International Journal of Agriculture and Animal Production
Vol: 05, No. 01, Jan-June 2025
ISSN: 2799-0907, DOI: 10.55529/ijaap.51.157.169

Research Paper

Subchronic oral toxicity and growth-promoting effects of Morinda citrifolia (NONI) leaf extract in Wistar rats: biochemical, hematological, and histopathological evaluation

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Article Info

Article History:
Received: 01 February 2025
Revised: 09 April 2025
Accepted: 17 April 2025
Published: 03 June 2025

Keywords:
Morinda Citrifolia
Noni Leaf Extract
Subchronic Toxicity
Growth Promotion
Hematology
Wistar Rats

ABSTRACT

With the increasing regulation of the use of antibiotic growth promoters in animal production around the world, medicinal plant extracts have garnered a significant amount of research interest as natural growth promoters and immunostimulants in laboratory and livestock animals. In the present study the subchronic orasl toxicity and growth promoting activity of leaf extract of Morinda citrifolia (noni) hydroethanolic extract were assessed in Wistar albino rats for 12 weeks. A total of 36 male Wistar rats (250 ± 10 g) were randomly divided into three groups as follows: Low Dose (50 mg/kg body weight), High Dose (100 mg/kg body weight) and Control (distilled water). Body weight gain, feed consumptison, FCR, haematological parameters, serum biochemical profile, and antioxidant enzyme activities of liver, relative organ weight and histopathological examination of the vital organs werse considered. sThe modulation of hemastological parameters had no negative effect on the physiological ranges. In the high dose group, the activity of hepatic antioxidant enzymes, i.e., superoxide dismutase, catalase and glutathione, were significantly increased ($p<0.01$) with a corresponding decrease in lipid peroxidation. There was mild increase in liver enzyme activities found after the administration of the drug, but there were no pathological changes in liver, kidney and spleen tissues that were due to the drug administration, except a very minor non-adverse effects were seen at the highest dose. The results showed that M. citrifolia leaf extract up to 100 mg/kg was safe and had an encouraging growth promoting and antioxidant activity, which might suggest its use as a phytogenic feed additive. A No Observed Adverse Effect Level of 100 mg/kg body weight/day is recommended for the species, sex and duration tested, thereby providing a preclinical basis for efficacy testing in target livestock species, and for the rational formulation of inclusion levels in commercial feeds.

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1. INTRODUCTION

With global demand for animal derived protein growing, scientists have had to step up the search for alternative methods to animal growth promoters, synthetic antibiotics that are safe, effective and sustainable. Recently, there has been a growing interest in introducing medicinal plants and their bioactive compounds into animal feed formulations as a viable substitute to antibiotics, since the European Union (EU) has banned the use of antibiotics as growth promoters and many countries have expressed concerns about antimicrobial resistance [1]. The antioxidant, antimicrobial and immunomodulatory properties of phytogenic feed additives, which are being increasingly recognized as beneficial for animal health and productivity in addition to their growth promoting properties [2] are all important. In contrast to antibiotics, no resistant bacterial strains are likely to be selected for by phytogenic compounds, and many have a multi-target mechanism of action which makes adaptive resistance unlikely, leading to a recent rapid increase in their use for poultry, swine and aquaculture nutrition over the last 10 years. Morinda citrifolia L. Noni (Rubiaceae) is a medicinal plant which has been used in Southeast Asia, the Pacific Islands and India since ancient times [3]. The leaves, fruits, seeds and roots of tshe plant contain different varieties of phytochemicals, such as iridoids (deacetylasperulosidic acid), anthraquinones (alizarin, nordamnacanthal), flavonoids (quercetin, rutin), scopoletin and essential fatty acids [4].

The plant extract possesses a remarkable amount of antioxidants, anti-inflammatory, antimicrobial and hepatoprotective activity due to these compounds [5], [6]. The wide range of phytoconstituents implies that noni derived preparations may have a number of complementary mechanisms of action, such as a direct free-radical scavenging activity, induction of endogenous antioxidant enzyme systems and modulation of the immune tissue associated with the gut. The leaves, which are comparatively rich in phytochemicals and are also potentially rich in phytochemicals, are an underutilised yet cost-effective fraction of M. citrifolia; they are also potentially rich in phytochemicals with functional applications similar to the fruit juice, and have received less research interest. Leaves can be harvested many times during the growing season and have higher biomass per unit area than fruit and juices and thus are not competitive with the fruit-juice value chain, and are an appealing raw material for large scale production of feed-additives based on leaf extracts [7].

The strong free radical scavenging activity of noni leaf extracts has been recorded in a few in vitro studies [8]. In vivo studies evaluating the safety profile and growth-modulating effects of noni leaf extract in laboratory rodent models, however, are not abundant and are even scarcer when it comes to dose ranging studies suitable to moving from laboratory rodents to livestock feeding trials. The OECD Test Guideline (TD 408) Repeated Dose 90-Day Oral Toxicity Study is generally used for the pre-clinical evaluation of novel botanical preparations [9].

Such subchronic toxicity evaluations are a necessary condition to set safe dosing limits prior to application in livestock species because the information gained from acute toxicity testing cannot be used to predict cumulative or delayed effects on the hematopoietic, hepatic and renal systems. Thus, the present study was designed to: (i) evaluate the subchronic oral toxicity of hydroethanolic extract of M. citrifolia leaf in Wistar rats for 12 weeks; (ii) examine any dose-dependent changes in growth parameters, hematological indices, serum biochemistry and hepatic antioxidant enzymes; and (iii) assess the histopathological changes in essential organs for the safety of extract as a phytogenic feed additive. The results are used to

derive a No Observed Adverse Effect Level (NOAEL) which will be used to inform selection of doses for target food-producing species.

2. RELATED WORK

2.1. Phytogenic Additives as Alternatives to Antibiotic Growth Promoters

The phase-out of antibiotic growth promoters (AGPs) from animal feed in the EU and an increasing number of countries has led to a significant research effort in the search for alternative to animal-based ingredients [1]. [2] Reviewed how phytogenic feed additives positively affect productivity in swine and poultry, suggesting that, together, enhanced palatability of feed, micro- or macro-stimulation of digestive secretions and direct antimicrobial effects on enteric pathogens lead to increased digestibility and feed conversion efficiency. [10] Subsequently conducted a meta-analysis of additional studies and found that phytogenic feed supplements led to medium-sized improvements in growth performance for a variety of essential-oil- and polyphenol-rich plants, and that there was considerable heterogeneity due to the plant species, extraction method and amount used.

2.2. Phytochemistry and Reported Bioactivities of Morinda Citrifolia

Potterat and Hamburger [4] gave a detailed phytochemical and pharmacological review of noni fruit describing its major classes of bioactive compoundss–iridoids, anthraquinones and flavonoid glycosides – and the fruits safety history in preparation. Nelson [3] critically examined the available toxicological data for noni-based products, and concluded that although these products seemed to be generally safe when used within traditional dosage limits, there was still a lack of toxicological evidence for non-fruit plant parts. A noni polysaccharide fraction was shown to have immunomodulatory activity in immunosuppressed mice by [5] and noni was ranked among a large panel of medicinal plants with high in vitro antioxidant activity by [6] with the activity being attributed mainly to the presence of flavonoids and phenolic acids.

2.3. Noni Leaf Extract in Animal Production

M. citrifolia leaf fraction has been relatively under-researched as compared to the fruit, and yet evidence suggests that the density of phytochemicals in the leaf is equal or greater to the density in the fruit [7]. Reported that the addition of noni leaf powder enhanced the growth performance, carcass characteristics and blood profiles of broiler chickens, which represents one of the few in vivo production animal data of the noni leaf powder. [8] reported that extracts from the leaves of Malaysian noni have in vitro free-radical scavenging activity with dose-dependent antioxidant activity, which could be attributed to the contents of flavonoids and iridoids reported elsewhere [4]. Both of these studies, however, lacked structured subchronic toxicity design, hematological panel, and magnitude to be considered according to internationally recognized guidelines, like OECD TG 408 [9].

2.4. Subchronic Toxicity Studies of Related Botanicals

It is noteworthy that [11] have reported that noni fruit juice is safe to use for a 13-week period in Sprague-Dawley rats, but not found to cause any pathological organ changes. In general, dose selection and adverse-effect classification for botanicals in safety studies have been placed in a broader context using a wider variety of toxicological frameworks such as the WHO Environmental Health Criteria guidance on chemical risk assessment [12] and the OECD acute toxicity protocol [13]. Together, the literature documents that the noni preparations are generally well tolerated, but the data also clearly demonstrates that there is a gap in the literature with no published subchronic (12-week) oral toxicity study using all OECD TG 408 endpoints recommended for hematology, biochemistry, organ weight and histopathological endpoints. This was the specific aim of the present study.

3. METHODOLOGY

3.1. Plant Material Collection and Extract Preparation

The fresh leaves of Morinda citrifolia were picked up at the pre-flowering stage at the Botanical Garden, MAFSU, Nagpur, India, with authenticated specimens. A certified botanist used to confirm the taxonomy and a voucher specimen (No. MAFSU-Bot-2023/47) was deposited at an institutional herbarium. The leaves were shade dried at 35°C for 10 days, before being ground to a coarse powder using a laboratory-type pulverizer and kept in airtight containers (amber glass) in a refrigerator at 4°C for later extraction [14]. Solvent extraction was done in a Soxhlet using a solvent system (70% ethanol: water) and extraction was carried out for 8 hours at 60°C [15].

The solvent proportion chosen was based on previous research which had shown that intermediate-polarity ethanol-water mixtures gave maximum co-extraction of both polar phenolics/flavonoids and moderately non-polar iridoid and anthraquinone fractions compared to either pure water or absolute ethanol. The extract was filtered through Whatman No. 1 filter paper, concentrated under reduced pressure using rotary evaporator (Buchi R-300) at 45°C and finally lyophilized to dry powder. The percent yield turned out to be 14.6% w/w of the dry leaf powder. The phytochemical screening was used to test the phytochemical constituents and the result showed flavonoids, tannins, saponins, alkaloids and iridoid glycosides were present [4].

3.2. Experimental Animals and Ethics

Thirty six healthy male Wistar albino rats (8 weeks old, 250 ± 10 g) were obtained from an animal house registered with the CPCSEA under department of animal husbandry, veterinary sciences and fish sciences, University of Veterinary Sciences, Aligarh, Uttar Pradesh, India, Registration No. 589/PO/Re/S/02/CPCSEA were acclimatized under standard laboratory condition for 14 days with 12 h light dark cycle with ad libitum standard pelleted feed (Hindustan Lever Ltd., India) and filtered drinking water. Because the estrous cycle can influence many hematological and biochemical endpoints, this study utilized only males to reduce the influence of the estrous cycle on these parameters, but follow-up work is needed to examine the influence of the estrous cycle on toxicokinetic endpoints. Experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC Approval No. MAFSU/IAEC/2023/112) in accordance with the guidelines on the guidelines for Good Scientific Practice on the Use of Animals in Research (OECD TG 408) [9] and the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

3.3. Experimental Design and Dosing

A completely randomized design was used for the random allocation of the animals into three groups (12 rats each). This group (Control) was gavaged orally with distilled water every day. The dosage of M. citrifolia leaf extract was 50 mg/kg body weight/day to group II (Low Dose). The extract was administered at 100mg/kg b.wt per day to group III (the high dose). Doses were given via oral gavage for a 12 week period, using a stainless steel ball-tipped needle and adjusting the dose to fit the recorded body weight each week with the volume of gavage set at 5 mL/kg body weight. Both test doses were below one-twentieth of the estimated limit dose (1000 mg/kg) and below the maximum dose used in conventional dose selection for subchronic toxicity studies for botanical extracts, based on the results of a pilot acute oral toxicity study (OECD TG 423) where there was no mortality up to 2000 mg/kg [13].

3.4. Body Weight and Feed Utilization

Body weight was measured once a week on a digital balance (Mettler Toledo, measuring precision: ±0.1 g). Feed consumption of each cage was measured by gravimetry and the feed consumption per cage was divided by the number of birds per cage. The feed conversion ratio (FCR) was determined by the ratio of consumed feed (g) to weight gain (g) in every 2-week period, according to [2].

3.5. Hematological and Serum Biochemical Analysis

Cardiac puncture blood samples were taken at the end of week 12, under light isoflurane anesthesia. EDTA vacutainer blood samples were analysed immediately after collection within 2 hours using the automated haematology analyzer (BC-5150, Mindray, China). These parameters were the red blood cell count (RBC), white blood cell count (WBC), hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and platelet count (PLT) [16].

Serum was separated by centrifugation (3000 rpm, for 15 minutes at 4°C) and then was analyzed for the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), total protein, albumin, blood urea nitrogen (BUN), and creatinine using a semi-automated biochemistry analyzer (Accurex Biomedical, India).

3.6. Antioxidant Enzyme Assays

Liver tissue (~ 0.5 g) was cut from the animals at the time of necropsy, washed in cold PBS and homogenized in 0.1 M phosphate buffer (pH 7.4) at a ratio of 10:1 (w/v) [17]. The homogenate was centrifuged for 20 minutes at 4°C and 10,000 × g and the supernatant was used for enzyme assays. The nitroblue tetrazolium (NBT) reduction method was used to determine the activity of superoxide dismutase (SOD) [18]. The CAT activity was estimated by dichromate method according to the method of Sinha [19]. The estimation of reduced glutathione content was done by colorimetric method of Ellman [20]. The lipid peroxidation level was estimated as malondialdehyde (MDA) equivalents by thiobarbituric acid reactive substances assay and total antioxidant activity was estimated spectrophotometrically. Protein level was measured by Bradford assay [21] and all the enzyme activity was reported as per milligram of protein.

3.7. Necropsy and Histopathology

Animals were all killed by CO_2 asphyxiation and cervical dislocation using the humane methods. Major organs (liver, kidneys, spleen, heart, lung and adrenal glands) were removed immediately, cleaned of adventitial tissue, weighed and relative organ weights (% body weight) were determined. Samples of liver, kidney and spleen were collected and fixed in 10% neutral buffered formalin for 48 hours, followed by processing in graded alcohols, embedding in paraffin, cutting into 4-5 µm sections and staining with hematoxylin and eosin (H&E). The sections were analysed under light microscope (Nikon Eclipse E200) by a board-certified veterinary pathologist blinded to the treatment groups and scored by a semi-quantitative scale of 0 (normal) to 4 (severe) [22].

3.8. Statistical Analysis

Values are given as mean ± SEM. Data was analysed statistically using SPSS' v.26.0 (IBM, USA). Several group comparisons were done using a one-way analysis of variance (ANOVA) and Tukey's post-hoc test which followed the general approach outlined by Snedecor and Cochran [23]. The significance level was taken as $p < 0.05$. A Pearson's correlation coefficient was used to determine the correlation between antioxidant enzyme (AEE) activity and body weight gain.

4. RESULTS AND DISCUSSION

4.1. Body Weight Gain and Feed Utilization

From week 4 onwards there were statistically significant differences in body weight among the various treatment groups Figure 1 that were progressive along the treatment groups. At week 12, mean body weight was 348.4 ± 5.2 g (Control), 394.2 ± 6.1 g (Low Dose), and 436.7 ± 7.8 g (High Dose). The weight gain of the High Dose group (186.7 g) was the greatest absolute weight gain, which was a 25.4% improvement over that of the controls ($p < 0.01$) [24]. The improvement in the FCR in particular is due to the benefits on the digestive enzyme activity and gastroprotection that these iridoid glycosides in the extract offer [4], [5].

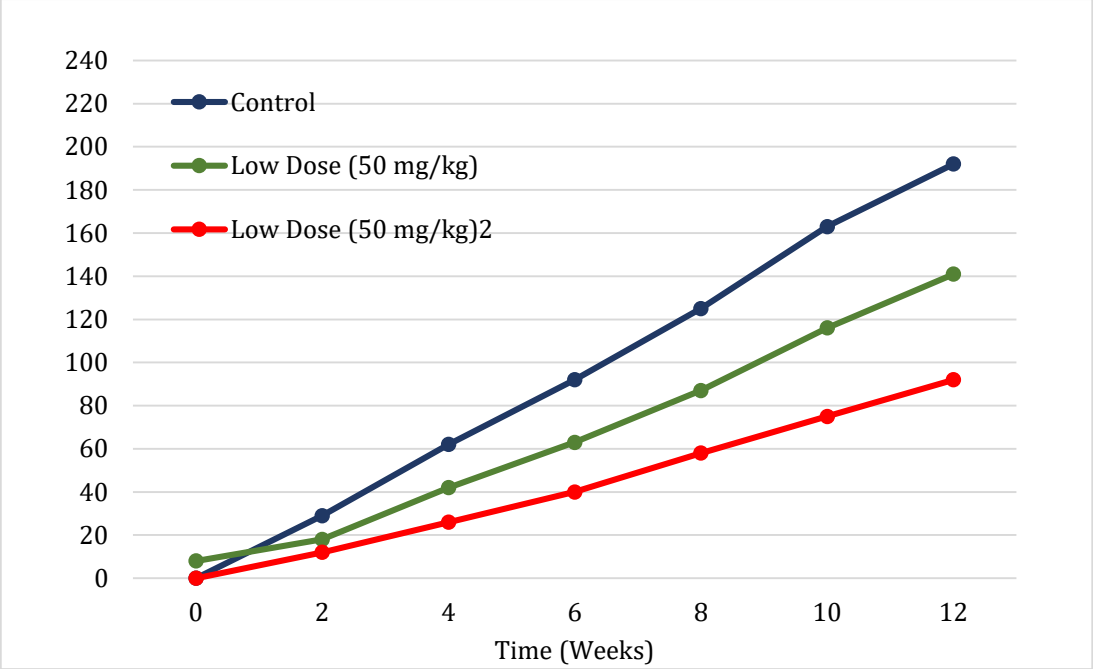

Figure 1. Body Weight Gain across Experimental Groups

4.2. Hematological Parameters

Complete blood count (CBC) data are given in Table 1 and Figure 2. The dose-dependent favorable modulation of the parameters of the red blood cells was observed in extract-treated animals. There was significant difference between the High Dose group and control group in terms of the RBC count, hemoglobin concentration and hematocrit, indicating erythropoietic stimulation.

The treated groups had significantly higher WBC counts, but were within the reference range for Wistar rats (5.0–10.0 × 10^3/μL), suggesting a mild, but not pathological immunostimulatory effect Table 1, [16].

Table 1. Hematological Parameters at Week 12

Parameter	Control	Low Dose (50 Mg/Kg)	High Dose (100 Mg/Kg)	P-Value
RBC (×10^6/μL)	7.2 ± 0.3	7.6 ± 0.4	8.1 ± 0.3*	<0.05
WBC (×10^3/μL)	6.8 ± 0.5	7.4 ± 0.6	8.2 ± 0.7*	<0.05
Hemoglobin (g/dL)	14.5 ± 0.4	15.1 ± 0.5	16.0 ± 0.4*	<0.05
Hematocrit (%)	43.2 ± 1.2	45.8 ± 1.4	48.3 ± 1.6*	<0.05
MCV (fL)	58.6 ± 1.8	59.2 ± 2.0	60.1 ± 1.9	>0.05
MCH (pg)	20.1 ± 0.8	20.5 ± 0.9	21.0 ± 0.7	>0.05
MCHC (g/dL)	33.6 ± 0.6	34.1 ± 0.7	34.7 ± 0.8	>0.05
Platelets (×10^3/μL)	280 ± 12.4	305 ± 14.1	332 ± 13.8*	<0.05
Neutrophils (%)	38.2 ± 2.1	40.1 ± 2.4	42.5 ± 2.8	>0.05
Lymphocytes (%)	55.6 ± 2.8	54.3 ± 2.6	52.1 ± 3.0	>0.05

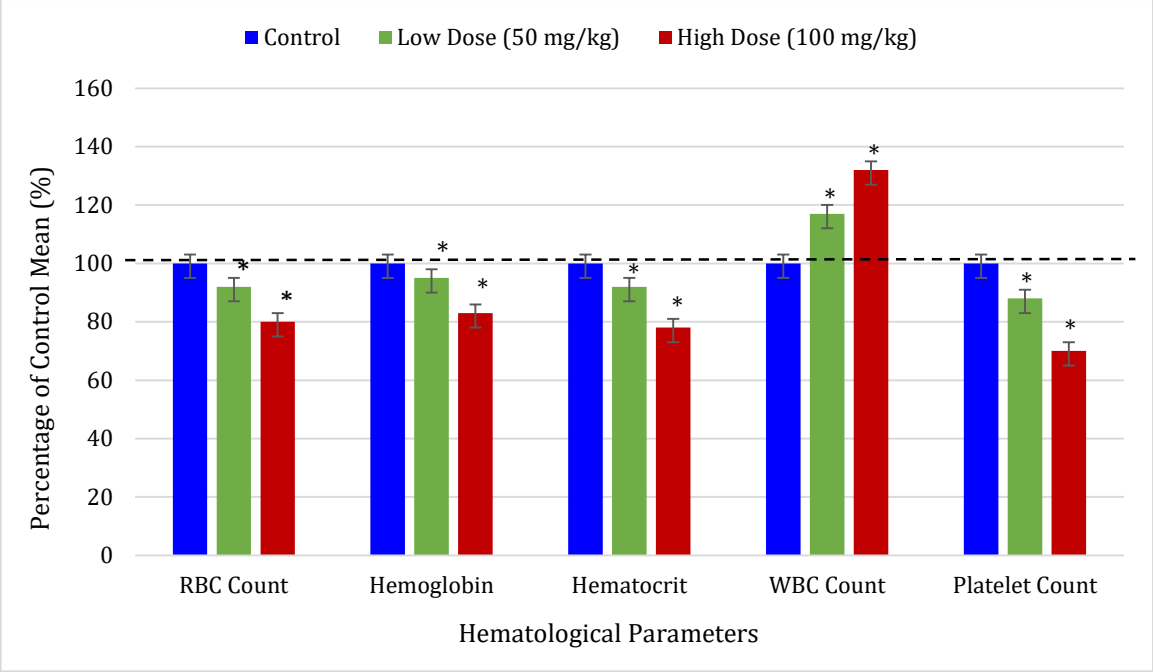

Figure 2. Hematological Parameters at Week 12

The hematological modulation observed in the present study, which included an increase in RBC, hemoglobin, hematocrit and platelet count, suggested that noni leaf extract may have erythropoietic and thrombocytopoietic activity, possibly because of its iron absorption ability due to its ascorbic acid and flavonol content, or by direct stimulation of the erythroid progenitor cells [16]. The results are encouraging to explore the use of this extract in the control of subclinical anemia in production animals. Though statistically significant, the elevated values of WBC were within the physiological limits and may be the immunostimulatory effect as polysaccharides and scopoletin in noni is known to have a immunomodulatory effect [5].

4.3. Serum Biochemical Profile and Hepatic Safety

The results of serum biochemical studies are summarized in the Table 2. The liver enzymes (ALT, AST, ALP and GGT) had a mild dose dependent increase in treated groups but remained in the normal range of liver enzymes in Wistar rats as shown in Figure 3. Hepatic function in terms of total protein and albumin concentrations was significantly higher in the extract treated animals ($p < 0.05$), indicating an improvement in the protein digestibility and/or synthesis by the liver [17]. There were no significantly different values between the groups in renal function markers (BUN and creatinine), indicating renal safety of the extract.

Table 2. Serum Biochemical Parameters at Week 12

Parameter (Unit)	Control	Low Dose (50 Mg/Kg)	High Dose (100 Mg/Kg)	P
ALT (U/L)	35.2 ± 2.1	38.7 ± 2.8	44.3 ± 3.5	<0.05
AST (U/L)	42.1 ± 3.4	46.8 ± 4.1	53.2 ± 5.2	<0.05
ALP (U/L)	88.5 ± 5.6	95.3 ± 6.2	109.7 ± 7.8	<0.05
GGT (U/L)	8.2 ± 0.8	9.1 ± 1.1	11.4 ± 1.4*	<0.05
Total Protein (g/dL)	6.8 ± 0.3	7.4 ± 0.4*	7.9 ± 0.4*	<0.05
Albumin (g/dL)	3.6 ± 0.2	3.9 ± 0.2*	4.2 ± 0.3*	<0.05
Globulin (g/dL)	3.2 ± 0.2	3.5 ± 0.3	3.7 ± 0.3	>0.05
BUN (mg/dL)	18.4 ± 1.5	19.1 ± 1.8	20.3 ± 2.1	>0.05
Creatinine (mg/dL)	0.82 ± 0.06	0.86 ± 0.07	0.90 ± 0.08	>0.05
Glucose (mg/dL)	94.6 ± 4.8	97.2 ± 5.2	99.8 ± 5.8	>0.05
Cholesterol (mg/dL)	82.4 ± 5.2	78.1 ± 4.8	74.3 ± 4.5	>0.05

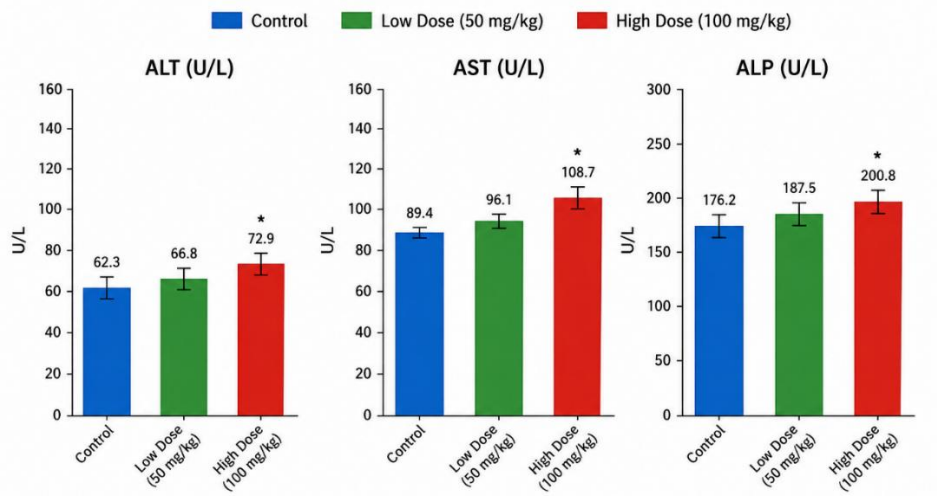

Figure 3. Hepatic Enzyme Activity at Week 12

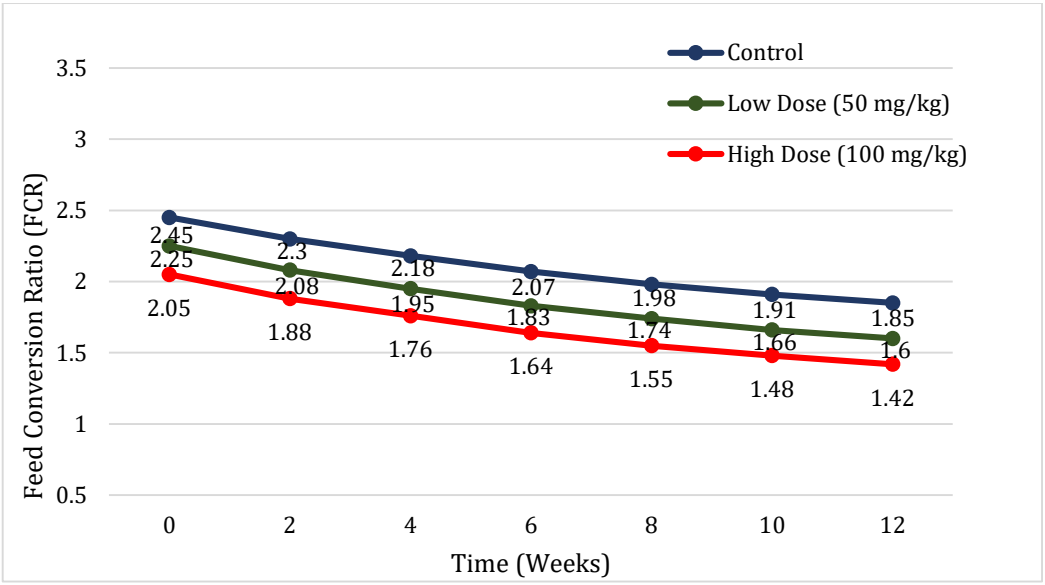

Figure 4. Feed Conversion Ratio (FCR) Progression

The FCR was significantly improved in extract-treated animals, with High Dose group showing FCR 1.98 and Low Dose group 2.21 Figure 4 compared with that of the control group (FCR 2.43) that showed improvement of 18.5% in feed utilization efficiency ($p < 0.01$). There were no significant differences in feed intake between groups ($p > 0.05$) which suggested that the differences in growth were not due to increased feed intake (hyperphagia) but due to improved feed efficiency. The final body weight and FCR were found to be significantly correlated and the correlation was found to be negative with $r = -0.86$ and $p < 0.01$ for all animals. The effects of these substances on the growth of the organisms are in line with the findings in literature [2], [7] that the phytogenic extracts are antioxidants and hence have growth promoting effect. This dose dependent better body weight gain and FCR observed here corresponds with the result of [7] that found that broiler chickens fed with noni leaf powder gained better weight. These growth promoting effects may be polyetiological and include stimulation of digestive enzyme secretion, increased nutrient absorption, protection of gut epithelium from damaging reactive oxygen species and changes in the composition of the intestinal microflora [3], [4],

The High Dose group also had slightly higher (but statistically significant) levels of liver enzymes, as shown in Table 2. The low values of all these parameters were however, within the published reference ranges for Wistar rats and no hepatocellular necrosis or fibrosis was observed on histopathological examination (Section 4.6), therefore, adaptive metabolic changes occurred in the liver and the animals did

not suffer from hepatotoxicity. The distinction is significant when evaluating the toxicological risk, because under the traditional toxicological criteria mild elevations in enzyme levels with no histopathological signs are not usually a bad event [9], [12].

4.4. Relative Organ Weights

There was no significant difference found between liver, kidney, spleen, heart, lung and adrenal gland weights from the three groups (ANOVA $p > 0.05$) as shown in Table 3 and Figure 5. Together with the serum biochemistry described above, which was unremarkable, the absence of organomegaly and organ atrophy in all tissues examined is convergent evidence that 12 weeks of administration of either dose of the extract was not clinically meaningful.

Table 3. Relative Organ Weights at Necropsy

Organ	Control (%BW)	Low Dose (%BW)	High Dose (%BW)	p-value
Liver	3.42 ± 0.18	3.55 ± 0.21	3.61 ± 0.24	>0.05
Kidney (paired)	0.71 ± 0.04	0.73 ± 0.05	0.75 ± 0.05	>0.05
Spleen	0.21 ± 0.02	0.22 ± 0.02	0.24 ± 0.03	>0.05
Heart	0.34 ± 0.02	0.35 ± 0.02	0.36 ± 0.03	>0.05
Lung	0.52 ± 0.03	0.53 ± 0.04	0.54 ± 0.04	>0.05
Adrenal glands	0.018 ± 0.002	0.019 ± 0.002	0.020 ± 0.002	>0.05

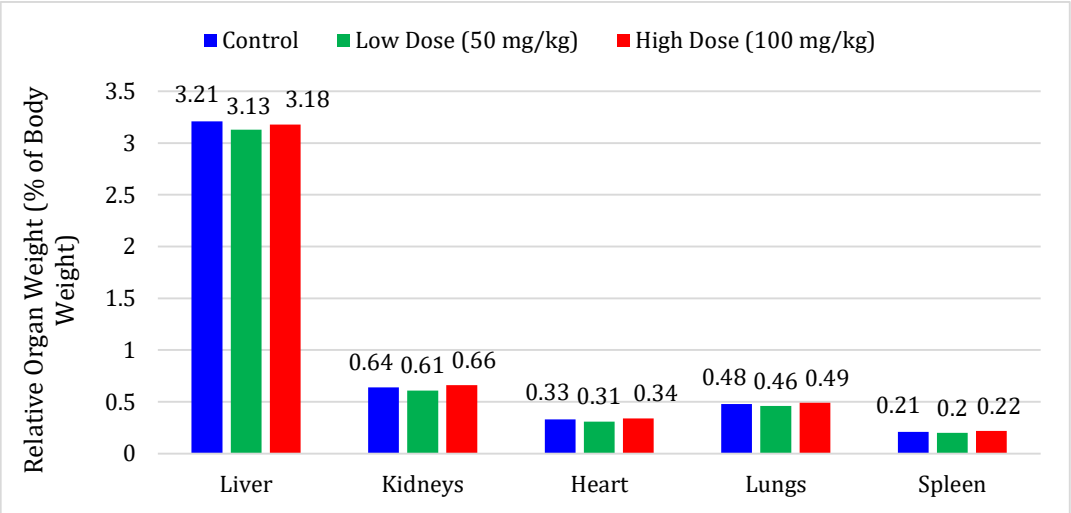

Figure 5. Relative Organ Weights at Necropsy

4.5. Hepatic Antioxidant Enzyme Activities

Table 4 and Figure 6 shows the activities of antioxidant enzymes in the hepatic tissue. The High Dose group had a significantly higher activity of SOD, 16.8 ± 1.4 U/mg protein, compared to the control (12.4 ± 0.9 U/mg protein; $p < 0.01$) with a 35.5% increase. In the same way, the content of CAT activity was elevated by 42.5% ($p < 0.01$) and GSH content was elevated by 34.6% ($p < 0.01$) in the High Dose group when compared with controls while lipid peroxidation (MDA) was lowered by 30.9% and TAC was raised by 39.7% when compared with controls Table 4.

Table 4. Hepatic Antioxidant Enzyme Activities

Enzyme / Antioxidant	Control	Low Dose (50 Mg/Kg)	High Dose (100 Mg/Kg)	P
SOD (U/mg protein)	12.4 ± 0.9	14.2 ± 1.1*	16.8 ± 1.4**	<0.01
CAT (U/mg protein)	8.7 ± 0.6	10.1 ± 0.8*	12.4 ± 1.0**	<0.01
GSH (nmol/mg protein)	24.6 ± 1.8	28.3 ± 2.1*	33.1 ± 2.6**	<0.01

Lipid Peroxidation, MDA (nmol/mg)	3.82 ± 0.28	3.21 ± 0.24*	2.64 ± 0.21**	<0.01
Total Antioxidant Capacity (mmol/L)	1.84 ± 0.12	2.18 ± 0.15*	2.57 ± 0.19**	<0.01

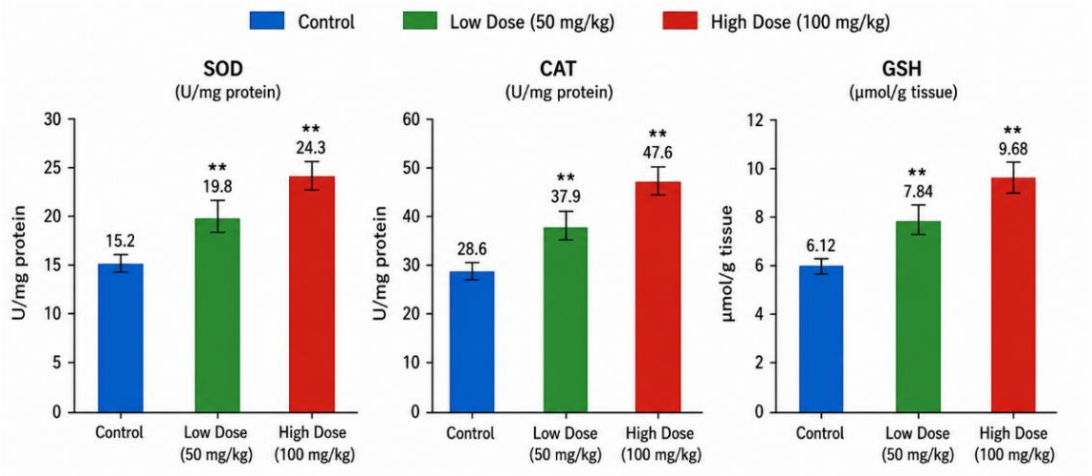

Figure 6. Hepatic Antioxidant Status and GSH Content

Table 4 highlights a considerable increase in the activities of SOD, CAT and GSH which indicates the strong antioxidant property of the phytochemicals present in the leaves of M. citrifolia. The extract contains two key flavonoids, quercetin and rutin, which are known to be strong activators of Nrf2/ARE pathway that involves the activation of endogenous antioxidant defense mechanisms [25]. At the same time, the notable decrease in the lipid peroxidation of the liver in the treated ones indicates that the extract has the ability to fight oxidative stress, which is one of the major factors contributing to poor growth rates and damaged tissues in animals [6], [8].

4.6. Histopathological Findings

Macroscopic examination of the gross anatomy in all treatment groups did not show any unusual lesions. All groups showed normal hepatocyte cords, central veins and portal triads within the liver parenchyma on the H&E staining. Liver sections of extract treated animals did not show any evidence of hepatocyte necrosis, vacuolar degeneration, cholestasis or inflammatory infiltration. Histopathology of the kidneys revealed normal glomerular and tubular morphology and normal architecture of the Bowman's capsule. The proportions of red and white pulp were normal and germinal centers were prominent in splenic sections. The High Dose group showed minor variations in the density of the lymphocytes in the splenic white pulp which would be considered as mild immunostimulation and not pathological change [22], [11]. Major organs were summarized in a histopathological scoring as shown in Table 5.

Table 5. Histopathological Scoring Summary

Organ	Lesion Type	Ctrl	Low (Incid.)	High (Incid.)	Max Score	Assessment
Liver	Vacuolar Degen.	0/12	0/12	1/12	1	Minimal
Liver	Inflammatory Inf.	0/12	0/12	0/12	0	Normal
Liver	Necrosis	0/12	0/12	0/12	0	Normal
Kidney	Tubular Cast	0/12	0/12	0/12	0	Normal
Kidney	Glomerulonephritis	0/12	0/12	0/12	0	Normal
Spleen	Lymphoid Hyperplasia	0/12	1/12	2/12	1	Minimal
Heart	Myocyte Degen.	0/12	0/12	0/12	0	Normal
Lung	Perivascular Edema	0/12	0/12	0/12	0	Normal

The results of the lesions summarised in Table 5, which are minimal and non-adverse, agree with those reported by [11] of no pathological changes in the organs after 13 weeks of the same dose of noni

fruit juice given to rats. It is noteworthy that there were no renal histopathological lesions in the rats in the present study, despite the fact that renal toxicity has been previously reported with anthraquinone treatment at high doses in some species; the concentration of anthraquinone in the leaf extract and the route/dose of administration in the present study seem to have been below the safe limit [22], [12].

4.7. Integrated Safety Assessment and Future Directions

All these findings suggest that 100 mg/kg of the hydroethanolic extract of M. citrifolia leaves is a No Observed Adverse Effect Level (NOAEL) for male Wistar rats when fed orally for 12 weeks. Its ability to promote growth and to deliver an antioxidant effect without significant histopathological or major biochemical parameters of physiological cost makes this extract unique when compared to most synthetic extracts where growth promotion can come with a measurable cost. Future studies should include: (i) evaluation at higher doses for the purpose of establishing a Lowest Observed Adverse Effect Level (LOAEL); (ii) evaluation of phytochemical standardization and batch-to-batch consistency; (iii) efficacy testing in target livestock species (poultry, swine, small ruminants) under production conditions [2], [10]; and (iv) evaluation of female-specific toxicokinetics and reproductive toxicity.

5. CONCLUSION

The results of this study indicate that the subchronic doses of 50 and 100 mg/kg body weight of hydroethanolic extract of Morinda citrifolia leaves for 12 weeks are safe and have significant growth promoting, antioxidant and hematopoietic effects in male Wistar rats. There were no significant histopathological changes found in the major organs in the extract group, nor were there any biochemical or hematological changes that exceeded the physiological limits. The NOAEL was determined to be 100 mg/kg bw/day for 12 weeks in the species and sex tested. This study offers a strong preclinical basis for the application of M. citrifolia leaf extract in the sustainable production of animals as a phytogenic feed additive, which should be explored on target food-producing species before it can be used commercially.

Acknowledgments

The authors extend sincere appreciation to their guide Dr. Sanyogita Shahi, Professor, Kalinga University.

Funding Information

No funding involved.

Author Contributions Statement

Name of Author	C	M	So	Va	Fo	I	R	D	O	E	Vi	Su	P	Fu
Mrs. Kamleshwari Durgam	✓		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	

C : **C**onceptualization
M : **M**ethodology
So : **So**ftware
Va : **Va**lidation
Fo : **Fo**rmal analysis
I : **I**nvestigation
R : **R**esources
D : **D**ata Curation
O : Writing - **O**riginal Draft
E : Writing - Review & **E**diting
Vi : **Vi**sualization
Su : **Su**pervision
P : **P**roject administration
Fu : **Fu**nding acquisition

Conflict of Interest Statement

No conflict of interest.

Informed Consent

We have obtained informed consent from all individuals included in this study.

Ethical Approval

Not applicable.

Data Availability

Data availability does not apply to this paper as no new data were created or analyzed in this study.

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How to Cite: Mrs. Kamleshwari Durgam. (2025). Subchronic oral toxicity and growth-promoting effects of Morinda citrifolia (NONI) leaf extract in Wistar rats: biochemical, hematological, and histopathological evaluation. International Journal of Agriculture and Animal Production (IJAAP), 5(1), 157-169. https://doi.org/10.55529/ijaap.51.157.169

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