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RESEARCH ARTICLE

Ameliorative potential of *Crataegus songarica* against Isoniazid and Rifampicin Induced Hepatotoxicity in Albino Wistar Rats

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Study Area: Bhopal, Madhya Pradesh, India
Coordinates: 23.2599° N, 77.4126° E

Key words: Liver cells, Hepatotoxic Control

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Abstract

Six rats for every five groups were selected randomly for this study. Ethanolic extract of *Crataegus songarica* and Liv-52 were given orally once daily for the time period of 28 days in Isoniazid and Rifampicin treated groups. Further, the level of serum alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin and total protein was evaluated followed by the histological examination of liver tissues. Results found considerably raised in almost all the tested parameters. Total bilirubin due to Isoniazid and Rifampicin treatment were ameliorated near to standard level in a concentration-dependent manner when the rats were given admittance to ethanolic extract of *C.songarica*. Meanwhile, the decreased level of total protein was also restored near to the normal level. The biochemical markers were fully augmented with histological examinations of the rat liver sections. The ethanolic extract of leaves of *C.songarica* showed potent hepatoprotective potential against Isoniazid and Rifampicin-induced hepatic damage in rats.

Introduction:

Drug-related hepatotoxicity is a severe reaction to anti-tubercular drugs (Tandon *et al*., 2008). According to DOTS (Directly Observed Treatment Shortcourse) procedure various drugs viz; Isoniazid, Pyrazinamide, Rifampicin and Ethambutol are used in the treatment of tuberculosis. Antitubercular drugs due to oxidative stress cause hepatotoxicity and the generation of free radicals damages hepatocytes (Chowdhury *et al*., 2006). A dose of 50 mg/kg/day showed maximum hepatotoxicity in comparison to other doses, hence, this concentration of Isoniazid (INH) and Rifampicin (RIF) was selected to administer to the rats (Brown *et al*., 2005). Reports about changes in enzymatic and nonenzymatic components of several cell defensive mechanisms have emerged due to Isoniazid (INH) and Rifampicin (RIF) caused hepatotoxicity (Tasduq *et al*., 2005). Treatment of tuberculosis is associated with hepatotoxicity and the acetylation of Isoniazid by liver enzyme N-acetyltransferase 2 (NAT2) followed by hydrolysation results in the formation of isonicotinic acid and acetyl-hydrazine, the later is activated by cytochrome P_{450} (Santhosh *et al*., 2006, Kalra *et al*., 2007). The phenyl-hydrazine undergoes metabolic oxidation followed by the formation of reactive acetylating species dimethylhydrazine. The binding of acetyl hydrazine and diacetyl-hydrazine with the macromolecules of liver cells provokes liver injury (Wang *et al*., 2016). Further liver damage is induced due to the accumulation of bile acid inside the liver which is a consequence of injury to hepatocytes and bile duct cells (Shah 2010). All the drugs used in the treatment of tuberculosis are shown to have hepatotoxic effects, natural herbal drugs, and synthetic compounds have been used to prevent toxicity, without interfering with the restorative action of the drugs. Garlic, Silymarin, N-acetylcysteine and several other herbal drugs are proved to have hepatoprotective effects. The mechanism of action of herbal drugs is due to the inhibition of CYTP450 2E1 and antioxidant activity (Ko *et al*., 2017). Liv-52, an ayurvedic multi herbal formulation is broadly used in various hepatic disorders (Handa *et al*., 1986).

Crataegus songarica is a deciduous shrub or tree, small, hard, ornamental, spiny up to 9 m tall. It has various vernacular names viz., Ban-Sangli, (Hindi), Ban-Sangli (Punjabi), Ring (Kashmiri) (Khan *et al*., 1979).

C.songarica is found on limestone, granite, mountain slopes, in woodlands, and along rivers at an altitude of 800-2700 m in countries like Iran, Tadzhikistan, Uzbekistan,

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Kyrgyzstan, Kazakhstan, Afghanistan, northern Pakistan, northern India, and Sinkiang (Pojarkova, 1939; Aziz *et al.*, 2020). The distribution of C.songarica in the Jammu and Kashmir region is in Lolab, Sindh valley, Gulmarg, Jehllum, Pahalgam and Pirpanjal Range (Lone 2013). It possesses various traditional uses; its edible fruits are regarded as cardiotonic and its bark extract reduces labour pain in pregnant women as reported from Ghoni Pakistan (Haq, 2012; Shah & Hussain 2012).

Consequently, this study aimed to scrutinize the protective potential of ethanolic extract of *C.songarica* (EECS) leaves to counter the Isoniazid and Rifampicin caused hepatotoxicity in albino Wistar rats.

Materials and methods

Plant material: the leaves of *Crataegus songarica* were collected during the month of May from the local area of village Banhama, Tehsil lar, Dist. Ganderbal, Jammu, and Kashmir, India. A voucher herbarium specimen (2225-KASH Herbarium) has been submitted to the Department of the Taxonomy, the University of Kashmir for future references.

Extract preparations: the leaves were shade dried for a period of one week at room temperature after cleaning with distilled water for the removal of dirt and soil. Drying was followed by mechanical grinding which reduced the leaves to a moderately coarse powder. During the next 18 hours soxhlation was done in 90% ethanol; under vacuum drying of the extract was achieved with rotavapor, and the resultant semi-solid material was stored in a deep freezer at -4 °C for further processing.

Experimental animals: Institute Animal Centre provided 150 – 200 gram Wistar albino rats which were kept at the surrounding temperature, relative humidity of 25°C and 45-55% respectively with a dark and light cycle of 12-hrs each at the Pinnacle Biomedical Research Institute animal house for acclimation and fed with pellet diet and water ad-libitum. Experiments were carried out in agreement with the Indian legislation on the use and care of laboratory animals.

Chemicals and drugs: Isoniazid and rifampicin were acquired from Sigma- Aldrich Company (USA). Liv-52 was procured from Himalaya drug company, Makali Bengaluru. Other chemicals used in this experimental study were of the scientific brand. Kinetic method grade kits from Auto span (Span Diagnostics Ltd.) and Accura (Lab Care Diagnostics Pvt. Ltd.) India was used to determining the serum ALT, AST and ALP by double beam spectrophotometry.

Preliminary phytochemical screening: the ethanolic extract of *Crataegus songarica* was tested for the presence of several phytoconstituents such as glycosides, alkaloids, tannins, saponins, flavonoids and reducing sugars (Evans *et al.*, 2002).

Isoniazid and rifampicin induced hepatotoxicity: separate suspension of 50 mg/kg body wt. p.o of Isoniazid and rifampicin each were prepared in carboxy methyl cellulose (CMC). Hepatotoxicity was induced with a 28 days treatment of isoniazid (INH), co-administered with rifampicin (RIF) (Ravinder *et al.*, 2006).

Experimental design: Albino Wistar rats were divided into five groups, each having six animals.

Group I : Normal control.

Group II : Hepatotoxic control dosed with INH+RIF each (50 mg/kg) (Ravinder *et al.*, 2006).

Group III : Standard group received INH+RIF+Liv-52 100 mg/kg (Sandhir & Gill, 1999; Ibrahim *et al.*, 2011).

Group IV : Treated with EECS 200mg/kg + INH+RIF (50 mg/kg).

Group V : Treated with EECS 400mg/kg + INH+RIF (50 mg/kg).

Distilled water alone for 28 days was used in all treatments given orally through an orogastric cannula. No lethality was observed up to 2000 mg/kg in the animals and on this basis of acute oral toxicity studies, 200 mg/kg and 400 mg/kg doses were selected for further experimentation which is $1/10^{th}$ and $1/5^{th}$ of the highest dose tested.

Blood samples of all animals taken at the completion of experiments were allowed to clot in sterile centrifuge tubes. These samples were centrifuged at 3000 rpm using a cooling centrifuge for 10 min to separate the serum for biochemical studies.

Biochemical estimation: the biochemical markers like serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were quantitated by using scientific grade kits from Auto span (Span Diagnostics Ltd.) and Accura (Lab Care Diagnostics Pvt. Ltd.). The results were stated as units/litre (IU/L) (Schumann *et al.*, 2002; Kind & King, 1954). The level of Total protein (TP) and Total bilirubin (TB) were estimated according to standard methods using scientific grade kits from Ebra (Transasia Bio-Medicals Ltd.) and Auto span (Span Diagnostics Ltd.) respectively (Koller, 1984; Jendrassik & Grof, 1938).

Histopathological studies: liver samples from each animal of all the groups were processed for light microscopy. Fixation was done in 10% formalin, thereafter the liver tissue sections were embedded in paraffin wax. The rotary microtome was used to cut the sections of 4-5 microns and staining was done with haematoxylin-eosin (H&E) (Raghuramulu *et al.*, 1983). The liver sections were examined under a light microscope by a liver pathologist for centrilobular necrosis, fibrosis, lymphocyte infiltration, pyknosis, ballooning, fatty infiltration etc. which are the histological markers of liver toxicity.

Statistical analysis: the results obtained were stated as Mean ± SE. The data evaluation was done by one way ANOVA test and variation is estimated by Bonferroni's multiple comparisons test. The difference below $p<0.001$

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implied significance.

Results and Discussion:

Phytochemical screening: the ethanolic extract of *C.songarica* showed the presence of saponins, terpenoids, flavonoids, alkaloids, carbohydrates, glycosides, amino acids, tannins and phenolic compounds.

Effect of ethanolic extract of *C.songarica* on biochemical markers: introduction of INH + RIF at a concentration of 50 mg/kg bw p.o. each raised the ALT, AST, ALP, activities significantly ($p< 0.001$) in comparison to the normal control (Table-1). Ethanolic extract treatment of *C.songarica* 1hr prior to INH+RIF administration significantly ($p<0.01$) protected the increase in ALT, AST, ALP, levels towards normal when compared to hepatotoxic controlled rats at a concentration of 200mg/kg and 400 mg/kg bw.

Effect of ethanolic extract on Total Protein and Total Bilirubin: the levels of Total protein (TP) and Total bilirubin (TB) are given in the Table-2 (Plate-1a,b,c). The total protein level was significantly ($p< 0.001$) decreased upon dosage at 50 mg/kg bw p.o. of INH + RIF each, as compared to normal control. This level of Total protein was significantly ($P<0.001$) increased as compared to hepatotoxic controlled rats with the treatment of ethanolic extract of *C.songarica* at a concentration of 200 mg/kg and 400 mg/kg. At the same concentration of 50 mg/kg bw p.o. INH + RIF each significantly ($p< 0.001$) elevated the level of Total bilirubin when compared to normal control. In contrast to hepatotoxic controlled group of rats, *C.songarica* significantly ($p<0.001$) reduced the Total bilirubin at a concentration of 200 mg/kg and 400 mg/kg (Plate-1d,e).

Table-1: Effect of ethanolic extract of *Crataegus songarica* (EECS) leaves on ALT, AST and ALP in Isoniazid and Rifampicin (INH, RMP) induced hepatotoxic rats.

Groups	ALT IU/L	AST IU/L	ALP IU/L
Normal Control	23.541±1.517	26.152 ±2.054	52.058±2.620
INH, RMP+VEHICLE	47.788±2.712	49.336±2.211	145.955±4.184
INH, RMP+STD DRUG	25.632 ±1.310*	28.015±1.770*	56.266±2.077*
INH,RMP+200mg C.S	35.132±1.561*	37.201 ±1.750*	84.983±4.154*
INH,RMP+400mgC.S	28.798±1.612*	31.065±2.415*	61.493±2.472*

Data are expressed as Mean ± SD (n=6) and * $p<0.001$ as compared to INH, RMP treated group. INH, RMP treated group was compared with control and rest of the groups.

Table-1: Effect of ethanolic extract of *Crataegus songarica* (EECS) leaves on total protein (TP) and total bilirubin (TB) in Isoniazid and Rifampicin induced hepatotoxic rats.

Groups	TP g/dL	TB mg/dL
Normal Control	11.163±2.404	0.541 ±0.225
INH,RMP+VEHICLE	5.393±0.997	1.269±0.217
INH, RMP+STD DRUG	10.07±0.715*	0.596±0.158*
INH,RMP+200mg CS	8.207±0.779*	0.812±0.208*
INH,RMP+400mg CS	9.803±1.037*	0.615±0.127*

Data are expressed as Mean ± SD (n=6) and * $P<0.001$ as compared to INH, RMP treated group. INH, RMP treated group was compared with control and rest of the groups.

Effect of ethanolic extract of *Crataegus songarica* on histopathology of liver: histological examination of the liver sections depicts normal central vein (CV), usual hepatocytes (H), expected kupffer cell (K) number, standard sinusoids (S), conventional lobular architecture in normal group (Plate-1a), whereas the hepatotoxic control group treated with INH+RMP, liver injury shows ballooning (B), increased number of kupffer cells (K), pyknotic nuclei (PN), microvesicular steatosis (MS), sinusoidal inflammation, low hepatocyte count (Palte-1b). The standard group treated with INH-RMP+LIV-52 showed nearly normal liver morphology such as hepatocytes (H), central vein (CV), sinusoids (S), normal kupffer cells (Plate-1c). The treatment group, treated with INH + RMP + 200 mg/kg ethanolic extract of *C.songarica* showed slight recuperation and promise of regeneration in most hepatocytes, although pyknosis (PN) and ballooning (B) of some hepatocytes and little macrovesicular steatosis (MS) was still observed (Plate-1d). The treatment group administered with INH-RMP + 400 mg/kg of *C.songarica* showed minimal hepatocyte damage, mild ballooning and less steatosis, least sinusoidal infiltration (Plate-1e).

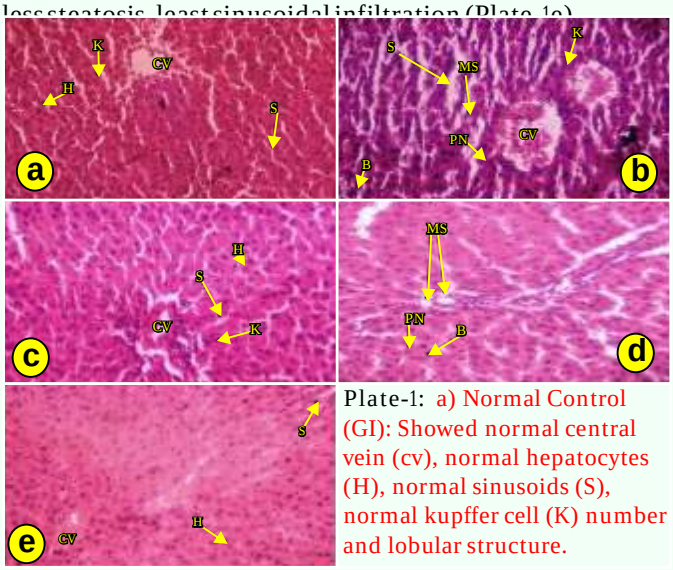

Plate-1: a) Normal Control (GI): Showed normal central vein (cv), normal hepatocytes (H), normal sinusoids (S), normal kupffer cell (K) number and lobular structure.

b) Hepatotoxic Control (GII): Rats treated with INH+RMP.

c) Standard Control (G3): Rats treated with INH-RMP+LIV-52.

d) Treatment group (GIV): Rats treated with INH+RMP+ 200mg/kg/b.w. ethanolic extract of *Crataegus songarica*.

e) Treatment group (GV): Rats treated with INH-RMP+400mg/kg/bw of *Crataegus songarica*.

Some patients on long-term treatment for tuberculosis have shown hepatic injuries due to well-known complications of the core drugs like Isoniazid (INH) and Rifampicin (RIF) used in the treatment of this disease (Saukkonen *et al.*, 2006). INH–RIF dose, 50mg/kg/day each administered in animals showed maximum hepatotoxicity

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as compared to other doses (Brown *et al*., 2005). The lipids and proteins get accumulated in hepatocytes which results in chronic liver disease (Ashakumary & Vijayammal, 1993) with an abnormal protein secretion by hepatocytes (Vilstrup & Tygstrup,1985). During liver damage enzymes ALP, ALT, AST present in the liver cells escape into the serum, resulting in their increased concentration (Deb, 1998). Few reports have stated the hepatotoxicity provoked by Rifampicin (RIF) and Isoniazid (INH) determined by the increased concentration of liver biomarkers and histological deformities (Ravinder *et al*., 2006).

Oral treatment of *C.songarica* at a concentration of 200 mg/kg and 400 mg/kg significantly ameliorated the liver damage by minimizing AST, ALT and alkaline phosphate levels in rats, which is in correspondence with the group of rats dosed with a potent hepatoprotective drug; Liv-52 which is used as reference standard.

Isoniazid and rifampicin consumption increased the bilirubin level in the serum of hepatotoxic rats due to a cirrhotic liver condition which in turn increased the bilirubin release (Nithiyanandam & Evan, 2020). The decrease in the level of albumin and total protein is due to decreased synthetic capacity as a result of hepatic dysfunction (Thapa & Walia, 2007).

Ethanolic extract treatment of *Crataegus songarica* reduced the total bilirubin concentration and increased the proportion of total protein in INH + RIF caused hepatotoxicity groups which indicates its protective ability on the liver and amend its functional effectiveness. The histopathological screening revealed the inflammation in the liver towards centrilobular region and hepatocellular disintegration in INH + RIF treated groups (Kaplowitz, 2002; Jain et al., 2011; Ravikumar & Gnanadesigan, 2011, Sengupta *et al*., 2011, Hussain *et al*., 2011), Thirumalai *et al*., 2011, Madhu *et al*., 2012, Singh *et al*., 2012).

The above results strongly indicate the potential of ethanolic extract of Crataegus songarica in amelioration of hepatotoxicity induced by INH+RIF in rats.

The hepatoprotective activity elicited by the ethanolic extract of *Crataegus songarica* leaves might be due to its free radical scavenging activity and its ability to activate antioxidant enzymes due to the presence of phytoconstituents, flavonoid and phenolic contents.

Conclusively, this study showed that ethanolic extract of leaves of *Crataegus songarica* has a significant potential to protect against the hepatotoxicity induced by Isoniazid and Rifampicin. Thus, this experimentation constitutes a novel and fascinating proposal to prevent the INH+RIF induced hepatic damage. More detailed research is required to derive the mechanism of action and develop a hepatoprotective drug from the leaves of *Crataegus songarica*. Refinement of extracts and detection of the principle may yield active hepatoprotective ingredients.

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