EFFECTS OF ROOT EXTRACT OF *SAUSSUREA LAPPA* ON CHEMICAL INDUCED LIVER DAMAGE IN RATS

THESIS

By

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(V-2018-30-017)

Submitted to

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CERTIFICATE – I

This is to certify that the thesis entitled "**Effects of root extract of *Saussurea lappa* on chemical-induced liver damage in rats**" submitted in partial fulfillment of the requirements for the award of the degree of **Master of Veterinary Science** in the discipline of **Veterinary Pathology** of CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur is a bonafide research work carried out by **Abhishek Kumar (V-2018-30-017)** son of Sh. Sanjeev Kumar and Smt. Poonam Varshnay under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been duly acknowledged.

(Dr. R.K. Asrani)

Major advisor

Place: Palampur
Dated: 10/09/2020

CERTIFICATE – II

This is to certify that the thesis entitled "**Effects of root extract of *Saussurea lappa* on chemical-induced liver damage in rats**" submitted by **Abhishek Kumar (V-2018-30-017)** son of **Sh. Sanjeev Kumar** to the CSK Himachal Pradesh KrishiVishvavidyalaya, Palampur in partial fulfillment of the requirements for the degree of **Master of Veterinary Science** in the discipline of **Veterinary Pathology** has been approved by the Advisory Committee after an oral examination of the student in collaboration with an External Examiner.

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Abhishek

Place: Palampur ***(Abhishek Kumar)***

Date: 10/9/2020

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ABBEREVIATIONS USED

ABBEREVIATION	MEANING
%	Per cent
SLE	*Saussurea lappa* extract
SL	*Saussurea lappa*
DEN	N-Diethylnitrosamine
IHR	Indian Himalayan Region
HAB	High Altitude Biology
CSIR-IHBT	Council of Scientific and Industrial Research – Institute of Himalayan Bioresource Technology
UPLC-DAD	Ultra performance liquid chromatography-diode array detector
IAEC-CPCSEA	Institutional Animal Ethics Committee for Committee for the Purpose of Control and Supervision of Experiments on Animal
Ml	Millilitre
G	Gram
Kg	Kilogram
Mg	Milligram
b.w.	Body weight
DMSO	Dimethyl sulfoxide
DGCN COVAS	Dr. G.C. Negi College of Veterinary and Animal Sciences
EDTA	Ethylenediaminetetraacetic acid
PCV	Packed cell volume
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ALP	Alkaline Phosphatase
CRT	Total Creatinine
TP	Total protein
GLB	Globulin

ALB	Albumin
PBS	Phosphate Buffered Saline
ANOVA	Analysis of variance
SE	Standard Error
CX	Control group
DX	DEN alone group
SX	DEN + Silymarin group
SA	DEN + 100 mg/Kg of SLE
SB	DEN + 250 mg/Kg of SLE
SC	DEN + 500 mg/Kg of SLE

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ABSTRACT

The present study was designed to study the effect of 70% ethanolic extract of *Saussurea lappa* (SLE) against DEN-induced hepatic damage in rats. The UPLC examination of the plant collected from Lahaul & Spiti district showed the presence of costunolide and dehydrocostus lactone. For the present study male albino Wistar rats were used and were divided into 6 groups, Group 1(CX): control; Group 2(DX): 0.01% oral DEN administered rats; Group 3(SX): DEN along with silymarin; Group 4(SA): DEN along with 100 mg/Kg of SLE; Group 5(SB): DEN along with 250 mg/Kg of SLE; Group 6(SC): DEN along with 500 mg/Kg of SLE; Group 7: Group treated with 2000 mg/Kg of the SLE to check acute toxicity of plant extract, if any. The animals were monitored for any clinical sign and mortality during the experiment. Only the group treated with DEN alone showed decline in food and water intake. The result of ultrasound of rats treated with DEN showed presence of hyperechoic and hypoechoic areas with increased echogenicity of liver which was improved in SLE treated groups in dose dependent manner. The result of haematology after DEN treatment showed significant increase in leucocytes and erythrocytes count which revealed significant improvement with results more or less comparable to the control group in SLE treated groups in dose dependant manner. Similarly, serum ALT, AST, ALP, TP and globulin levels showed improvements in the SLE treated groups in comparison to the group treated with DEN alone. The increase in absolute and relative liver weights treated with DEN showed decline with SLE treatments. The gross lesions in the group treated with DEN alone were largely characterized by enhanced lobulation, whitish coloured nodular parenchyma and hepatis peliosis which decline considerably in ameliorative groups. The microscopic examination of DEN treated group showed severely enhanced lobular pattern, dyplastic changes, necrosis, bile duct proliferation along with significant increase in cirrhotic changes declined significantly in the group treated with SLE in dose dependent manner. The immuno-histochemical expression of NF-κB and Capase-3 showed significant increase in the liver of DEN treated group in comparision to the silymarin and SLE treated groups. The results of the present study showed the hepato-protective nature of the aqua-ethanolic extract of *Saussurea lappa* based on haematobiochemical and immunopathological studies.

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CHAPTER-1

INTRODUCTION

The traditional health care system is one of the various medication systems which have least offshoot and reasonably low cost thus around 70% of the world population depends on it for treatment of different diseases. The medicinal plant trade in India is anticipated to be around $1 billion per year and is expected to grow in the future. India is known to be one of the most important centers for collection of medicinal plants as it constitutes 27% of the world medicinal plant species. The major contributor of the medicinal plant material of the country comes from Indian Himalayan Region (IHR) which contains around 47513 plant species. India is one of the foremost nations to use the traditional medicines by utilizing around 2500 medicinal plant species. The medicinal use of plants was known to ancient Indians since much old times and the knowledge was clearly evident in various ancient books. The indigenous population of India has a wide knowledge on the use this medicinal treasurer of the Indian Himalayan Region (Naseer et al. 2017)

Various plants have been used for medicinal purposes from past thousands of years especially in India and China. In the past, various diseases like heart diseases, infections and cancer have been treated by use of different plant remedies. In the developing nation, around 80% of the primary health care facility is dependent on the herbal drugs while in the developed nation 25% out of total medicines prescribed to individual comes from the plants. Consequently, the demand of drugs derived from natural medicinal plants is increasing steeply across the world (Lin et al. 2015; Chen et al. 2016; Kim and Choi 2019).

The region of Indian Himalayas ranging from an altitude of about 2000 to 8000 meters above sea level comprises of various medicinal plants which are studied for their use in Unani, Ayurvedic and various traditional systems of medicines like the Tibetan system (Samant et al. 2007). Out of so many medicinal plants found in the Himalayan region one of the important plants is “Kuth”. *Saussurea lappa* generally called costus or kuth root in English and have different vernacular names such as Kustha (Sanskrit), Kust (Arab and Persian), Kut, Kur, Pachak (Hindi and Bengali), Kostum, Putchuk, Gostham (Tamil), Upaleta, Kur (Gujarati), Kot, kust(Punjabi), Changala (Telugu), Sepuddy (Malayalam),

Kostha (Kannada), Kuth, Postkhai (Kashmiri) and the trade name is Kuth (Madhuri et al. 2012).

The seeds are sown manually in the spring season after which the plant completes it 2-3 years of life cycle. After maturation, the plant is harvested in the month of August or September. After harvesting the leaves can be stored and fed to the animals as fodders. The roots are cut separately, dried and marketed to larger cities. During whole growth there is no requirement of adding fertilizers.

Kuth is known for its multiple uses in various tradition of the human civilization especially centered on the Himalayan region. In the tradition of Ayurvedic medicine various diseases like epilepsy, headaches, leucoderma, vomiting, leprosy, toothache etc. were treated by the plants root. The roots are also used as antispasmodic agent for the treatment of cough and asthma. The plant parts especially the roots are still being used in the northern part of India for its medicinal aspects either in powdered form as a decoction for the treatment of disease conditions or as a whole root for prevention of damage to clothes by insects. In the Unani tradition, the plant is used as a cure to some serious conditions like liver and kidney diseases along with some ocular conditions and also as a tonic that stimulate the brain activity. In Siddha, the plant is used to treat mainly the diseases related to the inflammatory response of the body like asthma, rheumatoid arthritis and bronchitis and also the oil from the roots of the plant is a major ingredient in the perfumes and hair oils. Tibetan medicine also uses the root for the treatment of the chronic inflammatory diseases related to lungs (Pandey et al. 2007; Madhuri et al. 2012; Amara et al. 2017).

One of the important areas of work for the production of drugs against diseases is the use of various secondary metabolites of different plants as they pave the way for unearthing vital steps for the discovery of new drugs. The Asteraceae family which contain *Saussurea* species are known to have various compounds like sesquiterpene lactones, flavonoids, lignans, triterpenes, steroids, glycosides. One of the main phytoconstituents for this species is Sesquiterpene lactones while other molecules which are known for their biochemical potential are Terpenes which includes Costunolide, Dehydrocostus lactone and Cynaropicrin (Singh et al. 2017). Various extracts of the kuth root have been found to have antibacterial activity and could be well used as a new antimicrobial agent. Some of the

compounds in the root of the plant like cynaropicrin, reynosin and santamarin show anti-inflammatory activity (Cho et al. 1998) while some compound like Dehydrocostus lactone and Costunolidehave been shown to have antiviral (Gautam and Asrani 2018).

Since *Saussurea lappa* has been known to have incredible medicinal applications, the present study is proposed to evaluate the aqua-ethanolic root extract of *Saussurea lappa* for its hepatoprotective activity against N-Diethylnitrosamine (DEN) induced liver damage in rats. The objectives for the proposed study are as follows:

1. To study the protective effect of *Saussurea lappa* on certain biochemical changes in chemical-induced hepatic damage.
2. To study the protective effect of *Saussurea lappa* on pathological changes in chemical-induced hepatic damage.

CHAPTER-2

REVIEW OF LITERATURE

2.1 *Saussurea lappa*

Since the past hundreds of years, the various parts of different plants are being used in variety of pharmacological preparations which are known to us till date. One of so many different medicinal plants is *Saussurea lappa.* Known by different vernacular names, one of which is "Kuth" in Hindi, *Saussurea lappa* is found in cool temperature habitats of the continents of Asia, Europe and North America. In Asia, Kuth is found at an altitude of 2500-3000 meters in the Himalayan region. This well recognized herbal plant had been found in various medicinal disciples like in Unani and Ayurvedic medicines. The local use of plant part especially the root in the treatment of various disease conditions like toothache, asthma, Cough, old fever, inflammation, dysentery, scabies, headache, epilepsy and various other innumerable conditions has been reported from time to time. This medical efficiency of the plant is mainly attributed to its various bioactive compounds (Kamalpreet et al. 2019).

2.1.1Taxonomic Classification (Gautam and Asrani 2018)

Kingdom:	Plantae
Subphylum:	Euphyllophytina
Infraphylum:	Radiatopses
Subclass:	Asteridae
Superorder:	Asteranae
Order:	Asterales
Family:	Asteraceae
Genus:	Saussurea
Species:	*S. lappa*

2.1.2 Local/vernacular names of *Saussurea lappa*.

Some of the commonly used vernacular names for the plant are as following (Chadha 1972)

Hindi: Kuth

Urdu: Minal

English: Costus

Tamil: Kostum

Sanskrit: Amayam, Puskara

Gujrati: Upleta

Marathi: Kustha

Kannad: Changalkustha

Malayalam: Kottam

Bengali: Kudo

Chinese: Mu Xiang

German: Practigekostwurz

French: Costus elegant

2.1.3 Acute toxicity studies.

Ambavade et al. (2009) studied the anti-convulsant properties of various extracts of *Saussurea lappa* and conducted an acute toxicity trail on Swiss male albino mice using petroleum, alcoholic, and water extract of the plant. The trail was performed as per the OCED guidelines 425 with the limiting dose of 2000 mg/kg of the procedure. The doses of 175, 550 and 2000 mg/kg of body weight were administered intraperitoneally for a period of 14 days and therefore the mortality was recorded. They recorded no mortality and found that all the extracts of the plant were safe up to the highest dose of 2000 mg/kg.

Sutar et al. (2011) performed the acute toxicity study on albino mice weighing around 22-25g using methanolic extract of S*aussurea lappa* as per the OECD-423 guidelines. The mice were first treated with 5 mg/kg of the extract through oral intubation and were observed for mortality. They conducted the study till the dose rate of 2000 mg/kg of body weight. The result of their study showed no toxic symptoms and mortality.

Saleem et al (2013) reported that the aqueous extract of the root of *Saussurea lappa* did not show any mortality or any signs of toxicity in albino Wistar rats when treated with a dose of 2000 mg/kg of body weight according to up and down procedure.

Garg et al. (2016) conducted a study in order to find out the LD_{50} of some of the indigenous herbal plants which included root of *Saussurea lappa*. They examined various

extracts of the plant like water, ethanol, chloroform and petroleum ether by injecting them in swiss albino mice intraperitoneally at the increasing dose rate of 250, 500, 750, 1000, 1500 and 2000 mg/kg of body weight. They reported no mortality or any significant change in any body parameter.

2.1.4 Phytochemical evaluation

Duan et al. (2010) studied the various constituents of the *Saussurea lappa* roots and found 9 compounds i.e. dehydrocostus lactone (1), β-sitosterol (2), daucosterol (3),5-hydroxymethyl-furaldehyde (4), santamarine (5), β-cyclocostunolide (6), 4α-hydroxy-4β-methyldihydrocostol (7), trans-syingin (8) and 10α-hydroxyl-artemisinic acid (9). Out of the compounds present five represent sesquiterpenes. They detected a new compound known as 10α-hydroxyl-artemisinic acid which is an artemisinic acid and also observed that the presence of dehydrocostus lactone was the highest among the found compounds.

Liu et al. (2012) in their study derived the essential oils from the root of *Saussurea lappa* by means of hydrodistillation and then used gas chromatography (GC) and GC-Mass spectrometry (MS) in order to identify the component of the essential oil. The result of their study showed that the oil from the root of the plant contained 39 components with the highest content of sesquiterpenoids (79.80%) followed by monoterpenoids (13.25%). They also showed that the main component of the essential oil being dehydrocostus lactone (46.75%) and costunolide (9.26%) followed by 8-cedren-13-ol (5.06%), and α-curcumene (4.33%).

Ahmed et al. (2016) in a phytochemical investigation found that when different category of the solvents was used to extract the phenolic compounds and flavanoids from the *Saussurea costus* roots like absolute methanol, absolute ethanol and the various aqueous composition of both the methanol and the ethanol, the efficacy of the aqueous ethanolic (70%) was found to be the best out of the different solvents. The results also showed that the highest extraction value came from the oven dried (40°C) roots. They also observed that the antioxidant activity of the oven dried 70% aqueous ethanolic extract in the *in vivo* antioxidant assay also showed the best result out of the different remaining extracts.

Omer et al. (2019) used gas chromatography/ mass spectrometry (GC/MS) analysis to find out various active components in the aqueous and ethanolic extracts of the root of *Saussurea lappa* costus and also used the extracts to find out their effects on the Gram-

negative *Salmonella species* and Gram-positive *Staphylococcus aureus* bacteria. During analysis of the extract, they found 37 compounds in the ethanolic extract which were double the compounds found in the aqueous extract i.e. 18 compounds with the most abundant compound being Sesquiterpene lactones in both the extracts. The ethanolic extract also showed to inhibit the growth of *Staphylococcus* while no effect was observed in *Salmonella.* On the other hand, the aqueous extract of the root showed weak effect against *Staphylococcus* while the effects were negligible against *Salmonella* species.

2.1.5 Hepatoprotective activity

Yaeesh et al. (2010) studied the hepatoprotective effect of the aqueous methanolic extract of *Saussurea lappa* roots against the D-galactosamine (D-GalN) and lipopolysaccharide (LPS)-induced hepatitis in Balb-C mice of either sex. They administered the chemicals and the extract intraperitoneally. The simultaneous administration of the D-galactosamine (700mg/Kg) and lipopolysaccharide (1μg/Kg) showed a significant rise in the serum ALT and AST levels. While the groups treated with the methanolic extract of the plant (150, 300, 600 mg/Kg) showed a significant decrease in the serum biochemical levels and showed a very limited damage to the hepatic parenchyma as compared to the group administered with the hepato-toxins.

Alnahdi et al. (2016) studied the hepatoprotective activity of Methanolic extract of *Saussurea lappa* on the induced liver toxicity by deltamethrin. For this study, they took 60 male albino rats of weight around 150-180 gm and were divided into 6 groups for an experimental duration of 28 days. After the experiment, the level of the biomarkers presents in the serum i.e. ALT, AST, ALP, GGT and total protein for the hepatic damage showed a significant increase for the group treated with deltamethrin while the levels of SH-protein and the antioxidant enzymes were significantly on a lower side than the control group. The histopathological examination of the deltamethrin group showed degeneration of the hepatocytes along with congestion while the examination of the group supplemented by the extract showed much better result in the protection of the liver parenchyma. Hence, the results concluded the hepatoprotective effect of the plant extract.

Kadhem (2019) investigated the effect of 70% ethanolic extract of *Saussurea lappa* against paracetamol-induced hepatic and renal toxicity in male rabbits. For the study 18 rabbits were divided in three groups having six rabbit each. First group was taken as

negative control, second group was taken as positive control administered with a dose of 300 mg/kg body weight of paracetamol for 14 days while the third group received the same dose of paracetamol along with ethanolic extract of S*assurea lappa* at the dose rate of 300 mg/kg bw for a period of 2 weeks. Feeding of 70% ethanolic extract of *Saussurea lappa* showed a significant hepato and nephro protective activity by rendering the various biochemical and haematological parameter towards normal. The histopathological examination of the liver and kidneys also confirmed the finding.

2.1.6 Anti-Neoplastic activity

Ko et al. (2004) studied the anti-tumour effect of 4 plants i.e. *Saussurea lappa*, *Pharbiti nil*, *Plantago asiatica* and *Taraxa cummongolicum* by using human gastric adenocarcinoma cell-line for proliferation or expression of cell apoptosis related molecules. *Saussurea lappa* and *Pharbitis nil* showed apoptotic activity of AGS cells by increasing the Bax related apoptosis and decreasing the active caspase-3 protein. Hence, they concluded that *Saussurea lappa* and *Pharbitis nil* can be used as a potential anti cancerous medicine.

Oh et al. (2004) studied the effect of sesquiterpene lactones present in the root of *Saussurea lappa* on the human leukemia HL-60 cell line. The study was based on blocking or inhibiting the response of NF-κB as it plays a crucial role in prevention of the apoptosis in the cancer cell by the anticancer drugs. It also prevents the apoptosis by the tumour necrosis factor-α (TNF-α) hence preventing the treatment of the tumour. But in the cell line experiment showed that the dehydrocostus lactone, one of the important and abundant sesquiterpene lactones present in the root of the plant showed promising result in treatment of the tumour by inhibiting the activation of NF-κB by degradation of its inhibitory protein I-κBα and thus make the cancer cells prone to apoptosis by TNF-α by increasing the activities of Caspase 3 & 8.

Hsu et al. (2009) conducted a study which showed the anti-cancerous effects of the plant derived sesquiterpene lactone i.e Dehydrocostuslactone (DHE) on the hepatocellular carcinoma in both in-vivo and in-vitro studies. DHE inhibited the cell line proliferation by inducing apoptosis through stimulating the endoplasmic reticulum (ER) stress. The study was also supported by the fact that the tumour volume in the nude mice was decreased significantly by 50% after 45 days of treatment.

Sunkara et al. (2010) conducted an experiment in order to study the cytotoxic effect of chloroformic extract of the *Saussurea lappa* root on different cancer cell lines like HT-29 (Colon cancer), A549 (Lung cancer) and MDA-MB (Breast Cancer). The result of their study concluded that for the breast cell line the effect of the chloroformic extract was significant while the remaining two cell lines did not respond significantly to the extract.

Choi and Kim (2010) studied the anti-proliferative effects of dehydrocostuslactone, a sesquiterpene lactone derived from the roots of *Saussurea lappa* against the human breast cancer (MDA-MB-231, MDA-MB-453 and SK-BR-3) and ovarian cancer (SK-OV-3 and OVCAR3) cell lines by incorporating thiazolyltetrazolium assay. Their study showed that the compound dehydrocostus lactone resulted in dose-dependent decline for the proliferation of cells and the IC_{50} value for the cell lines were recorded to be 21.5, 43.2, 25.6, 15.9 and 10.8 µM in MDA-MB-231, MDA-MB-453, SK-BR-3, SK-OV-3 and OVCAR3 cells, respectively which wasmainly due to the effect of the compound to induce cell cycle arrest and apoptosis. Hence, their study showed the anti-tumorous property of the compound.

Moon et al. (2013) showed the anti-cancerous effect of *Saussurea lappa* in the human oral cancer cell line. The dried *Saussurea lappa* roots were subjected to methanolic extraction. They studied the viability of the KB cells which was evaluated by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide assay after being treated by the methanolic extract at the dose of 30 mg/ml. They found that the use of IC value of 30 mg/ml significantly reduced the cell viability. The methanolic extract of *Saussurea lappa* was shown to inhibit the cell proliferation by stimulation of apoptotic pathway in the human oral cell line culture.

Kumar et al. (2014) isolated various compounds from the root of *Saussurea lappa* which was collected at an altitude of 3000-3500 meters above sea level from the farms of Lahaul and Spiti. They then isolated various compounds using UPLC-DAD and then the isolated compounds were checked for their cytotoxicity against human lung carcinoma cell line (A549) and rat glioma cell line (C-6) by the use of Sulphorhodamine B assay. Through DAD analysis, they found 6 compounds out of which 4 compounds showed significant activity against the cell lines and also showed that the concentration of alantolactones was more than the isoalantolactone in the western Himalayan region. Hence, their study proved that the root of *Saussurea lappa* can be a potential source of anti-neoplastic compounds.

2.1.7 Anti-inflammatory activities

Cho et al. (2000) studied the anti-inflammatory effect of a sesquiterpene lactone from *Saussurea lappa i.e.* cynaropicrin on RAW264.7 cells and U937 cells. They studied its effect ontumour necrosis factor (TNF)-α, nitric oxide (NO) release, and lymphocyte proliferation when the cells were stimulated by lipopolysaccharide and interferon- γ. In their study they showed that the sesquiterpene downgraded the inflammatory response by discouraging the production of inflammatory mediators and multiplication of lymphocytes.

Gokhale et al. (2002) showed the anti-inflammatory effect of some traditionally used Indian medicinal plants like *Saussurea lappa, Argyreia speciosa* and *Achyranthes aspera.* They used the ethanolic extract of these plants at the dose rate of 50, 100 and 200 mg/kg p.o. for the treatment against the carrageenan and Freunds complete adjuvant induced paw oedema and carrageenan induced peritonitis. Their study showed that the ethanolic extract of *Saussurea lappa, Argyreia speciosa* and *Achyranthes aspera* showed anti-inflammatory and anti-arthritic activity which was largely in support with their traditional uses by the indigenous treatment.

Damre et al. (2003) showed the anti-inflammatory effect of the sesquiterpene lactone fraction of *Saussurea lappa* against the cotton pellet granuloma assay of the albino Wistar rats. They used the fraction at the dose rate of 25–100 mg/kg, p.o. which showed the dose dependent result for the transudative, exudative and proliferative phases of inflammation and also showed significantly lowering the increased biochemical parameters. The result of their study was mainly due to the upholding of the lysosomal memberane by the sesquiterpene lactone fraction of *S. lappa.*

Chandur et al. (2011) performed the phytochemical evaluation along with the effects of the various extracts from the root of *Saussurea lappa* on the inflammatory condition i.e. arthritis in rats. Arthritis was induced in rats by the use of Complete Freund's Adjuvant. Out of the used extracts of petroleum ether, chloroform and alcoholic extracts the activity of former one outcast the other two extracts in anti-inflammatory properties. Hence, the plant is widely used in the herbal medicine for its anti-inflammatory activities.

Choodej et al. (2018) in their study revealed that the hexane and ethylacetate (EtOAc) extract from the root of *Saussurea lappa* showed anti-inflammatory effects by

decreasing the activity of tumour necrosis factor- alpha (TNF-α) which is a pro-inflammatory cytokine. TNF-α in an important mediator for the inflammatory response in the body and is released by the activated immune cells. The result of their study showed that the hexane and EtOAc extract of the root showed anti-inflammatory effects while the methanolic extract did not. The results were determined by measuring the ability of the extracts to decrease or inhibit the production of TNF-α in RAW 264.7 macrophage cells. The column chromatography of the active extracts revealed the presence ofsesquiterpenes, costunolides and dehydrocostus lactone. Thus, the hexane and ethylacetate (EtOAc) extract from the root of *Saussurea lappa* showed anti-inflammatory effects.

2.1.8. Other uses

i) Antibacterial property

Li et al. (2005) in their study conducted researchon about 30 Chinese medicinal plants including *Saussurea lappa* which were being used for the treatment of gastric ulcer and other gastric ailments. They studied the ethanolic extract of the various plants in-vitro against 5 clinic strains of *Helicobacter pylori* and found that the ethanolic extract of the *Saussurea lappa* showed the third best results among the various plants used for its treatment with the minimum inhibitory concentration of 40µg/ml thereby showing the strong anti-microbial nature of the plant.

Hasson et al. (2013) studied the 99.9% ethanolic extract and the water extract of *Saussurea lappa* against the isolates of specific microbes like methicillin resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli, Klebsiella pneumonia* and *Acinetobacter baumannii*for its antimicrobial activity. The extract was tested by agar gel diffusion method as of commercial antibiotic as the standard. The results of their study showed that the higher dose of absolute ethanolic extract showed inhibitory zone against all microbes as compared to the lower dose of the same while the water extract did not show any inhibitory effect at all. Hence, they concluded that the higher dose of absolute ethanolic extract of *Saussurea lappa* shows much better anti-microbial activity.

Alaagib and Ayoub (2016) carried a study in order to show the anti-microbial activity of various extracts of *Saussurea lappa* against various microbes. For their study they examined the petroleum ether, chloroform, methanol and water extracts against the two standard Gram-positive bacteria i.e. *Bacillus subtilis* & *Staphylococcus aureus* and

three standard Gram-negative bacteria i.e. *Escherichia coli*; *Pseudomonas aeruginosa* and *Klebsiella pneumonia* for their anti-microbial activity. The result of their study showed that the Chloroform extract of *Saussurea lappa* showed the highest potent activity against bacteria as compared to others.

ii) Anti-ulcer property

Mitra et al. (1996) studied the anti-ulcerative effect of an herbal formulation "UL-409" which comprised of 6 medicinal plants i.e. *Glycyrrhiza glabra* L. (Papilionaceae; Root), *Saussurea lappa* C.B. Clarke (Compositae; Root), *Aegle marmelos* Corr. (Rutaceae; Fruit), *Foeniculum vulgare* Mill. (Umbeliferae; Seed), *Rosa damascena* Mill. (Rosaceae; Flower Petals) and *Santalum album* L (Santalaceae; Stem) against the aspirin and alcohol in Wistar rats and histamine and cysteamine induced ulcers in guinea pigs. Their study showed that the formulation greatly reduced the ulcer formation in dose dependent manner.

Sutar et al. (2011) studied the anti-ulcerative properties of the ethyl acetate extract from the roots of *Saussurea lappa*. In their study, the chemicals used to produce the gastric ulcers were aspirin and ethanol along with pyloric ligation. On the other hand, the duodenal ulcers were produced by the use of cysteamine hydrochloride given to the albino Wistar rats orally. The results of their study showed that the extract given at the dose rate of 400 mg/kg b.w. showed the most declines in production of gastric acid, free acid and total acid when compared to lower dose of 200 mg/kg b.w. Thus, root of the plant has anti-ulcerative properties according to their study.

iii) Cardiovascular activity

Saleem et al. (2013) studied the protective effect of aqueous extract of *Saussurea lappa* (AESL) root against the isoproterenol induced myocardial injury in male albino Wistar rats. The extract was orally administered to the rats at the dose of 100, 200 and 300 mg/kg. The result for the only isoproterenol treated rats showed an increase in serum biochemicals related to the muscle damage like lactate dehydrogenase (LDH), creatine kinase (CK), and aspartate transaminase (AST), increased myocardial thiobarbituric acid reactive substances (TBARS) level, and decreased myocardial glutathione (GSH) level and the damage was later confirmed by the histopathological examination while the result for the administration of AESL showed significant improvement in the serum biochemical's and was also confirmed by the histopathological examination. However, out of the three

doses, the effect of 200 mg/kg showed the best results by decreasing the oxidative stress caused by the chemical on the myocardium.

Akhtar et al. (2013) in their study evaluated the cardiovascular activity for the methanolic extract of root of *Saussurea lappa* against the Langendorff's technique in isolated perfused rabbit heart. The effect of the extracts with the dose of 0.5/µg, 2.5/µg and 5.0/µg showed positive ionotrophic on the various parameters of cardiac activity like Heart rate, contractility and coronary flow while the remaining doses showed negative effects. Thus, it was concluded that the root contains the active compounds which show cardiotonic activity in rabbits.

iv) Anticonvulsant activity

Ambavade et al. (2009) in their study showed the anticonvulsant activity of different extracts of *Saussurea lappa* in mice. The extracts used for the experiment were petroleum ether extract (SLP), alcoholic extract (SLA), and water extracts (SLW) against the pentylenetetrazole and picrotoxin-induced convulsions and the maximal electroshock (MES) test. The result of their study showed that the petroleum ether extract of *Saussurea lappa* showed significant anticonvulsant activity.

Gupta et al. (2009) conducted a study in order to determine the anticonvulsant activity of the 95% ethanolic extract of *Saussurea lappa* root at the dose of 50, 100, 200 mg/kg in the swiss albino mice. In their study, they used maximal electro-shock induced convulsions and pentylenetetrazol-induced clonic convulsions methods for the estimation of the anticonvulsant activity. Their result showed that the ethanolic extract of the plant showed noteworthy anticonvulsant activity for both the experiments.

v) Antiparasitic activity

Rhee et al. (1985) investigated the antiparasitic property of the water extract of *Saussurea lappa* against *Clonorchis sinesis* in rabbit. They treated the infected rabbits with the plant extract for duration of 30 days. The result of their study showed that the plant extract causes changes in the viscera of the worms and the recovery percent of rabbits infected with the parasite was 2.8%. Thus, the study showed the antiparasitic nature of the plant extract.

vi) Antihyperlipidemic activity

Anbu et al. (2011) studied the anti-hyperlipidemic effect of the ethanolic extract from the root of *Saussurea lappa*. The rats were fed with diet containing high amount of cholesterol which leads to accumulation of excessive triglycerides in tissues and serum. The results of the group treated with the ethanolic extracts showed significant decrease in the body weight along with decrease in serum and liver tissue cholesterol levels.

vii) Antidiarrheal activity

Negi et al. (2013) studied the anti-diarrhoeal property of the methanolic extract of *Saussurea lappa* against the castor oil induced diarrhoea in rats. For this study, Wistar rats were divided in 5 groups from which first group was provided saline. Second, third and fourth group was treated with the dose of 100, 300 and 500 mg/kg of the extract which was provided to the animals orally while the fifth group was maintained on the standard drug i.e. loperamide at the dose of 5 mg/kg body weight. The result of their study showed a significant decrease in the diarrhoea with the inhibition by the extract being 26.33, 32.2 8 and 66.77% respectively.

2.2 DEN (N-diethylnitrosamine)

Park et al. (2009) conducted a study in order to study the criteria for the relationship between the carcinogen and cell cycle. The effect of the chemical carcinogen diethylnitrosamine (DEN) was studied in three-week-old male Wistar rats by studying the various parameters including histopathological examination, immunohistochemistry along with cell cycle regulatory proteins like Cyclin D1, cyclin E, cdk4, and $p21^{CIP1/WAF1}$. The chemical was injected intraperitoneally for the period of 12 and 16 weeks at the dose of 50 mg/kg body weight. The DEN treated group showed increase in liver weight along with the presence of nodules on the hepatic parenchyma. Cyclin E, cdk, $p2^{CIP1/WAF1}$, cyclin B, and *p*-cdk1 were expressed in considerable amount in DEN treated group when were compared to the control group. The result of their study showed that the protein cdk4 showed a vital role in the passage to the neoplastic condition from the pre-neoplastic condition.

Costa et al. (2014) studied the timeline of the malignancy associated with Diethylnitrosamine (DEN) induced hepatic carcinogenesison the male ICR mice which were divided in twelve groups comprising of six control groups and six groups exposed to

DEN. The control group animals were injected with normal saline solution while the exposed groups were intraperitoneally injected with DEN at the rate of 35 mg of DEN/Kg body weight per mouse for a period of eight weeks repeatedly. They then sacrificed two groups simultaneously i.e. DEN and control at the time of 8, 15, 22, 29, 36 and 40 weeks of experimentation since the administration of first dose. The result of the experiment showed the development of hepatic damage on histopathological examination. The lesions like necrosis, hydropic degeneration and apoptosis were induced for eight week of DEN administration while the other lesions like hyperplastic foci and hepatocellular adenoma were observed from 29 to 40 weeks onwards. The specific histological feature of Hepatocellular carcinoma (HCC) was noted at 40 weeks of DEN administration having characteristic feature of malignancy.

Khan et al. (2015) aimed a study in order to evaluate the combined chemo-protective effect of Lopramine as combined with niacin against the single dose administration of diethynitrosamine (DEN) at the dose of 160 mg/kg body weight. The male albino Wistar rats were divided into 8 groups having 6 rats each for duration of 12 weeks. The result of their study showed the increase in the level of serum biochemical's like glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), acid phosphatase (AP), cholesterol (C), triglycerides (TG) and high-density lipoproteins (HDL), total proteins (TPR), bilirubin and the specific marker for hepatocellular carcinoma such as alpha fetoprotein (AFP). The caspase-3 activity of the therapeutic group however increased after the treatment when compared to the DEN treated group. Hence their study concluded that the combination of lopramide along with niacin showed improved effect against the chemical induced carcinogenesis cause by DEN

In a study conducted by Patial et al. (2015) on the male Wistar rats of age around 6-weeks and approximately weighing 150-200 g in order to investigate the synergistic effect of the curcumin along with piperine to suppress the growth of hepatocellular carcinoma (HCC) which was induced in rats by providing them diethylnitrosamine (DENA) in the drinking water at the concentration of 0.01%. After the completion of the experiment, the only DENA administered group showed the presence of multiple grey nodules along with enlargement of liver. The rats were treated with curcumin and piperine for 4weeks after the induction of HCC. The experiment showed promising results when treated with curcumin in combination with piperine in order to suppress the DENA induced HCC.

Tolba et al. (2015) studied the diethylnitrosamine (DEN) induced tumourogenic liver injury. In their study they showed that the liver tumour which was induced in mice by the use of DEN was showing high resemblance to the hepatocellular carcinoma in humans. They also showed that the single small dose of DEN may not develop tumour in mice but with the use of single high dose or continuous use of the chemical can induce hepatic tumour. Their study revealed that the tumour formation in an experimental model can be influenced by the sex, age and strain of mice.

Chooi et al. (2016) produced liver fibrosis in male Wistar rats by intraperitoneally injecting diethylnitrosamine for 3 continuous days in a week for 4 weeks. During the experiment, they measured serum ALT, AST of the affected and control group. After the end of trial, they measured the liver weight and compared it with the body weight of rat and performed Masson's trichrome stain in order to determine the liver fibrosis. The result of the study showed that the liver of the DEN treated group showed smaller and harder liver when compared to the control group along with increased ALT and AST levels. The histological liver sections showed increased level of collagen by Masson'ss trichrome stain.

Fathy et al. (2017) studied the analysis and staging of DEN induced hepatocellular carcinoma. For the study, 240 adult mature male albino Wistar rats weighing around 150-170 g were divided into 16 groups (8 control groups and 8 DEN administered groups) having 15 rats each. The rats were provided with DEN in drinking water at the dose of 100 mg/L for a period of 27 weeks. The result of administration of chemical showed decline in growth pattern along with significant decline in erythrocytic count, haemoglobin concentration, Haematocrit percentage, platelet count, WBC count and lymphocyte percentage while they show a significant increase in the lipid profile. The result also showed the increase in the oxidative stress, liver function parameters and hepatocellular tumour markers along the progress of the experiment.

Kumar et al. (2018) conducted a study in order to find the hepato-protective effect of R-phyco-erythrin (R-PE) -rich protein extract which was acquired from *Portieria hornemannii* (Lyngbye) Silva against H_2O_2-induced hepatotoxicity using HepG2 cells line and male Wistar albino hepatocellular carcinoma (HCC) rats. The rats treated with DEN showed increase in ALT and AST levels when compared to other groups. However, the level of the antioxidant in the liver homogenate showed significant decline in the group treated with the carcinogen but for the groups treated with the extract the antioxidant level

showed a visible rise in its level. The findings were also confirmed by the histopathological examination in which the cancer induced group showed the production of hepatocytes showing pleomorphism along with fibrous tissue proliferation and lymphocytic infiltration. While the group treated with the extract showed significant reduction in these cancerous parameters. Their study showed that the effect of the R-PE-rich protein extract is due to its nature to reduce the cell damage caused by free radicals.

2.3Ultrasonographic examination

Lee et al. (2005) in their study evaluated the non-invasive method for diagnosing the hepatic fibrosis and cirrhosis induced by CCl_4 by relating the histopathology and mean grey level in B- mode ultrasound. They used 45 male Wistar rats divided into 3 groups which were treated with olive oil, CCl_4, and CCl_4 + silymarin and were sacrificed at 4, 8 and 12 weeks of the trial after B-mode ultrasonography examination. These rats were later analyzed histopathologically for hepatic changes like fatty change and fibrosis. The results of the study showed that the B-mode grey level histogram is a handy tool for the diagnosis of diseases like hepatic inflammation, fibrosis and cirrhosis.

Dias et al. (2008) used ethanol (5.5 %) and carbon tetrachloride (0.05, mL/kg, ip) as chemical agents to induce hepatic cirrhosis in 11 female Wistar rats for 15 weeks and then out of 11, 5 were sacrificed while rest 6 were kept for additional 8 weeks without the treatment with the chemical agents. Upon ultrasonographic examination, the animals treated with DEN showed significant increase in the echogenicity and portal vein dilation as compared to the control group. They also studied the histomorphometry which showed the increase in the fibrous tissue specifically in the type I and III collagen. Tissue transglutaminase also showed an increase as compared to the control group. Hence, they showed that the ultrasonography can be a very important tool to study the advance hepatic alteration.

Lessa et al. (2010) in their study find the reliability of ultrasound for assessing the various diseases like fatty liver disease and cirrhosis by the use of Wistar rats and then compared the results with histological examination. They treated 30 rats for liver injury by exposing them to CCL_4 and ethanol for 4, 8 and 15 weeks and at the same time keeping 10 control rats. Then the rats were evaluated for the presence of the ultrasonographic changes like portal vein diameter or presence of ascites. Their finding showed that the portal vein

diameter was significantly increased in cirrhotic and steototic rats. Hence, they showed that non-invasive technique like ultrasound can prove beneficial for any liver disease diagnosis.

Akshatha (2016) performed the ultrasonographic examination of the hepatocellular carcinoma induced by N-nitrosodiethylamine in the Wistar rats. He found that the rats treated with the chemical carcinogen produced large lesions of carcinoma which being hyperechoic having heterogenous appearance along with small focal HCC nodules which appeared hypoechoic upon examination. The liver also showed increase in size of lobes along with irregular margins.

D'Souza et al. (2019) studied the quantitative analysis of computer-extracted features of B-mode ultrasound for the non-invasive technique to help in diagnosis of hepatic fibrosis. They treated 22 rats with DEN per os in order to induce hepatic fibrosis and kept 4 rats for control. They then studied the various quantitative parameters representing brightness and variance of the liver. The results showed that the DEN treated rats showed increased in echo intensity from 37.1±SD 7.8 to 53.5±5.7 (10 w) to 57.5±6.1 (13 w) while that of the control rats did not change and was maintained at 34.5±4.5. They also showed that when the ultrasound results were compared with METAVIR grades in histologic analysis for fibrosis, the result showed the strongest correlation. Hence, they concluded that ultrasound can be used as significant non-invasive tool for hepatic study.

Jeevan et al. (2020) chemically induced hepatic tumours in 10 male albino Wistar rats with 0.01% v/v concentration of diethylnitrosamine (DEN) in drinking water and then performed ultrasound of the liver in the end of the trial. They observed that the nodules of the tumour can be differentiated well from the surrounding hepatic tissue having indistinct borders and can be easily differentiated from the surrounding parenchyma.

CHAPTER-3

MATERIAL AND METHOD

3.1 Collection of plant material

Saussurea lappa locally known as Kuth is cultivated in whole of the districts of Lahaul and Spiti and Kinnaur where there is human settlement. The wild useful variety is not known tooccur without the interference of the humans. The plant is grown from seeds which are preserved from the last harvest. The freshly cultivated roots were collected from the village Muring, near the city of Udaipur in Lahual and Spiti, District of Himachal Pradesh in the month of September-October, 2019 (Plate 1 A & B). The village is situated at an altitude of 3050 meters from Mean Sea Level and at 32° 39' 20.6424" N latitude and 76° 48' 26.8236" E longitude. The plant was being cultivated in an area of around 1 acre while a lot of other peoples were cultivating this plant in a diffuse manner. The time for sowing the seeds is in the season of spring i.e. around March or April. The plant has a life cycle of 2 or 3 years and the harvest is done in two shifts and is locally known as two harvesting seasons in which first the matured seedpods are cut about 1 month prior from the second main harvest of the roots during themonth of August or September. The freshly collected whole plant was submitted to the High Altitude Biology (HAB) Division of the Council of Scientific and Industrial Research – Institute of Himalayan Bioresource Technology (CSIR-IHBT) to confirm the identity of the plant species. The roots of the plant were then dried and grounded to fine powder and stored in air tight container until further use.

3.2 Preparation of Extract

The extract was prepared by using fine dried powder of the *Saussurea lappa* root which was weighed and kept in the 70% ethanol for overnight (Plate 1 C). On the following day, the supernatant was collected in a flask by filtering through double layers of muslin cloth**.** The filtrate was concentrated by the use of Rotary Evaporator (Model- Buchi Rotavapor R-210) at around 50ºC until thick slurry was obtained (Plate 1 D). The slurry was subjected to lyophilization for around 24 hours until powder form of the extract was obtained. The formed extract was scraped out of the container and was stored at 4 ºC till further use. The percentage yield was recorded which was around 7.5% of the total raw material used.

3.3 Ultra Performance Liquid Chromatography (UPLC) Analysis

The various polyphenolic and other bioactive chemical compounds are known to be present in the roots of *Saussurea lappa* plant. Out of these, a few specific biochemical compounds were quantified using the UPLC analysis. Two standards i.e. Costunolide and Dehydrocostus lactone which were procured from Sigma Aldrich St Louis, MO, USA were used for qualitative and quantitative analysis using UPLC-DAD technique in CSIR-IHBT, Palampur.

3.4 Preparation of Experimental room

Before the start of the experiment, the allotted room of the Laboratory Animal House Facility of the college was washed thoroughly by 2.5% phenol solution. The same phenolic solution was also used to clean the room, cages, water bottle and the trays. The washed cages and water bottles after their thorough drying under the sunwere placed in the experimental room along with other accessories followed by fumigation of the room using potassium permanganate and formalin solution 3-4 days prior to the arrival of the experimental animals.

3.5 Experimental animal

The present study was conducted on 46 male Albino Wistar rats (weighing between 150-180 grams) were purchased from CSIR-IHBT, Palampur. The experimental design was approved by the Institutional Animal Ethics Committee - Committee for the Purpose of Control and Supervision of Experiments on Animal (IAEC-CPCSEA) prior to the start of the experimental trail. The rats were divided into different groups in such a way that the mean weight of the groups does not vary considerably and then were placed under the hygienic facility in the polypropylene cages with 12 hours day/night cycles. The temperature of the room was maintained at around 25ºC for the whole length of the experiment.

3.6 Maintenance of Animals

The animals were grossly examined for any abnormality or any disease condition prior to their arrival. Upon arrival, the rats were individually weighed and divided accordingly into 7 groups having 2 cages in each group and each cage having a maximum of 4 rats in it. They were then left for acclimatization for a week before the start of the

Plate 1: Photographs of the plant and Extract

<table>
<tr><td>  </td><td> 
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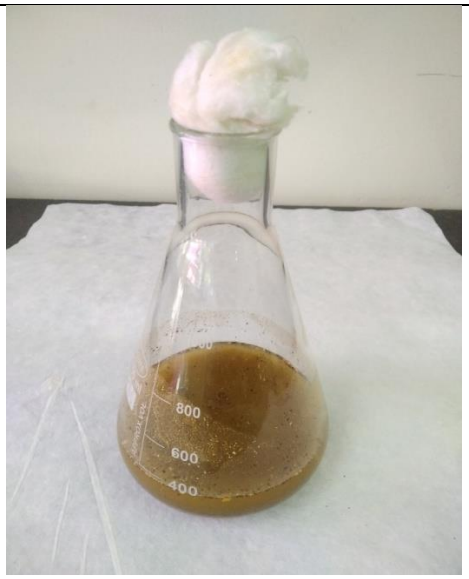
<tr><td>Fig A: Field of mature Saussuea lappa in Lahaul&Spiti.</td><td>Fig B: Freshly harvested roots of Saussurea lappa.</td></tr>
<tr><td> 
</td><td> 
</td></tr>
<tr><td>Fig C: Mixture of powdered root and 70% ethanol for overnight extraction</td><td>Fig D: BuchiRotavapor R-210</td></tr>
</table>

experiment. Each cage of the group was properly labeled with the number of animals, dose to be provided and group number allotted and in each cage the animals were ring marked on the tail for easy recoginition.

The standard feed was procured from CSIR-IHBT, Palampur and was provided ad libitum after autoclaving the feed at 15 lbs pressure for 15 minutes. The same feed was provided to the animals until the end of the experiment in order to prevent any stress condition due to the change of feed. The water was also provided ad libitum only after boiling and then cooling to room temperature. Autoclaved rice husk was used as bedding material for rats which was changed on alternate days until the end of experiment. On day of changing the bedding, the cages were also washed with potassium permanganate solution in order to prevent any disease condition. No medication of any kind was given to the animals during the entire duration of the experiment.

3.7 Acute toxicity

The acute toxicity study of the extract was done on 5 male albino Wistar rats given root extract of *Saussurea lappa* at a dose rate of 2 g/KG of body weight. This dose was administered through oral route for three alternate days after which the rats were closely examined for up to next week and sacrificed. They were thoroughly examined for gross and microscopic lesions for evidence of plant toxicity.

3.8 Experimental design

The experimental study was conducted in the 6 groups of rats for a period of 10 weeks during which time the rats were placed in temperature maintained environment. Five groups were given N-Diethylnitrosamine (DEN) (Sigma-Aldrich) @0.01% in their drinking water which was properly measured before making the solution in their drinking bottles. The various doses of 70% ethanolic extract of *Saussurea lappa* were included at 100, 250 and 500 mg/KG of body weight. Silymarin (Sigma-Aldrich) was used as positive control against the protective effect of the *Saussurea lappa* root extract. The summed up experimental design indicating various treatments is mentioned in the following table:

Table 1: Experimental design

Group	Treatment	No. of Animals	Dose	Route
1/CX	Control	6	Vehicle (DMSO)	Oral intubation
2/DX	DEN only	7	@0.01%	Oral
3/SX	DEN + Silymarin	7	@0.01% + 25 mg/Kg B.W.	Oral intubation
4SA	DEN + SLE	7	@0.01% +100 mg/Kg B.W.	Oral intubation
5/SB	DEN + SLE	7	@0.01% +250 mg/Kg B.W.	Oral intubation
6/SC	DEN + SLE	7	@0.01% +500 mg/Kg B.W.	Oral intubation

Note: DMSO = Dimethyl sulfoxide, DEN = N-diethylnitrosamine, SLE = *Saussurea lappa* extract

3.9 Dose preparation and administration

The dose of SLE and silymarin to be administered to the rat models were prepared afresh for each day. The quantity of extract was measured precisely by the help of electrical balance and was mixed with 10% solution of Di-methyl sulfoxide (DMSO) in such a way so that the desired proportion of the extract was obtained which was to be gavaged to the animal. The solution was mixed by the help of shaker and was incubated at 37ºC about 8-10 hours prior to the gavaging. The dose was prepared according to the weekly body weight of each animal and was administered at the rate of 0.5ml/100g BW of rat.

The specific quantity of the prepared extract solution was gavaged orally after proper handling of rat by using of 18G curved gavaging needle attached to 2ml syringe so that the exact amount of solution was provided to the rat according to their body weight. The gavaging was done in the morning hour before feeding and watering of the animals.

3.10 Monitoring of animals

The rats were closely monitored for development of any clinical sign throughout the period of experiment. They were observed for at least three times a day for any abnormality. The animals were also specifically monitored for clinical signs such as hair loss, diarrhoea, reduced appetite, dullness, and decrease in weight gain. Any mortality

during the experiment was recorded. The body weight of each individual rat was taken weekly.

3.11 Ultrasonographic examination

At the end of 10 weeks of experiment, the rats were subjected to ultrasonographic examination in order to determine the extent of damage done by DEN in rat liver. For this, the rats were taken to the Department of Veterinary Surgery and Radiology, DGCN COVAS, Palampur. The rats were provided with the mask induction of Isoflurane gaseous anesthetic and later on maintained on the same at the constant infusion rate (Plate 2 A). After the rat was in deep stage of anesthesia, the abdominal cavity was shaved and examined (Plate 2B). The changes in the liver were recorded.

3.12 Haematology and serum biochemical evaluation

At the end of the experiment i.e. after 10 weeks, blood was collected from the posterior vena cava of individual rats after sacrificing. Approximately 1ml blood from individual rat was collected in the Potassium-EDTA vials for the haematological studiesviz. Total and Differential Leucocyte count, Haemoglobin, PCV and Total erythrocyte count by using automatic haematology analyzer (Mindray BC-2800).

Approximately 2-3 ml of the blood from each rat was collected in a clean, dry glass vial. The blood sample was allowed to stand undisturbed at one place in order to harvest serum. The collected serum was placed at -20ºC until further estimation. The serum sample from each animal was subjected to the estimation of enzyme activities which included Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and Alkaline Phosphatase (ALP). In addition, Total Creatinine (CRT), Total protein (TP), Globulin (GLB) and Albumin (ALB) levels were estimated in the serum samples. All the biochemical estimations were carried out by using commercially available kits (AGAPEE Diagnostic Ltd, India) and Automatic Biochemical Analyzer (AGAPEE MISPA-NEO, India).

3.13 Gross pathological examination

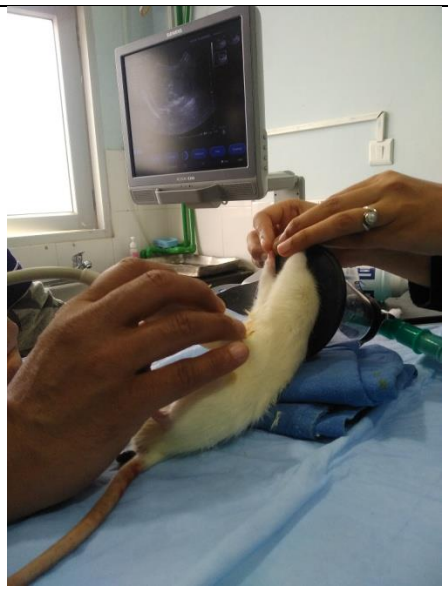
The sacrificed rats were subjected to detail gross pathological examination and pathological changes in various organs were recorded systematically. The body weight of the rat was recorded prior to sacrifice. The liver weight of the same individual rat was

measured using an electronic balance and the relative liver weight was calculated. The gross lesions of the liver were classified and scored according to the lesions which are tabulated below:

Table 2: Scoring pattern of gross lesions

Intensity	Type of Lesions	Intensity classified	Intensity Score
Mild	Liver surface appearing smooth to slightly rough/granular. Often isolated nodule(s) and/ or multifocal paler or whitish areas involving <25% of the liver parenchyma. Occasional or no hepatic peliosis.	+	1
Moderate	Liver surface appearing rough, granular, nodular with slightly enhanced lobulation pattern. Hepatic peliosis not uncommon. Multifocal projecting nodules and/or paler or whitish foci involving >25% to <50% of the liver parenchyma	++	2
Severe	Lesions involving >50% of the liver parenchyma with multiple paler foci and multifocal to coalescing nodules usually projecting over the hepatic surface. Nodules over 3mm size frequently present in addition to severely enhanced lobular pattern often accompanied with peliosis hepatis.	+++	3

Plate 2: Induction and Ultrasonography of Rats

	
Fig A: Mask Induction by Isoflurane.	Fig B: Ultrasonography of Liver.

3.14 Histopathology

After sacrifice, small representative samples of approximately 5 mm thickness of liver tissues were collected in 10% neutral buffered formalin solution. The formalin solution was changed after 24 hours from the time of collection of tissue. The formalin fixed tissues were subjected to histopathological processing (Luna, 1968) after their thoroughly washing under running tap water for 8-10 hours followed by dehydration in ascending grades of alcohol, clearing in benzene and paraffin embedding. The blocks were sectioned at a thickness of around 3-4 µ using a microtome. The obtained sections were then stained by Haematoxylin and Eosin stain for histopathological examination.

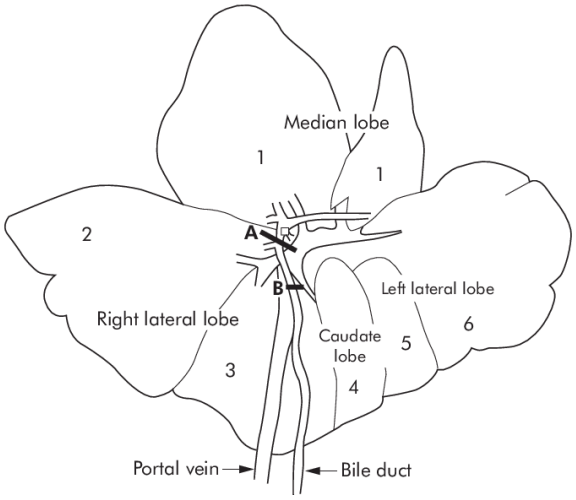


Fig 1: Diagrammatic representation of the rat liver

For the microscopic evaluation of the rat liver, the tissue sections were taken from the representative 3 lobes of each rat for the scoring reason. The lobes selected for the purpose were the left lateral, right medial and ventral caudal lobe as represented in the above figure. Then each liver tissue was examined and scoring was done according to the below mentioned criteria's in the Table 3. After scoring the lesions, the average score for whole liver of individual rat was done for each lesion and after which from each individual score, the mean lesion score of the group was calculated.

Table 3: Scoring pattern of microscopic lesions

	Type of microscopic evaluation and its description	Classified intensity	Intensity score
Proliferative lesions	Enhanced Lobular Pattern		
	1 Occasionally evident	Mild	1
	2 Lobular pattern fairly common	Moderate	2
	3 Diffuse or more than 50% surface area involvement	Severe	3
	Bile Duct proliferation		
	1 Mild hyperplasia at few places	Mild	1
	2 Moderately common	Moderate	2
	3 Diffuse often accompanied with cystic dilatation of bile ducts and numerous small bile ductules around fibrous tissue	Severe	3
	Cirrhosis		
	1 Scant fibrous tissue only around portal areas	Mild	1
	2 Fibrous tissue moderately increased but largely restricted to the portal areas	Moderate	2
	3 Fibrous tissue causes bridging between various portal areas and forming island of hepatocytes	Severe	3
	Dysplasia		
	1 At few places with hepatocytes showing megakaryocytes	Mild	1
	2 Fairly common dysplastic changes showing indistinct nodules of hepatocytes with moderately increased megakaryocytes, double nuclei, at times prominent nucleoli and slightly pleomorphic cells.	Moderate	2
	3 Severe dysplastic changes showing largely dictinct hepatic nodule showing characterized by increase number of megakaryocytes, hepatocytes with double nuclei, largely hyperchromatic and a marked variation in the size and shape of hepatocytes and their nuclei	Severe	3
	Lymphocytic infiltration		
	1 Occasional	Mild	1
	2 Fairly common at focal places in portal areas	Moderate	2
	3 Infiltration throughout the portal tracts	Severe	3
	Peliosis hepatis		
	1 Occasional small area	Mild	1
	2 At few places involving smaller area	Moderate	2
	3 Focal or multifocal involving relatively larger area	Severe	3

Toxic hepatic changes	Necrosis		
	1 Occasional involving individual hepatocytes	Mild	1
	2 Fairly common involving small cluster of hepatocytes	Moderate	2
	3 Usually involving bigger areas to the extent of 50% of the hepatic lobules at some places	Severe	3
	Vacuolar changes		
	1 Occasional	Mild	1
	2 Fairly common	Moderate	2
	3 More than 50% of hepatic lobule showing cells with hydropic degeneration and fatty change	Severe	3
	Clear cell foci		
	1 At few places	Mild	1
	2 Fairly common	Moderate	2
	3 Diffuse area of hepatic parenchyma	Severe	3
	Apoptotic cells		
	1 0-1 apoptotic cell/HPF	Mild	1
	2 1-2 apoptotic cell/HPF	Moderate	2
	3 >2 apoptotic cell/HPF	Severe	3
	Mitotic figure		
	1 0-1 mitotic figure/HPF	Mild	1
	2 1-2 mitotic figure/HPF	Moderate	2
	3 >2 mitotic figure/HPF	Severe	3

3.15 Masson's's trichrome stain (Luna 1968)

The trimmed and formalin fixed liver tissue section of 3-5µm were cleared in xylene and were hydrated by the descending grades of ethyl alcohol to water. After hydration, the liver tissues were left in the Bouin's solution for overnight in order to fix them. The fixed tissues were subjected to washing until the yellow colour of the tissue due to the Bouin's solution gets removed. After this the tissues were placed in Weigerts haematoxylin solution for 10 min. Then again washing was done for a period of 10 minutes in order to remove excess of haematoxylin. The tissues were then dipped in Biebrich scarlet-acid fuschin solution for duration of 2 minutes and then were subjected to washing. The liver tissues then subjected to treatment of 2.5% phosphomolybdic acid-phosphotungstic acid for 15 minutes and then were directly transferred to aniline blue solution for 5 minutes. The excess of the aniline solution was washed by running water for

5 minutes. After washing, the sections were treated for 1% Glacial acetic acid for time of 5 minutes. The liver sections were then dehydrated, cleared and mounted.

3.16 Immuno-histochemistry

The procedure for immunohistochemistry was done using Vector ImmPRESS^{TM}*Excel* staining kit for certain antibody like NF-κB and caspase 3. For this procedure, the paraffin embedded liver sections of 3-5 µm in thickness were collected on Poly-L-lysine coated slides. These sections were cleared by xylene and hydrated to water by descending of ethanol to water. The hydrated sections were then subjected to antigen retrieval step by 3 cycles of boiling in microwave and resting by submerging the slides in citrate buffer solution and then for the final rest the sections were left in citrate buffer solution for 20 minutes to cool. Then after washing the sections in PBS, they were incubated with BLOXALL blocking solution in a humidified chamber at room temperature for 10 minutes. Then after washing with PBS, they were incubated with 2.5% normal Horse serum in a humidified chamber at room temperature for duration of 20 minutes. After this the previous reagent is wiped and the liver tissues were then subjected to treatment with Primary antibody i.e. NF-κB and Caspase 3 (Biogenuix) in PBS with dilution of 1:100 and were then kept overnight at 4º C. Then the slides were allowed to come at room temperature. After washing with PBS, the liver tissues were treated by Secondary amplifier antibody (Goat anti-rabbit IgG) were incubated at room temperature in humidified chamber for 15 minutes. Then again after washing with PBS, the sections were treated by ImPRESS^{TM}*Excel* Reagent and then were again incubated at room temperature in humidified chamber for 30 minutes. Washing with PBS, the sections were treated by ImmPACT^{TM}DAB *EqV* after mixing the equal volume of Reagent I and II until the desired intensity of stain is not developed. Then after washing with PBS, the sections were then counterstained by 1:3 dilution of Mayer's haematoxylin for a time of 2-3 minutes and were washed under running water for 5 minutes. The stained sections were then dehydrated by two changes of absolute alcohol and then cleared with xylene and mounted with DPX.

3.17 Statistical analysis of data

Data generated from various experiments were suitably analysed. Data pertaining to body weight, relative liver weight and biochemical analysis subjected to ANOVA by the InStat statistical program. Gross lesion scoring and microscopic lesion scoring were

compared by Kruskal- Walis test (Chi square aproximation) ($P \leq 0.05$). For Immunohistochemical study, ImageJ software was used.

CHAPTER-4

RESULTS AND DISCUSSION

The present study was conducted in order to access the hepato-protective effect of the 70% ethanolic extract of the root of *Saussurea lappa* plant against N-Nitrosodiethylamine (DEN) induced chemical damage in male albino Wistar rats. Freshly harvested roots were collected from the Lahual district of Himachal Pradesh and were identified by HAB division of CSIR-IHBT and a specimen was submitted at the herbarium with a voucher number #PLP-15389. The root was then subjected to the preliminary phytochemical screening and the quantitative analysis of the various bioactive molecules mainly Costunolide and Dehydrocostus lactone. The present study was conducted on 46 male albino Wistar rats which were kept in the Laboratory Animal Facility of Dr. G.C.Negi College of Veterinary and Animal Sciences, Palampur. The rats were divided randomly into 6 groups. The group 1 (CX) was kept as controlwhile in the remaining groups, DEN was given ad-libitum in the drinking water at the concentration of 0.01% for a time period of 10 weeks. Group 3 (SX) was treated with silymarin @25mg/Kg of body weight and the root extract SLE was given at the dose rate of 100, 250, 500 mg/Kg body weight of rat. Before the sacrifice of the rats, an ultrasonography was conducted in order to estimate the extent of the hepatic damage caused by the chemical agent. During the entire course of the experiment, the animals were regularly checked for any clinical signs and their individual body weights were taken at weekly intervals for the purpose of daily SLE dosing according to their respective body weight. During sacrifice the blood without anticoagulant was collected from individual animal in the EDTA vial for the haematological examination while blood was also collected for the serological examination for certain biochemical parameters such as ALT, AST, Total proteinetc. The various other parameters like liver weight, gross and histopathology examination including immunohistochemical studies were carried out at the termination of the experiment.

4.1 Extract recovery

The freshly collected roots were weighed and subjected to oven drying after which the roots were ground to fine powder. Before extraction the powder was weighed in order to determine the per cent recovery of the extract. In the present study, the percentage recovery for *Saussurea lappa* turned out to be around 7.5% of the weight of raw plant

material used. Ahmed et al. (2016) also showed that the oven dried ethanolic extract of the plant root showed highest extract recovery when compared with the other extract and methods in order to recover the various phytochemical compounds. The studies with involvement of 20-40% of water along with ethanol also showed increased extraction of the bioactive compounds from the root (Chan et al. 2009 and Katsube et al. 2009).

4.2 Phytochemical evaluation

The dried root of the collected *Saussurea lappa* was subjected to the phytochemical screening and quantification for its active components especially for Costunolide and Dehydrocostus lactone. The UPLC chromatogram shows the matching of the peaks for the standard Costunolide and Dehydrocostus lactone (Fig 2) against the peak in the given sample of the plant (Fig 3). Hence, it was concluded that the two active ingredients were present in the collected plant. Furthermore, the quantitative estimation of the given two compounds was found to be 13.381µg/ml for Costunolide and 9.267µg/ml for Dehydrocostus lactone.

The secondary metabolites present in plant roots have shown various segment of bioactive compounds present in plant which were affected by various factors like ecotype, chemotype and environmental factors like temperature, irradiance etc. (Ren et al. 2007; Zahara et al. 2014; Qi et al. 2020). The major constituents of the root in the western Himalayan region in India were mainly Costunolide and Dehydrocostus lactone (Madhuri et al. 2012). The result of the UPLC of the plant extract used in the study also showed the presence of these two bioactive molecules. The level of terpenoids present in the plant root are regulated by various factors like temperature, amount of photoperiod, pedoclimatic condition and the time of harvest of plant even if the plants are harvested from same farmer (Marotti et al. 1994 and Tomassini et al. 2016). According to the study of Kumar (2020), the amount of Sesquiterpene lactones follows a seasonal pattern and the accumulation of these compounds depends on other factors like biotic and abiotic factors.

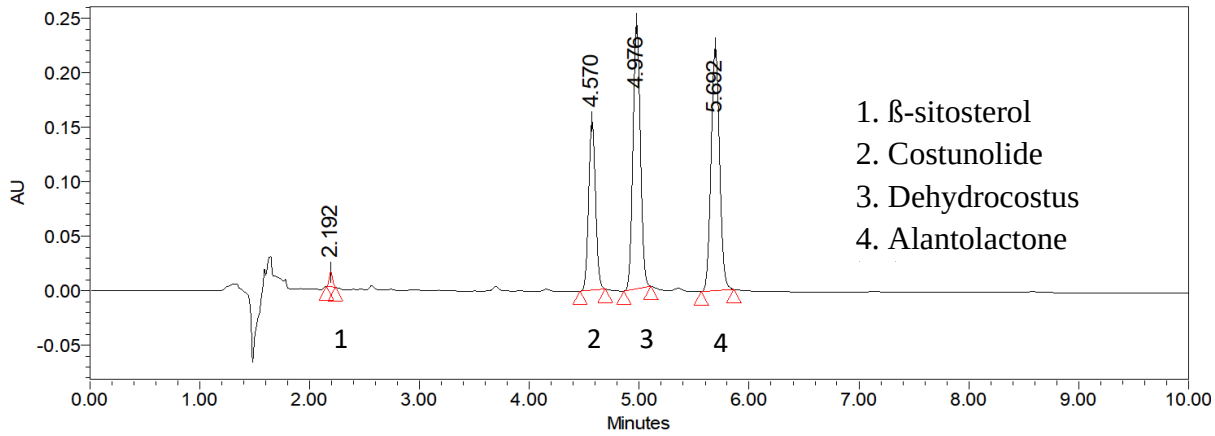


Fig 2: UPLC chromogram of standard Costunolide and Dehydrocostus lactone

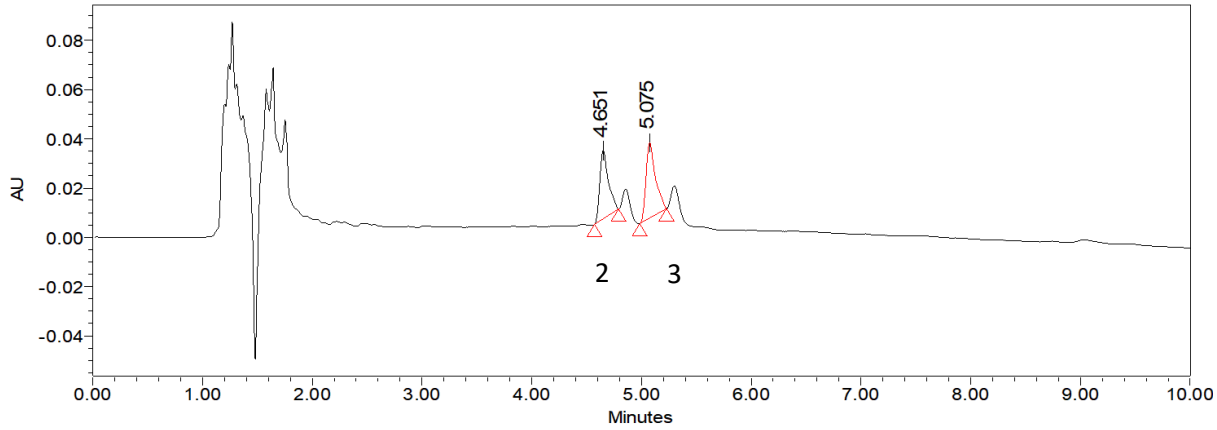


Fig 3: UPLC chromogram of the *Saussurea lappa*

4.3 Clinical signs and mortality

The control group i.e. CX animals did not show any clinical sign and were normal in feed and water intake. The animals in DX group showed a decrease in appetite since 6th week of experiment as they were starting to leave the feed material in the litter after eating just a few bites. In addition, a reduction in water intake was also noticed in DX group. The hair coat appeared slightly rough during the end stage of the experimental trial. The litter was continuously found to have a thin layer of fallen hairs since 8th week of the experiment. The decrease in feed and water intake was, however, comparatively less in rest of the groups than in the DX group kept only on the DEN. These clinical signs were also shown by the animals during the experiment of Patial et al. (2015) and Il'nitskaya (2016), however no such signs were observed in any other groups. These signs can be attributed to the

damage to liver causing disturbance in digestion. No mortality of rats was recorded in any group throughout the experiment.

4.4 Ultrasonographic examination

The ultrasonography of the liver in case of control group (CX) rats showed the normal hepatic parenchyma having a homogenous echotexture and uniformity in distribution. The hepatic vasculature also showed normal size and distribution. The margins and the capsule of the liver lobes also appeared normal (Plate 3 A & B). However the ultrasound of the liver in the group treated with DEN alone (DX) showed significant enlargement of the liver with a thickened hepatic capsule and irregular edges (Plate 3 C). The echotexture of the hepatic parenchyma also revealed a marked increase as compared to the control group. The animals in the DX group also showed the presence of large hyperechoic, well circumscribed lesions along with presence of smaller hypoechoic vacuolationsin the hepatic parenchyma (Plate 3D&F). The thickness of the portal vessels showed increase in thickness along with significant fat deposition (Plate 3E). While on the other hand the ultrasonic examination of the liver in the group treated with silymarin along with DEN (SX) showed a relative normal liver size as compared to group DX with moderate number of hyperechoic lesions present on the liver surface. The portal vessel appeared to be normal with fewer amounts of fat deposition and also revealed less systemic hypertension. The liver parenchyma also showed less number of distinct multiple hypoechoic areas with clear margins (Plate 4 A & B).

The ameliorative groups treated SLE showed improved results when compared to the DX group. The intensity of the lesions showed a decline with increase in the dose rate of the extract. The group treated with 100 mg/kg of the extract (SA) revealed thickened edges and capsule of the liver with the presence of distinct hyperechoic well circumscribed areas and increased echogenicity of the liver (Plate 4 C & D). The group treated with 250 mg/kg of extract (SB) showed mixed echogenicity of the hepatic parenchyma having a non-uniform echotexture along with the presence of hyperechoic and hypoechoic lesions (Plate 5 A & B). However, the group which was treated with the highest dose of the extract (SC) showed the best result for the entire ameliorative groups as the hepatic parenchyma appeared to be normal with much fewer occurrences of hyperechoic lesions. The intensity of hypoechoic lesions also decreased when compared to the previous extract treated groups (Plate 5 C & D).

Ultrasonography is a cost effective technique which can be used in gauging the damage to the liver with the ease of being non-invasive in nature and also duplicable (Akshatha, 2016). In the present study, the CX group showed hepatic parenchyma having homogenous echotexture which were also reported by lessa et al. (2010). DX group showed presence of hepatomegaly along with thickened hepatic capsule and irregular edges. The liver also showed increased echotexture along with large well circumscribed hyperechoic lesions and small hypoechoic lesions in the hepatic parenchyma. The inhomogeneous echogenicity of the parenchyma can be due to the various changes in liver like fibrosis, necrosis and increased vascular supply which were later confirmed by histopathological examination while the altered echotexture of the tumorous nodule can be due to the necrosis of the hepatocytes in the nodule caused by the chemical (Song et al. 2013, Akshatha 2016 and Jeevan et al. 2020).

4.5 Haematological parameters.

The total leucocyte count (TLC) in group DX was significantly higher ($P \leq 0.05$) in comparison to the control groups CX and SX. Among the ameliorative groups treated with *Saussurea lappa* plant extract (SLE), the increase in TLC was lower in all the combination groups when the corresponding values were compared with that of Group DX but the difference was significantly lower ($P \leq 0.05$) in group SB in comparision to DX. The increase in the TLC in group DX was found to be due to a significant increase ($P \leq 0.05$) in the number of lymphocytes, monocytes, and granulocytes in comparison to the control groups CX & SX. The increase in the number of lymphocytes, monocytes and granulocytes was however significantly lower in the groups given *Saussurea lappa* root extract treatment over 250 mg/kg i.e. Groups SB and SC (Table 4 & Fig. 4). The finding of the present study was also shown in the trials of Gnanaraja & Prakash (2014) and Singh et al. (2018) which is due to pathological condition of the liver.

Plate 3: Ultrasonographic images of rat liver

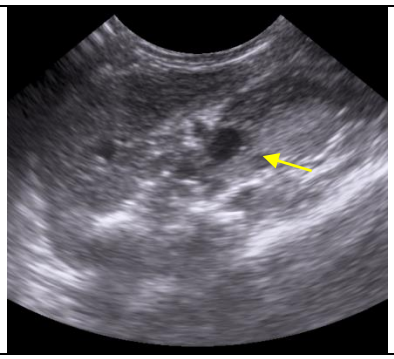
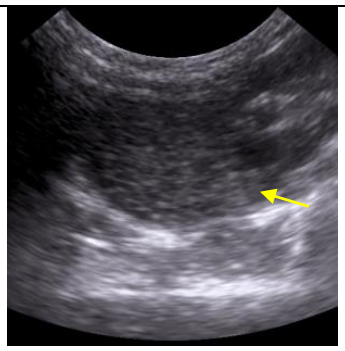
	

Fig A: Ultrasonographic examination of control group revealed normal hepatic vasculature (yellow arrow)	Fig B: Ultrasonographic examination of control group showing normal hepatic parenchyma having homogenous echotexture (yellow arrow)

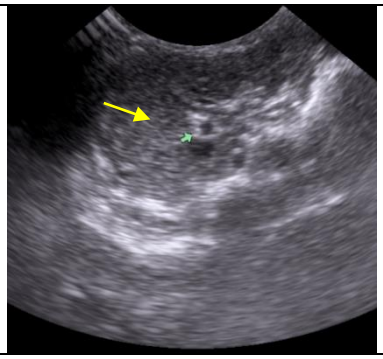
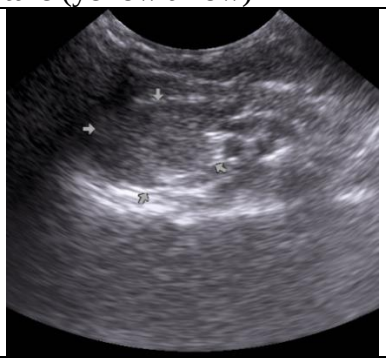

	

Fig C: Ultrasonographic examination of DEN only group showed increased echotexture of parenchyma (yellow arrow) and thickening of portal vein (small arrow).	Fig D: Ultrasonographic examination of DEN only group also showed the presence of well demarcated hyperechoic foci in liver parenchyma (small arrows).

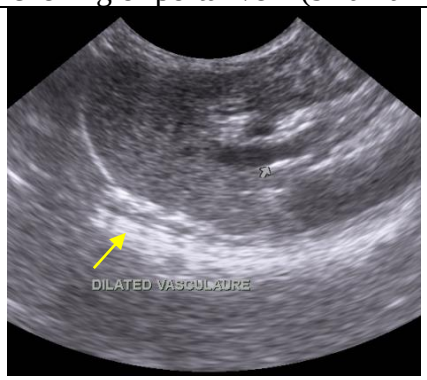
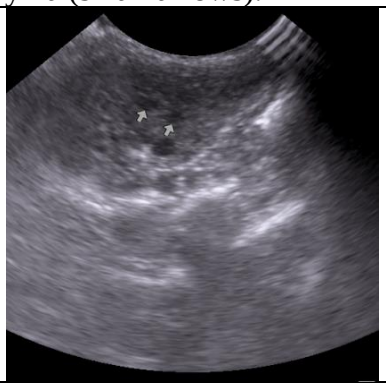

	

Fig E: Ultrasonographic examination of DEN only group revealed thickened hepatic capsule (yellow arrow) and dilatation of portal vein (small arrow).	Fig F: Ultrasonographic examination of DEN only group showed presence of vacuolation which were hypoechoic than remaining hepatic parenchyma (small arrows).

Plate 4:Ultrasonographic images of rat liver

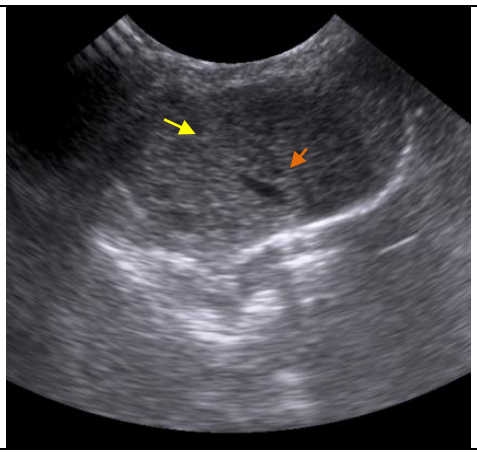
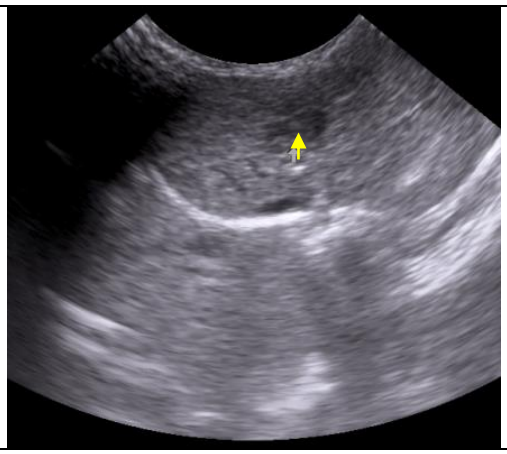
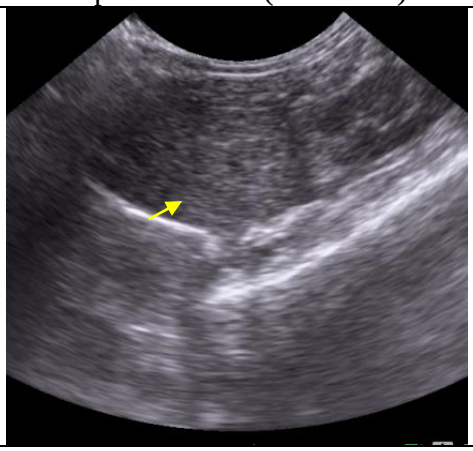
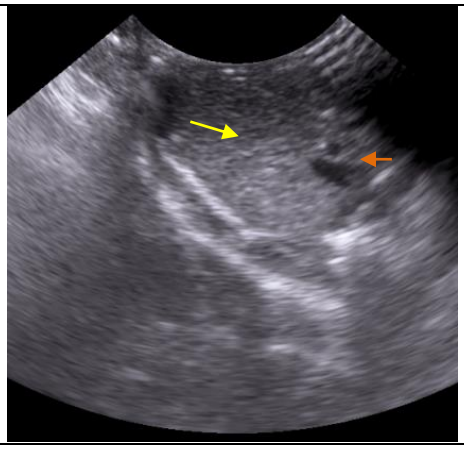
<table>
<tr><td>  </td><td>  </td></tr>
<tr><td>Fig A: Ultrasonographic examination of group treated with silymarin showed slight increase in echogenicity of parenchyma (yellow arrow) with dilated portal vessel (red arrow).</td><td>Fig B: Ultrasonographic examination of group treated with silymarin also revealed distinct hypoechoic lesion (yellow arrow)</td></tr>
<tr><td>  </td><td>  </td></tr>
<tr><td>Fig C: Ultrasonographic examination of group treated with 100 mg/kg of extract showed thickened liver margins (yellow arrow)</td><td>Fig D: Ultrasonographic examination ofgroup treated with 100 mg/kg of extract also showed increased echogenicity (yellow arrow) along with dilated blood vessels (red arrow)</td></tr>
</table>

Plate 5: Ultrasonographic images of rat liver

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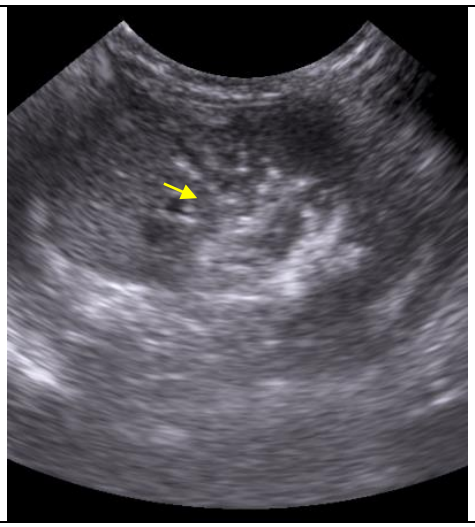
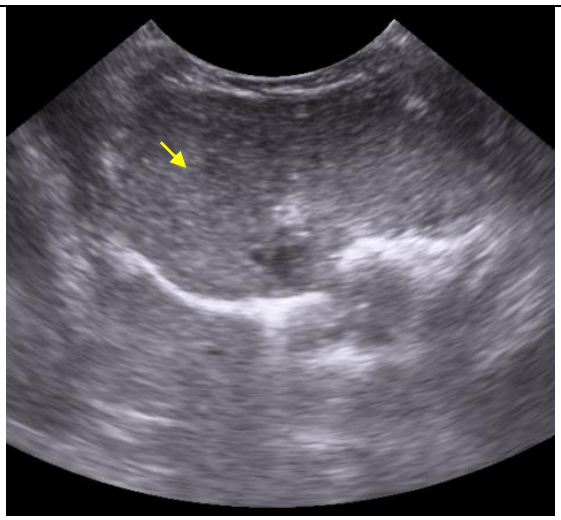
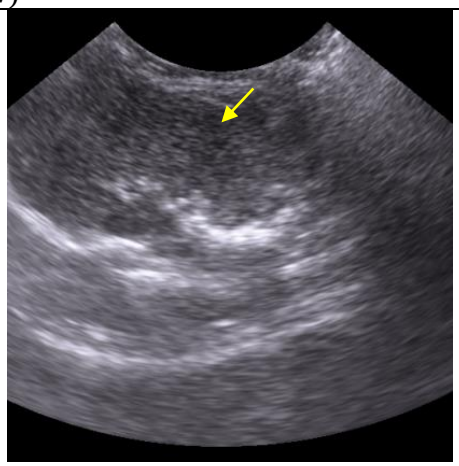
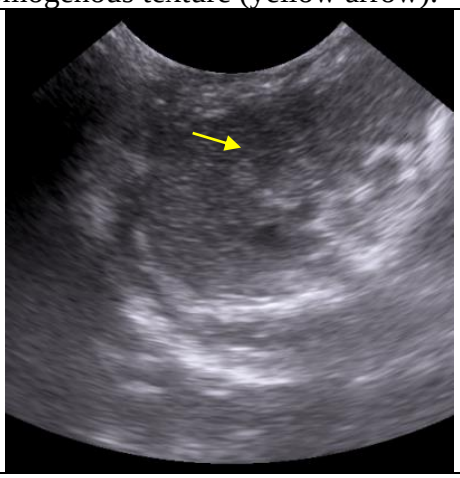
<tr><td>  </td><td>  </td></tr>
<tr><td>Fig A: Ultrasonographic examination of group treated with 250 mg/kg of extract showed hypoechoic lesions in liver (yellow arrow)</td><td>Fig B: Ultrasonographic examination of group treated with 100 mg/kg of extract showed liver parenchyma showing inhomogenous texture (yellow arrow).</td></tr>
<tr><td>  </td><td>  </td></tr>
<tr><td>Fig C: Ultrasonographic examination of group treated with 500 mg/kg of extract showed increased echogenicity of liver parenchyma but much less severe than only DEN treated group
(yellow arrow)</td><td>Fig D: Ultrasonographic examination of group treated with 100 mg/kg of extract also showed decline in hypoechoic areas in hepatic parenchyma (yellow arrow)</td></tr>
</table>

Table 4: Haematological parameters of rats after experiment

Haematological parameters	Groups					
	CX	**DX**	**SX**	**SA**	**SB**	**SC**
Total Leucocyte Count ($x10^3$ /µL)	11.15±0.98^{a}	18.18±2.86^{bc}	10.55±1.33^{a}	12.95±1.61^{ab}	11.60±1.61^{a}	12.97±1.24^{ab}
Lymphocyte ($x10^3$ /µL)	7.57±0.65^{a}	11.98±1.21^{bc}	7.57±1.22^{a}	8.42±1.17^{ab}	7.78±1.10^{a}	9.18±0.99^{ab}
Monocyte ($x10^3$ /µL)	0.37±0.33^{a}	1.42±0.57^{b}	0.45±0.08^{a}	0.60±0.07^{ab}	0.40±0.09^{a}	0.47±0.06^{a}
Granulocyte ($x10^3$ /µL)	3.22±0.34^{a}	6.58±1.89^{b}	3.15±0.54^{a}	3.93±0.50^{ab}	2.93±0.35^{a}	3.32±0.28^{a}
Erythrocyte ($x10^6$ /µL)	8.57±0.23^{a}	10.30±0.66^{b}	10.29±0.53^{b}	10.24±0.34^{b}	9.45±0.35^{a}	9.34±0.29^{a}
Hb (g/dL)	15.63±0.57 ^{a}	18.58±1.38^{a}	18.77±0.95^{a}	18.55±0.70^{a}	18.07±0.68^{a}	17.38±0.56^{a}
PCV (%)	54.12±1.91^{a}	64.18±4.78^{a}	64.38±3.40^{a}	65.10±2.53^{a}	62.42±2.14^{a}	59.90±2.19^{a}
MCV(fl)	62.43±0.46^{a}	62.55±0.83^{a}	62.60±0.75^{a}	63.57±0.68^{a}	63.50±0.44^{a}	64.12±0.49^{a}
MCH(pg)	17.97±0.13^{a}	18.05±0.23^{a}	18.22±0.24^{a}	18.05±0.20^{a}	18.28±0.23^{a}	18.55±0.08^{a}
MCHC(g/dl)	28.85±0.09^{a}	28.90±0.07^{a}	29.10±0.10^{a}	28.45±0.19^{a}	28.92±0.21^{a}	29.00±0.17^{a}
Platelet count ($x10^3$ /µL)	1186.67±70.05^{a}	932.83±133.46^{a}	1148.67±133.07^{a}	1034.00±131.02^{a}	1112.33±162.82^{a}	1315.83±98.64^{a}

*Data represent Mean ± S.E. (n=6). **CX**: Control group i.e. No DEN & No plant extract; **DX**: DEN @ 0.01% in drinking water only; **SX**: DEN + Silymarin @ 25mg/Kg of Body weight; **SA**: DEN + *Saussurea lappa* extract @ 100 mg/Kg of Body weight; **SB**: DEN + *Saussurea lappa* extract @ 250 mg/Kg of Body weight; **SC**: DEN + *Saussurea lappa* extract @ 500 mg/Kg of Body weight. Different superscripts within same row are significantly different at P≤0.05%.

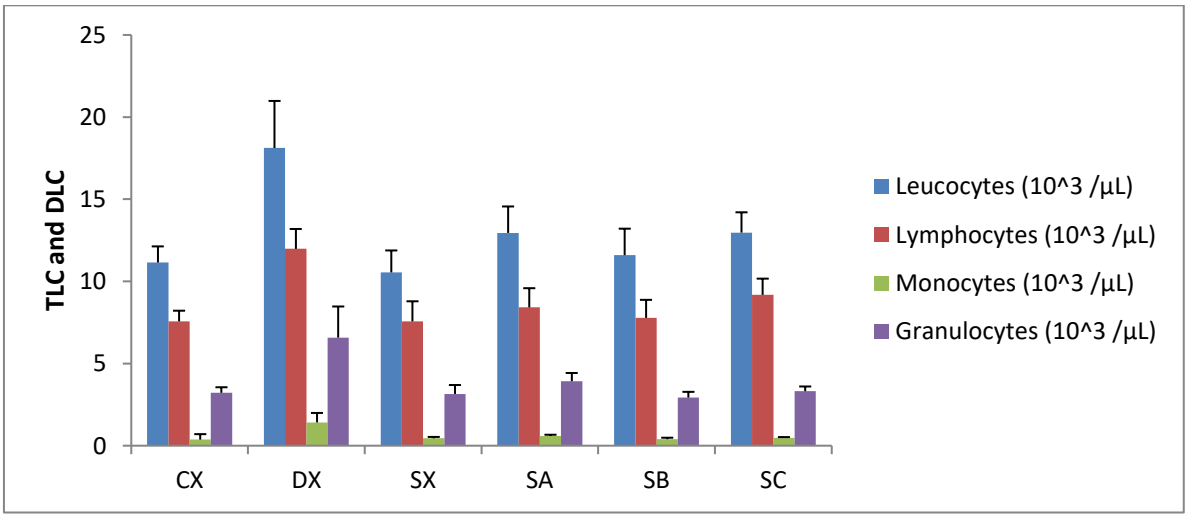


Fig 4: Effect of 70% ethanolic extract of *Saussurea lappa* on total leucocyte count of rats

The addition of *Saussurea lappa* plant extract did not seem to influence the erythrocyte count in the treatment groups kept on DEN even though the total erythrocyte count showed a significant increase ($P \leq 0.05$) in DX groupwhen the corresponding values were compared to those of combination Groups SB and SC. The haemoglobin concentration, PCV, platelet count and the values of erythrocytic indices did not differ significantly when their mean values were compared between different groups (Table 4& Fig. 5, 6, 7). The increase in the packed cell volume (PCV) and haemoglobin concentration and the other results were in accordance to the study of Amin et al. (2017).

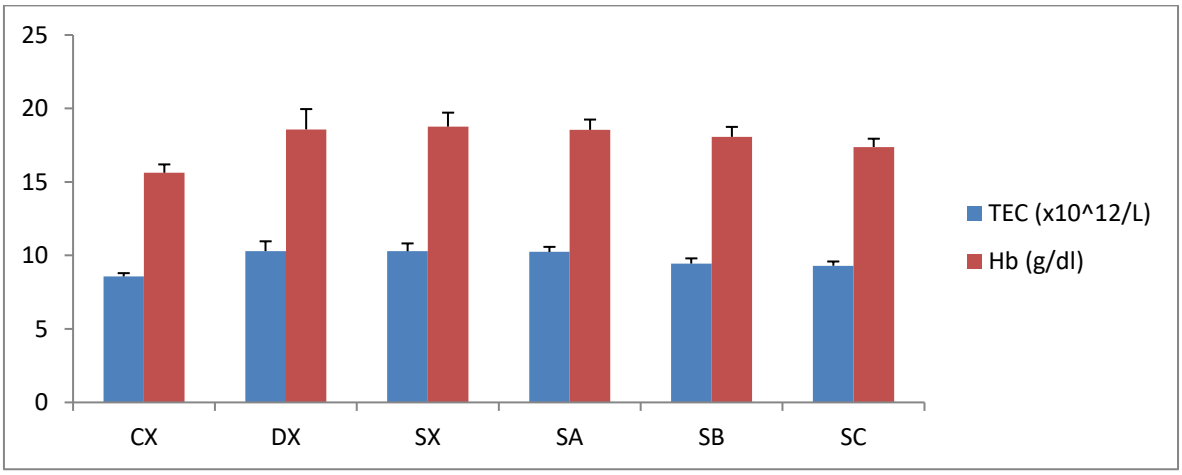


Fig 5: Effect of 70% ethanolic extract of *Saussurea lappa* on total erythrocyte count (TEC) and haemoglobin concentration (Hb) of rats

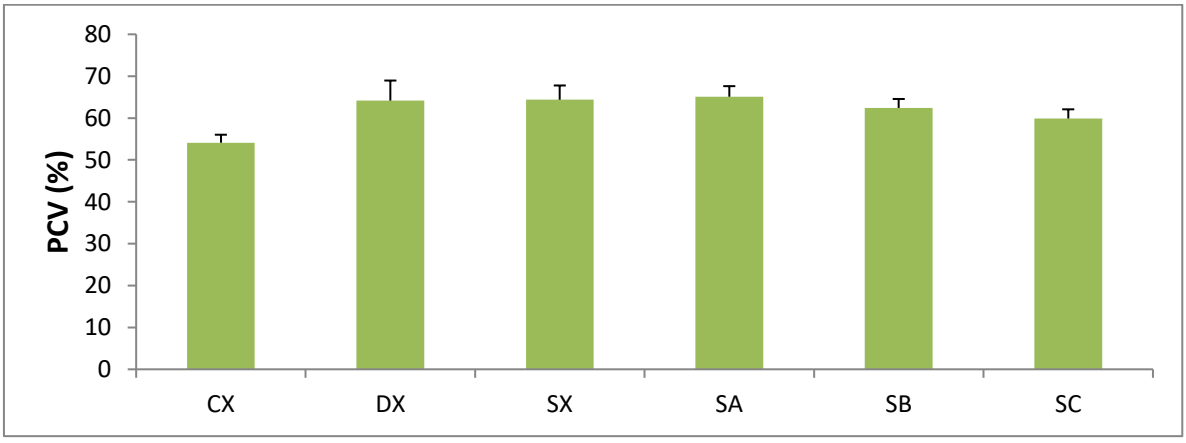


Fig 6: Effect of 70% ethanolic extract of *Saussurea lappa* on blood packed cell volume (PCV) of rats

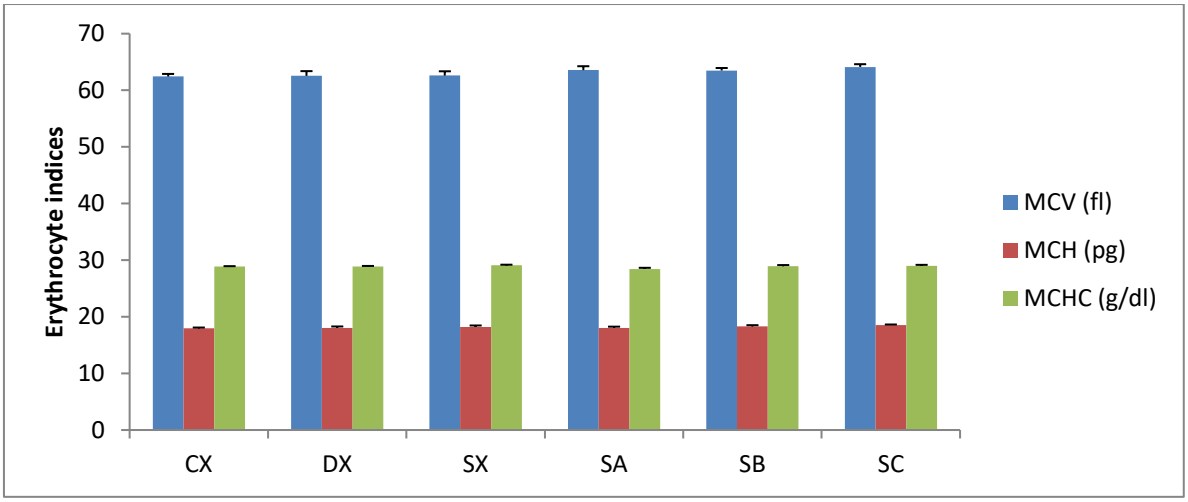


Fig 7: Effect of 70% ethanolic extract of *Saussurea lappa* on erythrocyte indices of rats

4.7 Serum biochemical evaluation

Alanine aminotransferase (ALT):

The serum ALT activity in the group kept on DEN and treated with plant root extract given at the dose rate of 500mg/kg b wt. (SC) or silymarin (SX) showed a significant decrease ($P \leq 0.05$) in comparison to that of the DX group (Table 5& Fig 8).

Table 5: Effects of plant extract on the biochemical parameters of the rats.

Groups	ALT(U/L)	AST(U/L)	ALP(U/L)	CRT(g/dl)	TP(g/dl)	ALB(g/dl)	GLB(g/dl)
CX	43.79±5.95^{a}	132.25±15.45^{a}	605.9±74.89^{a}	1.02±0.12^{a}	5.08±0.35^{a}	1.19±0.25^{a}	3.39±0.49^{a}
DX	76.53±10.81^{b}	156.53±10.40^{a}	1034.22±117.04^{b}	0.81±0.04^{ac}	6.52±0.62^{ab}	1.04±0.23^{a}	5.48±0.64^{ab}
SX	63.43±7.41^{a}	142.12±9.60^{a}	759.24±64.69^{ab}	0.88±0.04^{ab}	7.09±0.72^{ab}	1.07±0.17^{a}	6.03±0.71^{ab}
SA	77.71±3.71^{b}	150.17±14.77^{a}	915.75±120.52^{ab}	0.65±0.08^{bc}	7.99±0.51^{b}	0.92±0.22^{a}	7.07±0.44^{b}
SB	71.72±13.84^{ab}	147.95±13.60^{a}	678.05±46.44^{a}	0.71±0.03^{bc}	7.46±0.69^{b}	0.86±0.31^{a}	6.60±0.93^{b}
SC	50.74±5.52^{a}	137.23±12.28^{a}	616.96±81.64^{a}	0.59±0.02^{c}	6.90±0.21^{ab}	1.32±0.14^{a}	5.58±0.29^{ab}

*Data represent Mean ± S.E. (n=6). **CX**: Control group i.e. No DEN & No plant extract; **DX**: DEN @ 0.01% in drinking water only; **SX**: DEN + Silymarin @ 25mg/Kg of Body weight; **SA**: DEN + *Saussurea lappa* extract @ 100 mg/Kg of Body weight; **SB**: DEN + *Saussurea lappa* extract @ 250 mg/Kg of Body weight; **SC**: DEN + *Saussurea lappa* extract @ 500 mg/Kg of Body weight; **ALT**: Alanine aminotransferase; **AST**: Aspartate aminotransferase; **ALP**: Alkaline phosphatase; **CRT**: Creatinine; **TP**: Total protein; **ALB**: Albumin; **GLB**: Globulin. Different superscripts within same column are significantly different at P≤0.05%.

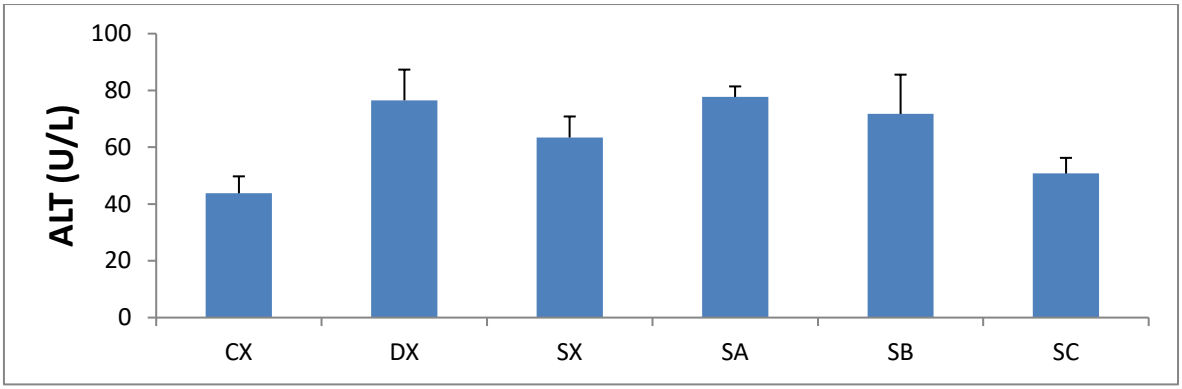


Fig 8: Effect of 70% ethanolic extract of *Saussurea lappa* on serum ALT activity of rats

Aspartate aminotransferase (AST):

The serum AST levels did not reveal any significant variation between different groups which were more or less comparable to the untreated control group (CX) (Table 5&Fig. 9).

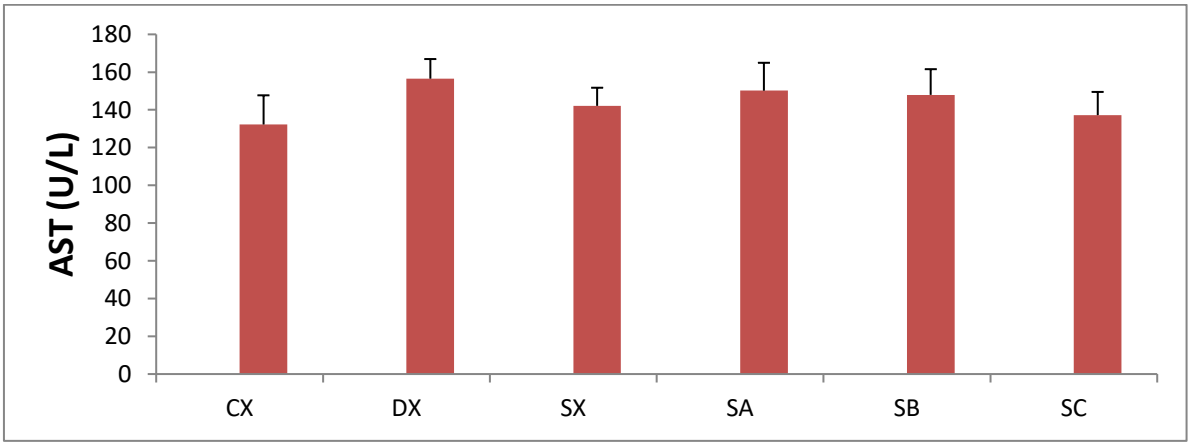


Fig 9: Effect of 70% ethanolic extract of *Saussurea lappa* on serum AST activity of rats

Alkaline phosphatase (ALP):

The serum ALP activity was significantly higher ($P \leq 0.05$) in the DX group in comparison to the combination groups kept on the root extract of the plant in Groups SB and SC. Though the increase in serum ALP activity in SX group animals treated with silymarin was much lower when compared with corresponding values in the DX group but the difference was not statistically significant (Table 5& Fig. 10).

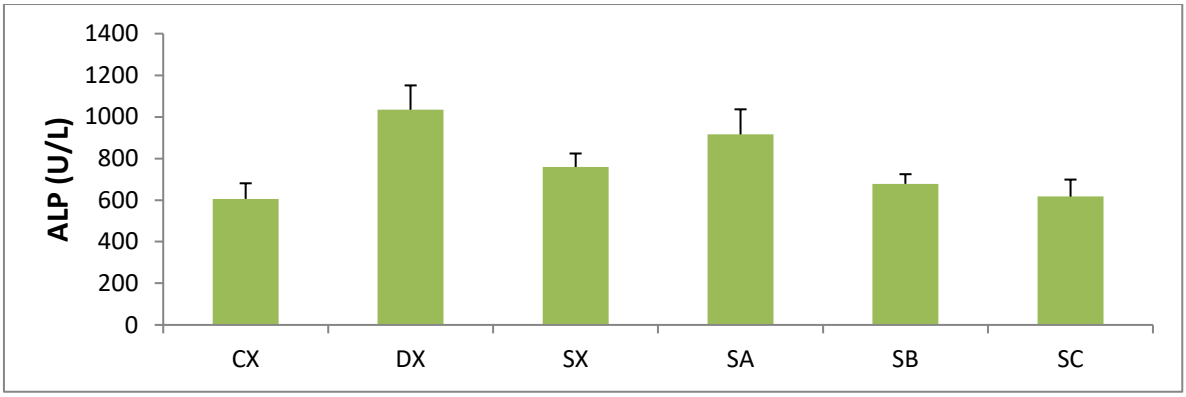


Fig 10: Effect of 70% ethanolic extract of *Saussurea lappa* on serum ALP activity of rats

Serum Creatinine:

The serum creatinine levels did not show any significant variation between different groups kept on DEN either alone or in combination with different treatments. The values were, however, slightly lower in the DX group when the corresponding values were compared with CX group (Table 5 &Fig. 11).

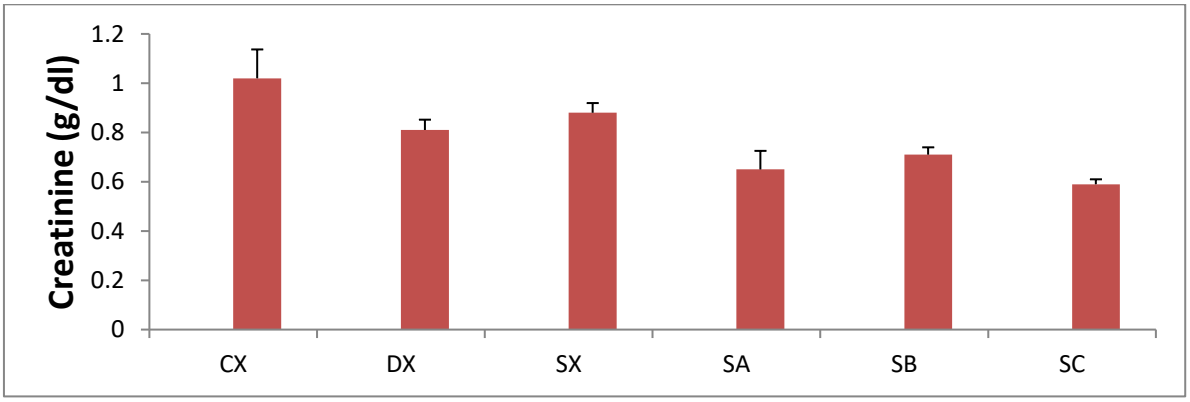


Fig 11: Effect of 70% ethanolic extract of *Saussurea lappa* on serum creatinine levels in different groups of rats

Total Serum protein:

In general, the total serum protein values were higher among all the DEN treated groups in comparison to the CX group. However, the values in the DEN treated groups kept on root plant extract of *Saussurea lappa* (Group SA & SC) were significantly higher ($P \leq 0.05$) as compared to the values in CX Group (Table 5 & Fig 12).

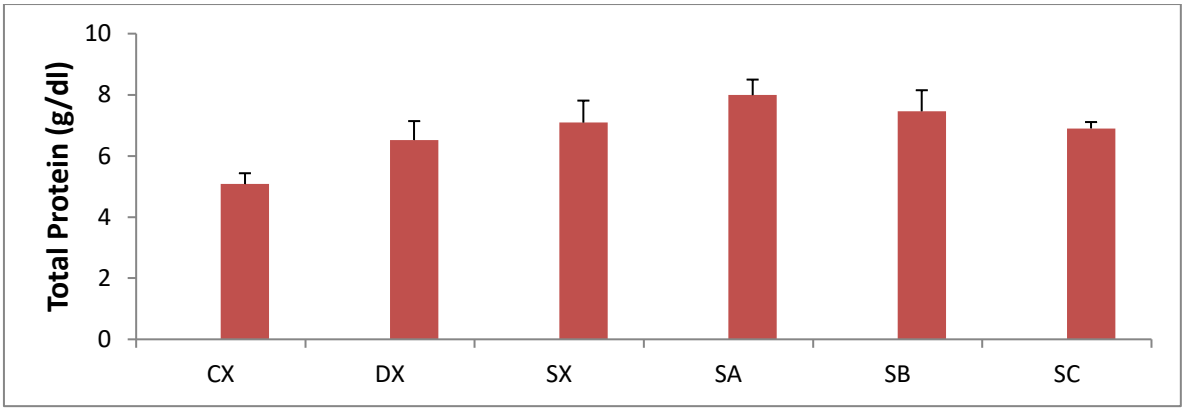


Fig 12: Effect of 70% ethanolic extract of *Saussurea lappa* on serum total protein levels in different groupsof rats.

Albumin:

The values of serum albumin did not show any significant variation between different groups though the values in general were lower in all the treatment groups except group SC in comparison to the CX group (Table 5 & Fig 13).

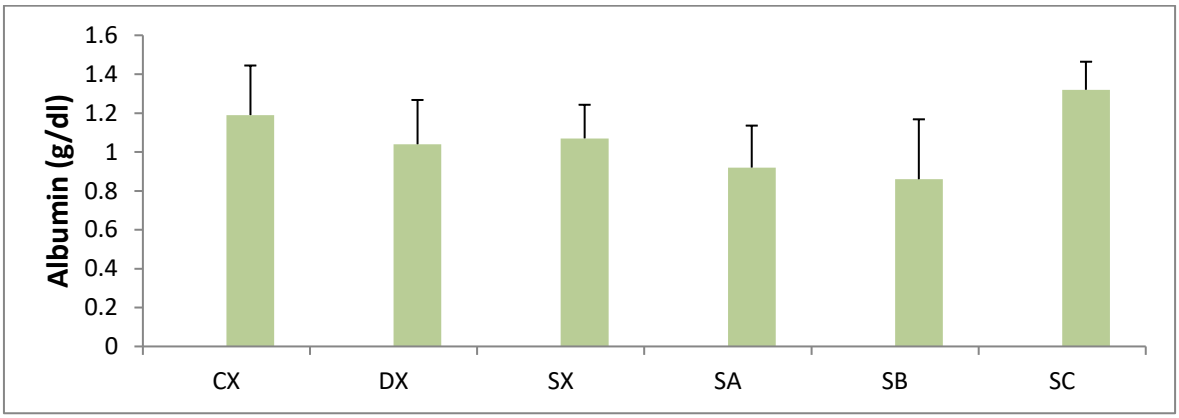


Fig 13: Effect of 70% ethanolic extract of *Saussurea lappa* on serum albumin level in different groups of rats

Globulin:

The Serum globulin values were, in general higher in the DEN treated groups in comparison to the control group (CX), the difference was however, statistically significant ($P \leq 0.05$) when the corresponding mean values in groups administered with the plant extract at 100 and 250 mg/kg b.w. (Groups SA & SB) were compared with control group CX (Table 5 &Fig. 15).

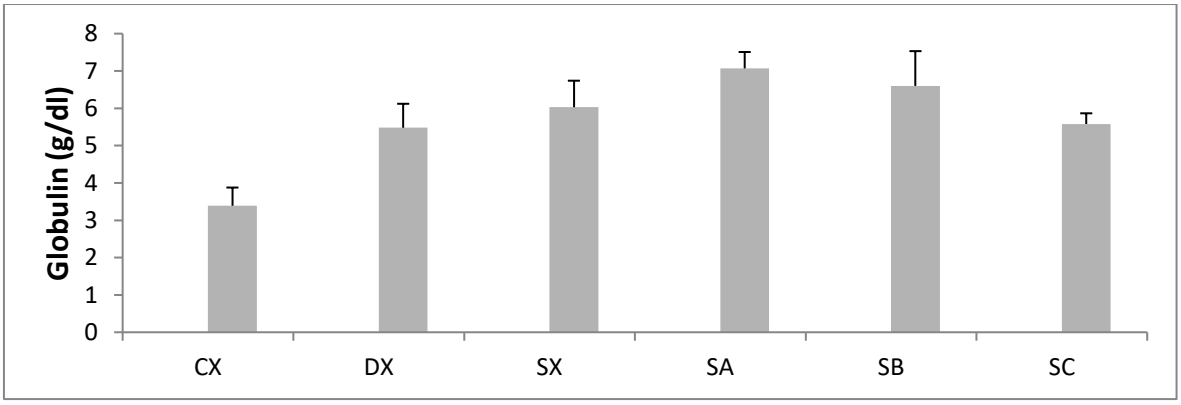


Fig 14: Effect of 70% ethanolic extract of *Saussurea lappa*on serum globulin level of rats

The serum biochemical parameters especially the liver marker enzymes like ALT, AST and ALP are well advised to be important parameters in order to access hepatic damage in rodents (Galle et al. 2014). The result of the present study showed significant increment in the level of serum ALT, AST and ALP for the DX group treated with DEN when compared to CX group which were in agreement with the work of Dhanasekaran et al. (2009), Jin et al. (2013) and Roy and Gadad (2016). The treatment with silymarin showed respectable reduction in these serum enzymes while the ethanolic extract of *Saussurea lappa* root also showed considerable reduction in serum biochemical enzymes showing the hepatoprotective nature of the extract. In the present study, the serum total protein and globulin level in the rats of group DX showed increase their levels which were in accordance with the study of Ding et al. (2017) and Singh et al. (2018) which can be attributed to increase in the physiological function of the liver (Murugan and Pari 2007) while the doses of extract and silymarin showed normalizing the serum protein level.

4.8 Liver weight

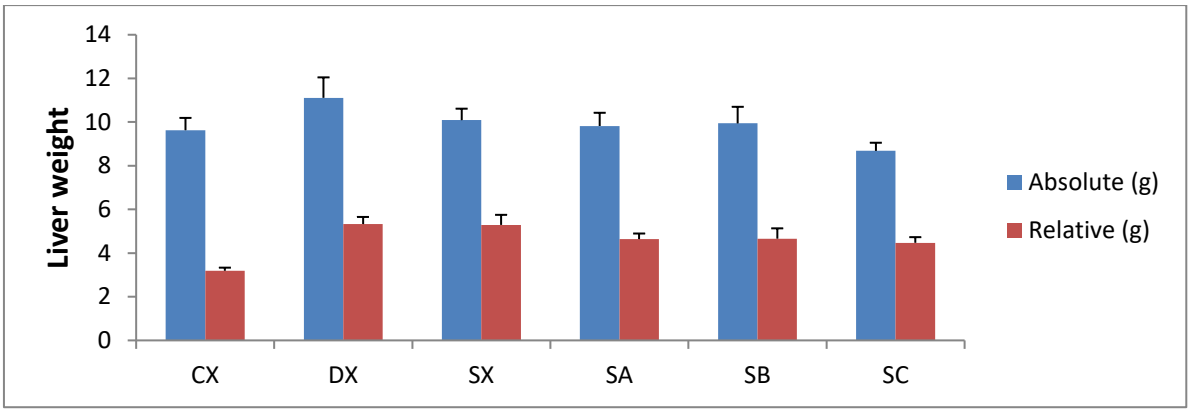
The liver weight of the rats was taken immediately before sacrifice. The absolute liver weight of the rats did not show any statistically significant variation between different groups though it was slightly higher in the group kept only on DEN (DX) in comparison to the controls or plant extract treated groups. In contrast, the relative liver weight of the DX group was significantly higher ($P \leq 0.05$) in comparison to the CX group. The relative liver weight in the plant extract treated groups was lower in comparison the group kept on DEN only (Group DX) (Table 6 &Fig. 15).

Table 6: Outcome of 70% ethanolicextract of *Saussurea lappa* on the liver weight of Rats

S.No	Liver weight (g)	Experimental groups					
		CX	DX	SX	SA	SB	SC
1	Absolute (g)	9.63±0.56[a]	11.11±0.94[a]	10.09±0.52[a]	9.82±0.60[a]	9.94±0.76[a]	8.68±0.37[a]
2	Relative (g)	3.19±0.14[a]	5.33±0.32[b]	5.29±0.46[b]	4.64±0.26[b]	4.66±0.47[b]	4.47±0.26[b]

*Data represent Mean ± S.E. (n=6). **CX**: Control group i.e. No DEN & No plant extract; **DX**: DEN @ 0.01% in drinking water only; **SX**: DEN + Silymarin @ 25mg/Kg of Body weight; **SA**: DEN + *Saussurea lappa* extract @ 100 mg/Kg of Body weight; **SB**: DEN + *Saussurea lappa* extract @ 250 mg/Kg of Body weight; **SC**: DEN + *Saussurea lappa* extract @ 500 mg/Kg of Body weight. Different superscripts within same row are significantly different at P≤0.05%.

Fig 15: Graphical representation of relative and absolute liver weight of rats



The relative liver weight can be attributed as an important factor in order to evaluate the pathological condition of the liver in an animal (Kumar et al. 2016). The results of the study were in accordance with the results of study from Ghosh et al. (2012) and Patial et al. (2015). The increase in the liver weight in DEN treated animals could be due to the chemical injury causing hypertrophy and hyperplasia of hepatocytes which further causes development of tumor nodules and also initiation of cirrhosis (Basak et al. 2001 and Roy and Gadad, 2016).

4.9 Gross Pathology

Group 1 (CX)

The control group did not show any significant gross pathological change in any of the body organs. The liver revealed a smooth shining surface on gross examination indicating normal hepatic parenchyma (Plate 6 A & B).

Group 2 (DX)

The gross examination after the sacrifice of the only DEN administered group showed variable type of lesions. The prominent lesion affecting the liver was its swollen parenchyma characterized by rounding of its edges and a pale discoloration. The liver showed a prominent lobular arrangement which on an average covered around 60-75% of the hepatic parenchyma. The nodules were white, raised, prominent, and well-demarcated and at few places showed a coalescing pattern (Plate 7 A). The nodules were sizing variably around <1 to >3 mm in diameter and were firm in consistency. The hepatic parenchyma invariably showed the presence of solitary to multifocal black coloured, well-demarcated, raised, projecting mass over the liver parenchyma (Plate 7 B). The overall gross examination of the liver in this group of rats exhibited a severe hepatic damage while the other organs grossly revealed no pathological changes. The gross lesion score recorded in the liver of individual animal of group 2 are presented in Table 7 and Fig. 16. These results were also reported by the work of Patial et al. (2015), Fathy et al. (2017), Akshatha et al. (2018) and Mo'men et al. (2020). The gross lesions were also evident on the ultrasonographic examination as the tumorous nodule being the hyperechoic area while the hyopechoic areas on ultrasound represent the areas of peliosis hepatis with the liver showing irregular margins both on ultrasound and gross examination (Akshatha 2016).

Group 3 (SX)

This group was kept on silymarin as a hepato-protectant against the damaging effects of the chemical DEN. The hepatic surface presented a slightly rough appearance with nodules usually ranging between 1 and 3 mm though it was less granular and nodular as compared to the DEN treated group (Plate 8 B). In two animals, however, the whitish coloured nodules were around 5 mm or more (Plate 8 A). In some animals, most of the liver parenchyma revealed a smooth surface though it was slightly swollen and granular with occasional whitish multifocal paler areas between 1 and 2 mm. The black-coloured foci viz. hepatic peliosis were evident in only two animals. Only one rat in this group presented a diffuse rough surface. The gross hepatic lesion score in this group showed a significant decrease in the severity of the gross lesions as compared to the DX group (Table 7& Fig.16).

Group 4 (SA)

This group received 70% ethanolic extract of *Saussurea lappa* at the dose rate of 100 mg/Kg of body weight along with DEN in the drinking water. On gross examination, the liver revealed multifocal paler areas over its rough surface with an enhanced lobular pattern. The overall lesion intensity was classified as moderate to severe with involvement of nearly 35-70% of the liver parenchyma (Plate 9 A). The occurrence of nodules was more frequent in this group as compared to the SX group. The nodules were multifocal in distribution and mostly present around the margins of the liver having a variable diameter ranging from <1 mm to 3 mm while occasionally the nodules tend to be bigger i.e. >3 mm in few animals. In some animals, the nodules were not as sharply demarcated as were evident in the Group 2 animals. The cystic blood-filled cavities were also seen in the majority of rats of this group (Plate 9 B). The gross hepatic lesion score was slightly lower when compared with DEN group 2 but it was significantly higher than that of the SX group (Table 7& Fig. 16).

Group 5 (SB)

This group was treated with 70% ethanolic extract of *Saussurea lappa* at the rate of 250 mg/Kg of bodyweight along with DEN in the drinking water. The intensity of gross lesions was classified as mild to moderate with apparently 20 to 50% surface area involved in this group. The liver of most of the animals appeared slightly swollen and congested. The majority of the liver surface was smooth in most of the animals. However, a slightly rough and granular surface with occasional nodules or multifocal paler areas around margins was evident in two animals. In one animal, however, multifocal areas >3 mm were observed on the ventral aspect of the left side lobe of the liver. The solitary area of hepatis peliosis was evident in only one animal in this group. The severity of the lesions was much less when it was compared with Group DX, SX, and SA (Plate 10 A& B) (Table 7 & Fig. 16).

Group 6 (SC)

The group was treated with 70% ethanolic extract of *Saussurea lappa* at the dose rate of 500 mg/Kg of bodyweight along with DEN in the drinking water. On gross examination, the hepatic parenchyma appeared largely smooth with a mild lobular appearance at places in the majority of animals. The mild degree of hepatomegaly was evident (Plate 11 B). The hepatic parenchyma showed whitish areas at focal places but the raised, well-demarcated, white-coloured nodules were uncommon. Most of the animals

indicated either isolated nodules or whitish foci with no apparent nodularity. The intensity of the gross lesions was mostly classified as mild with apparent 10-20% surface area involvement except in one animal where it was rated as severe with nearly 70% surface area involvement. The blood-filled cystic spaces (hepatic peliosis) were occasionally evident in this group (Plate 11 A). Overall examination of the liver tissue revealed that it was more or less comparable to the CX group with a decline in the severity of gross lesions in comparison to the other treated groups (Table 7 & Fig. 16).

Table 7: Mean gross lesion score

CX	DX	SX	SA	SB	SC
0.00 ± 0^{a}	2.71 ± 0.18^{bc}	1.57 ± 0.3^{ab}	2.00 ± 0.22^{b}	1.43 ± 0.3^{ab}	1.43 ± 0.3^{ab}

*Data represent Mean ± S.E. (n=6). **CX**: Control group i.e. No DEN & No plant extract; **DX**: DEN @ 0.01% in drinking water only; **SX**: DEN + Silymarin @ 25mg/Kg of Body weight; **SA**: DEN + *Saussurea lappa* extract @ 100 mg/Kg of Body weight; **SB**: DEN + *Saussurea lappa* extract @ 250 mg/Kg of Body weight; **SC**: DEN + *Saussurea lappa* extract @ 500 mg/Kg of Body weight. Different superscripts within same row are significantly different at P≤0.05%.

Fig 16: Graphical representation of gross lesion score

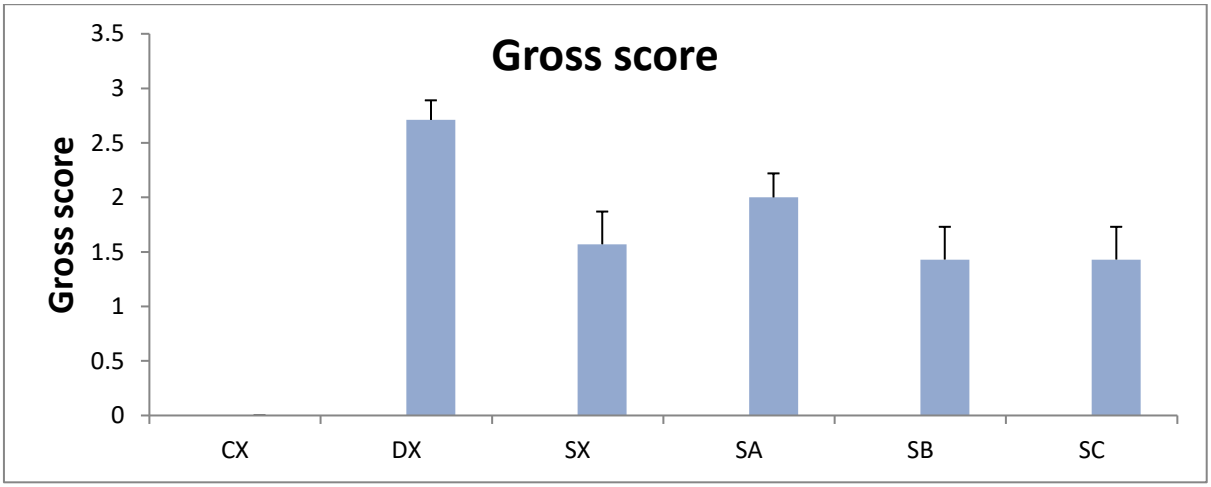


Plate 6: Gross pathological examination of rats (Group CX)

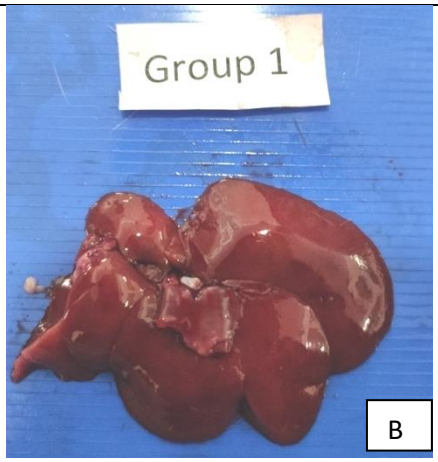
	
A: Normal surface of liver.	B: Normal surface of liver.

Plate 7: Gross pathological examination of rats (Group DX)

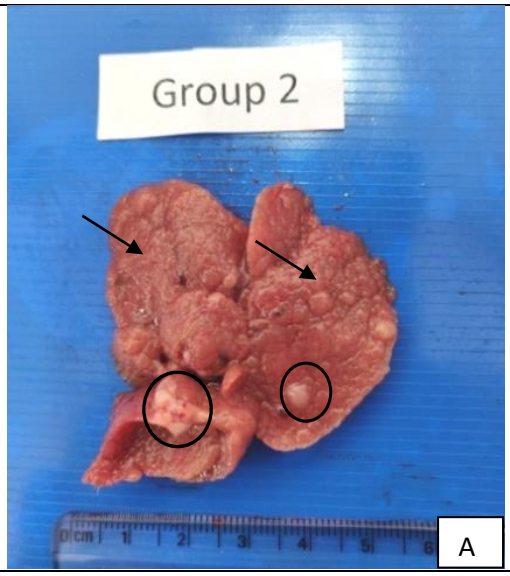
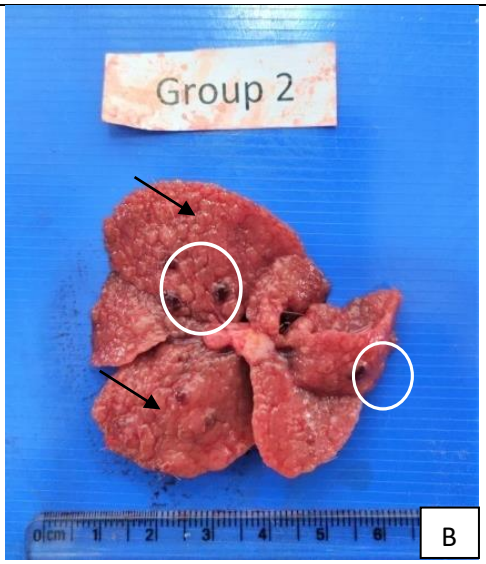
	
A: Rough hepatic parenchyma (Black arrow) along with raised white coloured nodules of variable size (black circle)	B: Enhanced lobulation of the liver with well demarcated nodules throughout the hepatic parenchyma (Black arrow) and multifocal areas of hepatis peliosis (white circle).

Plate 8: Gross pathological examination of rats (Group SX)

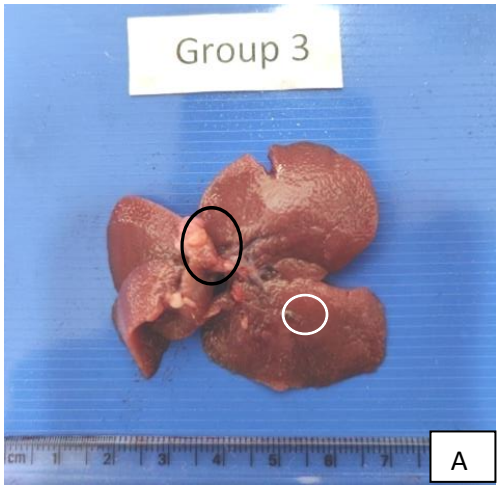
	
A: Enhanced lobular pattern with isolated white coloured nodules (black circle) and focal peliosis hepatis (white circle)	B: Liver parenchyma showing enhanced lobular pattern having slightly granular appearance (arrow).

Plate 9: Gross pathological examination of rats (Group SA)

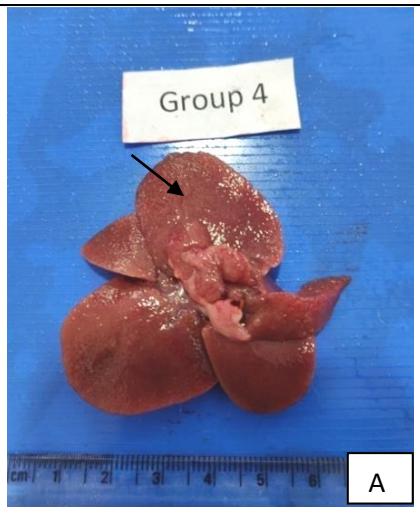
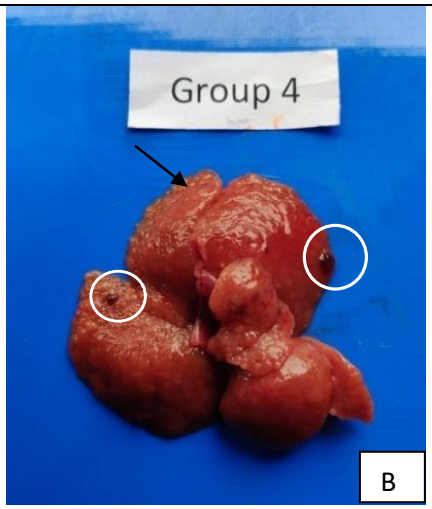
	
A: Mild rough hepatic parenchyma with enhanced lobular pattern and multifocal paler areas (arrow).	B: Liver parenchyma showing raised nodular appearance in nearly 50% of its parenchyma including the margins (Black arrow). Focal areas of peliosis hepatis are also visible (white circle)

Plate 10: Gross pathological examination of rats (Group SB)

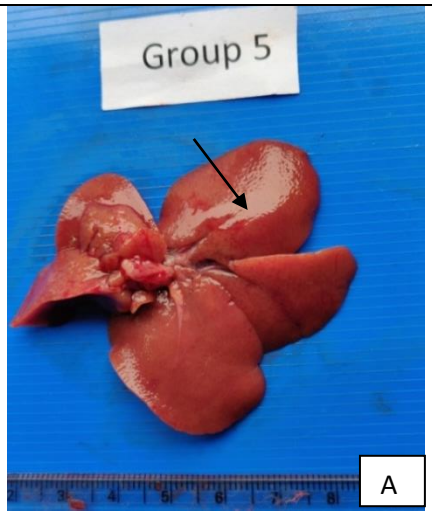
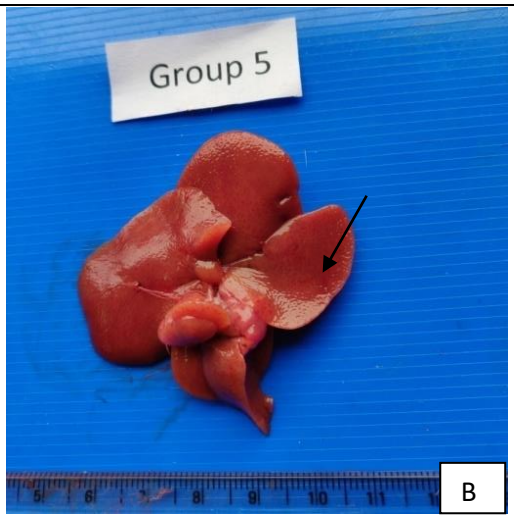
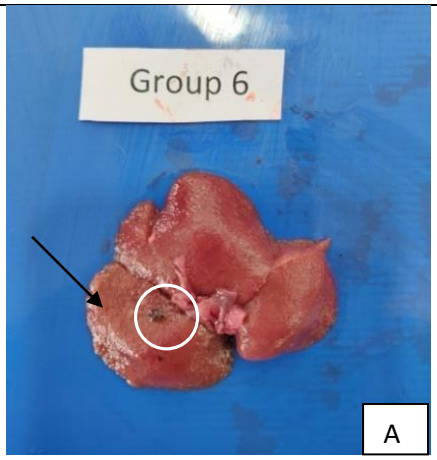
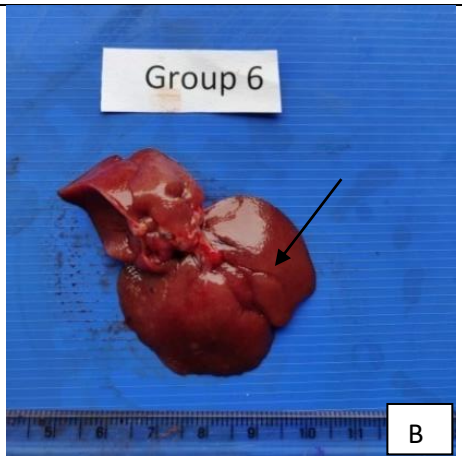
	
A: The liver parenchyma appearing largely smooth though focal areas of enhanced lobulation pattern with indistinct scattered paler foci are visible (arrow).	B: Mild granular appearance due to enhanced lobular pattern (arrow).

Plate 11: Gross pathological examination of rats (Group SC)

	
A: Solitary area of peliosis hepatis (white circle) along with small white nodules appearing especially towards the margin (arrow)	B: Mild lesion of granular parenchyma along with mild hepatomegaly (arrow).

Microscopic evaluation: -

Group 1 (CX)

The liver sections in this control group showed normal hepatic parenchyma with an intact hepatic cord arrangement. The portal areas appeared normal with no bile ducts proliferation. The normal hepatic cord arrangement was maintained throughout the parenchyma (Plate 12 A & B).

Group 2 (DX)

The predominant microscopic lesions included dysplastic changes characterized by a marked variation in the size and shape of hepatocytes appearing round, polyhedral, oval or triangular along with a marked variation in the size of hepatocyte nuclei with multiple nucleoli commonly present (Plate 13 A& B). The cells showed karyomegaly along with nuclear membrane appeared dense and hyperchromatic with margination of chromatin material and the nucleoli also stained dense and pyknotic. The degenerative changes were indicated by an increased eosinosphilic granularity in the cytoplasm of hepatocytes, varying degree of hydropic degeneration and lipid accumulation. Areas of coagulative necrosis were observed at focal places which either involved group of hepatocytes or were limited to a single hepatocyte. Some hepatocytes presented eosinophilic inclusion body like structures often surrounded by clear halo around them whilesome of thehepatocytes appeared either totally clear or with occasional remnants of eosinophilic granularity (Plate 13 C & D). At some places, however, boundaries of hepatocytes were intact and were sharply defined. Apoptotic cells with multiple apoptotic bodies were also evident at some places whilemitotic activity was mild but evident at places (Plate 13 E). Focal areas of haemorrhages with severe congestion were also evident in the hepatic parenchyma. Peliosis hepatis was characterized by focal areas of dilatation in the hepatic parenchyma which were filled by blood elements which resulted in the atrophy of the adjoining hepatocytes (Plate 13 E & 14 A).

The portal areas at the first instance revealed a marked increase in the number of bile ducts and fibrosis (Plate 14 B). The boundaries of the hepatic lobules were well defined due to moderately thick fibrous septa (Plate 14 C). Bridging portal to portal areas and fibrosis was accompanied by mononuclear cells and golden brown coloured pigment at certain places (Plate 14 D). At many places, the fibrous connective tissue appeared to

invade the hepatic parenchyma producing lobules of small islands of hepatocytes which were devoid of central vein and also exhibited mononuclear cells infiltration which were mainly lymphocytes. The hepatocytes in these pseudolobules appeared to be atrophied, shrunken or hyperchromatic with disruption in the normal hepatic cord arrangement (Plate 14 E).

The bile ducts were also evident at unusual location in the hepatic lobules. In addition, the bile ducts revealed massive cystic dilatations having centrally directing papillary projections along with eosinophilic debris in between the cavity which appeared to displace and flatten the bile duct epithelium by turning it into low cuboidal type. The epithelial lining of bile ducts revealed communication channels (Plate 14 E). The number of affected rats and their lesion score is given in Table 8 and 9. These results were parallel to the results of Kushida et al. (2011), Patial et al. (2015), Chang et al. (2016) and Ahmed et al. (2019). The liver showing these changes are due to the damage caused by the chemical on the function of hepatocytes causing increase in the level of oxidative metabolites which further cause DNA damage and thus resulting in the pre-neoplastic lesions in the liver (Da Costa, 2014).

Group 3 (SX)

The microscopic lesions in this group were characterized mostly by mild dysplastic changes as compared to the DEN treated group however, many hepatocytes in the individual lobules showed moderate intensity of degenerative changes characterized by increase in cytoplasmic granularity and moderate degree of vacuolar changes (Plate 15 A). The hyaline like bodies were occasionally observed. The size of the nuclei and hepatocyte showed variation in size. The nuclei stained hyperchromatic but these changes were significantly less pronounced than the group treated with DEN alone (Group DX) (Plate 15 B). The clear cells were also much less as compared to the previous group. Hyperplastic hepatocytes containing double nuclei were not uncommon. The lesions were accompanied with mild degree of apoptosis and mild mitotic activity. Multifocal areas of coagulative necrosis involving small group of hepatocytes were also evident. The lesions in Group SX were in general much less severe in intensity than in DX group (Table 10).

In one animal, however, a focal nodular area revealed severe lesion which was indistinguishable from Hepato-cellular Carcinoma. The hepatocytes showed loss of trabicularity along with marked pleomorphism, basophilic cytoplasm and variation in

nucleoli. At places, the hepatocytes showed glandular structure along with massive coagulative necrosis, karyorrhexis and occasional neutrophils along with sporadic presence of mitotic figures and apoptotic bodies (Plate 15 C& D). The varying degree of fibrous tissue was also present. At many places, however, apparently normal hepatic cord structure which was comparable to the control group. The hepatic cord arrangement structure in mid zonal to peripheral areas of hepatic lobules was intact. The cirrhotic changes were mild and the portal areas generally did not show much proliferation of the bile duct in comparison to the kept-on DEN alone (Group DX) even though at some places the number of bile ducts appeared increased (Plate 15 E). The margins of the hepatic lobules in most of the liver parenchyma were not sharply defined but, in some areas, the fibrous connective tissue didappear to wander into the hepatic parenchyma resulting in pseudolobulation. In some areas, the bile ducts were cystically dilated with eosinophilic mass in the lumen causing the lining epithelium to become low cuboidal type. At some places proliferated connective tissue in the portal areas was accompanied by mild to moderate infiltration of mononuclear cells (Plate 15 F). However, these changes were less frequent than in Group DX (Table 10). The number of affected rats and their lesion score is given in Table 8 and 9.The rats in this group showed decline in these microscopic changes which can be due to the decline in the oxidative damage caused by DEN to the hepatocytes due to the ability of silymarin in order to maintain the oxidant and antioxidant imbalance caused by the chemical (Pradeep et al. 2007).

Group 4 (SA)

The microscopic lesions in this group showed the areas of the hepatic parenchyma with dysplastic changes appeared slightly paler and were present at focal places (Plate 16 A). In suchplaces the hepatocytes showed variation in size of their nucleus, some hepatocytes were appearing as polyhedral cells with some representing karyorrhexis. At some places the nucleus also showed hyperchromasia. The hepatocytes in the areas appearing paler also showed eosinophilic granularity in their cytoplasm. Hepatocytes appearing as clear cells were also not uncommon (Plate 16 B & C). However, the dysplastic changes in this group were comparable to the DX group but the intensity of vacuolar changes, hepatocytic necrosis and clear cells were statistically ($P \leq 0.05$) comparable to the CX group (Table 10). The mitotic and apoptotic cell activity of the group was of mild to moderate intensity which showed a significant increase than the CX group.

The animals in this group showedenhanced lobular pattern on a moderate scale as compared to DX group as they showed thin fibrous connective tissue running from the portal areas which was later confirmed by the special staining of the hepatic tissue (Plate 16 D). The bile duct proliferation was usually mild to moderate as compared to the DX group though occasionally it also showed cystic dilatation along with eosinophilic material present in the cavity (Table 10) (Plate 16 E & F). At few places golden brown coloured pigment was also present along with moderate intensity of leucocytic infiltration. At places the portal areas showed moderate to severe congestion with sporadic areas of Peliosis hepatis. On an average, nearly 30-50% of the hepatic parenchyma in this group showed normal hepatic cord structure. The number of affected rats and their lesion score is given in Table 8 and 9.

Group 5 (SB)

The lesions in this group were much less severe in intensity as compared to the above-mentioned groups (Table 10). The liver of the rats consistently revealed about 50% of the intact hepatic parenchyma. The hepatic cord arrangement appeared distorted only at focal places by the areas of the dysplastic changes in the parenchyma (Plate 17 A & B). Dysplastic changes were characterized by paler areas with slightkaryomegaly along with cells showing invariable shape and size. The hepatocytes stained eosinophilic, revealedpyknotic and at few places showed prominent nuclei. The hepatocytes at many occasions revealed the presence of pseudonuclei. The hepatocytes also revealed areas of vascular changes along with the dysplastic areas in the parenchyma and at places the areas of clear cell with eosinophilic boundaries were also appreciated. But these changes were less severe than the lesions in group DX (Table 10) (Plate 17 C). On a few occasions, the hepatic cord arrangement was severely disturbed due to the necrosis of the hepatocytes either involving an individual hepatic cell or a group of them. The mitotic and apoptotic cell activity was increased than the DX group.

The hepatic parenchyma in the animals of this group was not differentiated into distinct lobules which were significantly less than group DX. The bile ducts largely revealednormal structures in the portal areas which were comparable to the control group animals although at a few places showed mild to moderate proliferation usually associated with a very thin fibrous connective tissue element (Plate 17 D). Cirrhotic changes were not as severe as compared to the DX group but were on a milder scale and can be compared to

the control group (Plate 17 E). Also, the lymphocytes infiltration of the portal areas also showed a marked decrease in comparison to the group kept only on DEN (group DX) and was statically comparable to the silymarin treated group SX (Table 10) (Plate 17 F). The number of affected rats and their lesion score is given in Table 8 and 9.

Group 6 (SC)

The group treated with the highest dose of the ethanolic extract showed by far the best result of the different doses used for the treatment of the chemical injury. The liver tissue in this group were characterized by normal hepatic parenchyma with intact cord arrangement of the hepatocytes though at places paler eosinophilic stained dysplastic areas were present at focal places in the parenchyma with not so sharply demarcated boundaries (Plate 18 A & B). The dysplastic nodules were focal and mild in nature characterized by slight pleomorphism, megakaryocytes, and cells showing eosinphilic granularity. Hepatocytes with pseudonuclei and cytoplasmic inclusions were also not uncommon (Plate 18 C). But when compared to group DX, these lesions were on a much milder scale hence showing the protective effect of the high dose of the extract (Table 10). The vacuolar changes in the hepatocytes showed a significant decline in occurrence in this group when compared to the DX group while the clear cells also showed considerable decline and was comparable to the CX group's hepatic parenchyma (Table 2) Hepatic cells showing necrosis were bound to individual places and the occurrence of the mitotic cells was comparable to the CX group as well. The presence of apoptotic cells were also not uncommon showing the comparable activity as with the control group and were significantly less as compared to the DX group (Table 10). However, there were few areas showing peliosis hepatis. The group was also characterized by the regular presence of regenerating nodules with hyperchromatic hepatocytes.

The hepatic lobulation did not appear enhanced and the portal areas were indistinct and were recognizable by the presence of bile duct which showed significant decline in the proliferation than the only DEN treated group DX (Plate 18 E). Only at few places the fibrous connective tissue was evident but the intensity was on a much milder scale with mild infiltration of the mononuclear cells as compared to the DX group or other groups treated with silymarin or plant extracts (Table 10) (Plate 18 F). On an average, the rats in this group showed maximum hepatic protection by the extract against the chemical agent as more than 75% of the hepatic parenchyma was intact as the result of the microscopic

evaluation was much comparable to the control group CX. The number of affected rats and their lesion score is given in Table 8 and 9.

The ameliorative groups treated with ethanolic extract of *Saussurea lappa* showed considerable decline in the dose dependent manner for the microscopic damage to the liver. The hepatoprotective effect of the ethanolic extract can be attributed for the anti-oxidative nature of the ethanolic extract of the root which has been shown to have highest scavenging nature for the DPPH radical among the various extracts for the same plant as shown by Chang et al. (2012) which further demonstrated an increase in the effect of the extracts when the concentration was increased. The extract of plant decreases the damage to the hepatocytes due to its ability to decrease the membrane damage and membrane fluidity due to its various bioactive constituents (Saleem et al. 2013 and Abdel-Rahman et al. 2020).

Table 8: Number of rats showing microscopic lesions of variable intensity in the liver

Type of lesion	**Intensity**	**CX**	**DX**	**SX**	**SA**	**SB**	**SC**
Enhanced Lobular pattern	Mild	0	0	3	1	3	4
	Moderate	0	3	2	6	1	0
	Severe	0	4	1	0	0	0
Bile duct proliferation	Mild	0	1	5	2	3	5
	Moderate	0	2	2	4	1	1
	Severe	0	4	0	0	1	1
Cirrhosis	Mild	0	2	4	1	5	6
	Moderate	0	0	3	3	0	0
	Severe	0	5	0	2	1	0
Dysplasia	Mild	0	0	5	3	4	4
	Moderate	0	4	2	4	2	2
	Severe	0	3	0	0	1	1
Lymphocytic infiltration	Mild	0	4	7	5	5	5
	Moderate	0	2	0	2	1	0
	severe	0	1	0	0	0	0
Peliosis hepatis	Mild	0	1	2	3	1	1
	Moderate	0	2	0	1	0	0
	Severe	0	1	0	0	0	0
Necrosis	Mild	0	4	6	3	5	6
	Moderate	0	1	1	2	2	0
	Severe	0	2	0	0	0	0
Vacoular changes	Mild	0	0	4	5	3	5
	Moderate	0	4	3	2	4	1
	Severe	0	3	0	0	0	0
Clear cell foci	Mild	0	0	4	5	4	5
	Moderate	0	4	3	2	3	1
	Severe	0	3	0	0	0	0
Apoptotic cells	Mild	0	2	7	3	5	5

	Moderate	0	5	0	4	2	0
	Severe	0	0	0	0	0	0
Mitotic figure	Mild	0	5	5	1	3	5
	Moderate	0	0	2	4	3	2
	Severe	0	0	0	0	0	0

Table 9: Total score for microscopic evaluation of rat liver

Microscopic lesion		**CX**	**DX**	**SX**	**SA**	**SB**	**SC**
Proliferative lesions	Enhanced Lobular pattern	0	18	10	13	5	4
	Bile duct proliferation	0	17	9	10	7	10
	Cirrhosis	0	17	10	13	8	6
	Dysplasia	0	17	9	11	11	11
	Lymphocytic infiltration	0	11	7	9	7	5
	Peliosis hepatis	0	8	2	5	1	1
Toxic hepatic changes	Necrosis	0	12	8	7	9	6
	Vacular changes	0	17	10	9	11	7
	Clear cell foci	0	17	10	9	10	7
	Apototic cells	0	12	7	11	9	5
	Mitotic figure	0	5	9	9	9	9
Grand Total		**0**	**151**	**91**	**106**	**87**	**71**

Table 10: Statistical evaluation of the Mean microscopic lesions of rat liver

Microscopic lesion		**Experimental groups**					
		CX	**DX**	**SX**	**SA**	**SB**	**SC**
Proliferative lesions	Enhanced Lobular pattern	0.00 ± 0.00^{a}	2.71 ± 0.14^{cd}	1.62 ± 0.38^{abc}	1.71 ± 0.20^{c}	0.76 ± 0.31^{ab}	0.66 ± 0.25^{ab}
	Bile duct proliferation	0.00 ± 0.00^{a}	2.48 ± 0.26^{bc}	1.43 ± 0.16^{ab}	1.43 ± 0.28^{b}	1.14 ± 0.40^{ab}	1.28 ± 0.26^{ab}
	Cirrhosis	0.00 ± 0.00^{a}	2.52 ± 0.31^{bc}	1.43 ± 0.17^{b}	1.76 ± 0.35^{bc}	1.04 ± 0.29^{ab}	0.90 ± 0.14^{ab}
	Dysplasia	0.00 ± 0.00^{a}	2.43 ± 0.20^{bc}	1.24 ± 0.16^{ab}	1.52 ± 0.16^{bc}	1.52 ± 0.22^{bc}	1.52 ± 0.24^{bc}
	Lymphocytic infiltration	0.00 ± 0.00^{a}	1.71 ± 0.26^{bc}	1.09 ± 0.06^{ab}	1.33 ± 0.18^{b}	1.05 ± 0.20^{ab}	0.81 ± 0.22^{ab}

	Peliosis hepatis	0.00±0.00[a]	1.05±0.47 [a]	0.28±0.20 [a]	0.71±0.27 [a]	0.14±0.14 [a]	0.09±0.09 [a]
Toxic hepatic changes	Necrosis	0.00±0.00[a]	1.81±0.33[bc]	1.24±0.14[b]	1.05±0.27[ab]	1.24±0.16[b]	0.71±0.14[ab]
	Vacular changes	0.00±0.00[a]	2.33±0.25[c]	1.38±0.15[bc]	1.33±0.15[abc]	1.52±0.16[bc]	0.95±0.15[ab]
	Clear cell foci	0.00±0.00[a]	2.48±0.19[bc]	1.43±0.17[b]	1.38±0.17[ab]	1.52±0.25[b]	1.09±0.16[ab]
	Apototic cells	0.00±0.00[a]	1.71±0.15[c]	1.09±0.06[abc]	1.47±0.18[bc]	1.33±0.18[bc]	0.71±0.15[ab]
	Mitotic figure	0.00±0.00[a]	0.76±0.24[ab]	1.19±0.18[b]	1.28±0.28[b]	1.19±0.28[b]	1.14±0.21[b]

*Data represent Mean ± S.E. (n=6). **CX**: Control group i.e. No DEN & No plant extract; **DX**: DEN @ 0.01% in drinking water only; **SX**: DEN + Silymarin @ 25mg/Kg of Body weight; **SA**: DEN + *Saussurea lappa* extract @ 100 mg/Kg of Body weight; **SB**: DEN + *Saussurea lappa* extract @ 250 mg/Kg of Body weight; **SC**: DEN + *Saussurea lappa* extract @ 500 mg/Kg of Body weight. Different superscripts within same row are significantly different at P≤0.05%.

Plate 12: Microscopic pictures of Group CX

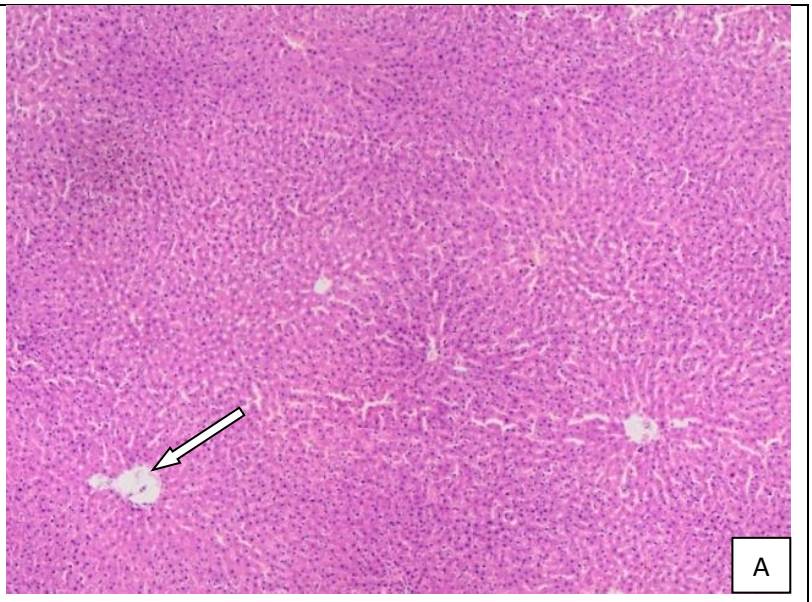


Fig A: The hepatic parenchyma showing normal portal area (white arrow) (4X).

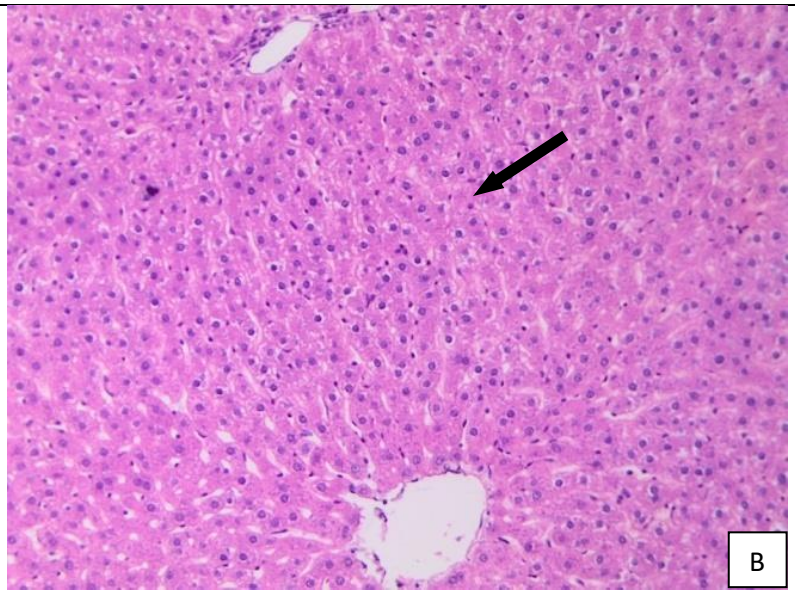


Fig B: Normal hepatic cord arrangement in the hepatic parenchyma (Black arrow) (10X).

Plate 13: Microscopic pictures of Group DX

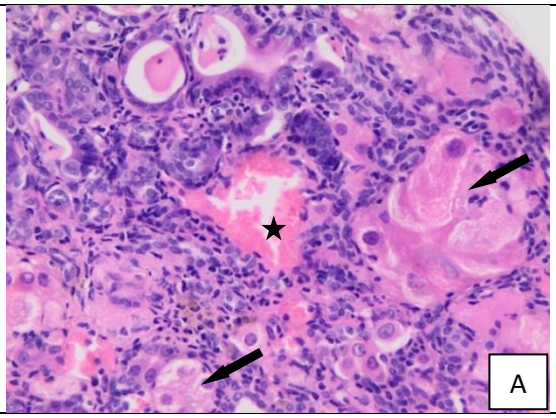


Fig A: Polyhedral hepatocytes along with severe fibrous tissue (Black arrow) and congestion of blood vessel (*) (20X).

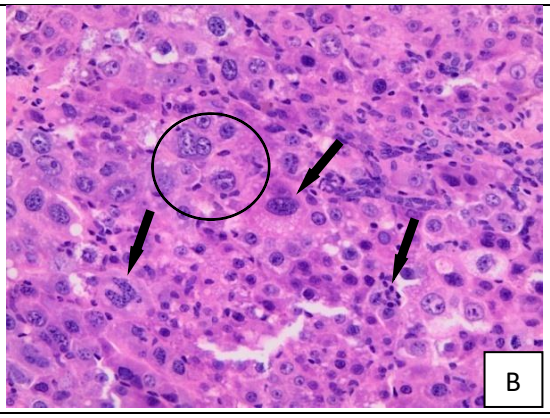


Fig B: Hepatocytes showing hyperchromasia as well as variation in nuclear size (Black arrow) and increased number of nucleoli (Black circle) (20X).

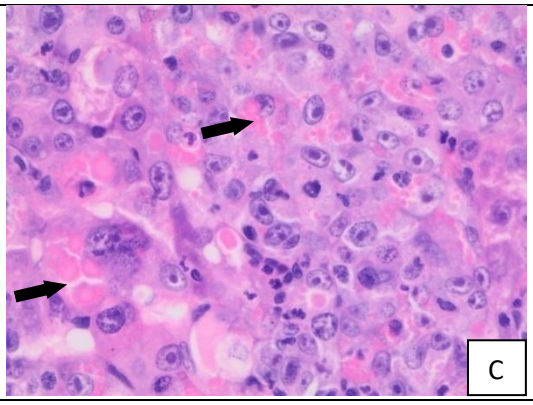


Fig C: Eosinophilic inclusion bodies in the cytoplasm of hepatocytes (Black arrow) (40X).

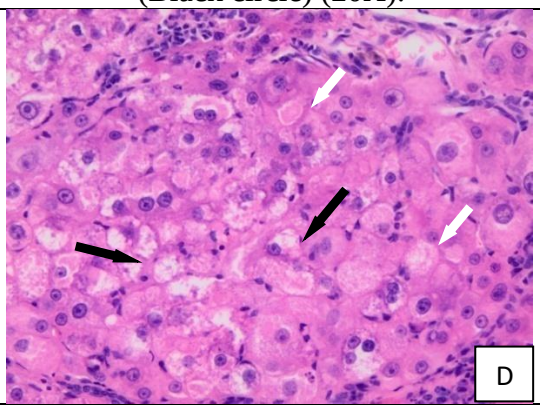


Fig D: Presence of clear cell (Black arrow) along with eosinophilic cytoplasmic inclusions (White arrow) of hepatocytes in a pseudolobule (20X).

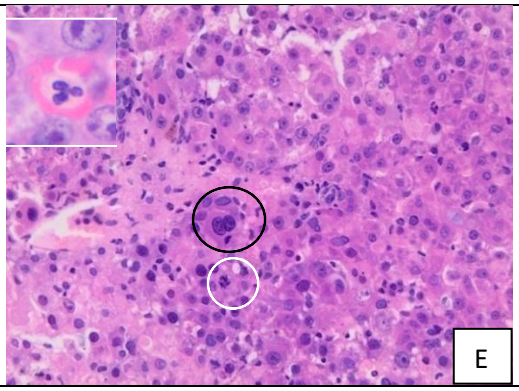


Fig E: Hepatocytes showing double nuclei (black circle), mitotic figure (white circle), and apoptotic body (insat) (20X).

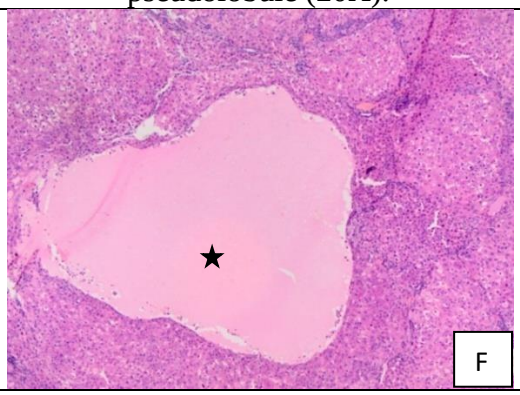


Fig F: Hepatic parenchyma showing area of Hepatis peliosis (*) (4X).

Plate 14: Microscopic pictures of Group DX

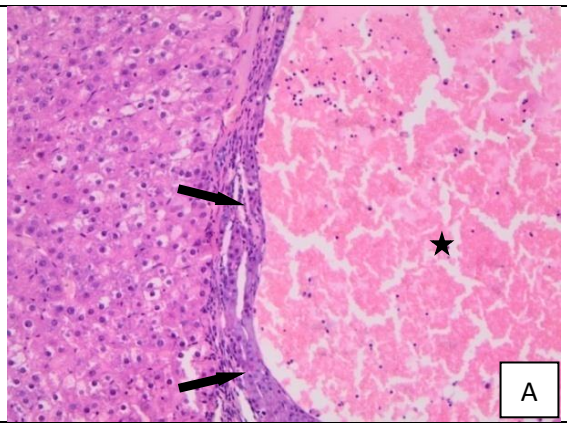


Fig A: Hepatocytes surrounding the area of blood cavity (*) showed pressure atrophy (Black arrow) (10X).

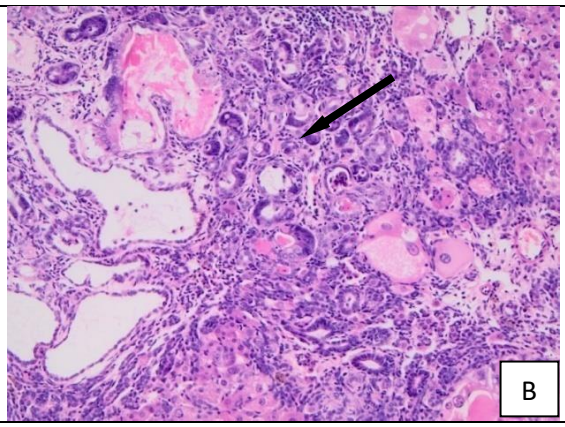


Fig B: Liver section showing extensive bile duct proliferation (Black arrow) along with severe lymphocytic infiltration (4X).

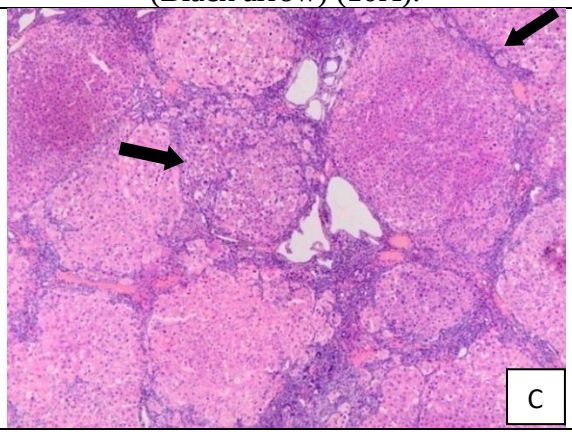


Fig C: Hepatic parenchyma showing pseudolobule formation without central vein due to extensive fibrous tissue proliferation (Black arrows) (4X).

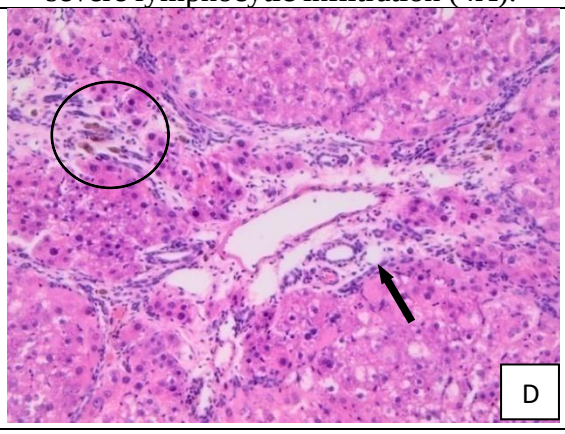


Fig D: The bile ducts showing proliferation into small ductules (black arrow) along with golden brown bilirubin pigment (circle) (10X)

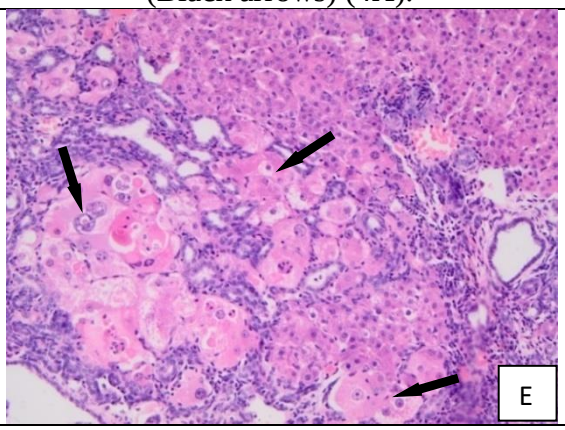


Fig E: Island of hepatocytes separated by fibrous connective tissue (Black arrows) (10X).

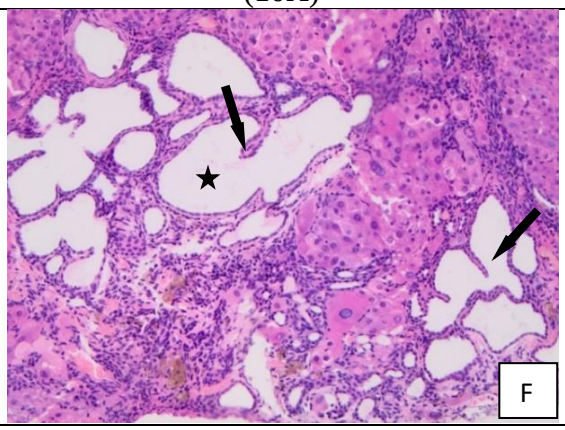


Fig F: Bile ducts showing papillary projection (Black arrow) along with eosinophilic debris present in the cavity (*) (10X).

Plate 15: Microscopic pictures of Group SX

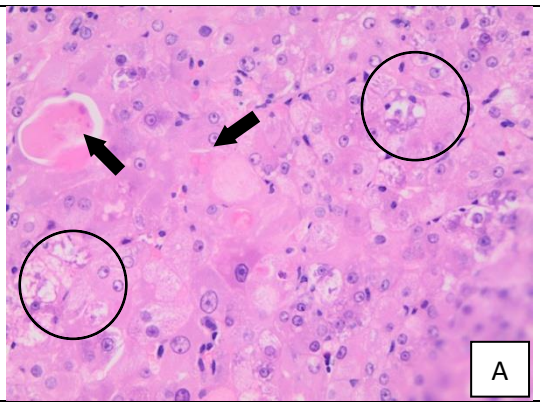


Fig A: Hepatocytes showing eosinophilic cytoplasmic inclusions (Black arrow) accompanied by vacuolar changes (circle) (20X).

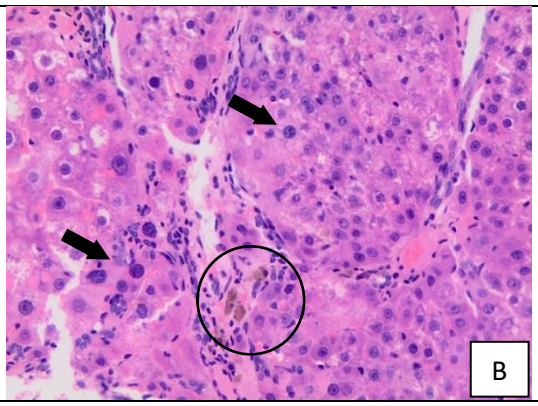


Fig B: Hepatocytes showing evident karyomegaly (Black arrow) accompanied with bilirubin pigment (circle) (20X).

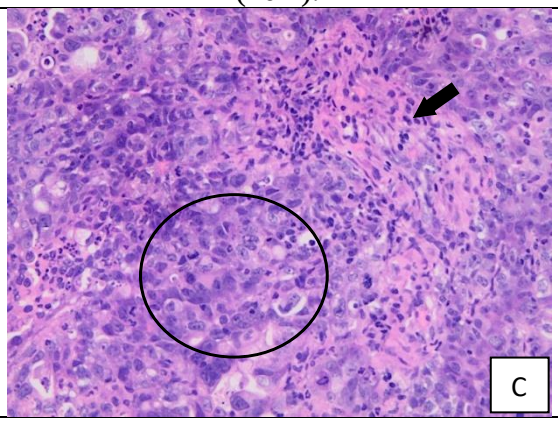


Fig C: Hepatocytes showing marked pleomorphism (black circle) and at places showing glandular arrangement (black arrow) (10X).

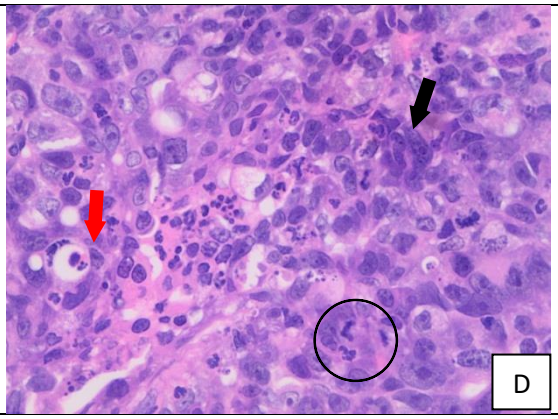


Fig D: Smaller hepatocytes showing basophilic cytoplasm along with bizarre shaped mitotic figures (circle) and apoptotic cell (red arrow) (20X).

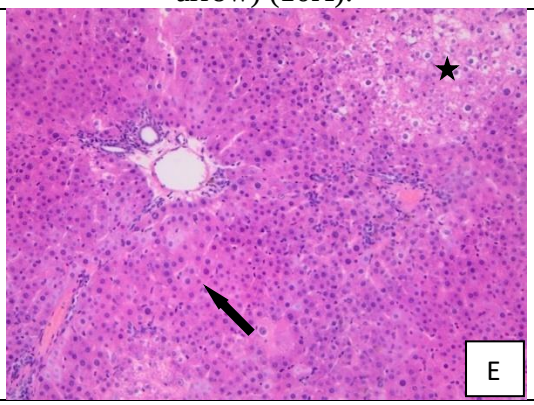


Fig E: Hepatocytes showing towards normal hepatic cord structure (Black arrow) with focal dysplastic area (*) (10X).

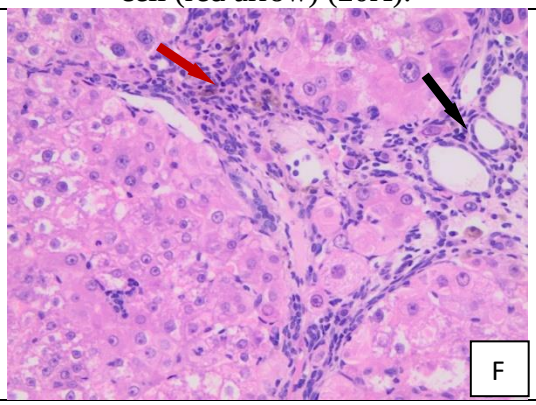


Fig F: The bile ducts showing mild proliferation (Black arrow) along with accompanied mild mononuclear cell infiltration (Red arrow) (20X).

Plate 16: Microscopic pictures of Group SA

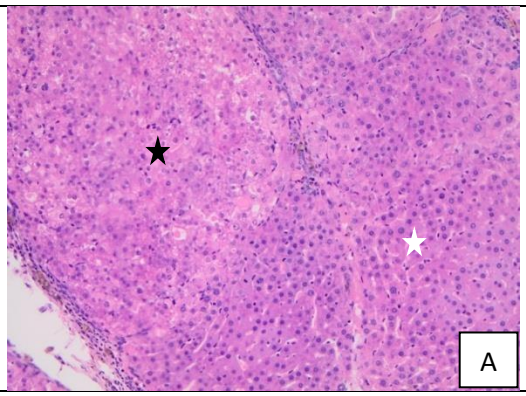


Fig A: Hepatic parenchyma showing paler eosinophilic dysplastic area (black *) along with areas showing normal hepatic parenchyma (white *) (10X).

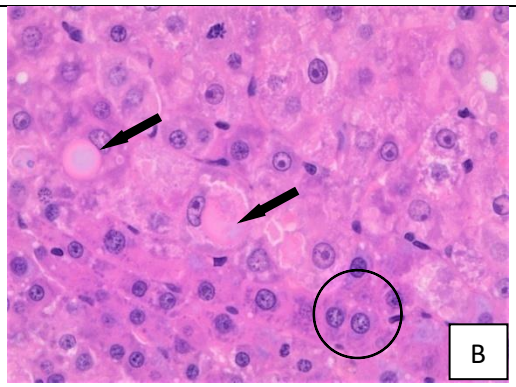


Fig B: Hepatocytes showing less frequent eosinophilic cytoplasmic inclusions (Black arrow) along with double nuclei (circle) (40X).

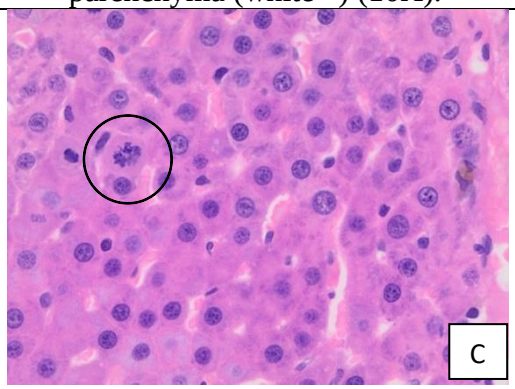


Fig C: Hepatocytes showing hyperchromasia with some mitotic activity (circle) (40X).

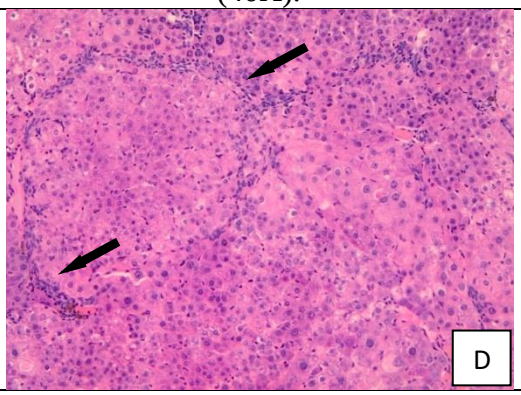


Fig D: Hepatic parenchyma showing thin fibrous connective tissue forming pseudolobule (Black arrow) (10X)

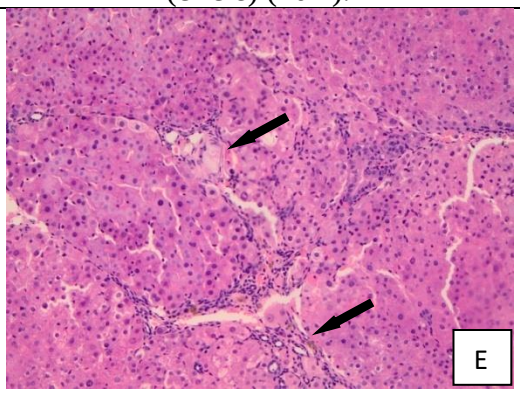


Fig E: Bile ducts in this group revealed Mild to moderate proliferation (Black arrow) (10X).

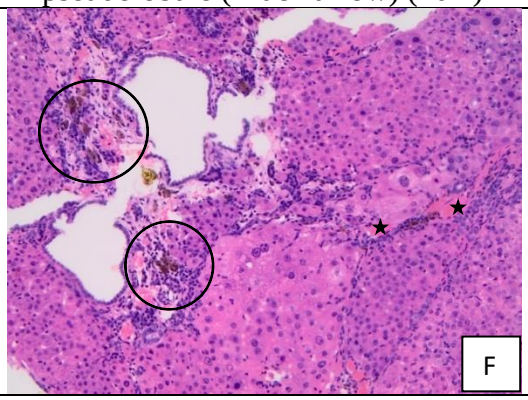


Fig F: Hepatic tissue also revealed presence of bile pigment (circle) along with moderate congestion of portal vessel (*) (10X).

Plate 17: Microscopic pictures of Group SB

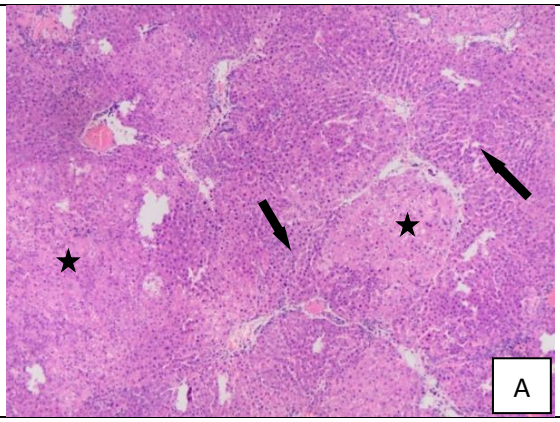


Fig A: Hepatic parenchyma showing intact hepatic cord arrangement (Black arrow) with occasional not so well clearly demarcated dysplastic areas (*) (4X).

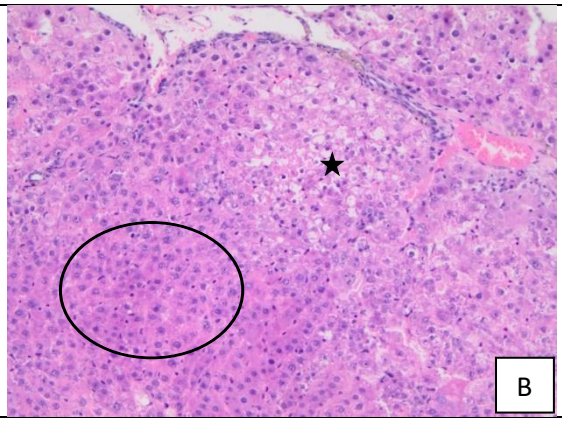


Fig B: Hepatic parenchyma showing paler dysplastic area (*) surrounded by regenerative nodule (Black circle) (10X).

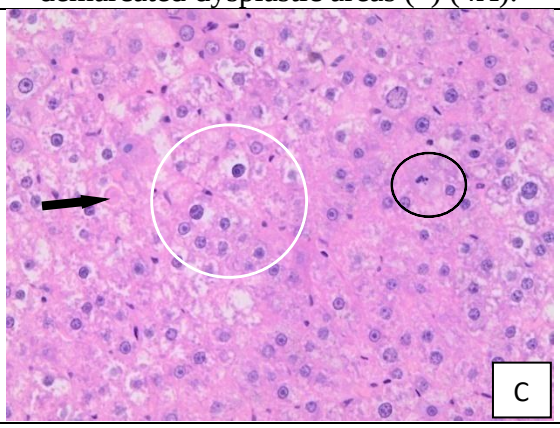


Fig C: Hepatocytes showing apoptotic body (Black arrow), mitotic figure (Black circle) and karyomegaly (White arrow) (20X).

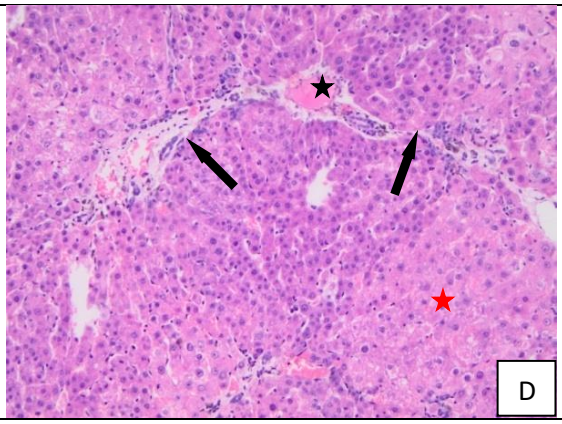


Fig D: Bile duct showing mild proliferation (Black arrow) along with portal congestion (black *) and areas of dysplasia (red *) (10X).

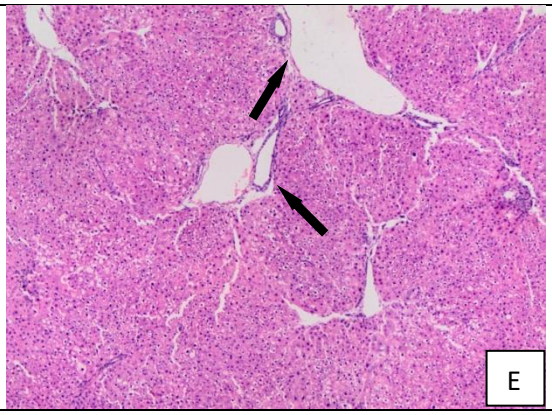


Fig E: Hepatic parenchyma showing mild cirrhotic changes (4X).

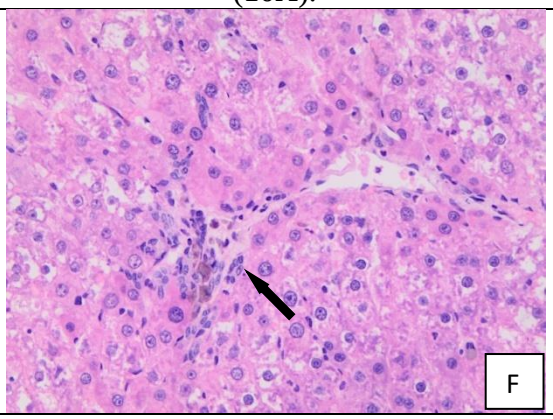


Fig F: The portal areas revealed decrease in mononuclear cell infiltration along with the presence of bilirubin pigment (Black arrow) (20X).

Plate 18: Microscopic pictures of Group SC

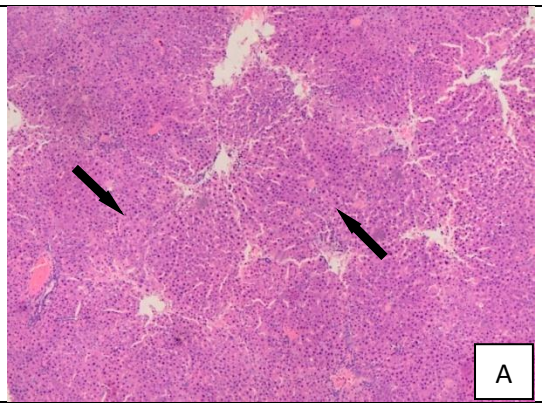


Fig A: Hepatic parenchyma showing intact hepatic cord arrangement with congestion of the portal areas (4X).

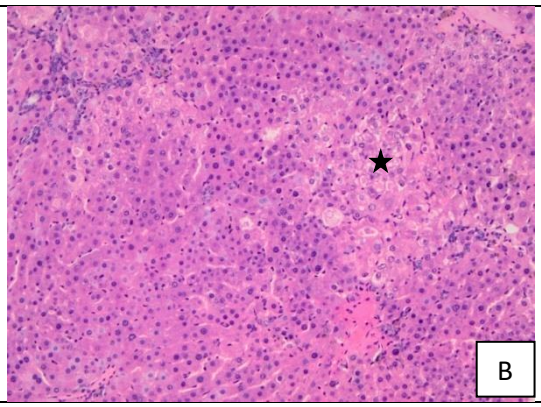


Fig B: Hepatic parenchyma showed the presence of solitary present dysplastic nodule in the hepatic parenchyma (*) surrounded by intact hepatic parenchyma (10X).

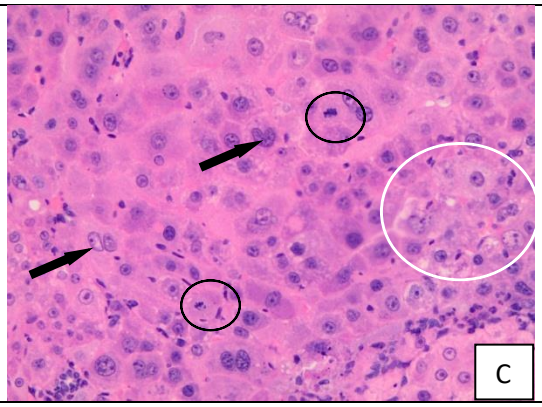


Fig C: Hepatocytes showing double nuclei (black arrow), mitotic figure (Black circle) and dysplastic changes (White circle) (40X).

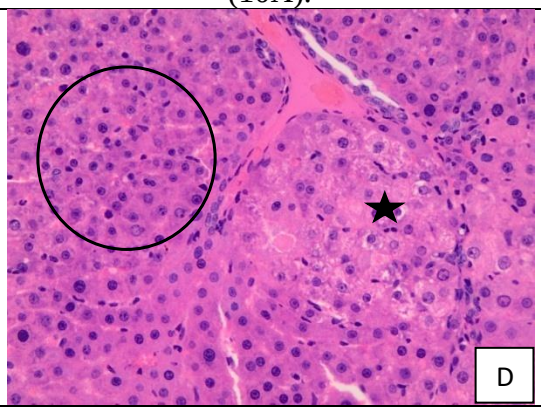


Fig D: Hepatic parenchyma showing regenerating nodule (circle) along side with dysplastic area (*) (20X).

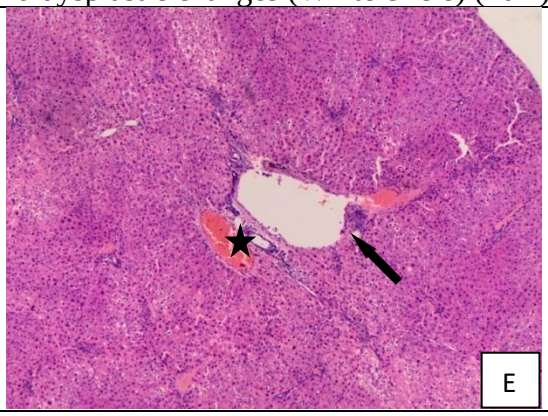


Fig E: Portal areas showing mild fibrous connective tissue and bile duct proliferation (black arrow) along with congestion (*) (4X).

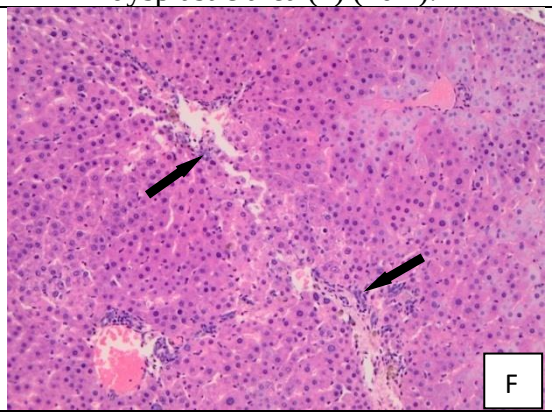


Fig F: Hepatic parenchyma showing mild mononuclear cell infiltration (Black arrows) (10X).

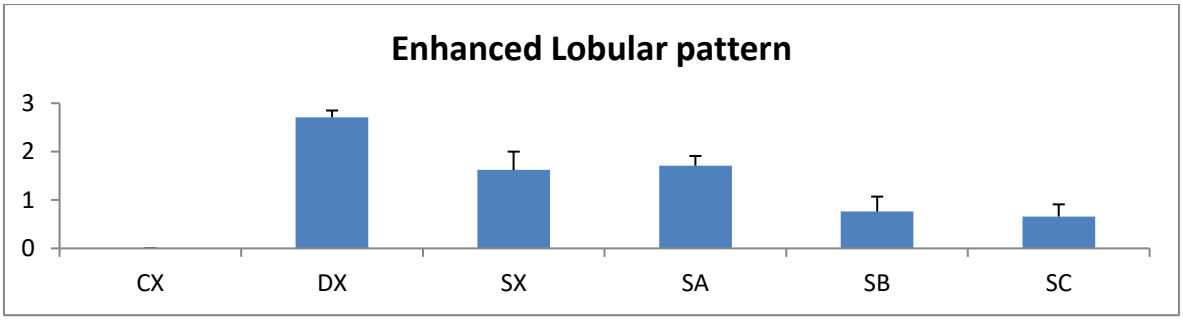


Fig 17: Mean lesion score of enhanced lobular pattern in the liver of rat

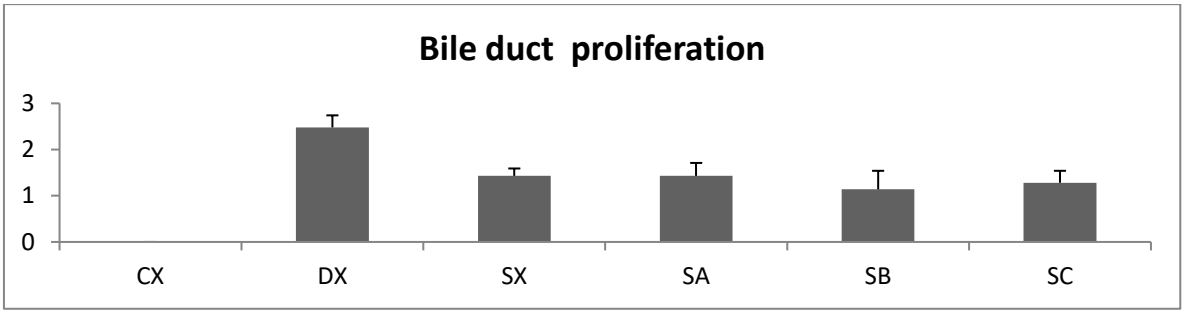


Fig 18: Mean lesion score of bile duct proliferation in the liver of rat

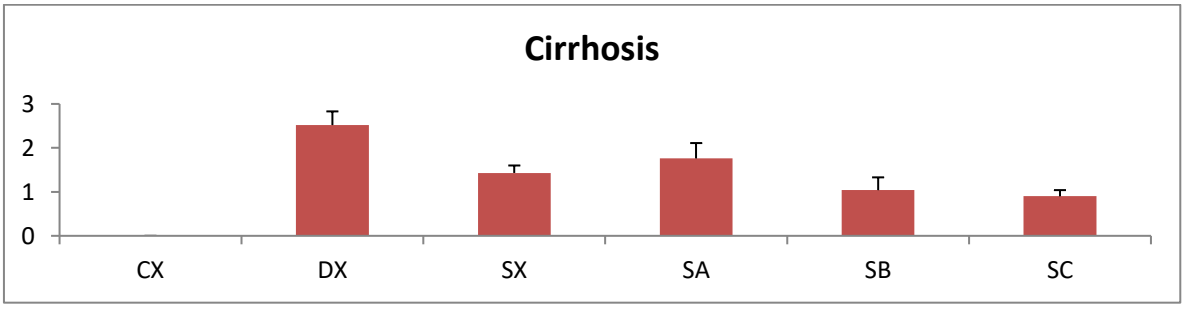


Fig 19: Mean lesion score of cirrhosis in the liver of rat

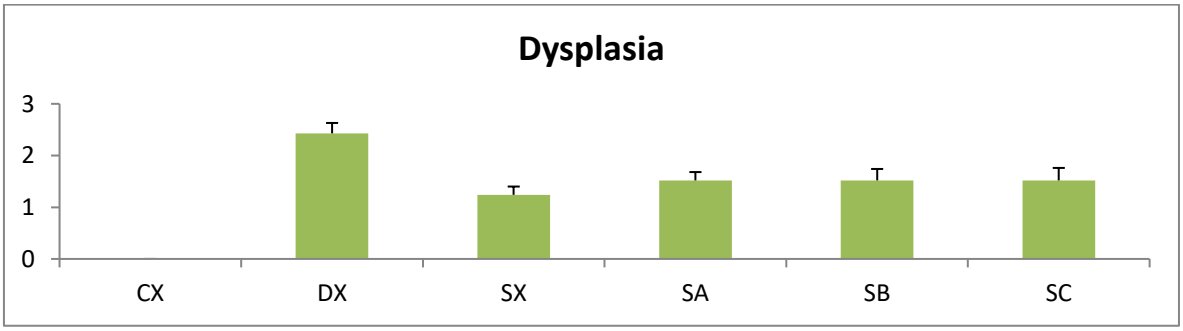


Fig 20: Mean lesion score of dysplasia in the liver of rat

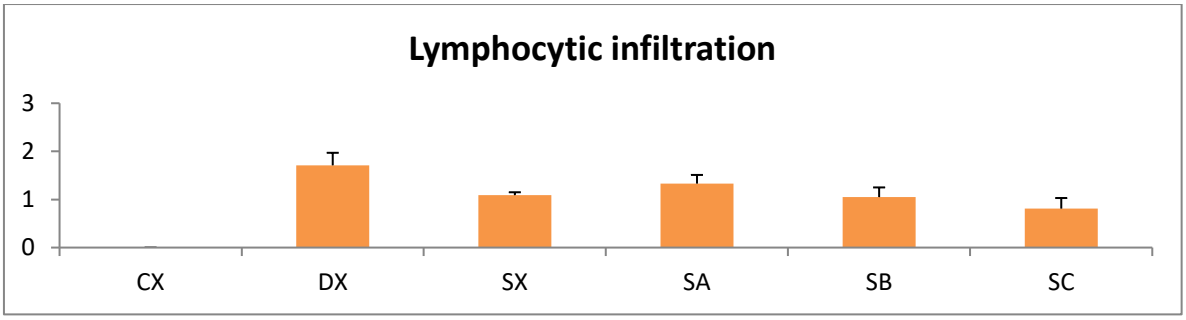


Fig 21: Mean lesion score of lymphocytic infiltration in the liver of rat

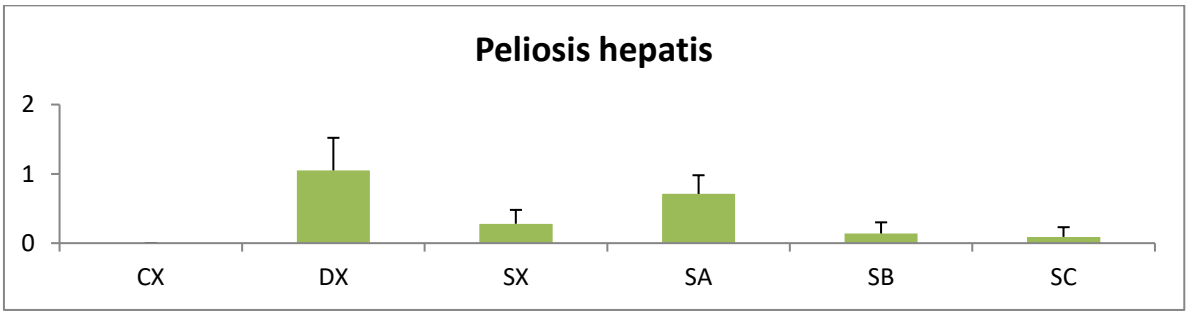


Fig 22: Mean lesion score of peliosis hepatis in the liver of rat

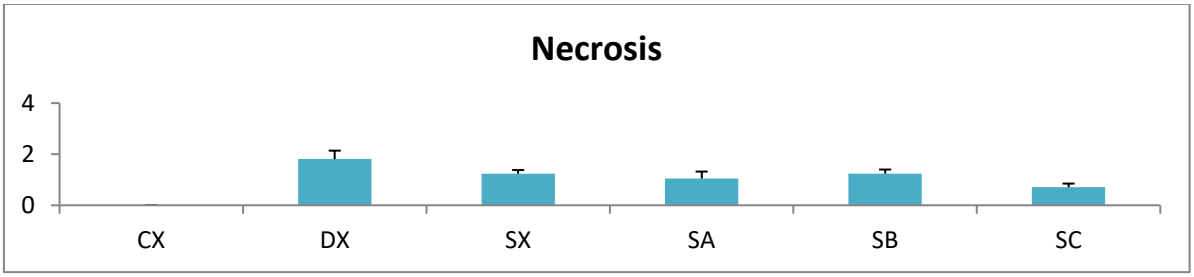


Fig 23: Mean lesion score of necrosis in the liver of rat

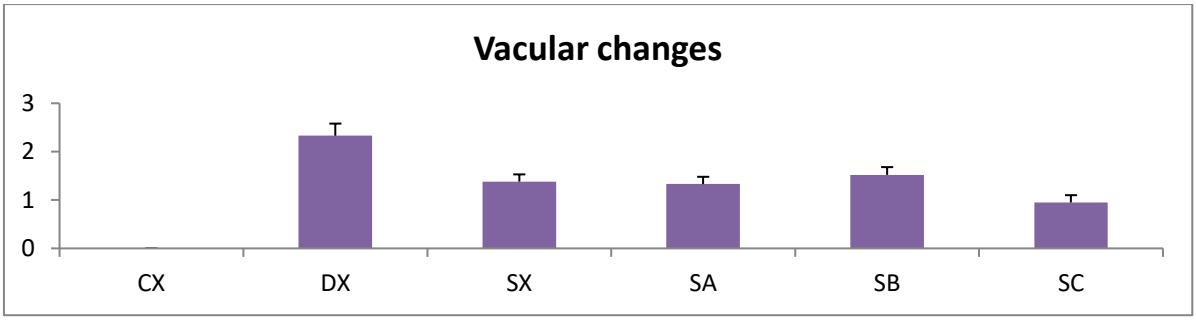


Fig 24: Mean lesion score of vacuolar changes in the liver of rat

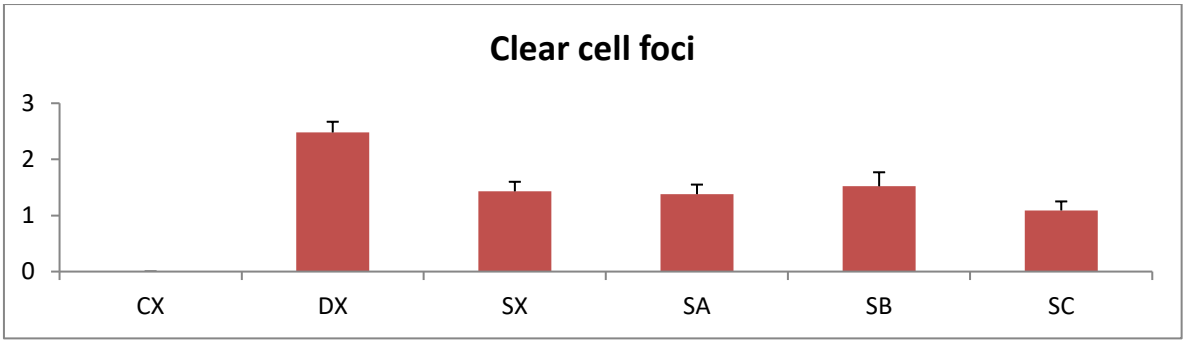


Fig 25: Mean lesion score of clear cell foci in the liver of rat

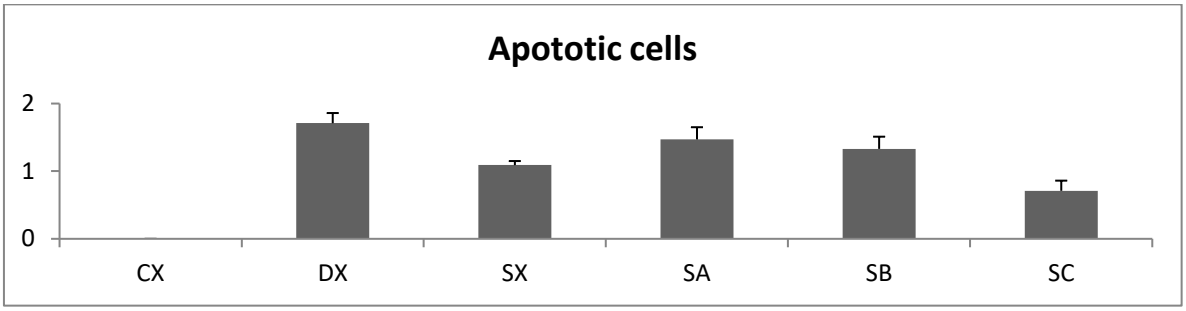


Fig 26: Mean lesion score of apoptotic cells in the liver of rat

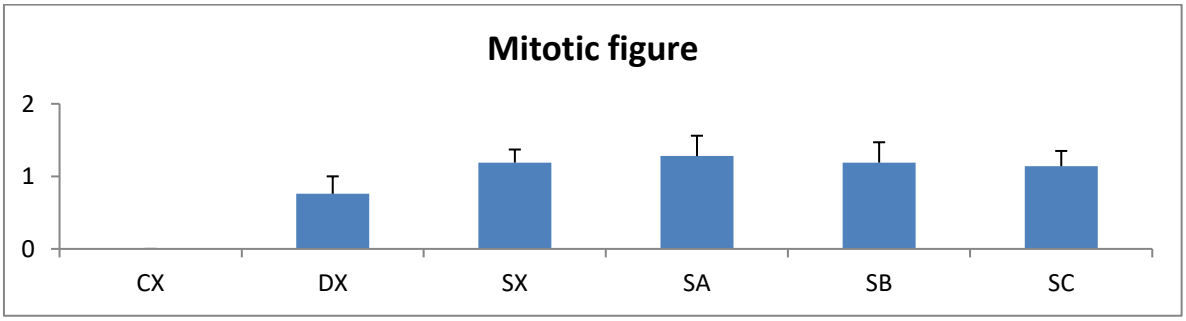


Fig 27: Mean lesion score of mitotic figures in the liver of rat

4.11 Masson's trichrome staining result

The tissue sections from the Control Group CX did not reveal any indication of the fibrous connective tissue proliferation around the portal areas when the tissue sections were stained with Masson's trichrome stain (Plate 19 A & B). Conversely, the DX group revealed an intense fibrous tissue proliferation around the portal areas which was consistently demarcated by the bridging pattern of the fibrous tissue joining the portal to portal and portal to central areas. The fibrous connective tissue was seen wandering into the hepatic parenchyma which further divided the parenchyma into pseudolobules that were devoid of central vein at places and were also seen surrounding the individual hepatocytes

quite often (Plate 19 C & D). In Group SX however, the fibrous connective tissue in the portal areas was much less with an occasional bridging pattern from portal to the central or portal to portal (Plate 19 E & F).

Similarly, the ameliorative groups showed a tremendous amount of improvement when the results of Masson's trichrome staining were compared with the DEN alone treated group DX. The SA group treated with 100 mg/kg of body weight revealed moderate to severe degree proliferation of fibrous connective tissue with frequent connective tissue proliferation around the portal areas and showing the bridging connections with the nearby portal areas (Plate 20 A& B). In comparison, the SB group treated with 250 mg/kg of body weight of the ethanolic extract of *Saussurea lappa* showed mild to moderate proliferation of the connective tissue. The connective tissue was mainly centred on the portal areas with the occasional wandering of the fibrous strands into the hepatic parenchyma (Plate 20 C & D). On the other hand, the SC group of rats treated with the highest dose showed the best result for the three groups. The hepatic tissue showed mild proliferation of the connective tissue which was mainly restricted around the bile ducts while portal areas at many places do not reveal any fibrous tissue at all (Plate 20 E & F). Thus, the ameliorative groups showed hepatoprotective activity by decreasing the proliferation of the connective tissue in the hepatic parenchyma as compared to the groups treated with the chemical agent only. The severe proliferation of the fibrous connective tissue upon the administration of DEN was also reported by Chooi et al. (2016) by using Masson's trichrome stain.

Plate 19: Masson's trichrome stained rat liver tissue sections

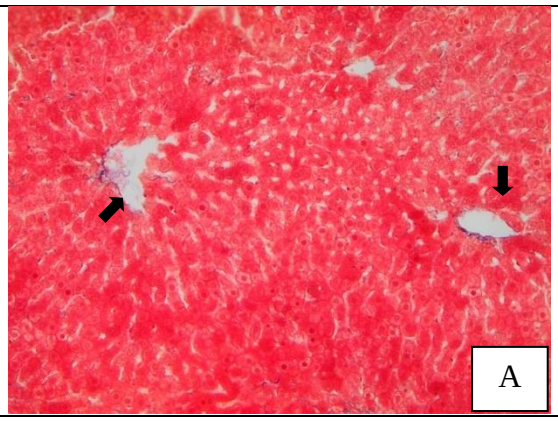


Fig A: Absence of connective tissue around bile ducts (Black arrow) (10X).

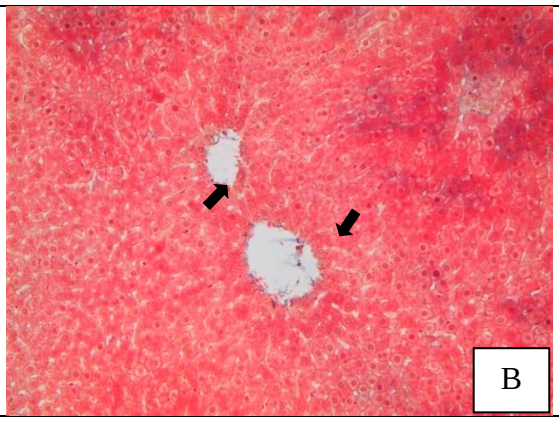


Fig B: Absence of connective tissue around portal area (Black arrow) (10X).

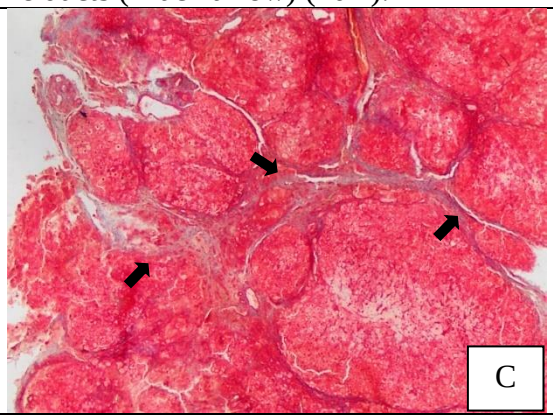


Fig C: Severe proliferation of connective tissue showing portal to portal bridging (Black arrows) (4X).

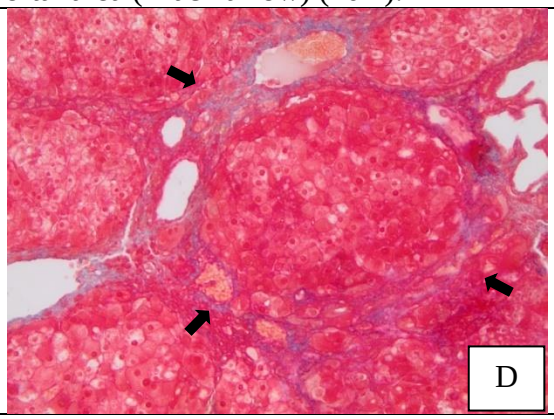


Fig D: Fibrous tissue surrounding hepatic parenchyma forming pseudolobule (Black arrows) (20X).

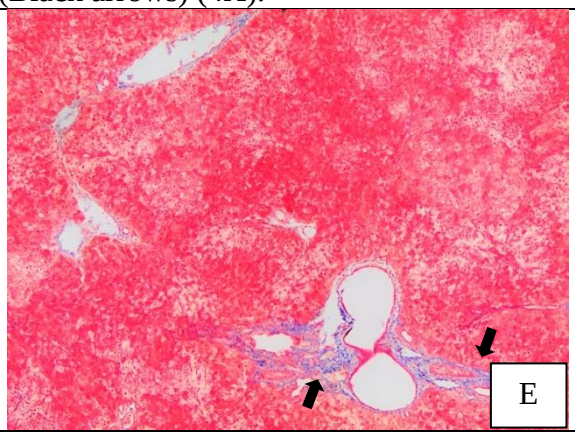


Fig E: Connective tissue proliferation around bile duct (Black arrows) (10X).

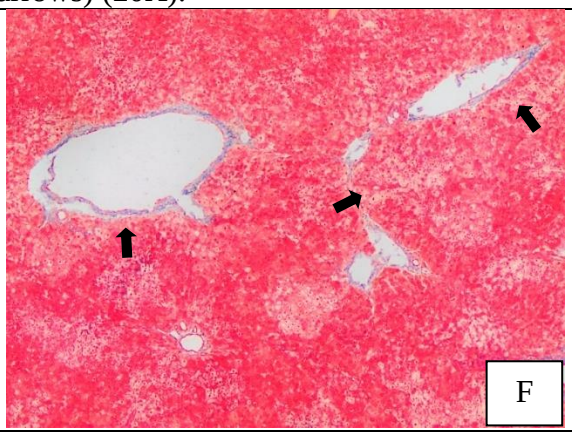


Fig F: Mild bridging of fibrous tissue (Black arrow) (10X).

Plate 20: Masson's trichrome stained rat liver tissue section

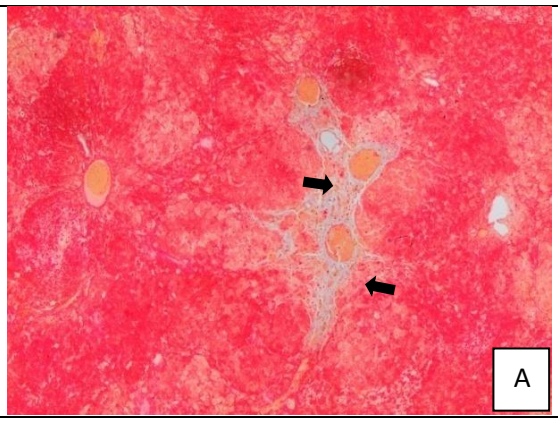


Fig A: Hepatic tissue showing vessel to vessel bridging (Black arrows) (10X).

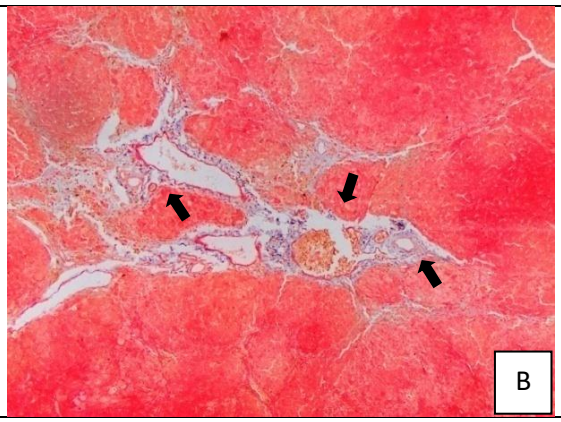


Fig B: Fibrous tissue proliferation around portal area (Black arrow) (10X).

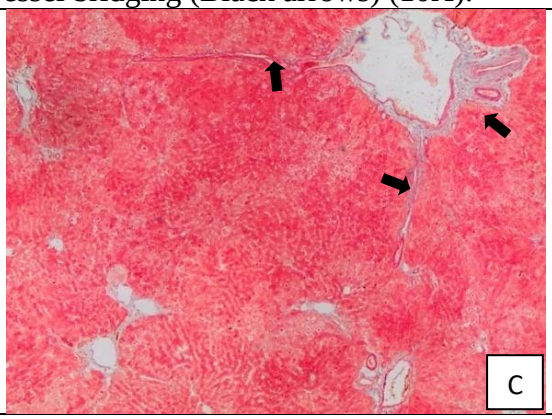


Fig C: connective tissue proliferation around bile duct and thin strand invading hepatic parenchyma (Black arrows) (10X).

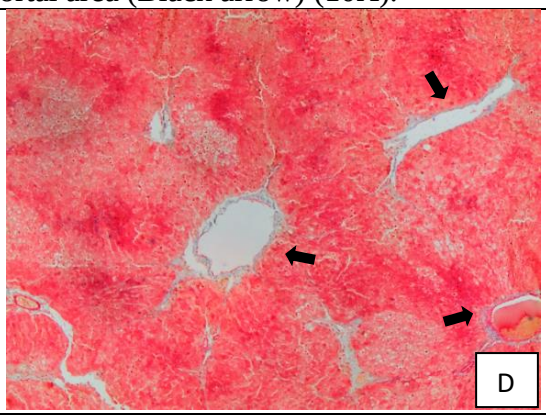


Fig D: Connective tissue proliferation around bile duct (Black arrows) (10X).

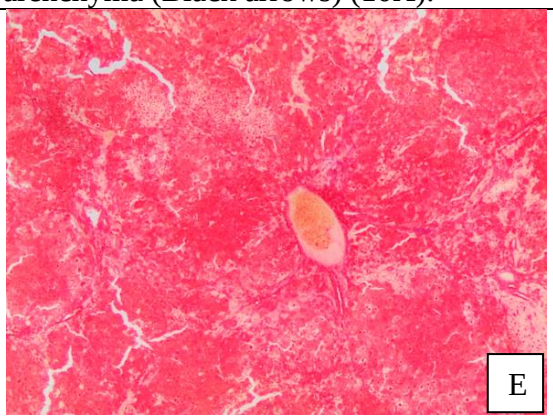


Fig E: No connective tissue proliferation a round blood vessel (10X).

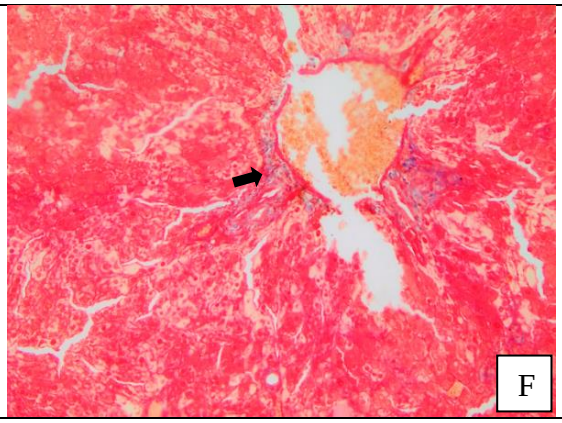


Fig F: Mild connective tissue proliferation a round blood vessel (Black arrow) (20X).

4.12 Immuno-histochemistry

The liver sections of the rats were also subjected to the immuno-histochemical examination for NF-kB and Caspase-3 protein which are crucial mediators for release of inflammatory cytokines and apoptosis in cell respectively. The result showed significantly ($P \leq 0.05$) higher immunoexpression of these proteins in the liver of rats treated with only DEN (Group DX). On the other hand, the groups treated either with Silymarin or *Saussurea lappa* root extract along with DEN showed a significant decrease in the expression cells when compared to DX alone and also the ameliorative groups showed a dose dependent decline in the expression for these proteins (Table 11 & 12, Fig 28 & 29, Plate 21 & 22).

Table 11: NF-κB immunoexpression

CX	DX	SX	SA	SB	SC
1.26 ± 0.10^{a}	21.98 ± 0.74^{e}	8.89 ± 0.9^{cd}	12.58 ± 1.77^{d}	6.39 ± 1.65^{bc}	4.03 ± 0.27^{ab}

*Data represent Mean ± S.E. (n=6). **CX**: Control group i.e. No DEN & No plant extract; **DX**: DEN @ 0.01% in drinking water only; **SX**: DEN + Silymarin @ 25mg/Kg of Body weight; **SA**: DEN + *Saussurea lappa* extract @ 100 mg/Kg of Body weight; **SB**: DEN + *Saussurea lappa* extract @ 250 mg/Kg of Body weight; **SC**: DEN + *Saussurea lappa* extract @ 500 mg/Kg of Body weight.NF-κBprotein levels were detected by immunohistochemistry as the DAB-stained percentage area in the liver sections.Different superscripts within same row are significantly different at P≤0.05%.

Fig 28: NF-κB immunoexpression

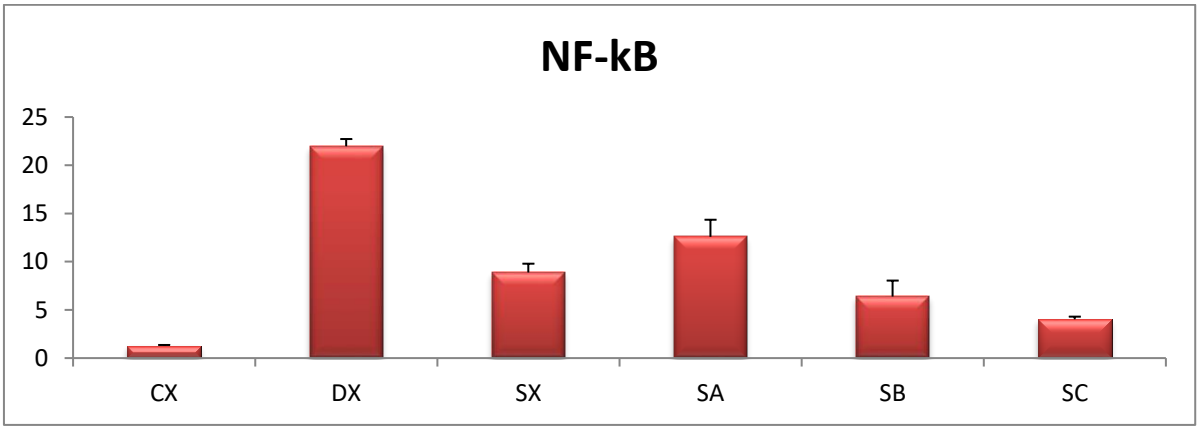
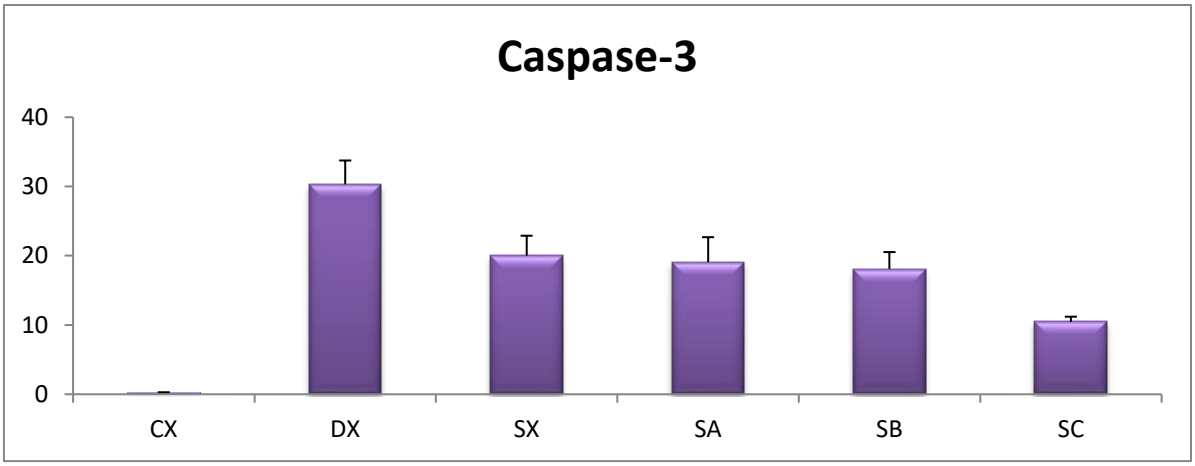


Table 12: Caspase-3 immunoexpression

CX	DX	SX	SA	SB	SC
0.23 ± 0.06^{a}	30.33 ± 3.42^{c}	20.01 ± 2.88^{b}	19.09 ± 3.59^{b}	18.11 ± 2.42^{b}	10.46 ± 0.74^{b}

*Data represent Mean ± S.E. (n=6). **CX**: Control group i.e. No DEN & No plant extract; **DX**: DEN @ 0.01% in drinking water only; **SX**: DEN + Silymarin @ 25mg/Kg of Body weight; **SA**: DEN + *Saussurea lappa* extract @ 100 mg/Kg of Body weight; **SB**: DEN + *Saussurea lappa* extract @ 250 mg/Kg of Body weight; **SC**: DEN + *Saussurea lappa* extract @ 500 mg/Kg of Body weight.Caspase-3 protein levels were detected by immunohistochemistry as the DAB-stained percentage area in the liver sections. Different superscripts within same row are significantly different at P≤0.05%.

Fig 29: Caspase-3 immunoexpression



The decline in the NF-κB can be explained as during the progression of the hepatic damage, cytokines play an important role for inflammatory response and for the defense of host cells (Li et al. 2014). These cytokines have shown a close relation with the up-regulation of NF-κB pathways for the inflammatory response but the action of NF-κB is regulated by the presence of a protein i.e. IkB which prevents the translocation of NF-κB from cytoplasm to nucleus for the transcription of the inflammatory cytokines (Vallabhapurapu and Karin, 2009). However, in the Study of Wang et al. (2017), they showed that the costunolide down-regulated the phosphorylation of IκB protein which in turn suppresses the activation of NF-κB, hence preventing the liver damage by suppressing the inflammatory response. Pyun et al. (2018) also showed that Dehydrocostus lactone inhibits the Nf-κB pathway, which plays an important role in regulating the inflammatory cytokines like IL-4, IL-5 etc by inhibiting the phosphorylation of IκBα in the lung tissue of rats against inflammatory response. In a research related to the anti-inflammatory property, the ethanolic extract of *Saussurea lappa* showed a considerable decline in the release of pro-inflammatory cytokines like TNF-α and IL-1β from the activated macrophages (Tag et al. 2015).

Caspase-3 is a protein and a part of cysteine-aspartic acid protease family (caspase) having an important role in execution of the apoptotic cells (Crawford and well 2011). The protein exists as a pro-enzyme in cytoplasm and gets cleaved in response to the stimuli for apoptosis (Zhang and Yu 2016). In the present study, the immunohistochemical analysis of the liver tissue for Caspase-3, DX group showed significant increase in the immunoreactivity when compared to any other group of study. This can be due to the genetic damage caused by DEN to the hepatocytes which leads to the cell death and as caspase 3 is an important factor in apoptosis, the expression of this protein increases in the DEN treated group (Luedde et al. 2014 and Mo'men et al 2020). The group treated with silymarin along with DEN showed a significant decline in Caspase-3 activity. While the groups treated with ethanolic extract also showed a significant decline in the apoptotic cells when compared to DEN treated group. The *in-vitro* studies of the extract of *Saussurea lappa* showed anti-cancerous activity in various cell lines like lung (Sunkara et al. 2010), liver (Hsu et al. 2009) and gastric cell (Ko et al. 2004) lines by the increase in the apoptotic index of the cancerous cell hence resulting in its anti-cancerous property which could be attributed to its main component i.e. Dehydrocostus lactone. However, in the present study the expression of Caspase-3 indicating apoptosis in the liver cell significantly declined in the groups treated with ethanolic extract in a dose dependent manner, which was also shown by the study of El-Rahman et al. (2020). This was also shown by Zhao et al. (2018) in their study in which *Saussurea lappa* constituents revealed a significant decline in the Caspase dependent apoptotic pathway in the nerve cells which can be attributed to the free radical scavenging activity of the extract thereby showing the anti-apoptotic nature of the plant.

Plate 21: Immuno-histochemical examination for NF-κB

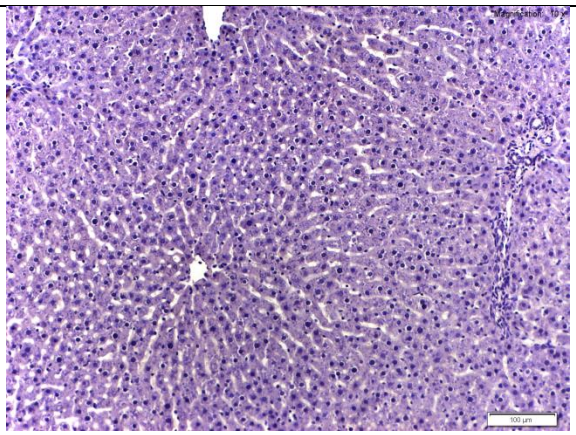
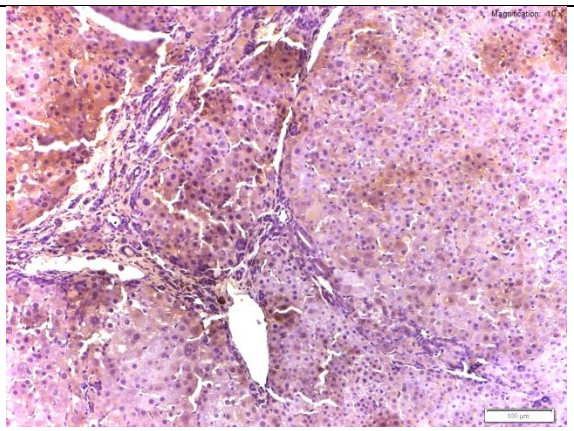
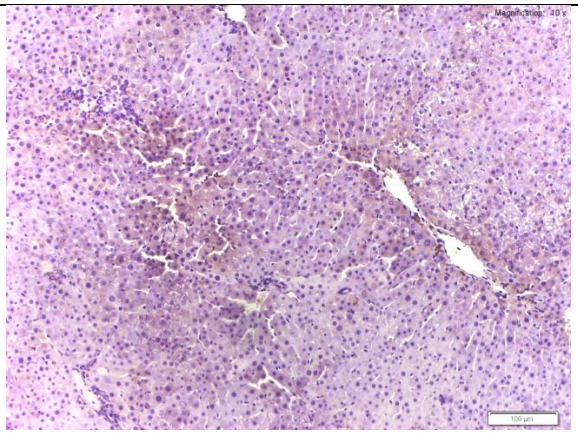
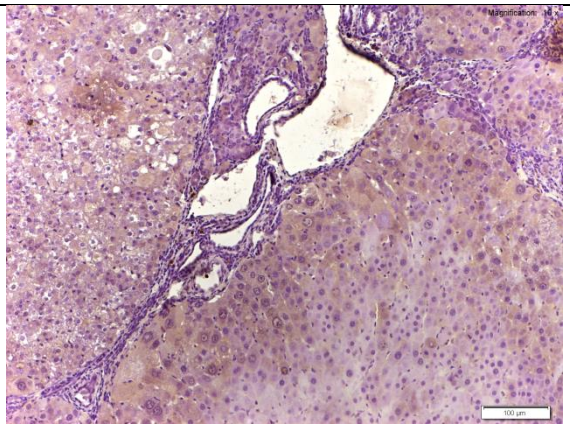
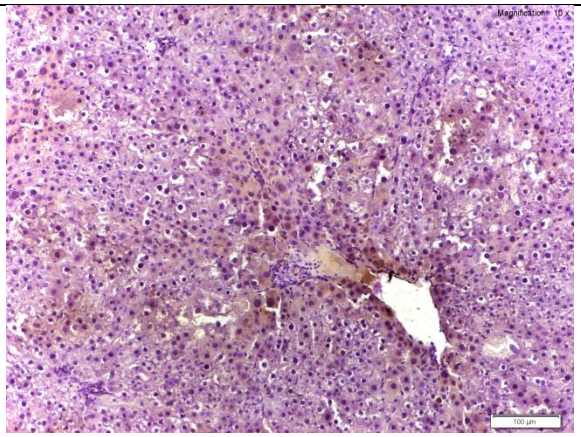
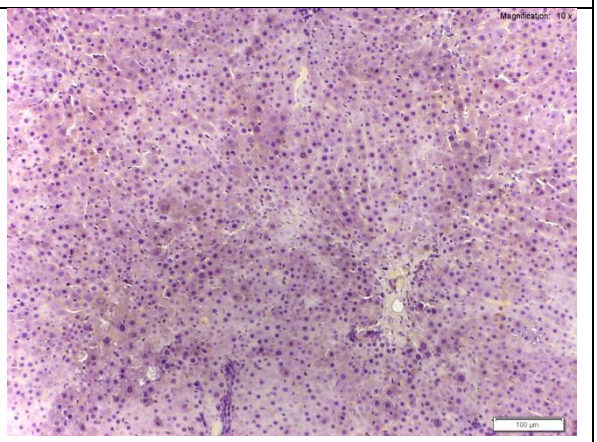
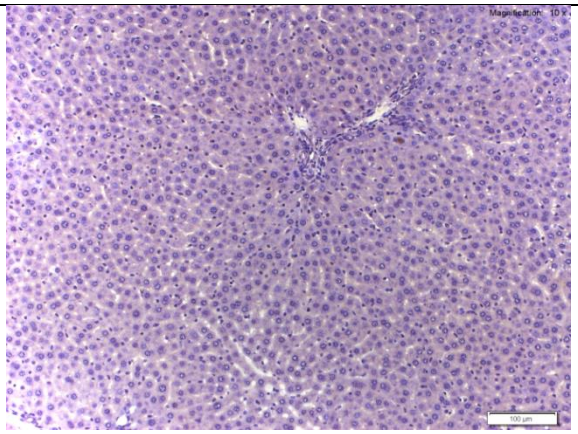
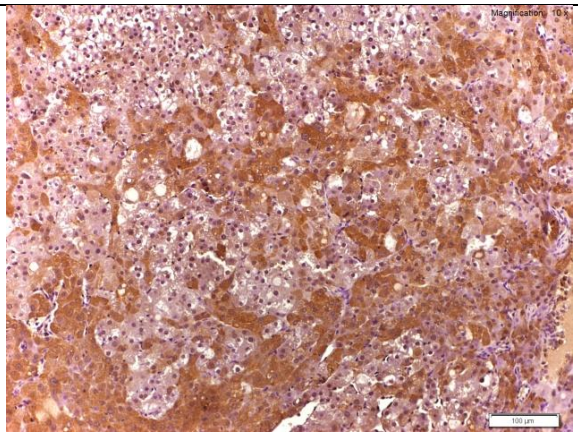
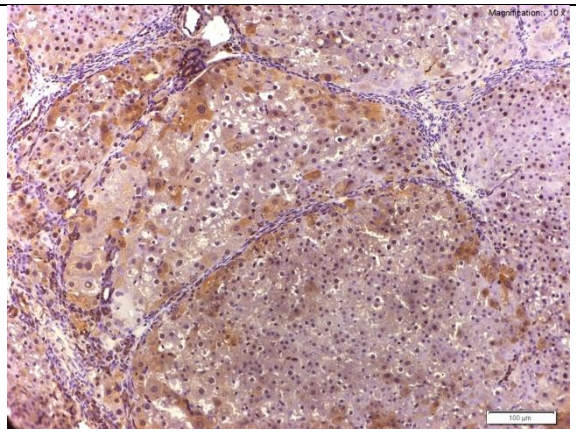
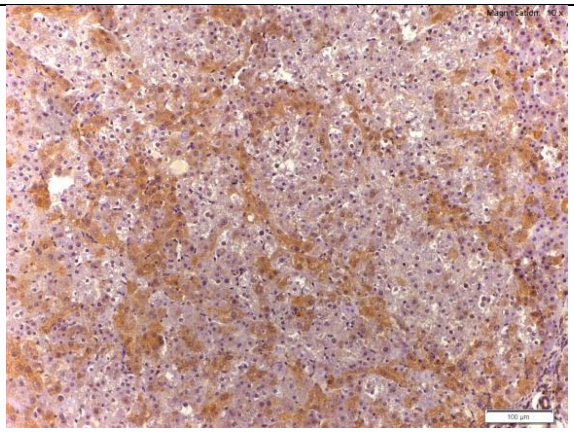
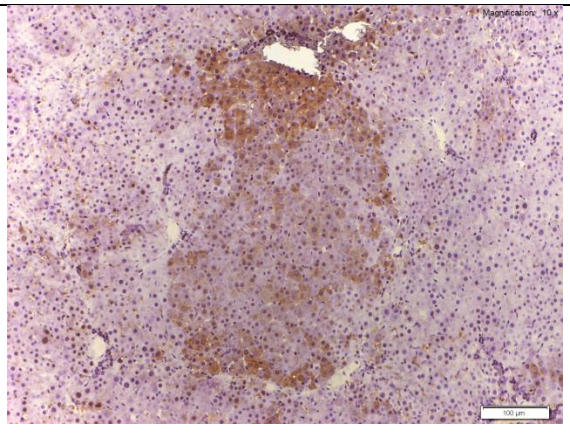
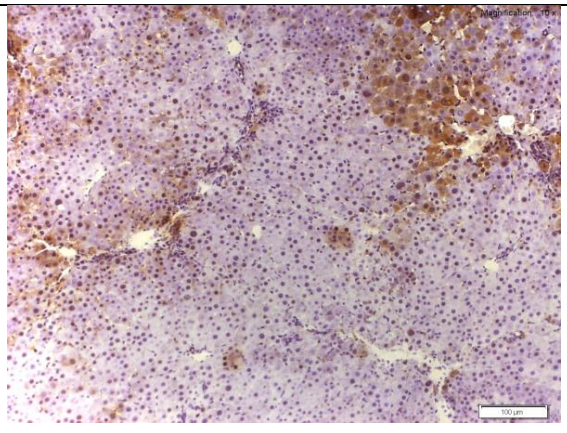
	
Group 1 (10X)	Group 2 (10X)
	
Group 3 (10X)	Group 4 (10X)
	
Group 5 (10X)	Group 6 (10X)

Plate 22: Immuno-histochemical examination for Caspase-3

	
Group 1 (10X)	Group 2 (10X)
	
Group 3 (10X)	Group 4 (10X)
	
Group 5 (10X)	Group 6 (10X)

CHAPTER-5

SUMMARY AND CONCLUSIONS

Saussurea lappa (Kuth) is a plant found in the north western Himalayas and is known for its medicinal properties due to its various photochemical constituents. For the present study, we enumerated the effect of the 70% ethanolic extract of its plant's root against the DEN-induced hepatic damage in rats. Before the start of the experiment, the plant was subjected to UPLC analysis for its phytotchemical compound especially Costunolide and Dehydrocostus lactone both qualitatively and quantitatively. The result of the analysis showed the presence of both the compounds in the freshly collected plant material for Lahaul and Spiti district of HP.

The present conducted study was conducted on 46 male Albino Wistar rats for duration of 10 weeks and the rats were divided into 6 groups which were Group 1: Control group, Group 2: rats treated with 0.01% DEN in drinking water, Group 3: Rats treated with DEN and 25 mg/Kg of Silymarin, Group 4: DEN along with 100 mg/Kg of the plant extract, Group 5: DEN along with 250 mg/kg of the plant extract, group 6: DEN along with 500 mg/Kg of the plant extract. Freshly prepared silymarin and the plant extract were provided to the animals orally by the help of gavaging needle and according to the weekly body weight of the individual animal. The animals were provided with hygienic condition and were monitored regularly for any mortality and clinical signs. The animals treated with DEN alone showed anorexia and decrease water intake after 6^{th} week of the trail accompanied by hair fall while the other groups did not show any adverse effect of the treatment evident clinically.

The acute toxicity of the plant extract did not produce any significant signs and lesions of toxicity in rats. Before sacrificing the rats on completion of 10^{th} week of trial; these were subjected to ultrasonographic examination in order to determine the extent of the damage caused by the chemical. The animals were anaesthetized by using gaseous anaesthetic and the liver of each animal was carefully examined. The examination of rats treated with DEN alone showed increased ecogenicity of the liver along with irregular liver edges and hyperechoic and hypoechoic lesions. The rats in other group showed similar lesions but the intensity of the lesions was greatly reduced.

The rats were euthanized and blood was collected for the estimation of different haematological and biochemical constituents. The liver was examined grossly and the sections were fixed in 10% neural buffered formalin. The haematological examination showed the increase in the leucocyte count in the group treated with DEN alone along with the erythrocytes as compared to control group while the group treated with the ethanolic extracts showed decline in these parameters. The serum biochemical parameters showed an increase in the ALT, AST, ALP, TP and Globulin values while serum albumin level showed a decline in group 2 animals when compared to control group. On the other hand, the groups treated with silymarin and the root extracts showed a tendency towards normalization of these parameters.

The gross lesions in the liver treated with DEN alone showed presence of prominent lobular pattern along with well demarcated white nodules rising from hepatic parenchyma and black-coloured areas of hepatis peliosis. These lesions were also present in the other groups treated with either silymarin or plant root extracts but the severity of these lesions was on decline with the increase in the dose rate of the extract. The group treated with highest dose of the extract showed almost normal hepatic parenchyma which was later on confirmed on histopathological examination.

The result of the histopathological examination of the liver for the group treated with DEN alone showed presence of severe damage to the hepatic cord structure with dysplastic nodules comprising of hyperchromasia and karyomegaly along with severe cirrhotic changes, proliferation of the bile ducts, increased lymphocytic infiltration and vacuolar changes. These lesions showed declined in group treated with silymarin and the ameliorative groups when scored individually. The cirrhotic changes were later confirmed by special stain i.e. Masson’s trichrome stain in which the DEN treated group 2 showed severe fibrous connective tissue proliferation having portal to portal proliferation at times fibrous connective tissue wandering into the hepatic parenchyma. The extract treated groups showed considerable decline in the proliferation of the connective tissue.

The immunohistochemcial analysis of the liver tissue in the group treated with DEN alone showed a significant increase in the expression of inflammatory protein NF-κB and Caspase-3 protein which is crucial mediator in apoptosis of cell. On the other hand, the expression of these proteins declined significantly in the group treated with either silymarin

or ethanolic extract of the plant thereby indicating the hepatoprotective nature of the plant extract used in the present study.

Conclusions:

- 70% ethanolic extract of *Saussurea lappa* at the dose rate of @2g/Kg of b.w. does not produce any acute toxicity.
- Costunolide and Dehydrocostus lactone were present in the samples collected from Lahaul & Spiti (H.P.).
- The plant extract showed a decline in severity of the serum biochemical parameters in dose dependent manner when compared with group treated with DEN alone.
- The results of gross and microscopic studies in the DEN treated group kept on higher dose of *Saussurea lappa* plant root extract showed hepatoprotective effects better than the group kept on silymarin.
- The result of immunohistochemistry studies further substantiated the hepatoprotective nature of the plant extract in dose dependent manner and also better than silymarin treatment.
- Hence it can be concluded from the results of the present study that *Saussurea lappa* roots have hepatoprotective potential and thus, in the future can be exploited for this property in human and veterinary medicine.

CHAPTER-6

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Matric	2011	Carmel Convent School, Nahan	ICSE	81%	1st
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B.V. Sc. & AH	2018	Dr. GC Negi College of Veterinary & Animal Sciences	CSKHPKV, Palampur	67.15%	2nd
M.V. Sc	2020	Dr. GC Negi College of Veterinary & Animal Sciences	CSKHPKV, Palampur	70.15%	1st

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Others

1. Participated in Veterinary Pathology Congress-2019 from 6-8 November, 2019 at CAU, Aizwal, Mizoram.
2. Research paper on **Studies on spontaneously occurring pathological conditions in goats of north western Himalayan region**, 2020, *Ruminant Sciences***.**
3. Research paper on **Clinico-pathological study of chronic nephropathy associated with prostatic hyperplasia in dog**. 2020. *International Journal of Livestock Research,* 10 (8), 205-208