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HEPATOPROTECTIVE ACTION OF A POLYHERBAL AQUEOUS ETHANOLIC EXTRACT AGAINST NIMESULIDE INTOXICATED ALBINO RATS

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Abstract: Kingdome Plantae is full of natural herbs and plants, most of which are constantly used in ethno-medical practice. In this study we used aqueous ethanolic extract of three plants (*Cuminum cyminum* seeds, *Cichorium intybus* leaves *and Solanum nigrum* aerial parts) to achieve hepatoprotective activity in Nimesulide intoxicated albino rats. Total of 49 albino rats were divided into seven groups with seven animals in each. Group-I and II were administered Normal saline and dimethyl sulfoxide respectively, in dose of 5 ml/Kg p.o., while Nimesulide (100 mg/Kg p.o.) was given to Group-III to induce hepatotoxicity. Group-IV was given Silymarin in dose of 25 mg/Kg p.o. Experimental control groups (Groups V-VII) were given plants extract in dose of 100, 200 and 300 mg/Kg p.o., respectively. After 15 days, all the animals were sacrificed and blood was collected by cardiac puncture. Livers were isolated after dissection and preserved in 10% formalin for histopathological studies. Serum was subjected to find out level of liver enzymes i.e., ALP (Alkaline Phosphatase), SGOT (Serum Glutamic Oxaloacetic Transaminase), SGPT (Serum Glutamic Pyruvic Transaminase) and TB (Total Bilirubin), by using Human diagnostic kits. Statistical and histopathological analysis indicated that poly herbal formulation produced marked reduction in level of all four parameters with significance level of $p < 0.001$ in comparison of intoxicated control group which indicates the excellent hepatoprotective activity of poly herbal extract

Keywords: Nimesulide, Silymarin, Hepatoprotective activity, ALP, SGOT, SGPT, TB.

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INTRODUCTION

Hepatoprotection is essential to cope with adverse events occurring due to intake of various chemicals and intoxicants in the body. A plenty of formulations are used from centuries to avoid hepatotoxicities [1,2]. In this concern, more or less 600 plants and herbal remedies are in practice around the world. About 170 constituents have been isolated from different plant families which are available in various formulations[3]. However, only a few no. of plants and herbs have been evaluated ethno-pharmacologically for their safe and effective use [4]. Three plants (*Cuminum cyminum, Cichorium intybus* and *Solanum nigrum*) used in this study have been known since a long ago for the treatment and prevention of hepatic illness. Out of which, *C. intybus* and *S. nigrum* have been pharmacologically approved as hepatoprotective agents in preventing Nimesulide induced hepatotoxicity in albino rats [5,6]. Phytochemical constituents like carbohydrates, proteins, calcium and phosphorus along with vitamin-A, vitamin-C along with variable fractions of various volatile oils are enriched in cumin seeds[7] due to which it exhibits both anti-oxidant and free radical scavenging activities [8]. It has been scientifically approved that plant extracts of *Solanum nigrum* and *Cichorium intybus* prevent the hepatocytes from damage by inhibiting free radical-mediated DNA damage [9]. *S. nigrum* plant extract has been extensively used as a hepatoprotective agent because it significantly reduces the hepatotoxicity by lowering the level of certain hepatic enzymes [10].

MATERIALS AND METHODS

Preparation of crude extract

We used three plants in this study. Fresh green leaves of *Cichorium intybus* as well as aerial parts of *Solanum nigrum* were collected from local green fields of Sahiwal division and Cumin seeds were purchased from Bahawalpur. All three plant materials were then identified by the botanist and specimen were preserved in the herbarium at the Faculty of Pharmacy and Alternative medicine, the Islamia University of Bahawalpur, Pakistan. Plant materials were then properly washed with water and dried properly. Completely dried materials were then ground separately to coarse powder by using electric grinder (National, Japan). 100 g of each ground powder was macerated in 70% aqueous ethanol for five days. Soaked material was thoroughly stirred thrice daily. At the end of 5th day of maceration, the soaked materials was filtered first by muslin cloth and then through Whatmann filters paper no. 1. Residue was again macerated to get more filtrate. This whole process was repeated thrice at five days interval and filtrate so obtained after three soakings was subjected to rotary evaporator at 30-40°C to concentrate the extract. In the end, semisolid paste having thick and viscous consistency was obtained. The paste was weighed out to find percentage yield. The poly herbal extract (C.E) so obtained was

49 g and percentage yield calculated was 16.3% which was packed in air tight container with proper labeling (C.E) and put into refrigerator for future use [11].

Equipments

Digital electronic balance (AY 62 Shimadzu Corporation, Japan) Centrifuge machine (EBA 20 Heltich D-7853), Vortex Mixer (SLV 6 Serulin Bioscience, Korea), Grinder (National, Japan), Merck Microlab 300 (Merck Germany), Rotary evaporator (Heidolph Laborota 4000, efficient, Germany) and Microscope (Micron).

Pharmacological Materials

Ethanol, Xylene, Formalin, Diagnostics kits for assessing ALP, SGOT, SGPT and TB levels in serum, Paraffin Wax, Eosin, Hematoxylin dye, Canada balsam, Silymarin and Nimesulide. All the chemicals of analytical grade were purchased from Merck, Human-Germany and Nimesulide was donated by Sami Pharmaceuticals, Pakistan upon request. Silymarin was purchased from Abbott Laboratories, Pakistan. Ketamine and Diazepam were purchased from local Pharmacy.

Experimental Animals

Albino Sprague-Dawley rats of both sexes weighing 180-200 g were selected for study after approval by Pharmacy Research Ethics Committee with Ref. No: (54-2012/PREC). Albino rats and mice are available in animal house of Faculty of Pharmacy and Alternative Medicine. All animals were kept in polycarbonated cages of size 47x34x18 cm^3. They were provided temperature controlled hygienic, neat and clean environments in animal house. The standard conditions of temperature (25 ± 2˚C) and humidity (50-55%) along with exposure of 12:12 hours light and dark cycle, were provided to animals till end of study. The rats were acclimatized for one week before initiation of experiments.

Phytochemical Analysis

Crude extracts were subjected to phytochemical analysis for identification of alkaloids, cardiac glycosides, steroids, tannins, and saponins. Following methods were used for analysis and results are shown in Table 1.

Tests for Saponins

Foam test: 500 mg of crude extract was dissolved in boiling water in test tube. Then it was cooled down and vigorously shaken to produce the forth [12]. Presence of forth indicated the saponins.

Tests for Tannins

Ferric chloride test: Extract was dissolved in 10 ml of distilled water and then filtered. 1% aqueous or alcoholic FeCl3 was added in filtrate which produced intense green, purple, blue or black colour which indicated the tannins.

Iodine test: Extract was treated with dilute iodine solution. Formation of transient red colour indicated the presence of tannins.

Nitric acid test: extract was treated with dilute nitric acid and the formation of raddish to yellow colour indicated the presence of tannins.

Gelatin test: 0.5 g of extract was mixed with 1% gelatin solution containing 10% NaCl. Formation of white precipitates indicated the tannins [13].

Test for Alkaloids

500-600 mg of crude extract was treated with 8 ml of 1% HCl, warmed on water bath and then filtered and divided in to four test tubes.

Hager's test: 2 ml of filtrate was mixed with few drops of Hager's reagent (saturated aqueous solution of picric acid). Appearance of turbidity or yellow precipitates indicated the presence of alkaloids.

Wagner's test: 2 ml of filtrate was mixed with few drops of Wagner's reagent. Appearance of reddish brown precipitates indicated the presence of alkaloids.

Dragendroff's test: 2 ml of filtrate was mixed with Dragendroff's reagent. Appearance of turbidity or precipitates indicated the presence of alkaloids.

Mayer's test: 2 ml of filtrate was mixed with Mayer's reagent. Appearance of turbidity or precipitates indicated the presence of alkaloids [14].

Tests for Glycosides

Keller-Kiliani Test: Took extract solution in test tube and added few drops of $FeCl_3$ in it. Concentrated CH_3COOH and concentrated H_2SO_4 were added carefully along the wall of test tube. Reddish brown coloration at the junction of both layers and bluish green color at the upper layer indicated the presence of glycosides.

Table 1: Phytochemical constituents present in Poly herbal formulation

S. No.	Phytochemical Tests	Phytochemical Constituents
	Saponins	
1	Foam Test	+ve
2	Haemolysis Test	+ve
	Tannins	
1	Iodine Test	-ve
2	Ferric Chloride Test	+ve
3	Nitric Acid Test	+ve
4	Gelatin Test	+ve
	Alkaloides	
1	Hager's Test	+ve
2	Wagner's Test	+ve
3	Mayer's Test	+ve
4	Dragendorff Test	-ve
	Cardiac glycosides	
1	Keller Killani test	+ve
	Terpenes and sterols	
1	Libermann-Burchard test	+ve

Note: (+) and (-) signs repot the relative presence and absence of constituents in formulation

Induction of Hepatotoxicity

Hepatic toxicity was induced by Nimesulide. Nimesulide was administered orally on daily basis in suspension form. Nimesulide is a 4-nitro-2-phenoxy methanesulphonamide, non-steroidal anti-inflammatory Drug (NSAID) preferential COX-2 inhibitor, which acts by inhibiting leukocyte function, PAF synthesis, TNFα release, metalloproteinase activity in cartilage and has strong free radical scavenging effect. Although this is very effective NSAID yet it is associated with severe adverse effects like hepato-biliary, cutaneous and gastrointestinal system. Acute hepatitis, fulminant hepatic failure, cholestatic liver injury, multiple enterocolic perforations and end stage renal failure with nimesulide intake have been reported in various case reports of hepatotoxicity. Even fatal hepatic failure leading to withdrawal of drug in various countries but this is still in practice in some developing countries [15].

Study Design

Animals were divided into seven groups with seven animals in each group. Group-I received normal saline in dose of 5ml/Kg p.o. once daily. Group-II was given DMSO at dose of 5ml/Kg p.o. Group-III received Nimesulide 100 mg/Kg p.o. for seven days to produce hepatotoxicity. Group IV was Standard Control and given Silymarin alone for first eight days in dose of 25 mg/Kg p.o. and then along with Nimesulide (100 mg/kg p.o.) for further seven days. Group V-VII was given poly herbal crude extract alone in dose of 100, 200 and 300 mg/Kg p.o., respectively for first eight days and then Nimesulide in dose of 100 mg/kg p.o. along with crude extract for further seven days. 24 hours after the last treatment dose, the animals were given anesthesia by administration of diazepam (5 mg/kg i.p.) and ketamine (50 mg/ kg i.p.). Animals were dissected and 3ml of blood was taken by cardiac puncture from each rat. Livers were isolated and preserved in 10% formalin for histopathological evaluation. Serum was collected by centrifugation of each sample of blood and then levels of serum enzymes were monitored by using diagnostic kits.

Table 2: Study design for assessment of hepatoprotective activity

Groups	Day1 to 7	Day 8 to 15
Group I (Normal Control)	Normal Saline 5ml/kg p.o	Normal Saline 5ml/kg p.o
Group II (Vehicle Control)	Dimethyl sulfoxide 5ml/kg p.o	Dimethyl sulfoxide 5ml/kg p.o
Group III (Intoxicated Control)	Nil	Nimesulide 100 mg/kg p.o.
Group IV (Standard Control)	Silymarin 25 mg/kg p.o.	Silymarin 25 mg/kg p.o. + Nimesulide 100 mg/kg p.o.
Group V (Experimental Control)	Poly Herbal Extract (C.E) 100 mg/kg p.o.	C.E 100 mg/kg p.o. + Nimesulide 100 mg/kg p.o.
Group VI (Experimental Control)	Poly Herbal Extract (C.E) 200 mg/kg p.o.	C.E 200 mg/kg p.o. + Nimesulide 200 mg/kg p.o.
Group VII (Experimental Control)	Poly Herbal Extract (C.E) 300 mg/kg p.o.	C.E 300 mg/kg p.o. + Nimesulide 300 mg/kg p.o.

Histopathology

Liver sections cut by microtome were dehydrated in ethanol, cleared in xylene and then fixed in paraffin. 4-5 µm sections were cut to prepare slides and hematoxylin and eosin dye was used for staining slides [16].

Statistical Analysis of Results

Results were expressed as Mean ± SEM (n=7). Student t test was applied. P values were considered as $P > 0.05$ non-significant (ns), and $P < 0.05$ as significant.

RESULTS

Effects of C.E on ALP, SGOT, SGPT and TB level in albino rats

The values for all parameters (ALP, SGOT, SGPT and TB) as shown in Table 3, indicates that there is no difference between values of Group I and Group II, thus it represents the least effect of DMSO on serum enzyme markers. Highly elevated values of Group III indicate marked hepatotoxicity produced in rats who were administered only Nimesulide. On the other hand, there is significant ($P < 0.001$) reduction in the level of all four serum enzyme markers in experimental control groups which were given poly herbal extract in dose of 100, 200 and 300 mg/Kg p.o. However, values of Group IV and Group VII are in nearest range to normal control group. This indicates that poly herbal extract has highest hepatoprotective potential in 300 mg/Kg dose, almost identical to standard control group.

Table: 3. Effects of different doses of poly herbal extract (C.E) on ALP, SGOT, SGPT and TB level in Nimesulide intoxicated albino rats.

No.	Groups	Level of ALP (IU/L)	Level of SGOT (IU/L)	Level of SGPT (IU/L)	Level of TB (IU/L)
1	Group I	220.77±15.56	112.24±5.27	51.60±4.35	0.85±0.07
2	Group II	219.17±15.82	108.81±4.22	51.04±4.35	0.86±0.08
3	Group III	889.01±24.71$^{###}$	223.29±7.57$^{###}$	115.57±5.67$^{###}$	3.60±0.16$^{###}$
4	Group IV	260.16±17.81	116.69±5.76	58.03±3.34	0.95±0.15
5	Group V	393.94±21.35***	159.66±8.22***	68.04±5.58***	1.85±0.17***
6	Group VI	370.97±19.01***	135.21±5.68***	65.03±5.11***	1.47±0.14***
7	Group VII	262.23±18.56***	109.31±5.73***	57.54±4.34***	0.96±0.09***
(Values are mean ± SE with 7 animals in each group)					

P-values: $^{###}$≤ 0.001vs. vehicle control, ^{ns}>0.05, *<0.05, ** <0.01, ***<0.001 vs. intoxicated

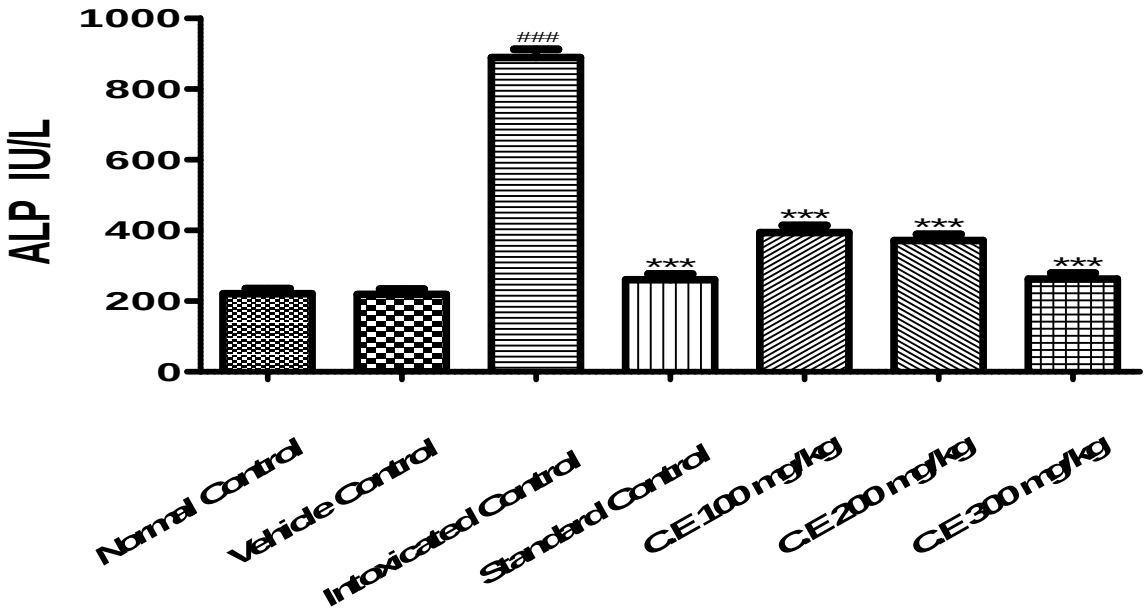


Figure 1: Effects of different doses of poly herbal extract (C.E) on ALP level in Nimesulide intoxicated albino rats.

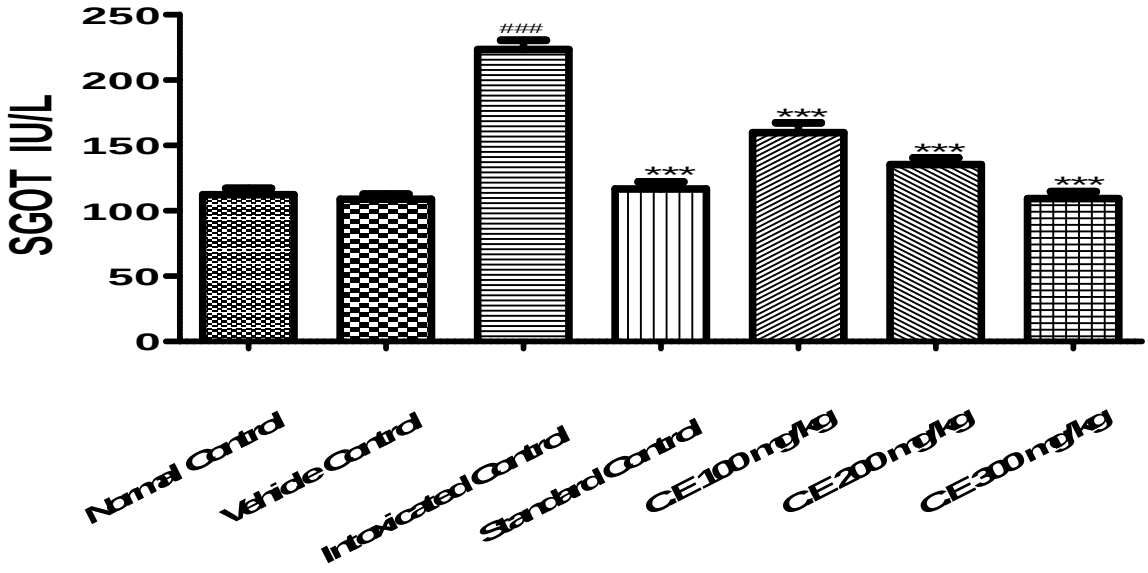


Figure 2: Effects of different doses of poly herbal extract (C.E) on SGOT level in Nimesulide intoxicated albino rats.

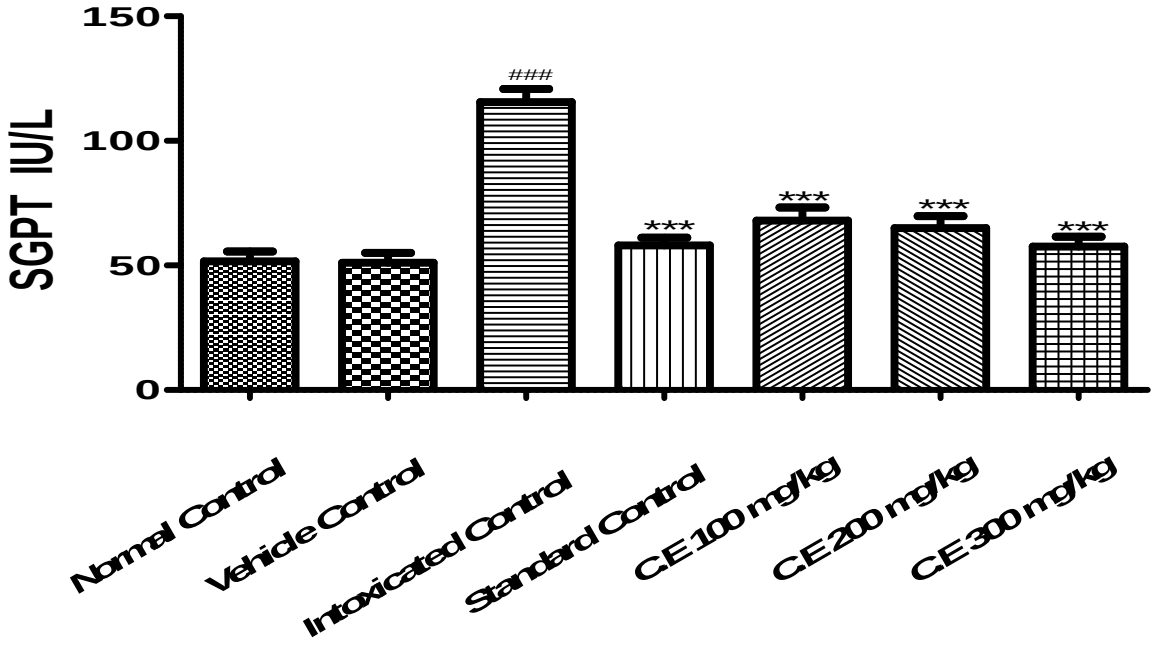


Figure 3: Effects of different doses of poly herbal extract (C.E) on SGPT level in Nimesulide intoxicated albino rats.

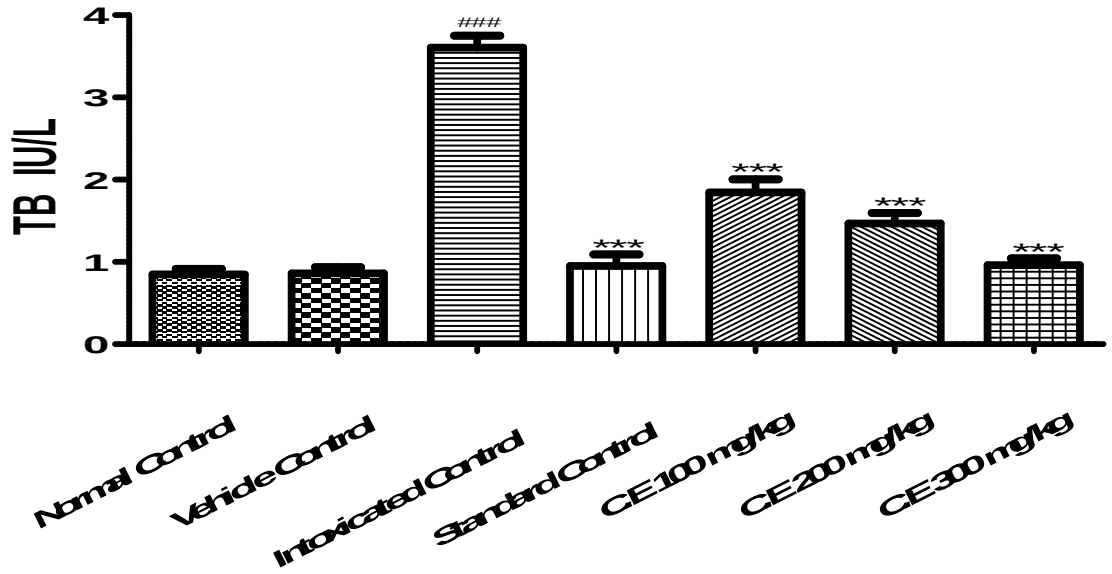


Figure 4: Effects of different doses of poly herbal extract (C.E) on TB level in Nimesulide intoxicated albino rats.

DISCUSSIONS

Different chemicals like CCl_4, acetaminophen and thioacetamide produce hepatic damages and are extensively used in hepatoprotective studies as intoxicated agent [17]. Nimesulide is selective cox-2 inhibitor, extensively used as analgesic but it is associated with hepatotoxic risks. It impairs the production of ATP from mitochondria by hepatocellular mitochondrial damage. It releases its nitro group by bio-reductive metabolism of its nitroarene group which produces hepatic damage. Moreover, its reactive metabolites also involved into oxido-reductive stress and immune mediated chemical reaction. Target proteins present in hepatocytes are modified covalently by Nimesulide. It has been proposed that it also impairs hepato-biliary export [15].

Hepatotoxicity is assessed by monitoring serum enzyme markers. The monitoring of the leakage of liver enzymes into the serum has proven to be very useful tool in assessing liver injury [18]. Any damage to membrane and hepatic necrosis leaks the enzymes into blood which results in elevation of serum enzyme levels. Increase in the level of serum bilirubin reflects the severity of jaundice [19].

Aspartate amino transferase (AST) is involved in transamination of amino acids and elevation in its level represents hepatic damage while elevated level of Alanine aminotransferase (ALT) more specifically represents the hepatic injury [20].SGPT is more sensitive and highly specific marker enzyme of acute hepatotoxicity [10].

In this study, we induced hepatic damage in albino rats by the use of Nimesulide. It significantly ($p < 0.001$) elevated the level of all four enzyme markers (ALP, SGOT, SGPT and TB). This indicated that it has marked hepatotoxic potential. There was marked reduction in level of all these enzyme levels in experimental control groups which were given poly herbal extract in three different doses (100, 200 and 300 mg/kg p.o.). On the basis of results, it is clear that different phytochemical constituents present in plant extract are responsible for hepatoprotective activity.

The main protective action is achieved by antioxidant enzymes, including Super oxide dismutase (SOD), catalase and glutathione peroxidases [21].Anti-oxidants have been reported to have hepatoprotective activity [22].

Nonspecific lipid transfer protein (nsLTP1) is present in cumin seeds which is responsible for transportation of lipids between cellular membranes [23]. Cumin seeds are also enriched with monoterpenes, sesquiterpenes, aromatic aldehydes and aromatic oxides[24] Saponins, tannins, glycosides, terpenes, sterol and tannins which exhibit hepatoprotective activity [25]. Saikosaponins are also found in *S. nigrum* which inhibit peroxidation of lipids by scavenging toxic and reactive metabolites [26]. Phytochemical studies indicated that saponins, tannins,

alkaloids, cardiac glycosides, terpenes and sterols are found in cumin which is responsible of hepatoprotective action [27]. *intybus* is acting as an antioxidant due to presence of both prooxidant and biological antioxidant constituents[28] and it also prevents nitrosamine induced oxidative damage of hepatocytes [29].

C. intybus and *S. nigrum* can also be used alone to prevent Nimesulide induced hepatic cell damage because they might prevent hepatotoxicity by scavenging free radicals which are produced in liver by metabolism of Nimesulide [5,6].

Histopathological studies as shown in figure: 5 represented that hepatocytes were extremely damaged in intoxicated control group but least pattern of cell destruction was observed in experimental control group. Histopatholigal study was based on interpretation of photomicrographs with presence of relative proportions of hepatocellular damaging areas. Different chemicals like (CCl_4 Nimesulide and Paracetamol) generate histopathological abnormalities like steatosis and fibrosis in hepatic cells [30].Photomicrographs of intoxicated livers showed alterations (necrosis, fibrosis and lymphocyte infiltration) in cellular pattern [16].

CONCLUSION

It is concluded that poly herbal formulation has marked hepatoprotective effectiveness in Nimesulide induced hepatic cell damage in albino rats. All the four serum enzymes (ALP, SGOT, SGPT and TB) levels were significantly ($P < 0.001$) reduced with use of its different doses (100, 200 and 300 mg/kg p.o.).

This poly herbal extract has been extensively used in traditional medicine as a remedy of hepatic disorders like jaundice. So, present study strongly supports its effectiveness in conventional treatment of hepatic dysfunctions. Further studies could be brought about to explain exact mechanism of hepatoprotection and margin of safety of this formulation.

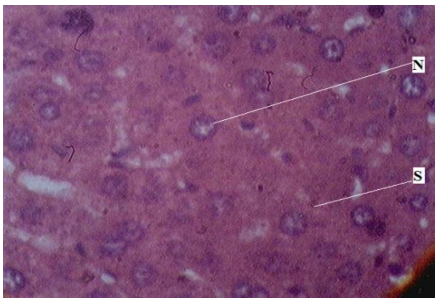
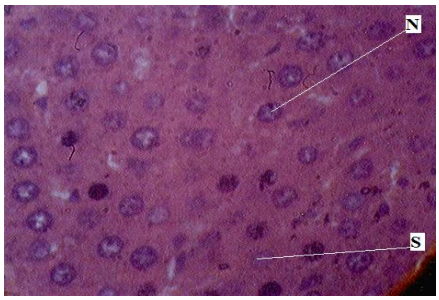


Fig: 5 (A)



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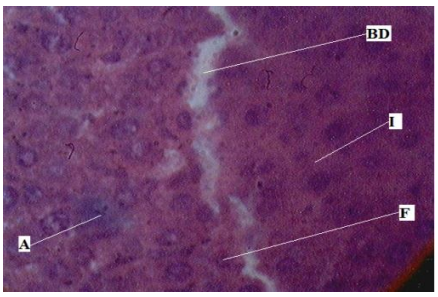

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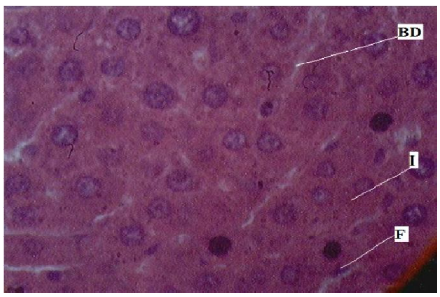

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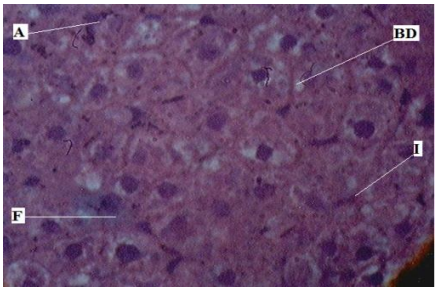

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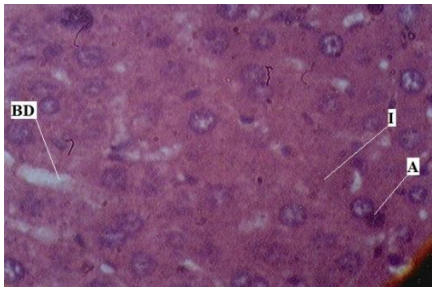

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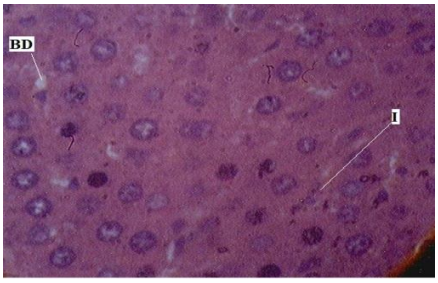

Fig: 5 (G)

Figure: 5. Photomicrographs (100X) of liver tissues of different groups of albino rats.

5(A) Normal control; 5(B) Vehicle control; 5(C) Intoxicated control; 5(D) Standard control;

5(E) C.E 100 mg/Kg; 5(F) C.E 200 mg/Kg; 5(G) C.E 300 mg/kg.

(N= Nucleus, S= Sinusoid, BD= Ballooning and Degeneration, F=Fibrosis, I= Inflammation, A= Apoptosis)

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