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## ASSESSMENT OF SYNERGISTIC HYPOGLYCEMIC EFFECTS OF *TETRACARPIDIUM CONOPHORUM* AND *PIPER GUINEENSE* IN ALLOXAN- INDUCED HYPERGLYCEMIC RATS

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### ABSTRACT

*In traditional practice, most medicinal plants and food supplements are normally used in combinations in order to increase the potency of the agents. However, there is need to provide scientific basis which underscores the practice. This study was therefore conducted to investigate the synergistic hypoglycemic effects of (1:1) Tetracarpidium conophorum and Piper guineense mixture in hyperglycemic rats. An analysis of the phytochemical and heavy metal (mineral) compositions of the plants was carried out. In addition, the acute oral toxicity test of the mixture was conducted following the OECD guidelines 423. The hypoglycemic potentials of ethanol extracts of the plants (300mg/kg body weight) was assessed in wistar albino rats administered with 100mg/kg body weight of alloxan intraperitoneally for 5 days and also assessed in normoglycemic rats. The results of the combined ethanol extract showed a significant decrease in glucose levels in hyperglycemic treated rats which compare favourably with the reference drug treated group. It was inferred from this study therefore that combined ethanol extract of T.conophorum and P.guineense (1:1) contained phytochemical and mineral constituents which may work in synergy to forestall the occurrence of hyperglycemia or reduce it where it already exists. The phytochemical constituents of the combined extracts contain appreciable quantities (in  $\mu$ /ml) of Saponin, Sapogenin, Tannin, Oxalate, Kaempferol and Catechin. Dominant mineral components present (in ppm) are Calcium, Iron, Magnesium, Sodium, Zinc, Selenium, and Potassium. Heavy metals like, Silver, Lead, Arsenic, Barium, Vanadium and Nickel were not detected.*

**KEYWORDS:** *Tetracarpidium conophorum Piper guineense, hyperglycemia, mineral,*

## INTRODUCTION

Hyperglycemia is described as an excess of glucose in the blood. Chronic Hyperglycemia often results to diabetes and its related complications. The damaging effects of increased glucose levels may cause increased oxidation of glucose resulting in the generation of superoxide radicals, a decrease in activity of key antioxidant enzymes, activation of protein kinase C (PKC) irreversible glycation of proteins also known as advanced glycation end-products, AGEs (Campos, 2012).

Medicinal plants containing several active ingredients from crude extracts of different plant products have been used either independently or in combination to manage and control hyperglycemic conditions (Odugbemi *et al.*, 2008). *Tetracarpidium conophorum* (Mull. Arg.) Hutch. & Dalziel belongs to the Euphorbiaceae family. It is popularly known as African walnut. (Ukpa in Igbo). The plant is cultivated mainly for its nuts which are cooked and taken as snacks (Amaeze *et al.*, 2011). The leaves, bark and fruits of *T.conophorum* have been used medicinally to treat toothache, syphilis, dysentery and as an antidote to snakebite (Akomolafe *et al.*, 2015). *Piper guineense* (Schumach) belongs to the Piperaceae family. It is a spice plant commonly called guinea pepper, black pepper, 'Uziza' in Igbo and 'Iyere' in Yoruba (Morufu *et al.*, 2016). It provides medicinal, insecticidal, culinary and dietary benefits to humans. (Echo *et al.*, 2012). The leaf has also been recognised for its anti-microbial, anti-fungal and antioxidant properties (Kiin-kabari *et al.*, 2011, Ojinaka *et al.*, 2016). Nutritional and mineral contents of the seeds of *P.guineense* have been investigated (Obomanu *et al.*, 1991). In traditional medical systems, poly therapy is a common practice and several of these formulations have been reported to exhibit antioxidant, hypoglycemic, antimicrobial, and antimalaria effects (Baldi and Goyal, 2011, Petchi *et al.*, 2014). This study is aimed at assessing the hypoglycemic effects of ethanol extracts of *Tetracarpidium conophorum* and *Piper guineense* leaves in alloxan- hyperglycemic albino rats.

## MATERIALS AND METHODS

### Plant Collection and Preparation of Extracts

Fresh leaves of *Tetracarpidium conophorum* and *Piper guineense* were collected from Oke-oko village in Owena L.G.A of Ondo State and Mile 1 Market Port Harcourt, Rivers State respectively. The plants were authenticated at the Plant Science and Biotechnology Department, Rivers State University, Port Harcourt Nigeria. The plants were shade-dried for 5 days in the laboratory at room temperature (29-30°C) and milled to a coarse powder using a grinder.

A known weight, 70 g of the powder was extracted by maceration with 600 ml of 80 % ethanol at room temperature in a rubber-corked bottle for 4 days. This was filtered with a clean muslin cloth and the filtrate evaporated to dryness in an oven at a temperature of 40-50°C. The extracts were resuspended in distilled water and dose administered according to the body weight of animals (Erhirhie *et al.*, 2014).

Alloxan monohydrate was procured from Sigma Company, St. Louis, USA. All other reagents and chemicals used were of analytical grade supplied by the Department of Biochemistry, Rivers State University, Port Harcourt, Nigeria

Thirty- five wistar albino rats, weighing (100 – 200 g) were used for this study. They were obtained from the animal house of University of Port Harcourt and allowed to acclimatize in the animal house of the Department of Biochemistry Rivers State University, Port Harcourt, Nigeria for seven (7) days. Rats' feeds and water were provided *ad libitum*. The experiment was conducted according to the University's ethical guidelines of the use of laboratory animals.

Phytochemical investigation of the plant extracts was conducted using a GC (Buck Scientific-GC M910, USA) equipped with Flame Ionization Samples Detector. A RESTEK 15 meter MXT-1 column (15m× 250µm ×0.15µm) was used. The injector temperature was 280°C with splitless injection of 2 µl of sample and a linear velocity of 30cms-1. Helium 5.0 Pas was the carrier gas with a flow rate of 40ml/min. The oven operated initially at 200°C, was heated to 330°C at a rate of 3°C/min and kept at this temperature for 5 min. Separation is governed by the more volatile nature of each component present in the sample and its interaction with the column stationary phase. Every separated component is brought to the detector system and transformed by the detector to an equivalent electronic signal which is collected and recorded as data.

Extraction of Sample for GC-FID: 1g of the crushed sample of each plant was weighed and transferred into a test tube containing 15 ml of ethanol and 10 ml of 50% w/v potassium hydroxide. The content of the test tube was allowed to stand in a water bath at a temperature of 60°C for 60 minutes after which it was carefully transferred into a separating funnel and rinsed with 10ml of cold water, 10ml of hot water, 20ml of ethanol and 3ml of hexane. The extract in the test tube was washed three times with 10ml of 10% v/v ethanol solution and dried with anhydrous sodium sulphate and the solvent evaporated. A sample of the extract was dissolved in 1000µl of pyridine then 200µl was transferred into a

vial in the Gas Chromatograph for phytochemical analysis.

### **Mineral/ Heavy Metal Composition**

Mineral/Heavy Metals were analyzed using the Varian AA240 Atomic Absorption Spectrophotometer.

### **Sample Preparation for AAS**

The plant sample were sorted, cleaned, dried and ground into fine powder and directly subjected to analysis. Each sample (0.25g) was placed in 50 ml flask and to this was added 6.5 ml of mixed acid solution comprising Nitric acid (HNO<sub>3</sub>), Sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) and Perchloric acid (HClO<sub>4</sub>) in the ratio (5:1:0.5) respectively. The sample was boiled in the acid mixture in a fume hood on a hot plate till the digestion was completed. This was indicated by white fumes issuing out from the flask. Thereafter, few drops of distilled water were added and the mixture was allowed to cool. The digested samples were transferred in 50 ml volumetric flasks and the volume was made up to 50ml by adding distilled water. The extract was filtered with filter paper (Whatmann No.42) and the filtrate collected in labeled plastic bottles. The solutions were analyzed for the elements of interest utilizing Atomic Absorption Spectrophotometer with suitable hollow cathode lamps. The percentages of different elements in these samples were determined by the corresponding standard calibration curves obtained by using standard AR grade solutions of the elements (Zafar *et al.*, 2010).

### **Preparation of Standard Drug**

Glibenclamide tablets, an oral hypoglycaemic drug were used as reference drug. This was freshly prepared in distilled water and administered at a dose of 2.5mg/kg body weight orally.

### **Acute Oral Toxicity Test of *T. conophorum* and *P. guineense***

Acute oral toxicity study was conducted according to the OECD guidelines 423.

Normal healthy rats were divided into Four groups containing three (n=3) animals each. Group 1 served as control and was given rat feeds and water. Groups 2 and 3 were given relatively high doses of 3000mg/kg body weight of ethanol extracts of *T. conophorum* and *P.guineense* respectively while group 4 was given combined extracts. Animals were observed individually after dosing at least once during the first 24 hours and daily thereafter, for a period of 7days for behavioral, neurological, autonomic responses and death.

### **Hyperglycemic rats**

The rats were kept fasting for 18 h and injected with 100mg/kg of alloxan monohydrate in normal saline intraperitoneally using insulin syringes. The rats were kept for 24hour on 10 % glucose solution to prevent fatal hypoglycemia. After one hour, the animals were provided feed ad libitum. After 48hours of administration, animals were considered hyperglycemic when the blood glucose level was raised above 200 mg/ dl using a glucometer. The selected hyperglycemic rats were grouped into five containing three rats in each (n=3) group.

### **Normoglycemic Rats**

The rats were fasted for 18 h. At the end of the fasting period time, the normal rats were then divided into five groups of three animals each (n=3). Blood glucose levels were monitored after 0, 2, 4, 6 and 8 hours of administration of single dose of test samples for 5 days.

The grouping of the animals and administration of extracts via oral route is presented in Table 1.

**Table 1: Groups and Treatment of Animals**

S/N	Groups	Treatment
1	Normal Control (NC)	1ml of distilled water only
2	Normal + Reference drug	received glibenclamide (2.5mg/kg)
3	Normal + Ethanol Extract of <i>P. guineense</i>	300mg/kg body weight of S1
4	Normal + Ethanol Extract of <i>T. conophorum</i>	300mg/kg body weight of S2
5	Normal+ Ethanol Extract of <i>P. guineense</i> and <i>T. conophorum</i> (ratio1:1)	300mg/kg body weight of S1 and S2
6	Hyperglycemic Control (HC)	100mg/kg B.W of alloxan only
7	HC + Reference Drug (RD)	received glibenclamide (2.5mg/kg) + 100mg/kg B.W of alloxan only
8	HC + Ethanol Extract of <i>P. guineense</i>	300mg/kg body weight of S1+ 100mg/kg body weight alloxan
9	HC + Ethanol Extract of <i>T. conophorum</i>	300mg/kg body weight of S2 + 100mg/kg body weight alloxan
10	HC + Ethanol Extract of <i>P. guineense</i> and <i>T. conophorum</i> (ratio1:1)	300mg/kg body weight of S1 and S2 + 100mg/kg body weight of alloxan

**Determination of Body Weight:** Body weights of animals were determined before and after

treatment of the extracts. The percent change in body weight was calculated using the formula:

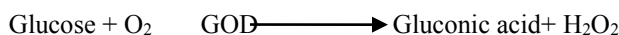
$$\text{Percentage change in body weight} = \frac{\text{Final body weight} - \text{Initial Body weight}}{\text{Initial body weight}} \times 100\%$$

### Collection of Blood Samples

At termination of the experiment, animals were fasted overnight, sacrificed by cervical dislocation. Incisions were quickly made in cervical region with a sterile blade and blood samples collected from the heart into fluoride oxalate bottles for glucose analysis.

### Glucose Estimation (Trinder, 1969)

Glucose was estimated by GOD-POD method. Glucose oxidase (GOD) converts the sample glucose into gluconate. The hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) produced in the reaction is degraded by peroxidase (POD) and this gives a colored product Phenol and 4-aminoantipyrine which is measured using Trinder indicator at 505nm. The increase in absorbance correlates with glucose concentration of the sample.



$$\text{Percentage Glycemic Change} = \frac{\text{Final glucose level} - \text{Initial glucose level}}{\text{Initial glucose}} \times 100$$

### Statistical Analysis

Data were expressed as mean  $\pm$  SEM. The data were subjected to one way analysis of variance (ANOVA) followed turkey post hoc test for comparison. Tests were performed to assess difference between groups. The values of  $p < 0.05$  were considered statistically significant.

### RESULTS

The phytochemical and mineral analyses of *P. guineense* and *T. conophorum* are presented in Table 2. Combined *P. guineense* and *T. conophorum* (1:1) showed higher compositions of phytochemical which include Saponins (17.6627  $\mu$ /ml), Sapogenin (11.8607  $\mu$ /ml), Tannin (5.1447  $\mu$ /ml), Oxalate (4.3083  $\mu$ /ml), Kaempferol (3.6228  $\mu$ /ml) and Catechin (3.20  $\mu$ /ml).

**Table 2: Phytochemical Composition of *P. guineense* and *T. conophorum* Leaves (1:1)**

Phytochemical Components	Retention Time	Concentration ( $\mu$ /ml)
Anthocyanin	1.006	0.6328
Oxalate	4.100	4.3083
Tannin	9.146	5.1447
Rutin	12.013	2.5310
Phenol	14.310	2.7482
Lunamarine	20.116	2.3090
Saponin	25.573	17.6627
Sapogenine	29.456	11.8607
Phytate	35.140	0.7305
Kaempferol	40.080	3.6228
Catechin	45.173	3.2029

The result for the mineral/heavy metal analysis presented in Table 3 showed the presence of important minerals Calcium (21.682ppm), Iron (15.709ppm), Magnesium (14.932ppm), Sodium

(9.904ppm), Zinc (9.212ppm), Selenium (7.312ppm) and Potassium (4.821ppm). No detectable amount of Silver, Lead, Arsenic, Barium, Vanadium, and Nickel was recorded.

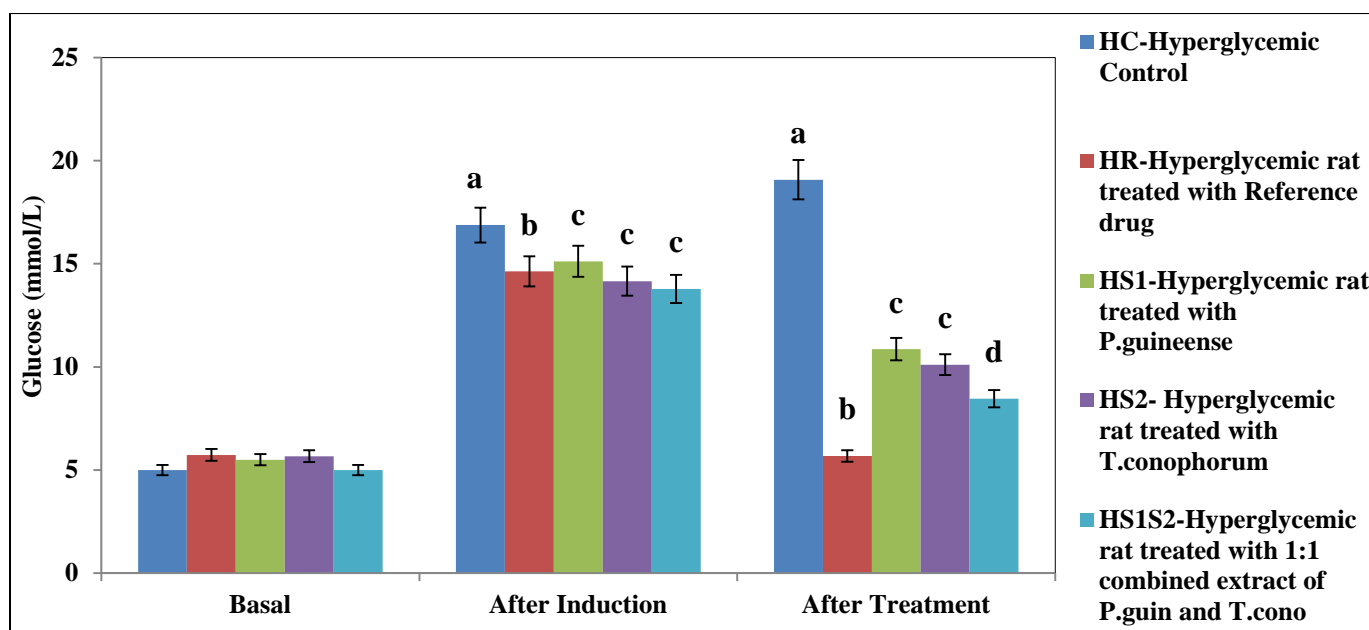
**Table 3: Mineral/ Heavy Metal Composition of *P. guineense* and *T. conophorum* Leaves (1:1)**

Mineral/Heavy Metal	Composition (ppm)
Copper	1.013
Chromium	0.057
Cobalt	0.084
Zinc	9.212
Molybdenum	0.390
Aluminium	2.715
Calcium	21.682
Selenium	7.312
Sodium	9.904
Silver	0.00
Lead	0.00
Iron	15.709

<b>Cadmium</b>	0.053
<b>Mercury</b>	0.234
<b>Potassium</b>	4.821
<b>Arsenic</b>	0.00
<b>Barium</b>	0.00
<b>Magnesium</b>	14.932
<b>Vanadium</b>	0.00
<b>Nickel</b>	0.00

Glucose levels before and after extract treatment is presented in Figure 1. Before induction (basal), all rats had similar glucose levels compared to the control. After induction, all alloxan treated rats showed significant ( $p < 0.05$ ) increase in glucose

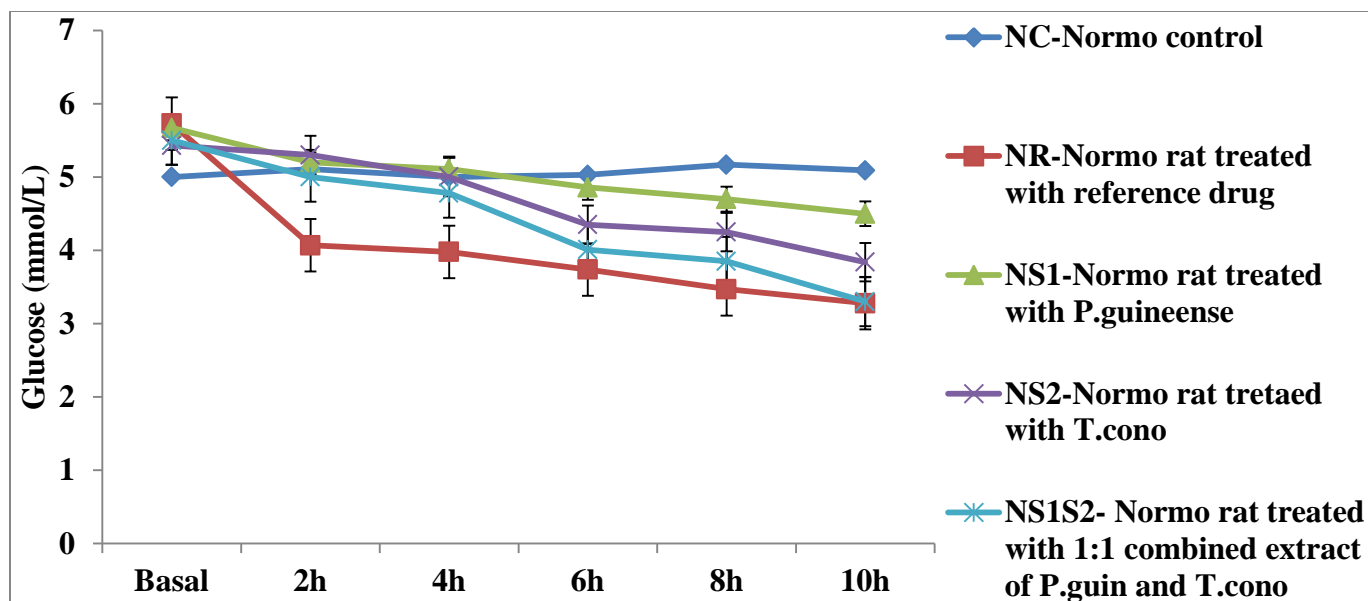
levels compared to control which is an indication of hyperglycemia. After treatment, all extracts treated group and reference drug group had significant ( $p < 0.05$ ) decrease in glucose levels compared to the hyperglycemia control group.



**Figure 1: Effect of Treatment on Glucose Concentration (mmol/L) in Hyperglycemic Rats.** Values are expressed as Mean  $\pm$  S.E.M (n=3). Means with different superscript (a-d) are significantly different (Turkey HSD,  $p < 0.05$ ).

Effect of extracts on glucose level of normoglycemic rats is presented in Figure 2. Normal rats treated with reference drug showed significant ( $p < 0.05$ ) decrease in glucose level from 2-10 hours compared to control.

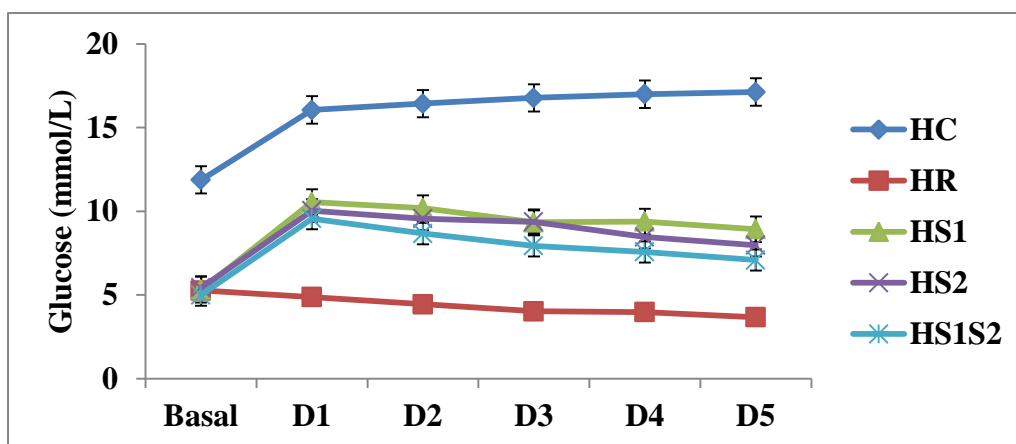
However normal rats treated with the extracts showed significant ( $p < 0.05$ ) decrease in glucose level at 6, 8 and 10 hours compared to the control.



**Figure 2: Effect of Treatment on Glucose Concentration (mmol/L) in Normoglycemic Rats.** Values are expressed as Mean± S.E.M (n=3).

The result of the effect of treatment on hyperglycemic rats is presented in Figure 3. Hyperglycemic control group showed significant ( $p < 0.05$ ) increase in glucose levels compared to hyperglycemic groups treated with the reference drug (HR) and extracts. Comparison between the extract treated groups showed that hyperglycemic rats

treated with the combined extracts (HS1S2) had significant ( $p < 0.05$ ) decrease, however not comparable to the reference drug treated group (HR), as it showed significant decrease in glucose levels. The results obtained for HS1, HS2 and HS1S2 respectively were not significantly different at D3, D4 and D5.

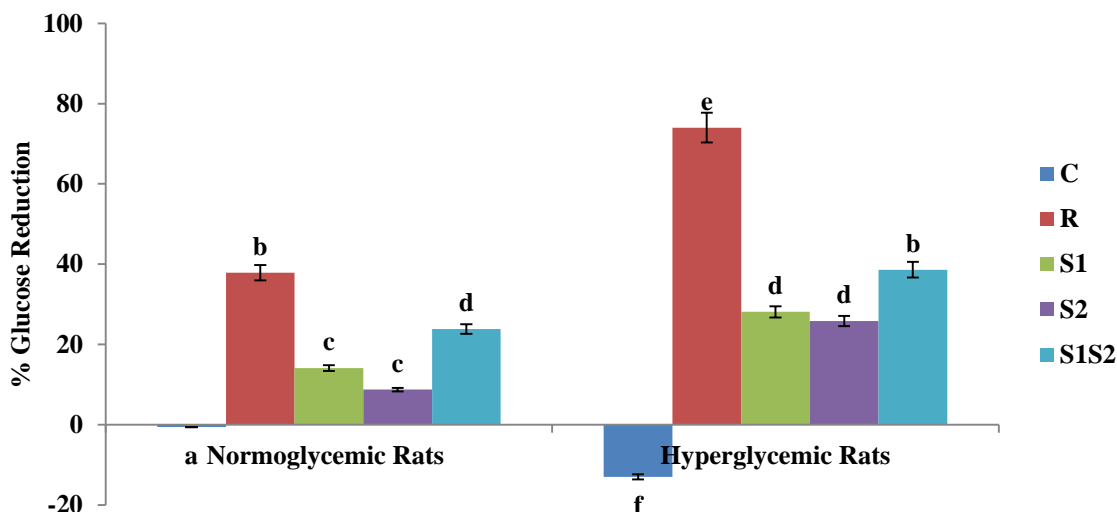


**Figure 3: Effect of Treatment on Glucose Concentration (mmol/L) in Hyperglycemic Rats.** Values are expressed as Mean± S.E.M (n=3).

The result obtained from the percentage glycaemic change is presented in Figure 4. In the hyperglycemic group, the percentage reduction for the combined extract (HS1S2) treated group, showed a total glucose reduction of 38.61 % which is statistically

similar to normoglycemic rats treated with the reference drug (NR) at 37.87%. Also total glucose reduction in hyperglycemic rats treated with extracts HS1 (28.1%) and HS2 (25.8%) results were also statistically similar.

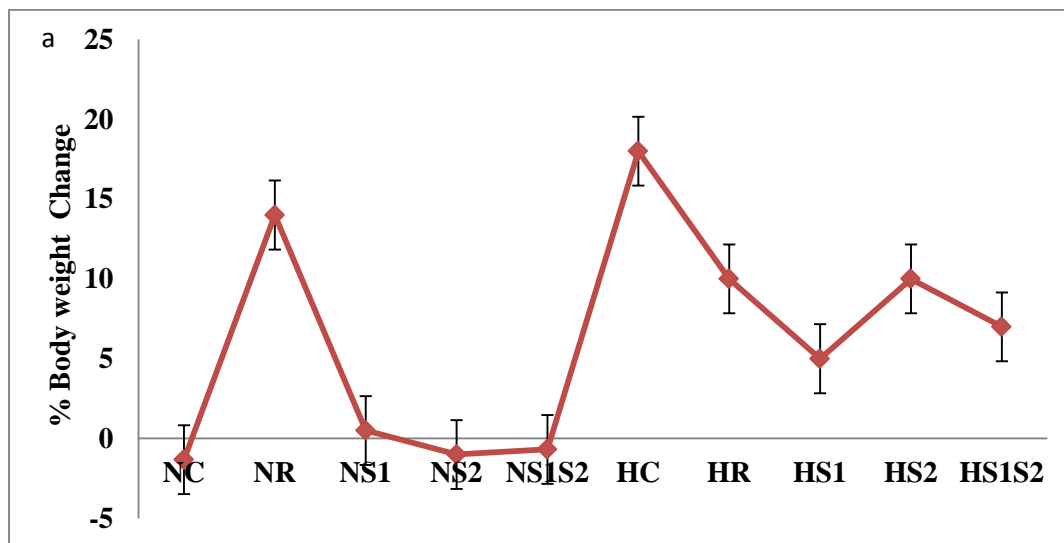




**Figure 4: Percentage Glucose Reduction in Rats after Treatment.** Values are expressed as Mean± S.E.M (n=3). Means with different superscript (a-f) are significantly different (Turkey HSD, p<0.05).

Effect of treatment on percentage mean body weight change in normoglycemic rats and hyperglycemic rats is presented in Figure 5. All hyperglycemic rats and normal rat treated with reference drug showed significant (p<0.05) decrease in percentage mean

body weight change compared to the hyperglycemic control. However, normal rats treated with extracts showed no significant (p>0.05) difference in percentage mean body weight change compared to the control.



**Figure 5: Effect of Treatment on Percentage Body Weight Change of Rats after Treatment.** Values are expressed as Mean± S.E.M (n=3).

## DISCUSSION

Plants of diverse origins have been exploited for their therapeutic values over many generations due to potent phytochemicals also known as bioactive compounds that are contained in them. The World Health Organization Expert Committee

on diabetes has recommended the implementation of traditional methods of treatment and management due to the adverse side effects associated with chemo-therapies, their increasing costs and high rate of treatment failures (Agunbiade *et al.*, 2012). The quest for pharmacologically active principles from

medicinal plants has led to various investigations and sustained search for new and readily available therapies with little or no side effects from plants and their products. Understanding of the chemical interactions between these compounds with humans and other organisms which can help to address specific health related issues (Odugbemi and Akinsulire, 2008). The phytochemical and mineral analyses of *P. guineense* and *T. conophorum* (1:1) are presented in Table 2 and Table 3 respectively. The tables show the presence of important bioactive components which may prevent or ameliorate the occurrences of chronic diseases. A combination of phytochemicals and minerals is beneficial in preventing the occurrence of diseases. Some of them are involved in insulin signaling and key enzymes of glycolytic pathways (Agunbiade *et al.*, 2012). Combined *P. guineense* and *T. conophorum* showed higher content of phytochemical which include Saponin (17.6627 $\mu$ /ml), Sapogenine (11.8607  $\mu$ /ml), Tannin (5.1447  $\mu$ /ml), Oxalate (4.3083  $\mu$ /ml), Kaempferol (3.6228  $\mu$ /ml) and Catechin (3.20  $\mu$ /ml) and mineral which include Calcium (21.682ppm), Iron (15.709ppm), Magnesium (14.932ppm), Sodium (9.904ppm), Zinc (9.212ppm), Selenium (7.312ppm) and Potassium (4.821ppm). The non-detection of Silver, Lead, Arsenic, Barium, Vanadium, and Nickel shows the safety profile of the extracts because these minerals are known to be toxic to biological systems even at very low concentrations. Combined *P. guineense* and *T. conophorum* is a good source of mineral elements and phytochemical constituents. Zinc and Magnesium have been shown to be involved in blood sugar control mechanism and therefore protect against diabetes (Agunbiade *et al.*, 2012). High composition of Alkaloids like Saponin, Sapogenine, Tannin, Oxalate Kaempferol and Catechin have also been shown to be helpful in alleviating hyperglycaemia and related diseases such as diabetes, hypercholesterolemia and bacterial infections (Stratton *et al.*, 2000, Lavie *et al.*, 2016). The *in vivo* acute toxicity study showed no sign of toxicity or death recorded of high doses at 3000mg/kg body weight (B.W) of ethanol extracts of *P. guineense* and *T. conophorum*. Thus one tenth of this dose, (300mg/kg B.W) was selected and used as fixed dose for this study. In the study, intraperitoneal administration of 100mg/kg body weight of alloxan caused significant increase in glucose level which is an indication of hyperglycemia. Chronic hyperglycemia is associated with diabetes. Alloxan is a toxic glucose analogue that is well known for its selective destruction of insulin producing pancreatic beta cells. It is used widely to induce diabetes in experimental animals by free radical generating mechanism formed in redox reaction. Induction of

diabetes gives elaborate information of the biochemical derangements and complications associated with diabetes (Ramadan *et al.*, 2017). Alloxan causes reduction of insulin secretion by damaging pancreatic  $\beta$  -cells in rats. The deficiency of insulin triggers the influx of a variety of enzymes, metabolic derangements, biochemical transformations and alteration of membranes and organs in the body. Reduction or deficiency in insulin secretion makes glucose to be liberally available in blood (Hyperglycemia) while unable to enter into cells for production of ATP via glycolysis. This finding is in agreement with previous reports conducted by Baldi and Goyal, (2011), Agunbiade *et al.*, (2012). The decrease in body weight observed in the hyperglycemic treated group may be due to toxicity imposed by alloxan and reference drug, reducing the ability of animals to eat food.

Administration of 300mg/kg body of ethanol leaf extracts of *P. guineense* and *T. conophorum* decreased glucose level significantly, thus indicative of hypoglycemic potentials. In normoglycemic study, significant reduction in glucose level was observed after 6, 8 and 10 hours of administration. Normal rats treated with the reference drug (glibenclimide) showed significant glucose reduction. This is an indication of a strong hypoglycemic agent hence it is used as standard reference drug. Insignificant difference in percentage body weight change may be attributed to safety profile of the extracts on the body weight as well as inhibition of alloxan -induced body weight reduction in the animals.

The combined extracts of *P. guineense* and *T. conophorum* (1:1) caused 38.61% fasting blood glucose level reduction over a period of 7 days. This hypoglycemic effect was comparable with the reference hypoglycemic agent used which had a total reduction of 37.87% in normal rats. Groups treated singly with ethanol extract of *P. guineense* and *T. conophorum* had glucose reduction of 28.1 % and 25.8% respectively which was statistically similar. Thus the 1:1 combination of *T. conophorum* and *P. guineense* has a demonstrable synergistic hypoglycemic effect on alloxan-induced wistar rats.

A number of mechanisms of actions have been proposed for the hypoglycemic effect of plant phytochemicals and minerals which include direct stimulation of glycolysis in peripheral tissues, inhibition of accelerated hepatic production of glucoses via gluconeogenesis, facilitation of peripheral absorption of glucose (Emeka and Oludare, 2011, Agunbiade *et al.*, 2012.). In support of these findings, further research has also shown that enhanced insulin activity and prevention of glucose autoxidation could also have offered a possible mechanism in prevention of hyperglycemia and related complications (Bnouham *et al.*, 2006, Emeka

and Oludare, 2011, Ezejiofor *et al.*, 2013, George and Uwakwe, 2014). Minerals may also have contributed to this observed hypoglycemic effect of the leaf extracts.

In conclusion, combined *P. guineense* and *T. conophorum* (1:1) is a good source of mineral elements and phytochemicals that may prevent the occurrence of diseases like diabetes. The Interplay of the potent phytochemicals in the combined extracts may have played a synergistic role in alleviating progression to hyperglycemia. Thus, such a combination could be used in medicinal formulation for the treatment and control of diabetes.

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