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ORIGINAL ARTICLE

Protective effects of *Ficus religiosa* stem bark extracts against isoniazid and rifampicin induced hepatotoxicity in albino rabbits

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Abstract

Tuberculosis (TB) is a worldwide pandemic disease and its death ratio is highest after HIV/AIDS. Rifampicin (RIF) and Isoniazid (INH) are the first line antituberculosis drugs that are administered for 6-8 months depending upon the severity of disease. This standardized therapy is associated with a number of problems including neuropathy, gastrointestinal and hematological disorders. As plants are used for the management of many ailments so the current study was planned to evaluate protective effects of *Ficus religiosa* against adverse effects associated with antituberculosis therapy. A total of forty rabbits were divided into five groups. Group 1 was kept as control. Group 2 was given antituberculosis drugs while groups 3, 4 and 5 were given silymarin, ethanolic ficus extract, and aqueous ficus extract along with antituberculosis drugs orally for the experimental period. Blood samples were taken before drug administration and at 7^{th}, 14^{th}, 21^{st}, and 28^{th} days post treatment for hematological and biochemical analysis. Liver tissues were taken for histopathological studies. Results have indicated that group treated with INH+RIF exerted synergistic effects causing hepatocellular damage thus induces the release of aminotransferases. Group treated with ficus alcoholic extract significantly reduced this elevation in enzymes showing hepatoprotective properties against INH+RIF induced toxicity. Hematolgoical studies indicated non-significant differences in treated versus control groups. Histopathological results showed necrosis, hepatic vascular degeneration and congestion as an adverse effect associated with concomitant use of INH+ RIF. In conclusion, *F. religiosa* extracts have hepatoprotective effects when administered along with the RIF+INH comparable to that of silymarin.

Keywords

Ficus religiosa
Hepatoprotective
Isoniazid
Rifampicin
Silymarin
Tuberculosis

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Introduction

Tuberculosis (TB) is a worldwide pandemic disease produced by *Mycobacterium tuberculosis*. According to a study, tuberculosis is annually estimated to cause about 8 million new disease cases and about 3 million deaths, more than half of which are reported in Asia (Khan et al., 2000). Pakistan has population above 160 million and was approximately ranked at position 6 in global TB burden list (Hasan et al., 2009). Tuberculosis is second commonest death causing disease after HIV/AIDS. In Pakistan, tuberculosis incidence rate is 181/100,000 and a prevalence rate of 263/100,000 (WHO, 2002). In Punjab 51% cases were observed, in Sindh 23%, followed by 15% in Khyber Pakhtunkhwa region and in Baluchistan 3.5% cases were reported. The remaining proportion is being distributed in the northern and tribal areas in Azad Kashmir (Hasan et al., 2009).

Standardized short course chemotherapy (SSC) is the effective therapeutic regimen for patients with TB, usually treatment include drugs such as isoniazid

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(INH), ethambutol, streptomycin, rifampicin (RIF) and pyrazinamide taken for 6–8 months depending upon patients' severity of disease (Dye et al., 1998; Chaulk & Grady, 2000). INH metabolism is through acetylation by the hepatic enzyme N-acetyltransferase. It is acetylated into acetylisoniazid and then hydrolyzed into acetylhydrazine and isonicotinic acid. Acetylhydrazine is either hydrolyzed in hydrazine, or acetylated into diacetylhydrazine. It is reported that acetylhydrazine is the toxic metabolite of INH (Mitchell et al., 1976). RIF is deacetylated into deacetylrifampicin and hydrolyzes separately to produce a 3-formyl rifampicin. RIF may be responsible for hepatocellular dysfunction at the start of treatment, which can be resolved without discontinuing the drug (Acocella & Conti, 1980). RIF has unknown mechanism of hepatotoxicity. There is no clear evidence for the presence of toxic metabolite. RIF is responsible for the hepatic CYP450 system in the liver and intestine (Kolars et al., 1992). The use of RIF and INH in combination increases the risk of hepatotoxicity. RIF induces INH hydrolase, increasing hydrazine production when RIF is used in combination with INH especially in slow acetylators as illustrated in Figure 1. RIF also interacts with many antiretroviral drugs and affects the plasma levels of these drugs and increases the risk of hepatotoxicity (Kwara et al., 2005). Adverse effects which are very common with the use of INH include drug induced hepatitis, elevated liver transaminases, peripheral neuritis, skin eruption, vasculitis, arthritic syndrome, hematological problems like agranulocytosis, anemia, thrombocytopenia, hypersensitivity like fever and skin rashes. RIF causes hepatitis and hypersensitivity reactions, hematological disorders including thrombocytopenia, hemolytic anaemia and transient leukopenia, rash, fever, nausea, vomiting, chills, myalgia, nephritis, tubular necrosis and shock. Marked elevations of serum alkaline phosphatase are also recorded (Zaleskis, 2005; Brunton et al., 2006).

Medicinal plants are extensively used for the management and treatment of various diseases (Raza et al., 2015; Raza et al., 2016). Plant drugs are considered nontoxic and devoid of side effects (Bhawna & Kumar, 2009). A large number of plants and formulations have been claimed to have hepatoprotective activity. Nearly 160 phytoconstituents from 101 plants have been claimed to possess liver protecting activity. The evaluation of protective effects of plant extracts against antituberculosis treatment has not been studied so far. Due to their protective effects, it is the demand of time to utilize medicinal plants to cope with serious adverse effects associated with the use of standard antituberculosis drugs. *F. religiosa* (family *Moraceae*) is cultivated worldwide and inhabitant of India, Bengal, sub-Himalayan tract and central India. It is commonly known as peepal plant. It is traditionally used for the management and treatment as antidiabetic, anticonvulsant, wound healer, anti-inflammatory, analgesic, antianxiety, antimicrobial, hypolipidemic, antiviral, immunostimulatory, antioxidant, parasympathetic modulator, antiasthmatic, antitumor, estrogenic, antiulcer, apoptosis inducer, hypotensive and antihelminthic (Singh et al., 2011; Hashmi et al., 2013).

In spite of the vast pharmacological activities of this plant, its protective effects against hepatotoxicity associated with the use of antituberculosis drugs have not been documented. Hence, the current study was designed to evaluate the hepatoprotective effects of peepal plant extract against antituberculosis drugs (INH + RIF).

Materials and Methods

Plant material: Stem bark of *F. religiosa* was collected from the vicinity of University of Agriculture Faisalabad. The shade dried bark stem was powdered by the use of mechanical grinder, passed through mesh sieve and then stored in airtight container for further experimental use.

Preparation of plant extract: For the preparation of aqueous extract, powdered *F. religiosa* stem bark was macerated with distilled water for 48 hours along with occasional stirring at room temperature. After maceration, it was filtered through Whatman filter paper (Pandit et al., 2010). For the preparation of ethanolic extract, soxhlet apparatus was used, powdered *F. religiosa* (300 g) was filled in thimble and kept in thimble chamber of soxhlet apparatus. Four hundred milliliter (400 ml) of ethanol was added in flask and extraction was carried out. After 3 days fluid extract was separated and kept in a petri dish. It was then placed in freezer for solidification. After that it was subjected to lyopholization so that moisture content was evaporated. Procedure was carried out repeatedly (Mounnissamy et al., 2010).

Experimental protocol: A total of 40 rabbits were divided into 5 groups (n=8) as follows: Group 1 (Control), given seasonal fodder along with water *ad libitum* for 28 days; Group 2, routine diet + INH (50 mg/kg body weight) + RIF (250 mg/kg body weight) orally for 28 days; Group 3, routine diet + INH + RIF + Silymarin (100 mg/kg body weight) orally for 28 days; Group 4, routine diet + INH + RIF + Aqueous bark extract of *F. religiosa* (250 mg/kg body weight) orally for 28 days; Group 5, Routine diet + INH + RIF + Ethanolic bark extract of *F. religiosa* (250 mg/kg body weight) orally for 28 days.

Blood sampling: Blood samples were collected in heparinized tubes. For each rabbit five blood samples were drawn, first (1^{st}) blood sample was collected one day before drug administration followed at 7^{th}, 14^{th}, 21^{st} and 28^{th} day after drug administration. All the experiments were carried out in accordance with the

guidelines of the directorate of graduate studies and institutional animal ethical committee.

Biochemical analysis: The alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the serum sample was measured by commercially available kit of Randox Laboratories, United Kingdom. The standard curve for ALT and AST was obtained by plotting the measured absorbance of increasing amount of pyruvate standard against the known transaminase activities in U/L by using spectrophotometer (Biosystem, BTS-330, Biosystem, S.A. Costa Brava, Barcelona, Spain) at 546 nm wavelength. Increased concentration of pyruvate was allowed to react with 2, 4- dinitrophenyl hydrazine. These standard curves were used to determine the activity of ALT and AST.

Hematological analysis: Blood samples were collected for the analysis of hematological parameters; platelet count, RBCs count, WBCs count, PCV, Hb and ESR by using standard methods.

Histopathological examination: Formalin fixed liver biopsies were processed in graded ethanolic concentrations and fixed in paraffin blocks. Liver fragments were arranged vertical to the plane of the section in the block and 6 µm thick transverse fragments were cut and mounted on glass slides and stained with hematoxylin and eosin (H & E stain). Microscopy was completed on automatic light microscope with a 40X objective.

Statistical analysis: The results were expressed as mean±SEM. Statistical analysis was conducted by two way analysis of variance (ANOVA) followed by Duncan's Multiple Range test (DMR) at 5% level of significance (p<0.05) (Steel & Torrie, 1960).

Results

Biochemical analysis: Results showed that mean ALT and AST level (U/L) was increased significantly (p<0.05) in INH + RIF treated rabbits as compared to control group. While, the administration of silymarin reduced ALT and AST level towards normal. Whereas, the administration of alcohol and aqueous extract of *F. religiosa* further reduced this enzyme level, almost close to normal value while its hydro extract could not reduce the enzyme level significantly as presented in Figure 2 (a, b).

Hematological analysis: Mean values of hematological parameters showed that group treated with INH+RIF significantly increased platelet count, RBCs count, WBCs count, PCV and Hb levels while reduces ESR value when compared with control group. While these parameters showed improvement in groups treated with *F. religiosa* extracts comparable to group treated with silymarin as presented in Table 1.

Histopathology: Liver from control groups of animals indicated hepatocytes with normal hepatic lobular architecture. Normal hepatic sinusoids with portal tract were seen in the slides. Nucleus were prominent with nucleolus as shown in Figure 3a. Liver cells of INH+RIF treated rabbits showed severe degree of hepatic vascular degeneration. Hepatic cells showed individual necrosis. Cell swallowing and mild degree of congestion was also observed throughout the cytoplasm of the cells (Figure 3b). Hepatocytes of INH+RIF+ ethanolic extract treated rabbits showed pyknotic nuclei in few spaces. Mild degree of cellular degeneration was present around the blood vessels and mild degree of hyperplasia was also observed in biliary cells. Nuclei were normal having nucleolus (Figure 3e). Hepatocytes of INH+RIF+aqueous extract treated rabbits showed pyknotic nuclei. Moderate degree of cellular degeneration was also present and moderate degree of hyperplasia was also observed in biliary cells (Figure 3d). Nuclei were normal having nucleolus in some hepatocytes while some hepatic cells do not have normal nuclei. Liver cells of silymarin treated groups indicated normal, lobular architecture having no degeneration (Figure 3c).

Table 1: Haematological parameters (Mean±SEM) with oral drugs and *F. religiosa* extracts daily administration for 28 days in rabbits (n=8).

Parameters	Groups				
	1	2	3	4	5
Platelet count (10^3/µl)	249163±1902.49^{A}	168285±36954.29^{A}	126035±35004.47^{B}	262692±48870.70^{AC}	190313±53340.62^{A}
RBC count (10^6/µl)	5.27±0.19^{A}	6.02±0.27^{A}	5.88±0.30^{A}	5.70±0.19^{A}	5.92±0.27^{A}
WBCs count (10^3/µl)	16179±252.49^{A}	27726±85019.07^{A}	24825±90354.01^{A}	22386±6990.53^{A}	10574±1478.61^{A}
PCV (%)	36.23±0.85^{A}	40.51±1.64^{A}	39.49±1.23^{A}	38.79±1.26^{A}	39.77±1.47^{A}
Hb (g/dl)	12.04±0.13^{A}	12.31±0.51 ^{A}	12.60±0.47^{A}	11.08±0.21^{A}	12.24±0.31^{A}
ESR (mm/hr)	4.16±0.18^{A}	3.63±0.30^{B}	3.58±0.19^{BC}	4.10±0.39^{AB}	3.08±0.30^{C}

Means sharing similar letter in a column are statistically non-significant (p>0.05). 1=Control and given seasonal fodder along with water *ad libitum* for 28 days; 2=routine diet + INH (50 mg/kg body weight) + RIF (250 mg/kg body weight) orally for 28 days as hepatotoxic drugs; 3=routine diet + INH + RIF + Silymarin (100 mg/kg body weight) orally for 28 days; 4=routine diet + INH + RIF + Aqueous bark extract of *F. religiosa* (250 mg/kg body weight) orally for 28 days; 5=routine diet + INH + RIF + Ethanolic bark extract of *F. religiosa* (250 mg/kg body weight) orally for 28 days.

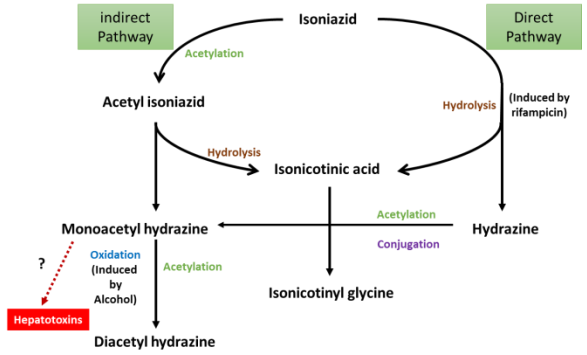

Figure 1: Metabolism of isoniazid and its influence by rifampicin and alcohol (Adopted from Yew & Leung (2006).

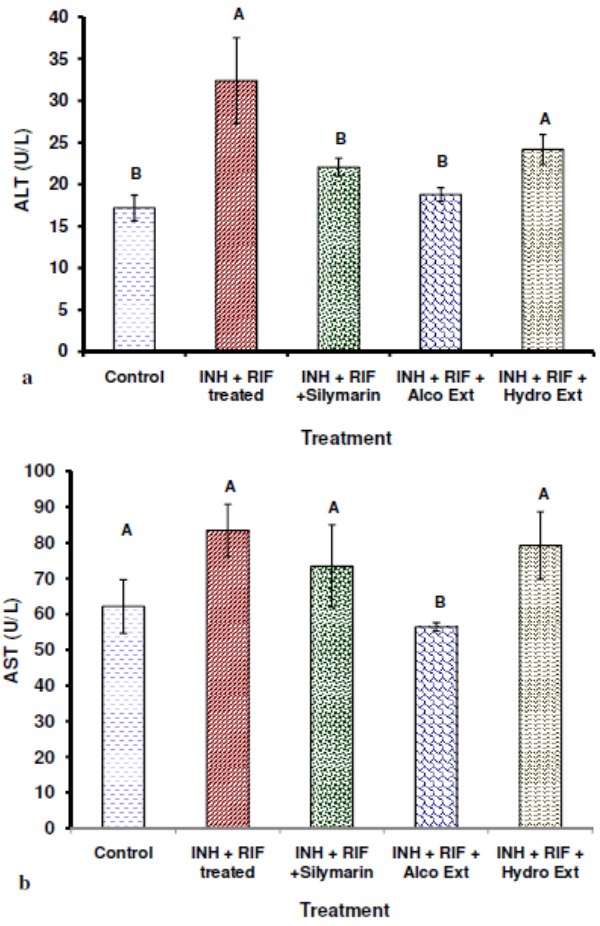

Figure 2: Mean±SEM serum (a) ALT level (b) AST level (U/L) before and after treatment with per oral drugs and *F. religiosa* extracts in rabbits (n=8) for 28 days.

Discussion

TB is an infectious and hazardous disease, declared as global emergency by WHO in 1993, reduces humans' life span day by day (Muthulingam, 2008). As liver is the vital metabolizing organ so it is affected foremost. Hepatotoxicity is the prominent adverse effect faced worldwide due to prolonged treatment protocol and high doses. Acute hepatic failure is frequently caused by drug toxicity in US (Chang & Schiano, 2007). INH and RIF are reported hepatotoxic. *F. religiosa* is reported to have antioxidant and antiulcer effect, so this study was conducted to record observations regarding the effectiveness of aqueous and alcoholic extract against INH and RIF induced hepatotoxicity. Biochemical analysis was kept as an indicator to conclude hepatic injury (Yimer et al., 2008; Ozer et al., 2010). Combined administration of INH and RIF prominently disturbed hepatic function as visualized by the level of serum enzymes along with liver histopathology studies.

In the current study, it has been shown that INH and RIF administration increases ALT and AST levels which showed hepatic damage while administration of alcoholic and aqueous extract of *F. religiosa* reduces ALT and AST levels comparable to synthetic drug (silymarin) proved it to be hepatoprotective. Earlier studies have shown that hepatocytes and bile duct (epithelium) are targeted by antituberculosis drugs toxicity which results in raised serum profile (Tasduq et al., 2006). However, the proposed mechanism by which the hepatotoxicity occurred might be due to bioactivation induced by CYP 450 2EI and disturbance in membrane integrity would possibly be responsible for this toxicity (Tasduq et al., 2007). In an extensive study, Wu et al. (1990) suggested that the oxidative stress might be one of the reasons for INH + RIF induced hepatotoxicity. Oxidative stress is the major contributor of INH and RIF induced hepatotoxicity (Sodhi et al., 1998). INH is much more hepatotoxic or RIF, it is not clearly described yet so far, but the presence of both provides synergistic response (Wu et al., 1990; Steele et al., 1991). The combination of INH + RIF was reported to result in higher rate of inhibition of bile secretion and increased in liver cell lipid peroxidation. So, in case of increased lipid peroxidation oxidative stress occurs which induced the hepatic damage. Hepatotoxicity is reported to occur more frequently in patients receiving INH + RIF in combination compared to those receiving only isoniazid (Steele et al., 1991). Drug-drug interaction with cytochrome P450 induced by rifampicin can be a possible cause of this (Mitchell et al., 1975; Sarma et al., 1986). Results of current studies are in accordance with previous research studies (Maryam et al., 2010; Uduman et al., 2011).

Hematological parameters has also been affected by anti-tuberculosis drugs, as these may cause decrease in platelets count thus leads towards thrombocytopenia (Koju et al., 2005; Garg et al., 2007). Speculated mechanism is that thrombocytopenia may be caused by

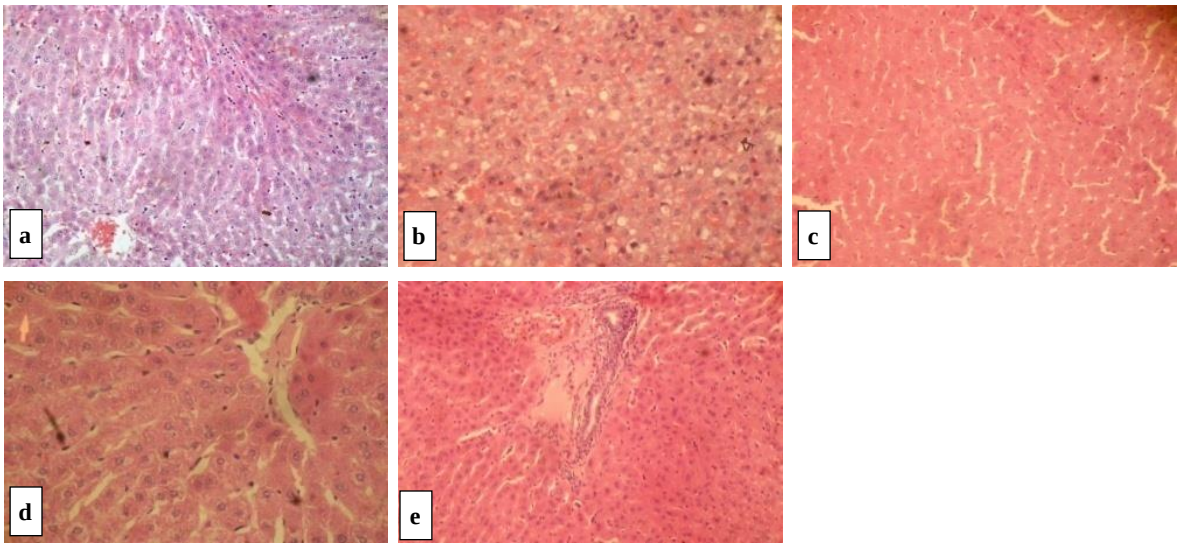

Figure 3: Liver of rabbit (a) control group, (b) routine diet + INH (50 mg/kg body weight) + RIF (250 mg/kg body weight), (c) routine diet + INH + RIF + Silymarin (100 mg/kg body weight), (d) routine diet + INH + RIF + Aqueous bark extract of *F. religiosa* (250 mg/kg body weight), (e) routine diet + INH + RIF + Ethanolic bark extract of *F. religiosa* (250 mg/kg body weight) with daily oral administration for 28 days (H & E, x 20).

RIF as its major side effect. (Nagayama et al., 2004) which is also linked with hepatic diseases (Spivak, 2000). Garg et al. (2007) reported in his study that platelet count was normalized during treatment with INH along with other second line antitubercular drugs (except RIF) but as soon as the RIF therapy was started platelet count dropped which confirms drug induced etiology. Two mechanisms are reported in earlier research studies that either it suppress platelet production or secondly it destroy them immune-logically by compliment activation which causes the formation of drug and antibody complex. While, earlier researches also show Hb and RBC level increased in a patient who was on antitubercular therapy up till 2 years time frame which was in accordance with our results. Research proposed possible mechanism in TB like bone marrow suppression, nutritional deficiencies, decreased uptake and iron utilization, hemolysis and malabsorption syndrome (Kuo et al., 2001). Another case study supports this hypothesis, as RIF is reported to be associated with hemolysis massively. An antibody related to drug gets fixed with the surface of RBCs and activates compliment in the availability of drug. This results in the destruction of RBCs. Direct antiglobulin test confirmed the presence of compliment (Lakshminarayan et al., 1973). In current study, WBCs count in INH + RIF treated group is increased as compared to control group in statistically non-significant fashion. No evidence is available to justify this. Suggestively chronic infection can be the possible cause of leukocytosis as in lung diseases such as tuberculosis. Might be it is a defensive response of our body to overcome infection. This hematological abnormality is associated with tuberculosis (Glasser et al., 1970; Ursavas et al., 2010). Suggestively it can be an adverse effect of drugs.

Histopathological studies in rabbits with INH+RIF induced hepatotoxicity shows higher degree of hepatic vascular degeneration along with necrosis. Cell swallowing and mild degree of congestion throughout the cytoplasm of hepatic cells was observed. While, concomitant administration of ethanolic extract of *F. religiosa* prevented INH+RIF induced histopathological injuries. This was evident from mild degree of degeneration in alcoholic extract treated rabbits as compared to INH+RIF treated groups. While aqueous extract treated group exhibited moderate degree of degeneration. The silymarin treated group has shown normal nuclei with nucleolus. Studies have reported that *F. religiosa* (alcoholic extract) exhibits antiulcer activity possibly due to existence of flavonoids and sterols. In experimental studies, Shetty et al. (2008) suggested that a constituent of *F. religiosa* possesses scavenging abilities that contributes towards preventing mucosal injury. In another study it was proposed that anti-inflammatory properties may be responsible for gastroprotective effects of *F. religiosa* ethanolic bark extract of *F. religiosa* mimic gastric acid secretion because of acid metabolizing and anti-secretory potential (Khan et al., 2011). The results of current study are in accordance with the research study conducted by Khan et al. (2011), however in his study ethanolic bark extract mimic gastric acid secretion because of acid metabolizing and antisecretory potential. The proposed mechanism by which it reduces hepatotoxicity may be due to its antioxidant properties

because of flavonoids, saponins and tannins. *F. religiosa* extract promotes prostaglandins synthesis and enhances mucosal secretion and antagonizes the effect of hepatotoxins (Jain et al., 2002) which could be related to the presence of various polyphenolic substances like saponins, flavonoids and tannins (Borrelli & Izzo, 2000; Shokunbi & Odetola, 2008; Abdulla et al., 2010). These compounds possesses scavenging ability thus contributes towards preventing mucosal injury also (Shetty et al., 2008).

Conclusion: *F. religiosa* stem bark extract proved efficient in lowering hepatotoxicity induced by the concomitant use of INH+RIF as evidenced by significant decrease in ALT and AST. Histological findings have also indicated the protective effect of alcoholic extract of plant against INH+RIF induced hepatotoxicity.

Conflict of interest: All authors declare no conflict of interest.

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