"Phytochemical, antioxidant and hepatoprotective effect of plant *Tribulus terrestris* and *Phyllanthus Niruri* against paracetamol induced liver injury in rats."

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Abstract:

Hepato- protective herbsplaya vital role for the treatment of Liver disease In order to overcome this difficulty a novel attempt has been made to standardize the drug *P. niruri & Tribulus terrestris* for its hepatoprotective properties by using analytical, preclinical studies. The drug P. niruri & Tribulus terrestris was selected from the to validate the safety and its efficacy of CCL4 and Paracetamol induced hepatotoxicity. According to the OECD guideline 423, it was concluded that the test drug P. niruri & Tribulus terrestris is a safest drug. No mortality was obtained. Toxicological study of both acute and sub-acute toxicity study were carried out in animal model Wistar albino rat according to the OECD guidelines. The test drug showed no acute toxicity as there was no mortality seen. The sub-acute toxicity after the repeated dose of 28 days was done. The mortality, functional observations, hematological and biochemical investigations were made. There was no significant change seen in the normal values. Thus the toxicological study of the test drug greatly establishes the safety and gives the justification for long time administration. In Conclusion, no toxic effect was observed up to 200mg/kg of *P. niruri & Tribulus terrestris* treated over a period of 28 days (OECD 423). So, it can be concluded that the *P. niruri & Tribulus terrestris* can be prescribed for therapeutic use in human with the dosage recommendations of up to 100mg/kg body weight p.o

Keywords: Hepatotoxicity, *P. niruri & Tribulus terrestris,* OECD 423, Morbidity, Antioxidant activity.

Introduction

Medicinal plants play a key role in the human health care. About 80% of the world population rely on the use of traditional medicine which is predominantly based on plant materials.[1] Liver is considered to be one of the most vital organs that functions as a center of metabolism of nutrients such as carbohydrates, proteins and lipids and excretion of waste metabolites.[2] Additionally, it is also handling the metabolism and excretion of drugs and other xenobiotics from the body thereby providing protection against foreign substances by detoxifying and eliminating them.[3] The bile secreted by the liver has, among other things, plays an important role in digestion. Liver cell injury caused by various toxicants such as certain chemotherapeutic agents, carbon tetra chloride, thioaceta amide etc., chronic alcohol consumption and microbes is well-studied. Enhanced lipid peroxidation during metabolism of ethanol may result in development of hepatitis leading to cirrhosis.[4]

Hepatoprotective herbs

Herbal-based therapeutics for liver disorders has been in use in India for a long time and has been popularized world over by leading pharmaceuticals.[5] Despite the significant popularity of several herbal medicines in general, and for liver diseases in particular, they are still unacceptable treatment modalities for liver dis-eases. The limiting factors that contribute to this eventuality are (i) lack of standardization of the herbal drugs; (ii) lack of identification of active ingredient(s)/principles(s); (iii) lack of randomized controlled clinical trials (RCTs), and (iv) lack of toxicological evaluation.[6]

Collection of Plant material and Authentication

P. niruri & Tribulus terrestris was collected in July- August 2022 from the Botanical Garden Balaghat, The plant material was identified and authenticated taxonomically at Nirmal Institute of Agriculture Technology Gondia 441614.

Chemicals

DPPH (1,1-diphenyl,2-picryl hydrazyl), nitro tetrazolium blue (NBT), ethylene diamine tetra acetic acid (EDTA), 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB), 2,4-Dinitro phenyl hydrazine (DNPH). All other chemicals were of analytical grade.

Plant Preparation

The plant preparation was done, about 20 grams of dried leaves of *P. niruri & T. terrestris* were placed in thimbles in the water as solvent then, followed by transferring the thimbles into Soxhlet extractors, and 300 ml grade water was added to flask, which was linked to the extractor and condenser.[7] The extraction was performed in 24 hours. After the extraction, solvent were evaporated to dryness in a rotary evaporator to obtain the crude extracts of *P. niruri & T. terrestris* The extracts were finally lyophilized and stored at 20^0C until the tests were performed

Phytochemical Screening

The phytochemical components of *P. niruri & T. terrestris* leaves were screened using standard method described by Harborne Alkaloids, tannins, flavonoids, saponins, steroids, lignans, glycoside and anthroquinones were screened qualitatively for its presence in leaves extract. The color intensity of the precipitate was measured.[8]

Table 1: Qualitative analysis of Phytochemical in leaf extracts of *P. niruri & Tribulus terrestris*

Phytochemical	**Aqueous Extract of *P. niruri & Tribulus terrestris***
Lignan	++
Anthraqunoles	+
Flavanoids	++
Sapnonin	++
Tanins	++
Alkaloids	-
Glycosides	+
Steroids	++

Strongly Present ++; Present +; Absent –

Table 2: Antioxidant activity of *P. niruri & Tribulus terrestris*

Concentration (µg/ml)	SOD Activity % Inhibition		Hydrogen Peroxide scavenging % Inhibition		DPPH Scavenging %	
	Ascorbate	Aqueous Extract	Ascorbate	Aqueous Extract	Ascorbate	Aqueous Extract
20	22.13	27.63	22.12	24.25	31.15	27.93
40	39.57	54.58	34.93	31.45	49.11	50.63
60	68.48	74.89	50.16	49.83	75.85	74.09
80	82.29	83.61	67.26	69.43	91.31	86.01
100	73.82	91.64	74.53	74.35	98.71	89.66

ACUTE ORAL TOXICITY – OECD GUIDELINES - 423

Acute toxicity study was carried out as per OECD guideline. (Organization for Economic Co - operation and Development, Guideline-423 Studied carried out at six female rats under fasting condition, signs of toxicity were observed for every one hour for first 24 hours and every day for about 14 days from the beginning of the study.[9]

Principle:

It is the principle of the test that based on a stepwise procedure with the use of a minimum number of animals per step; sufficient information is obtained on the acute toxicity of the test substance to enable its classification. The substance is administered orally to a group of experimental animals at one of the defined doses. The substance is tested using a stepwise procedure, each step using three animals of a single sex.[10]

METHODOLOGY

Selection of animal species:

The preferred rodent species was rat, although other rodent species may be used. Female

should be nulliparous and non-pregnant. Each animal at the commencement of its dosing should be between 8 and 12 weeks old and its weight should fall in an interval within ±20 % of the mean weight of the animals [11]

Housing and feeding conditions:

The temperature in the experimental animal room should be 22ºC (+3ºC). Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hrs light, 12 hrs dark. For feeding,conventional laboratory diets may be used with an unlimited supply of drinking water. Animals may be grouped and tagged by dose, but the number of animals per cage must not interfere with clear observations of each animal.[12]

Condition of animals in laboratory.

The animals are randomly selected, marked to permit individual identification, and kept in their cages for at least 7 days prior to dosing to allow for acclimatization to the laboratory conditions.

EXPERIMENT PROCEDURE

Administration of doses

P. niruri & Tribulus terrestris prepared as per the literature was suspended in 2% CMC with uniform mixing and was administered to the groups of wistar albino rats. It was given in a single oral dose by gavages using a feeding needle. Animals were fasted prior to dosing. Following the period of fasting, the animals were weighed and then the test substance was administered. After the substance has been administered, food was withheld for a further 3-4 hours. The principle of laboratory animal care was followed. Observations were made and recorded systematically and continuously observed as per the guideline after substance administration. The visual observations included skin changes, mobility, and aggressiveness, sensitivity to sound and pain, as well as respiratory movements.

Number of animals and dose levels

Since this test drug has been under practice for long time and likely to be non- toxic, a limit test at one dose level of 2000 mg/kg body weight will be carried out with6 animals (3 animals per step).

Duration of Study : 48 hrs

Evaluation : 14 Days

TOXICITY STUDY RESULTS

Table: 3. Dose finding experiment and its behavioral Signs of Toxicity for *P. niruri & Tribulus terrestris*

Observation done:

Group	**Day**
Body weight	Increased
Assessments of posture	Normal
Signs of Convulsion Limb paralysis	Absence (-)
Body tone	Normal
Lacrimation	Absence
Salivation	Absence
Change in skin color	No significant colour change
Piloerection	Normal
Group	**Day**
Defecation	Normal
Sensitivity response	Normal
Locomotion	Normal
Muscle gripness	Normal
Rearing	Mild
Ur	No

Table: 4. Dose finding experiment and its behavioral Signs of Toxicity for *P. niruri & Tribulus terrestris*

Dose mg/kg	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**

2000	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-

1. Alertness 2. Aggressiveness 3. Pile erection 4. Grooming 5. Gripping 6. Touch Response 7. Decreased Motor Activity 8.Tremors 9 Convulsions 10. Muscle Spasm 11. Catatonia 12. Musclerelaxant 13. Hypnosis 14. Analgesia 15. Lacrimation 16. Exophthalmos 17. Diarrhea 18. Writhing 19 Respirations Mortality

Interpretation:

In the acute toxicity study, the rats were treated with different concentration of *P. niruri & Tribulus terrestris* from the range of 5mg/kg to 200mg/kg which did not produce signs of toxicity, behavioral changes, and mortality in the test groups as compared to the controls when observed during 14 days of the acute toxicity experimental period. These results showed that a single oral dose of the extract showed no mortality of these rats even under higher dosage levels indicating the high margin of safety of this extract. In acute toxicity test the *P. niruri & Tribulus terrestris* was found to be non-toxic at the dose level of 2000mg/ kg body weight.

Weight gain of wistar albino rat

Table: 5. Body weight (g) changes of rats when exposed to *P. niruri & Tribulus terrestris*

Dose (mg/kg/day)	***Days***				
	0	**7**	**14**	**21**	**28**
Control	120.59±0.92	122.79±0.87	123.52±1.18	127.24±1.12	131.25±1.05
100 mg	121.53±0.93	124.14±0.58	127.24±1.15	128.92±1.40	132.23±1.05
200 mg	122.65±0.91	127.83±0.90	128.23±1.15	130.59±1.59	134.05±0.98

*Values are expressed as mean ± S.E.M; N=3; *P<0.05, **P<0.01, ***P<0.001 vs control.*

Interpretation

The total body weight of the animals was weighed on 1st, 7th, 14th, 21st, 28th day and is shown in the table. It was found that the test drug produced significant weight gain than control, with administration of the drug. Similarly the test drug at all dose levels induced weight gain and we could see longer the duration of administration of drug higher was the weight gain.

Results of organ weight in albino wistar rat

Table. : 6. Effect of *P. niruri & Tribulus terrestris* on organ weight in rats

Organ	Control	100 mg/kg	200 mg/kg
Liver (g)	3.07±0.20	4.41±0.32	4.72±0.32
Heart (g)	0.32±0.04	0.37±0.01	0.42±0.02
Lung (g)	0.28±0.05	0.32±0.01	0.38±0.01
Spleen (g)	0.74±0.07	0.68±0.17	0.78±0.08
Brain (g)	0.37±0.05	0.47±0.02	0.54±0.03
Kidney (g)	0.76±0.05	0.89±0.01	0.91±0.02

*Values are expressed as mean ± S.E.M; N=3; *P<0.05, **P<0.01, ***P<0.001 vs control.*

Interpretation

Results of Haematological parameters

Table: 7. Effect of *P. niruri & Tribulus terrestris* on Haematological parameters in rats

Parameter	Control	100mg/kg	200 mg/kg
RBC(x 10^6/mm^3)	8.29±0.43	8.27±0.44	8.26±0.44
PCV (%)	49.66±0.77	47.98±1.04	47.20±0.91
Hb (%)	15.13±0.39	14.83±0.40	15.03±0.39
WBC(x 10^3/mm^3)	11.75±0.85	11.73±0.85	11.74±0.85
Neutrophils (%)	23.29±0.73	21.94±1.03	22.96±0.49
Eosinophills (%)	4.10±0.23	3.8±0.25	3.97±0.23
Lymphocyte (%)	85.5±0.46	83.7±0.72	84.87±0.32

Platelets(x 10^3/mm^3)	425.73±1.35	423.43±1.47	425.03±1.26

*Values are expressed as mean ± S.E.M; N=3; *P<0.05, **P<0.01, ***P<0.001 vs control.*

Interpretation

The hematological investigation results of the rats conducted on 28th day after the repeated dose of the drug revealed the values of different parameters. There is a slight variation in the values of RBC count values in the dose group of 100 and 200 when compared with that of the control. The increase and decrease in the values obtained were all within the normal biological and laboratory limits.

Results of Biochemical Parameters

Table.No:8. Effect of *P. niruri & Tribulus terrestris* on biochemical parameters in rats

Parameters	Control	100 mg/kg	200 mg/kg
Protein (g/dl)	8.58 ± 0.68	7.56±0.61	6.76±0.44
Albumin (g/dl)	5.34 ± 0.40	5.29±0.44	3.5±0.54
BUN (mg/dl)	22.06 ± 1.55	22.72±1.9	25.53±1.8
Urea (mg/dl)	64.24 ± 3.11	66.7±5.3	69.2±2.9
Creatinine (mg/dl)	0.85 ± 0.07	0.6±0.24	0.71±0.25
Total Cholesterol (mg/dl)	93.21 ± 1.16	92.17±1.13	91.53±1.35
Triglycerides (mg/dl)	52.58 ± 1.56	52.16±1.3	52.93±1.7
Glucose (mg/dl)	108.63 ± 0.81	107.97±1.12	109.4±0.51
Total Bilirubin (mg/dl)	0.205 ± 0.04	0.16±0.08	0.10±0.03
SGOT (U/L)	73 ± 2.4	72.67±1.64	65.52±2.4
SGPT(U/L)	28.4 ± 1.2	25.77±0.64	23.99±0.70
Alkaline phosphatase(U/L)	102.4 ± 3.6	101.3±1.5	91.33±4.26

*Values are expressed as mean □ S.E.M; N=3; *P<0.05, **P<0.01, ***P<0.001 vs control.*

Interpretation

The biochemical investigations were conducted on 28th day and the results are produced. The results revealed that there is a slight significant change in the values of different parameters with that of the control. All the values were within the normal biological and laboratory limits.

Results of Urine parameters

Table.No:9. Effect of *P. niruri & Tribulus terrestris* on Urine parameters in rats

Parameters	Control	100 mg/kg	200 mg/kg
Color	Yellow	Yellow	Yellow
Transparency	Clear	Clear	Clear
Specific gravity	1.01	1.02	1.02
PH	7.2	7.4	7.3
Protein	Nil	Nil	Nil
Glucose	Nil	Nil	Nil
Bilirubin	-ve	-ve	-ve
Ketones	-ve	-ve	-ve
Blood	Absent	Absent	Absent
RBCs	Nil	Nil	Nil
Epithelial cells	Nil	Nil	Nil
Casts	Nil	Nil	Nil

Interpretation

Urine analysis data of control group and the test groups of animals taken on 28th day showed no abnormal results.

The above results showed that all parameters remained within normal limits.

Interpretation

The above slides show the histopathology studies of sub-acute toxicity. There is no toxicological abnormality seen in the vital organs after administration of the test drug *P. niruri & Tribulus terrestris* Thus the safety of the drug is revealed so that it can be administered for long time without any side effects.

Pharmacological Result: Paracetamol

INDUCED HEPATOTOXICITY

Table: 10. Effects of Serum Enzymes on *P. niruri & Tribulus terrestris*

Group	Treatment	SGPT U/L	SGOT U/L
1	Control	24.185 ±1.62	28.95 ±1.46
2	Paracetamol + LP	102.8 ±1.25	167.84 ±2.94
3	Standard (Silymarin – 100 mg)	30.95 ±2.34	43.8 ±2.35**
4	Paracetamol + low dose *SPC* – 100 mg	68 ±2.51	70.76 ±1.43
5	Paracetamol + high dose *SPC* – 200 mg	34.53 ±3.38*	40.05 ±1.72*

*Values are expressed as mean S.E.M; N=6; *P<0.05, **P<0.01, ***P<0.001 vs control.*

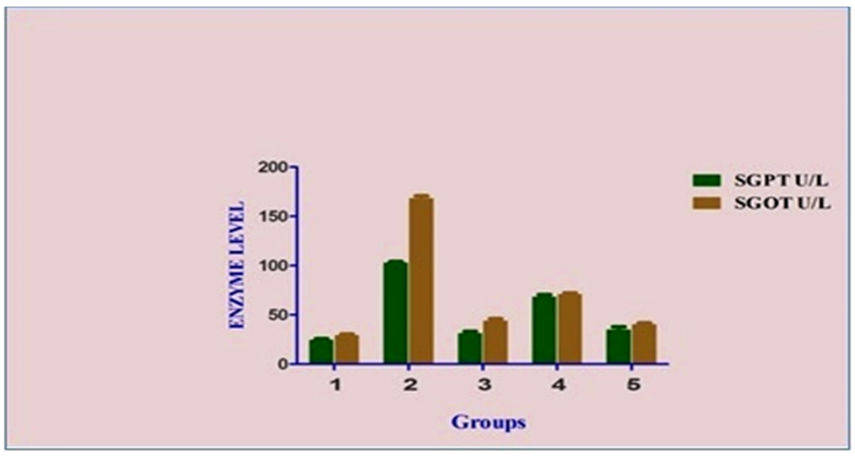

Figure no. 1. Effect of Serum Enzymes on *P. niruri & Tribulus terrestris*

Table: 11. Effect of Serum Enzyme (ALP) on *P. niruri & Tribulus terrestris*

Group	Treatment	ALP (mg/dl)
1	Control	39.86 ±3.46
2	Paracetamol + LP	89.33 ±0.97
3	Standard (Silymarin -100mg)	47.81 ±4.69**
4	Paracetamol + low dose *SPC* – 100 mg	76.05 ±2.96*
5	Paracetamol + high dose *SPC* – 200 mg	54.31 ±3.84*

Values are expressed as mean S.E.M*; N=6; *P<0.05, **P<0.01, ***P<0.001 vs control*

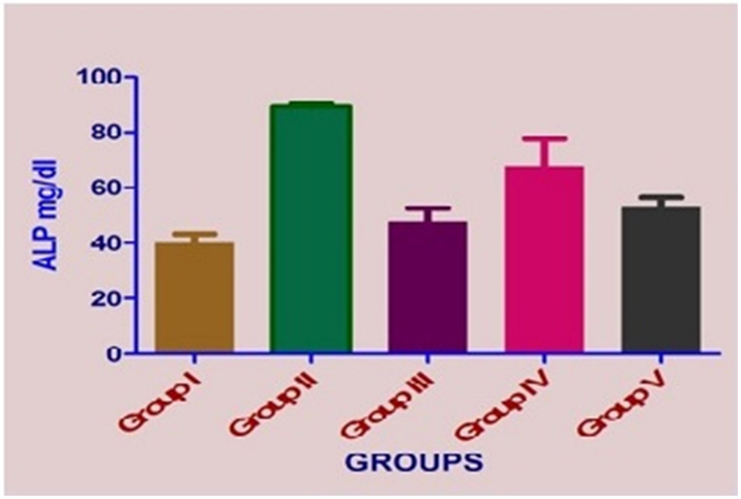

Figure no 2. Effect of Serum Enzyme (ALP) on *P. niruri & Tribulus terrestris*

Table: 12. Effects of Bilirubin Levels on *P. niruri & Tribulus terrestris*

Group	Treatment	Total Bilirubin mg/dl	Direct Bilirubin mg/dl
1	Control	0.253 ±0.003	0.17 ±0.003
2	Paracetamol + LP	1.5 ±0.1	1.53 ±0.01
3	Standard (Silymarin – 100mg)	0.44 ±0.007	0.24 ±0.004
4	Paracetamol + low dose *SPC* – 100 mg	1.25 ±0.015	1.21 ±0.040
5	Paracetamol + high dose *SPC* – 200 mg	0.52 ±0.002	0.20 ±0.018

*Values are expressed as mean S.E.M; N=6; *P<0.05, **P<0.01, ***P<0.001 vs control.*

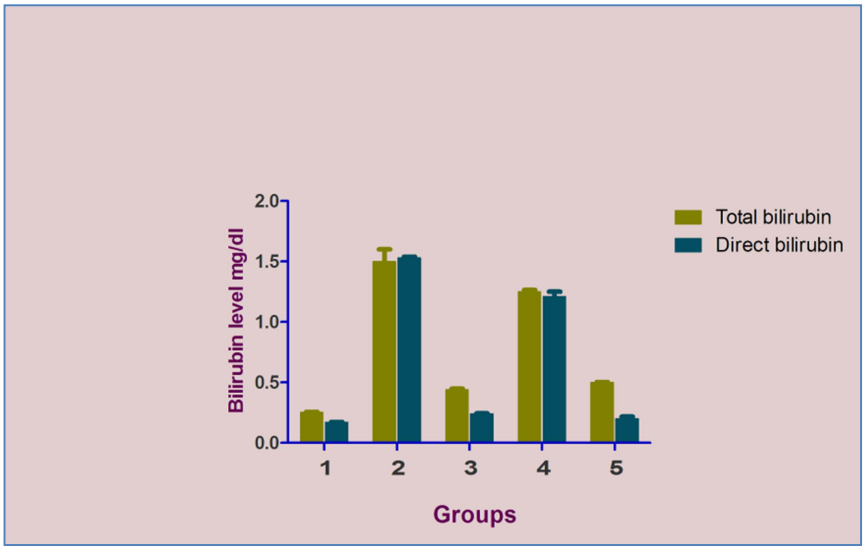

Figure no 3. Effects of Bilirubin Levels on *P. niruri & Tribulus terrestris*

- Group I: Control rat showing normal central vein and normal hepatocytes
- Group II: Showing dilated central vein and hepatocytes with degeneration
- Group III: Liver tissue of rats treated with *P. niruri & Tribulus terrestris* at 100mg/kg showing milddegree of necrosis (N) with normal cells (C)
- Group IV: Central vein showing normal hepatocytes with regenerating hepatocytes and mild inflammation in the portal area

- Group V: Photomicrograph of liver tissue treated with Silymarin showing normal hepatocytes, portal vein (V) and portal artery.

Conclusion

Liver diseases are the most common health problem in the world. The Liver is quantitatively the most important site of drug metabolism. However many drugs are known to cause hepatic injury. Conventional and synthetic drugs used in the treatment of liver diseases are sometimes inadequate and can have serious adverse effect. In order to overcome this difficulty a novel attempt has been made to standardize the drug *P. niruri & Tribulus* terrestris for its hepatoprotective properties by using analytical, preclinical studies. The drug P. niruri & Tribulus terrestris was selected from the to validate the safety and its efficacy of Paracetamol induced hepatotoxicity

Hepatoprotective activity against, Paracetamol:

The present study showed that *P. niruri & Tribulus terrestris* produce protective against the hepatotoxicity induced by Paracetamol. The hepatoprotective role of *P. niruri & Tribulus terrestris* might be due to its chemical constituent. Hence *P. niruri & Tribulus terrestris* may be act as prophylactic as well as curative drug in treating hepato toxic conditions. Further studies needs to isolate the active constituents and mechanism of action. Thus the author validates *P. niruri & Tribulus terrestris* as a new hepato-protective drug which is cost effective and without any side effect.

Reference

1. Ali BH, Blunden G, Tanira MO, Nemmar A. Some phytochemical, pharmacological and toxicological properties of ginger (Zingiber officinale Roscoe): a review of recent research. Food Chem Toxicol 2008; 46:409-420.
2. Farnsworth, N.R., Akerele, O. Bingel, A.S., Soejar to, D.D. and Guo, Z. Medicinal plants in therapy. Bulletin of World Health Organization, 965-981, 1985.
3. Handa SS, Sharma A, Chakraborti KK. Natural products and plants as liver protecting drugs. Fitoterapia,57:307-51, 1986.
4. Jezequel AM Mancini R, Rinaldesi ML et al. A morphological study of the early stages

of hepatic fibrosis induced by low doses of dimethyl nitrosamine in the rat. J Hepatol 1987; 5; 174-181.

5. K. George Mathew and Praveen Agrawal, Prep Manual for Undergraduates, Medicine, Elsevier, 2nd Edition; 349, 2014
6. Luper, S.N.D, A review of plants used in the treatment of liver disease: Part 1. Alternative Medicine Review, 410-421, 1998
7. Orient Longman Indian medicinal plants, a compendium of 500 species, Orient Longman pvt ltd, 1st volume, 1st edition, Annasalai, Chennai-600002, page no: 191, 1994.
8. Pessoa MG, Terrault NA, Detmer J. Quantitation of hepatitis G and C viruses in the liver: evidence that hepatitis G virus is not hepatotropic. Hepatology1998; 27:877.
9. Rathod Jaimik D, Patak Nimish L, Patel Ritesh G, Jivani Nuruddin Patel, Laxman D, ChauhanVijay, Ameliorative effect of bambusa arundinacea against adjuvant arthritis-with special reference to bone erosion and tropical splenomegaly, Journal of Drug Delivery and Therapeutics; 2012, 2(3): 141-145.
10. S.Venkatrajan, Sarabendra Vaidhiya Muraigal, Soolai, Moola, Kusta, Pitharogauraigal, Saraswathi Mahal Noolagam, 2nd edition, 194-195, 2000.
11. Schuppan D., Jia, J.D., Brinkhaus, B. and Hahn, E.G. Herbal products for liver diseases: A therapeutic challenge for the new millenium. Hepatology, 30(4), 1099-1104, 1999.
12. Shanmugavelu M, Noikaluku Siddha Parigaram, Department of Indian Medicine and Homoeopathy. Part -1, 3rd Edition. Oct 1999; pg no: 88, 93.