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Original Research Article

Protective role of *Moringa oleifera* leaf-based diet on protein- energy malnutrition induced skeletal muscle degeneration

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ABSTRACT

Background: Sequel to the diverse diseases resulting in muscle mass degeneration and its key role in the prognosis of the diseases. For instance, currently, only resistance exercise can be used to promote recovery of mass/strength following disuse atrophy. But in contrast, many patients are unable or unwilling to exercise at a sufficient intensity to promote muscle growth. It would therefore be of great advantage to develop natural compounds that counteract the negative effects of skeletal muscle degenerative diseases especially in the form of therapeutic feed). The present study was aimed at elucidating the mechanism by which *M. oleifera* ameliorates skeletal muscle degeneration caused by malnutrition.
Methods: The experimental animals were malnourished with low protein iso-caloric diets for four weeks after which they were treated with 25% *M. oleifera* leaf – based diet, vitamin E supplemented feed and soy bean based- diet for another four weeks.
Results: There was a significant reduction in the activity of calcium ATPase in the skeletal muscle of animals induced with skeletal muscle degeneration. However, this activity was significantly increased following treatments with the most significant increase observed in the skeletal muscle of animals treated with *M. Oleifera* leaf based diet followed by those fed with vitamin E supplemented diet.
Conclusions: In summary, the study revealed that the mechanism by which *M. oleifera* leaf corrects muscle degeneration caused by PEM might be by increasing calcium ATPase activity and/ or its synthesis and by preventing oxidative stress due to its antioxidant properties.

Keywords: Calcium ATPase, Mechanism, Iso-caloric diet, *M. oleifera*, Muscle atrophy

INTRODUCTION

Muscle degeneration occurs following alterations in normal balance between protein synthesis and degradation which results in down-regulation of protein synthesis pathways, and an activation of protein degradation.[1] An important ion that maintains this strict balance in the cell is calcium ion. Diseases and conditions which cause decrease in muscle mass include HIV/AIDS, cancer, infections and tuberculosis.[2-5] Most of these diseases negatively affect calcium homeostasis, ATPase activity and redox state of cells causing oxidative stress.[4] Inhibition of calcium adenosine triphosphatase (Ca^{2+}-ATPase) prevents pumping of calcium in the muscle cell which would otherwise be used for maintaining overall health of the muscle, thus resulting in a wasting and degeneration of (muscle) tissue.[4]

There is a very large trans-membrane electrochemical gradient of calcium ions driving its entry into cells, yet it is very important for cells to maintain low concentrations of calcium ions for proper cell signalling. Thus, it is necessary for cells to employ ion pumps to remove

excess calcium ions.[6] Calcium ATPase is a form of P-ATPase that transfer calcium after muscle contraction. The enzyme exits in two forms namely, plasma membrane calcium ATPase (PMCA) and sarcoplasmic endoplasmic reticulum calcium ATPase (SERCA).[6] They are located on various membrane types and serve to translocate calcium ions across these membranes against very steep concentration gradients.[7,8] PMCA is a transport protein in plasma membrane of cells that serves to remove calcium ion from the cell. It is vital for regulating the amount of Calcium ion within cells.[9] SERCA resides in the sarcoplasmic reticulum (SR) within muscle cells where it transfers Ca^{2+} from the cytosol of the cell to the lumen of the SR at the expense of ATP hydrolysis during muscle relaxation.

Malnutrition is a medical condition caused by improper or insufficient diet.[9] It is a category of diseases that includes under- nutrition, obesity and overweight, and micronutrient deficiency among others. It is frequently used to mean just under nutrition from either inadequate calories or inadequate specific dietary components for whatever reason.[10] The term "severe malnutrition" is however often used to refer specifically to protein-energy malnutrition (PEM) which is often associated with micronutrient deficiency.

Moringa oleifera is an angiosperm belonging to the family *Moringaceae.* Its English names include horseradish tree, drumstick or ben oil seed tree and locally known as '*Zogalegandi'* in Hausa, '*Ewe igbale'* in Yoruba and '*Okweoyibo'* in Igbo.[11] It is a fast growing, drought resistant tree native to the southern foot hills of the Himalayas in north western India. However, it is now cultivated in all regions of the world. Several biological properties ascribed to various parts of this plant have been reviewed in the past.[12] These include its use as an antioxidant, anticarcinogenic, antiulcer, antibacterial, and antifungal. Phytochemical analyses have shown that its leaves are particularly rich in potassium, calcium, phosphorous, iron, vitamins A and D, essential amino acids as well as antioxidants such as β-carotene, vitamin C, and flavonoids.[13] Studies on proximate and phytonutrient analysis of the leaf by Bamishaiye et al, 2011 also showed that it has high percentage of carbohydrate and protein and compared favourably with other high protein/ carbohydrates food crops. It is however a potential leaf source of food that is suitable for fortification of foods and their use as nutritional supplements is highly promising.[14] Moreover, its fruit pod and leaves have been used to combat malnutrition, especially among infants and nursing mothers for enhancing milk production.[15] Dietary consumption of its part is therein promoted as a strategy of personal health preservation and self-medication in various diseases.

In view of the aforementioned effects of muscle degeneration on calcium homeostasis in disease conditions, it would therefore be of great advantage to develop natural compounds that counteract the negative effects of skeletal muscle degenerative diseases without adversely affecting calcium levels or the redox state of the muscle cells. Although *M. oleifera* has been used effectively in cases of malnutrition, this study evaluates its mechanism of ameliorating skeletal muscle degeneration caused by malnutrition. This might be a useful tool in formulating drugs for the management and treatment of skeletal muscle degeneration resulting from different diseases of global concern.

METHODS

Chemicals and reagents

Adenosine triphosphate (ATP) is a product of Sigma Aldrich Chemical Company Poole England. All other chemicals and reagents used were of analytical grade.

Feed materials

Dried blended *M. oleifera* leaf was purchased from Faculty of Agriculture university of Ilorin. DL-methionine, vitamin- mineral mix, corn chaff, sucrose, yellow corn, soy bean, and soy oil were purchased from Ilorin. These were formulated as presented in Table 1.

Table 1: Components of the control and test diets.

Diet composition	Control diet (g/kg) (25%)	Test diet (4% soy bean) (g/kg)	Test diet (4% *Moringa* leave) (g/Kg)
Soy bean	250	40	---
***Moringa* leaves**	---	---	40
Corn starch	516	100	100
Soy bean oil	40	40	40
Cellulose	40	400	400
Sucrose	100	366	366
DL-methionine	4	4	4
***Vitamin/ mineral mix**	50	50	50

*Vitamin/ Mineral mix: Vitamin A 4,000,000 i.u; Vitamin D3, 800,000 i.u; Tocopherols, 400 i.u; Vitamin K3 800mg, Folacin, 200 mg; Thiamine, 600mg; Riboflavin 1,800 mg; Niacin, 6000 mg; Calcium pathothenate, 4 mg; Biotin, 8 mg; Manganese, 30,000 mg, Zinc, 20,000 mg; Iron, 8,000 mg; Choline chloride 80,000 mg; Copper, 2,000 mg; Iodine, 480 mg; Cobalt, 80 mg; Selenium, 40 mg; BHT, 25,00 mg Anti- caking agent 6000 mg.

Experimental animals

Forty female weanling albino rats (*Rattus norvegicus*) with mean weight of 65± 0.26 g were used for this research. They were procured from the Animal Holding Unit of the Department of Biochemistry, University of Ilorin, Ilorin. All the animals were fed with commercially prepared feed and clean water *ad libitum* for one week to acclimatize.

Induction of muscle degeneration

Skeletal muscle degeneration was induced by feeding the animals with low protein (4%) iso-caloric diet *ad libitum* for four weeks (a slight modification of the method used by Nadia et al.[16]

Animal grouping

The animals were randomly divided into four groups of ten animals each. Control animals constituted Group 1. Group 2 and Group 3 animals were fed low protein isocaloric diet (4% soy meal- based diet) while Group 3 feed was supplemented with vitamin E (40 mg/Kg body weight). Group 4 animals were also fed low protein iso-caloric diet (constituted with 4% *M. oleifera* leaf- based diet).

After 4 weeks of induction, the animals were subdivided into treatment groups. Group 2a and 2b were treated with 25% soy and 25% *M. oleifera* leave based diet respectively. Group 3a and b were treated with 25% soy supplemented with vitamin E and 25% soy alone respectively while group 4 animals were treated with 25% *M. oleifera* leaf- based diet. These animals were treated for another 4 weeks before they were sacrificed for analysis.

Anthropometric measurements

Anthropometric measurements taken include; weight, circumference of the head and length of the whole rat. The anthropometric study was done before the animals were induced with skeletal muscle atrophy (by PEM), and during the administration of treatment feeds.

Collection of blood and preparation of homogenates

The rats were sacrificed by cervical dislocation and blood was collected by jugular puncture. Blood samples were collected into plain and some into EDTA coated sample bottles (to prevent clotting) for serum and haematological analysis respectively. Skeletal muscle from the hind limbs was quickly extracted into iced cold solutions of 250 mM sucrose buffer (250 mM sucrose, 10 mMtris, pH 7.4).

Serum was thereafter prepared by centrifuging the blood samples at 3000 rpm for 5 minutes.[17] The skeletal muscle was homogenized in an iced cold mortar and pestle using the buffer as the homogenizing medium. The suspension of tissue homogenate was stored in aliquot units in Eppendoff tubes and stored in the freezer. The homogenate was kept frozen overnight to ensure maximum release of the enzymes [18] and thereafter used for enzyme assay.

Biochemical assay

The protein concentration in the tissue homogenates was determined using Biuret method described by Gornall et al, using bovine serum albumin as the standard protein.[19] Serum albumin concentration was quantified by the method described by Doumas et al. [20] Ca^{2+}-ATPase was assayed in the skeletal muscle tissue homogenate after the forth and eight weeks using the procedure described by Bewaji.[21]

Haematological analysis

The haematological parameters analyzed include, red blood cell count, white blood cell count, blood haemoglobin, full blood count. These were analyzed using automated haematological analyzer.

Statistical analysis

Data were analyzed using one-way ANOVA and differences were considered significant when $P < 0.05$. Values presented are mean±SEM. SPSS Version 16.0 software for windows was used to analyse the data.

RESULTS

Animal morphology

The weekly mean weight of the animals showed progressive decrease except in the control group as shown in Figure 1. The reverse was however the case when treatments commenced as in Figure 2. Group 1 (control) animals grew well, had smooth body fur, an oblong face and tails covered with fur; no loss of fur was observed in any part. In contrast, the malnourished animals (i. e Groups2, 3 and 4) experienced loss of appetite which could have led to the observed loss of body fur, developed moon face, circumference of the head remaining the same, scaly tails, bulged eyes, muscle wasting. There was a gradual improvement in the aforementioned morphological changes as the treatment progressed.

Haematological analysis

The result of the haematological analysis is presented in Tables 2 and 3. All the haematological parameters assessed had significant reduction during malnutrition in all the test groups. However the indices increased significantly after treatment and could be compared to the control.

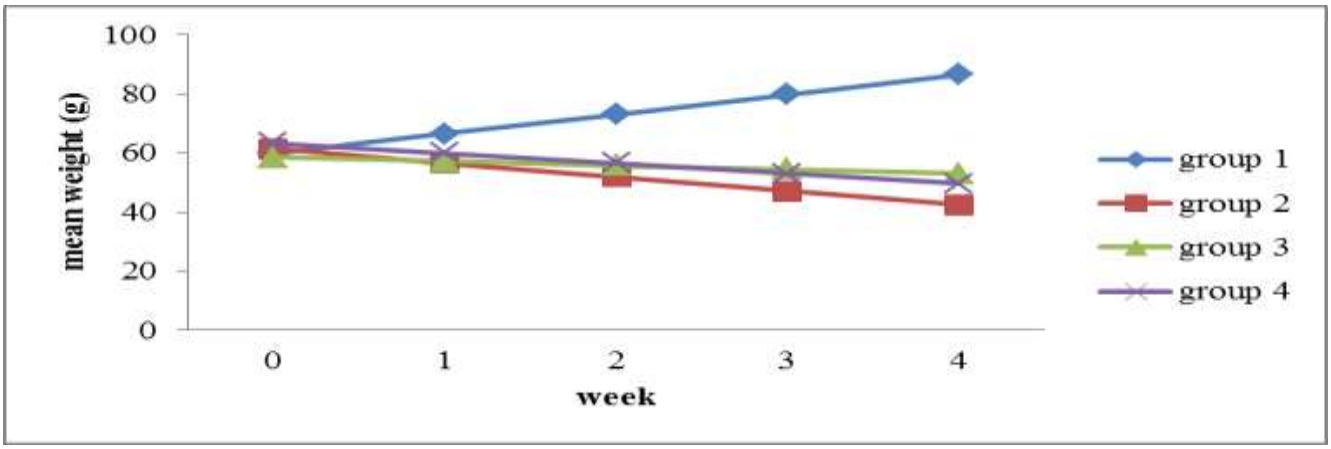
Serum albumin

The serum albumin concentration was significantly reduced in all the groups compared with the control

during weeks of malnutrition. However, a significant increase was observed after treatments across all the groups with the highest increase in group 4 animals as shown in Table 4.

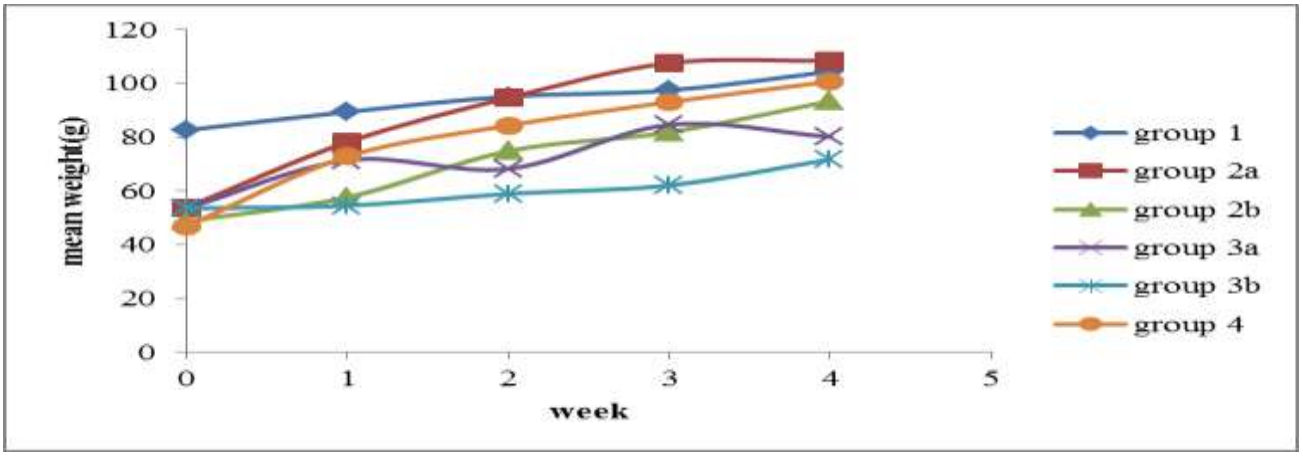
ATPase activity

The specific activity of Ca^{2+}-ATPase of the animals was used to plot Michealis- Menten curve as depicted in Figure 3 to Figure 8. These curves showed that the calcium ATPase of the malnourished animals had lower activities compared with the control, except for group 4 where which the enzyme activity curve was seen not to have a similar characteristic with Michealis- Menten curve though with higher activity. However, ATPase activity in all the groups had significant increase in activities after treatment though with varying activities.



Group 1= control animals, Group 2= malnourished with 4% soy, Group 3= malnourished with 4% soy + vit. E, Group 4 = malnourished with *M. oleifera.*

Figure 1: Average weight of malnourished rats per week.



Group 1= control animals, Group 2a= treated with 25% soy, Group 2b= treated with 25% *M. oleifera*, Group 3a= treated with 25% soy + vitamin E, Group 3b= treated with 25%soy, Group 4= treated with 25% *M. oleifera* leaf.

Figure 2: Average weight of treated rats per week.

Table 2: Haematological indices of rats before treatment.

	Group 1	Group 2	Group 3	Group 4
RBC (x10^6/µl)	7.46 ± 0.01^a	6.16± 0.08^b	6.22±0.02^b	6.36±0.07^b
WBC (10^3/µl)	17.8 ± 0.35^a	12.3± 0.10^b	Xxx	12.9±0.05^b
HGB (g/dl)	12.7 ± 0.15^a	10.1 ± 0.05^b	11.2 ± 0.15^c	11.5 ± 0.10^c
PCV (%)	48.4± 0.20^a	35.0 ± 0.25^b	39.0 ± 0.30^c	38.1± 0.40^c

*Data with different superscript across the same row are significantly different at $p < 0.05$
RBC = Red blood cells, WBC = White blood cells, HGB= haemoglobin, PVC = Pack cell volume
Group 1= control animals, Group 2= malnourished with 4% soy, Group 3= malnourished with 4% soy + vit.E, Group 4 = malnourished with 4% *M. oleifera.*

Table 3: Haematological indices of rats after treatment.

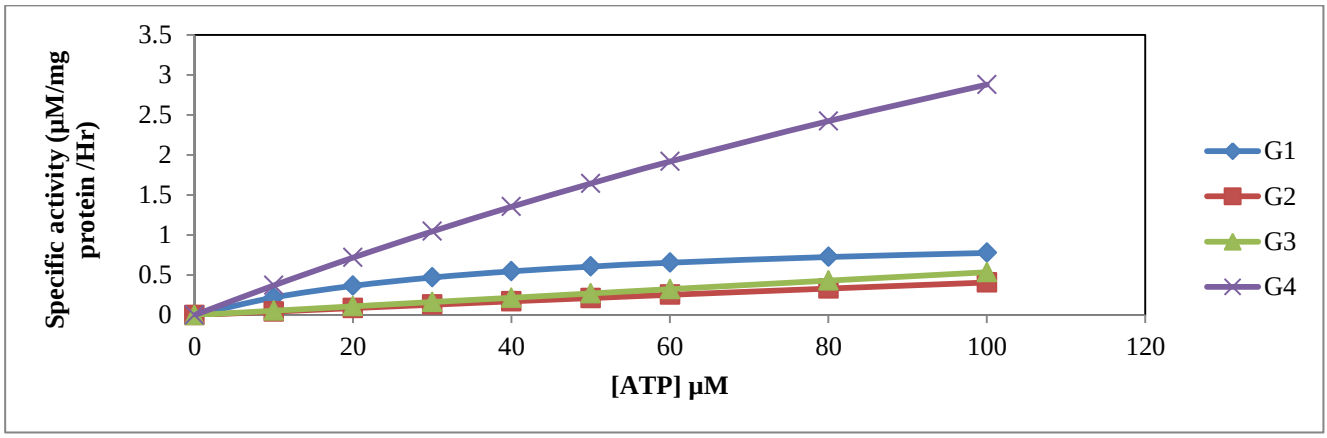
	RBC ($x10^6$/µl)	WBC (10^3/µl)	HGB (g/dl)	PCV (%)
Group 1	6.75 ± 0.03 [a]	14.75± 0.38 [a]	11.60± 0.10 [a]	40.20 ± 0.10 [a]
Group 2a	7.39 ± 0.05[c]	21.40 ± 0.20 [b]	12.70 ± 0.15 [a]	51.80 ± 0.10 [c]
Group 2b	6.58 ± 0.01[b]	14.30 ± 015 [a]	11.30 ± 0.10 [a]	39.30 ± 0.35 [a]
Group 3a	7.45 ± 0.03 [d]	21.30 ± 0.40 [b]	12.00± 0.15 [a]	47.50 ± 0.10[bc]
Group 3b	7.03 ± 0.01[b]	18.70 ± 0.50[ab]	12.30 ± 0.10 [a]	48.65 ± 0.33[c]
Group 4	6.66 ± 0.06 [a]	14.50 ± 3.20 [a]	11.60 ± 0.95 [a]	45.10 ± 5.20[bc]

*Data with different superscript along the same column are significantly different at p <0.05.
RBC = Red blood cells, WBC = White blood cells, HGB= haemoglobin, PVC = Pack cell volume
Group 1= control animals, Group 2= malnourished with 4% soy, Group 3= malnourished with 4% soy + vit. E, Group 4 = malnourished with 4% *M. Oleifera.*

Table 4: Effects of muscle degeneration on blood serum concentration of rats.

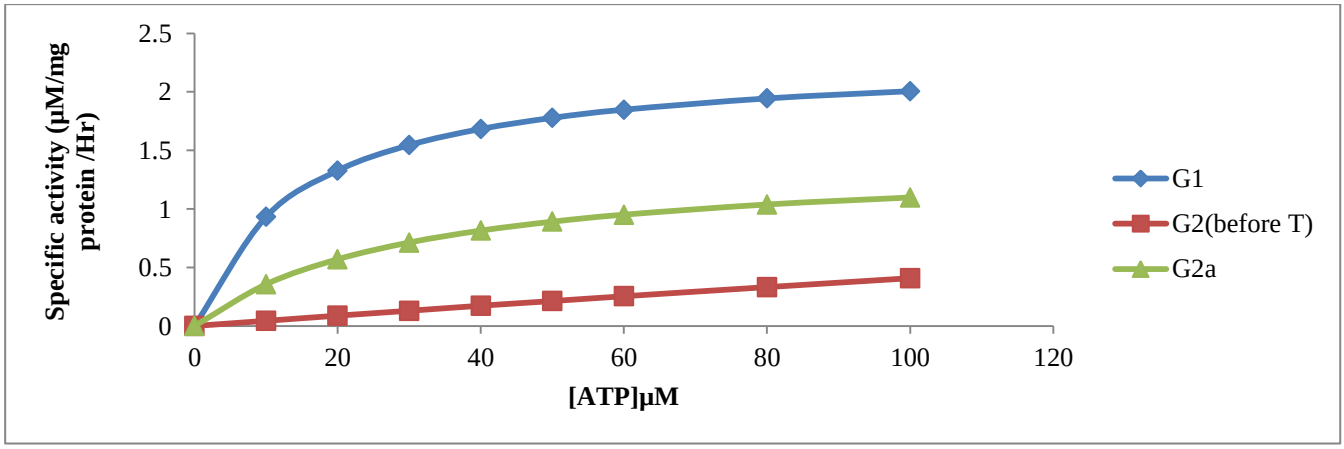
Serum albumin conc (g/l)	Group 1	Group 2a	Group 2b	Group 3a	Group 3b	Group 4
Before treatment	3.690±0.05 [a]	1.407±0.00 [b]	1.407±0.00 [b]	0.352±0.00 [c]	0.352±0.00 [c]	-1.583±0.01 [d]
After treatment	14.39±0.05 [a]	15.37±0.01 [c]	19.62±0.04 [e]	18.64±0.07 [d]	13.08±0.06[b]	23.06±0.13 [f]

*Data with different superscript across the same row are significantly different at p < 0.05
Group 1= control animals, Group 2= malnourished with 4% soy, Group 3= malnourished with 4%soy + vit. E, Group 4 = malnourished with 4% *M. oleifera.*



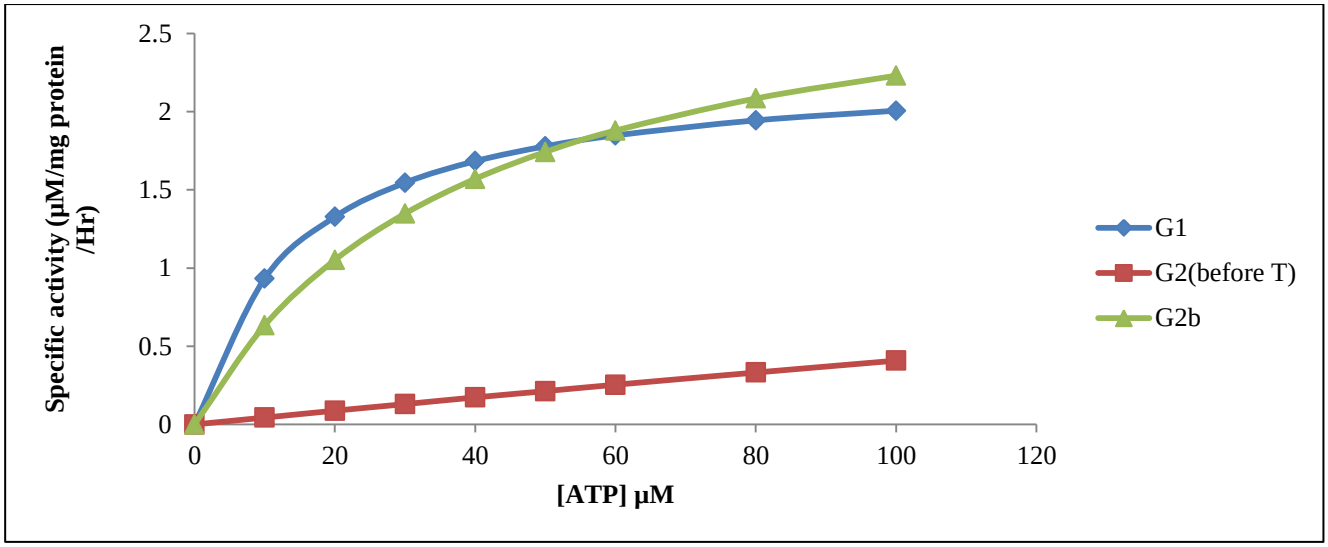
Group 1= control animals, Group 2= malnourished with 4% soy, Group 3= malnourished with 4%soy + vit. E, Group 4 = malnourished with 4% *M. oleifera.*

Figure 3: Ca^{2+} ATPase activity in the skeletal muscle of malnourished rats.



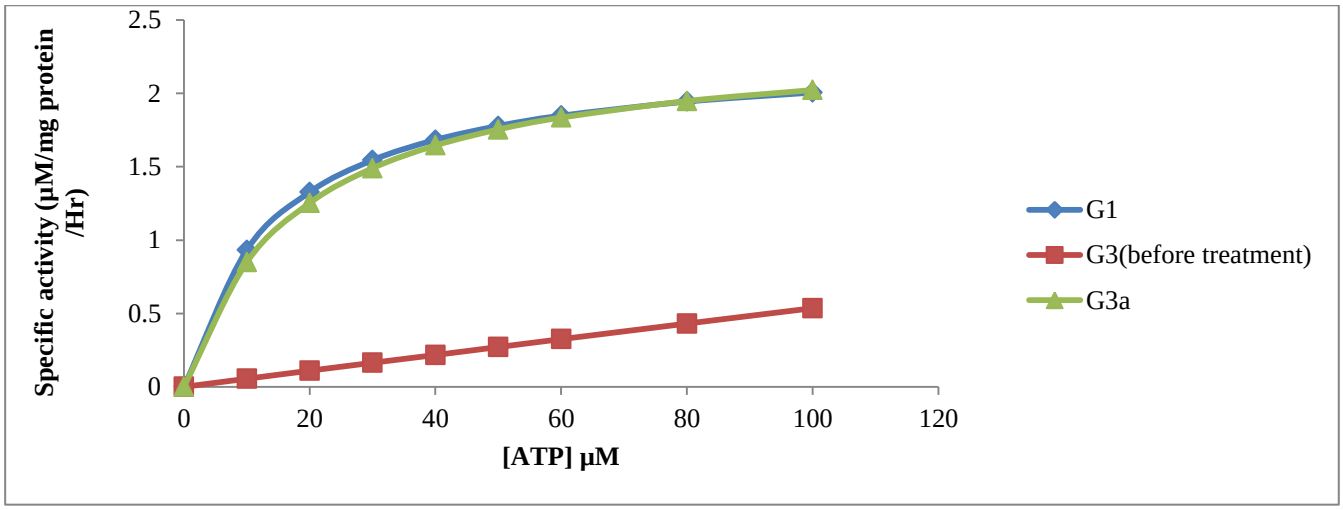
Group 1= control animals, Group 2= malnourished with 3% soy, Group 2a= treated with 25% soy.

Figure 4: Ca^{2+} ATPase activity in the skeletal muscle of group 2a rats.



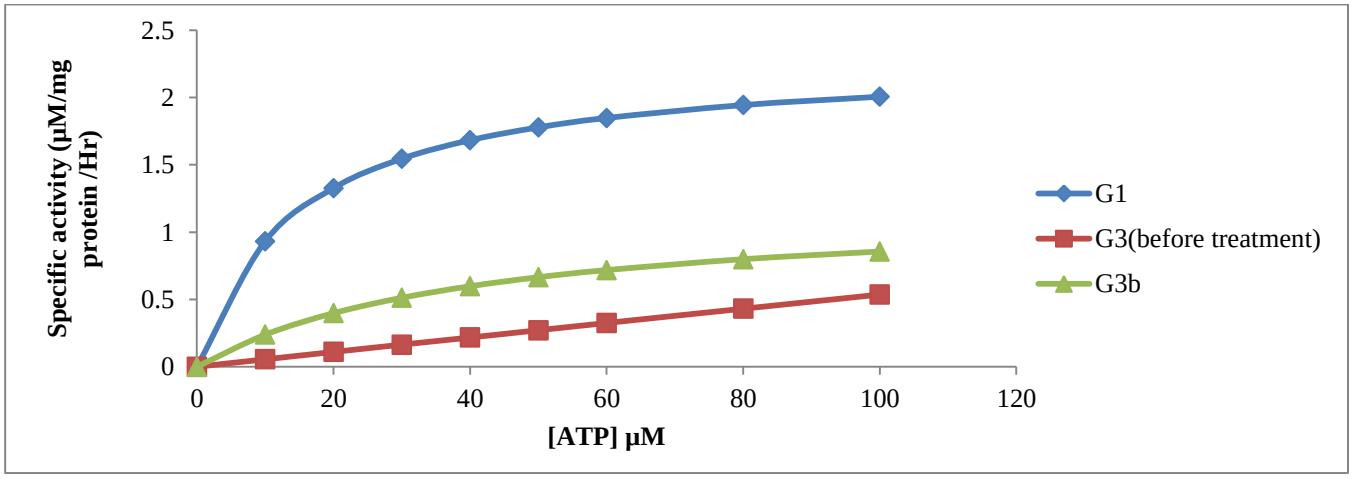
Group 1= control animals, Group 2= malnourished with 3% soy, Group 2b= treated with 25% *M. oleifera*.

Figure 5: Ca^{2+} ATPase activity in the skeletal muscle of group 2b rats.



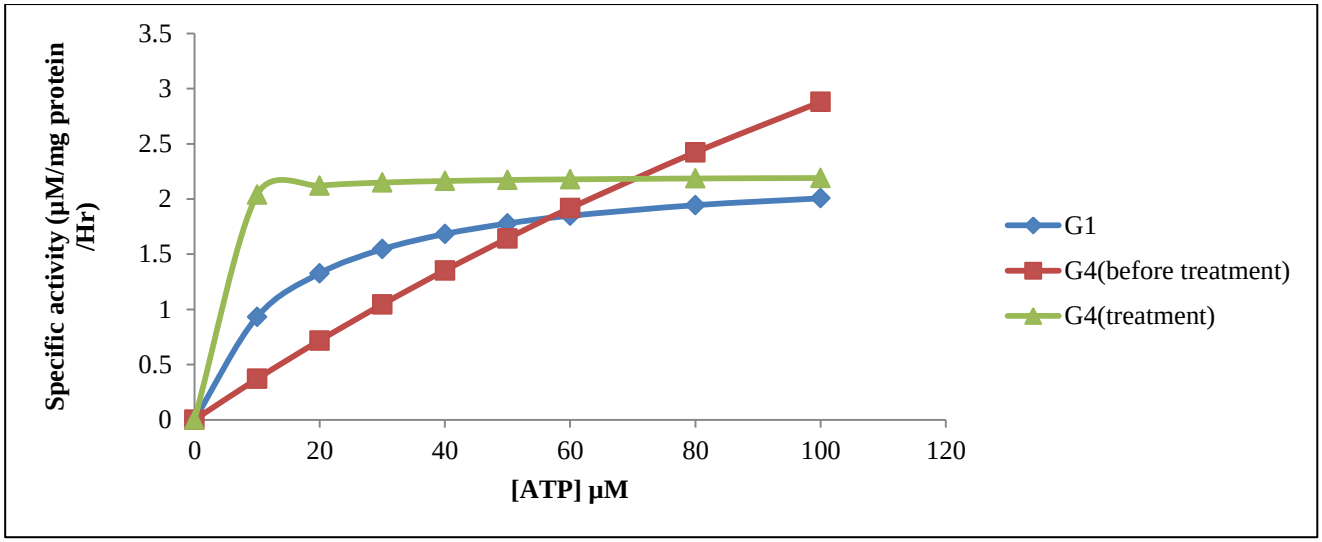
Group 1= control animals, Group 3= malnourished with 3% soy+ vitamin E, Group 3a= treated with 25% soy.

Figure 6: Ca^{2+} ATPase activity in the skeletal muscle of group 3a rats.



Group 1= control animals, Group 3= malnourished with 3% soy + vitamin E, Group 3b= treated with 25% soy.

Figure 7: Ca^{2+} ATPase activity in the skeletal muscle of group 3b rats.



Group 1= control animals, 4= malnourished with 3% *M. Oleifera* leaf and treated with 25% *M. oleifera* leaf.

Figure 8: Ca^{2+} ATPase activity in the skeletal muscle of group 3b rats.

DISCUSSION

Malnutrition (PEM) results into a lot of morphological changes which includes; muscle wasting (especially in the thigh and buttocks), alopecia (loss of fur), oedema, anaemia, infections and the subject becomes apathetic and lethargic among others, hence the morphological changes observed in the test animals.[22,23] However, morphological changes occurring as a result of malnutrition are often reversed by improved nutrition. The result of this study showed that all the adverse morphological changes observed in the test animals was improved with the administration of the improved diets though with different capacity. Animals fed only 40% soy- based diet had the lowest improvement when compared with those fed vitamin E supplemented diet and those fed *M. oleifera*- based diet. The leaves of *M. oleifera* is a source of both macro- and micronutrients such as β-carotene, protein, vitamin C, calcium, and potassium [15] and its use as an antioxidant is documented.[24] Higher growth pattern observed in group 3 and group 4 may have been because of their antioxidant properties they have in common added to other nutritional qualities. However the highest improvement was observed in *M. oleifera*- based diet fed animals.

One of the common complications of protein-energy malnutrition is anaemia.[25] Animals fed on protein calorie malnourished diets had been reported to have significant reduction in haemoglobin concentrations.[26] It has also been well documented that kwashiorkor and marasmus (protein energy malnutrition) patients had low levels of haematological indices.[27-29] Similarly, in this report, there was reduction in the haematological parameters observed during malnutrition. However, after treatment, all the parameters were significantly increased in all animal groups. This is also in accordance with the report of researchers which have shown that several protein-rich foods increase Hb concentrations in human and animal studies.[26]

In 1991, Bolarinwa et al reported a significant reduction in the levels of total protein in protein-calorie malnourished rats. [26] Similarly, a correlation had earlier been on drawn between total protein levels and severity of protein energy malnutrition.[8] Serum albumin test being a liver function test, decrease in its concentration following the malnutrition suggests impaired absorption of protein in the intestine or even liver damage.[30] Impaired intestinal absorption of protein may provide the liver with inadequate supply of amino acids to synthesize serum proteins (such as albumin), leading to a drop in serum protein level. Also, liver damage may impair the synthesis of serum proteins in the liver thereby leading to low serum levels.[30] The reduction in the serum albumin concentration during malnutrition could be a sign of low protein.

Kinetic analysis of enzymes permits scientists to reconstruct the number and order of the individual steps by which enzymes transform substrates into products.[31] Kinetic data combined with detailed information about an enzyme's structure and its catalytic mechanisms, provide some of the most powerful clues to the enzyme's biological function(s) and may suggest ways to modify it for therapeutic purpose.[32] After the induction of skeletal muscle degeneration, Ca^{2+} ATPase activities in the skeletal muscle of all the Groups except Group 4 were significantly reduced. This might imply that *M. oleifera* leaf-based diet fed animals were able to resist the effects of malnutrition on the enzyme activity. The significant reduction in the activity might be as a result of a reduction in the synthesis or inactivation of the enzyme during the malnourished condition. Since the main function of the Ca^{2+} ATPase is to pump out Ca^{2+} from the

cell thereby keeping the concentration of Ca^{2+} low, impaired or reduced activity of the enzyme will result in an unhealthy high concentration of Ca^{2+} within the cell which might finally result in cell death.[6]

The significant increase in the activity of Ca^{2+} ATPase across all the groups after treatment could be as a result of activation or increase synthesis of the enzyme during treatment and however implies that the effect of malnourishment on Ca^{2+} ATPase is reversible. There was slight increase in calcium ATPase activity in groups fed 40% soy-based diet but that of *M. Oleifera* leaf-based diet was significantly higher than other groups. This could as well imply that one of the corrective mechanisms of muscle degeneration by *M. oleifera* is by activating the Ca^{2+} ATPases in the skeletal muscle thereby reinstalling Ca^{2+} homeostasis. A pioneering study has demonstrated association between an immobilized rodent skeletal muscle with increased level of oxidative stress, which could partially be arrested by vitamin E supplementation. However, subsequent investigation provided the definite mechanistic link between acute atrophy and oxidative stress.[33,34] Vitamin E supplemented diet also resulted into a much significant increase in activity similar to those of *M. Oleifera* leaf-based diet, as this might imply some relationship between the two feeds probably because of their antioxidant properties, perhaps calcium ATPase activity and oxidative stress are both key factors affected in muscle atrophy.[35]

Conclusively, this research shows that though malnutrition induced muscle degeneration has adverse effects on Ca^{2+} ATPases of the skeletal muscle; the effects were most significantly improved by 25% *M. oleifera* leaf- based diet. However, the activity of Ca^{2+} ATPases in the skeletal muscle of rats malnourished in the presence of *M. oleifera* leaves was not significantly affected. Moreover, the parameters assessed shows that supplementing animal feed with Vitamin E has no significant effect on the changes induced by malnutrition.

Summarily, the mechanism by which *M. oleifera* leaf corrects muscle degeneration caused by PEM might be by increasing Ca^{2+}-ATPase synthesis and/or activity, preventing oxidative stress, and by possibly preventing energy depletion in the skeletal muscles.

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Conflict of interest: None declared
Ethical approval: The study was approved by the institutional ethics committee

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