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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

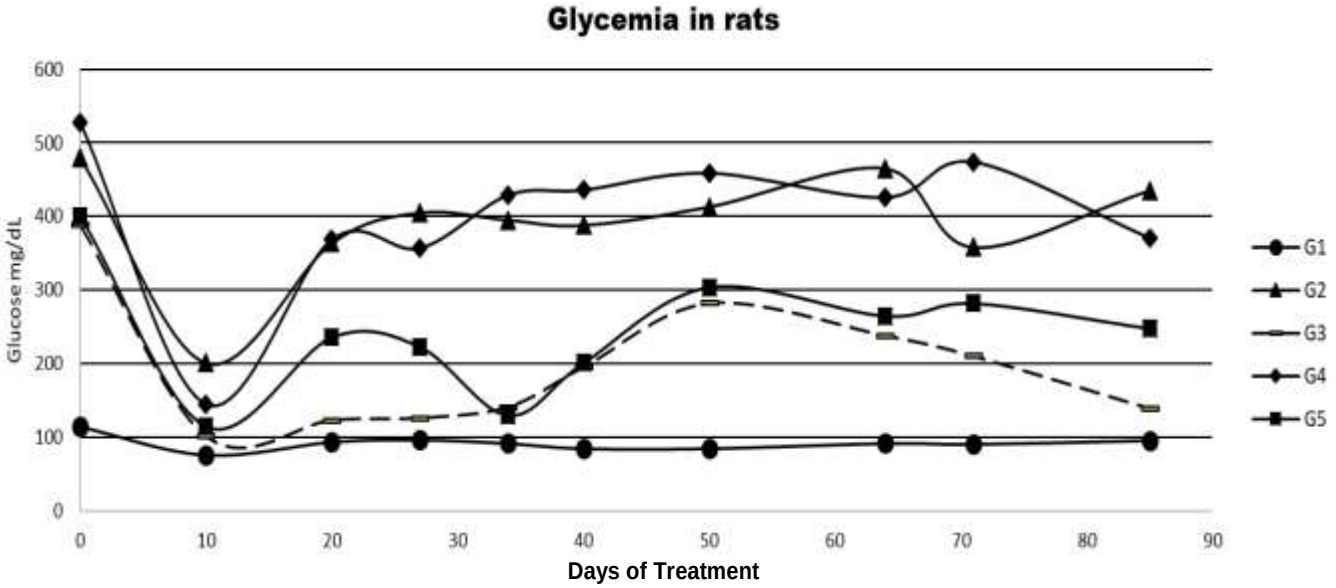

Figure 1. Average glucose tendency throughout the experiment.

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

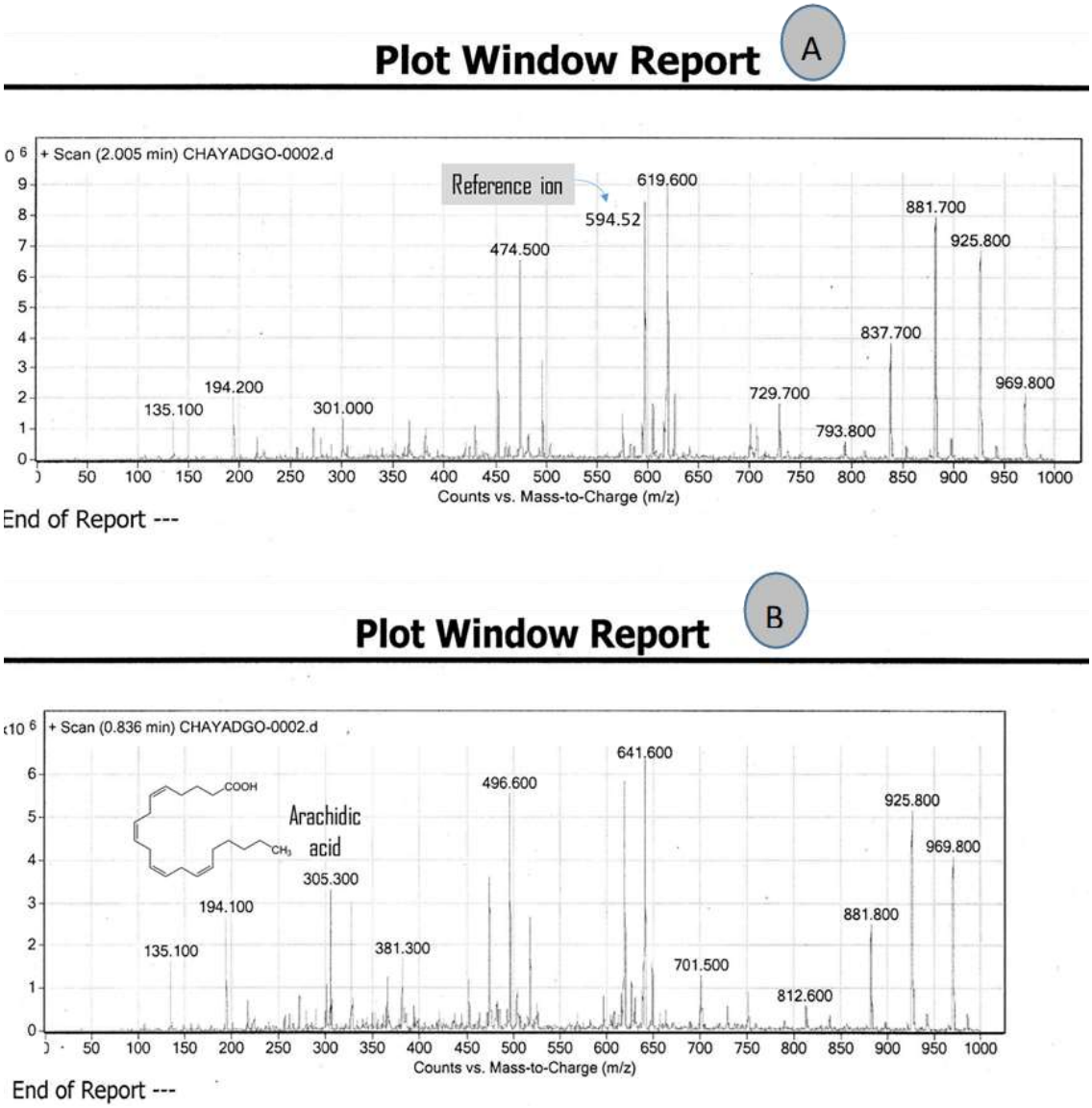

Figure 2. Representative chromatograms of the extract analysis.

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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