Evaluation of the Antihyperglycemic Activity of *Juglans Mollis* and *Hamelia Patens* Through *in Vitro* and *in Vivo* Methods

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Abstract

Objectives: Diabetes mellitus is one of the most common noncontagious diseases in the world. The International Diabetes Federation (IDF) reported that in 2017, approximately 425 million people worldwide suffered from diabetes. Drugs used for diabetes treatment have unwanted side effects, so new safe drugs are needed. Some natural products have antihyperglycemic activity and are less toxic than currently used drugs. In this work, we evaluated the antihyperglycemic activity of extracts of *Juglans mollis* and *Hamelia patens* as well as their cytotoxicity through *in vitro* and *in vivo* methods.

Materials and methods: Five extracts of each plant were subjected to *in vitro* amylase and glucosidase inhibition tests and subsequently analyzed by the *ex vivo* everted sac test. Additionally, their *in vivo* antihyperglycemic activity was evaluated.

Results: Each of the extracts of *J. mollis* and the polar extracts of *H. patens* showed antihyperglycemic activity in the *in vivo* model, but in the *in vitro* model, the extracts showed different effects; some of the extracts inhibited one or both digestive enzymes, and others reduced the absorption of glucose through the intestine.

Conclusions: In this article, we contribute to elucidating the antihyperglycemic mechanism of *H. patens*, and we report for the first time the antihyperglycemic activity of *J. mollis* and its possible mechanisms of action.

Introduction

Diabetes mellitus is one of the most common noncommunicable diseases, along with cardiovascular diseases and cancer[1]. Approximately 463 million people between the ages of 20 and 79 worldwide have diabetes[2]. The main characteristic of the disease is the inability to regulate blood glucose levels due to insulin resistance in peripheral tissues and/or due to the quantity or quality of insulin secreted by the beta cells of the pancreatic islets[3]. Long-term uncontrolled diabetes mellitus can cause ketoacidosis, retinopathy, nephropathy, neuropathy, hypertension, cerebrovascular accident, etc[4]. Treatments for lowering or controlling blood glucose levels include insulin (obtained from different sources) and hypoglycemic agents with various mechanisms of action that either inhibit the action of digestive enzymes or inhibit the action of enzymes responsible for absorption, carbohydrate reabsorption, etc. Almost all of these medications generate side effects and are associated with a high cost of treatment[5,6]. For this reason, it is necessary to identify new affordable products that can help control hyperglycemia with fewer side effects.

Results obtained in *in vivo* models are widely accepted in both the laboratory and the clinic since these models involve the entire organism and therefore consider the entire metabolic process. However, the use of *in vivo* models is expensive, is time consuming and requires a large number of products to be evaluated and a large number of animals[7]. On the other hand, *in vitro* tests are faster, simpler and more

economical. Such tests have been used to predict *in vivo* activity and help elucidate the mechanism of action of drugs.

Natural products have been shown to be good sources of molecules with various biological activities and fewer side effects[8]. A number of authors have reported the efficacy of plant extracts in lowering blood glucose using different *in vivo* models, while others have demonstrated the potential hypoglycemic or antihyperglycemic activity of extracts or natural products using *in vitro* or *ex vivo* models[6].

Juglans mollis commonly known as walnut or white walnut, has anti-infective properties. The genus *Juglans* has been reported to have antidiarrheal, antidiabetic, hepatoprotective, anticancer activity[9], against urinary tract infections[10], antihypertensive activity[11], as well as antioxidant, lipolytic, antihyperglycemic, antilipidemic, neuronal stimulant and antiproliferative properties[12–15].

Hamelia patens belongs to the Rubiaceae family and is commonly known as chacloco, coral plant or fire bush; it is a species of large evergreen shrub from subtropical and tropical America. It is found from Florida in the southern United States to Argentina, mainly from central and southern Mexico to Costa Rica[16,17]. The poultices of this plant are used to treat superficial wounds, and the plant can also decrease inflammation caused by external injury. Infusion of this plant are used to reduce the discomfort of abdominal bloating and menstrual cramps. Few studies out on this species have been carried. Extracts of this plant have been determined to have anti-inflammatory, antioxidant, hepatoprotective, hypoglycemic and antihyperglycemic activity[18,19].

In the present work, we evaluate the antihyperglycemic activity of different extracts of *J. mollis* and *H. patens* through *in vitro*, *ex vivo* and *in vivo* methods and suggest possible mechanisms of action of these plants.

Results

The cytotoxic activity of the extracts was evaluated by a cell viability assay using MTT as an indicator of growth. None of the extracts except the methanolic extract of *J. mollis*, which showed a CC_{50} of 285.7 ± 9.1 µg/mL, showed cytotoxicity at the maximum concentration of 500 µg/mL. The doxorubicin positive control showed a CC_{50} of 12.8 ± 0.5 µg/mL.

The inhibitory effects of the extracts on the activity of enzymes involved in the digestion of carbohydrates is shown in Table 1. All the extracts of *J. mollis* except the hexane extract inhibited the activity of glucosidase. Furthermore, the butanolic extracts and the aqueous residue inhibited amylase activity.

Table 1
Mean Inhibitory Concentration (IC_{50}) of digestive enzymes.

	Juglans mollis		*Hamelia patens*	
	Glucosidase	Amylase	Glucosidase	Amylase
Methanol	33.2 ± 0.3	> 50	> 330	3.7 ± 0.2
Hexane	> 330	> 50	> 330	> 50
Ethyl acetate	53.3 ± 4.8	> 50	> 330	> 50
Butanol	27.9 ± 0.2	12.3 ± 0.5	> 330	6.3 ± 0.8
Aqueous residue	74.5 ± 0.6	8.8 ± 0.2	> 330	> 50
Acarbose	164.3 ± 1.7	2.9 ± 0.1	164.3 ± 1.7	2.9 ± 0.1

Interestingly, the *H. patens* extracts were not able to inhibit glucosidase activity at the maximum concentration of 330 µg/mL, although the methanol and butanol extracts strongly inhibited amylase activity.

To evaluate glucose absorption through the inverted intestinal sac, glucose absorption kinetics were first analyzed in the absence of inhibition, that is, in the negative control condition. In the absence of inhibition, the kinetics showed a correlation coefficient of 0.9943, with an approximate flux of glucose absorption through the intestinal wall of 50 mg/dL every hour (not shown). When 5 mg/dL empagliflozin, an inhibitor of glucose transport through SGLUT, was added, the kinetics showed an excellent correlation coefficient of 0.9932, with an absorption flux of approximately 33 mg/dL every hour (not shown). To determine the reduction in glucose uptake induced by the positive control, the area under the curve was calculated. The area under the curve of the negative control condition was taken as 100%, and it was determined that at the concentration used, empagliflozin, reduced glucose absorption through the intestine by 38.4%.

The extracts were subjected to the inverted sac absorption test at three different concentrations, and their absorption kinetics were determined. The area under the curve was determined for each experiment and statistically compared to that obtained for the negative control. Compared to no treatment, the methanolic, butanolic and aqueous residue extracts of *J. mollis* significantly inhibited absorption. The ethyl acetate and butanolic extracts of *H. patens* at low concentrations and the aqueous residue of *H. patens* at high concentrations also caused a significant reduction in intestinal absorption (Fig. 1). The concentration of glucose absorbed after half of the kinetics, that is, at 90 min, is shown in Fig. 2.

All *in vivo* tests were carried out following the Good Practices for the Care and Management of Experimental Animals. The kinetics of oral starch tolerance was first determined. The hyperglycemic peak was reached 30 min after the administration of starch; subsequently, the blood glucose levels returned to

the initial values. When acarbose (positive control) was administered together with starch, the hyperglycemic peak (30 min) significantly decreased by approximately 65.7% (not shown).

To evaluate the effect of acarbose on kinetics, the reduction in hyperglycemia induced was determined by calculating the area under the curve, with that of the starch curve being considered 100%. Acarbose was determined to induce an approximately 29.3% reduction in all kinetics.

On the other hand, the control PPHT extract behaved statistically the same as acarbose, with reducing the hyperglycemic peak at 30 min by approximately 65.7% and the total kinetics by approximately 33.4%.

All the extracts were evaluated at three different doses under the same conditions, and the results are shown in Fig. 3. For all extracts except the hexanic extracts of both *J. mollis* and *H. patens*, a significant decrease in the area under the curve compared with that of starch was found. Interestingly, most of the *J. mollis* extracts induced a significant reduction in the area under the curve similar to that induced by the positive controls.

The normalized glucose level during the hyperglycemic peak (30 min) for each extract at each concentration is shown in Fig. 4. Four extracts had statistically the same effect as starch, but most exhibited significantly reduced the hyperglycemic peak.

Limitations

In the present work, only extracts of different polarity obtained from plants were included, not compounds was isolated. Hexane extracts could not be evaluated in the *ex-vivo* assay.

Discussion

The extracts used in this work were obtained from *J. mollis* and *H. patens*, both of which were collected in Nuevo León, Mexico.

J. mollis bark extract has been reported to have powerful antioxidant, hepatoprotective, and antimycobacterial activity[20,21]. To date, its use as an antihyperglycemic agent has not been reported, but recently, the use of *J. nigra* and *J. neotropica* as antidiabetic agents was reported[22,23].

The methanol extract of *J. mollis* had a moderate cytotoxic effect on the Vero cell line. For this reason, the residue was fractionated with solvents of increasing polarity. The butanolic, methanolic, ethyl acetate extracts and the residue more strongly inhibited glucosidase activity than acarbose as a control, while only the butanolic extract and the residue inhibited amylase activity. The residue and the methanolic and butanolic extracts significantly reduced glucose uptake both in full and half kinetics (90 min). In the inverted sack test, the activity of the hexane extract could not be evaluated because the solvent damages the integrity of the intestine. On the other hand, all the extracts were subjected to an *in vivo* oral starch tolerance test. Interestingly, all the extracts except hexanic at low concentrations decreased the area

under the curve in the starch tolerance test. The hexane and butanolic extracts at low concentrations and the residue and the hexane extract at high concentrations failed to reduce the hyperglycemic peak (at 30 min), while the methanolic and ethyl acetate extracts showed a similar inhibitory effect as the control.

To our knowledge, this is the first time that the antihyperglycemic activity of *J. mollis* has been reported. The methanolic extract exerted a potent antihyperglycemic effect similar to that of the positive control, possibly by inhibiting glucosidase and reducing absorption through the intestine; however, the extract induced moderate cytotoxicity, although none of the animals treated with this extract showed signs of intoxication in the *in vivo* experiment. The ethyl acetate extract also had a strong effect *in vivo*, and the findings suggest that it exerted this effect through glucosidase inhibition. The butanolic residue and extract also exhibited *in vivo* antihyperglycemic activity comparable to that of the positive control. According to the findings, both the residue and extract exert this effect through inhibiting digestive enzymes and delaying intestinal absorption.

On the other hand, various authors have demonstrated that *H. patens* has antidiabetic and hypoglycemic activity. Andrade-Cetto *et al.*[24]. postulated that the hypoglycemic activity of this plant is due to the inhibition of glucosidase; however, we found that none of the extracts inhibited glucosidase at concentrations below 330 µg/mL, which was the maximum concentration evaluated. As we found for the first time that the methanolic and butanolic extracts of *H. patens* inhibit amylase, these findings suggest that enzyme inhibition could be the mechanism underlying the antihyperglycemic activity of *H. patens* extracts. To our knowledge, this is the first time that glucose absorption was evaluated as a mechanism of the antidiabetic activity of *H. patens*. Both the residue and the butanolic and ethyl acetate extracts of *H. patents* significantly reduced the absorption of glucose, indicating that reduced glucose absorption could be the mechanism through which these extracts exert their antihyperglycemic activity. Taken together, these findings could suggest that the *H. patens* exerts hypoglycemic or antihyperglycemic effect through inhibition of amylase and a reduction in carbohydrate absorption.

In conclusion, in this article, we contribute to elucidating the antihyperglycemic mechanism of *H. patens* and report for the first time the antihyperglycemic activity of *J. mollis* and its possible mechanisms of action. We are currently working on isolating the compound(s) responsible for this activity.

Methods

Plants

Juglans mollis bark was collected in Villaldama, NL, Mexico, and *Hamelia patens* leaves and stems were collected from the northern part of the state of Veracruz, Mexico. In both cases, the relevant institutional, national, and international guidelines and legislation were complied. Specimens of both plants were deposited in the Institutional Herbarium of the Faculty of Biology of the Autonomous University of Nuevo León (Monterrey, Mexico) where they were authenticated by the M.C. Ma. Del Consuelo González de la Rosa with folios: UAN-23894 and UNL-01458, respectively.

Both plants were dried at laboratory temperature for 7 days. Two hundred grams of powdered material was removed with methanol at room temperature on a shaker for 60 min (3 times). The extract obtained was dried under reduced pressure. The methanolic extract was kept at -4°C until use. Twenty grams of the methanolic extract was resuspended in 250 ml of water and subjected to differential extraction with hexane (100 ml x 3), ethyl acetate (100 ml x 3) and butanol (100 ml x 3). The hexane, ethyl acetate and butanol extracts were evaporated under reduced pressure to dryness and stored at -4°C until use.

α-Glucosidase inhibition assay

The optimized and validated method described by Granados-Guzmán *et al.*[25]. was followed exactly.

α-Amylase inhibition assay

The optimized and validated method described by Granados-Guzmán[26] was followed exactly.

Cytotoxicity

Vero cells were maintained in DMEM supplemented with 10% FBS, 1% L-glutamine and 1% antibiotics and incubated at 37 ºC and 5% CO_2. When they reached 90% confluence, the cells were detached with trypsin, and after centrifugation at 3500 rpm for 5 min, the cells were counted in a Neubauer chamber. Approximately 50,000 cells were seeded in each well of a 96-well microplate and incubated overnight under the same conditions[27]. Subsequently, the medium was discarded, and 200 µL of medium (negative control), medium supplemented with doxorubicin (positive control) or medium supplemented with extract (final concentration between 0.5 and 500 µg/mL) was added to each well. The plates were incubated under the same conditions for 48 hours. Growth was checked under a light microscope to rule out contamination, and the contents of the plate were decanted. Cell growth was evaluated by adding 200 µL of MTT solution (0.5 mg/mL in buffer) to each well and incubating the plates for 3 hours under standard conditions. The supernatant was decanted, and the crystals were dissolved in 200 µL of DMSO[28]. The plates were shaken, and the absorbance of each well at 540 nm was measured. All experiments were repeated five times on 3 different plates. The absorbance of each well was compared to that obtained of the negative control (100% viability or 0% inhibition), and the percentage of cytotoxicity was calculated. The data for each plate were handled separately. Cytotoxicity percentages were plotted as a function of extract or doxorubicin concentration, and the mean cytotoxic concentration (CC_{50}) was determined by interpolation.

Intestinal glucose absorption assay

A protocol recently reported by our working group[29], which was designed and reported for the first time by Wilson and Wiseman[30], was followed. Male Wistar weighing 400 g rats were used and provided water and food ad libitum. All the experiments were carried out in accordance with the relevant guidelines and regulations approved by the Committee for the Care and Use of Laboratory Animals (CCUAL-FM-UAEM) with the protocol 005/2018, in addition the recommendations of NOM-062-ZOO-1999[31] (Technical specifications for the production, care and use of laboratory animals) were considered. The experiments

were carried out in the Laboratory of Electrophysiology and Pharmacological Bioevaluation of the Faculty of Medicine of the UAEM, following the recommendations of the ARRIVE guidelines.

The animals were sacrificed by cervical dislocation. An abdominal incision was made, and the small intestine was removed. Approximately 5 cm long pieces of intestine from the duodenum and the first part of the jejunum were isolated. Each piece was washed with saline solution, and one end was tied with surgical thread. With the help of a glass rod, the ligature was moved to the opposite side of the tissue, and once the piece of intestine had been inverted, the other end was tied to a Pasteur pipette. The intestinal sac was filled with physiological solution (0.15 M NaCl, pH 7.4), and absence of leaks was verified.

One of the intestinal sacs was incubated in saline at 37°C with constant bubbling for 3 hours to obtain baseline absorption data (negative control). The other sacs were incubated in saline solution supplemented with empagliflozin (5 mg/dL, positive control) or test extract (5, 10 and 20 mg/dL) under the same conditions. During the incubation, aliquots were taken from the inside of the bag at 0, 30, 60, 90, 120, 150 and 180 min. The glucose concentration absorbed by the intestine was measured with an Accu-chek Performa portable glucometer (Roche). A graph of the intestinal glucose absorption kinetics was obtained. The area under the curve was calculated using the midpoint Riemann sum to determine the effect of the extracts on the kinetics. In addition, the absorption percentage was determined at each time point, and a significant difference with respect to the negative control was observed at 90 min.

Oral starch tolerance test

Normoglycemic male Wistar rats with an average weight of 220 g were used. All animals were kept in the vivarium throughout the study period at a temperature of 25°C and a relative humidity of 40 to 50% on a 12-hour light and dark cycle and were also provided food and water ad libitum. All the experiments were carried out in accordance with the relevant guidelines and regulations approved by the Committee for the Care and Use of Laboratory Animals (CCUAL-FM-UAEM) with the protocol 005/2018, in addition the recommendations of NOM-062-ZOO-199931 (Technical specifications for the production, care and use of laboratory animals) were considered. The experiments were carried out in the Laboratory of Electrophysiology and Pharmacological Bioevaluation of the Faculty of Medicine of the UAEM, following the recommendations of the ARRIVE guidelines.

The procedure reported by Yusoff *et al.*[32]. was followed with slight modifications. Briefly, the rats were divided into groups of 5 rats. Each group received oral treatment: Group 1 was administered water (1 mL/kg) Group 2 was given corn starch (1 g/kg), group 3 was administered corn starch plus acarbose (0.5 mg/kg), and Group 4 was given starch plus protein hydrolysate from the legume *Mucuna pruriens* (PPHT; 0.5 mg/kg), which was included as a control extract for comparison[29]. Each of the 8 extracts was evaluated at concentrations of 0.5, 2.5 and 5 mg/kg. After a 5-hour fast, basal glucose levels in whole blood (drawn from the tip of the tail) were recorded using an Accu-chek Performa portable glucometer (Roche). Subsequently, carbohydrate (1 g/kg corn starch) with or without inhibitor (acarbose, PPHT or extract) was administered orally, and glucose levels were measured at 15, 30, 45, 60 and 120 min.

Normalized glycemic curves (ratio of the glucose concentration at each time point and at time zero for each rat) were constructed for each group, and the area under the curve was calculated using the midpoint Riemann sum to determine the effect of each agent on the kinetics. In addition, the percentage reduction in the glucose peak was determined after 30 min, with the peak generated by starch taken as 100% elevation.

Statistical analysis

In vivo experiments were performed five times, those in vitro and ex vivo models were done in triplicate. Results are reported as the mean ± SD. The effect of each extract or positive control was compared that of the negative control by one-way analysis of variance (ANOVA) followed by Dunnett's test. A p value < 0.05 indicated statistical significance.

Declarations

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AUTHOR CONTRIBUTIONS

GGG and SJMA performed the experiments and analyzed the data. JPM obtained and provided the plant extracts. JJAF conducted the in vivo experiments and reviewed the paper. NWM supervised the plant extraction and reviewed the manuscript. RSA designed the study, conducted and supervised all the experiments, wrote the manuscript.

Competing interests

The authors have no conflicts of interest to declare.

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Figures

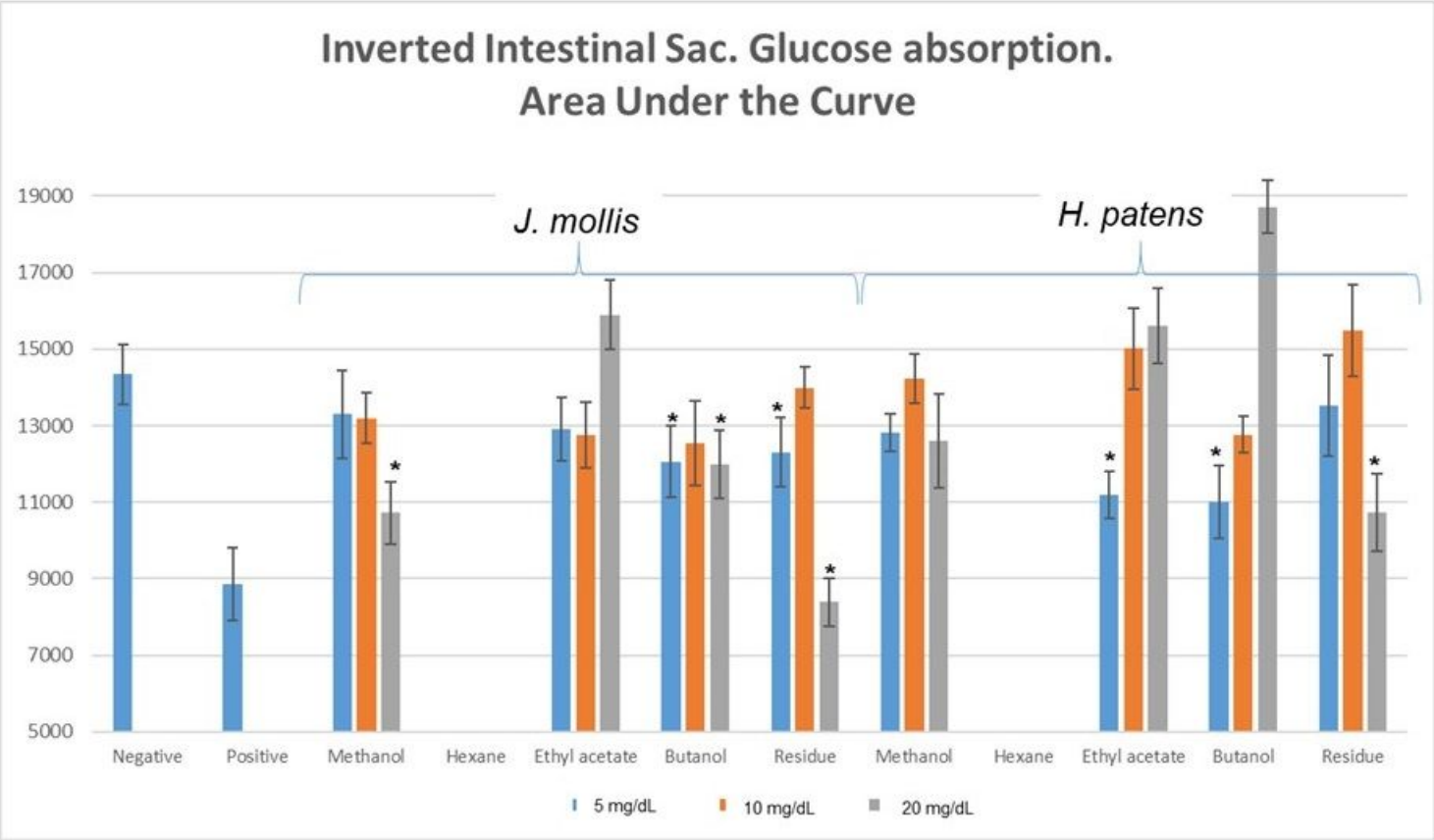

Figure 1

Area Under the Curve glucose uptake and control extracts. (* p <0.05)

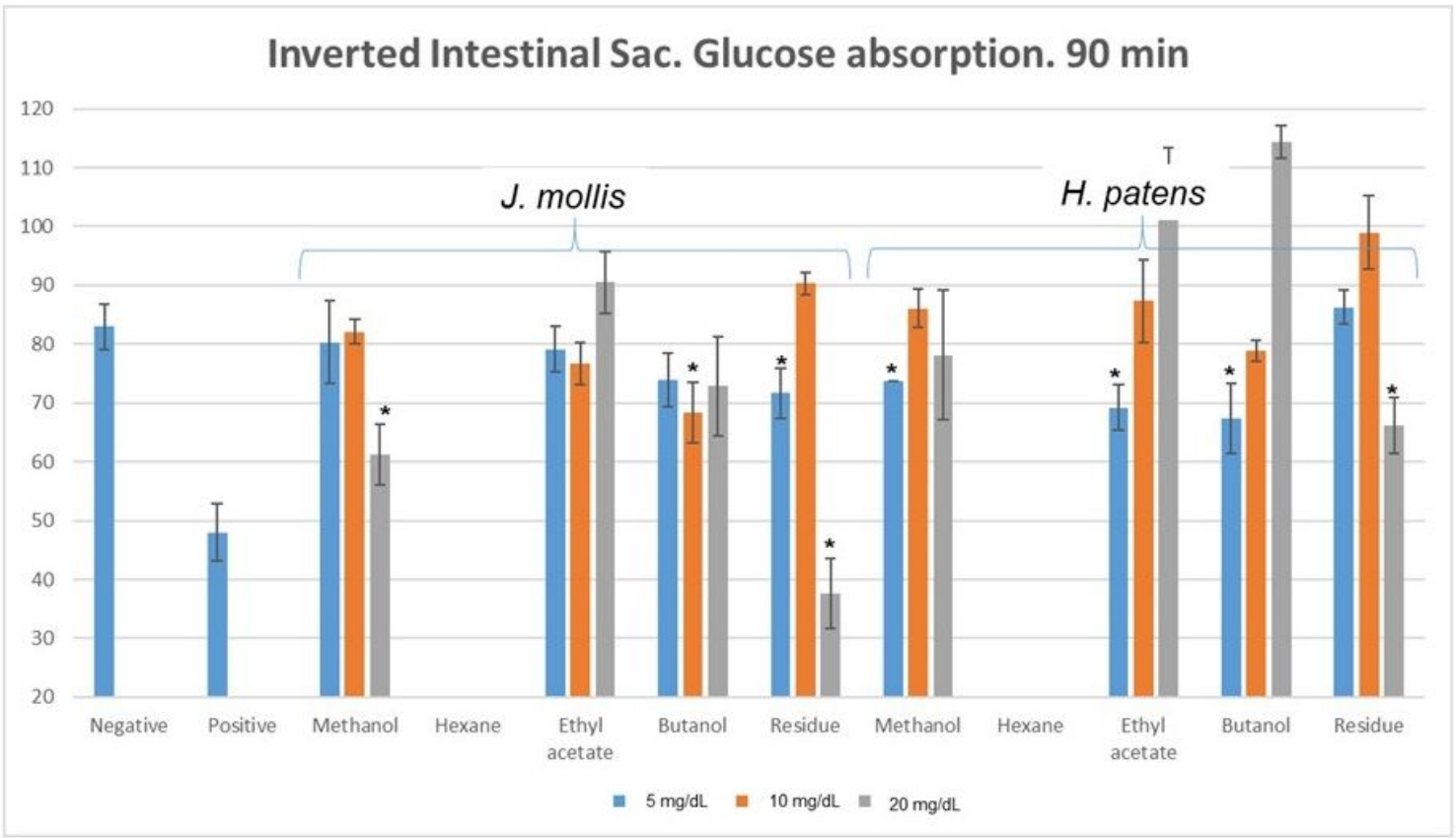

Figure 2

Glucose absorption at 90 min of exposure of controls and extracts. (* p <0.05)

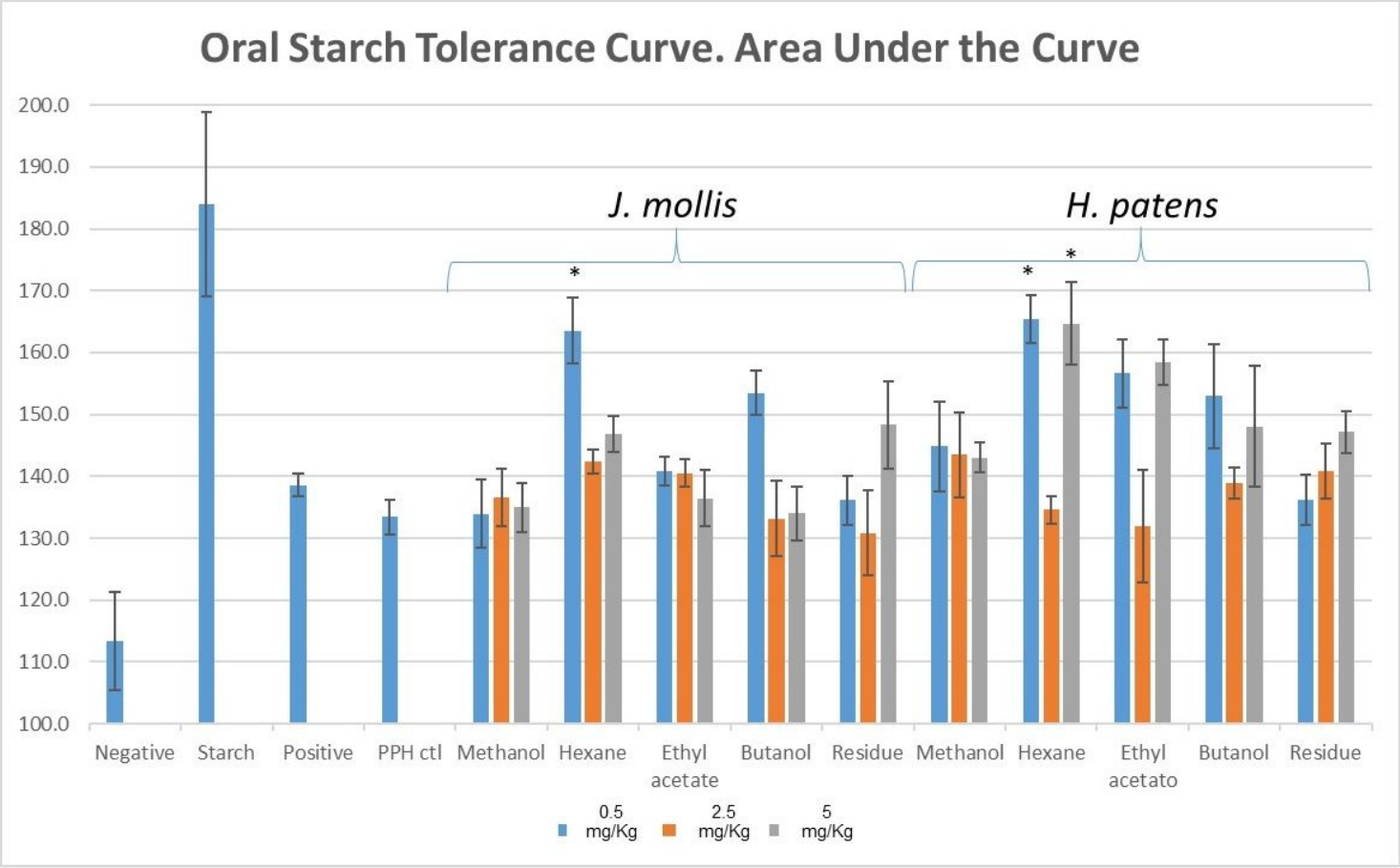

Figure 3

Area Under the Curve Oral Tolerance to the controls and starch extracts. * no significant difference (p <0.05)

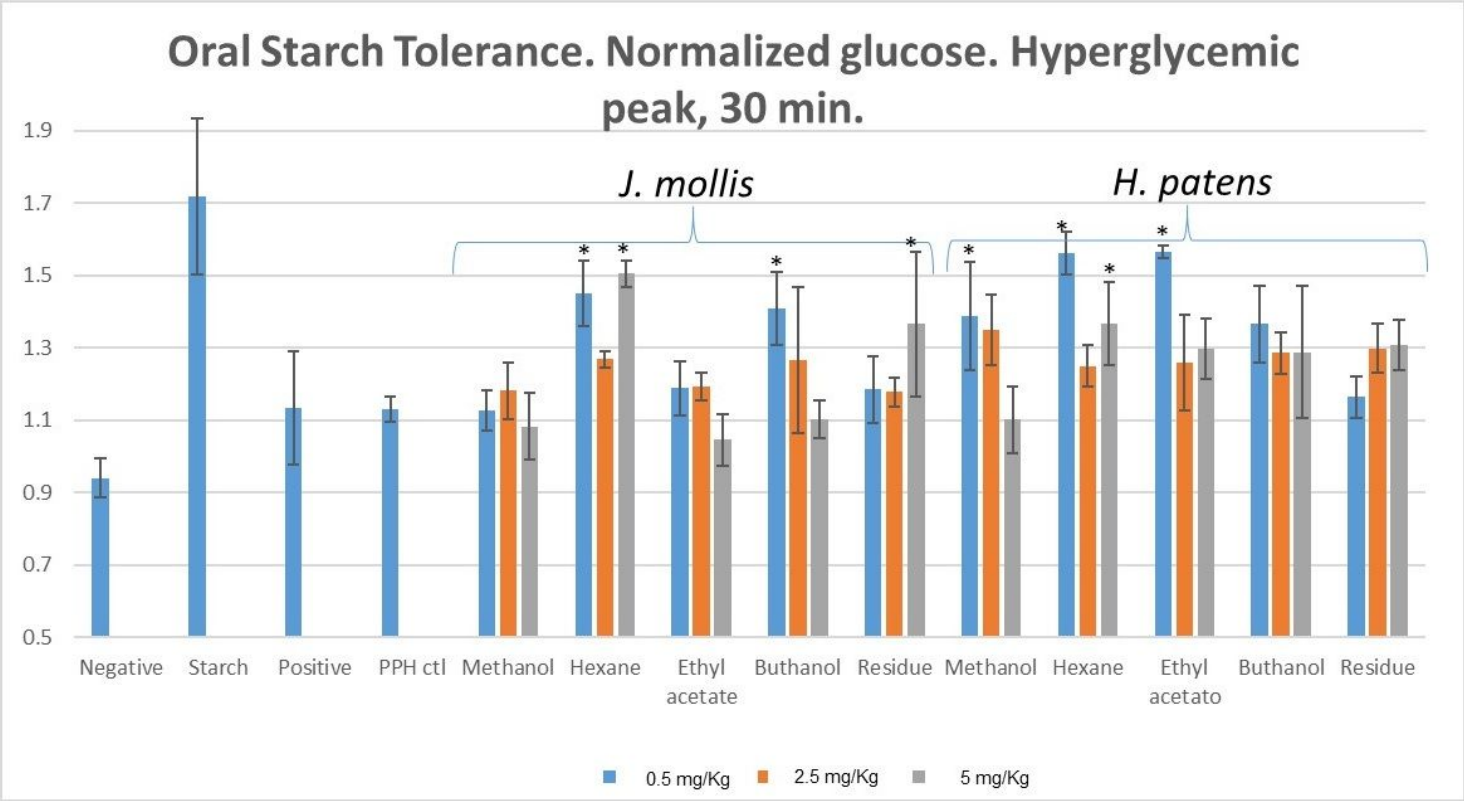

Figure 4

Normalized Glucose 30 min after exposure to controls or extracts. * no significant difference ($p < 0.05$)