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ORIGINAL RESEARCH

Modulation of Antioxidant parameters and Esterases activities in Tissues of Streptozotocin-induced diabetic rats

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Abstract:

Introduction: Enormous complications associated with diabetes contribute to the therapeutic challenge confronting most of the world, including developing countries.

Aim: This study was carried out to investigate diabetes mellitus on esterases and antioxidant enzymes in different tissues compartments of rats.

Materials and Methods: Animals were divided into two groups of 10 animals each. The experimental group was confirmed diabetic by a single dose of streptozotocin injection (STZ, freshly dissolved in citrate buffer, pH 4.5, 50 mg/kg) intraperitoneally. In contrast, the control group was injected with citrate buffer only. The blood glucose and weight of the animals were monitored for 7 days. Blood, liver and brain were removed, and biochemical parameters determined spectrophotometrically.

Results: Diabetes produced various degrees of alterations in antioxidant defense mechanism and esterase's activities that are compartment specific. Acetylcholinesterase (AChE) activity was inhibited to different extents. While AChE was inhibited to the tune of 39% in plasma, 33% in the brain and 30% in the liver, activity activation was observed in the red blood cell (RBC). The same trend of significant ($p < 0.001$) inhibition was observed with arylesterase in the plasma, brain and liver, and activation in the RBC. Diabetes induced significant ($p<0.001$) inhibition in catalase, Glutathione-S-transferase, and superoxide dismutase in the brain and liver, respectively, compared to the control. However, activation was also observed in the RBC of these enzymes except for catalase and nitric oxide.

Conclusion: The distinct compartmental effects of diabetes observed in this study could suggest new approach for effective and safer therapeutics.

Keywords: Antioxidants, Esterases, Acetylcholinesterase, Diabetes, Streptozotocin, Compartmentalisation, Hyperglycemia.

All co-authors agreed to have their names listed as authors.

1. INTRODUCTION

Diabetes Mellitus (DM) is a non-communicable chronic disease characterised by increased blood glucose [1, 2]. Pathogenesis of Diabetes Mellitus can occur due to: the autoimmune destruction of the pancreatic beta-cells [1], hormones opposing the action of insulin [3], diseases of the exocrine pancreas [3] and drug-induced diabetes [3, 4]. DM is fast becoming a common health problem worldwide [5-7], causing a massive burden for individuals and families, especially in developing countries [5, 7, 8]. Saeedi and his colleagues [6] concluded that the number of people living with diabetes worldwide is projected to increase by 25% in 2030 and 51% in 2045 [6]. The International Diabetes Federation (IDF), in a paper published in 2011, says, "This paper builds on previous IDF estimates and shows that the global diabetes epidemic continues to grow" [9]. Other researchers also agree that there is a continued increase [8, 10].

Esterases are enzymes that split esters into acid and alcohol through hydrolysis. Acetylcholinesterase (AChE) (EC 3.1.1.7) belongs to the carboxylesterase family of esterase enzymes. It is mainly responsible for the hydrolysis of acetylcholine [11]. AChE can be found in high concentrations in the red blood cells, the brain and nerve [12, 13]. AChE has been associated with disorders like Alzheimer's Disease (AD), tumorigenesis, and diabetes [12-14].

Apart from the autoimmune destruction of the pancreatic beta-cells, diabetes can be induced using drugs like streptozotocin and alloxan. They work by inducing apoptosis in the beta-cells [4, 15]. AChE is also believed to have this effect. Furthermore, it was found to have increased activity in the pancreatic islets of the Langerhans [13, 16].

Though the main characteristic of type-2 DM is hyperglycemia [1-3, 17-19], its complications can include hyperinsulinemia, hyperlipidemia and inflammation [20-23]. In addition, these complications induce and aggravate oxidative stress [23, 24], proinflammatory cytokines [25] and cardiovascular abnormalities [18, 23, 26, 27].

Oxidative stress is associated with increased Reactive Oxygen Species (ROS) production or a significant decline in antioxidant activity. Antioxidants are involved in the defense mechanism of aerobic organisms against ROS. However, in pathological complications like cancer, neurological disorders [28, 29], diabetes, atherosclerosis [27], hypertension, asthma [30-32] etc., the antioxidant system can be overwhelmed.

Studies have shown that a defect in the body's antioxidant system causes ROS concentration to multiply. contributes, directly or indirectly, to damages an organism's cellular components [33, 34]. It also contributes to diabetic complications and many diseases [19, 35, 36], including AIDS [37].

Antioxidants, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), sulfiredoxin and catalase (CAT), are tasked with eliminating ROS by donating an electron.

Some examples of ROS include radicals like hydroxyl (OH) and superoxide anion (O2-), and non-radical species like hydrogen peroxide (H_2O_2) and hydrochloric acid (HCl) [38, 39]. They are usually highly reactive due to one or more unpaired electron(s) in their molecular outskirt. They are formed as by-products of metabolic processes in the mitochondria, peroxisomes, endoplasmic reticulum, etc., by several enzymes. These enzymes are cyclooxygenase, lipoxygenase, NO synthase, heme oxygenase, peroxygenase and NADH/NADPH oxidase.

For example, superoxide anion is produced in the mitochondria during the Electron Transport Chain; Hydrogen peroxide by xanthine oxidase, amino acid oxidase, and NAD(P)H oxidase [40, 41]. Hydrogen peroxide can also be found in the peroxisomes [42]. These enzymes act in varying degree from organ to organ [43]. However, little effort has been directed to checking if there is a varying effect of diabetes on the natural antioxidant system in different tissues of an organism. Also, it is believed that there is a close relationship between AChE and Insulin-Dependent DM [14]. Therefore, this study aims to evaluate and compare antioxidant and esterase levels in different compartments of diabetic rats.

2. MATERIALS AND METHODS

2.1 Chemicals

All chemical used for this study were Sigma-Aldrich products, Missouri, USA. Streptozotocin (STZ), Coomassie blue, tris buffer (prepared with distilled water and tris base), phenylacetate solution (containing sodium phenylacetate and sodium benzoate), acetylcholinesterase buffer, 5,5′-dithionitrobenzoic acid (DTNB), and acetylcholine iodide (ATC). All other chemicals used were of analytical grade.

2.2 Animals and Treatment

Healthy adult male albino Wistar rats weighing between 120-220g were gotten from the College of Veterinary Medicine, the Federal University of Agriculture, Abeokuta, Nigeria. They were housed in an animal room with average controlled temperature (22±2˚C) and a regular 12h light-dark cycle (06:00-18:00h). They were allowed 14 days to acclimatise before the commencement at Lagos State University, Lagos, Nigeria. Animals were cared for following the principles for the care, use and protection of experimental animals as set by the EEC Council Directive 86/609/EEC.

2.3 Experimental Design and Diabetes Induction

After acclimatization, animals were divided into two groups of 10 animals each. The experimental group was established by a single injection of streptozotocin (50mg kg−1; freshly dissolved in citrate buffer solution (0.1 M, pH 4.5) administered intraperitoneally), whereas the

control group were injected with citrate buffer (1mL/kg) only. Seven days after injection with streptozotocin, all animals in the diabetics' group have fasting blood glucose above 120 mg/dl. The fasting blood glucose levels of all the rats are shown in Table 1.0.

2.4 Blood and Tissue Collection

At the end of the 7 days treatment, the control and diabetics group animals fasted overnight. Blood was collected by cardiac puncture under light anaesthesia (5% ketamine and 2% xylazine) into heparinised bottles. The liver, kidney and brain were removed from the animals for biochemical analysis. Blood samples were centrifuged to separate plasma and red blood cells. All samples were stored at -20˚C until analysed

2.5 Determination of Acetylcholinesterase Activity

Acetylcholinesterase (AChE) activity in the different compartments was determined according to Ellman GL, Courtney KD [44], using acetylcholine iodide as a substrate. In this method, AChE in samples hydrolyses acetylthiocholine iodide into thiocholine and butyric acid. The thiocholine reacts with 5,5′-dithio-bis-2- nitrobenzoic acid to form 5-thio-2-nitrobenzoate. The yellow colour developed was measured spectrophotometrically at 412 nm.

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2.6 Determination of Arylesterase Activity

PON1 arylesterase activity was measured against the substrate phenylacetate as described by [45]. First, 1ml of Phenylacetate solution was added to 1.0ml of 100mM Tris/acetate buffer pH 7.4 containing 10mM calcium chloride. Then, it was allowed to stand for 10mins at 37°C. Briefly, the assay was run in a cuvette which included 250 µl of phenylacetate solution, 250µl of Tris/acetate buffer. The reaction was initiated by the addition of the enzyme (5µl of the sample). The increase in absorbance at 270 nm was recorded every 30secs for 3mins. A molar extinction coefficient of 1480 M-1cm-1 was used to calculate enzyme activity. 1 unit of arylesterase activity is defined as the enzyme quantity that disintegrated 1 millimole of phenylacetate substrate in 1 minute.

2.7 Determination of GSH

The levels of reduced Glutathione (GSH) were determined by Mak, Ip [46]. Freshly prepared plasma was deproteinised by mixing with an equal volume of 4% sulfosalicylic acid. Deproteinized plasma and tissue homogenates were centrifuged at 4,000 g for 5 minutes. The supernatants were assayed for total glutathione content, using GSH as standard. After that, 0.5ml of the supernatant was added to 4.5ml of Ellman reagent. Finally, a blank was prepared with 0.5ml of the diluted precipitating agent and 4.5ml of Ellman's reagent. Reduced Glutathione, GSH, is proportional to the absorbance at 412nm.

2.8 Determination of Glutathione-S-Transferase Activity

GST activity was assayed by measuring the conjugation of reduced Glutathione with 1-chloro-2,4-dinitrobenzene, as described by Mak and his coworker [47]. The medium was prepared by a mixture of 10µL reduced Glutathione, 50µL CDNB (20mM), 930µL 0.1M Phosphate buffer (pH 6.5) and 10µL sample. The reaction was allowed to run for 60 seconds each time before the absorbance was read at 340nm. The temperature was maintained at approximately 31^0C. Enzyme activity was expressed as µmol substrate-GSH conjugate produced per min/mg protein at 31oC, using the extinction coefficient of 9.6 mM for CDNB.

2.9 Determination of Nitric Oxide (NO) Activity

The nitric oxide assay was by modification of the Griess method [48]. The medium was prepared by mixing 75µL distilled water and 125µL 0.3M NaOH with 50µL of sample and was incubated at room temperature for 5minutes. After which, 62.5µL 10% $ZnSO_4$ was added and centrifuged for 30 minutes at 4000rpm and supernatant was removed. Next, 200µL of Griess reagent was added to 200µL supernatant then absorbance was read at 540nm.

2.10 Estimation of Catalase (CAT) Activity

The catalase activity in the plasma, erythrocyte and homogenates of the liver and brain were estimated spectrophotometrically and expressed in units/ml for the plasma and erythrocyte. In contrast, that of the brain and liver were expressed in units/g tissue as described by Claiborne [49]. The used reaction mixture (1 ml) contained 100 mM phosphate buffer (pH 7.4), 50 mM H_2O_2 and plasma or erythrocyte or tissues homogenate. The reaction was started by adding H_2O_2, and its decomposition was observed by following the decrease in absorbance at 240 nm for 3 min

2.11 Estimation of Superoxide Dismutase (SOD) Activity

Superoxide dismutase activity in the plasma, erythrocyte and homogenate of liver and brain was estimated spectrophotometrically and expressed in units/ml for the plasma and erythrocyte. In contrast, that of the brain and liver were expressed in units/g tissue as described by Zou and colleague based on the inhibition of the autoxidation of pyrogallol [50]. Briefly, an aliquot of sample was mixed with Tris–cacodylic buffer (50 mM Tris–HCl, 50 mM cacodylic acid, 1 mM diethylenetriamine pentaacetic acid, pH 8.2) and 2 mM pyrogallol. The autoxidation of pyrogallol and the inhibition of this reaction were monitored spectrophotometrically.

2.12 Quantification of Protein Concentration

The protein content of the blood and all organs were determined using the Bradford method [51]

2.13 Statistical analysis

The differences among the control and experimental groups were determined using GraphPad Prism (version 6) software package. Analysis of data was performed using the unpaired student's t-test. All data are expressed as Mean ± SEM, and the level of significance was considered as $p<0.05$.

Table 1: Blood glucose levels in the STZ-induced rat

Animals	1	2	3	4	5	6	7	8	9	10
Blood Glucose (mg/dl)	233	146	254	138	157	238	257	245	147	121

3. RESULTS

The catalase activity in plasma, red blood cells, brain and liver, are presented in Fig 1. A significant decrease (P<0.001) was observed in the catalase activities in plasma and RBC and the brain and liver when compared to control. Diabetes induced significant inhibition of 8, 3, 10 and 13-fold on catalase activities across all compartment, respectively.

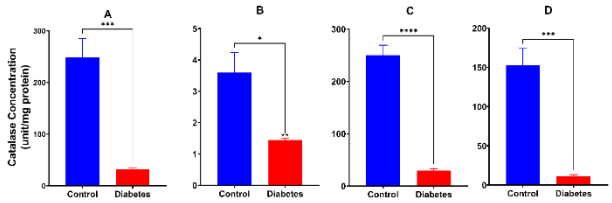

Fig 1: Effects of diabetes on catalase activities in plasma (A), red blood cell (B), brain (C) and liver (D). *Each bar represents the mean ± SEM of 10 rats. Bars with *, **, *, **** are significantly different at P < 0.05, 0.01, 0.001,* and *0.0001 respectively*.**

Superoxide dismutase activities in the four-compartment (plasma, RBC, brain and liver) of the animals are depicted in fig 2. While diabetes induced significant (*P < 0.001*) inhibition of above 70% on the activity of superoxide dismutase (SOD) in the plasma, brain and liver compartments, a significant (*P < 0.001*) activation of the activity of SOD was observed in the RBC compartment.

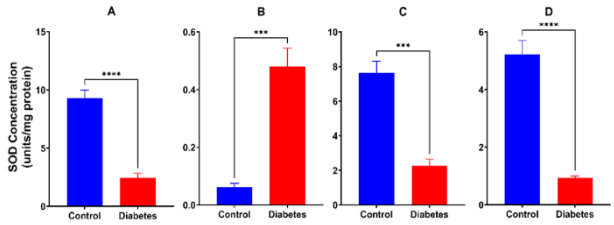

Fig 2: Effect of diabetes on superoxide dismutase activities in plasma (A), red blood cell (B), brain (C) and liver (D). *Each bar represents the mean ± SEM of 10 rats. Bars with *, **, *, **** are significantly different at P < 0.05, 0.01, 0.001,* and *0.0001 respectively.***

As shown in Fig 3, only in the RBC was an activation of Glutathione-S-transferase (GST) activity observed in all the compartments studied. However, all other compartments, plasma, brain, and liver, reveal significant inhibition (*P < 0.001, 0.0001, 0.01*) of 76, 90 and 67% of GST.

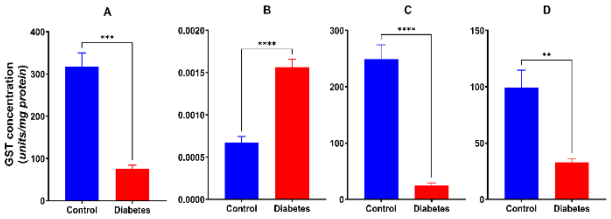

Fig 3: Effect of diabetes on Glutathione-S-transferase activities in plasma (A), red blood cell (B), brain (C) and liver (D). Each bar represents the mean ± SEM of 10 rats. *Bars with *, **, *, **** are significantly different at P <0.05, 0.01, 0.001, 0.0001 respectively.***

Glutathione (GSH) concentration of the animals are depicted in Fig 4. While diabetes does not affect the GSH in most of the compartments studied (plasma, brain and liver), a significant increase (*P < 0.01*) of 35% in GSH concentration was noticed in the RBC.

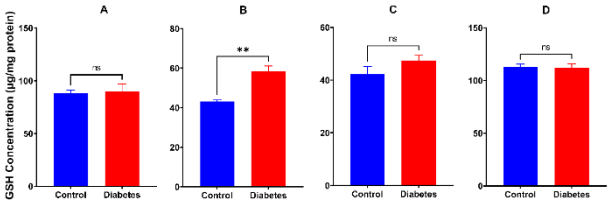

Fig 4: Effect of diabetes on GSH activities in plasma (A), red blood cell (B), brain (C) and liver (D). Each bar represents the mean ± S.E.M. of 10 rats. *Bars with *, **, *, **** are significantly different at P < 0.05, 0.01, 0.001,* and *0.0001 respectively*.**

Fig 5 shows the nitric oxide level of animals used in this study. Diabetes caused a significant decrease (*P < 0.0001,0.001*) of 57 and 64 % in the nitric oxide of plasma and RBC without any significant changes in the brain. Meanwhile, a significant increase (p<0.001) of 92% was observed in the liver.

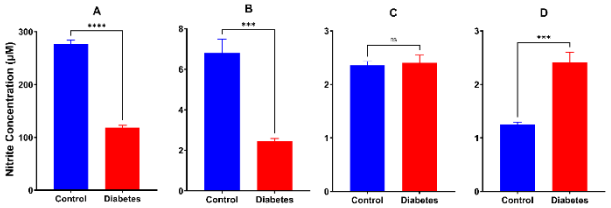

Fig 5: Effect of diabetes on nitric oxide in plasma (A), red blood cell (B), brain (C) and liver (D). Each bar represents the mean ± SEM of 10 rats. *Bars with *, **, *,**** are significantly different at P < 0.05, 0.01, 0.001,* and *0.0001 respectively.***

Depicted in Fig 6 is the acetylcholine esterase activities of rats used in this study. A significant inhibition (*P < 0.01, 0.0001*) in acetylcholine esterase activity was seen in plasma, brain and liver, with the highest inhibition of 39% in the plasma. While an expected significant increase was seen in the RBC of the animals.

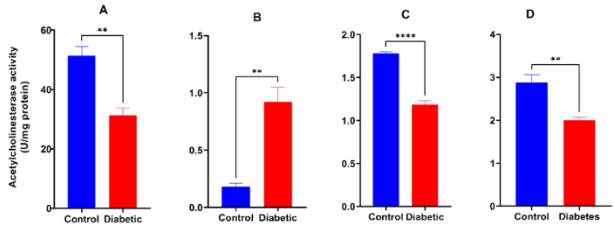

Fig 6: Effect of diabetes on AcChE activities in the plasma (A), red blood cell (B), brain (C) and liver (D). Each bar represents the mean ± S.E.M. of 10 rats. Bars with *, **, *,**** are significantly different at *P<0.05, 0.01, 0.001,* and *0.0001* respectively.**

Arylesterase activities in the plasma, RBC, brain and liver of the animals studied are shown in Fig 7. Diabetes caused significant inhibition of above 44% in the arylesterase activity of all the compartments; plasma, RBC, brain and liver.

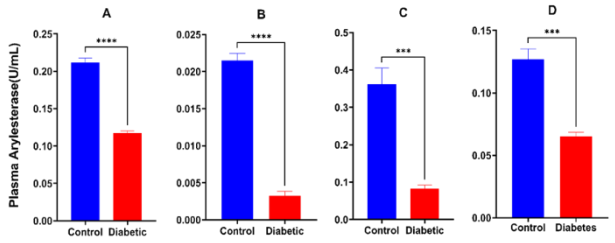

Fig 7: Effect of diabetes on arylesterase activities in plasma (A), red blood cell (B), brain (C) and liver (D). Each bar represents the mean ± S.E.M. of 10 rats. *Bars with *, **, *, **** are significantly different at P < 0.05, 0.01, 0.001,* and *0.0001 respectively.***

4. DISCUSSIONS

Due to prolonged hyperglycemia that induces oxidative stress [52], diabetic patients are believed to have an imbalance between ROS formation and degradation due to deficiency in their antioxidant systems [38]. This has also been confirmed using animal models [53]. It is believed to be more severe than in healthy persons [54]. Antioxidants can be found in various locations in cells and organs to varying degrees. In this study, we examined how diabetes affects acetylcholinesterase, arylesterase, and oxidative stress biomarkers (GSH, GST, SOD, CAT, NO) in the brain and liver erythrocytes and plasma of rats.

Our study revealed a significant reduction in catalase (CAT) activity in the RBC, liver, brain, and plasma of diabetic rats compared to normoglycemic rats. This result appears to be consistent with other research conclusions [19, 52, 55].

We also observed a significant reduction in SOD activity in the brain, liver, and plasma of diabetic animals but a significant increment in RBC activity. These findings are consistent with research carried out with Pan and Arab Sadeghabadi results [38, 52]. Reduction in SOD was also confirmed by Sadeghabadi *et. al* [38] and Pan *et. al* [52] diabetic rats. However, it was measured in the plasma and serum, respectively. We believe the reduction in SOD might be due to decreased levels of its cofactors (Zn2+ and Cu2+) due to hyperglycaemia [38]. Ahmed et. al [56] recorded high SOD activity in the liver, heart, and pancreas of diabetic rats. In contrast, we recorded an increase only in the RBC.

No significant difference was recorded when comparing Nitric Oxide (NO) level in the brain of diabetic and normoglycemic rats. However, there was a significant increase in the liver but a significant decrease in the RBC and plasma. The increased NO observed in the liver compartment of our study is consistent with the findings of Stadler and his co-worker [57]. Where he attributes the increased NO to an early sign of premature aging in diabetes.

Glutathione (GSH) concentration in the brain and liver of the diabetic animals in this study significantly reduced. At the same time, an increase was observed in the plasma and RBC. Though not completely consistent, the depletion of GSH agrees with a couple of researches [54, 58-61]. Hyperglycemia can promote ROS accumulation by activating various pathways [24], one of which includes pumping glucose to the polyol pathway. The polyol pathway promotes the conversion of glucose to polyalcohol sorbitol. This pathway consumes a high amount of NADPH (an antioxidant neutralising species). This causes a ripple effect on Glutathione Reductase, an enzyme that needs NADPH to generate GSH [24, 56]. So, ROS accumulation causes a reduction in NADPH's intracellular concentration and, indirectly, the depletion of GSH – two important antioxidising agents [56, 58].

Although not investigated in this study, Glutathione Peroxidases (GPx) are also critical antioxidant enzymes that scavenge free radicals. They catalyse hydrogen peroxide (H_2O_2) reduction to water and oxygen via glutathione oxidation to glutathione disulphide. Catalase also break H_2O_2, but GPx has a higher affinity for it. Different studies have confirmed a decrease in GPx activity in diabetic models [19, 38, 52, 56, 58]. The diminished activity of GPx in diabetes may be attributed to protein glycation, the inactivation of GPx due to hyperglycaemia, or the effect of polyol pathway on depleted GSH concentration, which is the substrate and cofactor of GPx. This will further lead to a reduction in the antioxidant ability of GPX against H_2O_2, an increase in the H_2O_2 burden, and a final inhibition of catalase activity by H_2O_2. This further confirms the GSH concentration and catalase activity finding of the compartments investigated in this study.

Cognitive impairment and neurodegenerative diseases have been reported by many studies to be associated with complications of diabetes [62, 63]. The esterases (AChE and arylesterase) have also been associated with the erythrocyte membrane's integrity [64, 65]. Paraoxonase has two substrates, paraoxon and phenylacetate. They detoxify a wide range of organophosphate anticholinesterase nerve gases and insecticides by hydrolysis. They are responsible for the selective toxicity of such compounds in various mammalian species, including man [66]. Our investigation showed a significant reduction in the esterases (AChE and arylesterase) in the RBC, liver, brain and plasma of diabetic rats. This result corroborates various studies [12, 16, 67]. Therefore, it is possible that paraoxonase is playing a role in

preventing the delayed neuropathy due to organophosphates or other dietary neurotoxins and thus be a factor of potential aetiological significance in the development of diabetic neuropathy. Also, paraoxonase has been reported to play a critical role in hydrolysing toxic lipids ester, like oxidative modification of circulating low-density lipoprotein reported in diabetes.

5. CONCLUSION

The findings of this study indicate that diabetes inhibitory effects on the antioxidants and esterases activities in the rats are compartment specific. There is a need for more investigation in other compartments which are not investigated in this study. In conclusion, distinct compartments effects of diabetes seen in this study could suggest a new approach for effective and safer therapeutics.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Adedoja D. Wusu designed the study and wrote the final draft of the manuscript. Olukorede A. Liadi did the first draft of the manuscript. Taiwo H. Saliu performed the analyses. While Oluwatimileyin T. Ayeni managed the literature searches, Olusegun K. Afolabi wrote the protocol and carried out the statistical analysis.

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