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Research Article

In vivo Studies: Effects of *Arnica montana* Linn. Extract on Blood, Urine and Histo-Pathological Parameters of Albino Rabbits

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ABSTRACT

Arnica montana Linn. (Asteraceae) is well known for curing mental, emotional, spiritual shocks and physical injuries. Our present research is carried out to investigate the toxicological effect on albino rabbits of low dose of *Arnica montana* when administered orally for a period of three months. Hematological, biochemical, histo-pathological studies and urine analysis were carried out to evaluate effects of low dose of extract when given for 90 days. In male test group, all the blood parameters were observed to be elevated while in female were found to be lowered except platelet count that was elevated. Creatinine and uric acid levels were found raised in female test group as compared to male test group. Cardiac enzymes were elevated in male test group and were lowered in female test group. Male test group revealed decreased lipid profile and liver enzymes parameters whereas, female test group exhibited raised lipid profile and liver enzymes levels, respectively. Urine volume and specific gravity were found lowered in both male and female test group. Blood cells were observed in urine of female test group. No significant toxic effects were observed with low dose treatment of *Arnica montana* on the stomach, kidney and liver tissues of male rabbits. Whereas, areas of myocytolysis in right ventricular wall and inter-ventricular septum was observed on the heart tissues. Our results justify the well-documented uses of *Arnica montana*.

Keywords: histopathology, biochemical parameters, hematology, urine analysis

INTRODUCTION

Arnica montana belongs to family Asteraceae and is commonly known by the following common names; Leopard's bane, common arnica, mountain arnica, mountain daisy, wolfsbane, mountain tobacco. The active components in arnica are sesquiterpene lactones, which are known to reduce inflammation and decrease pain. Other active principals are thymol (an essential oil), flavonoids, insulin, carotenoids, and tannins. *A. montana* is used topically for a wide range of conditions, including bruises, sprains, muscle aches, wound healing, superficial phlebitis, joint pain, inflammation from insect bites, and swelling from broken bones. *A. montana* is useful for patients suffering from dialysis complications. It is of great help in hemodialysis procedure in preventing clotting that might lead to complications such as subdural hematoma and intra-cerebral hemorrhage[1-3]. An extract of *A. montana* increased the resistance of animals to bacterial infections by activating phagocytosis of the bacteria involved. The sesquiterpene lactones helenalin acetate and 11, 13-dihydrohelenalin exhibited platelet aggregation, as well as anti-bacterial and anti-fungal activities. Acidic polysaccharides derived from an extract of *A. montana* have revealed in *invitro* studies immune-stimulating activities. The constituents of *A. montana* possess significant analgesic and anxiolytic effect[4]. *A. montana* can increase blood pressure; therefore, it should be used with caution in patients suffering from hypertension, heart disease or circulatory disorders[5,6]. The objective of our research work is to evaluate the effect of *A. montana* extract on hematological, biochemical and histo-pathological parameters of rabbits.

MATERIALS AND METHOD

Chemicals

Ethanol, acetic acid, formalin, diagnostic kits of biochemical parameters, xylene, paraffin wax, eosin, hematoxylin and Canada balsam were purchased from Merck, Germany. All the chemicals were of analytical grade.

Plant Extract

A. montana mother tincture (Lot # 4050409) was purchase from William Schawabe suppliers. Extracts was concentrated by rota-evaporator (Buchi-Rotary Evaporator, Switzerland, model # B490) at 40°C. The extract obtained was stored in cool, dry place for further studies.

Experimental Animals

Twenty-four male and female rabbits weighing between 1000 and 1,200 g were purchased from Animal House of Dow University of Health Sciences, (DUHS) Karachi and kept in animal house for a period 15 days to acclamized in separate cages. They were fed commercial feed and water *ad libitum*. Their weights were checked on random basis.

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Table 1: Chronic toxicity test: Effect of A. montana extract on complete blood count of rabbits.

Blood Parameter	Control Female	Test Female (AF)	Control Male	Test Male (AM)	Reference Range
Haemoglobin	12.15±0.0836	10.96±0.0408*	10.05±0.0836	11.835±0.0739	10.75±0.689
RBC (Erythrocyte Count)	5.895±0.00836	5.82±0.0063	5.485±0.0083	5.735±0.00836	3.916±0.277
Hematrocrit (HCT/PVC)	42.835±0.0739	38.835±0.0739*	34.2±0.0632	40.35±0.0836*	38.67±1.932
MCV	72.416±0.0658	66.75±0.0836*	62.5±0.836	70.35±0.0836*	89±3.183
MCH	20.835±0.0739	18.835±0.07395*	18.15±0.0836	20.35±0.0836*	30.167±1.180
MCHC	28.783±0.0658	28.316±0.0658	29.05±0.0836	29.23±0.201	32.5±0.836
Total Leucocyte Count (WBC)	6.05±0.0836	7.15±0.0836	5.5±0.0632	9.25±0.0836*	11±1.673
Platelet Count	353.5±0.836	327.67±0.7302**	140.5±0.836	526.83±0.658**	275±41.83

AF = female rabbit treated with drug; AM = Male rabbit treated with drug All values are mean ± SEM; n = 6; * = Significant ($p<0.05$), ** = Highly significant ($p<0.01$).

Table 2: Chronic toxicity test: Effect of A. montana extract on kidney function parameters of rabbits.

Biochemical Parameters	Control C (female)	Test Animal (AF)	Control C (male)	Test Animal (AM)	Reference Range
Urea	72.5±0.83	55.08±0.63**	23.5±0.83	29.08±0.63*	29.167±6.39
Creatinine	0.85±0.008	0.98±0.024	0.85±0.0083	0.775±0.0083	0.8167±0.127
Calcium (serum)	14.59±0.063	15.17±0.009	14.17±0.0083	14.94±0.0203	10.03±0.318
Phosphorus	3.825±0.068	3.405±0.0083	6.195±0.0083	4.73±0.017*	3.53±0.318
Uric acid	0.0175±0.004	0.065±0.0083*	0.165±0.0083	0.08±0.006**	3.916±0.639
Total proteins	8±0.02	8.275±0.008	7.495±0.0083	7.235±0.0083	7.467±0.347
Albumin	5.83±0.013	5.515±0.0083	4.305±0.0083	4.645±0.0083	4.5±0.28
Globulin	2.153±0.0096	2.771±0.019	3.185±0.0083	2.585±0.0083*	2.35±0.146
A/G ratio	2.715±0.0083	1.94±0.025*	1.35±0.016	1.795±0.0083	0.75±0.052

AF = female rabbit treated with drug; AM = Male rabbit treated with drug All values are mean ± SEM; n = 6; * = Significant ($p<0.05$), ** = Highly significant ($p<0.01$).

Blood (6 ml) was collected from rabbits for analyses of

Blood (6 ml) was collected by cardiac puncture with 10 ml

Table 3: Chronic toxicity test: Effect of A. montana extract on cardiac enzymes of rabbits.

Biochemical Parameters	Control C (female)	Test Animal (AF)	Control C (male)	Test Animal (AM)	Reference Range
LDH	163.5±0.836	130.5±0.83**	270.5±0.83	135.5±0.83**	331.67±40.34
CPK	729.5±0.83	675.5±0.83**	421.5±0.83	818.5±0.83**	90.33±25.03
CK-MB	852.5±0.83	378.83±18.81**	194.5±0.83	473.5±0.83**	16.67±2.46

AF = female rabbit treated with drug; AM = Male rabbit treated with drug All values are mean ± SEM; n = 6; * = Significant ($p<0.05$), ** = Highly significant ($p<0.01$).

hematological and biochemical parameters by cardiac puncture at the end of three months. Blood samples collected into clean non-heparinised bottles were allowed to clot and serum was separated from the clot and centrifuged according to groups into clean bottles for the biochemical analyses. After the collection of blood samples, urine analysis and histopathology was carried out.

Animal grouping

Four groups were made (male control – 6 rabbits), (female control – 6 rabbits), (male test (AM) – 6 rabbits) and (female control (AF) – 6 rabbits). Male and female control groups were given distil water, while test groups AM and AF were given 25mg/kg *A. montana* extract. All the administrations were given orally. The treatment continued for 90 days.

sterile syringe using 1mg/1ml EDTA as anticoagulant for the determination of blood and biochemical parameters. After blood collection only male control group animals and test group (AM) rabbits were sacrificed and liver, heart, kidney and stomach were dissected out for histo-pathological studies.

Hematological Evaluation

Hematological examination of the collected blood samples was performed according to standard procedures listed as follow. Total erythrocyte counts were counted using a Neubar chamber under a light microscope at 40 x 10 magnifications. Blood samples were diluted to 200 times by Hayem's reagent before counting. Blood hemoglobin concentration was determined using a Sahli's hemometer. Micro Wintrobe hematocrit tubes and hematocrit centrifuge were used to determine the (PCV). Total

Table 4: Chronic toxicity test: Effect of A. montana extract on lipid function parameters of rabbits.

Biochemical Parameters	Control C (female)	Test Animal (AF)	Control C (male)	Test Animal (AM)	Reference Range
Cholesterol	30.5±0.83	87.5±0.83**	58.5±0.83	13.5±0.83**	109.16±22.24
Triglycerides	0.5±0.83	173.5±0.83**	131.5±0.83	67.5±0.83**	111.67±13.68
HDL	12.5±0.83	21.5±0.83**	6.5±0.83	5.5±0.83	19.67±3.18
LDL	16.5±0.83	42.5±0.83**	38.5±0.83	2.83±0.65**	103.33±15.14
VLDL	7.5±0.83	33.5±0.83**	26.5±0.83	12.5±0.83**	30±5.83

AF = female rabbit treated with drug; AM = Male rabbit treated with drug All values are mean ± SEM; n = 6; * = Significant ($p<0.05$), ** = Highly significant ($p<0.01$).

leucocyte counts were detected using a Neubar chamber under a light microscope at 10 x 10 magnification after diluting blood samples to 10 times with Turk's solution. Mean erythrocyte volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) for particular blood samples were also calculated[7-11].

Biochemical Evaluation

Serum samples were obtained by centrifugation of blood at 1300 x g for 15 min. The Menarini Classic Chemistry Analyzer was used to determine the calcium (Ca), phosphorus (P), blood urea, creatinine, total bilirubin, total protein, albumin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine phosphokinase (CPK), cholesterol, glucose, amylase, and gamma-glutamyl transferase (GGT). The globulin concentration was determined by subtracting the albumin concentration from the total protein concentration[12,13].

Urine Analysis

Voided sample of urine was collected by placing a clean, empty litter box in the site where the animals usually urinates[14].

Histo-pathological Analysis

After blood collection, the liver, kidney heart and stomach of the male control and test group were carefully dissected from the abdominal region and were immediately fixed in 10% neutral buffered formalin. Fixed samples were trimmed and processed for paraffin embedding. Sections (5–7 μm) were cut and the tissues were dehydrated with alcohol of graded concentrations and allowed to dry. The sample slides were subsequently stained in haematoxylin-eosin and examined under a light microscope; photomicrographs of the samples were recorded[15-17].

Statistical Analysis

All the results were presented as a mean plus or minus standard error of mean (M ± SEM). Differences between control and treatment groups were analyzed by student t-test[18].

RESULTS

Hematology

In female test group (AF), $p<0.05$ level of lowering was observed in following blood parameters: hemoglobin, hematocrit, MCV and MCH. Platelet count was significantly lowered ($p<0.01$), in AF group. In male test group, $p<0.05$ level of elevation was found in hematocrit, MCV, MCH and total leucocyte count; while platelet count was found to be significantly elevated ($p<0.01$). Both test groups comparison was done with respect to their control groups respectively (Table 1).

Kidney Function Test

In female test group (AF), urea level was observed to be significantly lowered ($p<0.01$). A/G ratio was slightly reduced ($p<0.05$); while uric acid level was raised $p<0.05$ level. In male test group (AM), phosphorus and globulin levels were lowered, whereas urea level was elevated ($p<0.05$). Uric acid level was found elevated ($p<0.01$) in male test group. Male and female test groups' comparisons were done with their respective control groups (Table 2).

Cardiac Enzymes

Cardiac enzymes (LDH, CPK and CK-MB) were found to be significantly lowered ($p<0.01$) in female test group (AF). In male test group (AM), LDH level was significantly reduced while CPK and CK-MB enzymes were found elevated ($p<0.01$). both gender groups comparison was done with respect to their control groups (Table 3).

Lipid Profile

All the lipid profile parameters were found to be elevated ($p<0.01$) in female test group (AF) as compared to its respective control group. In male test group (AM), following lipid profile parameters were observed to be significantly lowered ($p<0.01$): cholesterol, triglycerides, LDL and VLDL in comparison with its respective control group (Table 4).

Liver Enzymes

In female test group (AF), $p<0.05$, elevation was observed in SGOT, alkaline phosphatase and gamma GT parameters, whereas, SGPT was found to be $p<0.01$ raised. In male test group (AM), direct bilirubin was found lowered and gamma GT was observed to be raised ($p<0.05$). While $p<0.01$ lowering of SGOT, SGPT and alkaline phosphatase levels were observed in AM group as compared to the respective control group (Table 5).

Urine analysis

In female test group (AF) and in male test group (AM) as compared with their respective female and male control groups, volume and specific gravity of urine was observed to be decreased. Blood cells were found in the urine of male test group (AM). No other changes were seen in the urine (physical, chemical and microscopy) of the both test groups (AF and AM) respectively (Table 6).

Histopathology

In the control male group, no significant pathology was observed in heart and stomach tissues respectively

Table 5: Chronic toxicity test: Effect of A. montana extract on liver enzymes parameters of rabbits.

Biochemical Parameters	Control C (female)	Test Animal (AF)	Control C (male)	Test Animal (AM)	Reference Range
SGOT	26.5±0.83	31.5±0.83*	42.5±0.83	20.5±0.83**	21.83±3.11
Total Bilirubin	0.275±0.0083	0.275±0.0083	0.265±0.0083	0.255±0.0083	1.75±0.083
Direct Bilirubin	0.021±0.005	0.03±0.0063	0.041±0.0065	0.0265±0.0073*	0.029±0.0008
SGPT	41.5±0.83	78.5±0.83**	68.5±0.83	40.5±0.83**	27.5±4.18
Alkaline Phosphatase	37.5±0.83	42.5±0.83*	228.5±0.83	103.5±0.83**	91.67±17.30
Gamma GT	6.5±0.83	8.5±0.83*	9.5±0.83	13.16±0.65*	29.16±6.39

AF = female rabbit treated with drug; AM = Male rabbit treated with drug All values are mean ± SEM; n = 6; * = Significant ($p<0.05$), ** = Highly significant ($p<0.01$).

Table 6: Chronic toxicity test: Effect of A. montana extract on urine parameters of rabbits

Urine Parameters	Control Animal C (female)	Test Animal (AF)	Control Animal C (Male)	Test Animal (AM)	Reference Range
Urine Physical					
Volume	30.08±0.11	10.25±0.2	25.01±0.136	15.1±0.11	179.17±61.81
Colour	Yellow	Yellow	Yellow	Yellow	Pale yellow-red brown
Appearance	Turbid	Turbid	Turbid	Turbid	Clear
Sp. Gravity	1.0045±0.00037	1.003±0.0007	1.0045±0.00037	1.003±0.0007	1.019±0.007
pH	9±0.063	9.07±0.096	9±0.063	9.07±0.096	8.53±0.195
Urine Chemical					
Protein	Nil	Nil	+1 (30 mg/dL)	Nil	Negative
Glucose	Nil	Nil	Nil	Nil	Negative
Ketone Bodies	Negative	Negative	Negative	Negative	Negative
Urobilinogen	Normal	Normal	Normal	Normal	Negative-weak positive
Blood	Negative	Negative	Negative	Positive +	Negative
Bilirubin	Nil	Nil	Nil	Nil	Negative
Urine Microscopy					
RBC	Nil/ HPF	Nil/ HPF	Nil/ HPF	Nil/ HPF	Nil/ HPF
WBC	Nil/ HPF	Nil/ HPF	Nil/ HPF	Nil/ HPF	Nil/ HPF
Epithelial Cell	Nil/ HPF	Nil/ HPF	Nil/ HPF	Nil/ HPF	Nil/ HPF

AF = female rabbit treated with drug; AM = Male rabbit treated with drug

(Figures 1 & 7). Mild portal inflammation and peri-portal fibrosis was found in liver tissues (Figure 5) and chronic non-specific pyelonephritis was seen in tissues of kidney (Figure 3). The following histo-pathological findings were observed in the heart, liver and kidney tissues of the male test group (AM) respectively. In heart tissues focal areas of myocytolysis in right ventricular wall and inter-ventricular septum was seen (Figure 2). Patchy chronic non-specific pyelonephritis was seen in kidney tissues, but no evidence of granuloma or malignancy was witnessed (Figure 4). Mild portal inflammation and fibrosis was observed in liver tissues (Figure 6). No significant pathology was found in examined stomach tissues of the male test group (Figure 8). Male control group heart tissue - Sections show wall of heart composed predominantly of thick myocardium consists of bundles of cardiac muscle fibers separated by fibrous band, forming syncytium. Nuclei of myocytes are centrally located. Endocardium is lined by single layer of mesothelial cells resting on a basement membrane. No significant pathology is seen in any of the sections examined. *A. montana* male test group heart tissue (AM): Focal areas of myocytolysis in right ventricular wall and inter-ventricular septum. Sections show wall of heart composed predominantly of thick myocardium consists of bundles of cardiac muscle fibers separated by fibrous band, forming syncytium. Nuclei of myocytes are centrally located. Focal areas of myocytolysis are seen in right ventricular wall and inter-ventricular septum. Endocardium is lined by single layer of mesothelial cells resting on a basement membrane. No significant pathology is seen in any of the sections examined. Figure 3: Control male Kidney tissue. Sections show renal tissue composed of cortex and medulla. Glomeruli are within normal limits. Tubule-interstitial compartment shows focal lymphocytic infiltrate. Vascular structures are distributed evenly. No evidence of granuloma or malignancy is seen in any of the sections examined. *A. montana* test male group kidney tissue (AM): Patchy chronic nonspecific pyelonephritis. No evidence of granuloma or malignancy seen. Sections show renal tissue composed of cortex and medulla. Glomeruli are within normal limits. Patchy areas of lymphocytic infiltrate are seen in the interstitium. Vascular structures are distributed evenly. No evidence of granuloma or malignancy is seen in any of the sections examined. Male Control Liver tissue: Sections show liver tissue with overall preserved lobular

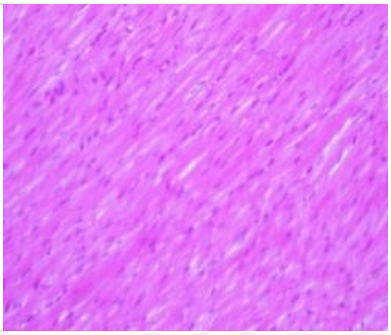

Figure 1: Histo-pathological slide of heart tissues of male control group

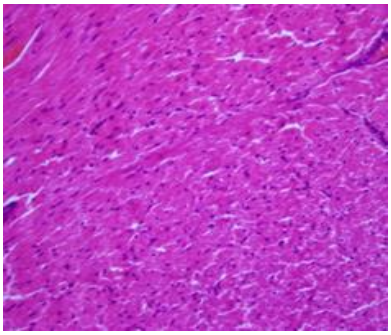

Figure 2: Histo-pathological slide of heart tissues of male test group (AM)

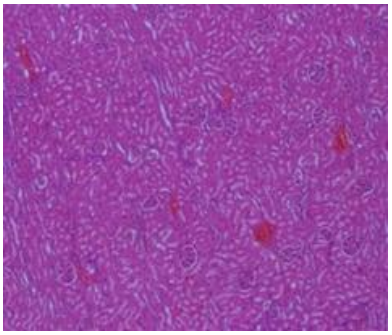

Figure 3: Histo-pathological slides of kidney tissues of male control group

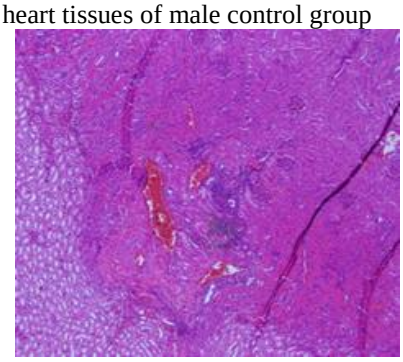

Figure 4: Histo-pathological slides of kidney tissues of male test group (AM)

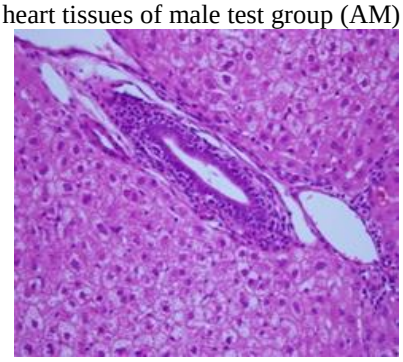

Figure 5: Histo-pathological slides of liver tissues of male control group.

Figure 6: Histo-pathological slides of liver tissues of male test group (AM).

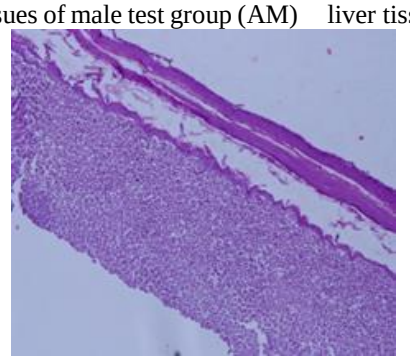

Figure 7: Histo-pathological slides of stomach tissues of male control group.

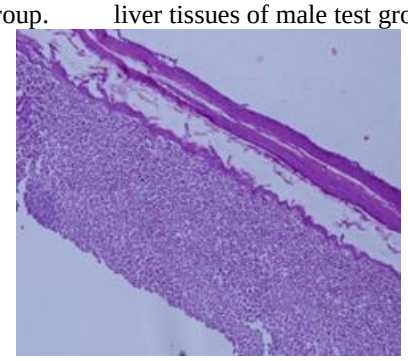

Figure 8: Histo-pathological slides of stomach tissues of male test group (AM).

architecture. Portal tracts are mildly dilated with lymphocytic infiltrate and minimal fibrosis. Centri-lobular hepatocytic degeneration also noted. No siderosis. No cholestasis. No evidence of granuloma or malignancy is seen. *A. montana* male test group liver tissue (AM): Mild portal inflammation and fibrosis. Sections show liver tissue with overall preserved lobular architecture. Portal tracts are mildly expanded with lymphocytic infiltrate and minimal fibrosis. No significant lobular inflammation seen. No siderosis. No cholestasis. No evidence of granuloma or malignancy is seen. Male Control Stomach tissue: Sections show wall of gastric mucosa with intact architecture. The gastric mucosa is thrown into gastric pits and folds revealing well organized glandular structures. Underlying submucosa is scanty and in unremarkable. Well organized muscular layer is seen beneath, lined externally by serosa. No significant pathology is seen in any of the sections examined. *A. montana* male test group stomach tissue (AM): Sections show wall of gastric mucosa with intact architecture. The gastric mucosa is thrown into gastric pits and folds revealing well organized glandular structures. Underlying submucosa is scanty and in unremarkable. Well organized muscular layer is seen beneath, lined externally by serosa. No significant pathology is seen in any of the sections examined.

DISCUSSION

The medicinal uses and efficacy of plants is well-known but the lack of the quality control data and toxicological studies is a major hurdle in the acceptance of the plant-based drugs. *A. montana* has well-documented data concerning its use in the treatment of mind and body pain and injuries. *A. montana* contains sesquiterpene lactones, flavonoids, volatile oil, mucilage and polysaccharides, tannins, miscellaneous substances. The flowers do not contain tannin and contain more arnicin than the rhizome. The active constituents of *A. montana*, helenalin and dihydrohelenalin are responsible for its anti-inflammatory, analgesic and antibiotic effect[19-21]. Our present research work is based on toxicological studies (Hematology, biochemistry, urine analysis and histopathology) carried over the period of three months with the daily

administration of 25mg/2ml dose of *A. montana*, in order, to determine the efficacy and safety of *A. montana*. The toxic effects exhibited by *A. montana* by the administration of low dose of the extract for 3 months may be due to the presence of flavonoids and helenalin present in it. Hematological studies help in diagnosis and in investigation of extent of damage caused by a particular pathological state[22-24]. Analysis of serum biochemistry (cardiac enzymes, lipid profile, liver enzymes and kidney function test) assists in the diagnosis and prognosis of heart, liver and kidney diseases[25]. The female test group animals (AF) that had been administered orally 25mg/kg of drug/ day, have reduced level of hemoglobin, RBCs count and MCV, maybe due to depression of erythropoiesis or anemia[26]. Results of urine analysis (decreased volume and specific gravity of urine) provided evidence of the presence of acute tubular necrosis (ATN) in kidney tissues along with the following kidney function enzymes levels, reduced level of urea and increased levels of creatinine and uric acid. Severe ATN is the causative factor of acute kidney injury[27]. The liver function enzymes biochemical findings (increased SGOT, bilirubin direct, SGPT and alkaline phosphatase may be suggestive of mild acute hepatitis[28,29]. Decrease levels of cardiac enzymes and elevated levels of the parameters of lipid profile were observed in the female test group. In the male test group animals (AM) all hematological parameters were found to be elevated which might also be the reason of the presence of red blood cells in urine on urine analysis. Histo-pathological findings of the test male group (AM) revealed patchy chronic non-specific pyelonephritis. These findings may be supported with biochemical results (lowered urea, creatinine, and uric acid levels) and urine analysis (decreased volume of urine, decrease specific gravity with the presence of blood cells in urine). Chronic pyelonephritis eventually leads to end-stage renal failure[30]. In male test group decreased LDH and significantly increased CPK and CK-MB may have caused focal areas myocytolysis in right ventricular wall and inter-ventricular septum is the source of ischemic cardiomyopathy[31,32]. Significantly decreased lipid profile parameters (cholesterol, triglycerides and LDL) may also have facilitated the damaging of heart. Decrease in the level of all liver enzymes except Gamma-GT may have assisted in the development of mild portal inflammation and fibrosis, which is the cause of mild acute hepatitis[33].

CONCLUSION

The present work details comprehensively the effect of low dose of *A. montana* on hematological, biochemical, urine and histo-pathological parameters. All the results support the already documented medicinal uses of *A. montana*. The toxic effects that appeared might be due to dose or frequency of administration. The safety profile of this plant extract should be verified by further experimentation including clinical studies.

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