Protective Effect of Pyridoxine and N-Acetylcysteine in Isoniazid Induced Hepatotoxicity

Arooj Javaid[1], Haji Bahadar[1*], Ghazala Bibi[1]

1. Institute of Pharmaceutical Sciences, Khyber Medical University, Peshawar, Pakistan

ABSTRACT

Isoniazid is used as a first-line drug for the treatment of tuberculosis in combination with other drugs. Long-term use of isoniazid has been known to cause liver toxicity. Some drugs have been approved to treat the isoniazid induced side effects. The purpose of the current study was to evaluate hepatoprotective effect of pyridoxine and compare it with N-acetylcysteine alone or in combination, in isoniazid-induced liver toxicity. The study was carried out on 30 Wistar rats equally divided into 5 groups. Isoniazid at 50 mg/kg dose was used to induce hepatotoxicity in rats. Pyridoxine (50 mg/kg) and N-acetylcysteine (100 mg/kg), either alone or in combination, were orally administered for four weeks. Body weight was checked weekly. After receiving N-acetylcysteine and pyridoxine, blood and liver tissues were collected for biochemical analysis and histological findings. The biochemical analysis included alanine transaminase, aspartate aminotransferase, total bilirubin, and hepatic glutathione levels. Rats treated with N-acetylcysteine and pyridoxine, in combination, showed significant changes in the level of various liver enzymes and hepatic glutathione. Conversely, as compared to the positive control group, in rats treated with N-acetylcysteine and pyridoxine alone, the effect of N-acetylcysteine was found more than pyridoxine. In conclusion, pyridoxine can be used as a hepatoprotective drug in isoniazid induced hepatotoxicity, either alone or in combination with N-acetylcysteine. Both pyridoxine and N-acetylcysteine possess positive effect on glutathione level.

Keywords: Drug induced liver injury, hepatotoxicity, isoniazid, N-acetylcysteine, pyridoxine

1. INTRODUCTION

Isoniazid is the hydrazide of isonicotinic acid (INH), which resembles pyridoxine in terms of structure. It was introduced into medicine in 1952 for the treatment of tuberculosis (TB). About 85% of TB cases are treated and cured, but some undesired effects such as liver injury (hepatotoxicity), GIT complications, sympathetic and parasympathetic nervous system disorders, and dermatological reactions also occur (1). Isoniazid is a pro-drug. The enzyme catalase-peroxidase (KatG) is responsible for its activation. After activation, it prevents the production of mycolic acid, which halts the development of the bacterial cell wall. Phase-II acetylation conjugation is employed to metabolize it. Slow acetylation of INH results in the production of harmful metabolites and causes hepatotoxicity, whereas fast acetylation generates no toxic metabolites (2). Isoniazid is extensively metabolized in the liver. Along other unwanted effects, hepatotoxicity is one of the major adverse effects caused by isoniazid. On the other hand, the old aged population is more susceptible to the adverse effects of isoniazid which may lead to death. Another cause of the incidence of hepatotoxicity in TB patients is poly pharmacy, as other co-administered drugs like rifampicin also induce liver injury (3). As liver is the main metabolizing organ for majority of drugs, hence it is more prone to the toxic assault of many chemicals. Drug induced liver injury includes hepatocyte injury, cholestatic injury, or combined liver cell (hepatocyte) injury/cholestatic injury. Due to bursting, hepatocyte death lead to increased liver enzyme levels such as alanine transaminase (ALT) and aspartate aminotransferase (AST) (4). Hepatotoxicity is also increased by newly recruited leukocytes such as neutrophils and monocytes as well as kupffer, sinusoidal endothelial and stellate (fat-storing) cells in the liver. Kupffer cells and neutrophils generate reactive oxygen and nitrogen species, as well as pro-inflammatory cytokines and chemokines, which are the basis of redox imbalance in hepatic injury due to the toxicants and ischemia/reperfusion (5). N-acetyl transferase-2 and cytochrome P4502E1 are the enzymes responsible for hepatotoxicity of isoniazid. Isoniazid is converted into acetyl isoniazid and ultimately in acetyl hydrazine by N-acetyltransferase-2. Another enzyme cytochrome P4502E1 transforms acetyl hydrazine into N-hydroxy acetyl hydrazine and finally to more toxic radical acetyl diazine. These reactive acetyl radicals produced from the breakdown of acetyl diazine bind with hepatic macromolecules like DNA, proteins, and cellular lipid causing hepatic damage (6). Reactive oxygen species (ROS) build up and adenosine triphosphate (ATP) is depleted as the mitochondrial respiratory chain is blocked, making the mitochondria a prominent target in drug-induced hepatotoxicity (7).

In some cases of hepatotoxicity, drug like N-acetylcysteine (NAC) is used. It serves as a precursor for the synthesis of an antioxidant glutathione (8, 9). It also minimizes the tissue damage caused by free radicals. In addition, NAC has been reported as a hepatoprotectant against isoniazid in the experimental studies (10).

Pyridoxine, also known as vitamin-B6 is used in acute toxicity caused by isoniazid overdose, mushroom poisoning, monomethylhydrazine (MMH) poisoning, and hydrazine poisoning (11). It is a cofactor that is responsible for the synthesis of various amino acids, neurotransmitters including serotonin and norepinephrine, and is involved in numerous enzymatic reactions in body (12). Additionally, it is vital in the

management of peripheral neuropathy caused by isoniazid. It is mostly recommended for individuals with liver cirrhosis who have anemia, edema, or ascites (13). Both in pre-clinical and clinical studies, pyridoxine has not been used as a hepatoprotectant in isoniazid induced hepatotoxicity so the aim of this study was to evaluate the ameliorative effect of pyridoxine alone, and in combination with a well known hepatoprotective drug, N-acetylcysteine.

2. MATERIALS AND METHODS

2.1. Chemicals

The chemicals used in this study were purchased from Bio-Lab and Drug Testing Laboratory, Pakistan. ALT/AST and other instrument assay kits were obtained from Sigma-Aldrich and ThermoFisher Scientific. Hematoxylin-Eosin (H&E) staining kit was purchased from Elabscience for histological examination.

2.2. Animals

Healthy male Wistar rats (N=30), each weighing about 200 grams were obtained from the animal house of Institute of Pharmaceutical Sciences, Khyber Medical University, Pakistan. Rats were adapted to the laboratory conditions two weeks before initiating the study. Rats were kept in an environment at standard room temperature 25 °C and humidity levels. The animals were provided with adequate food and water at all times and a 12-h light-dark cycle as part of their natural photoperiod. All steps were performed according to the rules and regulations regarding animal ethics. This study was approved by Basic Medical Research and Bioethics Committee, Khyber Medical University, Pakistan.

2.3. Experimental Design

A 30-day sub-chronic toxicity study was the intended design of the project. Total 30 rats were used in this study and were divided equally into 5 groups of six rats (Table 1). Pyridoxine, N-acetylcysteine and isoniazid were dissolved in water and administered orally.

Animal groups	Dose Description
Group 1	Negative Control group. No drug
Group 2	Positive Control group. Received isoniazid 50 mg/kg per day
Group 3	Received isoniazid 50 mg/kg and pyridoxine 50 mg/kg per day
Group 4	Received isoniazid 50 mg/kg and N-acetylcysteine 100 mg/kg per day
Group 5	Received isoniazid 50 mg/kg, pyridoxine 50 mg/kg and N-acetylcysteine 100 mg/kg per day

Table 1: Animals grouping and drug dose description.

2.4. Biochemical Analysis

Cardiac puncture was used to withdraw blood from all five groups of rats for biochemical tests. By centrifuging the tubes for 30 minutes at 4 °C at 5000 rpm, the serum was separated. After centrifugation, serum aliquots were collected, and the levels of the liver enzymes aspartate aminotransferase (AST), alanine transaminase (ALT), and glutathione (GSH) were determined using the commercial test kits mentioned above. An automated COBAS C111 chemical analyzer was used for the analysis.

2.4.1. Determination of Alanine Transaminase (ALT)

The ALT levels were assessed using the Sigma-Aldrich kit. 1.0 mL of alanine-KG substrate was pipetted into the test tubes for the treated and control samples, and warmed in a 37 °C water bath. 0.2 mL serum was added and mixed in it. After 30 minutes, 1.0 mL of the sigma color reagent was added. It was thoroughly mixed and allowed to stand at room temperature for 20 minutes. After 20 minutes, 10 mL of 0.40 N sodium hydroxide solution was added, mixed by inversion, and allowed to stand at room temperature for 5 minutes. Absorbance was read and recorded at the same wavelength (505 nm), used to generate a standard curve using water as a reference (14).

2.4.2. Determination of Aspartate Aminotransferase (AST)

AST levels were measured using the Sigma-Aldrich kit. 1.0 mL of aspartate substrate was pipetted into test tubes for the treated and control samples, and warmed in a 37 °C water bath. 0.2 mL serum was added and mixed in it. 1.0 mL of the sigma color reagent was added, mixed gently and left at room temperature for 20 minutes. After 20 minutes, 10 mL of 0.40 N sodium hydroxide solution was added, stirred upside down, and allowed to stand at room temperature for 5 minutes. Absorbance was measured and recorded at the same wavelength (505 nm) used in the experiment to construct a standard curve using water as a reference (14).

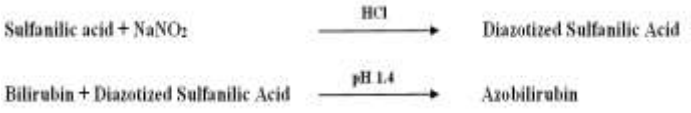
2.4.3. Determination of Glutathione (GSH)

Levels of glutathione in liver tissue were determined through Ellman's method. With the help of a homogenizer, uniform homogenate of liver tissue was obtained. Normal saline was added to the homogenate and stored in eppendorf tubes at −80 °C for further analysis. For glutathione analysis, homogenates were centrifuged at 2000 rpm for 10 minutes at 4 °C. Glutathione levels in the control and treatment groups were assayed using supernatants. Tissue homogenates were mixed in equal volumes with 0.1 M phosphate buffer (pH: 7.4), 20% trichloroacetic acid (TCA), and 1 mM EDTA was used to precipitate tissue proteins. After 5 minutes, it was centrifuged at 2000 rpm for 10 minutes. 50 µL of tissue supernatant and 1.7 mL of 0.3 M disodium hydrogen phosphate solution were mixed. Ellman's reagent (DTNB reagent): 4 mg of 5, 5-dithiobis (2-nitrobenzoic acid) in 10 mL of 1% (w/v) sodium citrate was added to make it up to the final volume i.e. 2 mL (250 µL). At 412 nm, the sample's absorbance was measured in comparison to a blank solution.

A standard curve generated from GSH was compared with the GSH concentrations in the samples (15).

2.4.4. Determination of Total Bilirubin

Bilirubin concentration was analyzed using COBAS C111 chemical analyzer. Sulfanilic acid combined with sodium nitrite in the presence of a strongly acidic media (pH: 1.4). The sample's bilirubin reacted with the diazotized sulfanilic acid to form azobilirubin, a red pigment. A photometric measurement was made of the azobilirubin's color intensity, which was proportional to the total bilirubin concentration (16).



2.5. Histopathological Findings

Slices of the liver tissues from each of the five groups were fixed in 10% formalin. To dehydrate the fixed tissues, ethanol was used. Tissues were infiltrated into molten paraffin wax at 55 °C, then passed through a solution of xylene, and immersed in the paraffin wax block. Using rotary microtome, tissues were cut into 5-micron-thick paraffin sections. Hematoxylin and Eosin (H&E) stains were used to stain the sections. A light microscope examined stained slides as photomicrographs of the tissue samples (17).

2.6. Statistical Analysis

This study's results were presented as mean standard deviation (SD) and $p = 0.05$ or less was regarded as statistically significant when performing the statistical analysis by applying the paired t-test and one-way ANOVA, followed by the Tukey Kramer post-hoc analysis test.

3. RESULTS

3.1. Effects of Isoniazid, Pyridoxine, and N-Acetylcysteine on weight

As shown in Figure 1, isoniazid only treated rats show reduction in weight as compared to other groups.

3.2. Confirmation of isoniazid induced liver toxicity

3.2.1. Effect of isoniazid on AST and ALT

AST and ALT levels were significantly ($p = 0.01$) increased in isoniazid treated group in comparison with the normal control group (Table 2).

3.2.2. Effect of isoniazid on bilirubin

The level of bilirubin was significantly ($p = 0.01$) increased in isoniazid treated group in comparison with the normal control group (Table 2).

3.2.3. Effect of isoniazid on glutathione

Glutathione level was significantly ($p = 0.01$) decreased in isoniazid treated group in comparison with the normal control group (Table 2).

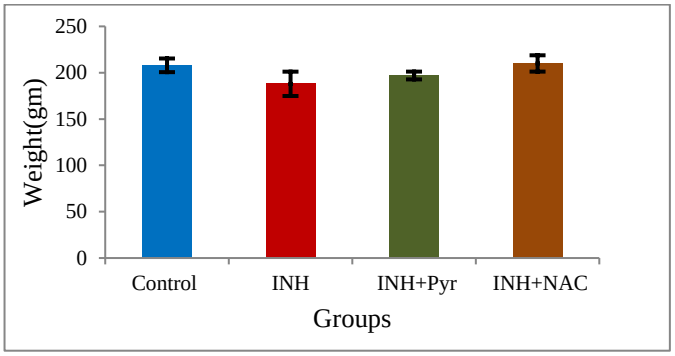


Figure 1: Effect of isoniazid, pyridoxine, and N-acetylcysteine on the mean body weight. Drugs were administered for four weeks to all groups. All values represent mean ± SD of six rats (*$p < 0.05$).

Biochemical Parameters	Control	INH
AST (U/I)	75 ± 5.63**	443 ± 2.16**
ALT (U/I)	48 ± 5.46**	278 ± 3.48**
Bilirubin (mg/dL)	0.4 ± 0.14**	6.56 ± 0.03**
Glutathione (µM/g)	7 ± 0.43**	2.705 ± 0.002**

Table 2: Effect of isoniazid on AST, ALT, bilirubin, and GSH. All values are expressed as mean ± SD between the control group and isoniazid, **$p = 0.01$

3.3. Effect of pyridoxine on biochemical parameters

3.3.1. Effect of pyridoxine on AST and ALT

As mentioned in Table 3, rats pre-treated with pyridoxine 50 mg/kg shows significant ($p = 0.01$) reduction in liver enzymes, as compared to the positive control group.

3.3.2. Effect of pyridoxine on bilirubin

As mentioned in Table 3, rats pre-treated with pyridoxine 50 mg/kg shows significant ($p = 0.01$) reduction in serum bilirubin, as compared to the positive control group.

3.3.3. Effect of pyridoxine on glutathione

As mentioned in Table 3, rats pre-treated with pyridoxine 50 mg/kg shows significant ($p = 0.01$) increase in total glutathione level, as compared to the positive control group.

Biochemical Parameters	INH	INH+Pyr
AST (U/I)	443 ± 2.16**	254 ± 5.91**
ALT (U/I)	278 ± 3.48**	90 ± 3.63**
Bilirubin (mg/dL)	6.56 ± 0.03**	2.0 ± 0.04**
Glutathione (µMg)	2.705 ± 0.002*	3.14 ± 0.01*

Table 3: Effect of pyridoxine on AST, ALT, bilirubin, and GSH. All values are expressed as mean ± SD between isoniazid and isoniazid + pyridoxine, $^{**}p = 0.01$

3.4. Effect of N-acetylcysteine on biochemical parameters

3.4.1. Effect of N-acetylcysteine on AST and ALT

As mentioned in Table 4, rats pre-treated with N-acetylcysteine 100 mg/kg shows a very significant ($p = 0.01$) reduction in liver enzymes in comparison to the positive control group.

3.4.2. Effect on N-acetylcysteine on bilirubin

As mentioned in Table 4, rats pre-treated with N-acetylcysteine 100 mg/kg shows a very significant ($p = 0.01$) reduction in serum bilirubin in comparison to the positive control group.

3.4.3. Effect on N-acetylcysteine on glutathione

As mentioned in Table 4, rats pre-treated with N-acetylcysteine 100 mg/kg shows a very significant ($p = 0.01$) increase in total glutathione level in comparison to the positive control group.

Biochemical Parameters	INH	INH+NAC
AST (U/I)	443 ± 2.16**	84 ± 2.16**
ALT (U/I)	278 ± 3.48**	28 ± 2.85**
Bilirubin (mg/dL)	6.56 ± 0.03**	0.3 ± 0.19**
Glutathione (µM/g)	2.705 ± 0.002**	9.765 ± 0.003**

Table 4: Effect of N-acetylcysteine on AST, ALT, bilirubin, and GSH. All values are expressed as mean ± SD between isoniazid and isoniazid + N-acetylcysteine, $^{**}p = 0.01$

3.5. Effect of pyridoxine + N-acetylcysteine on biochemical parameters

3.5.1. Effect of pyridoxine + N-acetylcysteine on AST and ALT

As mentioned in Table 5, rats pre-treated with a combination of pyridoxine 50 mg/kg and N-acetylcysteine 100 mg/kg shows a very significant ($p = 0.01$) reduction in liver enzymes in comparison to the positive control group.

3.5.2. Effect of pyridoxine + N-acetylcysteine on bilirubin

As mentioned in Table 5, rats pre-treated with a combination of pyridoxine 50 mg/kg and N-acetylcysteine 100 mg/kg shows a very significant ($p = 0.01$) reduction in serum bilirubin in comparison to the positive control group.

3.5.3. Effect of pyridoxine + N-acetylcysteine on glutathione

As mentioned in Table 5, rats pre-treated with a combination of pyridoxine 50 mg/kg and N-acetylcysteine 100 mg/kg shows a very significant ($p = 0.01$) increase in total glutathione level in comparison to the positive control group.

Biochemical Parameters	INH	INH+Pyr+NAC
AST (U/I)	443 ± 2.16**	106 ± 1.87**
ALT (U/I)	278 ± 3.48**	53 ± 4.36**
Bilirubin (mg/dL)	6.56 ± 0.03**	0.5 ± 0.12**
Glutathione (µM/g)	2.705 ± 0.002*	3.18 ± 0.02*

Table 5: Effect of pyridoxine and N-acetylcysteine combination on AST, ALT, bilirubin, and GSH. All values are expressed as mean ± SD between isoniazid and isoniazid + pyridoxine + N-acetylcysteine, $^{**}p = 0.01$

3.6. Histopathological Findings

The histological observations validated the results obtained from the serum liver biomarkers testing. Figure 2a demonstrates the normal hepatic architecture in the control group, which radiates from the central vein to the lobular periphery. There was a substantial hepatic damage in the group that received isoniazid, as shown by the presence of inflammatory infiltrates in parenchyma along with the inflammation of portal triad and bile duct (Figure 2b). Dose of 50 mg/kg, isoniazid + pyridoxine reduced or even stopped the growth of histopathological damage (Figure 2c). Isoniazid + N-acetylcysteine at a dose of 100 mg/kg completely altered hepatotoxic characteristics of the liver that showed a homogenous cytoplasm in compact form with no inflammatory infiltrates as well as no inflammation of a central vein, portal triad, and bile duct (Figure 2d). Isoniazid + pyridoxine + N-acetylcysteine revealed a normal periportal region similar to the control liver (Figure 2e).

4. DISCUSSION

Hepatotoxicity is a common adverse effect caused by many drugs. Anti-Tb drugs have been documented to cause liver damage followed by rise in liver enzymes (18). On clinical side, anti-TB drugs induced hepatotoxicity is a critical issue as it can significantly increase morbidity and mortality. Liver injury is indicated by the enzymes AST and ALT. Increased cell membrane permeability can result from the hepatocyte damage-induced degeneration, inflammation, and necrosis. Thus, through the cell membrane, AST and ALT are released into the body, increasing their blood concentration (19).

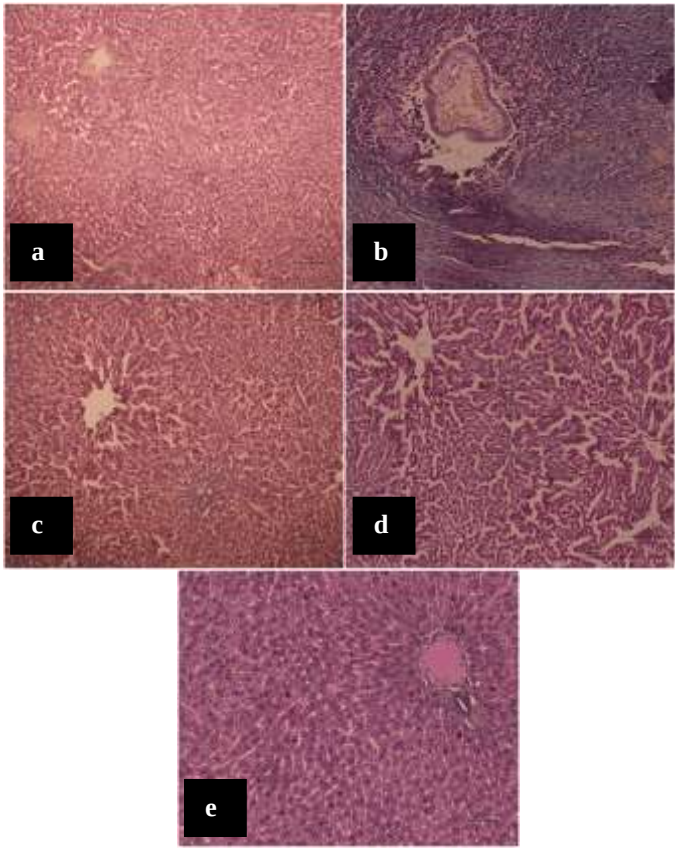


Figure 2: Photomicrographs of rat liver showing (a) normal morphology of control animal, (b) isoniazid 50 mg/kg damaged liver morphology, (c) effect of pyridoxine 50 mg/kg on isoniazid induced alterations in liver, (d) effect of isoniazid + NAC 100 mg/kg, (e) effect of isoniazid + pyridoxine + NAC.

In our study, dose of 50 mg/kg of isoniazid was selected as hepatotoxic dose as previously reported in several other relevant studies (20, 10). According to Adikwu and Deo, vitamin C has antioxidant properties with a hepatoprotective effect. In animals, vitamin C has been seen to possess strong hepatoprotective effect as blood level of alanine aminotransferase, aspartate aminotransferase, ALP, and bilirubin return to normal after vitamin C administration (21). Depending on the etiology, there are several well-established treatments available for the few particular DILI indications, such as silymarin (22).

N-acetylcysteine has been shown to have hepatoprotective effects. By preventing the generation of pro-inflammatory cytokines, NAC may also help to reduce liver inflammation and damage induced by toxins such as acetaminophen and alcohol. Studies have shown a hepatoprotective effect of NAC along with isoniazid (10). In addition to NAC, N-acetylcysteine amide (NACA) has been reported in recent years for its potential hepatoprotective properties (23, 24).

A study by Lheureux demonstrated that pyridoxine is most commonly used in isoniazid induced peripheral neuropathy. Due to limited information about hepatoprotective effect of pyridoxine, and its combination therapy with NAC, our study aimed to comparatively analyze pyridoxine and its interaction with NAC in isoniazid induced hepatotoxicity (11).

In the current study, pre-treatment with pyridoxine revealed a significant reduction in the levels of AST, ALT, and bilirubin as compared to the positive control. Moreover, pyridoxine also increased glutathione level that shows its hepatoprotective effect in isoniazid induced hepatotoxicity. Pyridoxine has been reported to protect the liver from oxidative damage by scavenging free radicals and increasing the levels of antioxidant enzymes. It is also involved in the synthesis of various amino acids. Moreover, pyridoxine has also been shown to reduce liver damage caused by certain toxicants such as carbon tetrachloride and ethanol. On the other hand, pre-treatment with N-acetylcysteine also restored serum levels of rats to normal.

The results of this study demonstrated that the combination of pyridoxine and NAC can reduce liver damage induced by isoniazid, improves liver function and morphology. These findings are evident due to the fact that NAC is a precursor of glutathione, a vital antioxidant that protects against oxidative stress in cells (25), and pyridoxine is a cofactor that is responsible for the synthesis of amino acids (11) therefore their combination leads to a positive hepatoprotective effect on isoniazid induced hepatotoxicity.

In summary, both pyridoxine and NAC have hepatoprotective effects, and may be beneficial in protecting the liver from oxidative damage by reducing inflammation. The combination of pyridoxine and NAC may have synergistic effects in protecting the liver and improving liver function. However, it's important to consult with a healthcare professional before using any supplements or medications to ensure their safety and efficacy.

5. CONCLUSION

The administration of pyridoxine and N-acetylcysteine revealed significant changes in the level of various liver enzymes and hepatic glutathione by exerting their hepatic defense through several mechanisms such as reducing oxidative stress, exhibiting anti-inflammatory and anti-apoptotic effects. Conversely, as compared to control, in rats treated with N-acetylcysteine and pyridoxine alone, the effect of N-acetylcysteine was found more than pyridoxine. The findings of this research suggest that pyridoxine can be used as a hepatoprotective drug in isoniazid induced hepatotoxicity, either alone or in combination with N-acetylcysteine.

Acknowledgements

The authors would like to thank Khyber Medical University for providing the necessary facilities for conducting experiments and analyzing results.

Funding

This research was funded by Office of Research Innovation and Commercialization (ORIC), Khyber Medical University, Pakistan.

Disclosure of interest

The authors report no conflict of interest.

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AUTHORS

First Author – Arooj Javaid, M.Phil, Institute of Pharmaceutical Sciences, Khyber Medical University, Peshawar, Pakistan 25000
Second Author, Correspondence Author – Haji Bahadar, Assistant Professor, Institute of Pharmaceutical Sciences, Khyber Medical University, Peshawar, Pakistan 25000
Third Author – Ghazala Bibi, M.Phil, Institute of Pharmaceutical Sciences, Khyber Medical University, Peshawar, Pakistan 25000