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An Evaluation of Acute Toxicity and Subchronic Toxicity of Hydroethanolic Extract of "*Shirishadi*"- A Polyherbal Ayurvedic Compound in Rodents

Kajaria Divya[1]*, J.S. Tripathi[1], S.K.Tiwari[1] and B.L. Pandey[2]

[1]Faculty of Ayurveda, Department of Kayachikitsa, IMS, BHU, Varanasi, India.
[2]Department of Pharmacology, IMS, BHU, Varanasi, India.

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This study was aimed at evaluating the safety profile, acute and subchronic toxicity of *Shirishadi* polyherbal ayurvedic compound in rodents, commonly used in the treatment of Bronchial Asthma. Acute toxicity was evaluated in wister albino rats by administering orally graded doses of extract of *Albezzia lebbeck*, *Cyperus rotandus* and *Solanum xanthocarpum* (*Shirishadi* Compound) in the dose of 2000, 5000 mg/kg, 10, and 20 g/kg body weight of animal and observed continuously for first 4 h and hourly for next 12 h, then 8 hourly for next 56 h (72 h, acute toxicity study). The study had been strictly followed the OECD guidance. Adult Wister albino rats were divided into 3 groups with 5 animals in each group and a control group, fed with doses of 5, 100 mg and 150 mg/ml once in a day for 30 days. Effect of drug was evaluated on hematological parameter after every 7 days and histological study was done after sacrificing the animals on 31st day. The median acute toxicity value (LD50) of the extract was found to be infinite and drug was found to be totally non toxic as after ingestion of 20 g no animal found to be dead. Hematological profile shows no abnormal elevation in level of Serum-Bilirubin, GOT, GPT, urea and creatinine. There was no any evidence of toxicity in histopathological studies.

Keyword: Asthma, LD 50 value, Histopathological study, Polyherbal compound.

Bronchial asthma is a chronic inflammatory disease of airways. It may be allergic or nonallergic in origin. Sustain inflammation leads to remodeling of airways and cause diminution of their proper functioning.

Asthma is said to be only prevented and not cure and this fact underlies its pathophysiological distinctiveness. Asthma proves to be lethal in its acute manifestation as acute onset of severe bronchoconstriction may cause grave respiratory distress due to lack of proper oxygen saturation. Asthma whether acute or chronic always associated with permanent damage of normal architecture of airways and thus need a careful and proper management. Although contemporary medicines proves beneficial and life saving in bronchial asthma but ultimate dependence on corticosteroids and wide range of toxic side effects forces researcher to think in different dimension and search for some alternative remedy that prove beneficial in preventing as well as cure asthma.

In an attempt to search some cheap, effective, and fast acting remedy of bronchial asthma, some ayurvedic compound are prepare and planned to be given in aerosol form through nebulization. Preclinical study of aforementioned

* To whom all correspondence should be addressed.
E-mail: divyakajaria@gmail.com

said research trial consists of toxicity study of ayurvedic compound in animal model. The route of administration of drug selected consists of both oral and Inhalation route. Whenever an investigator administers a chemical substance to a biological system, different types of interactions can occur and a series of dose-related responses result. In most cases these responses are desired and useful, but there are a number of other effects which are not advantageous. These may or may not be harmful to the patients. The types of toxicity tests which are routinely performed bypharmaceutical manufacturers in the investigation of a new drug involve acute, sub-acute and chronic toxicity. Acute toxicity is involved in estimation of LD50 (the dose which has proved to be lethal (causing death) to 50% of the tested group of animals). Determination of acute oral toxicity is usually an initial screening step in the assessment and evaluation of the toxic characteristics of all compounds. This article reviews the methods so far utilized for the determination of median lethal dose (LD50) and the new changes which could be made. This has to go through the entire process of validation with different categories of substances before its final acceptance by regulatory bodies. The following toxicity studies were performed in the present research trial:

i) Acute toxicity study
ii) Sub chronic toxicity study

Acute Lethality(Barlow et al., 2002; Bürger et al., 2005; Bruce, 1985)

Objectives

1. To determine Maximum Tolerated Dose (MTD) and No Observable Effect Level (NOEL).
2. To determine the Median Lethal Dose (LD50) after a single dose administered through oral route which is the intended route of administration in humans.
3. To identify potential target organs for toxicity, determine reversibility of toxicity, and identify parameters for clinical monitoring.
4. To help select doses for repeated-dose toxicity tests.

Duration

Mortality within 72 hours was cut-off time for acute toxicity however survival of animals were observed up to 2 weeks following single dose administration.

Parameters(Combes, 2004)

§ Mortality.
§ General clinical observation of behavior.
§ Weight change.
§ Gross autopsic examination of dying animals.

Experimental animal (Crook, 2006 Daswani et al., 2006)

Adult Charles Foster Albino rats (70 $\pm$ 30g) of either sex were obtained from the Animal

Table 1. Body weight variation of treated rats with various doses of the Hydroethanolic extract of *Shirishadi* during Acute Toxicity Study

Groups	Body weight of rats (Before Treatment)	Dose of Drug administered	Dose of Drug/ 100g body weight	Body weight of Rats (After treatment)		
				After 24hrs	After 48hrs	After 72 hrs
A	Ist - 90g	0.9ml	200mg/ ml	110g	110g	110g
	Iind - 70g	0.7ml		95g	100g	105g
	IIIrd - 65g	0.65ml		85g	87g	100g
B	Ist - 70g	0.7ml	500mg/ml	90g	90g	95g
	IInd - 70g	0.7ml		75g	90g	95g
	IIIrd - 50 g	0.5ml		60g	62g	65g
C	Ist - 95g	0.95ml	1g/ml	115g	115g	120g
	Iind - 75g	0.75ml		95g	97g	100g
	IIIrd - 75g	0.75ml		100g	100g	100g
D	Ist - 70g	0.75ml+ 0.75ml	1g/ml twice within 1/2hrs interval to make total of 2g.	85g	90g	90g
	Iind - 75g	0.75ml + 0.75ml		90g	90g	90g
	IIIrd - 65g	0.65ml + 0.65ml		65g	65g	65g

Table 2. Effect of Hydroethanolic extract of Shirishadi Extract on various organ weights after 3days of acute toxicity study.

Dose/ 100gm of animals	Lung/ 100g bwtN= 3	Liver/ 100g bwtN= 3	Stomach/ 100g bwtN=3	Kidney /100 g bwt.N= 3	Heart/ 100g bwtN=3	Adrenal gland/ 100g bwtN= 3	Testis/ 100g bwt.N=3	Spleen/ 100g bwtN=3
Control	948+ 1.56	3200+ 0.08	1078+ 1.78	350+ 2.05	330+ 1.98	7.1+ 3.67	884+ 0.56	281+ 1.05
200mg	875+ 2.34	3600+ 1.23	1136+2.3	340+1.56	320+ 2.08	7.8+ 0.68	865+ 1.78	286+ 0.56
500mg	890+ 0.54	3450+ 1.54	1010+ 0.01	300+ 0.04	305+ 3.56	6.9+ 2.36	840+ 0.47	261+ 1.23
1gm	930+ 0.90	3545+ 3.21	1080+ 2.78	365+ 0.64	315+ 0.01	7.5+ 1.67	884+ 0.02	290+ 0.56
2gm	950+ 1.04	3711+ 0.21	1178+ 0.08	370+ 1.02	327+ 1.36	8.0+ 2.98	870+ 0.05	280+ 0.01

All the values are Mean +SDE , where n=3.

Research Branch of the Institute of Medical Sciences, Banaras Hindu University, Varanasi . The animals were housed in polyvinyl cages and were fed on commercial pellet diet (Amrut, Pranav Agro Industries Ltd, India). They were grouped & housed under standard conditions of temperature ($22 \pm 2C^0$), relative humidity ($60 \pm 5\%$) and 12:12 light/dark cycle, where lights on at 0700 and off at 1900 h). The saline fed group served as control and one group was treated with a standard drug in each protocol. Before experimentation, the animals were kept on fast for 24 h but water was given *ad libitum* except during experimental test period. During experiments, animals were also observed for any alteration in their general behavior.

All the experiments and the care of the laboratory animals were according to current ethical guidelines by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India, New Delhi.

The research protocol was approved by Institutional Research committee of Institute of Medical Sciences, BHU- India.

Plant Material and Extraction

Theplants*Albezzia lebbeck,Cyprus rotandus & Solanum xanthocarpum*, were collected from local market of Varanasi. The identification of the drugs was done by Prof.A.K. Singh, Department of Dravyaguna, S.S.U., Varanasi (Identification number DG/ AKS / 604). Hydroalcoholic Extraction (Distilled water: Ethanol = 2:1) of drugs was carried out by hot percolation method through soxhlet appratus. Thereafter extracts were dried using rotatory evaporator and dried extracts were put to the process of standradization.

Drug Treatment

Standradized extract of Ayurvedic compounds dissolved in distilled water was orally administered as graded doses of 50mg, 100mg, & 150mg/ 100g of bwt of animal once daily for 30 days for Subchronic toxicity study. Control rats were treated with equal volume of normal saline. The test substance is administered in a single dose by gavage using a stomach tube or a suitable intubation canula. For Acute toxicity study, standradization extract of*Shirishadi* Polyherbal Ayurvedic compounds was administer as escalated doses of 200mg, 500mg, 1g & 2g/ 100g bwt to

know the LD 50 value. At each step (i.e. for each dose, starting from lowerone) three rodents were used as per OECD guideline (Guideline No.425).

Method Employed(W.J. Dixon,1965 , R.D. Bruce,1985 & R.D. Combes, 2004)

OECD guidelines number 425was followed for Acute Oral Toxicity study with Up-and –Down- Procedure (UDP) .The concept of the up-and-down testing approach was first described by Dixon and Mood

The method is easiest to apply to materials that produce death within one or two days. According to guideline minimum number of animal should be used for the experiment at each step. The test is divided into two parts: Limit test & Main test.

Limit dose at 2000mg /Kg

Step Ist:

One Albino rat of group A weighting was administered 200mg of*Shirishadi* Hydroalcoholic extract dissolved in 1ml of distilled water orally through intubation cannula.

Step IInd:

The rats were observed for 24 hour and when no toxic side effects appears, four more albino rats were taken in group A and administered 2000mg / Kg body weight of *Shirishadi* extract. The animals were watch for 48 hours. After 48

Table 3. Acute Toxicity study of Hydroethanolic Etract of *Shirishadi* in rodents;Death after 7days of treatment.

Group	No. of Albino Rats	Dose of Extract	No. of Dead rats	% Cumulative dead of rats
A	3	200mg/ 100g bwt	0.00	0%
B	3	500mg/100g bwt	0.00	0%
C	3	1g/100g bwt	0.00	0%
D	3	2g/ 100g bwt	0.00	0%

Table 4. Variation in body weight of normal and treated rats with various doses of the *Shirishadi* hydroethanolic extract during 30 days of subchronic toxicity study

Dose/ Kg bwt	Day 1 Bwt(gms)	Day 5 Bwt(gms)	Day 10 Bwt(mgs)	Day 15 Bwt(mgs)	Day 20 Bwt(mgs)	Day 25 Bwt(mgs)	Day 30 Bwt(mgs)
Control	100	101	105	110	112	113	115
	105	106	108	110	113	115	118
	110	112	114	116	118	120	122
	102	103	105	108	110	111	112
	100	100	105	106	110	110	112
50mg	115	120	122	125	128	130	134
	100	105	108	115	120	122	126
	110	115	120	125	130	132	135
	105	108	112	115	118	122	125
	110	115	118	120	125	128	132
100mg	110	112	115	120	125	130	132
	105	110	118	120	122	125	128
	100	105	108	110	112	115	118
	115	120	125	128	130	132	135
	120	124	130	132	135	140	142
150mg	100	102	105	108	110	112	115
	115	115	117	119	122	125	128
	120	122	124	128	130	133	135
	122	125	126	130	132	135	138
	108	110	112	115	118	120	122

hours all the rats were survived with no toxic side effect.

Limit Test at 5000 mg/kg

Step I[st]:

One albino rat of Group B 100gm waas treated with 5000mg/ Kg of *Shirishadi* extract dissolved in distilled water and observed for 48 hrs.The ratsurvived and found healthy after 48 hrs of treatment.

Step II[nd]:

When no observable toxic sign and symptoms appears in the albino rats treated with 5000mg/Kg of polyherbal extracts, two more albino rats in group B were added and administered polyherbal drugs in the dose of 5000mg/Kg. The rats were observed for 48 hours.

As no sign and symptom of toxicity appeared in limit test, 12 more albino rats were taken, divided into two groups and selected for main test.

Next escalated dose of extract (*Shirishadi*) was decided to be 10gm/Kg body weight administered in albino rats of Group C The rats were observed for 48 hrs and when no sing and symptom of toxicity appears six more albino rats were taken and divided into two groups namely D and were given next escalated dose of 20gm/ Kg body weight in two divided dose at the interval of 30 minutes.

As even after administration of trial drug in the dose of 20gm/ Kg body weight, no rat die, LD50 of present trial drug is found to be infinite.

To confirm that the present trial drug is non toxic OECD guidline, Page No. 40, was followed. According to the guideline if 2gms oral administration of drug dose not kill the rodent it should be considered as non –toxic.

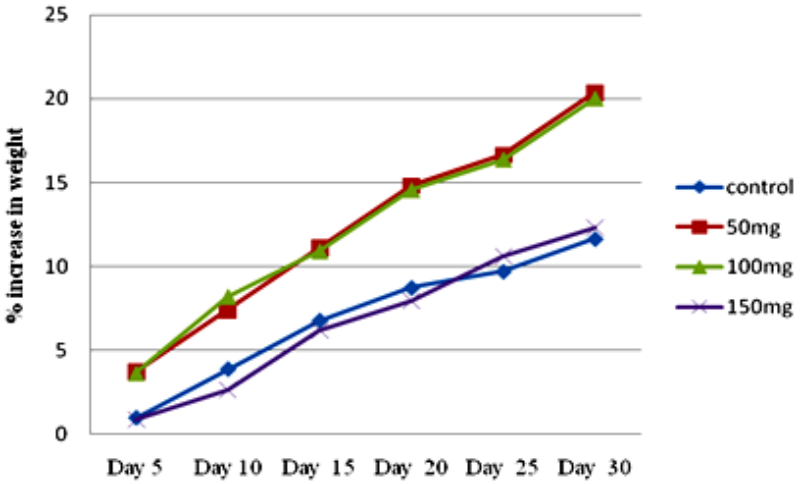

Fig. 1. Percentage increase in weight of the control and animals treated withdifferent doses of *Shirishadi* hydroethanolic extract

Table 5. Body weight variation of normal and treated rats with various doses of the extract during 30 days of subchronic toxicity study.

Dose/ Kg bwt	Day 1	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30
Control	103± 1.88	104±2.16	107±1.74	110±1.67	112± 1.46	113±1.77	115± 1.98
50mg	108± 2.54	112± 2.69	116 ±2.607	120± 2.36	124± 2.28	126±2.05	130±2.06
100mg	110± 3.53	114±3.44	119± 3.83	122±3.79	126±3.89	128± 3.86	132± 3.70
150mg	113±4.04	114±4.14	116± 3.86	120±4.08	122±4.02	125±4.23	127±4.20

All the values are Mean ±SDE , where n=5.

Table 6. Effect of *Shirishadi* Compound on Biochemical profile

Parameter	After 15 days				After 30 days			
	Control	50mg/Kgbwt	100mg/Kgbwt	150mg/Kbwt	Control	50mg/Kgbwt	100mg/Kgbwt	150mg/Kbwt
B.Urea	30.2 ± 2.78	36 ± 5.86	33.6± 2.73	44.6± 4.82	46.7± 4.87	39.2 ± 4.89	30.4± 1.43	50.6± 6.54
S.Creatinine	0.5± 0.05	0.5 ± 0.45	0.6± 0.09	0.5± 0.02	0.68± 0.09	0.52 ± 0.02	0.5 ± 0.05	0.68± 0.08
S.Bilirubin (Total)	0.6± 0.78	0.5± 0.45	0.74± 0.08	0.78± 0.12	0.5± 0.02	0.68± 0.04	0.5± 0.02	0.70± 0.12
S.Bilirubin(Direct)	0.5 ±0.54	0.5 ± 0.05	0.42 ± 0.06	0.56 ± 0.20	0.52± 0.03	0.48 ± 0.09	0.5± 0.08	0.5± 0.05
SGOT (AST)	150± 4.57	100 ± 1.25	143± 7.40	163.7 ± 14.12	150.8± 3.35	123.3± 9.40	107.1± 8.67	168.8± 5.89
SGPT (ALT)	60± 4.38	73.6 ± 2.56	43.4± 5.41	68± 1.90	40.3± 5.36	65.48± 5.29	69.4± 10.63	70± 4.35

All the values are Mean ±SDE , where n=5.

Sub- chronic toxicity study(R.D.Combes,2004 & S.M.Barlow,2002)

Objectives

1. To establish a "no observable effect level" (NOEL).
2. To characterize dose-response relationships following repeated doses.
3. To identify and characterize specific organs affected after repeated administration.
4. To predict a reasonable and appropriate dose for chronic exposure studies (maximum tolerated dose or MTD).

Duration: 30 days.

Test System/Animal System: Rodent

Dose Administration : Male and female Wistar albino rats weighing 100 ± 20 g were maintained on standard animal feeds and provided with water *ad libitum*. The animals were weighed and divided into seven groups of five animals each. After overnight fasting of the rats, the control group received a dose of 0.5 ml of normal saline solution orally once a day for 30 days. The treated groups respectively received the following doses: 50, 100, 150 mg/kg body weight of the hydroalcoholic extracts (*Shirishadi* &*Bharangyadi*) orally once daily for 30 days (Pieme *et al*., 2006; Joshi *et al*., 2007; Mythilypriya el al., 2007).The animals were then weighed every five days, from the start of the treatment, to note any weight variation.

Parameters

1. Mortality
2. Weight change
3. Signs of toxicity
4. Clinical pathology
5. Pathology and histopathology

RESULTS AND DISCUSSION

The result shows that *Shirishadi* polyherbal compound is innocuous and very safe for therapeutic use (Yashada, 2006). There were no abnormalities detected in histopathological study nor any biochemical abnormalities were identified in organ sample of sacrificed rodents after acute toxicity study. In chronic treatment with higher doses, death rate showed 20 and 40% cumulative increase. However there is no evidence of organic toxicity in the animals. Studies may be performed in very exclusive conditions to evaluate the issue of long term safety. The acute toxicity study of the extract (*Shirishadi* compound) indicated no changes in the behaviour and in the sensory nervous

system responses in the animals. Also no adverse gastrointestinal effects were observed in the albino rats. The LD50 calculated for the drug is found to be infinite (according to OECD guidelines) as no rat died even after oral administration of dose 20 g/kg bwt (Table 1). Histopathalogical study of viscera's showed no microscopic or macroscopic abnormality without any change in colour, no congestion, no necrosis or any other sign of toxicity (Plates 6, 7, 8, 9 and 10).

Biochemical profile including –Liver function test (LFT), Renal function test (RFT) show

Table 7. Sub-Chronic Toxicity study in albino rats with *Shirishadi* extract

Groups	No. of Rats	Dose of extract	No.of Dead Rats	% Cumulative dead of Rats
A	5	Control	0	0%
B	5	50mg/ Kgbwt	0	0%
C	5	100mg/Kgbwt	1	20%
D	5	150mg/ Kgbwt	2	40%

Plate 1. Showing different groups of animal

Plate 2. Measuring diet for animals during for toxicity studies sub- chronic toxicity study

Plate 3. Showing animals taking diet during toxicity studies

Plate 4. Different concentration of drugs

no abnormal change substantiated that drug has no renal or hepatotoxicity (Table 2). Percentage cumulative dead after acute toxicity study was found to be 0% (Table 3).

Body weight of albino rat's increases propionate with dose of drug but become stabilized at higher doses (Figure 1). As there was increase intake of feed with increasing dose of drug, the

Plate 5. Samples of organ specimens collected after dissection for histopathological studies

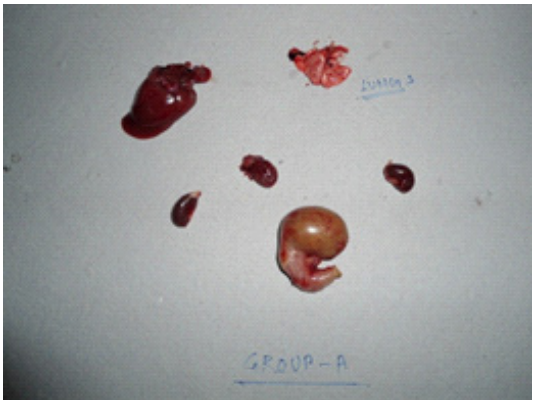

Plate 6. Different organ after dissection of albino

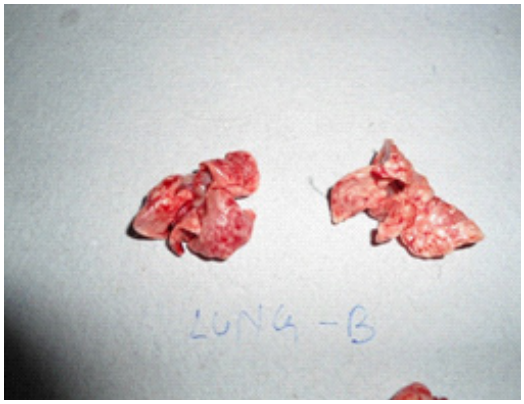

Plate 7. Sample of lungs showing no macroscopic rats in Acute toxicity study. changes (colour, texture etc.) in albino rat treated with 2gm of *Shirishadi* Compound

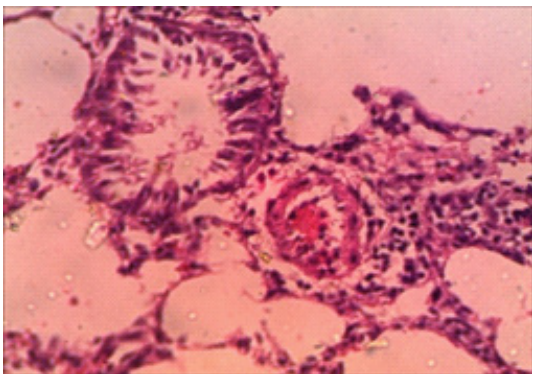

Plate 8. Microphotograph of rat's lung (*Shirishadi* compound treated group) Showing normal alveoli & lung parenchyma (40x) in Acute toxicity study.

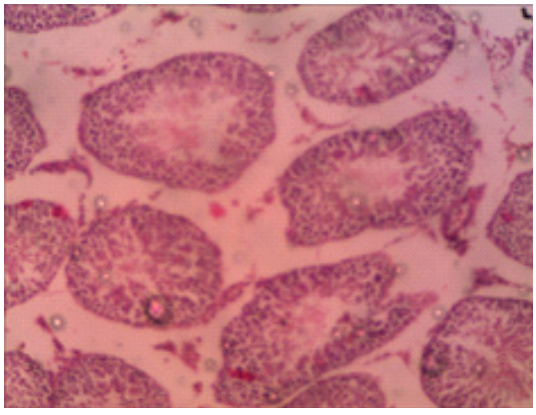

Plate 9. Showing normal Histology of testis tissues treated with *Shirishadi* compound in Acute toxicity study

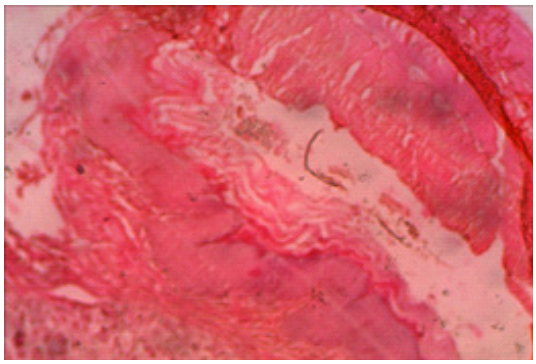

Plate 10. Showing normal histology of rat's Stomach tissuse treated with *Shirisahdi* compound in acute toxicity study(40x)

increase in body weight was indicative of anabolic effect (Tables 4 and 5). Biochemical analysis showed no toxic or abnormal changes suggesting that drug is safe for long duration internal use (Table 6). There was 40% cumulative death in group treated with 150 mg of *Shirishadi* extract during subchornic study whereas 20% (Table 7) in group treated with 100 mg of extract.

As there were no morbid signs found prior to death and in addition to this there was no any evidence of toxicity in histopathological studies (Plates 11, 12, 13 and 14) excludes the possibility of death due to toxic effect of drug.

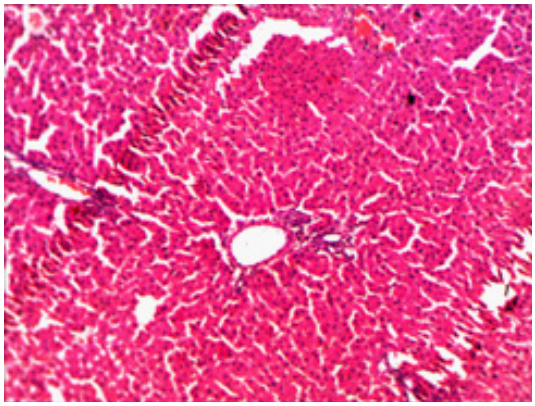

Plate 11. Showing histology of liver tissuses of albino rat treatedwith *Shirishadi* compound in Acute toxicityStudy. Microphotograph reveals normal hepatocytes, central vein & portal tract (40x)

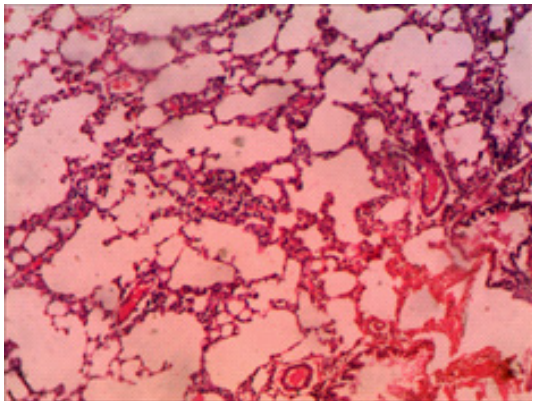

Plate 12. Showing normal histology of lung tissuse of albino rat treated with *Shirishadi* Compound in Sub-chronic toxicity study (40x)

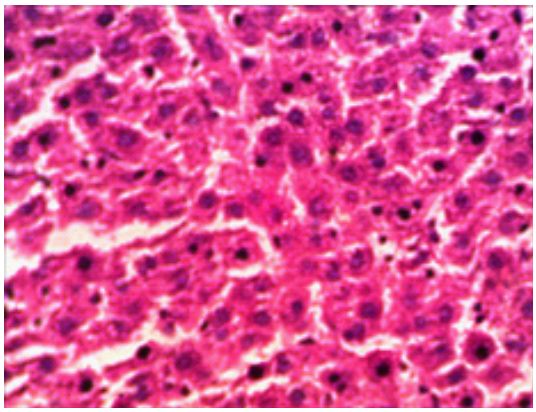

Plate 13. Showing histology of Kidney tissues of albino rat treated with*Shirishadi* compound in Sub- chronic toxicity study (40x)

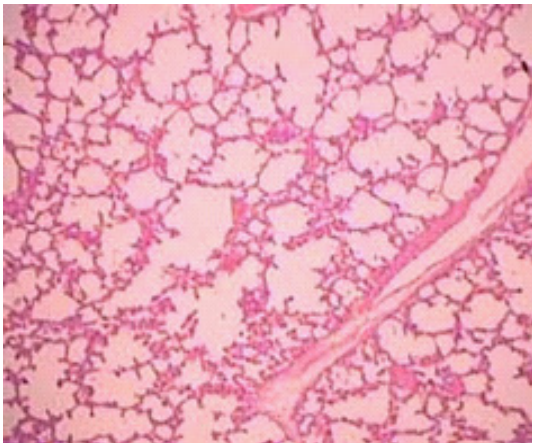

Plate 14. Showing histology of lung tissue after acute toxicity study.Many small, bubble-like alveoli can be seen in the thistissue, as well as a small bronchiole running obliquely along the right side of the specimen

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