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Hepatoprotective and antioxidant activity of *Dicliptera bupleuroides* Nees. extracts on paracetamol induced hepatotoxicity in rats

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Abstract: Aim of this study to evaluate the safety profile, hepatoprotective and *in-vivo* antioxidant activities of *Dicliptera bupleuroides* Nees. Toxicity studies were conducted in human RBCs and DNA by using standard procedures. Acute hepatoprotective investigation was carried out in albino rats by treated with all six fractions of *D. bupleuroides* 350 mg/kg/day. ALT, AST, ALP and total bilirubin (TB) were performed. The n-hexane fraction (200 mg/kg/day) exhibited appropriate hepatoprotective activity hence subjected to chronic study (14 days). Paracetamol induced the hepatotoxicity (350mg/kg) and silymarin (50 mg/kg) was standard drug. Liver function tests, liver peroxidation tests and histopathological examination were performed at the end. Hexane fraction showed significant decrease in the level of ALT (88.1±7.8), AST (93.8±7.6), ALP (136.3±8.4) and TB (0.6±0.03) as compared to the standard drug (p>0.05). Rats treated with ethyl acetate fraction showed decrease in MDA (42.8±0.7) while GSH was found to be increased (107.7±1.8) against the toxic group (51.3±2.9), (73.6±4.0) respectively. All the drug extracts decreased the oxidative stress and protect the DNA from free hydroxyl radicals. DNA damage protection activity of these fractions is due to phytochemicals present in these fractions. These results indicate that the plant fractions possess significant hepatoprotective and antioxidant activities with no toxic effects.

Keywords: *Dicliptera bupleuroides* Nees, DNA protection assay, hepatoprotective, hemolysis, histopathology, *in vivo* antioxidant.

INTRODUCTION

Liver is the main site for the metabolism of the carbohydrates, proteins and lipids. Its role in health maintenance is well described as regulates the processes including hemostasis, disposition of endogenous substances (xenobiotics), drugs and detoxification of toxic substances (Almazroo *et al*., 2017; Han *et al*., 2016). Beyond the advances in health care, still there is no effective medicine available that offers the protection against the hepatic cells damage. Day to day increase in exposure to chemicals has resulted in more incidences of hepatotoxicity and other liver ailments, therefore it is a need of time to find out new drugs from natural sources for liver protection (Mehvish and Barkat 2018; Ali *et al*., 2019). Medicinal plants are thought to contain rich antioxidants constituents with ability to reduce oxidative stress and ultimately secure the body against various chronic diseases like aging, diabetes, cancer, inflammatory and cardiac dysfunction (Kurutas, 2015). Use of medicinal plants has gained prime importance in developing countries owing to their safety, cost effectiveness and efficacy. Pakistan has excellent medicinal plants reserves and local communities of different areas are well aware to the traditional utilization of these plants (Mishra *et al*., 2019). Conventionally, medicinal plants were used to treat different types of illnesses ranging from headache to stomachache to cut and wound (Thapa and Walia, 2007).

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Therapeutic significance of medicinal plants is due to the presence of phytochemicals like tannins, alkaloids, flavonoid, and phenolic compounds. *Dicliptera bupleuroides* Nees. of family Acanthaceae is a perennial herb commonly known in Urdu as Kaali booti (Ajaib *et al.,* 2015). It is found in the planes of Pakistan and Afghanistan as a flowering plant. It is an erect herb, height up to 90cm, bushy, twigs are hairy and leaves ovate or elliptic acute (Singh *et al*., 2014). This species is also arrogated to be used in traditional medicines for applying on wound of snake bite, in fever, in stomach troubles and also used in bone fracture (Panigrahi and Dubey 1983). *Dicliptera bupleuroides* possessed antioxidant, hepatoprotective, antimicrobial and other biological activities. It contains phenols, flavonoids, ascorbic acid, lipids, starch, glycosides and many other compounds (Ahmad *et al.,* 2013, Bahuguna *et al.,* 1987; Luo *et al*., 2002).The present study focused on identifying the in vivo hepatoprotective properties against the paracetamol induced hepatotoxicity in Wistar albino rats.

MATERIALS AND METHODS

Plant material collection and extraction

The plant was collected from Bhimber (Shamani), Kotli, Azad Kashmir and got authenticated by Dr. Uzma Hanif, Department of Botany, Government College University (GCU) Lahore, Pakistan. A specimen of plant was deposited in herbarium of GCU under voucher No.GC. Herb.Bot.3402.The plant was dried under shade and then

the whole herb was powdered. This powdered herb was macerated in methanol solvent for 7 days, filtered and evaporated using rotary evaporator (Heidolph, model Laborata 4000, Schwabach, Germany). After extraction fractionation was done with different solvents according to the polarity. Active fraction would then be separated by small column chromatography and preparative TLC (Giri *et al*., 2020).

Animals

Wistar albino rats of either sex (170-200g) were used for *in vivo* investigation. These animals were fed on diet with free access of water under controlled conditions of temperature 22±4°C, humidity (50-60%) and light (12 hours light/12 hours dark cycle). All animals were 24 hours fasted prior to perform all assays. Animals were randomly grouped as normal, paracetamol group, silymarin group and treated groups. The experiments were carried out as per recommendations of Institutional Ethical Committee for Animal care and Experimentation, under voucher no: 416 College of Pharmacy, University of the Punjab, Lahore, Pakistan.

Toxicity studies

In vitro Haemolytic activity

Fresh human blood (3mL) was taken in EDTA vial and centrifuged (H-200 NR, Kokusan, Japan) for 5min at 850 rpm. Clear supernatant was decanted and sedimented pellets were washed three times with chilled sterilized isotonic phosphate buffer saline (PBS) followed by the formation of suspension in 20mL of chilled sterilized PBS. Cells were counted in hemocytometer (BioTek, model µ-Quant^{TM} Winooski, USA). Triton-X was used as positive control and PBS served as negative control. Reaction mixture contains 20µL of plant extract and 180µL of blood cells suspension. They were incubated at 37°C for 40min. After incubation tubes were placed in ice cold PBS for 5min and then centrifuged at 1500 rpm for 5min. 100 µL of supernatant was collected in Eppondrof tubes and diluted with 900 µL of chilled sterilized PBS. All these samples including positive and negative control (200 µL) were loaded into 96 well plate. Following expression was used to calculate % hemolysis (Zubair *et al*., 2017).

$$\%\,\text{Hemolysis} = \frac{\text{Abs (Sample absorbance)}}{\text{Abs (Control absorbance)}} \times 100$$

DNA damage protection assay

DNA was isolated from human blood using DNA isolation kit. Isolated DNA was quantified by nano drop technique. The ability of different fractions of plant extract to protect genomic DNA was determined using the previously described method with slight modifications (Bhatt *et al*., 2018). In this method, DNA damage was induced by hydroxyl radicals generated from Fenton's reagent. Reaction mixture contained 4 µL of genomic DNA, 3µL of Fenton's reagent and 4µL of different fractions of plant and make up the volume up to 20µL using deionized water. Positive and negative controls were also prepared. Reaction mixtures were incubated at 37°C for 30 min. Bromophenol dye was added in each sample after incubation. The reaction mixture (10µL) was loaded in the wells of 1% agarose gel and electrophoresis was allowed to run horizontally for 1hour at 100V followed by staining with ethidium bromide. DNA was visualized under gel documentation system.

Experimental Design

The hepatoprotective activities were studied in paracetamol-induced liver injury model according to the documented method (Verma, 2018). After an adaptation period, rats were divided randomly into nine groups of six animals each. Treatments were then carried out according to the following:

Acute hepatoprotective study

Study design was comprised of 9 groups, Group 1: Control group, received the vehicle (distil water) only, Group 2: Paracetamol group (1.5g/kg), Group 3: Silymarin group (100 mg/kg per day), Group 4: Methanolic treated (500 mg/kg per day), Groups 5–9: Received the aqueous, n-Hexane, Chloroform, Ethyl acetate, n-Butanol fractions (350mg/kg per day) respectively.

Chronic hepatoprotective study

Rats were divided in to four groups as; Group 1: Control group, which received the vehicle (dist water) only, Group 2: Paracetamol group (350mg/kg), Group 3: Silymarin (50 mg/kg per day), Group 4: n-hexane fraction group (200 mg/kg per day).

For induction of liver injury, all groups except control group, paracetamol dose of 1.5g/kg was post administered after 30 minutes of standard drug and plant extract fractions. LFTs were performed in each animal group (Merghem *et al*., 2019). After this acute phase, chronic study was conducted on the n-hexane fraction for 14 days, on the 15th day, blood samples were collected for determining enzyme serum level, and livers were taken for histopathology as well for *in-vivo* antioxidant studies.

Biochemical study

Liver function Analysis

After paracetamol intoxication the blood was collected by heart puncture. ALT, AST, ALP and TB were analyzed with Global laboratory Kits (London) (Whitfield *et al*., 2019).

Antioxidant parameter study

Liver homogenate was used to measure level of superoxide dismutase (SOD) and reduced glutathione (GSH) (Sun *et al*., 2017; Xu *et al*., 2019).

Histological Examination

Liver tissues were embedded in paraffin wax. Fine sections were stained with eosin and hematoxylin, examined microscopically for pathological changes in tissues.

STATISTICAL ANALYSIS

Results are presented as mean ± SEM. ONE WAY ANOVA followed by Tukey post hoc test during statistical analysis of data. Graph prism pad 6 is used for making curves. Value of $p<0.05$ was considered to be significant (Moutal *et al*., 2018).

RESULTS

Toxicity studies

The extracts did not display any obvious toxic symptoms or mortality even in high doses. Hemolytic and DNA protection assay showed the safe use in animal as well in human (fig. 1 and 2). Hydroxyl radicals generated by Fenton's reagent causes DNA damage as DNA is absent in 3rd lane and only smear of degraded DNA can be observed. Aqueous extract was not effective at low concentration but effectiveness was considerably increased at higher concentration. Activity of ethyl acetate extract was decreased with increase in concentration. Although all extract showed activity but n-hexane, aqueous and butanol extracts were found to be most effective, methanol chloroform and ethyl acetate were possess little activity at high concentration.

Biochemical examination

Paracetamol was responsible to raise ALT, AST, ALP, TB and MDA values while decreased GSH level as shown in table I, II and III. While n-hexane drug fraction effectively restored these changes which was similar to

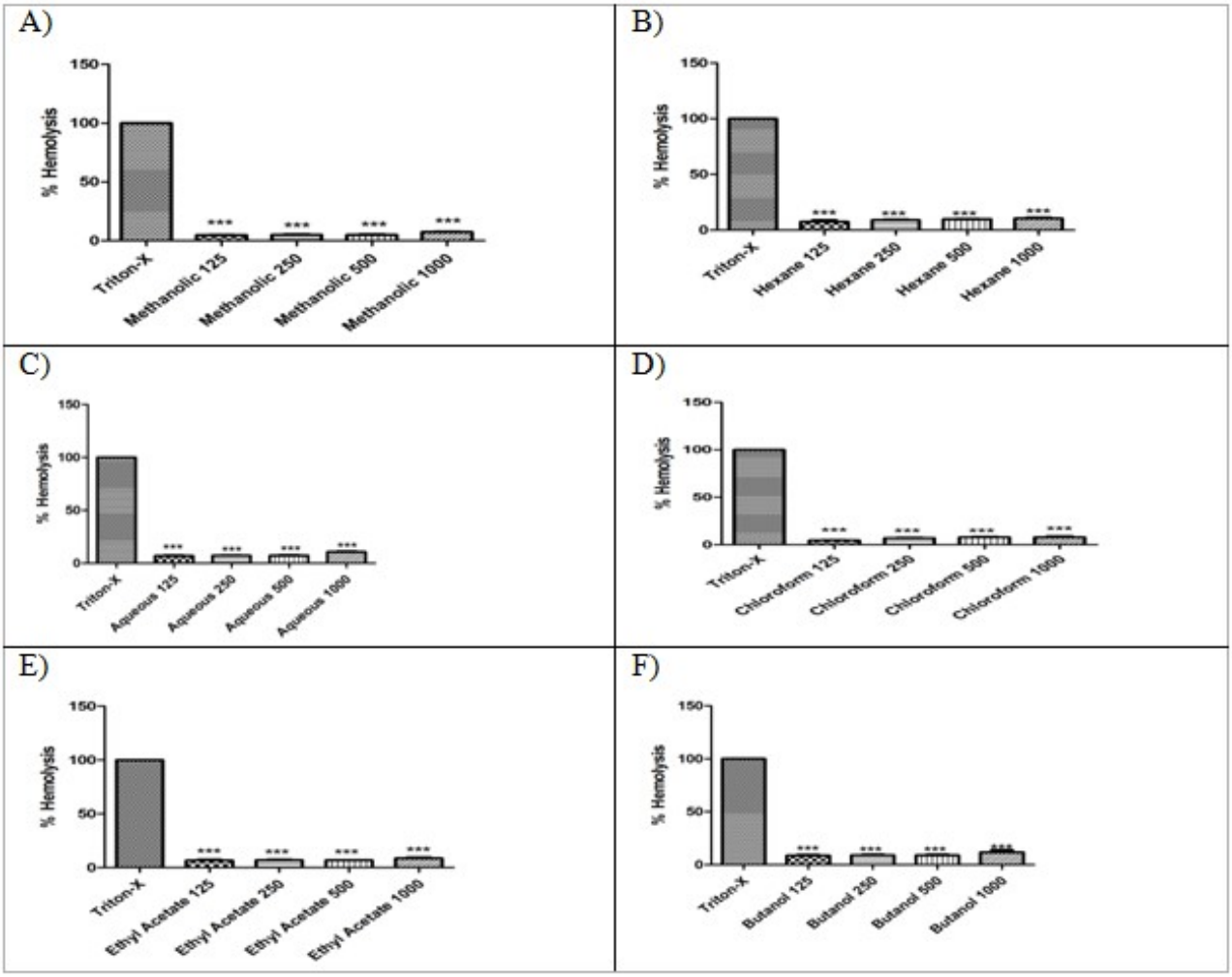

Fig. 1: Effect of extract of *Dicliptera bupleuroides* Nees. percentage hemolysis indicated by level of significant as *** $p>0.001$,** $p>0.01$,* $p>0.05$ (A) its crude methanolic extract; (B) n-hexane extract; (C) its Aqueous extract; (D) Chloroform extract; (E) Ethyl acetate extract; (F) n-butanol extract

Table 1: Acute Hepatotoxic effect on serum enzyme levels of different fractions of *Dicliptera bupleuroides* Nees.

Treatment	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	Total Bilirubin (mg/dL)
Control	57.83±5.58***	78.83±9.24***	109.7±1.76***	0.7±0.04
Paracetamol	1558.5±45.11	1606.67±42.74	301.0±43.38	0.73±0.03
Silymarin	83.33±8.21***	103.83±11.77***	126.67±20.95***	0.68±0.06
Methanol	111±15.56***	122.00±45.95***	145.33±13.59***	0.53±0.04*
Aqueous	129.67±14.36***	148.33±5.95***	179.67±30.30**	0.7±0.04
n-Hexane	91.5±10.03***	101.17±13.43***	135.5±11.70***	0.65±0.04
Chloroform	184.83±14.37***	204.67±22.79***	201.83±20.04*	0.6±0.04
Ethyl acetate	194.17±38.27***	201.5±28.56***	193.83±20.65*	0.67±0.06
n-Butanol	152.67±13.85***	171.17±27.70***	163.00±20.19***	0.72±0.05
(level of significance *** p>0.001,** p>0.01,* p>0.05)				

Table 2: Effect of n-hexane fraction of *Dicliptera bupleuroides* Nees. Serum enzymes in paracetamol induced chronic hepatotoxicity (level of significance *** p>0.001,** p>0.01,* p>0.05)

Treatment	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	Total Bilirubin (mg/dL)
Control	57.83±5.58***	78.83±9.23***	109.7±1.76***	0.7±0.04
Paracetamol	275±64.33	224.67±27.96	251±15.32	0.73±0.03
Silymarin	115±15.05**	95±6.64***	126.67±20.95***	0.7±0.04
n-Hexane	88.17±7.85**	93.83±7.62***	136.33±8.46***	0.67±0.03

Data presented as mean ± SEM, Significant at*** p<0.001,** p<0.01,* p<0.05 as compared to control group, n=6

Table 3: Antioxidant parameters in different studied groups

Treatment	MDA (nmol/g)	GSH (µmol/g)
Control	24.85±1.46***	104.25±7.71***
Paracetamol	51.34±2.91	73.67±4.04
Silymarin	34.44±1.94***	105.94±2.59***
n-Hexane	42.83±0.767***	107.74±1.833***
Data presented as mean ± SEM, Significant at*** p>0.001,** p>0.01,* p>.05 as compared to control group, n=6		

silymarin, graphically presented in fig. 3, 4 and 5.

Histopathological studies

Examination under light microscope showed the normal hepatocytes emitted from central vein and blood sinusoids with prominent nucleus in control group (fig. 6A). Paracetamol group showed disordered in hepatic articulator associated with central infiltration that led to severe necrosis. Additionally, dilation, hemorrhage of sinusoids were also observed (fig. 6B). Silymarin group showed least hepatic damage and reduction of hepatic necrosis. Few activated kupffer cells and dilated blood sinusoid were observed (fig. 6C). Ethyl acetate group showed restored histological structure and found cytoplasmic vacuoles with minimum activated kupffer cells (fig. 6D).

DISCUSSION

Red blood cells (RBCs) are the major cells in circulation and easy to isolate from blood. Their membrane resembles with structure of other membranes in the body and has complex structure that maintains the morphology, elasticity and deformability of cells. Exposure to toxic agents can change the membrane structure that results in hemolysis of RBCs. Anything that causes hemolysis is cytotoxic to RBCs and other body cells. Hence, to assess the cytotoxicity of molecules erythrocytes are widely used as biological model (Farag and Alagawany, 2018). DNA maintains the growth and repair by different metabolic reactions. Various factors like radiations, chemicals, hydroxyl radicals, and oxidative stress can damage DNA. Oxidative stress is one of the major causes of DNA damage in Human. This damage to DNA consequently onset different diseases including early age diabetes, cancer, Alzheimer disease, Parkinson's disease etc. Medicinal plants are considered to be the rich source of bioactive components. These bioactive components may have DNA damage protection activity. Hence they played an important role in providing better health to living beings (Kaur *et al*., 2019). In the present study, DNA damage protection

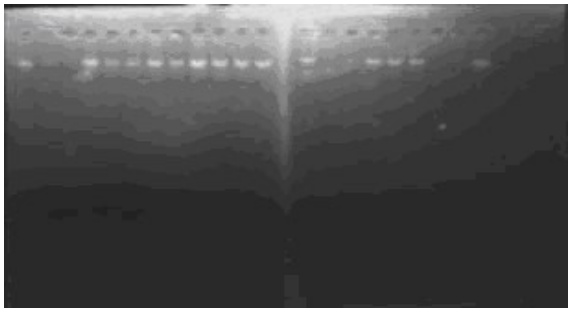

Fig. 2: DNA damage protection activity of Dicliptera. Lane 1: 4µl DNA+16µl DW, Lane 2: 3µl FR+17µl DW, Lane 3: 4µl DNA+3µl FR+13µl DW, Lane 4: 4µl DNA+4µl Butanol extract (50µg/µl) +3µl FR+9µl DW, Lane 5: 4µl DNA+4µl Butanol extract (100µg/µl) +3µl FR+9µl DW, Lane 6: 4µl DNA+4µl Butanol extract (200µg/µl) +3µl FR+9µl DW, Lane 7: 4µl DNA+4µl Hexane extract (50µg/µl) +3µl FR+9µl DW, Lane 8: 4µl DNA+4µl Hexane extract (100µg/µl) +3µl FR+9µl DW, Lane 9: 4µl DNA+4µl Hexane extract (200µg/µl) +3µl FR+9µl DW, Lane 10: 4µl DNA+4µl Methanol extract (50µg/µl) +3µl FR+9µl DW, Lane 11: 4µl DNA+4µl Methanol extract (100µg/µl) +3µl FR+9µl DW, Lane 12: 4µl DNA+4µl Methanol extract (200µg/µl) +3µl FR+9µl DW, Lane 13: 4µl DNA+4µl Ethyl acetate extract (50µg/µl) +3µl FR+9µl DW, Lane 14: 4µl DNA+4µl Ethyl acetate extract (100µg/µl) +3µl FR+9µl DW, Lane 15: 4µl DNA+4µl Ethyl acetate extract (200µg/µl) +3µl FR+9µl DW, Lane 16: 4µl DNA+4µl Chloroform extract (50µg/µl) +3µl FR+9µl DW, Lane 17: 4µl DNA+4µl Chloroform extract (100µg/µl) +3µl FR+9µl DW, Lane 18: 4µl DNA+4µl Chloroform extract (200µg/µl) +3µl FR+9µl DW, Lane 19: 4µl DNA+4µl Aqueous extract (50µg/µl)+3µl FR+9µl DW, Lane 20: 4µl DNA+4µl Aqueous extract (100µg/µl)+3µl FR+9µl DW, Lane 21: 4µl DNA+4µl Aqueous extract (200µg/µl)+3µl FR+9µl DW

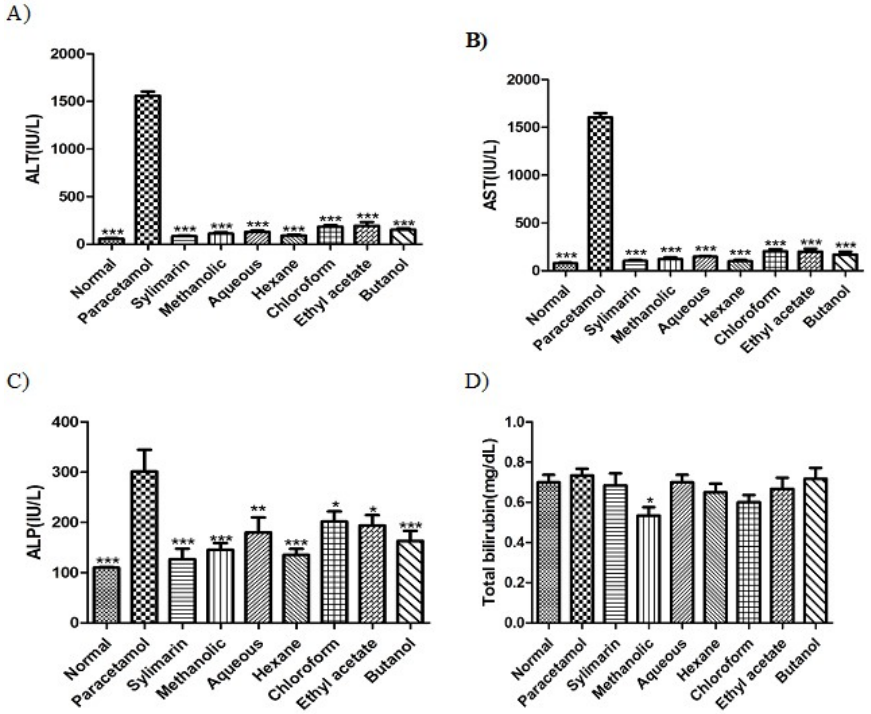

Fig. 3: Effects of different fractions of *Dicliptera bupleuroides* Nees. on blood serum levels of ALT, AST, ALP and total bilirubin in acute hepatotoxicity model.

activity of different fractions of *Dicliptera* was previously analyzed using Fenton's reagent as damaging agent. All the extracts lessen the oxidative stress and protect the DNA from hydroxyl radicals generated by Fenton's reaction. Butanol extract was comparatively found to be the most effective to protect DNA bands followed by n-hexane, aqueous and methanol extract. Butanol and aqueous extract showed dose dependent protection. DNA damage protection activity of these fractions is due to phytochemicals like phenolic and flavonoids present in these fractions. These phytochemicals scavenge free radicals and protect DNA from damaging effects of these radicals. Butanol and hexane fractions significant activity shown by extract is attributed to their inability to quench hydroxyl radicals.

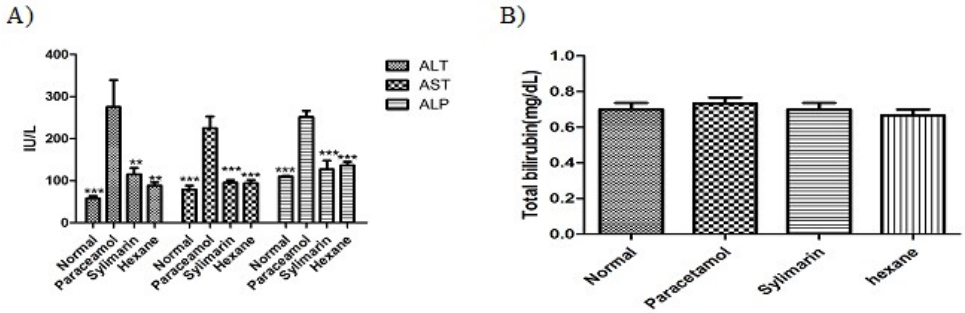

Fig. 4: Effect of n-hexane fraction of drug on serum activity of ALT, AST, ALP and (TB) in chronic hepatotoxicity model.

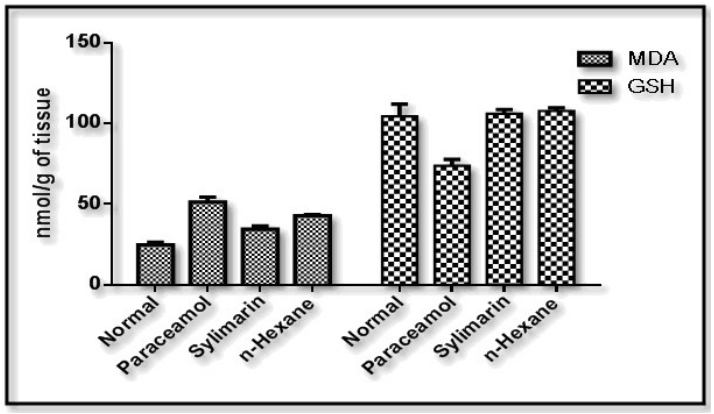

Fig. 5: Effect of n-hexane fraction of *Dicliptera bupleuroides* Nees. on the MDA and GSH levels in the liver homogenate.

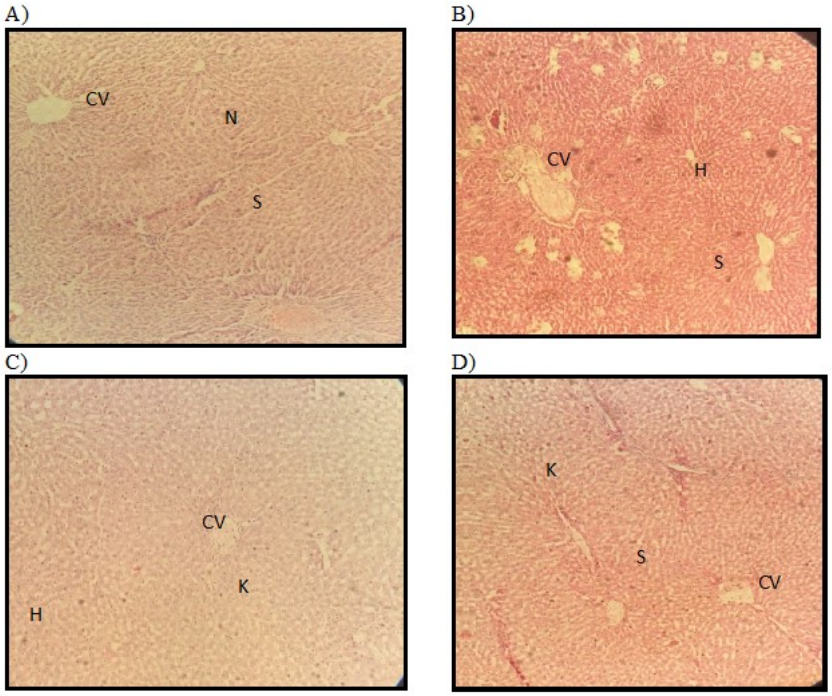

Fig. 6: Liver section stained hematoxylin and eosin. A: Normal control group showing central vein (CV), hepatocyte with prominent nuclei (N) and hepatic sinusoids (S); B: Paracetamol group showing inflammatory cells infiltrating around central vein (CV), hepatocellular necrosis with hemorrhage between hepatocytes (H); C: silymarin group showing that hepatocyte (H) mostly had normal appearance less dilated blood sinusoids and activated kupffer cells (K); D: n-hexane extract group showing restored hepatocytes architecture, with less dilated blood sinusoids (S) with activated kupffer cells (K).

The hepatoprotective studies on rats were performed to prove the claim to be used in a number of therapeutically assiduities such as for the treatment of ulcer and tumors; due to generation of free radicals in the body. Paracetamol has been prescribed widely as pain killer in our region, but it is well documented that the overdose may cause hepatic necrosis, hemorrhage in human and experimental animals as well (Wong and Graudins 2017; Caparrotta *et al*., 2018). For studying the hepatoprotective effect of medicinal plants the most commonly used injury was hepatic injury induced by paracetamol. Assessment of hepatic damage was assessed by histopathological evaluation as well as liver function tests along with serum markers in circulation. Free radicals were produced when paracetamol undergone metabolism in liver, yields peroxide radicals upon reaction with free oxygen anions. Integrity of membranes was destroyed when it making covalent bonds with lipids. Hepatotoxicity produces due to alteration in membranous structure of experimental rats. It was obvious from the results of table 1 that all plant fraction (methanol, aqueous, n-hexane, chloroform, ethyl acetate and n-butanol) reduced the level of biochemical parameters in acute hepatotoxic model. n-Hexane fraction was chosen for chronic study on the basis of result. Reduction in liver enzymatic levels towards normal values gave the indication of regenerative process of membrane. n- Hexane fraction showed protective effect when compared with results of toxic group (treated with paracetamol), showed normal articulator when compared with standard drug silymarin (Wong and Graudins, 2017).

Lipid peroxidation is a destructive process, auto catalytic and free radical mediated process in cellular membrane that convert polyunsaturated fatty acids into lipid hydro peroxides (Pund *et al.,* 2012). During this unsaturation process free radical are produced, responsible for formation of malondialdehyde. It is a reactive aldehyde capable of oxidative stress in different tissues and referred as advanced lipoxidation end product (Vurmaz *et al*., 2019). Production of this aldehyde is used as a biomarker level for oxidative stress in experimental animal (Del Rio *et al*., 2005). *Dicliptera* had strong antioxidant activity in Chinensis spp (Xu *et al*., 2017). While *D. bupleuroides* showed significant therapeutic values due to polyphenols (Riaz *et al*., 2019). Decrease level of MDA controls the oxidative stress and hepatic damage resulting in tissue health. Glutathione is one of the major tripeptides found in the liver tissue, its main function is to remove free radicals such as hydrogen peroxide, superoxide radical and maintenance of membrane protein thiols. It is a substrate for glutathione peroxidase, increase in level of GSH caused to decrease cellular stress and maintains the membranous health (Pawar and Abhang, 2017).

CONCLUSION

Our findings showed that histopathological hepatic damage is suppressed by administration of n-hexane fraction of *Dicliptera bupleuroides* Nees. These results were also confirmed from biochemical tests of serum as well as antioxidant levels of MDA and GSH of liver homogenate. Therefore it is concluded that all drug fractions could be used to protect liver cells damage with higher safety and n-hexane drug fraction has enhanced antioxidant activity to inhibit the paracetamol induced liver damage.

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