while significantly increased to 1.42 and 2.34 fold, respectively at day-6 and 8 compared to control (Fig. 5a). However, with acetaminophen + honey + picrorhiza feed, the serum SGOT enzyme activity showed decreased by 1.06 fold at day-2 but increased by1.17 and 1.41 fold at day-6 and 8 (Fig. 5a).

Liver lysate SGOT enzyme activity

With only acetaminophen feed, the liver lysate SGOT enzyme activity was increased by 1.06, 1.82 and 3.15 fold at day-2, 6 and 8, respectively compared to control (Fig. 5b), while with acetaminophen+honey feed, it was 1.10, 1.45 and 1.87 fold increase (Fig. 5b). With acetaminophen+picrorhiza feed, it showed increase by 1.05, 1.49 and 1.64 fold at day-2, 6 and 8, respectively (Fig. 5b), and with acetaminophen + honey+picrorhiza feed, it was 1.04, 1.34 and 1.49 fold increase at day-2, 6 and 8, respectively (Fig. 5b).

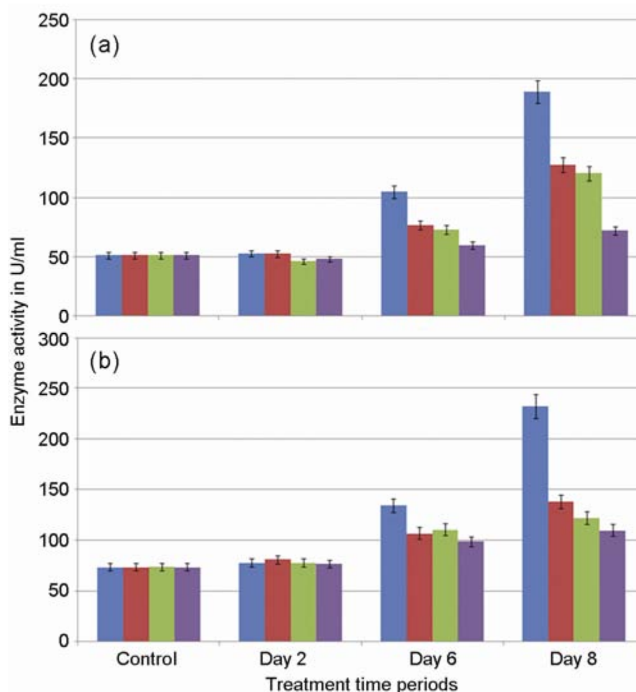

Fig. 5—(a) Serum; and (b) Liver lysate SGOT enzyme activity at day-0 (control), day-2, day-6 and day-8 in mice Groups I-IV fed with A, A+H, A+P, and A+H+P, respectively. [A, H, P represents acetaminophen, honey and picrorhiza. Data reported as the mean ± SEM for n=5 per group and compared against PBS control mice by using a Student t-test. *P* <0.05 were considered significant].

Discussion

Acetaminophen is a common analgesic effectively used to reduce fever and also control mild pain. The overdose (625 mg/kg body wt.) of acetaminophen causes severe hepatotoxicity[25,26] while sub-lethal dose is used to prepare mice model for experimental purposes[27]. The hepatotoxic effects *viz*., congestion, hemorrhage, necrosis, and distorted architecture as observed in our histopathological slides reflect this (Fig. 3). Such effects could be damaging, leading to liver failure both in animals and in humans[28,29]. In experiment performed with spleenocytes, we observed that sublethal dose of acetaminophen selected for *in vivo* mice experiment (625 mg/kg body wt.) was similar to the dose (60 µg/mL) used for *in vitro*

experiment, showing 78% viability (Fig. 1A). Moreover, selection of acetaminophen dose *in vivo* to develop hepatotoxic mice model is based on the earlier report of Ali *et al*.[27] Experiment performed to check the viability effects of honey, picrorhiza and honey + picrorhiza *in vitro* on spleenocytes gave interesting results, that apparently enhanced the proliferation of cells by 11, 33 and 77%. It indicates the synergistic effects of both drugs (Fig. 1B). Increase in the cell numbers might be due to growth promoting ingredients present in the honey and picrorhiza aqueous extract. The rapid proliferation of cells i.e., 77% within 1 h with honey and picrorhiza aqueous extract needs further investigation.

In our present finding, we showed that acetaminophen causes liver injury in Balb/c mice which is evident in hepato-histopathological slides of day-4, day-6 and day-8 (Fig 2). After day-8, maximum damage tissues were observed with marked distorted hepatic architecture, congestion, diffused areas of hemorrhage, extensive necrosis and inclusion of nuclear population of cells in fair numbers. Similar impact was observed at day-6 in comparison to day-0 acetaminophen which was observed at day-8 fed mice group (Fig. 2) and hence, day-6 results were presented with honey, picrorhiza and picrorhiza+honey, along with acetaminophen only not with day-8 fed mice (Fig. 2). The harmful effects of acetaminophen were reduced at substantial level with honey, picrorhiza and picrorhiza + honey, as evident in normal hepatic architecture with disappearance of necrosis, congestion and hemorrhage (Fig. 3). More pronounced recovery effects with honey and picrorhiza suggests the synergistic hepatoprotective role of picrorhiza with honey in combination.

There was increase in the overall activity of hepatic injury marker enzymes SGPT and SGOT compared to control. Increased activity was the sign of damage of liver tissues, which was well supported by the damages, as seen in the hepato-histopathological slides. The SGPT and SGOT enzyme activity in the serum showed significant increase, with 4.53 and 3.68 fold increase compared to the control. However, nearly equal increase in activity of SGPT and SGOT was observed in liver lysate which is 3.12 and 3.15 fold respectively, in comparison to the control (Figs. 4b and 5b).

In acetaminophen fed mice, the overall activity of tissue damaging marker enzymes SGPT (Fig. 4) and SGOT (Fig. 5) increased both in serum and liver lysate with increased treatment days while significant decrease in enzyme activity of both enzymes occurred after treatment of honey, picrorhiza and honey + picrorhiza. *P. kurroa* has been used for the treatment of liver damage as reported in the literature[30]. Similarly, honey is used for its antioxidant, antimicrobial and antipathogenic acitivity[18,19,31]. Here, we report its hepatoprotective and hepatoregenerative activity as well. The results and observations suggest that increase in SGPT enzyme activity is more in serum than the liver lysate most probably due to secretary nature of enzyme after damage of tissues into the blood stream. However, the effectiveness of picrorhiza was more on the SGPT enzyme activity of liver lysate which is evident by 1.70 fold decrease compared to the SGPT enzyme activity of serum where it showed only 1.26 fold decrease. Moreover, the effectiveness of drug might be more on the damaged liver tissues enzyme but less on secretary proteins or enzymes as our data suggests. This we hypothesized because we observed higher decrease in the activity in liver lysate enzyme compared to serum enzyme. This has not been reported earlier.

Effectiveness of picrorhiza could be due to the compound picroliv present which has been reported to have antihepatotoxic, hepatoregenerative, choleretic, and hypolipidemic effects[29,33]. The hepatoprotective and hepatoregenerative effects with honey, picrorhiza and honey + picrorhiza in the acetaminophen induced liver damage as observed in the present study (Fig. 4) could be aligned with the earlier report[32,33]

With the above study, we conclude that honey can be used with ayurvedic medicines to add palatability, decreasing bitterness and increasing potency.

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