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ORIGINAL ARTICLE

Sclerocarya birrea Fruit Peel Ameliorates Diet-Induced Obesity and Selected Parameters of Metabolic Syndrome in Female Wistar Rats

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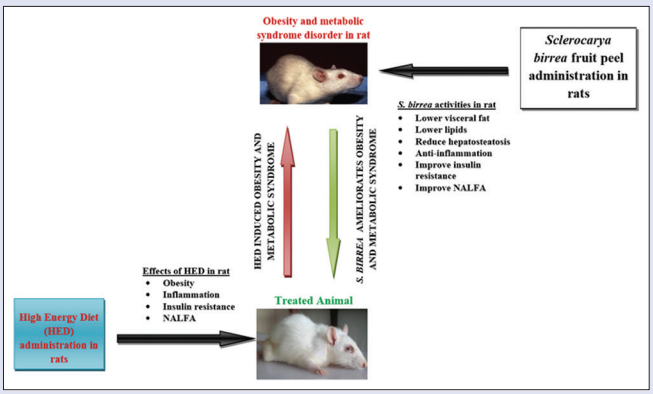
ABSTRACT

Background: We have shown *Sclerocarya birrea* fruit peels to possess *in vitro* antioxidant activity but yet to demonstrate its medicinal potential *in vivo*. **Objectives:** To investigate the effect of *S. birrea* fruit peel on diet-induced obesity and metabolic syndrome (MetS) in female Wistar rats. **Materials and Methods:** *S. birrea* fruit peels extract was profiled for phytochemicals by liquid chromatography-mass spectrometer. Total polyphenols, flavonoid, and total antioxidant capacity was determined by the colorimetric methods. Four groups of female rats (n = 6/group) were administered high energy diet (HED) formulation for 15 weeks then treated daily for 4 weeks as follows: normal diet and HED control groups received distilled water; HED treated with *S. birrea* hydroethanolic (70% ethanol) extract at 100 mg/kg BW (HED 100) and 200 mg/kg BW (HED200). Fasting glucose and body weights were monitored weekly. Oral glucose tolerance test and blood pressure (BP) were measured before and after treatment. After termination, visceral fat, total liver fat, lipid profiles, adiponectin, leptin, insulin, and homeostatic model assessment of insulin resistance (HOMA-IR) were determined. **Results:** *S. birrea* fruit peel was rich in polyphenols and had higher antioxidants activity than the fruit pulp. Untreated HED-fed rats showed increased body weight gain, visceral fat deposition, increased total cholesterol, glucose tolerance, serum insulin and HOMA-IR, increased BP and inflammation (increased serum leptin, leptin: adiponectin ratio and reduced adiponectin) as compared to normal control. Treatment with *S. birrea* extract at both doses fully or partially stabilized all these parameters except BP, triglycerides and low-density lipoprotein cholesterol which remained elevated after the 4-week treatment period. Histological examination showed reduced hepatic steatosis, thereby reducing non-alcoholic fatty liver disease. **Conclusion:** *S. birrea* fruit peel extract ameliorated obesity and MetS by reversing diet-induced visceral fat accumulation, hepatosteatosis, hypercholesterolemia, improving insulin resistance and inflammation and stabilizing leptin: adiponectin balance.

Key words: Insulin resistance, metabolic syndrome, non-alcoholic fatty liver disease, obesity, *Sclerocarya birrea* fruit peel

SUMMARY

- *Sclerocarya birrea* fruit is most famous for its use to manufacture a traditional alcoholic beverage "mukumbi" and a commercial alcoholic beverage, "Amarula." Although *S. birrea* fruit peel is discarded during the manufacturing of mukumbi and Amarula, we have previously shown the peel to be rich in polyphenols and flavonoids and possess *in vitro* antioxidant activity. In this present study, we showed *S. birrea* fruit peel extract to ameliorate metabolic syndrome (MetS) by reducing visceral fat accumulation, reducing dyslipidemia, improving insulin resistance, inflammation and non-alcoholic fatty liver disease and stabilizing leptin: adiponectin balance in HED-induced obesity and MetS in rats. We showed leptin: adiponectin ratio to be a potential marker to assess insulin resistance.



Abbreviations used: BW: Body weight; ELISA: Enzyme-linked immunosorbent assay; FFAs: Free fatty acids; HED: High energy diet; HFD: High fat diets; HOMA-IR: Homeostasis model assessment of insulin resistance; IHTG: Intra-hepatic triglyceride; IL-6: Interleukin; LDL-c: Low-density lipoprotein cholesterol; MetS: Metabolic syndrome; NALFD: Non-alcoholic fatty liver disease; ND: Normal diet; NO: Nitric oxide; OGTT: Oral glucose tolerance test; SPCA: Society for the Prevention and Cruelty for Animals; T2DM: Type-2 diabetes mellitus; TC: Total cholesterol; TG: Triglycerides; TNF-α: Tumour necrotic factor-alpha; VLDL-c: Very low-density lipoprotein cholesterol.

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INTRODUCTION

Metabolic syndrome (MetS) is a cluster of factors that are linked to increased risk for the development of cardiovascular diseases (CVDs) and type-2 diabetes mellitus (T2DM).[1] The metabolic factors include obesity, dyslipidemia, hyperglycemia, hypertension and insulin resistance, and the concurrence of at least three of these five risk factors defines a state of MetS.[1] Evidence suggests that obesity is primary to

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the origin of MetS and the consumption of high fat diets or high energy diets (HED) are known to promote the development of obesity.[2] Obesity is an abnormal accumulation of body fat, usually above 20% above the ideal body weight.[3] Hypertrophy of adipocytes and the consequent hypoxia results in increased expression and secretion of pro-inflammatory cytokines such as interleukin-6 and tumor necrosis factor-alpha which are associated with the development of local adipocyte and peripheral tissue insulin resistance.[4] Adipocyte insulin resistance results in lipolysis and increases free fatty acids (FFAs) into the circulation and subsequently the liver, where FFAs are stored as triglycerides (TGs) in a state of hepatosteatosis.[5] Increase in liver FFAs leads to increased synthesis of TGs, total cholesterol (TC), and production of apolipoprotein B containing cholesterol-rich very low-density lipoprotein cholesterol (VLDL-c) in the liver.[6] Increase in VLDL-c is associated with reduction in high-density lipoprotein cholesterol, a protective non-atherogenic lipid thus, leading to MetS-associated dyslipidemia.[6] Adipocytes synthesize adipokines such as adiponectin and leptin, among others, which are involved in various physiological functions including glucose and lipid metabolism.[7] Leptin is known to regulate energy consumption and expenditure through the control of food intake and glucose metabolism.[8] As a pro-inflammatory adipokine, leptin at elevated levels contributes to the development of insulin resistance.[9] On the other hand, adiponectin possesses anti-MetS effects through anti-obesity and anti-diabetic effects and alleviates insulin resistance by inhibiting inflammatory responses and atherosclerosis.[10] Furthermore, adiponectin is associated with increased endothelial nitric oxide (NO) synthase activity and a decrease in oxidative stress leading to increased synthesis and availability of NO thus protecting against hypertension through improvement in endothelial function.[11] Previous studies have shown that elevated leptin: adiponectin ratio is strongly related with CVDs and MetS than isolated leptin or isolated adiponectin concentrations with suggestions that leptin: adiponectin ratio could be a useful diagnostic index for insulin resistance and marker for assessing the effectiveness of antidiabetic therapy.[12,13] Based on the preceding discussion, there is ample evidence that suggest obesity is associated with chronic low-grade inflammation which contributes directly to the development of insulin resistance[14] and T2DM.[15] Insulin resistance is a metabolic condition in which cells fail to adequately respond to insulin action.[16] This impaired insulin response results in decreased glucose uptake by the adipose and muscle tissues.[16] Insulin resistance, characterized by hyperglycemia and hyperinsulinemia leads to the development of T2DM and MetS-related diseases.[17] Obesity and insulin resistance which are components of MetS can also lead to non-alcoholic fatty liver disease (NAFLD), a liver abnormality which has become an important problem of public health concern because of its increasing prevalence.[18] NAFLD is characterized by an increase of over 5% liver fat (hepatic steatosis), increase in intra-hepatic TG (IHTG), fibrosis along with the presence or absence of inflammation.[18] NAFLD is often associated with dyslipidemia and if not treated, it can progress to severe liver disease, T2DM, hypertension, and coronary heart disease.[19]

Several studies have shown that dietary polyphenols can be protective against MetS and reduce chronic low-grade inflammation.[20-22] *S. birrea* (Marula; or Mafura) forms an integral part of the diet, culture, and tradition of some rural communities in Southern Africa. Almost all parts of the plant are utilized either for food or medicine, but it is most famous for the use of its fruit to manufacture a traditional alcoholic beverage "mukumbi" and a commercial alcoholic beverage, "Amarula."[23] Although *S. birrea* fruit peel is discarded during the manufacturing of mukumbi and Amarula, we have shown that marula peel contains higher amount of flavonoids and polyphenols accompanied by higher *in vitro* antioxidant activity compared to the fruit pulp.[24] Although the mainstay for the treatment of obesity and associated MetS is lifestyle modification, reports show that more than 30% of individuals who initially succeeded to significantly lose weight, had rebound weight gain over 2 years.[25] This is a clue for researchers toward the development of new multi-target adjuvant pharmacological therapy to support lifestyle modifications. Several studies have demonstrated the potential of several fruit peels for MetS intervention including pomegranate,[26] apple,[27] and passion fruit.[28] Owing to the fact that *S. birrea* fruit peel is rich in polyphenols[24] which are known to be protective against MetS,[20-22] it was therefore of interest to investigate the effect of *S. birrea* fruit peel on diet-induced obesity and selected parameters of the MetS in female Wistar rats.

MATERIALS AND METHODS

Chemicals and reagents

Methanol (99.9%), acetonitrile (99.9%), sodium formate (99.0%), formic acid (95%), ethanol (95%) and glucose (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), gallic acid, quercetin, and ascorbic acid (Anmol Chemicals, Taloja Mumbai and Ankleshwar, India), polyalanine and chloroform (Rutland Industries PTY Ltd, Johannesburg South Africa), hematoxylin and eosin (Vector Laboratories, Inc., Burlingame, CA 94010, United States), TGs kit (catalogue no: TR212, Randox Laboratories, UK); low density lipoprotein cholesterol kit (catalogue no: CH2656, Randox Laboratories, UK), Blood cholesterol monitoring meter (Easy Touch®, VivaChek Biotech, China), rat ultrasensitive insulin Enzyme-linked immunosorbent assay (ELISA) kit (catalog no: NC9919398, Mercodia, Uppsala, Sweden), rat adiponectin ELISA Kit (catalog no: E-EL-R0329, Elabscience Biotechnology Co., Ltd, Wuhan, Hubei, China) and rat leptin ELISA kit (catalogue: E-EL-R0582, Elabscience Biotechnology Co., Ltd, Wuhan, Hubei, China). All the reagents were of analytical grade.

Plant collection

Fresh ripe *S. birrea* (marula) fruit was collected from Limpopo Province of South Africa and transported to Walter Sisulu University, Mthatha. The plant fruit was authenticated by Dr. Immelman of the Botany department at Walter Sisulu University and an herbarium voucher specimen (Manaka 1 KEI) was prepared and deposited in the Walter Sisulu University Kei Botany Herbarium. The fruits were cleaned and the peels were separated from the fruit pulp. The peels were air dried, crushed to a crude powder using a household blender.

Phenolic profiling by liquid chromatography-mass spectrometer

Phenolic compounds from powdered *S. birrea* peels (2 g) were extracted with 1:1 methanol: water solution with 1% formic acid and analyzed using the Waters Synapt G2 quadrupole time of flight (QTOF) (Milford, USA) as described by Stander *et al.*[29] Briefly, the powder was soaked in extraction solution overnight prior to sonication at 0.5 Hz at room temperature for 60 min. The mixture was then centrifuged for 5 min at 3000 g and filtered through a Whatman No. 1 filter paper. The filtrate was transferred to 2 ml crimp top vials for chemical characterization by the ultra-performance liquid chromatography coupled with QTOF mass spectrometer. The mobile phase comprised 0.1% formic acid as solvent A and 0.1% acetonitrile as solvent B and a 2.1 mm × 100 mm Waters high strength silica T3, 1.7 µm column was used. An injection volume of 2 µL was used and the gradient changed from 100% solvent A to 28% solvent B in 22 min, and went

to 40% solvent B in 50 s with a 1.5 min step wash with 100% solvent B. re-equilibration took 4 min and the column temperature was maintained at 55°C and the flow rate was 0.3 mL/min. A negative electrospray ionization mode was used with a 15 V cone voltage, and data were obtained from mass to charge ratio (m/z) above 150 and below 1500. Leu-enkaphalin was used as reference compound and sodium formate was used for machine calibration.

Plant extract preparation for antioxidant assays

The dried peel powder was extracted with hydroethanol (70% ethanol + 30% water). The mixture was left overnight with continuous agitation in a platform shaker (Labcon) at room temperature. The mixture was then filtered with Whatman No. 1 filter paper and ethanol was evaporated from the filtrate in a rotary evaporator. Water was removed from the filtrate using a fan oven at 40°C to obtain a dried extract which was used for animal treatments. Fresh *S. birrea* fruit juice pulp was dried in a fan oven and used for the analysis.

Acute toxicity study

Assessment of acute toxicity was done according to the Lorke's method[30] with slight modification as reported by Tata *et al*.[31] The study was conducted in two phases. In the first phase, 12 rats were divided into four groups of three rats each. Groups 1, 2, and 3 animals were treated orally with 10, 100, and 1000 mg/kg of the extract, respectively, whereas the fourth group (control) was given distilled water. The second phase which constituted three groups of 1 rat each was done after 24 h based on the findings of phase 1. No death was recorded in Phase 1, thus in phase II, higher doses (1600, 2900 and 5000 mg/kg) of the extract were administered to the three groups of animals respectively to determine the lethal dose (LD_{50}) value. The LD_{50} was calculated using the equation: $LD_{50} = \sqrt{(D_0 \times D_{100})}$. Where D_0 is the maximum dose that caused no mortality and D_{100} is the lowest dose that caused 100% mortality.

Antioxidant assay for *Sclerocarya birrea* fruit pulp and peel

Colorimetric total phenolic content (mg gallic acid equivalent/g dry extract) and total flavonoid content (µg quercetin equivalent/g dry extract) were assayed according to Pontis *et al*.[32] Quantification of total antioxidant capacity (TAC) using ferric reducing antioxidant power (FRAP; µg ascorbic acid equivalent/mg dry extract) was performed according to Benzie and Strain[33] as previously described by us.[34]

Experimental animals and ethics

Ethical clearance for this study was obtained from the Research Ethics Committee of the Faculty of Health Sciences, Walter Sisulu University with approval number: 072/2017. A total of twenty-four, 16 weeks old female Wistar rats weighing between 200 g and 250 g were purchased from the South African Vaccine producers (Johannesburg, South Africa) and kept in the Human Biology animal holding facility, Walter Sisulu University for 1 week. Animals were kept and housed in polypropylene cages and maintained on a 12 h day: 12 h night cycle at 22°C ± 2°C. The cage bedding was changed twice a week. The rats had access to food rodent pellets (Epol-SA, South Africa) and water *ad libitum*. The rats were handled in a humane manner by abiding to and following the guidelines specified by the National Council of the Society for the Prevention and Cruelty for Animals and the South African National Standard[35]

High energy diet preparation

The HED was prepared from the rodent pellets (Epol SA) designed as normal rat feed or normal diet (ND) as described previously by Oliva *et al*.[36] HED (3.5 kcal/g) was prepared from ND (1.2 kcal/g) by soaking in vegetable oil for 24 h and then mixed with sweetened full cream condensed milk (Nestle, South Africa). After air drying, this was the HED used for the study.

Study design and treatment

This study design was adopted from Oliva *et al*.[36] with slight modification. The HED was administered to 18 animals for 15 weeks to induce obesity and insulin resistance before initiation of *S. birrea* fruit peels extract treatment. Six rats were on ND. After 15 weeks on HED, the 18 female rats were randomly divided into three HED treatment groups (n = 6 rats/group), with 6 remaining on ND as follows:

- ND) = ND control treated with distilled water
- HED = High energy diet control treated with distilled water
- HED100 = High energy diet treated with 100 mg/kg BW *S. birrea* peel extract
- HED200 = High energy diet treated with 200 mg/kg BW *S. birrea* peel extract.

The 200 mg/kg BW dose was based on our previous study by Sewani-Rusike.[37] Treatment was administered orally once daily by gavage for 4 weeks. Weekly fasting blood glucose and body weights were measured. Oral glucose tolerance test (OGTT) and blood pressure (BP) were measured during the 15th week before the start of treatment and after 4 weeks of treatment, in the 19th week, before terminal procedures.

Measurement of blood pressure

BP was determined using the non-invasive tail-cuff plethysmography method as per manufacturer's instructions (CODA™ 8 BP System, Kent Scientific Corporation, USA). Mean BP was recorded and compared between groups.

Measurement of fasting glucose and oral glucose tolerance test

Fasting glucose (FG) was measured using a glucometer (Accucheck Active, Roche, Mannheim, Germany) in mmol/L. The OGTT was done as previously described by Sewani-Rusike *et al*.[38] to determine glucose clearance from the blood after a glucose load to reflect insulin response. After a 12 h fast, glucose levels were measured (time 0), and then, animals were given a glucose load of 3 g/kg p. o. in 1 ml volume. After the glucose load, blood glucose was measured at 30, 60, and 120 min using a glucometer (Accucheck Active, Roche, Mannheim, Germany).

Terminal procedures

At the end of the 4-week treatment period, rats were euthanized by CO_2 inhalation. Blood was collected by cardiac puncture into EDTA vacutainer test tubes (sterile VACUCARE ® SST GEL). Blood sample was centrifuged at 3000 RPM for 10 min at 15°C (Eppendorf 5810 R) to obtain plasma which was stored at-70°C (Skadie ultra freezer) for biochemical assays. The visceral fat was harvested and weighed. The right lobe of the liver was immediately frozen for total lipid determination. The left lobe of the liver tissue was harvested and stored in 10% formalin for the histological analysis. The percentage visceral fat to body weight ratio was calculated and expressed as weight indices from the formula: visceral fat weight index = (weight of visceral fat/final body weight) ×100.

Lipid profile determination

TC (mmol/L) was measured using a calibrated blood cholesterol monitoring meter (Easy Touch®, VivaChek Biotech, China). TGs (mg/dL) and low density lipoprotein cholesterol (mmol/L) were assayed using commercial kits (Randox Laboratories UK; reagents TR212 and CH2656, respectively) as per manufacturer's instructions.

Total liver lipid determination

The procedure used was adopted and modified as described by Folch *et al.*[39] Samples were run in duplicate. Approximately 500 mg frozen liver tissues were homogenized in 1000 µL of deionized water. Four milliliters (4 mL) of chloroform/methanol (2:1 vol/vol) mixture (Sigma-Aldrich) was added to the homogenate and thoroughly mixed for 10 min. Samples were centrifuged for 10 min at 16,000 ×g, to separate the organic phase (bottom) from the aqueous phase (top). The organic phase was carefully collected and transferred into a pre-weighed glass tube and dried in a fume hood with extraction fan for 48 h at room temperature. The tubes were reweighed and the change in weight constituted the total lipid content, which was expressed as percentage of liver tissue.

Plasma insulin, adiponectin, and leptin determination

Commercial ELISA kits were used for the determination of insulin (Mercodia, Uppsala, Sweden; rat ultrasensitive ELISA, catalogue NC9919398), adiponectin and leptin (Elabscience Biotechnology Co., Ltd, catalog E-EL-R0329 and E-EL-R0582, respectively) as per manufacturers' instructions.

Calculation of homeostatic model assessment of insulin resistance

Plasma insulin and FG concentrations were computed in the HOMA2 calculator v2.2.3 (https://www.dtu.o × .ac.uk/homacalculator/download.php) to determine homeostasis model assessment of insulin resistance (HOMA-IR), a measure for insulin resistance using the following formula:

$$\text{HOMA - IR} = \frac{\text{insulin}(\text{pMol}) \times \text{fasting glucose}\left(\frac{\text{mg}}{\text{dL}}\right)}{405}$$

Histological analysis

A piece of liver was placed in 10% buffered formalin and fixed for histological studies as previously described by Tiya *et al.*[40] Liver tissue was cut, placed into cassettes and processed using an automatic processor (Leica TP 1020, Wetzlar, Germany). Each portion of the tissue was embedded in paraffin wax (Leica EG1150, Wetzlar, Germany). The embedded tissues were cut into 5 µm sections using a sledge microtome (Leica SM2400, Wetzlar, Germany). The sections were placed in a water bath (Leica HI1210, Wetzlar, Germany) at 55°C to avoid folding of the sections and collected on glass slides. The slides were placed in an oven (Labcon 2085K) at 60°C overnight to remove excess wax. The slides were prepared and stained with eosin and hematoxylin and viewed under a light microscope for qualitative evaluation of lipid deposition consistent with NAFLD. Images were captured using a digital microscope (Leica DMD108, Wetzlar, Germany).

Statistical analysis

GraphPad Prism version 8 software (Graph-pad Software Inc., San Diego, CA, USA) was used for the statistical analysis and data presentation. The area under the curve (AUC) for OGTT assay was determined using GraphPad prism version 8 which employs the trapezoid method. Data were presented as mean ± standard error of the mean. Analysis of variance was used to compare mean differences of continuous variables between groups followed by Tukey's *post hoc* test. $P \leq 0.05$ was considered statistically significant.

RESULTS

Acute toxicity

The calculated LD_{50} value of the hydroethanolic extract of the *S. birrea* peel was 4235 mg/kg BW. The highest dose used in the current study was 200 mg/kg, which was 20 times less than the LD_{50}.

Phytochemical profile by liquid chromatography-mass spectrometer analysis

Profiling by liquid chromatography-mass spectrometer (LC-MS) showed the presence of diverse phytochemicals including polyphenols with the electrospray ionization negative mode (ESI–) profiling more phytochemicals than the ESI positive mode (ESI+), as shown in Figure 1. The LC-MS ESI – and ESI + were rich in polyphenols and other non-phenolic compounds [Figure 2]. ESI revealed the presence of more polyphenols compared to ESI–.

Antioxidants in fruit pulp and peels

Comparison of the antioxidant content of the fruit pulp and peels showed the fruit peel extract to have higher amounts ($P < 0.05$) in total phenolics and flavonoids than the fruit pulp. Furthermore, the TAC was higher ($P < 0.05$) in the fruit peels than in the pulp [Table 1].

Body weights of treated animals

Body weights were similar at initiation of feeding; however, animals fed with HED ($n = 18$) had consistently higher body weights compared to the ND control (ND; $n = 6$) animals especially at weeks 10–15 of the feeding period [Figure 3a]. At initiation of treatment with *S. birrea* peel extract, all HED-fed animals had similar body weights which were higher than ND controls. After 4 weeks of *S. birrea* extract treatment, both treatment groups (HED100 and HED200) showed lower body weights compared to HED ($P < 0.001$) but were comparable to ND [Figure 3b]. The net body weight after the 4 week treatment with *S. birrea* showed that there was a decrease in body weight for both treatment groups (HED100 and HED200) as compared to ND ($P < 0.05$; $P < 0.01$) and also compared to HED ($P < 0.001$) [Figure 3c].

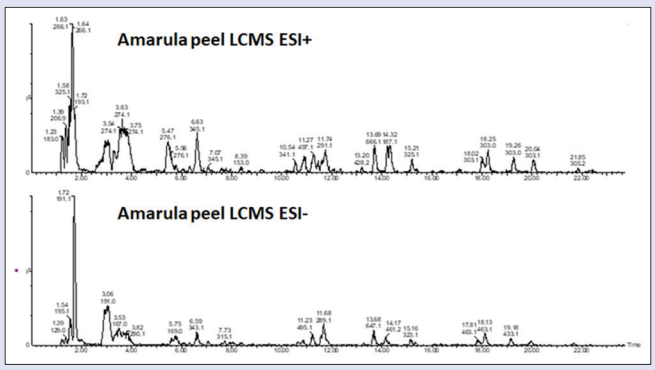

Figure 1: Liquid chromatography-mass spectrometer electrospray ionization positive (+) and negative (–) of 2 µl injections of *Sclerocarya birrea* fruit peel extract (1:1 methanol: water; 1% formic acid)

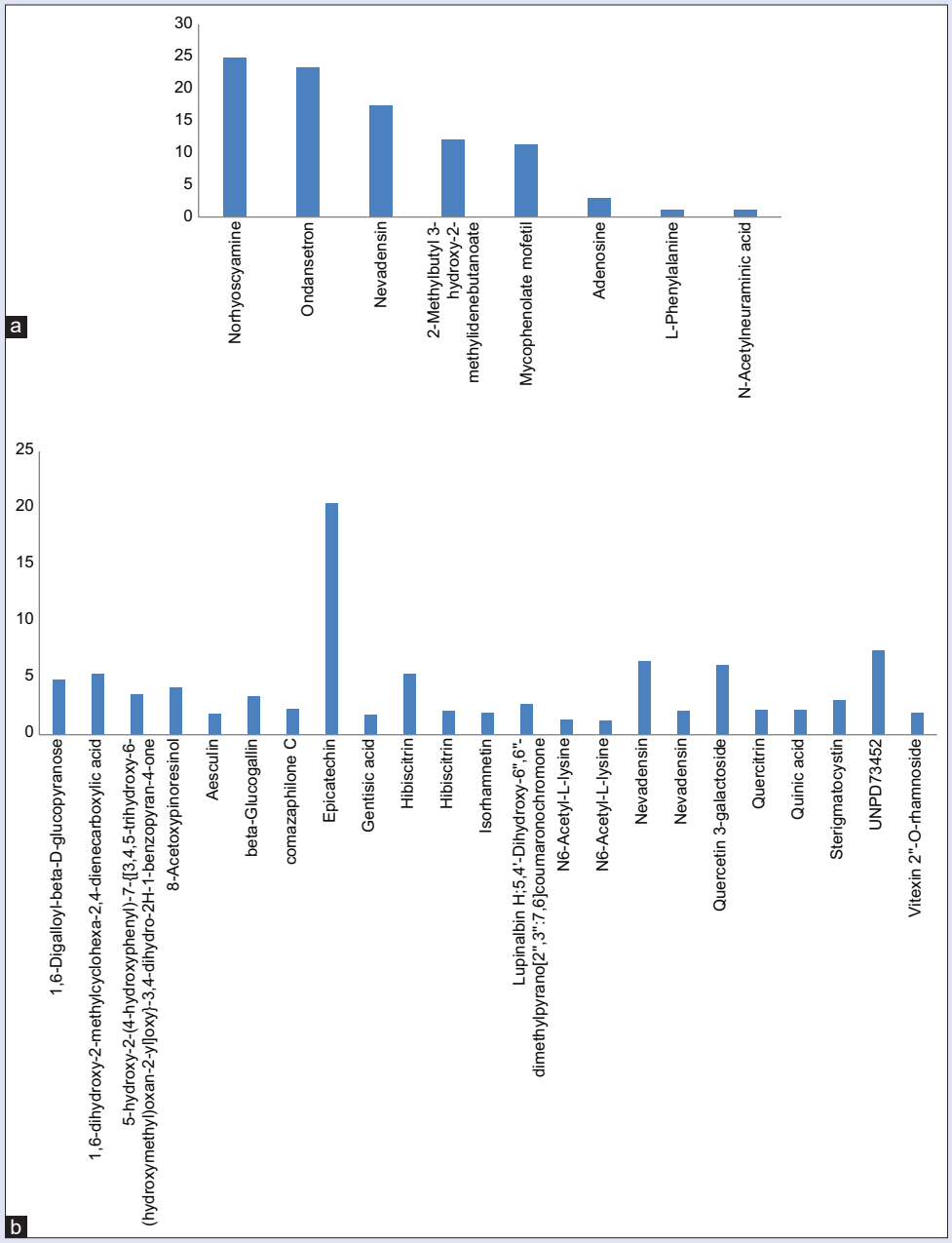

Figure 2: Relative abundance of phytochemical profile by Liquid chromatography-mass spectrometer ESI+ (a) and ESI-(b). ESI: Electrospray ionization

Table 1: Antioxidants in *Sclerocarya birrea* fruit pulp and peels

Parameters	Fruit pulp	Fruit peel
Total phenolics (mg GAE/g extract)	262.3±7.6	476±7.1***
Flavonoid (µg QE/g extract)	53.36±3.35	74.84±2.12***
TAC (µg AAE/mg extract)	50.6±2.3	146.1±4.3***

***$P < 0.001$ compared fruit pulp. Data were presented as mean±SEM. SEM: Standard error of the mean; TAC: Total antioxidant capacity; GAE: Gallic acid equivalent; QE: Quercetin equivalent; AAE: Ascorbic acid equivalent

Effect of *Sclerocarya birrea* fruit peels extract on feed intake, adiposity, and plasma lipid profiles

Visceral fat was higher ($P < 0.01$) in HED group as compared to ND group. However, treatment with *S. birrea* reduced ($P < 0.05$) visceral fat in HED100 and HED 200 groups compared to HED group but still remained higher than the ND controls. A similar trend was observed for TC which was higher ($P < 0.01$) in the HED group compared to ND and lowered ($P < 0.01$) in both *S. birrea*-treated groups compared to HED group but not compared to ND. There was a trend toward reduced LDL-c and TGs levels in the higher dose *S. birrea* treated animals (HED 200) "but the difference was not significant ($p<0.05$) compared to the HED control [Table 2].

Effect of *Sclerocarya birrea* treatment on plasma leptin, adiponectin concentrations, and leptin: adiponectin ratio

Food intake was higher ($P < 0.01$) in HED group as compared to ND group. However, treatment with *S. birrea* reduced ($P < 0.05$) food intake in HED100 and HED 200 groups as compared to HED group but still

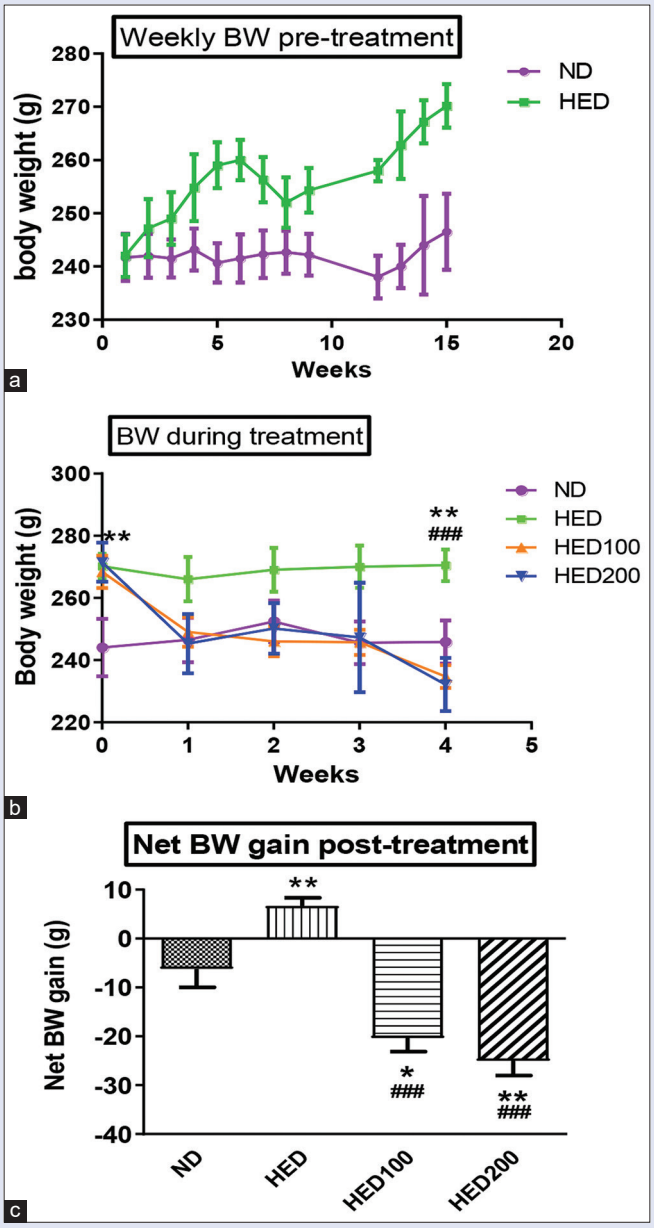

Figure 3: Effect of *Sclerocarya birrea* treatment on animal body weights. (a) Mean weekly body weights prior to treatment. (b) Mean weekly body weights during 4-week treatment with *Sclerocarya birrea*. (c) Mean net body weight change during 4-week treatment period with *Sclerocarya birrea*. Data were presented as mean ± SEM. SEM: Standard error of the mean; ND: Normal diet control; HED: High Energy diet control; HED100: High energy diet treated with 100 mg/kg *Sclerocarya birrea*; HED200: High energy diet treated with 200 mg/kg *Sclerocarya birrea*. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to ND; $^{\#\#\#}P < 0.001$ compared to HED

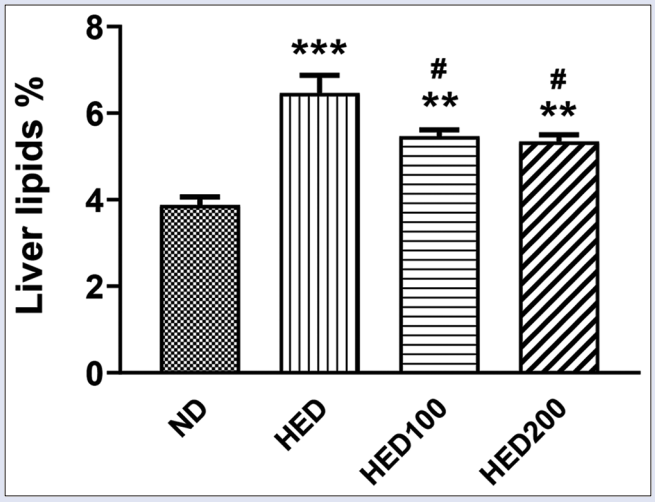

Figure 4: Effect of *Sclerocarya birrea* treatment on total liver lipids. ND: Normal diet control; HED: High Energy diet; HED100: High energy diet treated with 100 mg/kg *Sclerocarya birrea*; HED200: High energy diet treated with 200 mg/kg *Sclerocarya birrea*. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to ND; $^{\#}P < 0.05$ compared to HED

Table 2: Effect of *Sclerocarya birrea* treatment on food intake, adiposity and lipid profiles

Parameter	Treatment groups			
	Con	HED	HED100	HED200
Visceral fat (% BW)	1.20±0.17	2.46±0.22**	1.77±0.18*,#	1.44±0.18##
Cholesterol (mmol/L)	142±1.5	199±1.5***	175±2.7**,##	171±2.6**,##
TGs (mg/dL)	18.8±2.0	19.6±3.5	17.5±2.9	14.9±1.2
LDL-c (mmol/L)	1.8±0.04	1.89±0.03	1.83±0.02	1.82±0.01

*P<0.05; **P<0.01; ***P<0.001 compared to ND; #P<0.05; ##P<0.01 compared to HED. Data was presented as mean±SEM. SEM: Standard error of the mean; TG: Triglycerides; LDL-c: Low density lipoprotein cholesterol; Con: Normal diet control; HED: High energy diet control; HED100: HED treated with 100 mg/kg *S. birrea*; HED200: HED treated with 200 mg/kg *S. birrea; S. birrea: Sclerocarya birrea*; BW: Body weight

Table 3: Effect of *Sclerocarya birrea* treatment on leptin and adiponectin

Parameter	Treatment groups			
	Con	HED	HED100	HED200
Food intake (g)	142±1.5	199±1.5***	175±2.7**,##	171±2.6**,##
Leptin (ng/ml)	3.15±0.27	5.11±0.24***	3.68±0.07##	3.55±0.21##
Adiponectin (ng/ml)	2.50±0.17	1.64±0.09***	1.86±1.13**	2.47±0.14##
Lept/Adp ratio	1.29±0.14	2.74±0.20***	2.01±0.12*,#	1.33±0.11###

*P<0.05; **P<0.01; ***P<0.001 compared to ND; #P<0.05; ##P<0.01 compared to HED. Data was presented as mean±SEM. SEM: Standard error of the mean; Lept: Leptin; Adp: Adiponectin; Con: Normal diet control; HED: High energy diet control; HED100: HED treated with 100 mg/kg *S. birrea*; HED200: HED treated with 200 mg/kg *S. birrea; S. birrea: Sclerocarya birrea*

remained higher than the ND controls. HED increased plasma leptin and lowered adiponectin concentrations with resultant increased ($P < 0.001$) in leptin: adiponectin ratio compared to ND group. Treatment with both doses of *S. birrea* extract reduced plasma leptin, increased adiponectin and lowered leptin: Adiponectin ratio ($P < 0.05$) compared to HED fed rats. This effect was dose dependent with a greater effect observed for the higher *S. birrea* dose (HED200) that stabilized all parameters to be similar to ND control. In the lower *S. birrea* dose (HED100), adiponectin remained similar to HED group [Table 3].

Effect of *Sclerocarya birrea* treatment on total liver lipids

NAFLD is diagnosed when there is ≥5% total lipid in liver tissue equivalent to hepatic steatosis. All untreated and *S. birrea* treated HED exposed rats had total lipid above 5% (HED = 6.46% ± 0.41%; HED100 = 5.46% ± 0.15%; HED200 = 5.34% ± 0.16%) and higher than ND control. However, treatment with *S. birrea* at both doses reduced total hepatic lipid to lower than HED control [Figure 4].

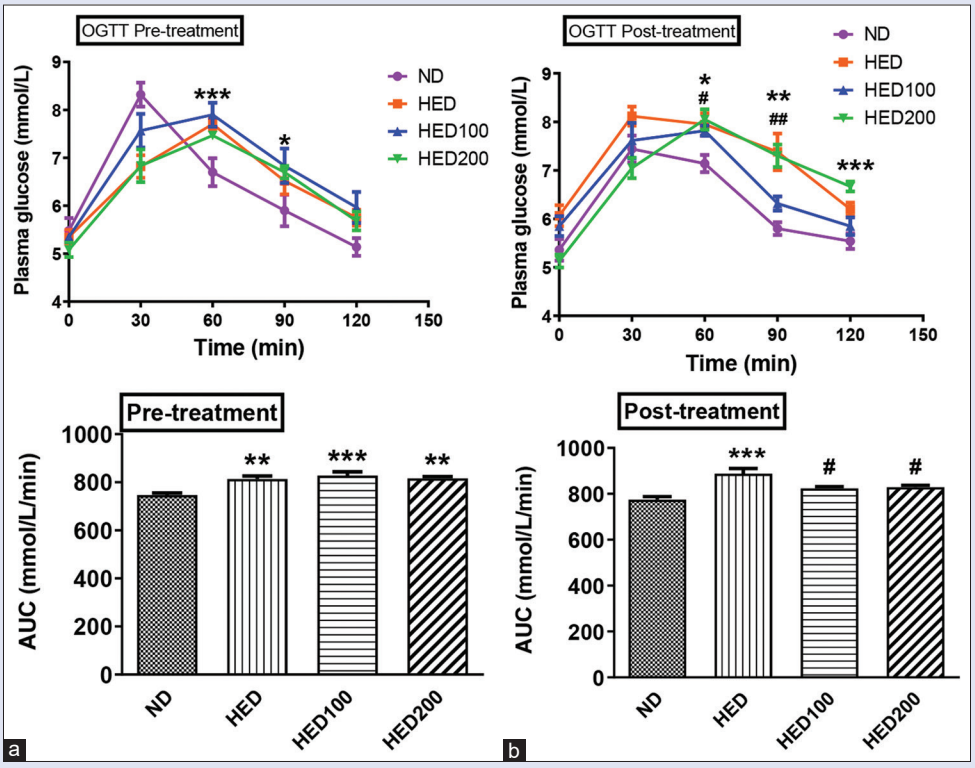

Figure 5: Effect of *Athrixia phylicoides* tea infusion on oral glucose tolerance showing dose response curves and area under the curve (AUC. [a] before treatment with *Sclerocarya birrea*. [b] after treatment with *Sclerocarya birrea*. Data were presented as mean ± SEM. SEM: Standard error of the mean; ND: Normal diet Control; HED: High Energy Diet; HED100: High energy diet treated with 100 mg/ml *Sclerocarya birrea*; HED200: High energy diet treated with 200 mg/ml *Sclerocarya birrea*. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to ND; $^{\#}P < 0.01$, $^{\#\#}P < 0.01$ compared to HED

Effect of *Sclerocarya birrea* fruit peels extract on oral glucose tolerance

OGTT curves as well as the AUC showed that all HED groups had developed insulin resistance after the 15-week obesity induction period [Figure 5a]. Treatment with both doses of *S. birrea* showed improvement in glucose tolerance which was comparable to the ND control with lower AUC ($P < 0.05$) compared to the HED control group [Figure 5b].

Effect of *Sclerocarya birrea* fruit peels extract on fasting glucose, insulin, and homeostasis model assessment of insulin resistance

The HED group showed impaired glucose homeostasis as shown by increased FG ($P < 0.05$), higher insulin ($P < 0.01$), and HOMA-IR index ($P < 0.001$) compared to the ND controls after the 19-week study period. Treatment with *S. birrea* at both doses stabilized glucose homeostatic parameters with FG and insulin concentration similar to ND controls. The HOMA-IR index was effectively reduced to ND control level by treatment with the higher dose of *S. birrea* (HED200) but not at the lower dose [Table 4].

Effect of *Sclerocarya birrea* treatment on blood pressure

Exposure to HED for 15 weeks during the obesity induction period resulted in increased mean BP ($P < 0.001$) in HED rats as compared to ND control. Treatment with *S. birrea* had no effect on mean BP, which remained higher than ND for all treatment groups [Figure 6].

Table 4: Effect of *Sclerocarya birrea* treatment on fasting glucose, insulin and homeostatic model assessment of insulin resistance index

Parameter	Treatment groups			
	Con	HED	HED100	HED200
Fasting glucose (mmol/L)	5.36±0.22	6.77±0.22*	5.85±0.20$^{\#}$	5.13±0.14$^{\#}$
Insulin (ng/ml)	0.071±0.08	1.26±0.12**	1.08±0.15	1.01±0.14
HOMA-IR index	0.93±0.2	2.70±0.3***	2.27±0.2*	1.29±0.3$^{\#\#}$

*P<0.05; **P<0.01; ***P<0.001 compared to ND; $^{\#}P$<0.05; $^{\#\#}P$<0.01 compared to HED. Data were presented as mean±SEM. SEM: Standard error of the mean; Con: Normal diet control; HED: High energy diet; HED100: HED treated with 100 mg/ml *S. birrea*; HED200: HED treated with 200 mg/ml *S. birrea; S. birrea: Sclerocarya birrea; HOMA-IR: Homeostatic model assessment of insulin resistance*

Effect of *Sclerocarya birrea* on liver histology

The liver of the ND group showed that all cells of the liver were intact with normal architectural arrangement [Figure 7a] while the liver of HED group showed clear micro-steatosis shown as fat droplets scattered in the liver, characteristic of NAFLD [Figure 7b]. The liver of animals HED100 and HED200 treated with 100 and 200 mg/kg BW *S. birrea* showed resolution of HED-induced micro-steatosis and liver histology was comparable to control [Figure 7c and d].

DISCUSSION

Sclerocaraya birrea is a plant whose fruit peel is discarded during the manufacturing of mukumbi and Amarula local drinks. However, we have previously shown that *S. birrea* contain higher content of polyphenols and flavonoids and has higher *in vitro* antioxidant activity as compared to the fruit pulp.[22] This present study confirms *S. birrea* fruit peel to

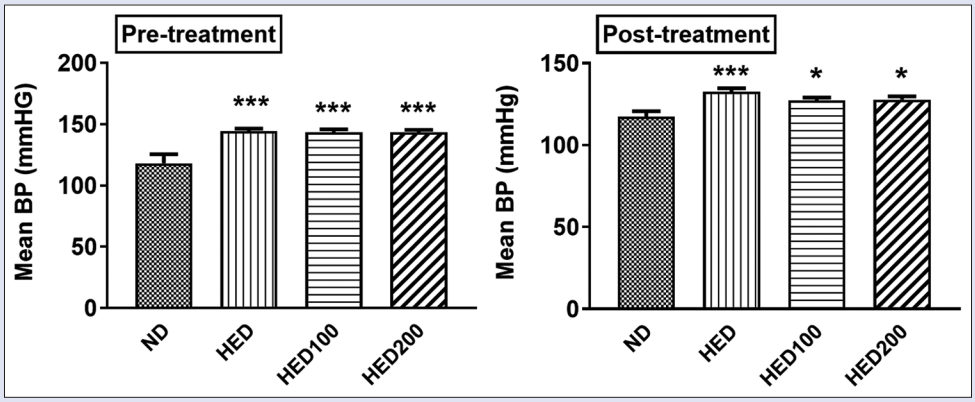

Figure 6: Effect of *Sclerocarya birrea* treatment on mean blood pressure. Data were presented as mean ± SEM. SEM: Standard error of the mean; BP: Blood pressure; ND: Normal diet control; HED: High energy diet; HED100: High energy diet treated with 100 mg/ml *Sclerocarya birrea*; HED200: High energy diet treated with 200 mg/ml *Sclerocarya birrea*. *$P < 0.05$, ***$P < 0.001$ compared to ND; #$P < 0.05$ compared to HED

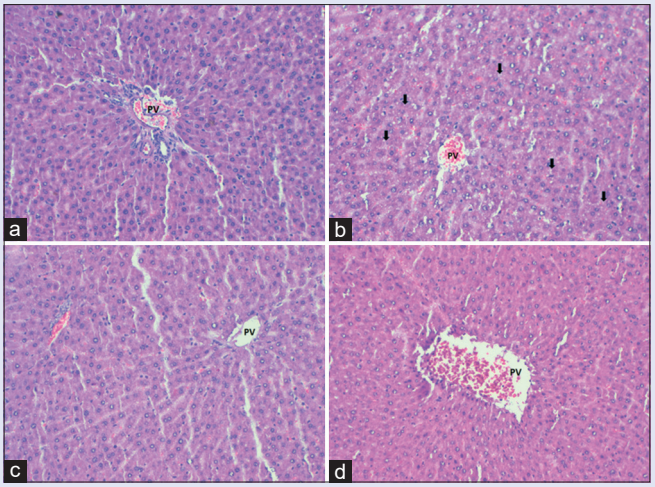

Figure 7: Representative photomicrographs showing histopathological features of the hematoxylin and eosin (Magnification × 20) stained liver sections of male rats from each treatment group. Black arrows represent micro-steatosis. (a) is ND: Normal diet control; (b) is HED: High energy diet; (c) is HED100: High energy diet treated with 100 mg/kg BW *Sclerocarya birrea*; (d) HED200: High energy diet treated with 200 mg/kg BW *Sclerocarya birrea*

be richer in polyphenols and flavonoids and had higher antioxidant activity than the fruit pulp. The colorimetric antioxidant activity was associated with the many polyphenols from LC-MS. Since polyphenols and flavonoid have been shown to possess antioxidant activity[41] with other medicinal potential in managing obesity,[42] insulin resistance and diabetes,[43] we hypothesized that *S. birrea* fruit peel could have some medicinal potential in the management of obesity and MetS. To achieve this, we sought to induce obesity and MetS in animals and subsequently treated them with *S. birrea* peels extract. In this study, HED which contained high amount of carbohydrates and fats and known to cause MetS[44] was used to induce obesity and insulin resistance in rats. This resulted to increased food intake, increased visceral fat leading to obesity which is evident by increased weight gain in animals. Treatment with *S. birrea* peel extract resulted in a marked decrease in visceral fat and body weights of both treated groups comparable to the ND control. Obesity promotes lipolysis of fat in the adipose tissue thereby increasing FFAs in circulation which is subsequently metabolized to other forms of lipids, especially increasing TGs, TC as well as VLDL-c and LDL-c. As such, obesity is often associated with dyslipidemia.[45] Furthermore, *S. birrea* peel extract treatment reduced cholesterol and also showed a trend to reduce TG and LDL-c. These findings suggest that *S. birrea* peels possess anti-obesity and anti-lipidemic effects and could prevent dyslipidemia. This is in accordance with other studies which have shown other fruit peels to possess obesity and lipid lowering effects.[46-48]

Obesity is known to promote insulin resistance by indirectly promoting low-grade inflammation.[49] In the adipose tissue, there exist some anti-inflammatory molecules such as adiponectin which helps to prevent inflammation. However, obesity-induced inflammation impairs adiponectin secretion.[15] Conversely, leptin is a pro-inflammatory molecule that promotes inflammation. Apart from its roles in regulating food intake, leptin is known to regulate food intake and energy expenditure.[50] Increase in leptin level increases food intake casing hypertrophy of adipocytes and hypoxia which in turn promotes the release of inflammatory molecules[51] and eventually leading to inflammation. There exist evidence that obesity promotes chronic low-grade inflammation which contributes directly to insulin resistance[52] and T2DM.[53] More so, increase leptin level increases food intake thereby increasing glucose load (hyperglycemia) which often leads to insulin resistance. Therefore, the leptin to adiponectin ratio has been considered as a marker for assessing insulin resistance. Also, increased leptin: Adiponectin ratio has been previously reported to be strongly associated with MetS as well as CVDs than their independent concentrations.[12,13] Our findings showed that adiponectin level was increased in animals treated with the higher dose of *S. birrea* (HED200) while leptin was reduced in animals treated with *S. birrea*. Moreover, the net leptin to adiponectin ratio was reduced in animals treated with *S. birrea* as compared to the HED untreated group. More so, *S. birrea* treatment was shown to reduce food intake in the HED100 and 200 treated animals. These findings suggest that *S. birrea* ameliorated inflammation and insulin resistance by stabilizing the leptin: adiponectin balance and lowering food intake which may correspond to lowering glucose load in the animals. To confirm the involvement of *S. birrea* in ameliorating insulin resistance, its role on glucose clearance and insulin action was further assessed.

Insulin is a key hormone that regulates glucose clearance from the circulation. In a normal homeostatic state, insulin reaches its peak after a meal which corresponds to increase blood glucose. This is followed by a gradual decrease of blood glucose which eventually returns to a fasting state as a result of insulin action.[16] Obesity can promote insulin

resistance by preventing glucose tolerance, and therefore, increased HOMA-IR and hyperinsulinemia are the characteristic markers of insulin resistance.[16] In the current study, the FG level was reduced in the *S. birrea* treated animals as compared to the HED untreated animals. Furthermore, treatment with *S. birrea* increased glucose clearance in the treated animals, especially the lower dose treatment (HED100). More so, blood insulin and HOMA-IR were reduced in *S. birrea* treatment groups as compared to the HED-fed untreated animals. These findings confirmed *S. birrea* to possess glucose lowering effect and to ameliorate insulin resistance. In support of these findings, studies have shown Jaboticaba berry peel which is rich in polyphenols to have positive effects on insulin sensitivity,[54,55] suggesting a possible role of these plant peels in improving glucose and insulin metabolism. Furthermore, insulin resistance could be as a result of obesity-induced NAFLD; a condition characterized by an increase in IHTG content with inflammation and fibrosis.[56] In a study conducted by Hong *et al.*,[57] animals fed with HED resulted in NAFLD, characterized by mark increase in visceral fat, hepatic lipids, and insulin resistance. At the moment, weight reduction remains the main known means to prevent or reverse NAFLD. However, studies have shown that reduction of fat could reverse insulin resistance and liver steatosis in NAFLD animals.[58] Treatment with *S. birrea* fruit peel reduced total liver lipid content which was also demonstrated by histological analysis that showed the resolution of hepatosteatosis. This finding suggests that *S. birrea* treatment could ameliorate NAFLD as well as insulin resistance.

Obesity, dyslipidemia, and insulin resistance are associated with hypertension. Obesity as well as dyslipidemia can alter adiponectin which negatively affects endothelial function by lowering NO causing vasoconstriction and high BP.[59] Furthermore, hyperglycemia and hyperinsulinemia activates the rennin-angiotensin system which may lead to hypertension[60] while insulin resistance can increase kidney sodium reabsorption, increases cardiac output with resulting arterial vasoconstriction leading to hypertension.[61] Findings in this study showed *S. birrea* not to have an effect in lowering BP as the BP was similar between the *S. birrea* treated and untreated animals. This finding suggest that *S. birrea* peels may not possess anti-hypertensive effect, though previous studies have shown *S. birrea* leaf extract to lower BP in animals.[62,63] This finding does not concur with other studies which have shown dietary polyphenol-rich plants to lower BP in human and animal studies.[64,65] A possible mechanism for the effect of *S. birrea* fruit peel on MetS is that the polyphenols in the fruit peels lowered visceral fat and lipids in the liver, thereby preventing NAFLD and hepatosteatosis. Furthermore, the reduced visceral fats and lipids maintained the adiponectin/leptin preventing inflammation and insulin resistance. Although this study showed *S. birrea* fruit peel extract to ameliorate MetS, it was limited in that no specific positive control drug was used in the study to control for independent MetS parameters.

CONCLUSION

The present study showed that *S. birrea* fruit peel extract ameliorated MetS by lowering body weight, lipids, improving insulin resistance, inflammation and NAFLD, and stabilizing leptin: adiponectin balance in HED-induced obesity and MetS in rats. Leptin: Adiponectin ratio was shown to be a potential marker to assess insulin resistance. The presence of polyphenols in the *S. birrea* fruit peel may be responsible for the observed biological effects linked to its medicinal properties. Therefore, *S. birrea* fruit peel has potential as a one-step dietary supplement to reverse visceral obesity and associated metabolic complications.

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Conflicts of interest

There are no conflicts of interest.

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