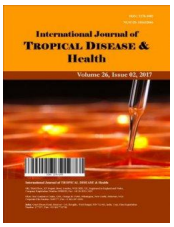

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Growth Performance, Metabolic Efficiency and Nutrient Utilization of BALB/C Mice Infected with *Leishmania major* Fed with Standard Rat Pellets or *Annonaceae* Fruit Pulp Pellets

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Authors' contributions

This work was carried out in collaboration between all authors. Authors LMC, SMK, JAO and COA designed the study, developed the protocol and managed the literature searches the study. Author LMC performed the experimental analyses of the study. Authors LMC and SE performed the statistical analysis and participated in the development of the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

This study was aimed at evaluating the clinical, biochemical, and hematological changes in male BALB/C mice infected with *Leishmania major* fed with standard rat pellets (RP) and Annonaceae Fruit Pulp pellets (AFPP) in different experimental exposures. The results of the study showed good

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palatability, acceptability and normal behaviour of the mice during the whole experimental period except in the infected groups. Furthermore, the results achieved with low levels of Annonaceae fruit pulp pellets crude proteins (3.81±0.14) and lipids (2.95±0.18) were comparable with that of RP at a level of 25.28±0.04 and 18.60±0.06 respectively. The growth parameter, metabolic efficiency and feed utilization parameters between the rat pellets and Annonaceae fruit pulp pellets in non-infected, infected non-treated and infected treated groups of mice did not differ. At the same time haematological changes, liver and kidney function parameters and lipid profile of the mice that were feed with rat pellets and Annonaceae fruit pulp pellets in non-infected, infected non-treated and infected treated were also comparable same. Other parameter measured in the study such as weight of organs (liver and spleen) parasite burden (LDU) followed the same trend. However, some of the infected non-treated groups had parameters beyond the normal ranges than the non-infected and infected treated groups at the end of the experimental period. It can be concluded that Annonaceae fruit pulp pellets can be utilized as source of raw material in the manufacture of animal feeds and neutraceuticals upon further research in higher animal model.

Keywords: BALB/C; leishmania major; Annonaceae; growth performance; metabolic efficiency; feed utilization.

1. INTRODUCTION

One of the world's most devastating neglected tropical diseases of documented epidemiological; and experimental public health importance is Leishmaniasis [1]. In developing countries where it is manifested, it often co-exists with chronic malnutrition, one of the main risk factors for its development [2,3,4]. In most of the study conducted, only a few emphasized on the relationship between leishmaniasis progression and malnutrition. Malnutrition is a serious health problem that remains common in many parts of the world [5,6].

In the developing world, Leishmaniasis combined with malnutrition is a public health problem. The most frequent form of malnutrition is Protein energy malnutrition (PEM). Globally, about 800 million people, including over 150 million children under 5 years of age, most of them in developing countries, are affected by PEM [7]. The excess morbidity and mortality associated with malnutrition is due to the impairment of sufferers' defense mechanisms, which predisposes them to infectious diseases [8]. Further, the poor understanding of the impact of PEM on immune response against *Leishmania* infection coupled with neglected nutritional status of the host [9,10] worsen the situation. Studies have revealed that good protein energy (PE) promote adaptive immune response in mice infected with *L. chagasi* [11]. The BALB/C mice provide a unique opportunity to study human cutaneous leishmaniasis (CL) in its active form due to their susceptibility to L. major infection. As a model, it reproduces clinical and pathological features of human CL.

Food insecurity, especially in the developing world, has necessitated research in alternative source of food substances. Therefore, natural products, notably those derived from plants, have been used to help mankind sustain its nutrition and health since the dawn of medicine. The importance of plants in agriculture and medicine has stimulated significant scientific interest [12]. However, a restricted range of plant species, with regard to their medicinal importance, has experienced detailed scientific inspection, resulting into comparatively insufficient knowledge concerning their potential role in nutraceutical application [13]. Therefore, to attain a reasonable perception of medicinal values/benefits, comprehensive investigations on the role of plants in the management of human ailments are needed.

In a nutraceutical landscape, plants with a long history of use in ethno medicine are a rich source of active phyto-constituents that provide medicinal or health benefits against various ailments and diseases [14]. One such plant family with extensive traditional use in Kenya and mostly grown in the coast region is *Annonaceae* family which include; *A. cherimola*; Mill (cherimoya), *A muricata*; L (soursop), *A. squamosa*; L (sugar apple) *A. senegalensis*; L (wild soursop) and *A. reticulata*; L (custard apple). The fruits of these plants contain numerous bioactive substances [15]. These fruits are also possible contributors of the carotenoids, vitamins, mineral salts, fibres and bioactive compounds [16,14]. Thus they can be incorporated as components of diets to provide a delicate balance of food security. The fact that they are used as food by other parts of the world

[16] with Kenyans having similar gastronomic habits [17] and also exhibit medicinal potency is a significant aspect of modern trends in research focusing on food-medical interface (Neutraceuticals).

Nonetheless, these plants are neglected especially as sources of alternative foods for reasons not well understood. The present paper reports the influence of diet made from Annonaceae fruit pulp on growth performances, metabolic efficiency and nutrients utilization on BALB/C mice infected with *L. major*. This might reflect on proper utilization of these neglected crops for development of nutraceutical products. It might also contribute towards curbing food insecurity in the region where they are grown.

2. MATERIALS AND METHODS

2.1 Plant Material

The ripe fresh fruits of Annonaceae (*A. muricata* and *A. squamosa*) were collected from farms in coast province (Kilifi and Kwale Counties) of Kenya. The harvested fruits collected were then transported to the Jomo Kenyatta University of Agriculture and Technology (JKUAT) in the Department of Food Science Technology, Food Biochemistry laboratories. In the laboratory, the fruits pulps were dried using a constant temperature and humidity chamber (Tokyo Thermo Tech Co. Ltd, Japan) set at 25ºC. Thereafter, feed pellets were made from the dried fruit pulp. The pellets made were taken to the Centre for Biotechnology Research and Development (CBRD), Kenya Medical Research Institute (KEMRI) leishmaniasis laboratories for *in vivo* trials.

2.2 Animal Feeds

Standard rat pellets (Rate pellets®, Unga Feeds Ltd, Kenya) and Annonaceae fruit pulp (AFP) were used in the study. To ensure palatability of the AFP formulated feed, cubes were made from the powdered AFP by mixing with wheat flour as a binder after addition of water using a pellet-making machine. Proximate composition analysis of the feeds was performed as described in the Association of Official Analytical Chemists (AOAC) manual [18].

2.3 Parasites

Metacyclic promastigotes of *L. major* (strain IDUB/KE/83=NLB-144) maintained by cryopreservation and *in vitro* culture, and periodic passage in BALB/c mice at KEMRI were cultured in Schneider's insect medium (Gibco, Invitrogen), supplemented with 20% fetal calf serum (FBS) (Life Technologies), glutamine (2 mM), penicillin G (100 U/mL), and streptomycin (100 µg/mL). The parasites were harvested in the stationary phase after 8–10 days of culture, centrifuged (at 1000rpm for 5 min), washed twice with Schneider's insect medium, counted, and used to inoculate mice.

2.4 Animals

Male BALB/c mice (3-4 weeks old) obtained from Kenya Medical Research (KEMRI) animal house were fed with standard rat pellets (Rate pellets®, Unga Feeds Ltd, Kenya), Annonaceae fruit pulp (AFP) and water ad libitum and kept under standard temperature (26ºC and 60% humidity) and a natural light-darkness cycle. All *in vivo* experiments with mice were performed according to the established bioethical standards of the KEMRI's Animal Care and Use Committee (ACUC), Scientific and Ethics Review Unit (SERU).

2.5 Experimental Design

The mice were equally and randomly allocated into experiment groups assigned to groups of six mice per group as follows; Non-Infected fed with Rat pellets (NI-RP), Infected Non-Treated fed with Rat pellets (INT-RP), Infected Treated fed with Rat pellets (IT-RP), Non-Infected fed with Annonaceae fruit pulp (NI-AFP), Infected Treated fed with Annonaceae fruit pulp (IT-AFP), Infected Non-Treated fed with Annonaceae fruit pulp (INT-AFP). After the different experimental exposures, the mice were closely monitored on a daily basis for agility, hair ruffling, appetite, vomiting, urine colour, skin turgor, ocular tension, limb paralysis, convulsions and roll-over movements [19]. Body weight and temperature were measured on a weekly basis. After lesion have developed, density of parasites was determined by counting the number of amastigotes form of parasite from smears made from ulcer stained with Leishman's stain [20]. The infected footpads were measured using a direct reading vernier caliper and lesion size calculated [21]. Means of weekly readings were calculated to facilitate comparison of lesion progression. Growth performance and nutrient utilization was assessed at the end of the experimental period (12 weeks) described by as follows; Body mass gain (BMG) = [(Final Body

Mass [FBM]) - (Initial Body Mass [IBM])]/IBM] x 100, Specific growth rate (SGR% per day) = [(FBM) - (IBM)/number of trial days] x 100, Metabolic growth rate (MGR g/kg$^{0.8}$/day) = (BMG)/{[(IBM/1000)$^{0.8}$ + (FBM/1000)$^{0.8}$]/2}/ duration of the trial days, Feed conversion ratio (FCR) = Dry feed fed (g)/BMG (g), Protein efficiency ratio (PER) = fresh BMG (g)/Crude Protein (CP) fed (g), Protein productive value (PPV)% = [(final mice body protein in g – initial mice body protein in g)/total protein consumed in g] x 100 and Apparent lipid conversion (ALC)% = [(final mice body lipid in g - initial mice body lipid, g)/total crude lipid consumed in g] x 100 [22]. At the end of the experimental period (12 weeks), the animals were fasted for about 4 hours with free access to water and sacrificed by inoculation with 100µL of pentabarbitone sodium (Sagatal®). 2 mL of blood sample was collected for determination of haematological and biochemical parameters, renal and liver functions and blood metabolites using a Retroflon® Plus automated analyzer. Liver and spleen impression smears were used to quantitate the parasite loads [23].

2.6 Data Analysis

Haematology and biochemical parameters, blood metabolites, growth performance parameters and body weights data were analyzed using mean separation by GenStat program [24]. Mean separation was done through Fischer least significance difference. Comparisons between two treatments were done by means of unpaired Student's t-test and significance established by ANOVA. A difference of $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1 Proximate Composition Analysis and Palatability of the Experimental Feeds

Good palatability, acceptability and normal behaviour were observed during the whole experimental period except in the infected group. In the non-infected group, minimal residual feeds were observed in the cages. The feed composition parameters in % Dry Weight Basis (DWB) of the two experimental feeds (Table 1) differ significantly ($p < 0.05$). The two experimental feeds were analyzed for proximate feed composition parameters in % Dry Weight Basis (DWB); carbohydrates, crude lipids, crude proteins, moisture content, ash content, dry matter and fibre content (Table 1). The analyzed proximate composition parameter of the two feeds; RP and AFPP differed significantly ($P < 0.05$). The RP had a higher value of crude protein and crude lipids, 25.28±0.04 and 18.60±0.06 respectively against the AFPP; 3.81±0.14 and 2.95±0.18 respectively. However, the AFPP had higher values of carbohydrates, moisture content, ash content, dry matter and fibre content of 40.12±0.38, 6.20±0.12, 5.93±0.50, 93.80±0.12 and 59.56±2.64 against the RP of 27.14±2.05, 2.17±0.98, 3.51±0.04, 1.42±0.24 and 25.67±1.28 respectively. Good palatability, acceptability and normal behaviors were observed during the whole experimental period in all groups of BALB/C mice except in the infected groups.

Table 1. Proximate composition analysis of diets used in feeding BALB/C mice

Parameter	Experimental diets	
	Rat pellets (RP)	Annonaceae fruit pulp pellets (AFPP)
Crude Proteins	25.28^{a}±0.04	3.81^{b}±0.14
Crude Lipids	18.60^{a}±0.06	2.95^{b}±0.18
Carbohydrate	27.24^{a}±2.05	40.12^{b}±0.38
Moisture content	2.17^{a}±0.98	6.20^{b}±0.12
Ash content	3.51^{a}±0.04	5.93^{b}±0.50
Dry matter	1.42^{a}±0.24	93.80^{b}±0.12
Fibre content	25.67^{a}±1.28	59.56^{b}±2.64

Mean values (n=3) ± SEM. Values appended by different small letters within a row are significantly different (P < 0.05).

3.2 Growth Performance and Nutrient Utilization

The body mass changes in the different groups of BALB/C mice measured weekly. Generally, the BMG was observed in the two NI groups of BALB/C mice; NI-RP and NI-AFPP. However, the BMG in the NI-RP group was higher (56.10±1.49) compared to that of NI-AFPP (49.11±1.39). A significant decrease in body weight was observed in both the IT-RP and IT-AFPP before treatment (Figure 1). However, this reversed after treatment with Pentostam in week 4 with the IT-RP and IT-AFPP groups having almost the same BMG values of 27.31±0.66 and 27.56±1.37 respectively at the end of the experimental period. There was also a significant decrease in BMG in both INT-RP and INT-AFPP groups ($P < 0.05$) (Fig. 1) with the INT-AFPP group having greater decrease in BMG (-40.71±0.68) than the INT-RP group (-34.62±0.79).

Other growth performance, metabolic efficiency and nutrient utilization parameters such as IBM, FBM, SGR, FCR, PER, PPV and ALC followed the same trend. Higher values of these growth performance, metabolic efficiency and nutrient utilization parameters were observed in the RP groups as compared to the AFPP groups except in the INT where low values were observe in the RP groups than in the AFPP groups. Statistically, there was a significant difference in most of the parameters ($p < 0.05$) among the different groups in different experimental treatment in the growth performance, metabolic efficiency and nutrient utilization parameters. The onset of the diseases decreased all the growth performance, metabolic efficiency and nutrient utilization parameters investigated (Table 2). Slightly high SGR was observed in the NI-RP group (13.24±0.11) than in the NI-AFPP group (11.60±0.23). However, in the infected treated (IT) groups, both the RP and AFPP groups had almost the same SGR. Although both the INT groups had a negative SGR, the negativity was more in the AFPP group than the RP group.

3.3 Haematological Changes

Hematological changes are presented in Table 3. A decrease in Hemoglobin (Hb) below the normal range was observed in all experimental groups except in NI groups. Red Blood Cells (RBC), Hematocrit (HCT) or Packed Cell Volume (PCV), Mean Capsular Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Hemoglobin Concentration (MCHC) decrease in all the experimental groups. However, a significant decrease ($p < 0.05$) in Hb was observed in the INT groups as compared to the NI groups. After treatment with pentostam in the IT groups, Hb increased was significant ($p < 0.05$), although it was still below the physiological range. The RBC, HCT, MCV, MCH and MCHC followed the same trend as Hb. Total white blood cells (WBC) or total leucocytes count (TLC) increased significantly ($p<0.05$) in infected groups compared to NI groups. Differentially, Leukocytes, platelets (PLT) and leukocyte populations (neutrophils, lymphocytes, eosinophils, monocytes and basophils) increased significantly in the INT groups compared to the NI groups. The increase in these parameters was within normal range in all the experimental groups. However after treatment in the IT groups, these parameters increased returning almost to the values in the NI groups. On comparison, the groups showed at least one significant difference among the averages of Hb ($p=0.001$), HCT ($p=0.05$), MCV ($p=0.001$), MCH ($p=0.001$), RBC ($p=0.001$), leukocytes ($p=0.008$), lymphocytes ($p=0.013$), neutrophils ($p=0.029$), monocytes ($p=0.002$) and basophils ($p=0.005$).

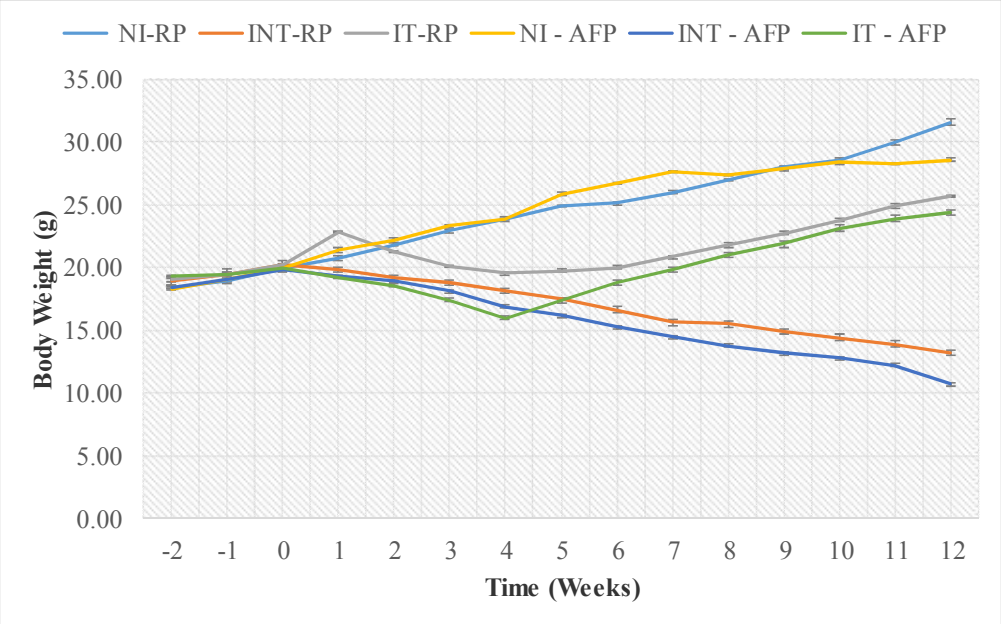

Fig. 1. Body weight changes of BALB/c mice infected with *L. Major* fed with the experimental diets for 12 weeks

3.4 Liver Function Tests

The function test changes are presented in Table 4. Alanine aminotransferase (ALT) also called Serum glutamic pyruvic transaminase (SGTP), aspartate aminotransferase (AST) also called Serum glutamic oxaloacetate transaminase (SGOT), lactate dehydrogenase (LDH), Alkaline phosphatase (ALkP) sorbitol dehydrogenase (SDH) creatine kinase (CK) and bilirubin activity increased significantly in the INT groups compared to the NI groups. After treatment, the activity ALT (SGTP), AST (SGOT), LDH, SDH, CK and bilirubin decreased almost returning to that of NI groups. The total protein, albumin, globulin and ALkP decreased significantly in INT animals compare to the NI animals. Most of the liver function tests were found to be within normal range in all IT and NI groups. A significant increase in ALT (SGTP) beyond the physiological range (183.49±3.18) was observed in INT-AFPP group. Also a higher activity of AST (SGOT) beyond the physiological range (218.20±3.56 and 184.87±5.99) was observed in INT-RP and INT-AFPP groups respectively. The activity of amylase increased beyond the physiological range whereas that of LDH decreases below the range in NI-RP (24.33±0.67), NI-AFPP (17.90±0.69) groups but increased significantly beyond the physiological range (40.17±0.29) in the INT-AFFP group. After comparison, there was at least one significant difference among the averages of all biochemical parameters used to ascertain liver function of the mice.

3.5 Kidney Function Tests and Lipid Profiles

The kidney function tests and lipid profile changes are presented in Table 5. Creatinine, urea and blood urea nitrogen (BUN) increased significantly ($p<0.05$) in the INT group compared to NI groups. In all the IT groups, creatinine, blood Urea and BUN levels decreased significantly ($p<0.05$) compared to the INT groups. Creatinine levels beyond the physiological range were observed in all BALB/C mice groups while BUN decreased in NI-AFPP (25.16±0.11) and IT-AFPP (23.82±0.10) groups. However, the increased urea levels in all the BALB/C mice groups were found to be within normal range. Serum sodium, chloride, potassium and phosphorous increased significantly in the INT groups compared to the NI groups although values remained within the physiological ranges. After treatment, a decrease in these ions was observed in the IT groups. Cholesterol, triacylglycerides (TGs), high-density lipoproteins (HDL), low density lipoprotein (LDL) and cholesterol/HDL ratio increased significantly ($p<0.05$) in the INT groups in NI groups. This is an indicative of liver damage and risk of development of cardiovascular diseases. All lipid profile values were within the physiological range except HDL in INT-RP (2.73±0.11), IT-RP (1.23±0.07), INT-AFPP (3.03±0.06) and IT-AFPP (1.90±0.13) groups suggesting a relationship between leishmaniasis and cardiovascular diseases. Statistically, there was no significant difference between lipid profiles of NI-RP and NI-AFPP (0.09) and INT-RP and INT-AFPP ($p=0.08$) but there was significant differences between NI and INT ($p=0.003$) and INT and IT ($p=0.001$) groups.

3.6 Lesion Size and Liver and Splenic Length, Size and Parasite Burden

The lesion sizes (mm^2) for the INT and IT groups were determined and are presented in Fig. 2. Generally, there was an increase in the size of the lesions with the progression of the disease. After the initiation of treatment on the IT groups, the lesion sizes decreased. However, the rate of decrease of the size of the lesions was higher in the IT-RP group than in the IT-AFPP group (Fig. 2). The lesion sizes of the INT groups increased significantly until the end of the experiment, with the INT-AFPP having larger lesion sizes than the INT-RP group. The hepatic and splenic length (mm) and size (mg) for the NI, INT and IT groups (Table 6) determined at the end of the experiment varied significantly ($p<0.05$). The relative mean weight and length of the liver and the spleen increased with increasing the time of infection. A significant differences ($p<0.05$) was observed between the weight and length of infected (both IT and INT) and non-infected (NI) BALB/C mice groups. However, no significant difference ($P>0.05$) was observed between NI-RP and NI-AFPP. The same trend was observed between ITC-RP and IT-AFPP.

The parasite density of *L. major* amastigotes in liver and spleen for infected not treated (INT) and IT groups of BALB/C mice (Table 6) determined after the experimental period (12 weeks) showed significant variations. In all the IT groups of BALB/C mice, parasite load decreased significantly ($p<0.05$) as compared to the INT groups. The reduction in parasite load was significantly ($p<0.05$) more in the IT-RP group as compared to the IT-AFPP group. After the

experimental period (12 weeks), hepatomegaly and splenomegaly was observed on the INT groups of BALB/C mice. Impression smears of liver and spleen on the INT groups of BALB/C determined at the end of the experimental period (12 weeks) showed dissemination of *L. major* amastigotes inside and outside macrophages.

4. DISCUSSION

The results of the present investigation demonstrate that AFPP may be a good dietary protein source for BALB/C mice feed because growth performance and feed utilization of Annonaceae group was similar to control group (RP fed group). No significant difference in growth performance (BMG, SGR and MGR) and feed utilization parameters (FCR, PER and ALC) show that digestion and absorption of nutrients from RP and AFPP were similar. Nutrient utilization values observed in this study signify excellent utilization of the diets. Growth performance of RP fed group was similar to AFPP group indicating that nutrients and energy availability from the AFPP for the protein synthesis were similar to animal protein (RP). Further, the growth performance and feed utilization parameter of the infected and treated groups feed with RP and AFPP returned to normal. This shows that AFPP can be used as an alternative source of feed for BALB/C mice.

In terms of haematological changes, lesion size and organ length, size functioning and parasite burden, *Leishmania major* is an aetiological agent of cutaneous leishmaniasis, which is a parasite of the skin on humans. However, in BALB/C mice, it attacks visceral organs in addition to the local lesion at the point of inoculation [25]. The parasites can be deposited in major visceral organs involved in the synthesis of major macromolecule after the digestion, absorption and transport. Nonetheless, BALB/C mice model was chosen for this study as a suitable model for the investigation of interaction between malnutrition and leishmaniasis. In earlier studies, malnourishment contributed to higher parasite loads found in the blood, skin, bone marrow, lymph node, liver and spleen and favoured the development of leishmaniasis [6]. Based on these observations, this model provides an excellent opportunity to elucidate the factors implicated in severe malnutrition. Moreover, as it has been shown in this work, the model can be used for further investigation on the relationship between severe malnutrition and leishmaniasis

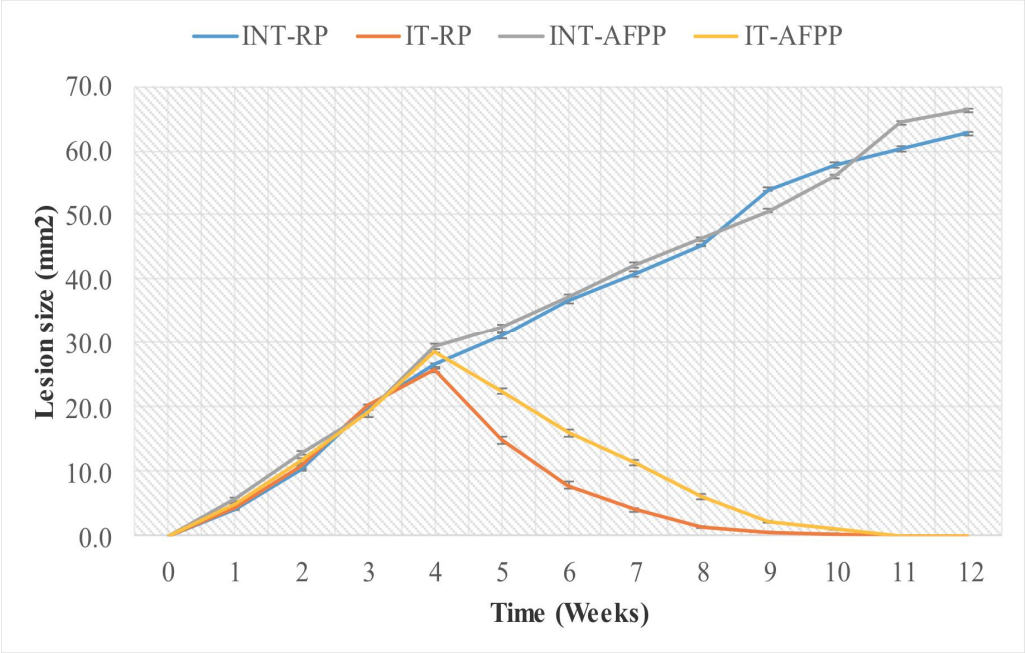

Fig. 2. Lesion sizes of BALB/C mice infected with *L. major* and feed with RP and AFPP on 12 weeks

Table 2. Growth performance and nutrient utilization in BALB/C mice infected with *L. major* fed with experimental diets of 12 weeks

Parameter	Rat pellets (RP)			Annonaceae fruit pulp pellets (AFPP)		
	NI-RP	INT-RP	IT-RP	NI-AFPP	INT-AFPP	IT-AFPP
IBM	19.89^{a}±0.11	20.18^{b}±0.11	20.18^{b}±0.11	19.99^{a}±0.19	19.76^{a}±0.07	19.93^{a}±0.13
FBM	31.02^{b}±0.21	13.18^{d}±0.13	25.68^{a}±0.10	29.74^{b}±0.12	11.71^{c}±0.13	25.38^{a}±0.19
BMG	56.10^{c}±1.49	-34.62^{a}±0.79	27.31^{b}±0.66	49.11^{e}±1.39	-40.71^{q}±0.68	27.56^{b}±1.37
SGR	13.24^{c}±0.30	-8.34^{a}±0.22	6.54^{b}±0.14	11.60^{d}±0.23	-9.58^{a}±0.17	6.49^{b}±0.29
FCR	3.05^{b}±0.07	-4.93^{c}±0.11	6.28^{a}±0.18	3.51^{b}±0.10	-4.16^{c}±0.07	6.56^{a}±0.32
PER	0.33^{a}±0.01	-0.21^{b}±0.01	0.16^{c}±0.04	0.29^{a}±0.01	-0.24^{b}±0.004	0.16^{c}±0.01
PPV	0.66^{a}±0.06	0.02^{b}±0.01	0.46^{a}±0.16	0.46±0.01	0.01^{b}±0.04	0.46±0.01
ALC	52.67^{a}±3.31	22.17^{b}±2.60	40.17^{c}±6.60	49.36^{a}±4.49	18.7^{d}±6.60	38.70^{e}±6.60

Mean values (n=3) ± SEM. Values appended by different small letters within a row are significantly different (P < 0.05)

Table 3. Hematological changes of BALB/C mice infected with *L. Major* fed with RP and AFPP for 12 weeks

Parameter	Rat pellets (RP)			Annonaceae fruit pulp pellets (AFPP)			Ref. Range
	NI-RP	INT-RP	IT-RP	NI-AFPP	INT-AFPP	IT-AFPP	
HB (g/dL)	12.76^{a}±0.67	8.07^{b}±0.10	10.40^{a}±0.10	12.09^{a}±0.14	8.58^{b}±0.08	9.89^{ab}±0.10	12.60 - 20.50
WBC x 10^{9}/L	6.11^{a}±0.12	9.08^{b}±0.09	6.58^{a}±0.16	5.91^{a}±0.05	11.97^{c}±0.12	9.91^{b}±0.05	3.48 - 14.03
RBC x 10^{12}/L	8.39^{b}±0.06	5.96^{a}±0.12	8.05^{b}±0.12	7.04^{c}±0.09	6.10^{ac}±0.11	8.08^{b}±0.05	6.93 – 12.24
HCT/PCV (%)	48.30^{a}±0.88	43.13^{c}±0.79	46.80^{b}±0.08	46.97^{b}±0.88	44.60^{c}±0.63	44.80^{c}±0.47	42.10 - 68.30
MCV (FL)	54.28^{c}±0.97	46.28^{d}±0.53	51.44^{a}±0.62	51.28^{a}±0.66	42.30^{e}±0.53	49.61^{b}±0.38	50.70 – 64.40
MCH (FL)	30.98^{b}±0.44	23.22^{a}±0.19	26.38^{c}±0.38	26.82^{c}±0.54	22.03^{a}±0.19	26.72^{c}±0.42	13.20 – 17.60
MCHC (pg)	31.04^{c}±0.58	23.20^{b}±0.31	28.37^{ad}±0.19	27.54^{a}±0.16	25.30^{c}±0.12	27.70^{a}±0.97	23.30 – 32.70
PLT x 10^{12}/L	511.98^{a}±19.54	695.32^{b}±7.39	611.98^{c}±19.54	449.58^{d}±23.47	727.40^{e}±19.54	357.66^{i}±12.23	420.00 – 1698.00
Neutrophils (%)	24.89^{b}±0.51	30.06^{a}±1.15	23.39^{ac}±0.62	23.73^{c}±0.37	36.50^{d}±0.79	26.06^{e}±0.40	9.86 – 39.11
Lymphocytes (%)	74.62^{a}±0.35	83.95^{b}±0.61	73.57^{a}±0.39	61.95^{c}±0.65	78.20^{d}±0.95	65.62^{e}±1.41	50.00 – 96.00
Monocytes (%)	3.72^{c}±0.13	4.05^{b}±0.13	3.72^{c}±0.06	3.97^{c}±0.08	4.00^{b}±0.17	5.38^{a}±0.33	3.29 – 12.48
Eosinophils (%)	0.72^{a}±0.06	0.85^{a}±0.08	0.63^{c}±0.05	0.58^{d}±0.05	0.60^{b}±0.02	0.61^{b}±0.07	0.11 – 4.91
Basophils (%)	0.10±0.00	0.07^{a}±0.00	0.08^{ab}±0.00	0.09^{b}±0.01	0.05^{c}±0.01	0.09^{b}±0.00	0.00 – 1.84
Abs Neutrophils	3.15±0.24	4.77±0.19	4.75±0.17	3.85±0.17	7.05±0.29	4.07±0.19	0.00 – 3.83
Abs Lymphocytes	3.13±0.11	5.09±0.17	3.79±0.07	4.10±0.07	6.14±0.05	4.84±0.09	2.22 – 9.83
Abs Monocytes	0.90±0.03	1.27±0.06	0.63±0.04	0.79±0.04	1.78±0.04	0.68±0.07	0.21 – 1.25
Abs Eosinophils	0.24±0.04	0.43±0.02	0.13±0.02	0.17±0.02	0.38±0.15	0.28±0.05	0.01 – 0.49
Abs Basophiles	0.01±0.0	0.07±0.00	0.13±0.00	0.02±0.00	0.15±0.02	0.05±0.01	0.00 - 0.18

Mean values (n=3) ± SEM. Values appended by different small letters within a row are significantly different (P < 0.05)

Table 4. Liver functions in BALB/C mice infected with *L. major* fed with RP and AFPP for 12 weeks

Parameter	Rat pellets (RP)			Annonaceae fruit pulp pellets (AFPP)			Ref. Range
	NI-RP	INTC-RP	IT-RP	NI-AFPP	INT-AFPP	IT-AFPP	
Glucose (mg/L)	122.71±2.12	123.87±1.95	124.37±1.91	126.17±0.45	124.27±0.65	125.20±0.41	106.00 – 278.00
T-Bilirubin (g/dL)	9.02±0.17	11.18±0.10	9.52±0.29	10.20±0.06	12.68±0.35	9.68±0.07	7.12 -12.05
C-Bilirubin (g/dL)	2.57±0.11	3.43±0.05	2.57±0.11	3.07±0.26	4.23±0.35	2.80±0.08	0.00 – 2.00
U-Bilirubin (g/dL)	3.67±0.17	4.83±0.13	3.17±0.13	3.83±0.16	4.33±0.20	3.00±0.11	0.00 – 3.00
ALT (IU/L)	77.49±2.40	115.99±1.02	98.66±1.31	83.49±3.18	183.49±3.18	81.82±2.86	41.00 – 131.00
AST (IU/L)	119.87±2.16	218.20±3.56	111.53±0.69	134.87±5.86	184.87±5.99	129.87±5.66	28.00 – 191.00
GGT (IU/L)	25.43±0.55	28.77±0.48	24.60±0.46	23.77±0.60	31.43±0.56	23.77±0.60	20.00 – 40.00
ALKP (IU/L)	132.36±1.53	127.36±1.33	134.03±1.21	157.36±4.40	140.70±1.70	155.70±3.03	118.00 – 187.00
T-Protein (g/dL)	51.77±0.90	45.94±0.04	47.12±0.33	42.95±0.51	35.02±0.51	45.27±0.51	43.00 – 70.00
Albumin (g/dL)	26.46±0.49	23.34±0.58	35.27±0.51	24.50±0.32	22.27±0.40	26.35±0.14	27.00 – 46.00
Globulin (g/dL)	34.56±0.64	25.22±0.47	35.34±0.35	26.34±0.38	23.18±0.42	26.68±0.10	27.00 – 42.00
Alb/Glob ratio	0.77^{d}±0.02	0.93^{d}±0.03	1.98^{bc}±0.05	1.10^{a}±0.02	1.06^{a}±0.02	2.12^{b}±0.04	0.00 – 1.09
CPK (IU/L)	3.48^{b}±0.22	4.02^{c}±0.08	1.79^{a}±6.86	1.79^{a}±0.07	2.46^{d}±0.05	1.60^{a}±0.04	2.50 – 3.70
Amylase (U/L)	796.97^{b}±17.47	1580.31^{a}±54.65	896.97^{d}±7.90	813.64^{e}±23.68	1413.64^{e}±36.61	896.97^{f}±10.69	210.43 -323.57
LDH (IU/L)	24.33a±0.67	34.66b±0.39	27.17c±0.68	17.90d±0.69	40.17e±0.29	24.83a±0.52	26.8 – 34.00
SDH (IU/L)	29.15^{a}±0.90	34.50^{c}±0.53	31.16^{ab}±0.54	23.83^{d}±0.39	43.67^{e}±0.44	29.17^{a}±0.25	27.00 - 37.00

Mean values (n=3) ± SEM. Values appended by different small letters within a row are significantly different (P < 0.05)

Table 5. Kidney functions and lipid profile in BALB/C mice infected with *L. Major* fed with RP and AFPP for 12 weeks

Parameter	Rat pellets (RP)			Annonaceae fruit pulp pellets (AFPP)			Ref. range
	NI-RP	INT-RP	IT-RP	NI-AFPP	INT-AFPP	IT-AFPP	
Creatinine (mg/L)	2.57±0.16	3.32±0.15	2.49±0.19	2.12±0.11	2.87±0.25	2.48±0.10	0.50 - 0.80
Blood Urea Nitrogen	26.66±0.16	31.32±0.15	27.49±0.19	25.16±0.11	27.66±0.25	23.82±0.10	7.00 – 26.00
Urea (mg/L)	7.88±0.11	9.11±0.11	7.38±0.18	6.88±0.22	10.03±0.06	6.88±0.15	7.00 – 26.00
Sodium (meq/L)	143.48±0.74	147.13±1.04	142.96±0.46	135.63±1.33	143.63±0.93	137.29±0.78	125.30 – 187.40
Chloride (meq/L)	116.23±0.82	121.90±1.49	117.40±0.64	121.23±1.10	131.23±1.56	122.90±1.91	109.6 – 138.80
Potassium (meq/L)	6.16±0.13	6.49±0.17	5.49±0.19	7.14±0.06	6.82±0.17	6.32±0.21	7.30 – 12.07
K/Na Ratio	0.04±0.00	0.04±0.00	0.04±0.00	0.05±0.00	0.05±0.00	0.05±0.00	0.06 – 0.09
Phosphorus (meq/L)	3.77±0.09	3.72±0.09	3.65±0.08	4.22±0.16	5.22±0.19	4.22±0.02	8.20 – 14.70
Calcium (mg/dL)	2.59±0.06	2.30±0.03	2.74±0.04	2.76±0.11	2.68±0.09	3.43±0.06	9.40 – 12.70
Cholesterol (g/dL)	3.75±0.18	7.13±0.16	4.81±0.16	4.22±0.14	7.13±0.16	5.31±0.26	11.10 – 24.60
Triglycerides (g/dL)	1.16±0.04	2.48±0.05	1.68±0.05	1.34±0.06	3.11±0.08	1.81±0.03	1.050 – 5.35
HDL Cholesterol (g/dL)	0.77±0.02	2.73±0.11	1.23±0.07	1.00±0.09	3.03±0.06	1.90±0.13	0.40 – 1.00
LDL Cholesterol (g/dL)	1.34±0.11	3.10±0.09	2.12±0.15	1.51±0.11	3.10±0.09	2.12±0.15	
Cholesterol/ HDL Ratio	4.23±0.08	2.97±0.39	4.37±0.07	4.65±0.07	2.97±0.14	4.30±0.13	

Mean values (n=6) ± SEM. Values appended by different small letters within a row are significantly different (P < 0.05)

Table 6. Body weight (g) and relative weight (mg) and length (mm) of the liver and spleen in BALB/C mice infected with L. major and feed with RP and AFPP on 12 weeks after infection

Parameter	Organ	NI-RP	INT-RP	IT-RP	NI-AFP	INT-AFP	IT-AFP
Body Weight	Day 0	19.89^{a}±0.11	20.18^{a}±0.11	20.18^{a}±0.11	19.99^{a}±0.19	19.76^{a}±0.07	19.93^{a}±0.13
	Day 84	31.02^{b}±0.21	13.18^{c}±0.13	25.68^{d}±0.10	29.74^{ab}±0.12	11.71^{c}±0.13	25.38^{d}±0.19
Relative length (mm)	Liver	22.49^{c}±0.14	47.50^{b}±0.24	26.07^{a}±0.53	22.08^{c}±0.11	51.10^{d}±0.38	28.15^{ae}±0.28
	Spleen	15.83^{b}±0.44	40.33^{a}±0.64	19.50^{c}±0.31	18.83^{d}±0.32	43.17a±0.44	20.17^{c}±0.19
Relative weight (mg)	Liver	1.36^{b}±0.02	2.66^{d}±0.04	1.59^{a}±0.02	1.44^{ab}±0.06	3.16^{c}±0.06	1.61^{a}±0.04
	Spleen	0.14^{a}±0.00	2.48^{c}±0.07	0.15^{a}±0.00	0.14^{a}±0.00	2.34^{c}±0.03	0.21^{b}±0.01
LDU (10^{6})	Liver	ND	564.59^{a}±13.24	ND	ND	645.44^{b}±12.58	ND
	Spleen	ND	13.28^{a}±0.42	ND	ND	19.02^{a}±0.24	ND

Mean values (n=6) ± SEM. Values appended by different small letters within a row are significantly different (P < 0.05). ND – not done

Malnutrition resulting from dietary protein deficiency decreases weight as age progresses [11]. Further, cure of diseases depends upon the development of an effective immune response that activates macrophages [26] which can be promoted and enhanced by good protein nutrition in diets. During Leishmaniasis, there is a profound immunosuppression in the host that promotes the survival of parasites [1]. In this study, it is revealed that treatment of infected animals coupled with good nutrition brought the levels of most biological parameters such as Hb and total leucocytes count (TLC) to normal range as compared to the abnormal levels in infected animals.

Kidney function tests include estimation of urea, blood urea nitrogen and creatinine. Renal abnormalities caused by Leishmania have been well documented in experimental animal studies and are comprised of interstitial and glomerular abnormalities [27]. Sodium, chloride and potassium and K/Na ratio values give an indication of electrolyte/water balance, whereas high levels of calcium implies thyroid or parathyroid, intestine, pancreas, kidney and borne metastasis. Although in the current study there were elevated levels of sodium, chloride and potassium and K/Na ratio, the role of infection caused by *L. major* on damage of these organs causing the elevated levels of these parameter is yet to be established. The reason may be that these parameters can vary with mouse strain/stock, age, sex; blood sampling method, environmental conditions pathogen status and the laboratory as well as nutrition [28].

The task of establishing a range of reference values for rodents is very difficult. This is because of very many variables, such as gender, age, genetic variation, diet and environmental conditions to which these animals are subjected, must be considered [29]. In this study, mice of the same sex and age infected with the same strain of *L. major* were used. Therefore, they may provide a useful starting point to investigate the effect of leishmaniasis and nutrition on hematological parameters. Since significant variation of biological parameters may occur between individual mice strain, stock, laboratories and method of sampling, individual laboratories should establish normal reference values for their facility [30].

The most important organ concerned with majority of biochemical activities in the human body is the liver. Since it has a great capacity to detoxify toxic substances and synthesize useful biological molecule, damage inflicted by hepatotoxic agents is of grave consequence. The increased level of ALT, AST, ALP, and bilirubin is conventional indicator of liver injury. In Leishmaniasis liver damage due to high parasitic load in infected groups resulting in elevated levels of ALT (SGTP), AST (SGOT), ALP and SDH. An increased level of bilirubin was observed in this study suggesting loss of functionality of the liver in Leishmaniasis. Albumin is used to detect liver damages whereas globulin and total protein content is used to detect and immunoglobulin status, a key indicator in fighting of infections in organisms which is affected by nutrition. The stabilization of serum bilirubin, ALT (SGPT), AST (SGOT), and ALP levels is a clear indication of the improvement in the functional status of the liver cells [31]. The increase in AST (SGOT) and ALT (SGPT) levels has been reported in the visceral leishmaniasis (VL) patients [32] which is depicted in this study for cutaneous leishmaniasis (CL) using mice model. In the current study, there was no significant elevation in the levels of liver function tests in all the treated groups as compared to infected controls.

The mononuclear phagocyte system (spleen, liver, bone marrow, intestinal mucosa and mesenteric lymph nodes) is the normal habitat of Leishmania parasite [33]. However, the parasite may be found in endothelial cells of the kidneys, suprarenal capsules, lungs, meninges and in cerebrospinal fluid [34]. In leishmaniasis there can be possibilities of multiple organ damage as indicated by elevated levels of non-specific markers such as lactate dehydrogenase (LDH) indicating possible damage of liver, heart, skeletal muscles and lungs and creatine kinase (CK) commonly elevated in heart and skeletal muscle damage and muscular dystrophies.

These effects are consequences of the stimulation of the immune system by *L. major*, which promotes the inflammatory components of atherosclerosis, which are primarily the parasite-activated macrophages [35]. Several studies suggest that pathogenic bacteria, viruses and protozoa, contribute to the atherogenesis process [36,37,38,39,40]. The increased inflammation caused by these pathogens promotes macrophage activation and migration to the atheroprone site. Alternatively, proatherogenic status may be attributed to the systemic oxidative stress induced by infection, which enhances lipoprotein or endothelium oxidation. Pathogens involved in atherosclerosis development usually induce a systemic infection

instead of a localized infection such as an *L. major* infection. However, even localized infections, such as odontologic ones, may be associated with the development of atherosclerosis [41].

In this study, microscopic examination of stained impression smear of liver and spleen showed the density of amastigotes in two organs that demonstrated the pathological effect of parasite. The results of this study have demonstrated that the infected mice show the hepatosplenomegaly sign of pathological effect of *L. major* promastigote in the infected mice. The weight of liver was increased with increasing days of infection in the INT mice compared with NI mice on 12 weeks. For both the liver and spleen, NT mice had the highest LDU. Significantly ($p < 0.05$) higher parasite load in the liver and spleen occurred in NT mice and the lowest LDU occurred in the IT mice. There were no significant difference between the following groups, NI-RP and NI-AFPP ($p > 0.05$), IT-RP and IT-AFPP ($p > 0.05$), INT-RP and INT-AFPP ($p > 0.05$), indicating that there is no difference between the two feeds.

5. CONCLUSION AND RECOMMENDATION

From the study it can be demonstrated that there was no difference in growth performance, metabolic efficiency and nutrients utilization parameters between RP and AFPP among the same treatment of BALB/C mice. This suggests that Annonaceae fruit pulp can be used as an alternative source of raw material in the manufacture of animal feeds. It has been observed that the data obtained from present investigation related to different growth performance, metabolic efficiency and nutrients utilization make Annonaceae fruit a good candidate for manufacture of neutraceuticals. The choice of animal model (BALB/C mice) may be a major limiting factor that could have influenced the applicability of the results of this study since different laboratories have different reference range values. Thus, there is a need to study these parameters in another higher animal model preferable primates such as monkey and baboons to validate the findings of this study for utilization of Annonaceae fruits in the nutraceutical manufacturing industry.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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