

Evaluation of the ex vivo and in vitro antidiabetic and antioxidant activities of the hydroalcoholic extract of *Psidium guajava* L. leaves (Myrtaceae)

ABSTRACT

Objective: Antidiabetic activity was assessed by the skeletal muscle glucose uptake test and antioxidant potential by the total antioxidant capacity, reducing power and DPPH test.

Materials and methods: Same-sex Sprague Dawley rats weighing 150 ± 20 g and approximately 3 months old and different concentrations of the extract obtained from 500 g of *P. guajava* leaves powder were used for the following methods: reduction, free radical scavenging, glucose uptake by skeletal muscle. **Results:** In terms of antioxidant potential, the extract showed significant activity in a dose-dependent manner. In addition, the extract potentiated glucose uptake in the presence and absence of insulin. Compared with metformin and glibenclamide, the extract increased glucose uptake to a greater extent. **Conclusion:** This study shows that the hydroethanolic extract of guava leaves is one of the best recipes for helping to delay the complications associated with diabetes.

Keywords: Diabetes, *Psidium guajava*, antioxidant activity, uptake of glucose

Introduction

Diabetes is one of the most common non-communicable diseases in the world (Fah et al., 2013). It is a condition defined by an abnormal increase in blood sugar levels (Gbekley et al., 2015). Worldwide, the number of diabetics (aged 20 to 79) was estimated at 463 million in 2019 and this number will reach 700 million in 2045 if urgent preventive measures are not taken to reduce its incidence (IDF, 2019). The number of deaths due to this disease was around 4.2 million and the total expenditure recorded was estimated at 673 million US dollars (Ogurtsova et al., 2017) and could reach 760 million in 2019 (IDF, 2019). Of all the continents, Africa is the most affected by this disease (Erasto et al., 2005). In 2019, around 19 million (31.12 %) people (aged 20 to 79) had diabetes, and if nothing is done by 2045, this figure will rise to 47 million among the African population. The number of deaths was 366,200, or 6.8 % of all-cause mortality, and total expenditure on the condition was US\$9.5 million (IDF, 2019). In Togo, diabetes is on the rise due to the population's changing lifestyle and the prevalence rate was 6.2 % (IDF, 2017). According to the WHO, it is a metabolic disease characterised by chronic hyperglycaemia resulting in specific organ complications

(WHO, 2006), leading to death. The positive diagnosis of diabetes can also be confirmed by a fasting blood glucose superior or equal to 1.26 g/l (7 mmol/l) checked on two occasions and/or at least 2.0 g/l (11 mmol/l) at any time of the day; or the 2-hour blood glucose level of an oral glucose tolerance test (75 g OGTT) (Scheen et al., 2010). Hyperglycaemia is the common cause of all types of diabetes. There are three main types and other secondary causes, all of them give rise to almost the same problem: complications (acute and chronic). Acute complications are often metabolic emergencies or coma due to hyperglycaemia and ketoacidosis, but also hypoglycaemia due to inadequate amounts of insulin administered (Gbekley, 2017). Chronic complications are essentially linked to chronic hyperglycaemia via increased activation of the polyol pathway and oxidative stress, increased formation of glycation end products, and accumulation of acylglycerol, activation of protein kinase C (Meziani et Lebsir, 2015). Therapeutic education and drug treatments are therefore essential. Treating diabetes with oral medication (Metformin (Glucophage), Acarbose (Glucobay)); insulin does not replace healthy lifestyle habits. It is used to support a healthy diet, physical activity and weight control. These remain the recommendations of first choice (Auger et al., 2016). The side effects produced by these sometimes-ineffective drug treatments are not negligible. In addition, the relatively high cost of these treatments means that developing African countries are unable to afford adequate care (Fah et al., 2013). To achieve this, people are turning in particular to phytotherapy (treatment using plants), which offers remedies that are better tolerated by the body and more accessible given to the plant biodiversity and wealth of medicinal plants (Eddouks et al., 2007). People can then refer to certain plants that have anti-diabetic properties in Africa, and more specifically in Togo. Data collected during an ethnobotanical survey by Gbekley et al. (2015) identified 112 plant species belonging to 51 families including *Psidium guajava*, an exotic fruit tree species from the Myrtaceae family used in Togo in the maritime region. Scientific data on the plant's mechanism of action is almost non-existent in Togo. This is the background to the current study, the general objective of which is to evaluate the anti-diabetic activity of the hydroethanol extract of the leaves of *Psidium guajava*, a plant with bioactive properties, using methods developed in an animal model.

Materials and methods

Plant material

Guava leaves were collected from the Badougbé farm in the Maritime region. A specimen was identified and authenticated by the plant ecology laboratory and registered under lot 10866 at the herbarium of Université de Lomé. The harvested leaves were then dried in the laboratory at a temperature of 22°C for 5 to 6 days and then ground using a crushing mill.

Animal material

We used Sprague Dawley rats of the same sex with an average weight of 150±20 g, aged approximately 3 months. The animals were reared in the animal house of the Faculty of Science at University de Lomé. The animals were subjected to the natural alternation of day and night, corresponding to 12±1 h of day and night. The ambient temperature in the animal house was 23±1 °C and humidity was between 35 and 60%. They were fed a complete diet consisting of cereals, soya cake, cereal by-products, soya oil, wheat flour, sucrose, casein and vitamins. Water and food were provided ad libitum.

Traditional use

In traditional medicine in Togo, *P. guajava* leaves (Gbèbèti in the vernacular) are used to treat diarrhoea and dysentery. To do this, the leaves are crushed and infused in a glass of hot water, or chewed as they are. A decoction of young guava leaves and corn husks, to which ash is added, is recommended to relieve stomach aches and cramps. A decoction of a mixture of lemon and guava leaves is used to treat coughs. A concentrated decoction of guava leaves is recommended for treating fever, flu symptoms, malaria and fatigue. It is also a commercial plant used to treat diabetes and inflammation.

Hydroalcoholic extraction

A mass of 500 g of the powder obtained was extracted by percolation in 5 litres of a 50 % (v/v) Ethanol/water mixture for 72 hours. The mixture was intermittently homogenised. After 72 h, the macerate collected was filtered using cotton wool and filter paper, then the filtrate obtained was evaporated under vacuum at 45 °C using a Büchi rotary evaporator. The extraction yield obtained was 12.03 % using the following formula: [(Weight of extract/Weight of dry plant matter powder) *100]. The dry extract was stored at 4 °C until use.

Preliminary phytochemical screening

The qualitative determination of flavonoids, tannins, alkaloids, triterpenes, saponosides and carbohydrates in the extract was carried out by chemical reactions using the Harbonne (1973) method, with a few slight modifications.

Phenolic compound content

Total phenols were determined using the Folin-Ciocalteu method, and tannins were determined secondarily after fixation by PVPP (polyvinylpolypyrrolidone) using the method of Maksimovic et al. (2005).

Assay:

Step A: 500 μ L of each extract (stock solution at 1 mg/mL) was transferred to tubes containing PVPP and methanol. The resulting mixture was vortexed and incubated on ice for 30 min (2 times). After centrifugation, 200 μ L of the supernatant was transferred to dry tubes for assay with folin calcium reagent. The blank was prepared with 1mL of methanol in place of the extracts.

Step B: To 200 μ L of the extract solution (stock solution at 1mg/mL) or 200 μ L of the Gallic Acid solutions (200, 150, 100, 50, 25 and 0 μ g/mL) or 200 μ L of the solution obtained in A (extract + PVPP), were added 200 μ L of 10% Folin-ciocalteu. 800 μ L of sodium carbonate solution (700 mM) was added. After two hours incubation in the dark, the optical density (OD) was read at 735 nm.

The amount of total phenol was expressed in terms of mg Gallic Acid equivalent/g extract.

The total amount of tannin was calculated using the formula:

$$DO_T = DO_{Ext} - DO_{Ext+PVP}$$

$$DO_T = DO_{tannin}; DO_{Ext} = DO_{extract}; DO_{Ext+PVP} = DO_{extract + PVP}$$

The determination of total flavonoids is based on the property of flavonoids to form aluminium chelates with aluminium chloride (Mimica- Dukic, 1992) with slight modifications.

Dosage: To 1 mL of extract (1 mg/mL) or rutin (1 mg/mL), was added: 1 mL of aluminium chloride (20 mg/mL). The blank was made with 2 mL ethanol in place of the sample. Different concentrations were used: 0; 5; 25; 50; 100 and 200 μ g/mL. The optical density was read at 440 nm after 150 min incubation at laboratory temperature.

Antioxidant activity of *P. guajava* hydroalcoholic extract

Total antioxidant capacity (CAT)

In an acid medium, the extract containing antioxidants reduces Mo (+6) to Mo (+5) and consequently the green phosphate-Mo (+5) complex is formed.

Dosage: To 0.3 mL of different concentrations (25 to 500 µg/mL) of ascorbic acid and to 0.3 mL of the extract solution (1 mg/mL) prepared with methanol, was added, 3 mL of the prepared reagent (0.6 M sulphuric acid; 28 mM sodium phosphate and 4 mM ammonium molybdate). For 100 mL: 494 mg ammonium molybdate ((NH₄)₆Mo₇O₂₄ · 4H₂O), + 1003 mg disodium sodium phosphate (HNa₂PO₄ · 12H₂O) + 50 mL distilled water; after complete dissolution, 3.33 mL sulphuric acid (18 M) was added and the final volume adjusted to 100 mL. The absorbance of the reaction medium was read at 695 nm against a blank (methanol) after incubation at 95°C for 90 min (N=3). Antioxidant activity was expressed as mg ascorbic acid equivalent/g extract (Prieto et al., 1999).

DPPH (2,2-diphenyl-1-picrylhydrazyl) test

DPPH is a purple-red radical in methanolic solution that turns yellow in the presence of an electron donor (a reductant or antioxidant).

To 1.5 mL of DPPH solution (100 µmol/L) was added 0.25 mL of methanol (blank) or 0.25 mL of extract methanolic solution at different concentrations (5; 10; 25; 50; 100; and 200 µg/mL) in a vat. Ascorbic acid at different concentrations (5; 10; 25; 50; 100 and 200 µg/mL) was used as a positive control. The optical density was read after 10 min at 517 nm (Misseboukpo et al., 2013).

Test of glucose uptake by isolated rat skeletal muscle

This test consists of bringing muscle, a glucose solution and the extract into contact in order to measure the effect of the latter on the mechanism by which skeletal muscle manages to regulate post-prandial glycaemia.

After 18h of fasting, rats were sacrificed by cervical dislocation and the abdominal muscles of the left and right sides were exposed. These muscles were cut into small square pieces (30 in total) weighing 200 to 250 mg and transferred to Erlenmeyer flasks containing 20 ml of Krebs-Ringer's bicarbonate buffer (KRB) at 37°C for 10 min. The KRB solution alone (reference solution) was then replaced with KRB containing 11.1 mM glucose for all samples. We then added the glucose-containing KRB solution + 1 mg/ml extract and 1 mg/ml metformin in the presence or absence of 100 mU/ml insulin to each Erlenmeyer flask. The KRB solution was aerated for 5 min before incubation for 3 h on a shaker at 96 rpm and aliquots of solution were taken after 30, 60, 120 and 180 min to assay glucose and weigh the

piece of muscle. This assay was carried out in the solution samples used for baseline measurements and those taken at T30, T60, T120 and T180 min using GOD-POD (Lawson-Evi et al., 2020).

Lot composition (n = 3 for each lot)

- Batch I: KRB only for 1 to 3 hours
- Lot II: KRB + 100 mU/ml insulin
- Lot III: KRB + 100 mU/ml insulin + Total extract (0.5 mg/ml)
- Lot IV: KRB + Extract (1 mg/ml)
- Lot V: KRB + 100 mU/ml insulin + Total extract (1 mg/ml)
- Lot VI: KRB + Extract (2 mg/ml)
- Lot VII: KRB + 100 mU/ml insulin + 1 mg/ml metformin
- Lot VIII: KRB + 1 mg/ml metformin
- Lot IX: KRB 100 mU/ml insulin + Glib (0.6 µg/ml)
- Lot X: KRB + Glib (0.6 µg/ml)

Preparation of the solutions

Dilution solvent: Distilled water

- ✓ Total extract at a final concentration of 1 mg/ml: 250 mg extract in 5 ml
- ✓ Total extract at a final concentration of 2 mg/ml: 500 mg extract in 5 ml
- ✓ Insulin at a final concentration of 100 mU/ml: Actrapid one pack
- ✓ Metformin Denka at a final concentration of 1 mg/ml: one tablet (500 mg)
- ✓ Glibenclamide at a final concentration of 6 µg/ml: one tablet
- ✓ Glucose stock solution in mM for the standard range
- ✓ Krebs-Ringer's bicarbonate buffer [KRB: 118 mM NaCl (6.89 g in 1 L), 5 mM KCl (0.372 g in 1 L), 1.28 mM CaCl₂ (0.19 g in 1L), 1.2 mM KH₂PO₄ (0.163 g in 1 L), 1.2 mM MgSO₄ (0.14 g in 1 L), 25 mM NaHCO₃ (2.1 g in 1 L)] in the presence of 95 % O₂ and 5 % CO₂.

Use of results

The loss of glucose in the incubation solution is assumed to represent the glucose absorbed by the muscle. Absorbed glucose = (Initial glucose - Final glucose) / 250 where 250 is the fresh weight of the muscle. Absorbed glucose was expressed in mM/g fresh tissue.

Statistical analysis

GraphPad Prism 8.4.2 software was used to carry out statistical analyses of the results obtained. All results were expressed as the mean with the standard error of the mean ($M \pm$

SEM). Analysis of variance (ANOVA) followed by Tukey's and Dunnett's test which was used to define the statistical significance of the results. A value of $p < 0.05$ was considered statistically significant.

Results

Phytochemical study of the hypdroethanolic extract of guava leaves

Preliminary phytochemical screening

The qualitative study (Table 1) shows the presence of phenolic compounds (tannins, flavonoids) and saponosides, which are the most abundant; alkaloids, carbohydrates, reducing compounds and triterpenes.

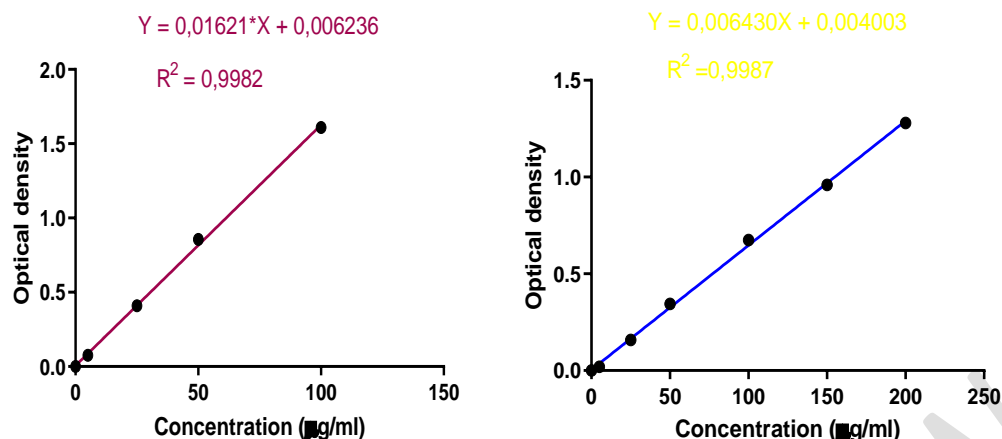
Table 1: Phytochemical compounds present in the extract

Phytochemical compounds	Reagents	Results
Total phenols	FeCl ₃ 10%	+
Tannins	FeCl ₃ 1%	+
Flavonoids	NaOH 1/10 ; FeCl ₃	+
Alcaloids	Mayer	+
Saponosides	Distilled water	+
Carbohydrates	Naphtol + Alcool	+
Reducing compounds	(C ₂ H ₄ O ₂ , FeCl ₃) ; (CuSO ₄ , FeCl ₃)	+
Triterpenes	C ₂ H ₄ O ₂ + CuSO ₄	+

+ = Presence

Content of phenolic compounds in the extract

Total phenols and tannins were determined from the gallic acid calibration range and flavonoids from the rutin calibration range (**Figure 1**).



A (Total Phenols and tannins) B (Flavonoids)

Figure 1 (A ; B) : Calibration curves for the determination of phenolic compounds

Total phenols and tannins are expressed in mg Eq AG /g and flavonoids in mg Eq R /g with AG = gallic acid, R=rutin and Eq= equivalent (n=3).

Tannins were more abundant than flavonoids in *Psidium guajava* leaves: 79.56% compared with 59.85% of total phenols (**Table 2**).

Table 2: Phenolic compound content in the extract

Phytochemical compounds	Polyphenols	Tannins	Flavonoids
Concentration($\mu\text{g/mL}$)	$85,920 \pm 0,308$	$68,358 \pm 2,794$	$51,425 \pm 1,1,607$

In-vitro antioxidant activity of hypdroethanol extract of *P. guajava* leaves

Total antioxidant capacity (CAT)

CAT increased with extract concentration. The antioxidant capacity value: 711.585 ± 38.067 represents all the antioxidant compounds present in the extract and is expressed in mg Eq AA/g.

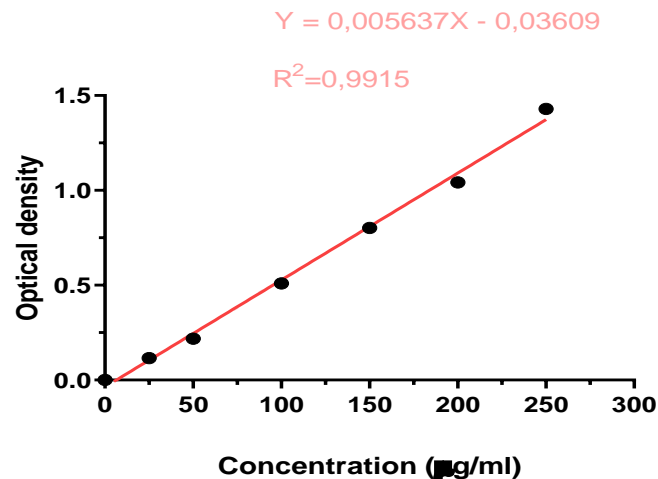


Figure 2: Ascorbic acid calibration curve for CAT determination.

Values are expressed as mean \pm SEM (n=3)

Reducing power

The absorbance of the control and extract increased with concentration, showing an increase in reducing power. However, the absorbance of the extract was lower than that of the control (ascorbic acid) (**Figure 3**).

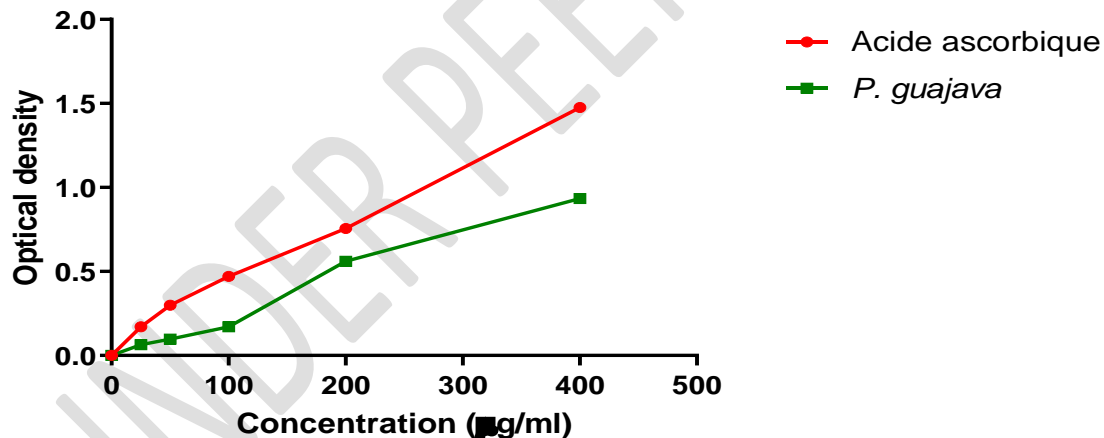


Figure 3: Comparison of the reducing power of the extract compared to ascorbic acid. Values expressed as mean \pm SEM (n=3).

Determination of DPPH trapping activity (1, 1-diphényl-2-picryl hydrazyl)

Table3: Effect of extract on DPPH \cdot radical scavenging

Drugs	Ascorbic acid	Extract
IC ₅₀	48,211 ± 0,847	41,718 ± 0,918

IC₅₀ (µg/mL) expressed as mean ± SEM were determined by Fit Spline/LOWESS with GraphPad (n=3). The IC₅₀ of the extract is lower than that of ascorbic acid.

The ability to trap the DPPH radical is dose-dependent.

The lower the IC₅₀, the greater the trapping capacity. The extract therefore traps the DPPH radical more than ascorbic acid (standard).

Evaluation of the anti-diabetic activity of hypdroethanolic extract of guava leaves

Guava leaves extract significantly increased glucose uptake both in the presence and absence of insulin. Compared with metformin and glibenclamide, the extract increased uptake more. However, it should be noted that this uptake was potentiated in the presence of insulin (Table 4).

Table 4: Effect of extract (P.G) on glucose uptake by isolated rat skeletal muscle

Lots	Glucose absorbed by tissu (mM/gof freh tissu)			
	30 min	60 min	120 min	180 min
Glucosemedium (MG)	5,637±0,226	8,382±0,672	8,090±0,691	9,315±0,585
MG+Insulin(10mU/L)	9,563±0,498*	9,960±0,907	11,319±1,462	11,808±0,000
MG + P.G (0,5mg/mL)	30,733±0,453****	33,646±1,100****	33,084±1,931****	30,263±0,353****
MG+P.G(0,5mg/mL) +Insulin(100mU/L)	30,682±0,000****	33,067±1,478****	37,215±1,226****	31,186±0,571****
MG + P.G (1mg/mL)	32,563±0,507****	34,058±0,319****	33,848±1,021****	32,731±1,511****
MG + P.G (1mg/mL) +Insulin(100mU/L)	34,797±1,259****	36,980±0,890****	37,265±1,175****	36,207±0,353****
MG+Met(0,2mg/mL)	9,930±0,232**	14,069±0,067***	12,918±0,606**	13,616±0,069**
MG+Met (0,2mg/mL) + Insulin(100mU/L)	11,438±1,411***	14,427±0,269***	14,432±0,273****	15,429±0,140****

MG+Glib (0,6µg/mL	11,869±1,652***	13,211±0,030**	15,451±0,051*****	15,539±0,538*****
MG+Glib (0,6µg/mL)	13,080±0,400***	13,536±0,155***	14,528±1,444*****	17,003±0,000*****
+ Insulin (100mU/L				

Values were expressed as mean \pm SEM (n=2); Two-way ANOVA followed by Dunnett's multiple comparisons test; significantly different from control: * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$; **** $p < 0,0001$.

Discussions

The preliminary phytochemical tests carried out during this study showed the presence of total phenols, flavonoids, tannins, alkaloids, carbohydrates and reducing compounds. These results are in agreement with those obtained by Feng et al. (2015); Correa et al. (2016); Müller et al. (2018).

The determination of polyphenolic compounds (total phenols, flavonoids and tannins) in the extract revealed that the extract contains a high amount of total phenols but is less rich in flavonoids (51.425 ± 1.607 mg Eq R/g) than in tannins (68.358 ± 2.794 mg Eq AG /g). These results are in line with those obtained by Mariana et al. (2016). Nevertheless, some slight differences were observed in flavonoids, which were in high quantities unlike tannins. This could be explained by the diversity of *P. guajava* chemotypes depending on the sampling location, the harvesting season and the nature of the extraction solvent. In diabetic patients, chronic hyperglycaemia remains a highly alarming condition due to the excessive production of free radicals, which cause damage to the body's antioxidant systems through the oxidation of membrane lipids, DNA fragmentation and other biomolecules. Ighodaro (2018) has demonstrated this increased production of superoxide anion radical and hydrogen peroxide in the glucose oxidation pathway during hyperglycaemia. The high total antioxidant capacity of the extract and its reducing power highlighted its ability to trap free radicals by DPPH, thanks to the flavonoids in the oxidation-reduction chain. The antioxidant capacity and reducing power of the extract may enable the body to reduce the peroxidation of membrane lipids, the generation of reactive species and prevent damage to DNA by the NADPH oxidase system. This reduction could be explained by the presence of total phenols, flavonoids and tannins that interact directly with activated oxygen species to inhibit the formation of free radicals (Martin-Nizard et al., 2004). Compared with ascorbic acid (AA), the extract (PG) showed a greater capacity to trap free radicals ($IC_{50}AA=48.21\pm0.847$ µg/mL; $IC_{50}PG = 41.718\pm0.918$ µg/mL). Antioxidant activity combined with a reduction in glycaemia would be an asset for

diabetic patients. Glucose uptake by skeletal muscle (the most abundant tissue in the body) thanks to GLUT1 and GLUT4 (St-Amand,2015) thus constitutes one of the therapeutic approaches for the treatment of diabetes. Indeed, it remains the crucial site for post-prandial glucose utilization (Saisree et al., 2019). In diabetes, the general pathological state results from the failure of skeletal muscle to absorb glucose despite stimulation by insulin (Scheen et Paquot, 2006; Saisree et al., 2019). To correct this defect, the effect of the extract was measured in vitro on glucose uptake by rat skeletal muscle in the presence or absence of insulin. This effect was observed by the decrease in the concentration of glucose in the incubation medium with time. In the presence of the extract, we observed a significant uptake of glucose by skeletal muscle compared with the control. By increasing the concentration of the extract, the uptake of glucose was more significant at 30, 60, 120 and 180 min, with a slight variation, as was the case when the extract was combined with insulin. In combination with oral antidiabetics (metformin and glibenclamide), insulin resulted in significant glucose uptake, but this was low compared with the extract. We deduced that the extract would induce translocation of the GLUT4 glucose transporters that carry glucose to the plasma membrane and to the transverse tubules, and would therefore have a mechanism of action similar to that of insulin. These results confirm those obtained by Saisree et al. (2019).

Conclusion

This study identified the phytochemical compounds contained in the hydroalcoholic extract of guava leaves and their quantities. These compounds form the basis of the antioxidant activity determined by total antioxidant capacity, reducing power and free radical scavenging (DPPH[•]) and therefore delay the complications associated with diabetes. The effect of the extract on glucose uptake by skeletal muscle ex vivo showed satisfactory results, significantly increasing glucose uptake in the presence and in the absence of insulin compared to metformin and glibenclamide. All these results justify the use of this plant in traditional medicine for the treatment of diabetes.

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