http://dx.doi.org/10.22161/ijeab/2.1.44 *ISSN: 2456-1878*

Evaluation of the euglycemic effect of oral administration of *S. rebaudiana* B. cultivated in Mexico in normoglycemic and induced-diabetic rats

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Abstract— *Stevia rebaudiana is a plant widely used as sweetener and food supplement. There are some reports of the euglycemic effect of S. rebaudiana extracts, which has been attributed mainly to stevioside for the results obtained by other authors. However, studies with extracts lack the precise quantification of glycosides and it is known that several agricultural and environmental factors affect the steviol glycosides content. The aim of this study was to evaluate the euglycemic effect of oral administration of extracts of S. rebaudiana varieties cultivated in Mexico in normoglycemic and induced-diabetic Wistar rats and quantify their glycosides content. Aqueous leaves extracts of Criolla and Morita II varieties of S. rebaudiana were used to quantify glycosides by a validated HPLC method or lyophilized for in vivo experiments. Hypoglycaemic effect was evaluated in normoglycemic fasting rats, subjected to intragastric administration of Criolla or Morita II (100 or 200 mg/kg) and glucose measured at time 0, 1, 3, 5 and 7 hours. The antihyperglycemic effect was first evaluated with Streptozotocin / Nicotinamide-induced diabetic rats following the methodology previously described, and further evaluated with glucose load in normoglycemic rats. Control groups were distilled water and glibenclamide (5 mg/kg). Stevioside content was 2.08 ± 0.11 and 5.22 ± 0.23 (g/100 g of dry leaves) in the Morita II and Criolla variety, respectively. Acute administration of both varieties of S. rebaudiana had no euglycemic effect in normoglycemic or induced-diabetic rats (p> 0.05) at any of the doses tested.*

Keywords— Antihyperglycemic, Hypoglycemic, glucose, Stevia rebaudiana Bertoni, Steviol glycosides.

I. INTRODUCTION

Stevia rebaudiana Bertoni is a plant native of Central and South America whose leaves contains steviol glycosides with high sweetening capacity. [1] The steviol glycosides are compounds derived from steviol, which is glycosylated at C-19 and C-13 replacing the carboxyl hydrogen with glucose, xylose or rhamnose. [2]

In 2010, the Joint FAO/WHO Expert Committee for Food Additives (JECFA) identified nine steviol glycosides: Rebaudioside A, Rebaudioside B, Rebaudioside C, Rebaudioside D, Rebaudioside E, Rebaudioside F, Stevioside, Dulcoside A and Steviolbioside, [3] however there are many more steviol related-molecules which have been reported elsewhere. [4] In *Stevia* varieties usually cultivated, Rebaudioside A and Stevioside are the glycosides found in greater quantities whereas other glycosides are found in lesser amounts. [1, 5]

Since 2008 the Food and Drug Administration in the United States (FDA) granted the status of "Generally Recognized As Safe" (GRAS) to Rebaudioside A for use as a sweetener and in the same year, the JECFA and The Food Standards of Australia and New Zealand (FSANZ) established the acceptable daily intake (ADI) to steviol glycosides at 0-4 mg/kg bw/day (based on steviol equivalents). [6] The FDA does not allow the use of crude whole-leaf extracts of *S. rebaudiana*, and only isolated steviol glycosides with a purity of at least 95% are

permitted. [7] However, in the local market dried leaves of *S. rebaudiana* can be found to prepare homemade extracts or to sweeten hot beverages and even as capsules to be consumed as food supplements.

Some studies have shown that *S. rebaudiana* extracts have an antihyperglycemic activity in diabetic rats, [8-10] and subsequent studies have attributed this to stevioside as it increases insulin production and lowers blood glucose in rats. [11-13] However, those studies performed with extracts of *S. rebaudiana* have not quantified total steviol glycosides including stevioside, and it is known that several factors contribute to the amount and type of glycosides in *Stevia rebaudiana*, such as plant variety, weather conditions, soil and extraction method. [14,15]

In a previous work, we evaluate the effect of acute administration of 10 mg/kg of extract of *S. rebaudiana* Morita II grown in Mexico, without finding any hypoglycemic effect; [16] however the dose used could be very conservative and do not represent regular consumption as it was administered intraperitoneally.

Since regular consumption of *S. rebaudiana* could be as crude whole-leaf extracts or food supplement, which glycoside content is unknown, the aim of this study was to evaluate the euglycemic effect of oral administration of extracts of *S. rebaudiana* varieties cultivated in Mexico in normoglycemic and induced-diabetic Wistar rats and quantify their glycosides content.

II. MATERIALS AND METHODS

1. Materials and reagents

1.1 Reagents

Standards of Rebaudioside B (ASB-00018227), Rebaudioside C (ASB-00018228), Rebaudioside D (ASB-00018229), Steviolbioside (ASB-00019349) and Dulcoside A (ASB-00004949) were purchased from Chromadex (Irvine, CA, USA), and Stevioside (Sigma S3572) and Rebaudioside A (Sigma 01432) were purchased from Sigma-Aldrich (USA). The standards were lyophilized under vacuum pressure of 133 x 10^{-3} bar at -40 ºC (Labconco, Kansas City, MO, USA), suspended in HPLC grade water, filtered with 0.45 um membrane and frozen at -20 °C until use. Acetonitrile and HPLC grade water were purchased from J.T. Baker (Phillipsburg, NJ, USA). Streptozotocin, Nicotinamide and Glibenclamide were purchased from Sigma-Aldrich (St. Louis, MO) and commercial Metformin was used (Glucophage ®).

1.2. Experimental animals

Wistar rats (200-250 g) were purchased from Regional Research Center "Dr. Hideyo Noguchi" at the Universidad Autónoma de Yucatán. The animals were kept under controlled temperature (24 °C) with light-dark cycle. Food and drinking water was administered *ad libitum*. All procedures were conducted in accordance with the Official Mexican Standard "Norma Oficial Mexicana NOM-062-ZOO-1999, Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio". [17]

1.3. Plant material

First cut leaves of two varieties of *Stevia rebaudiana* were collected. Morita II variety was grown in experimental field from INIFAP in Tizimín, Yucatán, México, whereas Criolla variety was grown in a farm field located in Quintana Roo, México. Leaves obtained were dried in the shade and powdered in Willey mill mesh of 1 mm and protected from light for later use.

2. Procedures

2.1. Aqueous extracts of *S. rebaudiana* Bertoni

Five hundred milligrams of leaves of each *S. rebaudiana* variety was extracted three times with 5 mL of HPLC grade water each time in a boiling water bath (100 ºC) for 30 min; extracts were cooled to room temperature and subsequently centrifuged for 10 minutes at 2,500 *x g* and 10 °C. The supernatant was transferred to a 25 mL volumetric flask and filled to capacity after the last extraction. [18] Extracts were filtered through a membrane filter (0.45 μm) to remove any solid residue before HPLC analysis or lyophilized (133 x 10^{-3} bar and -40 ºC) for *in vivo* tests on rats.

2.2. Diabetes Mellitus induction

DM Induction was performed in Wistar rats after 12 hours of fasting. Intraperitoneal injection of 65 mg/kg of streptozotocin dissolved in citrate-phosphate buffer (0.1 M, pH 4.5) was performed 15 minutes after administration of 120 mg/kg (i.p.) of nicotinamide. [19] Two weeks later, the blood glucose was measured from the tip of the tail and only animals with glucose ≥200 mg were included. [20]

2.3. Evaluation of hypoglycemic effect in normoglycemic Wistar rats

After 16 hours of fasting, lyophilized extracts of *S. rebaudiana* Criolla, Morita II (100 and 200 mg/kg), distilled water (2.5 mL) or glibenclamide (5 mg/kg) were administered to rats through an intragastric tube. Lyophilized extracts and glibenclamide were weighted at corresponding dose and suspended in 2.5 mL of distilled water immediately before being administered. Glucose was measured in the blood of the tip of the tail using a commercial glucometer Optimun Xceed (Abbott) at time 0 and 1, 3, 5 and 7 hours. [21] Glucose value at 0 h was considered as basal and percentage of change in glucose at each time were later calculated.

2.4. Evaluation of antihyperglycemic effect in induced-diabetic Wistar rats

The evaluation of the antihyperglycemic effect was made

only with the highest dose (200 mg/kg) of *S. rebaudiana* Criolla or Morita II. In induced-diabetic rats, procedure was repeated. After 16 hours of fasting, distilled water, glibenclamide and *S. rebaudiana* Criolla or Morita II were administered to rats through an intragastric tube and glucose measured at time 0 and up to 7 hours. [21] Glucose value at 0 h was considered as basal and percentage of change in glucose at each time were later calculated.

2.5. Evaluation of antihyperglycemic effect in normoglycemic Wistar rats by a glucose load

This evaluation was also performed with the highest dose (200 mg/kg) of *S. rebaudiana* Criolla or Morita II. In normoglycemic rats and after 16 hours of fasting, a load of glucose (2 g/kg) was administered through an intragastric tube 10 min before treatments. Treatments administration was set as 0 h and glucose was measured in the blood of the tip of the tail at time 0, 0.5, 1, 2, 3 and 4h. [20] Glucose value at 0 h was considered as basal and percentage of change in glucose at each time were later calculated.

2.6. Chromatographic conditions and quantification of steviol glycosides

According to JECFA (2010), [3] liquid chromatography was performed using an Agilent 100 HPLC system with a UV-Vis detector set to a wavelength of 210 nm. Chromatographic method was carried out using a Luna C18(2) 100A (Phenomenex Co., Ltd., CA, USA) column (250 mm length, 4.6 mm internal diameter and 5 μm particle size) with mobile phase of 32:68 (v/v) acetonitrile and buffer sodium phosphate 10 mmol/L (pH 2.6), and isocratic flow at 1 mL/min. The results were analyzed with the Clarity program version 2.7.3.498 (2000-2009). Standard solutions were prepared at concentration ranges of 100-500 μg/mL for Rebaudioside A and Stevioside whereas the rest of glycosides at 25–150 μg/mL. Each concentration was injected a total of six times onto the HPLC equipment. Quantification of the steviol glycosides content was based on peak area, from linear regression curves of the standards. [22, 23]

3. Statistical analysis

The analysis was performed using the statistical package Statgraphics, by t-test or ANOVA Student. Differences were considered significant with a p-value <0.05.

III. RESULTS AND DISCUSSION

Single oral administration of both varieties of *S. rebaudiana* at any dose tested, had not hypoglycemic effect in normoglycemic rats (Fig. 1). In this assay, as can be seen in Figure 1, there is a slight decrease in the percentage of change in glucose at 5 hours in all the experimental groups. As this phenomenon also occurred in the control group it could be attributed to the period of fasting reached at that time (21 hours approximate) or also be the result of mild stress caused by repeated measurement of glucose and handling of animals. [24] As expected, the group treated with glibenclamide had a marked hypoglycemia from the first hour, reaching the peak (approximately -60%) at 3 hours and remaining constant up to 7 hours.

As in normoglycemic rats, administration of *S. rebaudiana* varieties in induced-diabetic rats had no antihyperglycemic effect (Fig. 2). No variety of *S. rebaudiana* at the dose tested had any effect on glucose. As expected, glibenclamide exerted his glucose lowering effect but it is north worthy that the effect of glibenclamide was more powerful in normoglycemic rats compared to induced-diabetic rats, as glucose lowering was greater (-60 vs. -40%) and in a shorter time (1 vs. 5 hours).

Finally, as seen in figure 3, any of the varieties at the highest dose had an antihyperglycemic effect in normoglycemic rats subjected to glucose load. The only effect was in the group treated with glibenclamide, with a similar response (-60%) at 3 h as observed in single administration (Fig. 1).

The steviol glycoside content of *S. rebaudiana* B. varieties grown in the Yucatán is presented in Table 1.

It is noteworthy that the quantification of glycosides was obtained from a previously validated HPLC method, which chromatographic data and validation parameters can be consulted elsewhere. [22, 23] In this regard, all steviol glycosides are at lower concentration than previously reported, [22, 23] possibly attributed to a different batch. Comparing the varieties Morita II and Criolla, there are no differences in the content of minor glycosides, except for Rebaudioside B and Rebaudioside D; however, the most important difference are the content of Stevioside and Rebaudioside A. Morita II variety had higher content of total glycosides as compared to the Criolla variety (16.74 vs. 9.65 g/100 g) with a higher content of Rebaudioside A (4.5 times more). It is known that Stevioside is 250-300 times sweeter than sugar but with a slightly bitter aftertaste, while Rebaudioside A has more sweetener capacity (350-450 times more than sugar) without bitter taste. [1] The Rebaudioside A to Stevioside ratio is a commonly measure used of sweetness quality, [4] and as expected Morita II had a better profile with a ratio of 5.9, unlike Criolla which ratio was 0.52. Taking into account the total content of glycosides, particularly Rebaudioside A and its sweetness quality, Morita II variety cultivated in Mexico is suitable for the food industry.

http://dx.doi.org/10.22161/ijeab/2.1.44 *ISSN: 2456-1878*

The quantification of steviol glycosides in *S. rebaudiana* extracts through a validated chromatographic method, allowed to set the steviol glycoside content administered in 100 and 200 mg/kg of lyophilized extracts. Steviol glycoside administration expressed in mg/kg is detailed in table 2, considering the highest dose used (200 mg/kg).

There are studies that have evaluated the hypoglycemic effect of *S. rebaudiana* extracts, with varying results. In Wistar rats with alloxan-induced diabetes, chronic administration had hypoglycemic effect, [8, 9] but the administration in normoglycemic Wistar rats had no antihyperglycemic effect during a glucose tolerance test. [25]. Aqueous extract of *S. rebaudiana* administered (400 mg/kg) during 28 days to Sprague-Dawley rats with streptozotocin-induced diabetes had an antihyperglycemic effect through PPAR-γ mechanism. [10]

However, studies by Misra *et al* (2011) [9] as well as the Kujur *et al* (2010) [8] used extracts with another polarity and not aqueous. On the other hand, Assaei *et al* (2016) [10] although they used and aqueous extract and elucidated a mechanism, used a higher dose of extract without quantification of steviol glycosides and therefore, no comparisons can be made.

Stevioside is the molecule that has been attributed the euglycemic effects of *Stevia* as has been shown to inhibit gluconeogenesis and increase production of insulin *in vivo* [11-13] and *in vitro.* [26]

It has been reported that acute administration of a dose of 200 mg/kg of stevioside in normoglycemic Wistar rats, increases the plasma insulin concentration but has no effect on glucose tolerance during the test. [11] In this context, the administration of 100 mg/kg *of S. rebaudiana* is equivalent to 4.58 mg/kg of stevioside in Morita variety, while in Criolla variety it is equivalent to 15.21 mg/kg of stevioside (Table 2) which in both cases are much less than that the dose used by Jeppesen *et al* (2002). [11]

Administration of stevioside in diabetic rats at doses of 20 mg/kg for 14 days, [27] 25 mg/kg for 6 weeks, [12] or 30 mg/kg for 3 weeks [13] has shown antihyperglycemic effect during oral or intravenous glucose tolerance tests. In these studies the dose of stevioside are higher than that used in this study, administration was for several days and with a different diabetes model.

Nevertheless, high amounts of stevioside do not represent the usual consumption of this steviol glycoside as a sweetener since its sweetening power has been calculated as 300 times sweeter than sugar. [1] That is, the sweetness of even the small amount of 1 mg/kg of stevioside would be equivalent to using 18 grams of sugar for a 60 kg person.

The administration of Criolla variety extracts, despite its relative high content of stevioside had no effect on blood glucose levels in normoglycemic and induced-diabetic rats, suggesting that the presence of this compound in varieties grown in southeast Mexico is not sufficient to achieve an *in vivo* effect on glucose. However it cannot be ruled out that chronic consumption could have a different effect.

It is known that *S. rebaudiana* leaves also contain protein, fats and carbohydrates, diterpenes, flavonoids, tannins, among other phytochemicals, [28] not quantified in this project and that might have some effect on the regulation of glycaemia. Further studies are needed to fully characterize the extracts in addition to glycosides content.

The administration *S. rebaudiana* extracts had no effect on glucose in normoglycemic or diabetic-induced Wistar rats. The relevance of the results are that the diabetes model used in this study was more close to clinical reality, since it is considered Type 2 diabetes unlike alloxan, [29] whose model is considered type 1 diabetes; type 2 diabetes model is more relevant since this type affects 90-95% of people with diabetes. [30] Furthermore, the intragastric administration of *S. rebaudiana* resembles oral consumption through food, infusions (as aqueous extract) or even food supplements. This might suggest that the consumption of *S. rebaudiana* extract varieties cultivated in southeast Mexico is safe to be consumed by healthy or diabetic individuals, however further studies are needed.

IV. FIGURES AND TABLES

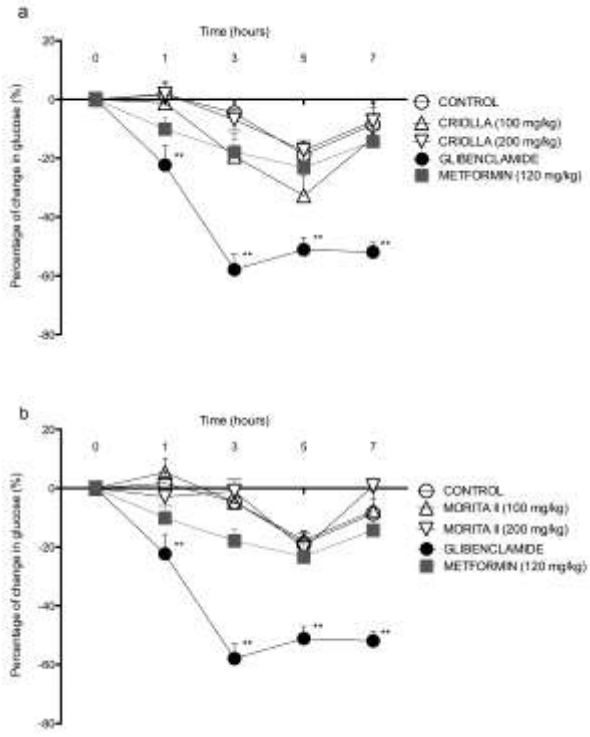

Fig.1: Evaluation of hypoglycemic effect of S. rebaudiana extracts in normoglycemic rats. Each point represents the

http://dx.doi.org/10.22161/ijeab/2.1.44 *ISSN: 2456-1878*

*average of variation in the percentage of glucose after administration of extracts from Criolla (a) or Morita II (b) variety of 5 animals ± SEM. Statistical significance by One-way ANOVA and post-hoc Tukey HSD vs controls denoted by ** p <0.01.*

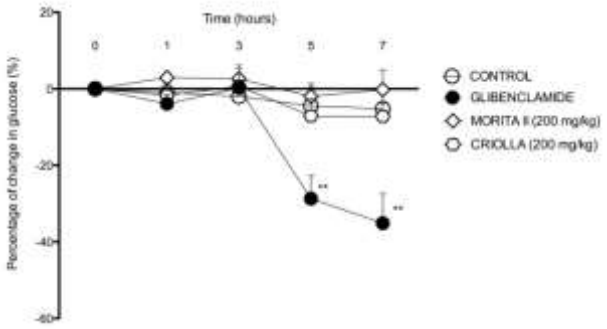

*Fig.2: Evaluation of antihyperglycemic effect of S. rebaudiana extracts in induced-diabetic rats with induced diabetes mellitus. Each point represents the average of variation in the percentage of glucose after administration of extracts from Criolla or Morita II variety of 5 animals ± SEM. Statistical significance by One-way ANOVA and post-hoc Tukey HSD vs controls denoted by ** p <0.01.*

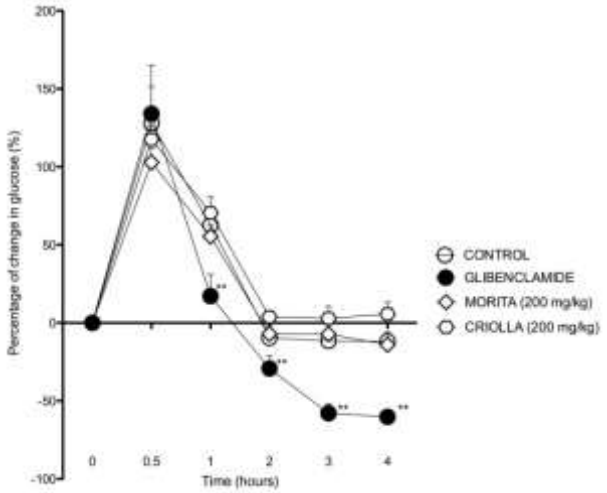

*Fig.3: Evaluation of antihyperglycemic effect of S. rebaudiana extracts in normoglycemic rats with glucose load. Each point represents the average of variation in the percentage of glucose after administration of glucose load and extracts from Criolla or Morita II variety of 5 animals ± SEM. Statistical significance by One-way ANOVA and post-hoc Tukey HSD vs controls denoted by ** p <0.01.*

Table.1: Contents of glycosides in g/100 g of dried leaves of S. rebaudiana Morita II and Criolla

Steviol glycoside	Morita II Mean ± D.E.	Criolla Mean ± D.E.
Dulcoside A	0.11 ± 0.000	0.13 ± 0.03
Rebaudioside B	0.46 ± 0.01 **	0.16 ± 0.03 **
Rebaudioside C	0.81 ± 0.07	0.56 ± 0.17
Rebaudioside D	0.67 ± 0.04 *	0.49 ± 0.06 *
Steviolbioside	0.32 ± 0.15	0.38 ± 0.11
Rebaudioside A	12.29 ± 0.27 **	2.71 ± 0.08 **
Stevioside	2.08 ± 0.11 **	5.22 ± 0.23 **

Values are expressed as mean ± standard deviation. The significant differences found in the same row are denoted denote by *p <0.05, **p <0.01 (Student's t-test).

Table.2: Steviol glycosides dose (mg/kg) administered in 200 mg/kg of lyophilized extract of Stevia rebaudiana B.

Steviol glycosides	Dose (mg/kg) according *to Stevia rebaudiana* variety	
	Morita II	Criolla
Dulcoside A	0.48	0.76
Rebaudioside B	2.03	0.93
Rebaudioside C	3.57	3.26
Rebaudioside D	2.95	2.86
Steviolbioside	1.41	2.21
Rebaudioside A	54.14	15.79
Stevioside	9.16	30.42
TOTAL	**73.74**	**56.23**

Values are expressed as mean of steviol glycoside content.

V. CONCLUSION

The highest dose orally administered (200 mg/kg) of lyophilized *Stevia rebaudiana* extract contains 30.42 and 9.16 mg/kg of stevioside and about 56 or 74 mg/kg of total steviol glycosides in Criolla and Morita II varieties, respectively, with no acute euglycemic effect in normoglycemic or induced-diabetic rats. These results suggest that it is unlikely that the use of *S. rebaudiana* leaves as sweetener or food supplement could decrease glucose in normoglycemic or diabetic people since the acceptable daily intake of glycosides is 4 mg/kg.

VI. ACKNOWLEDGEMENTS

The authors acknowledge the funding provided by the Fondos Fiscales-INIFAP and CONACyT for the scholarship provided to Aranda-González for her postgraduate studies.

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