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GC/MS assisted phytochemical analysis of *Ajuga parviflora* leaves extract along with anti-hepatotoxic effect against anti-tubercular drug induced liver toxicity in rat

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Abstract: Owing to its traditional applications, the current study focuses on *Ajuga parviflora* (*A. parviflora*) leaves extract for phytochemical and pharmacological analysis. The principle constituents were identified through gas chromatography (GC), and gas chromatography/mass spectroscopy (GC/MS), these includes phthalic acid, squalene, α-tocopherol, vitamin E, phytol, 2-methylenecholestan-3-ol, stigmasterol, cholest-22-ene-21-ol and 3,5-dehydro-6-methoxy. Hepatoprotective effect of *A. parviflora* was evaluated through isoniazid and rifampicin (INH and RFP) induced hepatotoxicity in rat. Animals in group A were treated with INH and RFP 50 mg/kg. Animals in group B, C, and D were pre-treated with *A. parviflora* extract at 100, 200 and 300 mg/kg dose prior drug administration. *A. parviflora* extract at 200 and 300 mg/kg in group C and D significantly reduced aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and bilirubin ($p<0.001$) as compare to group B (100mg/kg). Total protein (TP) was also significantly ($p<0.01$) reduced in group C and D at dose of 200 and 300 mg/kg, respectively. The extract pre-treated animals with (*A. parviflora*, 200, and 300 mg/kg) showed that the epithelium of the central portal vein is intact with replete glucagon. The pre-treatment with *A. parviflora* protected the liver from INH and RFP induced hepatotoxicity. The results of pre-treated animals with *A. parviflora* 200, and 300 mg/kg dose prettily revert the severely disturb parameters like, cytolysis, lymphocytic infiltration, and lymphoid aggregate in portal vein and hydropic degeneration. The decrease peroxisome proliferator-receptor activator-δ (PPAR-δ) gene expression by INH, and RFP was significantly up regulated by *A. parviflora* extract in pre-treated animals at 200 and 300 mg/kg dose. These findings provide baseline pharmacological uses of *A. parviflora* in liver disorders. Further investigations are required for identification and isolation of biologically active components responsible for pharmacological activity.

Keywords: *Ajuga parviflora,* phytochemical analysis, GC/MS, hepatoprotection, gene expression, PPAR-δ

INTRODUCTION

Liver diseases gain much attention in recent years (Asrani *et al*., 2019), and are considered one of the leading global health problem in developing countries. Detoxification and metabolism of xenobiotic is one of the cause associated with liver injury, and impaired liver function (Ranawat *et al*., 2010). Number of liver disease such as hepatotis, chronic or acute hepatitis, fibrosis or cirrhosis, are due to toxins, heavy metals, alcohol intake, self-medication and malnutrition. Free radicals and reactive oxygen species are also involve in lipid peroxidation that overt into liver injury, nevertheless, liver diseases gain much attention in recent years. Due to scarcity of potent liver protective agent's, scientific community focuses on search of novel, and safe anti-hepatotoxic agent. In modern therapeutics system, green medicines are considered as safe and potent alternative anti-hepatotoxic agents (Okaiyeto *et al*., 2018).

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Ajuga parviflora (*A. parviflora*) belongs to *Ajuga* genus and Lamiaceae family. This plant is native to Pakistan mostly found in northern areas (South Waziristan) of Khyber Pukhtunkhwa (KPK) province. Locally this plant is known as trakha boti (bitter herb). *A. parviflora* leaves has number of ethnopharmacological uses. Traditionally people use *A. parviflora* in management of diabetes, cure of malaria, and digestive problems (Ibrar *et al*., 2015). The local population also use this plant in skin infections. Previously this plant has been evaluated for its antiviral activity (Yousaf *et al*., 2018). In current study *A. parviflora* has phytochemically evaluated to standardize the crude methanol extract of this plant and evaluate it for its hepatoprotective effect.

MATERIALS AND METHODS

Plant sample collection

The plant was identified via its local name Trakha boti and was collected from hilly region of South Waziristan, KPK, Pakistan. The plant was identified by a National Agriculture Research Center (NARC) Islamabad,

Pakistan. The voucher of specimen (RAW101001) was submitted to NARC and Faculty of Pharmacy, Federal Urdu University of Arts, Science and Technology (FUUAST).

Extraction

The leaves of the *A. parviflora* were rinsed with tap water and dried under shed. One kg leaves were pulverized to fine powder, and were macerated in commercial grade methanol for 15 days. After 15 days of maceration the obtained extract was filtered with Whatmann filter paper, concentrated with rotary evaporator (B-490, Buchi) at 45 ºC, resulted in greenish extract of 132 gm.

Gas chromatography (GC) analysis

The GC analysis of *A. parviflora* was carried out on (Agilent USB-393752, USA) with capillary HHP-5MS (5%) phenylmethylsiloxane capillary 0.25µm) assembled with FID-detector. The experimental conditions and sample running was in accordance of previously reported protocol (Burki *et al*., 2018).

Gas chromatography mass spectroscopy (GC/MS) analysis

The GC/MS of *A. parviflora* was carried out on (Agilent HP-5973, USA). A HHP-5MS 5% phenylmethylsiloxane capillary column (30 m×0.25mm×0.25µm) and FID detector was used for analysis. The sample was run on the instrument as described earlier (Burki *et al*., 2019).

FTIR analysis

The FTIR of *A. parviflora* was analyzed on (Thermo Nicolet FT-IR Nexus) in the mid-IR region i.e. 4000-400 cm^{-1} at resolution $4cm^{-1}$ as previously reported by (Oliveira *et al*., 2016).

Animals

The two months old male Wister albino rats (180-200 gm) average body weights were purchased from Dow University of Health Sciences (DUHS). The rats were maintained under ordinary cage condition of 12hrs day/light at 22±5 ºC temperature, with free access to food and water. An ethical approval (AP-SM-19B) was obtained from institutional ethical review committee.

Experimental design and isoniazid and rifampicin induced toxicity

Liver toxicity in rat was induced *via* administering 50mg/kg dose of each INH and RFP in suspension by oral route for 21 days as explained by (Pal *et al*., 2006). The experimental design for the study was performed as described by (Khan *et al*., 2012). Briefly animals for the study were divided in five groups, group A animals were administered isoniazid and rifampicin, group B, C and D were supplied with *A. parviflora* extract (100, 200 and 300 mg/kg) dose 1-h prior to the administration of INH and RFP. To group E animal's normal saline was administered. In each group (n=6) animals were placed.

Blood collection and serum preservation

Biochemical assays

The important liver biomarkers like ALT, TP, AST, bilirubin, and ALP were assayed as per standard protocols recommended by manufacturers and suppliers of diagnostic kits as previously reported (Zakaria *et al*., 2011). The assays were performed on UV/visible double beam spectrophotometer (Lambda 25, Perkin Elmer USA).

Histological study

The animal liver was harvested after 21 days of treatment. Each animal liver was collected in (10%) formalin and incubated for 24-h. Further the tissue was dehydrated with ethanol of different percentage (50,70, 80, 90 and 100%) and then cleared with 100 % xylene. The tissue was embedded in paraffin wax using embedding machine. The section cutting of tissue block was performed at 4µm using microtome (Germany, SALEE, Mainz-CUT, 5062). Tissue sections were stained with eosin and Harris Hematoxylin, in order to observe the tissue architecture microscopic analysis were performed (Optik: AC-5, Digital camera OMAX, A3590U China). The changes in histopathology were scored as non (- -), very mild (-), mild (+), moderate (++), and severe (+++).

Detection of gene expression (PPAR-δ) by real time PCR in liver tissue

In order to better understand and the possible heptoprotective mechanism of *A. parviflora*, peroxisome proliferator-activated receptor- delta (PPAR-δ) gene expression was analyzed in isoniazid and rifampicin induced toxicity in rat liver. Tissues were harvested from experimental group animals and the liver were pulverized into small pieces and the total RNA was extracted by Trizol reagent method as previously described (Laukova *et al*., 2018). The total RNA was quantified and its purity was determined by Colibri Micro volume spectrophotometer (Titertek Berthold) in a ratio of 260/280. One µg RNA from each sample was reverse transcribed into complementary DNA by cDNA synthesis kit. Gene expression of PPAR-δ in rat liver tissue was quantitatively measured *via* real time-PCR (Applied biosciences, CA, USA and Rotor Gene Q real time PCR detection system) as per manufacturer instructions. The primer sequences for PPAR-δ as follows; forward: GCCAAGAACATCCCCAACTTC, reverse: GCAAAGA TGGCCTCATGCA. GAPDH, forward: AACTCCATTC CTCCACCTT, reverse: GAGGGCCTCTCTCTTGCTCT, was used as an internal control for qRT-PCR. PCR was performed according following cycle; 1 cycle at 95 ºC for 5 minutes for initial denaturation, followed by 35 cycles at 95, 60 and 72 ºC for 30s at for denaturation, annealing and extension, respectively. Results were analyzed using $2^{-\Delta\Delta CT}$ method for gene expression.

STATISTICAL ANALYSIS

Statistical analysis of Data was obtained using GraphPed prism 6.lnk. Results are presented as mean±SEM, statistical investigation was performed by applying one-way ANOVA, followed by Tukey *post hoc* test. Group of different doses were compared with each other. For relative expression of PPAR-δ, one-way ANOVA and Tukey-Kramer as a post of ANOVA test was applied at ($^{\dagger}p<0.001$, $^{\ddagger}p<0.01$, $^{*}p<0.05$).

RESULTS

Phytochemical analysis

The GC and GC/MS (figs. 1a and 1b) spectral analysis of *A. parviflora* methanolic extract on comparing with NIST library showed the presence of important bioactive compounds like 2-piperidinone, N-[4-bromo-n-butyl]-Phthalic acid, mono-(2-ethylhexyl) ester, squalene, α-tocopherol, vitamin E, phytol, 2-methylenecholestan-3-ol, stigmasterol, cholest-22-ene-21-ol, 3,5-dehydro-6-methoxy-, pivalate, 3-ethyl-3-hydroxyandrostan-17-one, and dihydro-actinidiolide. The FTIR analysis (fig. 1c) showed the important functional groups, OH of phenol and formic acid, C=C-H, H-C-H, C≡C, C=O, N=O, N-H, C-O.

Anti-hepatotoxic activity

Biochemical analysis

After treating the animals with INH and RFP 50mg/kg of both drug, the AST, ALT and ALP significantly elevated. Billirubin was significantly increased, while total protein was attenuated as compare to negative control animals treated with saline. Pre-treatment with *A. parviflora* crude extract at dose 200 and 300 mg/kg in group C, and D significantly reduced AST at ($p<0.001$), ALP ($p<0.001$), ALT ($p<0.001$) and bilirubin ($p<0.001$) as shown in table 1. Likewise, total protein (TP) were also significantly ($p<0.01$) inhibited in group C and D at (200 and 300mg/kg) dose. The serum AST in group B treated with *A. parviflora* (100 mg/kg) dose was significantly ($p<0.01$) reduced, while, ALT and ALP reduced at ($p<0.05$). Billirubin was also significantly inhibited at ($p<0.01$) with *A. parviflora* (100 mg/kg) dose (table 1).

Histopathological assessment

Histopathological assessment was performed after 21 days of administration of INH and RFP. The epithelium of central vein (CV) was disrupted, and the central hepatic lobules were shrieked as shown in (fig. 2A) and specified with large arrow, while infiltration in the picture is specified with arrow head. The asterisk represents the dilatation of sinusoidal spaces. The central vein is appeared with normal histology with intact endothelium indicated with (large arrow), the hepatocytes pointed with (small arrows), sinusoidal spaces mentioned with (asterisks), the arrow heads showed the endothelial cells in groups of rat treated with *A. parviflora* in (figs. 2B, 2C and 2D). The hepatocytes appeared to be glucagon depleted. Around the necrotized central vein there is focal aggregation of lymphocytes. The image of extract pre-treated with (*A. parviflora*, 200 and 300 mg/kg) showed that the epithelium of the CV is intact with repleted glucagon. The 21 days' pre-treatment with *A. parviflora* significantly protected the liver from isoniazid and rifampicin induced hepatotoxicity (figs. 2C and 2D). The 100 mg/kg dose of *A. parviflora* comparatively less protect the CV epithelium.

The endothelium was severely disrupted in isoniazid and rifampicin induce hepatotoxic animals. Glucagon depletion, lymphocytes infiltration, and lymphoid aggregate in portal vein were severe in INH and RFP as shown in (table 2). In the animals pre-treated with *A. parviflora* 200, and 300 mg/kg dose prettily revert the severely disturb parameters like, glucagon depletion, lymphocytes and lymphoid infiltration as shown in (table 2).

Fig. 3 showed the effect of INH plus RFP and *A. parviflora* methnolic extract 100, 200, and 300 mg/kg dose on rat liver tissue PPAR-δ gene expression. In INH plus RFP treated group the PPAR-δ gene expression was decreased significantly ($p<0.001$) by 0.5 fold (fig. 3a) compared to control. The administration of *A. parviflora* 200, and 300mg/kg dose in combination with INH plus RFP displayed significant ($p<0.01$, $p<0.001$) increase in PPAR-δ expression levels by 2.3 fold.

DISCUSSION

Xenobiotic metabolism is an important process for their excretion from the body, that may result in free radical's generation. The free radicals and xenobiotic metabolites causes acute hepatocellular and hepatic parenchymal damages. Additionally, viral infections and various drugs also causes hepatic injuries (Jaeschke *et al*., 2002). The minimal pharmacological treatment options confined health practitioners to treat liver disease toward hemostasis. Therefore, people globally accept and corroborate the use of medicinal plant in health care system. Indeed, medicinal plants bear pharmacologically active constituents that are responsible for biological activities (Wahid *et al*., 2016). Since, *A. parviflora* was evaluated for antiviral activity (Yousaf *et al*., 2018). Keeping in view, this study was aimed to evaluate mechanistically *A. parviflora* in INH and RFP induced liver toxicity in rat. Hepatotoxicity could be induced in animals with various interventions. CCl_4 induced hepatotoxicity leads to formation of free radicals and oxidative stress that damage hepatocytes (Abouzied *et al*., 2016). Acetaminophen induce hepatotoxicity through mitochondrial oxidative stress (Yan *et al*., 2018).

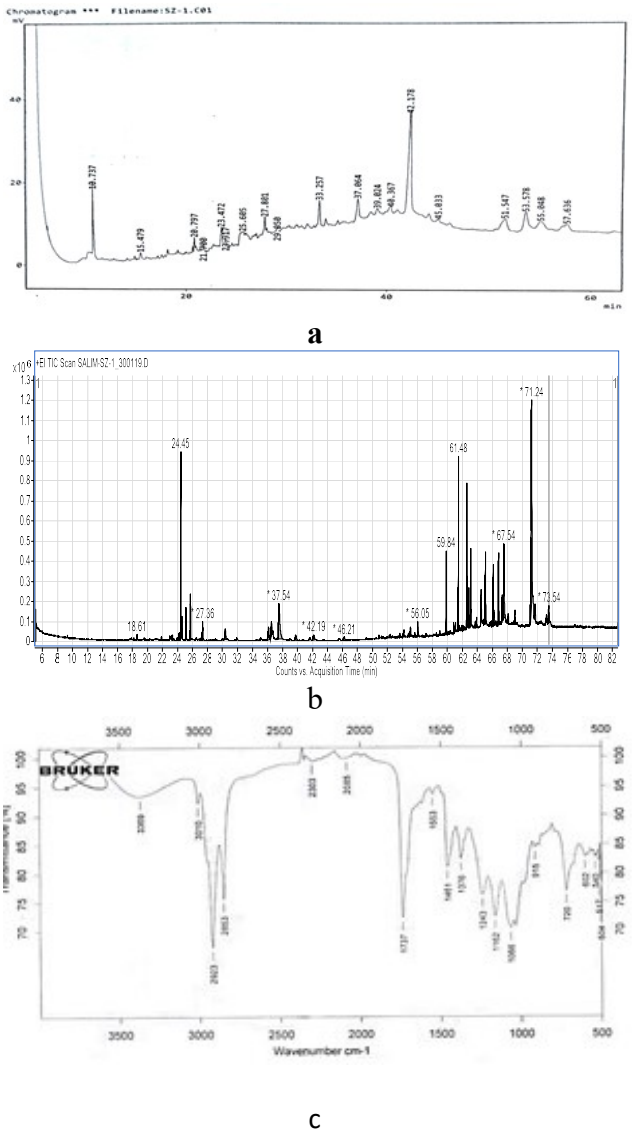

Fig. 1: (a) GC, (b) GC/MS and (c) FT-IR chromatogram of *A. parviflora* leaves extract

INH being an important choice of drug in tuberculosis induce liver toxicity at high dose that overt in to liver failure (Harrington *et al*., 2010). Rifampicin synergistically impaired liver function probably by producing hydrazine's formation (Rao *et al*., 2015). Nonetheless, this method is frequently employed in the screening, investigating of hepatoprotective drugs and medicinal plants. Liver toxicity and liver malfunctioning can be evaluated through measuring liver biomarkers. In this study the liver enzymes like AST, bilirubin and ALT were severely ameliorated in INH and RFP treated drugs as compare to negative control animals. The ALP levels in all animals were also ameliorated with sufficient reduction in total protein level. The sever increase of liver enzymes indicate liver injury. However, in *A. parviflora* pre-treated animals 200 and 300 mg/kg dose significantly attenuates the AST, ALT, ALP, and bilirubin level, while profoundly improved the total protein level.

Glucagon level was repleted after pre-treatment with *A. parviflora* 200 and 300mg/kg as compare to INH and RFP treated animals. Histopathological investigation showed remarkable recovery of endothelium disruption with *A. parviflora* extract treated animals as compare to positive control group. Lymphocytic infiltrations and lymphoid aggregates in portal vein were comparatively reduced in *A. parviflora* treated animals. The changes were also observed morphologically in INH and RFP induced liver toxicity. The *A. parviflora* (200 and 300 mg/kg) dose inhibited the morphological changes significantly in INH and RFP induced hepatotoxicity. In addition, this study suggest that 300 mg/kg dose have more significant anti-hepatotoxic effect over 200 mg/kg dose. This tremendous hepatoprotective activity may be due to presence of pharmacologically active ingredients in the crude methanolic extract of *A. parviflora*.

These active compounds include squalene, α-tocopherol, stigmasterol, 2-piperidinone, methylenecholestan-3-ol, lupeol, linolenic acid pivalate, and dihydro-actinidiolide. Regarding squalene (Zuhan *et al*., 2015) (Sivakrishnan and Muthu, 2014) reported previously that squalene isolated from *A. procera* showed promising anti-hepatotoxic effect in CCl_4 induced hepatotoxicity. α-Tocopherol an isoform of vitamin E, reported as a strong antioxidant and exhibits potent liver enzymes reducing capability and reverse hepatic toxicity (Sodhi *et al*., 2008) (Palipoch *et al*., 2014) (Kothekar *et al*., 2004). Linolenic acid has been reported to have cardio protective, neuroprotective, anticancer, anti-inflammatory, and antioxidant activity (Kim *et al*., 2014). Phytol also have antioxidant activity as well as act as a precursor of vitamin E (Serafini *et al*., 2011). PPARs are group of nuclear protein receptors that regulate several genes. PPARs greatly involve in metabolism, crucial cellular functions in muscles cells, and lipid oxidation. PPAR-δ over expression also appears to have anti-inflammatory affect and also protect normal cells from metabolic syndromes like hepatosteatosis, dyslipidemia and insulin resistance. (Liu *et al*., 2018). PPAR-δ expression in this study was attenuated significantly in INH and RFP induce hepatotoxic group as compare to control. Similarly, another study reported attenuation of PPAR-δ level following isoniazid administration (Mahmoud *et al*., 2014). The administration of *A. parviflora* 200 and 300 mg/kg dose significantly over express PPAR-δ. The PPAR-δ over expression suggest that the mechanism of liver protective effect of *A. parviflora* may be by eliminating damaged hepatocytes through apoptosis process. These hepatoprotective activities of *A. parviflora* may be attributed to the active compound present in the extract. This study also authenticates the use of *A. parviflora* in hepatitis. The biochemical parameters were

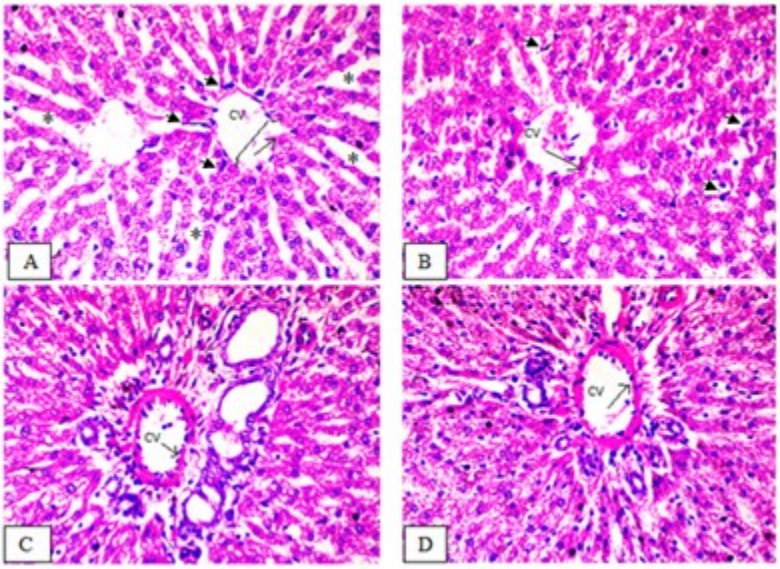

Fig. 2: Histopathological images of rat at (x400 magnification). (A) rat liver treated with INH and RFP showing central vein congestion (B) *A. parviflora* 100mg/kg (C) *A. parviflora* 200mg/kg (D): *A. parviflora* 300mg/kg 1-h prior INH and RFP administration

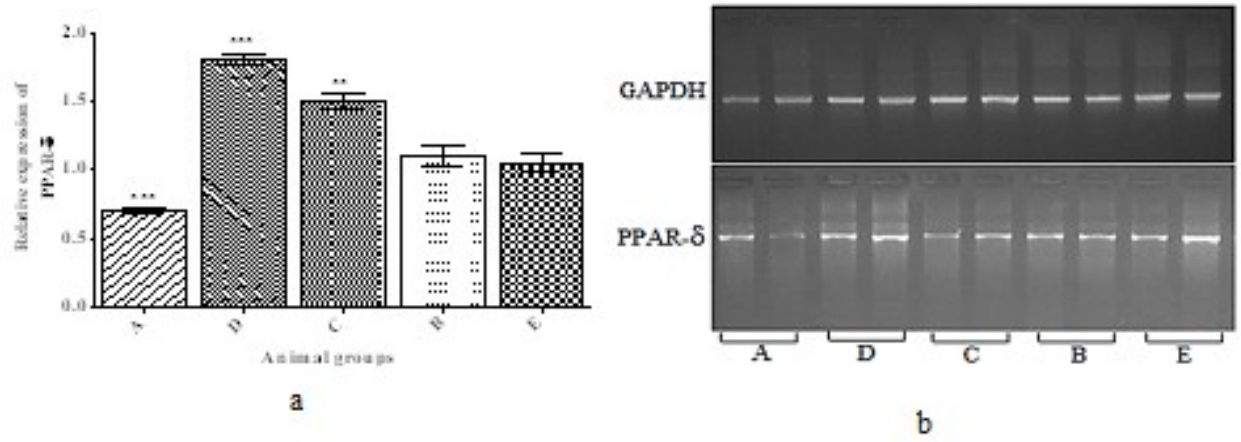

Fig. 3: (a) level of gene expression. (A) INH plus RFP on gene expression. Combination of different doses of *A. parviflora* group B, 100 mg/kg, group C, 200 mg/kg, group D, 300 mg/kg and group E, negative control on rat liver. (b) agarose gel electrophoresis GAPDH as in internal qRT-PCT, and PPAR-δ as a test respectively of all groups, at $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, ANOVA followed by as a Tukey-Kramer post ANOVA test

Table 1: Effect of *A. parviflora* on isoniazid and rifampicin-induced hepatotoxicity in rats

Treatment		Dose (mg/kg)	AST (U/L)	ALT (U/L)	ALP (U/L)	Billirubin (mg/dL)	Total proteins (g/dL)
Group A (Isn and rif)		50	143 ± 4.8	134 ± 5.4	1.4 ± 0.2	189 ± 8.5	3.1 ± 0.5
Group B	*A. parviflora*	100	109 ± 5.20 ‡	114 ± 5.0*	0.82 ± 0.2*	116 ± 6.3 ‡	6.1 ± 1.0*
Group C		300	64.0 ± 3.5†	93.5 ± 6.0†	0.71 ± 0.3*	110 ± 5.5†	7.2 ± 0.8 ‡
Group D		500	41.8 ± 3.1†	78.0 ± 4.3†	0.63 ± 0.1 ‡	102 ± 6.2†	8.3 ± 1.1 ‡
Group E control (NS)		1 ml	46.1 ± 2.0	81.51 ± 3.1	0.36 ± 0.08	113 ± 3.5	6.4 ± 0.3

NS (normal saline), Isn and rif (isoniazid and rifampicin), Values are expressed as mean ± SEM; ANOVA followed by Tukey's post hoc test; at $^{\dagger}p<0.001$, $^{\ddagger}p<0.01$, $^{*}p<0.05$. Group B, C and D were compared with group A and E, (n = 6)

Table 2: Effect of *A. parviflora* extract on 21-days administration of INH and RFP induced liver toxicity

Histopathological findings	Group				
	A	B	C	D	E
Endothelium disruption	+++	+	-	-	-
Hydropic changes	++	-	-	-	- -
Cytolysis	+	-	-	-	- -
Lymphocytes infiltration	++	+	-	-	-
Lymphoid aggregate in (portal vein)	+++	+	-	-	- -
Glucagon depletion	++	++	-	-	-
Congestion	++	+	-	-	+

(- -) none, (-) very low, (+) mild, (++) moderate (+++) severely damaged

supported by histopathological studied of liver. The histopathological results suggest that *A. parviflora* have liver regenerative effects. Broadly *A. parviflora* is considered as the safe green medicine with negligible deleterious effects.

CONCLUSION

In conclusion, the crude methanol extract of *A. parviflora* exhibited hepatoprotective effect by reducing the toxicity of isoniazid and rifampicin in healthy animals. This significant hepatoprotective activity of *A. parviflora* may be due to compounds present in the crude extract. In future, the mechanism of its hepatoprotective effect may be evaluated either by over expression of PPAR-δ, by preventing lipid peroxidation and/or inhibition of free radicals.

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