Objective: To quantify some major pharmacologically important flavonoids and phenolic acids in *Brachystegia eurycoma* seed flour (BESF) and evaluate its antidiabetic activity in type 2 diabetic rats.

Method: Flavonoids and phenolic acids were quantified using a reverse-phase high pressure liquid chromatography coupled with diode array detection. Type 2 diabetes was induced in rats using high-fat diet, low-dose streptozotocin (HFD/STZ) model, by feeding the rats with HFD for 2 weeks followed by single dose administration of STZ (40 mg/kg body weight, intraperitoneally). The diabetic rats were later fed BESF-supplemented (10% and 20%) diets, or administered with metformin (25 mg/kg b.w.) for 21 days; the control rats were fed basal diet during this period. After the dietary regimen, the rats were sacrificed, and their blood, liver and pancreas samples were collected for biochemical assays.

Results: The flavonoids (catechin, rutin, quercitrin, quercetin and kaempferol) and phenolic acids (gallic acid, caffeic, chlorogenic and ellagic acid) were abundant in BESF. BESF-supplemented diets (BESF-SD) significantly (*P* < 0.05) decreased the levels of fasting blood glucose (FBG); ameliorated the lipid profile, and significantly (*P* < 0.05) increased the hepatic glycogen of the diabetic rats. BESF-SD also significantly (*P* < 0.05) attenuated the oxidative stress induced by STZ in the liver and pancreas of the diabetic rats. The ameliorative effects of 20% BESF-SD compared (*P* > 0.05) with metformin administration in some of the biomarkers.

Conclusion: The flavonoids and phenolic acids in BESF may have acted synergistically to produce the observed antidiabetic effects. BESF could therefore be an effective and affordable dietary therapy for the management of T2DM; and an excellent source for drug discovery.

1. Introduction

Type 2 diabetes mellitus (T2DM) or non–insulin dependent (NIDDM) is the most common form of diabetes, accounting for 90–95% of diabetes cases[1]. It is characterized by chronic hyperglycemia arising from insulin resistance and loss of pancreas β-cell function[2]. Chronic hyperglycemia is often associated with a variety of complications, such as retinopathy, nephropathy and neuropathy; and these constitute threats to the life of T2DM patients[3]. Dyslipidemia[4] and oxidative stress[5] are among the prominent features of T2DM.

Globally, diabetes mellitus (DM) has become a major cause of death in people less than 60 years of age. The International Diabetes Foundation estimated that it caused 5.1 million deaths and cost USD 548 billion in healthcare spending by the end of 2013[6]. Despite this huge capital investment, diabetes remains a major global health problem, having no satisfactory therapy in modern medicine, in terms of safety and efficacy. The currently available antidiabetic drugs for the treatment of T2DM have certain limitations ranging from adverse side effects and development of resistance to lack of responsiveness in many diabetic patients. In addition to these drawbacks, none of the hypoglycemic agents adequately control the hyperlipidemia that frequently characterizes the disease[7]. These
drawbacks, coupled with the emergence of the disease into global
epidemic have necessitated research into safer and more efficient
alternative management strategies for T2DM, including use of
nutraceutical dietary therapy.

Plant phenolics are increasingly attracting research interest due
to their possible health benefits in humans. As a prominent group
of secondary metabolites, phenolic compounds have diverse
pharmacological activities including anti-diabetic activity[8], and the
consumption of polyphenols-rich diets has been reported to protect
against development of degenerative diseases including diabetes[9].
Hence, as natural components of the human diet, plant polyphenols
could offer a safer and more affordable management strategy for
T2DM than synthetic antidiabetic drugs.

*Brachysetgia eurycoma* is a leguminous tree crop mostly grown
in the tropical rain forest of West Africa. It is an economically
valuable tree crop, having applications that range from food to
ethnomedicine. The edible seeds are used as a natural additive for
soup thickening among the rural dwellers in West Africa, particularly
in the South-Eastern part of Nigeria[10,11]. The nutrients composition
of the seeds as earlier reported by Uhegbu et al.[11], shows that they
are a good source of nutrient supplement. The seed flour has good
water absorption capacity, and so, is useful as functional agent in
processed foods such as bakery products and meat formulations
[10]. In ethnomedicine, some natives of Nigeria use the extracts of
*B. eurycoma* to cure infections such as toothache, scabies, asthma,
tuberculosis, bronchitis, catarrh, sore throat, phlegm, guinea worm
infections and other inflammatory conditions[12]. To further explore
*B. eurycoma* seeds as a potential nutraceutical, this study quantified
the major flavonoids and phenolic acids of pharmacological
importance in the seeds flour and evaluated its antidiabetic properties
in high-fat diet, low-dose streptozotocin-induced T2DM in rats.

2. Materials and methods

2.1. Sample collection and preparation

Sample of *B. eurycoma* seeds (Figure 1) was purchased in a local
farm settlement in Ibadan, Oyo State, Nigeria. The seeds were later
authenticated at the Department of Botany, University of Ibadan,
Nigeria. Subsequently, the seeds were sorted, sun-dried, manually
shelled and milled to fine particle size to produce the flour (BESF).
The BESF sample was packed in an airtight container and stored
shelled and milled to fine particle size to produce the flour (BESF).

2.2. Experimental animals and induction of T2DM using the
HFD/STZ model

Adult male Wistar rats weighing 180-200 g were used for this
study. The rats were procured from the experimental animal breeding
unit of Department of Veterinary Medicine, University of Ibadan,
Nigeria, and were handled in accordance with the Guide for the Care
and Use of laboratory animals, as approved by the Animal Ethics
Committee of our institution. Rats were housed in experimental
cages under a 12 h light-dark cycle at an ambient temperature of (25
± 2°C), and were allowed to acclimatize for 7 days, during which
they were fed rodent chow and water *ad libitum*. To induce T2DM,
rats (excluding the normal control group), were fed HFD (40% fat,
18% protein and 41% carbohydrate, as a percentage of total kcal)
for 2 weeks[13], and then injected with single dose STZ (40 mg/
kg body weight, intraperitoneally, in citrate buffer; pH 4.5)[14]. The
development of hyperglycemia in rats was confirmed by testing
fasting blood glucose (FBG) 72 h post-STZ injection. Rats that
maintained FBG higher than 200 mg/dL were considered diabetic,
and selected for the study.

2.3. Tissue preparation

The liver and pancreas tissues, excised immediately after rats
were sacrificed, were perfused with ice-cold saline. They were
homogenized at 4°C with 10 times w/v 0.1 M phosphate-buffer, pH
7.4, in a polytron homogenizer. The homogenate was centrifuged
at 800 rpm for 5 min at 4°C to separate the nuclear debris, and an
aliquot of the supernatant was used for estimation of malonaldehyde
(MDA) level. The supernatant was further centrifuged at 10000 rpm

2.4. Animal groups and experimental design

Rats were divided into 5 groups of 5 animals each as follows:
group I (normal control) rats were fed basal diet throughout the
experiment; group II (diabetic control; HFD + STZ) rats were fed
HFD throughout the experiment; group III (diabetic, metformin-
treated; HFD + STZ + metformin) rats were administered metformin
(25 mg/kg bw), while continuing on the HFD; group IV (diabetic,
10% BESF diet-fed; HFD + STZ + 10% BESF) rats were fed HFD
supplemented with 10% BESF; group V (diabetic, 20% BESF diet-
fed; HFD + STZ + 20% BESF) rats were fed HFD supplemented
with 20% BESF. The dietary regimen lasted for 21 days, during
which FBG was measured at 0, 7th, 14th, 21st day of the study using
blood from rats’ tail vein. At the end of the experiment, the rats were
fasted overnight, and sacrificed. Then their blood, liver and pancreas
samples were collected for biochemical assays. The plasma portion
of the blood was separated by centrifuging at 800 rpm for 10 min.
for 20 min at 4°C to get the post-mitochondrial supernatant, which was used for antioxidant biochemical assays.

2.6. Quantification of flavonoids and phenolic acids using HPLC-DAD

Major flavonoids and phenolic acids content of BESF was quantified using reverse phase HPLC-DAD. The HPLC-DAD instrumentation comprised a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector and LC solution 1.22 SP1 software.

Chromatographic quantification of BESF extracts was carried out at a concentration of 20 mg/mL under gradient conditions using C₁₈ column (4.6 mm × 150 mm) packed with 5 μm diameter particles; the mobile phase was water containing 1% formic acid (A) and acetonitrile (B), and solvent gradient elution program used was as described by Kamdem et al.[15] with slight modifications. Samples extracts and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. The flow rate was 0.7 mL/min, injection volume 50 μl and the wavelength were 257 for gallic acid, 281 for catechin, 325 nm for chlorogenic, caffeic and ellagic acids, and 366 nm for quercetin, quercitrin, rutin and kaempferol. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.030–0.250 mg/mL for kaempferol, quercetin, quercitrin, rutin and catechin; and 0.050 – 0.450 mg/mL for ellagic, gallic, caffeic and chlorogenic acids. Chromatography peaks of sample were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200 to 600 nm). Both the sample extract and the standards were analyzed in triplicates.

2.7. Biochemical assays

Fasting blood glucose was estimated using a portable glucometer (Accu check Active). Liver glycogen was estimated following the method reported by Ong and Khoo[16], using Anthrone reagent. Plasma Lipid profile, namely, triglycerides (TRIG), total cholesterol (TC) and high-density lipoprotein (HDL) levels were estimated using commercially available standard kits (Randox Laboratories), according to manufacturer’s instruction. Low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) levels were calculated using Friedewald formula[17], as follows: LDL = TC – (HDL + VLDL); VLDL = TG/5.

Enzymatic and non-enzymatic biomarkers of oxidative stress in liver and pancreas tissues were determined according to the following methods: superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) activities were estimated using standard kits (Randox Laboratories); catalase (CAT) activity[18]; reduced glutathione (GSH) level[19]. Liver and pancreas malondialdehyde (MDA) level as an index of lipid peroxidation was determined according to the method described by Okhawa et al.[20]. The protein content of the liver and pancreas was estimated following the method of Lowry et al.[21].

2.8. Statistical analysis

Results of replicate experiments were expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) and least significance difference (LSD) were carried out on the result data at 95% confidence level using SPSS statistical software package, version 17.

3. Results

3.1. Flavonoids and phenolic acids composition of BESF

Table 1 presents the major flavonoids and phenolic acids of pharmacological importance in BESF. A representative chromatogram of the reverse phase HPLC-DAD analysis of the sample is shown in Figure 2. Flavonoids including catechin, rutin, quercitrin, quercetin and kaempferol were in appreciable levels in BESF in the order of quercitrin > kaempferol > quercetin > rutin > catechin. Similarly, phenolic acids including gallic acid, caffeic acid, chlorogenic acid and ellagic acid were abundant in BESF such that caffeic acid > chlorogenic acid > ellagic acid > gallic acid.

Table 1

| Phenolic compound | Quantity (mg/g dry weight) |
|------------------|---------------------------|
| Gallic acid      | 1.17 ± 0.03               |
| Catechin         | 3.69 ± 0.01               |
| Chlorogenic acid | 18.05 ± 0.01              |
| Caffeic acid     | 18.13 ± 0.02              |
| Ellagic acid     | 9.26 ± 0.01               |
| Rutin            | 5.92 ± 0.01               |
| Quercitrin       | 14.87 ± 0.03              |
| Quercetin        | 6.04 ± 0.01               |
| Kaempferol       | 9.11 ± 0.02               |

Results are expressed as mean ± SD of triplicate determinations.

Figure 2. Representative HPLC chromatogram of B. eurycoma seed flour.

3.2. Fasting blood glucose level

The effect of BESF-SD on the FBG level of diabetic rats is shown in Figure 3. The FBG levels of diabetic rats increased significantly
Results are expressed as mean ± SD of replicate determinations (n = 5). Values with different letters along the same row differ significantly (P < 0.05).

Table 3

| Parameter/Group          | Control (C) | HFD + STZ | HFD + STZ + Metformin | HFD + STZ + 10% BESF | HFD + STZ + 20% BESF |
|--------------------------|-------------|-----------|-----------------------|----------------------|----------------------|
| Hepatic MDA (nmol/g Prot) | 136.7 ± 10.3 | 140.9 ± 12.6 | 143.8 ± 13.2 | 146.5 ± 14.0 | 149.2 ± 14.8 |
| Pancreatic MDA (nmol/g Prot) | 140.8 ± 15.4 | 144.9 ± 16.5 | 148.0 ± 17.4 | 151.1 ± 18.5 | 154.2 ± 19.7 |
| Hepatic GSH (nmol/mg Prot) | 4.78 ± 0.32 | 5.01 ± 0.35 | 5.24 ± 0.38 | 5.47 ± 0.40 | 5.69 ± 0.43 |
| Pancreatic GSH (nmol/mg Prot) | 4.92 ± 0.35 | 5.15 ± 0.38 | 5.38 ± 0.41 | 5.61 ± 0.44 | 5.83 ± 0.47 |
| Hepatic GPx (U/mg Prot) | 2.87 ± 0.21 | 3.09 ± 0.24 | 3.31 ± 0.27 | 3.53 ± 0.30 | 3.75 ± 0.33 |
| Pancreatic GPx (U/mg Prot) | 3.05 ± 0.23 | 3.28 ± 0.26 | 3.50 ± 0.29 | 3.73 ± 0.32 | 3.95 ± 0.35 |
| Hepatic GR | 2.48 ± 0.19 | 2.71 ± 0.22 | 2.94 ± 0.25 | 3.17 ± 0.28 | 3.40 ± 0.31 |
| Pancreatic GR | 2.65 ± 0.21 | 2.88 ± 0.24 | 3.11 ± 0.27 | 3.34 ± 0.30 | 3.57 ± 0.33 |
| Hepatic SOD (U/mg Prot) | 7.84 ± 0.45 | 8.18 ± 0.48 | 8.52 ± 0.51 | 8.86 ± 0.54 | 9.19 ± 0.57 |
| Pancreatic SOD (U/mg Prot) | 8.02 ± 0.48 | 8.36 ± 0.51 | 8.70 ± 0.54 | 9.04 ± 0.57 | 9.37 ± 0.60 |
| Hepatic CAT (U/mg Prot) | 2.26 ± 0.16 | 2.49 ± 0.19 | 2.72 ± 0.22 | 2.95 ± 0.25 | 3.18 ± 0.28 |
| Pancreatic CAT (U/mg Prot) | 2.44 ± 0.18 | 2.67 ± 0.21 | 2.90 ± 0.24 | 3.13 ± 0.27 | 3.36 ± 0.30 |

Results are expressed as mean ± SD of replicate determinations (n = 5). Values with different letters along the same row differ significantly (P < 0.05).
Discussion

Plant products rich in polyphenols could offer a safer, cheaper and more effective strategy for managing T2DM than synthetic antidiabetic drugs. Flavonoids and phenolic acids constitute the main classes of polyphenols in addition to stilbenes and lignans[22]. These two classes of polyphenols have been reported to be of pharmacological importance[23], having several pharmacological activities including antidiabetic activity[8]. Plant extracts rich in these two classes of phenolic compounds were potent in inhibiting some key enzymes implicated in the pathology and complications of T2DM in vitro[23]. Flavonoids are prominent for their multifaceted pharmacological activities including antidiabetic efficacy. For instance, rutin has been reported to lower blood glucose and increase the activities of the antioxidant enzymes SOD, CAT and GPx in diabetic rats[24]; quercetin also lowered blood glucose, reduced lipid peroxidation and increased the activities of SOD and CAT and GSH level in diabetic rats[25]. It is interesting to report that the levels of the flavonoids and phenolic acids in BESF are much larger than the levels in the edible parts of vegetables. For instance, the quercetin in BESF (6000.04 mg/kg) in this study is about 600 times larger than the quercetin level in the edible parts of vegetables[26]. Phenolic acids of plant origin such as caffeic acid and chlorogenic acid have also been reported to exhibit antidiabetic activity[27].

The induction of T2DM using HFD, low-dose STZ model had been reported by previous studies[13,14]. STZ is a known diabetogen[28], being able to cause the destruction of β-cells of the islets of Langerhans, thereby leading to a massive reduction in insulin release. Deficiency of insulin consequently leads high glucose level in the blood (hyperglycemia)[29] and other metabolic aberrations such as increased cholesterol[30] associated with T2DM. Insulin deficiency is usually accompanied with decreased utilization of glucose by the tissues, and excessive hepatic glycogenolysis and gluconeogenesis, which constitute the fundamental mechanism underlying hyperglycemia in DM[31]. The ameliorative effect of BESF-SD on the hyperglycemic conditions of the diabetic rats observed in this study could be attributed to the flavonoids and phenolic acids present in the BESF, which may have exerted their antihyperglycemic activity through reduction of intestinal absorption of dietary carbohydrate, inhibition of glucose-metabolizing enzymes, improvement of pancreas β-cell function, stimulation of insulin secretion and action, and antioxidative action[32,33].

Liver glycogen level is a good index for assessing antihyperglycemic activity of therapeutic agents[34]. Glycogen metabolism is regulated through reciprocal modulation of glycogen phosphorylase and glycogen synthase, such that activation of glycogen phosphorylase is tightly linked to inhibition of glycogen synthase, and vice versa[35]. Insulin enhances intracellular glycogen deposition by stimulating activities of glycogen synthase and inhibiting glycogen phosphorylase[36]. However, under a condition of insulin deficiency as triggered by the diabetogenic activity of STZ, this regulatory function of insulin is jeopardized; this could be responsible for the depleted hepatic glycogen level in the diabetic rats observed in this study. This finding is in agreement with that of Ahmed et al.[37], who reported that STZ-induced diabetes reduces hepatic glycogen content in diabetic rats. Pari and Murugan[38] also reported that insulin deficiency resulted in the activation of glycogenolitic and gluconeogenic pathways. This anomaly was however reversed in diabetic rats fed BESF-SD. This reversal may have resulted from increased insulin release; increased synthesis of glycogen synthase and prevention of its inactivation[39], due to the BESF-SD.

Diabetic hyperglycemia is usually accompanied by changes in the levels of plasma lipids. These alterations in plasma lipid profile arise from abnormalities in lipid metabolism, and contribute to the development of coronary heart disease (CHD) in diabetic patients[40]. Under normal metabolic conditions, insulin activates lipoprotein lipase, the enzyme that hydrolyzes triglycerides to release fatty acids and glycerol. The fatty acids are then taken up by body tissues where they are oxidized for fuel or re-esterified for storage. However, deficiency in insulin results in inactivation of this enzyme thereby leading to elevated plasma TRIG (hypertriglyceridemia). In this study, elevation in plasma TRIG, TC, LDL-C and VLDL-C; and reduction in HDL-C were observed in the diabetic rats. These results are in accordance with alterations in lipid profiles of HFD-fed, STZ-induced diabetic rats reported by other researchers[41,42]. LDL transports cholesterol from the liver to body tissues[43]. Hence, increase in LDL level promotes deposition of cholesterol in the arteries and aorta, and consequently the development of CHD in diabetic patients. On the other hand, HDL transports endogenous cholesterol and cholesteryl esters from other body tissues to the liver and steroidogenic tissues for metabolism and excretion. Therefore, HDL is a beneficial lipoprotein which protects the system from cholesterol deposition, and hence atherosclerosis[44]. The observed reduction in the elevated plasma TRIG, TC, LDL-C and VLDL-C; and increase in HDL-C levels of diabetic rats fed BESF-SD could be attributed to improvements in the level of insulin secreted by the pancreas, which signals lipid utilization and metabolism. It is also possible that the BESF-SD inhibited fatty acid synthesis to exert its hypocholesterolemic effect[30].

Chronic hyperglycemia in diabetes is usually accompanied by increased production of ROS and impaired antioxidant defense system, which precipitate oxidative stress. This, in turn, promotes the onset, progression, and pathological consequences of diabetes[45]. Furthermore, diabetic monocytes are induced by oxidative stress to generate large amount of superoxide anion (O2−) which in turn leads to peroxidation of plasma and tissue lipids to generate peroxidation products[46]. Hence, increased level of lipid peroxidation in tissues of STZ-induced diabetic rats is one of the characteristic features of chronic diabetes[47]. The products of polyunsaturated fatty acids peroxidation such as MDA, can impair membrane function; inactivate membrane-bound receptors and enzymes, and increase tissue permeability[48]. If unmitigated, lipid peroxidation will in turn result in increased production of free radicals that are deleterious
to the body cells[49]. In particular, the accumulation of Fe$^{2+}$ in the acinar cells and in the islets of Langerhan, has been reported to result in the oxidative destruction of the $\beta$-cells of the pancreas associated with DM[50]. In this study, increased lipid peroxidation as indicated by elevated MDA level, was observed in the liver and pancreas of the diabetic rats. This was however mitigated in diabetic rats fed BESF-SD, as well as in those treated with metformin. This therefore suggests that BESF has the potential to protect the pancreas cells and hepatocytes from oxidative damage.

The depleted GSH level (a non-enzymatic antioxidant) and the attenuated activities of antioxidant enzymes (SOD, CAT, GPx and GR) in the diabetic rats observed in this study indicate impaired antioxidant status arising from exacerbated ROS formation. Increased production of ROS promotes the depletion of non-enzymatic antioxidants, and reduces the activities of antioxidant enzymes[51]. This is in accordance with the findings of other studies[13-15].

Increased lipid peroxidation as indicated by elevated lipid peroxidation in HFD-fed and low dose STZ-induced diabetic rats[52,53]. GSH, SOD, CAT, GPx and GR are part of the antioxidant defense system of the body that protects the body cells from oxidative damage. Among these, SOD, CAT and GPx constitute the front line of the body’s endogenous antioxidant enzyme defense system, for scavenging free radicals[54]. SOD dismutates O$_2^-$, the most reactive of the ROS, into H$_2$O$_2$, and is usually the first antioxidant defense mobilized by the cell against oxidative stress. Then the H$_2$O$_2$ is detoxified by CAT and GSHPx[55]. The significant increase in GSH level and the activities of antioxidant enzymes in the liver and pancreas of diabetic rats fed BESF-SD suggests that BESF has the potential to ameliorate oxidative stress and its associated damages that characterize T2DM.

This study has demonstrated that BESF is a rich source of pharmacologically important flavonoids and phenolic acids. Diets supplemented with BESF displayed potent antidiabetic activity in HFD, low dose STZ-induced diabetic rats[16-18] in rats, by ameliorating the FBG, hepatic glycogen, lipids profile and antioxidant status of the diabetic rats. The flavonoids and phenolic acids in BESF possibly acted synergistically to produce the observed antidiabetic effects. Therefore, BESF could be an effective and affordable nutraceutical for the management of T2DM; and an excellent source for drug discovery.

Conflict of interest statement

We declare that we have no conflict of interest.

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