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RESEARCH ARTICLE

Hepatoprotective Activity of *Nigella sativa* and *Piper nigrum* against Concanavalin A-Induced Acute Liver Injury in Mouse Model

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ABSTRACT

Nigella sativa and *Piper nigrum* are implicated in the treatment of various disorders, especially in the management of metabolic, infectious and hepatorenal ailments. However, data on the mechanism behind therapeutic potential of *N. sativa* and *P. nigrum* in liver diseases is scarce. The present study investigated the hepatoprotective potential of 70% methanolic extract of *N. sativa* (NSE) and *P. nigrum* (PNE) at varying dose levels (100 to 400 mg/kg body weight) against concanavalin A (conA)-induced liver injury. Qualitative phytochemical analysis of plant extracts was performed. Acute hepatic injury was induced by administering intraperitoneally 12mg/kg conA in Balb/c mice. The extent of hepatic injury was measured by analysing serum biochemical parameters, liver antioxidant stress assay and histopathology. Data were analysed statistically. NSE and PNE showed dose-dependent hepatoprotective efficacy by lowering the conA-dependent rise in liver transaminase level. Treatment with NSE (400 mg/Kg) and PNE (400 mg/Kg) ameliorated conA-induced alterations in serum oxidative stress markers, biochemical parameters, liver function markers and histopathology. NSE indicated greater effectiveness to ameliorate the acute hepatic injury in comparison with PNE at the same dose. Collectively, pretreatment with NSE and PNE attenuated the liver injury induced by conA, might be through alleviating the antioxidant capacity of experimental mice.

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INTRODUCTION

Liver diseases are the main cause of various public health problems globally. According to World Health Organisation (WHO), Pakistan has the highest incidence of hepatitis C after Egypt (WHO, 2017). Viral hepatitis, drug-induced hepatitis and autoimmune hepatitis (AIH) are the most common liver pathophysiological conditions (Asrani *et al.*, 2018). Presently, more than hundred drugs are known to cause hepatic damage and numerous hepatotoxic agents are responsible for liver impairment (Adam *et al.*, 2016). AIH is related to abnormal stimulation of immune cells in liver, leading to cirrhosis, hepatocellular carcinoma, and even death. Although, currently available therapeutic options for AIH are the use of immunosuppressant and liver transplantation; these are not considered ideal (Sucher *et al.*, 2019). These outcomes highlight the crucial requirements for understanding the pathophysiological changes in hepatic impairment and need for developing effective therapy for the treatment of hepatic ailments.

Some herbal treatments have potential immunological effects to alter hepatic immune balance towards therapeutic side as in case of AIH. The studies conducted to check immunological effects of herbal treatments indicated that they might involve in the regulation of immunoglobulin and cytokines secretion, lymphocyte proliferation, histamine release and cytotoxic activity (Balaban *et al.*, 2017). Moreover, natural antioxidants present in medicinal plants help in curing various diseases, including hepatic ailments, as they possess strong free radicals scavenging potentials and provide rational and safe curative strategies to prevent or treat oxidative stress caused liver injuries (Li *et al.*, 2015). In this regard, herbal medicines from traditional system provide safe and effective options in the management of liver ailments (Hong *et al.*, 2015).

Nigella sativa (*N. sativa*) is generally recognised as black seeds or black cumin belonging to *Ranunculaceae* family. The seeds of *N. sativa* have about 34 to 39% oil, and have valuable nutritional components such as high contents of essential fatty acids, alkaloids, glycolipids, flavonoids, phytosterol, essential oils and polyphenols (Mazaheri *et al.*, 2019). This plant is indigenous to South-western parts of Asia and the Middle East (Adam *et al.*, 2016), and has been cultivated in countries like Pakistan, Saudi Arabia, India, Iran and Syria (Mazaheri *et al.*, 2019). In traditional system of medicines, *N. sativa* has following basic therapeutic uses as an immune-modulatory, antimicrobial, antioxidant and hepatoprotective (Ahmad *et al.*, 2013)

Piper nigrum (*P. nigrum*) commonly called 'king of spices' belonging to *Piperaceae* family and seeds recognised as 'black pepper'. *P. nigrum* is indigenous to Southern India (Joshi *et al.*, 2018). The seeds have nutritional value owing to alkaloids, lignans, flavonoids and essential oils. In Ayurvedic system, ripe fruit of *P. nigrum* is used for various ailments like asthma, fever, tumours, pain, inflammation and liver disorders while in Unani system, its roots are used in hepatitis, arthritis and metabolic disorders (Agbor *et al.*, 2006). Piperin is the main bioactive constituent of *P. nigrum*, has many traditional therapeutic uses as anti-inflammatory, antioxidant and analgesic (Gorgani *et al.*, 2017).

Concanavalin A (conA), a lectin obtained from jack beans plant, initially used to investigate immune cell-mediated hepatic injury (Tiegs *et al.*, 1992), induces hepatitis mimicking viral and autoimmune hepatitis involving interleukin 1, 6, 10, interferon-γ and tumor necrosis factor-α (Boutsikou *et al.*, 2018). The objective of current study was to investigate hepatoprotective potential of *N. sativa* and *P. nigrum* in conA-induced oxidative stress. In this study, we have explored the antioxidant potential and hepatoprotective efficacy of methanolic extracts of *N. sativa* (NSE) and *P. nigrum* (PNE) against conA-induced liver injury.

MATERIALS AND METHODS

Preparation of plant extracts: *N. sativa* and *P. nigrum* seeds were identified and verified by Department of Botany, University of Agriculture, Faisalabad, Pakistan and the samples were deposited in the herbarium of Botany Department, against voucher number 245-1-19 and 246-1-19, respectively. Preparation of plant seed extracts were made by macerating 100 grams of each plant dried powder in methanol and water (7:3) for seven days with occasional shaking and concentrated by using rotatory evaporator. The final extracts were used to make optimum doses in normal saline.

Qualitative phytochemical analysis: Phytochemical analysis of NSE and PNE was performed by methods described previously (Ramamurthy and Sathiyadevi, 2017) for the detection of alkaloids, carbohydrates, glycosides, proteins, tannins, steroids, saponins, fixed oils, flavonoids, and phenols.

Acute oral toxicity testing: Acute oral toxicity testing were performed to determine safe dose range of NSE and PNE, which tested according to guideline no. 425 as provided by Organization of Economic Cooperation Development (OECD, 2008). Doses of NSE and PNE in range of 100, 200, 400, 800, and 2000 mg/kg to Balb/c mice group (n=3) were administered orally and after 24 hours, mortality was recorded. Particularly, after administration of plant extracts, gross behavioural alteration were observed for first one hour (Barua *et al.*, 2011).

Experimental animal and design: Balb/c mice, male sex weighing 27-32 gram were used. Mice were kept in the animal house at Institute of Physiology and Pharmacology, University of Agriculture, Faisalabad, Pakistan. Mice were fed with normal diet and water *ad libitum*. Firstly, mice were divided into eight groups (n=6) to determine the effective hepatoprotective dose of NSE and PNE against conA-induced acute liver injury. Group A was normal control. Group B was conA group. Group C-E were NSE treatment groups. Group F-H were PNE treatment groups. After determining the effective hepatoprotective dose of NSE and PNE mice were divided into four groups (n=6). Group I was normal control. Group III and IV were pretreated with NSE (400 mg/Kg) and PNE (400 mg/Kg) intragastrically on daily basis, respectively. ConA injection (12 mg/Kg) administered intraperitoneally in mice Group II, III and IV on the 7th day of treatment. Sampling was done on 7th day after 8 hours of conA administration.

Liver tissue homogenate preparation: The liver tissue homogenate was prepared for antioxidant profiling. For this, excised liver pieces were cleaned and washed with phosphate buffer saline (PBS) solution of pH 7.4. Liver pieces were homogenized with ice-cold PBS and centrifuged at 10000 rpm at 4ºC for 10 minutes (Adam *et al.*, 2016). Supernatants were separated and stored at -20ºC in biomedical freezer.

Liver function parameters: Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) levels were measured in serum samples by using commercially available kits SBio by Singapore Biosciences PTE Ltd. Total bilirubin, total protein and albumin levels were measured by using commercially available kits (Fluitest® BIL T/D provided by Analyticon® Biotecnologies AG, Germany).

Oxidative stress markers: Total oxidative stress (TOS) and total antioxidant capacity (TAC) were measured in serum samples using colorimetric method described by Erel (2005) and (Erel, 2004), respectively. Malondialdehyde (MDA) level was calculated following method mentioned by Ohkawa *et al.* (1979). Serum catalase enzyme activity was calculated according to method described earlier by Goth (1991). Superoxide dismutase (SOD) enzyme activity was determined by a method given by Giannopolitis and Ries (1977).

Histopathological examination of liver: Liver tissues were dehydrated in ascending grades of alcohol and then sections were fixed in the paraffin wax. Tissues were cut in thin sections, mounted on glass slides and stained with

hematoxylin and eosin (H & E). Slides were observed for histopathological investigation under light microscope. Scoring of liver tissue for histopathological analysis was performed according to Arsad *et al.* (2014). The histopathological parameters that were measured for scoring of liver tissue, included perivascular and portal cell infiltration, centrilobular necrosis and sinusoidal dilatation. The level of histopathological alterations was examined for each group (Table 2) and the extent of liver injury was graded such as: (0) indicating absence of changes, (1), (2) and (3) indicating mild, moderate and severe alterations, respectively.

Statistical analysis: Data was statistically analysed using one way-analysis of variance (ANOVA) and Duncan multiple range (DMR) test to check significance of difference between the experimental groups (Steel *et al.*, 1997). Kruskal-wallis test was applied on histopathological parameters. All the results were presented as mean±S.E. and the level of significance was set at 5% ($P<0.05$) using Statistical Package for the Social Sciences (SPSS) version 23.0.

RESULTS

Qualitative phytochemical characterization of NSE, PNE: Initial screening indicated the presence of valuable phytochemicals such as alkaloids, phenols, flavonoids, carbohydrates, glycosides, fats, fixed oils and tannins in NSE and PNE while steroids and terpenoids were only present in NSE, as shown in Table 1.

Acute oral toxicity test results: Oral administration of NSE and PNE up to 2 g/Kg revealed no toxic effects on treated mice as they showed normal behaviour, no other significant change in neurological responses and no mortality was found. It was found that both NSE and PNE at given doses (100, 200, 400, 800 and 2000 mg/kg) were non-toxic. After execution of acute oral toxicity testing, optimum safe dose (100-400 mg/Kg) of NSE and PNE was selected for the assessment of hepatoprotective activity.

Selection of effective hepatoprotective dose: For the determination of most effective dose, a dose-dependent hepatoprotective efficacy of NSE group (100-400 mg/Kg) and PNE group (100-400 mg/Kg) against conA for ALT was measured as shown in Fig. 1. As compared to other liver enzymes, ALT is more specific and sensitive to liver injury. In conA-induced group ALT (U/L) level was considerably ($P<0.05$) high, when compared with normal control group. On the other side, pretreatment with NSE (100, 200 and 400 mg/Kg) and PNE (100, 200 and 400 mg/Kg) during seven days lowered the conA-dependent rise in serum ALT levels. The 400 mg/Kg dose of NSE as well as PNE showed maximum hepatoprotective potentials by restoring ALT level almost near to normal control group. Accordingly, the dose of 400 mg/Kg of NSE and PNE was selected for further experiments.

NSE and PNE decrease conA-dependent rise in serum liver function markers: The conA-induced acute hepatic injury in mice as evident from significantly ($P<0.05$) raised levels of ALT, AST, ALP and bilirubin as compared to normal control group (Fig. 2, A-D). The pretreatment with 400 mg/Kg of NSE and PNE on a daily basis for seven days significantly ($P<0.05$) protected the mice from conA-induced acute liver injury by restoring liver enzyme markers. The total protein and albumin serum levels in conA group were declined significantly ($P<0.05$) as compared to pretreatment groups (Fig. 2, E, F), while NSE and PNE considerably ($P<0.05$) restored the total protein and albumin levels. Hence, pretreating mice with NSE (400 mg/Kg) and PNE (400 mg/Kg) during seven days prevented conA-induced alterations in liver function markers.

Table 1: Qualitative phytochemical analysis of NSE and PNE

Phytochemical	NSE	PNE
Alkaloids	+	+
Carbohydrates	+	+
Flavonoids	+	+
Fats and Fixed oils	+	+
Glycosides	+	+
Phenols	+	+
Saponins	+	+
Steroids	+	-
Tannins	+	+
Terpenoids	+	-

(+) symbol specifies the presence of the phytochemical, while (-) symbol specifies the absence of the phytochemical.

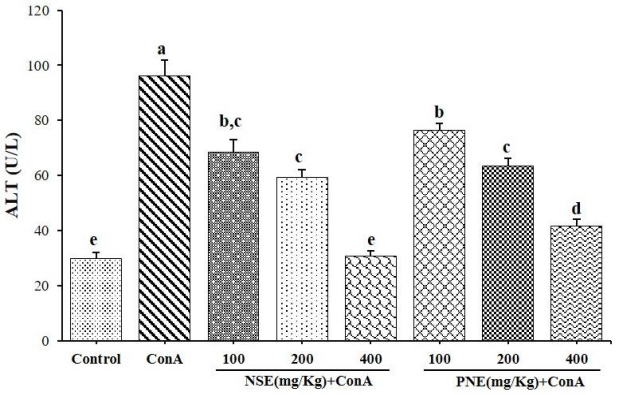

Fig. 1: NSE and PNE at dose level (100-400 mg/Kg) showed dose-dependent hepatoprotective against conA-induced acute hepatic injury in mouse model. Pretreatment with NSE (100, 200, 400mg/Kg) and PNE (100, 200, 400mg/Kg) for seven days after 8 hours exposure with conA (12 mg/Kg) protects experimental mice from acute hepatic injury due to conA as evaluated by measuring; serum ALT. The data are represented as Mean±S.E. Means with different letters (a, b, c, d, e) are significantly different at $P<0.05$. ALT, alanine aminotransferase.

Table 2: Effect of NSE and PNE pretreatment on histopathological alterations after 8 hours of conA-induction in experimental mice liver. The values are expressed as Mean±SE

Histopathological Parameters	Control group	ConA group	ConA+NSE group	ConA+PNE group	Kruskal-Wallis Test for global comparison of organ lesion among groups Asymptotic Significant ($P<0.05$)
Perivascular and portal cell infiltration	0.27±0.07	1.27±0.09	0.60±0.08	0.73±0.06	0.03
Centrilobular necrosis	0.13±0.08	1.2±0.13	0.47±0.16	0.60±0.11	0.005
Sinusoidal dilatation	0.20±0.13	1.40±0.02	0.53±0.07	0.67±0.12	0.002

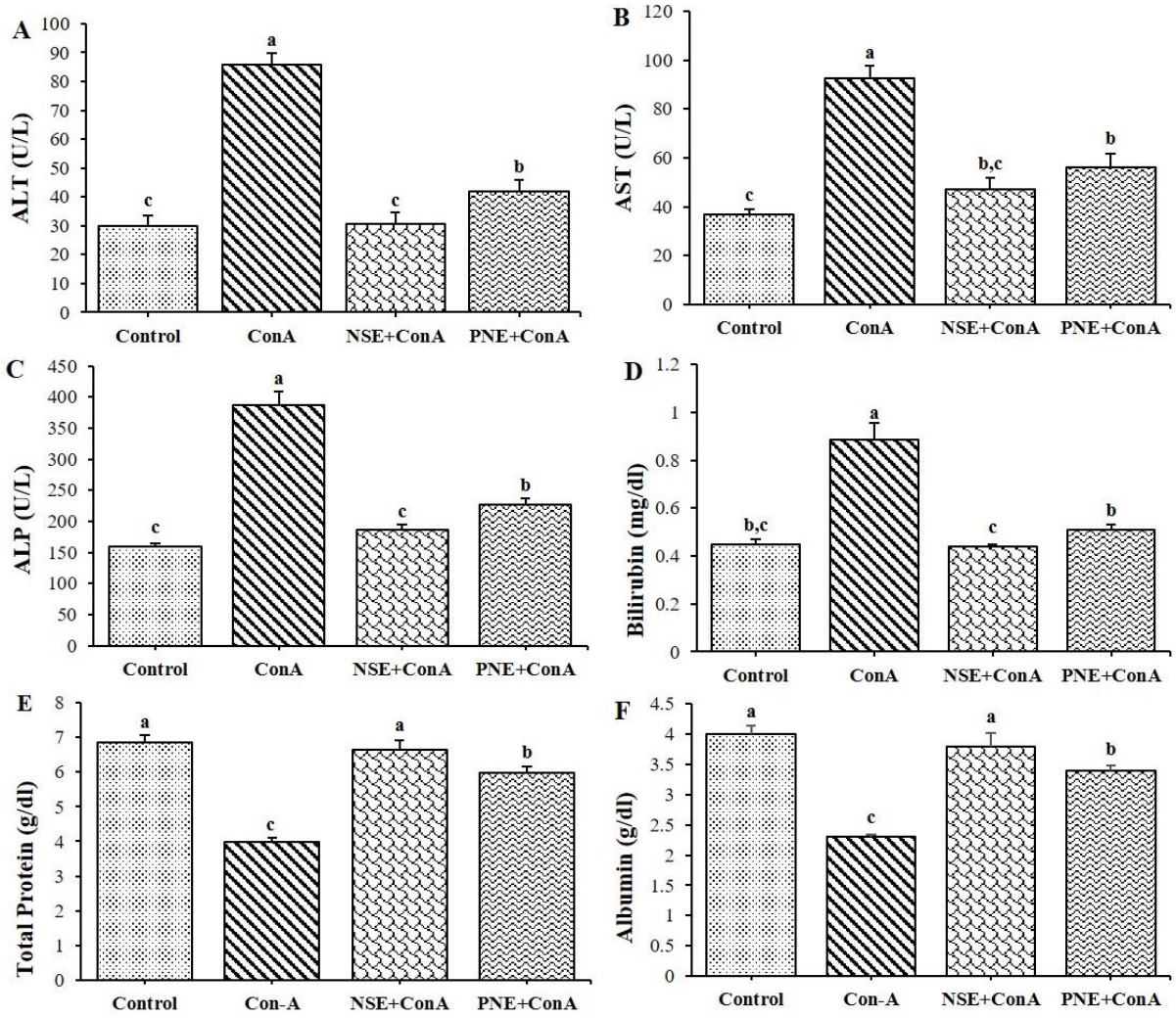

Fig. 2: NSE and PNE protect mice from conA-induced acute hepatic injury. Pretreatment with NSE (400mg/Kg) and PNE (400mg/Kg) for seven days after 8 hours exposure with conA (12 mg/Kg) protects experimental mice from acute hepatic injury due to conA as evaluated by assessing; (A) serum ALT, (B) serum AST, (C) serum ALP, (D) Bilirubin, (E) Total Protein, (F) Albumin. The data are represented as Mean ± S.E. Means with different letters (a, b, c) are significantly different at P <0.05. ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase.

NSE and PNE exert antioxidant effects in serum and tissue levels: ConA treatment in the vehicle group significantly (P<0.05) decreased the level of TAC, CAT and SOD and significantly (P<0.05) increased TOS and MDA level in serum and liver tissue homogenate as compared to the negative control group. Pretreatment of mice with NSE and PNE significantly (P<0.05) increased TAC, CAT and SOD and significantly (P<0.05) decreased TOS and MDA level in serum samples and in liver tissue homogenate, when compared with the conA group (Fig. 3, A-E).

Effect of treatments on histopathological parameters: Macroscopic analysis of mice livers showed morphological alteration in color suggesting conA-induced acute hepatic injury (Fig. 4). The scoring of liver histopathological parameters is presented in Table 2. Microscopically, hepatic tissue from negative control group showed the normal architecture (Fig. 4A). Whereas, hepatic parenchyma in the conA-induced group represented acute liver injury, indicated significant (P<0.05) mild to moderate degree of vascular degeneration (hazy appearance), condensed and pyknotic nuclei at some places showed a mild degree of necrotic changes as well as mild to moderate degree of congestion and inflammation (Fig. 4B). NSE group indicated significant (P<0.05) normal hepatocytes with no evidence of the hepatocyte inflammation as compared to conA group (Fig. 4C). Liver tissue of PNE treated group also indicated significant (P<0.05) improvement in the degenerative effects as compared to conA-treated group (Fig. 4D).

DISCUSSION

Liver is prone to various hepatotoxic agents because of its key role in metabolizing xenobiotics and regulating immune system. Autoimmune, viral, and metabolic disorders proceed towards end stage liver disease (Balaban *et al.*, 2017). Disturbance in immune system due to environmental or genetics factors lead towards AIH. In Pakistan, there is scarcity of data on current situation of AIH, however, a survey analysis showed that AIH predominantly affects female as compared to males. While the incidence of AIH with hypergamma-globulinemia is commonly occurred in male population of

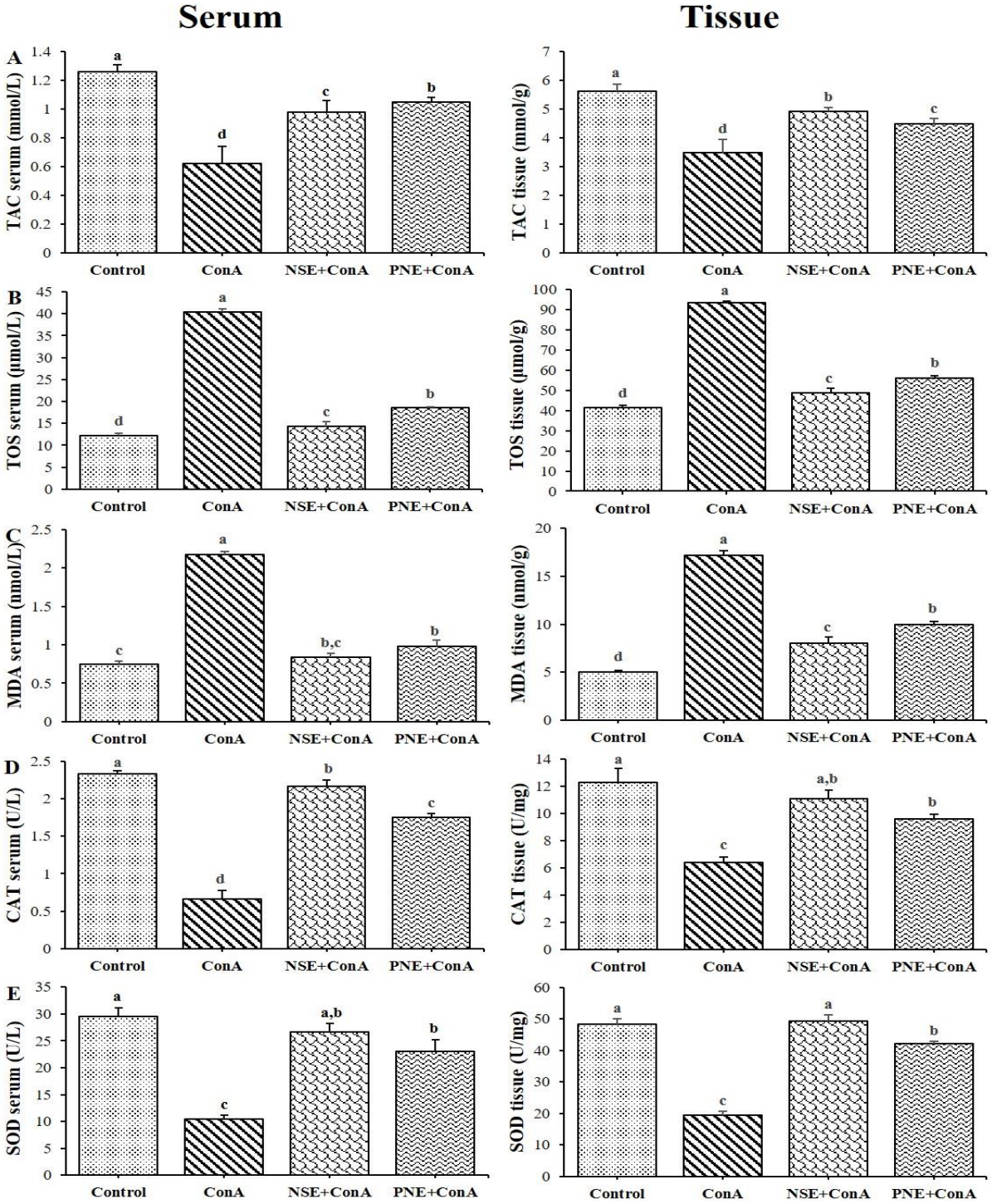

Fig. 3: NSE and PNE improve antioxidant capacities in experimental mice in conA-induced acute hepatic injury. Pretreatment with NSE (400mg/Kg) and PNE (400mg/Kg) for seven days after 8 hours exposure with conA (12 mg/Kg) reverse oxidative stress in experimental mice from acute hepatic injury due to conA as evaluated by measuring; (A) TAC, (B) TOS, (C) MDA, (D) CAT, (E) SOD in serum and liver tissue samples. The data are represented as Mean ± S.E. Means with different letters (a, b, c, d) are significantly different at P <0.05. TAC, total antioxidant capacity; TOS, total oxidative stress; MDA, malondialdehyde; CAT, catalase; SOD, superoxide dismutase. U/mg, Unit/mg of tissue protein.

Pakistan (Somroo *et al.*, 2018). Some medicinal plants have potential to modulate immune system. It is well-described in literature that *N. sativa* possesses beneficial immunomodulatory characteristics by reducing the secretion of pro-inflammatory cytokines, histamine and immunoglobulins (Koshak *et al.*, 2018).

Inflammation or injury of hepatocytes triggers oxidative stress in the liver. Oxidative stress plays significant role in pathogenesis of various hepatic diseases often associated with a reduction in the antioxidant defence system (Cichoż-Lach and Michalak, 2014). Phytoconstituents, as natural antioxidants, are vital plant components having the capacity to defend human health against diverse ailments (Ajuru *et al.*, 2017). In the current study, we have demonstrated the hepatoprotective potential of *N. sativa* and *P. nigrum* having valuable phytoconstituents, there is scarcity of experimental studies that could have established their hepatoprotection involving antioxidant mechanisms against conA-induced oxidative stress model.

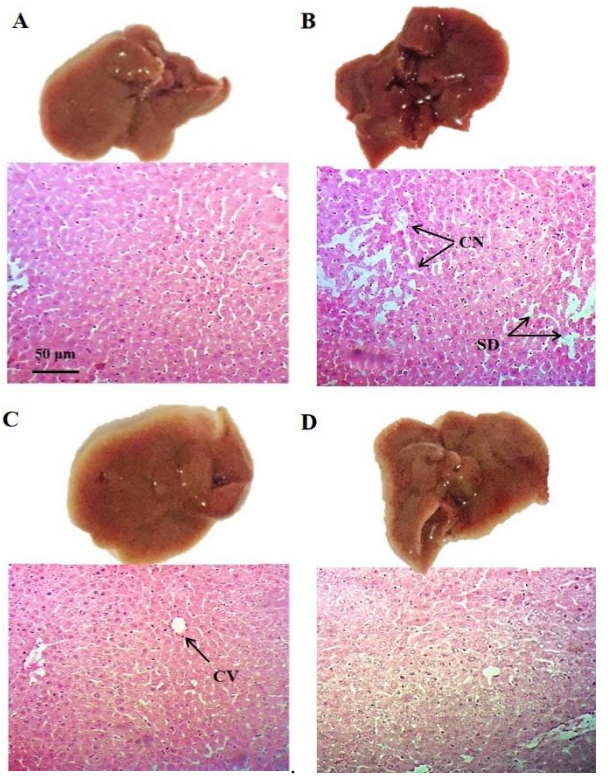

Fig. 4: Macroscopic examination and histolopathological analysis of liver tissues exposed to conA and pretreated with NSE and PNE. (A) control (B) ConA (12 mg/Kg) group (C) ConA + NSE (400mg/Kg) group (D) ConA + PNE (400mg/Kg) group. H and E, 100× magnification. CN: centro-lobular necrosis; CV: central vein; SD: sinusoidal dilatation.

Our study indicated the presence of essential phytoconstituents in the extracts of *N. sativa* and *P. nigrum.* Alkaloids, tannins and saponins are important phytoconstituents with diversified pharmacological properties. Phenolic and flavonoids compounds are secondary plant metabolites acting as natural antioxidants owing to free radical scavenging properties, thereby reduce oxidative stress in cells arising from environmental or chemical risk factors (Ajuru *et al.*, 2017).

Elevated liver enzymes are an indicator of hepatic cell injury that could lead to tissue damage and leakage of intracellular enzymes from the cell cytosol into the general circulation (Jaswal and Shukla, 2015). ALT is very specific and sensitive biomarker of hepatotoxicity even at acute hepatic damage. Raised level of ALP, an enzyme of cell membrane is a basic indicator of hepatobiliary injury (Schefer *et al.*, 2011). Our study results indicated that conA-induced acute liver injury after 8 hours exposure in experimental mice, which was evidenced by significant raise in the activity of serum transaminase such as ALT, AST and ALP. The results remain consistent with previous study conducted by Yu *et al.* (2018) who reported that intravenous administration of conA (25 mg/kg) significantly increased the serum transaminase activity (ALT and AST) at 8th hour in Balb/c mouse model. Further, our results revealed that pretreatment with NSE and PNE considerably restored the level of serum transaminases induced by administration of conA in experimental mice. These findings were verified by macroscopic and histopathological analysis of hepatic tissues suggesting that NSE and PNE provide protection against conA-induced hepatic injury. This indicates that functional integrity of hepatocytes was maintained by NSE and PNE pretreatment in the same way as restoration of hepatic enzyme levels.

Liver is a chief site of protein synthesis, particularly for albumin which is a basic marker to assess synthetic functions of the liver (Hamza and Al-Harbi, 2015). The present study indicated that after conA-induced hepatic injury level of total protein and albumin were significantly decreased. While the levels of total protein and albumin were restored in the pretreatment groups of NSE and PNE. Our findings are in accordance with Zahak *et al.* (2015). They reported that NSE provides protection against D-galactosamine-induced hepatic injury by restoring the levels of serum transaminases and albumin.

Antioxidant/oxidant markers were assessed by measuring TOS, MDA, CAT and SOD activity in serum and liver tissue samples. The level of TAC, SOD and CAT was significantly decreased while the level of TOS and MDA was significantly increased in conA-induced hepatic injury. The low level of TAC, SOD and CAT in conA group might be an indication of decreased antioxidant capacity which might be attributed to consumption of these antioxidant enzymes in detoxification of ROS. An earlier study conducted by Shirin *et al.* (2010) verified the presence of ROS in conA-induced acute hepatic injury mouse model. Pretreatment with NSE and PNE significantly increased CAT, SOD and TAC level while the level of TOS and MDA levels significantly decreased in serum and in liver tissue samples. Previous studies also indicated that NSE has potential to recover hepatotoxicity and metabolic disturbance due to oxidative stress and also has the ability to enhance the level of antioxidants by reducing the generation of reactive oxygen species (Adam *et al.*, 2016; Hamza and Al-Harbi, 2015). A previous study by Vijayakumar *et al.* (2004) reported that PNE has potential to overcome oxidative stress induced by high fat diet, and it significantly decreased the oxidative stress (Vijayakumar *et al.*, 2004). It has been demonstrated that application of antioxidant rich diet reduces many risks linked with liver pathologies (Li *et al.*, 2015).

Although, the present study reveals the hepatoprotective effects of NSE and PNE against conA-induced hepatic injury in mouse model for the first time, certain limitations exist in the current work. Further analysis is required of isolated phytoconstituents of *N. sativa* and *P. nigrum* to define the role of each component exclusively against conA-induced hepatic injury. These investigations may help in understanding the underlying mechanisms of hepatic ailments and also make appropriate therapeutic approaches utilizing plant constituents.

Conclusions: Pretreatment of mice with NSE and PNE during seven days significantly ameliorated the conA-induced alterations in liver function markers (ALT, AST, ALP), oxidative stress indicators (TOS, TAC, MDA, SOD, CAT) and liver histopathology presumably through antioxidant mechanism. Taking diet with high contents of antioxidants (i.e. fruits and vegetables) could provide protection against oxidative stress-induced ailments.

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Authors contribution: Conceived and designed the experiments: Aqsa Mushtaq, Bilal Aslam and Junaid Ali Khan. Performed the experiment: Aqsa Mushtaq. Contributed reagents/materials/analytical tools: Bilal Aslam, Faqir Muhammad and Junaid Ali Khan. Analyzed the data: Aqsa Mushtaq, Bilal Aslam and Junaid Ali Khan. Wrote the paper: Aqsa Mushtaq, Bilal Aslam and Junaid Ali Khan.

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