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AN ANTI-DIABETIC POLY-HERBAL MEDICINE PREPARED FROM EXTRACTS OF *ANNONA STENOPHYLLA*, *CITRUS LIMON* AND *ZINGIBER OFFICINALE*

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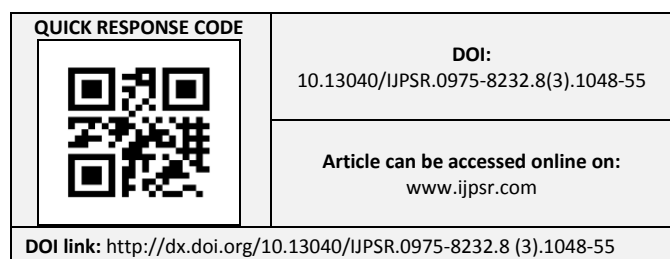
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ABSTRACT: Background: Diabetes poses a burden on the limited health care delivery system. Current pharmacological anti-diabetic agents cause undesirable side effects and are very expensive. Herbal remedies offer the potential for alternative treatment strategies. This study was therefore conducted in an effort to formulate a poly herbal medicine for management of diabetes. **Methods:** A stenophylla root bark was extracted with aqueous ethanol (70%) and quantitative analysis for total phenolic content, total alkaloids and flavonoids was carried out on the dried extract. The hypoglycaemic effects of aqueous *A. stenophylla* root bark extract, powdered *Zingiber officinale* and powdered *Citrus Limon* peels was determined on alloxan-induced diabetic rats singly and in combinations over 14 days. Glibenclamide administered at 0.2 mg/kg b. wt was used as the positive control. **Results:** All the plants decreased plasma glucose levels in a dose-dependent manner. The glucose levels of diabetic rats treated with all the plants combined showed the greatest reduction (59.2%). *A. stenophylla* and *Z. officinale* showed a glucose level reduction of 47.7%, *A. stenophylla* and *C. limon* (42.9%) whilst *Z. officinale* and *C. limon* (42.7%). The reductions in glucose levels were comparable to glibenclamide positive control which showed a 61% reduction. **Conclusion:** Combining the three plants (*A. stenophylla*, *Z. officinale* and *C. limon*) led to the greatest reduction in blood glucose levels of alloxan induced diabetic rats. The formulation demonstrates more potential in combinational herbal medicines therapy in diabetes management.

INTRODUCTION: Diabetes mellitus (DM) is a metabolic disorder indicated by the presence of hyperglycemia due to defective insulin secretion, defective insulin action or both. The chronic hyperglycemia of diabetes is associated with relatively specific long-term micro-vascular complications affecting the eyes, kidneys and nerves, as well as an increased risk for cardiovascular disease (CVD).

The diagnostic criteria for diabetes are based on thresholds of glycemia (Fasting plasma glucose > 7.0 mmol/l, Fasting = no caloric intake for at least 8 hours or Random plasma glucose > 11.1 mmol/l, Random = any time of the day, without regard to the interval since the last meal)¹. Diabetes mellitus can either be immune-mediated (Type 1 diabetes), insulin resistance (Type 2), gestational or others (environment, genetic defects, infections, and certain drugs)². The prevalence of DM is high and rising worldwide mainly due to the global increases in obesity and a detrimental lifestyles. The increase in diabetic cases gives rise to colossal financial burdens and medical care policy³. The management of diabetes is still a major challenge. The main problems encountered in Sub-Saharan Africa include diagnosis; medical care; insulin and



other drug supplies; monitoring; infections associated with diabetes, especially the diabetic foot; dietary advice; diabetes education; and the low priority placed on non-communicable diseases⁴. Many synthetic hypoglycaemic agents that are currently available are either too expensive or have undesirable side effects on chronic use⁵.

Folklore anti-diabetic herbal medicines can be used to prepare new oral anti-diabetes drugs, which can counter the average cost of the current treatment options and low availability of the existing medicines for many rural populations. World Health Organization (WHO) has defined herbal medicines as finished, labelled medicinal products that contain active ingredients, aerial or underground parts of the plant or other plant material or combinations⁶. Herbal medicines have been in use in all cultures throughout history. Herbal remedies or medicines contain portions of plants or non-purified plant extracts comprising of a number of constituents, which usually work together synergistically⁷. Medicinally, the value of these plants lies in certain chemical substances that yield a definite physiological action when administered to humans. The most essential of these bioactive constituents found in plants are alkaloids, tannins, flavonoids and phenolic compounds⁸.

Herbal medicines are considered to be very cost effective and they are readily available. Herbal medicines are generally regarded as being harmless since they are of natural origin (come from natural sources). However, this notion is not true as there are several reported cases of adverse reactions caused by herbal drugs mentioned in published literature⁹. Extracts from the Annonaceae family have been reported to have therapeutic effects in the treatment of diabetes. *Annona stenophylla* has been used as a remedy for hyperglycemia by communities and traditional medical practitioners in Zimbabwe for a very long time¹⁰. Studies have demonstrated hypoglycaemic effects, antioxidant and antimicrobial effects of the plant extracts^{11, 12}. *Zingiber officinale* commonly known as ginger is well known as a remedy for travel sickness, nausea and indigestion and is used for wind, colic, irritable bowel, loss of appetite, chills, cold, flu, poor circulation, menstrual cramps, dyspepsia (bloating, heartburn, and flatulence), indigestion and gastrointestinal problems such as gas and stomach

cramps. Ginger has also been shown to reduce elevated blood sugar levels.

C. limon contains a class of natural substance called polyphenols that are available in large quantities in the lemon peel¹³. When fed to obese experimental animals, lemon polyphenols significantly curbed different features of obesity. They decreased high blood glucose levels, prevented weight gain, development of hyperlipidemia, fat accumulation and insulin resistance.

Polyphenols have also been implicated in spontaneous regeneration of β -cells for the secretion of insulin. *C. limon* peels have high levels of polyphenols hence they exhibit anti-diabetic effects¹⁴. The current study confirmed the hypoglycaemic effects of each plant and further determined the combined hypoglycaemic effect of the plants to find out the extent to which the three plants influence each other.

MATERIALS AND METHODS:

Plant collection: *Annona stenophylla* Engl. (Annonaceae) (45570) roots were collected from Mazowe in October 2015. *Zingiber officinale* and *Citrus limon* were purchased at a local market in Harare, Zimbabwe. The plants were authenticated by a botanist from the National Herbarium and Botanic Gardens of Zimbabwe.

Preparation of plant material: Air dried root bark from *A. stenophylla* was first ground into a coarse powder using a mortar and pestle. The coarse powder was further ground into fine powder using an electric grinder. 500g of fine powdered root bark was mixed with redistilled ethanol (70% v/v). The mixture was kept in conical flask away from light for 48 hours with occasional stirring. The mixture was filtered and the filtrate evaporated to dryness using a rotary evaporator, freeze dried in a Heto FD3 freeze dryer. The obtained powdered extract was kept in a desiccator until time of use. Percentage yield was calculated using the equation below:

$$\text{Percentage yield} = \frac{\text{Final dry plant extract weight}}{\text{Dry starting plant material weight}} \times 100$$

Fresh *Zingiber officinale* (ginger) rhizomes were finely cut into small pieces to facilitate drying.

Dried rhizomes were ground into a fine powder using a grinder. Fresh *C. limon* (lemon) peels were separated and cut into small pieces with a knife. These were dried and ground into a fine powder. Powders were kept in khaki papers in cupboards away from light, till use.

Animals: Male Sprague–Dawley rats (250-300g) were obtained from the University of Zimbabwe animal house and kept in cages under standard laboratory conditions. The rats were kept with free access to water and a certified pelleted rodent supplemented with high fat. Permission to undertake the research was sought from the Joint Parirenyatwa Hospital and College of Health Sciences Research Ethics committee.

Induction of diabetes: Diabetes in rats was induced by an intra-peritoneal injection with alloxan monohydrate (120 mg/kg b. wt) dissolved in sterile normal saline solution. After 7 days, rats with moderate diabetes (blood glucose levels of 6.0 mmol/ L-11.0 mmol/L), were used for the experiment.

Hypoglycaemic activity tests: Rats were randomly divided for each plant sample tested into three groups of 3 rats per group. Samples were suspended in distilled water and administered by oral gavage once daily. Blood from the treated rats was collected from the tail vein and fasting blood glucose content was measured using a glucometer at 0, 1, 3 and 7 days. Three different concentrations were constituted and tested for each plant sample.

FIG. 1: THE RATS WERE DIVIDED INTO THE FOLLOWING GROUPS

Group	<i>A. stenophylla</i>	<i>Z. officinale</i>	<i>C. limon</i>
Group 1	(A1) -150 mg/kg	(G1) -250 mg/kg	(L1) -100 mg/kg
Group 2	(A2) -300 mg/kg	(G2) -500 mg/kg	(L2) -200 mg/kg
Group 3	(A3) -900 mg/kg	(G3) -1000 mg/kg	(L3) -300 mg/kg

Effect of plant extract combinations on fasting blood glucose levels: Rats were randomly divided into six groups of 6 rats per group. Different plant samples combinations were prepared and administered by oral gavage once daily. Blood from the treated rats was collected from the tail vein and fasting blood glucose content measured using a glucometer at 0, 1, 3, 7 and 14 days. The rats were divided into the following groups:

Group 1: Diabetic control (distilled water)

Group 2: Glibenclamide (GLIB) 0.2mg/kg b.wt

Group 3: *A. stenophylla*, *Z. officinale* and *C. limon* (AGL)

Group 4: *A. stenophylla* and *Z. officinale* (AG)

Group 5: *A. stenophylla* and *C. limon* (AL)

Group 6: *Z. officinale* and *C. limon* (GL)

Total phenolic compounds in *A. stenophylla* root extract: Extract (100mg) of the sample was weighed accurately and dissolved in 100 ml of double distilled water (DDW). Solution (1ml) was transferred to a test tube and 0.5 ml of 2N Folin Ciocalteu reagent together with 1.5 ml 20% of Na₂CO₃ solution was added. The volume was made up to 8 ml with DDW followed by vigorous shaking. The solution was finally allowed to stand for 2 hours after which the absorbance was read at 765 nm. These data were used to estimate the total phenolic content using a standard calibration curve¹⁵. Total Phenolic Content was calculated from the following equation:

$$T=C \times V/M$$

Where, T= Total Phenolic Content (mg/g) of extract as GAE, C= Concentration of GA established from the calibration curve in (mg/ml), V= Volume of the extract solution in ml and M= weight of extract in g.

Determination of flavonoids in *A. stenophylla* root extract: Ten grams of plant sample was repeatedly extracted with 100ml of 80% aqueous methanol at room temperature. The mixture was filtered through a filter paper into a pre-weighed 250ml beaker. The filtrate was transferred into a water bath and allowed to evaporate to dryness and weighed. The percentage flavonoid was calculated by difference¹⁶.

Determination of total alkaloids in *A. stenophylla* root extract: Five grams of the plant sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added, covered and allowed to stand for 4 h. The mixture was filtered and the extract concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added in a drop wise manner until the precipitation was complete. After the precipitate settled, it was collected and washed with dilute ammonium

hydroxide and filtered. The residue was the alkaloid, which was dried and weighed¹⁷.

Statistical Analysis: Data was entered into Microsoft office excel 2013 and analyzed using Microsoft office excel 2013 and graph pad prism version 5.1. Results were expressed as mean \pm SD. Differences between groups were analysed using the Student's t-test and among groups using Anova significance set at $p < 0.05$. Bonferroni post hoc test was done for multiple comparisons against the control.

RESULTS:

A. stenophylla plant extraction: The ethanolic plant extraction gave a coffee brown extract (3.5 %) yield.

Total phenolic content of A. stenophylla root extract: The absorbance of the plant extract was 2.67 nm which translated to a total phenolic content of 17.37 mg/g of gallic acid equivalence.

Determination of total flavonoids: The amount of total flavonoids from the 10 g of starting plant sample material of *A. stenophylla* was 1.04 g (10.4%).

Determination of total alkaloids: The amount of total alkaloids from the 5 g starting plant sample material of *A. stenophylla* was 1.28g (2.56%).

Effect of different dosages of A. Stenophylla on plasma glucose levels:

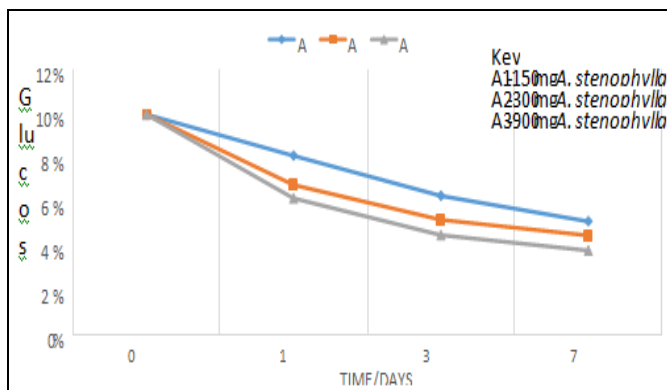


FIG. 1: EFFECT OF A. STENOPHYLLA ON PLASMA GLUCOSE LEVELS

Annona stenophylla plant extract showed a dose dependent decrease in plasma glucose levels of the alloxan diabetic rats. 150mg of *A. stenophylla* decreased plasma glucose levels by 48.4% after a period of 7 days. 300mg of the plant extract

decreased plasma glucose levels by 54.8% whilst the 900mg concentration showed a decrease of 61.6%. The differences shown amongst the different concentrations were however not significant when compared on the different test days ($p > 0.05$).

Effect of different dosages of Z. officinale on plasma glucose level:

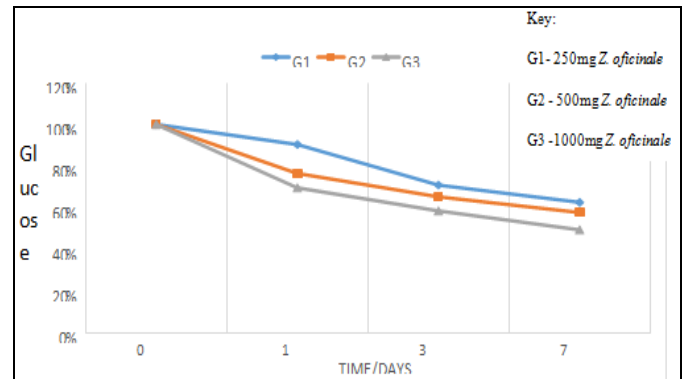


FIG. 2: EFFECT OF DIFFERENT DOSAGES OF Z. OFFICINALE ON PLASMA GLUCOSE LEVELS

Z. officinale powdered sample showed a dose dependent decrease in plasma glucose levels over time. 250mg of powder produced a 37.5% decrease in plasma glucose level, 500mg and 1000mg of powdered plant material gave a 42.3% and 50.7% decrease respectively. Differences shown amongst the different concentrations were not significant when compared on the different test days ($p > 0.05$).

Effect of different dosages of C. limon on plasma glucose levels:

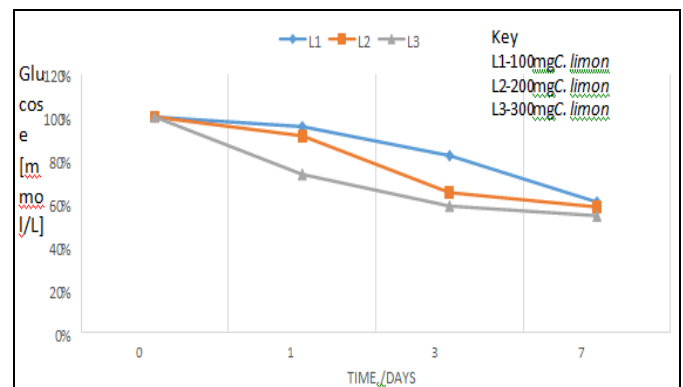


FIG. 3: EFFECT OF DIFFERENT DOSAGES OF C. LIMON ON PLASMA GLUCOSE LEVELS

The most effective dosage of *C. limon* was found to be 300mg, at this dose plasma glucose levels were reduced by 45.8%. 100mg and 200mg powdered plant reduced plasma glucose levels by 39.4% and 45.8% respectively.

Effect of plant extract combinations on fasting blood glucose levels:

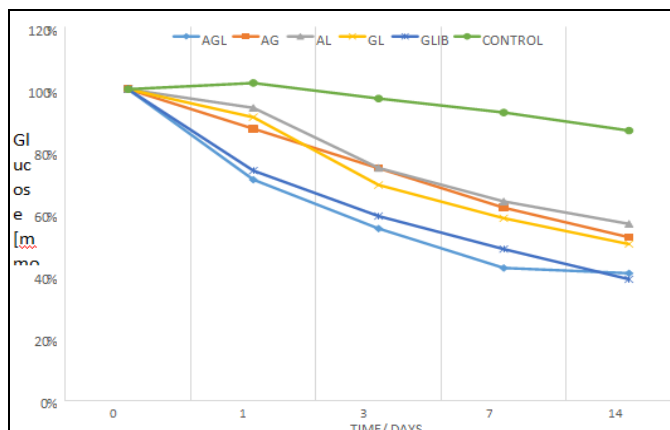


FIG. 4: EFFECT OF PLANT EXTRACT COMBINATIONS ON FASTING BLOOD GLUCOSE LEVELS.

Key: AGL- *A. stenophylla*, *Z. officinale* and *C. limon*; AG- *A. stenophylla* and *Z. officinale*; AL- *A. stenophylla* and *C. limon*; GL- *Z. officinale* and *C. limon*; GLIB- Glibenclamide; Control.

Combination of all the plant samples showed the most decrease in plasma glucose levels (59.2%). The second most effective combination was that of ginger and *C. limon* (47.7%). Percentage decrease in blood glucose levels for the combination of *C. limon* and *Z. officinale* (GL) was almost exactly the same as that of *Z. officinale* (G1) on day 1 and day 3. The anti-diabetic effects of *Citrus limon* only become significant 3 days after the first dose is taken.

DISCUSSION: In the present study the percentage yield for the ethanolic extract of *Annona stenophylla* (3.46%) was lower than the 4.62% percent yield reported in another study 18. This difference might have been due to disparities in the extraction procedures. Quantitative analysis of the *A. stenophylla* root extract showed the total phenolic content (mg/g) to be 17.37mg/g of plant sample, the percentage flavonoid to be 10.4% and the percentage alkaloid to be 2.56%. *A. stenophylla* root extract has been previously reported not to contain any flavonoids or alkaloids and the Total Phenolic Content was reported to be much lower than the reported value 18. Differences in the extraction procedure, the seasons the plant was collected in and the method used to determine the phytoconstituents present, could be the reason for the conflicting data. Seasons and the geographical location have been shown to cause variations in the amount of poly-phenols found in plants. The concentration of active principles is high in full

bloom period, it is the best period for collection for high percentage yields 19. The amounts of phytochemicals and antioxidant activities are significantly affected by cultivar, plant part, and season 20.

In the current study, *Annona stenophylla* root extract showed a dose dependent decrease in plasma glucose levels. The ethanolic root bark extract of *A. stenophylla* has been reported to inhibit α -amylase and α -glucosidase activities, it was also shown to decrease plasma glucose levels by as much as 60% in an oral glucose tolerance test within an 8hour period 11. In another study, 100mg/kg of boiled root extract showed a 60% decrease in serum glucose level after 4 hours of extract administration 21. All these findings were consistent with the findings of the current study. *Z. officinale* is being used as diet-based therapy due to its wide range of therapeutic potential against management of type 2 diabetes mellitus and diabetic complications. It has been shown to act by directly interacting with different molecular and cellular pathways that provoke the pathogenesis of type 2 diabetes mellitus. It is likely that 6-gingerol, the predominant pungent compound in ginger, is responsible for its benefits in diabetes 22. In the present study different doses of *Z. officinale* powder was evaluated for their hypoglycaemic properties.

A dose dependent decrease in plasma glucose levels was observed. A review by Daily and colleagues found that studies administering 1,600-3,000 mg of ginger powder per day for 8-12 weeks lowered fasting serum glucose levels and HbA1c levels in patients with Type 2 diabetes 23. A study conducted in Saudi Arabia showed that raw ginger was significantly effective in lowering serum glucose, cholesterol and triacylglycerol in diabetic rats. Ginger oil was shown to give the best anti-diabetic activity results followed by ginger extract and finally ginger powder 24.

In another study ginger supplementation significantly reduced the levels of fasting blood sugar, hemoglobin A1c, apolipoprotein B, apolipoprotein B / apolipoprotein A-I and Malonyldialdehyde (MDA), and increased the level of Apo A-I in type 2 diabetes mellitus patients 25. Another group of researchers investigated the anti-diabetic effects of *Z. officinale* evaluating the

carbohydrate metabolism in STZ-induced diabetic rats using three different doses (100, 300, 500 mg/kg body weight) of *Z. officinale* for 30 days. *Z. officinale* exhibited its hypoglycaemic effects in a dose-dependent manner by ameliorating the body, liver and kidney weights along with increasing the activities of hepatic glycolytic enzymes in STZ-induced diabetic rats as compared to the non-treated STZ-induced diabetic rats²⁶.

All these findings support the results obtained in the current study. It has been reported in patients with coronary artery disease that 4g of ginger powder for 3 months neither affected the level of blood glucose nor lipid²⁷, these findings contradict the findings of the present study. Such discrepancy of results may be attributed to the variation in chemical composition of the administered ginger extracts, the preparation method, and product origin, duration of storage or disparity in patient's response. *C. limon* showed glucose lowering effects in this study. *Citrus limon* peels are rich in flavonoids and research has shown significant antioxidant, anti-inflammatory, anti-diabetic, cardio-protective and hepatoprotective effects of flavonoids²⁸⁻³³. Flavonoids found in *Citrus limon* ([L.] Burm.f.) include hesperidin, quercitrin, eriocitrin, didymin and naringin³⁴. Naringin and its aglyconenaringenin are two flavonoid compounds with promising anti-diabetic effects.

In recent studies conducted by Mahmoud AM and colleagues they demonstrated the ability of naringin to improve blood glucose levels through its ability to improve insulin sensitivity and secretion, attenuate inflammation and oxidative stress, enhance peripheral glucose uptake, decrease intestinal glucose and cholesterol absorption and suppress hepatic glucose production. In addition, naringin showed marked modulatory effect on adiponectin and resistin levels, adipose tissue peroxisome proliferator activated receptor gamma (PPAR γ) expression, and muscle glucose transporter (GLUT)-4 translocation^{32, 35, 36}. *Citrus limon* have been shown to have antioxidants that help to reduce free radical damage in the body. Many people with diabetes mellitus have low levels of vitamin C in their system and *C. limon* could possibly increase the levels. Some recent studies have shown that vitamin C may also help decrease triglyceride, cholesterol, inflammation and even

HbA1c levels³⁷. The combined plants decreased blood glucose levels and gave maximal response among all treated groups in the study.

The triple combination was probably the most effective because the plants enhanced each other's activities. The plants most probably work through different mechanisms and the best way to combat DM has been shown to be through using different combinational drugs or drugs that exhibit multiple mechanisms. All the plants have been reported to contain anti-oxidants which help in scavenging the hyperglycaemia generated free radicals. The diabetes control group showed slight decrease in plasma glucose level. The decrease could possibly be as a result of partial destruction of the pancreatic islets by alloxan monohydrate. The remaining functional beta cells could have possibly led to the decreased glucose levels.

The plants used in this study have been shown to be safe to use up to doses as large as 1gram³⁸⁻⁴¹. Giving such high doses chronically may however have dire consequences. Small animals excrete compounds more rapidly than larger animals in a rather systematic manner, therefore studies conducted in animals would need dose adjustments when they are applied to humans^{42, 43}. A non-toxic dose in rats can be toxic to humans if safety factor is not accounted for.

CONCLUSION: This study was carried out to determine the anti-diabetic effects of combining three herbal plants, *Annona stenophylla*, *Zingiber officinale* and *Citrus limon* on alloxan induced diabetic rats. The poly-herbal medicine gave maximal responses in reducing the fasting plasma glucose levels and the formulated product can be standardised and used as a potential anti diabetic drug.

CONFLICTS OF INTEREST: The authors declare that there is no conflict of interest in the research.

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