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Liver mitochondrial membrane permeability modulation in insulin-resistant, uninephrectomised male rats by *Clerodendrum volubile* P. Beauv and *Manihot esculenta* Crantz

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Abstract

Background: Non-alcoholic fatty liver disease, which occurs in people who are not alcohol drinkers, describes some of the pathogenic conditions that may be in the least characterized by simple steatosis or can be as serious as non-alcoholic steatohepatitis and cirrhosis. Its mechanistic pathogenesis has been said to arise from insulin resistance and oxidative stress, which may be compounded by obesity. An experimental model showing, systemic insulin resistance, obesity and accumulated hepatic fatty acids was created in adult male rats using high-fat diet manipulation and surgical removal of the left kidney (uninephrectomy). This study sought to identify the impact of these multiple burdens on the liver mitochondrial membrane permeability transition pore opening, and the possible in vitro effects of the extracts of *Clerodendrum volubile* and *Manihot esculenta* leaves on the membrane permeabilization.

Results: The results indicated that the methanolic extract of *Clerodendrum volubile* leaf inhibited mitochondrial membrane pore opening in the insulin resistance condition or when it is followed by uni-nephrectomy, while the ethanolic extract of *Manihot esculenta* leaf does the same in the insulin resistance condition both prior to and following uni-nephrectomy.

Conclusion: Since the vegetable extracts were able to abrogate mitochondrial pore opening at low concentrations, the structural integrity of the mitochondria can possibly be restored over time if treated by the vegetable extracts. Research efforts should, therefore, be made to harness the drugability of the bioactives of these vegetables for use in the treatment of non-alcoholic fatty liver disease arising from insulin resistance and renal failure.

Keywords: Insulin resistance, Uninephrectomy, Non-alcoholic fatty liver disease, Mitochondria, Natural products

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Introduction

Multiple burden diseases including diabetes, obesity, inflammation, and infection are often consequences of reduced liver functions [1, 2]. It is widely known that a reduction in liver function is a result of the bidirectional relationships in the course of the development of these diseases [2, 3]. Evidence from established studies in both humans and animal models suggest that an immediate reduction in liver functions will likely contribute to metabolism dysfunction, inflammation and oxidative stress [4] and thus the development of the disease. The non-alcoholic fatty liver disease has been strongly linked to metabolic syndrome [5], and together they are associated with a state of chronic low-level inflammation and stress [6]. It encompasses a wide spectrum of pathological conditions, ranging from simple steatosis to non-alcoholic steatohepatitis and cirrhosis [7]. Non-alcoholic steatohepatitis is the most prevalent cause of chronic liver disease in the Western world and has no approved treatment [8]. Ischemic hepatopathy, a condition of acute liver injury caused by insufficient blood flow due to cardiogenic shock (*arrising from* the leftsided failure of the heart), or other hemodynamic collapses, may lead to massive hepatocellular necrosis [9, 10]. The situation arising from sinusoidal stasis leads to cell death among other clinical manifestations [11] [12]. Mitochondrial dysfunction and oxidative stress associated with metabolic syndrome have been proposed as major pathophysiological disturbances that contribute to these pathologies [13, 14].

Mitochondrial dysfunction can cause ATP depletion and overproduction of reactive oxygen species (ROS), resulting in protein and lipid oxidation, oxidative damage to injured tissues, and loss of ATP characterize typical events of cell death [15]. The mitochondrial membrane potential dissipates, and the capacity of the organelle to accumulate Ca^{2+} becomes abrogated as a consequence of the inhibition of the respiratory chain. The molecular manipulation of the mitochondrial pathways has been embraced as a convenient way to investigate mitochondrial functional integrity [16]. Although mitochondria abundance is not a concern for functional studies, the certainty of protein purity and structural integrity are important barriers that can encumber mitochondrial research [17]. Liver morphological and ultrastructural integrity may be of concern among kidney donors who suffer from non-alcoholic fatty liver disease [18, 19].

The mitochondrial membrane permeability transition (MMPT) represents a sudden increase of inner mitochondrial membrane permeability to solutes with molecular mass up to 1500 Da. The MMPT pore is as a result of the opening of a voltage- and Ca^{2+}-dependent, spermine-sensitive, high-conductance protein channel [15, 20]. In its fully open state, the apparent diameter of the MMPT pore is about 3 nm. When mitochondrial swelling is prolonged, the membrane potential dissipates as a result of equilibration of the proton gradient across the inner mitochondrial membrane of ions, following the further massive release of the Ca^{2+} stored in the matrix consequent upon the colloidal osmotic pressure exerted by the elevated concentration of matrix proteins [21]. However, it is imperative to note that permeability transition pore opening can induce the outer mitochondrial membrane rupture only through the matrix swelling and therefore cytochrome c and the other apoptogenic molecules do not exit mitochondria through the permeability transition pore itself [22].

The permeability transition-based model for the outer mitochondrial membrane permeabilization is supported by a number of observations, among which are studies with experimental models showing evidence of the collapse of membrane potential before caspase activation [23, 24]; the protective potential of specific inhibitors against pore opening and several other apoptotic responses [25].

Experimentally, mitochondria swelling can be easily investigated by light scattering measurements [26]. Also, when mitochondria swell, their refraction index decreases, thereby decreasing the intensity of scattered light. The correlation between the amount of light scattered by a mitochondrial suspension and the volume of the mitochondrial matrix was extensively exploited for qualitative studies of solute transport across the inner membrane. The mitochondrial swelling, due to the matrix permeation to external solutes, can be detected as the decrease in the light scattering of the mitochondrial suspension at 540 nm [21, 22]. *Percentage swelling was calculated based on the optical density decrease between non-treated and 20 μM Ca2+ or 200–1400 μg/ml extract treated mitochondria.*

Clerodendrum volubile P. Beauv belongs to the family Lamiaceae. It is found growing in many deciduous forests across Africa [23]. It is generally known among the Urhobo and Itsekiri tribes of the Niger-Delta of Nigeria as 'Obenetete', and among the Yorubas in Ondo state as 'marugbo' [24, 25]. In Nigeria, notably, the southern area, among the Ijaws, Urhobos and Itsekiris, the leaf is consumed as a delicacy in foods. In folklore medicine, the plant is useful for the treatment of several ailments such as diabetes, rheumatism, arthritis, edema and gout [26, 27]. The reported pharmacological properties of *C. volubile* leaf includes anti-inflammatory [27], antioxidants [28, 29], antihypertensive [25, 30], antidiabetic [31, 32], neuroprotective [33], hepatoprotective [34, 35] and cancer chemopreventive [36, 37] activities. In a recent study by Erukainure et al. [38], the antidiabetic effect of acute treatment with the ethyl acetate fraction of *C. volubile* leaves was investigated and this property was attributed to the protocatechuic acid which was observed to be the active

compound of the plant. The authors further reported that this fraction suppressed hyperglycemia in type 2 diabetic rat via synergetic attenuation of phagocytic oxidative burst as well as molecular interactions with α-glucosidase and TNF-α [38]. This plant is often termed the magic leaf owing to its wide use in folklore medicine to treat several diseases especially diabetes mellitus. Several polyphenolic compounds (flavonoids and phenolic acids) have been reported [25, 29, 30, 32] to be present in *C. volubile* leaves which has been shown to confer these medicinal and health benefits of this plant.

Manihot esculenta Crantz, an edible rainforest plant is a perennial crop native to tropical America with its centre of origin in North-eastern and Central Brazil [39, 40]. Its common names include cassava, yuca, tapioca, manioc, Brazilian arrowroot, dang noi, man sum palung, pearks sakhoo, huacamote, gbaguda, paki, and ege. The leaves have also been used against many disorders such as rheumatism, fever, headache, diarrhea and loss of appetite [41]. The leaves also possess anti-inflammatory, antipyretic, anti-diarrhoeal and antihaemorrhoid activities [42]. The seed oil is found to have antimicrobial activity [43]. The leaves of the bitter variety of cassava are used to treat hypertension, headache, and pain [44]. The Cubans commonly use cassava to treat irritable bowel syndrome by eating the paste in excess during treatment [45]. As cassava is a gluten-free, natural starch, it is embraced in Western cuisine as a wheat alternative for sufferers of celiac disease [46]. The leaves have also been shown to have antioxidant properties and hypoglycemic effect and the ability to close the mitochondrial membrane permeability transitionn pore in high-fat diet-manipulated, low-dose STZ-induced diabetic male Sprague-Dawley rats [47]. A few researchers have reported the antioxidant activities of cassava tubers including Omar et al. [48], who showed that the antioxidant activities of organically grown cassava tubers were higher than those of mineral-base fertilized roots. They found that total phenolic and flavonoid contents were significantly higher for organic cassava tubers compared to those grown with inorganic fertilizers. Increased intake of cassava leaves in diet has also been reported to decrease the risk of metabolic syndrome in type 2 diabetic patients [49]. Topical application of the ethanolic extract of Manihot esculenta to wound area on type 1 diabetic rats was shown to have little effect on collagenation, re-epithelization and granulation resulting in no significant effect on wound closure rate compared to Povidone Iodine [50].

Both plants are used as delicacies and in traditional medicine and interestingly, certain bioactive compounds have been identified in these vegetables. The possible medicinal effects of the plant leaves were studied in our animal model to see how they would impact MMPT as they possess known anti-inflammatory, antioxidant, antihypertensive, antidiabetic, neuroprotective, hepatoprotective and cancer chemopreventive activities. Therefore, owing to the pharmacological potentials of these two plants (*C. volubile* and *M. esculenta*) as used in folklore medicine in treating several diseases especially diabetes, we hypothesized that the plants may have the ability to inhibit liver MMPT pore opening should the liver be exposed to damage following uni-nephrectomy in the rats, a response that would be important in diabetes for the prevention of cell death primarily via the mitochondrial pathway, and tissue wasting from oxidative stress. We seek to investigate the effects of uni-nephrectomy on mitochondrial membrane permeability transition pore status of control and insulin-resistant rats and determine if their liver mitochondria can still take up Ca^{2+} and retain their morphological integrity.

Materials and methods

Plant materials and sample preparation

Matured leaves of *Clerodendrum volubile* P. Beauv were purchased at the Oja-Oba Market, Akure, Ondo State, Nigeria purchased, authenticated and assigned the voucher specimen number FUTA/BIO/0121. The leaves of red stalk *Manihot esculenta* (Crantz) were collected at the botanical garden of the National Institute of Pharmaceutical Education and Research, Mohali, Punjab, India, and given the voucher specimen number NIPER-S/NPTM/0298. The leaves were air-dried and pulverized and 50 g of each was soaked in 500 ml of 95% methanol and ethanol, respectively for 24 h. The extracts were concentrated under pressure using a rotavapor. The resulting crude extracts were used to assess the extent of mitochondrial membrane permeability transition in the liver of insulin resistant, uni-nephrectomized male rats (Figs. 1 and 2).

Chemicals

Mannitol, sucrose, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), sodium succinate, and other chemicals were products of Sigma Chemical Co. (St. Louis, MO USA). Except otherwise stated, all other solvents used were of analytical grade.

Experimental animals

Twenty-four Sprague-Dawley rats weighing 180–250 g were used for the experiment. All animals were maintained under standard conditions. The control groups were fed rodent normal pellet diet ad libitum, while test groups manipulated for T2DM were fed on high-fat diet. All experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC 12/11). The animals were allowed access to water during a fast for

12 h before sacrifice by decapitation. Experiments were conducted using 5 animals per group.

Experimental design

The experimental animals were randomly divided into six groups: (1) a control non-nephrectomized group fed normal pellet diet; (2) a uni-nephrectomized group fed normal diet; (3) a non-nephrectomized 'sham' group fed normal pellet diet (surgically opened, but no ligations and excision of kidneys were performed); (4) a non-nephrectomized group fed high fat diet; (5) a uni-nephrectomized group fed high-fat diet; (6) a high-fat-diet group subsequently uni-nephrectomized at week 9. High-fat diet – HFD (58% fat, 25% protein and 17% carbohydrate, as a percentage of total kcal) was given to animals in groups 4–6 ad libitum throughout the study that lasted 18 weeks (except Group 5 animals, which commenced HFD feeding immediately after uni-nephrectomy). This serves as a model for obesity and insulin resistance [51].

The surgical procedure of uni-nephrectomy

The rats underwent uni-nephrectomy of the left kidney in an aseptic condition, under ketamine (70 mg/kg, i. p.)/ xylazine (7 mg/kg, i. p.) anesthesia, before injection with saline (20 ml/kg, s.c.) according to the method described by Holte et al. [52] with slight modifications [53]. Briefly, a 2 cm flank incision penetrating the abdominal cavity was made after the area had been cleaned and shaved. Using forceps, the kidneys were gently pulled out by holding the perirenal fat and the renal artery, vein, and ureter tied by a non-absorbable thread. The left kidney was then excised and the muscular layer sutured with absorbable suture thread, while the skin layer was closed with non-absorbable thread. The animals were left in a solitary cage under the heat of 60 W lamp till recovery from anesthesia.

Isolation of rat liver mitochondria

The liver mitochondrial fraction was prepared according to the method described by Hogeboom et al. [54] and Johnson and Lardy [55] with slight modifications. Rat liver was weighed and washed in isolation buffer containing 210 mM mannitol, 70 mM sucrose, and 1 mM EGTA in 5 mM HEPES-KOH, pH 7.4. A 50% suspension was prepared by homogenizing the liver in a glass-Teflon homogenizer. The homogenate was centrifuged at 2300×g for 5 min twice in a SIGMA-6 K15 refrigerated centrifuge to sediment the nuclear fraction and cell debris and to remove unbroken cells by low-speed centrifugation. The supernatant was centrifuged at 13000×g for 10 min to pellet the mitochondria. The brown mitochondria pellet was washed twice by re-suspending in isolation buffer containing 0.5% BSA and centrifuged at 12000×g for 10 min. Mitochondria were suspended in buffer containing 210 mM mannitol, 70 mM sucrose, 5 mM HEPES-KOH, pH 7.4 and 0.5 mM KH_2PO_4 and dispensed in microcentrifuge tubes kept on ice.

Protein content determination

Protein content was determined using the method of Lowry et al. [56] using bovine serum albumin as standard.

Procedure for incubation of mitochondria

One mg/ml liver mitochondrial protein was incubated with the assay buffer containing 210 mM mannitol, 70 mM sucrose in 5 mM HEPES-KOH, pH 7.4. The incubation of mitochondria was done with 20 μM rotenone for 3 min (to block the Complex I and limit the source of electron supply to the electron transport system to Complex II) prior to the addition of 12 mM $CaCl_2$, 30 s after which the assay was energized by 50 mM sodium succinate [57].

Assessment of mitochondrial permeability transition

Mitochondrial swelling as the indicator of MMPT was spectrophotometrically measured by continuous time scan of the change in absorbance at 540 nm for 12 min [58]. *Percentage swelling was calculated based on the optical density decrease between non-treated and 20 μM Ca2+ or 200–1400 μg/ml extract treated mitochondria.*

Transmission electron microscopy (TEM) of mitochondria

For TEM (FEI Tecnai G2 F20, Thermo Scientific™, USA), mitochondria pellets were recovered from – 80 °C freezer and dispersed in 20% DMSO containing 1% FAF-BSA. Mitochondria were fixed in 2.5% glutaraldehyde prepared in sodium phosphate buffer (pH 7.4, 1:1 v/v) and kept at 4 °C overnight. This was washed with 500 μl sodium phosphate buffer and spun at 7000×g for 10 min. Stepwise dehydration was achieved using 20%, 40%, 70% and 90% ethanol while spinning at the same speed each time. Final dehydration was done with 100% ethanol at 4 °C over 15 min. The mitochondria pellet was dispersed in the embedding medium (1:3, 2:2, 3:1 and 4:0, v/v resin: ethanol) for 15 min, respectively spinning each time as with the dehydration steps. At absolute resin embedding, the spur was incubated at 65 °C overnight to achieve solidification. The block was then removed for processing by tissue sectioning to 60 nm using a glass knife on an RMC Boekeler PT-PC PowerTome ultramicrotome. The ultra-thin sections were mounted on Pioloform filmed copper grids prior to staining with 4% uranyl acetate prepared in 50% methanol. Ultrathin sections were examined using FEI Tecnai G2 F20 transmission electron microscope at 120 KV, and digital micrographs were captured by a Gatan CCD camera.

Statistical analysis

The results were presented in the form of percentages. The data were analyzed by ANOVA (SPSS 20) at a significance level of *p*-value less than 0.05 and if significant, the comparisons between the groups were performed using Tukey's post hoc test.

Results and discussion

The degree of Ca^{2+} uptake (percentage induction of the pore opening) exhibited by the mitochondria significantly varied across the groups (Table 1; Additional files 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12). Methanolic extract of *Clerodendrum volubile* leaf induced opening in normal pellet diet-fed, uni-nephrectomized rats (Additional file 3) at 200 μg/ml, and in high-fat-diet-fed at 200 μg/ml, 600 μg/ml, and 1000 μg/ml, but showed late-onset opening around 15 s at 1400 μg/ml (Additional file 4). Similarly, ethanolic extract of *Manihot esculenta* leaf induced pore opening in high-fat-diet-fed rats at 200 μg/ml and 600 μg/ml (Additional file 10) and in uni-nephrectomized, high-fat-diet-fed only at 1400 μg/ml (Additional file 11). These observations show that the crude vegetable extracts of these edible plants are capable of modulating mitochondrial pore opening in rat liver.

From the TEM images, the observations in the differences in the architectural ultrastructure of the double membranes were compared to that of the mitochondria in the apparently normal control group. Following similar preparation steps for each sample, same volume of mitochondria pellets were fixed, ultra-thin slices of the same size were prepared, stained and the images were taken at the same resolution to make comparison easy. Simple observation of the TEM images showed the state of both the inner and outer mitochondrial membranes. The modulation of pore opening by the extracts followed a concentration-dependence, revealing which concentration may be considered efficient in preserving the mitochondrial against possible oxidative damage, even if the native state was not recovered. The TEM images revealed the morphological changes of the mitochondrial population across the study groups (Fig. 3). These observations confirmed that high-fat diet lifestyle and/or uni-nephrectomy predispose to the development of non-alcoholic fatty liver disease, which invariably affects the structural integrity and functional capacity of liver mitochondria (Fig. 3: B-F). Following the mitochondrial isolation protocol, no whole cells or nuclei were seen by TEM and the mitochondria were essentially free of other contaminants. Only a small membranous artifact attributable to microsomes was observed. Across the treated groups, the isolated mitochondria population displayed morphological signs of damage to differing degrees. The observations from the in vitro study support the hypothesis that the extracts may be able to reverse damages caused by the development of non-alcoholic fatty liver disease in insulin-resistance with or without nephrectomy.

A low-amplitude swelling was observed in the energized mitochondria by the extracts in the presence of exogenous $CaCl_2$. The ability of *Clerodendrum volubile* leaf extract to abrogate the progression of mitochondrial membrane depolarization and leakage only in uni-nephrectomized normal rats at high concentrations, and in insulin-resistant, uni-nephrectomized rats at all concentrations suggests its protective role against complications that may arise from nephrectomy in normal and previously insulin-resistant conditions before kidney donation or damage. This observation is similar to the submissions of [59] that insulin

Table 1 Percentage induction of mitochondrial membrane pore opening in rat liver mitochondria incubated with methanolic extract of *Clerodendrum volubile* leaf and ethanolic extract of *Manihot esculenta* leaf, *in vitro*

% Induction	NPD-C		NPD-UNX		HFD-Sham		HFD		UNX-HFD		HFD-UNX	
	Cv	Me	Cv	Me	Cv	Me	Cv	Me	Cv	Me	Cv	Me
NTA	13.98 ± 6.53	13.98 ± 6.53	23.49 ± 8.30	23.49 ± 8.30	26.38 ± 7.30	26.38 ± 7.30	23.59 ± 15.58	23.59 ± 15.58	3.10 ± 0.80	3.10 ± 0.80	50.55 ± 5.87	50.55 ± 5.87
TA	[a]86.02 ± 10.75	[a]86.02 ± 10.75	[a]76.51 ± 19.85	[a]76.51 ± 19.85	a73.62 ± 10.05	[a]73.62 ± 10.05	[a]76.41 ± 15.71	[a]76.41 ± 15.71	[a]96.90 ± 14.36	a96.90 ± 14.36	49.45 ± 13.73	49.45 ± 13.73
200 μg/ml	[b]30.19 ± 6.15	[b]16.36 ± 9.66	[b]96.64 ± 10.60	[b]158.24 ± 14.71	[b]84.64 ± 10.22	[b]44.34 ± 11.73	[b]195.23 ± 24.72	[b]207.58 ± 27.08	[b]44.34 ± 15.91	[b]41.82 ± 14.54	[b]65.56 ± 10.00	[b]53.00 ± 10.17
600 μg/ml	[b]37.38 ± 16.06	[b]9.10 ± 1.94	[b]38.43 ± 14.30	[b]70.31 ± 12.09	[b]217.93 ± 22.51	[b]11.87 ± 1.77	[b]225.84 ± 24.74	[b]63.57 ± 9.79	[b]11.87 ± 7.75	[b]80.47 ± 10.07	[b]46.69 ± 9.13	[b]38.54 ± 7.24
1000 μg/ml	[b]45.68 ± 16.45	[b]8.74 ± 2.97	[b]23.49 ± 9.99	[b]78.73 ± 16.80	[b]175.79 ± 22.91	[b]32.57 ± 16.97	[b]221.52 ± 28.46	[b]75.18 ± 11.45	[b]32.57 ± 14.05	[b]67.16 ± 17.82	[b]142.46 ± 17.47	[b]185.85 ± 23.59
1400 μg/ml	[b]37.26 ± 16.13	[b]53.08 ± 18.96	[b]14.19 ± 6.15	[b]143.88 ± 13.63	[b]47.27 ± 14.42	[b]17.13 ± 8.06	[b]131.38 ± 18.61	[b]148.04 ± 17.21	[b]17.13 ± 3.52	[b]133.85 ± 17.29	[b]120.22 ± 15.54	[b]129.78 ± 16.94

Cv Clerodendrum volubile P. Beauv, *Me Manihot esculenta* Crantz (red stalk), *NPD-C* Normal Pellet Diet-fed, Control, non-nephrectomized, *NPD-UNX* Normal Pellet Diet-fed, Uni-nephrectomized, *HFD-Sham* High Fat Diet-fed, surgically opened, non-nephrectomized, *HFD* High Fat Diet-fed, uni-nephrectomized, *UNX-HFD* Uni-nephrectomized before High Fat Diet-fed, *HFD-UNX* High Fat Diet-fed before uninephrectomized, *NTA* No triggering agent, negative control (no treatment with exogenous calcium, only energized with sodium succinate), *TA* Triggering agent, positive control (treatment with exogenous calcium to represent calcium overload which triggers pore opening). [a]significantly different when compared with NTA effect across all groups at $p < 0.05$. [b]Significantly different from TA effect across all groups at $p< 0.05$

Fig. 1 *Clerodendrum volubile*. Molehin et al., 2017 [29]. Molehin OR, Oloyede OI, Boligon AA. Comparative study on the phenolic content,antioxidant properties and HPLC fingerprinting of the extracts of *Clerodendrum volubile*. P. Beauv. J App Pharm Sci. 2017a; 7:135–140

resistance (IR) predisposes to the development of chronic kidney disease (CKD) in non-diabetic patients, and those with mild-to-moderate stage CKD. It is also interesting that the aqueous extract of *C. volubile* leaves has earlier been identified to contain phytochemicals that exert antidiabetic effects in Wistar rats [32].

Inhibition of the mitochondrial membrane pore opening by the ethanolic extract of *Manihot esculenta*leaf was observed at intermediate concentrations in high-fat-diet-fed rat liver, at the lower concentrations in uni-nephrectomized,

Fig. 2 *Manihot esculenta* Crantz. From: Richard Wong Garden & Woodwork In: Poisonous Yet Nutritious [64]. (http://rwgardenwoodwork.blogspot.com/2017/07/)

high-fat-diet-fed rats, and at all the extract concentrations in the high-fat-diet-fed, uni-nephrectomized rats. These findings were consistent with the results of another study which revealed that the crude ethanolic extract of *Manihot esculenta* leaf inhibited mitochondrial pore opening in both normal and type 2 diabetic Sprague-Dawley rats, both in vivo and in vitro, which may be due to the significant antioxidant properties of the extract [47, 60]. These observations suggest that the protective role of *Manihot esculenta* leaf extract can be harnessed both when insulin-resistance develops following kidney donation or damage, and when there has been insulin resistance before nephrectomy.

As depicted from the TEM results (Fig. 3), it can be observed that high-fat diet resulted in the shrinking of mitochondria while nephrectomy resulted in the swelling of mitochondria. The latter event, which is characteristic of fatty liver, would ultimately lead to mitochondrial pore opening that will lead to the escape of calcium into the cytosol, marking the progression of cell suicide via the mitochondrial pathway. Previous research findings have established increased lipid peroxidation and decreased active biliary transport in hepatocytes following nephrectomy. This is usually a result of uremic toxins affecting liver cells, probably by competing at the hepatocellular level, with the endogenous substances, which are normally excreted in urine [61] Damage to the liver, a remote organ, has also been reported following renal failure, especially when insulin resistance is present [62]. Indeed, according to Filozof et al. [8], accumulation of fat in the liver exerts several cellular and metabolic effects that can, in the long run, culminate in steatohepatitis and fibrosis. These include a high preponderance of apoptotic cell death in hepatocytes, mitochondrial dysfunction characterized by the increased generation of reactive oxygen species, accompanied by lipid peroxidation of membranes structures as well as a cascade of activation of pro-inflammatory genes, which will further exacerbate the induction of several other inflammatory mediators involved in the development of liver fibrosis.

Conclusions

We showed uni-nephrectomy mimicked the situation of kidney donation, especially in the presence of insulin resistance developed by a life style of high fat diet. We also endeavored to see if there is/are any possible attending effects on the liver mitochondria architecture, by the two extracts on the mitochondrial membrane, in vitro by the simple swelling assay and ex vivo using TEM. As the vegetable extracts were able to abrogate mitochondrial pore opening at low concentrations, the structural integrity of the mitochondria can possibly be restored over time if treated by the vegetable extracts. This can be a good recommendation for insulin-resistant, living kidney

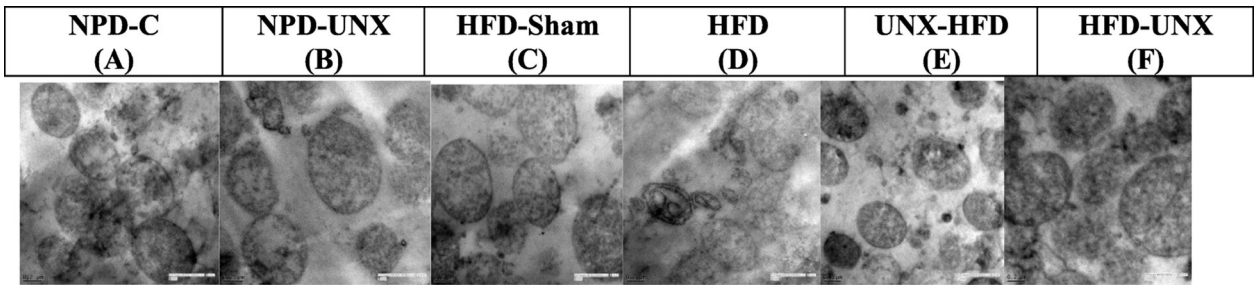

Fig. 3 TEM images of ultra-thin sections showing the morphological architecture of insulin resistant. uni- nephrectomized male rat liver mitochondria. **a** NPD-C: Normal Pellet Diet-fed,Control, non-nephrectomized; **b** NPD-UNX: Normal Pellet Diet-fed, Uni-nephrectomized; **c** HFD-Sham: High Fat Diet-fed, surgically opened, non-nephrectomized; **d** HFD: High Fat Dietfed,uni-nephrectomized; **e** UNX-HFD: Uni-nephrectomized before High Fat Diet-fed; **f** HFDUNX:High Fat Diet-fed before uni-nephrectomized. The TEM images are also shown in the samesection size (0.2 μm) and magnification (× 5000) using high resolution transmission electron microscope (HRTEM, 120 kV)

donors, who may be predisposed to non-alcoholic fatty liver disease following nephrectomy. Thus, the knowledge about the ability of these vegetable extracts to modulate mitochondrial pore opening which precedes the occurrence of cell death can be applied for useful drug development for the management of the complications of insulin-resistance, especially when kidney disease is co-morbidity as typified by nephrectomy [63].

Limitations

This study was carried out in vitro. The observations may not be extended to explain what might happen in vivo as the administration of the plant extract by oral gavage may show a different response in the animals.

Additional files

Additional file 1: Liver MMPT pore opening in the normal male rat using the methanolic extract of *Clerodendrum volubile leaf*, in vitro (TIF 7009 kb)

Additional file 2: Liver MMPT pore opening in 'Sham' male rat using the methanolic extract of *Clerodendrum volubile leaf*, in vitro (TIF 7438 kb)

Additional file 3: Liver MMPT pore opening in normal diet fed, uni-nephrectomized male rat using the methanolic extract of *Clerodendrum volubile leaf*, in vitro (TIF 7094 kb)

Additional file 4: Liver MMPT pore opening in high-fat diet fed male rat using the methanolic extract of *Clerodendrum volubile leaf*, in vitro (TIF 7363 kb)

Additional file 5: Liver MMPT pore opening in uni- nephrectomized, high fat diet fed male rat using methanolic extract of *Clerodendrum volubile leaf*, in vitro (TIF 7016 kb)

Additional file 6: Liver MMPT pore opening in high-fat diet fed uni-nephrectomized male rat using the methanolic extract of *Clerodendrum volubile leaf*, in vitro (TIF 7105 kb)

Additional file 7: Liver MMPT pore opening in the normal male rat using the ethanolic extract of *Manihot esculenta* leaf, in vitro (TIF 6993 kb)

Additional file 8: Liver MMPT pore opening in 'Sham' male rat using the ethanolic extract of *Manihot esculenta* leaf, in vitro (TIF 7499 kb)

Additional file 9: Liver MMPT pore opening in normal diet fed, uni-nephrectomized male rat using the ethanolic extract of *Manihot esculenta* leaf, in vitro (TIF 7294 kb)

Additional file 10: Liver MMPT pore opening in high-fat diet fed, male rat using the ethanolic extract of *Manihot esculenta* leaf, in vitro (TIF 7221 kb)

Additional file 11: Liver MMPT pore opening in uni- nephrectomized, high-fat diet fed, male rat using the ethanolic extract of *Manihot esculenta* leaf, in vitro (TIF 7265 kb)

Additional file 12: Liver MMPT pore opening in high-fat diet fed, in uni-nephrectomized male rat using the ethanolic extract of *Manihot esculenta* leaf, in vitro (TIF 7114 kb)

Abbreviations

ATP: Adenosine Triphosphate; BSA: bovine serum albumin; $CaCl_2$: Calcium chloride; CKD: chronic kidney disease; Cv and MLE: *Clerodendrum volubile* leaf extract; EGTA: Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′- tetraacetic acid; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HFD: high fat diet; HFD: High Fat Diet-fed, uni-nephrectomized; HFD-Sham: High Fat Diet-fed, surgically opened, non-nephrectomized; HFD-UNX: High Fat Diet-fed before uni- nephrectomized; KH_2PO_4: potassium hydroxide; KOH: potassium hydroxide; Me and CLE: *Manihot esculenta* leaf extract; MMPT: Mitochondrial Membrane Permeability Transition; NPD-C: Normal Pellet Diet-fed, Control, non-nephrectomized; NPD-UNX: Normal Pellet Diet-fed, Uni-nephrectomized; NTA: No triggering agent, negative control (no treatment with exogenous calcium, only energized with sodium succinate); ROS: reactive oxygen species; SPSS: Statistical Package for Social Sciences; STZ: Streptozotocin; T2DM: Type 2 diabetes mellitus; TA: Triggering agent, positive control (treatment with exogenous calcium to represent calcium overload which triggers pore opening); TEM: Transmission electron microscopy; TNF-α: Tumor necrosis factor alpha; Treatment groups: Extracts tested at different concentrations (200, 600, 1000 and 1400 μg/ml) for each group.; UNX-HFD: Uni-nephrectomized before High Fat Diet-fed

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Authors' contributions

AEIO conceived the study, and participated in its design and execution and helped to draft the manuscript; ORM participated in the design, coordination and helped to draft the manuscript; OIO participated in the coordination of the study; VK participated in the execution of the study; VRA participated in the execution of the study; JK participated in the execution of the study; PK participated in the execution of the study; KBT participated in the design and coordination of the study. All authors read and approved the final manuscript.

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Availability of data and materials
Please contact corresponding author for data requests.

Ethics approval and consent to participate
All experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC 12/11).

Consent for publication
All the authors contributed, agreed, submitted, revised and re-submitted this manuscript.

Competing interests
The authors declare that they have no competing interests.

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References
1. Rosário M, Isabel A. Chronic inflammation in obesity and the metabolic syndrome. Mediators Inflamm. 2010;2010:1–10. https://doi.org/10.1155/2010/289645.
2. Beicheng S, Michael K. Obesity, inflammation, and liver cancer. J Hepatol. 2012;56:704–13. https://doi.org/10.1016/j.jhep.2011.09.020.
3. Jadeja RN, Devkar RV, Srinivas N. Oxidative stress in liver diseases: pathogenesis, prevention, and therapeutics. Oxid Med Cell Longev. 2017; 2017:1–2. https://doi.org/10.1155/2017/8341286.
4. Karina R-G, Ruchi S, Pablo M. Oxidative stress and inflammation in hepatic diseases: current and future therapy. Oxid Med Cell Longev. 2017/2017:1–2. https://doi.org/10.1155/2017/3140673.
5. Galicia-Moreno M, Gutiérrez-Reyes G. Papel del estrés oxidativo en el desarrollo de la enfermedad hepática alcohólica. Rev Gastroenterol Mex. 2014;79:135–44. https://doi.org/10.1016/j.rgmx.2014.03.001.
6. de Samuele M, Brenner David A. Oxidative stress in alcoholic liver disease: role of NADPH oxidase complex. J Gastroenterol Hepatol. 2008;23:S98–S103. https://doi.org/10.1111/j.1440-1746.2007.05277.x.
7. Giovanni T, Day Christopher P, Enzo B. Risk of cardiovascular disease in patients with nonalcoholic fatty liver disease. N Engl J Med. 2010;363:1341–50. https://doi.org/10.1056/nejmra0912063.
8. Claudia F, Goldstein Barry J, Williams Richard N, Arun S. Non-alcoholic steatohepatitis: limited available treatment options but promising drugs in development and recent Progress towards a regulatory approval pathway. Drugs. 2015;75:1373–92. https://doi.org/10.1007/s40265-015-0437-3.
9. Monjur A. Non-alcoholic fatty liver disease in 2015. World J Hepatol. 2015;7: 1450. https://doi.org/10.4254/wjh.v7.i11.1450.
10. Paolo B. Mitochondrial transport of cations: channels, exchangers, and permeability transition. Physiol Rev. 1999;79:1127–55. https://doi.org/10.1152/physrev.1999.79.4.1127.
11. Alicia A, Debabrata M. Liver abnormalities in cardiac diseases and heart failure. Int J Angiol. 2011;20:135–42. https://doi.org/10.1055/s-0031-1284434.
12. Nikolaou M, Mebazaa A. Cardiohepatic interactions in heart failure: clinical and therapeutic implications. Continuing Cardiol Educ. 2017;3:117–20. https://doi.org/10.1002/cce2.63.
13. Samantha H, Hsu Walter H. The etiology of oxidative stress in insulin resistance. Biom J. 2017;40:257–62. https://doi.org/10.1016/j.bj.2017.06.007.
14. Francisqueti Fabiane Valentini, Universidade Estadual Paulista "júlio de Mesquita Filho", Brazil, Chiaverini Lidiana Camargo Talon, Unesp, Brazil. The role of oxidative stress on the pathophysiology of metabolic syndrome. Rev Assoc Med Bras. 2017;63:85–91. https://doi.org/10.1590/1806-9282.63.01.85.
15. Martin C. The mitochondrial permeability transition pore and its role in cell death. Biochem J. 1999;341:233. https://doi.org/10.1042/0264-6021:3410233.
16. Naoufal Z, Guido K. The mitochondrion in apoptosis: how Pandora's box opens. Nat Rev Mol Cell Biol. 2001;2:67–71. https://doi.org/10.1038/35048073.
17. Martin P, Kathryn W, Turnbull Douglass M. Mitochondrial morphology, topology, and membrane interactions in skeletal muscle: a quantitative three-dimensional electron microscopy study. J Appl Physiol. 2013;114:161–71. https://doi.org/10.1152/japplphysiol.01096.2012.
18. Rajkumar C, James R, Darren G, Kalra Philip A. Non-alcoholic fatty liver disease and clinical outcomes in chronic kidney disease. Nephrol Dial Transplant. 2018. https://doi.org/10.1093/ndt/gfx381.
19. Giovanni T, Byrne Christopher D. Non-alcoholic fatty liver disease: an emerging driving force in chronic kidney disease. Nat Rev Nephrol. 2017;13: 297–310. https://doi.org/10.1038/nrneph.2017.16.
20. Sten O. Reactive oxygen species in mitochondria-mediated cell death. Drug Metab Rev. 2007;39:443–55. https://doi.org/10.1080/03602530701468516.
21. Paolo B, Alexandra K, Emy B, Forte Michael A. The mitochondrial permeability transition from in vitro artifact to disease target. FEBS J. 2006; 273:2077–99. https://doi.org/10.1111/j.1742-4658.2006.05213.x.
22. Mario Z, Ildikò S. The mitochondrial permeability transition. Biochim Biophys Acta Rev Biomembr. 1995;1241:139–76. https://doi.org/10.1016/0304-4157(95)00003-a.
23. Burkill HM. The useful plants of west tropical Africa. Kew: Royal Botanic Gardens; 1985. p. 5.
24. Erukainure OL, Ebuehi OAT, Choudhary IM, Adhikari A, Hafizur Perveen RMS, Muhammad A, et al. Iridoid glycoside from the leaves of *Clerodendrum volubile* beauv shows potent antioxidant activity against oxidative stress in rat brain and hepatic tissues. J Diet Suppl. 2014;11(1):19–29.
25. Adefegha SA, Oboh G. Antioxidant and inhibitory properties of Clerodendrum volubile leaf extracts on key enzymes relevant to noninsulin dependent diabetes mellitus and hypertension. J Taibah Univ Sci. 2016;10: 521–33.
26. Erukainure OL, Oke OV, Ajiboye AJ, Okafor OY. Nutritional qualities and phytochemical constituents of *Clerodendrum volubile*, a tropical non-conventional vegetable. Int Food Res J. 2011;18:1393–9.
27. Fred-Jaiyesimi A, Adekoya Y. Pharmacognostic studies and anti inflammatory activities of *Clerodendrum volubile* P Beauv leaf. Int J Phytomed. 2012;4:414–8.
28. Ogunwa TH, Adeyelu TT, Fasimoye RY, Oyewale MB, Ademoye TA, Ilesanmi OC, et al. Phytochemical evaluation and in vitro antioxidant status of Clerodendrum volubile (an indigenous medicinal plant). Pak J Pharm Res. 2016;2:77–88.
29. Molehin OR, Oloyede OI, Boligon AA. Comparative study on the phenolic content, antioxidant properties and HPLC fingerprinting of the extracts of Clerodendrum volubile P Beauv. J App Pharm Sci. 2017a;7:135–40.
30. Erukainure OL, Hafizur RM, Kabir N, Choudhary MI, Atolani O, Banerjee P, et al. Suppressive effects of *Clerodendrum volubile P Beauv*. [Labiatae] Methanolic extract and its fractions on type 2 diabetes and its complications. Front Pharmacol. 2018a. https://doi.org/10.3389/fphar.2018.00008.
31. Erukainure OL, Narainpersad N, Singh M, Olakunle S, Islam MS. *Clerodendrum volubile* inhibits key enzymes linked to type 2 diabetes but induces cytotoxicity in human embryonic kidney (HEK293) cells via exacerbated oxidative stress and pro-inflammation. Biomed Pharmacother. 2018b;106: 1144–52.
32. Molehin OR, Oloyede OI, Adefegha SA. Streptozotocin-induced diabetes in rats: effects of white butterfly (*Clerodendrum volubile*) leaves on blood glucose levels, lipid profile and antioxidant status. Toxicol Mechanisms Meth. 2018;28(8):573–86.
33. Oboh G, Ogunruku OO, Oyeleye SI, Olasehinde TA, Ademosun AO, Boligon AA. Phenolic extracts from Clerodendrum volubile leaves inhibit cholinergic and monoaminergic enzymes relevant to the management of some neurodegenerative diseases. J Dietary Suppl. 2017;14:358–71.
34. Molehin OR, Oloyede OI, Idowu KA, Adeyanju AA, Olowoyeye AO, Tubi OI, et al. White butterfly (*Clerodendrum volubile*) leaf extract protects against carbon tetrachloride-induced hepatotoxicity in rats. Biomed Pharmacother. 2017b;96:924–9.
35. Molehin OR, Oloyede OI. Attenuation of oxidative stress and hepatic damage by white butterfly (*Clerodendrum volubile*) leaves in streptozotocin-

induced diabetes in rats. J Basic Clin Physiol Pharmacol. 2018. https://doi.org/10.1515/jbcpp-2018-0083.
36. Erukainure OL, Mesaik AM, Muhammad A, Chukwuma CI, Manhas N, Singh P, et al. Flowers of *Clerodendrum volubile* exacerbate immunomodulation by uppressing phagocytic oxidative burst and modulation of COX-2 activity. Biomed Pharmacother. 2016;83:1478–84.
37. Erukainure OL, Mesaik MA, Atolani O, Muhammad A, Chukwuma CI, Islam MS. Pectolinarigenin from the leaves of *Clerodendrum volubile* shows potent immunomodulatory activity by inhibiting T- cell proliferation and modulating respiratory oxidative burst in phagocytes. Biomed Pharmacother. 2017a;93:529–35.
38. Erukainure OL, Hafizur RM, Choudhary MI, Adhikari A, Mesaik AM, Atolani O, et al. 2017. Anti-diabetic effect of the ethyl acetate fraction of *Clerodendrum volubile*: protocatechuic acid suppresses phagocytic oxidative burst and modulates inflammatory cytokines. Biomed Pharmacother. 2017b;86:307–15.
39. Allen AC. The origins and taxonomy of cassava. In: Hillocks RJ, Tresh JM, Belloti AC, editors. Cassava: biology, production and utilisation. Oxon and New York: CABI Publishing; 2002. p. 1–16.
40. Miller AJ, Gross BL. From forest to field: perennial fruit crop domestication. Am J Bot. 2011;98(9):1389–414.
41. Hidayat A, Zuraida N, Hanarida I. The cyanogenic potential of roots and leaves of ninety-nine cassava cultivars. Indonesian J Agric Sci. 2002;3:25–32.
42. Okpuzor J, Oloyede AM. Anti-inflammatory, antipyretic and anti-diarrhoeal properties of an antihaemorrhoid tri-herbal pill. Nature Sci. 2009;7:89–94.
43. Popoola OP, Yangomodou OD, Akintokun AK. Antimicrobial activity of cassava seed oil on skin pathogenic microorganisms. Res J Med Plant. 2007;1:60–4.
44. Chiwona-Karltun L, Katundu C, Ngoma J, Chipungu F, Mkumbira J, Simukoko S, et al. Bitter cassava and women: an intriguing response to food security. LEISA Magazine. 2002;18(4):1-15.
45. Agro-Hub. Cassava (manioc); 2013. Available online at http://www.agro-hub.com/blog/cassava-manioc
46. Horstman SW, Lynch KM, Arendt EK. Starch characteristics linked to gluten-free products. Foods. 2017;6(4):29.
47. Ajayi EIO, Modo EU, Adebamowo AO, Banerjee UC, Tewe OO, Olorunsogo OO. Inhibitory activity of ethanol extract of *Manihot esculenta* on mitochondrial membrane permeability transition pore and caspase 3 in type 2 diabetes mellitus. Int J Biochem Res Rev. 2016;9(4):1–10.
48. Omar NF, Hassan SA, Yusoff UK, Abdullah NAP, Wahab PEM, Sinniah UR. Phenolics, flavonoids, antioxidant activity and cyanogenic glycosides of organic and mineral-base fertilized cassava tubers. Mol. 2012;17(3):2378–87.
49. Mvitu MM, Longo-Mbenza B, Tulomba MD, Nge OA. Reduced risk of metabolic syndrome due to regular intake of vegetables rich in antioxidants among African type 2 diabetics. Diabetes Metab Syndr Clin Res Rev. 2010;4:132–6.
50. Ajayi EIO, Popoola G, Ojediran E. Wound healing potential of Nauclea latifolia and *Manihot esculenta* leaf extracts in type 1 diabetic rats. Afr J Tradit Complement Altern Med. 2015;13(1):1–5.
51. Lukas M, Hui T, Haumesser Jens K, van Christoph R. High-fat diet-induced obesity and insulin resistance are characterized by differential beta oscillatory signaling of the limbic cortico-basal ganglia loop. Sci Rep. 2017;7. https://doi.org/10.1038/s41598-017-15872-x.
52. Holte K, Sharrock NE, Kehlet H. Pathophysiology and clinical implications of perioperative fluid excess. Br J Anaesth. 2002;89:622–32. https://doi.org/10.1093/bja/aef220.
53. Rao AV, Kumar SS, Kulbhushan T. Dysregulation of microRNAs and renin-angiotensin system in high salt diet-induced cardiac dysfunction in uninephrectomized rats. PLoS One. 2017;12:e0180490. https://doi.org/10.1371/journal.pone.0180490.
54. Hogeboom GH, Schneider WC, Pallade GE. Cytochemical studies of mammalian tissues; isolation of intact mitochondria from rat liver; some biochemical properties of mitochondria and submicroscopic particulate material. J Biol Chem. 1948;172:619–35.
55. Diane J, Henry L. Isolation of liver or kidney mitochondria. Methods Enzymol Oxidation Phosphoryl. 1967:94–6. https://doi.org/10.1016/0076-6879(67)10018-9.
56. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193:265–75.
57. Lapidus RG, Sokolove PM. The mitochondrial permeability transition: interactions of spermine, ADP, and inorganic phosphate. J Biol Chem. 1994;269:18931–6.
58. Lapidus RG, Sokolove PM. Spermine inhibition of the permeability transition of isolated rat liver mitochondria: an investigation of mechanism. Arch Biochem Biophys. 1993;306:246–53. https://doi.org/10.1006/abbi.1993.1507.
59. Liao M-T, Chih-Chien S, Kuo-Chin H, Kuo-Cheng L. Insulin resistance in patients with chronic kidney disease. J Biomed Biotechnol. 2012;2012:1–12. https://doi.org/10.1155/2012/691369.
60. Satisheknath B, Ranjanasushil K. Evaluation of antioxidant activity of Manihot esculenta Crantz in wistar rats. J Pharm Bioallied Sci. 2016;8:119. https://doi.org/10.4103/0975-7406.171697.
61. Fleck C, Börner A, Kretzschmar M, Bräunlich H. Liver function after bilateral nephrectomy. Liver. 2008;12:319–25. https://doi.org/10.1111/j.1600-0676.1992.tb00580.x.
62. Behjat S, Mehri K, Atefeh N, Atefeh M. Protection of liver as a remote organ after renal ischemia-reperfusion injury by renal ischemic Postconditioning. Int J Nephrol. 2014;2014:1–4. https://doi.org/10.1155/2014/120391.
63. Maria D'a, Xueliang D, Haihong Z, Ida G. Urea-induced ROS generation causes insulin resistance in mice with chronic renal failure. J Clin Investig. 2010;120:203–13. https://doi.org/10.1172/jci37672.

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