

## Evaluation of *In Vivo* Toxicity of Dichloromethane: Methanolic Leaf Extracts of *Prosopis juliflora* in Female Wistar Albino Rats

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### Abstract

*Prosopis juliflora* (Mathenge) is an exotic, evergreen leguminous plant found in the dry Coastal, Rift Valley and Northern parts of Kenya. It is tolerant to extreme environmental conditions, rated among top 100 most invasive species worldwide. The species leaf extracts is used in folk cure to various ailments and have promising pharmacological properties however; information on their toxicity in animals and human is insufficient. The study assessed phytochemical composition of *P. juliflora* leaf extracts, effects on body weights, organ weights, hematological parameters, liver function markers and histopathology in major organs of Wistar albino rats. Acute toxicity test was carried out at 2000 mg/kg body weight followed by a 28 days sub chronic toxicity study at 100, 350 and 1000 mg/kg body weight extracts dosages. The control animals were administered with normal saline. Animals were monitored for physical and behavioral changes including death. They were fasted overnight on 28th day and sacrificed on anesthesia on 29th day. Blood was collected by cardiac puncture. Hematological and liver functions tests were done. Tissue sample of selected organs were processed for histopathology. Data from control and treated animals groups were analyzed by ANOVA and Dunnett's test. Phytochemicals confirmed included alkaloids, flavonoids, phenols, tannins, terpenoids and saponins but no cardiac glycosides. Median lethal dose was estimated at above 2000 mg/kg body weight. Dose related transient toxicity symptoms included wheezing, decreased activity, and pilo erection. No significant toxicity effects on hematological parameters were noticed except in mean platelets volume. Similarly, no significant adverse effects occurred in liver function tests except at 1000 mg/kg body weight dosage. No significant adverse changes in plasma proteins, body weights and absolute organ weights were observed except in kidneys and spleen. Histological examination of sample tissues showed mild effects in spleen and kidneys but no adverse pathology in other organ tissues.

**Keywords:** *Prosopis juliflora*; Toxicity; Haematology; Histopathology

### Introduction

The medicinal properties of plants is linked to phytochemicals synthesized to protect them from infections, herbivory, injuries, photosynthetic stress, competition for available resources among other functions [1,2]. A number of secondary metabolites including tannins, phenols, flavonoids, terpenoids, saponins and alkaloids have been identified in extracts from different parts of *P. juliflora* such as leaves, pod, roots, flowers and the bark, all with varied pharmacological properties. From the leaves, several piperidine alkaloids have been isolated and structures illustrated. They are juliprosinene, juliflorine, julifloridine, julifloricine, 3'-oxojuliprosopine, sceojuliprosopinol, 3-oxojuliprosine and 3-oxo-juliprosine [3].

Different studies have reported that the piperidine alkaloids from *P. juliflora* leaves have better antibacterial activity compared to some first line antibiotics. For example, alkaloids from the species leaf extracts demonstrated better active against *S. aureus*, *S. lactis*, *S. faecalis*, *S. payogenes* and *C. diphtheria* compared to bacitracin, gentamycin, chloromycin and trimethoprim [4]. Correspondingly, the extracts from the leaves have showed higher inhibitory activity against *E. coli*, *S. aureus*, *B. cereus*, *P. putida*, Klebsiella, Salmonella, Acinetobacter, and Alcaligen when compared to ampicillin, tetracycline, streptomycin, chloramphenicol, rifampicin [5,6]. Separately, the pathogens Acinetobacter, Alcaligen and *E. coli* were reportedly resistant to all the

standard antibiotics used in a study except ofloxacin, similar to leaf and pod extracts of *P. juliflora* species. Comparison of extracts composition from the two parts of the species showed just a slight variation in the number of alkaloids present [1,7]. Similarly, Piperidine alkaloids from the leaves are active against clinical isolates of *Cryptococcus neoformans* compared to environmental isolates, making it a future potential therapy for cryptococcosis and cryptococcal meningitis [8,9]. Meanwhile, juliflorine in the leaves is active against *Trichophyton mentagrophytes* which causes dermatophytic lesions in rabbits [1].

Apart from the antimicrobial properties, *P. juliflora* alkaloids also possess anticancer and cytotoxic activities against human epithelial tumour cells (HeLa) and human liver tumour (HeG2) as well as two other fibroblast lineages F26 and F57. Similar activity has been reported against Molt-4 cells *in vitro* [4,10]. The leaf extracts have acetylcholinesterase inhibiting properties, while polyphenols have demonstrated free radical scavenging activity. The bark extracts have anti-inflammatory action in both acute and chronic study models; and inhibit serotonin and histamine [1]. In addition, flavonoids from the plant's pollen possess antioxidant properties similar to mesquite from the heartwood. Comparison between mesquite, probucol and  $\alpha$ -tocopherol shows that the former has better anti-inflammatory activity [4].

Compared to synthetic medicine; and despite wide spread use of plants in herbal remedies, feed and even food, scientific research to

establish their safety and quality is still a challenge; more so in the developing countries which have higher dependency on phytomedicine in primary healthcare [1,11]. Toxicity test for plants of value is important because a number of studies have reported various adverse biochemical and toxicological effects of secondary metabolites of plant origin [12]. Cases of human poisoning by higher plants often occur in children and involve plants that produce attractively colored but toxic fruits [13]. On the other hand, poisoning of livestock by plants is prevalent in extensive grazing areas where poisonous weeds are seldom controlled and as such occur together with feed [14]. While toxicity effects of most metabolites are intrinsic, in some instances, it results from catabolic process of the ingesting organisms on the secondary metabolites. For example, it is reported that monogastric animals are affected by trypsin inhibitors whereas ruminants, capable of digesting trypsin in rumen are not affected [15]. The decision to perform toxicity studies on *P. juliflora* was informed by reported use in folk medicine, the immense potential for research in pharmaceutical and nutraceutical industry; in addition to perceived adverse effects in animals [16].

## Materials and Method

### Collection and preparation of plant material

Fresh leaves of *Prosopis juliflora* were collected from Kenya Forestry Research Institute located in Kitui, Eastern part of Kenya. The plant was botanically identified by an acknowledged taxonomist and a voucher specimen deposited at the Kenyatta University herbarium. The collected fresh leaves were washed thoroughly in tap water and shade dried at room temperature for two weeks. They were ground into small particles using a blender and thereafter, an electric mill to generate a fine powder. The powdered samples were labeled using stickers, weighed and recorded ready for extraction [17].

### Extraction

The complexity of compounds in plant parts necessitates use of solvents of different polarities when extracting the various phytochemicals. Moreover, medicinal properties of plant extracts have been associated largely with unique combinations between the various individual component metabolites [18]. Cold extraction was carried out using DCM: Methanol mixture (ratio of 1:1). A 500 gms quantity of the powdered leaves of the species material was put in a beaker containing one litre of the solvents so that the dry powder was totally submerged. The mixture was kept for 24 hrs. The mixture was then decanted, filtered using muslin cloth and then with Whatman filters paper No.1. The whole process was repeated three times and supernatant collected and pooled together. The extract was concentrated by rotary evaporator at 40°C to obtain the dry extract which was kept in bottles [19].

### Laboratory animals and experimental design

Thirty healthy female Wistar albino rats aged between 8 and 10 weeks and weighing between 140 gms and 196 gms were obtained from Kenyatta University Animal House, Department of Biochemistry and Biotechnology. The animals were kept under standard laboratory conditions; 25°C, 12 hrs light and 12 hrs dark cycle. The rats were freely supplied with conventional rodent laboratory pellet diet from Unga Feeds Kenya limited and continuous supply of drinking water. Five rats were randomly selected, marked to permit individual

identification and housed in a standard cage. They were allowed to acclimatize for five days before starting the limit test at a dose of 2000 mg/kg body weight and 28 days sub-chronic toxicity studies. All protocols followed were in line with Organization for Economic Co-operation and Development guidelines on animal studies [20,21].

### Acute oral toxicity test

A limit test at 2000 mg using the Up-Down method was performed based on information from previous preliminary work [16]. Five animals were fasted overnight, only water provided and weighed using electronic balance (Shimadzu Corporation: BL-220H). The fasted body weights were used to calculate individual dosages of the test substance for oral administration. Animals were sequentially dosed at 48 hrs intervals by oral gavages using specialized needles. After administering the dosages, the animals were denied access to food except water for the initial 4 hrs. They were observed for fatality within the initial 12 hrs, and up to 48 hrs. The results were recorded as Survival (0) or death (X) [20].

Sub-chronic toxicity study: For sub-chronic toxicity tests, a modified guideline for main test was applied [21]. The experiment consisted of four groups (I, II, III and IV) of animals. Groups II, III and IV, were orally administered with the extracts at doses 100, 350, and 1000 mg/kg body weights daily for 28 days respectively. The rats had free access to food and water throughout the study period. The doses were based on a progression factor of 3.2 starting from 1000 mg which is the lower limit for a substance to be considered nontoxic or safe [16]. Group I, the control group was orally administered with normal saline and similarly, had unlimited access to water and pellets. The rats were observed within the first 4 hrs, followed by 12 hrs and daily for any abnormal clinical reactions including mortality during the study period. Their body weights were measured at the end of every 7 days and recorded. The animals were fasted overnight on the 28th day and the final body weights taken [22].

On the 29th day, all animals were sacrificed and the blood was collected by cardiac puncture using sterile needle syringe. One portion of the blood was put in bottles containing EDTA to prevent coagulation. The EDTA blood samples were immediately used to determine hematological parameters (full haemogram) using automated analyzer (Model: Changchun Dirui Industrial Co., LTD). Blood sample for biochemical tests was collected in bottles without EDTA and kept at 4°C for 4 hrs to let it clot and centrifuged at 1500 rpm for 15 minutes to obtain serum. The serum was refrigerated at -22°C and used for biochemical assays. The sacrificed animals were laid on a dissecting board after blood sample collection. Vertical mid-line was cut with a pair of scissors running from the neck to the pelvis to open the peritoneum. The rats' organs including liver, kidneys, heart, spleen, lungs and brain were excised, collected and weighed using the digital weighing balance. The values were expressed as absolute organ weights [23,24].

### Determination of haematological parameters

The hematological factors were analyzed based on guidelines in the manufacturer's operational manual of the auto-analyzer (Changchun Dirui Industrial Co., Ltd.). They included absolute White Blood Cell count (WBC), Red Blood Cell count (RBC), Hemoglobin (HB), Hematocrit (HCT), Mean Cell Volume (MCV), Mean Corpuscular Hemoglobin Concentration (MCHC), Platelets Count (PLT), Mean Platelet Volume (MPV), Platelets Distribution Width, (PDW) Red cell

Distribution Width (RDW), Absolute Lymphocytes Count (ALC), Absolute Neutrophils Count (ANC), Absolute Monocytes Count (AMC), Absolute Eosinophils Count (AEC) and Absolute Basophils Count (ABC).

### Determination of liver function (LF) parameters

The following markers of liver function were tested by automated chemical analyzer using the refrigerated animals sera; Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP), Aspartate Aminotransferase (AST), Gamma-Glutamyltransferase (GGT), Total Bilirubin (TBIL), (DBIL), Total Protein (TP) and Total Albumin (TALB) [25,26].

### Histopathological analysis

The organ tissues initially preserved in 40% formalin were washed in running tap water for 8 hrs to remove excess formalin. The tissues were then processed using automated tissue processor which involved serial dehydration in increasing concentrations of ethanol at hourly intervals. The dehydrated tissues were then cleared of excess alcohol in two changes of xylene. The tissues were embedded in paraffin wax with the help of Electro-thermal Wax Dispenser to form tissue blocks in squared metallic plates block moulds. Rotary microtome was used to section the tissue blocks manually at a thickness of 4  $\mu$ m and floated on warm water. Unfolded thin sections were picked by clean microscopic glass slides and stained using hematoxylin-eosin dyes and covered with DPX. The slides were examined under light microscope for any pathological changes due to oral administration of the extracts; and photomicrographs for both the treated and control tissues were taken [19,22,27].

### Qualitative phytochemicals screening

Standard phytochemicals screening procedures were applied to confirm the presence of the following secondary metabolites; alkaloids, phenols, tannins, terpenoids, cardiac glycosides, and flavonoids [19,28].

### Data management and analysis

The raw data was entered in spread sheet by columns based on dosages. They were fed into Minitab version 17.0 and data inspection performed through scatter gram and Bartlett's test followed by visual inspection to check out for any outliers. Outliers noted were winsorized by G-1 method [25]. The final values were expressed in mean  $\pm$  SEM (Standard Error of Mean). The data was analyzed using one way ANOVA followed by Dunnet's test to obtain significant values

among the various groups. The tools determined whether there were significant differences between the control animals and the animals administered with the leaf extracts at the various dosages. All  $P < 0.05$  values were considered significant [29]. Photomicrographs of slides prepared were printed and comparisons done between the control group tissues and the tissues of the groups orally administered with the extracts.

## Results

### Acute oral toxicity test

**After the sequential animals treatment with a single oral dosage at 2000 mg/kg body weight of DCM:** Methanolic leaf extracts of *Prosopis juliflora*, each animal was observed initially for 4 hrs thereafter 12 hrs up to 48 hrs for signs of toxicity. The noted transient symptoms included wheeze, decreased animals' activity, salivation, pilo-erection and loss of appetite. All the animals survived the initial 48 hrs observation period. However, the second and the fourth animal in the treatment sequence died after 58 and 70 hrs respectively. Correspondingly, the animals which survived the entire study period exhibited decreased severity of toxicity symptoms noted.

### Sub-chronic toxicity tests

**Effects of DCM: Methanolic leaf extracts of *Prosopis juliflora* on erythrocytic parameter profiles in Wistar albino rats:** The DCM: Methanolic leaf extracts of *Prosopis juliflora* induced changes in erythrocytes and related parameter profiles in treated mice (Table 1). After 28 days oral administration of the extracts at the dosage level of 100 mg/kg body weight, there was insignificant increase in Red Blood Cells (RBC), Mean Corpuscular Hemoglobin (MCH) and Mean Cell Volume (MCV) compared to the control ( $p > 0.05$ ; Table 1). Alternatively, there was insignificant decrease in Hemoglobin (HB), Mean Erythrocytic Width (RDW), and Mean Corpuscular Hemoglobin Concentration (MCHC) at the same dosage level compared to the control group ( $p > 0.05$ ; Table 2).

At 350 mg/kgbw dosage level, the extracts induced insignificant increase in RBC, HB, MCH, MCHC and MCV, after twenty 28 days of oral administration ( $p > 0.05$ ; Table 1). Conversely, there was insignificant decrease in RDW after the same duration and dosage. Similarly, at 1000 mg/kgbw dosage level, there was insignificant increase in RBC and RDW parameters; while HB, MCH, MCHC and MCV decreased insignificantly compared to the control group ( $p > 0.05$ ; Table 1).

Parameter	Control	100 mg/kg	350 mg/kg	1000 mg/kg
RBC ( $\times 10^3 \mu$ L)	4.10 $\pm$ 0.38a	4.34 $\pm$ 0.31a	5.30 $\pm$ 0.42a	5.18 $\pm$ 0.57a
HB ( $\times 10^3 \mu$ L)	10.72 $\pm$ 1.19a	10.02 $\pm$ 0.75a	11.92 $\pm$ 1.03a	8.86 $\pm$ 0.95a
RDW (g/dl)	16.60 $\pm$ 0.40a	16.42 $\pm$ 0.34a	15.58 $\pm$ 0.55a	17.86 $\pm$ 0.21a
MCH (pg)	21.82 $\pm$ 0.99a	23.02 $\pm$ 0.60a	23.06 $\pm$ 0.97a	20.72 $\pm$ 0.50a
MCHC (%)	26.36 $\pm$ 0.47a	26.34 $\pm$ 0.79a	27.52 $\pm$ 0.89a	25.26 $\pm$ 0.40a

MCV (fl)	83.72 ± 4.42a	85.06 ± 1.60a	80.42 ± 1.68a	82.06 ± 2.66a
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**Table 1:** Effects of DCM: Methanolic leaf extracts of *P. juliflora* on erythrocytic parameter profiles in Wistar albino rats. All values are expressed as mean ± SEM for five animals per group. Values with the same superscript are not significantly different by ANOVA followed by Dunnett's post hoc test ( $P > 0.05$ ).

**Effects of DCM: Methanolic leaf extracts of *Prosopis juliflora* on white blood cells and absolute differential count parameters profiles in Wistar albino rats:** The oral administration of Methanol: DCM leaf

extracts of *Prosopis juliflora* led to changes in both total and differential White Blood Cells (WBC) counts in the treated Wistar albino rats compared to the control (Table 2).

Parameter	Control	100 mg/kg	350 mg/kg	1000 mg/kg
WBCS ( $\times 10^3 \mu\text{L}$ )	20.31 ± 2.03a	14.50 ± 1.35a	17.72 ± 2.30a	18.16 ± 1.75a
ANC ( $\times 10^3 \mu\text{L}$ )	3.22 ± 0.54a	2.52 ± 0.28a	2.60 ± 0.28a	2.96 ± 0.32a
ALC ( $\times 10^3 \mu\text{L}$ )	11.82 ± 3.01a	9.54 ± 1.04a	8.98 ± 2.84a	11.64 ± 1.08a
AMC ( $\times 10^3 \mu\text{L}$ )	3.02 ± 0.46a	2.44 ± 0.39a	2.74 ± 1.08a	3.48 ± 0.48a
AEC ( $\times 10^3 \mu\text{L}$ )	0.64 ± 0.03a	0.61 ± 0.03a	0.65 ± 0.02a	0.66 ± 0.03a
ABC ( $\times 10^3 \mu\text{L}$ )	0.12 ± 0.01a	0.11 ± 0.03a	0.13 ± 0.02a	0.12 ± 0.03a

**Table 2:** Effects of DCM: Methanolic leaf extracts of *P. juliflora* on white blood cells and absolute differential counts parameters profiles in Wistar albino rats. All values are expressed as mean ± SEM for five animals per group. Values with the same superscript are not significantly different by ANOVA followed by Dunnett's post hoc test ( $P > 0.05$ ).

Following 28 days of extracts administration at 100 mg/kgbw dosage level, there was insignificant decrease in the total White Blood Cells counts (WBC), Absolute Neutrophils Count (ANC), Absolute Lymphocytes Count (ALC), Absolute Monocytes Count (AMC), Absolute Eosinophils Count (AEC) and Absolute Basophils Count (ABC) ( $p > 0.05$ ; Table 2). Further, at 350 mg/kgbw dosage level, there was insignificant increase in WBCs, ANC, AMC, AEC and ABC save for insignificant decrease in ALC compared to the control group ( $p > 0.05$ ; Table 2). Finally, at 1000 mg/kgbw dosage level, there was

insignificant increase in total WBC, ANC, ALC, AMC and AEC, but no change in ABC compared to the control ( $p > 0.05$ ; Table 2).

**Effects of DCM: Methanolic leaf extracts of *Prosopis juliflora* in platelets counts and related parameters profiles in female Wistar albino rats:** The DCM: Methanol leaf extracts of *Prosopis juliflora* resulted to effects in platelets and their related parameters profiles in the treated rats compared to the control group after daily oral dosing for 28 days (Table 3).

Parameter	Control	100 mg/kg	350 mg/kg	1000 mg/kg
PLATS ( $\times 10^3 \mu\text{L}$ )	738.00 ± 66.50a	643.40 ± 90.80a	860.20 ± 19.70a	674.60 ± 55.9a
PCT	0.55 ± 0.08a	0.56 ± 0.08a	0.64 ± 0.12a	0.64 ± 0.09a
MPV	6.52 ± 0.78a	8.40 ± 0.39b	7.42 ± 0.24a	7.80 ± 0.19a
PDW	19.78 ± 0.36a	20.20 ± 0.44a	19.68 ± 0.26a	18.88 ± 0.21a

**Table 3:** Effects of DCM: Methanolic leaf extracts of *P. juliflora* in platelets counts and related parameters profiles in Wistar albino rats. All values are expressed as mean ± SEM for five animals per group. Values with the same superscript are not significantly different by ANOVA followed by Dunnett's post hoc test ( $P > 0.05$ ).

At 100 mg/kgbw dosage level, there was unimportant decrease in platelet counts (PLAT), insignificant increase in Plateletcrit (PCT) and mean Platelet Width (PDW) ( $p > 0.05$ ; Table 3). In the same group, there was significant increase in mean platelet volume (MPV) compared to the control group ( $p < 0.05$ ; Table 3). Further, at the 350 mg/kgbw dosage, the *Prosopis juliflora* leaf extracts led to insignificant increase in PLATs and PCT but insignificant decrease in both MPV and PDW ( $p > 0.05$ ; Table 3). After 28 days of daily oral extract administration at 1000 mg/kgbw, there was insignificant decrease in

PLATs and PDW but, insignificant increase in MPV and PCT compared to the control group ( $p > 0.05$ ; Table 3).

**Effects of DCM: Methanolic leaf extracts of *Prosopis juliflora* in liver function test parameters profiles in Wistar albino rats:** The DCM: Methanolic leaf extracts of *Prosopis juliflora* induced changes in liver function test markers and related parameter profiles in normal treated mice compared to control group (Table 4).

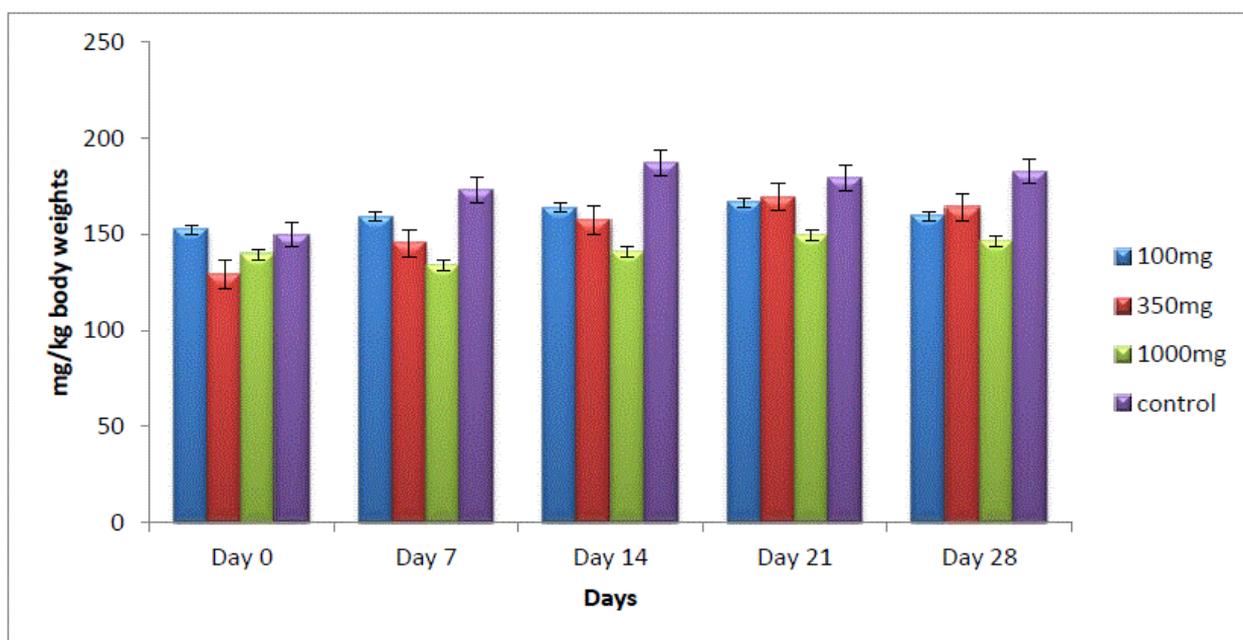
Test (Units)	Control	100 mg/kg	350 mg/kg	1000 mg/kg
ALT (U/L)	109.60 ± 1.60a	114.80 ± 2.06a	59.40 ± 10.80b	41.60 ± 10.60c
AST (U/L)	141.80 ± 8.93a	152.00 ± 5.91a	163.00 ± 6.42a	252.40 ± 5.98b
TP (g/L)	56.62 ± 2.38a	54.92 ± 1.35a	58.82 ± 1.67a	50.86 ± 1.01a
ALB (g/L)	27.78 ± 0.86a	26.98 ± 0.44a	29.54 ± 0.82a	25.92 ± 0.62a
TBIL(μmol/L)	4.42 ± 0.29a	4.00 ± 0.08a	5.36 ± 0.54a	5.00 ± 0.20a
DBIL(μmol/L)	8.44 ± 0.18a	10.08 ± 0.64a	8.74 ± 0.70a	9.62 ± 0.68a
ALP (U/L)	3.80 ± 0.58a	3.40 ± 0.25a	2.60 ± 0.25b	6.40 ± 0.51b

**Table 4:** Effects of DCM: Methanolic leaf extracts of *P. juliflora* on liver function test markers in Wistar albino rats. All values are expressed as mean ± SEM for five animals per group. Values with the same superscript are not significantly different by ANOVA followed by Dunnett's post hoc test ( $P > 0.05$ ).

Twenty eight days after oral administration of the extracts at 100 mg/kgbw, there was insignificant increase in plasma levels of Alanine Aminotransferase (ALT) and Direct Bilirubin (DBIL). At the same dosage, there was insignificant decrease in plasma concentration of Aspartate Aminotransferase (AST), Total Protein (TP), Albumin (ALB), Total Bilirubin (TBIL) and Alkaline Phosphatase (ALP) ( $p > 0.05$ ; Table 4). Further, at 350 mg/kgbw, there was significant decrease in ALT, insignificant increases in AST, TP, ALB and TBIL compared to the control ( $p > 0.05$ ). Similarly, insignificant decrease in DBIL and ALP was noted at the same dosage compared to control ( $p > 0.05$ ; Table 4). Finally, at 1000 mg/kgbw, there was a significant

decrease in ALT but significant increase in AST and ALP ( $p < 0.05$ ; Table 4). In addition, the extract led to unimportant decrease in TP, ALB and TBIL; and insignificant increase in DBIL at 1000 mg/kgbw ( $p > 0.05$ ; Table 4).

**Effects of DCM: Methanol leaf extracts of *Prosopis juliflora* in body weight and absolute organ weight parameter profiles in Wistar albino rats:** The DCM: Methanolic leaf extracts of *Prosopis juliflora* after 28 days daily oral administration induced changes in body weight and absolute organ weights in the administered rats compared to the control groups (Figure 1).



**Figure 1:** Changes in body weights of Wistar albino rats after oral administration with normal saline (control), and DCM: Methanolic leaf extracts of *P. juliflora*. Values are represented as mean ± SEM of 5 replicates.

After 28 days of oral administration of normal saline to the control group rats, there was insignificant increase in body weights on 7th, 14th, 21st and 28th compared to day 0 ( $p > 0.05$ ; Figure 1).

Furthermore, at 100 mg/kgbw, there was insignificant decrease in body weights on 7th, 14th and 28th day. Alternatively, there was insignificant increase in body weights on day 21 at the same dosage

level ( $p > 0.05$ ; Figure 1). At a higher dosage of 350 mg/kgbw, the extracts lead to insignificant rise in the animal's body weights on 7th and 28th day but insignificant decrease in body weights on the 14th and 21st day ( $p > 0.05$ ; Figure 1). Finally, at 1000 mg/kgbw dosage level, the extract induced insignificant rise in body weights on the 7th

day followed by steady but insignificant drop in weights on the 14th, 21st and 28th day.

Likewise, the DCM: Methanolic leaf extracts of *Prosopis juliflora* induced varied changes in absolute organ weights (Table 5).

Absolute organ Weight (%)	Control	100 mg/kg	350 mg/kg	1000 mg/kg
Brain	1.27 ± 0.06a	1.26 ± 12a	1.26 ± 0.90a	1.60 ± 0.16a
Liver	5.46 ± 0.15a	5.35 ± 0.37a	4.96 ± 0.29a	6.66 ± 0.50a
Kidneys	1.05 ± 0.07a	0.85 ± 0.03b	0.82 ± 0.03c	1.11 ± 0.07a
Spleen	0.60 ± 0.02a	0.50 ± 0.04a	0.47 ± 0.03b	0.51 ± 0.03a
Lungs	1.49 ± 0.21a	1.35 ± 0.07a	1.31 ± 0.18a	1.57 ± 0.18a
Heart	0.41 ± 0.02a	0.45 ± 0.02a	0.43 ± 0.04a	0.46 ± 0.05a

**Table 5:** Effects of DCM: Methanolic leaf extracts of *P. juliflora* in absolute organ weights parameter profiles in Wistar albino rats. Values are expressed as mean ± SEM for five animals per group. Values with the same superscript are not significantly different by ANOVA followed by Dunnett's post hoc test ( $P > 0.05$ ).

After 28 days of daily oral administration of the extracts at 100 mg/kgbw, there was insignificant decrease in absolute weights of brain, liver, spleen, lungs and heart but a significant decrease in absolute weight of the kidney ( $p < 0.05$ ; Table 5). At 350 mg/kgbw dosage, the extract induced insignificant decrease in absolute weights of brain, liver, lungs and heart ( $p > 0.05$ ; Table 5). Besides, there was significant decrease in absolute weights of both kidneys and spleen compared to the control group ( $p < 0.05$ ). Finally, at 1000 mg/kgbw dosage level, the extracts led to insignificant increase in absolute weights of brain, liver, kidneys, spleen, lungs and heart ( $p > 0.05$ ; Table 5).

### Histopathological examination of organs

The prepared tissue slides were stained using Hematoxyline-eosin and examined under light microscope. Photomicrographs of the various organ tissues were printed and comparison done between control and treated animals [19]. The architecture of liver from the control group rats administered with 1.0 ml normal saline daily for 28 days was normal with hepatocytes well organized (plate 1).

Similarly, examination of histological sections of the liver from rats administered with DCM: Methanolic leaf extracts of *P. juliflora* at 100, 350 and 1000 mg/kgbw for 28 days showed no fatty change, necrosis or hepatic jaundice (plate 2).

Examination of photomicrographs of the kidneys from the rats administered with 1.0 ml normal saline daily for 28 days were all normal (plate 3).

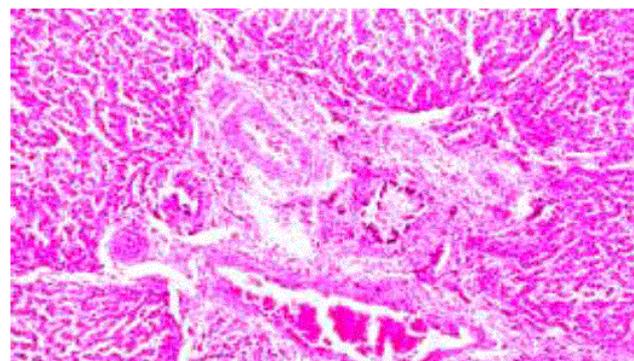
Equally, examination of histological sections of the kidneys from rats administered with DCM: Methanolic leaf extracts of *P. juliflora* at 100, 350 and 1000 mg/kgbw for 28 days did not reveal inflammatory symptoms, apoptosis but mild hydropic degeneration and necrosis of cells (plate 4).

The architecture of heart tissues from the control group of rats administered with 1.0 ml normal saline daily for 28 days revealed normal myocardial fibres (plate 5).

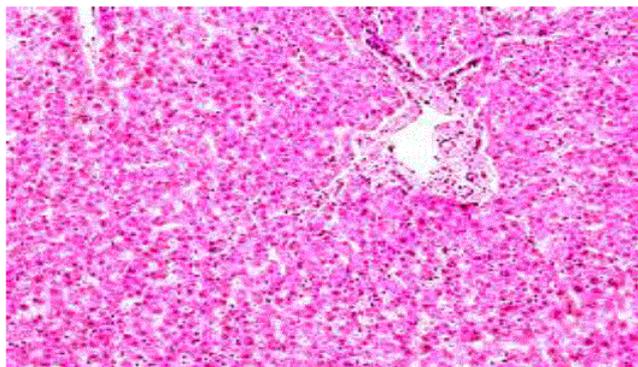
Likewise, examination of histological sections of the heart of rats administered with the leaf extracts at 100, 350 and 1000 mg/kgbw for 28 days showed no pathological myocardial degradation (plate 6).

The structure of spleen tissues from the control group of rats administered with 1.0 ml normal saline daily for 28 days was normal (plate 7).

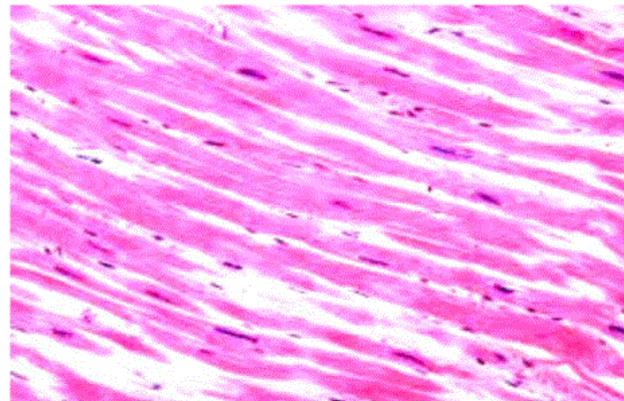
Further, examination of tissue sections of the spleen of rats administered with DCM: Methanolic leaf extracts of *P. juliflora* for 28 days revealed mild steatitis at 350 and 1000 mg/kgbw dosages but the tissues were intact at 100 mg/kg body weights (plate 8).



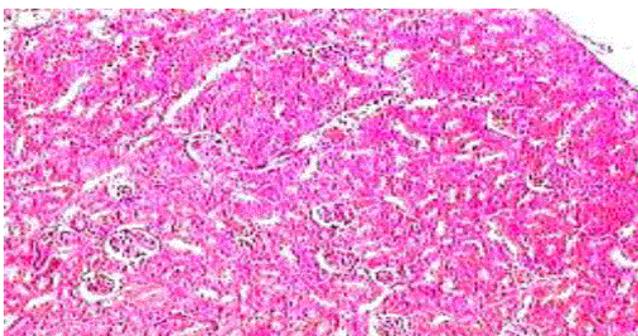
**Plate 1:** Photomicrograph of a section of liver architecture of Wistar albino rats treated with 2 ml normal saline showing normal features. Duration: 28 days. Hematoxylin-eosin, mag: × 100.



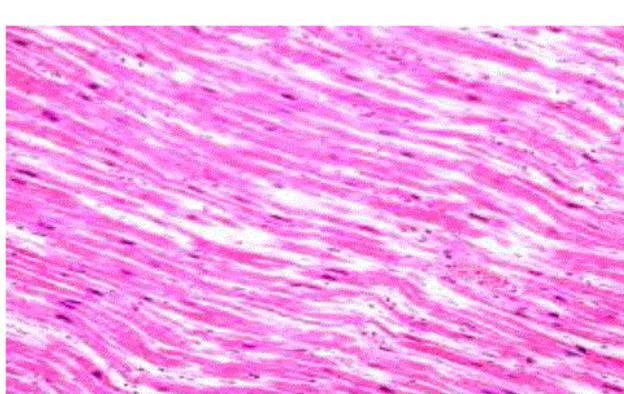
**Plate 2:** Photomicrograph of a section of liver architecture of Wistar albino rats treated with 350 mg/kg body weight of DCM: Methanolic leaf extracts of *P. juliflora* showing normal features. Duration: 28 days. Hematoxylin-eosin, mag:  $\times 100$ .



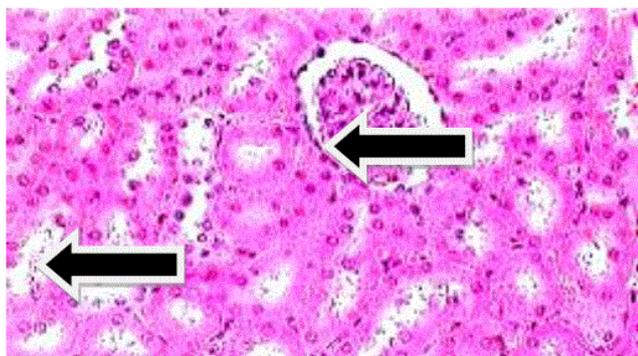
**Plate 5:** Photomicrograph of a section of heart architecture of Wistar albino rats administered with 2 ml normal saline showing normal features. Duration: 28 days. Hematoxylin-eosin, mag:  $\times 400$ .



**Plate 3:** Photomicrograph of a section of kidney architecture of Wistar albino rats treated with normal saline (2 ml) showing normal features. Duration: 28 days. Hematoxylin-eosin, Mag:  $\times 100$ .



**Plate 6:** Photomicrograph of a section of heart architecture of Wistar albino rats administered with 1000 mg/kg body weight of DCM: Methanolic leaf extracts of *P. juliflora* showing normal myogenic fibres. Duration: 28 days. Hematoxylin-eosin, mag:  $\times 400$ .



**Plate 4:** Photomicrograph of a section of kidney architecture of Wistar albino rats administered with 350 mg/kg body weight of DCM: Methanolic leaf extracts of *P. juliflora* showing mild hydropic degeneration and necrosis (arrow). Duration: 28 days. Hematoxylin-eosin, mag:  $\times 400$ . Plate.

### Qualitative phytochemicals screening

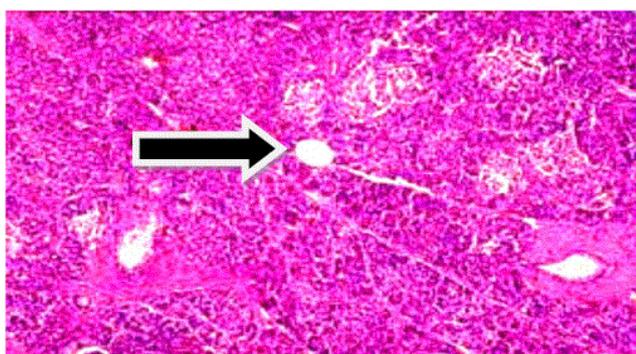
Qualitative phytochemicals tests of the DCM: Methanol leaves extract of *Prosopis juliflora* established the presence of the following compounds; alkaloids, flavonoids, phenolics, terpenoids, phenols tannins. However, steroids and cardiac glycosides were not detected in the leaf extracts (Table 6).

### Discussion

Limit test at 2000 mg/kg body weight is used to estimate LD<sub>50</sub> when there is information that the test material may not be very toxic. It is an important test often recommended only if the results to be obtained will have direct role of either human, animal health or the environment protection (OECD 425). Since all the test animals survived for at least 48 hrs after sequential oral administration of the extract at 2000 mg/kgbw, and only 2 animals died afterwards in the test, it was concluded that LD<sub>50</sub> for the extracts is greater than 2000 mg.



**Plate 7:** Photomicrograph of a section of spleen architecture of Wistar albino rats administered with 2 ml normal saline showing normal features. Duration: 28 days. Hematoxylin-eosin. Mag: × 100.



**Plate 8:** Photomicrograph of a section of spleen architecture of Wistar albino rats treated with 350 mg/kg body weight of DCM: Methanolic leaf extracts of *P. juliflora* showing mild fat vacuolation. Duration: 28 days. Hematoxylin-eosin, mag: × 400.

higher than the test dose at that level. The deduction was further supported by the observation that symptoms of toxicity which included decreased activity, salivation, pilo-erection and decreased appetite were dose related and transient.

Hematological analysis is important to risk evaluation since observed variations in animal studies have a high predictive index for human toxicity if the tests were to be carried out in human subjects [30]. The changes observed after Sub-chronic oral administration of DCM: Methanolic leaf extracts of *Prosopis juliflora* to Wistar albino female rats in dosages of 100, 350, and 1000 mg/kg body weights were attributed to extracts components. The insignificant increase in RBC and HB parameters at 100 and 350 mg/kg body weight doses was attributed to mild stimulation of erythropoietin activity by the extracts. Under normal physiological conditions, a rise in RBC and related components is attributed to the increased activity of erythropoietin in the stem cells of an animal. Erythropoietin is a glycoprotein hormone produced by the kidneys, which activates stem cells in the bone marrow to form red blood cells. Stimulation, synthesis or secretion of erythropoietin directly or indirectly by substances including plant secondary metabolites has been reported [31,32]. For example, the antioxidants flavonoids and terpenes detected in the leaf extracts of *Prosopis juliflora* have been reported to induce production of erythropoietin [33,34]. Previous research showed that prophylactic and therapeutic oral administration of antioxidant supplements from plant extracts significantly increased cells of hematopoietic origin in animals exposed to potentially lethal doses of radiation. They also protected erythrocytes from oxidative damage [35,36].

However, the insignificant decrease in HB, MCH, MCHC and MCV at 1000 mg/kg dose showed that the extracts at higher concentrations may inhibit erythropoietin activity. Similarly, such slight decrease may be attributed to kidneys injury or normal physiological readjustments of the rats due to the high dose oral administration of the extracts. The MCH, MCHC and MCV parameters relate to individual erythrocytes. Therefore, the overall insignificant increase in values observed in the parameters and factors attributed to them at 100 and 350 mg/kg dosages were correlated to the similar trends observed in the RBC count [37,38]. The insignificant decrease in WBC and the differential leukocytes counts (ANC, ALC, AMC, AEC and ABC) at 100 mg/kg body weight showed that at low dosages the active components of DCM: Methanolic leaf extracts of *Prosopis juliflora* could not stimulate hemopoietic activities. This is because at higher dosages of 350 and 1000 mg/kg body weights, the trend changed to insignificant increase in the leucocytic parameters.

Granulocyte-macrophage colony stimulating factor, macrophage colony stimulating factor, interleukins IL-2 IL-4 and IL-5 control the proliferation, differentiation and maturation of committed stem cells responsible for the production of white blood cells [39,40]. These factors are affected by phenolics and terpenoids which, as isolated compounds, have ability to initiate immune response. Terpenoids alone have been reported to activate protein kinase C [37]. Probably, the full stimulatory effects of phenolics and terpenoids were masked by other metabolites in the total leaf extracts as used in the study.

The observed insignificant but consistent decrease in the leucocytic components was also attributed to effects of unknown components in the extracts other than the phenolics and the terpenoids with the ability to inhibit production of the regulatory factors. Such components may have affected sensitivity of the committed stem cells responsible for the production of white blood cells. Secondly, that the treated animals' leucocytic parameters remained low even after twenty

Phytochemicals	Leaf extracts
Alkaloids	+
Flavonoids	+
Steroids	-
Saponins	+
Cardiac glycosides	-
Phenolics	+
Terpenoids	+
Tannins	+

**Table 6:** Phytochemical composition of DCM: Methanolic leaf extracts of *P. juliflora*. The (+) sign indicates a positive phytochemical test result while (-) denotes a negative phytochemical test result.

This is in line with OECD 425 guidelines which state that if 3 or more animals survive the limit test then, LD50 of the test substance is

eight days may as well imply that other than inhibition of immune stimulatory factors, the extract may have protected the animals against microbial infections. This was attributed to the exemplary antimicrobial activity of *Prosopis juliflora* leaf extracts as reported by several researchers [3,8].

The insignificant fluctuation in PLATS and PDW observed at 100 mg/kg body weight oral administration of the leaf extracts reported in the results was attributed to normal physiological readjustments in the Wistar albino rats [16]. However, the consistent rise in PCT and MPV though insignificant, was attributed to mild production (or activation) of hematopoietic regulatory factor (thrombopoietin) by stromal cells in the bone marrow. Production of thrombopoietin could have been stimulated by certain phytochemicals components in the leaf extracts which also created a favourable environment for hematopoiesis.

Platelets are involved in maintenance of normal blood homeostasis in organisms [30,41]. Mean platelet volume as a platelet function relies on the release of thromboxane A<sub>2</sub>, beta thromboglobulin, platelet aggregation factor and more importantly, the expression of glycogen Ib and IIb / IIIa receptors. Therefore, the observed insignificant rise in MPV at 100 and 1000 mg/kg body weights doses implied that certain components of the leaf extracts mildly stimulated the factors. This was attributed to the presence of tannins in the leaf extracts which have been reported to possess anti-hemorrhagic properties. Tannins have been linked to formation of a protective cover over open wounds by *Prosopis juliflora* extracts leading to healing as reportedly used in folk medicine [42]. The insignificant decrease in platelet counts (PLATS), after oral administration of *Prosopis juliflora* leaf extracts at 100 and 1000 mg/kg body weights, was attributed to physiological changes and cytolytic properties of the saponins in the extracts respectively [43].

Assessment of the effects of sub-chronic administration of extracts on biochemical parameters in animal studies is useful in determining overall toxicity of such extracts [30]. From the results obtained after 28 days of daily oral administration of DCM: Methanolic leaf extracts of *Prosopis juliflora* to Wistar albino rats, the insignificant increase in ALT; and the insignificant decrease in AST and ALP at 100 mg/kg body weights suggested lack of toxicity effects on hepatocytes. It also implied that other cells and organelles which contain AST and ALP were largely preserved at the dosage level [25]. This is because the plasma concentration of ALT that occurs exclusively in hepatocytes usually increases only due to hepatocellular injury. ALT is thus considered a specific liver injury marker.

The observed significant decrease in serum ALT concentrations at 350 and 1000 mg/kg body weight dose levels similar to ALP at 350 mg/kgbw suggested no liver toxicity by the extracts. Rather, it implied that components of the leaf extracts may have inhibited dietary uptake or activity of Pyridoxal-5-phosphate (Vit.B6) which is a key cofactor for full function of aminotransferases [26]. Direct or indirect negative effect on Vit.B6 results in low serum aminotransferase activity. Therefore, decreased serum aminotransferase concentrations are not directly associated with toxicologically significant effect on liver. Certain diseases, for example, alcoholic liver disease may lead to deficiency in pyridoxal-5'-phosphate, thus a great reduction in serum ALT concentrations [26,30].

The significant rise in AST and ALP at 1000 mg/kgbw pointed out that though the hepatocytes were largely intact, there could have been injury to unknown tissue(s) at the dosage level possibly, the cytolytic effects of saponin component of the extracts. AST and ALP are present in the heart, skeletal muscle, kidney, brain, pancreas and blood cells

[13,25]. Similarly, at 100, 350 and 1000 mg/kg dosage levels, the insignificant rise and fall in plasma proteins (TP and ALB) showed no marked toxicity effects of the extracts on the rats' physiological processes. Several reasons have been linked to increased serum total proteins during animal studies [26].

They include; dehydration of treated animals which result in clinical symptoms such as excessive salivation, reduced water consumption and gastrointestinal fluid losses among other symptoms. Other reported causes are; inflammatory conditions that stimulate production of globulins (acute phase proteins) and decreased protein synthesis or increased protein loss. Decreased food consumption has been reported to result in low protein synthesis, maldigestion or malabsorption and hepatic dysfunction. The insignificant fluctuations in TP, ALB, TBIL and DBIL at 100, 350 and 1000 mg/kg body weight dosage levels suggested that liver functions were largely preserved.

In absence of haemolysis, hyperbilirubinemia indicates liver dysfunction [26]. This is because intact hepatocytes efficiently remove unconjugated bilirubin from the plasma by uptake, conjugation, secretion and excretion. On the other hand, conjugated bilirubin is freely filtered through the glomerulus. *Prosopis juliflora* alkaloids have been reported to reverse *Staphylococcus aureus* induced hepatotoxicity in rats [44]. It was suggested that the effects must have been responsible for the gross preservation of the liver functions observed in the study despite oral administration of the extracts at higher than normal doses.

Variations in animal body weight and more so absolute organ weights following administration of test substances is key factor to consider in evaluation of toxicity of substances [26]. This is because under normal physiological conditions, organ weights change in proportion to change in body weight except the brain and in extreme conditions of obesity and starvation. Toxicants including phytochemicals have been reported to result in deviation from this standard norm [25]. The observed overall insignificant changes in body weights of the treated animals compared to the control group following the 28 days oral administration of the extracts reflected but increased physiological activities in the rats. It was also attributed to the frequency of administration of the extracts hence, decreased calorific intake (malnutrition), dehydration and gastrointestinal disturbances [30,45]. The fact that the all animals were fasted before the start of the experiment and on the 28th day before the final weights were measured was also considered a contributing factor to the observations.

The liver, being a very important organ in metabolism and detoxification of xenobiotics is by nature of its role exposed to injury due to different types of toxicants [13,46]. However, the observed insignificant increase in absolute liver weight particularly at 1000 mg/kg body weight dose level was attributed to increased metabolic activity in the organ as a result of the administration of the leaf extracts containing different phytochemicals. The significant decrease in kidney weight at 100 and 350 mg/kg body weights, similar to the spleen at 350 mg/kg body weights were attributed to toxicity effects of the leaf extracts. Saponins have been reported to cause contraction of the kidney vascular system while both saponins and tannins have been reported to cause hepatotoxicity. The significant decrease in absolute kidney weight was attributed to the earlier observed insignificant decrease in total protein [19,25].

Similarly, the significant decrease in spleen weight was associated with decreased hemopoietic activity which could explain the

insignificant decrease in WBC and differential counts as observed in the study. The insignificant changes noted in lungs, heart and brain weights showed that the leaf extracts did not have any adverse effect on these organs. Under normal circumstances, the brain does not undergo any significant changes during toxicity studies in rats, mice, rabbits and dogs [25,26].

There were no histopathological changes in all sampled organ tissues obtained from rats in the control group. This is attributable to the standard handling and feeding procedures which the animals were subjected to during the entire study period leading to no infections, physical injuries or stress [20,21]. Since there was no fatty change or necrosis noted after histological examinations of the liver tissues from the rats administered with DCM: Methanolic leaf extracts of *P. juliflora* at all the dosage levels (100, 350 and 1000 mg/kg body weights) implied that the leaf extracts was not toxic to liver. It showed that extracts did not initiate any of the toxicant related liver damage mechanisms including: calcium homeostasis and cell membrane injury, canalicular and cholestatic injury, stimulation of apoptosis, stimulation of autoimmune system or mitochondrial injury [30].

Likewise, that there was no hepatic jaundice observed supported the insignificant changes noted in the TBIL and DBIL values at all dose levels in the study. Further, the findings meant that the insignificant variations in ALT, ALP and AST as observed in LFTs were not toxicity effects of the extracts administration on the organ. As a result, it was suggested that the liver rapidly metabolized the phytochemical components of the extract into less harmful products which were cleared from the system. Equally, it was proposed that processing of plant materials, particularly drying before extraction might have led to inactivation of certain secondary metabolites reducing toxicity effects. It has been reported that drying of plant materials used as feed known to contain phytotoxic metabolites when fresh rendered them safe [47].

The mild pathological effects in the kidney tissues including hydropic degeneration and necrosis observed were associated with extracts administration particularly at 100 and 350 mg/kg body weight dosage levels. It further supported the suggestion that decreased TP levels observed may have resulted from loss through extracts damaged kidneys [25]. The intact architecture of heart tissues showing normal myocardial fibers both in the control and the treated groups after 28 days indicated no pathological effect on the organ. Consequently, the variations in absolute weight of the spleen were attributed to increased physiological activities due to the key role it plays in removal of foreign materials from the circulation (leaf extracts) but not actual phytochemical toxicity [48,49]. The alkaloids, flavonoids, phenolics, terpenoids, phenols tannins detected in the extracts were correlated to the significant and insignificant *in vivo* changes observed in various tests during the study.

Tannins are reported to have erythrolytic effects *in vitro* and have been associated with severe central hepatonecrosis similar to terpenes [19]. Isolated alkaloids of *Prosopis juliflora* have been reported to cause adverse effects to neurons at high doses *in vitro*, while other types of alkaloids may lead to veno-occlusive disease of the liver resulting to cancer. Likewise, terpenoids are reported to stimulate immune response, activate protein kinase C, form adducts with the DNA and cause carcinogenesis. Saponins have been established to cause hemolysis of both human and animals RBCs *in vitro*, and certain saponins may cause enlargement of pancreas.

Despite the potential toxicity effects of individual phytochemicals *in vitro* studies reported, there were no severe adverse effects of the

extracts reported during the study except for the kidneys and spleen. This was attributed to oral route of extracts administration which may have subjected the secondary metabolites to detoxification by the gut microbial, sequestration and binding, as well as biotransformation reactions of the liver through fast pass mechanism [50]. Non severe toxicity was also linked to un-established interactions between the various phytochemicals in the extracts as well as drying of materials before extraction [51].

## Conclusion

In summary, the DCM: Methanolic leaf extracts of *P. juliflora* was positive for alkaloids, tannins, saponins, phenols, flavonoids and terpenoids but not steroids and cardiac glycosides. The presence of the secondary metabolites was linked to the effects noted in rats administered with the leaf extracts during the study period. It was established that the extracts had an LD50 above 2000 mg/kg body weight. The daily oral administration of the extracts did not lead to severe specific toxicity effect in major organs tissues including liver, heart, lungs and brain. Moderate toxicity effects were observed in kidneys and spleen. The leaf extracts of *P. juliflora* did not have toxicity effect on absolute organ weights studied except kidneys and spleen. Similarly, there were insignificant body weight changes in the rats over the study period. Finally, there was insignificant toxicity effects of the extracts on hematological parameters examined. From the results of this study, the DCM: Methanolic leaf extracts of *P. juliflora* may lead to adverse effect in kidneys and spleen on medium term oral administration (28 days). This supports the various *in vitro* study findings reports that certain phytochemical extracts of the plant species may be toxic. However, severity of the adverse effects was low, attributed to the route of administration and the unestablished interactions (polyvalent effects) between the different secondary metabolites in the leaf extract. All the same, caution should be observed on dosage if the species leaf extracts must be used in phytomedicine specifically by oral administration.

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