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LIFAGO DIELSII SCHWEINF AND *MUSCHL* (ASTERACEAE) AMELIORATES LIVER INJURY INDUCED BY ISONIAZID AND RIFAMPICIN IN RAT

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ABSTRACT

The *Lifago dielsii* has been used in Algerian folk medicine for the treatment of various disorders since ancient time. In this study we aimed to investigate the possible hepatoprotective effects of n-BuOH extract of *Lifago dielsii* (BELD) in isoniazid (INH) and rifampicin (RIF)-induced liver injury model. Administration of INH and RIF for 14 days resulted in hepatic failure as evidenced by the elevation of aspartate aminotransferase (AST), alanine aminotransaminase (ALT), and hepatique oxidative stress which was associated with extensive hepatic parenchyma alteration. The co-treatment of rat with BELD (200 mg/kg; 14 days) attenuated the increase of hepatic dysfunction markers (AST, ALT) and suppressed malondialdehyde (MDA). BELD co-treatment restored GSH level and reversed the antioxidant parameter SOD as compared to the quercetin co-treatment in suppressing MDA level and in GSH, SOD levels preservation. The histopathological protection was clearly confirmed by the reduction of hepatic alteration. Conclusion: The data obtained in the present study revealed that *Lifago dielsii* butanolic extract has significant

hepatoprotective activity against INH and RIF-liver injury that may be attributed to its bioactive constituents.

KEYWORDS: *Lifago dielsii*, Isoniazid, Rifampicin, oxidative stress, liver injury.

INTRODUCTION

Hepatotoxicity is one of the most serious adverse effects of anti-tuberculosis drugs.[1,2] It is associated with high morbidity and mortality.[3] Isoniazid (INH) and rifampicin (RIF), two front-line drugs used in antituberculosis therapy, have been known to be potentially hepatotoxic and may lead to drug-induced liver injury[4,5] by production of many metabolic and morphological aberrations in the liver due to the fact that the liver is the main detoxifying site for these antitubercular drugs.[6] The risk of hepatic injury is enhanced when the two drugs are used in combination.[7] It was reported in their meta-analysis that INH and RIF given together produce hepatotoxicity more frequently than INH without RIF.[8] The increased risk of INH and RIF combination induced hepatotoxicity has been attributed to the interaction between the metabolism of INH and RIF.[9,10] INH could directly or indirectly generate acetyl hydrazine and hydrazine by N-acetyl transferase and amide hydrolysis enzyme, they are important toxic metabolites which are connected with liver toxicity of rats or rabbit.[11] RIF has liver toxicity and it is a kind of strong liver inducers of drug metabolizing enzymes.[5]

Oxidative stress is one of the mechanisms for INH and RIF induced hepatic injury.[12] Alterations of various cellular defence mechanisms including enzymatic and non-enzymatic components have been reported to be involved in INH and RIF-induced hepatotoxicity.[13]

Plants are the gifts of nature and have been utilized by human beings for basic preventive and curative health care since time immemorial. Secondary metabolites derived from plant extracts have been reported scientifically for biological activities and can also protect humans against several diseases.[14] Biologically active components with antioxidant properties are contributed to the protection of cells and tissues against deleterious effects of reactive oxygen species and other free radicals.[15,16,17] The genus Lifago which is a monotypic genus belonging to the Asteraceae family and represented by only one species: *Lifago dielsii Schweinf* & *Muschl* is an endemic scrub to westerner Sahara.[18] In south Algeria, the aerial parts of *L. dielsii* are used in folk medicine as antidiabetic. It is also demonstrated that flowers and stems of plant contained several phyto-constituents such as

triterpenes, saponins, coumarins, tannins, alkaloids and flavonoids.[19] These phyto-constituents can be used for the treatment of various diseases due to wide range of biological activity.[20]

The current study was carried out to investigate firstly the *In vitro* antioxidant activity such reducing power of n-BuOH extract of *L. dielsii* (BELD) and the potential hepatoprotective effect of BELD against INH and RIF induced liver injury in Albino wistar rats. To the best of our knowledge, no reports are available on the in vivo antioxidant potential and hepatoprotective effect of BELD.

MATERIALS AND METHODS

Drugs and chemicals

INH and RIF used for the experiments were obtained from Centre of Tuberculosis chemotherapy, Constantine. All other reagents used in the analysis were of analytical grade or of the highest purity and obtained from sigma chemicals.

Plant material and extraction

L. dielsii. (Asteraceae) was collected at the flowering stage in April 2011 in Southern Algeria and authenticated by Mr. Abdelhakem (Director of forest, Bechar, Algeria) on the basis of Ozenda.[21] A voucher specimen has been deposited, under N LDC /2011, in the Herbarium of the varenbiomol unit research, University of Constantine 1. The aerial parts of L. dielsii. (2000 g) were macerated with MeOH/H_2O (80:20 v/v) for 48 hr three times. The crude extract was concentrated at room temperature and diluted with 900 ml H_2O. The remaining aqueous solution was extracted successively with $CHCl_3$, EtOAc and n-BuOH and the water insoluble MeOH part. The n-butanolic extract of plant was served for the in vivo experiments.

In vitro antioxidant activity

Reducing power assay

Reducing power assay is well known method to assess total antioxidant capacity and considered as a useful indicator of the body's antioxidant status to counteract the oxidative damage due to ROS. The reducing power of the n-BuOH extract was estimated using the method of Oyaizu[22]. Various concentrations of 0.2 mL extract (0-800 µg/ml) were mixed with 2.5 mL of phosphate buffer (0.2 mol/L, pH 6.6) and 2.5 mL of potassium ferricyanide (1%). After incubation at 50 °C for 20 min, 2.5 mL of TCA (10%) was added; each mixture

was centrifuged at 1000 rpm for 10 min. Subsequently, 2.5 mL of the supernatant was collected and mixed with 2.5 mL of deionized water and 0.5 mL of ferric chloride (0.1%). The absorbance was measured at 700 nm. The increased absorbance of the reaction mixture indicates an increase in reducing power. Quercetin are used as standard for comparison.

In vivo hepatoprotective analysis

Animals and experimental design

A total of 24 adult male Albino wistar rats, weighing 180-250 g were used as experimental animals in this study. The animals were housed in well ventilated large spacious polypropylene cages and had 12 h light and dark cycle throughout the experimental period and they received a balanced diet of commercially available pellet rat feed and water and libitum. The study was approved by the Local Ethics Committee for animal experiments. The research complied with the current European laws, and procedures involving the animals and their care conformed to the institutional guidelines, in compliance with national and international laws and Guidelines for the Use of Animals in Biomedical Research.[23]

The experimental animals were randomly divided into six groups of six animals, each as follows.

Group 1 normal control untreated rats received orally a volume of normal saline. Hepatotoxicity was induced in Group 2 by the intraperitoneally injection of INH and RIF combination [INH (75 mg/kg) + RIF (150 mg/kg)] once daily for 14 days. In Group 3 rats were orally administered with quercetin (50 mg /kg) and with intraperitoneally injection of antitubercular drug described in group 2, for 14 days. Group 4 rats were orally administered with BELD (200 mg /kg) and with intraperitoneally injection of antitubercular drug described in group 2, for 14 days. Rats had free access to food and drinking water during the experiment. At the final stage of the experiment, on the 15th day, the animals were sacrified by decapitation. Blood was collected from the retro-bulbar vessels into dry clean tubes for determination of liver function markers.

Assessment of liver function markers

Levels of various liver marker enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) were determined in the sera using the commercial assay kits. The results were expressed as an international unit/liter (IU/l).

Assessment of hepatic oxidative stress parameters

Preparation of liver homogenate

The liver samples were homogenized in KCL (10 mM) phosphate buffer (1.15 %) with EDTA (pH 7.4) and centrifuged at 8000 rpm for 10 min for the measurement of MDA level, the supernatant was centrifuged again at 12,000 g for 60 min for the measurement of antioxidant status.

Determination of lipid peroxidation level

According to the method of Ohkawa *et al.* [24], 0.5 ml of tissue homogenate were added to 0.5 ml 25 % trichloroacetic acid and 1 ml of 0.67 % thiobarbutiric acid solution. The mixture was heated for 45 min in boiling water bath. After cooling, 4 ml of n-butanol were added and mixed vigorously. The mixture was centrifuged for 10 min at 3000 rpm. The resultant butanol layer was separated and MDA content was determined by measuring the absorbance at 535 nm. The concentration of MDA was calculated by the absorbance coefficient of MDA-TBA complex 1.56 x 10^5 $M^{-1}cm^{-1}$ and expressed in nmol/g tissue.

Determination of reduced glutathione (GSH)

GSH level was performed according to the method of Ellman.[25] An aliquot of homogenate was precipitated with 0.05 ml of 50 % (w/v) trichloroacetic acid and then centrifuged at 1000 g for 5 min. The reaction mixture containing 0.25 ml of supernatant,1 ml of 0.2 M Tris-HCl (containing 1 mM EDTA, pH 8.9) and 0.05 ml of 10 Mm DTNB in absolute methanol was kept at room temperature for 5 min, and the yellow color developed was measured spectrophotometrically at 412 nm. Results were calculated from a standard GSH curve as µg/mg protein.

Determination of superoxide dismutase (SOD)

SOD activity was measured as previously described by Marklund and Marklund.[26] This method is based on the ability of SOD to inhibit the autoxidation of pyrogallol. Briefly, 0.1 ml of liver homogenate was mixed with 1.5 ml of 20 mM Tris-HCl (containing 1 mM EDTA, pH 8.2), then 0.1 ml of15 mM pyrogallol was added. Thereafter, the change in OD per min was determined by monitoring the increase in OD at 420 nm for 3 min for the samples. The percentage of inhibition for the samples was calculated by the aid of running a control with no sample under the same conditions. SOD enzyme activity was expressed as U/mg protein, where one unit was defined as the amount of the enzyme that inhibited the rate of pyrogallol

autoxidation by 50 %. The protein concentration was determined by the method of Lowry et al.[27] using bovine serum albumin (BSA) as the standard.

Histopathological evaluation

Liver tissue for histopathological analysis was fixed in 10 % buffered formalin saline, processed by routine histology procedures and embedded in paraffin. Tissue sections (4-5 µm) were stained with haematoxylin-eosin (H&E400 x, H&E, 1000 x), and examined for possible histopathological changes.

Statistical analysis

The results were expressed as Mean ± S.D. Statistical analysis was carried out by Student's test. The obtained Results were considered statistically significant when p values were $P<0.05$ or $P<0.01$.

RESULTS

***In vitro* antioxidant activity**

Reducing power

Reducing power measure the reductive ability of antioxidant, and it is evaluated by the transformation of Fe^{+3} to Fe^{+2} in the presence of the sample extract.[28,29] Figure1 shown that the reducing power of the BELD increased with increase in concentration of the extract and the significant increasing absorbance was found to be 1,35±0,04 at 800 µg/ml, quercetin as standard reference achieve a maximum effect (1,44±0,03) at 400 µg/ml.

In vivo hepatoprotective effect

Liver function marker enzymes

The administration of INH and RIF for 14 days produced severe liver damage characterized by significant ($P<0.01$) elevation in serum ALT, AST. However a significant ($P<0.01$) reduction (66.69 % - 65.34 %) in serum ALT and AST activities was observed in the groups co-treated with BELD (200 mg/kg) and in the group co-treated with quercetin (50 mg /kg) (8513 % - 87.83 %), compared with INH and RIF-groups (Fig. 2).

Hepatic oxidative stress parameters

Administration of INH and RIF caused a significant increase ($p<0.01$) in MDA concentration (2 folds) and significant ($p<0.01$) depletion in the status of hepatic GSH level and in the activity of SOD as hepatic antioxidants parameters when compared with the normal control

group. However the co-retreatment with 200 mg/kg of BELD significantly (P<0.01) reduced hepatic MDA level (80.09 %) and significantly (P<0.01) raised the levels of liver antioxidant parameters (GSH; 70.63%) and, (SOD; 71.45 %) as compared to the INH/RIF-rats. Quercetin as standard drug supressed (87.21 %) significantly (P<0.01) the hepatic MDA level and also significantly (P<0.01) helps in the preservation of antioxidants parameters, GSH (88.82 %) and SOD (88.61 %) (Fig. 3).

Histopathologic examination

Histological observation of the liver supported the results obtained above from biochemical index and antioxidant profile measurements and further confirmed hepatoprotective potential of BELD. A normal cellular architecture and a central vein normal with portal triad and sinusoidal spaces were observed in the control group (Fig. 4A; a). However, INH and RIF intoxicated treatment exhibited severe histopathological changes, such as hepatic necrosis and dilation of sinusoids, fatty accumulation (steatosis) and massive inflammation (mononuclear inflammatory infiltrates in portal triad region) (Fig. 4B; b). Co-retreatment with 200 mg/kg of BELD prevented the histopathological changes as a minimal degenerative changes, minimal centrilobular fatty, granular degeneration of hepatocytes and the absence of necrosis (Fig. 4C; c). quercetin co-treatment ameliorate also the histopathological changes from INH and RIF-induced liver injury in the experimental rats (Fig. 4 D; d).

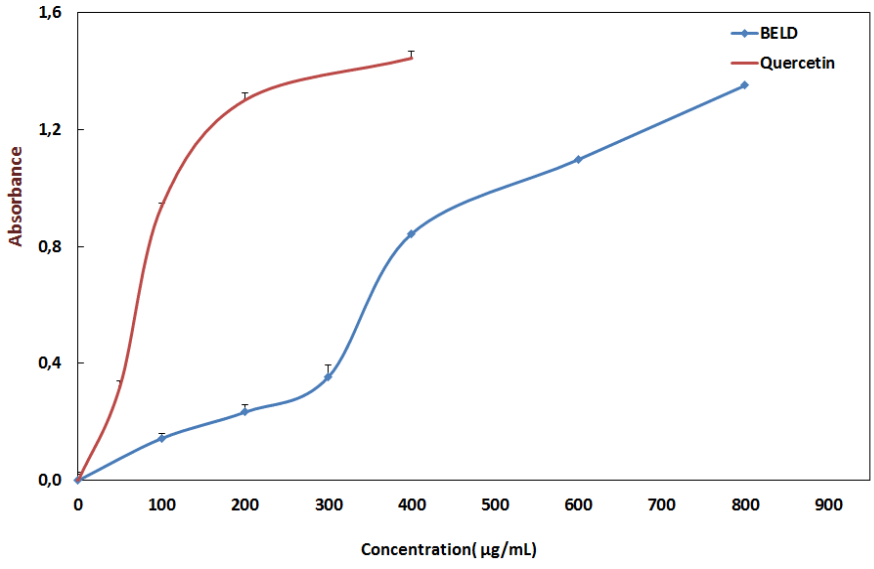


Fig 1: Reducing power of BELD and quercetin. Each value represents a mean ± SD (n=3), P<0.05.

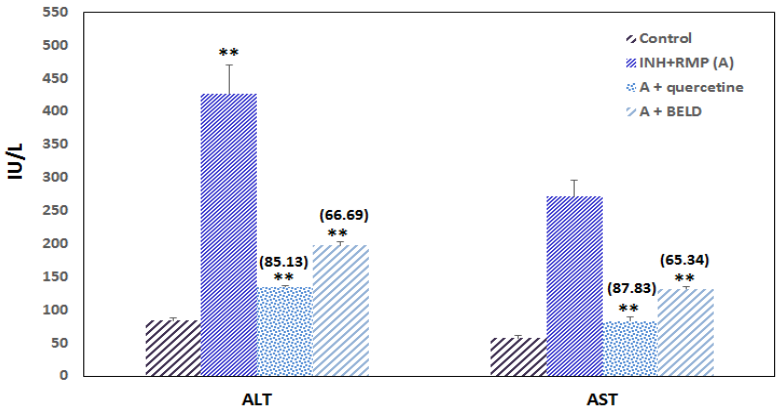


Fig 2: The effect of BELD (200 mg/Kg) on hepatic function markers in INH-RIF-rats. Each value is the mean ± SD (n=6), **p< 0.01:INH-RIF vs control; **p< 0.01: INH-RIF + BELD vs INH-RIF; **p< 0.01: **p< 0.01 NH-RIF + Quercetin vs INH-RIF. Values in parentheses : % of protection.

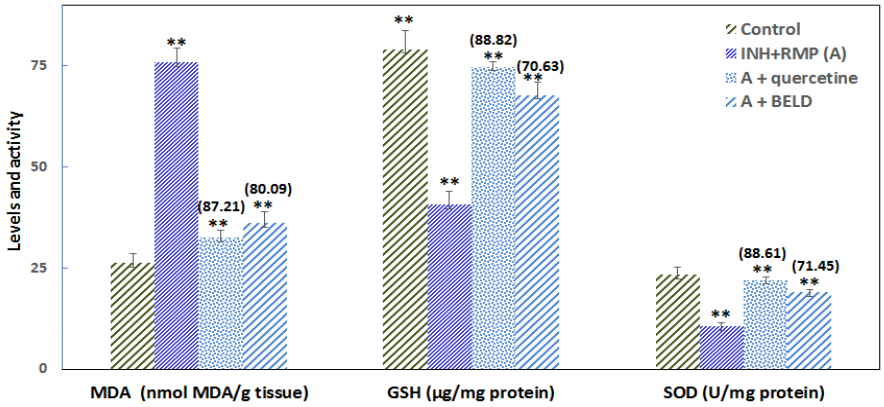
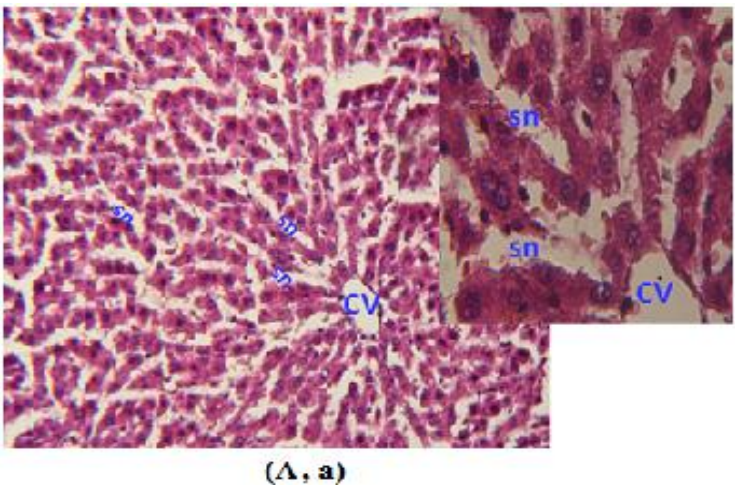


Fig 3: The effect of BELD (200 mg/Kg) on hepatic oxidative stress markers in INH-RIF-rats.Each value is the mean ± SD (n=6), **p< 0.01:INH-RIF vs control; **p< 0.01: INH-RIF + BELD vs INH-RIF; **p< 0.01: **p< 0.01 NH-RIF + Quercetin vs INH-RIF. Values in parentheses : % of protection.



(A, a)

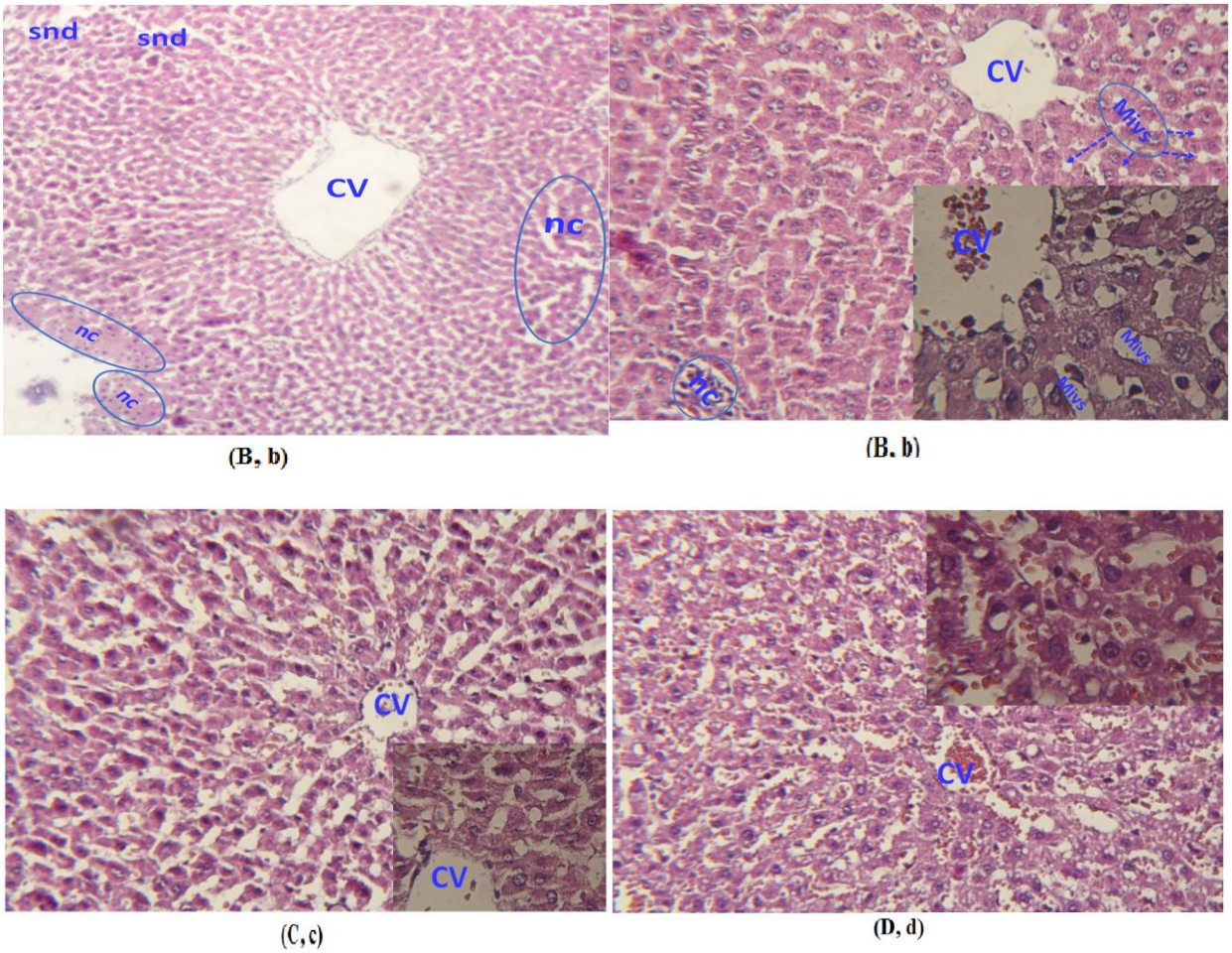


Fig 4. Micrographe of histopathological examination (H&E,400 x, 1000x).

A,a: (H&E, 400 x, H&E,1000x) control: normal cellular architecture, compact lobular arrangement, sinusoidal (sn)spaces, central vein (cv). B,b:(H&E,400 x, H&E,1000x) INH and RIF-treated rats ,sinusoidal dilation(snd), balloning degeneration, loss of cell boundaries and fatty accumulation (microvesicular steatosis; Mivs) wich was predominant in mid-zones, centrilobular hepatic necrosis(nc) with massive inflammation (leukocyte inflammatory infilterates in portal triad region) C,c: (H&E,400 x, H&E,1000x), INH and RIF+ BELD: a moderate degree of steatosis ,slight degeneration in hepatic parenchyma. D,d. :(H&E,400 x, H&E,1000x), INH and RIF+ quercetin: preserved hepatic architecture, regular hepatocyte cords, less fatty change.

DISCUSSION

Isoniazid and rifampicin being the first line drugs used as antituberculous chemotherapy, are known to be associated with hepatotoxicity.[30,1] The frequency of this hepatotoxicity is increased when these drugs are used in combination.[10,31,32] INH could directly or indirectly

generate acetyl hydrazine and hydrazine by a series of enzymes including cytochrome P450.[33] and could induce oxidative stress to cause hepatotoxicity.[34] RIF is thought to enhance this effect by enzyme induction. [35] In the present investigation, animals treated with INH and RIF developed hepatic injury that was manifested by a significant increased of serum level of hepatic enzymes AST, ALT, compared to control animals. This increase can be attributed to hepatic structural damage.[36]

On concurrent, administration of BELD extract at a dose of 200 mg/kg improved liver function by decreasing significantly the serum levels of AST and ALT, these indicating the hepatopprotective effect of BELD that could be possibly due to the stabilization of plasma membrane as well as repair of hepatic tissue damage caused by INH and RIF administration. Previous studies have demonstrated that oxidative stress is a major mechanism in the development of INH and RIF induced hepatotoxicity.[37] In our study INH and RIF administration produce MDA in the liver tissue, affecting cellular structure and function, increased amount of MDA in INH and RIF-induced liver signifies the enhance degree of lipid peroxidation, which can lead to liver damage. Antitubercular drug mediated oxidative damage is generally attributed to the formation of the highly reactive oxygen species, which act as stimulators of lipid peroxidation and source for destruction and damage to the cell membrane leading to its rupture and subsequent release of the cytosolic contents.[38,39]

In the present study, BELD (200 mg/kg) co-treatment was found to prevent the progression of hepatic injury in rats intoxicated with INH and RIF for 14 days as confirmed by decreasing the elevated MDA contents. It was shown that the changes in lipid peroxidation in rats treated with INH and RIF were also accompanied by a concomitant decrease in the activities of the antioxidant involved in the removal of superoxide anions and peroxides, namely GSH and SOD. SOD is the first antioxidant enzyme to deal with oxyradicals by accelerating the dismutation of superoxide to hydrogen peroxide. The decrease in SOD activity could cause the initiation and propagation of lipid peroxidation in the INH and RIF-treated rat.[40] It is well acknowledged that excessive lipid peroxidation increased GSH consumption.[41] SOD plays an important role in scavenging toxic intermediates of incomplete oxidation, which is regarded as one of the most primary antioxidant enzymes in the enzymatic defense mechanism.[42] SOD scavenges the superoxide anion radical ($O_2^{\bullet -}$) to oxygen and hydrogen peroxide (H_2O_2). H_2O_2 and lipid peroxides may then be converted and reduced by both CAT and GPx. A decrease in the both activities in liver tissue of INH and RIF-induced group was

largely due to the impairment of antioxidant enzymes that safeguard cells against reactive oxygen.

The GPx is able to protect the cell against oxidative stress via the reduction of H_2O_2 and lipid peroxides, using glutathione as an electron donor.[43] GSH, an abundant tripeptide in liver, is an important defense molecule against such as free radicals, peroxides and akylating agents[44]. It plays a pivotal role in the detoxification processes. The enhanced levels of GSH protect the cells against a potential oxidative damage.[45]

BELD (200 mg/kg) was able to normalize the elevated biochemical oxidative stress markers, and restored GSH normal level that plays an important role in the antioxidant defense mechanism in liver of in INH / RIF-rats. In the present study, histological evaluation was undertaken to support the biochemistry profiles. The presence of injuries in livers of INH/RIF-rats was revealed by histopathological examinations; hepatocellular disintegrate, mild necrosis, fatty accumulation, and inflammation in the liver cells was observed. These morphological changes reflecting enhancement of pathological alterations in livers by INH and RIF which have shown that liver It might be due to the formation of highly reactive radicals because of oxidative threat induced by INH/RIF. The hepatoprotective effect of BELD was associated with preservation of GSH concentration, upregulation of SOD, prevention hepatic parenchyma from increased MDA and reduction of the pathological changes in the INH–RIF -treated liver.

A plausible justification for hepatoprotective effects of BELD may be due to its antioxidant effect as the reducing power that proved in the current study, in which the antioxidant action of reductones is based on the breaking of the free-radical chain by donating a hydrogen atom.[29] Reductones also react with certain precursors of peroxide, thus preventing peroxide formation. Also this justification may be on the base of our previous study that reported the antioxidant capacity of BELD, by using the *In vitro* DPPH radical model flavonoids.[19] These antioxidant effects of BELD may be due to the presence of bioactive compounds such as flavonoids and phenolic acids, which were highly detected in BELD, together other several phytoconstituents such as triterpenes, saponins, coumarins, tannins, alkaloids[19] that have been reported to possess hepatoprotective effects[46] due to their wide range of biological activity.[47] These phytoconstituents may acted as reducing agents, hydrogen donors, singlet oxygen quenchers and potential metal chelators.[48,49,50]

CONCLUSION

Our finding suggests that the n-butanolic extract of *L. dielsii* has potent antioxidant activity against free radicals, prevent oxidative damage to the altered liver membrane by reducing the amount of lipid peroxides, minimized disruption of structure of hepatocytes and afford significant hepatoprotection against INH and RIF induced liver injury in rats by enhancing the levels of antioxidant parameters which were possibly at least in part a mechanism in hepatoprotective effect exhibited by *L. dielsii*. However, further studies are still needed to elucidate possible molecular mechanisms underlying the protective effects of *L. dielsii*.

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