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

Okorie Pamela¹, Agu Francis¹, Ani Celestine²*, Alozie Ifeoma², Nworgu Choice¹, Anyaeji Pamela¹, Ugwu Princewill¹, Uzoigwe Jide¹, Igwe Uzoma³, Ejim Nnamdi² and Nwachukwu Daniel¹

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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, saponis, 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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Møller and Ts. The solution was filtered using

...tuitary hormones involved in the regulation of
dehiscent seeds and powdery pulp... T

tel is a small group depending on the soil type (Jitin et al., 2005). It
grow as solitary individuals though it can be found in
...brooklands of African savannahs, it is a very long
...rns 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,
Agban 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 absence 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 cm³) and another 5 g portion was treated with Wengers reagent (1.0 cm³) 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% FeCl₃ 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 of 1% NH₃ 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 CHCl₃ in a test tube. 3 ml of concentrated H₂SO₄ was carefully added to mixture to form a layer, an interface with a reddish-brown coloration is formed if terpenoid constituent is present (Sofowora, 1982).

Test for cardiac glycoside

Two milliliters of concentrated H₂SO₄ was prepared in attest tube. 5 g of plant sample was mixed with 2 ml of glacial acetic acid containing 1 drop of FeCl₃. The mixture was carefully added to the 1 ml of concentrated H₂SO₄ so that the concentrated H₂SO₄ 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.

\[
\% \text{ Alkaloid} = \frac{\text{Weight of precipitate}}{\text{Weight of the sample}} \times 100
\]

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.

\[
\% \text{saponin} = \frac{\text{weight of the extract}}{\text{weight of the sample}} \times 100
\]

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 FeCl₃, 0.1 ml N HCl and 0.008 M K₃Fe(CN)₆ (potassium ferrocyanide). The absorbance was measured at 120 nm within 10 min. Absorbance was traced against concentration using tannic acid standard graph.
% Tannin = \text{concentration} \times \text{dilution} \times 100

Flavonoid determination

The method of Bohm and Koczypa-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.

\[
\% \text{Flavonoid} = \frac{\text{Weight of extract}}{\text{weight of the sample}} \times 100
\]

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 \textit{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 cm³) 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 \textit{ad libitum}
- 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
of the these leaf groups are often associated with a characteristic loss of body weight as observed in these groups. It is noted that increase in body weight or decrease in body weight is observed after administration of increase or decrease. Moreover, the administration of Metformin showed a decrease in TSH levels brought as seen in Group 7 and 8. However, the significant difference in Group 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) showed a decrease in TSH whereas Groups 3, 5 and 6 showed a decrease in the serum level of TSH. These values observed in these groups were comparable 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 Groups 7 and 8 had a 4.98 and 5.48% decrease in body weight respectively, while group 9 had a 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.

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 beneficial 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 weight 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 leaf of *A. digitata*.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ethanol</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tannin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Glycoside</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Terpenoid</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Phenol</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Key: + = detected; - = not detected.

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloid</td>
<td>0.97</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>3.63</td>
</tr>
<tr>
<td>Glycoside</td>
<td>0.21</td>
</tr>
<tr>
<td>Saponin</td>
<td>1.84</td>
</tr>
<tr>
<td>Terpenoid</td>
<td>1.02</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>0.81</td>
</tr>
<tr>
<td>Protein</td>
<td>14.35</td>
</tr>
<tr>
<td>Crude fibre</td>
<td>7.22</td>
</tr>
<tr>
<td>Ash</td>
<td>1.43</td>
</tr>
<tr>
<td>Fats &amp; oil</td>
<td>10.00</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>66.19</td>
</tr>
</tbody>
</table>

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
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 intermedia 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 1](image-url)
Figure 2. Result of the percentage changes in body weight of the experimental animals.

Plate 1. Photomicrograph of control animal group administered 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/kg-bwt of Alloxan and 200 mg/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 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 600 mg/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).
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 medical 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 conducted 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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REFERENCES


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
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-Gómez 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 adjvant 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 Ospiña (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.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment 1 (Two months)</th>
<th>Treatment 2 (One month)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Metformin (mg/kg)</td>
<td>Chaya extract (%)</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>7.5</td>
<td>75</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>5</td>
<td>2.5</td>
<td>25</td>
</tr>
</tbody>
</table>

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 of 92.3 ± 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>α), 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

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

(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
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-κB, 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-κB, and whose expression increases in hyperglycemia. Metformin blocks these mechanisms by decreasing the expression of NF-κB 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

Figure 2. Representative chromatograms of the extract analysis.
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

REFERENCES


