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Original Research Article

Effects of Asheitu Adams Bitters Polyherbal Formulation on The Carbohydrate Biomarkers, Body Weight, and Blood Glucose Level of Diabetic Wistar Rats.

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ABSTRACT

The number of people with diabetes mellitus (DM) today has been growing and causing increasing concerns to the public. Africans are now using medicinal plants as an alternative to conventional drugs. The combination of this medicinal plant gives rise to polyherbal drugs in which Asheitu Adams bitter (AABs) polyherbal formulation is one. This drug has been claimed to treat DM without any empirical data to support such claim. Therefore, this study investigated the impacts of AABs formulation on the body weight, blood glucose level, and its expression pattern on Glucagon-like peptide-1 (GLP-1), Glucose-6-phosphate dehydrogenase (G6PD), and Glucose transporter 2 (GLUT2) genes of Wistar rats. AABs polyherbal was screened for various phytochemicals. Rats were given different treatments at different concentrations and their body weights and blood glucose levels were monitored for 28 days. Gene expression profiling was used to analyze the expression pattern of GLP-1, G6PD, and GLUT2 genes in the rats. The blood glucose level of streptozotocin-induced diabetic rats treated with AABs at 30mg/kg was reduced to almost normal. Though, the polyherbal has no significant effect on the body weight of the treated rats. AABs polyherbal treated rats showed up-regulation of GLP-1 and GLUT2 genes in the intestinal crypt and a down- and up-regulation of the G6PD gene in the kidney and liver respectively. AABs polyherbal formulation is able to regulate GLP-1, GLUT2, and G6PD genes and bring them close to normal in diabetics which may be due to its ability to lower blood glucose at 30 mg/kg body weight.

***Keywords*:** Asheitu Adams Bitters, Diabetes mellitus, Glucagon-like peptide-1, Glucose transporter 2, Glucose-6-phosphate dehydrogenase.

Introduction

Diabetes Mellitus (DM) is a chronic metabolic condition marked by high blood glucose (blood sugar) levels that can cause significant damage to the heart, blood vessels, skin, kidneys, and nerves over time.[1] DM signs include frequent urination, increased hunger, and increased appetite.[2] Type 1, type 2, and gestational DM are the three common types of diabetes.[3] Type 1 DM is due to insufficient production of insulin.[2] Type 2 diabetes is marked by high insulin resistance and an insulin deficiency. During pregnancy, some women develop DM known as gestational diabetes and the other types are a collection of a few individual causes such as monogenic diabetes.[4] DM is a global health problem that has reached unprecedented proportions: half a billion people now have DM. One of the top ten causes of death in the world is diabetes mellitus.[3] About a billion people are diabetic globally and the number is expected to rise by 51% in 2045.[5] More than 80% of diabetes deaths occur in low and middle-income countries around the world.[1] In Africa, 15.9 million adults are estimated to have diabetes, resulting in a regional prevalence of 3.1 percent. The African continent has the highest proportion of people with undiagnosed diabetes, and global forecasts indicate that by 2045, it would have experienced a 156 percent rise in the burden of DM.[6] Diabetes has a high prevalence, pathogenesis, a progressive mechanism, and multiple complications, all of which illustrate the urgent need for effective therapies.[7]

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According to Diabetes UK,[8] there is no treatment for DM. It's management, on the other hand, that focuses on keeping blood sugar levels as close to normal as possible without causing hypoglycemia. Dietary adjustments, exercise, weight loss, and the use of effective drugs (insulin, oral medications) are normally enough to achieve this.[9] Various treatments, such as hormone therapy, pharmacotherapy, and diet therapy, are also available to monitor diabetes. Despite tremendous improvement in the treatment of diabetes over the last three decades, the outcomes of treatment in patients are still far from optimal.[7] Drug resistance (lower efficacy), side effects, and even toxicity are some of the drawbacks of these therapies. Sulfonylureas, for example, lose their potency in 44 percent of patients after 6 years of treatment. It is also said that glucose-lowering medications cannot control hyperlipidemia.[10] Since modern/conventional medicines are often out of reach in Africa, up to 80% of the population relies on medicinal plants and polyherbal drugs to treat their ailments.[11] Most plants contain carotenoids, flavonoids, terpenoids, alkaloids, glycosides and some of these bioactive compounds have been found to have anti-diabetic effects[12] and the use of more than one herb in a medicinal preparation is known as polyherbal formulation.[13] According to,[7] plants' anti-hyperglycemic effect in the treatment of DM is often due to their ability to increase pancreatic tissue function, which is accomplished by increasing insulin secretion or reducing glucose absorption in the intestine.

Asheitu Adams bitters (AABs) is a polyherbal formulation gotten from medicinal plants and is claimed to be effective in the management and treatment of DM and other illnesses by the producer. People who do not have access to conventional anti-diabetic therapies in developing countries or those that have a strong belief in the potency of traditional medicines or herbal remedies use it freely without caution despite the lack of empirical data to validate these claims. The study investigated the effects of Asheitu Adams Bitters (AABs) polyherbal formulation on Glucose transporter 2 (GLUT2),

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Glucagon-like peptide-1 (GLP-1), Glucose-6-phosphate dehydrogenase (G6PD), the body weight, and blood glucose level of diabetic Wistar rats.

Materials and Methods

Drugs and chemicals
Streptozotocin (STZ) was obtained from Sigma Aldrich in the United States, and metformin was obtained from Teva Pharmaceutical in Wales. All diagnostic kits were purchased from Lab-care Diagnostics Ltd. in India, and all other chemicals used in this research were analytical grade.

Polyherbal formulation
AABs polyherbal formulation (300ml), manufactured by Asheitu Adams enterprise was purchased from the company's distributor at Boundary, Ajegunle, Lagos State, Nigeria. The polyherbal mixture had been approved for use in Nigeria by the National Agency for Food and Drug Administration and Control (NAFDAC) and has a NAFDAC Registration number: A7-1827L. The polyherbal mixture was lyophilized and then reconstituted to match the normal drug's unit (mg) (metformin). This allows us to use the same dosage unit for both the polyherbal mixture and the regular medication (i.e., mg/kg).

Phytochemical screening
This phytochemical screening was used to determine the presence of Saponins, Tannins, Alkaloids, Phlobatannins, Flavonoids, Steroids, Terpenes, Anthraquinones, Phenols, Cardiac glycosides, Cardenolides in the AABs polyherbal formulation.[14,15]

Experimental animals
Ethical approval was obtained from the Research and Development Center of Adekunle Ajasin University, Akungba Akoko, Ondo State, Nigeria. Twenty-five (25) male Wistar rats of average weight 96.51 ± 11.28 g were purchased from Ibadan, Oyo State, Nigeria. They were housed in an iron cage and acclimatized for two weeks in the Animal Unit of Centre for Bio-computing and Drug Development (CBDD), Akungba Akoko, Ondo State, Nigeria. The rats were fed ad libitum with normal rat pellet ration (Top Feeds, Nigeria) and portable water in a room with a temperature of 22^{o}C and relative humidity of 55.5% in a house with an equal cycle of light and darkness (i.e., 12 hours of light/12 hours of darkness). All procedures were in line with the National Institutes of Health guide for the care and use of laboratory animals.[16]

Induction of Type 1 Diabetes Mellitus
Diabetes was induced according to Islam and Wilson procedure with slight modification.[17]After an overnight fast, the animals received three intraperitoneal injections of streptozotocin dissolved in 0.1M of citrate buffer (pH 4.5) at doses of 40 mg/kg, 45 mg/kg, and 35 mg/kg of bodyweight. The presence of elevated fasting blood glucose levels established the induction of type 1 DM. Rats with a fasting blood glucose level of more than 200 mg/dl were chosen for the study.

Experimental Design
Five healthy male Wistar rats were chosen to be the Normal Control (i.e., non-diabetic (Ctr)) and were administered 1ml of distilled water orally. The diabetic Wistar rats were divided into four groups with five rats each; group one served as Negative Control (i.e., Diabetic Control (DC)) and were administered 1ml of distilled water orally, while group two served as Positive Control (i.e., Metformin (Met)) and were administered 15 mg/kg of metformin orally. Group three and four served as AABs polyherbal formulation treatment groups and were administered AABs at 15 mg/kg and 30 mg/kg bodyweight orally respectively.

Body Weight and Blood Glucose Determination
Ghasemi *et al*. procedure was used to assess blood glucose levels in the rats.[18] Rats' fasting blood sugar (FBS) levels and body weight (BW) were assessed at the end of each week for four weeks in a row. Animals fasted overnight and FSB levels were measured using a drop of blood from the rats' tail vein on an ACCU CHEK Strip fixed in a Glucometer (Mannheim, Germany) and readings were taken. The BW of the animals was determined by placing them on a weighing scale. At the end of the 28th day of administration of the AABs polyherbal formulation, metformin and distill water, rats were fasted overnight and were sacrificed the next morning. Tissues which included kidney, liver, and intestinal crypt were excised from all the animals into well-labeled Eppendorf tubes containing 50 µL molecular biology reagent (Trizol) across the groups including the Normal Control. Tissues were then stored under a temperature of 0^{0}C to 4^{o}C for 24 hours.

Ribonucleic acid (RNA) isolation
Each piece of isolated tissue (liver, intestinal crypt, and kidney) was put in an Eppendorf tube with 100 µL of RNA Snap kits that were identified. Tissues were homogenized and centrifuged at 17000 rpm for 30 minutes. All samples were treated with 100 µL of chloroform and centrifuged at 3000 rpm for 30 minutes. The supernatant (RNA phase) was separated into clean, sterile, and labeled Eppendorf tubes with care. Every sample was treated with 100 µL of Iso-amyl alcohol and centrifuged for 30 minutes at 3000 rpm to obtain the RNA in pellet form. After a 5 minutes centrifugation at 17000 rpm, the tubes containing the samples (RNA) were uncapped to enable the ethanol to evaporate. The RNA was then air-dried and resuspended in a sterile tube containing nuclease-free water, and the total RNA concentration was measured using UV absorbance spectrophotometry (JENWAY 6305). Finally, for RNA (mRNA) quantification, all samples were diluted to the same concentration.

Reverse transcription
To convert 20µl of total isolated RNA samples into Complementary deoxyribonucleic acid (cDNA), 2 µL of reverse transcriptase containing oligo primers, dNTPs, reverse transcriptase, reverse transcriptase buffer, and nuclease-free water was added. After which the samples were put in a thermocycler and heated to 65^{0}C for 4 hours.

Polymerase chain reaction (PCR) amplifications
Before starting the amplification phase, the reaction system was optimized. Nuclease-free water 3.5 µL, forward primers 2 µL and reverse primers 2 µL and master mix 2µL were all added to each sample for a complete enzymatic reaction. Multigenoptimax PCR machine was used and amplification conditions were: 94^{o}C pre-denaturation for 5 minutes, 94^{o}C for 30 seconds, Annealing 55^{o}C (Tm) for 30 seconds, Extension 72^{o}C for 30 seconds, and 5 minutes at 72^{o}C by 30 cycles.

Agarose gel electrophoresis
In a microwave, 0.5 mg agarose powder was added to 60 mL 1x TBE buffer and heated for 45 seconds until agarose dissolved, then allowed to cool for 5 minutes. To assist visualization, 0.5 µL of ethidium bromide was added to each sample, along with 3 µL of tracking dye. The agarose was poured into a gel tray with combs and allowed to solidify for 15 minutes before it was filled with a buffer inside the gel box. After carefully removing the combs, 8 µL of the amplified samples were carefully loaded into the wells, and the gel ran at 100 V for 6 minutes. A UV light box was used to visualize the results.

Statistical Analysis
Statistical analysis was carried out using IBM SPSS Statistics, Version 21 software. Mean ± Standard Error of Mean (SEM), and two-way analysis of variance (ANOVA) was used to assess statistical significance, followed by Duncan's New Multiple Range Test. Data were considered significant at $P<0.05$.

Results and Discussion

Since conventional drugs have too many negative side effects, research into safe treatments became imperative. Acupuncture, herbal remedies, homeopathy, traditional medicine, and other medical therapies, as well as complementary and alternative medicine, are thought to be beneficial in the treatment of DM.[19] The study investigated the effects of Asheitu Adams Bitters (AABs) polyherbal

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formulation on the body weight, blood glucose, GLP-1, G6PD, and GLUT2 genes of STZ-induced diabetic rats.

Considering week 0, Table 1 shows that there is a significant difference in blood glucose level of the control group and other groups at ($P<0.05$). However, at week 4, Asheitu Adams bitters (30 mg/kg) blood glucose levels do not differ significantly from that of the control at ($P<0.05$). This shows that the polyherbal formulation has an anti-diabetic effect. It was able to bring blood glucose levels closer to normal. This could probably be associated with its ability to stimulate the regeneration of beta cells, which stimulates insulin secretion from the regenerated beta cells and/or the remnant beta cells.[20]

Table 2 shows that tannins, phlobatannins, saponins, steroids, flavonoids, terpenoids, cardiac glycosides, cardenolides, anthraquinones, phenols, alkaloids are present in Asheitu Adams bitters polyherbal formulation, ranging from trace amounts to abundant amounts. According to a study by El Barky *et al*.,[21] Saponins are known to have anti-diabetic effects. Patel *et al*. also accounted that terpenoids, phenolics, flavonoids, alkaloids, and some other categories have shown anti-diabetic potential through the insulinomimetic activity of plant extract.[22] Hence, AABs polyherbal formulation contains phytochemical constituents that its anti-diabetic effect may be due to its saponin, phenol, flavonoid, terpenoid, etc. contents. The concentration of these phytochemicals also shows their effectiveness at a specific dose as seen in AABs polyherbal at 30 mg/kg which may be due to the increased concentration of these phytochemical constituents.

Figure 1 shows that Asheitu Adams bitters at 15 mg/kg and 30mg/kg body weight up-regulate intestinal crypt glucagon-like protein 1 (GLP-1) gene in comparison with control, diabetic control, and metformin groups. Incretins are gastrointestinal hormones that help the body release insulin after a meal.[23] GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) are two major incretins and are thought to be responsible for up to 70% of insulin secreted from the *β*-cells of the pancreas following food intake. However, up-regulation is seen in AABs treated animals as in comparison with the control animals agrees with the report made by Duttaroy *et al*.[24] Reports have previously shown that an increase in plasma GLP-1 level improved insulin production (i.e., increase in insulin level is directly proportional to an increase in GLP-1 level). Tonne *et al*. reported that pancreatic over-expression of GLP-1 prevented the *β*-cell loss upon STZ challenge and protected mice from developing hyperglycemia.[25]

Figure 2 shows that Asheitu Adams bitters at 15 mg/kg and 30 mg/kg body weight up-regulate intestinal crypt glucose transporter 2 (GLUT2) gene in comparison with other groups (control, diabetic control, and metformin groups) however, Asheitu Adams bitters at 30 mg/kg body weight slightly down-regulates GLUT2 gene expression in comparison with metformin group. The glucose transport gene expression is reduced when there is relative insulin deficiency, such as in STZ-induced diabetes which means that the expression of GLUT2 is directly proportional to the level of insulin. AABs polyherbal increases intestinal crypt GLUT2 of experimental animals in comparison with the normal and diabetic controls as shown in this study which may be due to increased insulin production as reported by Al-Shaqha *et al*.[26] GLUTs are involved in the absorption, distribution, and excretion of glucose and other hexoses.[27] Marks *et al*. reported over-expression of GLUT2 genes at the enterocyte brush border membrane (BBM) vesicles in diabetes in response to elevated glucose levels in the intestinal lumen as seen in rats administered treatments. The result suggests that GLUT2 accounts for the increased glucose absorption seen in diabetics, with increased glucose levels in the plasma or tubular fluid accelerate GLUT2 expression. The excessive increase in GLUT2 expression at the BBM in relation to the degree of glucose transport stimulation indicates that hyperglycemia also affects this transporter's intrinsic operation. The decline in glucose transport gene levels is one of the key causes of hyperglycemia in diabetics, which is caused by reduced glucose uptake. The contents of glucose transport gene mRNA were restored to near normal values after treatment with Catharanthus roseus (c. roseus), which may be one of the reasons for the increase in GLUT2 gene in AABs polyherbal treated rats.[28] The restoration of glucose transport gene levels will improve glucose absorption in the liver and thus aid in the treatment of hyperglycemia. Glucose insensitivity due to Glucose transporter gene dysfunction is commonly seen in people with type 2 diabetes.[26]

Figure 3 shows that Asheitu Adams bitters at 30mg/kg body weight up-regulates liver glucose 6 phosphate dehydrogenase (G6PD) gene in comparison with other treatments/groups (i.e., control, diabetic control, metformin groups, and Asheitu Adams at 15 mg/kg). G6PD is a crucial enzyme in the pentose phosphate pathway (PPP). The enzyme provides reduced energy to cells by maintaining co-enzyme nicotinamide adenine dinucleotide phosphate (NADPH).[29,30] AABs polyherbal treatment at 30 mg/kg up-regulates G6PD gene in the liver of experimental animals in comparison with the other treatment groups in this study agrees with Bhat *et al*. report,[30] that the enzyme activity in treated mice was significantly restored by a chloroform extract of *Azadirachta indica* and aqueous, methanolic extracts of *Bougainvillea spectabilis*. This may be attributed to an increase in insulin production, as this enzyme activity is dependent on insulin which is also seen in AABs polyherbal treated animals. This revealed improvement in the formation of Nicotinamide Adenine Dinucleotide Phosphate (NADPH), favoring lipogenesis and the use of an alternative channel to dispose of excess glucose via the HMP pathway in diabetic rats.[31]

Table 1: Blood Glucose (mg/dL) level of Experimental rats

Treatments Weeks	Normal Control	Diabetic Control	Metformin (15mg/Kgb.wt)	Asheitu (15mg/Kgb.wt)	Asheitu (15mg/Kgb.wt)
Week 0	72.00 ± 5.25^{a}	330.20 ± 26.42^{b}	342.40 ± 21.89^{b}	323.25 ± 24.36^{b}	325.00 ± 45.49^{b}
Week 1	74.80 ± 3.97^{a}	404.20 ± 52.47^{b}	374.20 ± 38.03^{b}	346.75 ± 73.10^{b}	399.25 ± 29.64^{b}
Week 2	68.40 ± 2.66^{a}	240.00 ± 44.83^{b}	385.25 ± 38.45^{b}	328.50 ± 84.89^{b}	283.67 ± 45.73^{b}
Week 3	72.75 ± 4.27^{a}	196.60 ± 55.17^{ab}	195.33 ± 57.71^{ab}	247.00 ± 17.62^{b}	213.33 ± 57.85^{ab}
Week 4	71.75 ± 2.25^{a}	249.80 ± 62.32^{bc}	291.50 ± 10.50^{c}	244.00 ± 33.00^{bc}	96.33 ± 21.65^{ab}

Values are mean ± S.E.M of 5 rats in each group; Means with different superscripts are significantly different at ($P < 0.05$).

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Figure 4 shows that Asheitu Adams bitters at 15 mg/kg and 30 mg/kg body weight down-regulate kidney glucose 6 phosphate dehydrogenase (G6PD) gene in comparison with the control group but, up-regulate the gene in comparison with diabetic control and metformin groups except for Asheitu Adams bitters at 30mg/kg bodyweight that is slightly down-regulated in comparison with metformin group. AABs polyherbal down-regulates G6PD gene in the kidney of experimental animals in comparison with the control group in this study is in contrast to the report by Adekomi *et al*. They reported that the administration of ethanolic leaf extract of *C. roseus* increased the activity of G6PD significantly in the kidney of rats. In addition to the synthesis of the precursor of DNA, G6PDH also generates NADPH. This is critical for maintaining Glutathione (GSH) in its reduced form. Glutathione is essential for the detoxification of reactive free radicals and lipid hydroperoxides. This may be one mechanism by which *C. roseus* mediates long-term normalization of glycemia in rodent models of diabetes mellitus.[32]

Table 3 shows that there is no significant difference in the body weight of animals between the control group and the other treatment groups (diabetic treatment groups) at ($P<0.05$) in week 0. However, at week 4, the diabetic and treatment groups' weight differs significantly from that of the normal control group at ($P<0.05$). Gandhi *et al*. reported the anti-diabetic effect of *Merremia emarginata* (*M. emarginata*). *M. emarginata* also improved body weight to a certain extent, indicating control over muscle wasting resulted from glycemic control which is also seen in this study.[20] Therefore, AABs polyherbal showed no remarkable increment in body weight of experimental animals in comparison with the normal control group. This may be due to the breakdown of tissue proteins in diabetic rats and animals trying to improve body weight to a certain extent, indicating control over muscle wasting resulted from glycemic control. [20,33]

Table 2: Phytochemical screening of Asheitu Adams bitters polyherbal formulation

Phytochemical	Results
Tannins	+
Phlobatannins	-
Saponins	+
Flavonoids	+
Steroids	+
Oxalate	+
Cardiac Glycosides	+
Cardenolides	-
Anthraquinones	-
Phenols	+
Alkaloids	+
Terpenoids	-

Key word: +: present, -: absent.

Table 3: Body Weight (g) of Experimental rats

Treatments Weeks	Normal Control	Diabetic Control	Metformin (15 mg/Kgb.t)	Asheitu (15 mg/Kgb.t)	Asheitu (30 mg/Kgb.t)
Week 0	99.28 ± 8.97^{a}	88.67 ± 6.1^{a}	94.41 ± 4.68^{a}	95.92 ± 9.28^{a}	94.11 ± 7.78^{a}
Week 1	124.23 ± 9.03^{b}	89.20 ± 7.0^{a}	100.93 ± 3.85^{a}	97.72 ± 8.46^{a}	91.60 ± 9.98^{a}
Week 2	135.97 ± 8.26^{b}	89.69 ± 7.7^{a}	98.76 ± 8.21^{a}	97.60 ± 9.59^{a}	96.67 ± 6.69^{a}
Week 3	145.85 ± 12.9^{b}	86.35 ± 6.0^{a}	97.29 ± 10.07^{a}	104.98 ± 7.30^{a}	97.80 ± 6.15^{a}
Week 4	148.30 ± 10.1^{b}	83.76 ± 6.1^{a}	106.39 ± 14.3^{a}	103.51 ± 7.57^{a}	96.50 ± 4.94^{a}

Values are Mean ± Standard Error of Mean (S.E.M) of 5 rats in each group; Means with different superscripts are significantly different at ($P<0.05$).

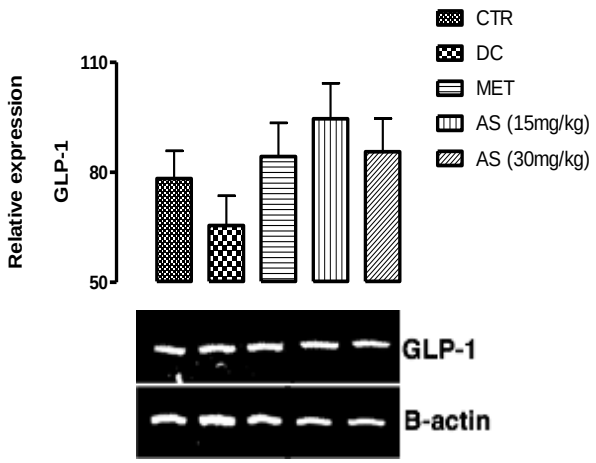

Figure 1: GLP-1 relative expression in the intestinal crypt of normal and diabetic induced rats.

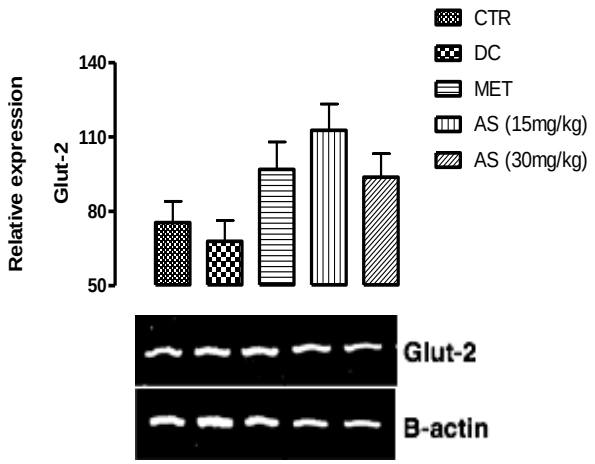

Figure 2: GLUT2 relative expression in the intestinal crypt of normal and diabetic induced rats.

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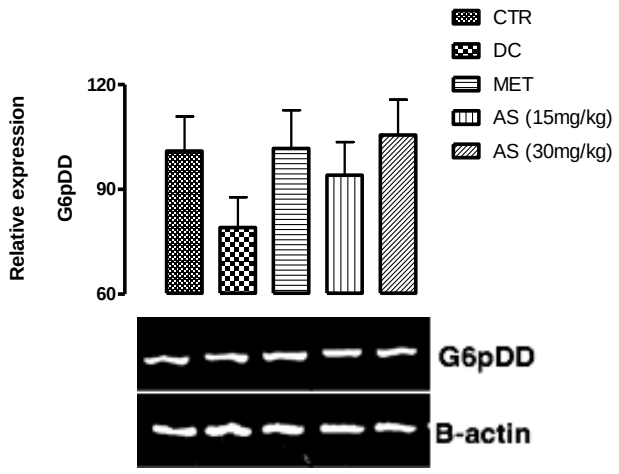

Figure 3: G6PD relative expression in the liver of normal and diabetic induced rats.

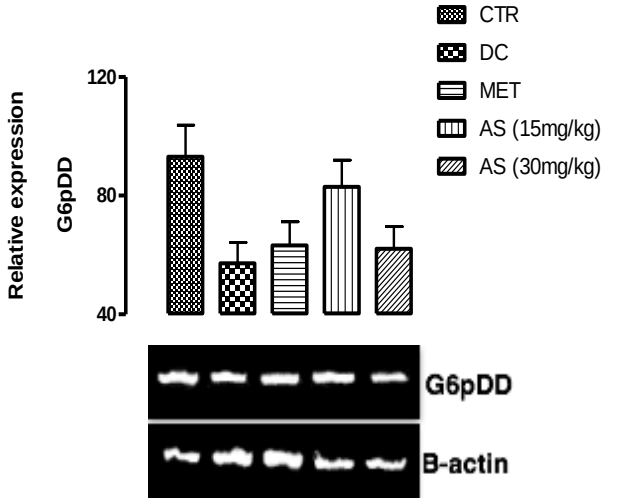

Figure 4: G6PD relative expression in the kidney of normal and diabetic induced rats.

Conclusion

The study has shown that Asheitu Adams Bitters (AABs) polyherbal can regulate GLP-1, GLUT2, and G6PD genes close to normal in diabetic rats which may be due to its ability to lower blood glucose. The increase in the GLP-1 may help to increase insulin production and its tendency to regenerate beta cells. The polyherbal has no remarkable effect on body weight.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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