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Annona muricata L. extract decreases intestinal glucose absorption and improves glucose tolerance in normal and diabetic rats

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ABSTRACT

Introduction: Poorly controlled hyperglycemia causes numerous health complications. Postprandial hyperglycemia is an important indicator of diabetic status. The aim of this research was to evaluate the effect of *Annona muricata* L. extract on the *in vitro* intestinal glucose absorption in diabetic rats and *in vivo* antihyperglycemic activity in both normal and diabetic rats.

Methods: Phytochemical screening of the aqueous extract from the leaves of *A. muricata* was carried out. Albino rats were randomly assigned into normal and diabetic groups. Each group was divided into three subgroups: control (vehicle), experimental (*A. muricata*) and standard (Metformin) groups, to determine antihyperglycemic activity at different times after glucose overload. The everted intestinal sac technique was used to study intestinal glucose absorption in diabetic rats.

Results: Aqueous leaf extract of Peruvian *A. muricata* exhibited statistically significant ($P < 0.05$) *in vivo* antihyperglycemic activity in both normal and diabetic rats when compared to control group. The magnitude of the effect was similar to metformin treatment. Moreover, the aqueous leaf extract of *A. muricata* significantly diminished *in vitro* intestinal glucose absorption, with a magnitude similar to metformin treatment. Phytochemical analysis of the aqueous extract revealed the presence of tannins, flavonoids, alkaloids, and leucoanthocyanidins, among others.

Conclusion: This study reveals that *A. muricata* aqueous extract is able to reduce *in vitro* intestinal glucose absorption and improve oral glucose tolerance in rats.

Implication for health policy/practice/research/medical education:
Annona muricata leaf extract exhibited significant antihyperglycemic activity by restricting intestinal glucose absorption, which suggests that *A. muricata* may be a source of new antidiabetic drugs.

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Introduction

Diabetes mellitus (DM) is a complex and chronic metabolic disease that requires continuous medical attention with multifactorial strategies to reduce risks beyond glycemic control (1). It is estimated that around 463 million people around the world, or 9.3% of adults aged 20 to 79, have DM. If the trend continues, by the year 2045, 700 million people will have DM (2).

Some medicinal plants have been shown to improve glycemic control by different mechanisms, making their phytoconstituents potential therapeutic or complementary medicine agents (3,4). *Annona muricata* L. (Family: *Annonaceae*), also known as Graviola, Soursop, and Guanabana in Hispanic America, is a tree that reaches up to 10 meters in height. It has compact foliage and simple leaves. Its fruit has a juicy white edible pulp containing glossy black seeds. It is indigenous in tropical areas in America including the Amazon basin (5). All parts of the *A. muricata* tree have ethnopharmacological uses. The leaves are used for the treatment of cancer (6,7), pain, inflammation (8), insomnia, diabetes, headache, and cystitis (9), the crushed seeds are used as anthelmintic,

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the fruits and the flowers are used for colds (10), the bark, roots and leaves are used for their antispasmodic, hypotensive, antihyperglycemic, and sedative properties (10).

Although studies have evaluated the effect of *A. muricata* on glucose metabolism, the causes of its hypoglycemic effect, such as its impact on intestinal glucose absorption, is lacking. The aim of this study was to determine the activity of *A. muricata* leaves on postprandial blood glucose levels in normal and alloxan-induced diabetic rats and *in vitro* intestinal glucose absorption in diabetic rats.

Materials and Methods

Chemicals and drugs

Alloxan monohydrate was purchased from Sigma-Aldrich (St. Louis, MO, USA) and metformin was purchased from Farmindustria (Lima, Peru). All other chemicals and solvents were of analytical grade.

Plant material

Annona muricata leaves were collected from the Rosa de Los Ríos Martínez Botanical Garden of Medicinal Plants of the Universidad Nacional de Trujillo (8°6'43.2" S 79°1'43.68" W) at 34 m elevation, located in Trujillo, Department of La Libertad, Peru. The identification of the leaves was confirmed by the Herbarium Truxillense (HUT) of the Universidad Nacional de Trujillo, Peru (Voucher specimen: 58837).

Preparation of aqueous extract and phytochemical screening

Annona muricata leaves were washed with water and dried under shade for 6 days until reaching a constant weight. After that, they were crushed and pulverized. The aqueous extract was obtained by decocting 50 g of leaves in 500 mL of boiling water for fifteen minutes. The resulting aqueous extract was filtered and evaporated in a rotary evaporator at 40°C (yield: 7.2%). The dried extract was stored at 4°C in an amber bottle.

Phytochemical screening was carried out according to the methodology described by Lock (11). Standard colorimetric and precipitation assays were performed to identify phytoconstituents such as flavonoids, saponins, tannins, alkaloids, anthraquinones, naphthoquinones, anthocyanidins, phenolic compounds, steroids, and triterpenes present in the extract (Table 1).

Table 1. Semi-quantitative phytochemical screening of aqueous extract from *A. muricata* leaves

Phytoconstituents	Test name	Results
Flavonoids	Shinoda	+++
Tannins	Jelly/Gelatin	++
Alkaloids	Mayer	+++
	Wagner	++
	Dragendorff	++
Leucoanthocyanidin	Rosenheim	+++
Saponins	Foam	-
Phenols	Ferric chloride	++
Steroids	Liebermann-Burchard	+
Quinones	Borntrager	-

(-): absence; (+): low; (++): moderate; and (+++): high levels.

Animals

Forty-eight Sprague-Dawley rats (8-9 weeks old,170-200 g) were purchased from the vivarium of the Universidad Peruana Cayetano Heredia (Lima, Peru). Specimens were maintained under controlled temperature (23±2°C), a 12/12 h light/dark cycle, 55 ± 10% humidity, and were fed with standard diet and water *ad libitum* for 15 days prior to the study. All experiments were performed in the animal laboratory of the School of Pharmacy and Biochemistry of the Universidad Nacional de Trujillo, Trujillo, Peru. The study protocol was approved by the Ethical Committee of the School of Pharmacy and Biochemistry of the same university (Ethical Code: PR006-2021/CEIFYB).

Experimental design

In vivo antihyperglycemic activity in normoglycemic rats

Twenty-four rats were randomly assigned into three groups of 8 animals each. After a 14 hours overnight fast, a basal blood glucose measurement was performed. Then, the treatments were administered by intragastric gavage, as follows: normal control group (NC) received water (3 mL/kg), normal *A. muricata* group (NAM) received aqueous extract (200 mg/kg), and normal metformin group (NMetf) received metformin (200 mg/kg) (12). After thirty minutes, an oral glucose tolerance test (OGTT) was performed. All animals were given a glucose solution (2 g/kg) orally by intragastric gavage (13). Blood samples were withdrawn from the tail vein at 30, 60, 90 and 120 minutes after glucose loading, and blood glucose levels were measured using a glucometer (Accu-Chek Active®, Roche Diagnostics, Germany).

In vivo antihyperglycemic activity in diabetic rats

Experimental diabetes induction

Diabetes was induced in 24 overnight fasted rats by a single injection (120 mg/kg) of a 3% cold saline alloxan (Sigma-Aldrich, St. Louis, USA) solution (14). An hour later, the rats were given free access to food and water. At 24 and 96 hours after alloxan treatment, blood glucose was measured using the glucometer. Maintenance of hyperglycemia (greater than 300 mg/dL) 10 days after the alloxan injection was considered as alloxan-induced pancreatic damage, evidenced by signs of diabetes mellitus in rats.

Alloxan-induced diabetic rats were randomly selected

and divided into three treatment groups of 8 rats each to perform OGTT. As described above, a basal blood glucose measurement was performed on overnight fasted rats and each group received a specific treatment as DC (Diabetic control: 3 ml/kg of water); DAM (Diabetic experimental: 200 mg/kg of *A. muricata* decoction) and DMetf (Diabetic standard: 200 mg/kg of Metformin). The animals were then treated the same as the normoglycemic group.

In vitro antihyperglycemic activity

Everted gut sac technique

Seven days after the OGTT study, all 24 rats with induced diabetes were sacrificed with sodium pentobarbital (40 mg/kg) (15) for *in vitro* analysis of intestinal glucose uptake. A cut was made in the midline of the abdomen, the small intestine was extracted and washed with physiological saline both internally and externally with a blunt needle and syringe. The jejunal-ileal portion of the intestine was cut into pieces 15 cm long. The intestinal segments were everted following the technique described by Wilson (16) and modified by Mahomoodally et al (17) and Viviyan et al (18). We used a polyethylene rod (300 mm × 1.5 mm) to push one end of the intestine into the intestinal lumen until it passed through the other end of the gut on the rod. The everted intestine was placed in saline in a petri dish. The intestine was weighed, one end was tied with thread and filled with 1.5 mL of glucose-free Krebs-Henseleit bicarbonate (KHB) buffer using a syringe with blunt needle. The composition of this aqueous pH 7.4 buffer was 118 mM NaCl, 4.7 mM KCl, 1.2 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 9.7 mg/L Na_2EDTA. After tying the ligature at the proximal end, the sac was weighed again and placed in a bath containing 100 mL of incubation medium. The bath was kept at 38° C with shaking at 110 rpm. A gas mixture of 95% O_2 and 5% CO_2 was bubbled through the solution during the incubation.

Mucosal compartment and glucose concentrations

In all three diabetic groups, the everted intestinal sac technique was performed. The incubation media used was KHB solution supplemented with 2.0 g/L glucose for the DC group. The incubation medium of the DAM group contained the same components as the control, but also contained 1.8 mg/mL of *A. muricata* extract. Likewise, the incubation medium of the DMetf group also contained 1 mg/mL of metformin. In each case, a sample of the incubation medium (glucose KHB solution) was taken before the experiment to quantify glucose using the Accu-Check Active® glucometer. Other samples of the incubation medium were taken after 15, 30, 45, and 60 minutes of incubation with the everted intestinal sac.

Serosal compartment and glucose absorbed from the intestine

At the end of the incubation, all of the intestinal sacs were removed from the incubation media and weighed. The fluid contained in the sac (serosal side) was collected by making a small incision in the sac. Glucose concentration in the fluid was quantified using the same glucometer to determine *in vitro* glucose uptake. The empty sac was reweighed. Before and after incubation, the weight of the empty intestinal sac was almost unchanged.

Statistical Analysis

Data analysis was carried out using Microsoft® Office Excel 2016 and IBM SPSS Statistics for Windows, version 20.0 (IBM Corp., Armonk, NY, USA). The results were expressed as the mean ± standard error of the mean (SEM). Statistical comparisons were made using one-way ANOVA test followed by Tukey's post hoc test. Values of $P < 0.05$ were considered statistically significant.

Results

Phytochemical screening

Preliminary phytochemical screening of the *A. muricata* decoction indicated the presence of alkaloids, flavonoids, steroids, tannins, and leucoanthocyanidin (Table 1). Among these, the most prominent components were flavonoids, leucoanthocyanidin, and tannins.

In vivo antihyperglycemic activity of *A. muricata* in normal rats

The OGTT test performed on normal rats (Table 2) resulted in a maximum increase in blood glucose at 60 minutes after starting the experiment for all 3 groups. The NC group showed a maximum increase from baseline of 90.67%, while maximum increases of blood glucose of 40.38% and 19.64% were achieved in the NAM and NMetf groups, respectively (Table 2). In absolute terms, the maximum glucose levels observed were 168.75 ± 3.61, 120.39 ± 2.81, and 108.89 ± 4.31 mg/dL for the NC, NAM and NMetf groups, respectively. At 120 minutes, the NAM and NMetf groups had blood glucose reductions of 24.42% and 31.08% respectively, when compared to NC group. At a $P < 0.05$ level, there was a statistically significant difference between the NAM and NC groups and between the NMetf and NC at times ≥ 60 minutes, but no statistically significant difference was found between the NAM and NMetf groups.

In vivo antihyperglycemic activity in diabetic rats

Table 2 also shows the maximum blood glucose increase in diabetic rats during the OGTT test. The DAM and DMetf groups achieved a lower percentage of glucose increase above baseline (14.19% and 11.41%, respectively) compared to the control group (43%). In absolute terms, the blood glucose concentration in the DAM and DMetf groups (393.25 ± 8.70 mg/dL and 374.63 ± 10.11 mg/dL) were significantly lower than that of the DC group (497.25 ± 4.47 mg/dL) at 60 minutes. At 90 minutes, the difference in blood glucose levels between the DAM and

Table 2. Effect of *A. muricata* leaf extract on blood glucose (mg/dL) and its maximum percent increase in normal and diabetic rats

Treatments	Blood glucose levels (mg/dL)					Maximum% increase glycemia from baseline
	0 min	30 min	60 min	90 min	120 min	
NC	88.50±1.23	125.12±1.24	168.75±3.61	143.25±4.73	112.63±3.60	90.67
NAM	85.75±2.80	102.75±1.81	120.39±2.81*	98.88±4.31*	85.12±2.70*	40.38*
NMetf	91.00±3.53	103.50±4.09	108.89±4.31*	87.00±3.13*	77.62±1.28*	19.64*
DC	362.5±15.33	427.89±4.95	497.25±4.47	518.00±4.81	500.89±5.19	43.00
DAM	344.38±7.18	376.27±10.10	393.25±8.70#	350.62±16.10#	312.5±18.11#	14.19#
DMetf	336.25±15.22	364.25±13.31	374.63±10.11#	333.00±5.24#	280.38±8.39#	11.41#

Values are expressed as Mean ±SEM (n=8); $P < 0.05$: significant difference, *$P < 0.05$: compared to NC group; #$P < 0.05$: compared to DC group. NC: normal control (vehicle); NAM: normal, *A. muricata*; NMetf: normal, metformin; DC: diabetic control (vehicle); DAM: diabetic, *A. muricata*; DMetf: diabetic, metformin.

DMetf groups with respect to the DC group became larger. At 120 minutes, the DAM and DMetf groups had reduced blood glucose levels by 37.61% and 44.02% respectively, compared with the control group. Glycemic values of the DC group remained higher over time in comparison to DAM and DMetf groups. At a $P<0.05$ level, there was a statistically significant difference between the DAM and DC groups and between the DMetf and DC groups at times ≥ 60 minutes, but no significant difference was found between the DAM and DMetf groups.

In vitro antihyperglycemic activity of *A. muricata* in diabetic rats

Glucose absorption in everted gut sac

A decrease in glucose concentration of the glucose-supplemented KHB incubation media indicates transport from the intestinal lumen (mucosa) to the serosa (Figure 1). In the DAM group, the glucose concentration was 183.25 ± 1.09 mg/dL at 15 minutes, 162.87 ± 2.72 mg/dL at 30 minutes, 153.25±2.60 mg/dL at 45 minutes and 148.5 ± 2.44 mg/dL at 60 minutes, unlike the control group whose glucose concentrations were lower: 145.5 ± 1.76, 123.25 ± 1.80 and 95.13 ± 1.33 mg/dL at 30, 45 and 60 minutes, respectively. The DMetf group showed similar trend to the DAM group. After 30 minutes, a statistically significant difference ($P<0.05$) was found between the DC and the DAM and DMetf groups. There was no significant difference between the DAM and DMetf groups ($P>0.05$).

Glucose concentrations in the serosal compartment of the everted intestines of diabetic rats were also measured. Significantly lower glucose concentrations were found inside the everted gut sacs after 60 minutes of incubation in the DAM and DMetf groups, (33.40 ± 0.49 mg/dL and 28.85 ± 0.79 mg/dL, respectively) compared with the control group (80.62 ± 0.73 mg/dL) ($P<0.05$). There was no significant difference between DAM and DMetf groups

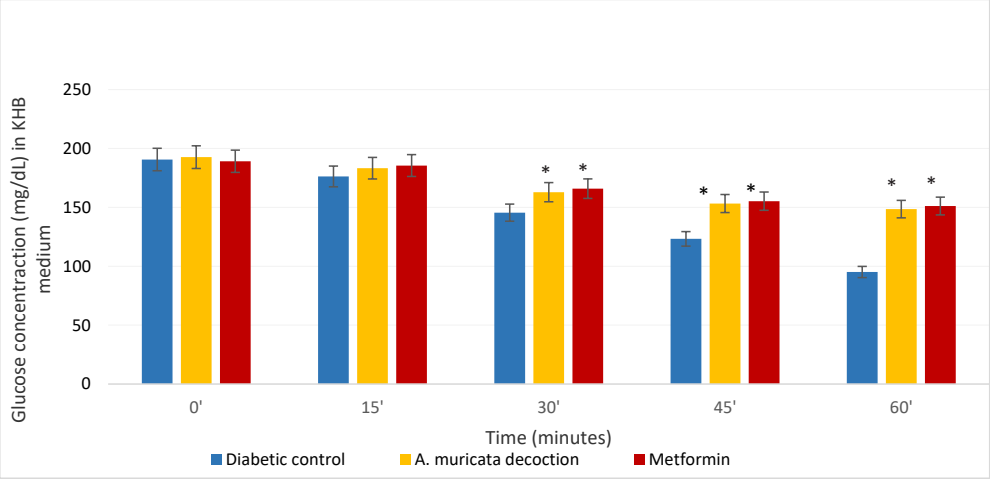

Figure 1. Time variation in glucose concentrations of the medium in contact with the mucosa of diabetic rat intestine. Incubations were carried out in glucose-containing Krebs-Henseleit Bicarbonate (KHB) medium (control, blue), in glucose-containing KHB medium supplemented with 1.8 mg/mL *A. muricata* decoction (yellow) and in glucose-containing KHB medium supplemented with 1 mg/mL metformin (red). * P <0.05: significant difference compared to diabetic control.

($P > 0.05$). The presence of glucose in the serosal fluid is indicative of glucose absorption and transport from the incubation medium.

Discussion

Hyperglycemia is not only a marker but also a mediator in the occurrence of adverse cardiovascular events and mortality (19). Therefore, failure to adequately control blood glucose favors progression of DM and associated chronic diseases (20). Thus, developing ways to control glucose can help treat DM. Fasting blood glucose in diabetic rats represents an important animal model of diabetic status for screening of potentially bioactive compounds (21) .

Medicinal plant extracts are traditionally used in many countries to control DM, making them potential drug candidates. One plant used in such a manner is the soursop (*A. muricata*). We find that aqueous extract from Peruvian *A. muricata* leaves contains several different bioactive secondary metabolites such as flavonoids, alkaloids, quinones, leucoanthocyanidins, steroids, phenols, and tannins. These phytochemicals were in accordance with those reported in other studies of *A. muricata* grown in different places (22,23).

Using a rat model, the effects of *A. muricata* decoction on glucose levels and uptake were measured and compared against controls. When challenged in the OGTT test, the control group of healthy rats showed a peak in blood glucose level which then decreased until returning to baseline. This response is physiologically expected, because the pancreas of these rats is functionally and structurally normal, which permits glucose and insulin homeostasis (24). The effectiveness of *A. muricata* leaf aqueous extract in decreasing glycemia was observed to be similar to metformin in non-diabetic rats. Metformin, a standard anti-DM drug, can attenuate glucose increase by acting in different ways, including decreasing liver glucose production and intestinal glucose absorption, and augmenting muscle glucose uptake, which is largely mediated through 5'-AMP-activated protein kinase AMP (25,26). However, metformin does not stimulate insulin secretion.

In rats with alloxan-induced DM, a higher glucose level was observed in the control group throughout the OGTT test, without a return to baseline. However, if the rats were treated with metformin or *A. muricata* decoction before OGTT, there was a statistically significant reduction in the blood glucose when compared to the control group. In the induced diabetic state, the capacity to metabolize glucose is altered, because the β-cells in the islets of Langerhans are selectively destroyed by the alloxan, so little to no insulin is produced and released. This prevents an appropriate insulin response to glucose overload (27). After entering β-cells, alloxan produces its pathological effect through two independent mechanisms, glucokinase inhibition and reactive oxygen species (ROS) cycle generation, resulting in necrotic β-cell death and a permanent diabetic hyperglycemic state (28).

During the OGTT test, diabetic rats treated with *A. muricata* decoction or metformin showed a significant delay in the increase of blood glucose and an evident suppression of the glucose peak at the end of two hours. Our results agree with those reported by Florence et al including significant decrease in blood glucose two hours post glucose gavage in both treated normal and diabetic rats. Moreover, the study suggested that the action mechanism of *A. muricata* could be different from that of the sulfonylurea control (29). *A. muricata* extract could be delaying the increase in blood glucose levels because bioactive compounds block or inhibit intestinal glucose absorption. It may also activate the pancreas leading to increased insulin secretion or have other extrapancreatic mechanisms, such as allowing for greater tissue utilization of glucose. These mechanisms have been suggested in other species of the *Annona* genus (30,31). Phytochemical compounds in *A. muricata* leaf extract cultivated in Peru, such as flavonoids, steroids, tannins, leucoanthocyanidin, phenolic compounds, and alkaloids, could produce similar effects to those produced by the African species *A. reticulata,* and *A. squamosa* (30,32). Furthermore, our results agree with those reported by Valdes et al, which attributed the phytoconstituents of the polar extract fractions of *A. diversifolia* as responsible for the antihyperglycemic activity (33). The antidiabetic properties of flavonoids are mainly from their effects on reducing apoptosis, improving proliferation of pancreatic β-cells and promoting insulin secretion, regulation of glucose metabolism in hepatocytes, and subsequent improvement of hyperglycemia (34). Cordero-Herrera et al, hypothesized that flavonoids might increase GLUT-4 expression and PI3K/Akt activity leading to a restoration of insulin sensitivity, which might be a viable DM treatment approach (35). Flavonoids mediate glucose metabolism by many mechanisms and pathways such as influencing insulin secretion, energy metabolism, and insulin sensitivity in peripheral tissues. Also, the role of flavonoids is important in enhancing glucose uptake by expression, up-regulation, and translocation of glucose transporter proteins such as GLUT-4 (34).

Reduction of postprandial hyperglycemia is one of the most effective DM management and treatment strategies (9). In the small intestine, glucose absorption occurs in two stages. First, glucose is transported by SGLT1 from the intestinal lumen into enterocytes moving in favor of its concentration gradient. Second, from the basolateral membrane glucose is transported by GLUT2 to blood plasma (36), thus increasing blood glucose. The goal of controlling postprandial hyperglycemia is to decrease and prevent unwanted increases in blood glucose. *In vitro* studies can evaluate bioactive compounds that prevent carbohydrate hydrolysis (α-amylase and α-glucosidase enzyme inhibitors) or that inhibit glucose transport and

absorption (SGLT1 and GLUT2 inhibitors) in the intestine (9).

In the current *in vitro* study, glucose concentration in the incubation medium in contact with the intestinal mucosa of the DC group was found to decrease as glucose was absorbed into the everted gut sac. This decrease was significantly different at 30 minutes to the end of the incubation, while the incubation media of the DAM and DMetf groups maintained a higher glucose concentration. On the other hand, the increase in glucose concentration in the serosal compartment of the DC group could be explained because, in DM, intestinal glucose absorption is increased due to increased expression of glucose transporters SGLT1 and GLUT2 in the enterocyte (37). The presence of *A. muricata* phytoconstituents in the incubation medium could decrease glucose transport. Valdes et al reported that *A. diversifolia* flavonoids inhibited the SGLT1 transporter (33), similar actions were attributed to flavonoids of *Phyllanthus amarus* (18), alkaloids of *Alstonia macrophylla* Wall, flavanones of *Sophora flavescens* Ait, *Malus domestica* Borkh and *Allium cepa*, which showed the inhibition of SLGT1 and GLUT2 transporters (37). Likewise, phytoconstituents of *Momordica charantia* extracts, exerted glucose transport inhibition due to the alteration of the Na^+-K^+ gradient in the enterocyte membrane (17). Recently, the ethanol extract of *A. muricata* leaves exhibited *in vitro* inhibition of α-amylase and α-glucosidase enzymes (38), and Soursop leaf infusion showed a slight inhibitory effect after *in situ* perfusions of glucose and soursop leaf infusion (39). On the other hand, the decrease in intestinal glucose absorption produced by metformin is consistent with that reported in other studies, which implicate inhibition of transepithelial glucose transport (26), which decreases glucose absorption into the everted intestinal sac (40). Based on the analysis of our results, the decrease in intestinal glucose absorption produced by the aqueous extract of *A. muricata* leaves, could be explained by the following mechanisms: inhibition of the SGLT1 and/or GLUT2 transporters, inhibition of hydrolase enzymes, and/or by affecting the Na^+/K^+-ATPase pump in the enterocyte, which results in the loss of the ion gradient necessary for secondary active transport of glucose. Classes of compounds found in the aqueous extract, such as flavonoids, alkaloids, tannins, and leucoanthocyanidins as well as other phytoconstituents may be responsible for these effects.

Conclusion

The aqueous extract from *A. muricata* leaves decreased *in vitro* intestinal glucose absorption and improved oral glucose tolerance in rats. Thus, such an extract may represent a potential treatment candidate for the control of hyperglycemia, particularly in cases of type 2 DM. Further studies that aim at elucidating the exact mechanism of action and identifying the active components would be additional steps in developing pharmaceutical candidates from *A. muricata*.

Authors' contributions

AGV, JCF, and JDC conceived and designed the research and carried out the collection of results. JVCF performed the data analysis. AGV and JDC wrote the manuscript. All authors critically reviewed and approved the final manuscript.

Conflict of interest

Authors declare they have no conflicts of interest, financial or otherwise.

Ethical considerations

Experiments on the albino rats were performed in accordance with the ethical guidelines and regulations set forth by the Ethics Committee for Animal Research of the School of Pharmacy and Biochemistry of the Universidad Nacional de Trujillo, Peru (Ethical code: PR006-2021/CEIFYB).

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