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IN-VIVO EVALUATION OF HEPATOPROTECTIVE PHARMACOLOGICAL ACTIVITY OF DIOSMIN LOADED PHYTOSOME

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ABSTRACT

To investigate the hepatoprotective activity of phytosome against CCL_4-induced liver injury in wistar albino rats. The degree of protection was evaluated by estimation of serum biochemical parameters, histopathology study.

Method: Phytosome was prepared with Diosmin and excipient by using solvent evaporation method. the pharmacological- activity was measured *in-vivo*.The albino wistar rats (150-200 gm) were divided into 6 group and 6 animals in each group were taken for the study, Group I: Normal Saline. Group II: Toxin control 1:1CCl_4+liquid paraffin IP Inducing group. Group III: Standard (25mg/kg) +CCl_4+liquid paraffin IP Inducing group. Group IV: Pure Drug (Diosmin 20mg/kg/day) +CCl_4+liquid paraffin IP Inducing group. Group V: Drug +Lipid physical mixture +CCl_4+liquid paraffin IP Inducing group. Group VI: Diosmin phytosome +CCl_4+liquid paraffin IP Inducing group for 14 days. Sample was collected by retro-orbital puncture and serum was isolated from blood for estimation of biochemical parameters and other parameters and histopathological studies were also performed.

Results: Experimental finding revealed that the carbon tetrachloride produce significant change in physical (increase liver weight), biochemical parameters (serum ALT,AST, and ALP and bilirubin)in blood circulation and histological (damage to hepatocyte) in liver .Pretreatment with Diosmin phytosome complex significantly improves physical, biochemical, histological and functional change induced by CCL_4 in liver.

Conclusion: Experimental data and analysis of different evaluation parameters showed that Diosmin phytosome has significant hepatoprotective potential. further study still needed to be done on exposure of phytosome to human beings.

Keywords: Diosmine-phytosome, Hepatoprotective activity, Carbontetrachloride, Pharmacologicl activity

INTRODUCTION

Natural resources from plants provide new opportunities for the treatment of various diseases which is a more effective approach with enhance effectiveness of dose with minimum dose size and reduced side effect .Different active plant content isolated in twenth century are used as therapeutic active agent as anticancer,anti-inflammatory,hepatoprotective,antioxidant with minimal side effects.

ACTIVE CONSTITUENTS

Diosmin is a natural flavonoid glycoside and obtained from pericarps of various citrus used for medical purpose. Disomin act as vascular protecting agent used to treat chronic venous

insufficiency,hepatoprotective activity. Pharmacological and therapeutic activity of diosmin shows that its more potent drug to treat liver disease , hepatoprotective action,antioxidant activity but possess practically very low solubility in water, low bioavailability, poor drug dissolution and higher dose (Bogucka–Kocka et, al 2013).it is also a nutrient used to improve metabolic disorders like glucose metabolism. Diosmin is worked as vascular protecting agents and used to improve hemorrhoids,lymphedema,chronic venous insufficiency ,and varicose veins. (Mustafa, et al.,2022)

Diosmin has molecular formula $C_{28}H_{32}O_{15}$.basic and clinical research studied that the diosmin (IUPAC name 5-hydroxy-2- (3-hydroxy-4-methoxyphenyl) -7- [(2S,3R,4S,5S,6R) -3,4,5-trihydroxy-6-[[(2R,3R,4R,5R,6S) -3,4,5- trihydroxy -6- methyloxan -2- yl] oxymethyl] oxan-2-yl] oxychromen -4-one.) (Mustafa, et al.2022), the Diosmin loaded phytosome is the one approach to increase the physicochemical properties and bioavailability of drug in comparison of pure drug. (Sangeetha et al.,2016).In the study of in-vivo pharmacology activity of drug hepatic damage caused by carbon tetrachloride is a widely used model for the assessment of hepatoprotective potential of herbal drug and phyto-formulation .According to concept firstly the CCL_4 is metabolized by the enzyme Cytochrome P450 presents in the endoplasmic reticulum of the liver cell to produce the trichloromethyl(CCL_3^-) free radical and then it(CCL_3^-) convert further into a highly reactive trichloromethyl peroxy radical (CCL_3OO^-) in the presence of oxygen.These metabolites of CCL_4 is very reactive and toxic in nature. it change the integrity and permeability of hepatic cell membrane by oxidation of polyunsaturated fatty acid (EL-Gazayerly et al.,2014).

These free radicals are capable of initiating lipid peroxidation reaction in liver and damage the liver. Several studies indicate that antioxidants act as protect the liver from oxidative damage, and they can prevent the risk of liver diseases (Ouassou.et al., 2021).These effect the release of liver enzyme such as ALT,AST and ALP in blood circulation.the level of these liver enzyme and Total Bilirubin(TB) concentration are the important tools for identification of status of liver function.In normal case low level of these biomarkers are normally present in plasma.in case of hepatotoxicity or CCl_4 induced hepatotoxicity the concentration of these biomarkers increased in plasma. (Mitra et al.,1998)

The present study involved the investigating of in-vivo activity of diosmin pure compound and Phytosome by using wistar albino rats and after applying the statistical methods like ANOVA and concluded the result.

MATERIALS

Diosmin (purchased from Sigma-Aldrich,Mumbai,India),Phospholipid and other solvents and chemical reagents are analytical grade.

METHOD

Phytosome is a chemical complex system can be prepared by using Solvent evaporation method .Diosmin phytosome was prepared by using round bottom flask.Diosmin were dissolve in Dimethylsulphoxide (DMSO) and taken with different molar ratio of phospholipid in round bottom flask. Mixture was refluxed on water bath for 3 hours at 45^0 C and after 3 hour the mixture was cooled and then pour to wide mouth petridish.the petridish was kept open overnight at room temperature for evaporation of solvent and after this dried under vacuum at 60^0 C for 3 hour. Dried diosmin phytosome residue were collected and stored in desiccator.(Freag et al., 2013).Optimized Formulation having Diosmin to phospholipid molar ratio (1:1.75) was used for pharmacological evaluation.

EXPERIMENTAL ANIMALS

Male Wistar albino rats weighing (150–200 gm) obtained from Department of pharmaceutical sciences ,Bhimtal campus ,Kumaun University,Nainital,Uttarakhand ,India.The animals were housed at Departmental Animal House under CPCSEA standard conditions, at a temperature 24 ± 2 °C and relative humidity 40–70%. For 12 hour,the light and dark cycle was followed. All animals had free access to pure water and standard laboratory animal diet .All the experimental methods and protocols used in this study were reviewed and approved by the authority (IAEC Approved Protocol No.- KUDOPS/100)of

Institutional Animal Ethical Committee and were in accordance with the guidelines and instruction of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA).the experiment was performed in same place.Total 14 days were taken for the study. On day 14 the animals were sacrificed and Histopathology study was done.

EXPERIMENTAL DESIGN

Male Wistar rats were divided into six groups of six animals in each group viz: Group I: Normal Saline. Group II: Toxin control 1:1CCl_4+liquid paraffin IP Inducing group. Group III: Standard silymarin (25mg/kg) +CCl_4+liquid paraffin IP Inducing group. Group IV: Pure Drug (Diosmin 20mg/kg/day) +CCl_4+liquid paraffin IP Inducing group. Group V: Drug +Lipid physical mixture +CCl_4+liquid paraffin IP Inducing group. Group VI: Drug phytosome +CCl_4+liquid paraffin IP Inducing group for 14 days. Animals received the test dose, respectively.(Singh, A. et al.,2018)

GroupI: Normal Saline.
GroupII: Toxin control (1:1v/v CCl_4+liquid paraffin) IP Inducing group.
GroupIII: Standard Drug silymarin (25mg/kg) +CCl_4+liquid paraffin IP Inducing group.
GroupIV: Pure Diosmin (20 mg/kg/day) +CCl_4+liquid paraffin IP Inducing group.
GroupV: Diosmin +Lipid physical mixture +CCl_4+liquid paraffin IP Inducing group.
GroupVI: Diosmin phytosome +CCl_4+liquid paraffin IP Inducing group.

This study was undertaken to determine the hepatoprotective potential of phytoconstituents by using carbon tetrachloride induced hepatotoxicity in rats screening model.

COLLECTION OF BLOOD SAMPLE AND ASSESSMENT OF HEPATOPROTECTIVE ACTIVITY

On day 14 all the animals were administered test, standard, $CCl_4$and normal saline to their respective groups, then within half an hr CCl_4 were administered. After 6 hrs all animals were anesthetized by using Thiopentone sodium 40 mg/kg IP and sleeping time were recorded. The blood collection were performed by retro-orbital puncture of experimental animal and allows standing for 30 min at 37^0C, then Serum was separated from it with the help of centrifugation in 2500 rpm for 30 minute and further estimation of Serum Biochemical Parameter (Transasia Bio Medical HP) like serum enzymes aspartate aminotransferase (AST),serum saline aminotransferase (ALT), serum alkaline phosphatase (ALP) and total bilirubin. The animals were sacrificed under ether anesthesia, liver from all animals were removed, washed with ice cold saline and weighed and used for further histopathological examination. The serum marker enzymes like serum enzymes used aspartate aminotransferase (AST),serum saline aminotransferase (ALT), serum alkaline phosphatase (ALP) and total bilirubin are important tool for evaluation of liver function.

HISTOPATHOLOGY

Histopathological method were carried by modified methods of "Luna" in brief the autopsied livers were washed with normal saline, the material were fixed in ten percent buffered neutral formalin for two days followed with bovine solution for six hours and liver were paraffin embedding section of liver 5µ thickness were sliced by using microtome, and processed in absolute alcohol solution –Xylene, serves and stand with Haematoxyline and Eosin-Blue, the slides were observed and found the changes under a light Microscope for any histological studies. (Prabhakar, P. V et al.,2012)

STATISTICAL ANALYSIS

For the liver function biomarker test the data were expressed as mean ± SEM. The statistical significance was determined by the one way analysis of variance (ANOVA) method followed by Dunnett's test. The statistical p value less than 0.05 were considered significantly and plot the comparison graph by graph-pad prism method.

RESULTS AND DISCUSSION

ESTIMATION OF BIOCHEMICAL PARAMETERS

The liver damage caused by administration of CCl_4 to the wistar albino rats resulted in significant increase (<0.05) of concentration of marker enzymes in table-1 and respective graph in Figure-1.

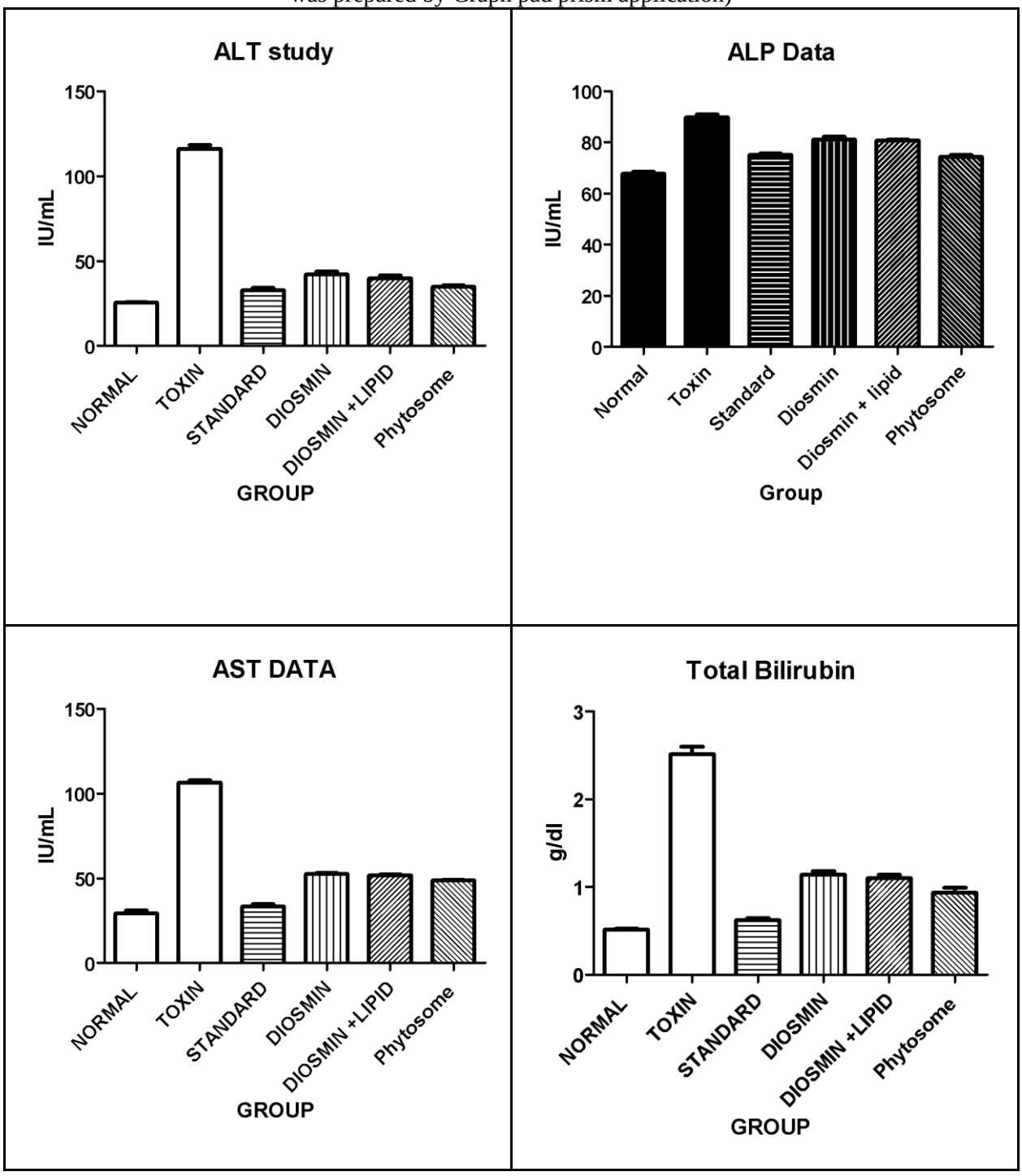
Table-1-Estimates of normal control ,disease control,standard control,diosmin,and phytosome showed different ranges of biomarkers.

GROUP	TREATMENTS	ALT(IU/ml)	AST(IU/ml)	ALP(IU/ml)	TB(mg/dl)
I	Normal	25.56±0.30**	29.32±1.51**	69.68±1.07**	0.51±0.01**
II	Toxin	116.06±2.41	106.62±1.15	89.70±1.12	2.51±0.08
III	Standard (silymarin)	32.83±1.23**	33.43±1.25**	75.01±0.07**	0.62±0.02**
IV	Diosmin	42.16±1.70*	52.57±0.65*	81.16±0.96*	1.14±0.03*
V	Diosmin + Phospholipid Physical Mixture	39.83±1.74**	51.66±0.64**	80.59±0.48**	1.10±0.03**
VI	Phytosome	34.83±1.01**	48.66±0.33**	74.22±0.92**	0.93±0.05**

The data are expressed as mean± SEM (N=6).The statistical data was observed as one way ANOVA followed by Dunnett's.*p<0.05 and **p<0.01 as cpmared to disease control group.

The animal group (toxin control) showed the range of all biomarkers as ALT 116.06±2.41, AST 106.62±1.15, ALP 89.70±1.12 &TB 2.51±0.08.Normal treated group showed the range of all biomarkers as ALT 25.56±0.30, AST 29.23±1.51, ALP 69.68±1.07 & TB 0.51±0.01.The standard group showed the range of all biomarkers as ALT 32.83±1.23, AST 33.43±1.25, ALP 75.01±0.07 &TB 0.62±0.02.The diosmin drug treated group showed the biomarker reading of ALT 42.16±1.70 ,AST 52.57±0.65, ALP 81.16±0.96 and TB in the range of 1.14±0.03 .The physical mixture of Diosmin and Excipient treated group showed ALT 39.83±1.74, AST 51.66±0.64, ALP 80.59±0.48 and TB in the range of 1.10±0.03 .The group of diosmin phytosome showed the significant difference in biomarkers level. As It expressed the biomarker reading of ALT 34.83±1.01, AST 48.66±0.33, ALP 74.22±0.92 and TB in the range of 0.93±0.05.

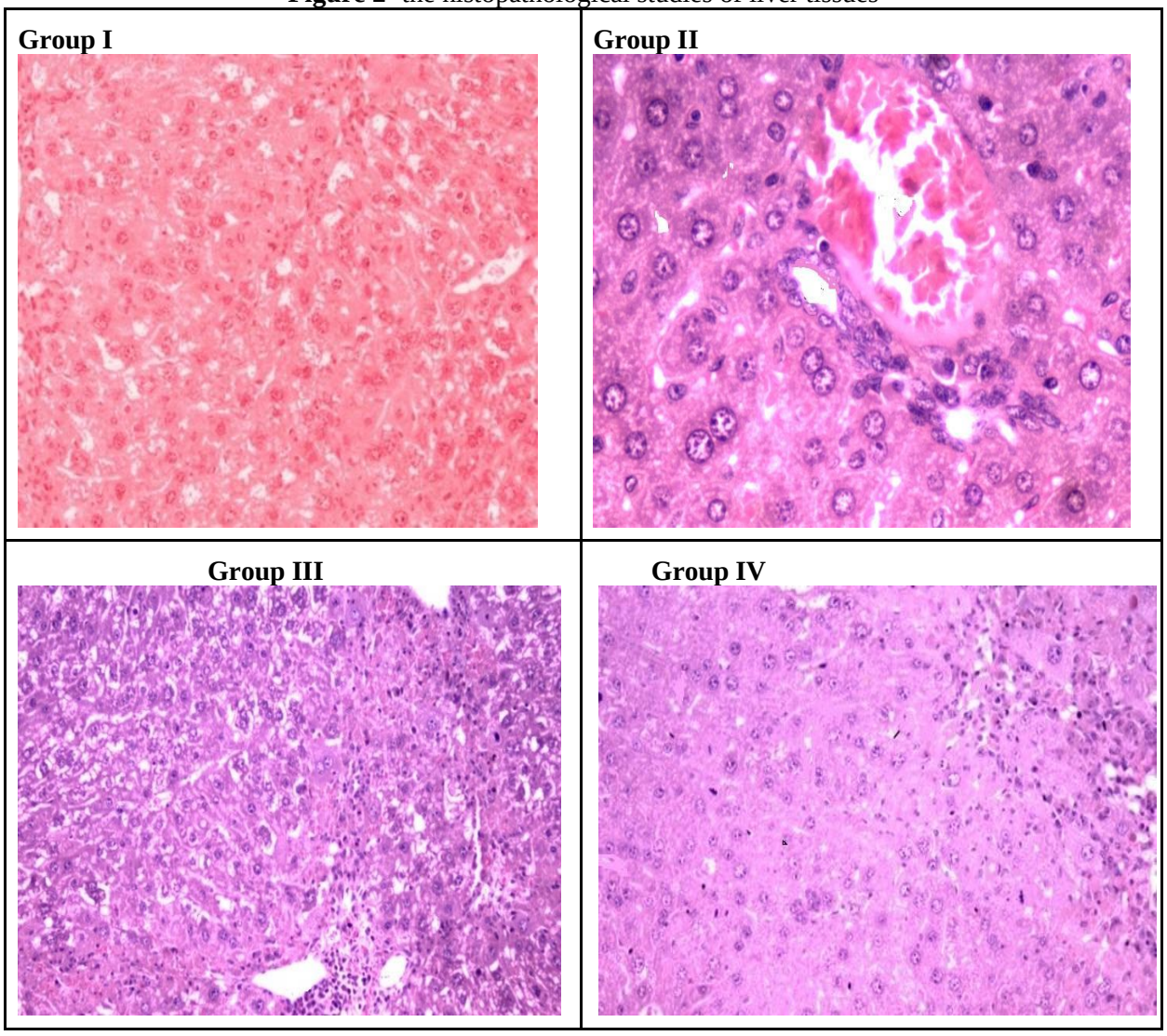
Figure 1- Effect of different treatments on serum ALT,AST,ALP and Total Bilirubin levels in rats.(Graph was prepared by Graph pad prism application)

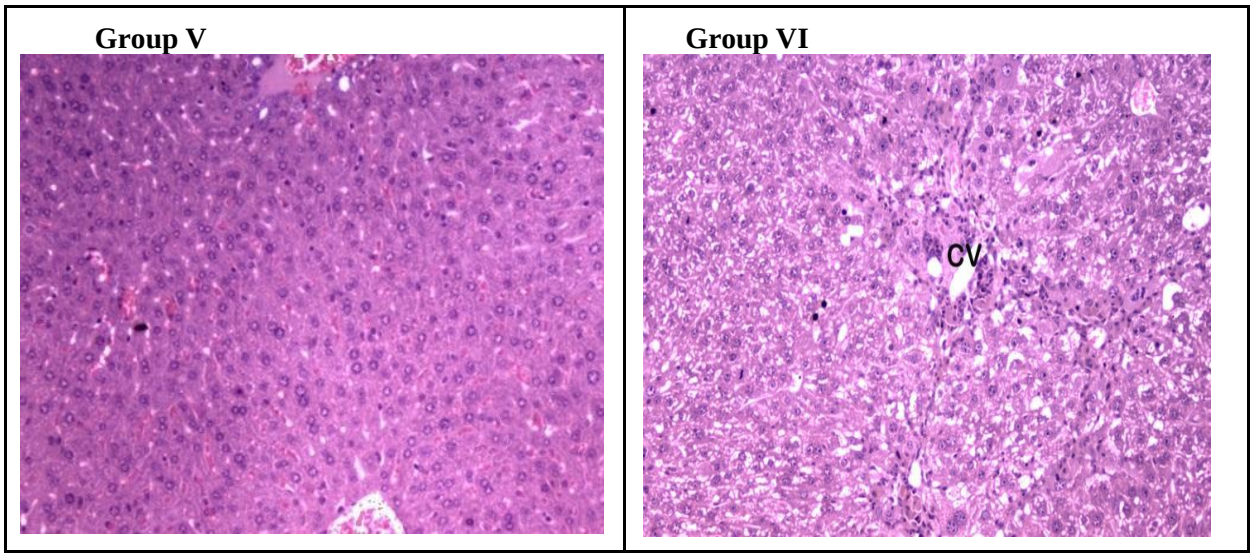


HISTOPATHOLOGICAL STUDIES

The histopathological studies of liver tissues showed the result in Figure 2

Figure 2- the histopathological studies of liver tissues





Histopathology of liver tissues at 10× showed following observation

(A)Group I slide- first is a liver of a normal control group showing normal histology of hepatic cell.showing no necrosis and no cytoplasmic vacuolation.

(B) Group II slide showing the Toxin control (CCl_4 Treated) Inducing group,showing a central vein necrosis,Hepatic degeneration with heavy necrosis in fatty acid vacuoles and inflammatory cells.

(C) Group III slide showed Standard Drug silymarin Inducing group and showing normal histology and portal triad showing regeneration of hepatocytes..

(D) Group IV slide Section of the liver tissue of animal treated with Diosmin drug treated animal showing the reduction of inflammation and decreases the cell necrosis in liver.

(E) Group V slide represent the Physical mixture of diosmine and phospholipids showing .reduces inflammation and cell necrosis and higher level of fatty regeneration.

(F) Group VI slide represents the Phytosome formulation showing the complete regeneration of an hepatocyte cell are found to be normal and better effect as comparison to Pure Disomin drug.

CONCLUSION

The present experimental work was aimed to develop Diosmin phytosome formulation and to evaluate hepatoprotective potential .The outcome of the study revealed that the phytosome preparation of Disomin significantly reduces the elevated hepatic serum enzymes level in experimental animal after administration of CCl_4 hepatotoxicity inducing agent. The biochemical testing it was clearly observed that the phytosome preparation were found to be more significant as comparison to other groups. It significantly reduced the serum level ALT,AST,ALP ($p<0.05$ and 0.01) & enhanced the hepatoprotective activity after phytosome administered. Furthermore the histopathological studies strongly backup the biochemical findings.

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