Antidiabetic activity of Annona crassiflora (Annonaceae)

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Researches have shown that marolo (Annona crassiflora Mart.) contains many biologically active phytochemical classes with antidiabetic properties. Therefore, in order to obtain scientific data that contribute to a better insight of the species, integrating both safety and efficacy to the practice of using the plant as medicine, the study of A. crassiflora Mart. leaves was chosen. To evaluate the possible effect of A. crassiflora Mart. in diabetic rats, blood glucose levels, concentrations of fructosamine, urea, creatinine, ASL and ALT was determined in serum samples from animals treated with 300 mg of hydroethanolic extract 70% (v/v) per kg of animal weight. Glucose levels were determined by Trinder reaction. Dosages of urea and creatinine were held using the Jaffé method for creatinine and the enzyme method colorimetric for urea in automated apparatus (HumaStar 80, Brazil/Alfenas). The concentration of fructosamine was measured by a commercial kit kinetic method (analyzes ®) and the reading was held in Bioplus® (Brazil-Alfenas). Based on the results obtained in this study, it can be concluded that administering the extract, according to the adopted protocol, did not change the biochemical parameters that were analyzed in the normal and diabetic rats’ serums.

Key words: Medicinal plants, phytochemical, diabetes mellitus, pharmacological, hydroethanolic extract.

INTRODUCTION

Medicinal plants are defined as those capable of producing active ingredients that treat several diseases and hence achieving an important role in global health (Lima et al., 2014). Studies show that, in Brazil, about 91% of the population makes use of some medicinal plants, and 46% of them keep these plants homegrown (Ethur et al., 2011). These data indicate that many Brazilians use natural sources as an alternative to health. Thus, the spreading of the necessary knowledge for a rational use of these medicinal plants is important (Vilar et al., 2008). According to Oliveira and Menini (2012), studying...
medicinal plants, starting from their employment by the communities, can provide useful information in the development of pharmacological and phytochemical studies about plant species. This way you can plan the research beginning with an already existing empirical knowledge, often enshrined by continuous use, which must have its actions known for being scientifically proven.

As many of the studies with medicinal plants are developed based on therapeutic information obtained from folk medicine, the *Annona crassiflora* species, belonging to the Annonaceae family, arouses the interest of researchers for being widely used in traditional medicine (Luzia and Jorge, 2013).

*A. crassiflora* is a native species from the Brazilian Cerrado and is widely distributed in this biome, being found in the states of Bahia, Minas Gerais, São Paulo, Mato Grosso do Sul, Mato Grosso, Goiás and Tocantins. It is popularly known as araticum, marolo, pinha-docerrado (cerrado pinecone), cabeça de negro, etc. The fruit of this species is highly appreciated by local people, being found in many domestic orchards and consumed fresh and/or used in the manufacture of compote, sweets, jams, ice cream, juice and liqueurs (Luzia and Jorge, 2013).

According to Luzia and Jorge (2013), the *A. crassiflora* leaves and seeds’ infusion is used in traditional medicine as a tonifier, astringent, antimicrobial, antidiarrheal as well as in fighting against syphilis, rheumatism, treating wounds, snake bites and pediculosis. Acetogenins were identified in this species, with cytotoxic, antimicrobial (Biba et al., 2014), antitumogenic and antiparasitic properties (Vilar et al., 2008); alkaloids with antimicrobial and leishmanicidal activity (Oliani, 2012); and phenolic compounds with antioxidant and hypoglycemic action (Barbalho et al., 2012).

Diabetes mellitus is a multiple etiology metabolic disorder characterized by elevated levels of blood glucose, which leads to carbohydrate, lipid and protein metabolism abnormalities, leading to acute and chronic complications due to failures in insulin secretion or action or in both situations (Surya et al., 2014).

Until 2010, the estimated number of diabetics in Brazil was 12 million and in 2012, about 4.8 million people died as a consequence of this disease. The high morbidity and mortality rates of diabetic patients are mainly attributed to the chronic complications induced by hyperglycemia, that compromise productivity, quality of life and survival of human beings (Sbd, 2012).

Glycemic control is involved in the pathogenesis of these changes. There are leukocyte dysfunctions, with adhesion, chemo taxis, phagocytosis abnormalities and intracellular killing. There is also a decrease in the spontaneous activation and neutrophilic response when compared with non-diabetic patients (Boronat et al., 2010).

Furthermore, there are studies saying that hyperglycemia or the presence of glycation end products (AGEs) lead to a state of persistent activation of polymorphonuclear leukocytes, which induces a spontaneous oxidative chain activation and myeloperoxidase, elastase, and other neutrophilic component release, being able to become the least responsive polymorphonuclears when stimulated by pathogens (Boronat et al., 2010).

Therefore, throughout the studies reported in literature and realizing the pharmacological potential of the substances derived from the Annonaceae family, as well as the reports of few studies, from the pharmacological point of view, of many of its species, and certifying the importance of the *Annona* gender, studying the *A. crassiflora* species was chosen in order to seek scientific data and contribute to a better knowledge of the species, adding safety and efficacy to the practice of using this plant.

**MATERIALS AND METHODS**

**Collection and identification of the plant**

The chosen plant material was the *A. crassiflora* leaves, collected at 16 o’clock, on October 2013, in the city of Paraguaçu-MG, Por do Sol neighbourhood. The plant was identified and confirmed by Prof. Dr. Geraldo Alves da Silva and a voucher specimen of the botanical material was deposited in the Herbarium of the Federal University of Alenas, Alenas Campus-MG and registered under number 2538.

**Plant extract preparation**

The collected *A. crassiflora* leaves were arranged in thin layers, being dried in an air circulation and renewal kiln at 45°C, for 72 h. The time required for a complete drying was determined when the sample weight of 10 g leaves remained constant over three consecutive weighing with an analytical balance. The oven temperature was controlled by a thermostat and a thermometer, assuring uniformity in the temperature used during drying. After drying, the plant material passed through a rough division followed by spraying in a Wiley mill.

Then, the obtained powder underwent a previous tumescence with 70% (v/v) ethanol, outside the percolator, for 30 min, after which the percolator containing the mixture (602.62 g of pulverized plant drug + 70% v/v ethanol) was packed. The packing was done as homogeneously as possible, avoiding bubble formation in the content allocated within the percolator.

Ethanol (70%) was gradually added until covering the powder surface and the mixture was left for 48 h maceration and after this period, percolation in flow was carried out at 2.0 ml/min. The hydroethanolic extract 70% (v/v) was obtained by simple percolation, according to Prista et al. (2008). After extraction, the leachate was concentrated on a rota evaporator at 50°C.

The concentrated hydroethanolic extract 70% (v/v) was transferred to tared glass bottles and stored in the refrigerator until the moment of drying it in a lyophilizer. The freeze-dried extract was used for carrying out a biological assay at a concentration of 0.3 g/ml, always prepared at the moment of administration.
Animals

Twelve week old, male Wistar rats, weighing between 300 and 250 g, obtained from the animal facilities of the Federal University of Alfenas (MG UNIFAL), were kept at a temperature of 23°C, throughout the trial period, with a light-dark period of 12 h, receiving commercial feed ad libitum and water.

The procedures were performed in accordance with the ethical principles for animal experimentation, adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by the committee of ethics in animal research at the Federal University of Alfenas, under number 523/2013.

Experimental design

After a week of acclimatization, 56 animals were divided into seven groups (n = 8): non-diabetic animals treated with distilled water (Group 1); diabetic animals treated with distilled water (Group 2); non-diabetic animals treated with the extract (Group 3); diabetic animals treated with the extract (Group 4); non-diabetic animals treated with metformin (Group 5); diabetic animals treated with metformin (Group 6); diabetic animals treated with both the extract and metformin (Group 7).

On the 22nd day, the animals that had fasted for 12 h were anesthetized using sodium pentobarbital in a concentration of 40 mg/kg and all of the blood was collected by cardiac puncture and distributed into silicon test tubes without any additives and with separator gel to obtain the serum. Then, the animals were euthanized by cervical dislocation.

Diabetes mellitus induction

The induction of diabetes mellitus was conducted in animals that kept fasting for 12 hours by administrating alloxan intraperitoneal in a dosage of 150 mg/kg and all of the blood was collected by cardiac puncture and distributed into silicon test tubes without any additives and with separator gel to obtain the serum. Then, the animals were euthanized by cervical dislocation.

Administration of 70% (v/v) hydroethanolic extract and standard metformin

The hydroethanolic extract 70% (v/v) was reconstituted in water and administered by gavage for 21 days, beginning the treatment, a week after the diabetes induction and its confirmation by blood glucose testing. The administrated amount of hydroethanolic extract 70% (v/v) was 300 mg of extract per kg of animal weight. The concentration of the final solution was 0.3 g/ml, where the appropriate volume for each animal’s weight was calculated (Dhanabal et al., 2004).

The standard hypoglycemic (Metformin 850 mg, Eurofarma®) was dissolved in 0.9% saline solution at a concentration of 10 mg/kg body weight. The employed metformin concentration was established based on a study by Kumar et al. (2011).

Biochemical parameters

The glucose concentration was monitored using blood glucose collected from the rat-tails, 7 days after the alloxan administration. After collecting the heart blood, by cardiac puncture, the fasting glucose level was determined by the enzymatic method based on the Trinder reaction in automated apparatus (HumaStar 80, Brazil/Alfenas). The fructosamine concentration was measured by a commercial kit through kinetic method (analyzes®) and the reading was performed in Bioplus® (Bio 2000).

The urea and creatinine dosages were carried out in a commercial kit, by the Jaffé method for creatinine and the colorimetric enzymatic method for urea in the automated apparatus (80 HumaStar, Human®) (Burtis and Ashwood, 2011). The liver function assessment was performed by evaluating the activity of aspartate amino transferase (AST) and alanine amino transferase (ALT) by the kinetic methods in an automatic analyzer, HumanStar 80 (Burtis and Ashwood, 2011).

Statistical analysis

The results were expressed as mean and standard deviation, submitted to the one way analysis of variance (ANOVA) and compared by the Scott-Knott (1974) test at a significance level of 5%. The analyses were performed using the system for variance analysis (SISVAR) statistical program.

RESULTS AND DISCUSSION

Blood glucose and fructosamine assessment

Treating the animals with alloxan induced hyperglycemia in the animals in groups 2, 4, and 6 however, the extract intake did not change the glycemic profile in the serum taken from the animals in this study (Figure 1). Several studies on Annona, have been conducted to evaluate the effects of the hydroethanolic extract on the glycemic profile, but the results are still controversial (Rout et al., 2013). These differences can be attributed, at least in a part, to variations in the harvest (regions of the country or time of year) and it is well known that the chemical constituents of plants vary according to soil moisture and climatic conditions. There is a possibility that the active hypoglycemic agents would be more focused in other parts of the plant. The adopted experimental models can also affect the obtained results (Pepato et al., 2001).

It is shown in literature that certain phenolic compounds, present in extracts, can reduce hyperglycemia. However, in this study, treating the animals with the extract did not induce significant changes in the hyperglycemia of the animals belonging to the different analyzed groups. This divergence could be attributed to differences in content, profile and bioavailability of polyphenols present in different extracts (Rao et al., 2001).

Metformin is an antihyperglycemic, from the biguanide class, used to treat type 2 diabetes mellitus. However, its action mechanism is not fully known yet. Studies have shown that metformin activates the AMP-activated kinase protein (AMPK) in rat hepatocytes and skeletal muscle (Misra and Chakrabarti, 2007). Recent researches indicate that AMPK plays a role in the glucose and lipid metabolism regulation (Zou et al., 2004).
Figure 1. Fasting glucose assessment in Wistar rats. The determinations were done on serum samples obtained after euthanasia. The results present the mean ± standard deviation for each group with n of 8 animals. Averages with different letters are statistically different. ANOVA was determined followed by the Scott-Knott test with p<0.05 as significance. Source: Carla Pereira Rosa

The AMPK activation, in the liver, results in an increase in the oxidation of fatty acids and decrease in the production of glucose, cholesterol and triglycerides. This effect contributes to the decrease in glycemia, improves the metabolic profile and causes an increase in the glycogen in the liver and muscles as well as promotes benefits to the blood lipid profile (Zou et al., 2004). Metformin administration did not bring blood glucose levels to near normal (Group 6) at a 10 mg/kg dosage. According to Rout et al. (2013), metformin at a concentration level of 300 mg/kg was able to reduce the levels in hyperglycemic rats. Group 6 showed statistically lower blood glucose than Group 4, that is, metformin was shown to be more effective than the extract. Although, statistically not less than or equal to Group 1, the results of group 6 when compared with those of group 2 suggest a hypoglycemic action of metformin, because diabetes presented itself in a severe form (plasma glucose greater than 400 mg/dl) which does not often produce hyperglycemia in a diabetic patient (Mazzanti et al., 2003). The determination of the glycation end products, such as frutosaminas, has been used to evaluate the metabolic control in diabetic patients, as it reflects the glycemic control in a range of 2 to 3 weeks. With the obtained results for the frutosaminas dosage, it can be seen that protein glycation has not occurred (Figure 2).

Renal function assessment

Urea is the main product of protein metabolism. It is synthesized in the liver from CO₂ and ammonia, circulates in the blood and is filtered in the kidneys, where most of it is excreted in the urine. For being more sensitive to primary changes in kidney conditions, it is a marker that has a strong significance in cases that involve them. Nevertheless, it is not as specific as creatinine in the assessment of renal function, since it is influenced by the person’s hydration degree and diet (Burtis and Ashwood, 2011).

Creatinine is a phosphocreatine degradation product in the muscle, that is, often produced at a constant rate, being directly proportional to the muscle mass. It assesses the glomerular filtration rate, since its concentration in the blood increases as renal filtration rate is reduced (Burtis and Ashwood, 2011). Both urea and creatinine levels in the serum of animals, belonging to the different groups, were evaluated. A significant difference between the levels of urea and creatinine in the control animals (Group 1) and diabetic animals’ (Groups 2, 4, 6 and 7) serums are as shown in Figures 3 and 4, respectively. No significant differences were observed related to urea and creatinine serum levels when Group 4 was compared to Group 2.

Renal damage is a chronic microvascular complication very common in diabetic patients (Bastos et al., 2010).
Figure 2. Assessment of frutosaminas concentration in Wistar rats. The determinations were carried out in serum samples obtained after euthanasia. The results present the mean ± standard deviation for each group of 8 (n) animals. Averages with different letters are statistically different. ANOVA was determined followed by the Scott-Knott test with p<0.05 as significance. Source: Carla Pereira Rosa.

Figure 3. Urea level assessment in the serum of Wistar rats. The determinations were carried out on serum samples obtained after euthanasia. The results present the mean ± standard deviation for each group of 8 (n) animals. Averages with different letters are statistically different. ANOVA was determined followed by the Scott-Knott test with p<0.05 as significance. Source: Carla Pereira Rosa.

and in animal models of diabetes induced by alloxan (Dornas et al., 2006). These damages have been associated with glycemia and/or elevated glycated protein concentrations (Moresco et al., 2013).

It is known that in cases of renal damage the urea and creatinine levels have to be high. In the present study, it has been observed that the extract administration was not able to maintain the serum creatinine levels in values comparable to those of non-diabetic animals (Group 1) suggesting that the extract did not show a beneficial effect in the maintenance of a glomerular filtration rate in diabetic animals. These findings could be attributed to the
Creatinine levels (mg/dl)

**Figure 4.** Evaluation of creatinine levels in serum from male Wistar rats. The determinations were carried out on serum samples obtained after euthanasia. The results present the mean ± standard deviation for each group of 8 (n) animals. Averages with different letters are statistically different. ANOVA was determined followed by the Scott-Knott test with p<0.05 as significance.

failure in the glycemic control, presented by these animals, as assessed by measuring blood glucose.

The extract administration has not been able to stop the increase in serum urea levels. Despite being more sensitive than creatinine, the urea determination is well known to be less specific in the glomerular filtration evaluation since its serum levels may rise due to non-renal causes such as dehydration, increased protein catabolism, reduction of blood volume, among others. Therefore, it is important to note that besides a possible dehydration, induced by polyuria as a result of the hyperglycemia in diabetes mellitus, protein catabolism may be more pronounced. These two situations could contribute to an increase in tubular reabsorption or urea synthesis, respectively (Burtis and Ashwood, 2011). Thus, the urea results observed in this study could be justified by the possible presence of such factors, associated with renal dysfunction induced by diabetes and lower specificity of this marker.

It is also observed that there was no significant increase in the plasma creatinine concentration in normal and diabetic animals, treated with the extract (Groups 3 and 4, respectively) compared to the diabetic (Group 2) and control (Group 1) groups suggesting lack of toxicity in the plant constituents since, they may produce toxic effects in the kidneys shown by the rise in plasma creatinine and urea concentrations in diabetic and non-diabetic animals as demonstrated in the study by Bwititi et al. (2000).

**Assessment of liver function**

ALT and AST enzyme activity dosage allows to check the presence of changes in the permeability of hepatocytes, since ALT is found mainly in the cytoplasm, whereas 80% of the AST is present in the mitochondria of these cells (Toledo et al., 2013). Given that the species of the genus *Annona* are rich in different chemical compounds, such as phenols, alkaloids, glycosides and sterols; the effect of hydroethanolic extract 70% (v/v) (ExHd) was also evaluated on the activity of AST and ALT enzymes (Table 1).

In the diabetic animals (Group 2), liver enzymes AST and ALT were elevated indicating liver dysfunction caused by the alloxan action, which in general can cause disruption or permeative action to the membrane of hepatocytes (Agbai and Nwanegwoe, 2013). In our data, it can be seen that the concentration of the extract cannot interfere in ALT and AST values, since it did not demonstrate a trend in reducing the levels of these enzymes and the ALT and AST values of Group 4 are statistically higher than Group 1.

Therefore, according to the results, it can be inferred that the ingestion of 300 mg/kg of extract from leaves of *A. crassiflora* for 21 days, cannot exert oxidative action in kidney and liver tissue. On the other hand, it suggests that there is no damage to hepatocytes in dose and extract of the period, based on the comparison of Group
with Group 3.

Conclusions

Treating the diabetic rats with the hydroethanolic extract 70% (v/v) from A. crassiflora leaves neither reduced blood glucose nor glycation of plasma proteins, estimated as fructosamins. The extract administration showed no beneficial effect on the maintenance of glomerular filtration rate in diabetic animals. The elevation of liver enzymes (AST and ALT) was observed in diabetic rats indicating hepatic dysfunction, and with the possibility of necrotic damage in this organ and the use of the extract did not cause statistically significant difference between diabetic groups for the activity of liver enzymes.

Due to the limitations in the methodology used in this study, the results and comments presented herein are based only on data obtained in the experimental conditions of this study, not to incur the risk extrapolations that are not relevant.

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REFERENCES


