Acute and Chronic anti-hyperglycemic effect of *Prosopis ruscifolia* extract in Normoglycemic and Alloxan-Induced Hyperglycemic Rats

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**ABSTRACT**

*Prosopis ruscifolia*, vinal, is used to treat diabetes. This work aims to study the influence of the hydro-alcoholic extract of this plant on alloxan induced diabetic rats. Hydroalcoholic extract from aerial parts of *Prosopis ruscifolia* Griseb. (Fabaceae) was prepared (Pr) and the safety was assessed to determine the acute toxicity in mice. The hypoglycemic activity of the extract was evaluated in normo- and hyperglycemic rats. Hyperglycemia was induced by intravenous administration of alloxan monohydrate (32 mg/Kg body weight). Rats with blood glucose level higher than 200 mg/dL were used for the experiment. The animals were assigned to different groups and treated with a single dose of solvent (water, *p.o.*), Pr (100 mg/Kg, *p.o.*), tolbutamide (100 mg/Kg, *p.o.*) or insulin (5 IU/kg, *i.p.*); Pr extract was also administered to normoglycemic rats (100 mg/Kg, *p.o.*). Fasted blood glucose level was measured at times 0, 1, 2, 4 and 24 h after treatment, in the acute test, and at days 0, 14, 21 and 28, in the chronic study. No evidence of acute toxicity in mice was observed. The results show that Pr extract significantly reduces blood glucose level in hyperglycemic rats (*p < 0.01*) 24 h after administration of a single oral dose of 100 mg/Kg. Treatment with Pr during 28 days showed a reduction in blood glucose level in experimentally hyperglycemic rats. Additionally, rats treated with the extract showed a reduced body weight gain. *Prosopis ruscifolia* hydroalcoholic extract showed low toxicity. After acute and chronic oral treatment was effective to reduce fasted blood glucose level, and the body weight gain was less after 28 days in the treated group.

**INTRODUCTION**

Diabetes is a chronic disease that occurs either when the pancreas does not produce enough insulin (type I diabetes) or when the secreted insulin cannot effectively be used by the body (type II diabetes). Hyperglycemia, or high blood sugar, is a common condition of uncontrolled diabetes and over time leads to serious damage, especially to the nerves and blood vessels (American Diabetes Association, 2014). Worldwide, 347 million people have diabetes. In 2014, 9% of adults, 18 years and older had diabetes. In 2012 diabetes was the direct cause of 1.5 million deaths. WHO projects that diabetes will be the 7th leading cause of death in 2030. More than 80% of diabetes related deaths occur in low and middle-income countries. Type 2 diabetes comprises 90% of people with diabetes around the world, and is largely the result of excess body weight and sedentarism (WHO, 2015). In Paraguay, diabetes showed a significant increase.

According to a survey conducted in 2011, 9.7% of the population was diagnosed with some form of diabetes, the prevalence in women being 11% and in men 7.9% (Cañete, 2011). This represents an increase, considering that in the survey conducted in 1992 it had been estimated that the prevalence of diabetes in Paraguay was 6.5% (Jiménez et al., 1998). These data demonstrate that diabetes is a disease that has major impact on the Paraguayan population, so the management of the disease demands...
lots of resources to be directed to the national health system.

The treatment of type 2 DM is based on oral hypoglycemic agents, which have characteristic profiles of adverse side effects (Lloveras Rubio, 2012). However, type II DM is also managed in Paraguayan communities using hypoglycemic medicinal plants that are affordable in rural areas and are claimed to be effective and safe. Among the plants traditionally used to control diabetes mellitus is vinal, *Prosopis ruscifolia* Griseb. (Fabaceae) (González Torres, 2012).

*Prosopis ruscifolia* (vinal) is a very thorny medium size tree that grows in subtropical regions of Bolivia’s Grano Chaco, the Paraguayan Chaco Boreal and the north of Argentina. It grows in arid sandy soils and resists drought, developing an extremely deep root system. It has arching down flexuous branches gray-green bark, strong and cylindrical, single, unimodal narrow thorns and lanceolate leaves. Its flowers are racemes, with rachis and pedicels pubescent or glabrous, yellowish to green and small. The fruit is a legume (Meloni et al., 2008).

A decoction of the leaves and stems of vinal is popularly used to cure eye irritation and conjunctivitis for ocular instillation (González Torres, 2012; Martínez Crovetto, 1981), while the indigenous population of Paraguay used this plant as hypoglycemic, to treat hypercholesterolemia (Polini and López Ramírez, 2013; Filipoy, 1994) and for weight loss (Arambarri et al., 2011).

Considering the health problems arising from diabetes in Paraguay, the high cost of pharmacological treatment and the reports about the traditional use of this plant claimed to be useful in diabetes, we proposed to study the influence of hydro-alcoholic extract of *Prosopis ruscifolia*, orally administered to normo- and hyperglycemic rats.

**MATERIAL AND METHODS**

**Plant material and extraction**

*Prosopis ruscifolia* Griseb. (Fabaceae) was collected in Laguna Hermosa, Presidente Hayes, in October 2011. A voucher specimen was deposited at the herbarium of Facultad de Ciencias Químicas, Universidad Nacional de Asunción under the number “Fátima Mereles No: 8.803”. The aerial parts were air dried and later concentrated, and the powder (950 g) was extracted three times with a mixture of ethanol:water (70:30) by a conventional reflux method for 1 h, filtered and evaporated under reduced pressure. The concentrated extract was frozen and finally freeze-dried, and used in all biological experiment.

**Experimental animals**

Female Swiss albino mice, weighing 20-30 g were used to study the acute toxicity and the general effects of the extracts. Male Wistar rats, weighing 200-250 g were used to test the hypoglycemic activity. They were fed with balanced pellet diet and water *ad libitum*, housed in plastic cage at a constant room temperature (23-25 °C), with 12:12 h light-dark cycle, in humidity controlled environment (50-60%). The animals were fasted for 12 h prior to the experiment, allowing access only to water, and deprived of both, food and water, during the experiment. All experiments were conducted in accordance with international standards of animal welfare and experimental protocol was approved by the Bioethical Committee of the Facultad de Ciencias Químicas (PI-0312). The minimum number of animals and duration of observation required to obtain consistent data were used, each animal was used once (Real Decreto, 2005).

**Drugs**

Alloxan, tolbutamide and sodium chloride were obtained from Sigma Chemical Company (St. Louis, MO, USA), Human recombinant insulin HUMULIN LILLY, pentobarbital (Nembutal) from Abbott (Japan), ethanol was purchased locally and distilled before use.

**Acute toxicity study**

The acute toxicity study was done according to OECD 420 guidelines (OECD 420; 2002). The extract was administered orally in different doses, and 24 h toxicity was recorded to identify the toxic effect, additionally all animals were observed during 14 days.

**Alloxan induced hyperglycemia and treatment**

Adult Wistar rats were kept fasted during 12 h and treated with a single intravenous injection of alloxan monohydrate (32 mg/Kg) in saline solution, through the tail vein (Dunn and McLetchie, 1943; Mason Lerco et al., 2003). After 48 h, fasting blood glucose concentration was measured by the method of the glucose oxidase enzyme. Animals with a blood glucose concentration higher than 200 mg/dL were considered to be hyperglycemic.

Before the assay, animals were left fasted since 12 h before the onset but received water during that time and during all the experiment. In the acute test, the basal glycemia (time = zero) was determined and afterwards each group received the assigned treatment. Each animal in the control group received distilled water 0.1 ml/10g of weight, p.o. and each animal in the treated group received hydro-ethanolic extract of Pr, (100 mg/Kg, p.o.); tolbutamide (100 mg/Kg, p.o.) or insulin (5 UI/kg, i.p.). The glycemia of each group was observed also at 1, 2, 4 and 24 h after the onset of the experiment (Tomita, 1963).

In the chronic test, each animal received once a day for 28 days, distilled water 0.1 ml/10g of weight, p.o. or Pr hydro-ethanolic extract (100 mg/Kg, p.o.), and were fed with 30g/day with balanced diet. The glycemia of each group was observed at 0, 14, 21 and 28 day (García et al., 2006).

**Animal blood collection and biochemical estimations**

All blood samples for fasting blood glucose level determination were collected by puncture in the tail vein. Blood was collected in heparin capillaries for micro hematocrit with capacity of 80 μl and centrifuged at 11000 g for 5 minutes.
in order to separate plasma. The plasmatic glycemia was achieved by the glucose/oxidase methodology. In this method, hydrogen peroxide formed by the catalytic action of the enzyme glucoseoxidase in D-glucose was measured by the oxidation of o-dianisidine in the presence of peroxidase (Trinder, 1969; De Sousa Menezes et al., 2007). After its separation, an aliquot of 20 μl was collected and glucose-oxidase (color reagent) was added in a glass tube. Standard solution (20 μl) + 2 ml of a glucose-oxidase solution were used as positive control and 2 ml of glucose-oxidase solution was used as control.

For estimating glycated hemoglobin, whole blood is mixed with a lysing reagent; the glycohemoglobin is separated by ion-exchange resin, the percentage is determined by measuring the absorbance at 415nm (Nuttall, 1998).

Effect of Prosopis ruscifolia on body weight

All animals were treated during 28 days with water or Pr and given 30g of food per animal; body weight was recorded every day in the early morning, before food consumption.

Statistical analysis

Results are expressed as mean ± S.D, and statistical analysis of the data was performed by Dunnet o Bonferroni’s Multiple Comparison test after one way ANOVA using GraphPad Prism 5.0 software (GraphPad Software, Inc. CA. USA). Differences were considered to be statistically significant when p < 0.05.

RESULTS

P. ruscifolia is used in Paraguayan traditional medicine as hypoglycemic. The hydro-ethanolic extract was prepared from 950 g of dried and ground leaves, yielding 108.3 g of dried material (11.4%), which was dissolved in water for the assays.

The acute toxicity was determined in female mice, treated by intragastric cannulation. After 24 h, no mortality was evidenced therefore LD50 is assumed to be greater than 2,000 mg/Kg.

Acute effect of Pr hydro ethanolic extract on glycemia of normo- and hyperglycemic rats

The acute effect of the hydroethanolic extract of Prosopis ruscifolia was established using the described methodology, six groups of rats (n=6) were used, Nv (normoglycemic, vehicle); Hv (hyperglycemic vehicle); NPr (normoglycemic, Pr); HPr (hyperglycemic, Pr); Ht (hyper- glycemic, tolbutamide) and Hi (hyperglycemic, insulin). Male Wistar rats were treated with 32 mg/Kg of alloxan in the tail vein, 48 h later the blood sugar level was determined and hyperglycemia was established (initial: 106 mg/dL ± 12.52; final: 347 mg/dL ± 59.95).

For determination of glycemia in healthy animals treated with vehicle (Nv), six males rats were previously held for a 12-hour fast. In these animals basal glycemia was determined, and kept unchanged at 1, 2, 4 and 24 h after (Table 1). A group of hyperglycemic animals was treated with vehicle (Hv). Glucose values at times 1, 2, 4 and 24 h show that there were no statistically significant changes compared to baseline (P> 0.05) (Table 1).

The influence of Pr extract on glycemia was observed in a group of normoglycemic (NPr) and in another of hyperglycemic animals (HPr). All animals were treated orally with a single dose of 100 mg/Kg of hydro ethanolic extract of Prosopis ruscifolia. The results show that the extract produced no significant variation of glycemia in normoglycemic animals. The group of hyperglycemic rats (HPr) showed a significant decrease of glycemia (p <0.01) 24 h after the treatment with Pr (Table 1).

Additionally, rats with experimentally induced hyperglycemia were treated with 100 mg/Kg of tolbutamide (Ht), glycemia was significantly reduced (p <0.05) after 4 h (Table 1).

In order to verify that the weak response to tolbutamide is due to the lack of insulin, a group of hyperglycemic rats was treated with recombinant human insulin, 5 IU per kg, i.p. (Hi). Considering that the action of insulin is quite fast, the measurement was made at 0.5, 1 and 2 h. A significant decrease of glycemia was observed (p <0.001) (Table 1).

Chronic effect of Pr hydro-ethanolic extract on glycemia of normo and hyperglycemic rats

The effect of Pr hydro-ethanolic extract on glycemia of normo- and hyperglycemic rats after chronic treatment was also determined. Four groups of rats (n=6) were used, cNv (normoglycemic, vehicle); cHv (hyperglycemic, vehicle); cNPr (normoglycemic, Pr, 100 mg/Kg, p.o.) and cHPr (hyperglycemic, Pr, 100 mg/Kg, p.o.). All animals received the treatment during 28 days and fasting blood glucose was performed on days 0, 14, 21 and 28.

The evolution of glycemia in cNv group show that no significant difference is produced between the initial glucose level (day 0) and the following (P> 0.05). Animals with induced hyperglycemia and treated with vehicle (cHv) exhibit a slight decrease in glycemia level, but this is shown as not statistically significant (P> 0.05) that is, the model of experimental diabetes is maintained for 28 days (Table 2).

The influence of a single daily oral dose of Prosopis ruscifolia in normoglycemic animals (cNPr) was then analyzed, the results show that administration of the extract did not induce statistically significant difference in fasting blood glucose levels (P > 0.05) during the period of 28 days. Therefore, Pr hydro-ethanolic extract does not affect the glycemia of normoglycemic animals; during the observation period (Table 2).
Finally the influence of chronic treatment with Pr extract on glycemia of experimentally hyperglycemic animals (cHPr) was evaluated. After 28 days of treatment, a statistically significant reduction on glycemia is observed (p < 0.001). The average value of glycemia at 14 days was 98 mg/dL and this level remained unchanged until the end of the experiment (Table 2).

### Effect on HbA1C and body weight

HbA1c values after 28 days indicated a statistically significant increase in hyperglycemic experimental groups treated with vehicle (cHv, P < 0.001) and treated with extract (cHPr, P < 0.001) and a statistically significant decrease in the group of normoglycemic animals (cNPr, P < 0.05) compared with the initial value. No difference was observed in the percentage of HbA1c in normoglycemic animals treated with vehicle (cHv) gained weight gradually, statistically significant at days 21 (P < 0.05) and 28 (P < 0.01). On the other hand, normoglycemic (cNPr) and hyperglycemic (cHPr) animals treated with the extract body weight remained unchanged (P > 0.05). It was also observed that the animals in both groups treated with the extract did not consume all the daily ration of food.

When comparing the weights of the different groups, with the cNv group, no statistically significant difference with cHv group was observed (P > 0.05), and there is a difference with cNPr groups (day 21: P < 0.05; day 28: P < 0.001) and cHPr (day 21: P < 0.01, day 28: P < 0.001; Figure 2).

**DISCUSSION**

Evaluation of acute toxicity in mice demonstrates that Pr extract does not show acute lethal toxicity, but the chronic toxicity should be evaluated.

Experimental models of induction of diabetes in rats are widely used by researchers around the world (Szkudelski, 2001). Alloxan has been found to be selectively toxic to pancreatic beta cells.
cells as it preferentially accumulates in the beta cells as glucose analogues. In addition, the cytotoxic action of alloxan is mediated mainly by the generation of reactive oxygen species (ROS). Alloxan and the product of its reduction, dialuric acid, has been noted to establish a redox cycle with the formation of superoxide radicals, which undergo dismutation to hydrogen peroxide ($H_2O_2$) and more highly reactive hydroxyl radicals are formed by the Fenton reaction. Further, the massive increase in cytosolic calcium concentration ultimately causes rapid destruction of beta cells of pancreatic islets (Rohilla and Ali, 2012).

The required dose of alloxan to induce diabetes depends on the species and strain of animal, the route of administration and nutritional status of the animals (Szukedelski, 2001; Rohilla and Ali, 2012). We established a dose of 32 mg/Kg administered by tail vein, to induce a hyperglycemia ranging 191-440 mg/dL. The success of hyperglycemia induction was about 40%, and it is in agreement with reported data (Dunn and Mcletchie, 1943). In all experimental groups male rats were used, to avoid hormonal interference.

The normal level of glycemia in Nv group kept unchanged during all the experiment as well as the hyperglycemia in Hv group.

The effect of Pr on glycemia of normo- (NPr) and hyperglycemic (HPr) rats was assessed using 100 mg/Kg, this dose was selected from the acute toxicity test, reducing twenty times the maximum dose used. After oral administration of Pr extract in normoglycemic animals, no statistical difference was observed at any time considered. It should be noted that there is not an effect of hypoglycemia in NPr animals and it has a significant value because we can assume that the effect occurs only with an excess of glucose. The results of Pr extract on hyperglycemic animals showed a significant reduction in blood glucose at 24 h (P <0.01). In the group of hyperglycemic animals treated with tolbutamide (Ht), in order to maintain the same experimental protocol, we proceeded to the measurement of blood glucose at 24 h and as expected, no significant changes was verified compared the initial value, because tolbutamide has a half-life shorter than 6 h (Powers and D’Alessio, 2011). After oral drug administration, experimentally hyperglycemic animals reduced significantly their glycemia only after 4 hours, the weak response to tolbutamide suggests the limited availability of insulin to be released by this drug. On the other hand, the response to insulin was good and according to what was expected, this highlights the limited availability of insulin to be released.

Additionally, while with tolbutamide the glycemia is reduced in 68.7 mg/dL (- 20%) at time 4h, with Pr extract this is reduced in 152.2 mg/dL (- 41.8%) at time 24. The common feature of both groups is the apparent lack of availability of insulin, this results indicates that Prosopis ruscifolia seems to improve the response to insulin. Comparing blood glucose levels of the different experimental groups in the acute test, as observed in **Figure 3**, there is a highly significant difference (P <0.001) between the level of glycemia between Nv and Hv, and a significant difference (P <0.001) is also found when compared HPr and Hv. However, besides there is significant difference (P <0.001) between Nv and HPr, after 24 h this difference is reduced (P <0.01). Additionally, the difference between Hv and HPr became extremely significant (P <0.001) after 24 h. On the other hand, no difference was found between Nv and NPr.

When comparing experimental groups in the chronic study, between cNv and cHv, a highly significant difference (P <0.001) is observed, indicating that both, normo- and hyperglycemia are maintained during the observation period. The statistical comparison between groups cNv and cNPr, showed no statistically significant differences, indicating that the treatment of normoglycemic animals with the extract does not affect the glycemia. The most encouraging results were seen in cHPr when compared with cNv; a statistically significant difference (P <0.001) was observed on days 14th, 21st and 28th, that is, a significant reduction of glycemia of animals in group cHPr is produced. Moreover, the glycemia drops to normal (Zúñiga et al., 2011) at this time, and it is the same until the end of the experiment on day 28th. This result shows that chronic treatment with Prosopis ruscifolia extract can reverse the hyperglycemia in alloxan induced hyperglycemic rats.

The initial value of HbA1c of the group is 7.74%, no difference was found in the cNv (8.298%) and cNPr group decreases (5.48%, P < 0.05). The cHv and cHPr groups significantly increased (13.58%, P < 0.0001 and 12.09%, P < 0.0001, respectively). Glucose combines with hemoglobin continuously and irreversibly during the half-life of erythrocytes; therefore, the percentage of glycosylated hemoglobin is proportional to the mean plasma glucose level of the last 6-12 weeks (Wallach, 1998). This would explain the high values of HbA1c of hyperglycemic group treated with extract.

The weight of the vehicle treated animals increased, unlike vinal treated animals, which maintained their weight during treatment. Prosopis ruscifolia extract prevents weight gain in both normoglycemic animals and those with experimental hyperglycemia. Apparently this happens by reducing appetite,
considering that these animals did not eat the entire daily ration of food. This finding is agreed with the popular use of this plant.

_Prosopis ruscifolia_ leaves show the presence of flavonoids, tannins, alkaloid and saponins (Arambarri _et al._, 2011; Chiale _et al._, 1982; Gianinetto _et al._, 1975; Cercos, 1951). All these compounds had exhibited hypoglycemic activity. Some triterpenoid saponins have been cited as having hypoglycemic effect, like _Calendula officinalis_ L. (Compositae) methanolic extract and butanol soluble fraction (Connolly and Hill, 2001; Yoshikawa _et al._, 2001). The flavone Isorarinetin together with chlorogenic acid, extracted from the _Cecropia obtusifolia_ (Cecropiaceae) exacerbate the hypoglycemic effect (Andrade-Cetto and Wiedendeld, 2001) and flavonoids were described as agents that increase insulin release of islets of Langerhans β in concentration-dependent manner (Koshy and Vijayalakshmi, 2001). Moreover, the extracted polyphenols in green tea, containing residues of tannins as gallocatechin, epicatechin, epigallocatechin and epicatechin gallate were reported with hypoglycemic effect (Sabu _et al._, 2002).

Finally, other _Prosopis_ species have been tested in search of hypoglycemic activity. _P. cineraria_ demonstrated antihyperglycemic potential in mice after 45 days of treatment (Sharma _et al._, 2010) and in rats treated for 12 weeks (Sharma and Si, 2013). On the other hand, _P. glandulosa_ efficacy was tested in a rat model of diabetes and insulin resistance, and showed effectivity increasing insulin levels and reducing blood glucose level (George _et al._, 2011).

**CONCLUSION**

The present study has shown that _Prosopis ruscifolia_ exhibited low toxicity after oral acute administration, and that it has a strong effect lowering fasting blood glucose level in alloxan induced hyperglycemic rats after acute and chronic treatment. The results found in this work agree with traditional use of this plant. The compound to which the activity is attributed has not been established. Further research is needed to allow us to completely understand the way the extract make the glucose blood level lower.

**Competing interests:** All authors have none to declare.

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