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African walnuts attenuate ectopic fat accumulation and associated peroxidation and oxidative stress in monosodium glutamate-obese Wistar rats

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ABSTRACT

Aims: African walnuts were previously shown to modulate hepatic lipid bio-accumulation in obesity. Herein, we investigated the impact of the nuts on fat accumulation in adipose and ectopic regions, and associated oxidatiive stress status in obese rats.
Materials and methods: Whole ethanol extract (WE) of the nuts, and its liquid-liquid fractions-ethyl acetate (ET) and residue (RES) were separately administered to obese rats for 6 weeks. The normal (NC) and obese (OC) controls received normal saline and the standard control (SC), orlistat (5.14 mg/kg b.w.), during the same period. Thereafter, the animals were euthanized and the adipose, brain, kidneys and heart tissues were studied.
Results: The change in body weight to naso-anal length which increased by 63.52 % in OC compared to NC ($p < 0.05$), decreased by 57.88, 85.80 and 70.20 % in WE, ET and RES-treated groups, respectively, relative to the OC ($p < 0.05$). Also, adipose tissue weights were lowered upon treatment with the extracts and fractions versus OC ($p < 0.05$). Total lipids, phospholipids, triacylglycerol and cholesterol concentrations in the studied tissues which were higher in OC ($p < 0.05$) were lowered ($p < 0.05$) and compared favorably with SC. Further, malondialdehyde levels in the tissues were lowered upon treatment, compared to the OC ($p < 0.05$). Glutathione level and activities of glutathione peroxidase, superoxide dismutase and glutathione-S-transferase which were decreased ($p < 0.05$) in OC, were restored upon treatment with the extracts, relative to the obese control ($p < 0.05$).
Significance: African walnuts assuaged lipogenesis, oxidative stress and peroxidation in extra-hepatic tissues of obese rats, hence, may attenuate ectopic fat accumulation and its associated pathogenesis.

1. Introduction

Tetracarpidium conophorum (Hutch. & Dalziel), also known as *Plukenetia conophora* (Müll. Arg.) is a woody perennial climber, which belongs to the plant family Euphorbiaceae [1,2]. It grows in the wild and is widely distributed across Africa and India [3,4], and produces nuts known as African walnuts or Nigerian walnuts. The plant is cultivated and valued mainly for these nuts [4], which are traditionally eaten after boiling. The nuts are sold at a relatively expensive rate compared to other common nuts because of their traditionally acclaimed nutritional and medicinal benefits. Previous studies have reported bountiful endowments of nutrients in the nuts [5,6].

The nuts of *T. conophorum* possess amongst other activities wound healing, antioxidant, anti-ulcer, anti-microbial, anti-inflammatory and anti-oxidant activities [7]. The African walnuts are reported to be rich in substances known to lower the risk of gaining unwanted weight [8], such as resveratrol, a phytochemical found in grapes and walnuts, with the ability to activate STRT1 gene, which in turn, boosts the body's

Abbreviations: BMI, body mass index; BW, body weight; ET, ethyl acetate fraction; GPx, glutathione peroxidase; GSH, reduced glutathione; GST, glutathione-*S*-transferase; MSG, monosodium glutamate; NAL, naso-anal length; NC, normal control; OC, obese control; RES, fractionation residue; SC, standard control; SOD, superoxide dismutase; TBAR, thiobarbituric acid reactive substances; WE, whole ethanol extract

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metabolic rate, leading to increased energy expenditure. Amongst other reported pharmacological properties of the nuts are; management of gastric abnormality and hypertension [9], anti-cholesterolemic activity [10], anti-hyperlipidemic activity in rats when used as feed supplements [11], and anti-peroxidative action on male reproductive organs [12].

In discussing the industrial potentials of African walnuts, previous studies have advocated its development into a weight management agent given its traditional uses [13]. In a study with Nigerian adults fed African walnuts (called "Nigerian walnuts" in that study), it was demonstrated that the nuts prevented complications of hyperlipidemia and improved lipid metabolism [14]. In diet-induced hyperlipidemia animal models, African walnuts were also found to reduce body weight gain along with other vital parameters in a dose-dependent manner [11]. Earlier, oil obtained from the African walnuts was shown to lower blood lipids in rats [15].

Against this background of reported anti-cholesterolemic, anti-lipidemic and traditional claim of weight and blood pressure regulation, our earlier study evaluated the anti-obesity effect of the nut, with emphasis on hepatic lipid accumulation in obese rat models, since the liver is the hub of cellular fuel metabolism, including lipid metabolism [16]. We reported that the nuts modulated hepatic lipid synthesis and bio-accumulation via reciprocal regulation of HMG-CoA reductase and paraoxonase 1 activities [16]. The question of the impact the nuts would have on lipid accumulation in the extra-hepatic tissues (ectopic fats), and the adipose tissue which is at the center of lipid storage both in physiological and obese conditions, was not addressed in that study. The present study was therefore undertaken to evaluate the effect of the African walnuts on lipid bio-accumulation in extra-hepatic tissues of MSG-obese rat models.

The adipose is endowed with the capacity to store excess triacylglycerols, thereby preventing other tissues (liver, heart and kidney) from tissue lipotoxicity, which usually results from exposure to excessive lipids [17]. The adipose also functions as an endocrine organ and produces tissue-derived factors with biological influence in health and disease [18]. These biologically active factors of the adipose, including the adipokines and the pro-inflammatory cytokines, relay information to other metabolically active organs such as the muscle, liver, pancreas, heart, kidneys and brain through endocrine mechanisms, thereby modulating systemic metabolism [19]. This underscored the need to evaluate the impact of our treatment on tissues other than the adipose and the liver, particularly in obese condition.

Additionally, the pro-inflammatory cytokines known to increase in obese condition, such as tumor necrosis factor alpha (TNF-α), are essential in instigating inflammatory response and suppressing anti-inflammatory processes, a combined action which precipitates oxidative stress: increased generation of superoxide anions and other reactive oxygen species (ROS) [20,21]. It is thought that these ROS aggravate excess lipids infiltration into non-specialized tissues such as liver, kidney and heart leading to further increases in ROS production. Therefore, it becomes biochemically compelling to study lipids accumulation in adipose and non-adipose tissues along with key indicators of oxidative stress, which may constitute part of the mechanism of some plant derived substances in combating the menace of obesity.

Consequently, the current study evaluated the impact of *T. conophrum* nut extracts and fractions, on lipid accumulation along with oxidative stress markers in extra-hepatic tissues. It is intended to deepen understanding of the reported anti-obesity action of the African walnut and its systemic effect. Studies such as this may lead to eventual discovery of the needed dietary adjunct, functional food or a nutraceutical for the prevention and management of obesity, with comparative advantage over the existing conventional therapies.

2. Materials and methods

2.1. Chemicals and reagents

Ammonium molybdate, sodium bisulphate, potassium chloride, trichloroacetic acid, Triton-X_{100}, thiobarbituric acid (TBA), glutathione (GSH), sucrose, mannitol, 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), pyrogallol and1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Sigma–Aldrich, USA. All other reagents and chemicals used not listed were of analytical grade and also purchased from Sigma–Aldrich, USA, unless otherwise stated.

2.2. Plant material

2.2.1. Preparation of whole ethanol extract

The African walnuts were obtained from Shagamu, Ogun State and brought to Biochemistry Laboratory of Evangel University, Akaeze, Ebonyi State, Nigeria. The nuts were authenticated by a botanist, Dr. Bronu Ado of the Department of Biological Sciences, Evangel University, Akaeze, Nigeria. Thereafter, the nuts were de-shelled, cut into bits and dried under tropical room temperature. The dried nuts were pulverized and suspended in absolute ethanol in the ratio 1:2 (sample to solvent) for 48 h at room temperature [22]. The suspension was then filtered firstly with a cheese material and afterwards with Whatman No. 2 filter paper, and the filtrates were concentrated in a rotary evaporator (45– 50 °C) to about 1/10th of the original volume, after which the concentrates were evaporated to complete dryness in a water bath (45– 50 °C). The percentage yield of extracts was calculated thus: (Wt of extract/Wt of starting material) × 100.

2.2.2. Liquid-liquid fractionation of the crude extract

Liquid-liquid fractionation was performed as described in our earlier study [16]. Briefly, a portion of ethyl-acetate was added to a known amount of the extract (re-solubilized in an aliquot of ethanol), shaken and allowed to stand for 5 − 10 min. in a separating funnel to separate into two visible layers. The denser lower layer was collected into a labeled beaker, separated from the less dense ethyl acetate soluble layer. The dense lower layer was re-suspended in ethyl acetate and the separation process repeated. This whole process was repeated several times until presumably all the components of the extract soluble in ethyl acetate were collected. All the ethyl acetate soluble portions were pooled and evaporated to dryness using a rotary evaporator, and this dried fraction was named ethyl acetate fraction (ET). The remainder of the extract not soluble in ethyl acetate was also concentrated *in vacuo* and the dried concentrate named residue (RES). The portion of the original ethanol extract not subjected to fractionation was labeled whole extract (WE). These three (3) plant materials constituted the test substances in this study.

2.3. Experimental animals

2.3.1. Induction of experimental obesity

Twenty Wistar rats (15 females and 5 males), aged 6– 8 weeks, were obtained from the College of Medical Sciences Animal House, University of Calabar, and co-habited in the ratio 3:1. The litters produced were used for induction of obesity using procedures described previously [23,24]. Briefly, on the day of delivery, pups were divided into two groups. The first group was intraperitoneally administered 4 mg/g body weight of monosodium glutamate (MSG) (reconstituted in normal saline) on postnatal days 2, 4, 6, 8 and 10 to produce the obese models. The second group was similarly treated, but with normal saline (normal control). These rats were weaned on the 21st day and raised normally thereafter and studied at the age of 12 weeks. Lee's index (LI) and body mass index (BMI) of the animals were measured and rats with Lee's indices ≥ 0.3 and/or BMI ≥ 20 % greater than the normal control were considered obese [24,25] and included in the study.

Table 1
Experimental design and animal groups treated with extracts and fractions of *T. conophorum* nut.

S/N	Group	Treatment
1	Normal control (NC)	Normal saline
2	Obese control (OC)	Normal saline
3	Standard control (SC)	Orlistat (5.14 mg/kg b.w.)
4	Test group 1(WE)	Whole ethanol extracts (2 g/kg b.w.)
5	Test group 2 (ET)	Ethyl acetate fraction (2 g/kg b.w.)
6	Test group 3 (RES)	Fractionation residue (2 g/kg b.w.)

2.3.2. Experimental design and procedures

The animals were divided into six (6) groups and treatment with extracts and fractions of the nuts, and orlistat, a standard anti-obesity drug was carried out for 6 weeks (Table 1). The rats were fed with rat pellets and tap water *ad libitum* under tropical room temperature (25 ± 3 °C). The animals' body weights and fasting blood glucose were measured prior to the commencement of treatment and at the end of the six (6) weeks, using electronic weighing balance (Life Assistance Scientific Co. UK) and glucometer (Lifescan Inc., California, USA), respectively. Also, naso-anal length, a functional indicator of obesity was measured using a calibrated measuring tape. At the end of the 6 weeks and after an overnight fast, the animals were anesthetized using ketamine (2 µL/kg b.w.). The euthanized animals were dissected and the organs and tissues of interest surgically excised. The excised tissues/organs – adipose (epididymal and perirenal), heart, kidneys and brain, were weighed and stored at −80 °C until used for tissue lipids and antioxidant/peroxidation assays. The protocol used in this study was in line with the guidelines of the National Institute of Health (NIH) publication, (1985) for laboratory animal care and use. Also, the procedures and the use of animals were approved by the Faculty of Basic Medical Sciences Animal Research Ethics Committee (FAREC- FBMS, Approval number: 032BCH3319), University of Calabar, Nigeria.

2.3.3. Relative organ weight

The animals' body weights and absolute tissues/organs weights were used to estimate the relative tissue/organ weights thus:

Relative weight (%) = [(Absolute organ weight (g))/(Final body weight (g))] × 100

2.4. Tissue preparation for lipids evaluation

Tissue lipids were extracted and purified as described previously [26]. Briefly, 0.2 g of tissue was homogenized in 1.8 mL of chloroform-methanol (2:1, v/v). Thereafter, the resultant homogenate was transferred whole into 2 mL Eppendorf tubes. The homogenate was allowed to stand for 10 min at freezing temperature (- 20 °C) and afterwards, centrifuged at 2400 rpm for 10 min. A biphasic system was observed viz: 25 % aqueous-methanol upper layer (containing non-lipid portions) and 75 % chloroform lower layer which contains lipids. The chloroform layer (lipid extract) was aspirated into fresh tubes and further washed with 0.2 mL of 0.05 M potassium chloride and centrifuged at 2400 rpm for 10 min. The upper non-lipid component was separated, and the final lower chloroform layer was stored at - 20 °C until required for the lipid analyses.

2.4.1. Determination of total tissue lipids

Total tissue lipids were determined according to the method of Folch, Lees and Stanley [26]. Briefly, 200 µL of the lipid extract was pipetted into pre-weighed Eppendorf tubes and the content was evaporated to dryness at 50 °C using Ecotherm heating/chilling dry bath. The weight of total lipids was recorded as the difference between the pre-weighed tubes and weight after drying. The result was expressed as mg/g of organ weight.

2.4.2. Determination of tissue total cholesterol

The method of [26] was used with minor modifications. Briefly, 50 µL of lipid extract was measured into clean tubes and evaporated to dryness at 60 °C using Ecotherm heating/chilling dry bath. This was followed by the addition of 20 µL of a mixture of triton-X100 and chloroform (1:1, v/v), and 1 mL cholesterol reagent (from Randox assay kit). The mixture was vortexed and incubated for 5 min. at 37 °C, and absorbance measured at 546 nm against reagent blank, within 60 min..

2.4.3. Determination of tissue total phospholipids

The phospholipids in the tissues were estimated by a method described previously [27], with minor modifications. Briefly, 100 µL of the lipid extract was evaporated to dryness at 60 °C using Ecotherm heating/chilling dry bath. Thereafter, 430 µL of distilled water was added and vortexed. Further, 50 µL of molybdate II reagent (2.5 % ammonium molybdate in 3 N dihydrogen sulphate) was added and allowed to stand for 10 min. at room temperature. Then, 20 µL of amino napthosulphonic acid (ANSA) reagent (0.5 g of ANSA in 195 mL of 15 % sodium bisulphite and 5 mL of 20 % sodium sulphite) was added and incubated at room temperature for 20 min.. Afterwards, the absorbance was read at 660 nm. The value obtained was multiplied by a factor of 25 to obtain phospholipids. Results were expressed in mg/100 mL.

2.4.4. Determination of tissue triacylglycerol

This was also determined by the method of Folch, Lees and Stanley [26], with modifications. Briefly, 50 µL of the lipid extract was measured into Eppendorf tube and evaporated to dryness at 60 °C using Ecotherm heating/chilling dry bath. This was followed by the addition of 200 µL of ethanol and vortexed. 1 mL of the reconstituted triacylglycerol reagent was added to the tubes, mixed and incubated for 5 min. at 37 °C and absorbance read at 546 nm against reagent blank within 60 min..

2.5. Tissue homogenization for lipid peroxidation/oxidative stress studies

Exactly 0.2 *g* each of heart, kidney, brain and adipose tissues were separately homogenized in 1.8 mL ice-cold homogenizing buffer, pH 7.2 (125 mM sucrose, 125 mM mannitol, 1 mM EGTA and 5 mM HEPES) with a Teflon pestle homogenizer. The whole content was transferred into a 2 mL Eppendorf micro tube and centrifuged at 3000 rpm for 10 min.. The supernatant was stored at - 20 °C until required for analyses of peroxidation/oxidative stress indicators.

2.5.1. Determination of superoxide dismutase activity

Superoxide dismutase (SOD) activity was determined according to a previously described method [28] which uses pyrogallol. In this method, SOD competes with pyrogallol for O_{22}^{-}; the percent inhibition of pyrogallol autoxidation is a measure of the amount of SOD present. Briefly, 100 µL of 100 mM bicarbonate buffer, pH 10.2 was added to clean Eppendorf tubes followed by 830 µL distilled water and 50 µL of sample and allowed to stand for 10 min. at room temperature. Thereafter 20 µL of pyrogallol was added and absorbance read immediately at 420 nm at 0, 1, 2 and 3 min., respectively, against a reagent blank. The SOD activity was expressed as unit/mg protein, where 1 unit is defined as the amount of SOD required to inhibit pyrogallol autoxidation by 50 %.

2.5.2. Determination of glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was assessed using a previously described method [29]. In the assay method, DTNB reacts with a free oxidized sulfhydryl group to yield a mixed disulfide and 2-nitro-5-thiobenzoic acid, whose yellow colour intensity is correlated with GPx activity. The reaction mixture comprised of 200 µL of 0.4 M phosphate buffer, pH 7.0, 100 µL of 10 mM sodium azide, 200 µL of 10 mM GSH, 100 µL of H_2O_2 and 200 µL of sample was incubated for 10 min. at room temperature. Thereafter, 200 µL of 10 % trichloroacetic

acid (TCA) was added, centrifuged for 15 min. at 3000 rpm and the supernatant collected. Exactly 400 µL of this supernatant was pipetted into a fresh tube containing 500 µL of 0.4 mM tris-acetate buffer, pH 8.9, followed by 20 µL of freshly prepared DTNB. The oxidation of GSH in the reaction mixture gives a yellow colouration whose absorbance is measured at 412 nm against distilled water blank. GPx activity was expressed as unit/mg protein.

2.5.3. Determination of glutathione-S-transferase activity

Glutathione-*S*-transferase (GST) activity was determined using the method described previously [30]. This assay utilizes CDNB which is suitable for the broadest range of GST isozymes. Upon conjugation of the thiol group of glutathione to the CDNB substrate, there is an increase in the absorbance at 340 nm. Hence, the rate of change in absorbance correlates with GST activity. The reaction mixture comprised of 1 mL of 0.1 M potassium phosphate buffer, pH 6.5, 50 µL of GSH, 50 µL of CDNB and 50 µL of sample, was vortexed and absorbance read immediately at 0, 1, 2, 3, 4 and 5 min. against a reagent blank at 340 nm. GST activity was expressed as unit/mg protein.

2.5.4. Determination of reduced glutathione

The level of reduced glutathione (GSH) was determined using the method previously described by Ellman [29] which employed DTNB. The reaction mixture containing 125 µL of sample, 100 µL distilled water and 25 µL of 50 % TCA, was vortexed and centrifuged at 5000 rpm for 5 min.. 200 µL of the supernatant was pipetted into a fresh tube, followed by the addition of 400 µL of 0.4 mM tris-acetate buffer, pH 8.9 and 10 µL of DTNB. The absorbance of this mixture was measured at 412 nm. The concentration of GSH was expressed as nmol/mg protein

2.6. Estimation of lipid peroxidation using TBARS-MDA reagent

Lipid peroxidation was determined using the method described by Buege and Aust [31]. Briefly, 1 mL of working TBARS reagent was added to Eppendorf tubes followed by 50 µL of sample and heated at 95 °C for 15 min. until the mixture turned yellow. It was then cooled in ice bath and centrifuged at 1000 rpm for 10 min.. The absorbance of the supernatant was read at 535 nm and TBARS concentration expressed as nmol/mg protein

2.7. Statistical analysis

All data are presented as the mean ± standard deviation (SD). The data obtained in this study was analyzed using one-way analysis of variance (ANOVA), followed by Tukey post-hoc test. The association between total lipids, total cholesterol and TBARS level was assessed using Pearson's correlation coefficient and differences were considered significant at 95 % level of confidence. GraphPad prism 7.0 for windows (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analysis.

3. Results

3.1. Effect of treatment on body weight, naso-anal length ratio

Table 2 indicates the effect of the treatment on the body weight: naso-anal lengths ratio. From the result, the naso-anal body lengths of treatment groups were higher ($p < 0.05$) than the obese control (OC), an indication that MSG had a significant effect on animal growth rate compared to NC. Further, there was 22.41 % increase in the body weight (cBW %) of OC animals compared to the NC at the end of the study period ($p < 0.05$), indicating successful induction of obesity. Treatment with the extract and fractions of African walnuts caused a decrease in body weight by 17.48, 69.97 and 16. 71 % in WE, ET and RES, respectively ($p < 0.05$). The ratio of change in body weight to change in naso-anal length (cBW: cNAL), a potent indicator of obesity which increased by 63.52 % in OC compared to NC ($p < 0.05$) was found to decreased by 57.88, 85.80 and 70.20 % upon treatment with WE, ET and RES, respectively, relative to the OC ($p < 0.05$). These effects of the extracts and fractions were similar to the effect of the standard drug, orlistat.

3.2. Effect of treatment on organ/tissue weights

Table 3 shows absolute and relative weights of the white adipose tissue (WAT) and other tissues measured at the end of the 6-week treatment. The relative adipose tissue weight was found to have increased by 60.04 % upon obesity induction compared to the normal control ($p < 0.05$). However, 6-week oral administration of WE, ET and RES of *T. conophorum* nuts caused 55.0, 41.0 and 52.3 % decreases in the relative adipose tissue weights, respectively, compared to the obese control ($p < 0.05$). From the result, the effect of the whole nut extract on the adipose tissue weight compared well with the effect of the standard drug, orlistat. The weights of other organs including brain, kidneys and heart were as well measured, but were not significantly affected by the treatment.

3.3. Effect of treatment on tissue lipids

The effect of the 6-week administration of extract and fractions of African walnuts on tissue lipids are shown in Table 4.

3.3.1. Total lipids

Tissue total lipids were found to increase significantly ($p < 0.05$) by 52.38 % in adipose tissue (5.3 ± 0.5 OC vs 3.5 ± 0.6 NC), 50 % in the brain (4.5 ± 0.5 OC vs 3.0 ± 0.9 NC), and 81.01 % in the kidneys (3.3 ± 0.8 OC vs 1.8 ± 0.9 NC) (Table 4). However, 6-week oral administration of WE caused significant reductions by 24.44, 8.23, 7.14 and 6.91 % in the adipose, brain, kidney and heart tissues, respectively, compared to OC ($p < 0.05$). The ET fraction caused 9.76, 28.57, 81.82 and 4.23 % reductions in the adipose tissue, brain, kidneys and heart, respectively compared to OC. Similarly, RES reduced total lipids by 17.42, 29.71, 14.34 and 8.72 % in the adipose tissue, brain, kidney and heart, respectively (Table 4). The effects of these treatments were most prominent ($p < 0.05$) in the adipose and the brain tissues, whereas only RES caused significant effect ($p < 0.05$) in the kidney. The effect of these treatments compared favorably with the effect of the standard drug (Table 4).

3.3.2. Total cholesterol

In OC, total cholesterol significantly increased ($p < 0.05$) across all studied tissues compared to NC i.e. 44.44 % in adipose, 21.87 % in the brain, 20.56 % in the kidneys, and 34.62 % in the heart (Table 4). Six-week administration of WE of *T. conophorum* nuts was found to cause 9.87, 35.59, 22.99 and 8.72 % reductions in the adipose, brain, kidneys and heart tissues, respectively (Table 4). Similarly, the ET fraction caused 15.71, 78.00, 42.39 and 62.47 % reductions in the adipose, brain, kidneys and heart, respectively. Moreover, RES attenuated hypercholesterolemia by 22.78, 17.91, 9.75 and 62.47 % decreases in cholesterol level of the adipose, brain, kidneys and heart tissues, respectively. Again, these effects compared well with the effect of the standard drug, orlistat (Table 4).

3.3.3. Total phospholipids

The phospholipids which significantly increased in OC compared to their respective NC values were positively attenuated upon 6-week administration of extract and fractions of *T. conophorum* nuts thus: WE decreased the phospholipids by 52.3, 4.98, 40.3 and 2.78 %, in the adipose, brain, kidneys and heart tissues, respectively and ET by 44.9, 50.8 and 44 % in the adipose, kidney and heart, respectively (Table 4). The ET fraction did not cause any significant change in measured phospholipid levels in the brain. In the same vein, RES administration

Table 2
Naso-anal length (NAL) in relation to body weight (BW) of the animals in the different study groups.

Group	NC	OC	SC	WE	ET	RES
*i*BW (g)	195.30 ± 18.21	208.53 ± 34.60	203.87 ± 34.85	202.20 ± 30.59	217.10 ± 27.53	186.25 ± 18.41
*i*NAL (cm)	14.67 ± 0.68	13.34 ± 1.42	13.06 ± 1.35	13.06 ± 1.35	12.80 ± 0.57	13.07 ± 1.47
*i*BW :*i*NAL (g/cm)	13.32 ± 1.10	15.61 ± 2.3	15.72 ± 2.87	15.61 ± 2.71	16.97 ± 2.18	14.43 ± 2.33
*f*BW (g)	223.83 ± 36.30	224.35 ± 40.11	230.97 ± 37.20	223.43 ± 30.90	233.63 ± 51.43	201.47 ± 20.45
*f*NAL (cm)	21.52 ± 1.59	17.50 ± 2.69[a]	21.50 ± 1.61[b]	21.86 ± 1.49[b]	21.75 ± 0.50[b]	21.58 ± 0.74[b]
*f*BW :*f*NAL (g/cm)	10.36 ± 0.98	13.89 ± 2.38	10.92 ± 1.33	10.42 ± 1.29	10.72 ± 2.23	9.33 ± 0.79
*c*BW (g)	11.78 ± 1.27	14.42 ± 2.4*	13.16 ± 3.91	11.91 ± 3.3[b]	4.33 ± 0.30*,[b]	12.01 ± 1.77
*c*NAL (cm)	31.62 ± 4.47	16.96 ± 3.68*	37.73 ± 8.99[b]	38.83 ± 7.92*,[b]	41.09 ± 3.88*,[b]	39.36 ± 3.88*,[b]
*c*BW :*c*NAL (g/cm)	3.92 ± 2.13	6.41 ± 2.46*	3.43 ± 0.58*,[b]	2.70 ± 1.49*,[b]	0.94 ± 0.07*,[b]	1.91 ± 1.18*,[b]
(*f*BW :*f*NAL) – (*i*BW : *i*NAL) (g/cm)	−2.96 ± 0.56	−1.73 ± 0.82	−4.80 ± 1.99	−5.19 ± 2.13	−6.26 ± 1.01	−5.12 ± 1.99

Data is Mean ± SD, n = 6, [a] $p < 0.05$ vs NC, [b] $p < 0.05$ vs OC. NC = normal control, OC = obese control, SC = standard control, WE = whole ethanol extract, ET = ethyl-acetate fraction, RES = residue fraction, BW = body weight, NAL = naso-anal length, *i* = initial, *f* = final, *c* = change.

Table 3
Organ/tissue weights (absolute and relative) of MSG-obese rats, treated with extract and fractions of *Tetracarpidium conophorum* nuts.

Organ/tissues		Adipose tissue		Brain		Kidneys		Heart	
Groups	*f*BW	AW (g)	RW (%)	AW (g)	RW (%)	AW (g)	RW (%)	AW(g)	RW (%)
NC	223.83 ± 36.30	2.30 ± 0.70	1.04 ± 0.34	1.55 ± 0.18	0.70 ± 0.09	1.25 ± 0.38	0.56 ± 0.17	0.83 ± 0.14	0.38 ± 0.06
OC	224.35 ± 40.11	5.90 ± 0.63[a]	2.74 ± 0.74[a]	1.55 ± 0.10	0.70 ± 0.10	1.22 ± 0.30	0.54 ± 0.06	0.78 ± 0.18	0.36 ± 0.10
SC	230.97 ± 37.20	4.93 ± 0.67[a,b]	2.17 ± 0.37[a]	1.55 ± 0.10	0.69 ± 0.14	1.22 ± 0.17	0.53 ± 0.03	0.70 ± 0.18	0.30 ± 0.04
WE	223.43 ± 30.90	3.67 ± 0.70[a,b,c]	1.67 ± 0.41[a,b,c,e,f]	1.70 ± 0.30	0.77 ± 0.16	1.58 ± 0.10[a,b,c,e,f]	0.72 ± 0.09[a,b,c,e,f]	0.95 ± 0.16[c,f]	0.43 ± 0.07[c,e,f]
ET	233.63 ± 51.43	4.58 ± 0.53[a,b,c,d]	2.03 ± 0.52[a,b]	1.40 ± 0.08[d]	0.62 ± 0.11	1.13 ± 0.15	0.49 ± 0.04	0.75 ± 0.17	0.32 ± 0.024
RES	201.47 ± 20.45	4.00 ± 0.41[a,b,cd,e]	2.00 ± 0.31[a,b]	1.42 ± 0.16[d]	0.71 ± 0.12	1.12 ± 0.16	0.55 ± 0.05	0.65 ± 0.10	0.33 ± 0.06

Data are presented as Mean ± SD, n = 6, [a] $p < 0.05$ vs NC, [b] $p < 0.05$ vs OC, [c] $p < 0.05$ vs SC, [d] $p < 0.05$ vs WE, [e] $p < 0.05$ vs ET, [f] $p < 0.05$ vs RES. AW: absolute weight, *f*BW: final body weight, NC: normal control, OC: obese control, SC: standard control, WE: whole ethanol extract, ET: ethyl-acetate fraction, RES: residue fraction, RW: relative weight.

caused a 66.66 % reduction in the adipose tissue, 30 % in the kidneys and 43.1 % in the heart but no significant effect in the brain (Table 4).

3.3.4. Triacylglycerol

Triacylglycerol (TG) concentration was significantly increased ($p < 0.05$) in all studied tissues of the OC compared with NC (Table 4). Upon oral administration of *T. conophorum* nut extract and fractions, the following observations were recorded: TG decreased by 7.80, 12.97, 42.41 and 6.79 % with WE in the adipose tissue, brain, kidneys and heart, respectively, by 10.07, 7.85, 3.78 and 4.99 % with ET in the adipose tissue, brain, kidneys and heart, respectively and by 10.63, 3.97, 3.78 and 40.63 % with RES in the adipose tissue, brain, kidneys and heart, respectively, with the only exception in the brain (Table 4).

Table 4
Tissue lipid profile of MSG-obese rats following treatment with extract and fractions of *Tetracarpidium conophorum* nuts.

Parameter	NC	OC	SC	WE	ET	RES
Total lipids (mg/g tissue)						
Adipose tissue	3.5 ± 0.6	5.3 ± 0.5[a]	3.7 ± 0.5 [b]	4.2 ± 0.8 [b]	3.8 ± 0.5 [b]	4.5 ± 0.6 [b]
Brain	3.0 ± 0.9	4.5 ± 0.6 [a]	3.5 ± 0.6 [b]	3.8 ± 0.8	3.4 ± 0.6 [b]	3.8 ± 0.8
Kidneys	2.0 ± 1.3	3.3 ± 0.8 [a]	2.5 ± 0.6	2.5 ± 0.6	2.6 ± 0.9	1.8 ± 0.8 [b]
Heart	1.3 ± 0.5	1.7 ± 0.8	1.5 ± 0.6	1.7 ± 0.5	1.4 ± 0.6	1.3 ± 0.5
Total cholesterol (mg/g tissue)						
Adipose tissue	274.0 ± 55.9	520.7 ± 32.2 [a]	388.1 ± 73.9 [b]	332.3 ± 51.4 [b]	371.2 ± 22.8 [b]	374.8 ± 44.4 [b]
Brain	243.8 ± 68.8	342.1 ± 48.5 [a]	240.1 ± 85.3 [b]	241.8 ± 71.7 [b]	192.2 ± 83.4 [b]	269.1 ± 89.9
Kidneys	271.0 ± 31.5	350.3 ± 28.8 [a]	229.9 ± 49.8 [b]	243.3 ± 35.4 [b]	212.6 ± 26.3 [b]	267.8 ± 46.9 [b]
Heart	221.6 ± 45.3	338.7 ± 91.7 [a]	223.5 ± 12.9 [b]	249.1 ± 52.6 [b]	244.3 ± 36.8 [b]	208.5 ± 19.9 [b]
Phospholipids (mg/g tissue)						
Adipose tissue	4.8 ± 2.8	7.3 ± 1.5 [a]	6.0 ± 1.2	4.7 ± 2.0 [b]	3.1 ± 0.9 [b]	5.3 ± 1.9
Brain	10.1 ± 0.9	11.6 ± 2.9	5.4 ± 2.2 [b]	9.3 ± 3.3	12.5 ± 2.1 [c]	13.0 ± 1.6 [c]
Kidneys	10.3 ± 1.8	13.2 ± 0.9 [a]	9.3 ± 0.6 [b]	9.7 ± 1.4 [b]	10.2 ± 1.2 [b]	11.5 ± 1.9 [b]
Heart	5.3 ± 1.4	6.2 ± 0.5	6.3 ± 1.2	6.4 ± 0.6	4.3 ± 0.4[b,c,d]	4.4 ± 0.9[b,c,d]
Triacylglycerol (mg/g tissue)						
Adipose tissue	1403.0 ± 5.2	1553.0 ± 131.5 [a]	1404.0 ± 55.7 [b]	1412.0 ± 39.9 [b]	1477.0 ± 89.3	1404.0 ± 112.6 [b]
Brain	196.3 ± 14.9	269.0 ± 58.9 [a]	185.8 ± 8.3 [b]	167.7 ± 13.8 [b]	203.3 ± 23.4 [b]	306.6 ± 68.0 [a,b,c,d,e]
Kidneys	1253.0 ± 177.0	1563.0 ± 222.6 [a]	1181.0 ± 398.6 [b]	1523.0 ± 275.2	1267.0 ± 297.7	1005.0 ± 83.5 [b,d]
Heart	597.4 ± 202.6	880.3 ± 65.7 [a]	626.0 ± 192.9 [b]	694.0 ± 112.2 [b]	810.6 ± 54.6	530.0 ± 56.5 [b,e]

Data are presented as Mean ± SD, n = 6, [a] $p < 0.05$ vs NC, [b] $p < 0.05$ vs OC, [c] $p < 0.05$ vs SC, [d] $p < 0.05$ vs WE, [e] $p < 0.05$ vs ET. NC: normal control, OC: obese control, SC: standard control, WE: whole ethanol extract, ET: ethyl-acetate fraction, RES: residue fraction.

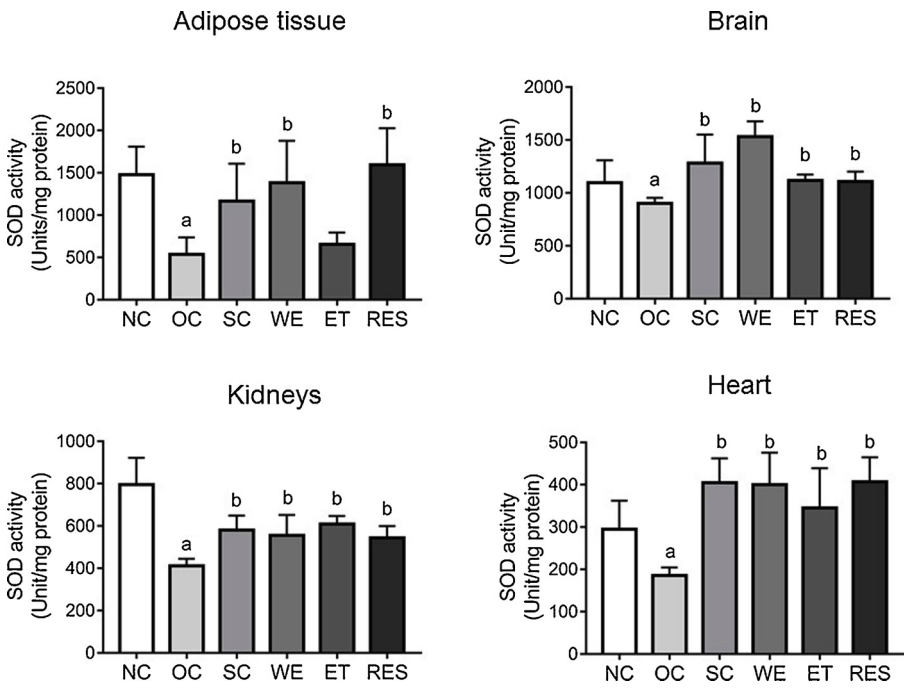

Fig. 1. Tissue SOD activity of MSG-obese rats treated with extract and fractions of *Tetracarpidium conophorum* nuts. Values are expressed as mean ± SD, n = 6. [a] $p < 0.05$ vs NC, [b] $p < 0.05$ vs OC.

3.4. Effect of treatment on indices of oxidative stress

The effect of the 6-week administration of extract and fractions of African walnuts on indices of oxidative stress are shown in Figs. 1–5 across various tissues/organs.

3.4.1. Superoxide dismutase activity

SOD activity was decreased ($p < 0.05$) in OC compared to NC group by 175.27 % in the adipose, 21.82 % in the brain, 100.74 % in the kidneys and 52 % in the heart (Fig. 1). The enzyme activity was up-regulated significantly ($p < 0.05$) in the treatment groups as follows; adipose (134.03, 20.53 and 134.71 %), brain (55.90, 24.11 and 22.97 %), kidney (34.79, 47.32 and 37.25 %) and heart (91.90, 78.09 and 109.84 %) corresponding to WE, ET and RES groups, respectively (Fig. 1).

3.4.2. Glutathione peroxidase activity

Obesity induction caused significant decrease ($p < 0.05$) in GPx

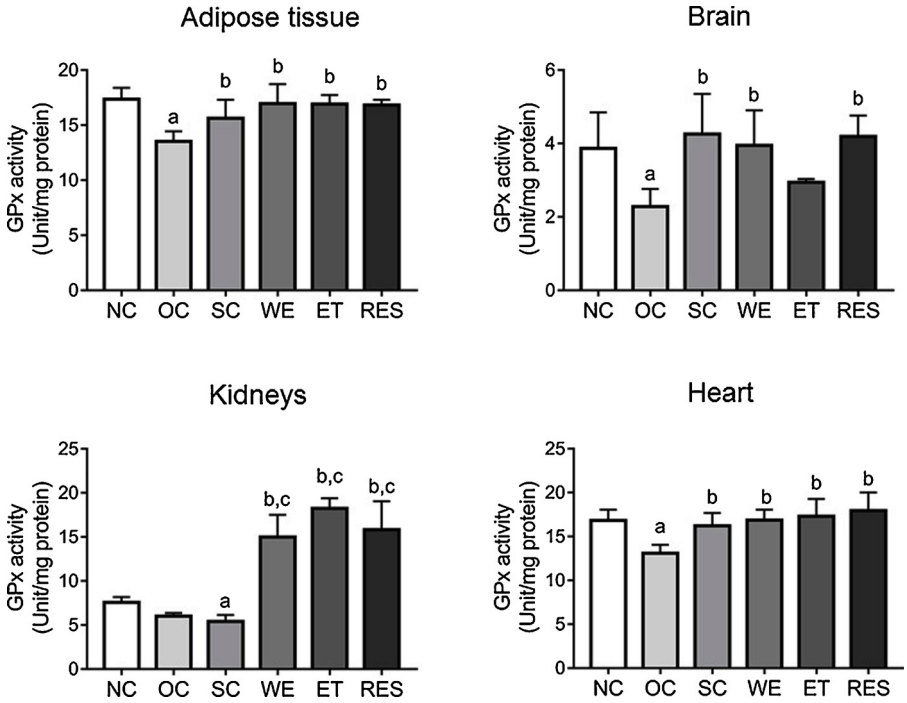

Fig. 2. Tissue GPx activity of MSG-obese rats, treated with extract and fractions of *Tetracarpidium conophorum* nuts. Values are expressed as mean ± SD, n = 6. [a] $p < 0.05$ vs NC, [b] $p < 0.05$ vs OC, [c] $p < 0.05$ vs SC.

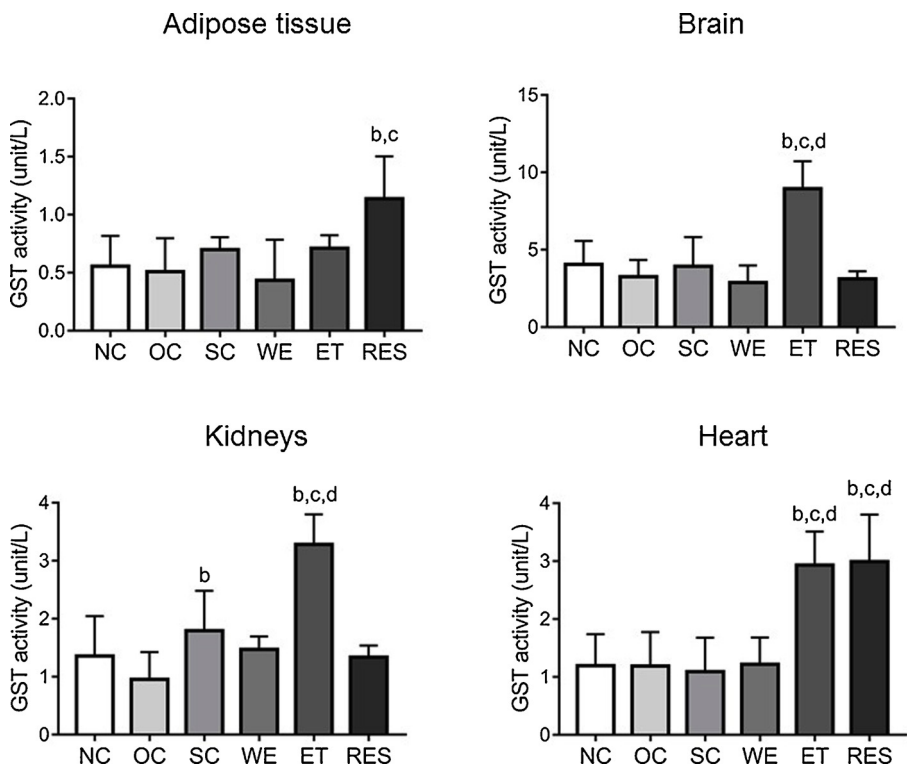

Fig. 3. Tissue GST activity of MSG-obese rats, treated with extract and fractions of *Tetracarpidium conophorum* nuts. Values are expressed as mean ± SD, n = 6. [a] $p < 0.05$ vs NC, [b] $p < 0.05$ vs OC, [c] $p < 0.05$ vs SC, [d] $p < 0.05$ vs WE.

activity by 19.31 % in adipose, 31.09 % in brain, and 53.40 % in the heart (Fig. 2). GPx activity decreased by 14.09 % in the kidneys of OC group, which was not statistically significant relative to NC group (Fig. 2). These hitherto decreased GPx activities were significantly increased ($p <$ 0.05) upon treatment thus: adipose (9.01, 16.31 and 15.64 %), kidney (12.39, 40.46 and 33.92 %) and heart (2.19, 17.97 and 0.18 %) corresponding to WE, ET and RES groups, respectively (Fig. 2). In the brain, only increases caused by WE and RES (16.19 and 98.64 %, respectively) were statistically significant relative to OC group, and were comparable with SC group (Fig. 2).

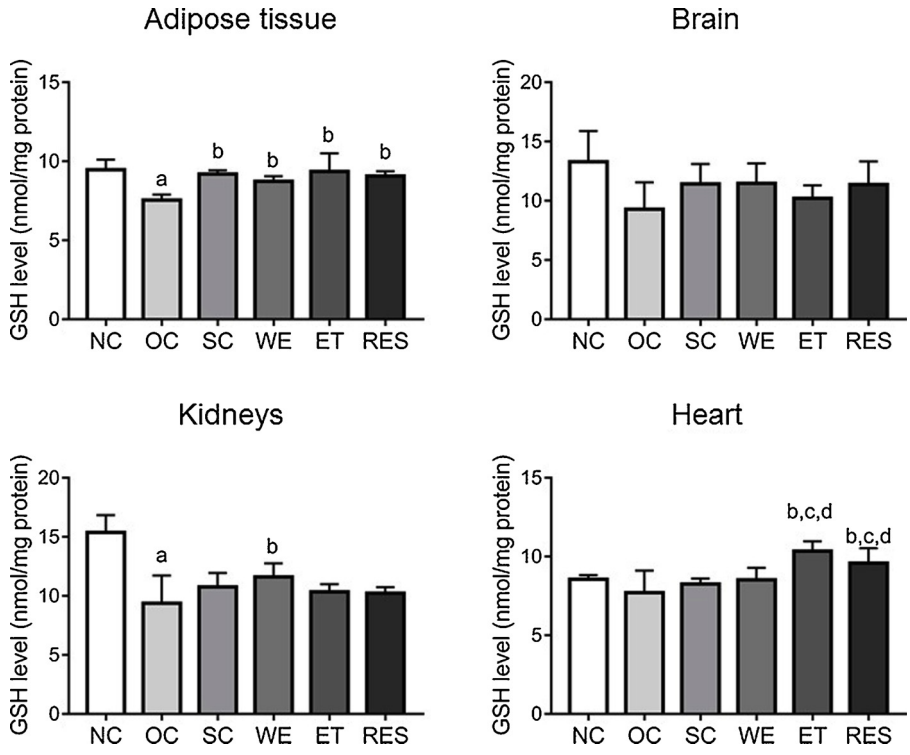

Fig. 4. Tissue GSH level of MSG-obese rats, treated with extract and fractions of *Tetracarpidium conophorum* nuts. Values are expressed as mean ± SD, n = 6. [a] $p < 0.05$ vs NC, [b] $p < 0.05$ vs OC, [c] $p < 0.05$ vs SC, [d] $p < 0.05$ vs WE.

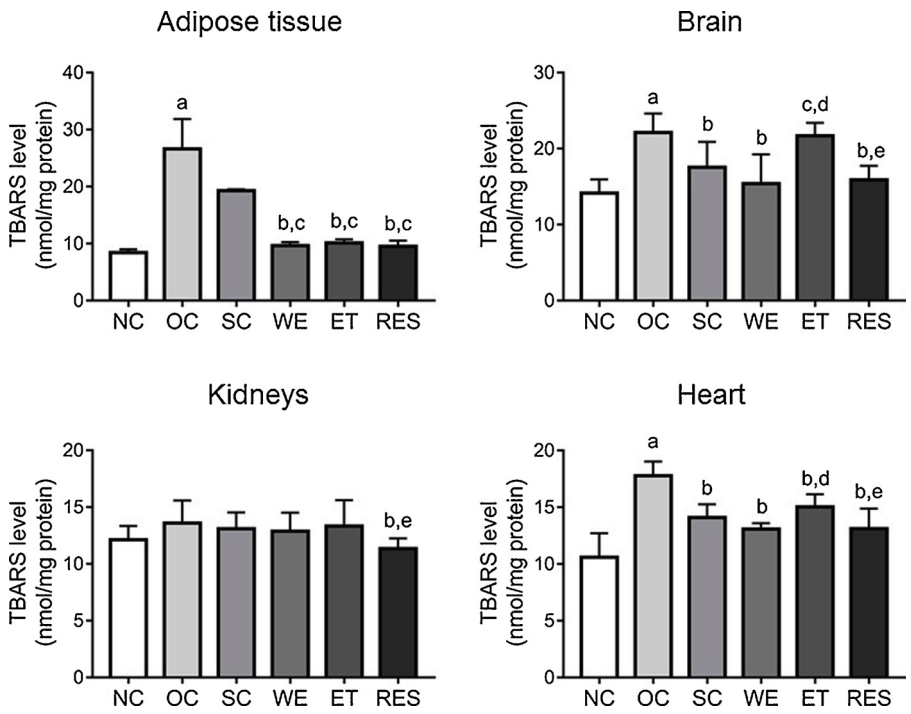

Fig. 5. Tissue TBARS level of MSG-obese rats, treated with extract and fractions of *Tetracarpidium conophorum* nuts. Values are expressed as mean ± SD, n = 6. [a] $p < 0.05$ vs NC, [b] $p < 0.05$ vs OC, [c] $p < 0.05$ vs SC, [d] $p < 0.05$ vs WE, [e] $p < 0.05$ vs ET.

3.4.3. Glutathione-S-transferase activity

The induction of obesity did not cause significant impact on GST activity in extra-hepatic tissues except in the brain (Fig. 3). In the brain, GST activity which decreased by about 18.56 % in OC relative to NC group, was increased by 141.06 % following treatment with ET fraction, when compared to OC group ($p < 0.05$). WE and RES administration showed non-significant effect ($p > 0.05$) on GST activity (Fig. 3).

3.4.4. Reduced glutathione level

Reduced glutathione was found to decrease by 22.83 % in adipose tissue (7.7 ± 2.3 OC vs 9.5 ± 0.6 NC), 35.71 % in the brain, 40.00 % in the kidneys, and 11.00 % in the heart (Fig. 4). Six-week administration of extract and fractions of *T. conophorum* nuts caused significant increase ($p < 0.05$) in GSH level thus; adipose (13.32, 21.36 and 17.61 %), brain (44.48, 30.89 and 45.78 %), kidney (21.70, 10.33 and 9.06 %) and heart (9.68, 31.58 and 26.48 %) for WE, ET and RES, respectively (Fig. 4).

3.5. Effect of treatment on lipid peroxidation

The TBARS-MDA level of OC group was significantly increased ($p < 0$ .05) by 36.72 % in adipose, 38.92 % in the brain, 23.82 % in kidneys and 7.74 % in the heart (Fig. 5). However, on treatment with extracts and fractions of African walnuts, there was observed decrease in the level of lipid peroxidation in all tissues/organs studied. In the adipose, there was 25.29 % decrease with WE, 23.90 % with ET and 26.71 % with RES. In the brain, there was 26.10 % decrease with WE, 11.68 % with ET and 8.48 % with RES, in the kidneys 29.17 % with WE, 31.35 % with ET and 41.64 % RES, and in the heart 1.83 % WE, 3.87 % with ET and 1.32 % with RES (Fig. 5).

3.6. Correlation/association between total lipids, total cholesterol and TBARS levels

The association between TBARS and total lipids, and TBARS and cholesterol concentration is shown in Fig. 6. The *r* value was found to be highest in the adipose tissue (r = +0.7251), indicating a strong positive correlation ($p < 0.05$) between the level of TBARS and cholesterol in the adipose tissue (Fig. 6). There were also positive correlations observed in the other tissues in both TBARS vs total lipids and TBARS vs total cholesterol (Fig. 6). In sum, a strong relationship between tissue peroxidation and increased lipid storage further authenticates the significance of the study (Fig. 6).

4. Discussion

The present study evaluated the effect of *T. conophorum* nuts on lipid accumulation (ectopic fat), associated peroxidation and oxidative stress status in extra-hepatic tissues of obese rats. The result revealed a reduction in the naso-anal lengths (cNAL %) of obese animals, indicating decreased growth rate, which was attenuated upon administration of the extract and fractions of *T. conophorum* nut. The reduction in cNAL % caused a significant increase in the other obesity indicators namely *f*BW: *f*NAL ratio, *c*BW (%), *c*BW: *c*NAL ratio and (*f*BW: *f*NAL) – (*i*BW: *i*NAL) compared to normal controls. Interestingly, these hitherto increase in obesity indicators were significantly modulated upon treatment with walnut extract and fractions. Our observation is in line with a previous study which showed that growth cessation and shortening of body length in obesity is likely an alteration that results from accumulation of fat in the abdominal region of the rats, with concomitant increase in body weight, without a corresponding increase in the body length [25]. In the present study, *T. conophorum* nut extract and fractions may have attenuated any of the central pathogenetic processes of MSG in obesity induction, such as; inhibition of lipid droplet accumulation in fat cells without affecting adipose conversion, inhibition of fatty acid synthase, or inhibition of adipocyte differentiation [32].

The concentrations of storage lipids namely; total lipids, triacylglycerols, phospholipids and cholesterols, in key organs and tissues (ectopic fat) of the body, were found to increase significantly with obesity induction. However, 6-week oral administration of *T. conophorum* nut extracts and fractions were found for the first time, to significantly modulate these storage lipids in the extra-hepatic tissues, corroborating the data on adipose tissue weights. In humans, these

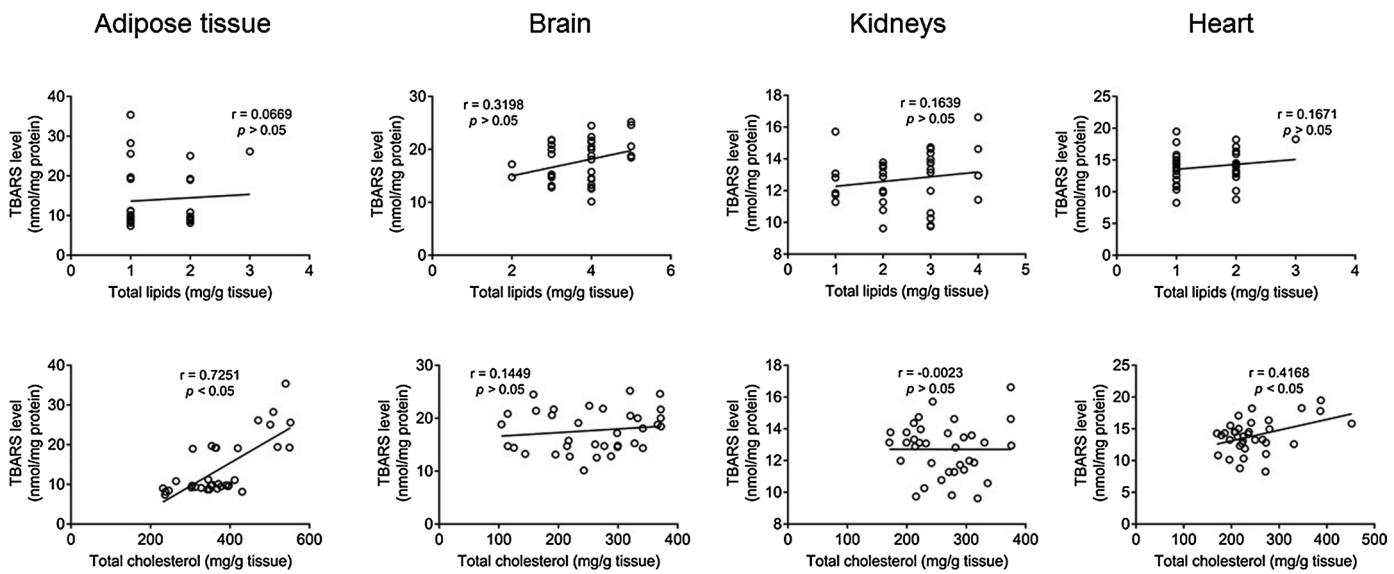

Fig. 6. Correlation between tissues total lipids, total cholesterol and TBARS levels of MSG-obese rats treated with extract and fractions of *Tetracarpidium conophorum* nuts. Pearson's correlation coefficient is denoted as "r". Correlation/association is significant if $p < 0.05$.

storage lipids are known to increase the risk of cardiovascular dysfunction, especially when total cholesterol level is elevated [33]. It is therefore a notable observation that the extracts used in this study were able to improve tissue dyslipidemia in MSG-induced obesity in rats. The presence of saponins, alkaloids, flavonoids, tannins and steroids in the nuts shown in our previous report [16] may be responsible for these observed anti-atherogenic and anti-hyperlipidemic effects of the nuts.

In several studies, the saponins have been shown to possess anti-adipogenic actions via pancreatic lipase inhibition [34], modulation of AMP-activated protein kinase (AMPK) [32], thereby inhibiting pre-adipocyte differentiation which could be useful agents in anti-obesity research [35]. Hence, the rich presence of saponins in *T. conophorum* nuts could decrease the overall lipid pool in circulation, thus, shifting the balance of lipids across tissues specifically towards catabolism than storage [36].

Our earlier study revealed significant presence of polyunsaturated fatty acids (PUFAs) in *T. conophorum* nut extract and fractions, with a low omega 6: omega 3 fatty acids ratio [16]. Omega-3 PUFAs have been shown to possess endothelial relaxation, anti-thrombotic and anti-fibrotic activities [37], which are beneficial in cardiac health. Peter and Jacob [38] have demonstrated a decreased triacylglycerol synthesis in hepatocytes, and increased clearance of circulating lipids from body tissues by omega-3 PUFAs. This implies that, PUFAs may have an important role in the management of dyslipidemia associated with obesity, while their length and increased number of double bonds, can influence the function of different membrane proteins and modulate the sodium channel function in cardiomyocytes, leading to antiarrhythmic effect [37]. It has also been reported that omega-3 PUFAs can interact with the main inflammatory signaling pathways and show suppressive effects on inflammatory cytokines production, hence their use as anti-inflammatory agents [39]. In the light of the above and given that *T. conophorum* nuts were found to be greatly endowed with PUFAs, it is plausible to hypothesize that the PUFAs in these nuts may be responsible for the observed tissue lipid mitigation, hence, a potential candidate for sourcing natural products for the fight against obesity.

The importance of omega-3 index defined as the percentage of eicosapentaenoic acid to docosahexaenoic acid in diets cannot be overemphasized [38]. An omega-3 index of less than 4 % is an indication of low cardio-protection associated with increased risk of ventricular fibrillation, along with complications which could result to sudden cardiac death [38], whereas levels higher than 8 % confer cardio-protection. *T. conophorum* nut oil belongs to the latter category since its omega-3 index is higher than 8 % [40], thus making it a cardio-protective candidate besides its anti-obesity effect.

Our study also assessed TBARS, a collection of peroxidized products, and other tissue antioxidant indicators including; GSH level, SOD, GPx and GST activities, as measures of lipid peroxidation and oxidative damage, respectively, in the studied tissues. Lipid peroxidation products which increased in obese rats across all studied tissues, and strongly correlated with adipose and organs lipid contents, were decreased upon treatment with *T. conophorum* nut extracts and fractions. This observation appears similar to previous reports which showed that lipid peroxidation in the liver, kidney and brain tissues of rats treated with cadmium was successfully attenuated upon administration of *T. conophorum* nut oil at 2.0 g/kg body weight [41]. It is however, worth noting that tissue target antioxidant action of the extracts for the whole nut in obese subjects is novel, to our knowledge. It is likely that this observed antioxidant property of the nuts is also achieved via the same mechanism employed against cadmium induced oxidative stress.

A strong relationship is known to exist between increased oxidative stress and hypercholesterolemia in obesity and diabetes [42], which is in line with our observations in this study. It has been shown that the African walnuts have great potentials to ameliorate lipid peroxidation *in vitro* [43]. Additionally, the flavonoids in these nuts [44,45] may also contribute to the observed antioxidant activity. Whereas the *in vitro* antioxidant properties of *T. conophorum* nuts may have been reported in previous studies, it is worthy to note that such studies, did not relate same to *in vivo* antioxidant activity nor obese situation, hence to our knowledge, this study reports *in vivo* antioxidant properties of the nuts for the first time, particularly as solvent fractions of the nut extract in obese animal models.

5. Conclusion

In conclusion, data from this study, in addition to validation of anti-obesity effect, showed that *T. conophorum* nuts can assuage the key pathogenesis and pathophysiological factors of obesity in ectopic sites, namely; lipid storage or lipogenesis, peroxidation and oxidative stress, hence a promising and prospective natural source of bio-actives for the prevention and treatment of obesity. Molecular mechanisms of action are however required to fully understand the role of *T. conophorum* nuts in attenuating obesity and its ameliorative effects at ectopic sites.

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Author's contribution

IJA, GEE and EUE conceived, designed and supervised the study. IJA and DEU performed literature searches, DEU and GUU carried out the animal experiments and the laboratory analyses. PAU participated in the laboratory analysis. DEU and VUN analyzed the data and prepared the manuscript. IJA proof read and edited the manuscript for intellectual content. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest

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