Combined effect of metformin and gallic acid on inflammation, antioxidant status, endoplasmic reticulum (ER) stress and glucose metabolism in fructose-fed streptozotocin-induced diabetic rats

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ABSTRACT

Over time, diabetes patients usually need combination therapy involving two or more agents, including phytonutrients to attain therapeutic targets. The purpose of this research is to elucidate the combined effect of metformin and gallic acid (GA) on glucose metabolism, inflammation as well as oxidative and endoplasmic reticulum (ER) stresses in fructose-fed diabetic rats. Thirty-five rats of Wistar strain were arbitrarily distributed into five groups, each containing seven animals as follows: normal control, diabetic control, groups administered 100 mg/kg bw metformin only, 50 mg/kg bw gallic acid only and a combination of both. Experimental animals were made diabetic by single injection of 40 mg/kg streptozotocin (intraperitoneally) subsequent to 14 days administration of 10 % fructose prior. Treatment of rats continued for 21 days following diabetes confirmation. Glucose and insulin levels as well as lipid profile were evaluated in the serum, while activities of catalase and superoxide dismutase were estimated in both liver and pancreas. In addition, levels of malondialdehyde, interleukin-6 and tumor necrosis factor-alpha, as well as expression of activating transcription factor-4 were evaluated in liver and pancreas of diabetic rats. Activities of glucose-6-phosphatase and glucokinase were also determined in liver of diabetic animals. Metformin only, GA only and combination of metformin and GA significantly improved antioxidant status and glucose homeostasis while inflammation and endoplasmic reticulum stress were significantly ameliorated in diabetic rats. Metformin/GA combination appeared to improve glucose metabolism by increasing insulin level and ameliorating the dysregulated activities of glucose metabolizing enzymes and ER stress better than either metformin only or GA only. It could be concluded that coadministration of metformin/GA produced a combined effect in ameliorating diabetes in Wistar rats and could be considered in treatment of diabetes.

1. Introduction

Diabetes mellitus is usually characterized by hyperglycemia and insulin resistance, which occurs as a result of deficiencies in insulin secretion and/or action, with progressive beta-cell failure [1]. Type 2 diabetes (T2D) has the highest prevalence, and it usually occurs due to compromised insulin secretion and sensitivity, obesity, lifestyle, ageing etc. [2]. Research has demonstrated that a combination of lifestyle modification and antidiabetic drugs could avert the occurrence of diabetes and its complications [3]. Despite this, the global incidence of diabetes rose by 102.9 % between 1990 and 2017 [4]. In addition, the prevalence rate of diabetes was recently reported to be 9.3 %, in 2019 and this is expected to become 10.2 % in 2030, with further increase to 10.9 % by 2045 [5].

Oxidative stress is critical to pathogenesis of type 2 diabetes and its complications [6]. Moreover, chronic inflammation has been identified as an essential feature of type 2 diabetes, thereby making inflammation a prospective drug target for treating type 2 diabetes [7]. Endoplasmic reticulum (ER) is a very important organelle in the cell in which protein synthesis and folding take place. Disruption in the balance of protein synthesis and in the capability of ER to fold protein activates a pro-survival pathway known as unfolded protein response (UPR). However, chronic activation of the UPR by factors such as oxidative stress, hyperglycemia, high lipid load and excess Ca^{2+} release from ER stores leads to a state of ER stress [8], which is usually associated with inflammation and apoptosis [9]. This occurs mostly in specialized cells such as pancreatic β-cells where synthesis, folding and processing of newly synthesized insulin occur [10].

Regulation of enzymes that catalyze reactions in glycolytic and gluconeogenic pathways such as hexokinase, phosphofructokinase, glucose-6-phosphatase plays a significant role in glycemic control [11], and it was established that effective glycemic control is vital in preventing and treating diabetes as well as its complications [12].

Fructose-rich diets have been identified to give rise to hepatic insulin resistance as well as metabolic syndrome both in experimental animals and humans [13,14]. Thus, combination of low dose streptozotocin and fructose which cause mild impairment of insulin secretion and insulin resistance, respectively, both of which are hallmarks of type 2 diabetes, have been severally employed in inducing type 2 diabetes [15].

Metformin is the most commonly recommended drug for type 2 diabetes, and it is presently prescribed as the therapy of first choice for type 2 diabetes patients [16]. Nevertheless, due to progressive loss of β-cell, attainment of glycemic control becomes a challenge for diabetes patients on monotherapy thereby necessitating combination therapy [17]. Moreover, high rates of secondary failure after successful initial metformin therapy has been reported, as with all other oral antidiabetic drugs [18]. Plants and plant products have been identified to confer protection against toxicity from chemicals and drugs [19,20]. Moreover, plants are reputed to contain substances that have antidiabetic effects [21]. Gallic acid (GA) (3,4,5 trihydrobenzoic acid) is a member of the class of plant phenolics that are increasingly being studied for their biological activities [22]. Via its antioxidant activity, GA was reported to counteract the toxic effects of chemical substances [23]. Moreover, its antidiabetic potential has earlier been reported [24].

Due to the fact that type 2 diabetes worsens with time in many patients, efficacy of initial monotherapy declines, and combination therapy eventually becomes a necessity [25]. Moreover, plant derived substances have been reported to possess antidiabetic potential [21]. This study therefore purposed to investigate the ameliorative effect of metformin and GA co-administration on the derangements in glucose metabolism, inflammation, antioxidant status and ER stress in diabetic rats.

2. Materials and methods

2.1. Chemicals

Gallic acid, metformin, streptozotocin, fructose, sodium citrate and citric acid were products of Sigma-Aldrich (St-Louis, Missouri, USA). Enzyme-linked immunosorbent assay (ELISA) kits for insulin, tumour necrosis factor-α and interleukin-6 were products of MyBioSource Biotechnology company (San Diego, U.S.A). The ELISA kits were rat specific. Other reagents and chemicals used in this study were of analytical grade.

2.2. Induction of type 2 diabetes and experimental design

Thirty-five male Wistar albino rats with weights of 190 ± 10 g were obtained from the animal breeding facility of Afe Babalola University, Ado-Ekiti, Nigeria. Experimental animals were kept under standard

conditions and fed standard laboratory chow and water without restriction for seven days before the experiment and while the experiment lasted. Experimental animals were made diabetic (type 2) by initial 14 days administration of 10 % fructose after which a one-off injection of 40 mg/kg bw body weight streptozotocin was given intraperitoneally [26]. Confirmation of diabetes was done by using Accu-check® glucometer 72 h after induction of diabetes. Blood glucose level ≥ 250 mg/dl was used as the inclusion criterion for diabetic rats in this study [27]. Of the thirty-five animals used in this study, twenty-eight were diabetic. The seven non-diabetic rats served as normal control group (group 1), while groups 2–5 contained diabetic rats. Animals in group 2 were treated with a combination of 100 mg/kg bw metformin and 50 mg/kg bw GA; animals in group 3 were administered metformin only (100 mg/kg bw); group 4 animals were administered 50 mg/kg bw GA only, and rats in group 5 were the diabetic control group. Groups 1 and 5 were not treated for diabetes throughout the course of the study. Each group contained seven animals, and distribution to groups was done arbitrarily. The concentrations of metformin and GA used in this study are 40 mg/ml and 20 mg/mL, respectively. Choice of doses for metformin and GA used in this study was based on findings from literature [28,29]. Treatment doses used in this study Means of extrapolating these doses to humans can be obtained from the work of Nair and Jacob [30]. Distilled water served as vehicle for both metformin and GA, while both normal and diabetic control groups were administered the same volume of vehicle throughout the period of the study. Treatment of experimental rats was via oral gavage, and went on for 21 days following diabetes confirmation. Animal studies followed the Principles of Laboratory Animal Care (NIH publication #85−23, revised in 1985). Ethical approval number - ABUAD-SCI19/03/096 was obtained from the Research Directorate of Afe Babalola University, Ado-Ekiti, Nigeria.

2.3. Serum and homogenates preparation

After twenty-four hours of administering the last dose of treatment, animals were sacrificed under mild chloroform anesthesia via inhalation, blood was withdrawn from the heart and centrifuged for 5 min at 3000 rpm in order to obtain serum that was used for evaluation of biochemical indices. Liver and pancreas of each rat were excised and were thereafter rinsed in chilled normal saline solution. The organs were blotted dry with paper towel, weighed and then homogenized in chilled Tris−HCl buffer (1:10 w/v). The homogenates were spun for 10 min at 3000 x *g* and the resulting supernatant was utilized for analysing various biochemical parameters [31].

2.4. Biochemical analyses

Glucokinase activity in the liver was estimated using the modified method of Brandstrup et al. [32], hepatic glycogen content and activity of glucose-6-phosphatase were evaluated with the methods of Roe and Dailey [33] and Harper, [34], respectively. Levels of glucose, HDL-cholesterol, total cholesterol, triglycerides, LDL-cholesterol, and total protein were determined by following instructions supplied by Randox Laboratories Crumlin, United Kingdom. Activity of catalase and level of malondialdehyde (MDA) were respectively ascertained by using the method of Sinha [35] and Varshney and Kale [36]. Activity of superoxide dismutase (SOD) was evaluated with the method of Misra and Fridovich [37], while activity of glutathione peroxidase (GPx) was evaluated according to the method of Rotruck et al. [38]. Serum insulin level and levels of both interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) were estimated following instructions from kit manufacturer. (Intra-assay coefficients of variation for insulin, IL-6 and TNF-α kits were <8%, 5.7 % and 5.1 %, respectively).

2.5. Gene expression analysis

Instruction of kit manufacturer (New England BioLabs,

Massachusetts, USA) was followed in isolating total RNAs from liver and pancreas of experimental animals and also for PCR amplification. Concentration and purity of extracted RNA was evaluated using NanoDrop 2000 spectrophotometer (Thermofisher CA, USA). The reaction was carried out using a Labgene thermocycler. The forward and reverse sequences for activating transcription factor-4 (ATF4) primer are GTTGGTCAGTGCCTCAGACA and CATTCGAAACAGAGCATCGA, respectively, while forward sequence and reverse sequence for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (housekeeping gene) primer respectively are AGACAGCCGCATCTTCTTGT and CTTGCCGTGGGTAGAGTCAT. Evaluation of amplicons was done by electrophoresis in agarose gel (0.2 %) using $0.5 \times$ TBE buffer adjusted to pH 8.3 with the sodium hydroxide pellet) with 5 µl EZ-vision (VWR Life Science). ImageJ software was used to quantify relative amount of complimentary DNA [39].

2.6. Data analyses

Results were written as mean value $\pm$ standard deviation (SD). Graphpad prism 5 was used to analyse data with one-way analysis of variance (ANOVA). Tukey test was used to compare between groups, while $p < 0.05$ was taken as the level of statistical significance.

3. Results

3.1. Co-administration of metformin and gallic acid modulated glucose and lipid levels in serum of diabetic animals

Table 1 shows that level of serum glucose in diabetic animals administered metformin only, GA only as well as combination of both for 21 days was reduced significantly ($p < 0.05$) relative to diabetic control animals. After 21 days, glucose level of rats administered metformin/GA combination had a sharp decrease, though not significant when compared to the decrease observed with metformin only or GA only. In Table 2, it was revealed that levels of total cholesterol, triglycerides and LDL cholesterol in diabetic animals administered metformin only, GA only and metformin/GA combination for 21 days were significantly ($p < 0.05$) attenuated in comparison with diabetic control animals, and also compared favourably with normal control animals. However, no significant differences were noted with individual treatment.

3.2. Treatment with metformin, GA and metformin/GA combination improves hepatic and pancreatic antioxidant capacity in diabetic animals

Table 3 shows that diabetic animals administered metformin only, GA only and metformin/GA combination of both significantly improved hepatic antioxidant status in comparison with diabetic control animals.

Table 1

Effect of metformin, GA and metformin/GA combination level of glucose in serum of experimental animals.

Groups	Glucose Level (mg/dl)		
	Day 0	Day 1	Day 21
Normal control	98.00 ± 3.26	96.00 ± 1.28	100.00 ± 1.71
Diabetic + met + GA	98.00 ± 5.34	$362.00 \pm 31.49^*$	$117.00 \pm 10.87^\#$
Diabetic + metformin	97.00 ± 2.36	$351.00 \pm 37.22^*$	$118.00 \pm 7.97^\#$
Diabetic + Gallic acid	99.00 ± 3.99	$321.00 \pm 29.10^*$	$133.00 \pm 9.39^\#$
Diabetic control	98.00 ± 1.15	$350.00 \pm 13.48^*$	$316.00 \pm 42.90^*$

Results are written as mean $\pm$ SD of seven replicates. In the same column, values with * are significantly ($p < 0.05$) different relative to normal control, while values with $\#$ are significantly ($p < 0.05$) different relative to diabetic control. Met = Metformin, GA = Gallic acid.

Day 0 = fasting blood glucose before STZ injection; Day 1 = first day of treatment after confirmation of diabetes; day 21 = 21st day of treatment after confirmation of diabetes.

Table 2

Effect of treatment with metformin, GA and metformin/GA combination for 21 days on lipid parameters in diabetic rats.

Groups	Total-Chol (mg/dl)	HDL-Chol (mg/dl)	Triglycerides (mg/dl)	LDL-Chol (mg/dl)
Normal control	117.39 ± 4.96	54.08 ± 4.15	48.79 ± 2.92	59.66 ± 6.44
Diabetic + met + GA	$115.46 \pm 3.76^\#$	$34.75 \pm 1.26^\#$	$40.86 \pm 4.94^\#$	$60.54 \pm 6.15^\#$
Diabetic + metformin	$115.84 \pm 10.4^\#$	$35.00 \pm 0.82^\#$	$51.94 \pm 3.64^\#$	$62.62 \pm 3.24^\#$
Diabetic + Gallic acid	$124.39 \pm 3.00^\#$	$34.50 \pm 1.91^\#$	$45.19 \pm 4.77^\#$	$66.80 \pm 2.51^\#$
Diabetic control	$241.82 \pm 2.55^*$	$17.42 \pm 2.07^*$	$89.20 \pm 5.48^*$	$97.53 \pm 6.48^*$

Results are written as mean $\pm$ SD of seven replicates. In the same column, values with * are significantly ($p < 0.05$) different relative to normal control, while values with $\#$ are significantly ($p < 0.05$) different relative to diabetic control. Met = Metformin, GA = Gallic acid.

Table 3

Effect of metformin, GA and metformin/GA combination on hepatic antioxidant parameters in diabetic rats.

Groups	Catalase (U/mg protein)	SOD dismutase (U/mg protein)	GPx (U/mg protein)	MDA (µmol MDA /mg protein)
Normal control	442.69 ± 3.74	89.17 ± 1.44	146.43 ± 1.06	2.05 ± 0.01
Diabetic + met + GA	$444.58 \pm 7.46^{\#a}$	$88.57 \pm 1.71^{\#a}$	$148.13 \pm 1.68^{\#a}$	$2.25 \pm 0.05^{\#a}$
Diabetic + metformin	$365.45 \pm 11.76^{\#b}$	$69.21 \pm 2.40^{\#b}$	$135.55 \pm 1.32^{\#a}$	$2.70 \pm 0.09^{\#b}$
Diabetic + Gallic acid	$298.61 \pm 4.70^{\#c}$	$29.03 \pm 3.48^{\#c}$	$108.34 \pm 1.43^{\#b}$	$3.97 \pm 0.02^{\#c}$
Diabetic control	$232.08 \pm 1.53^*$	$17.20 \pm 0.85^*$	$82.80 \pm 0.93^*$	$6.81 \pm 0.02^*$

Results are written as mean $\pm$ SD of seven replicates. In the same column, values with * are significantly ($p < 0.05$) different relative to normal control, values with $\#$ are significantly ($p < 0.05$) different relative to diabetic control. In the same column, values with dissimilar alphabet superscript are significantly ($p < 0.05$) different. Met − Metformin, GA − Gallic acid.

Activities of catalase, GPx and SOD in the liver were significantly ($p < 0.05$) higher with these treatments, while the level of MDA was significantly lower after 21 days relative to the diabetic control group. Moreover, it was observed that metformin/GA combination significantly ($p < 0.05$) improved antioxidant status of diabetic rats better than when administered separately. As revealed in Table 4, treatment with metformin, GA and co-administration of metformin and GA raised activities of SOD and catalase in pancreas of experimental animals significantly (p

Table 4

Effect of metformin, GA and metformin/GA combination on antioxidant parameters in pancreas of diabetic animals.

Groups	SOD (U/mg protein)	Catalase (U/mg protein)	MDA (µmol MDA /mg protein)
Normal control	79.17 ± 0.89	58.90 ± 5.91	1.16 ± 0.10
Diabetic + met + GA	$76.03 \pm 1.53^{\#a}$	$118.88 \pm 15.96^{\#a}$	$0.96 \pm 0.03^{\#a}$
Diabetic + metformin	$61.72 \pm 3.39^{\#b}$	$89.24 \pm 6.42^{\#b}$	$1.32 \pm 0.03^{\#b}$
Diabetic + Gallic acid	$25.25 \pm 1.65^{\#c}$	$95.81 \pm 9.82^{\#b}$	$1.65 \pm 0.02^{\#c}$
Diabetic control	$15.04 \pm 0.76^*$	$25.69 \pm 9.92^*$	$1.94 \pm 0.07^*$

Results are written as mean $\pm$ SD of seven replicates. In the same column, values with * are significantly ($p < 0.05$) different relative to normal control, values with $\#$ are significantly ($p < 0.05$) different relative to diabetic control. In the same column, values with dissimilar alphabet superscript are significantly ($p < 0.05$) different. Met − Metformin, GA − Gallic acid.

< 0.05) while the level of MDA was lowered significantly ($p < 0.05$) relative to the diabetic control animals. Antioxidant status was significantly ($p < 0.05$) improved in diabetic rats that were treated with metformin/GA combination than in those treated with either metformin only or GA only.

3.3. Glucose metabolism was improved in diabetic animals administered metformin only, GA only and metformin/GA combination for 21 days

Fig. 1 shows that diabetic rats administered metformin only, GA only and metformin/GA for 21 days showed significantly ($p < 0.05$) improved insulin level in serum relative to diabetic control animals. Moreover, it was observed in Fig. 2 that diabetic animals treated with metformin only, GA only and metformin/GA combination had a significantly ($p < 0.05$) lowered glucose-6-phosphatase activity relative to animals in diabetic control group, while a reverse effect was observed with glucokinase activity (Fig. 3). From Fig. 4, it was observed that administration of metformin only, GA only and metformin/GA combination to diabetic rats raised hepatic glycogen level significantly ($p < 0.05$) in comparison with animals in diabetic control animals.

3.4. Effect of metformin only, GA only and metformin/GA combination on inflammation in liver and pancreas of diabetic animals

Metformin, GA and metformin/GA combination significantly ($p < 0.05$) reduced levels of hepatic and pancreatic interleukin-6 of diabetic rats (Fig. 5) relative to animals in diabetic control group. Likewise, hepatic and pancreatic TNF-α levels (Fig. 6) were lowered significantly ($p < 0.05$) upon treatment with metformin only, GA only and metformin/GA combination. It is noteworthy that treatment with metformin/GA combination reduced pancreatic IL-6 and hepatic TNF-α significantly ($p < 0.05$) in diabetic rats relative to treatment with metformin only or GA only.

3.5. Metformin, gallic acid and co-administration of both modulates hepatic and pancreatic ER-stress in diabetic rats

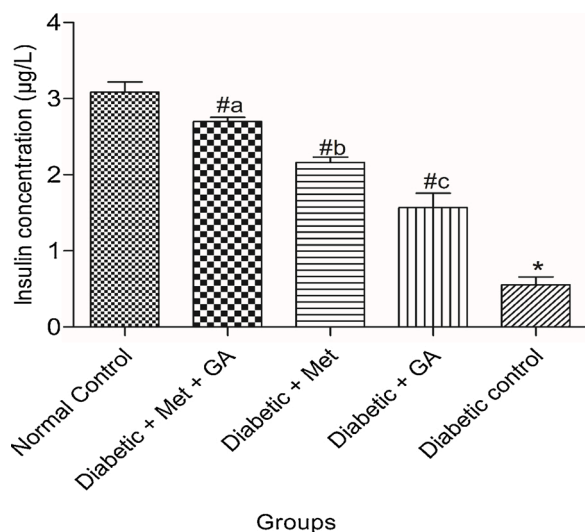Rats administered metformin only, GA only and metformin/GA

Fig. 1. Effect of metformin only, GA only and metformin/GA combination on insulin level in serum of diabetic rats.
Results are written as mean ± SD of seven replicates. Bars with * are significantly ($p < 0.05$) different relative to normal control, while bars with # are significantly ($p < 0.05$) different relative to diabetic control. Bars having dissimilar alphabets are different significantly ($p < 0.05$). Met = Metformin, GA = Gallic acid.
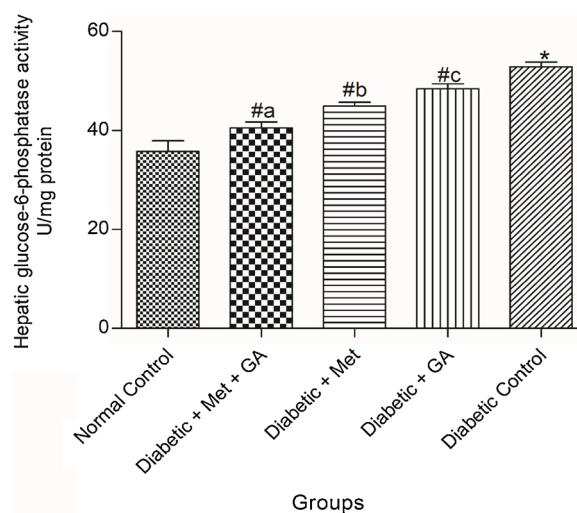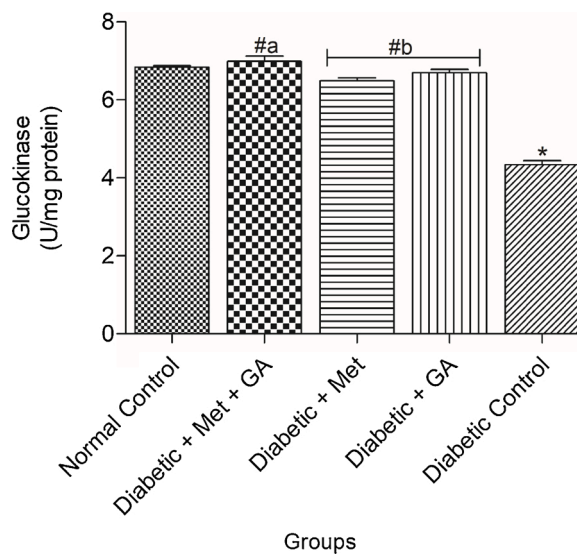

Fig. 2. Effect of metformin only, GA only and metformin/GA combination on hepatic glucose-6-phosphatase activity in diabetic rats.
Results are written as mean ± SD of seven replicates. Bars with * are significantly ($p < 0.05$) different relative to normal control, while bars with # are significantly ($p < 0.05$) different relative to diabetic control. Bars having dissimilar alphabets are different significantly ($p < 0.05$). Met = Metformin, GA = Gallic acid.

Fig. 3. Effect of metformin only, GA only and metformin/GA combination on hepatic glucokinase activity in diabetic animals.
Results are written as mean ± SD of seven replicates. Bars with * are significantly ($p < 0.05$) different relative to normal control, while bars with # are significantly ($p < 0.05$) different relative to diabetic control. Bars having dissimilar alphabets are different significantly ($p < 0.05$). Met = Metformin, GA = Gallic acid.

combination were observed to show a significantly ($p < 0.05$) attenuated expression of ATF4 in both pancreas and liver of diabetic rats (Fig. 7). Co-administration of metformin and GA was however observed to be more effective in reducing expression of hepatic ATF4 when compared to metformin only or GA only.

4. Discussion

Combination therapy in diabetes which is defined as treatment with two or more oral agents or injectables becomes necessary because chronic hyperglycemia and its associated complications arise from
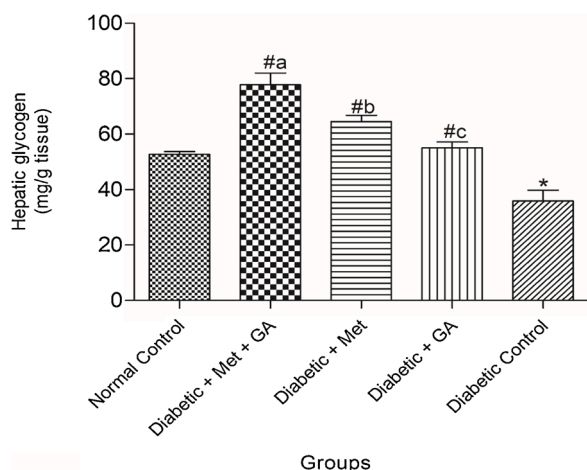
Fig. 4. Effect of metformin only, GA only and metformin/GA combination on hepatic glycogen level in diabetic rats.

Results are written as mean ± SD of seven replicates. Bars with * are significantly ($p < 0.05$) different relative to normal control, while bars with # are significantly ($p < 0.05$) different relative to diabetic control. Bars having dissimilar alphabets are different significantly ($p < 0.05$). Met = Metformin, GA = Gallic acid.

defects in several pathways [40]. Apart from coadministration of synthetic antidiabetic drugs, most times, herbs are sometimes administered together with conventional drugs, and this may enhance the possibility of herb-drug antioxidant activity [41,42]. This is important because diabetes-associated hyperglycemia is usually characterized by oxidative stress [43].

Combination of metformin and bioactive compounds from plants has been utilized in treating diabetes in both *in vitro* and *in vivo* models of the disease [44]. Moreover, previous studies have reported that GA ameliorates hyperglycemia in diabetes models [45,46]. A significantly lowered serum glucose level was observed when diabetic rats were administered metformin/GA combination for 21 days relative to diabetic control group. However, this reduction was neither significantly different from that of normal control nor the groups treated with metformin only and GA only. This observation is quite instructive as chronic hyperglycemia is one of the key factors leading to the onset of diabetic complications [47] as well as destruction of β-cell [48]. Moreover, attaining effective management of hyperglycemia is recognized as a top priority in achieving specific glycemic goals, and it might also be beneficial regarding cardiovascular risk factors [49]. Thus, onset of cardiovascular incidences in type 2 diabetics might be prevented or delayed by administration of metformin only, gallic acid only, and a combination of both via their antihyperglycemic effects.

Dysregulation of lipid profile is a critical attribute of type 2 diabetes, and is usually characterized by elevated total cholesterol (total-chol),
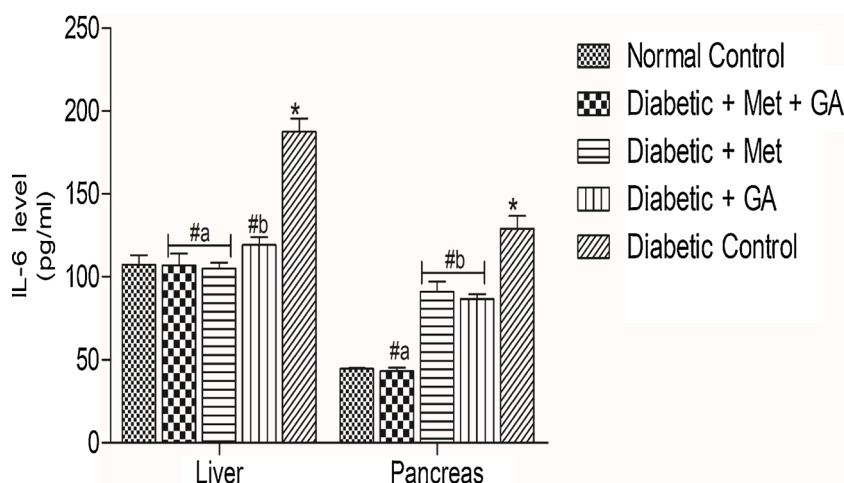

Fig. 5. Effect of metformin only, GA only and metformin/GA combination on hepatic and pancreatic interleukin-6 levels in diabetic animals.

Results are written as mean ± SD of seven replicates. Bars with * are significantly ($p < 0.05$) different in comparison with normal control, while bars with # are significantly ($p < 0.05$) different relative to diabetic control. Bars having dissimilar alphabets are different significantly ($p < 0.05$). Met = Metformin, GA = Gallic acid.
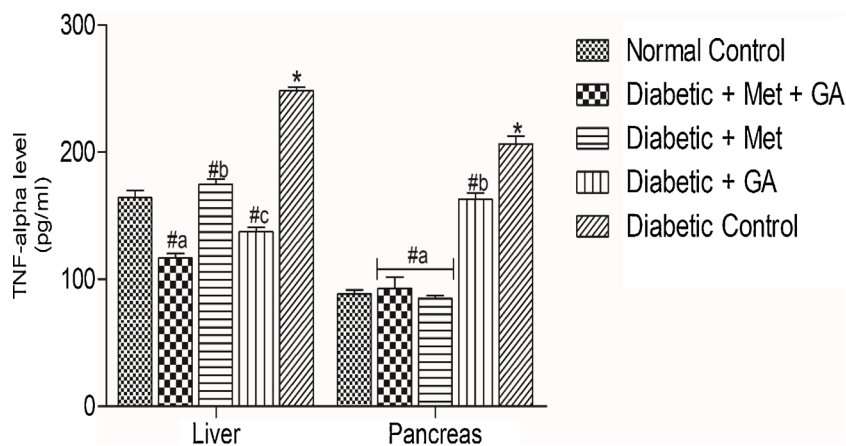

Fig. 6. Effect of metformin only, GA only and metformin/GA combination on level of tumour necrosis factor in liver and pancreas of diabetic animals.
Results are written as mean ± SD of seven replicates. Bars with * are significantly ($p < 0.05$) different relative to normal control, while bars with # are significantly ($p < 0.05$) different relative to diabetic control. Bars having dissimilar alphabets are different significantly ($p < 0.05$). Met = Metformin, GA = Gallic acid.
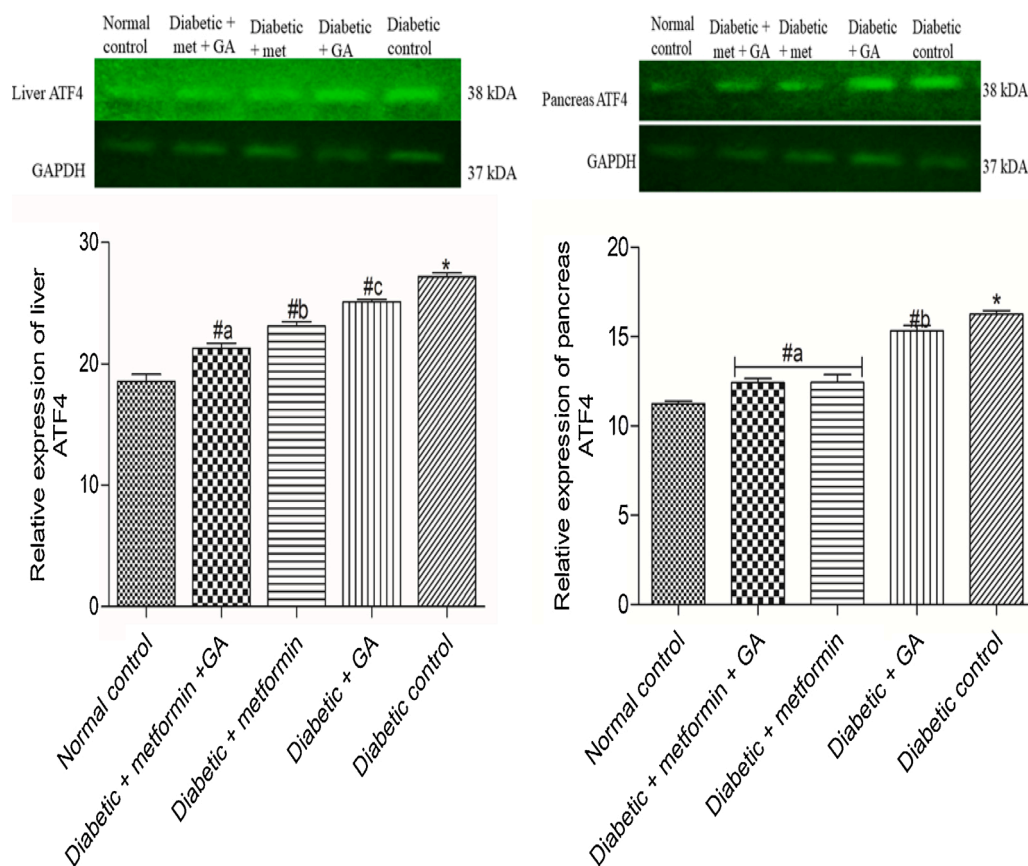
Fig. 7. Effect of metformin only, GA only and metformin/GA combination on expression of ATF4 in the liver and pancreas of diabetic animals.
Results are written as mean $\pm$ SD of seven replicates. Bars with * are significantly ($p < 0.05$) different relative to normal control, while bars with # are significantly ($p < 0.05$) different relative to diabetic control. Bars having dissimilar alphabets are different significantly ($p < 0.05$). Met = Metformin, GA = Gallic acid.

low-density lipoprotein cholesterol (LDL-chol) and triglycerides; and reduced high-density lipoproteins (HDL-chol) [50]. These alterations are critically involved in elevated risk of cardiovascular diseases and rapid onset of macrovascular atherosclerotic disease [51]. In this study, treatment with metformin only, GA only and metformin/GA combination for 21 days markedly modulated components of lipid profile relative to diabetic control animals. The significant lowering of serum total-chol, LDL-chol and triglyceriades, as well as the significant rise in serum HDL-chol agrees with a study by Huang et al. [24] and van Stee et al. [52] where GA and metformin, respectively, ameliorated the abnormalities in lipid metabolism in different models of diabetes. In addition, it could be suggested that incidence of cardiovascular events could be prevented after treatment with metformin, GA and a combination of both as cardiovascular complications associated with type II diabetes are attributable to reduced HDL-chol as well as elevated triglycerides, LDL-chol and total cholesterol levels [53]. In addition, hypertriglyceridemia as well as low level of HDL-chol adversely affect glucose metabolism [54,55]. Thus, improvement in these lipid components as observed in this study could also contribute to the observed reversal of hyperglycemia after treatment of diabetic rats with metformin, gallic acid ana a combination of both.

A link has been established between diabetes and oxidative stress, which is usually depicted by increase in lipid peroxidation products in addition to reduced levels and/or activities of antioxidant enzymes such as SOD, catalase and GPx [56,57]. This is especially so in the β-cells of the pancreas which have been reported to have reduced antioxidant capacity. Thus, increase processing of glucose in the β-cell leads to oxidative stress [58]. The liver is not spared from the deleterious effects of oxidative stress caused by hyperglycemia, which eventually leads to tissue injury [59]. In our study, it was observed that treatment with

metformin, GA and co-administration of both significantly improved antioxidant capacity in both liver and pancreas of diabetic rats. This is evident from the higher Gpx activity in the liver, as well as activities of SOD and catalase in both liver and pancreas, with concurrent reduction in malondialdehyde (MDA) level in both organs. MDA is a marker of lipid peroxidation, thus, increase in its level suggests a decline in protective ability of both enzymatic and non-enzymatic antioxidants [60]. It is worthy of note that significant improvement of antioxidant status in pancreas and liver of diabetic rats treated with metformin/GA combination relative to other treated groups suggests that a combined effect exists between the two compounds.

Defects in the both insulin secretion and its action prompts hyperglycemia and other metabolic anomalies associated with type 2 diabetes as a result of uncontrolled hepatic gluconeogenesis, as well as dyslipidemia due to dysregulation in the metabolism of fatty acids, triglycerides and lipoproteins [61]. Besides, it is generally acknowledged that inability of the β-cell to supply the quantity of insulin required for maintaining euglycemia causes type 2 diabetes to develop [62]. The impaired insulin secretory capacity of β-cell seen in type 2 diabetes has been linked to oxidative stress and accompanying inflammation in beta-cell islets [63], which altogether may culminate in beta-cell apoptosis and reduction in beta-cell mass [64]. A previous research reported that metformin restored insulin secretion induced by glucose in isolated rat pancreatic islets subjected to persistent elevated levels of glucose concentrations to levels comparable with that of controls. This improvement was ascribed to the improvement in β-cell metabolism [65]. Moreover, GA was also discovered to significantly increase insulin secretion in diabetic rats [66]. Our results revealed that metformin, GA and metformin/GA combination markedly ameliorated insulin secretion in diabetic rats. The increase in insulin level observed from this study

could be due to the impact of treatment on the secretory capacity of β-cell as alluded to in the research cited earlier [65], as well as via amelioration of oxidative stress and inflammation in β-cell islets. This could also account for the antihyperglycemic effect of treatment modalities as observed in this study.

Glucokinase catalyzes a rate limiting step in hepatic glucose utilization pathway in which glucose is phosphorylated to glucose-6-phosphate. As such, glucokinase plays a critical role in regulation of blood glucose levels [67]. Glucose-6-phosphatase on the other hand is a key regulator of gluconeogenesis pathway and also important in the pathophysiology of hyperglycemia [68]. Alteration of these glucose metabolizing enzymes portrayed by reduced glucokinase activity and elevated glucose-6-phosphatase activity has been reported in type 2 diabetic rats [29]. Such changes result in diminished liver glycogen and hyperglycemia [69]. Gallic acid was reported to improve the activity of glycolytic enzymes in a type 2 diabetes model in rats [70,24]. Moreover, metformin, an antidiabetic drug that modulates glucose metabolizing pathways, suppresses enzymes of gluconeogenic pathway such as glucose-6-phosphatase and phosphoenolpyruvate carboxykinase, and increases glycolytic flux by stimulating key glycolytic enzymes including hexokinase [71,72]. In this present study, diabetic animals treated with metformin, GA and metformin/GA combination had improved hepatic hexokinase activity, while activity of G6Pase was significantly attenuated. These modulations could contribute to the antihyperglycemic effect observed after treatment of diabetic animals as increased glucokinase activity is known to upregulate the rate of glucose utilization while reduction in G6Pase activity, which is the terminal enzyme in gluconeogenesis, reduces the rate of *de novo* glucose synthesis [73]. Likewise, the increased hepatic glycogen level after administering diabetic rats with metformin only, GA only and combination metformin and GA for 21 days as shown from results obtained in our study could be due to improved availability of glucose-6-phosphate as a result of reduced G6Pase activity and increased glucokinase activity [73].

Proinflammatory cytokines are implicated in pathophysiology of β-cells. It has been reported that TNF-α is involved in β-cell dysfunction and death, while raised levels of IL-6 is known to be a predictor of future development of type 2 diabetes and also strongly lowers insulin sensitivity in liver cells [74–76]. In addition, the biosynthesis of acute-phase proteins, which are involved in the pathogenesis and progression of type 2 diabetes, is under the influence of proinflammatory cytokines such as IL-6 and TNF-α [77]. Thus, increased inflammation inhibits insulin signalling and facilitates pathogenesis of type 2 diabetes [78]. Metformin lowered pro-inflammatory cytokines levels such as interleukins 1β, 6, 8 and TNF-α in blood and various tissues [79,7]. It was also earlier reported that GA ameliorated IL-6, IL-8 and TNF-α levels in metabolic syndrome brought about by fructose-rich diet in rats [80]. In this study, treatment of diabetic animals with metformin, gallic acid and a combination of both significantly lowered inflammation in liver and pancreas of experimental animals. Moreover, the notable decline in pancreatic IL-6 and hepatic TNF-α in rats treated with metformin/GA combination relative to metformin only or GA only, is an indication of a better anti-inflammatory activity of the combination therapy with attendant improvement in insulin signalling.

Increased demand for insulin biosynthesis consequent upon hyperglycemia, peripheral insulin resistance, elevated gluconeogenesis in the liver, and inflammation, promote both oxidative stress and ER stress in β-cell [81]. It was previously reported that activation of the c-Jun NH$_2$-terminal kinases JNK pathway which causes insulin resistance is associated with ER stress [8]. Previous studies have also identified a correlation between ER stress and inflammation, with a vicious cycle existing specifically between TNF-α and ER stress. Besides, blocking NF-κB/TNF-α signalling prevented ER stress induced cell death [82,83]. ER stress in β-cell can solely reduce both transcription and translation of insulin [84]. It has been established that during ER stress, there is usually an increased translation of ATF4 despite the global attenuation of translation [85,86]. ATF4 was therefore used as an indication of ER

stress in this study. This study showed that metformin, GA and a combination of both significantly downregulated expression of hepatic and pancreatic ATF4 in diabetic rats, an indication of declining ER stress with likely improvements in insulin synthesis and action. Moreover, the significant improvement of the antioxidant status in both tissues, especially after treatment with metformin/GA combination could also be linked to decreased ER stress, as ATF4 has been implicated in regulating genes responsible for resistance of cells to oxidative stress [87].

5. Conclusion

As observed from this study, the mechanisms of antidiabetic effect of metformin only, gallic acid only and combination of both include amelioration of inflammation, oxidative stress and ER stress, with concomitant improvement in glucose metabolism. It was also apparent from this study that combination of metformin and GA exhibited better antidiabetic activity than either metformin only or GA only in most of the biochemical parameters evaluated. This further gives credence to the fact that combination therapy might be more efficacious than monotherapy in treatment of diabetes mellitus. Further studies on the antidiabetic effect of co-administration of metformin and GA using graded doses of both should be conducted. This would shed more light on the dose-dependent nature of the combination treatment and also elucidate the best dose of the two components of the treatment.

Authors contribution

T.O Obafemi and K.F Jayesinmi designed the study; A. A Olomola, O. R Olasehinde and O.A Olaoye did most of the bench work; D.F. Adewumi, C. O. Akintayo and O.A Ojo analysed the results; B.A Afolabi and O.B Adewale wrote the original draft, reviewed the manuscript for technical content and edited the manuscript. All authors approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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