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IN VIVO AND *EX VIVO* EVALUATION OF *LUFFA ACUTANGULA* FRUIT EXTRACT AND ITS FRACTIONS FOR HEPATOPROTECTIVE ACTIVITY IN WISTAR RATS

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Keywords:

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ABSTRACT: The alcoholic extract of fruits of the plant *Luffa acutangula* previously reported for is hepatoprotective activity was fractionated into three parts to chemically identify the most potent bioactive fraction. The hepatoprotective potential of the fraction prepared from extract was studied in *vivo* in rats as well as *ex vivo* in isolated hepatocytes against paracetamol induced hepatotoxicity. The hepatoprotective activity was determined on the basis of their effects on parameters like direct bilirubin, serum aspartate transaminase, serum alanine transaminase and alakaline phasphatase. Ethyl acetate fraction (EAF) of alcoholic extract 100 mg/kg, p.o.) exhibited maximum hepatoprotective activity and is the active fraction for hepatoprotective activity of *L. acutangula* fruit.

INTRODUCTION: It is commonly recognized that reactive oxygen species (ROS) are involved in a variety of physiological and pathological processes, including cellular signal transduction, cell proliferation, differentiation and apoptosis, as well as ischemia - reperfusion, injuries, inflammation and many neurodegenerative disorders. In healthy individuals, ROS production is continuously balanced by natural antioxidant defense system. Oxidative stress is a process where the physiological balance between pro-oxidants and antioxidants is disrupted in favor of the former, ensuing in potential damage for the organism [1]. The liver play a major role in protecting our body against any ingested toxic substance by efficient detoxification mechanism.

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In an increasing toxic world, we place growing burdens on the body's detoxification system. Millions of compounds are detoxified within each liver cell or hepatocyte. Hepatotoxicity is fast becoming a major health issue and liver protecting agents have assumed a major role in the treatment options against liver diseases. Unfortunately, liver protection is one area where chemical drugs are not provided interesting options. At the same time bioactive leads from herbs are available in traditional system of medicine.

Herbs and spices have been known since ancient times for their use in the preparation of foodstuffs to enhance their flavor and organoleptic properties [2 - 5]. Nowadays, they have great potential in a growing nutrition industry because many plant-derived phytochemical preparations possess dual functionality in preventing lipid oxidation, a major cause of food quality deterioration and microbial spoilage. Furthermore, there is an increasing interest in antioxidants, particularly in those intended to prevent the presumed deleterious

effects of free radicals in the human body and to prevent the deterioration of fats and other constituents of food stuffs [6, 7].

Ridge, angled gourd, or *Luffa acutangula* (L.) Roxb. is a cucurbitaceous vegetable originated in sub-tropical region of Asia. India is considered as a primary centre of origin [8]. This crop is cultivated in India, Southeast Asia, China, Japan, Egypt and other parts of Africa. *Luffa acutangula* is commercially grown for its unripe fruits as a vegetable. The fruits *of L. acutangulla* cures 'vata, 'kapha', biliousness, anemia & different liver complaints [9]. It also used as folk medicine for leucoderma, piles, uterine and vaginal tumours, bronchitis and jaundice. The dried fruit is used as a snuff in jaundice [10]. It used as laxative, purgative, diuretic, in the treatment of asthma and spleen enlargement. The fruit juice is used in different traditional medicines in treatment of hepatic congestion, irritation & inflammation of gastric mucosa [11].

Leaves and fruit juice of *L. acutangula* are commonly used in tribal area of Madhya Pradesh as folk medicine for the treatment of Jaundice [12]. Fruits and seeds of *L. acutangula* contain bitter principles cucurbitacins -B, D, E, G & H and oleanolic acid [13]. It also contains elaterin 2-0-β-D glucopyranoside, cucurbitacin S, gypsogenin & sitosterol [14]. The alcoholic extract of *L. acutangula* seeds yielded a crude saponin which on hydrolysis gives oleanolic acid, while ether extract of fruit mesocarp yielded cucurbitacins –B&E [15]. Seven oleanane-type triterpene saponins, acutosides A--G, were isolated from the herb of *Luffa acutangula* Roxb [16]. Two trypsin inhibitors, LA-1 and LA-2, were isolated from the seeds of *Luffa acutangula* Linn (ridged gourd). Both of these inhibitors strongly inhibit trypsin by forming enzyme-inhibitor complexes at a molar ratio of unity [17].

MATERIALS AND METHODS:

Chemicals and Reagents:

Technical grade paracetamol (purity 99.4%) was obtained from Merck, India and silymarin was obtained from Sigma Chemicals, USA. Bilirubin, ALP, ALT and AST were assayed by using kits from Ranbaxy diagnostic, New Delhi. All other reagents used were of analytical grade and obtained from Qualigens Fine Chemicals, Mumbai, India.

Plant Material: The fruit samples of *Luffa acutangula* were collected from surrounding area of northern part of Uttar Pradesh and Madhya Pradesh, India in the month of June to July in which the plant grows widely under natural condition. The identification of these crude samples of drug was authenticated by taxonomist at National Botanical Research Institute, (NBRI) Lucknow, with voucher no (LWG-224813) and was preserved for further use.

Extraction and Fractionation of Extract: Drugs were chopped into small pieces and air-dried under shade and powdered after collection. The powdered plant material (1 kg) was extracted with petroleum ether (60° - 80°C) for defatation. The defatted powdered drugs were then extracted with 95% ethanol at room temperature (25± 2ºC). The extract was evaporated in a rotary evaporator below 50 ºC and further dried under high vacuum; thus solid residue 19.28% w/w was obtained. A part of the ethanol extract thus obtained was fractionated into three fractions toluene soluble fraction (LA- I, 8.41%w/w), chloroform soluble fraction (LA- II, 15.62% w/w) and ethyl acetate soluble fraction (LA-III, 33.32 % w/w).

In the separate study, ethanolic extract and different fractions of *L. acutangula* plant were then subjected to preliminary phytochemical screening, antioxidant activity and hepatoprotective activity to identify the most active fraction against PCM induced liver toxicity.

Animal Treatment: Albino rats (n=42) of either sex weighing 150-200 g were selected for hepatoprotective studies. Animals were kept in polypropylene cages in room under standard condition of temperature 23±2°C, relative humidity 55±10% & 12 hrs controlled light / dark cycle. The animals were maintained under standard pellet diet & water *ad libitum* in animal house, Initial body weight of each animal was recorded and they were given seven days time to get acclimatized with the laboratory condition.

All experiments were performed in the morning according to current guidelines for the laboratories animals and ethical guidelines for investigation of experimental of conscious animals[18].

The study protocol was approved by the Institutional Animal Ethics Committee of Central Drug Research Institute (CDRI), Lucknow.

Experimental Design: Rats were divided into seven groups containing six rats each. Ethanolic extract and all three fractions were dissolved in 5% acacia suspension and administered orally with the help of gastric canula.

Group 1 was administered 5% acacia suspension, served as positive control.

Group 2 was administered PCM, (3 gm/kg, p.o) served as negative control.

Group 3 was administered 100 mg/kg of EELA orally for 7 days.

Group 4 was administered 100mg/kg of TF orally for 7 days.

Group 5 was administered 100 mg/kg of CF orally for 7 days.

Group 6 was administered 100 mg/kg of EAF orally for 7 days.

Group 7 was administered 25 mg/kg of standard drug Silymarin for 7 days.

Group I rats received 5% acacia suspension for 7 days and served as a positive control. The animals in the group II served as paracetamol-intoxicated control and were given single oral administration of paracetamol (3 g/kg), 1 h after distilled water administration. Groups III, IV,V, VI and VII received extract of *L. acutangula* (100 mg/kg), its different fractions as mentioned above (100 mg/kg) and silymarin (25 mg/kg), respectively,[19] once daily for seven consecutive days followed by a single oral administration of paracetamol (3 g/kg), 1 h after the last dose administration.

After 48 h of paracetamol administration, rats were anesthetized with diethyl ether. Blood of each rat was collected by puncturing retro-orbital plexus in sterilized dry centrifuge tube and allowed to coagulate for 30 min at 37 °C. The clear serum obtained after centrifugation (3000 rpm for 15 min) were used for further biochemical estimation of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and direct billirubin (BL) according to the method of Bhakta *et al.*,[20]. After blood collection, the animals of all groups were sacrificed by cervical decapitation and liver specimens were collected to observe the *in vitro* hepatoprotective activity and histopathological analysis.

***Ex-vivo* Hepatoprotective Activity Using Rat Hepatocytes:** Fresh hepatocytes preparations and primary cultured hepatocytes were used to study antihepatotoxic activity of drugs. Hepatocytes were treated with toxins and the effect of the drug extract and ethyl acetate fractions were evaluated due to significant hepatoprotection. The activities of the transaminases enzyme released into the medium were determined.

Isolation of Rat Hepatocytes: The isolation of rat hepatocytes and *ex vivo* studies were performed with slight modification of a previously reported process [21]. The abdomen of the rat was opened under ether anesthesia and heparin (50 units in 0.1 ml) was injected into the heart to prevent blood coagulation. A mid line incision was made and the portal vein was cannulated about 2mm. inside with a Teflon catheter. Buffer I (NaCl 0.142 M; KCl 0.0067 M; HEPES 0.1M, was taken without enzyme and bubbled with oxygen for 10 min at pH 7.4). This was perfused through the liver at a flow rate of 2.5-3 ml/min/g of liver. The inferior vena cava near the heart was then cannulated for the escape of the perfusate. The liver was perfused in-situ through the portal vein with buffer I for 12-15 min followed by buffer II (NaCl 0.0667 M; KCl 0.0067 M; HEPES 0.1M, colagenase $1.6x10^{-9}$ g/ml-630 U/mg solid at 37 ^{0}C at pH 7.6. for 8-10 min. After ten minutes of perfusion when liver was completely bleached and freed from the blood, the inferior vena cava was tied off above the renal vein and the thorax portion of the superior vena cava was cannulated.

At the end of perfusion, the collapsed liver was transferred to a beaker containing buffer-I and tissues were teased with blunt forceps to separate hepatocytes from each other. The crude cell suspensions were then rotated in a rotator for 10 min. The cell suspension was filtered gently through cotton gauze into centrifuge tubes. The filtered solution was subjected to cold centrifuged (4^{o}C) thrice at 200 rpm for 1 min. The supernatant was removed and the loosely packed hepatocytes were gently settled in tubes.

Measurement of Cell Viability:

Trypan Blue Exclusion Test: One drop of hepatocyte stock suspension was mixed with three drops of trypan blue solution (0.02%). The unstained viable cells were counted under a microscope and could be easily distinguished from the damaged cells stained blue. The percent viable cells were calculated accordingly [22].

Oxygen Consumption: The oxygen uptake in the isolated hepatocytes was determined by Gilson oxygraph as described in previously reported method [23 - 24]. A voltage is imposed across the anode and cathode and oxygen undergoes electrolytic reduction. The current then generated is measured and 0, consumption calculated as microliter of 0, utilized per hour per milligram of protein. The reaction mixture contained 1.7 ml of HEPES buffer I and 0.1 ml of hepatocyte suspension.

RESULTS AND DISCUSSION:

***In vivo* Hepatoprotective Activity: Fig. 1** indicates marked increase in direct bilirubin levels from 0.45 mg dl^{-1} in control rats (group I) to 5.64 mg dl^{-1} in paracetamol (PCM) induced rats (group II) was observed. Paracetamol is known to be converted to highly reactive elecrophilic metabolites by the hepatic constituents (*e.g.* lipid, protein, DNA, RNA) to form alkylated or arylated derivatives. However, the direct bilirubin levels were reduced to 1.63 mg dl^{-1} with the treatment of 100 mg kg^{-1} (group III) of EELA. Interestingly, the comparatively same doses *i.e.* 100 mg kg^{-1} (group VI) of ethyl acetate fraction of EELA have effectively restored the direct bilirubin levels to 0.95 mg dl^{-1}. In addition, the standard silymarin (25 mg kg^{-1}), a hepatoprotective drug, has restored the direct bilirubin levels significantly *i.e.* 0.66 mg dl^{-1}. Serum AST (International unit l^{-1} or IU l^{-1}) levels were also elevated from 68.61 IU l^{-1} in the control group to 166.22 IU l^{-1} in the PCM treated group. Treatment with standard silymarin 25 mg kg^{-1} has brought back the AST to the near normal levels *i.e.* 75.43 IU l^{-1}. However, treatment with the 100 mg kg^{-1} of EELA and EAF restored the AST levels up to 96.03 and 78.24 IU l^{-1}, respectively.

In the case of ALT, there was a noticeable rise from 23.78IU l^{-1} in control rats to 93.96 IU l^{-1} in PCM induced rats was observed. Silymarin (25 mg kg^{-1}) decreased levels of serum ALT (23.42 IU l^{-1}) in the induced rats. In addition, the EELA and ethyl acetate fraction of EELA significantly reduced and effectively restored levels of persisting ALT activity up to 47.21and 36.05 IU l^{-1} with the single dose of 100 mg kg^{-1}, respectively.

Considerable increase in the serum ALP levels were observed in PCM induced rats *i.e.* 47.47 IUl^{-1} compared to 18.58 IU l^{-1} in control rats. However, the serum ALP levels were reduced to 32.28 and 23.30 IU l^{-1} with the treatment of 100 mg kg^{-1} of EELA and ethyl acetate fraction of EELA, respectively. In addition, the silymarin (25 mg kg^{-1}) has restored the serum ALP levels significantly *i.e.* 18.89 IU l^{-1} (**Fig. 2**). To sum up the above discussion altogether, it is proved that *L. acutangula* extract and fractions could inhibit PCM induced hepatitis by regulating various biochemical parameters such as serum AST, ALT, ALP and BL and liver metabolites. The maximum protection in ethyl acetate soluble fraction against PCM intoxication was observed in above hepatic biochemical parameters. These data are required not only for identification procedures that guarantee the utilization of the appropriate raw material, but also for quality-control standards demanded by health legislation.

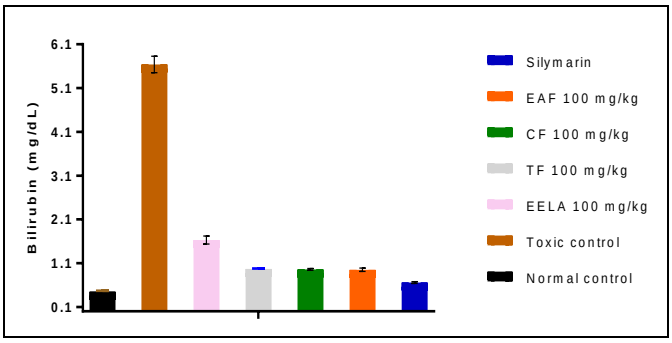


FIG. 1: EFFECT OF ETHANOLIC EXTRACT AND ITS FRACTIONS OF *L. ACUTANGULA* ON BILIRUBIN

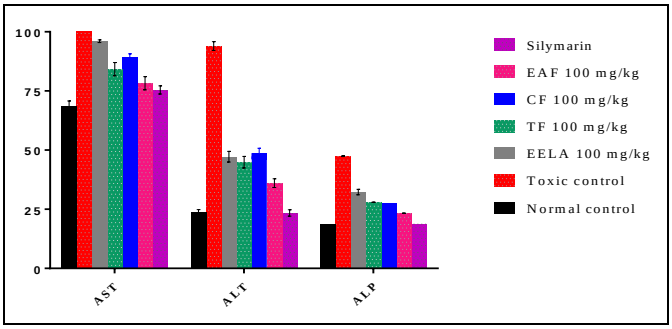


FIG. 2: EFFECT OF ETHANOLIC EXTRACT AND ITS FRACTIONS OF *L. ACUTANGULA* ON BIOCHEMICAL PARAMETERS

***Ex vivo* Hepatoprotection:** In the present investigation, we have studied the protective activity of ethanolic extract of *L. acutangula* and its ethyl acetate fraction against paracetamol in isolated rat hepatocytes. Paracetamol produced damage was evidenced by reduction in the viability of the isolated hepatocytes as assessed by the trypan blue exclusion and reduction in oxygen uptake. The change in certain biochemical markers of the haptocytes as well as of the serum also indicated the degree of hepatic damage. Pretreatment of the animals with extract and its fraction gave a significant hepatoprotective activity by reversing the altered values to normal levels. The viability of the hepatic cells was tested by trypan blue exclusion test and the rate of oxygen consumption. Incubation of isolated rat hepatocytes with PCM significantly reduced the viability indicating its cytotoxicity at the dose of 3g/kg. Pretreatment with ethanolic extract at the concentration of 1 mg/ml produced 78.7 ± 2.27% protection whereas 65.2 ± 0.84% protection was observed on treatment with ethyl acetate fraction at 1 mg/ml concentration. Other fractions like chloroform and toluene were found to be ineffective even after being tested at the higher concentration **Table 1**.

TABLE 1: CELL SURVIVAL ON PCM INDUCED RAT HEPATOCYTES INJURY

S. no.	Groups	Test drug conc. (mg/ml)	Trypan blue exclusion % Viability ± S.D.	Oxygen uptake µl/h/mg protein ± S.D.
1	Control		92.5±1.8	4.93 ± 0.07
2	Toxicant PCM	3g /kg	41.2±0.86**	1.96±0.01
3	EELA	0.01	52.9±2.86*	3.53±0.43 (52.8%)
		0.1	59.5±1.46*	4.10±0.89 (72.0%)
		1.0	78.7±2.27*	2.81±0.01 (28.6%)
4	EAF	0.01	49.8±0.16	2.88±0.04 (30.9%)
		0.1	53.4±0.96*	3.69±0.06 (58.2%)
		1.0	65.2±0.84*	4.54±0.15 (86.8%)

The value represents the mean ± S.D. for 6 rats per group. Values in parenthesis indicate percentage recovery. All groups were compared to toxic control by students-Newman-Keuls-test (*p<0.01), compared to control group (**p<0.001)

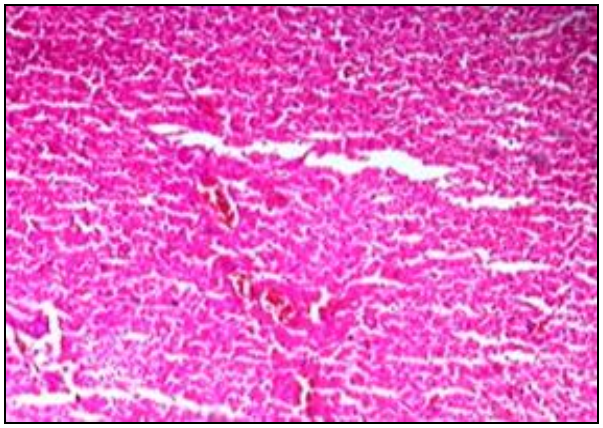
Histopathological Observation of Liver: The histopathological studies revealed the actual changes which occurred in the cellular structure of rat liver.

The section of liver of control group (normal healthy rat liver) (**Fig. 3A**) shows intact hepatocytes with central vein and distinct nucleus.
The section liver of toxin group intoxicated with PCM (**Fig. 3B**) indicated the presence of vacuoles and disturbed hepatocyte structures due to necrosis. Fatty degeneration was also observed in areas other than the centrilobular ones with lymphocyte infiltration.

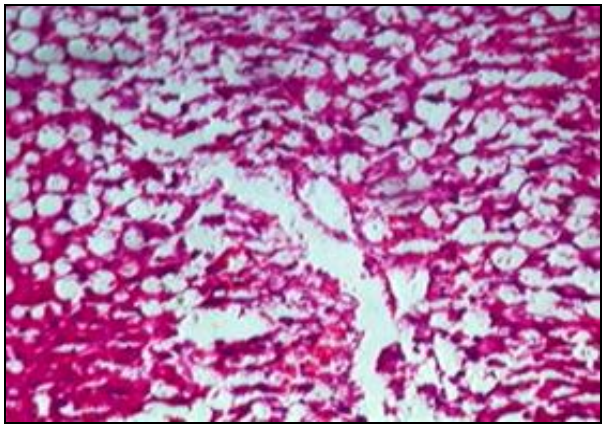
After the treatment of alcoholic extract of *L. acutangula* along with PCM (**Fig. 3C**) the sections of rat liver indicated the absence of vacuoles and necrosis with resembling structure to normal liver, thus confirming the protective effect. Liver sections of the rats intoxicated with PCM and then treated with active toluene fraction of ethanolic extract of *L. acutangula* showed marked regenerative activity without any necrosis with little lymphocytic infiltration and low fatty degeneration confirming its hepatoprotective effect against PCM intoxication (**Fig. 3D**). These observations were found to be in accordance with earlier reported studies. Similarly in the case of chloroform fraction a marked regenerative activity was observed; the section of rat liver indicated the normal hepatocyte cells without any vacuoles and necrosis. (**Fig. 3E**)

The ethyl acetate which could protect the PCM intoxication, as evidenced by absence of necrosis and fibrosis, low infiltration on inflammatory cells with less vacuole formation and marked regenerative process indicating the presence of normal hepatic cords confirmed its activity. (**Fig. 3F**)

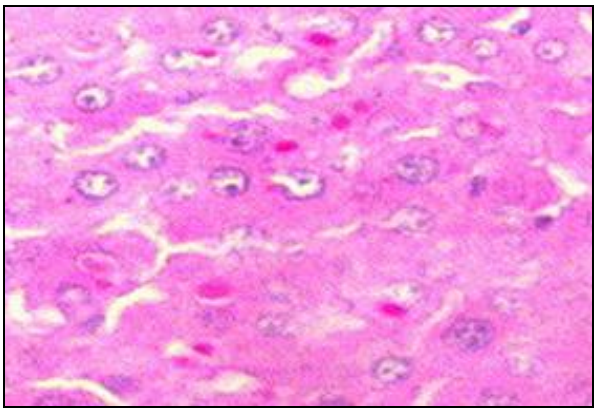
Liver sections of the rats treated with standard drug silymarin showed marked regenerative activity against PCM intoxication and also preserve the normal hepatocyte structures which confirm its significant hepatoprotective nature.(**Fig. 3G**)



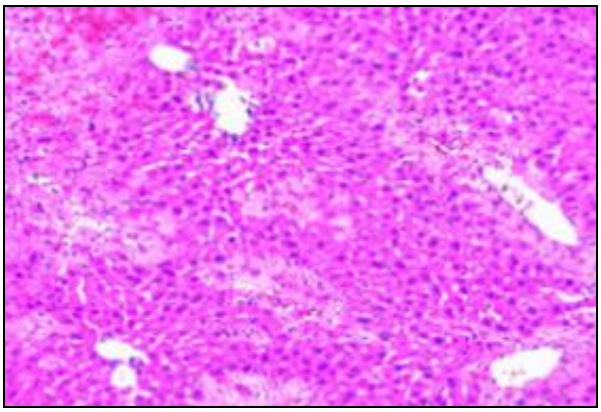
3A Control group



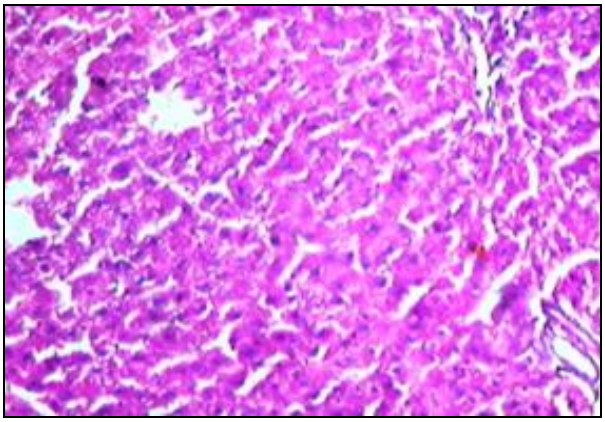
3B Toxic Control



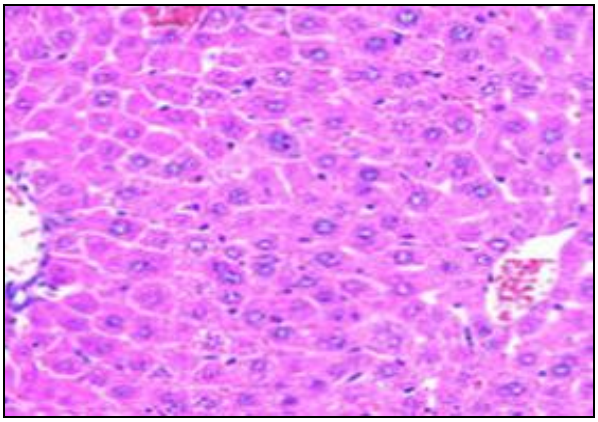
3C EELA treated



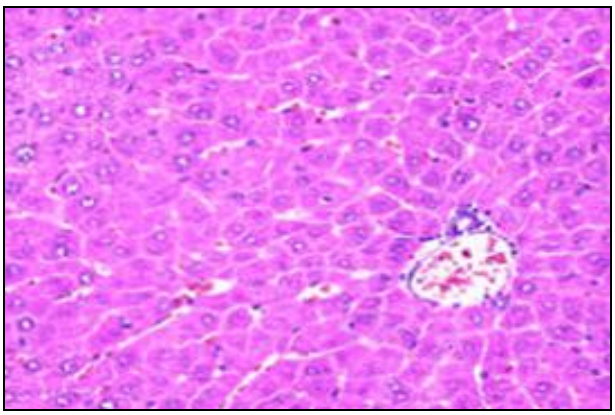
3DToluene fraction (TF) treated



3E chloroform fraction (CF) treated



3F Ethyl acetate fraction (EAF) treated



3G Silymarin treated

FIG. 3: HISTOPATHOLOGY OF LIVER

CONCLUSION: The results of the present investigation carried out on *L. acutangula* clearly indicated that these drugs possess significant hepatoprotective activity and highly efficient in terms of dosage, tolerability and restoring the liver. It substantiates the traditional claims about the use in the treatment of various liver disorders. These findings show the prophylactic and curative efficacy of *L. acutangula* in maintaining the integrity and functional status of hepatocytes. Considering the promising results obtained, the author perceives that further research work may be carried out to fully utilize the present investigation by formulating a suitable dosage form.

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CONFLICTS OF INTEREST: Nil

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