

# Sub chronic toxicity studies of Asena, a poly-herbal formulation for the treatment of arthritis in rat

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**Abstract.** Sub chronic toxicity studies of Asena, a poly-herbal formulation for the treatment of arthritis in Ghana, was carried out in the rat. In the sub chronic studies, rats were administered with Asena at 60, 600 and 1200 mg/kg daily for six weeks. Urinalysis, hematological and biochemical analyses were carried out on urine; blood and serum samples collected at the end of the six weeks treatment. Histological analysis of the liver, heart, kidney and lung tissues were also done at the end of the treatment period. The results showed that, there were no significant differences ( $p < 0.05$ ) in the urinalysis and biochemical analyses in Asena-treated animals compared to controls over the treatment period. Similarly, the administration of Asena to experimental rats did not adversely affect hematological indices assayed except platelets which were significantly elevated ( $p > 0.05$ ) in the treatment groups compared to controls. There was no noticeable morphological change in liver, kidney, lung and heart micrographs of Asena-treated and control animals. In conclusion, it can be said that prolonged administration of Asena may not affect the normal growth nor cause organ specific toxicity in the rat. These findings support its continual use and at least provide a basis for its assessment in clinical trials.

**Keywords:** Phytomedicine, Asena, sub chronic, micrograph, biochemical, hematological, herbal.

## INTRODUCTION

There is an increased use of herbal medicines worldwide probably due to their relative low cost, availability and perceived relative low adverse effects. Indeed phytomedicine has been identified as the most common form of alternative medicine (Ogbonnia et al., 2011). However there is growing concern about the safety of most herbal formulations because proper scientific safety evaluation has not been carried out on them (WHO, 2002). Indeed adverse side effects including illness and death have been reported as result of consumption of herbal medicines (Stewart et al., 1999; Ernst, 2002; Veiga-Junior et al., 2005; Saad et al., 2006; Park et al., 2010). Thus comprehensive pre-clinical studies especially toxicity studies are needed to subsequently pave the way for clinical trials in order to validate the continual use of these preparations in humans. These studies are needed to protect the population from harmful

herbal formulations.

Asena is a poly-herbal decoction prepared by the Centre for Plant Medicine Research (CPMR), Mampong-Akuapem for the treatment of arthritis. It is made from seven medicinal plants namely *Khaya senegalensis*, *Kigelia africana*, *Nuclea latifolia*, *Clausena anisata*, *Ptilostigma thonningii*, *Trichilia monadelpha* and *Strophanthus hispidus*. Phytochemical screening of most of the plants used in the preparation of the drug show the presence of indole-quinolizidine, alkaloids, glycoalkaloids, glycosides, saponins, flavonoids, phenolics, anthraquinones, terpenoids, and coumarin (Jimoh and Oladiji, 2005; Akpanabiatu et al., 2005). Some of these constituents depending on their concentration may have some toxic effects. For instance, glycosides are known to cause decreased heart rate, decreased sympathetic activity and decreased systemic vascular resistance

(Seigler, 1998). Strophanthin, the major constituent of *Strophanthus hispidus* is a muscle poison (Ellingwood, 1919) while anthraquinones have been implicated in causing death in humans (Millonig et al., 2005; Stadlbauer et al., 2005), however later observation suggests this may not be the case (Brett et al., 2006).

Recent studies have shown that Asena has anti-inflammatory and anti-nociceptive activities and phytochemical screening showed the presence of saponins, phenolics, reducing sugars and polyamides. Also the oral LD<sub>50</sub> of the preparation was found to be more than 5000 mg/kg in the rat (Donkor et al., 2013). As stated elsewhere, pre-clinical toxicity studies are paramount in validating the continual use of herbal formulations. It is also the basis for conducting clinical trials on medicinal agents. Thus the present work seeks to evaluate the toxicity of Asena in male Sprague-dawley rats upon sub chronic administration.

## MATERIALS AND METHODS

### Reagents and chemicals

Test Kits; aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl-transferase (GGT), bilirubin (direct and total), creatinine and urea were purchased from Cypress Diagnostics (Belgium). Urine test strips (UroColor™ 10) were supplied by Standard Diagnostics Inc. (Kyonggi-do, Korea). All other chemicals were purchased in their purest form available from British Drug Houses (BDH) Ltd. (Poole, UK).

### Preparation of Asena extract

A total of thirty bottles of Asena (330 ml) was obtained from the Production Store of the CPMR, Mampong-Akuapem in the Eastern Region of Ghana. They were emptied in to a clean vessel making a total volume of 9.90 L. It was lyophilized (Heto Power dry LL 3000, Denmark) to produce a dry weight of 66.36 g (0.67%, w/v). The lyophilized Asena extract was kept in a dry place and reconstituted in distilled water before administration.

### Animals

Male Sprague-Dawley rats (182 to 206 g) were obtained from the Animal Unit of the CPMR, Mampong-Akuapem, in the Eastern Region of Ghana. The animals were fed on animal chow obtained from Ghana Agro Food Company (GAFCO) Tema, Ghana and sterilized distilled water *ad libitum* throughout the study period. They were maintained at a room temperature of 23 to 25°C, with a 12 h light/dark cycle. The research was conducted in

accordance with the internationally accepted principles for laboratory animal use and care (NIH, No.85-23, revised 1985). Ethical clearance was obtained from the CPMR's ethics committee and the paper was written in accordance with the ARRIVE guidelines (Kilkenny, 2010).

### Treatment of animals

#### Sub chronic toxicity studies

Four groups of ten male rats each were kept in four separate metal cages. Group 1 was kept as control and received sterilized distilled water for six weeks. Groups 2, 3 and 4 were treated daily with 60, 600 and 1200 mg/kg body weight, respectively of lyophilized reconstituted Asena *ad libitum* for six weeks. The animals in each group were weighed on day zero (baseline), 3<sup>rd</sup> and 6<sup>th</sup> weeks after treatment.

### Urinalysis

Urine samples of the rats in each treatment group at baseline, the 3<sup>rd</sup> and 6<sup>th</sup> weeks were analyzed for glucose, bilirubin, ketones, specific gravity, pH, proteins urobilinogen, nitrate, blood and leukocytes using urine reagent strips UroColor™ 10 (Standard Diagnostic Inc., Korea).

### Blood sampling

Blood samples of rats in each treatment group were obtained by tail bleeding (at day 0, 3<sup>rd</sup> and 6<sup>th</sup> week) into Eppendorf tubes without anticoagulant, centrifuged at 4000 g for 5 min (Denley BS 400, England) and serum stored at -40°C for biochemical analysis. Other blood samples were collected into separate tubes already coated with trisodium citrate (Westergreen E.S.R, UK) for hematological analysis.

### Serum biochemical analysis

Serum ALT, AST, GGT, total and direct bilirubin, creatinine and urea of samples were determined using protocols from Cypress diagnostic kits (Belgium) with a semi-auto blood chemistry analyzer, photometer 4040 (Robert Riele G & Cole-2000, Germany).

### Hematological analysis

Red blood cells (RBC), mean cell volume (MCV), haematocrit (HCT), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration

**Table 1.** Effect of Asena on urine parameters at termination of treatment.

Parameter	Treatment Group			
	Control	ASENA		
		60 mg/kg	600 mg/kg	1200 mg/kg
Glucose (mg/dl)	-	-	-	-
Bilirubin (mg/dl)	-	-	-	-
Ketones (mg/dl)	±	±	±	±
S.G.(g/ml)	1.030	1.030	1.030	1.030
Blood (RBC/μl)	-	-	-	-
pH	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0
Protein (g/L)	±	±	±	±
Nitrite	-	-	-	-
Leukocyte (WBC/μl)	-	-	-	-
Urobilinogen (mg/dl)	N	N	N	N

Results are means of  $N = 6$ . (-): Absent; (N): Normal; (±): Trace.

(MCHC), red cell distribution width (RDW), white blood cells (WBC), hemoglobin (HGB), platelet counts (PLT) mean platelet volume (MPV), procalcitonin (PCT), platelet distribution width (PDW) and lymphocytes (LYMPH) were determined with Haema-screen 13 (Hospitex Diagnostics, Italy).

### Histology

At termination of treatment, four rats from each group were sacrificed by cervical dislocation and the heart, lungs, liver, kidney and spleen were excised and weighed. All the organs except the spleen were stored in 10% neutral formalin and dehydrated with a progressively increasing concentration of ethanol. The tissues were cleared with chloroform and impregnated with paraffin wax. Sections, 5 μm thick were stained with haematoxylin and eosin (Khalaf et al., 2010) and mounted on slides for light microscopic examinations.

### Statistical analysis

Results were expressed as the mean value ± standard error of mean (SEM). Differences between control and experimental groups were determined by one-way analysis of variance (ANOVA), followed by Holm-Sidak's multiple comparisons test. GraphPad Prism for Windows version 6 (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses and  $P$  values less than 0.05 were considered significant.

## RESULTS

### Urinalysis

Dipstick urinalysis data indicate (Table 1) that there were

no significant differences ( $p > 0.05$ ) in the levels of urine parameters between Asena-treated and control animals.

### Body weight

The effects of Asena on the percentage weight gain of test and control animals are presented in Figure 1. Both test and control animal gained weight steadily throughout the study period and there were no significant difference ( $p > 0.05$ ) between control and test animals.

### Organ/bodyweight ratio

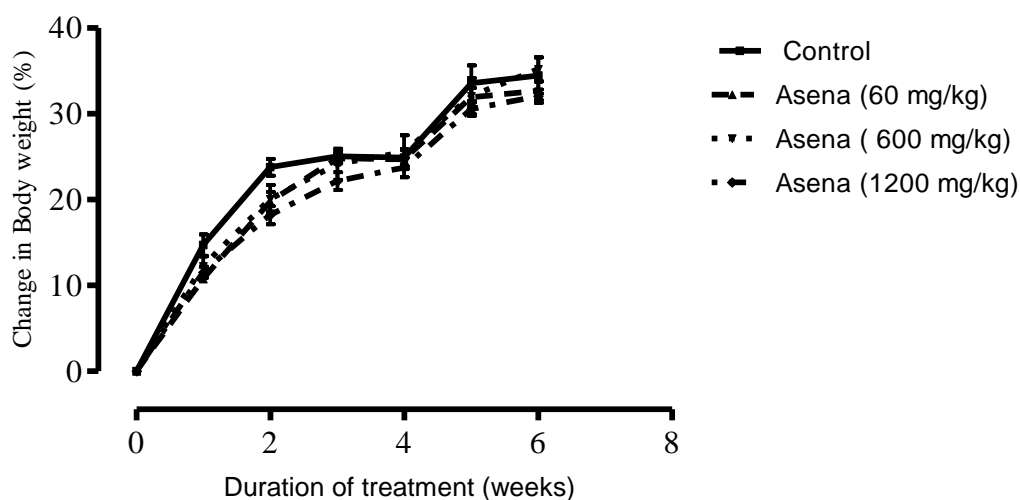
The percentage change in mean organ/body weight ratio at termination of treatment in control and Asena-treated animals are shown in Table 2. The results showed no significant changes ( $p > 0.05$ ) in the organ weights, expressed as percentage of body weight, at termination between control and test animals.

### Serum biochemistry

The effects of sub chronic administration of Asena to rats on selected serum biochemical parameters at termination of treatment are shown in Table 3. The results indicate that Asena did not cause significant changes ( $p > 0.05$ ) in the levels of all the parameters studied compared to the controls.

### Hematological studies

The effect of sub chronic administration of Asena on certain hematological indices at termination of treatment is shown (Table 4). The results show that there were no



**Figure 1.** Effect of Asena on the weight of rats with duration of treatment. Values are means  $\pm$  S.E.M. of  $N = 6$ .

**Table 2.** Effect of Asena on organ/body weight ratio at termination of treatment.

Organ	Treatment group			
	Control	Asena 60 mg/kg	Asena 600 mg/kg	Asena 1200 mg/kg
Heart	0.80 $\pm$ 0.00	0.67 $\pm$ 0.01	0.65 $\pm$ 0.01	0.65 $\pm$ 0.01
Lungs	0.37 $\pm$ 0.02	0.34 $\pm$ 0.00	0.41 $\pm$ 0.01	0.37 $\pm$ 0.01
Liver	3.04 $\pm$ 0.28	2.71 $\pm$ 0.03	2.89 $\pm$ 0.10	2.94 $\pm$ 0.09
Kidney	0.61 $\pm$ 0.12	0.59 $\pm$ 0.01	0.60 $\pm$ 0.02	0.56 $\pm$ 0.01
Spleen	0.33 $\pm$ 0.02	0.24 $\pm$ 0.02	0.29 $\pm$ 0.02	0.33 $\pm$ 0.00

Values are Mean  $\pm$  SEM (n = 6)

significant changes ( $p > 0.05$ ) in all parameters measured between control and Asena-treated animals except for platelets which was significantly higher ( $p < 0.05$ ) in all test groups.

### Histology

The effects of Asena on the histopathology of the kidney, lung, liver and heart tissues at termination of treatment are shown in Figures 2 to 5. Results showed that Asena did not affect the morphology of all the organs studied.

### DISCUSSION

Phytomedicines in the form of mono- and/or poly-herbal preparations are common place in most developing countries perhaps due to their ease of preparation, accessibility of plant parts used in their preparation, readily available traditional knowledge of medicinal plants, poor regulation by related government agencies

and a good source of income for producers and practitioners. Increased patronage of herbal medicines has exposed the toxicity of some of these preparations through reports of illness and fatalities (Stewart et al., 1999; Ernst, 2002; Veiga-Junior et al., 2005; Park et al., 2010) and organ specific toxicities including hepatotoxicity (Saad et al., 2006) and nephrotoxicity (Cosyns, 2003; Colson and De Broe, 2005; Debelle et al., 2008). Asena is a poly-herbal preparation by the CPMR, Mampong-Akwapim for the treatment of arthritis. Recent single oral dose (5000 mg/kg) acute toxicity evaluation of the preparation in rats showed no mortality or signs of toxicity such as piloerection, abnormal respiratory, locomotor and lachrymatory activities (Donkor et al., 2013).

In the present sub chronic toxicity studies, the administration of Asena did not affect the growth of the test animals in all the treatment groups as evidenced by normal weight gain by the experimental animals (Figure 1) over the six weeks treatment period. Studies have shown that relative organ/ bodyweight is more indicative of organ specific toxicity than absolute weight (Demma et

**Table 3.** Effect of Asena on some serum biochemical parameters at termination of treatment.

Parameter	Treatment group			
	Control	Asena		
		60 mg/kg	600 mg/kg	1200 mg/kg
ALT (U/L)	51.00 ± 1.98	46.83 ± 3.90	49.50 ± 3.30	53.83 ± 4.30
AST (U/L)	106.00 ± 7.60	109.67 ± 4.64	106.00 ± 2.38	112.50 ± 14.22
ALP (U/L)	147.33 ± 7.55	146.60 ± 11.75	158.80 ± 4.53	161.33 ± 16.56
GGT (U/L)	3.53 ± 0.77	3.13 ± 0.65	2.70 ± 0.37	2.80 ± 0.36
ALB (g/L)	34.00 ± 2.00	40.00 ± 1.48	37.40 ± 0.60	36.00 ± 1.53
T. bil (µmol/L)	3.35 ± 0.76	3.35 ± 0.65	3.13 ± 0.44	2.95 ± 0.15
D. bil (µmol/L)	1.03 ± 0.33	1.30 ± 0.36	1.00 ± 0.33	1.10 ± 0.23
Creat (µmol/L)	87.98 ± 3.43	73.95 ± 2.30	74.53 ± 3.10	83.83 ± 3.28
Urea (mmol/L)	8.68 ± 0.83	9.00 ± 0.34	8.23 ± 0.54	9.00 ± 0.43

Values are Mean ± SEM (n = 6). T. bil, total bilirubin ; D. bil, direct bilirubin ; Creat, creatinine.

**Table 4.** Hematological parameters at termination of treatment.

Parameter	Treatment Group			
	Control	Asena		
		60 mg/kg	600 mg/kg	1200 mg/kg
RBC (×10 <sup>6</sup> /µl)	7.38 ± 0.42	7.94 ± 0.14	7.59 ± 0.08	7.52 ± 0.17
MCV (µ <sup>3</sup> )	59.06 ± 1.03	58.48 ± 1.00	59.23 ± 0.93	60.08 ± 1.44
HCT (%)	43.73 ± 3.01	46.47 ± 1.28	44.93 ± 0.47	45.18 ± 1.39
MCH (pg)	18.25 ± 1.35	19.45 ± 0.240	19.55 ± 0.21	19.18 ± 0.19
MCHC (g/dl)	30.92 ± 2.32	33.30 ± 0.57	33.03 ± 0.40	32.03 ± 0.93
RDW (%)	34.55 ± 4.86	30.98 ± 1.61	30.83 ± 1.15	33.98 ± 5.75
WBC(×10 <sup>3</sup> /µl)	7.58 ± 0.94	6.86 ± 0.74	10.80 ± 0.64	6.92 ± 0.53
HGB (g/l)	13.70 ± 1.47	15.43 ± 0.26	14.83 ± 0.18	14.42 ± 0.25
PLT ( ×10 <sup>3</sup> /µl)	552.33 ±119.01	829.00 ± 42.22*	932.83 ± 34.00*	723.80 ± 53.04*
MPV (µ <sup>3</sup> )	6.53 ± 0.17	6.32 ± 0.05	6.18 ± 0.07	6.47 ± 0.13
LYMPH (#)	6.42 ± 0.87	5.30 ± 0.68	7.93 ± 0.45	6.02 ± 0.41

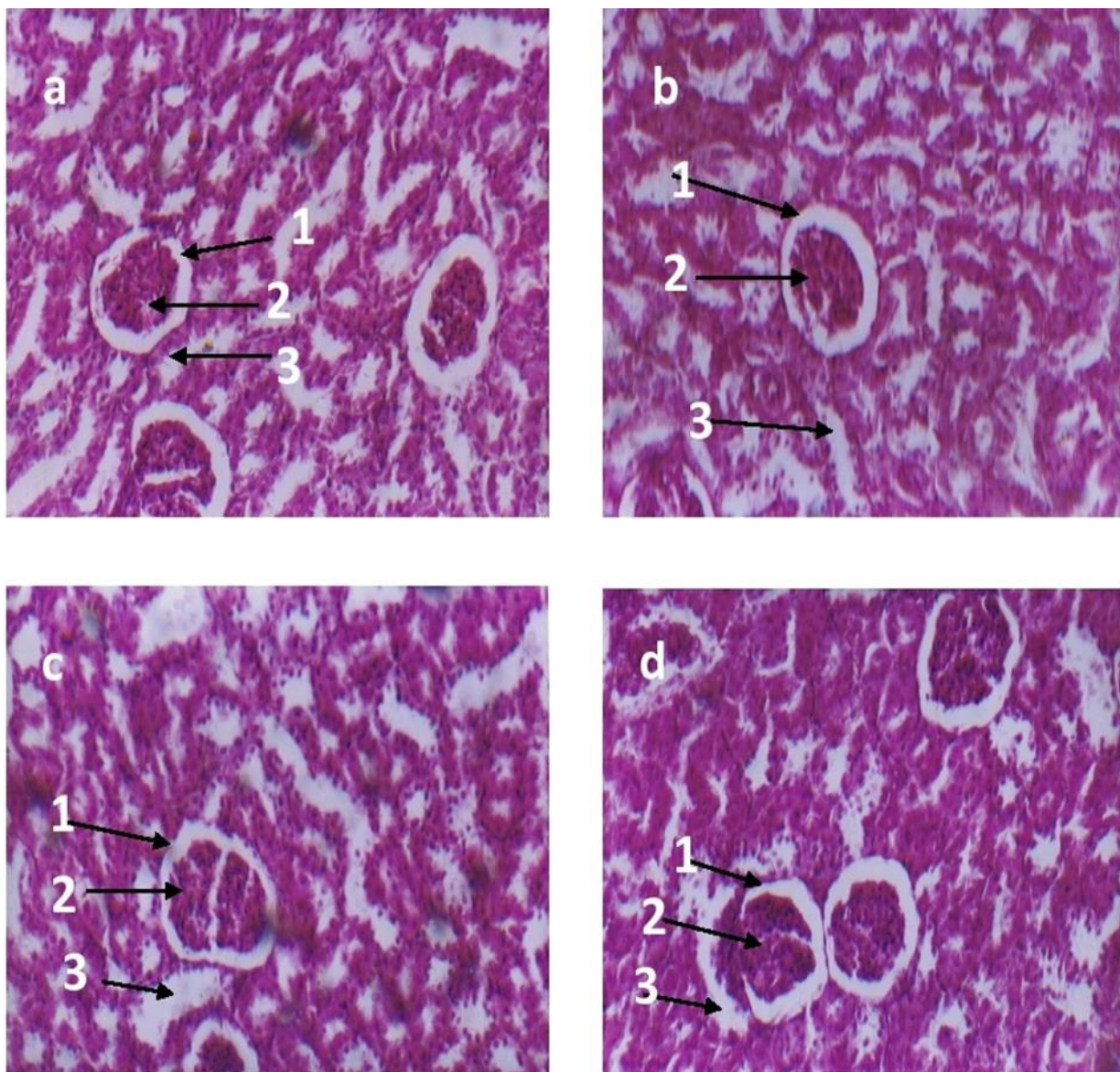
Values are means ± S.E.M. of N = 6. \*p < 0.05, compared to control.

al., 2006). The present result showed no significant change when excised heart, kidney, liver, lung and spleen were expressed relative to body weight in control and test animals (Table. 1) suggesting that the preparation did not have deleterious effect on these organs leading to them becoming edematous or hypertrophied.

The liver and kidney and to some extent muscle cell membrane are the major target organs of toxic substance because of their role in metabolism (David et al., 2000). ALT and AST are largely used in the assessment of liver damage by drugs or hepatotoxin (Ramaiah, 2011). The liver and heart release ALT and AST and an elevation in their plasma concentrations are indicators of liver and heart damage (Wasan et al., 2001; Mythilypriya et al., 2007). However, ALT is more specific to the liver and is thus a better parameter for detecting liver injury (Ozer et al., 2008). The results showed that Asena did not significantly affect the levels of ALT and AST suggesting

that the poly-herbal formulation may not possess hepatotoxic effect or adversely affect cardiac tissue (Crook, 2006). Albumin and ALP are markers of synthetic function of hepatocytes while GGT and bilirubin are indices of hepatocellular damage (Brett et al., 2006). Albumin is synthesized and degraded in the liver and measurable decreased amount reflect either an impaired hepatocellular production and/or an increased catabolism that may occur in various physiological or pathological processes (Killingsworth, 1979; Johnson, 1999). ALP on the other hand is related to the functioning of hepatocytes and increased activity may be due to increased synthesis in the presence of increased biliary pressure (Manjunatha et al., 2005). Several previous observations revealed that a significant elevation of a dose-response relationship in all these parameters correlates well with liver damage (Nyarko et al., 1999; Celik et al., 2002; Kutlu et al., 2005; Demir and Celik, 2006). Thus Asena may not affect synthetic hepatic function nor cause hepatocellular





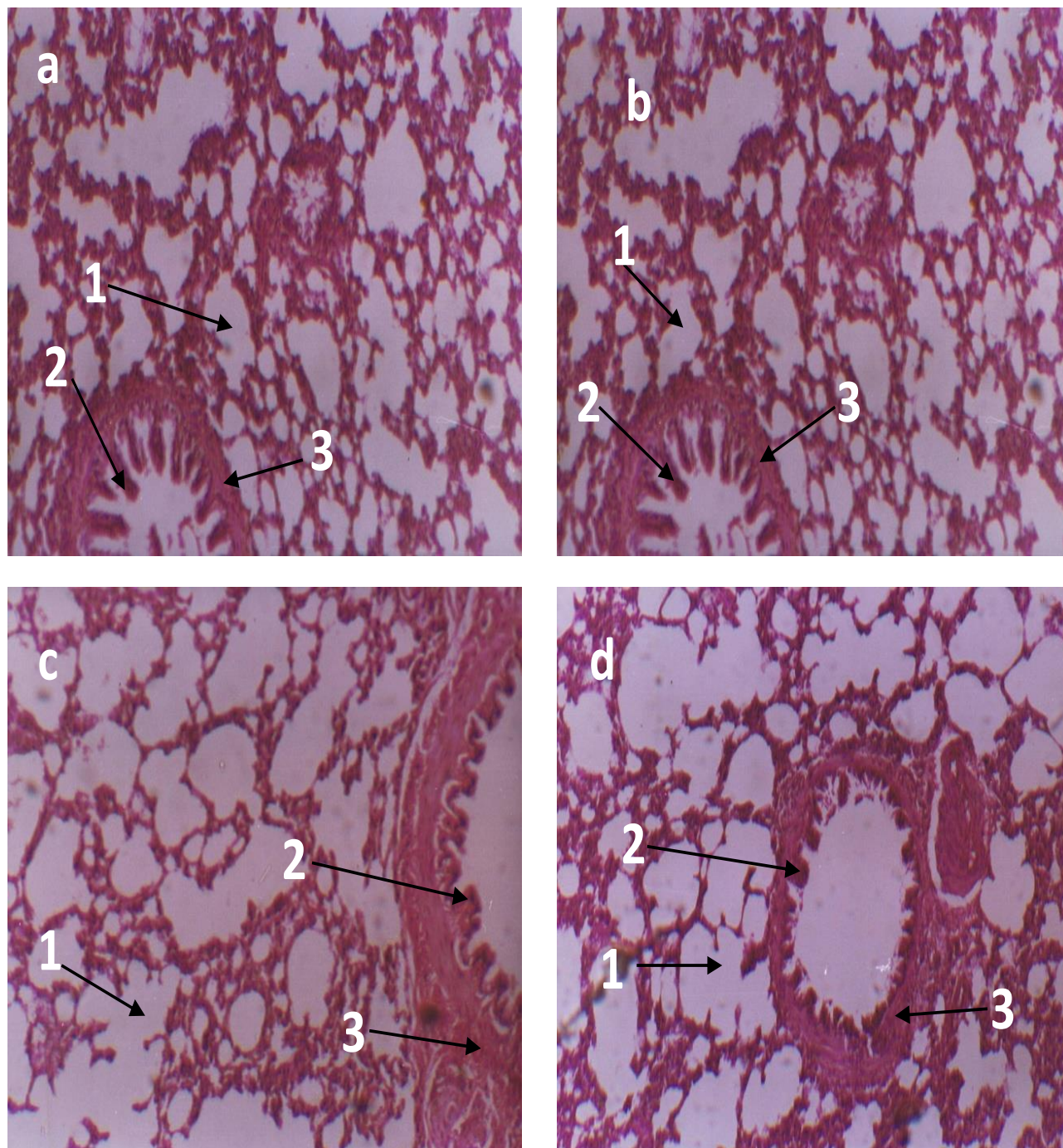
**Figure 2.** Histological appearance (H&E-stained, x132) of the kidney at termination of treatment for control (a), 60(b), 600(c) and 1200(d) mg/kg of Asena, showing no difference in morphology of glomerulus (1), bowman capsule (2), renal tubules (3) and adjoining cells.

damage since it did not significantly affect the levels of albumin and ALP; and GGT and bilirubin in test animals.

Elevated serum urea and in particular serum creatinine are highly specific for nephrotoxicity (Kutlu et al., 2005). Urea is the first acute renal marker upon renal injury and creatinine is the most trustable and increases only when the majority of renal function is lost (Borges et al., 2005). Previous studies have shown that nephrotoxicants such as cadmium increase serum levels of urea and creatinine in the rat while nephroprotective agents such as honey reduce serum urea and creatinine levels (Abdel-Moneim and Ghafeer, 2007). The present result reveals no significant change in the serum levels of urea and creatinine in all the dose levels compared to controls.

Similarly, the urine chemistry analysis showed no significant differences ( $P > 0.01$ ) between controls and test groups. This is indicative of no nephrotoxicity specifically by renal filtration mechanism (Crook, 2006) or probably suggests that Asena did not interfere with the renal capacity to excrete these metabolites.

The hematopoietic system is also a sensitive target of toxic compounds and is an important index of physiological and pathological status in man and animals (Adeneye et al., 2006). Alkaloids, a very common phyto-constituent have been implicated in the inhibition of phosphodiesterase leading to the accumulation of cAMP which in turn stimulates protein synthesis (Eteng et al., 2003) resulting in stimulation of erythropoiesis and leuko-

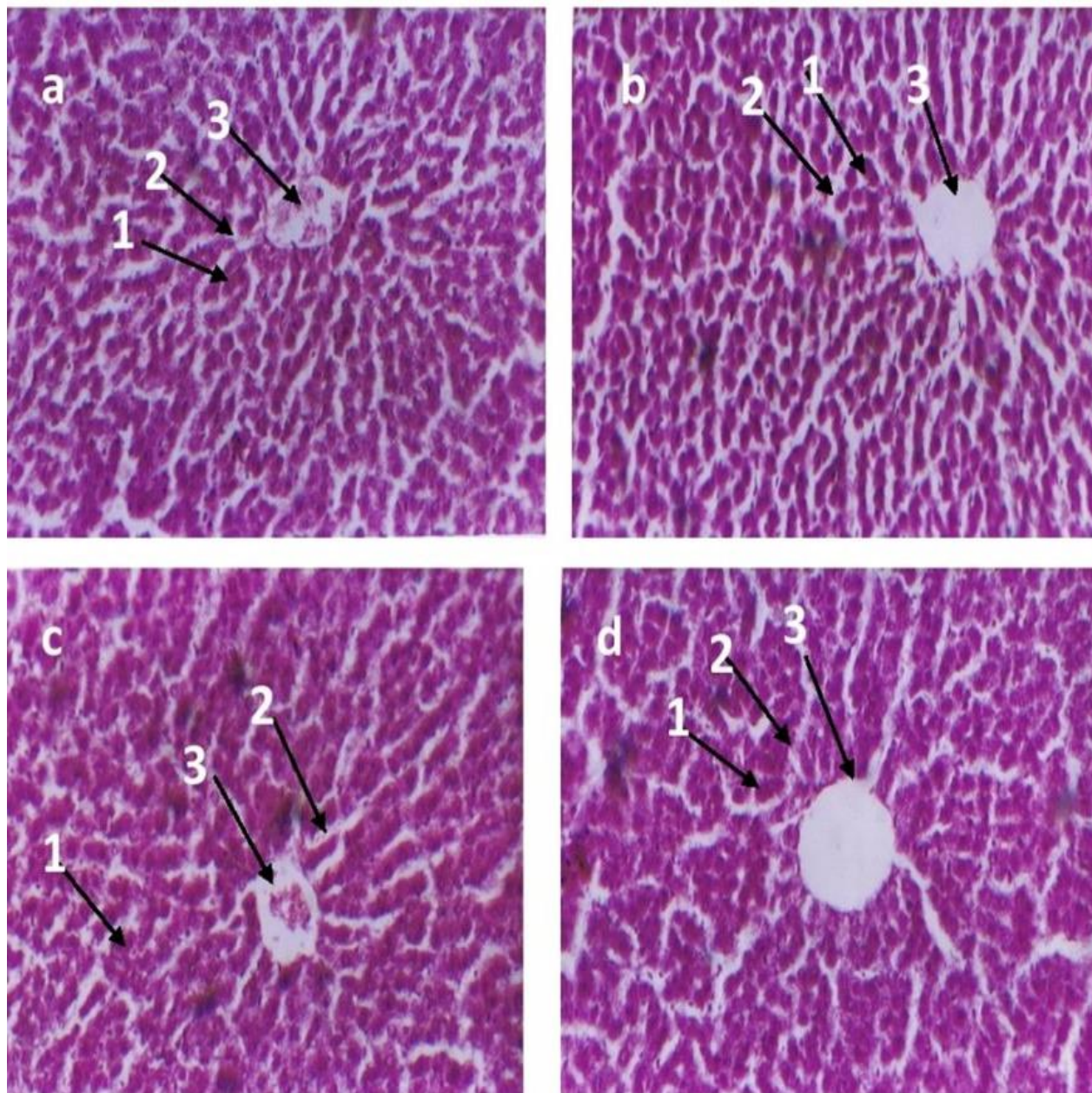


**Figure 3.** Histological appearance (H&E-stained, x132) of the lungs at termination of treatment for control (a), 60(b), 600(c) and 1200(d) mg/kg of Asena, showing normal alveolar areas (1), and normal Clara cells (2) lining a normal bronchiolar epithelial wall (3).

cytosis (Okokon et al., 2010). In the present studies, Asena significantly elevated the level of platelets in all test animals compared to controls. This may be attributable to enhanced production and secretion of thrombopoietin, the primary platelet production regulator, (Kaushansky, 1995) by Asena indicating that it may have hemostatic property (Olaleye et al., 1998).

Histological examination of Asena treated samples stained with hematoxyline and eosin revealed no morphological adverse effect at the microscopic level. In a previous study, *Mitragyna speciosa* extract have revealed the presence of Kupffer cells and karyomegaly in the liver of the rat (Harizala et al., 2010). Furthermore, hepatotoxins such as paracetamol cause hepatocyte





**Figure 4.** Histological appearance (H&E-stained, x132) of the liver at termination of treatment for control (a), 60(b), 600(c) and 1200(d) mg/kg of Asena, showing normal hepatocytes (1), interstitial spaces (2) and central veins (3).

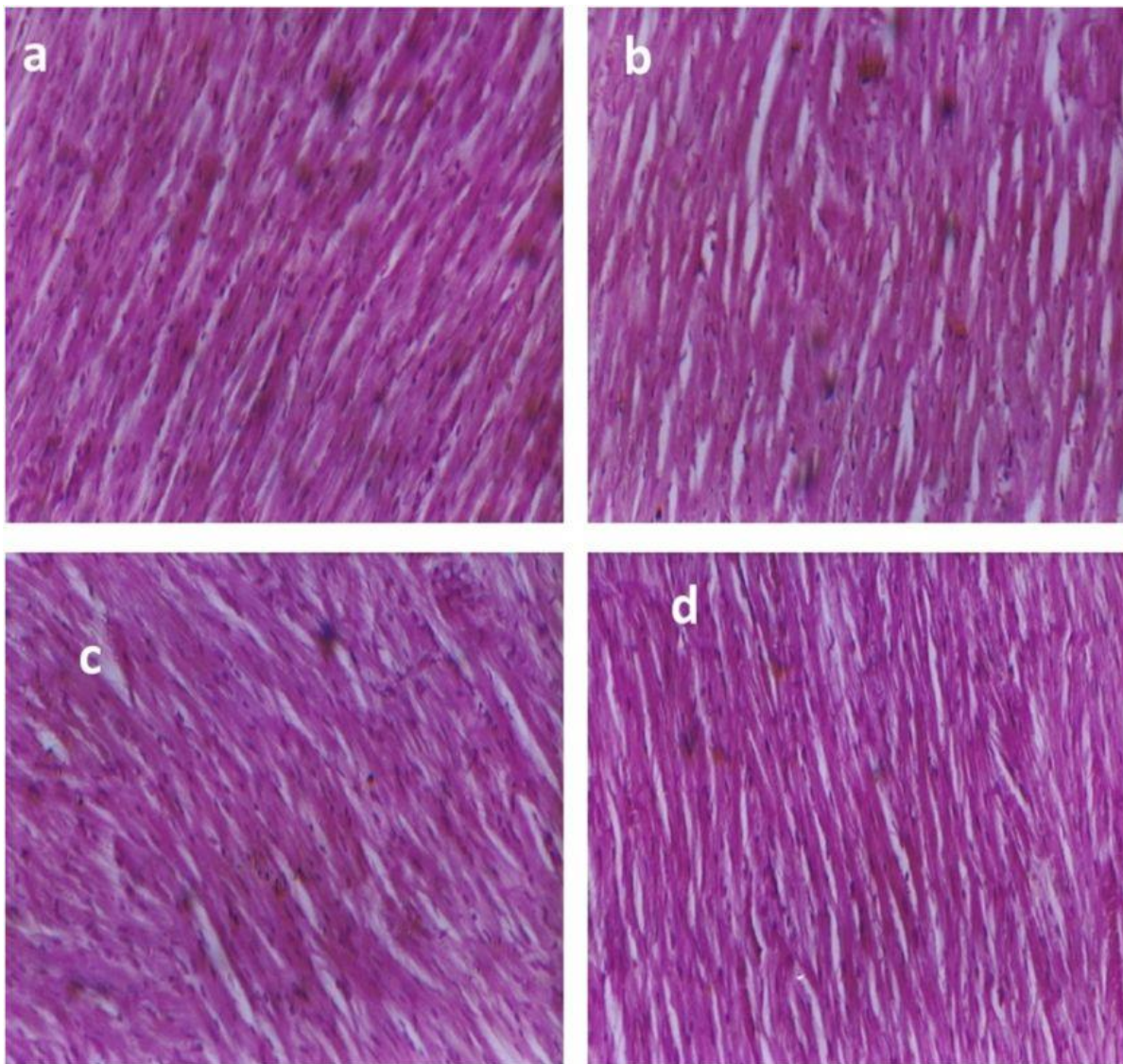
degeneration, centrilobular necrosis and hemorrhages (Iweala et al., 2011). Pneumotoxins on the other hand are known to cause inflammatory response such as alveoli collapse and interstitial inflammatory cellular infiltration in the lungs of rats (Zidan, 2011). Similarly, nephrotoxicity is associated with hypertrophied and necrotic cells, degenerated renal corpuscle with expanded mesangial matrix, hyalinization in the glomerulus (segmental glomerulosclerosis), and congested blood vessel between the tubules in the rat (Abdu et al., 2011). In the present studies, Asena did not adversely affect any of the organs. This observation coupled with the fact that the

formulation did not significantly affect the biochemical and hematological parameters assayed for in the studies confirms that the preparation may be safe in the rat upon prolonged administration.

### Conclusion

In conclusion, it can be said that the sub chronic administration of Asena may not affect the normal growth nor cause organ specific toxicity in the rat. These findings support its continual use and at least provide a basis for





**Figure 5.** Histological appearance (H&E-stained, x132) of the heart at termination of treatment for control (a), 60(b), 600(c) and 1200(d) mg/kg of Asena, showing no differences in morphology of cardiac cells.

its assessment in clinical trials.

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