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Article

Toxicological Assessment of Supplemental *Zanthoxylum Acanthopodium* Essential Oil in Rabbits: Evidence from Haemato-Biochemical and Histopathological Indices

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Abstract: This study investigated the toxicological assessment of supplemental *Zanthoxylum acanthopodium* essential oil in rabbits: evidence from haemato-biochemical and histopathological Indices. Thirty-two clinically healthy weaner male rabbits of about 7 to 8 weeks with an average initial live weight of 710 ± 0.68 kg were stratified by body weight and randomly allocated to one of four experimental groups (n = 8). The experimental diet consisted of a basal (control) group (0 mL/kg) and three levels of *Zanthoxylum acanthopodium* essential oil inclusion: 0.5 mL, 1.0 mL, and 1.5 mLper kg DM feed daily. Feed and water were made available at all times, and a completely randomized design was adopted throughout the 14-week experimental period during which rabbits were maintained under standard management conditions. The results suggested that *Zanthoxylum acanthopodium* essential oil influenced ($p<0.05$) several haematological parameters. Specifically, haemoglobin, red blood cell, mean corpuscular volume, mean corpuscular haemoglobin, and mean corpuscular haemoglobin concentrations. Conversely, pack cell volume, white blood cell, lymphocytes, monocytes, neutrophils, basophils, and eosinophils levels were not affected by the treatment. In serum analysis, total protein, albumin, globulin, glucose, creatinine, sodium, potassium, alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase remained stable across all groups ($p>0.05$); however, a significant effect was observed on cholesterol, calcium, phosphorus, and magnesium levels. Liver histology was not affected by *Zanthoxylum acanthopodium* essential oil across all treatments, showing no signs of toxicity or degeneration. Furthermore, all blood values remained within the recommended physiological ranges for healthy rabbits. The study concludes that *Zanthoxylum acanthopodium* essential oil can be safely incorporated into rabbit diets up to 1.5 mL as it supports normal physiological function and maintains liver integrity.

Keywords: *Zanthoxylum Acanthopodium* Oil, Rabbits, Haematology, Serum Biochemistry, Hepatic Histology, Cholesterol

Introduction

Globally, rabbit production is experiencing significant growth, particularly in developing countries, due to the high prolificacy, rapid growth rate, and superior nutritional quality of rabbit meat

[1]. To maximize these production benefits, nutritionists have shifted focus towards the use of phytogenic feed additives – natural plant extracts such as essential oils to improve animal sustainability, health, and performance [2]. This transition is largely driven by the worldwide trend of reducing synthetic antibiotics use in animals due to concerns over antimicrobial resistance and toxic residues in animal products [3], [4].

Essential oils are volatile, aromatic compounds extracted from herbal or medicinal plants that possess well documented anti-inflammatory, antioxidant, anti-helminthic, cytotoxic, hypolipidemic, antidiarrheal, antifungal, immune-modulatory, gastro-protective, dermato-protective, cardio-protective, antimicrobial, hepato-protective, and antidiabetic properties [1], [5]. While their potential to improve growth performance and feed efficiency is widely recognized, it is imperative to establish their safety profile regarding the internal physiology of the animal [6], [7]. Haematological and serum biochemical parameters are important indicators of the nutritional, physiological, and pathological status of animals [8]. Deviations in these parameters often serve as a systemic toxicity or metabolic stress [9], [10]. Furthermore, because the liver is the primary organ responsible for detoxification of ingested substances, its histology is a critical standard for evaluating the safety of any dietary inclusion [11], [12].

Despite the promising benefits of essential oils, there is a need to determine the optimal inclusion levels that can be tolerated without compromising the animal's health. Previous research by Bassiony et al. and Sameh et al. has shown that the supplementation of clove and eugenol oil at low doses below 500 mg/kg in the diet of rabbits revealed beneficial effects; high doses above 500 mg/kg can lead to liver damage [13], [14]. Therefore, some caution should be exercised in the use of essential oils as natural feed additives in rabbit diets. Recent studies on phytogenics, although very few, have been carried out using different plant extracts on animals' performance. Whereas essential oils from rosemary, thyme, clove, ginger, turmeric, amongst others, have been investigated [14], [15], [9]. However, no one has investigated the toxicological assessment of supplemental *Zanthoxylum acanthopodium* essential oil in rabbits: evidence from haemato-biochemical and histopathological Indices.

Materials and Methods

Experimental location, ethical Approval and animal care

The experiment was carried out at the Rabbit unit of the Gandhi College of Agricultural Teaching and Research Farm, Rajasthan, India. The study site lies between latitude 11° 250N and 16° 000E and longitude 4° 000N and 9° 070E. The mean annual rainfall and temperature range from 400 to 1000 mm and 21.83 to 32.57°C, respectively. Relative humidity is about 70 % during the rainy season and 45 % during the dry season. The regulations relating to animal care and the use of animals in this experiment were approved by the Research and Ethics Committee within the Department of Animal Nutrition and Biochemistry, Gandhi College of Agriculture, Rajasthan, India.

Sanitation commenced two weeks before the purchase of experimental animals with thorough cleaning and disinfection of pens and other equipment with disinfectant (Morigad). Feeders were properly washed, and nipple drinkers were flushed with antiseptic (Aquaclean). Thirty-two clinically healthy weaner male rabbits of about 7 to 8 weeks were sourced from a reputable source in Rajasthan, India. They have an average initial live weight of 710 ± 0.68 kg, and were stratified by body weight, such that the rabbits in each treatment group had similar average initial body weight, and randomly allocated to one of four experimental groups (n = 8). On arrival, rabbits were placed on a two-week adjustment period and dewormed with Ivermectin Plus (Needan Pharmaceuticals, Gujarat, India) at the dosage of 1 mL to 1 kg BW. Animals were housed individually in an all-wired battery cage equipped with galvanized feeders for each animal and nipples for supplying fresh water. The basal diet was compounded according to the nutritional standard described by NRC (1977). A completely randomized experimental design was adopted. The dietary treatments were: (A) Basal diet without a supplement (control); (B) Basal diet with *Zanthoxylum acanthopodium* essential oil supplement (per kg DM feed daily) at 0.5 mL; (C) Basal diet with *Zanthoxylum acanthopodium* essential oil supplement (per kg DM feed daily) at 1.0 mL; (D) Basal diet with *Zanthoxylum acanthopodium* essential oil supplement (per kg DM feed daily) at 1.5 mL. Feed was given three times daily *ad libitum* at 07:30 h, 12:00 h, and 17:30 h. The

feeding trial lasted for 14 weeks, including two weeks acclimatization period. Before the first feeding, each rabbit received a 100 g feed with a dose of essential oil to guarantee full consumption. Proximate analysis of the basal diet was ascertained according to the AOAC (2003) procedure.

***Zanthoxylum acanthopodium* leaf collection, extraction, and GC-MS analysis**

Fresh leaves of *Zanthoxylum acanthopodium* were harvested from different trees at Gandhi College of Agriculture, Rajasthan, India. It was sent to the department of Biological Sciences in the same institution, where it was identified by a certified taxonomist (Dr. Ram Vinod) and registered under voucher number HY/004/2023. Leaves were shade-dried for 10 days and pulverized using a mechanical grinder. Extraction of oil was done by hydrodistillation with an H –shaped Clevenger-type apparatus according to the procedures modified by [1], [16]. Briefly, 300 g of the pulverized *Zanthoxylum acanthopodium* was added to 1200 mL of water heated in a glass flask at 60 ℃ for 20 minutes, steam passes via the condenser and collected in a beaker. The oil was collected by decantation and then dried over a column of anhydrous sodium sulfate before it was introduced into glass bottles and stored in a refrigerator at 4 °C.

GC/MS analysis of *Zanthoxylum acanthopodium* essential oil was done using Claudus 5006 GC-MS Auto Sampler (China) equipped with two silica capillary columns, interfaced with a quadrupole detector (single quadrupole acquisition Method-MS parameters report), source temperature 230°C, Quadrupole temperature 150 °C; the temperature program was 60 °C for 2 min, 60-240 °C at 3 °C/min, then kept at 240 °C during 8 min; injector temperature, 240 °C. The mass spectrometry transfer line temperature, 250 °C; carrier gas, helium at a flow rate of 0.7 ml/min; injection type, split, 20:1; ionization voltage, 70 eV; electron multiplier 1000 eV; scan range 33-400 amu; scan rate, 1.56 scan/s.

Identification of components. Interpretation of mass spectrum GC-MS was conducted using the database of the National Institute of Standards and Technology (2001) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library.

Histology analysis

On the last day of the experiment, 3 animals were randomly selected for histological study. The animals were slaughtered, and their liver was removed, fixed in 10 % neutral-buffered formalin, dehydrated in 70% alcohol, cleared with methyl benzoate, and embedded in paraffin wax. Sections of 5μm were cut and stained on glass slides with hematoxylin and eosin stain for light microscopic examination. Stained sections were examined by light microscope and photographed using a digital camera.

Collection of blood for analysis

On the last day of the trial, 2 sets of blood were collected very early in the morning from four randomly selected rabbits in each treatment through the vein using a 5 mL syringe. A 5 ml blood sample was collected into labelled sterile bottles containing disodium salt of ethylene diamine tetra-acetic acid (EDTA) as anticoagulant for the determination of haematological parameters. Blood samples for serum analysis were collected into anticoagulant-free bottles. All samples collected were placed in an ice pack and sent to the laboratory for further analysis. Blood for haematology was analyzed using BC-5390 Auto Hematology Analyzer (China). The Kit utilizes the impedance method for pack cell volume, mean corpuscular haemoglobin concentration, red blood cell, mean corpuscular determination, Cyanide-free reagent for hemoglobin test, and Flow Cytometry (FCM) + Laser scatter + Chemical dye method for white blood cell and its differentials analysis. Blood for serum was analyzed using the Sysmex Auto Biochemical analyzer – HD -3040 Series. The sample rotor valve measures the required volume of sample precisely to provide accurate and reliable results for all serum biochemical parameters.

Data analysis

Data obtained were subjected to analysis of variance (ANOVA) for a complete randomized design using Statistical Package for the Social Sciences (SPSS version 25). When the ANOVA was significant, means were separated using Duncan's multiple range test at the level of $P \leq 0.05$.

Results

Table 1. Ingredient and chemical composition of the basal diet (kg of DM).

Ingredients	(kg of DM)
Corn	35.00
Wheat bran	20.00
Palm kernel meal	16.29
Soybean meal	20.06
Limestone	2.50
Bone meal	5.00
Lysine	0.20
Methionine	0.20
Min/Vitamin Premix	0.25
Salt	0.35
Toxin binder	0.15
Total	100.0
Chemical composition	
Dry matter	91.25
Organic matter	90.14
Crude protein	16.21
Crude fibre	13.87
Ash	9.86
Energy (Kcal/kg)	2891.5

Each 2.5 kg consists of: Vit A 12000, 000 IU; Vit D3, 2000, 000 IU; Vit. E. 10g; Vit k3 2 g; Vit B1, 1000 mg; Vit B2, 49g; Vit B6, 105 g; Vit B12, 10 mg; Pantothenic acid, 10 g; Niacin, 20 g, Folic acid, 1000 mg; Biotin, 50 g; Choline Chloride, 500 mg, Fe, 30 g; Mn, 40 sg; Cu, 3 g; Co, 200 mg; Si, 100 mg and Zn, 45 g.

Dominant bioactive compounds in *Zanthoxylum acanthopodium* oil are presented in Table 2. Twelve major bioactive compounds were identified: Limonene (16.85 %), β-Caryophyellene (14.37 %), Linalool (12.86 %), 9, 17-Octadecadienal (10.77 %), γ-Terpinene (8.18 %), α-Copaene (6.55 %), p-Cymene (5.72 %), β-Citronella (5.14 %), Terpinene-4-ol (4.43 %), α-Humulene (3.89 %), amongst others.

Table 2. Dominant bioactive compounds in Zanthoxylum acanthopodium oil.

Compounds	Retention time (minutes)	% Area
9,17-Octadecadienal	6.113	10.77
alpha-copaene	7.008	6.55
γ-Terpinene	7.072	8.18
p-Cymene	8.009	5.72

Linalool	10.76	12.86
α-Humulene	10.95	3.89
Terpinene-4-ol	10.99	4.43
Limonene	11.85	16.85
β-Caryophyellene	12.10	14.37
β-Citronella	12.94	5.14
cis-Vaccenic acid	13.88	2.09
9-Eicosenoic acid	14.12	3.11

Haematological parameters of weaner rabbits fed a diet supplemented with *Zanthoxylum acanthopodium* oil are presented in Table 3. Haemoglobin, red blood cell, mean corpuscular volume, mean corpuscular haemoglobin, and mean corpuscular haemoglobin concentration were higher ($p<0.05$) for diet B (DB), diet C (DC), and diet D (DD) than for diet A (control). Pack cell volume, white blood cell, lymphocytes, monocytes, basophils, and eosinophil values were similar ($p>0.05$) among the diets.

Table 3. Haematological parameters of weaner rabbits fed a diet supplemented with *Zanthoxylum acanthopodium* oil.

Components	DA	DB	DC	DD	SEM	P-value
Packed cell volume (%)	32.92	33.56	33.97	34.05	0.85	0.275
Hemoglobin (g/dL)	9.66^{b}	11.72^{a}	11.86^{a}	11.91^{a}	2.21	0.001
Red blood cells (10^{6}/ L)	13.87^{b}	14.25^{a}	14.37^{a}	14.40^{a}	0.56	0.899
MCHC (%)	28.17^{b}	34.86^{a}	34.98^{a}	35.07^{a}	0.71	0.002
MCH (pg)	12.31^{b}	18.74^{a}	18.82^{a}	18.93^{a}	3.08	0.007
Mean corpuscular volume(fL)	48.87^{b}	57.11^{a}	58.07^{a}	58.91^{a}	6.65	0.933
White blood cells (10^{9}/L)	13.55	13.65	13.93	14.04	2.76	0.406
Lymphocytes (%)	60.34	66.85	67.12	67.24	7.07	0.587
Monocytes (%)	2.95	2.76	2.80	2.86	0.41	0.665
Neutrophils (%)	45.17	47.08	47.11	47.23	5.94	0.872
Basophils (%)	0.07	0.06	0.06	0.06	0.02	0.961
Eosinophils (%)	4.00	4.03	4.11	4.15	1.1	0.751

Except for cholesterol concentration, which was influenced ($p<0.05$) by the treatment, the value was higher in DA than in other treatments. Total protein, albumin, globulin, albumin: globulin ratio, glucose, creatinine, and urea concentration showed no ($p>0.05$) significant difference (Table 4). Serum ions and enzymes of weaner rabbits fed a diet supplemented with *Zanthoxylum acanthopodium* oil are presented in Table 5. Except for calcium, phosphorus, and magnesium, which were influenced ($p<0.05$) by the treatments, other parameters (sodium, potassium, alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase) were not affected ($p>0.05$) by the treatments.

Table 4. Table 5: Serum biochemical indices of weaner rabbits fed a diet supplemented with *Zanthoxylum acanthopodium* oil.

Components	DA	DB	DC	DD	SEM	P-value
Total protein (g/L)	69.12	71.33	71.47	71.81	7.86	0.61
Albumin (g/L)	33.51	34.05	34.12	34.15	4.31	0.825
Globulin (g/L)	35.61	37.28	37.35	37.66	3.46	0.512
Albumin: globulin ratio	0.94	0.91	0.91	0.90	0.10	0.801
Glucose (mg/dL)	70.76	70.75	70.72	70.73	0.03	0.993
Cholesterol (mg/dL)	117.8a	89.34b	89.11b	88.75b	2.08	0.882
Urea (mg/dL)	4.16	4.05	4.11	4.09	1.05	0.772
Creatinine (mg/dL)	2.36	2.57	2.67	2.77	0.11	0.761

Means within a row with different letters and significantly different ($p< 0.05$); SEM: Standard error; T1: Experimental diet without oil (control); T2: experimental diet + 2.5 g Adriamycin® /kg diet; T3: experimental diet + 2.0 mL *Clinopodium brownei* oil/kg diet; T4: experimental diet + 2.0 mL *Clinopodium brownei* oil/kg diet

Table 5. Serum ions and enzymes of weaner rabbits fed a diet supplemented with *Zanthoxylum acanthopodium* oil.

Items	DA	DB	DC	DD	SEM	P-value
Calcium (mmol/L)	2.24b	3.56a	4.72a	4.81a	0.02	0.175
Phosphorus (mmol/dL)	4.77b	6.96a	7.02a	7.05a	0.31	0.811
Magnesium (mmol/L)	2.05b	3.81a	3.98a	4.01a	0.12	0.183
Sodium (mmol/L)	149.6	150.8	151.7	153.1	4.47	0.900
Potassium (mmol/L)	3.67	3.83	3.91	3.96	0.35	0.586
Alkaline phosphatase (IU/L)	29.11	28.59	28.07	28.08	0.12	0.834
Alanine aminotransferase (IU/L)	16.57	15.95	15.08	15.16	0.69	0.115
Aspartate amino transferase (IU/L)	58.55	51.03	51.25	51.20	4.04	0.806

Discussions

Bioactive compounds in *Zanthoxylum acanthopodium* oil have numerous medicinal properties, anti-inflammatory, dermato-protective, gastro-protective, immuno-stimulatory, cardio-protective, antioxidant, antimicrobial, hepato-protective, cytotoxic, anti-helminthic, antidiabetic, antidiarrheal, hypolipidemic, amongst others [17], [18]. These phyto-compounds help to improve the overall health of rabbits because they are eco-friendly, contain non-toxic compounds, and are generally regarded as safe [14], [19]. Additionally, it is well documented that dietary supplementation of essential oils in the diet of rabbits modulates the intestinal flora, immune activity, and prevents the incidence of antimicrobial resistance [20], [21].

All the haematological indices obtained in this study were within the normal physiological ranges for healthy rabbits [16]. The red blood cell, haemoglobin, and packed cell volume were within the normal range 8.99 – 16.00 (10^6/ L), 7.00 – 14.00 g/dL, and 29.00 – 39.00 % recorded by [14]. When red blood cell, haemoglobin, and packed cell volume are all within the normal range, it implies the absence of anaemia, oxygen sufficiency in the tissues, as well as efficient hydration [22], [18]. Mean corpuscular

volume, mean corpuscular haemoglobin, and mean corpuscular haemoglobin concentration were within 41.00 – 67.00 fl, 17.00 – 24.00 pg, and 30.00 – 36.00 % cited by [23]. These outcome indicates the absence of chronic iron deficiency, inflammation, as well as folate deficiency in the blood [24]. White blood cell protects the body's cells from damage and help to maintain proper immune function [25]. As earlier discussed, essential oils possess antioxidant properties, thus inhibiting the efficacy of pathogenic organisms in the gut as well as neutralizing the activities of free radicals, which are the cause of most chronic diseases [26]. However, their value was within 12.00 – 25.00 (10^9/L) reported by Bacova et al. (2020). This outcome further reveals that the animals used for this current study were healthy [27]. Lymphocytes, monocytes, neutrophils, basophils, and eosinophil counts were within the normal ranges, 40.00 – 78.00 %, 1.00 – 3.00 %, 30.00 – 50.00 %, 0.01 – 1.00 %, and 0.1 – 5.00 % reported by [28]. This result suggests a stability in the immune system as well as the absence of tissue inflammation [29]. The presence of limonene, β-caryophyellene, linalool, and other bioactive compounds in *Zanthoxylum acanthopodium* oil is probably the reason for the strong immune capacity in rabbits [29].

All the serum parameters were within the normal physiological ranges for healthy rabbits [6] . These results indicate a balanced metabolic state in rabbits. As previously noted, the bioactive compounds in *Zanthoxylum acanthopodium* oil have antimicrobial activities against some bacteria, ensuring that the animal's internal organs are functioning properly [30]. Total protein, albumin, and globulin values were within the reference range 40.00 – 85.00 g/L, 28.00 – 40.00 g/dL, and 20.00 – 50.00 g/L cited by [31]. Normal albumin and globulin levels in rabbits suggest efficient protein synthesis at a level that translates to better performance and weight gain [32]. Normal globulin range also indicates the absence of chronic inflammatory diseases [33]. This result concurs with earlier studies [34]. A normal glucose level of 70.00 – 155 mg/dL is a clear sign that the rabbits were not severely stressed due to malnutrition and other management practices [2]. The cholesterol level recorded in this study was within 97.00 – 180.5 mg/dL, as recorded by Omokore and Alagbe [34]. Recently, Alagbe and Hernandez observed that essential oils have the ability to reduce the concentrations of saturated fatty acids and prevent cardiovascular diseases when supplemented in the diet of animals [7], [8]. The lowered cholesterol levels among animals that received *Zanthoxylum acanthopodium* oil may be due to the presence of bioactive compounds, especially cis-Vaccenic acid and 9-Eicosenoic acid, which have been previously associated with hypolipidemic properties [34]. Creatinine and urea concentrations are the most reliable markers for kidney degeneration [28]. However, the urea and creatinine ranges of 4.09 – 4.11 mg/dL and 2.36 – 2.77 mg/dL recorded in this study were within 2.50 – 5.60 mg/dL and 2.00 – 4.50 mg/dL, respectively, as referenced by [30]. This outcome suggests the absence of chronic renal disease in rabbits [35].

The normal range of serum calcium is 1.88 – 4.33 mmol/L [34], phosphorus 4.00 – 8.50 mmol/L [36], magnesium (2.00 – 4.10 mmol/L [30], sodium (100 – 210 mmol/L [37], and potassium (2.10 – 3.80 mmol/L [38], for all treatments suggests that *Zanthoxylum acanthopodium* oil supplementation in the diet of rabbits did not interfere with the absorption of minerals in the tissues. This outcome is parallel with the reports of [39], [40], who recorded that the inclusion of phytogenics in the diet of growing rabbits did not affect the absorption and availability of nutrients in their system. Although alkaline phosphatase (ALP), alanine aminotransferase (AST), and aspartate aminotransferase (ALT) were not influenced ($p>0.05$) by the treatment diets, values were within the range of 18.00 – 30.00 IU/L, 12.00 – 25.00 IU/L, and 45.00 – 70.00 IU/L reported for healthy growing rabbits (Muritala et al., 2022).ALT, ALP, and AST normal levels suggest no hepatocellular damage [35].

Hepatic histology of weaner rabbits fed a diet supplemented with *Zanthoxylum acanthopodium* oil is presented in figures 1-4. Figure 1 (control) without oil supplement, figures 2, 3, and 4 contained basal diet supplemented with *Zanthoxylum acanthopodium* oil at 0.5 mL, 1.0 mL, and 1.5 mL/kg diet, respectively.

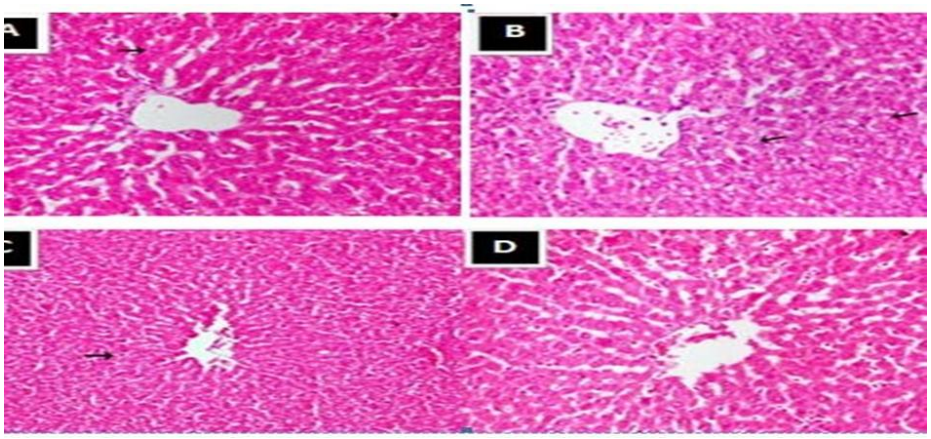

Figure 1. Basal diet without essential oil supplement (control).

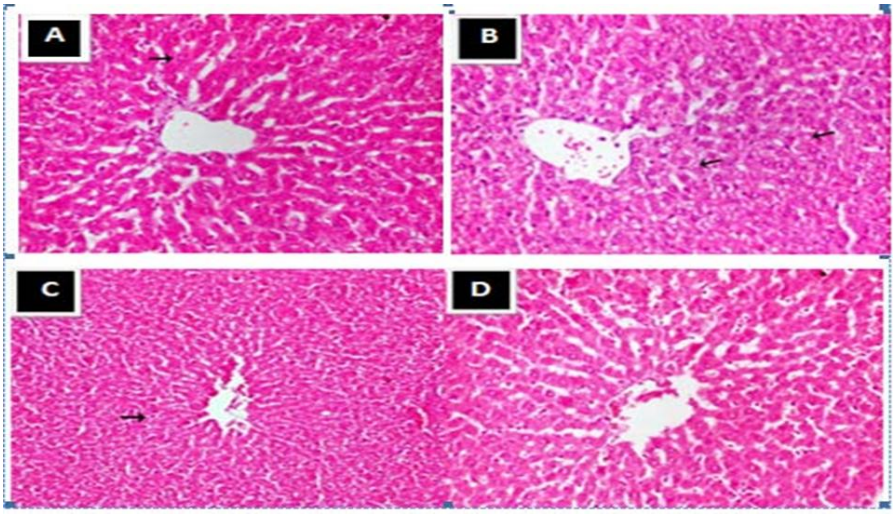

Figure 2. Basal supplemented with supplemented with Zanthoxylum acanthopodium oil at 0.5 ml/kg diet.

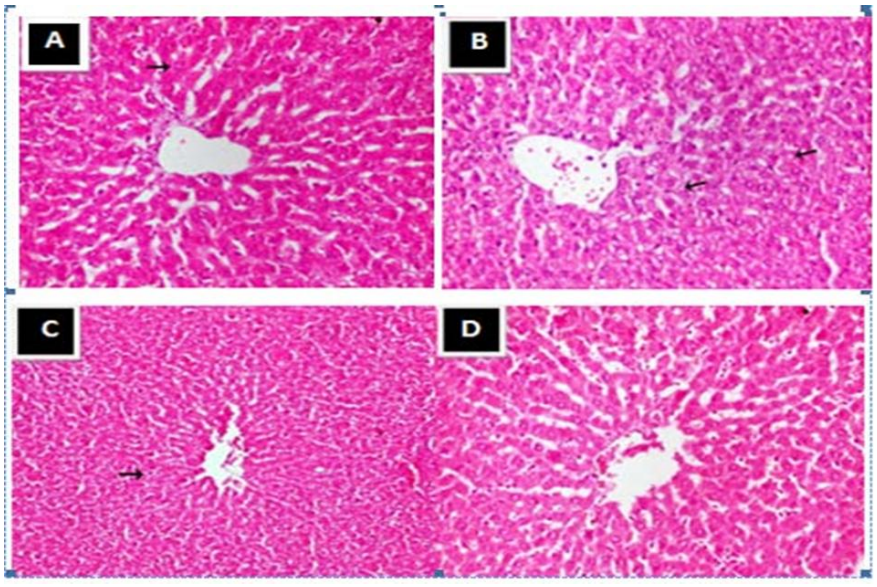

Figure 3. Basal supplemented with supplemented with Zanthoxylum acanthopodium oil at 1.0 ml/kg diet.

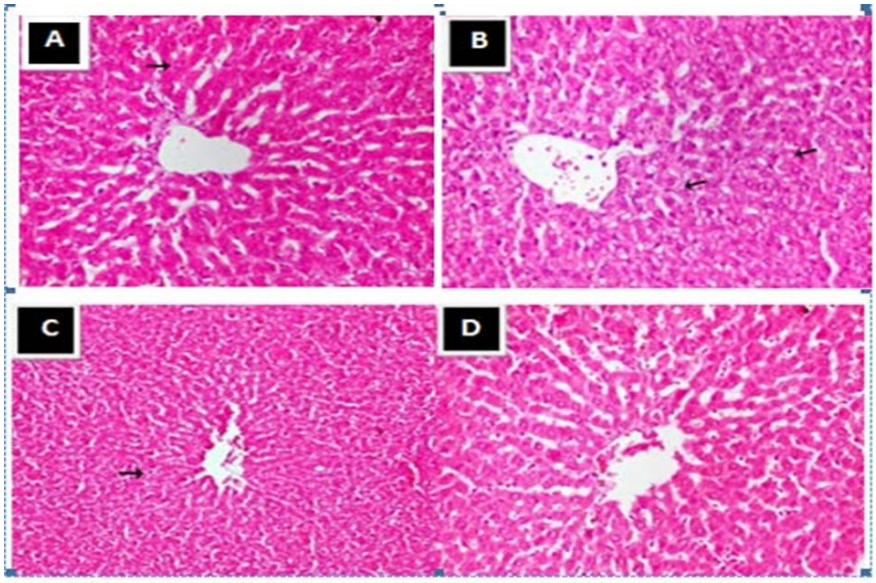

Figure 4. Basal supplemented with supplemented with Zanthoxylum acanthopodium oil at 1.5 ml/kg diet.

There was no significant difference ($p>0.05$) in their histological examinations; all slides showed normal hepatic architecture without congestion, there was normal or clear radial arrangement of hepatic cords around the central vein without any signs of inflammation or fatty infiltration, and well-formed sinusoids. The outcome of this result suggests that dietary supplementation of *Zanthoxylum acanthopodium* oil up to 1.5 ml/ kg diet was optimum for the animals and had no toxic effect on their liver histology. This result is in line with the observations of Nwaopara et al., who noted a normal central vein and hepatic cords without inflammation with the dietary supplementation of ginger, clove, red pepper, and black pepper in growing rabbits [41]. Additionally, it is well documented that supplementing the diets of animals with essential oils poses no vacuolar degeneration [42]. Ashour et al. Noted that the administration of mixtures of essential oils ensures that the hepatocyte plasma membrane and portal lobules are well organized [22]. Aside from toxicity, environmental stress has also been linked to one of the causes of minor shifts in hepatic histology [43]. However, bioactive compounds in *Zanthoxylum acanthopodium* oil have proven to possess protective or therapeutic effects when growing rabbits are exposed to toxins and environmental stressors.

Conclusion

The outcome of this study demonstrates that dietary supplementation of *Zanthoxylum acanthopodium* oil up to the level of 1.5 mL/kg is physiologically safe and well tolerated by growing rabbits, while the oil significantly influenced some blood parameters, it did not trigger an adverse immune response or systemic inflammation. Thus, this study warrants further investigation.

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