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A Study to Elicit Prophylactic Hepatoprotective Effect of *Phyllanthus niruri* Against the Paracetamol Induced Liver Toxicity in Albino Rats with an Insight into Its Mode of Action at Molecular Level.

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ABSTRACT

To investigate the mode of action of *phyllanthus niruri* (*P. niruri*) as a prophylactic hepatoprotective agent against paracetamol (PCM) induced liver toxicity in albino rats. Five groups of six animals in each group of wistar rats with a weight of 180- 210 gms were the experimental material. Group I - Served as normal control, administered sodium CMC for all the eight days. Group II rats were treated only with PCM at a dose of 2.5 gm/kg on 8th day. Group III animals were administered silymarin at a dose of 50 mg/kg for 8days and PCM at a dose of 2.5 gm/kg on 8th day, while group IV is the treated group which was given *P. niruri* aqueous extract at a dose of 200mg/kg followed by PCM of 2.5 gm/kg on 8th day. Group V rats were administered with *P. niruri* at a dose of 400mg/kg for 8days and PCM at a dose of 2.5 gm/kg on 8th day. Biochemical, hisological and immunohistological (IHC) examinations were performed. Histopathological picture is in line with the biochemical parameters and IHC study revealed that P. NIRURI acts by preventing the increase in NKT cells subsequently blocking FASL, by antiapoptotic and by increasing regeneration. *Phyllanthus niruri* aqueous extract at a dose of 400 mg/kg was more effective than at 200mg and silymarin 100mg

Keywords: Histological Processing, Paracetamol, Per Oral, *Phyllanthus niruri,* IHC and NKT cells.

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INTRODUCTION

Liver is in continuous cellular turnover, due to occurrence of careful removal of senescent and damaged cells with simultaneous repopulation. As liver cells are exposed to portal blood which is unfiltered, they are prone to insults from gut derived, diet derived in addition to blood borne insults. In drug induced liver diseases, viral hepatitis, alcoholic liver disease, non-alcoholic fatty liver disease, cholestasis and vascular liver diseases, the prominent feature is apoptosis [1]. While in massive ischemia, oxidative stress and xenobiotics, causing liver injury necrosis occurs [2]. Hepatic adaptation is a process which compensates the deteriorating effects on liver by inducing protective mechanisms in either antioxidant pathway or anti apoptotic pathways. Traditional systems of medicine like Ayurveda have served the medical needs of people of India, people of developed countries have also used native herbs since ages and even now in 21st century these are popular. According to the WHO estimates, around 80% of the population of the developing countries relies on traditional medicine with a progressing global market value of US $ 62 billion. International agencies like WHO, ICS and APCTT have emphasized the need of evaluating these herbs using the present day scientific techniques [3]. It is estimated that liver diseases are among the top ten killer diseases in India, causing lakhs of deaths every year, and considerable number of patients suffering from chronic liver problems, needing recurrent hospitalization and prolonged medical attention. *Phyllanthus niruri* (*P.niruri*) of euphorbiaceae family occurs as winter weed (kharif) draws researcher's interest presently for its hepatoprotective effect and studies confirming its positive role in hepatitis drug induced liver diseases. *P.niruri* is also mentioned in Ayurveda in the treatment of jaundice (kamala). The present study is to evaluate the efficacy of *P.niruri* aqueous extract as a pretreatment prophylactic drug in paracetamol induced hepatotoxcity in albino rats.

MATERIALS AND METHODS

Animals:

On obtaining clearance from Institutional as per CPCSEA guidelines bearing No: IAEC 1/11, Wistar albino rats weighing between 180 to 210 g from Animal House were the study material. They were housed in stainless steel cages and kept in a room where a 12-hour light/dark cycle was maintained. They were allowed to have free access to water and standard pellet feed throughout the period of the experiment.

Chemicals:

All the chemicals used were of analytical grade, 1% sodium carboxy methyl cellulose (Na.CMC) acquired from Sai chemicals and paracetamol (Lambert). Biochemical parameters were estimated by using the diagnostic kits purchased from various manufacturers. Serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP) from Excel Diagnositics Hyderabad, total serum bilirubin (TBIL) from Erba diagnostics and total serum protein (TPRO) from Autospan diagnostics which were all performed on a semi auto-analyser of Erba Company. Standard oro-gastric cannula was used for the oral administration of drug.

All the weights were taken by using the Laboratory analytical balance, volume of the liver was measured by water displacement method using syringe accurate up to 1/40th of millilitre.

Induction of liver damage:

Liver damage was induced by an oral administration of paracetomal (acetaminophen) of 2.5 gm/kg body weight [4]. Liver detoxifies paracetamol to a limit by the process of glucuronidation and sulphation, oxidation into toxic metabolite N-acetyl-p-benzoquinone occurs by the cytochrome P450 system. This increases the values of the biochemical parameters such as SGOT, SGPT, TBIL and TPRO.

Preparation of the aqueous plant extract:

Aqueous extract of *P. niruri* was obtained from GR Herbals, Indore.

Experimental design:

The rats were randomly divided into six groups and each group containing six animals.

Group 1: Normal control group received 1% sodium carboxy methyl cellulose (Na.CMC) 5 ml/kg p.o.
Group 2: Liver damage induced, by giving paracetamol (paracetamol control) 2.5 gm/kg p.o single dose on 8th day.
Group 3: Received silymarin at a dose of 100 mg/kg p.o. for 7days followed by a single dose of PCM at a dose of 2.5 gm/kg p.o. on 8th day after 30minutes of silymarin administration.
Group 4: Prophylactic group received *P. niruri* at doses of 200mg for 7days followed by a single dose of paracetamol 2.5 gm/kg on 8th day after 30 minutes of *P. niruri* administration.
Group 5: Prophylactic group received *P. niruri* at doses of 400mg for 7days followed by a single dose of paracetamol 2.5 gm/kg on 8th day after 30 minutes of *P. niruri* administration.

Analysis of biochemical parameters:

After 24 h of the last treatment, blood was collected from all the rats from retro-orbital plexus. Blood was allowed to clot for 1h at room temperature and serum was separated by centrifugation at 3000 rpm for 15 min. The serum was collected and analyzed for various biochemical parameters like SGOT/AST, SGPT/ALT, ALP, TB and total protein (TP) [5]. Serum transaminase activity was measured using "Rietman and Frankel method [6]." ALP and the serum bilirubin were determined by using "method of Scand."[7] The TP was measured by using Lowry method [8]."

Analysis of histopathological changes:

Animals in each group were euthanized; the livers were isolated, cleared off blood and were immersed in 10% neutral formalin solution and allowed to fix for 1 week. The liver bits of around 5 mm thickness were dehydrated with a sequence of ethanol solutions, and embedded in paraffin. Sections of 4 microns were made using rotary microtome and staining was done with "H&E" accordingly and the observations in slides and the blood investigations were recorded.

Immunohistochemistry:

Fixation of liver tissue followed by preparation of paraffin blocks was done as per protocol. Sections of 4µ thickness were prepared then fixed to defrosted slides and kept in an incubator for 45 minutes at 60^{0}C. Deparaffinisation of the these slides was achieved by 2 changes of xylene for 10 minutes each followed by a sequential process of hydration in descending grades of alcohol from a 100, 95, 80% then rinsed in running distilled water for 5 minutes. Slides were immersed in methanol (peroxidase) for 10 minutes then they were kept in water wash for 10 minutes. They were placed in citrate buffer and heated at 95-100^{0}c in microwave oven for 20minutes and was let at room temperature for 20minutes. The slides were washed in TRIS buffer for 5 times, immersed in primary antibody for 1 hr, a 5 time tris buffer wash repeated followed by immersion of slides in secondary antibody for 30 minutes. Another 5 washes of tris buffer followed by water wash brings the procedure to next step of dehydration in ascending grades of alcohol from 80, 90 and 100%. Two changes of xylene were performed before mounting with a cover slip. The antibodies used were Bcl2 (anti-apoptotic) [9], cd56 (membrane marker on NKT cells) [10], and Ki 67 (proliferative marker) [11] and tests were performed at Pathology department Apollo Hospitals Hyderabad. Please note that all the tests are done on Ventana Benchmark XT automated staining system.

1. Bcl2- antibody 100/5 MA5-11757 Thermo Scientific- Mouse monoclonal antibody 1:50
2. CD56- clone 56C04 MA5-11563 Thermo Scientific- Mouse monoclonal antibody at a concentration of 0.2mg/ml.
3. Ki-67- Dako- clone MIB-5 Mouse monoclonal antibody, 1:50 dilution.

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Statistical Analysis:

All the results were expressed as Mean ± SEM. The statistical analysis was carried by one-way Analysis of Variance (ANOVA) followed by Dunnett's multiple comparison tests using graph pad Prism software, $P < 0.05$ was considered as significant.

RESULTS

Significant variations were observed in weight, volume and biochemical parameters of the liver due to hepatotoxicity and hepatoprotective activity of PCM and *P. niruri*, silymarin respectively. A significant increase ($P < 0.001$ when compared to normal control group) in the weight, volume, serum SGOT, SGPT, ALP, bilirubin were observed in only paracetamol treated animals (group II), while serum protein levels were decreased ($P < 0.001$). In animals administered with *P. niruri* 200 mg/kg, 400 mg/kg, and silymarin 100 mg/kg, a decrease in SGOT, SGPT, ALP, bilirubin and an increase in the total protein that followed an ascending sequence, was observed. As a consequence of the hepatic damage, production of the proteins decreased, which was evident in the decreased values of the serum protein levels in only PCM treated group, unlike as *P. niruri* and silymarin treated groups. These changes were depicted in the table 1.

Table 1: Showing the biochemical parameters of evaluation of *phyllanthus niruri* for its prophylactic hepatoprotective effect in PCM toxicity in wistar albino rats.

Treatment group	Liv wt (g)	Liv vol (ml)	SGOT/AST (IU/L)	SGPT/ALT (IU/L)	ALP (IU/L)	TB (mg/dL)	TP (IU/L)
Control	6.93±0.12	6.21± 0.09	70.65±1.10	46.47±0.84	171.3±1.303	0.55±0.01	9.15±0.17
Paracetamol	7.9±0.09***	6.91±0.07***	255.7±3.40***	134.8±1.70***	440.8±3.07***	0.99±0.05***	5.59±0.09***
Silymarin (100 mg/kg)	7.37±0.04***	6.41±0.06***	125.3±1.17***	92.67±0.61***	234.5±1.80***	0.56±0.01***	8.37±0.03***
P. niruri (200 mg/kg)	7.58±0.10**	7.18±0.04**	131.3±0.49***	94.50±0.50***	251.8±2.99***	0.66±0.009***	7.90±0.03***
P. niruri (400mg/kg)	7.24±0.03***	6.23±0.03***	114.8±1.40***	88.17±0.70***	234.0±1.46***	0.56±0.01***	8.34±0.02***

The values are expressed as mean ± SEM. **P < 0.01, ***P < 0.001, as compared to the control group.

H&E stained slides of normal control Fig.1 (A) showed central vein without any congestion radiating hepatocytes with round nucleus and unremarkable cytoplasm indicating Grade 0 .In Fig.1 (B) massive degeneration of hepatocytes extending between two adjacent lobules indicating large scale necrosis, steotosis and congestion of blood vessels characteristic feature of Grade +3 hepatic damage observed in only PCM2.5 treated group . Fig. 1(C) couagulative necrosis extending till mid zone , vacuoles in hepatocytes and congestion corresponding to Grade +2 observed in silymarin100 +PCM2.5 group .In Fig. 1(D) PN200+PCM2.5 the level of necrosis relatively reduced when compared silymarin group moderate congestion of vessels noted.

In PN400+PCM2.5 group Fig.1 (E) minimal necrosis involving few cells around central vein, neither appearance of fat vacuoles nor congestion in the vessels was visible according to Humasom [12] these findings correspond to Grade +1.

Immunohistochemistry was performed using antiapoptotic marker Bcl2, NK cell marker CD56, proliferatory marker Ki67 the following are the findings.

The percentage of cells positive for antiapoptotic Bcl2 followed an ascending order from only PCM 2.5 treated group Fig.2(B) ,Silymarin 100 +PCM group Fig.2(C),further more Bcl2 positive cells seen in PN200+PCM2.5 group Fig.2(D) and a maximum Bcl2 positive cells noted in PN400+PCM 2.5 treated group

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Fig.2(E) . Fig.2 (A) represents the IHC marker control for Bcl2 antiapoptotic marker. Similar scenario was observed in Ki67 the IHC marker denoting the proliferation of hepatocytes amongst the various treated groups where the IHC control with maxsimum positive cells Fig.4(A). Though to a lesser extent the Ki67 activity of hepocytes seen increasing from only PCM 2.5 treated group Fig.4(B) to Silymarin +PCM group Fig.4(C) further increase noted in PN200+PCM 2.5 group Fig.4(D) and prominent positive cells was evident in PN400+PCM treated group liver tissue .

Fig.3(A) a IHC marker control for CD56 showed complete positive cells while there was a prominent descending trend of CD56 positive cells was noted from Only PCM group Fig.3(B) to silymarin 100 +PCM 2.5 treated group Fig.3 (C) still the positive cells decreased in PN200+PCM group Fig.3(D) and null staining of cells for CD56 marker was evident in PN400+PCM treated group liver tissue.

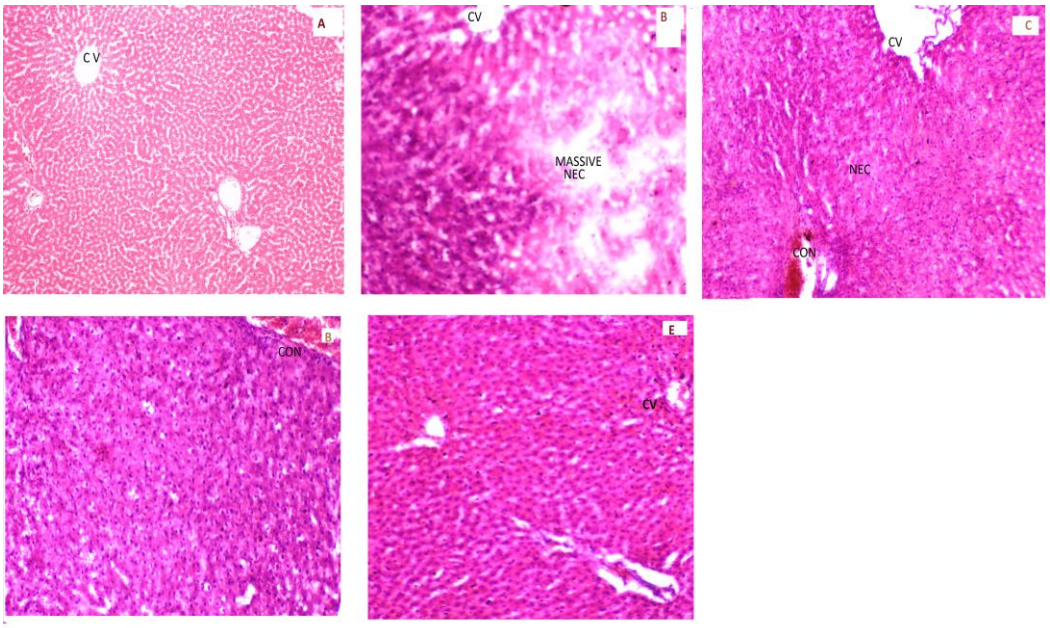

FIG.1: H&E 200 MAGN A.Showing normal hepatic architecture of normal group .B.showing massive necrosis in only PCM treated group .C.silymarin +PCM group showing dilated central vein (CV) congestion (CON)in vasculature and dropout necrosis (NEC). D.PN 200 + PCM treated group showing congestion (CON) less necrosis apoptosis is also evident .E. PN400+PCM treated group showing minimal necrosis without congestion in vasculature .

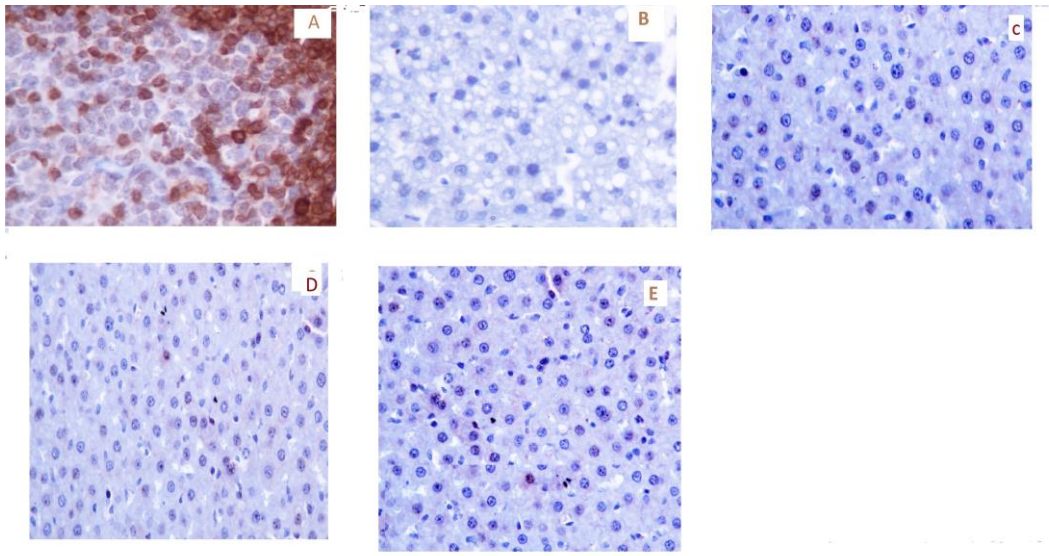

FIG.2: IHC Marker Bcl2 A.Bcl2 control showing complete positive area B. Bcl2 negitive seen in only PCM treated group.C. Appearence of Bcl2 positive cells in silymarin +PCM group.D. moderate number of cells are Bcl2 positive in PN 200+PCM treated group. E. maximum Bcl2 positive cells are in this group of PN 400+PCM.

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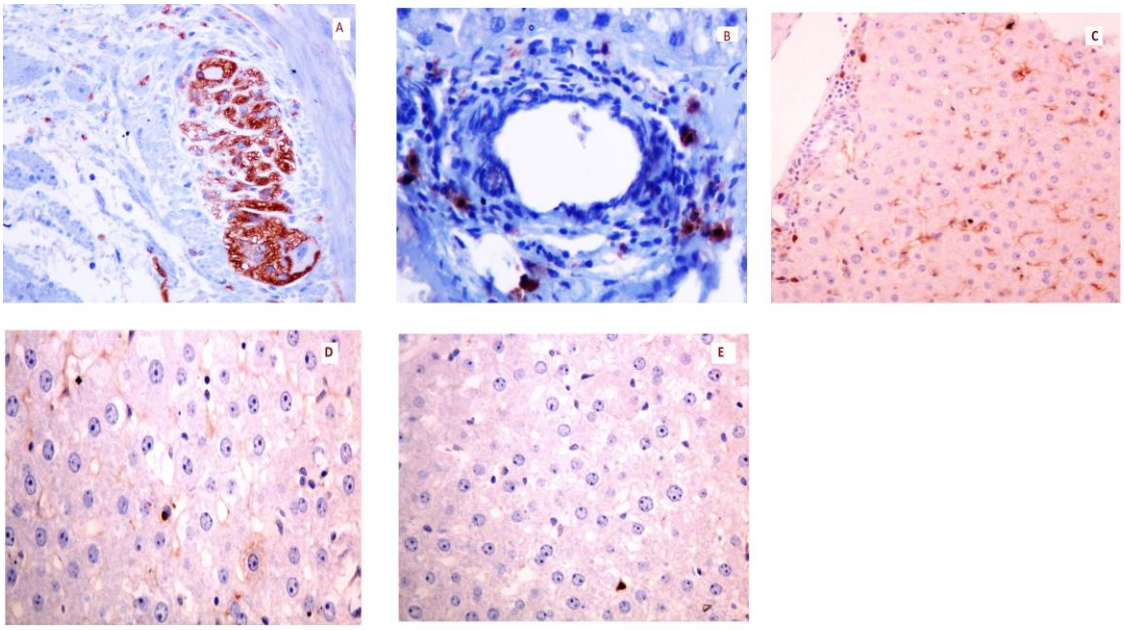

FIG.3: IHC marker CD56 . A. CD56 control showing complete positive cells . B. CD56 positive cells present moderately in only PCM treated group . C. CD56 positive cells of moderate grade present in silymarin +PCM treated group . D.minimal Cd 56 positive cells seen in PN200+PCM treated gorup . E. Negligible positivity observed in PN400 +PCM treated group .

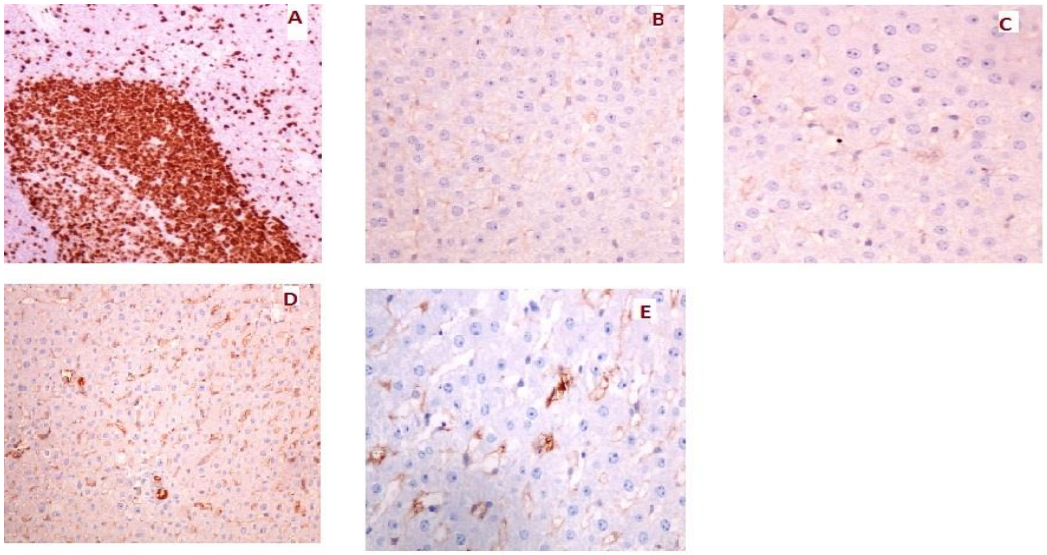

FIG.4: IHC marker Ki-67 . A.control showing complete positive cells . B.Only PCM group whowed no Ki-67 activity. C.Silymarin +PCM treated group Ki-67 positive cells evident, D.PN200+PCM treated group relatively more Ki-67 positive cells . E. PN400+PCM treated group showed moderate Ki-67 activity.

DISCUSSION

Though paracetamol is considered to be the safest non-steroidal anti-inflammatory drug available over the counter if used in recommended doses; it is also capable of producing hepatic damage on consuming single overdoses or chronic low dose [13,14]. Hence single dose PCM model is used to evaluate the prophylactic hepatoprotective effect of *P. niruri*. The normal levels of biochemical parameters are liver weight 6.983±0.074, liver volume 6.05±0.0143, SGOT 71.37±0.551, SGPT 44.82±0.25, ALP 169.7±1.03, TBIL 0.52±0.055 and TPRO 9.29±0.043.

Acetaminophen (APAP; *N*-acetyl-*p*-aminophenol) over dose manifests in incapacitation of hepatocyte cell membrane to retain the enzymes, which appear in the blood stream resulting in the elevation of the serum

values [5,14] but also produces a centrilobular hepatic ifnecrosis. APAP induced toxicity occurs by initial hepatic metabolism of APAP by cytochrome P450 to the reactive metabolite *N*-acetyl-*p*-benzoquinone (NAPQI). Further damage would result in the necrotic changes in the parenchyma of the liver. Except for the Serum total protein, which showed a decrease in serum values as hepatic damage resulted in the decreased production of protein[5], rest of all the parameters have shown significant increase. Histology of the control group showed normal architecture.

Tissues of animals treated with only paracetamol (group II) showed fatty changes and necrosis prominent in the perivenular region, while group IV and V showed relatively less necrosis and inflammatory changes**..** Immunohistochemistry would help in understanding the mechanisms of liver injury at molecular level. Liver injury results from multiple etiologies such as alcohol intake, viral infection, cholestasis, steatosis, drug abuse, and autoimmunity one amongst them is drug induced liver injury [15, 16, 17]. Liver being the key organ of detoxification has to bear constant stress, mechanism of cell death can be apoptotic or necrotic or necroapoptotic sharing common cell death machinery, including death receptor-dependent and mitochondria-dependent pathways [18, 19].

Apoptosis can be understood as three phases 1) pre-mitochondrial 2) mitochondrial 3) post mitochondrial. Amongst number of death receptors responsive in pre-mitochondrial trail and fas ligands are significant in liver injury. Cytototoxic T lymphocytes and, Natural killer cells have FasL expression. CD56 is an IHC marker for NKT cells .Mitochondrial phase of death mechanism is regulated by BCL2 family in the present study the BCL2 antiapoptotic marker is used to evaluate the antiapoptotic activity of *P.niruri*. IHC marker Ck18 is employed to locate the apoptosis in the hepatocytes. To evaluate the regeneration capabilities of the test drug Ki 67 is the marker. The results of the present study show that *P. niruri* 400 exhibited a significant antiapoptotic as bcl2 is predominantly positive followed by *P. niruri* 200 and silymarin showed minimal positivity of the marker. This is also supported by the fact that CD56 showed minimal reactivity with an increase in a sequence of *P. niruri* 400, *P. niruri* 200, silymarin, PCM control. Ki 67 a marker for scaling the regenerative property certainly showed positive activity more in PCM + *P. niruri* treated group of animals .Our study not only demonstrated prophylactic hepatoprotective efficacy of Phyllanthus niruri in Pcm liver toxicity, as was stated by previous studies of sabir and harish [20,21], but also propounded a possible molecular mechanism of action .

CONCLUSION

In the present study the morphometric, biochemical, histopathological and IHC markers findings strongly suggest that *P. niruri* acts by neutralising the innate immune cells of NKT, this in turn reduces the Fasl death pathway activation this occurs at pre-mitochondrial phase. While at mitochondrial phase administration of *P. niruri* at 200and 400mg promotes antiapoptotic activity which is evident by significant expression of antiapoptotic marker bcl2 and minimal expression of ck18. The present suggests another herbal hepato-protective herb with an insight on understanding of the mode of its action.

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