

## 28-DAYS SUB-ACUTE TOXICITY PROFILE OF ETHANOLIC EXTRACT OF *NYCTANTHES ARBOR-TRISTIS* CALYX IN RATS

Pawar, N.<sup>1,2\*</sup>; Gadgoli, C. <sup>1</sup>; Kamble, M.<sup>1</sup>; Panchal, S.<sup>2</sup>

<sup>1</sup>Saraswathi Vidya Bhavan's College of Pharmacy, University of Mumbai, Dombivli (E), Maharashtra, India

<sup>2</sup>Institute of Pharmacy, NIRMA University, Ahmedabad, Gujarat, India

[\\*panchalshital22@gmail.com](mailto:*panchalshital22@gmail.com) ; [unoneha@gmail.com](mailto:unoneha@gmail.com)

### Abstract

Nyctanthes arbour-tristis (NAT), Oleaceae, is one of the most popular medicinal plant in Ayurvedic and Homeopathic Systems of Indian medicine. NAT flowers possess multiple pharmacological activities and its calyx is reported to possess carotenoids which is responsible for rendering all its medicinal properties. The rationale behind present study involves NAT flowers consumption for various medicinal utilities and commonly as a food ingredient without any information about its associated risks. Therefore for the present study, OECD guideline no.407 of 28 days sub-acute toxicity study was followed critically by using 48 wistar rats of either sex (24 male and 24 female). The dose range of 250, 500 and 750 mg/kg body weight of NAT extract was selected based on preliminary dose determination experiments and administered by oral gavage to the respective groups. NAT calyx extract treatment induced significant alterations in essential haematological parameters like RBCs, MCV, MCHC and Platelets in atleast one treated group thereby indicating its toxic effect on haematological system after the exposure for 28 days. ALT levels were significantly increased by NAT treatment suggesting its possible hepatotoxic nature after exposure for 28 days. Mortality of four animals from treated groups was recorded at the intermediate stage of the study. Hence, caution should be exercised with NAT calyx treatment at the concentration of 250 mg/kg bw and above, when required to be consumed for the period greater than 14 days.

**Key words:** Nyctanthes arbour-tristis, Sub-acute toxicity, Carotenoids, OECD no. 407, OECD no.425

## Introduction

In recent years there has been an exponential growth in the use and development of herbal medicine in both developed and developing countries due to their natural origin accounting to less side effects. The most common reasons for using herbal medicine are that they are cost effective with reasonably less adverse effects than that of synthetic medicines, provides an opportunity for more personalized health care and allows greater public access to health information. However usage of traditional herbal remedies increase when conventional medicine fails in the treatment of disease conditions such as in advanced cancer or infectious diseases [1]. The non toxic property of herbal drugs may not appear true in every situation specially when herbs are taken with prescription drugs, over-the-counter medications, or other herbs as is very common [2,3]. In the above context World Health Organization (WHO) has recognized the role of traditional alternative medicine and has encouraged member nations to develop national policies suitable for their situations taking into consideration that herbal medicines are popular worldwide and are one of the commonest form of treatment. [4]. In India, herbal medicine is a common practice and about 960 plant species are used by the Indian herbal industry of which 178 species are of maximum utility exceeding 100 metric tons per year [5].

The statistics suggests that India is one of the largest producer of medicinal plants and 70 % of Indian population depend on herbal medicine to meet their health care need probably, because herbal drugs constitute a major share of all the officially recognised systems of health viz

Ayurveda, Yoga, Unani, Siddha, Homeopathy and Naturopathy.[6]

*Nyctanthes arbour tristis* (NAT) Family *Oleaceae*, is one of the well known medicinal plants in India often used in folklore, Ayurvedic and Homeopathic systems of Indian medicine. [7] It is widely distributed in sub Himalayan regions of India and many other parts of Asia. [8]. Each part of this plant is bestowed with medicinal property and thus it is considered as a valuable source of medicine against various chronic diseases [9,10] and also for the development of certain industrial products [11]. Biological activity of NAT has been reported from the crude extracts and their isolated fractions from leaf, bark, flower, root and seed [12]. Traditionally the powdered stem bark is given in rheumatic joints pain, malaria and also used as an expectorant [13].

The flowers are bitter in taste and useful as an astringent, ophthalmic, stomachic and carminative agent. Also the dried flowers constitutes a common food ingredient of the Northeastern states of India. The flower calyx is reported to contain carotenoids in it [14,15] and possibly the various medicinal properties of NAT flowers are due to the carotenoids present in the calyx. The carotenoids of NAT calyx are reported to have structural [14] and pharmacological similarity with the carotenoids of *Crocus Sativus* (Saffron) [16,17,18]. Therefore in the light of the fact that due to the various properties of NAT flowers, they are consumed for multiple purposes and most disease conditions demand long periods of drug administration wherein periodical dosage monitoring and possible side effects are difficult and often goes unnoticed. This study was therefore undertaken to elucidate the sub-acute toxic effects of ethanolic calyx extract containing carotenoids of *N. arbour-tristis* using rats.

## Methods

### Preparation of extract

Fresh NAT flowers were collected from Thane, India, during the months of July to October. The samples collected were authenticated by Agharkar Research Institute, Pune, India (voucher specimen no. AHMA L-035). Extract was prepared as described by Gadgoli and Shelke, 2010 [14]. Crushed dried calyx (10 g) was macerated in 60 ml of 70% v/v ethanol. The solvent was recovered until a syrupy consistency was achieved. The remaining solvent was allowed to evaporate under reduced pressure at a temperature of  $\leq 45^{\circ}\text{C}$  to yield the dried ethanolic extract (2 g). The occurrence of carotenoid is reported to be 36.57% w/w in the extract. [14]

### Isolation of Carotenoid

The ethanolic extract was subjected to column chromatography. The ethanolic extract (1 gm) was loaded on silica gel (60-120 mesh) column and isolation of the major carotenoid was achieved by eluting isocratically with the mobile phase Ethyl acetate: Isopropanol: Water (65:25:10). Eluents with identical TLC pattern were pooled together and concentrated under reduced pressure to get the desired compound (Carotenoid ~ 0.23 gm –Rf-0.36).

### Quantification of Carotenoid in NAT Extract

Content of Carotenoid in the NAT extract was determined by HPTLC (High Performance Thin Layer Chromatography) method.

### **Preparation of Standard Solution**

About 0.6 mg of the dried isolated carotenoid was accurately weighed and dissolved in 10 ml of methanol. The volume was made up to 10 ml to get an effective concentration of 600 ppm (stock I). From (stock I) 12 ppm, 24 ppm, 36 ppm, 48 ppm and 60 ppm concentrations were prepared, 20 µl from each concentration were applied on precoated silica gel GF - 254 plates (HPTLC plates, Merck KGaA). The standard curve was prepared by plotting area on Y-axis and concentration of carotenoid on X axis.

### **Preparation of Sample**

About 10 mg of the dried NAT extract was accurately weighed and dissolved in 5 ml of methanol. The volume was made up to 10 ml to get an effective concentration of 1000 ppm (stock I). From stock I, concentration of 60 was prepared and 20 µl of sample was applied using Linomat applicator V on the same plate. The HPTLC plates were developed using Ethyl acetate: Isopropanol: Water (65:25:10) [19] as the mobile phase and plate was scanned using CAMAG Scanner 3 Version 1.14.28, at 430 nm. The standard curve was prepared by plotting area on Y-axis and concentration of carotenoid on X axis thereby obtaining carotenoid concentration in NAT extract by extrapolation method. (Figure 1A and 1B)

### **Experimental animals**

28-days sub acute toxicity of NAT calyx extract in wistar rats was conducted in the Department of Veterinary Pathology at Central Laboratory Animal Facility of Bombay Veterinary College bearing Institutional Animal Ethics Committee (IAEC) approval no.MVC/IAEC/17/2012. Principles of Laboratory Animal Care (NIH publication #85-23, revised in 1985) Wistar rats (N=48) of either sex with body weight ranging from 150-160 g were obtained from Bharat Serum, Thane, India and maintained in an air conditioned room (25±1°C) with a 12 h light: 12 h dark cycle. Standard pellet diet and water were provided ad libitum.

### **Limit Test / dose selection assay**

As per OECD guideline no. 425, limit test using NAT calyx ethanolic extract was performed to determine dose range for 28 days toxicity study. 2000-mg of NAT extract was administered by oral gavage to five animals. Critical monitoring of the experimental animals were performed for fourteen days.

### **Experimental Procedure**

OECD guideline no. 407 was followed for performing 28 days repeated dose oral toxicity study. All 48 wistar rats (24 Male and 24 Female) were acclimatized in standard housing condition. Male and female rats were separated to avoid breeding and were grouped as given in table 1.

The NAT calyx extract was given orally for 28 days to understand the toxicity. Record of daily feed intake and weekly body weight was maintained during the study. Blood was collected by puncturing the retro-orbital plexus on 14<sup>th</sup> day and on the day of sacrifice (28<sup>th</sup> day) to estimate hematological parameters and serum biochemical parameters. After 28 days all rats were sacrificed and organs were observed for any gross changes. Organ weight of liver, kidney and spleen were recorded and visceral organs were fixed in 10% formalin for histopathological studies.

### **Determination Of Parameters**

#### **Body weights and relative organ weights (ROW)**

The changes in body weights were recorded every week and the organs (liver, kidney, and spleen) were weighed using sensitive weighing balance to calculate the ROW for different groups after sacrificing the animals on 28<sup>th</sup> day.

#### **Hematology**

Blood samples were collected on 14<sup>th</sup> and 28<sup>th</sup> day for analysis using Haematological auto-analyzer (Abacus diatron). The haematological parameters analyzed included: Packed cell volume(PCV), Red Blood Cell (RBC) count, White Blood Cell (WBC) count and Haemoglobin (Hb) concentration. Others included Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC), Platelets (PLT), Lymphocytes (Ly), Monocytes (Mo) and Granulocytes (Gr).

#### **Biochemical Parameters**

The blood samples were kept at room temperature for 30 min, allowed to coagulate and then centrifuged at 4000 rpm for 10 min to separate serum. Serum was used for evaluating the different biochemical parameters such as glucose, cholesterol, urea, ALT (Alanine Aminotransferase), AST (Aspartate Aminotransferase), triglycerides, total proteins, ALP (Alkaline Phosphatase), creatinine and bilirubin content using standard Erba estimation kits using auto analyzer (Erba Chem 7, Germany).

Standard procedure as specified in the kit literature was followed.

#### **Estimation of Cholesterol**

Cholesterol estimation was done by Erba Cholesterol Diagnostic Kit manufactured by Erba Diagnostics Mannheim, Germany, which was based on modified Roeschlau's method [20,21].

#### **Liver markers enzymes**

AST and ALT levels were determined by International Federation of Clinical Chemistry (IFCC) UV kinetic method as described by Erba Mannheim manufacturer kit. The principle includes monitoring the rate of NADH consumption which is measured photometrically and is directly proportional to the ALT and AST concentration in the sample.

#### **Estimation of ALP level**

ALP level was estimated by modified Lowry *et al.* method. The principle is based on the rate of formation of p-Nitrophenol as a measure in increase in absorbance, which is proportional to the ALP activity in the sample [22]

#### **Estimation of Total Protein**

The peptide bonds of protein react with copper II ions in alkaline solution to form blue-violet complex (Biuret reaction). Each copper ion complexing with 5 or 6 peptide bonds. Tartarate was added as a stabilizer whilst iodide was used to prevent auto-reduction of the protein concentration and was measured at 546 nm [23].

#### **Estimation of albumin level**

Albumin level was estimated by using Bromocresol green (BCG) method as described by Doumas *et al.*, 1971 [24]. Development of blue green color indicated the end point and was measured spectrophotometrically at 625 nm.

#### **Estimation of globulin level**

Globulin level was calculated by subtracting the amount of albumin obtained from the amount of obtained total protein.

#### **Estimation of Blood Urea Nitrogen (BUN) level**

BUN level was estimated by Tiffany *et al.*, 1972 (25), modified principle, utilising urease and glutamate dehydrogenase (GD)

#### **Serum creatinine level**

Creatinine reacts with alkaline picrate to produce an orange-yellow colour (Jaffe's reaction). The

absorbance of the orange -yellow colour formed is directly proportional to creatinine concentration and is measured photometrically at 500-520 nm.

#### **Estimation of glucose level**

Glucose level was estimated by GOD-POD method. Glucose is oxidised by glucose oxidase (GOD) to produce gluconate and hydrogen peroxide. The hydrogen peroxide is then oxidatively coupled with 4 amino antipyrene (4-AAP) and phenol in the presence of peroxidase (POD) to yield a red quinoimine dye that is measured at 505 nm. The absorbance at 505 nm is proportional to concentration of glucose in the sample (26).

#### **Histopathology**

All the animals under study were sacrificed by cervical dislocation to isolate vital organs viz liver, kidney, spleen, heart and lungs for histopathological studies. Tissue samples collected were fixed in 10% formalin for 24 h. They were dehydrated by treating with ascending grades of ethanol, cleared with xylene, and embedded in paraffin wax. The paraffin blocks with embedded tissues were sectioned using a microtome and stained with haematoxylin and eosin (H and E) and mounted on Canada balsam (27). All the sections were examined under a light microscope under different magnifications. Photomicrographs of lesions were taken with camera Olympus FE 4000 (12 mega pixels) for observations and documentation of histopathological lesions.

#### **Data analysis**

All results are presented as Mean  $\pm$  SD and analyzed using one way analysis of variance (ANOVA). The differences between the means were tested using Tukey's test and values of  $p < 0.05$  were considered statistically significant. Microsoft excel 2007 and Graphpad Instat 3 version was used for the statistical analysis.

## **Results**

#### **Standardisation of NAT extract**

Figure 1 (A and B) describes the results obtained for NAT calyx ethanolic extract standardisation by HPTLC method

#### **Dose determination assay**

NAT calyx ethanolic extract was found non-toxic upto 2000 mg/kg when evaluated by limit test. Based on these observations, dose range of 250 mg, 500 mg and 750 mg/kg were selected to conduct the present study

### **Clinical observation**

All the surviving rats were feeding well, urine and faeces appeared normal throughout the duration of study. Apparently no major alteration in physiological parameters were observed, although mortality of four extract treated animals were seen during the course of study. One animal (female) of Group B died on seventh day of treatment and three animals of Group D died on 10<sup>th</sup> (2 females) and 11<sup>th</sup> (male) day of treatment. These observations indicated that females are more susceptible to the toxic effects of NAT calyx extract than males.

### **Effect on body weight (Figure 2)**

Figure 1 indicates that 28 days of NAT calyx extract treatment (250 mg/kg, 500 mg/kg and 750 mg/kg/day p.o) has not altered the body weight significantly ( $p > 0.05$ ).

### **Relative organ weights (ROW) (Table 2)**

There exists no significant ( $p > 0.05$ ) difference in the ROW of liver, kidney and spleen of the treated group of rats when compared to the control group ROW.

### **Effect on haematological parameters (Figure 3)**

To understand the gradual effect of NAT treatment on haematological system, all the vital parameters were checked on 14<sup>th</sup> day and on the day of sacrifice (28<sup>th</sup> day). Vital parameters like RBCs, MCHC, MCV and platelet were altered by NAT treatment in atleast one treated group. The observed effects are independent of dose and duration of treatment.

### **Effect on biochemical parameters (Table 3)**

To further understand the toxic effects of NAT treatment *if any*, biochemical parameters were critically evaluated after 28 days of treatment. Significant increase in Alanine aminotransferase (ALT) was observed while the levels of Aspartate aminotransferase (AST) were found above the normal range in all the animals under study. Other essential biochemical parameters are found unaltered by NAT treatment.

### **Histopathology (Figure 4)**

Histopathological studies of the vital organs viz. heart, lungs, kidney, spleen and liver of rats under study were performed. The organs were found with varying degrees of lesions in all the animals under study suggesting it to be a spontaneous development and not induced by NAT treatment.

### **Discussion**

Traditional alternative medicines are popular in India. *Nyctanthes arbour-tristis* is a mythological plant referred as 'Kalpavriksha', meaning wish fulfilling divine tree due to the various medicinal properties shared by each part of the plant. NAT flowers are also found to possess several pharmacological properties for which it is consumed widely without any reports of its adverse effects on human system.. The sub-acute toxicity study of 28 days revealed the safety profile of the NAT extract in rats. Said profile of medicinal plants is important as herbal medicines used has recently been questioned due to reports of their toxicities and fatalities (28). Hydroethanolic (80% ethanol and 20% water) extract was used for the study since it improves the extractive value and shelf life. For traditionally used medicinal plants, use of alcoholic or aqueous solutions have been found popular. General behaviour and body weights are the critical parameters indicating initial signs of toxicity.(29). In the present study, it is observed that NAT extract did not altered the gradual increase in body weight attributable to the increase in age and subsequent improved feed consumption, as a result mean body weights of all the animals were found similar during the termination of the study. The mean relative organ weights of treated animals did not deviate significantly when compared to the control group, suggesting no observed adverse effect level (NOAEL) of NAT upto 750 mg/kg bw. Hematopoietic system is one of the most sensitive target for toxic compounds and important index of physiological and pathological status. Also blood profile usually gives vital information on the response of body to injury or stress (30). NAT treatment for 28 days at the concentrations of 500 mg/kg and 750 mg/kg inhibited RBCs production significantly although no significant reduction in the haemoglobin levels were noted. Yet caution should be exercised during chronic exposure to NAT flowers/calyx as it may induce anemia. These observations were in agreement with the result obtained for MCHC which increased significantly in group D animals upon 14 days treatment however such effect was not observed after treatment for 28 days. Similarly significant increase in MCV was observed in group C animals after 28 days of treatment. The platelet count in group A animals were noted significantly high upon 28 days of NAT treatment. Overall, the results of NAT treatment suggests certain degree of toxicity on the haematopoietic system since each selected concentration altered one or more vital parameters variably

In order to complete the toxicity profile of NAT calyx, it is essential to understand its effects on liver, since it is the major organ involved in drug biotransformation. Levels of serum liver biomarker enzymes are biochemical parameters usually performed in order to evaluate any toxic effects on the liver [30]. Increase in the levels of AST, ALT and ALP in the serum are associated with liver toxicity by drugs or any other hepatotoxic agent [31]. However, ALT is more specific to liver and thus a better parameter for detecting liver injury as AST is also associated with diseases of other organs such as heart and muscles. ALP is present mostly in cells which are lining the biliary duct of the liver and is used to diagnose obstruction to the biliary system. Therefore, its elevation in the blood indicates cholestatic diseases such as gall stone or tumor blocking the bile duct. In this study, 28 days exposure of rats to NAT extract at different doses (250, 500 and 750 mg/kg) caused dose dependent increase in ALT levels and significant rise was observed in group C and D animals while AST levels remained unaffected. Therefore, it suggests possibility of hepatotoxicity by NAT treatment. The AST values in all the groups were found three folds of the standard AST range which can be attributed to specific species/genetic variation. Mean total protein among all the groups did not show any significant variation. Urea and creatinine are considered as important markers of kidney dysfunction [30]. In the present study both these biomarkers are found unaffected by the test agent. The histopathological results of vital organs revealed various degrees of histopathological lesions in all animals (treated as well as untreated), viz. Lung (Congestion, Hemorrhages, MNC infiltration, Emphysema, BALT Hyperplasia), Liver (Congestion, Hemorrhages, Perivascular MNC infiltration, Degeneration, Regeneration, Bile Duct Hyperplasia, MNC infiltration), Kidney (Congestion, Hemorrhages, MNC infiltration, Tubular Degeneration, Glomerular Atrophy, Glomerular Swelling, Protein cast), Heart (Congestion, Hemorrhages, MNC infiltration), Spleen (Lymphoid Hyperplasia, Lymphoid Depletion) and the recorded lesions are observed consistently in all the groups which may be referred as incidental or spontaneous development as no specificity was noted in NAT treated groups. Although few animals of group D displayed bile duct hyperplasia but its direct correlation with NAT treatment cannot be ruled out. The spontaneous death of four animals, one from group B and three of group D were noted during the intermediate stage of the study which indicates

idiosyncratic nature of the test extract. In conclusion, the present study established the toxicity profile of NAT calyx as per OECD guideline no. 407 and has contributed to the fact that naturally occurring herbal drugs may not be non-toxic in every situation. Selection of NAT flowers at concentration of 250 mg/kg and above for chronic treatment needs close monitoring for the possible side effects, however at lower concentration it may be safe for the acute treatment. Further exploratory studies are required in complementary systems to illustrate the precise cellular and molecular mechanisms for the obtained effects and also to explore its interaction with common components like other drugs, food or altered body conditions.

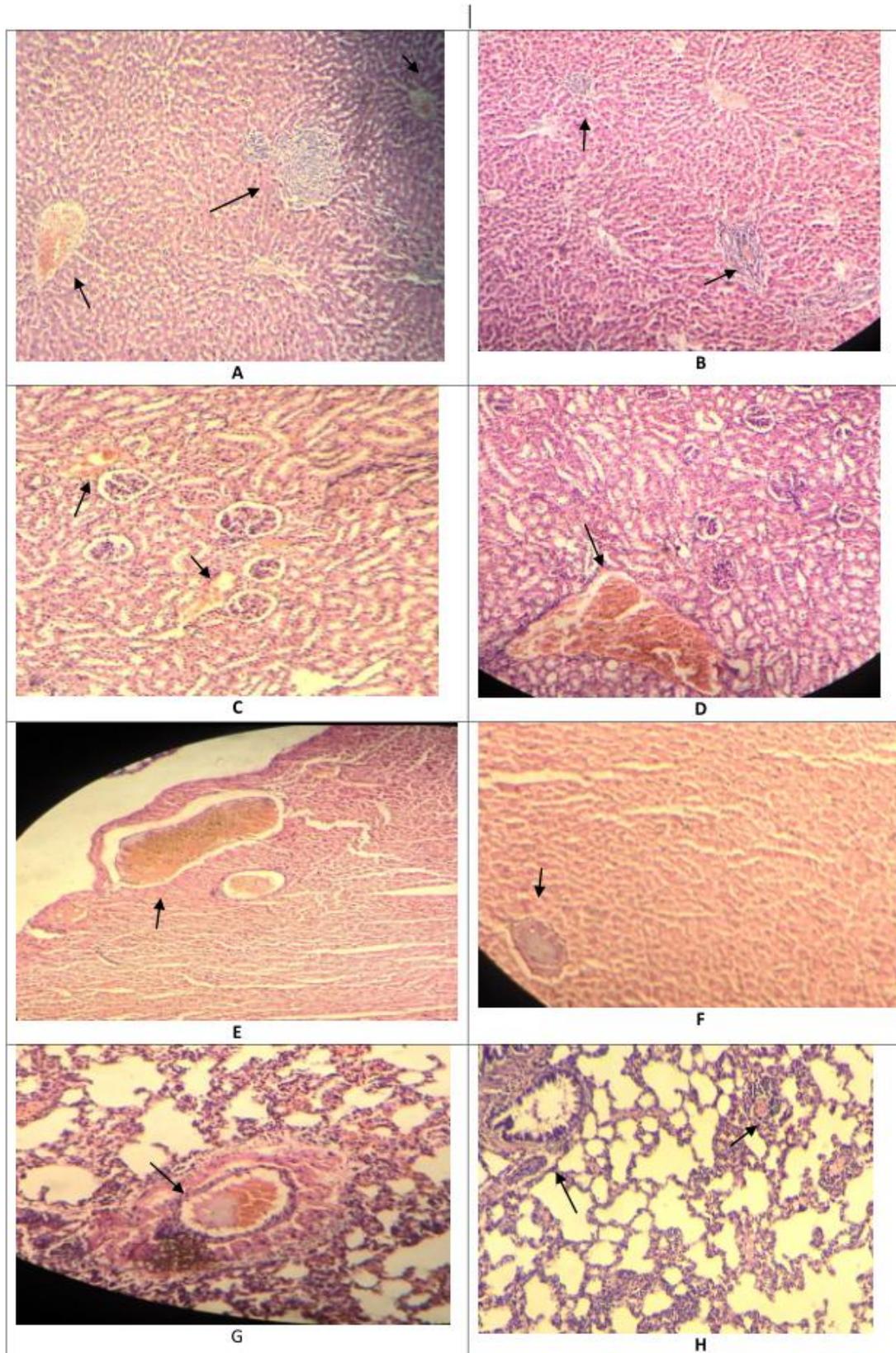
### Acknowledgments

The authors are thankful to National Medicinal Plant Board, Department of Ayush, Government of India for funding this project and Department of Pathology, Bombay Veterinary College, Parel, Mumbai, India for extending technical support.

### References

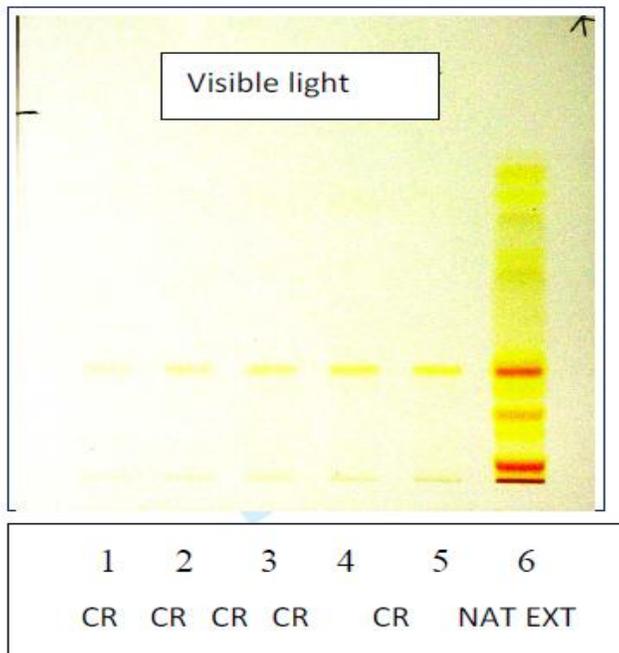
1. Sissi Wachtel-Galor, Iris F. F. Benzie, Herbal Medicine: Biomolecular and Clinical Aspects, Chapter 1 Herbal Medicine An Introduction to Its History, Usage, Regulation, Current Trends, and Research Needs, NCBI Bookshelf. NIH, 2nd edition, 2011;1-10
2. Canter P. H, Ernst E., Herbal supplement use by persons aged over 50 years in Britain: Frequently used herbs, concomitant use of herbs, nutritional supplements and prescription drugs, rate of informing doctors and potential for negative interactions. *Drugs Aging* 2004;21: 597-605
3. National Institutes of Health State-of-the-science conference statement: multivitamin/mineral supplements and chronic disease prevention, NIH State-of-the-Science Panel, *Ann Intern Med* 2006;145(5):364-71.
4. WHO, National Policy on Traditional Medicine and Regulation of Herbal Medicines. Report of WHO Global Survey. WHO, Geneva 2005:168.
5. Sahoo N, Manchikanti P, Dey S., Herbal drugs: Standards and regulation., *Fitoterapia* 2010;81(6):462-71
6. Ashok D.B. Vaidya, Thomas P.A. Devasagayam, Current Status of Herbal Drugs in India: An Overview, Recent Advances in Indian Herbal Drug Research, *J Clin Biochem Nutr* 2007;41:1-11.
7. Essential Drug List (EDL) Homeopathy, Department of Ayush, Drug Control Cell, Ministry of Health and Family Welfare, Government of India 2013:8.
8. Harleen Kaur Sandhar, Mohanjit kaur, Bimlesh Kumar, Sunil Prasher, An update on *Nyctanthes arbor-tristis* Linn, *Internationale Pharmaceutica Scientia* 2011;1(1):77-86.
9. Puri A, Saxena R, Saxena RP, Saxena KC, Srivastava V, Tandon JS, Immunostimulant activity of *Nyctanthes arbor-tristis* L. *J Ethnopharmacol* 1994;42 (1): 31-7.
10. Saxena RS, Gupta B, Lata S, Tranquilizing, antihistaminic and purgative activity of *Nyctanthes arbor tristis* leaf extract, *J Ethnopharmacol* 2002;181 (3): 321-5.
11. R.Saratha, V.G.Vasudha,, Inhibition of Mild Steel Corrosion in 1N H2SO4 Medium by Acid Extract of *Nyctanthes*

- arbortristis Leaves, E-Journal of Chemistry, 2009;6(4), 1003-1008.
12. Champa Rani, Sunaina Chawla, Manisha Mangal, A.K. Mangal, Subhash Kajla, A.K. Dhawan, 2012, *Nyctanthes arbor-tristis* Linn. (Night Jasmine) A sacred ornamental plant with immense medicinal potentials, Indian Journal of Traditional Knowledge 2012;11(3):427-435.
  13. Suresh V, Jaikumar S, Arunachalam G, Antidiabetic activity of ethanolic extract of stem bark of *Nyctanthes arbortristis* Linn Res J Pharmaceutical Biol Chem Sci 2010;1:311-317.
  14. Gadgoli Chhaya, Shelke Sandeep, Crocetin from the tubular calyx of *Nyctanthes arbortristis*, Nat Prod Res 2010;24:1610 – 1615.
  15. Pallavi Bhuskat, Omkar Amrite, Neha Patel and Chhaya Gadgoli, *Nyctanthes arbor-tristis* a Substitute for Saffron Color” Indian Drugs, 2007;44(8):640-642.
  16. Omkar Amrite, Jeeja Thurackal and Chhaya Gadgoli, Evaluation of Anti-inflammatory Activity of *Nyctanthes arbor-tristis* and *Onosma echioides*, Pharmacog Mag 2006;2(8):258-260
  17. Omkar Amrite, Pallavi Bhuskat, Neha Patel, and Chhaya Gadgoli, Evaluation of Antioxidant Activity of Carotenoid from *Nyctanthes arbor-tristis*” Int. J. Pharmacol. Biol. Sci 2007a;1(2):57-59.
  18. Omkar Amrite, Pallavi Bhuskat, Neha Patel, Sheetal Biyani and Chhaya Gadgoli, 2007b , Evaluation of Analgesic Activity of Carotenoid from *Nyctanthes arbor-tristis*, Adv. Pharmacol. Toxicol., 8(2), 55-58.
  19. Wagner, H., Bladt, S., Zgainski, E.M. ,1984), Plant Drug Analysis . Translated by Scott A., Springer Verlag Berlin Heidelberg GmbH , pp-274.
  20. Allain C.C., Poon, L.S., Chan, L.S., et al., Enzymatic determination of total serum cholesterol Clin Chem 1974;20:470–475.
  21. Roeschlau P., Bernt E., Gruber W.J. Enzymatic determination of total cholesterol in serum. J. Clin. Chem. Clin. Biochem. 1974; 12(5), 226.
  22. Burtis, C.A, Ashwood, E.R, Enzymes: In Tietz Fundamentals of Clinical Chemistry, 5th edition W.B. Saunders Company, New York, USA 2001;352–369.
  23. George R. Kingsley, The Determination Of Serum, Total Protein, Albumin and Globulin by The Biuret Reaction,. J. Biol. Chem 1939;131:197-200.
  24. Dumas, B.T., Watson, W.A., Biggs, H.G. Albumin standards and the measurement of serum albumin with bromocresol green. Clin. Chim. Acta 1971;31:87-96.
  25. Tiffany T. O. Jansen J.M. Burtis C.A. Overton J.B. and Scott. C.D. Clin. Chem 1972;18:829.
  26. Trinder, P., Determination of blood glucose using an oxidase-peroxidase system with a non-carcinogenic chromogen. J Clin Pathol 1969;22:158-161.
  27. Bancroft J.D., Stevens A., Theory and Practice of Histological Techniques, 1st edition W.B. Saunders, USA. 1977
  28. Park.M., Choi.H., Kim,J., Lee,H., Ku,S., 28 days repeated oral dose toxicity test of aqueous extracts of Mahwangyounpae teng, a polyherbal formula, Food Chem. Toxic. 2010;48:2477–2482.
  29. Sireatawong,S., Lertprasertsuke,N., Srisawat,U., Thuppia,A., Ngaamjariyawat,A., Suwannliho,N., Jaijoy,K. Acute and sub-chronic toxicity study of the water extract from *Tiliacora trianora* (Colebr.) Diels in rats. Songklan karin J.Sci.Technol 2008;30:729–737.
  30. Mukinda, J.T, Eagles, F.K., Acute and sub-chronic oral toxicity profile of the aqueous extract of *Pohy gala fruticosa* in female mice and rats. J Ethnopharma- col. 2010;128:236–240.
  31. Ramaiah, S.K., Preclinical safety assessment, Current gaps, challenges and approaches in identifying translatable biomarkers of drug-induced liver damage. Clin. Lab. Med. 2011;31:161–172.
  32. Sissi Wachtel-Galor, Iris F. F. Benzie, Herbal Medicine: Biomolecular and Clinical Aspects , Chapter 1 Herbal Medicine An Introduction to Its History, Usage, Regulation, Current Trends, and Research Needs, NCBI Bookshelf. NIH, 2nd edition, 2011:1-10

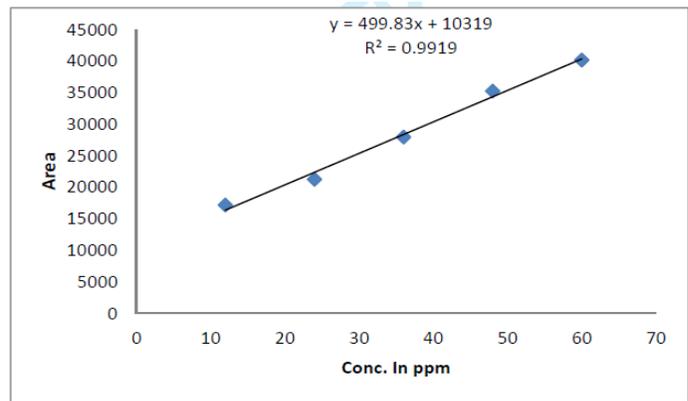


**Figure 4.** Representative histopathological images of all vital organs of A and D group

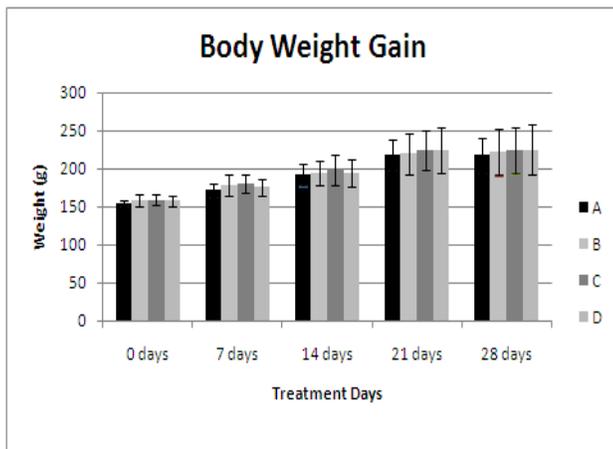
A and B: Photomicrograph of liver section of group A and group D respectively, both showing congestion and MNC infiltration marked by arrow, H and E ( $\times 100$ ); C and D: Photomicrograph of kidney section of group A and group D respectively, C shows haemorrhage surrounding a normal glomeruli while D shows significant haemorrhagic development without affecting glomeruli as shown by arrows, H and E ( $\times 100$ ); and F: Photomicrograph of heart section of group A and group D respectively, E shows haemorrhagic development while F shows MNC infiltration indicated by arrows, H and E ( $\times 100$ ); G and H: Photomicrograph of lung section of group A and group D respectively, both showing varying degrees of congestion as shown by arrow, H and E ( $\times 100$ )



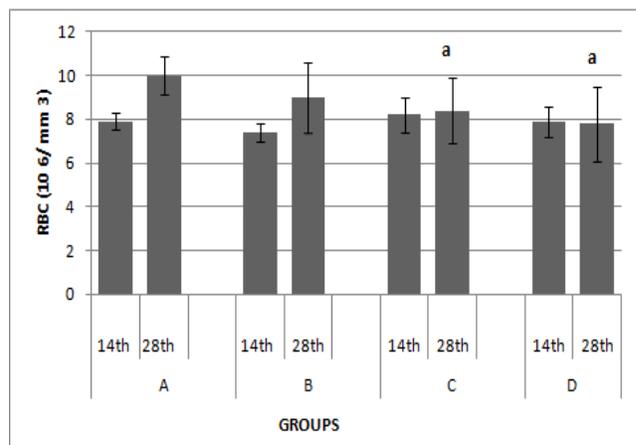
**Figure 1(A).** Standardisation of NAT ethanolic extract by HPTLC  
Visualization of Chromatogram



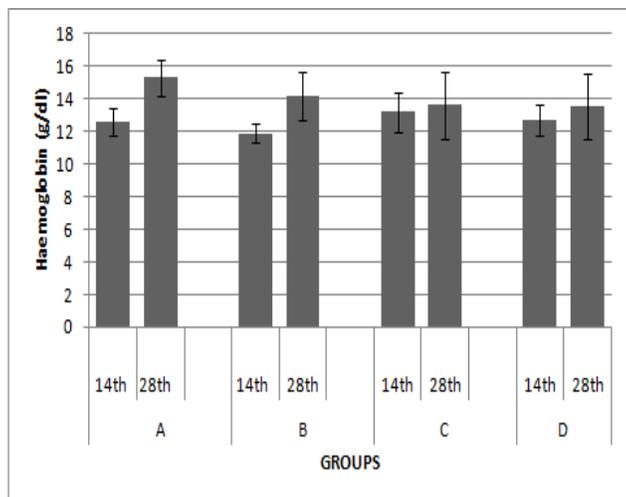
**Figure 1 (B).** Standard Curve for carotenoid



**Figure 2.** Effect of NAT treatment on body weight of wistar rats during the 28-days sub-acute toxicity study  
NAT treatment did not alter body weight ( $p > 0.05$ ) upto 28 days of treatment at the selected dose range. A: Control, B: 250 mg/kg, C: 500 mg/kg, D: 750 mg/kg

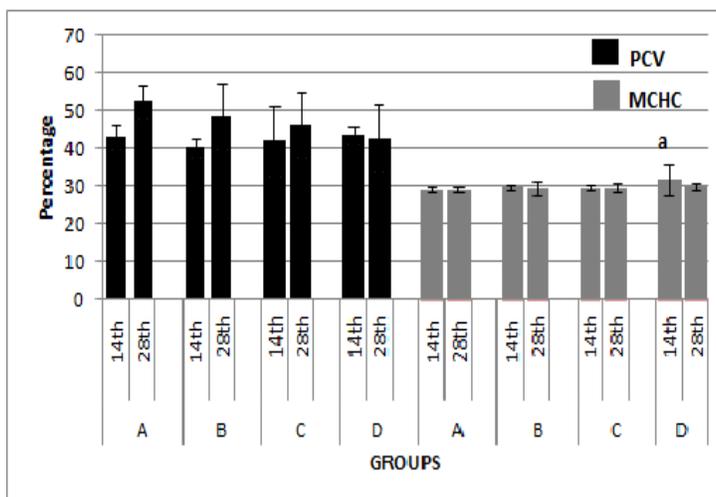


**Figure 3 A.** Effect of NAT treatment on Red Blood Cell Count (RBCs) at 14<sup>th</sup> and 28<sup>th</sup> day of study NAT calyx extract reduced RBCs significantly at mid and high doses  $a = p < 0.05$  upon 28 days of treatment. No significant ( $p > 0.05$ ) change noted at 14<sup>th</sup> day of treatment



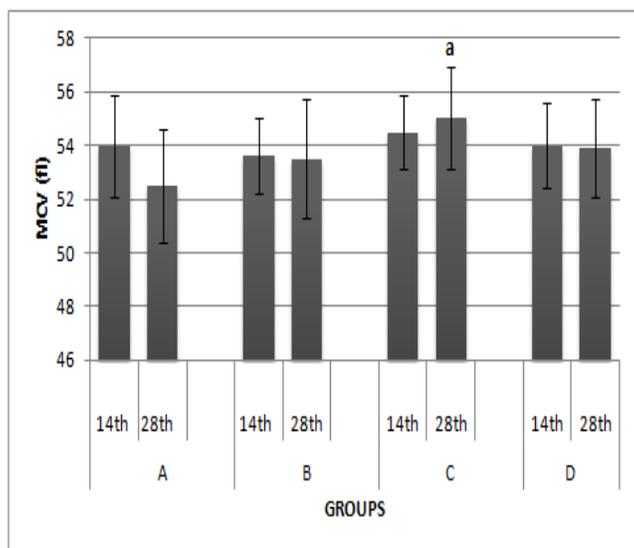
**Figure 3B.** Effect of NAT treatment on Haemoglobin at 14<sup>th</sup> and 28<sup>th</sup> day

No significant change observed with NAT treatment upto 28 days at all concentrations



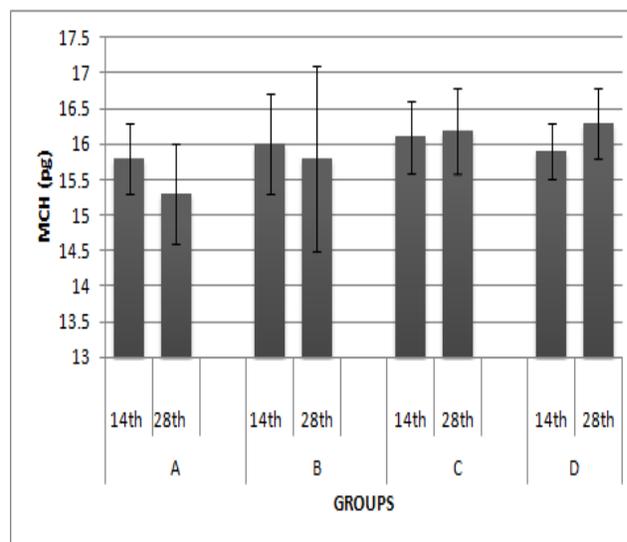
**Figure 3C.** Effect of NAT treatment on Packed Cell Volume (PCV) and Mean Corpuscular Haemoglobin Concentration (MCHC) at 14<sup>th</sup> and 28<sup>th</sup> day

NAT treatment did not induce significant change ( $p>0.05$ ) in PCV upto 28 days of treatment at all concentrations. 14 days treatment altered MCHC significantly ( $a=p<0.05$ ) at highest dose but the effect reversed by 28<sup>th</sup> day of treatment.



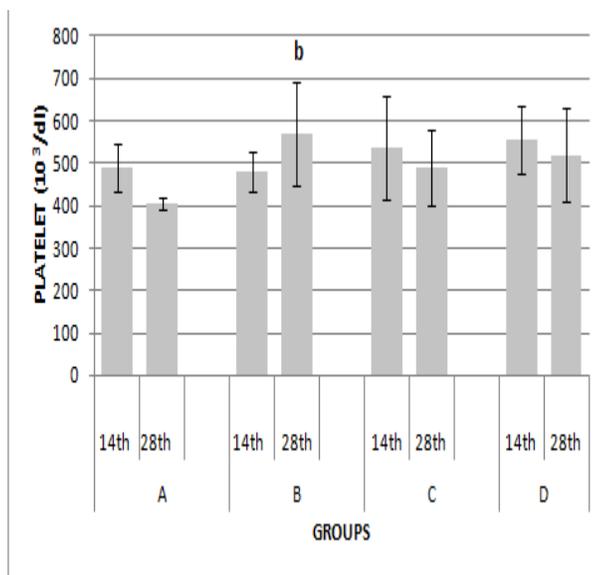
**Figure 3D.** Effect of NAT treatment on Mean Corpuscular Volume (MCV) at 14<sup>th</sup> and 28<sup>th</sup> day

NAT treatment induced no significant change in MCV upto 14<sup>th</sup> day of treatment, significant increase ( $a=p<0.05$ ) was noted on 28<sup>th</sup> day with mid dose (500 mg/kg)



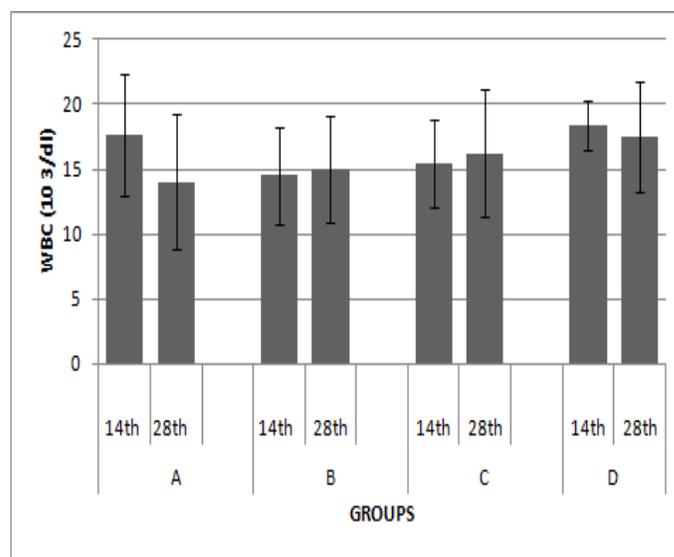
**Figure 3E.** Effect of NAT treatment on Mean Corpuscular Haemoglobin MCH at 14<sup>th</sup> and 28<sup>th</sup> day of treatment

No significant change observed in MCH throughout the study at all concentrations.



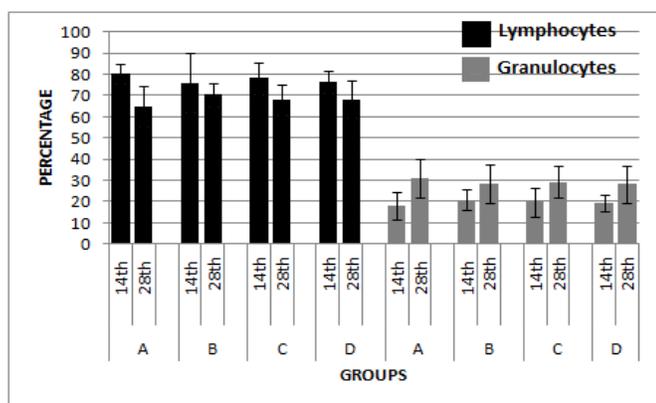
**Figure 3F.** Effect of NAT treatment on Platelet at 14<sup>th</sup> and 28<sup>th</sup> day

NAT treatment did not induce significant change in platelets upto 14<sup>th</sup> day, but at low dose significant increase (b= p<0.01) was observed with 28 days treatment.



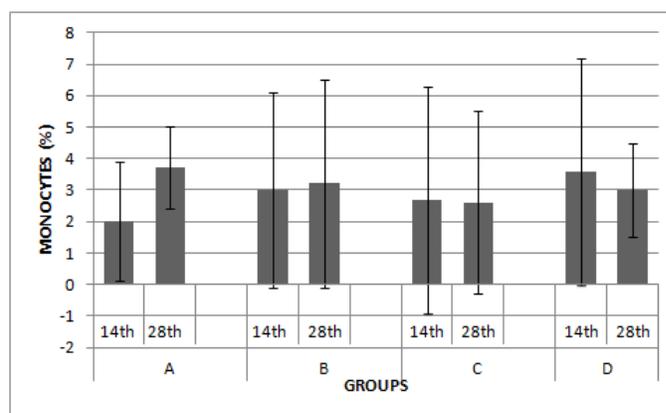
**Figure 3G.** Effect of NAT treatment on WBC at 14<sup>th</sup> and 28<sup>th</sup> day

No significant change observed with NAT treatment on WBC at all concentrations upto 28 days of treatment.



**Figure 3H.** Effect of NAT treatment on Lymphocytes and Granulocytes on 14<sup>th</sup> and 28<sup>th</sup> day

No significant change noted at all concentrations of NAT treatment upto 28 days.



**Figure 3I.** Effect of NAT treatment on Monocytes at 14<sup>th</sup> and 28<sup>th</sup> day

No significant change observed at all doses upto 28 days NAT treatment.

**Table 1.** Randomisation of 48 animals into four groups ; bw: Body Weight

| Group | Group     | Treatment for 28 days |
|-------|-----------|-----------------------|
| A     | Control   | Control (Water)       |
| B     | Low dose  | 250 mg/kg b.w. p.o.   |
| C     | Mid dose  | 500 mg/kg b.w. p.o..  |
| D     | High dose | 750 mg/kg b.w. p.o.   |

**Table 2.** Effect of 28 days NAT treatment on Relative Organ Weight (ROW) of vital organs

| Experimental group | Dose (mg/kg) | Organ weight (g) |          |           |          |           |          |
|--------------------|--------------|------------------|----------|-----------|----------|-----------|----------|
|                    |              | Liver            | % liver  | Kidney    | % kidney | Spleen    | % spleen |
| Control            | A            | 6.7 ± 1          | 3.1±0.3  | 1.5 ±0.1  | 0.7±0.04 | 0.6 ±0.1  | 0.3±0.07 |
| Extract            | B            | 6.9 ± 1.4        | 3.1 ±0.4 | 1.5 ± 0.2 | 0.6±0.07 | 0.6 ± 0.1 | 0.3±0.07 |
| Extract            | C            | 6.9 ±1.5         | 3.1±0.5  | 1.5 ± 0.2 | 0.7±0.06 | 0.7 ± 0.3 | 0.3±0.1  |
| Extract            | D            | 7.5 ±1.1         | 3.3±0.2  | 1.5 ± 0.7 | 0.7±0.05 | 0.8 ± 0.1 | 0.3±0.09 |

No significant change ( $p>0.05$ ) observed with NAT treatment at all doses upto 28 days. A: Control, B:250 mg/kg, C: 500mg/kg, D:750mg/kg

**Table 3.**Effect of 28 days of NAT treatment on biochemical parameters

| Serum Biochemical parameters | Experimental group (Mean ± sem) |            |                     |                         |
|------------------------------|---------------------------------|------------|---------------------|-------------------------|
|                              | A                               | B          | C                   | D                       |
| Glucose (mg/dl)              | 91.6 ±18.7                      | 91.8± 19.4 | 91.4±12.3           | 97.2 ± 18.5             |
| Cholesterol(mg/dl)           | 131.5 ± 26.2                    | 126.3±21.8 | 122.6±23.6          | 133 ± 35.1              |
| TP (g/dl)                    | 6.5 ±0.2                        | 6.6±0.4    | 6.6±0.3             | 6.7 ±0.3                |
| Alb. (g/dl)                  | 2.9 ±0.4                        | 3.1±0.3    | 3.1±0.3             | 3.1 ±0.2                |
| Glo. (g/dl)                  | 3.5 ±0.3                        | 3.4±0.5    | 3.5±0.2             | 3.5 ±0.1                |
| ALT(U/L)                     | 59.5 ± 22.5                     | 64.7±12.3  | 85.7±6 <sup>c</sup> | 83.2 ±11.3 <sup>b</sup> |
| AST( U/L)                    | 319.9 ± 67.5                    | 327.2±17.3 | 325.8 ± 55.2        | 326.2 ± 51.3            |
| ALP (U/L)                    | 113.7 ± 13.8                    | 107.6±31.2 | 102 ±17.9           | 110.1 ± 20.7            |
| BUN (mg/dl)                  | 73.7 ± 14.1                     | 64.4 ±17.9 | 69.4 ±25            | 76.4 ±16.7              |
| Creatinine. (mg/dl)          | 1.7 ± 0.3                       | 1.4±0.3    | 1.7 ±0.4            | 1.7 ± 0.3               |

NAT treatment induced significant increase in Alanine Aminotransaminase (ALT) at mid and high doses with 28 days of treatment. Other vital parameters remained unaffected at all concentrations.

A: Control, B:250 mg/kg, C: 500mg/kg, D:750mg/kg, <sup>b</sup>  $p < 0.01$ , <sup>c</sup>  $p < 0.001$