

REGULAR ARTICLE

A study of hypoglycemic effects of *Azadirachta indica* (Neem) in human blood cells

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Abstract

The present investigation was conducted with an objective to validate the effect of the plant *Azadirachta indica* (neem) in human blood cells in a normoglycemic medium. This hypoglycemic effect had been attributed by the communities to this plant. In this study, aqueous extract of the plant was used in a concentration of 6.4% m/v and from this, following doses were prepared: 0.01 mg; 0.05 mg (low doses); 0.1 mg; 0.175 mg (medium doses); 0.7 mg and 1.4 mg (high doses); also it was used insulin Humulin R[®] in different concentrations (0.1, 1, 10 nM) in order to compare both effects in a normoglycemic medium with human blood cells. The results demonstrated a hypoglycemic effect when the levels of glucose concentration went down in the normoglycemic medium in relation to the control. There was a significant effect ($p < 0.001$) for the high doses group. These findings validate the hypoglycemic effect of this plant attributed by the communities.

Key words: Aqueous extract, Human blood cells, Hypoglycemic effect, Neem, Normoglycemic medium

Introduction

Azadirachta indica, also called neem, is an Asiatic origin arborous plant, specifically from the region from India to New Guinea; it belongs to the meliaceae family, its height varies from 6 to 25 m, and the trunk diameter ranges from 1 to 1.6 m. This plant was introduced in Venezuela in 90's, and it's widely spread along the country (Reyes et al., 2003). In Aragua state is very abundant and used as an ornamental plant.

Popular belief attributes to this plant, *A. indica*, with a lot of pharmacologic effects such as fungicide (Viveros and Castaño, 2006), insecticide (Reyes et al., 2003; Iannacone et al., 2005), antimicrobial (López et al., 2007), antihelminthic (Avello et al., 2006), as well as hypoglycemic effects (Urdaneta, 2001). It's used frequently by

diabetic patients who are looking for therapeutic alternatives instead the conventional pharmacologic treatment (Urdaneta, 2001).

Several surveys about the neem hypoglycemic effect in animals had been carried out around the world, usually by using rats and mice, all the findings had shown significant hypoglycemic effect (Akinola et al., 2010; González et al., 2010; Isea et al., 2011; Manish et al., 2010), besides, Akinola et al. (2010), also found that lesions in pancreatic islet cells had improved in the neem treated experimental group.

In this research, the aim is to validate the hypoglycemic effect of this plant in human blood cells in normoglycemic medium, and verify the pharmacologic effects attributed by popular belief and surveys carried out in animals. Depending on this, there will be more reliable therapeutic alternatives that contribute to the diabetes mellitus treatment.

Materials and Methods

Plant sample collection

The botanical sample used in this studio was collected from the leaves taken from one single neem tree located in the garden of the University of

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Carabobo (Universidad de Carabobo) campus Aragua. It was identified by the "Victor M. Badillo" herbarium of the Central University of Venezuela (Universidad Central de Venezuela) campus Aragua.

Human blood samples

The blood samples were taken from adults in good health conditions under their approval.

Vegetal sample process

Once the sample was collected, the leaves were washed, dried at ambient temperature, and then were dried again at 45°C for 24 hours. After this, the leaves were triturated manually by using a ball mill.

Preparation of the aqueous extract

32 g of the pulverized vegetal material were dissolved in 200 ml of saline solution at 0.9%, and then cooking for 30 minutes in a heating iron at 150°C. It was keeping at ambient temperature to get colder, and then the sample was separated in small fractions, it was centrifuged for 10 minutes at 3000 rpm. The supernatant was filtered with Whatman paper N° 2 and then was stored and preserved at -20°C. The concentration of the extract was 6.4% m/v, from which were prepared the following doses: 0.01, 0.05, 0.175, 0.5, 0.7 and 1.4 mg (Martínez et al., 2010).

Leucocytes separation by centrifugation

To carry out the human blood cells separation, were taken 5 ml of blood from donors forearm vein in good health conditions, this samples were placed in a tube with ethylene diamine tetra acetic anticoagulant (EDTA), 4 ml of each sample were used for leucocytes preparation, and the remaining 1 ml was taken as a control and was not centrifuged. The process to obtain leucocytes was started with the centrifugation of 4 ml of blood for 5 minutes at 2700 rpm (Beckman® rotor JA-20), the plasma was dismissed and then it was taken 1 ml from the first layer of cells, in which there are the biggest quantity of leucocytes, it was divided in two 500 µl aliquots (A and B) which were washed with 600 ml of saline phosphate buffered to dismiss the plasma remainder. The count of cells was made with a Mindray BC-2300 hematological analyzer. To carry out the glucose consume test, was selected the aliquot (A or B) in which was obtained the highest leucocytes concentration.

Tests of glucose consume

Some tests of glucose consume were carried out using different quantities of *A. indica* aqueous

extract, insulin was used as a control, because is the most powerful endogenous hypoglycemic.

Some samples of 1×10^6 leucocytes, at 37°C were incubated for a range of time between 0 and 10 minutes, in 1 ml of a suitable ionic medium (Hepes-KOH 20 mM pH 7.5; buffered phosphate 5 mM, pH 7.5; KCl 2.5 mM, NaCl 70 mM and Mg_2SO_4 2.5 mM) with normoglycemic conditions (100 mg/dL) and an additive that could be insulin Humulin R® in different concentrations (0.1; 1 y 10 nM) or aqueous solution of the phytomedicine (3, 9, 18, 36 y 72 µg). Once the incubation started at 37°C, aliquots of 40 µl were taken in each essay at increasing times from 0 minutes until 10 minutes (0, 1.5, 3.5, 5 y 10 minutes), these aliquots were centrifuged at 4000 rpm for 2 minutes with a micro centrifugation machine Eppendorf® 5415. 5 µl of supernatant were taken and mixed with 500 µl of the glucose reactive (Bioscience®) following the manufacturer instructions, absorbency of the different media were registered by using a spectrophotometer Beckman DU 650.

Determination of the glucose concentration

The quantification of glucose was made using the method of Trinder (Braham and Trinder, 1972).

Data analysis

The spectrophotometric readings, to determine the glucose levels in the medium treated with insulin and aqueous extract of the plant *A. indica*, were tabulated using MS Excel software, and some comparative bar and curve graphics were done to show the glucose value obtained as well as the percentage of the consume of the insulin and the plant extract.

The test carried out corresponds to an experiment design with eight treatments, seven doses and a control, under a random blocks experimental design, the people sampled represents the blocks, repeated observations were taken at some times (0, 1.5, 3, 5 and 10 minutes). One strategy to analyze this experiment is the split-plot design analysis of variance, where the main plot is referred to the treatments and the secondary plot is referred to the repeated observations along the time. The multiple comparisons of means were carried out using the Tukey's test. These analyses were carried out by using the statistical software Statistix 8.0 and Minitab 14.0.

Results

Hypoglycemic effect of the *A. indica* aqueous extract

In these tests the insulin was used as an additive in the normoglycemic medium, to verify

the efficiency of the test designed to evaluate the glucose consume. In the figure 1A it can be noted a progressive increase of the glucose consume in the medium, when it was added the insulin in a concentration of 10 nM since 1.5 until 5 minutes, in which is observed a quick glucose consume. Since the minute 5 until 10 the glucose consume becomes constant. The figure 1B shows at 10 nM of insulin, the glucose consume is 27.7% at 3.5 min, whereas after 10 min the consume reach 40%.

In the Figure 2, a mild hypoglycemic effect of the aqueous extract de *A. indica* at different doses after 10 min of reaction is observed. The

hypoglycemic effect of the extract started to be observed at the dose 0.7 mg, but the biggest effect was obtained at 1.4 mg.

In the Figure 3, the action of the high doses of the aqueous extract at different times of reaction is observed, the consume with 0.7 mg of extract starts since the 3 minutes, decreasing the concentration of glucose at 89.8 mg/dL at 10 minutes, to 1.4 mg of the aqueous extract the consume starts at the minute 1.5, reaching 65.7 mg/dL at 10 minutes.

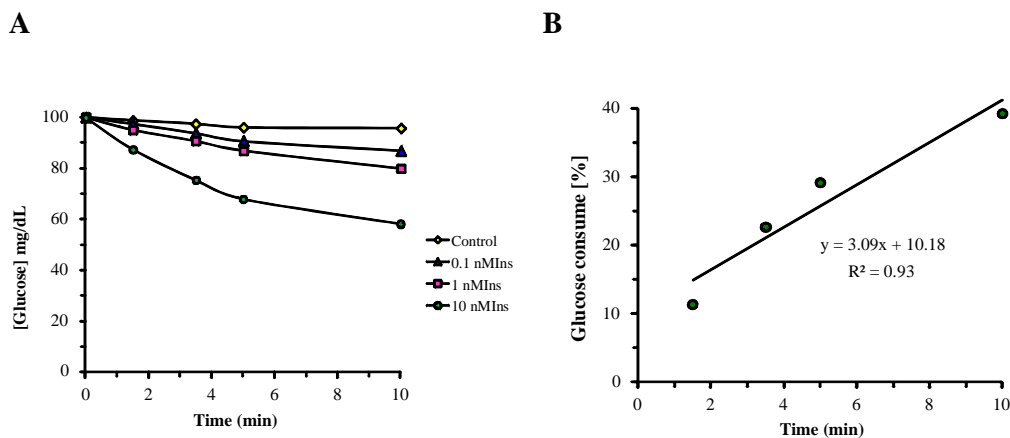


Figure 1. A) Glucose consume with different concentrations of insulin in relation to the control. B) Percentage of glucose consume with 10nM of insulin.

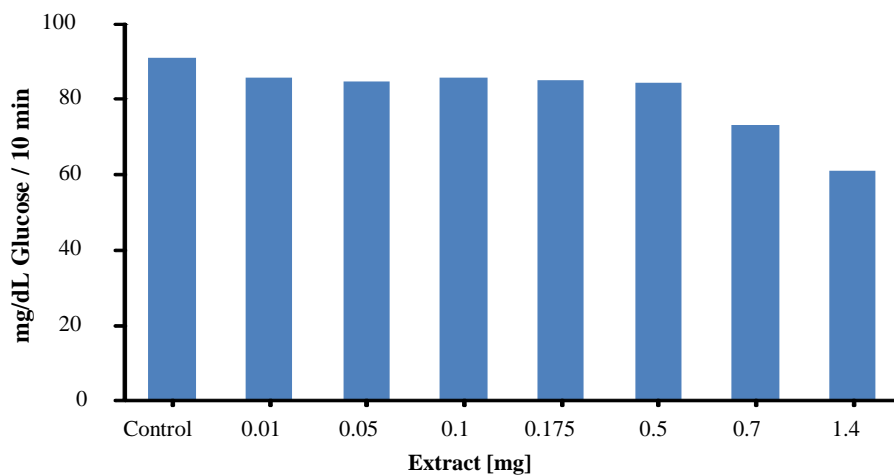


Figure 2. Effect of different quantities of *A. indica* aqueous extract at 10 min of reaction.

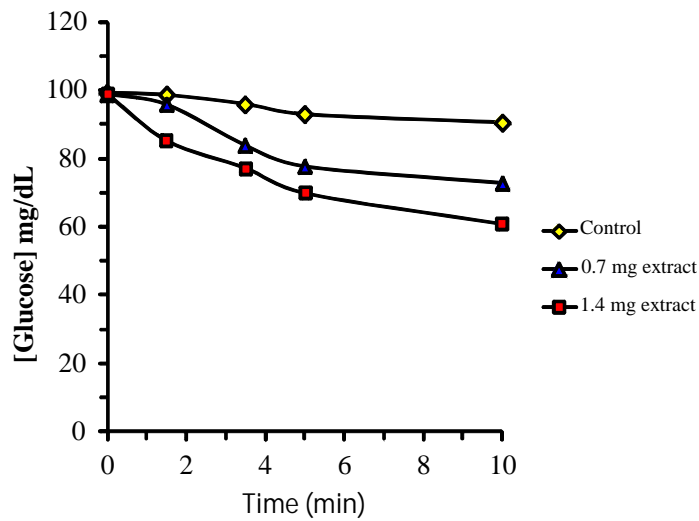


Figure 3. Hypoglycemic effect at 0.7 mg and 1.4 mg of *A. indica* extract.

This reduction in the concentration of glucose in the medium by the effect of the extract is represented as the consumed percentage of glucose in the figure 4, in which is observed 26.6% of the consume for the doses of 1.4 mg of the aqueous extract of neem at 10 min, which is an analogous result to the percentage of insulin consume, 27.7%

at the minute 3.5 and 29.2% at 10 min, seen in figure 1B. Once it was added the doses of 0.7 mg of the extract, it was obtained a consumed percentage of glucose of 15.4% at 10 min as it is shown on the Figure 5.

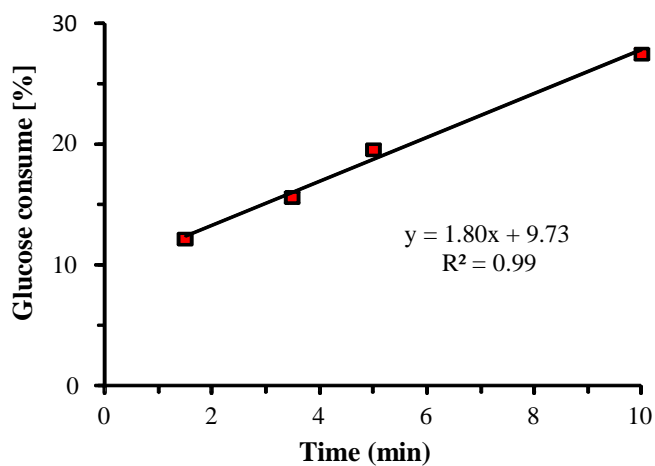


Figure 4. Percentage of glucose consume with 1.4 mg of *A. indica* aqueous extract.

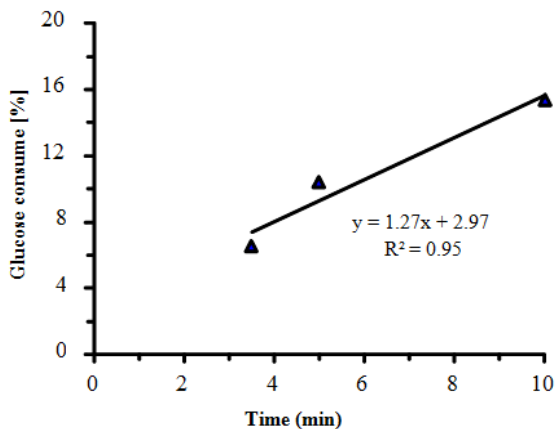


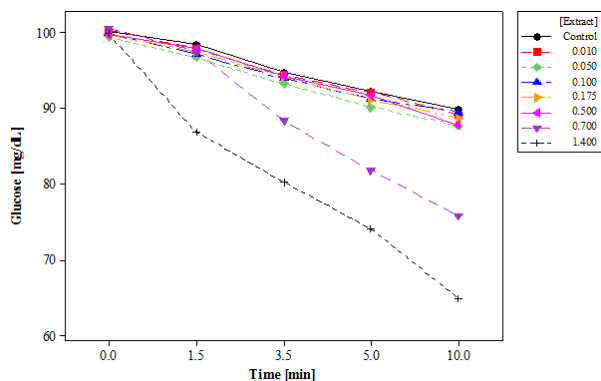
Figure 5. Percentage of glucose consume with 0.7 mg of *A. indica* aqueous extract.

The analysis of variance for the glycaemia concentration showed significant differences between the doses ($p < 0.0001$) which indicates that the glucose reduction was not homogeneous for the different treatments. The results of the means comparison with Tukey's test for the treatments are shown in the Table 1.

Table 1. Tukey's test comparisons for the treatments.

Doses (mg)	Average	Homogeneous group
0	95.180	A
0.01	94.840	A
0.1	94.400	A
0.05	94.360	AB
0.175	94.307	AB
0.5	93.493	B
0.7	88.860	C
1.4	81.220	D

Note: Treatments with the same letter are statistically homogeneous.



The first five doses 0, 0.01, 0.1, 0.05 and 0.175 mg, were statistically equals to the control sample, the three remaining samples, 0.5, 0.7 and 1.4 mg, showed a middle reduction of glucose, also it was found differences statistically significant for the different times of reaction ($p < 0.0001$), which indicates that the decrease of glucose levels resulted modified by the time factor, see Table 2.

Table 2. Tukey's test comparisons for time factor.

Time (minutes)	Average	Homogeneous group
0	100.03	A
1.5	96.29	B
3	91.73	C
5	88.14	D
10	84.22	E

Note: Treatments with the same letter are statistically homogeneous.

The average content of glucose reduction decreased along the time and a minimum apparently was not reached. The analysis of variance showed significant differences for the interaction doses-time ($p < 0.0001$), which indicates that the treatments were not homogeneous at different times and the behavior was not the same for the different concentrations of aqueous extract of the plant.

The Figure 6 shows that the treatments were homogenous until the doses 0.5 mg approximately. Only with the doses 0.7 and 1.4 mg it can be observed a significant change in the angle of the line, which it means that these two treatments are, specifically, which produces more quickly the biggest glucose reduction in the medium.

Figure 6. Interaction of glucose concentration and time.

Discussion

The test *in vitro* carried out with the aqueous extract of the plant *A. indica* in a normoglycemic medium with human blood cells evidenced a hypoglycemic effect, it was confirmed once did was observed the decrease of the concentration of glucose in relation to the control sample.

The extract doses applied, showed some differences in the glucose consume, so they were classified in groups according to the doses: small doses (0.01 and 0.05 mg), it was not observed glucose consume in the medium; middle doses (0.1, 0.175 and 0.5 mg), it was observed a non-significant decrease of the glucose levels in the medium; and as a high doses (0.7 and 1.4 mg), in this case, it was shown a continuous glucose consume, with a significant value since the five minutes until ten minutes. The concentrations 0.7 and 1.4 mg of the extract produced a hypoglycemic effect with a consume percentage of 15.4% and 26.6%, respectively in ten minutes.

As time passes, and increases the concentrations of the aqueous extract of the plant, the hypoglycemic effect is bigger. It may suggest that in high doses of the extract and in a longer time of reaction, the glucose consume will increase by the cells in the normoglycemic medium.

This results are different from the obtained in another research, in which was evaluated the hypoglycemic effect of the plant *Bixa orellana L.* in rabbits (Martínez et al., 2010). When taking the glycaemia measure, pre-test and post-test of the rabbits, it was not evidenced a significant difference; that's why it was studied the hypoglycemic effect of the plant with the realization of test *in vitro* in a hyperglycemic medium (11.1 mM glucose) in human blood cells, having as an observation a decrease in the glucose concentrations in relation to the control sample at small doses of the extract of the plant in the first minutes of reaction, whereas the doses 0.7 and 1.4 mg were considered not reliable, because they showed a decrease of the percentage of consumed glucose.

The results obtained with the aqueous extract of *A. indica* are different of other results, where the hypoglycemic effect of the extract of the plant *Petiveria alliacea L* was analyzed (Rojo et al., 2002), regarding the glucose consume of the erythrocytes in a cultivation medium, demonstrating there was not decrease in the glucose levels, due to the action of the extract of the plant, because it did not affected the glucose consume by the erythrocytes.

The hypoglycemic effect of the plant *A. indica* had been studied before in animals (Akinola et al., 2010; Ali et al., 2003; González et al., 2010; Isea et al., 2011; Manish et al., 2010), usually in rats and mice, finding statistical significant neem extract effect, but not in *in vitro* systems using human blood cells in medium with glucose, like the research done in this case, in which it was obtained a significant result, when the glucose levels decreased significantly in the medium, as wells as the obtained by the surveys carried out in animals.

Based on the results obtained in the tests with the aqueous extract of the plant *A. indica*, in a normoglycemic medium with human blood cells, it is considerate that some posterior test should be done in order to extend the research about the hypoglycemic effect of this plant, such as to use a hyperglycemic medium to evaluate the hypoglycemic effect of the aqueous extract of *A. indica* under this condition, or using concentrations and incubations with longer time intervals than the used in this research, and carry out researches that let know the action mechanism of the hypoglycemic effect of the *A. indica* aqueous extract.

Conclusions

It is concluded that the aqueous extract obtained from the leaves of the plant *A. indica*, produce a hypoglycemic effect observed through the determinations of glucose in the normoglycemic medium.

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