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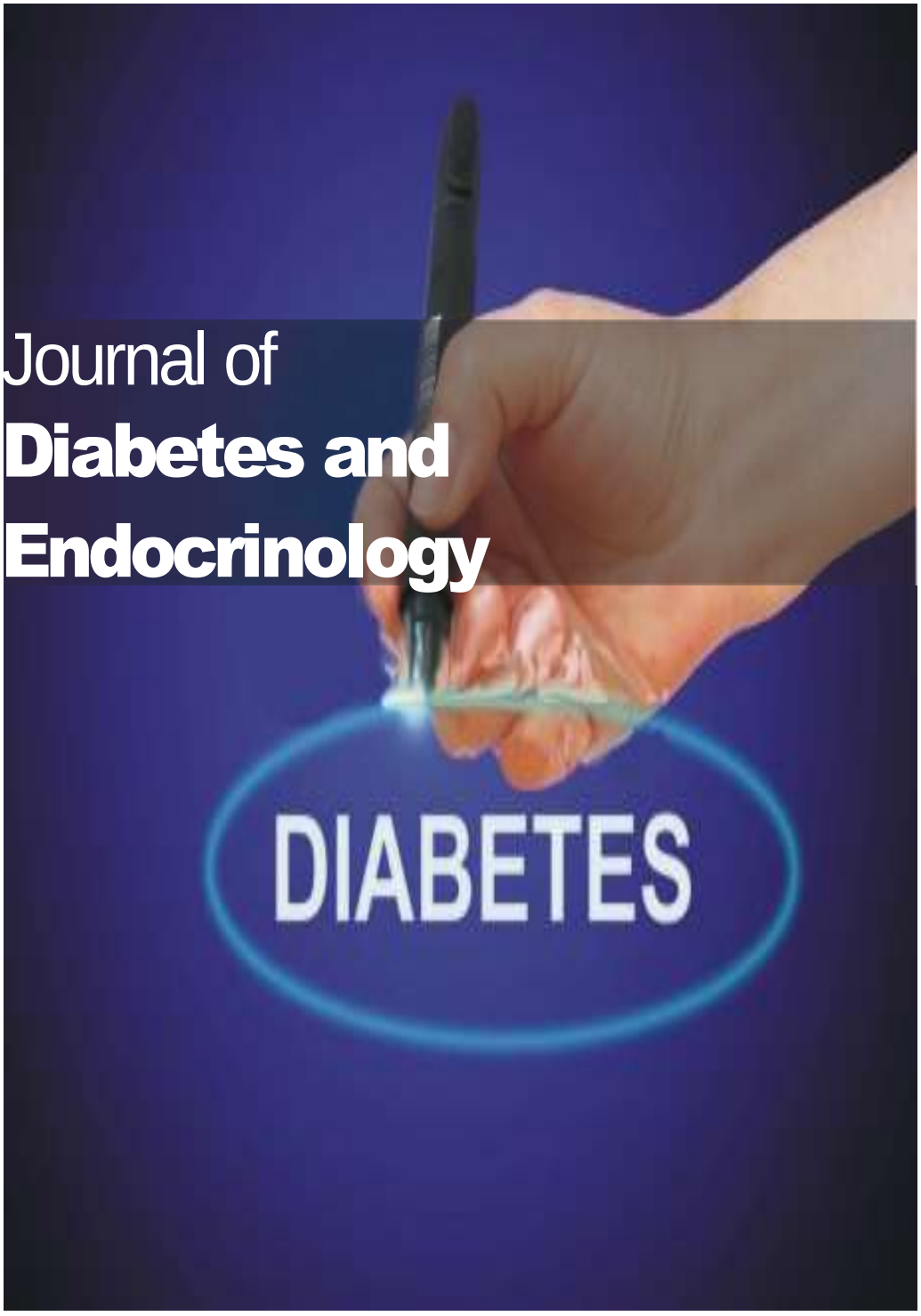

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Journal of Diabetes and Endocrinology

Full Length Research Paper

The effect of aqueous leaf extract of *Adansonia digitata* (baobab) on diabetes mellitus and the anterior pituitary of adult male wistar rats

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This study was carried out to evaluate the anti-diabetic properties of aqueous leaf extract of *Adansonia digitata* leaf (ALEAD) on blood glucose level. 36 of the rats were randomly distributed into 9. Group one served as the normal control and Group 2 rats were administered with alloxan (150 mg/kg) intraperitoneally. Groups 3, 4, and 5 were orally administered with alloxan (150 mg/kg) intraperitoneally and aqueous leaf extract of *A. digitata* (200, 400, and 600 mg/kg) once daily for 2 weeks. Group 6 were orally administered with metformin (150 mg/kg) once daily for 2 weeks. Groups 7, 8, and 9 were orally administered with aqueous leaf extract of *A. digitata* (200, 400, and 600 mg/kg) once daily for 2 weeks. The serum concentration of glucose of all the rats in each group was determined after the 8th and 15th dose of treatment. Groups 3, 4 and 5 showed a decrease after the first week of treatment but this decrease was not significant (P>0.05). The group treated with metformin (150 mg/kg) also showed a decrease which was also not significant (P>0.05). The result of the qualitative phytochemical analysis of aqueous leave extract of *A. digitata* indicated the presence of glycosides, flavonoids, tannins, saponins, terpenoid and steroids. These results suggest that the aqueous leaf extract of *A. digitata* possess anti-diabetic effect on alloxan induced diabetic rats.

Key words: Diabetes mellitus, *Adansonia digitata*, anterior pituitary, wistar rats.

INTRODUCTION

Diabetes mellitus is a group of metabolic disorders characterized by hyperglycemia and defective metabolism of glucose and lipids (Muhammed and Hauwa, 2013). It has been shown that diabetes is a heterogeneous syndrome characterized by an elevation of blood glucose level caused by relative or absolute deficiency of insulin (Mohammed and Hauwa, 2013).

Diabetes affects 177 million people worldwide in 2000

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and this figure is projected to increase to 300 million by 2025 (Chevenne and Fonfrede, 2007). It is a chronic condition which the body cannot properly convert food into energy and it is associated with long term complications that affect every part of the body (Bluestone et al., 2013). According to Williams and Randall (2015), diabetes mellitus alters the function of anterior pituitary gland either by hypo functioning or hyper functioning of the gland caused by lesion of the gland. The anterior pituitary gland secrete the following hormones adrenocorticotropic hormone (ACTH), Thyroid stimulating hormone (TSH), Luteinizing hormone (LH) and follicle stimulating hormone (FSH), Prolactin and Growth hormone (GH). A study by Hisayo et al. (1996) shows that there is failure of secretion of TSH, ACTH on insulin dependent diabetes mellitus in the anterior pituitary of rats. The anterior pituitary growth hormone stimulates the release and oxidation of free fatty acids which leads to decreased glucose and protein oxidation and preservation, thereby enhancing glycogen stores into the liver (Arun et al., 2013). Amounts of growth hormone have been found to cause no increase in blood sugar in normal subjects but may decrease the sensitivity to injected insulin (Møller and Jørgensen, 2009). Alloxan have been found to selectively inhibit glucose-induced insulin secretion through its ability to inhibit the beta cell glucose sensor kinase (Sigurd, 2008). This permits the selective study to potential antidiabetic agents in rodents. Several studies have consistently used alloxan induced diabetes as an animal model of experimentally induced diabetes (Mohammed and Hauwa, 2013). Medicinal plants, also called medicinal herb have been and used in traditional medicine practices since pre-historic times, plants synthesize hundreds of chemical compounds for functions which include the cure of disease illness, defense against insects, fungi, and herbivores mammals (Sharangi, 2009).

The medicinal value of these plants lies in some chemical substance that produces a definite physiological action on the human body, this chemical substance has a potential or established biological activity that has been identified and they are known as phytochemicals (Sinija et al., 2008). The baobab plants are tropical trees native to Africa, Australia and Madagascar but dispersed widely by humans. The members of the genus are united by several derived characters that serve to distinguish them from other *Bombacacea* including a characteristic indehiscent fruit with deiform seeds and powdery pulp (David, 1995).

Adansonia digitata is commonly found in the thorn woodlands of African savannah, it is a very long-lived tree with multipurpose use. *A. digitata* is commonly found in thorn wood lands of African savannahs, which tend to be at low altitudes with 4-10 dry months per year. It tends to grow as solitary individuals though it can be found in small group depending on the soil type (Jitin et al., 2005). Baobab as a multipurpose tree offers protection and serves as food, clothing and medicine as well as raw materials for many useful items (Chukwuma et al., 2017).

A. digitata plants parts are used to treat various ailments such as diarrhea, malaria and microbial infections. The plants and parts have interesting anti-oxidant and anti-inflammatory properties; hence, baobab is used extensively since ancient times in traditional medicines (David, 1995). *A. digitata* contain glycosides, saponins, steriods and flavonoids while alkaloids, tannins and resins were absent (Chukwuma et al., 2017). According to Muhammed and Hauwa (2013), baobab has been used in management of diabetes mellitus in Hausa land. According to WHO (1980) the number of people suffering from diabetes has risen from 108 million in 1980 to 422 million in 2014, furthermore in 2015 an estimated death of 1.6 million people were directly caused by diabetes. Being aware of all these, not excluding the side effects of most of the synthetic drugs used in the treatment of diabetes mellitus, arises the need to seek for an alternative in the herbal medicine. Although, studies have shown the antidiabetic effect of *A. digitata*, these studies only demonstrate the biochemical effect, and failure to access the histological effect the plant might have on the primary organ as well as the pituitary. *A. digitata* has been used in the management of diabetes and other metabolic activity. Traditionally *A. digitata* used to manage diabetes mellitus and a study by Muhammed and Hauwa (2013) have established the potential of these plant seed in the management of diabetes but no study have established on the effect of these extract on the pituitary hormones involved in the regulation of bodies carbohydrate metabolic activity. Hence this study is necessary to investigate the modulatory effect that the plant *A. digitata* might have on the pituitary histology and hormone secretion. The study investigated the effect of leaf extract of *A. digitata* (baobab) on alloxan-induced diabetes mellitus in adult male wistar rat.

MATERIALS AND METHODS

Collection and authentication of plant materials

Fresh leaves of *A. digitata* were procured from a local dealer in Kaduna state. It was identified at the Department of Agricultural Science, Enugu State University of Science and Technology, Agbani campus and a sample of it deposited at the herbarium unit.

Plant preparation and extraction

A sample of 5 g of each powdered plant materials was soaked in 100 ml of distilled water for 48 h. The solution was filtered using approximately 11 cm diameter whatman filter paper. The extract was subsequently collected after 24 h and immediately used for phytochemical analysis.

Phytochemical analysis

The aqueous extract of *A. digitata* was subjected to phytochemical

screening test to detect the presence or absent of carbohydrates, anthraquinones, flavonoids, tannins, alkaloids, saponins, glycosides, sterols and triterpenes. Also proximate analysis to detect the presence or absence of moisture, protein, crude fibres, ash, fats and and oil and carbohydrate at PRODA Emene Enugu state. Each of the tests was qualitatively screened; the presence or absence of the compound was expressed as positive or negative respectively.

QUALITATIVE ANALYSIS OF PHYTOCHEMICAL CONSTITUENTS

Test for alkaloids

The presence of alkaloid was determined as described by Zagga et al. (2018). A portion of the plant powder (5 g) was reacted with a few drops of hagers reagent (1.0 cm^3) and another 5 g portion was treated with Wengers reagent (1.0 cm^3) turbidity or precipitate with either of these reagents was taken as an evidence for the presence of alkaloids.

Test for tannins

A portion of the plant sample was diluted with distilled water in the ratio of 1:4 and a few drops of 10% ferric chloride was added to produce a blue black or green color. 5 g of dried powdered sample of the plant was boiled in 20 ml of distilled water in a test tube and then filtered using a hydrophilic filter (5.5 cm in size) and funnel (35 mm in size) placed in a conical flask. 0.1% $FeCl_3$ was added to the filtered samples and observed for brownish green or a blue-black coloration, which shows the presence of tannins (Trease and Evans, 1996).

Test for saponins

Five gram of powdered sample of the plant was boiled together with 20 ml of distilled water in a water bath and then filtered. 10 ml of the filtered sample was mixed with 5 ml of distilled water in a test tube and shaken vigorously to obtain a stable persistent froth. The frothing observed for the formation of emulsion which indicates presence of saponins (Odebiyi and Sofowora, 1978).

Test for flavonoids

A few drops 0f 1% NH_3 solution was added to the aqueous plant sample in a test tube, a yellow coloration was observed to indicate the presence of flavonoid (Sharma et al., 2013).

Test for terpenoid

Five gram of the plant sample was mixed with 2 ml of $CHCl_3$ in a test tube. 3 ml of concentrated H_2SO_4 was carefully added to mixture to form a layer, an interface with a reddish-brown coloration is formed if terpenoid consitituent is present (Sofowora, 1982).

Test for cardiac glycoside

Two milliliters of concentrated H_2SO_4 was prepared in attest tube. 5 g of plant sample was mixed with 2 ml of glacial acetic acid containing 1 drop of $FeCl_3$. The mixture was carefully added to the 1 ml of concentrated H_2SO_4 so that the concentrated H_2SO_4 is underneath the mixture. If cardiac glycoside is present in the sample, a brown ring will appear indicating the presence of the cardiac glycoside constituent (Zagga et al., 2018).

Test for phenols

Two milliliters of the extracts was mixed with ferric chloride solution. A green or dirty green precipitate indicates the presence of phenolic compounds.

QUANTITATIVE ANALYSIS

Alkaloid determination

This was done using the method of Harborne (1973). 10 g of the test sample was weighed into 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added. Beaker was covered and allowed to stand for 4 h, then it was filtered and the extract was concentrated on a water bath to one quarter of its original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitate was complete. The whole solution was allowed to stand till its settlement (24 h). The precipitate was filtered out from the solution using filter paper and washed with dilute ammonium hydroxide. The residue was the alkaloid which was weighed after complete dryness and the percentage was calculated.

$$\%\ Alkaloid = \frac{Weig\,ht\ of\ precipitate}{Weig\,ht\ of\ the\ sample} \times 100$$

Saponin determination

Method of Obadoni and Ochuko (2001) was used for the determination of saponin. 10 g of test sample was put into 250 ml conical flask and 100 ml of 20% aqueous ethanol was added. Then the flask was heated on a hot water bath for 4 h, with constant stirring at about 55°C. The mixture was then filtered and the residue was again extracted with another 200 ml 20% ethanol. The combined extract was reduced to 40 ml on a hot water bath at about 90°C. The concentrate was transferred into 250 ml separator funnel, added 20 ml diethyl ether in it followed by vigorous shaking. The aqueous layer (lower layer) was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added for washing two times. In both cases, the upper layer was collected while the lower layer discarded. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride, the remaining solution was heated in a water bath. After evaporation the samples were dried in oven, weighed and saponin content was calculated as percentage.

$$\%\ saponin = \frac{weig\,ht\ of\ the\ extract}{weig\,ht\ of\ the\ sample}\ x\ 100$$

Tannin determination

This was done using the method of Van-Burden and Robinson (1981). 500 mg of test sample in each case was taken in a plastic bottle and 50 ml of distilled water was added. Then it was shaken in a mechanical shaker for 1 h, and filtered in a 50 ml volumetric flask made up to mark. 5 ml of the filtrate was pipette out into the test tube and mixed with 2 ml of 0.1 M $Fecl_3$, 0.1 ml N HCl and 0.008 M $K_4Fe(CN)_6$ (potassium ferrocyanide). The absorbance was measured at 120 nm within 10 min. Absorbance was traced against concentration using tanic acid standard graph.

% Tannin = concentration × dilution × 100

Flavonoid determination

The method of Bohm and Kocipai-Abyazan (1994) was used. 10 g of test sample was extracted with 100 ml of 80% aqueous methanol repeatedly at room temperature using separating funnel. The whole solution was filtered through Whatman filter paper No. 42(125 mm). The filtrate was later transferred into a crucible and evaporated into dryness over a water bath, the weight of the material and percentage quantity were calculated.

$$\%\ Flavoniod\ = \frac{Weig\,ht\ of\ extract}{weig\,ht\ of\ the\ sample} x\ 100$$

Proximate analysis

Ash, moisture, crude protein, crude fiber, fat and oil, and carbohydrate were determined according to the methods of Association of Official Analytical Chemist (1990).

Experimental animals

A total of 45 adult male wistar rats were purchased from the Animal House Unit of the College of Medicine, Enugu State University of Science and Technology Parklane GRA Enugu Nigeria. The animals were housed fly-proof metal cages and were provided with food (growers mesh) and water *ad libitum*. The animals were maintained under standard laboratory condition (24°C) with relative humidity of 60-70% under 12 h light/ dark cycles and were acclimatized for two weeks prior to the experiment.

Induction of diabetes using alloxan monohydrate

Stock solution of alloxan monohydrate (Sigma- Aldrich Canada) was prepared by dissolving alloxan monohydrate (0.9 g) in distilled water (6 cm^3) and diabetes was induced by single intraperitoneal injection of alloxan monohydrate (150 mg/kg). The volume of the solution containing 150 mg/kg given to each rat was determined by its weight. After a period of two days (48 h), the rats with blood glucose level greater than 200 mg/dl was considered diabetic and used for the research work. The method of Mohammed and Hauwa (2013) was adopted in the study with slight modification.

Experimental design

The animals were randomly divided into nine groups of five animals each. They were labeled group 1-9 of which Group 1 served as the control group.

Group 1: Normal control group and received 0.1 ml/kg normal saline as placebo
Group 2: Diabetic untreated group + feed and water ad libitium
Group 3: Diabetic + 200 mg/kg ALEAD
Group 4: Diabetic + 400 mg/kg ALEAD
Group 5: Diabetic + 600 mg/kg ALEAD
Group 6: Diabetic + 400 mg/kg metformin as standard drug
Group 7: Non- Diabetic + 200 mg/kg ALEAD
Group 8: Non- Diabetic + 400 mg/kg ALEAD
Group 9: Non- Diabetic + 600 mg/kg ALEAD

Determination of blood glucose level

Glucometer strips were inserted into the strip compartment of the glucometer (Accu –Answer ZH–G01) and a sample of blood collected by tail snipping was used to touch the sensitive part of the strip and the values were displayed and recorded in mg/dl according to the method of Akpotu et al. 2018

Biochemical study

Hormonal assay

At the end of the experiment, blood samples were collected via cardiac puncture using 5 ml plane sample container. Sera were separated and stored at -20°C until ready for the analysis of the hormonal assay. Serum level of total growth hormone (GH) and thyroid stimulating hormone (TSH) were determined.

Method used for hormonal assay

TSH and GH ELISA (enzyme linked immune-solvent assay) by Uotila et al. (1981) procedure. The desired numbers of coated wells were secured in their holders' and 100 µl of standards, specimens and controls were dispensed into appropriate wells and thoroughly mixed for 30 s. They were later incubated at room temperature (18-25°C) for 60 min and the incubation mixtures were removed by flicking plate contents into a waste container. After, the micro titer wells were rinsed and flicked 5 times with distilled or deionizer water. The wells were sharply stroked onto absorbent paper or towels to remove all residual water droplets. Later 100 µl of TMB reagent was dispensed into each well and gently mixed for 10 s, the reaction was stopped by adding 100 µl of stop solution to each well and gently mixed for 30 s. Finally, absorbances were read at 450 nm with a micro titer well reader within 15 min.

Histopathology study

Tissue preparation

Twenty-four hours after the last treatment, all animals were sacrificed under anesthesia. The skull was opened and the brains of each rat were excised. The tissue was fixed in 10% neutral formal saline container with lids for 3 days to prevent autolysis, improve staining quality and aid optical differentiation of its cells. The tissues were subsequently trimmed, dehydrated in 4 grades of alcohol (70, 80 and 90% and absolute alcohol), cleared in 3 grades of xylene and embedded in molten wax. On solidifying, the blocks were sectioned, 5 µm thick with a rotary microtome, floated in water bath and incubated at 60°C for 30 min. The 5 µm thick sectioned tissues were subsequently cleared in 3 grades of xylene and rehydrated in 3 grades of alcohol (90, 80 and 70%). The sections were then stained with hematoxylin for 15 min. Bluing was done with ammonium chloride. Differentiation was done with 1% acid alcohol before counterstaining with Eosin. Permanent mounts were made on degreased glass slides using a mountant;DPX

Slide examination

The prepared slides were examined with a Motic™ compound light microscope using x4, x10 and x40 objective lenses. The photomicrographs were taken using a Motic™5.0 megapixels microscope camera at x160 and x400 magnification

Statistical analysis

Data obtained were expressed as the mean ± standard deviation. They were fed into the computer using statistical package for social

sciences (SPSS, version 20; IBM SPSS, Chicago, Illinois, USA) software package. One-way analysis of variance (ANOVA) with Tukey post- hoc test was used to compare the statistically significant difference at $P< 0.05$.

RESULTS AND DISCUSSION

This study investigated the effect of aqueous leaf extract of *A. digitata* on alloxan induced wistar rats. The result of this study investigated the effects of *A. digitata* on the serum concentration of growth hormone and TSH. From the result there was an elevation in the growth hormone concentration in group 3 with the diabetic untreated group showing the highest increase when compared with the negative control. This elevation agreed with the research conducted by Mamza et al. (2013) hormone has an insulin antagonistic effect. During hypoglycemia, it is being secreted to restore blood glucose levels by stimulating glucose increase from the liver and inhibiting glucose uptake in peripheral tissues elevation (Mamza et al., 2013). There was a significant increase in growth hormone concentration in Group 7 and also in Group 8 which were given 200 and 400 mg/kg of extract only when compared to the negative control. This elevation suggests that it may be due to the presence of flavonoid in the extract, which has a glucose lowering property by inhibiting (Mohammed et al., 2013). Flavonoid inhibits glucose 6-phosphatase activity in the liver thereby suppressing gluconeogenesis and glycogenolysis and consequently reduces hyperglycemia (Chen, 1998). The increase in growth hormone concentration was more pronounced in Group 7 which suggest that it might be dose dependent. There was decrease In TSH level in the diabetes treated group when compared to Group 1. The serum TSH levels in Groups 1 and 2 which served as the negative control and diabetic untreated group respectively showed normal level of this hormone. This suggests that the diabetic states of the animals in Group 2 may have had no direct effect on TSH secretion on the anterior pituitary gland. This fact might be supported by the normal histology of the pituitary gland in Group 2 when compared with the histology of the Group 1 animals. The diabetes treated groups (Groups 3, 4, 5 and 6 respectively) however, showed fluctuations in the serum concentration of TSH whereas Groups 3, 5 and 6 showed decrease in the serum level of TSH while Group 4 showed an increase in the serum levels of TSH. Within these values, only the decrease in TSH levels brought about by the administration of Metformin (Group 6) was statistically significant ($P<0.05$). This suggest that diabetes mellitus might have no direct stimulatory effect on TSH secretion but an anti-diabetic medications may pose an effect on the TSH level However, the mechanism or process of this effect is still unknown and there is need for further research on the mechanism of action .Moreover, the administration of increased doses of the aqueous leaf extract of *A. digitata* had a decreasing effect on TSH levels as seen in Group 7 and 8. However, the high doses of the aqueous leaf extract of *A. digitata* increased the secretion of TSH from the anterior pituitary gland. These values observed in these groups administered with the extract only were not significant ($P>0.05$). The general administration of the plant extract suggests that the aqueous leaf extract of *A. digitata* has the potency of decreasing the serum levels of TSH. Though their mechanism of action is not known, it suggests that these effects were not directly on the anterior pituitary as the normal histology of this gland was noted in all the groups. There is need for further research on the mechanism of action. As revealed in Group 1 animals which served as the negative control group and received only normal saline, had a 19.2% increase in body weight at the end of the experiment. In contrast, Group 2 animals that served as the positive control (diabetic untreated) group showed a 10.7% decrease in body weight at the end of the experiment. This percentage decrease noticed in the group might be due to the adverse effects of diabetes on the body. This is in agreement with Lau et al. (2003) who stated that diabetes is often associated with a characteristic loss of body weight which is partially due to increased muscle wasting. Groups 7, 8 and 9 which served as the extract-treated groups were given low, medium and high doses of the aqueous leaf extract of *A. digitata*. Groups 7 and 8 had a 4.98 and 5.48% decrease in their body weights respectively, while group 9 had 7.4% increase in body weight at the end of the experiment. This suggest that high dose of the extract can cause weight gain as a result of increased blood glucose level (63.45%) at the end of the experiment (Tables 1 to 3, Figure 1).

However, comparing the percentage changes in body weight of animals in Groups 7, 8 and 9 to Group 1, it can be suggested that the aqueous leaf extract of *A. digitata* had no benefiting effect on the body weight of the animals and even the weight gain noticed with high dose administration were not up to the normal control group. Among the diabetic-treated Groups (3, 4, 5 and 6) which were given low, medium, high dose of the aqueous leaf extract of *A. digitata* and Metformin respectively, only Group 3 showed a percentage increase in body weight while Groups 4, 5 and 6 showed a decrease respectively in their body weights at the end of the experiment. Since the animals in this Groups 4, 5 and 6 also had increased blood glucose levels respectively as seen in Table 4, it may also be suggested that they also experienced the weight-decreasing effect of diabetes just as noticed in the Group 2.

The fasting blood glucose profile shows that *A. digitata* leaf extract was capable of lowering plasma glucose levels, as seen in the diabetic groups treated with low and medium doses of the extract (200 and 400 mg/kg respectively) after the first week of the experiment. This supports previous studies on other parts of *A. digitata* which were shown to possess antidiabetic potential

Table 1. Qualitative results of phytochemical screening of the leave of *A. digitata.*

Parameter	Ethanol	Water
Alkaloids	+	+
Tannin	-	-
Flavonoid	+	-
Glycoside	+	+
Saponin	+	+
Terpenoid	+	-
Phenol	-	-

Key: + =detected; - =not detected.

Table 2. Quantitative results of phytochemical screening of the leaf of *A. digitata.*

Parameter	Values (%)
Alkaloid	0.97
Flavonoid	3.63
Glycoside	0.21
Saponin	1.84
Terpenoid	1.02

Table 3. Results of proximate analysis of leaf of *A. digitata.*

Parameter	Values
Moisture	0.81
Protein	14.35
Crude fibre	7.22
Ash	1.43
Fats & oil	10.00
Carbohydrate	66.19

Values were analyzed as Mean± SD using ONE WAY ANOVA with Tukey post hoc test. *P <0.05 compared with the control Group 1; βP <0.05 compared with the control Group 2; CDEFGHP <0.05 compared with the control Group 3, 4, 5, 6, 7, 8 and 9. Values without superscripts showed no significant difference among and between groups.

(Tanko et al., 2008; Saravanaraj et al., 2017). The blood glucose level of the groups treated with treated with a high dose of the extract (600 mg/kg), as well as the normal groups treated with different doses of the extract (Groups 7-9), on the other hand, were increased after administration. However, at the end of the second week of the experiment, the blood glucose levels increased in all the diabetic and non-diabetic groups treated with the extract. This increase in blood sugar might be as a result of the high carbohydrate content of the extract. As stated by Eizirik and Cnop (2010), carbohydrate consumption increases demand on the β-cell for insulin secretion, which may lead to endoplasmic reticulum stress, as well as oxidative stress (Sung et al., 2012) both of which can result in β-cell damage after a long period of time. The postprandial fluctuations of glucose increase gradually with increased proportions of carbohydrates, as well as an increase in mean blood glucose which take longer times to decrease back to normal levels (Kang et al., 2013). It is therefore virtually impossible to match carbohydrates and insulin which leads to unpredictable blood glucose levels; but by reducing the doses of

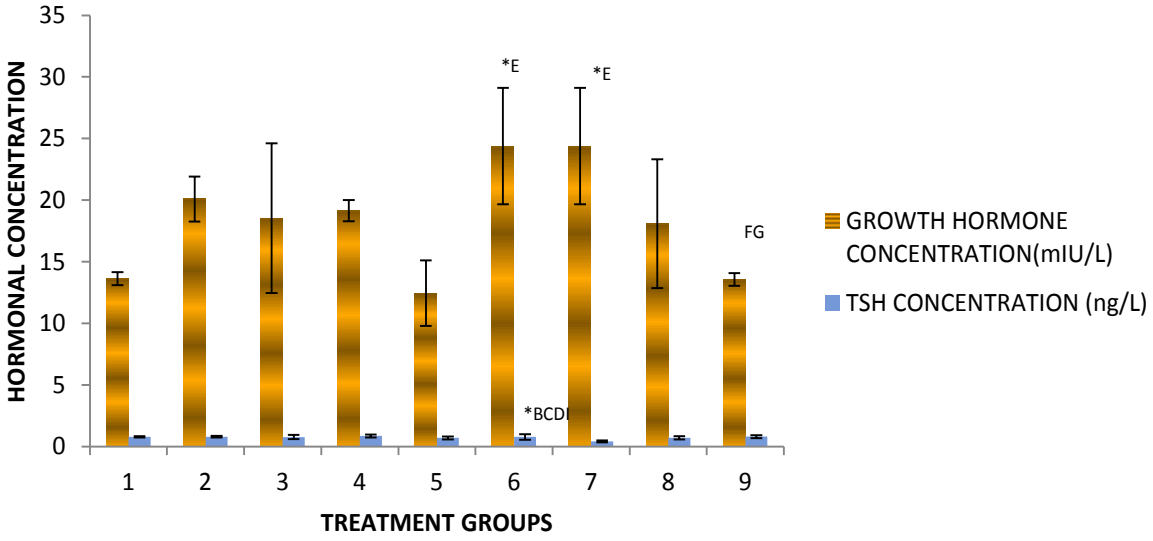

Figure 1. Result of the effect of the aqueous leaf extract of *A. digitata* on hormonal (GH and TSH) concentration. Values were analyzed as Mean± SD using ONE WAY ANOVA with Tukey post hoc test . *P <0.05 compared with the control Group 1; βP <0.05 compared with the control Group 2; CDEFGHP <0.05 compared with the control Groups 3, 4, 5, 6, 7, 8 and 9. Values without superscripts showed no significant difference among and between groups.

Table 4. Result of the effect of aqueous leaf extract of *A. digitata* on fasting blood sugar level in wistar rats.

Groups	Day 1	Day 8	Day 15	Changes (D15-D1)	% changes
1	85.5±3.7	95.2±6.8	110.0±4.3	24.5±0.6	28.7
2	422.7±135.9*	439.2±13.21	466.5±110.6*	43.8±25.3	10.4
3	181.8±71.3 $^{\beta}$	153.8±92.1	223.5±68.4	41.8±2.9	22.9
4	191.3±134.1	120.33±69.2	217.3±142.6	26.0±8.0	13.6
5	149.8±64.3 $^{\beta}$	199.0±200.9	254.5±117.9	104.8±53.6	69.9
6	389.5±185.2	198.8±77.5	309.8±111.0*	63.8±74.2	17.1
7	76.3±4.3* $^{\beta}$	84.3±5.5	108.3±47.5 ^{f}	37.5±43.2	49.4
8	80.5±5.5 $^{\beta}$	92.5±13.8	113.8±78.9 ^{f}	33.3±73.5	41.4
9	76.5±1.3 $^{\beta}$	82.0±2.5	120.0±30.0 ^{f}	43.5±28.7	63.4

Values were analyzed as Mean± SD using ONE WAY ANOVA with Tukey post hoc test. *P <0.05 compared with the control Group 1; $^{\beta}$P <0.05 compared with the control group 2; ^{CDEFGH}P <0.05 compared with the control Group 3, 4, 5, 6, 7 and 8.

carbohydrates and insulin, the size of the blood glucose fluctuations can be minimized (Bernstein, 1980). It is also possible that a high-carbohydrate/lower-fat diet such as contained in the extract, on a prolonged period of time could increase insulin sensitivity and lower fasting glucose levels, as reported by Gower et al. (2012). However, it was also observed that the blood glucose levels of the diabetic group treated with the extract were significantly lower than that of the untreated diabetic group as well as the group treated with the standard drug (metformin). This suggests that the extract has an antagonistic effect between its high carbohydrate content and its hypoglycemic property via unclear mechanisms (Figure 2).

Histological findings

Histology sections of the pituitary gland presented on all the slides (both in the control and treated groups) showed the normal histo-architecture of the rodent pituitary gland. The sections showed the bi-lobed pars distalis surrounding the pars intermidia and pars nervosa. The pars distalis is made up of 3 groups of cells; acidophilic chromophils, basophilic chromophils and chromophobes arranged in nests and cords interspersed within a rich fibro-vascular plexus. The acidophilic chromophils are characterized by small round to oval cells with a central nucleus with deeply eosinophilic cytoplasm while basophilic chromophils are more polyhedral with an

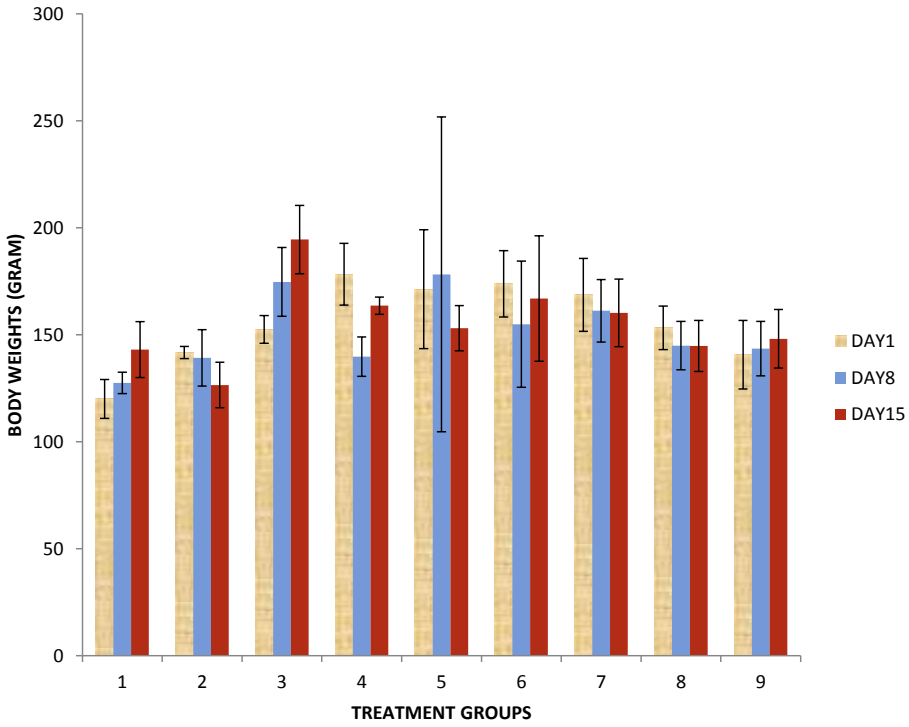

Figure 2. Result of the percentage changes in body weight of the experimental animals.

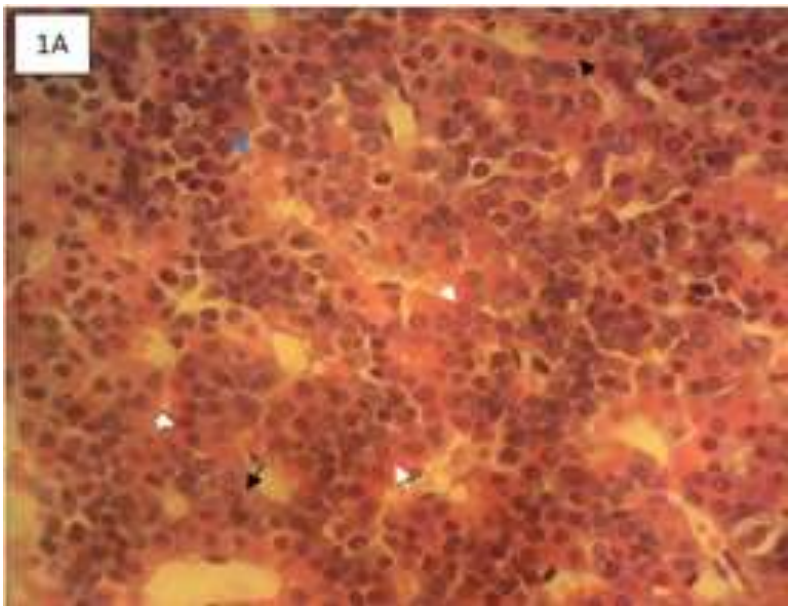

Plate 1. Photomicrograph of control animal groupadministered normal saline and given feed and water (H & E stain x 400) at high magnification showing the cells of the pars distalis; Basophilic chromophils (black arrow);acidophilic chromophils(white arrow);congested capillaries (blue arrow).

eccentric nucleus and pale basophilic cytoplasm. The chromophobic cells have a large nucleus with 1 or 2 nucleoli and abundant pale cytoplasm. The sections of the pituitary glands examined in this study did not show any deviation from their respective normal histopathologies (Plates 1 to 9).

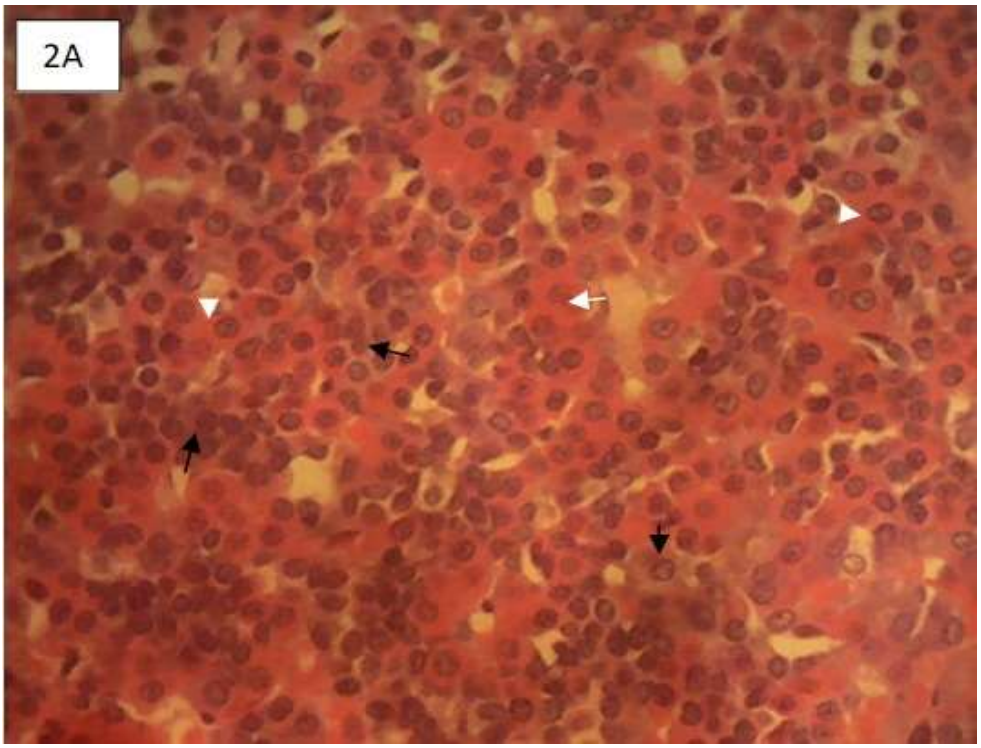

Plate 2. Photomicrograph of Group II given 150 mg/kg-bwt of Alloxan (H & E stain x 400) at high magnification showing the cells of the pars distalis; Basophilic chromophils(black arrow);acidophilic chromophils white arrow); congested capillaries (blue arrow).

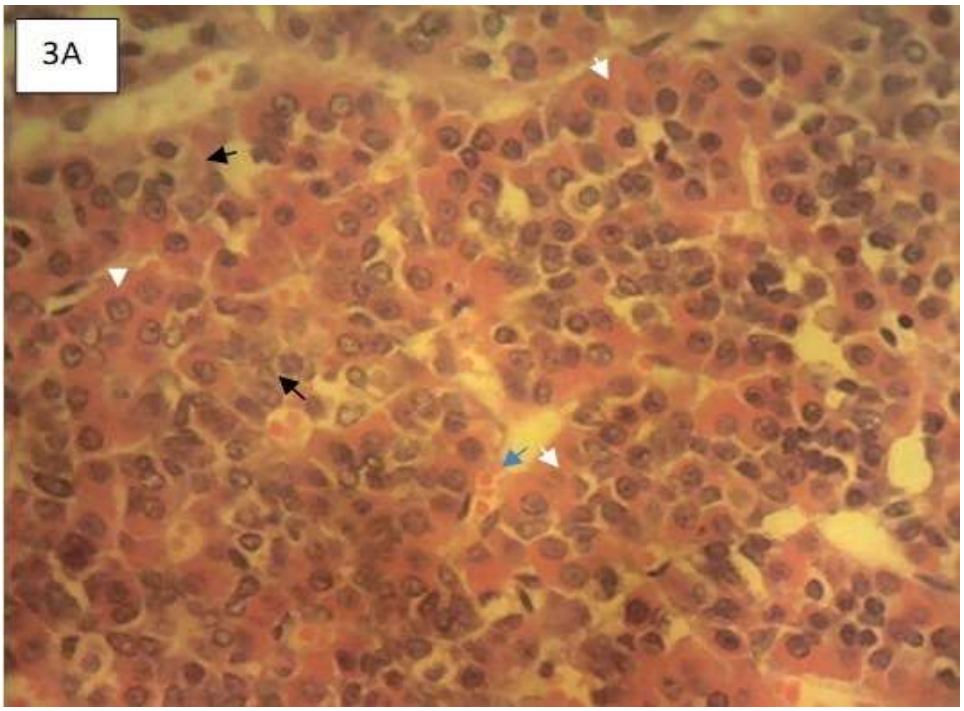

Plate 3. Photomicrograph of Group III given 150 mg/kgbwt of Alloxan and 200 mg/kg-bwt of extract (H & E stain x 400) at high magnification showing the cells of thepars distalis;Basophilic chromophils(black arrow);acidophilic chromophils (white arrow); congested capillaries(blue

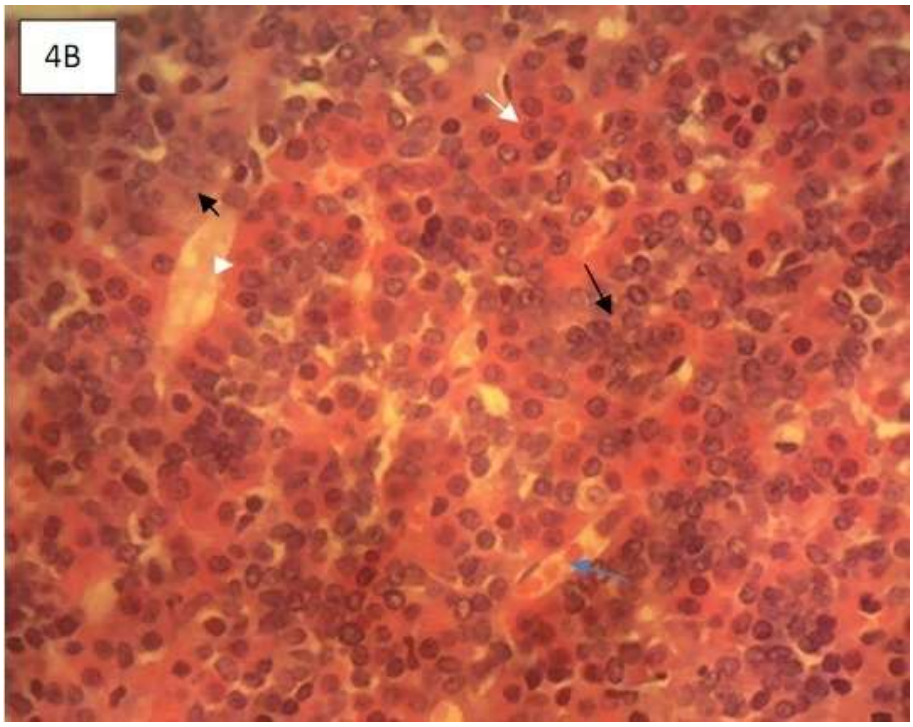

Plate 4. Photomicrograph of Group IV given 150 mg/kg -bwt of Alloxan and 400 mg/kg-bwt of extract (H & E stain x 400) at high magnification showing the cells of the pars distalis; Basophilic chromophobes (black arrow);acidophilic chromophils (white arrow);congested capillaries (blue arrow).

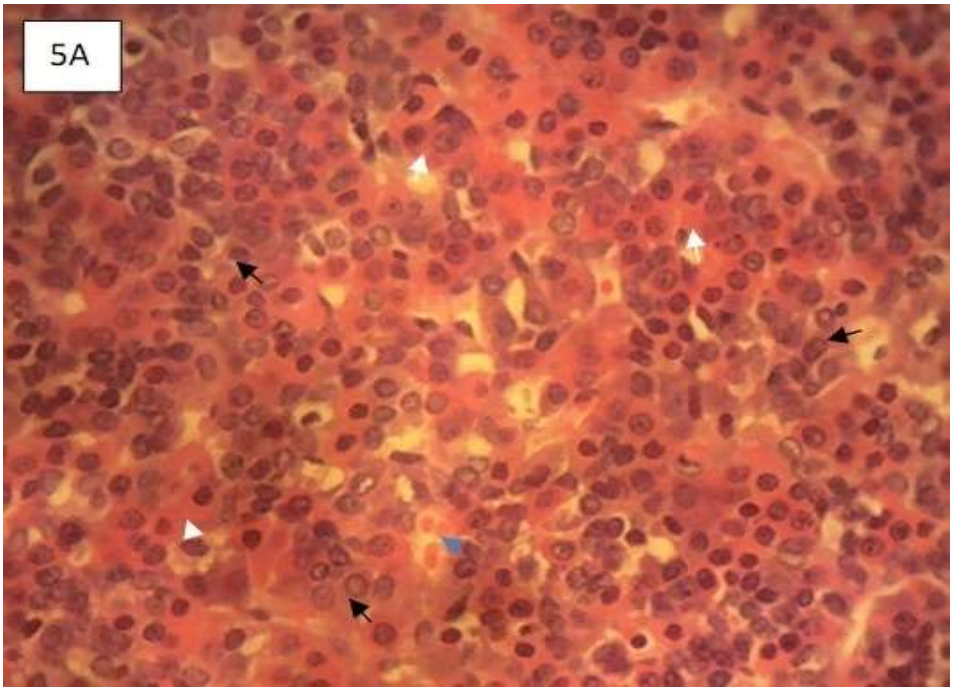

Plate 5. Photomicrograph of Group V given 150 mg/kg -bwt of Alloxan and 600mg/kg-bwt of extract (H & E stain x 400) at high magnification showing the cells of the pars distalis; Basophilic chromophils(black arrow);acidophilic chromophils(white arrow); congested capillaries (blue arrow).

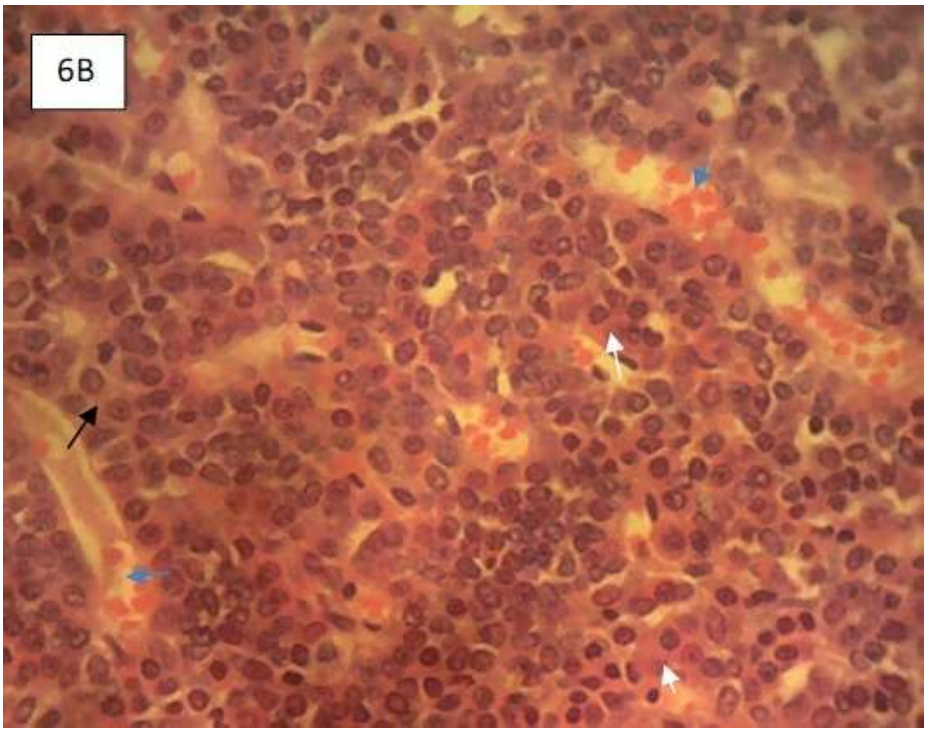

Plate 6. Photomicrograph of Group VI given 150 mg/kg -bwt of Alloxan and 150 mg/kg-bwt of metformin (H & E stain x 400) at high magnification showing the cells of the pars distalis; Basophilic Chromophils (black arrow);acidophilic congested capillaries; (blue arrow).

Conclusion

The results of this study provided evidence showing that ALEAD constitute viable phytochemical with anti-diabetic properties which accounts for its anti-diabetic potencies, which agrees with its anti-properties of its fruit pulp recorded in human and its use in traditional folk medicinal practices This research work also showed that the ALEAD has the ability to increase growth hormone level and thyroid stimulating hormone level. Further studies should be de carried out with longer time duration to confirm its mechanism of action as an anti-diabetic agent and its mechanism of action on its ability to decrease the level of TSH.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effects of the combination of *Cnidoscolus aconitifolius* and Metformin on the glycemia in streptozotocin-induced diabetes rats

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Currently, diabetes mellitus type two is a public health challenge worldwide. Even though there are many oral hypoglycemic agents, a large part of the population continues to use herbal remedies with proven benefits. However, there are few works aimed at evaluating combinations of drugs and herbal remedies. These combinations of drugs and herbal substances can lead to a decrease in the therapeutic effect of each of them. The present work was designed to evaluate the combination of Metformin with aqueous extracts of chaya (*Cnidoscolus aconitifolius*) in a group of Long Evans streptozotocin-induced diabetes rats. Several combinations of aqueous extracts of *C. aconitifolius* and Metformin were tested and glycemia was measured in streptozotocin-induced diabetes rats. Additionally, the chemical profile of the extracts was determined by high performance liquid chromatography coupled mass tandem detector (HPLC-MS / MS). Results revealed that the combinations tested suggested an antagonistic effect between both compounds since the glycemia remained high in three of the four treated groups. Some of the compounds detected in chaya extracts by HPLC-MS/MS could give a clue of the explanation of this behavior. Conclusively, the therapeutic effect of Metformin may decrease when chaya is regularly consumed as a complementary herbal remedy, as used in a part of the Mexican population. It is recommended to deepen in the future in the pharmacodynamic part to explain this behavior.

Key words: Chaya, Metformin, diabetes, antagonistic effect.

INTRODUCTION

Diabetes is a group of metabolic diseases characterized by hyperglycemia, which results from defects in insulin secretion, insulin resistance or the combined effect of both. Type 2 diabetes is the most common form of

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diabetes. It is estimated that between 90 and 95% of diabetic patients have type 2 diabetes (American Diabetes Association, 2018). To combat it, both plants and medicines have been used. It is known that many plants used in traditional medicine have hypoglycemic effects and that they help to control the effects of diabetes. Among these plants are *Ruta graveolens*, *Citrus aurantium*, *Cnidoscolus aconitifolius* and many others. In particular, it has been reported that *C. aconitifolius* has a high hypolipidemic power (Figueroa et al*.,* 2009). *C. aconitifolius,* well known as "Chaya" is a plant native to the Mayan regions of Mexico and Central America. For hundreds of years it has been used as food and as a remedy for various conditions, mainly against diabetes (Lorca-Piña et al., 2010). Valenzuela et al. (2015) have reported the use of aqueous extracts of *Cnidoscolus chayamansa* cultivated by hydroponics in a model in Wistar rats with demonstrated hypoglycemic benefits comparable to glibencalmide effects. Ramos-Gomez et al. (2017) also report finding a hypolipidemic and hypoglycemic effect.

On the other hand, the first-line drug to treat diabetes is Metformin, which has been shown to be effective both in monotherapy and in association with other oral drugs or with insulin (Salazar ÁLvarez, 2011). Additionally, it has been observed that patients treated with Metformin have a lower total and cardiovascular mortality than those treated with other oral drugs or insulin (Cases, 2008). The main mechanism of action of Metformin is the reduction of hepatic glucose production by decreasing hepatic gluconeogenesis, and in smaller proportion also increases the uptake of glucose in the muscle cell (Cases, 2008). Despite the abundant reports of Metformin and chaya as alternatives to treat diabetes, there are few studies focused on studying the possible synergism when combining them. Nowadays, many people usually consume chaya in the form of tea as an adjuvant for diabetes control, which is why we have found it important to study the effect that these extracts may have on Metformin. The objective of the present study was to evaluate the hypoglycaemic power of combination of various doses of Metformin and aqueous extracts of chaya in Long Evans rats induced to diabetes by streptozotocin.

MATERIALS AND METHODS

To determine the effect of the combination of aqueous extract of chaya with Metformin, Long Evans rats were used, of both sexes, which presented a weight of 189 ± 30 g at the time of the study. The rats were kept in individual cages with access to food and drink and cleaned daily. The ambient temperature was maintained at 25 ± 3°C respecting circadian cycles of 12 h. Throughout the experiment, the ethical guidelines for experimentation in laboratory animals established by NOM-062-ZOO-1999 "Technical specifications for the production, care and use of laboratory animals" were met. The fulfillment of the ethical aspects was certified by MVZ Gerardo del Campo G. (C.P. 975133-R. SAGARPA 10-0006). The chaya leaves were identified in the herbarium of the Interdisciplinary Center for Regional Integral Research and Development (CIIDIR) by Dr. Arturo Castro Castro (Voucher num 53,591) as *C. aconitifolius* (Mill.) I.M. Johnst from Euphorbiaceae family. (The name was confirmed in http://www.theplantlist.org/1.1/browse/A/Euphorbiaceae/Cnidoscolus/, July 5th 2019).

Fresh leaves were collected from a bush grown in a domestic garden in the city of Durango, Dgo. Mexico (25° 11' 00" N - 104° 34' 00" W and 1885 m of elevation). The bush has been cultivated directly on land. It is approximately 2 m high and shows abundant ramifications. The leaves were collected during the summer of 2018. They had an intense green color, lobed and 10 to 15 cm long. The leaves were dried in the shade naturally until a weight loss of 80% (500 ± 170 mg per dried leaf).

Chaya extracts and Metformin

Chaya extracts were obtained by boiling 7.5 mg of dry leaf in 1 L of water for 5 min. This procedure is the one that the population commonly uses. The concentration of this extract was taken as 100%. Dilutions of the extracts were made with water and administered *ad libitum*. Metformin was also used in tablets of 850 mg of PiSA brand, Code 010.000.5165.00 with registration 2992000 SSA, which were pulverized in mortar and adjusted to the required dose according to the weight. The recommended dose in humans (850 mg per day, Cases 2008) was used as the basis for calculation. Metformin was given daily at a single evening dose.

Treatments

The rats were randomly distributed into five groups of six rats each fed a Roden Chow specific diet of Purina® rodents. Group 1 served as a control group and water *ad libitum* was administered. The remaining groups were streptozotocin-induced diabetic. Streptozotocin (STZ) is an antibiotic that produces pancreatic islet β-cell destruction; therefore, it is widely used to induce type 1 and 2 diabetes in rats and mice (Furman, 2015). According to the protocol applied by Aragón and Ospina (2009), the rats were subjected to a 12-h fast, and then, intraperitoneally, they were injected with a single dose of 60 mg/kg of streptozotocin dissolved in a 0.1 M citrate buffer - pH 4.5. After checking the hyperglycemia (time zero) they were treated with combinations of Metformin and aqueous extract of chaya in two treatments as described below. Group 1 did not have any special treatment and remained healthy with food and water. Treatment 1 (T1) lasted two months counting from induction to diabetes, during which the doses specified in Table 1 were administered. At the end of this time, the second treatment (T2) was implemented for one more month as also specified in the same table.

As shown in Table 1 - Treatment 2, Groups 2 and 3 were treated only with undiluted chaya extract whereas Groups 4 and 5 were treated only with Metformin at a dose of 7.5 mg/kg. All groups were determined weekly alongside fasting glucose for 8 h. The consumption of water and food was also monitored. To determine intergroup differences during T1, an ANOVA was applied, and to determine if there were differences between the T1 and T2 treatments, the Student t test for dependent variables was applied, both with a confidence index of 95%, by using IBM Software SPSS v.22.

Chromatographic analysis

Additionally, the aqueous extract of chaya was subjected to analysis by high performance liquid chromatography coupled with triple quadrupole tandem mass detector (HPLC-MS/MS). An Agilent

Table 1. Doses of the combination of *Cnidoscolus aconitifolius* extract and Metformin in both treatments.

Group	Treatment 1 (Two months)		Treatment 2 (One month)	
	Metformin (mg/kg)	Chaya extract (%)	Metformin (mg/kg)	Chaya extract (%)
2	10	100	0	100
3	7.5	75	0	100
4	5	50	7.5	0
5	2.5	25	7.5	0

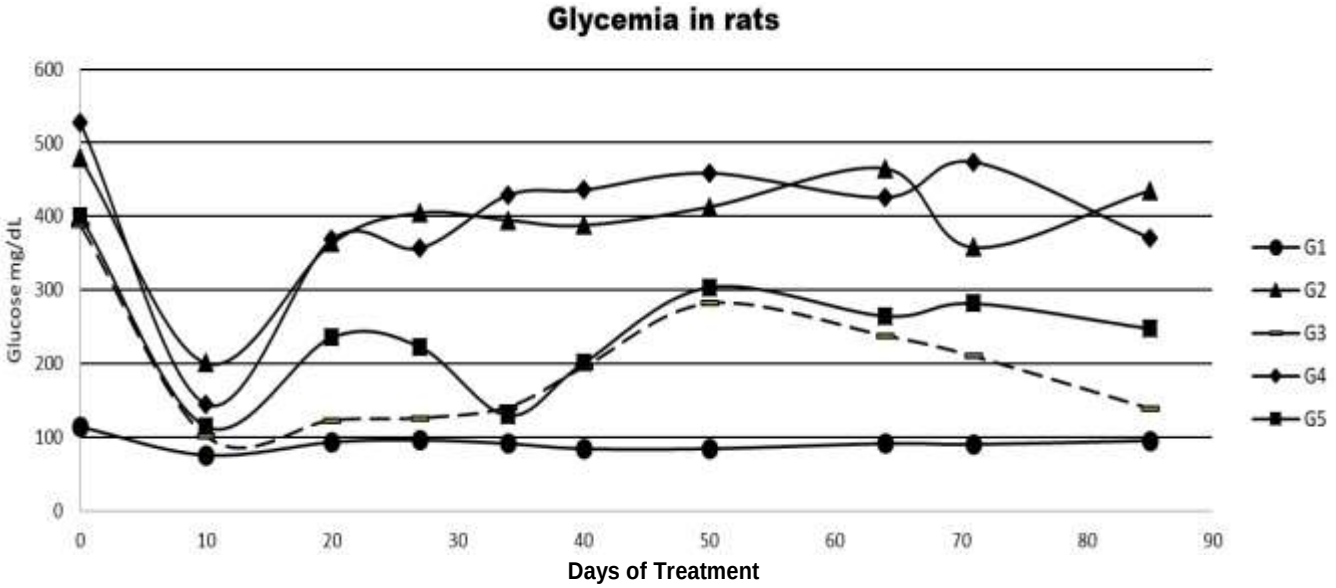

Figure 1. Average glucose tendency throughout the experiment.

1200 equipment with binary pump in isocratic regime and reverse phase was used. The mobile phase composed of an aqueous solution of 0.1% Formic Acid and Acetonitrile in proportion 65/35% v/v. To carry out the separation, a Zorbax Eclipse XDB-C18 4.5x150 mm 5 µm column was used. The extract was diluted in mobile phase in a ratio of 1:500 and was injected to the chromatograph 2 µL of the solution. We worked at an isocratic flow of 1 mL/min. The detector was used in "scan" mode at a rate of 500 scans per second. An ESI ionization chamber was used with a drying flow at 200°C with a flow rate of 13 l/min and a pressure of 35 psi. Fragmentation energy was maintained at 135 V. Both polarities, negative and positive, were used. From time zero, and at intervals of 5 min, a mass spectrum was obtained, from which the corresponding chromatograms were extracted. The total elution time was 15 min. Additionally, a sample of the water used in obtaining the extracts was injected in order to discard the masses present in the water. The compounds were identified in bases to their masses with the help of the software Merk-Index © 2001 (Cambridgesoft, Merck & Co Inc.).

RESULTS

Throughout the essay, no deaths were recorded in any of the groups. Figure 1 shows the results obtained whereas time zero (To) indicates the start of the experiment once hyperglycemia was verified by induction of diabetes. Treatment T1 covers days 0-60 whereas Treatment 2 covers day 60-90. Group 1 (healthy control) presented, throughout the experiment, an average glycemia of92.3 ± 10.1 mg/dl. However, the rest of the groups always maintained a hyperglycemia. In spite of this, it is noteworthy that Groups 3 and 5 showed a tendency to decrease glycaemia during Treatment 1. The ANOVA and Tukey tests used indicated that there was no significant difference between Group 3 and the control group ($P>\alpha$), so it could be inferred that the 75% combination of chaya + 7.5 mg/kg of Metformin seems to have a therapeutic effect, but a synergistic effect cannot be inferred.

On the other hand, the rest of the combinations seem to have no beneficial effect. During T2 treatment, in which Metformin and extracts were individually and separately administered, glycemia decreased by almost half with respect to the initial value at time zero. However, the applied Student's T test did not show differences between the T1 and T2 treatments for any of the groups. The water consumption in the control group did not present significant differences between the T1 and T2 treatments

Table 2. Phenolic compounds found in the Chaya extract

Name	Molecular weight (g/mol)	Abundance	%
Kaempferol-3-O-rutinoside	594.52	9.00E+06	100
Amentoflavone	538.45	4.50E+06	50.00
Ferulic acid	194.1	3.20E+06	35.56
Tiamine	337.27	3.00E+06	33.33
Rutine	610.5	1.90E+06	21.11
Riboflavine	376.3	1.50E+06	16.67
Arachidonic acid	304.4669	1.20E+06	13.33
Kaempferol-3-rhamnoside	481.373	1.10E+06	12.22
Naringenine	273.2	1.00E+06	11.11
Quercetin-3-O-rhamnosyl-11-glucoside	756.6587	1.00E+06	11.11
Oleic acid	284.4774	9.00E+05	10.00
Linoleic acid	280.4455	9.00E+05	10.00
Retinol	286.45	9.00E+05	10.00
Chlorogenic acid	354.3	8.00E+05	8.89
Beta carotene	356.8	8.00E+05	8.89
Catechin	290.26	6.00E+05	6.67
Astragalina	448.3	5.00E+05	5.56
Kaempferol-3-O-(2”-rhamnosyl-galactoside)-7-O-rhamnoside	740.6593	5.00E+05	5.56
Protocatechic acid	154.1	4.00E+05	4.44
Miristic acid	228.37	4.00E+05	4.44
Palimitc acid	256.4	4.00E+05	4.44
Stearic acid	256.4241	4.00E+05	4.44
Lairic acid	200.3178	2.00E+05	2.22
Ascorbic acid	176.12	7.00E+03	0.08
Caffeic acid	180.1	1.60E+03	0.02

(21 ± 8 and 24 ± 6 ml/day respectively). However, in the rest of the groups, water consumption was significantly decreased during T2 (77 ± 5 and 55 ± 10 ml/day respectively).

Thirty-two compounds present in the extracts were identified. Table 2 shows these compounds. The ionic abundance of each is presented in percentage in relation to the most abundant registered ion, Kaempferol-3-O-rutinoside (reference ion).

Figure 2 shows two of the chromatograms representative of HPLC-MS/MS analyzes. Panel A shows Kaempferol-3-O-rutinoside (Molecular weight 594.52), which was taken as a reference ion because it has the highest ionic abundance. Panel B shows the mass of arachidonic acid and its formula.

DISCUSSION

Since in Mexico there is a large number of people who use the consumption of chaya tea as an adjuvant to reduce hyperglycemia in addition to the treatment prescribed by the doctor, it is necessary to verify if this population is being treated with Metformin to indicate a better treatment. Karunaweera et al. (2015) explained that some polyphenols such as apigenin, quercetin and resveratrol have anti-inflammatory activity because they inhibit kinases by preventing the phosphorylation and translocation of factor NF-kB involved in the expression of COX-2. On the other hand, Yoshida et al. (2013) indicated that Toll-like receptors (TLR) are involved in fat-induced inflammation in adipose tissue, which contributes to the development of insulin resistance and type 2 diabetes. Therefore, the appropriate regulation of TLR expression or activation is an important strategy. In this work, Yoshida et al. (2017) demonstrated that naringenin inhibits the expression of TLR2 during the differentiation of adipocytes, suppresses the expression of TLR2 induced by the co-culture of adipocytes and macrophages and also inhibits the expression of TLR2 induced by necrosis factor. tumor α (TNF-α) by inhibiting the activation of nuclear factor-κB. It has also been shown that Naringenin inhibits the expression of TLR2 via PPAR activation. Considering these contributions, a decrease in the glycaemia was expected due to the relatively high concentration of phenolic compounds found in chaya extracts, including amentoflavone (50% based on the reference ion), which is an important hypoglycemic (Guilberth et al., 2017), naringenin and quercetin (11% based on the reference ion) as described what is the role played by Metformin when combined with

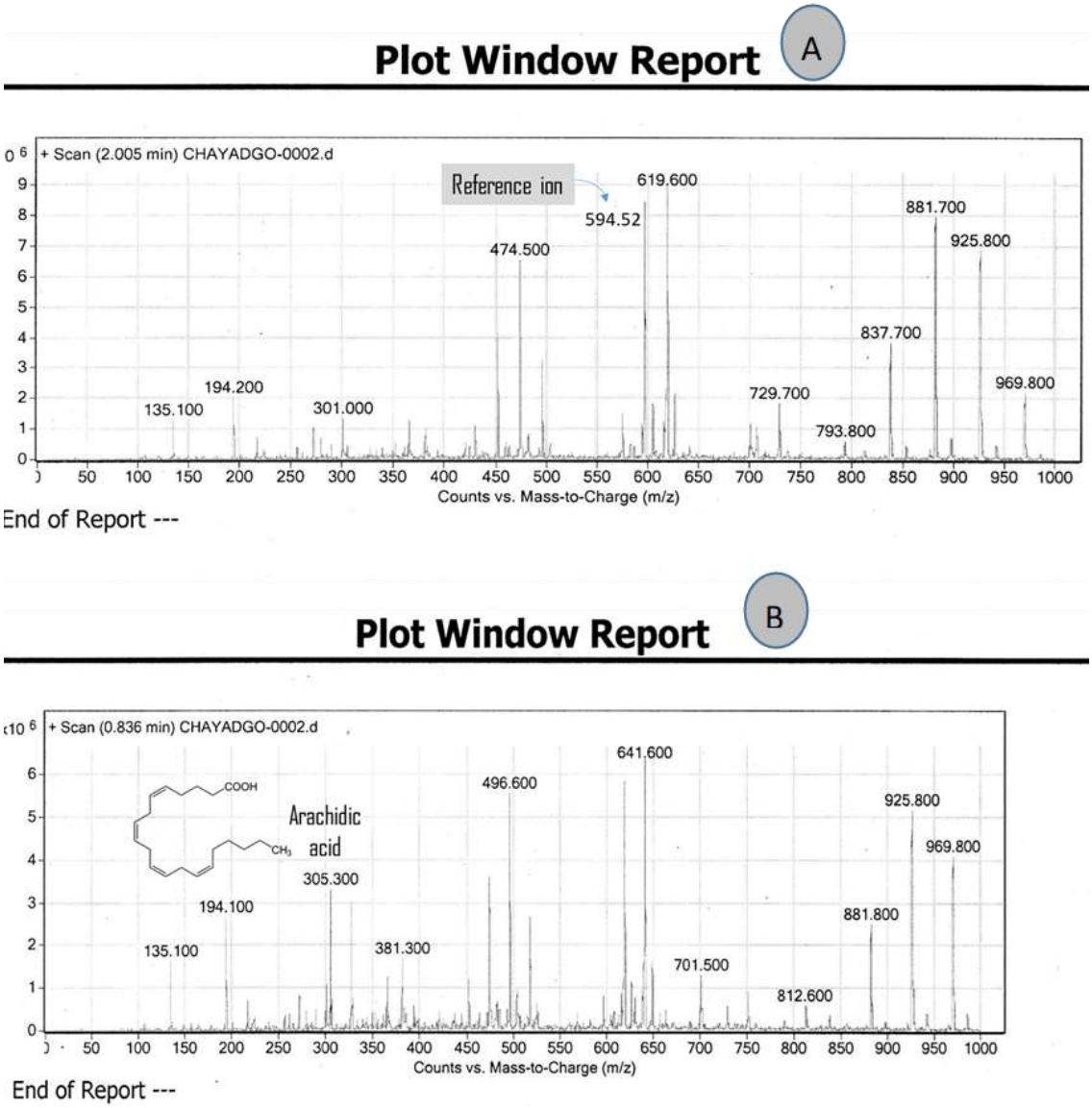

Figure 2. Representative chromatograms of the extract analysis.

Chaya extracts to prevent hyperglycemia? A clue can be found in the work of Yoshida et al. (2017) in which the effect of combinations of Naringenin with pioglitazone, which is a hypoglycaemic of the family of thiazolidinedione and which acts as a selective ligand for PPARγ, was studied. They found that narigenin attenuates the hypoglycaemic effect of pioglitazone since when combined with pioglitazone it behaves as a partial agonist of PPAR receptors, preventing its action, although it does not modify its pharmacokinetics. This means that the absorption, distribution and plasma concentration of pioglitazone is not altered; besides, it has therapeutic effect itself. Thus, avoiding the combination of foods rich in Naringenin and pioglitazone was recommended.

On the other hand, Caballero et al. (2017) has explained that oxidative stress and glycosylation of mitochondrial proteins involve the transcriptional factor NF-kB, NADPH-oxidase and the pro-apoptotic gene BAX. He explained that the NADPH generated from the metabolism of glucose plays an important role in oxidative stress through the reduction of hydrogen peroxide whose enzymatic mechanisms are associated with NF-kB, and whose expression increases in hyperglycemia. Metformin blocks these mechanisms by decreasing the expression of NF-kB and blocking the kinases involved in the activation of gluconeogenesis in the liver (Millán, 2003; Rena et al., 2017). It was observed that some of the phenols present in the extract like naringenin interfere in this action of Metformin in a similar way to that described by Yoshida et al. (2017) behaving as partial agonists in

these sites, as it was noticed in this study.

Until now, the described mechanisms of action of Metformin include the biochemical part, the action in the liver cells and at the intestinal level, but many of them remain unknown (Rodulfo et al., 2017). Although a deep search was done, many reports on the mechanisms of action between Metformin and phenolic compounds in the TLR receptors or in the activation of the nuclear factor KB and its role as enzymatic inhibitor were not found, so it is necessary to go deeper into this area. Thus, although many natural sources such as chaya have a proven hypoglycaemic power, we recommend caution in their use when combined with Metformin because Chaya could inhibit the therapeutic effect of Metformin.

Conclusion

The results obtained suggest a possible antagonistic effect when combining aqueous extracts of Chaya (commonly used in Mexican populations) with Metformin (a medicine widely used in the treatment of diabetes), so it is recommended to extend the study and alert the physician so that this is taken into consideration. Future researches are recommended in the future about the pharmacodynamics and interaction at the molecular level of the combinations as well as verify the behavior of other biomarkers.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest

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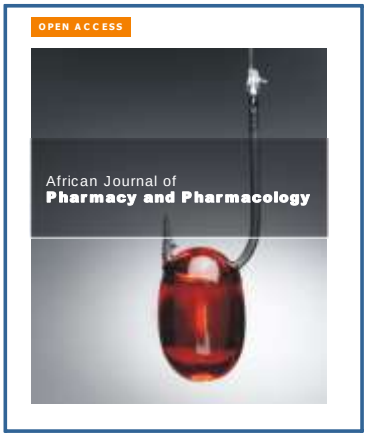

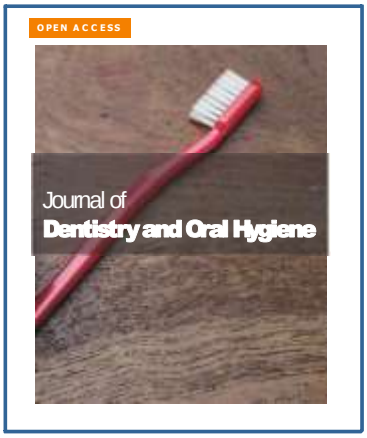

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