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Calcium and s100a1 protein balance in the brain–heart axis in diabetic male Wistar rats

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Abstract

Objectives: Calcium deregulation in diabetes mellitus (DM) is central to the brain–heart axis pathology. This has led to the use of medical plants in complementary medicine such as *Amaranthus hypochondriacus* (GA). The objective of the study was to establish the effects of grain amaranth feed supplementation on calcium, s100al protein and antioxidant levels on the brain–heart axis in diabetic male Wistar rats.

Methods: The study involved six groups (n=5) with DM being induced in 20 rats. To the diabetic rats, Group I received mixtard®, Group II was positive control, Groups III and IV received GA feed supplementation at 25 and 50%. In the nondiabetic rats (n=10), Group V received 50% grain amaranth while Group VI was the negative control. The brain and heart tissues were harvested after five weeks and processed using standard methods.

Results: Grain amaranth feed supplementation led to improved calcium levels in DM as compared to the positive control. This also led to increased s100a1, antioxidant levels in the brain–heart axis during DM. This then protected the tissues against oxidative damage, thus preserving tissue function and structure.

Conclusions: Grain amaranth's actions on calcium signaling subsequently affected s100a1 protein levels, leading to improved tissue function in diabetes.

Keywords: calcium in T2DM; ethnomedicine in T2DM; grain amaranth.

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Introduction

Neurocardiology is characteristic of diabetes mellitus (DM), following the deregulation of calcium signals in the primary tissues [1–3]. In cardiac muscle, DM has been associated with the accumulation of reactive oxygen species which lead to oxidative stress [4]. This subsequently predisposes the cell membrane to lipid peroxidation [5] and disruption of cellular integrity. This would lead to increased disruption of ion traffic, leading to dyshomeostasis within the affected tissues [2, 6]. In brain tissue calcium ions (Ca^{2+}) are essential for neural transmitter release from the synaptic vesicles [7], while in cardiac tissue, it's essential to sustain the power stroke, characteristic of ventricular depolarization [8, 9], showing the importance of this secondary messenger in neurocardiology [1]. In DM, calcium entry in the brain and heart tissues is disrupted, leading to poor calcium sequestration into the

sarcoplasmic reticulum and utilization by the mitochondria in calcium-dependent ATP production activities [10], and this leads to tissue inefficiency, failure and apoptosis [11, 12].

In the management of DM in the brain–heart axis, the emphasis has been placed on gene therapy, and the s100a1 calcium handling proteins have shown a lot of success [13–15]. The s100a1 proteins are part of the s100 family proteins which play a crucial role in calcium handling within the tissues [15]. Once expressed significantly within the tissues during DM, s100a1 proteins lead to improved patient outcomes due to their direct synergistic effects on key calcium transport proteins in the body [15–18]. On the other hand, a majority of healthcare providers in developing countries rely heavily on complementary medicine to manage complications associated with DM [19]. *Amaranthus hypochondriacus* (amaranthus, GA) is one with an international reputation [20] since it is a major vegetable food source in many African communities and it's commonly known as the 'Prince-of-Wales feather' [21]. Amaranthus has been shown to improve on cardio-pulmonary function due to its antioxidant properties [22, 23], bone density due to its high calcium content [24, 25] and hypoglycemic and hypocholesterolemic effects due to its phytochemical compounds [26, 27]. Also, processing of amaranthus leads to increased nutrient bioavailability [28], and recent findings [29] demonstrated the ability of GA to improve calcium homeostasis in the liver and the kidneys during DM. Information on the role of GA in the brain–heart axis continues to be scarce, although many advances in complementary medicine promoting the use of medical plants such as vegetables which have hypoglycemic effects [30] continue to gain momentum. The objective of the study was to gain basic insights into the actions of grain amaranth on calcium and s100a1 protein homeostasis during DM in male Wistar rats.

Materials and methods

Study design

This was an experimental study in which 30 adult male rats, two months of age were randomly assigned to six study groups each consisting of five animals. Rats in groups I–IV were diabetic and the research model was a nicotinamide/streptozotocin (STZ) model of DM [31, 32], and the induction protocol was as described previously [29, 33]. In brief, DM was induced (n=20 rats) using STZ (60 mg/mL) and nicotinamide (120 mg/kg) intraperitoneal as described previously and those with a hyperglycemic index ≥250 mg/dL were used for this study. Group I was treated with Mixtard® [34]. Group II the positive control (DM and no treatment on regular rat pellets), Groups III and IV were provided with feed supplementation at 25 and 50% w/w GA respectively, while Group V was nondiabetic and was provided with 50% GA feed supplement (comparative control). Group VI was the negative control (nondiabetic and on regular rat pellets).

Grain amaranth processing

This was done as previously described [29]. In brief, *A. hypochondriacus* was processed to create popped grain amaranth by heating at 260 °C for 5 s. This was weighed and mixed with regular rat feed to make 25% w/w and 50% w/w for low and high GA feed supplementation respectively. Water was added to the mixture to moisten it and pellets were formed and dried at for 24 h in an oven (WTE Binder, type 19240300002000, no. 950228, Germany) for preservation and stored in a sac at room temperature.

Laboratory analysis

At the end of five weeks, animals were euthanized using sodium pentobarbitone injected intraperitoneally as previously described [29]. The brain and heart were harvested from each rat and placed in sterile sample bottles. Samples for biochemical analysis were subsequently homogenized in 1M phosphate buffer saline, centrifuged at 3,000 rpm for 5 min and the filtrate was collected into sterile Eppendorf tubes, which was stored in a refrigerator at –20 °C. Heart samples for histological analysis were placed in 10% neutral buffered formalin, while the brain was placed in bouin's solution.

Determination of tissue calcium: This was done using an atomic absorbance spectrometry (AAS) method [35]. The AAS (Perkin-Elmer, model GBC932AA, USA) was set up according to manufacturer's recommendations, and an equation from the standard curve (absorbance=450 nm) was used to determine calcium concentrations for each on all samples.

Determination of s100a1 levels: This was done by using the ELISA standard protocol [36]. The s100a1 protein was determined using a commercial test kit (Santa Cruz, Biotechnology, USA, Texas) following the manufacturer's recommendations. The s100a1 variant used in this study was cataloged SC-71992 with a Gene ID of 6271 (1q21.3) in humans and that of 20193 (3F1) in rats. The optical density was measured at 450 nm for the s100a1 proteins [37] using an automatic ELISA plate reader (Biotech, USA) as previously described [29].

Determination of oxidative and antioxidant activity: This was done as previously described [29]. Briefly, 1 M of MDA reacts with 2M of 2-thiobarbituric acid (TBA) to yield a chromophore, and the absorbance was taken at 540 nm according to standard methods [38] using trichloro acetate, TBA, hydrochloric acid and sodium hydroxide. Glutathione peroxidase (GPx) activity was determined measured using the method of Yutaka [39] following the formation of GSSG using a coupled enzyme system with glutathione reductase (GRx). This was important since the formation of glutathione (GSSG) is catalyzed by GPx coupled with the recycling of GSSH back to GSH using GSSG-R (glutathione reductase). NADPH is oxidized to $NADP^+$. The change in absorbance due to NADPH oxidation was monitored and was indicative of GPx activity [40].

After making the reaction volumes, the mixture was vortexed at room temperature, incubated at 37°C for 15 min in a water bath. The activity of the samples was enhanced by adding 5% TCA. The samples were then centrifuged at 3,000 rpm for 5 min. The supernatant was collected and transferred into 96-well plates and an ELISA plate reader was used as described previously [39].

Histopathology determination: Sections of the brain and heart tissue blocks of each rat were analyzed according to a systematic random embedding, random sectioning and sampling method [29, 41]. Microscopic changes were assessed using light microscopy and described descriptively.

Data analysis

Quantitative data was generally being expressed as mean ± SD using Graph Pad Prism Version 6. ANOVA was conducted to determine group differences and this was followed by a Turkey's test to determine sources of variation against within experimental groups. Data from the histological analysis was summarized and presented in paragraphs. Photographs from some samples were also included.

Results

Calcium in brain and heart tissues following grain amaranth administration

In the brain and the heart, mean calcium concentration was found to be 0.60 ± 0.31 mg/dL and 0.60 ± 0.21 mg/dL as well as 0.41 ± 0.25 mg/dL under low and 0.38 ± 0.16 mg/dL under grain amaranth supplementation at low (25%) and high (50%) concentration respectively. No significant differences were seen ($p>0.05$) in the brain samples from individual groups; however, significant differences were shown to exist (Table 2) and this was mainly due to the GA supplementation administration groups as shown in Table 1.

S100A1 protein levels in brain and heart tissues

High levels of s100a1 proteins were seen in the brain during DM higher than those in the control group. Grain amaranth significantly lowered levels of s100a1proteins in the brain at higher concentrations (Table 2). On the other hand, levels of s100a1 proteins in the heart were too low in DM while these were elevated following grain amaranth supplementation as shown in Figure 1.

Table 1: Mean calcium concentrations in brain and heart tissues in male Wistar rats.

Experimental groups	N	Mean ± SD mg/dL	
		Brain	Heart
DM + Mixtard®	5	0.96 ± 0.52^{a}	1.01 ± 0.34^{a}
Positive control	5	0.78 ± 0.39^{a}	1.35 ± 0.36^{b}
DM + 25% GA	5	0.60 ± 0.31^{a}	0.41 ± 0.25^{c}
DM + 50% GA	5	0.60 ± 0.21^{a}	0.38 ± 0.16^{c}
Normal + 50% GA	5	0.68 ± 0.32^{a}	0.66 ± 0.21^{a}
Negative control	5	0.84 ± 0.31^{a}	0.65 ± 0.32^{c}

Tukey's multiple comparison's test conducted on brain and heart samples for each tissue amongst their experimental groups. Different superscripts (a, b, c) indicate $p<0.05$; DM, diabetes mellitus; GA, grain amaranths.

Malondialdehyde and glutathione peroxidase levels in brain and heart tissues

In both the brain and cardiac tissues, levels of MDA were elevated and this was associated with low GPx content in the positive control and this was characteristic of DM. Grain amaranth feeds supplementation in diabetic rats was associated with elevated GPx levels and low MDA levels (Figure 2). Furthermore, no significant differences ($p>0.05$) were found in MDA content, while significant differences ($p<0.05$) were associated with GPx content between the normal rats on 50% amaranth and the negative control as shown in Table 2.

Structural changes in the brain and heart tissue following grain amaranth administration

Severe vacouations were seen in the brain tissue of the rats of the positive control and these vacouations were widely distributed in the brain tissue of the cerebral cortex. Treatment with Mixtard® and 25% GA supplement led to mild vacouations in the neural tissue in the presence of DM. No vacouations were associated with DM + 50% GA as wells in the normal rats without GA as shown in Figure 3.

Mild myocardial atrophy in the positive control was detected though no significant lesions were in DM + GA as well as the nondiabetic rats as shown in Figure 4.

Discussion

The study showed that calcium deregulation in the tissues is a hallmark of brain–heart pathology during DM and this was in agreement with previous findings [1]. An onset of tissue pathology would lead to reduced neural [33] and cardiac function [11, 12]. Bearing in mind that grain amaranth has a high calcium content [24, 25], findings in this study show that the brain and heart tissues can metabolize calcium better under feed supplementation, especially following GA feed processing which has been associated with increased on nutrient bioavailability [28]. However, relatively higher levels of calcium were seen in the brain of the DM untreated group (positive control)

Table 2: p-Values showing multiple comparisons in experimental groups in brain and heart tissues for calcium, s100a1, MDA and GPx.

Group comparisons	Brain	Heart	Brain	Heart	Brain	Heart	Brain	Heart
	Calcium		S100a1		MDA		GPx	
DM + mixtard vs. positive control	0.9699	0.4961	0.9997	<0.0001	0.1020	<0.0001	0.0002	0.0744
DM + mixtard vs. DM + 25% GA	0.5097	0.0092	<0.0001	<0.0001	0.0422	0.5122	0.9569	0.3976
DM + mixtard vs. DM + 50% GA	0.5176	0.0061	<0.0001	0.9790	0.0002	<0.0001	0.0017	<0.0001
DM + mixtard vs. normal + 50% GA	0.7544	0.3208	0.0026	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
DM + mixtard vs. negative control	0.9929	0.2844	0.0006	0.5350	<0.0001	<0.0001	<0.0001	<0.0001
Positive control vs. DM + 25% GA	0.9659	0.0006	<0.0001	0.0006	0.0002	<0.0001	<0.0001	0.0020
Positive control vs. DM + 50% GA	0.9679	0.0004	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Positive control vs. normal + 50% GA	0.9978	0.0213	0.0055	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Positive control vs. negative control	0.9998	0.0183	0.0012	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
DM + 25% GA vs. DM + 50% GA	>0.9999	>0.9999	0.0606	<0.0001	0.4485	0.0044	0.0245	0.0111
DM + 25% GA vs. normal + 50% GA	0.9985	0.6412	0.0026	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
DM + 25% GA vs. negative control	0.8353	0.6881	0.0113	<0.0001	<0.0001	<0.0001	<0.0001	0.0003
DM + 50% GA vs. normal + 50% GA	0.9987	0.5419	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
DM + 50% GA vs. negative control	0.8414	0.5896	<0.0001	0.9144	0.0011	0.0076	0.0019	0.3218
Normal + 50% GA vs. negative control	0.9665	>0.9999	0.9927	<0.0001	0.0629	0.3808	0.0179	<0.0001

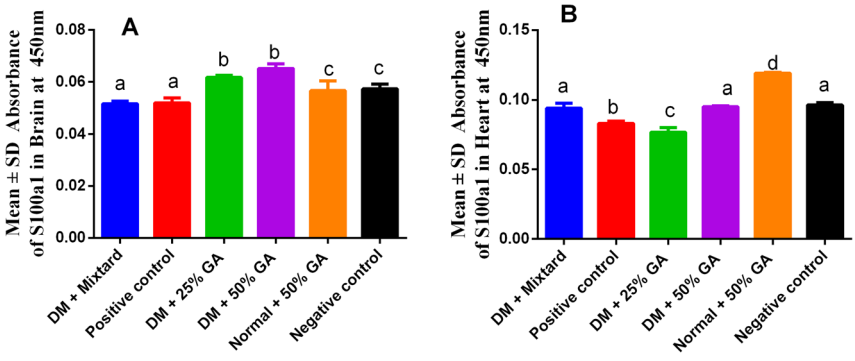

Figure 1: Diabetes was associated with low s100a1 levels in the brain while grain amaranth feed supplementation increased its expression in the brain (A) and the heart (B). Under physiological situations, grain amaranth feed supplementation was associated with increased s100a1 protein levels in the brain-heart axis.

comparable to the negative control (healthy animals). This may be explained by the fact that the brain needs Ca^{2+} for neurotransmitter release and in DM, neural Ca^{2+} overload in the cytosol occurs [2, 42] and this was similar to our findings in the positive control. This is important since elevated calcium levels are associated with brain lesions [43], thus suggesting the protective effects of GA in this particular study. Neural adaptation is triggered by calcium signals [44], and in synaptic activity Ca^{2+} signals for the release of neurotransmitters [45]. Improved calcium homeostasis is associated with improved glucose metabolism in the cells [12] since failure of the brain-centered glucoregulatory system (BCGS) is responsible for the development of DM in neural tissue [42], demonstrating the importance of GA in this study. Increased calcium metabolism was also associated with increased s100a1 protein content in the tissues following grain amaranth supplementation in DM. These findings suggest that grain amaranth actions in heart and brain tissues are synergistic to increased s100a1 protein expression, thus rescuing tissues from calcium deregulation, and this was in agreement with our previous findings [29], showing that observations in the study would be generalized to other body organs. This would subsequently lead to improved calcium transport in and out of the affected tissues, thus improving the prognosis of affected tissues [16–18]. This offers the basis for its rapidly growing international reputation as an ethnomedicinal plant in the management in DM [30, 46, 47] since improved cellular calcium metabolism would lead to balanced ATP production [9, 10].

The antioxidant activity in DM was enhanced by grain amaranth feed supplementation and this was dose-dependent. Findings in the study are in agreement with previous studies which have shown that grain amaranth has high antioxidant status [22, 23], thus leading to preservation of tissue calcium handling proteins such as the s100a1 proteins. This synergistic action is essential to guarantee tissue function in affected tissues in DM, thus

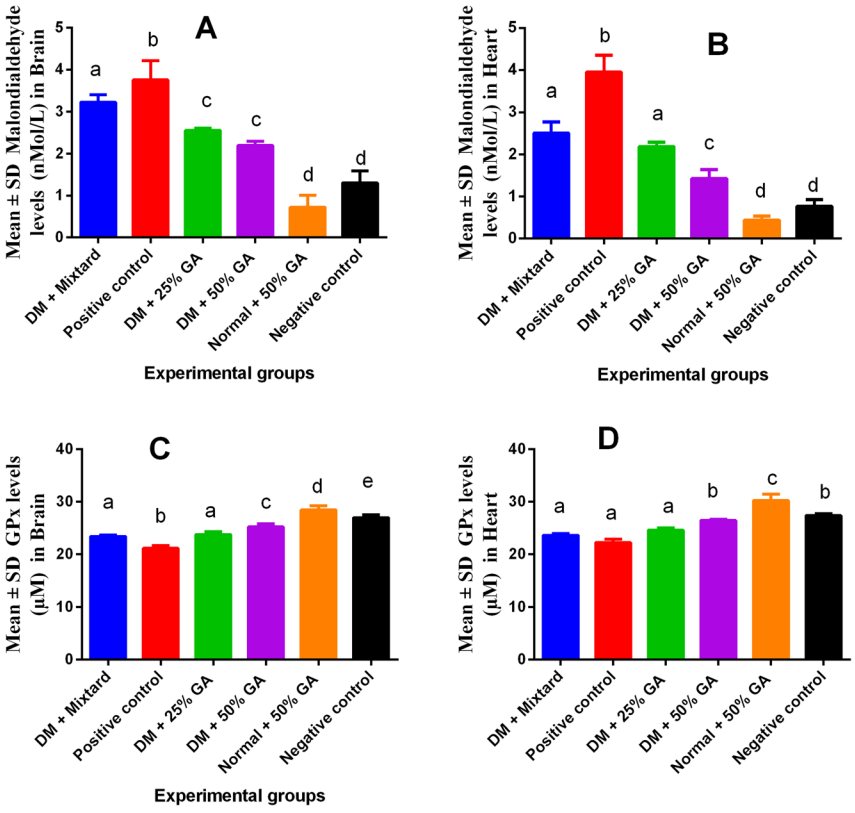

Figure 2: Diabetes led to an increase in malondialdehyde (MDA) in both the brain (A) and the heart (B), while grain amaranth feed supplementation reduced these levels. On the contrary, glutathione (GPx) levels increased with grain amaranth (C) and (D) demonstrating the strong antioxidant status in grain amaranth which helps to offer tissue protection in diabetes.

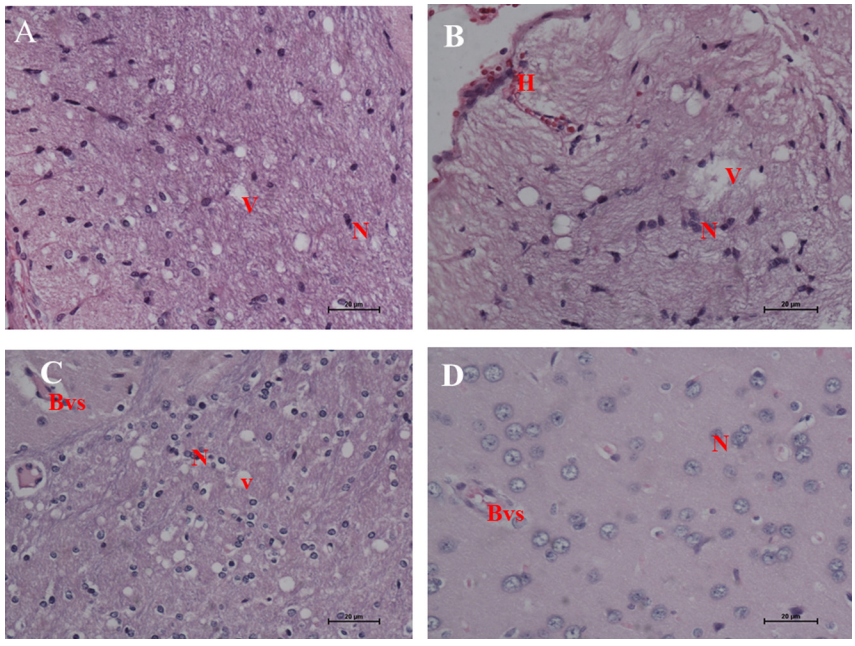

Figure 3: Neurological changes following grain amaranth supplementation in diabetic wistar rats. Photomicrographs in experimental animals showing histological lesions in the brain. A = treatment with Mixtard® in diabetes; B = positive control; C = 25% Grain amaranth with DM; D = Negative control. Bvs = blood vessel; N = Nuclei of cell body; V = Vacoulation in neural tissue; H = hemorrhage. Diabetes was associated with severe vacoulations (A) and the severity of the vacoulations reduced in diabetic rats under grain amaranth feed supplementation.

showing the relevance of grain amaranth in neuro-cardiology. Findings in the study re-emphasis the ability of vegetables to affect key cellular functions [30] and in particular, improved antioxidant activity during DM, which is crucial for improved gene expression. Furthermore, pathological lesions in DM rats were minimized as compared to the positive control in both the brain. In the cerebral cortex, there was diffuse tissue degeneration in DM which was associated with the high calcium levels, and these effects were reduced by insulin therapy and GA supplementation. It has been shown that DM leads to cerebral cortex degeneration leading to electro-physical

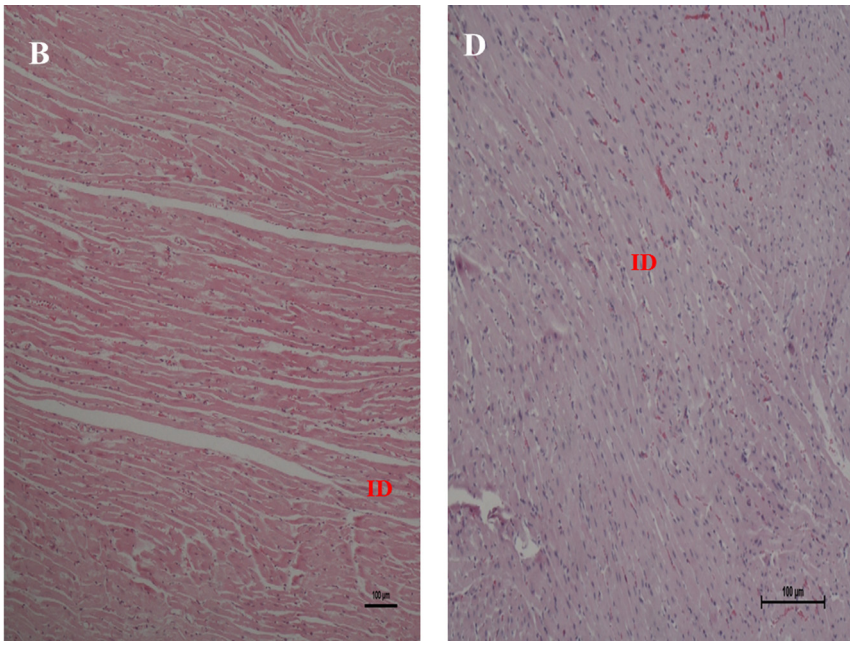

Figure 4: Myocardial changes following grain amaranth supplementation in DM wistar rats. Photomicrographs in experimental animals showing histological lesions in myocardium. B = positive control and D is the negative control. Myocardial atrophy was seen in the diabetic group (B). No significant changes were seen in the negative control and diabetic rats supplemented with grain amaranth demonstrating the protective effects in the myocardium.

(including calcium) and structural properties dysregulation [48]. GA due to its physio-chemical and phytochemical properties helps to replenish the tissue thus improving on its architecture. This subsequently leads to improved neural function due to improved tissue protection [49]. Cardiac tissue improved following GA supplementation due to its antioxidant properties. Tissue protection offered by the increased antioxidant action was able to protect affected tissues, thus improving on their prognosis, which is in agreement with previous findings [22–27]. Primary observations from our study indicate that improved calcium signaling is central to the management of DM due to improved physiological function in the brain–heart axis, thus offering a rationale for the community usage of grain amaranth in DM.

Conclusion

Grain amaranth was associated with increased s100a1 proteins and improved calcium levels in the brain–heart axis during DM. Increased tissue proteins were further protected by the increased antioxidant activity, thus leading to an improved prognosis in the brain–heart axis during DM. Prospective studies on other secondary messengers would yield more information that would guide therapy since these were beyond the scope of the current study.

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Author contributions: KIK, DN, AAS, MBV designed the study; KIK, HIN, KM, AAS, EO, FS conducted data acquisition, analysis while KIK, DN, HIN, JK, KM, AAS, EO, FS, AOO, MBV interpretation of data for the work; KIK drafted the work and KIK, DN, HIN, JK, KM, AAS, EO, FS, AOO, MBV revised it critically for important intellectual content. Approved final version to be published and are in agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy and integrity of any part of the work are appropriately investigated and resolved.

Competing interests: Authors state no conflict of interest.

Ethical approval: This was acquired from the Kampala International University Research and Scientific Review Board. The research related to animals' use has complied with all the relevant national regulations and institutional policies in Uganda for the care and use of animals.

Data availability statement: Data files used in the study can be found at https://figshare.com/s/6ebdf31242751b8eb726.

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