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Moringa oleifera Lam.-Enriched Diet Boosts Serum Protein Levels Independently of Dietary Protein Intake

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ABSTRACT

Moringa oleifera Lam. leaves (MOL) have been a staple food source in India for centuries. Recent reports have highlighted their potential as immunomodulators, hepato-protectants, and more. To verify these claims and provide a robust solution for protein-energy malnutrition, we conducted a study to shed light on the nutritional benefits of MOL consumption. This six-week study employed an isocaloric and isonitrogenous feeding trial, using a pelleted diet with 20% protein, supplemented with either 2% or 4% MOL. Healthy adult male rats were subjected to a unique forced exercise regimen. We analyzed the biochemical parameters of 30 rats distributed across five groups. It showed that the test groups had significantly lower serum urea and liver enzyme levels (AST, ALT, and ALP) than the control group. Total protein levels increased significantly (14-19%) in all test groups, with no significant difference in creatinine levels. This analysis concludes that administering MOL powder orally at 2% and 4% is biochemically safe and exhibits liver-protective and nephroprotective properties. Our study suggests that MOL powder can be safely incorporated into both human and animal diets in a dose-dependent manner. Additionally, the synergistic effect of exercise enhances these benefits. The study provides long-term results without altering animal behavior, making MOL a promising option for combating protein-energy and malnutrition in India.

Keywords: *Moringa oleifera Lam*., swimming, forced-exercise model, hepato-protective, nephroprotective.

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INTRODUCTION

Moringa oleifera Lam. (MOL) hails from the *Moringaceae* family. (Martínez-González *et al.*, 2017; Nwidu, Elmorsy, Aprioku, Siminialayi, & Carter, 2018; Paikra, Dhongade, & Gidwani, 2017; Sadek, Abouzed, Abouelkhair, & Nasr, 2017) and originates from the Indian subcontinent. Its uses span from traditional herbal medicine (López *et al.*, 2018) to a vegetable (Atawodi *et al.*, 2010) and staple food in various countries, including India.

Recent scientific reports emphasize the nutritional richness of dried *MOL* leaf (MOL) powder. (Paikra *et al.*, 2017), which falls into the superfood category due to its protein (19-35%), metabolizable energy (2273-2978 kcal/kg), fat (2.3-10%), crude fiber (9.14%), vitamins (A, B, C, and E), minerals (0.6-11.2%) - calcium (3.65%), phosphorus (0.3%), magnesium (0.5%), potassium (1.5%), sodium (0.164%), Sulphur (0.63%), zinc (13.03 mg/kg), copper (8.25%), manganese (86.8 mg/kg), iron (490 mg/kg) and selenium (363 mg/kg)(Moyo, Masika, Hugo, & Muchenje, 2011), and essential amino acid content (Lamou *et al.*, 2016). Furthermore, MOL contains various secondary plant compounds. (Martínez-González *et al.*, 2017; Paikra *et al.*, 2017) and bioactive substances (such as beta-sitosterol, caffeoylquinic acid, kaempferol, quercetin, and zeatin, as well as alkaloids, reducing sugars, steroidal aglycones, tannins, and terpenoids) known for their anti-inflammatory, antibacterial, antioxidant, anti-cancer, hepatoprotective, and neuroprotective properties (Nwidu *et al.*, 2018; Paikra *et al.*, 2017).

Protein-energy malnutrition (PEM) is a pressing concern, particularly in India, affecting a significant portion of the population in terms of disease susceptibility, severity, and mortality (Gonakoti & Osifo, 2021), especially children under five (Bhutia, 2014). PEM contributes to both non-communicable (Brahmbhatt, Brahmbhatt, & Boyages, 2001; Mohammed, Qadri, Molangiri, Basak, & Rajkumar, 2023) and communicable (Schaible & Kaufmann, 2007; Taylor *et al.*, 2013) diseases, making it a priority for researchers and policymakers. MOL has been traditionally used as a medicinal plant (S. K. Gavadiya, T. Sharma, & V. M. Bapna, 2022; Kumar, Meena, Mishra, & Yoga, 2023; Maurya *et al.*, 2021; Sonewane *et al.*, 2022) to address various health issues (Aremu *et al.*, 2018; Azad *et al.*, 2017; Zeng *et al.*, 2018), but validation is an ongoing process. The phytoconstituents of Moringa oleifera (*Shigru* in 'Sanskrit' language) are rich and diverse, contributing to its extensive therapeutic benefits. According to Rasa-Panchaka, the plant is characterized by a *Katu* (pungent) and *Tikta* (bitter) *Rasa* (taste), with *Guna* (qualities) described as *Laghu* (light), *Ruksha* (dry) and Teekshna (sharp). It possesses *Ushna* (hot), *Veerya* (potency), and *Katu Vipaka* (pungent post-digestive effect). Its Doshaghnata (dosha-balancing properties) primarily pacifies *Vaata* and *Kapha* doshas. The plant exhibits various therapeutic actions (Karma), including *Deepana* (digestive stimulant), *Hrudya* (heart tonic), *Vidahakruta* (causing mild heat), *Vishgna* (detoxifying), *Shukrala* (enhancing reproductive health), *Chakushya* (improving vision), and *Vaataghna* (relieving *Vata*-related disorders) (Kumar *et al.*, 2023).

Moringa oleifera is a versatile tree derived from various plant parts that have medicinal applications. The roots are used as an antilithic, rubefacient, vesicant, and stimulant, offering anti-inflammatory and antifertility effects and treating conditions like rheumatism, kidney pain, and constipation. The leaves serve as a purgative and are applied for ailments like headaches, bronchitis, and sore throats, while the stem bark helps with eye diseases, spleen enlargement, tumors, and ulcers. The gum treats dental caries, fevers, and intestinal issues and is mixed with sesame oil for various therapeutic uses. Moringa oleifera exhibits diverse pharmacological activities, including antihypertensive, hepatoprotective, and antitumor properties, making it a valuable resource in traditional and modern medicine(Maurya *et al.*, 2021).

Shigru is recognized in Ayurvedic literature as one of the few herbs that exhibit both *Balya* (nourishing) and *Medohara* (anti-obesity) qualities. (Sonewane *et al.*, 2022a). *Shigru* is also a widely available and therapeutically potent remedy in diverse external therapeutic applications; a comprehensive literature survey revealed 149 formulations of *Shigru*, presented in 145 dosage forms, recommended for treating 24 different diseases. (S. K. Gavadiya, T. Sharma, & V. M. Bapna, 2022). *Shigru* is a powerful antioxidant herb that protects the body from free radicals and helps combat various infections. Renowned for its exceptional nutritional value, it also boasts a broad spectrum of therapeutic applications. (Kumar *et al.*, 2023). Therefore, our experiment aimed to explore MOL's hepatoprotective and nephroprotective properties while developing lean mass.

Additionally, we investigated through a review of the literature for the potential of vegetarian diets using moringa leaves as an alternative protein source where we found adequate evidence supporting its use for protein built up in the fish study. (Richter, Siddhuraju, & Becker, 2003) and cattle study (Mendieta-Araica, Spörndly, Reyes-Sánchez, & Spörndly, 2011).

In addition, it also provides evidence that it has a low amount of anti-nutritional factors, which helps in meat quality(Su & Chen, 2020). Therefore, in this study, we looked at its capacity to enhance serum protein levels independently of protein content. The novelty about this study includes a unique forced-impaired rodent model with an isocaloric and isonitrogenous moringa enriched diet.

METHODS

The animal experiment was conducted at ICMR-NIN, Hyderabad, and was approved by Institutional Animal Ethics Committee (ICMR-NIN/IAEC-IV/02/001/2020). Male SD/NIN rats (n=30), 3 months (12 weeks) old, were used in the study. The rats were acclimatized to a reversed day-night cycle (12hr:12hr) and forced to swim by housing them in an experimental room for 7 days before the feeding trial. Randomization of the rats was based on their body weight, and they were housed individually in open typed cages with netted floors. The environment was controlled as per Committee for Control and Supervision of Experiments on Animals guidelines (Temperature: 24±4^{0}C and Relative humidity: 50-70%). *Ad libitum* food and water were provided to the rats, and their food intake and body weight were measured weekly. Daily inspections of the cages were conducted, and blood samples were taken three times ('0', '21', and '42' days) during the experimental phase. Assuming a moderate to large effect size (f = 0.55), based on pilot data and supporting literature, with a significance level (α) set at 0.05 (two-tailed) and statistical power (1−β) of 80%, the minimum required sample size was calculated to be 5 rats per group for a study involving five groups. This yielded a total sample size of 25 animals. However, to account for potential biological variability and unexpected dropouts, the sample size was increased to 6 rats per group, resulting in a total of 30 rats included in the study.

Plant material collection and Preparation of Diet

The dried MOL powder was procured from a local supplier [Medikonda Nutrients, Hyderabad, Telangana (Head Office: 17275 Old Tobacco Rd, Lutz, Florida 33558 USA)], which was shade-dried. The admixture of MOL in the feed was determined based on the proximate analysis of the powder. A standard protocol was followed to prepare pelleted feed, where raw materials were weighed and mechanically mixed for 15 minutes using a Hardcore Mixer™. The pellets were prepared using a pellet machine (Model No. 120 - Sanjeevani Agro-Machinery, Nagpur) with a mesh size of 8 mm and were then placed on sterilization trays. The pellets were sterilised in a double-door autoclave (Steri-Horizontal Rectangular Steam Autoclave, Yorco Steriliser, Gaziabad) at 121ºC for 20 minutes and subsequently dried at 100ºC for 3 hours in a pellet dryer (IDS-48 Trays-Drier, Industrial Drying Systems, Madras).

An earlier study examining extraction temperatures (25–200 °C) on Moringa leaf powder found that myricetin and kaempferol peaked at 100 °C (2699 mg/kg and 3440 mg/kg, respectively) but decreased at 150 °C. Quercetin levels, however, remained stable (1429–1488 mg/kg) across all temperatures (Matshediso, Cukrowska, & Chimuka, 2015). In this study, the dried pellets were aseptically weighed, packed, and stored in a cold room until further use. Later for the quality check of feed from both autoclaved and unautoclaved feed samples of three diet groups were sent for analysis about protein using Kjeldahl method (IS7219), quercetin and chlorogenic acid quantification using GCLC/MS and UV methods in an NABL accredited lab.

Component	Amount
Moisture	4.1 g
Ash	10.63 g
Fat	4.90 g
Protein	26.62 g
Dietary Fibre	31.4 g
Carbohydrates	22.35 g
Energy	299.33 kcal

Table 1a: Nutritional Profile of Moringa oleifera: Macronutrient and Mineral Content

Ingredient	Percent (%)	Protein (g)	Fiber (g)
Roasted Bengal Gram	59.00	12.71	0.59
Wheat	22.63	2.40	2.54
Casein	4.00	3.24	0.00
Moringa dried leaf powder	0.00	0.00	0.00
Skimmed Milk Powder	5.00	1.75	0.00
Groundnut Oil	4.00	0.00	0.00
Vitamin Mix	0.50	0.00	0.00
Mineral Mix	4.00	0.00	0.00
Cellulose	0.87	0.00	0.87
Total	100.00	20.10	4.00

Table 1b: Composition of Normal Diet without Moringa

The protein levels in a conventional rodent diet (20%) remained unchanged when crafting a customized diet by incorporating 2% and 4% MOL powder. This adjustment was made without any alterations to the calorie content (355 Kcal/100gm), fiber content (4%), or protein content (20%). The formulation of this custom diet was guided by the proximate analysis of MOL powder, as depicted in Table - 1a. A comprehensive breakdown of the custom feed composition (Standard maintenance diet and Moringa-enriched

pelleted diet composition for rodents) can be found in Table - 1b to Table - 1d.

Ingredient	Percent (%)	Protein (g)	Fiber (g)
Roasted Bengal Gram	58.20	12.54	0.58
Wheat	22.00	2.33	2.47
Casein	3.68	2.98	0.00
Moringa	2.00	0.50	0.32
Skimmed Milk Powder	5.00	1.75	0.00
Groundnut Oil	4.00	0.00	0.00
Vitamin Mix	0.50	0.00	0.00
Mineral Mix	4.00	0.00	0.00
Cellulose	0.62	0.00	0.62
Total	100.00	20.10	4.00

Table 1c: Composition of Isocaloric and Isonitrogenous Custom Diet -1 (2% Moringa

Ingredient	Percent (%)	Protein (g)	Fiber (g)
Roasted Bengal Gram	54.76	11.80	0.55
Wheat	23.91	2.53	2.69
Casein	3.72	3.01	0.00
Moringa	4.00	1.00	0.65
Skimmed Milk Powder	5.00	1.75	0.00
Groundnut Oil	4.00	0.00	0.00
Vitamin Mix	0.50	0.00	0.00
Mineral Mix	4.00	0.00	0.00
Cellulose	0.12	0.00	0.12
Total	100.01	20.10	4.00

Table 1d: Composition of Isocaloric and Isonitrogenous Custom Diet -2 (4% Moringa Enriched)

Diet Regimen

The animals were housed individually in an open grilled bottom cage where the diet was fed (standard and pelleted diet) to animals of respective groups during the complete duration of the study. Daily, 30 grams of diet was provided, and the weekly leftover pellets were weighed to calculate feed intake per week.

Exercise interventions

Based on the rationale that exercise may have a synergistic positive impact on the body during the feeding trial, two groups out of the five in the study design (Table-2) were required to undergo forced exercise. Therefore, twelve animals (2% and 4% MOL) from Group-3 and Group-5 were subjected to forced (intensive free-swimming) exercise for 30 minutes each day, six days a week, at a water temperature of 30±5C at the same time to avoid any variation and no stress related porphyrin markings were seen on animals during study phase.

Determination of Biochemical Parameters

After 6 weeks, the rats, including the control and MOL-fed groups, were fasted for 16 hours. Blood was collected from the retro-orbital plexus under isoflurane sedation for serum separation, which was performed by centrifugation, and aliquots were stored at -20°C temperature until biochemical profiling. The serum samples were used to determine kidney function tests, including serum creatinine and serum urea, as well as liver function tests, such as serum alkaline phosphatase (ALP), serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), and serum total protein (TP), using an automated biochemical analyzer (COBAS, model C-311 analyser, Roche, Switzerland) at the National Institute of Nutrition, Hyderabad -500007, India (ICMR-NIN). Then, animals were euthanized using CO_2 asphyxiation, and vital tissues were collected along with muscles for histology (data not published) and caecum of gut microbes (data not published). All analytical kits were obtained from Roche, Switzerland, to ensure consistency in testing. The chemical profile of MOL was assessed through GCLC/MS and UV methods for Chlorogenic acid and Quercetin. Chlorogenic acid is a polyphenol compound found in coffee, fruits, and vegetables, known for its antioxidant, anti-inflammatory, blood sugar regulation, weight management, and potential anti-cancer properties. Quercetin is a potent antioxidant, anti-inflammatory, cardiovascular support, immune booster, anti-cancer agent, natural antihistamine, and exercise enhancer.

Statistical methods

The experimental data was analyzed using GraphPad Prism software (version-10.1.0). An ordinary two-way ANOVA test with Tukey's multiple comparison test with a single pooled variance was applied to analyze the results. Tukey's test was used to classify the means of the groups at a significance level of ($P< 0.05$).

RESULTS

The animal hydration status was adequate as evident through TOBEC analysis, water consumption pattern, skin turgor and alertness in actimeter readings. The proximate analysis of MOL indicated high levels of dietary fiber (31.4%) and protein (26.62%), as presented in Table-1a.

Feed Intake

The Moringa-fed groups (Groups 2-5) exhibited an increase in feed intake (9.0%-18.2%) compared to the standard group (Group 1). Additionally, the forced exercise groups (Group 3 and Group 5) showed higher feed intake (18.2% and 17.5%, respectively) compared to the non-exercise Moringa diet groups (Group 2: 9.0% and Group 4: 11.0%).

This longitudinal analysis highlights how diet and physical activity affect feed intake in rats. Group 1 (standard diet, no exercise) consistently had the lowest intake. Groups 2 and 4 (2% and 4% Moringa, no exercise) showed only slight increases over Group 1. In contrast, Groups 3 and 5 (2% and 4% Moringa with daily 30-minute swimming) had significantly higher feed intake from Week 2 onward.

By Weeks 4–6, differences between exercising (Groups 3 and 5) and non-exercising groups (Groups 1, 2, and 4) were highly significant ($p < 0.0001$). These results confirm that Moringa supplementation combined with exercise synergistically enhances dietary intake, supporting its use in strategies aimed at improving appetite and nutrient use in both clinical and athletic applications (Figure 1a).

Body Weight Gain

During Weeks 1 and 2, no significant differences in body weight were observed across groups ($p > 0.05$), confirming a uniform baseline. By Week 3, Group 4 (4% Moringa, no exercise) showed significant weight gain over Group 1 ($p = 0.0490$) and Group 2 ($p = 0.0077$), indicating a diet-driven effect. This persisted into Week 4 (Group 2 *vs.* 4, $p = 0.0069$), supporting the role of 4% Moringa in weight gain.

In Week 5, Group 2 showed lower weight than Group 1 ($p = 0.0013$) and Group 5 ($p = 0.0131$), while by Week 6, Group 1 exceeded both Group 2 ($p = 0.0007$) and Group 4 ($p = 0.0355$). Group 2 also remained significantly lower than Group 5 ($p = 0.0007$), and Group 4 was lower than Group 5 ($p = 0.0355$), emphasizing exercise's additive effect.

The data show that while Moringa alone (Group 4) improves weight, the combination with exercise (Group 5) yields the best outcomes. Group 2 (2% Moringa, no exercise) had minimal weight gain, suggesting a limited effect without physical activity. Group 5 maintained the highest weight, indicating a synergistic benefit of higher Moringa and exercise (Figure 1b)

Biochemical parameters

All MOL-enriched diet groups (Gr. 2-5) exhibited a significant and consistent decrease in serum urea by 29-36%, whereas the control group showed an increase of 2.16% over 6 weeks (Figure 2a). The MOL-fed groups demonstrated a uniform decrease in AST (by 8% to 17%) and ALT (by 19.5% to 25.8%), while the control group showed a slight change in AST (2.1%) and ALT (1.5%) over 6 weeks (Figure 2b & 3a). The MOL-enriched diet groups also showed a significant and consistent decrease in serum ALP (22% to 35%), whereas the control group showed an increase of 7.4% over 6 weeks (Figure 3b). Conversely, serum total protein levels increased (14% to 19%) in all MOL groups, regardless of exercise, while water consumption was not measured in the study. These findings validate the results of a previous study (Ghasi, Nwobodo, & Ofili, 2000), but in this case, we are presenting the dose-dependent outcomes of MOL powder instead of extracts. These findings suggest that liver function is normal and water consumption during exercise did not influence the study's results, as two MOL groups without exercise (2M-WOE and 4M-WOE) yielded similar outcomes (Figure 3c).

Serum Total Protein Levels

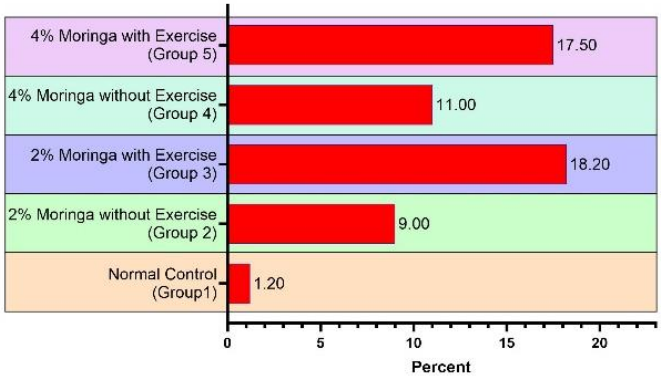
At baseline (Day 0), no significant differences were observed in serum protein levels across all groups ($p > 0.05$), indicating uniform nutritional status. By Day 21, Group 1 (control) had significantly lower protein levels than all others (Groups 2–5; $p < 0.0001$). Group 3 (2% Moringa + exercise) showed higher protein levels than Groups 2, 4, and 5 ($p = 0.0005$, 0.0003, 0.0396), indicating a synergistic effect of Moringa and exercise. By Day 42, differences deepened. Group 1 remained significantly lower than Groups 2–5 ($p < 0.0001$). Group 3 stayed significantly higher than Group 1 ($p < 0.0001$) and differed from Group 5 ($p = 0.0005$). Group 5 (4% Moringa + exercise) had the highest protein level (7.93 ± 0.11 g/dL), significantly exceeding Group 3 ($p = 0.0005$) and marginally higher than Group 4 ($p = 0.0655$).

The findings confirm that Moringa fortification boosts serum protein, and exercise amplifies this effect. Group 5's combination of 4% Moringa and swimming yielded the most robust outcomes, supporting its potential for addressing undernutrition and developing functional food strategies.

Serum Urea levels

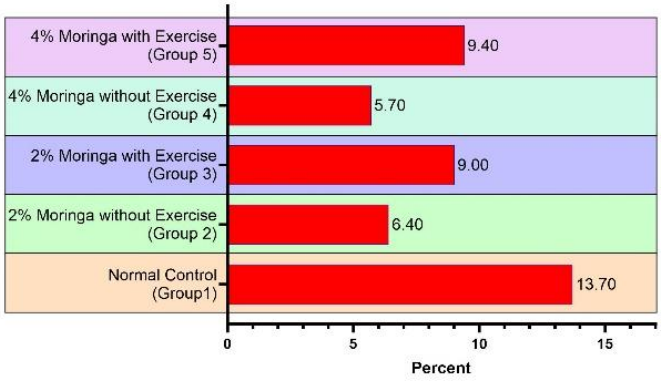
At Day 0, no significant differences in serum urea levels were observed across groups ($p > 0.05$), indicating a balanced baseline. By Day 21, Group 1 (control) showed significantly elevated urea compared to all Moringa or exercise-treated groups (Groups 2–5; $p < 0.0001$), suggesting improved nitrogen metabolism with both interventions. Group 4 (4% Moringa, no exercise) had lower urea than Group 3 (2% Moringa, with exercise), and Group 5 (4% Moringa + exercise) showed the greatest reduction (*vs.* Group 4, $p = 0.0001$), indicating a dose- and exercise-dependent effect.

Fig. 1a Feed Intake Across Dietary and Exercise Groups in Rodent Study



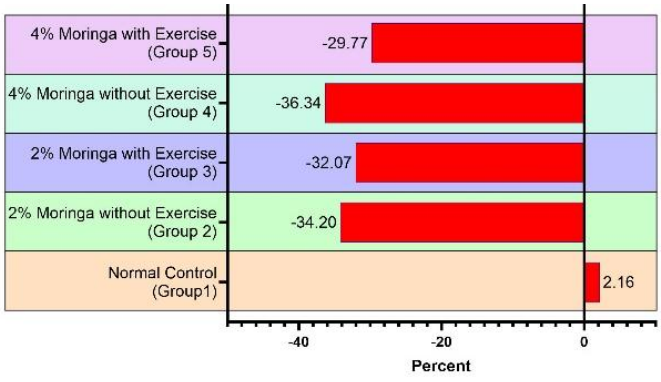
Legend: The graph depicts the percentage increase in feed intake across five experimental groups with varying diets and exercise regimens.

Fig. 1b: Weight Gain Across Dietary with and without Exercise Regimen



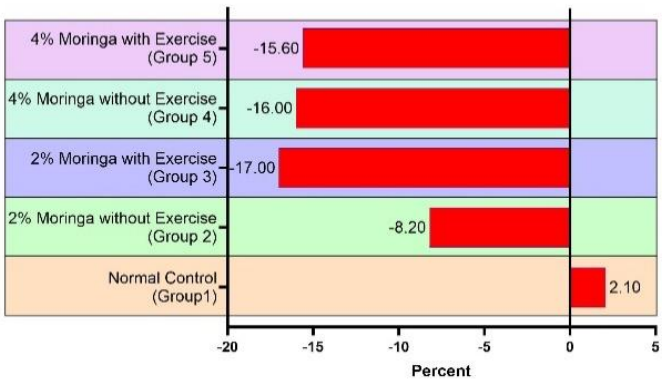
Legend: The graph illustrates weight gain percentage across five groups with varying diets and exercise.

Fig. 2a: Influence of Moringa-Fortified Diet and Exercise on serum urea in rodents



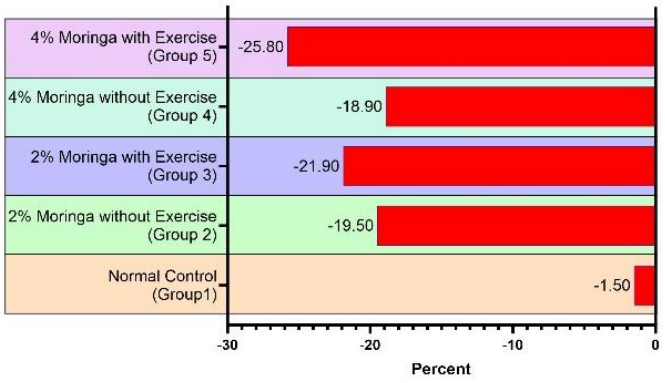
Legend: The graph depicts the percentage increase in serum urea levels across five experimental groups under different dietary and exercise conditions.

Fig. 2b: Effect of Moringa-Fortified Diet and Exercise on AST increase in Rodent Models



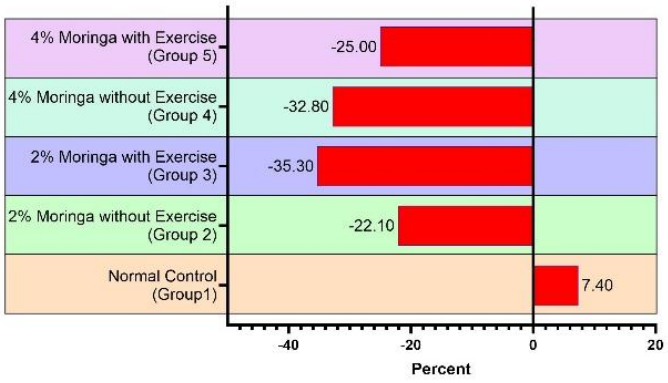
Legend: The graph illustrates the percentage increase in serum AST levels across five groups under varying dietary and exercise conditions.

Fig. 3a: Impact of Moringa-Fortified Diet and Exercise on serum ALT in Rodent Study



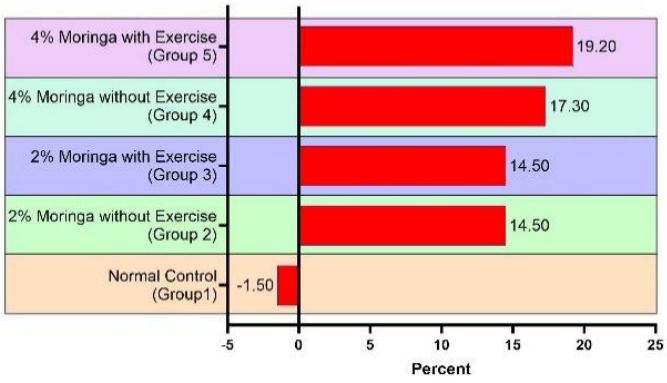
Legend: The graph depicts the percentage increase in serum ALT levels across five experimental groups with varying diet and exercise regimens.

Fig. 3b: Effect of Moringa-Fortified Diet and Exercise on serum ALP in Rodent Models



Legend: The graph depicts the percentage increase in serum alkaline phosphatase (ALP) across five groups under varying dietary and exercise conditions.

Fig. 3c: Impact of Moringa-Fortified Diet and Exercise on total protein in Rodents



Legend: This graph presents the percentage increase in total protein levels across five experimental groups with variations in diet and exercise.

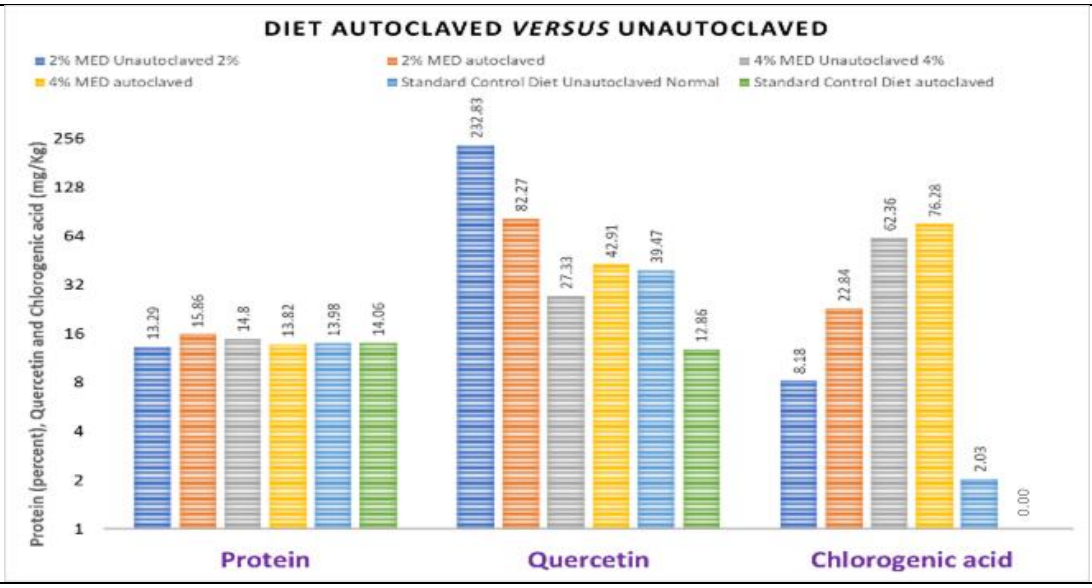


Fig. 4: Protein and Bioactive Profile of 2% and 4% Moringa-Enriched Diet Compared to Standard Diet: Effects of Autoclaving.

Legend: The bar graph compares protein content, quercetin, and chlorogenic acid levels in 2% and 4% Moringa-enriched diets and a standard control diet under autoclaved and unautoclaved conditions.

By Day 42, differences widened further. Group 1 maintained the highest urea levels, significantly higher than all others ($p < 0.0001$), while Group 5 had the lowest, outperforming Group 4 ($p = 0.0032$). These results support the conclusion that 4% Moringa supplementation, especially when combined with exercise, enhances protein utilization, reduces catabolism, and may protect renal function. The data suggest potential for Moringa-based dietary strategies to improve metabolic efficiency and kidney health in experimental and translational contexts.

Serum Creatinine Levels

At baseline (Day 0), Group 3 (2% Moringa + exercise) and Group 5 (4% Moringa + exercise) had significantly higher creatinine than Group 1 ($p = 0.0002$ and $p < 0.0001$), possibly due to early exercise-induced muscular stress or renal adaptation. By Day 21, Group 1 showed significantly lower creatinine than Groups 3, 4, and 5 ($p = 0.0001–0.0002$), suggesting increased muscle metabolism or altered renal function from interventions. Differences between Groups 3, 4, and 5 became statistically non-significant, indicating stabilization.

By Day 42, Group 1 remained significantly lower than Groups 2 and 4 ($p = 0.0043$), while Group 2 exceeded Group 5 ($p = 0.0043$). Group 5 maintained the lowest and most stable creatinine, reflecting improved renal efficiency and muscle metabolism. Overall, 4% Moringa with exercise (Group 5) demonstrated the best physiological resilience, supporting its protective, adaptive effect on renal and metabolic health without inducing burden.

Serum AST Levels

AST levels over time reveal the hepatoprotective effects of Moringa, enhanced by exercise. At baseline (Day 0), no significant differences were found among groups, indicating similar liver profiles. By Day 21, all intervention groups showed significantly lower AST levels than Group 1 ($p < 0.0001$), with Groups 4 and 5 (4% Moringa) showing the most reduction. Group 2 also had higher AST than Groups 4 and 5 ($p = 0.0128, 0.0174$), indicating a dose-dependent effect.

By Day 42, Group 1 remained significantly higher than all others ($p < 0.0001$). Group 2 still had higher AST than Group 3 ($p = 0.0115$), reinforcing the benefit of exercise. Though differences among Groups 3–5 were not statistically significant, Group 5 (4% Moringa + exercise) maintained the lowest AST, indicating strongest hepatic protection.

Overall, Moringa supplementation, especially at 4% with exercise, significantly lowers AST and supports liver health through dose-dependent and synergistic effects.

Serum ALT Levels

At baseline (Day 0), ALT levels were comparable across all groups ($p > 0.05$), confirming uniform liver function. By Day 21, Group 1 (standard diet, no exercise) showed significantly higher ALT than all other groups ($p < 0.0001$), indicating potential liver stress. While differences among Moringa-fed groups (2–5) weren't significant, better outcomes were noted in higher Moringa and exercise groups.

By Day 42, Group 1 still had the highest ALT ($p < 0.0001$), while Group 5 (4% Moringa + exercise) maintained the lowest. Though differences among Moringa groups remained non-significant, Group 5 trended lower than Group 2 ($p = 0.0805$), highlighting the additive benefit of higher Moringa and exercise. These results confirm that Moringa, especially at 4% with exercise, effectively lowers ALT, supporting liver health. Group 5 demonstrated the strongest and most consistent hepatoprotection.

Serum ALP Levels

At 1st Week, Group 1 (standard diet) had significantly higher ALP levels than Groups 3, 4, and 5 ($p = 0.0094, 0.0012, 0.0014$), indicating an early ALP-lowering effect of Moringa. The difference with Group 2 was borderline ($p = 0.0535$), suggesting a trend. By Day 21, Group 1 showed significantly higher ALP than all other groups ($p < 0.0001$), with Group 5 (4% Moringa + exercise) showing the greatest reduction. Group 2 *vs.* Group 5 was also significant ($p = 0.0147$), supporting the dose-dependent effect.

By Day 42, Group 1 remained significantly elevated ($p < 0.0001$). Group 2 showed higher ALP than Groups 3, 4, and 5 ($p = 0.0147, 0.0443, 0.0007$), indicating enhanced protection with increased Moringa and exercise. No significant differences were found among Groups 3, 4, and 5, suggesting a plateau in ALP reduction.

These results confirm that 4% Moringa with exercise (Group 5) offers the strongest, sustained protection of hepatic-biliary function, with effects becoming evident by Day 21 and persisting through Day 42.

Table 4: Biochemical Parameters						
Group	0th Day	21st Day	42nd Day	0th Day	21st Day	42nd Day
	Table 4a: Total serum protein (g/dL)			Table 4b: Urea levels (mg/dL)		
1	6.63 ± 0.30	6.81 ± 0.06	6.53 ± 0.13	35.58 ± 1.48	36.81 ± 1.36	36.35 ± 0.80
2	6.77 ± 0.07	7.44 ± 0.15	7.75 ± 0.21	34.88 ± 1.59	27.70 ± 1.01	25.35 ± 0.48
3	6.55 ± 0.12	7.87 ± 0.29	7.50 ± 0.10	35.70 ± 0.62	30.95 ± 1.80	28.25 ± 1.30
4	6.53 ± 0.10	7.42 ± 0.25	7.66 ± 0.15	35.28 ± 1.19	25.70 ± 0.46	24.46 ± 0.58
5	6.65 ± 0.13	7.58 ± 0.20	7.93 ± 0.11	36.88 ± 1.28	31.13 ± 2.11	27.23 ± 1.70
	Table 4c: Serum creatinine levels (mg/dL)			Table 4d: AST levels (U/L)		
1	0.40 ± 0.02	0.44 ± 0.01	0.48 ± 0.01	164.63 ± 3.82	167.31 ± 4.27	168.10 ± 4.90
2	0.39 ± 0.02	0.47 ± 0.02	0.52 ± 0.01	161.45 ± 4.34	139.45 ± 7.67	148.20 ± 5.01
3	0.45 ± 0.02	0.50 ± 0.03	0.49 ± 0.01	165.05 ± 1.09	135.43 ± 8.77	137.00 ± 8.85
4	0.43 ± 0.03	0.49 ± 0.01	0.52 ± 0.03	169.05 ± 3.48	128.38 ± 10.26	142.00 ± 5.14
5	0.46 ± 0.02	0.49 ± 0.01	0.48 ± 0.01	165.78 ± 1.29	128.73 ± 5.45	140.00 ± 4.23
	Table 4e: ALT levels (U/L)			Table 4f: ALP levels (U/L)		
1	91.06 ± 1.27	93.18 ± 1.81	89.71 ± 9.08	350.5 ± 8.89	355.6 ± 6.74	376.5 ± 35.1
2	94.18 ± 2.03	68.13 ± 3.87	75.81 ± 2.98	380.1 ± 7.19	278.5 ± 11.23	295.0 ± 11.2
3	93.11 ± 2.29	64.20 ± 4.66	72.70 ± 4.76	386.8 ± 21.6	257.3 ± 12.40	260.3 ± 9.80
4	91.68 ± 3.43	65.46 ± 5.06	74.33 ± 4.39	393.6 ± 34.8	258.3 ± 17.60	264.6 ± 26.10
5	92.90 ± 3.79	61.80 ± 8.05	68.91 ± 4.01	393.1 ± 17.6	243.8 ± 12.70	250.3 ± 9.80

Other Diet-related Parameters

The impact of autoclaving was also checked on the diet formulated by analyzing the protein, quercetin, and chlorogenic acid content (Figure 4) in the diet before and after autoclaving. The observed changes in the nutritional and bioactive profiles due to autoclaving can be attributed to its impact on moisture content and compound stability. Autoclaving significantly reduced the moisture content in the diets, as evidenced by the drop from 14% in the unautoclaved samples to 4% in the autoclaved ones. This reduction in moisture leads to a relative concentration of certain compounds, such as chlorogenic acid and quercetin, in the autoclaved Moringa-enriched diets (MED). The increase in chlorogenic acid content could be due to the release of bound forms or the conversion of precursor compounds during high-temperature processing.

The protein content showed minimal changes across all diets and conditions. In the 2% MED, autoclaving resulted in a slight increase in protein content (from 13.29% to 15.86%), potentially attributed to moisture reduction concentrating the protein. A similar trend was observed in the standard control diet, where protein increased slightly from 13.98% to 14.06% post-autoclaving. Interestingly, the 4% MED decreased protein content post-autoclaving (from 14.8% to 13.82%), suggesting that other factors, such as thermal denaturation or interaction with bioactive compounds, might influence protein stability at higher Moringa concentrations.

Quercetin, a sensitive bioactive compound, displayed significant degradation due to autoclaving. In the 2% MED, quercetin levels dropped from 232.83 mg/g in the unautoclaved diet to 82.27 mg/g in the autoclaved diet, indicating the susceptibility of quercetin to thermal degradation despite the moisture-driven concentration effect. In the 4% MED, quercetin levels followed an unexpected trend, increasing from 27.33 mg/g in the unautoclaved diet to 42.91 mg/g in the autoclaved diet. This increase might be attributed to the enhanced release of bound quercetin forms at higher Moringa concentrations, which partially counteracted the degradation caused by autoclaving. The standard control diet exhibited low quercetin levels, with substantial reductions observed post-autoclaving (39.47 mg/g to 12.86 mg/g), emphasizing the protective effects of Moringa bioactive against thermal stress.

Chlorogenic acid, another key bioactive compound, showed a remarkable increase in the autoclaved Moringa-enriched diets. In the 2% MED, chlorogenic acid content rose from 8.18 mg/g in the unautoclaved diet to 22.84 mg/g in the autoclaved diet, while in the 4% MED, it increased from 62.36 mg/g to 76.28 mg/g. These increases suggest that autoclaving might facilitate the release of bound chlorogenic acid or enhance its formation from precursor compounds during high-temperature processing. In contrast, the standard control diet exhibited negligible chlorogenic acid levels, with complete degradation observed post-autoclaving (2.03 mg/g to 0 mg/g), further emphasizing the importance of Moringa enrichment in preserving or enhancing bioactive during processing.

The consistent reduction in moisture content from 14% to 4% across all diets due to autoclaving highlights its impact on the concentration and stability of bioactive compounds. While moisture loss concentrates certain compounds, thermal stress induces degradation, particularly for sensitive components like quercetin.

In summary across all measured physiological, metabolic, and biochemical parameters—including feed intake, body weight, serum protein, urea, creatinine, and liver enzymes (AST, ALT, and ALP)—statistical analyses revealed a consistent pattern: Moringa oleifera fortification, particularly at 4%, and the inclusion of daily forced swimming exercise independently and synergistically contributed to significant improvements. Significant differences began emerging from Week 2 onward for feed intake ($p < 0.0001$), from Week 3 for body weight (Group 2 *vs.* 4, $p = 0.0077$), and from Day 21 for serum proteins (Group 1 *vs.* all other groups, $p < 0.0001$). By Day 42, Group 5 (4% Moringa + exercise) consistently outperformed others across parameters, indicating superior metabolic adaptation, protein anabolism, and organ protection.

Renal parameters demonstrated significant reductions in serum urea and creatinine levels in Moringa-treated and exercise-exposed groups by Day 21 and 42 ($p < 0.0001$ for Group 1 *vs.* Group 5). Hepatic markers followed a similar trend: AST, ALT, and ALP levels were markedly elevated in the sedentary standard diet group (Group 1) and were significantly lower in Groups 4 and 5 by Day 21 and Day 42, confirming both hepatoprotective and biliary-supportive effects of Moringa ($p < 0.0001$ across comparisons). Collectively, these findings provide strong statistical evidence for the dose-responsive and exercise-synergistic efficacy of Moringa fortification, establishing Group 5 as the most physiologically resilient and biochemically protected group across the study timeline.

DISCUSSION

Our study not only reaffirms the medicinal benefits of MOL but also highlights its potential to enhance serum protein levels. This increase falls within the normal range, indicating untapped potential for healthy individuals and potential benefits for those with protein-energy malnutrition (PEM). Additionally, none of the animals shown any signs of stress during exercise regimen as described earlier in other studies that animals undergo depression. This could be because moringa not only has multiple antioxidants, studies have shown that it has capacity to restore the melatonin levels (Saleh, & Sarhat, 2019).

Previous research has shown that MOL supplementation can improve various aspects of animal health, such as meat quality, body weight gain, feed conversion ratio, antioxidant levels, and more across multiple animal models (Cui *et al.*, 2018; Selim, Seleiman, Hassan, Saleh, & Mousa, 2021). Overall, it positively affects anthropometric growth. (Sun *et al.*, 2018), as well as improves the gut microbiome (Abu Hafsa, Ibrahim, Eid, & Hassan, 2020). Our study supports these findings and suggests that MOL can enhance food intake and assimilation efficiency due to change in gut bacteria proportion (unpublished data), particularly when combined with exercise. The study also investigated the effects of MOL on liver and kidney function, demonstrating significant differences in biomarkers. These findings and other data on makers for kidney damage (unpublished) suggest that MOL may protect against kidney and liver diseases and promote overall health.

In contrast, groups with 2% MOL had a 7.7-fold increase in feed intake, and those with 4% MOL had a 9.2-fold increase. Forced exercise resulted in a 6-8-fold increase in feed intake in both MOL-enriched and control groups. While weight gain was not significant across all groups, it was 52-56% lower in MOL groups without exercise and 33-28% lower in MOL groups with forced exercise. The decrease in serum urea was significant and consistent in MOL-enriched groups by 29-36% but increased by 2.16% in the control group over 6 weeks (Figure 1a-1b). These findings suggest that incorporating MOL in the diet, whether 2% or 4%, improves food intake and assimilation efficiency, with exercise providing a

positive synergistic effect. This could be attributed to the antioxidative and gut microbiota-improving properties of MOL (unpublished data) and the release of vital organ potentials, such as those in the liver, stomach, and kidneys.

This experiment aimed to investigate the effects of MOL powder on liver and kidney function in rats by evaluating the activities of liver enzymes and kidney function tests, which serve as biomarkers for liver and kidney function. The results indicated significant differences in the markers for liver and kidney function. The available literature also demonstrated a mechanism by which MOL may protect against kidney disease by mitigating various pathological factors associated with the condition, such as inflammation and oxidative stress. (Tsopanakis & Tsopanakis, 1998). Additionally, it is evident that as the body adapts to exercise, there is a noticeable decrease in urea levels. The lower mean serum urea levels (as shown in Table-4b) were observed in the MOL-enriched group with forced exercise (Gr. 4-5), followed by the MOL-enriched group without forced exercise (Gr.2-3), indicating reduced protein catabolism, which implies enhanced endurance.

The mean serum creatinine levels did not show significant differences between the groups at different time points. Literature suggested that the protective properties of MOL against kidney and liver tissue damage, such as anti-apoptotic, antioxidant, and anti-oxidative stress effects (Soliman *et al.*, 2020), may mitigate or reverse the effects of increased serum levels of ALT, AST, creatinine, and urea, as well as significantly decreased levels of total proteins, albumin, and globulin. Additionally, the literature suggests that MO's hepatoprotective and antidiabetic effects have been attributed to the phenolic bioactive compounds present in the methanolic leaf extracts of MO (Muzumbukilwa, Nlooto, & Owira, 2019).

The animals from the 4M-FE group exhibited the lowest mean values of AST (Table-4d), ALT (Table-4e), and ALP (Table-4f), followed by the 2M-FE group, indicating the anti-apoptotic and antioxidant properties of MO. These properties are associated with increased endurance performance and restoration of liver enzymes and liver tissue to normal levels, which is consistent with previous research (Saleh, & Sarhat, 2019). ALP is critical in calcium homeostasis, and elevated calcium levels are associated with various dental and skeletal disorders. However, the present study demonstrated that MO has a significant effect on ALP levels, reducing it significantly (by 27% to 50%). The reduction in ALP increases bone mineral content and decreases the risk of kidney stones. Although there was no significant change in body weight gain, an increase in lean mass, bone density, and bone content was observed in the MOL groups, which could be attributed to the reduced ALP activities. In addition to our unpublished findings demonstrating a positive alteration in gut microorganisms, several other studies have indicated that shifts in gut flora diversity and gene expression, both upregulation and downregulation, may be associated with the favorable increase in lean muscle mass. (Cohen-Zinder *et al.*, 2017; El-Rahman *et al.*, 2019; Moyo, Masika, Muchenje, & production, 2012; Nkukwana, Muchenje, Masika, Hoffman, & Dzama, 2014; Sebola, Mlambo, Mokoboki, Hugo, & Muchenje, 2018).

The 4M-FE group (Gr.5) exhibited high serum total protein content on average (Table-4a), followed by the 2M-FE group (Gr. 3), indicating that the consumption of 4% MOL as a protein diet with 30 minutes of forced exercise significantly increased total protein content. The same trend was observed for 2% MOL with forced exercise, suggesting that trained muscles' ability to utilize protein as an energy source was enhanced, leading to an increase in lean muscle mass (unpublished data). This significant increase in total protein content is crucial in enhancing swimming performance without fatigue. This finding is consistent with other studies (Muzumbukilwa *et al.*, 2019; Omara, El-Esawy, Riad, Mohi El-Din, & Feeds, 2018; Saleh and Sarhat, 2019). The 4M-FE and 4M-WOE had 19% and 17% increases in serum total protein, respectively. A similar pattern was observed in 2M-FE and 2M-WOE, with a 14% increase in serum total protein compared to the standard diet (control group). These results suggest that forced exercise can only lead to better outcomes than an additional group if the diet includes a sufficient dose of MOL, which is dose-dependent. However, 2M-FE did not improve better than 2M-WOE, which may be due to inadequate MOL causing required for anabolism compared with catabolism of proteins.

The protein digestibility usually increases after autoclaving (Fadhilatunnur, Dewi, & Technology, 2021). Based on these results, a study on the PEM animal model will be performed. Interestingly, the significant increase in total serum protein without an increase in dietary protein suggests that a high-protein diet may not be necessary for improving anthropometric indices. Instead, measures to promote protein absorption, assimilation, and homeostasis should be emphasized. This positive balance of serum proteins can help address protein-energy malnutrition, anthropometric failure, and communicable diseases such as tuberculosis. Additionally, the decrease in urea and ALP enzymes observed in the study can benefit individuals with kidney and liver diseases and iatrogenic hyperphosphatemia in childhood, dental caries, and postmenopausal mineral deficiency. Mass awareness campaigns, such as the Moringa Festival Week, could promote its use through the media. MOL-Ladoo, made from dried MOL and dates, could provide a simple solution to address malnutrition in all age groups.

In the end, as a quality check on diet, the analysis on diet samples of unautoclaved and autoclaved feed samples were analyzed and showed the contrasting trends in quercetin and chlorogenic acid

levels between Moringa-enriched and standard diets, highlighting the dual effects of autoclaving. Moringa supplementation provides a protective and enhancing impact, reducing the extent of thermal degradation for some bioactive while facilitating the release or formation of others. However, the sensitivity of specific compounds to thermal processing underscores the need to optimize autoclaving conditions to preserve the functional benefits of Moringa-enriched diets. These findings suggest that Moringa's bioactive compounds enrich the diet and mitigate the adverse effects of processing, making it a promising component in nutritionally enhanced diets. Interestingly, while quercetin—a thermolabile flavonoid with known antioxidant and performance-enhancing effects—showed degradation upon autoclaving, chlorogenic acid concentrations increased significantly. This compensatory shift in the bioactive profile could explain the continued biochemical improvements observed in MOL-fed groups, such as reduced serum urea and liver enzyme levels. Chlorogenic acid, in particular, may play a pivotal role in hepatoprotection and improved nutrient metabolism under these conditions. Thus, autoclaving, while reducing some phytochemicals, may simultaneously enhance the bioavailability or formation of others, warranting further exploration into optimizing thermal processing for maximal biological efficacy.

CONCLUSION

Our study highlights a critical insight: a 2% Moringa oleifera (MOL) diet can induce significant physiological changes in a sedentary lifestyle, effectively meeting dietary needs without the need for higher supplementation. However, when paired with physical exercise, the 4% MOL diet demonstrates a remarkable synergetic activity, enhancing serum protein levels by 2% and showcasing its superior benefits in active individuals. This underscores the dynamic role of MOL in optimizing endurance, protecting liver and kidney health, and promoting muscle strength, making it an unparalleled choice for vegetarians seeking to build muscle while maintaining dietary thresholds.

Contrary to preconceived notions about taste and utility, MOL emerges as a dietary powerhouse deserving of widespread adoption. With strategic mass awareness campaigns, its potential to combat malnutrition across various demographics, especially in vulnerable age groups, becomes undeniable. This study reinforces the call for MOL's resurgence as a human and animal nutrition cornerstone after extensive study trials.

ABBREVIATIONS:

MOL - Moringa oleifera Lam. dried leaves powder, PEM – Protein Energy Malnutrition, 2M-WOE – 2% Moringa oleifera Lam. dried leaves in the diet without forced exercise, 4M-WOE – 4% Moringa oleifera Lam. dried leaves in the diet without forced exercise, 2M-FE - 2% Moringa oleifera Lam. dried leaves in diet with forced exercise, 4M-FE - 4% Moringa oleifera Lam. dried leaves in diet with forced exercise

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Authors contribution:

P. B. Patil – Conceptualization, Methodology, Investigation, Data Curation, Formal Analysis Resources, Writing – Original Draft, Writing – Review & Editing, Supervision, Funding Acquisition, Project Administration, Ethics Approval
P. Alduri - Methodology, Investigation, Data Curation, Formal Analysis Resources
G. Purushottam – Methodology
A. Devarasetti – Methodology
Kumar Reddy – Biochemical test
S. Deshmukh – Phytochemical analysis
J. S. Rao – Proximate analysis
B. Tulja – Diet preparation
S.S.Y.H. Qadri – Draft overview

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