



Anti-diabetic Analysis of Ruzu Herbal Bitters and Asheitu Adams Bitters Polyherbal Medicines in Streptozotocin-Induced Diabetic Albino Rats

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Abstract

Keywords:

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Diabetes is still one of the major prevalent diseases of this century despite the advancement recorded so far in the field of medicine. Its treatment, management and prevention with synthetic drugs globally are of great financial burden coupled with some adverse side effects. All these problems in addition to prediction that incidence of diabetes would rise by 2030 led to the formulation of different polyherbal medicines. However, empirical data to support usage of these polyherbs as alternative therapy to conventional hypoglycemic drugs are lacking. This study therefore examined antidiabetic analysis of Ruzu Herbal Bitters (RHB) and Asheitu Adams Bitters (AAB) polyherbal medicines in streptozotocin-induced diabetic rats. Streptozotocin was used to induce diabetes in male Albino rats and the animals were treated with RHB and AAB polyherbs (15 or 30 mg/kg b.wt.) for 4 weeks. The relative expressions of pancreatic duodenal homeobox 1 (PDX-1), insulin, intestinal glucagon-like peptide 1 (GLP-1) and Glucose transporter 2 (GLUT-2) genes were then determined by quantitative polymerase chain reaction. Findings showed that blood glucose concentrations of RHB- and AAB-treated animals at week 3 and/or 4 did not differ significantly ($p > 0.05$) from that of the control group, except 15 mg/kg AAB-treated group at week 3. Furthermore, RHB and AAB down regulated PDX-1 and insulin gene expressions but up regulated GLP-1 and GLUT-2 gene expressions in blood glucose concentration-dependent manner. Sequel to the observed decrease in blood glucose concentrations, and gene expression-regulating potential of the polyherbs, both RHB and AAB are premised to have anti-diabetic properties.

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INTRODUCTION

Diabetes is a heterogeneous primary disorder of carbohydrate metabolism in which glucose is underutilized. It is a group of metabolic diseases characterized by hyperglycemia as a result of defects in insulin secretion, insulin action, or both (American Diabetes Association (ADA), 2009; 2012). Several pathogenic processes are involved in diabetes development. These range from autoimmune destruction of β -cells of the pancreas with resultant insulin deficiency to abnormalities that lead to resistant

insulin action, and consequently, impaired carbohydrate, fat and protein metabolism (ADA, 2012). Diabetes is a global public health problem with increasing prevalence. It's predicted that about 366 million people worldwide will be diabetic by the year 2030 (Wild *et al.*, 2004). Thus, diabetes still remains a major metabolic disease challenging the health of majority of the world's populace, most especially in the developing countries. It is one of the five leading causes of death in the world, with type 2 diabetes occurring

more frequently than type 1 diabetes according to Ghasemi *et al.* (2014).

Furthermore, management of diabetes without side effects is also a challenge, hence new strategies are needed to be examined (Ghasemi *et al.*, 2014). Despite the availability of medications for management of diabetes, the interest in alternative traditional remedies is increasing (Pari and Umamaheswari, 2000). Herbal treatment of disease is currently gaining momentum with varieties of plants (e.g. *Lamium album* and *Urtica dioica*) and plant-derived products being promoted as capable of reducing blood sugar levels (Mohammad *et al.*, 2015). Plants and their products have been used for medicinal purposes in Africa for many centuries (Adewunmi and Ojewole, 2004) without conducting therapeutic and/or regulatory studies with animals prior to exposure of man to such herbal or polyherbal products. According to Miller *et al.* (2004), until toxicological, pharmacodynamics, and pharmacokinetic data are available, traditional health practitioners and clinicians must exercise caution in recommending phytomedicine and/or prescribing polyherbs to their patients. Unfortunately, this seems to be neglected by most manufacturers of polyherbal medicines. They introduced their products into the markets and

recommend them for treatments of various diseases without scientific validations of efficacy, suitability, toxicology, and safety of these polyherbs. Consequently, this study was undertaken to determine the anti-diabetic properties of Ruzu Herbal Bitters (RHB) and Asheitu Adams Bitters (AAB) polyherbal medicines in streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

Drugs and Chemicals

Streptozotocin was obtained from Sigma Aldrich, USA, and metformin from Teva Pharmaceutical, Wales. All other chemicals used for the study were of analytical grade. All diagnostic kits were procured from Lab-care diagnostics Ltd., India.

Polyherb

Ruzu herbal bitters (RHB), a product of Ruzu Natural Health Products and Services, and Asheitu Adams bitters (AAB), a product of Asheitu Adams Enterprises were purchased from a pharmaceutical store within Ondo State and Boundary-Ajegunle, Lagos State respectively. Compositions of the two polyherbs are presented in Table 1 below:

Table 1: Major Constituents of RHB and AAB Polyherbal Medicines

RHB Constituents	Qty (%)	AAB Constituents	Qty (%)
Cluster pear/bush banana stem (<i>Uvaria chamae</i>)	20.0	<i>Pterygota macrocarpa</i>	30.0
African crocus root (<i>Curculigo pilosa</i>)	40.0	Bitter apple/cucumber (<i>Citrullus colocynthis</i>)	4.2
Bitter apple bark (<i>Citrullus colocynthis</i>)	40.0	<i>Strophanthus species</i>	7.0
		<i>Nauclea latifolia</i>	13.0

Source: Ruzu Natural Health Products and Services; Asheitu Adams Enterprises

RHB polyherbal mixture is approved for use in Nigeria by the National Agency for Food and Drug Administration and Control (NAFDAC) (Ruzu Natural Health Products and Services). Both RHB and AAB polyherbal mixtures were lyophilized and reconstituted to achieve the same unit (mg) with metformin, the standard drug. This is to enable the usage of same concentration/dosage unit (i.e. mg/kg) for the polyherbal mixtures and the standard drug, metformin.

Experimental Animals

Male Albino rats obtained from the Animal Facility, University of Ibadan, Oyo State, weighing averagely 96.37±11.42 g were used for this study after two weeks of acclimatization period. They were maintained in a temperature- and light-controlled environment (23 ± 2; 12 h light/12 h dark cycle) and were given free access to food and water in line with National Institutes of Health (2011) guide for the care and use of Laboratory animals.

Induction of Type 1 Diabetes Mellitus (T1DM)

The method of Sharma *et al.* (2012) with slight modifications was used to induce type 1 diabetes in Albino rats. Successive triple intraperitoneal (i.p.) injection of streptozotocin dissolved in 0.1 M citrated buffer (pH 4.5) at (40 mg/kg, 45 mg/kg and 35 mg/kg of body weight) were given to the animals at three days interval after overnight fasting. The induction of type 1 diabetes mellitus was confirmed by the determination of high fasting blood glucose level with polydipsia (increased thirst) and polyuria (increased urination). Rats with fasting blood glucose level >200 mg/dl were selected for the experiment.

Experimental Design

Experimental animals (Albino rats) were divided into seven groups of five animals each.

Group 1: Animals not induced with diabetes and orally administered distilled water (Normal control)

Group 2: Animals induced with diabetes and orally administered distilled water (Diabetic control)

Group 3: Animals induced with diabetes and orally administered 15 mg/kg metformin (Standard control)

Group 4: Animals induced with diabetes and orally administered 15 mg/kg RHB

Group 5: Animals induced with diabetes and orally administered 30 mg/kg RHB

Group 6: Animals induced with diabetes and orally administered 15 mg/kg AAB

Group 7: Animals induced with diabetes and orally administered 30 mg/kg AAB

Fasting blood glucose and weight of animals were monitored weekly for 28 days. Fasting blood sugar level was measured using drop of blood from tail vein of animals on ACCU CHEK Strip fixed with Glucometer (Mannheim, Germany) using the method of Ghasemi *et al.* (2014). Pancreas and intestinal crypt were excised from animals for pancreatic duodenal homeobox 1 (PDX-1), insulin, glucagon-like peptide 1 (GLP-1) and glucose transporter 2 (GLUT-2) gene expressions.

RNA Isolation with RNA Snap Kit

RNA isolation for gene expression was carried out using Timao (1996) method with some modifications. Target tissues were excised and homogenized in Eppendorf tubes containing RNA isolation reagent (RNA Snap kits). Tissue homogenates were centrifuged for 30 min at 17000 rpm and the supernatant (RNA phase) was carefully aspirated into new sterile labeled tubes. Sodium acetate (3 M) and iso-amyl alcohol were added to precipitate RNA. The samples were then incubated at -7 °C for an hour, centrifuged for 30 min at 17000 rpm and 70% ethanol added to recover pelleted RNA. Further centrifugation at 17000 rpm for 5 min was done and the tubes containing the samples (RNA) uncapped for ethanol to evaporate. The RNA was then :

Table 2: Gene Sequences of Primers Used

Gene	Forward 5'-3'	Reverse 5'-3'
B-actin	ACACTTCTACAATGAGCTGCG	ACCAGAGGCATACAGGACAAC
Insulin	GAGGCTCTGTACCGTGGTGTG	ACCTCCAGTGCCAAGGTTT
GLP-1	ACCGTTTACATCGTGGCTGG	CCCTGTGAATGGCGTTCTC
GLUT-2	CCTGGCGTCTTCAGAGAGTG	ACCGAGGAAGGAATCGGTTT
PDX-1	TCCGCTAGCCACCATGAATAGTGAGGAGCA	TTCGAAGCTTAAATCACCGGGGTTTCTGCGGT

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics, Version 21 Software. Data were statistically analyzed using Two-Way Analysis of Variance (ANOVA) and statistical differences were separated at $p < 0.05$. Means were separated using Duncan's New Multiple Range Test.

air-dried and re-suspended into sterile tube containing nuclease-free water and total RNA concentration was determined by UV absorbance spectrophotometry (JENWAY 6305). Thereafter, all samples were diluted to the same concentration for RNA (mRNA) quantification.

cDNA Synthesis

Successfully isolated and quantified RNA was immediately converted to complementary DNA (cDNA). A 2 μ l of super-enzyme (Reverse Transcriptase) containing random oligo primer, dNTP's, reverse transcriptase, reverse transcriptase buffer and nuclease-free water was added to 20 μ l of isolated RNA for cDNA synthesis. The sample was incubated at room temperature in a thermocycler running for 4 h at 65 °C/12 h.

Polymerase Chain Reaction (PCR) Amplifications

The reaction system was first optimized before amplification process began. Template (cDNA), nuclease-free water, forward and reverse primers, and master mix were used to carry out polymerase chain reaction (PCR) using multigen optimax PCR machine for complete enzymatic reaction. Amplification conditions were; 94 °C pre-denaturation for 5 min, 94 °C for 30 sec, annealing 55 °C (Tm) for 30 sec and extension 72 °C for 30 sec and then 5 min at 72 °C for further and final extension in 30 cycles. Thereafter, amplicons were electrophoresed in 0.5% agarose gel. The primers for the genes analyzed are presented in Table 2 below

RESULTS AND DISCUSSION

In this study, anti-diabetic property of RHB and AAB polyherbal medicines was investigated. Results indicated that blood glucose concentrations of all treatment groups differ significantly ($p < 0.05$) from control group at week 0, 1 and 2. However, there is

no significant difference ($p > 0.05$) in blood glucose concentrations between polyherbs groups (RHB- and AAB-treated groups) and control group at week 3

and/or week 4, except 15 mg/kg AAB-treated group that differs significantly ($p < 0.05$) from control group at week 3 as shown in Table 3 below.

Table 3: Blood Glucose Levels of Polyherbs-Treated Animals

Wk/Grp	Control	Diab Control	Metformin	RHB(15 mg/kg) mg/dl	RHB(30 mg/kg)	AAB(15 mg/kg)	AAB(30 mg/kg)
0	72.00±5.25 ^a	330.20±26.42 ^b	342.40±21.89 ^b	335.80±20.60 ^b	340.75±33.87 ^b	323.25±24.36 ^b	325.00±45.49 ^b
1	74.80±3.97 ^a	404.20±52.47 ^b	374.20±38.03 ^b	351.00±44.13 ^b	380.75±23.32 ^b	346.75±73.10 ^b	399.25±29.64 ^b
2	68.40±2.66 ^a	240.00±44.83 ^b	385.25±38.45 ^b	338.00±48.26 ^b	235.50±79.02 ^b	328.50±84.89 ^b	283.67±45.73 ^b
3	72.75±4.27 ^a	196.60±55.17 ^{ab}	195.33±57.71 ^{ab}	189.40±25.87 ^{ab}	193.33±62.83 ^{ab}	247.00±17.62 ^b	213.33±57.85 ^{ab}
4	71.75±2.25 ^a	249.80±26.32 ^{ab}	291.50±10.50 ^b	281.75±76.70 ^{ab}	197.67±85.86 ^{ab}	244.00±33.00 ^{ab}	93.33±21.65 ^{ab}

Note: Comparison is strictly across group. Mean ± SEM values with the same alphabets are not significantly different ($P > 0.05$) from others but values with different alphabets do. The means were only separated between groups for each period (week 0-4) using Duncan's New Multiple Range Test but no comparison was made across weeks. Key: Wk – Week; Grp – Group; Diab – Diabetic; RHB – Ruzu herbal bitters; AAB – Asheitu Adams bitters

The observed results imply that the polyherbs worked therapeutically in streptozotocin-induced diabetic rats, most especially at 30 mg/kg dosage to bring animals' final blood glucose levels near that of control animals after treatments. Polyherbal medicines, RHB and AAB exerted their hypoglycemic effect by decreasing blood glucose concentrations of treated animals from 340.75±33.87 mg/dl to 197.67±85.86 mg/dl and 325.00±45.49 mg/dl to 93.33±21.65 mg/dl respectively thus, bringing it closer to 71.75±2.25 mg/dl of the control animals. These results are in agreement with the findings of Emaleku (2019), who reported significant decrease in blood glucose of diabetic rats administered Asheitu Adams bitters. Although, the observed reduction in blood glucose level in this study is not significant ($p > 0.05$) however, results still showed that both RHB and AAB are not just supplements but are natural bitters that could help treat or manage diabetes due to their blood glucose-reducing (hypoglycemic) effect attributed to their constituents (Ogunlana *et al.*, 2018; Arifin, 2019). These polyherbs could exert their hypoglycemic effect by acting like antioxidant to mop up free radicals and reactive oxygen species and consequently reversing lipid peroxidation and cellular damage to pancreas, an insulin-producing organ (Ogunlana *et al.*, 2018). Streptozotocin (STZ), a β -cytotoxin, induces 'chemical diabetes' in animals

(Akbarzadeh *et al.*, 2007) by selectively damaging the insulin-secreting β -cells of the pancreas (Ghasemi *et al.*, 2014) through fragmentation of DNA of β -cells that stimulates poly (adenosine dinucleotide phosphate (ADP)-ribose) and depletes NAD^+ in the pancreas. This destruction of the β -cells is evidenced by clinical symptoms of hyperglycemia and hypoinsulinaemia (Rodrigues and McNeill, 1986; Siddiqui *et al.*, 2013). Therefore, the reversal of high blood glucose levels induced in experimental animals *via* STZ administration by RHB and AAB treatments at 30 mg/kg body weight suggests the anti-diabetic potential of these polyherbs. It is thus logical to infer that these polyherbs counteracted the effects of STZ by acting as antioxidant to mediate the observed hypoglycemic effect (reduction in blood glucose levels) in treated animals as shown in Table 3. It could also be that the medicinal herbs used to formulate these polyherbal medicines contain two or more natural constituents/compounds that acted synergistically to induce the observed hypoglycemic effect i.e. decrease in blood glucose levels of treated animals (Roy *et al.*, 2005; Sy *et al.*, 2005). Furthermore, results from this study showed that AAB and RHB regulate PDX-1, insulin, GLP-1 and GLUT-2 genes expression in accordance with the level of glucose in the blood. The obtained results are presented in Figures 1, 2, 3 and 4 respectively.

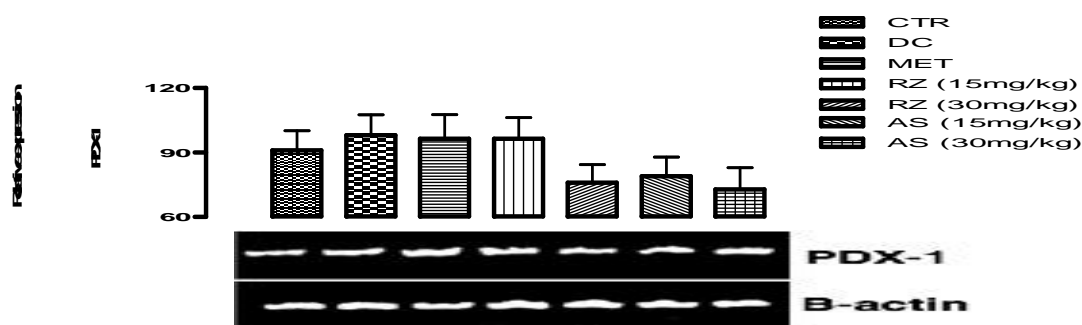


Figure 1: Pancreatic duodenal homoeobox 1 gene expression of polyherbs-treated animals

Figure 1 results showed down regulation in PDX-1 gene in glucose concentration-dependent manner in animals administered 30 mg/kg RHB and AAB (15 mg/kg and 30 mg/kg) in comparison with other groups. This down regulation is blood glucose dependent and also polyherbal concentration dependent as found out in this study. During pancreas development, PDX-1 is expressed in all pancreatic precursor cells and its expression is restricted majorly to beta cells in adult (Kiba, 2018). Its expression in this study after STZ has been used to induce type 1 diabetes in the animals by destroying the pancreas signals the polyherbs ability to regenerate already destroyed pancreas while its down regulation in glucose concentration-dependent manner (most especially by AAB) further indicates the polyherbs anti-diabetic potential. Reduction in blood glucose concentration of polyherbs-treated animals is opined to be the reason for the observed down-regulation in PDX-1 gene, and blood glucose reduction (hypoglycemia) could only have been possible because polyherbs made treated animals' pancreas to regenerate and/or recuperate from the devastating effects of STZ to become sensitive to blood glucose concentrations. Due to the sensitivity of PDX-1 to glucose concentrations as observed in Figure 1, the observed down regulation in PDX-1 genes cannot be a sign of pathogenesis of β -cell failure and/or type 2 diabetes suggested by Weir *et al.* (1997). The observed decrease in blood glucose concentrations (hypoglycemia) of polyherbs-treated

groups which is opposed to hyperglycemia typical of β -cell failure and type 2 diabetes, further proves that PDX-1 gene down regulation observed in this study is not due to β -cell failure/ β -cell dysfunction and/or type 2 diabetes (Fujimoto and Polonsky, 2009) but rather for regulatory purpose (low glucose concentration condition). It is therefore a good omen that RHB and AAB did not cause over expression of PDX-1 gene in animals because, recent findings revealed that aberrant over-expression or up regulation of PDX-1 gene resulted into human pancreatic, gastric, colon, breast, prostate, colorectal and kidney cancers as well as paediatric solid pseudopapillary tumor (Zhou and Brunicardi, 2011).

Pancreatic duodenal homeobox-1 is a homeodomain-containing transcription factor essential for pancreatic development, beta-cell differentiation and the maintenance of mature beta cell function (Kim and Hebrok, 2001; Fujimoto and Polonsky, 2009; Kiba, 2018). It performs all the above-stated functions by regulating the expression of key endocrine beta-cell-specific genes (Jonsson *et al.*, 1994) such as insulin, glucokinase, islet amyloid polypeptide and the glucose transporter type 2 (Zhou and Brunicardi, 2011). Zhou and Brunicardi (2011) found PDX-1 to be directly controlling insulin gene expression, hence similar trend was observed in the insulin gene expression shown in Figure 2 below.

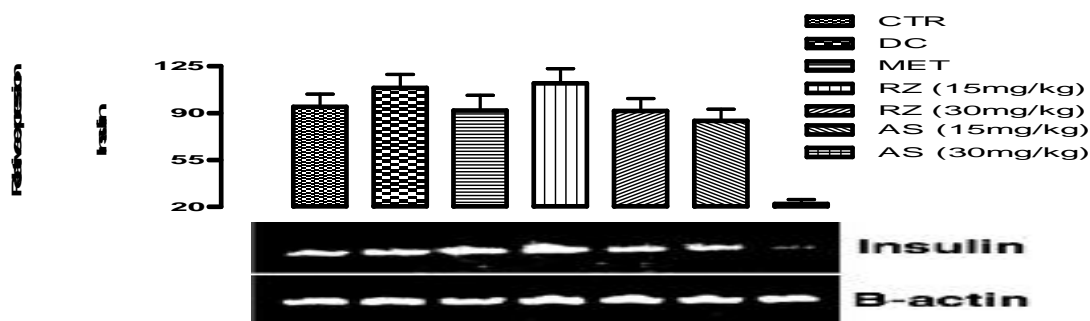


Figure 2: Insulin gene expression of polyherbs-treated animals

Figure 2 results showed that RHB (30 mg/kg) and AAB (15 mg/kg and 30 mg/kg) down regulate insulin gene in glucose concentration-dependent manner. The ability of polyherbs to stimulate and regulate insulin gene expression in accordance with blood glucose levels is noteworthy. It indicates restoration of the functional role(s) of β -cell of the pancreas. In a normal pancreatic β -cells, increase in blood glucose concentration triggers insulin secretion but inhibits its release when the concentration is low (Thorens and Mueckler, 2010). RHB and AAB are thought to have restored normalcy to the β -cell of the pancreas that benefitted insulin hormone, which enabled it to regulate insulin gene expression in glucose concentration-dependent manner.

According to Marty *et al.* (2007), glucose is the principal regulator of insulin secretion in intact pancreatic β -cells, and modulates the expression of insulin gene in β cells of the endocrine pancreas. And as observed in this study, insulin gene expression is high when blood glucose level is high (See 15 mg/kg RHB and 15 mg/kg AAB in Figure 2 and Table 3) but low when blood glucose level is low as seen in animals administered 30 mg/kg RHB and 30 mg/kg AAB in Figure 2 and Table 3.

The up-regulation of GLP-1 gene in glucose concentration-dependent manner by RHB and AAB further demonstrates the anti-diabetic potential of these polyherbal medicines.

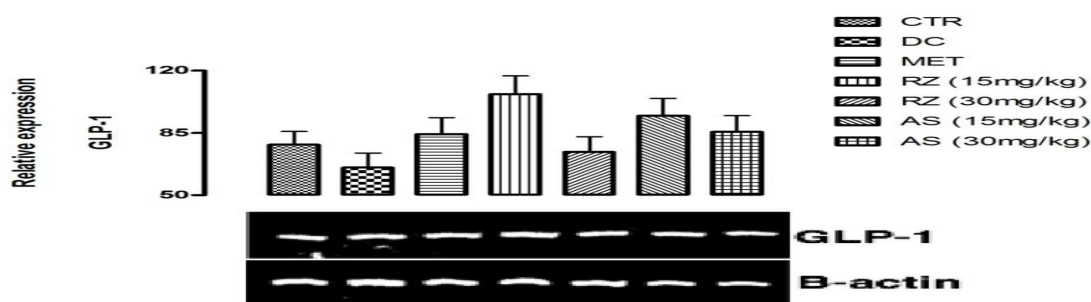


Figure 3: Glucagon-like peptide 1 gene expression of polyherbs-treated animals

Results from Figure 3 showed up regulation in GLP-1 gene in glucose concentration-dependent manner for RHB- and AAB-treated groups when compared with other groups. GLP-1, alongside glucose-dependent insulinotropic peptide (GIP) is the only known incretin with the ability to decrease blood sugar levels in a glucose-dependent manner by enhancing the secretion of insulin (Wikipedia, 2017). Therefore, the observed up regulation (high) and down regulation (low) of GLP-1 when blood glucose level is high and low respectively suggest the role of GLP-1 in augmenting/complementing insulin action. Dale *et al.*

(2009) reported that GLP-1 seems to be responsible for nearly half of the total postprandial insulin secretion and has also been shown to promote insulin gene transcription and biosynthesis. The observed results in Figure 3 suggest the polyherbs' ability to act like GLP-1 agonist that stimulates GLP-1 gene expression and activity, which in turn stimulates insulin secretion when blood glucose is high (Claudia *et al.*, 2010) but contrariwise (a likely antagonist) when blood glucose is low.

It is good to know that GLUT-2 gene expression also demonstrated the anti-diabetic tendency of the polyherbs in glucose concentration-dependent manner.

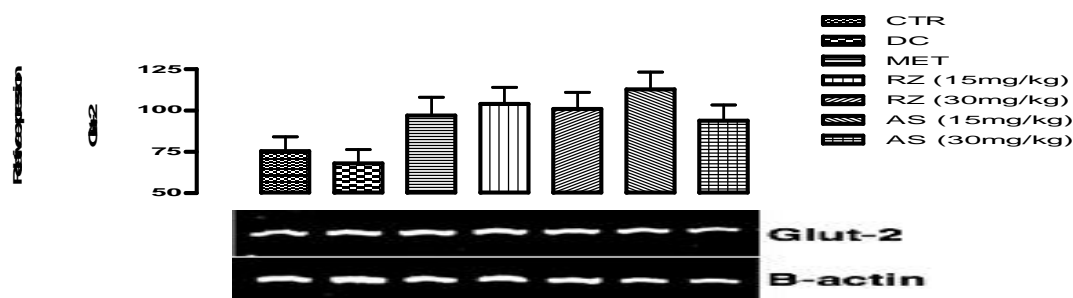


Figure 4: Glucose transporter 2 gene expression of polyherbs-treated animals

Figure 4 showed that RHB and AAB up regulate GLUT-2 gene expressions in glucose concentration-dependent manner in comparison with the control group. This up-regulation is necessary in order to facilitate the entrance of glucose from the blood into cells and peripheral tissues, and enhanced its clearance from the blood. GLUT-2 activity is essential for both glucose secretion and keeping intracellular glucose-6-phosphate (Glu-6-P) concentration low and could be needed to allow normal regulation for insulin secretion by glucose in β cells (Petchi *et al.*, 2014) to facilitate glucose clearance and usage thus reducing its concentration in the blood. According to Tobin *et al.* (2008), insulin has been reported to induce the internalization of apically expressed GLUT-2 in order to enhance glucose absorption, a process that is impaired in insulin resistance or insulin destruction.

CONCLUSION

Based on findings from this study, it is thus inferred that RHB and AAB polyherbal medicines have anti-diabetic potential and could be forms of alternative therapy to conventional or orthodox drugs in the treatment of diabetes.

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