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Cassia grandis fruit extract reduces the blood glucose level in alloxan-induced diabetic rats

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ABSTRACT

Introduction: *Cassia grandis* Lf fruits are ethnobotanically used for digestive disorders, anemia, and for reducing blood glucose. However, there are no studies about the antidiabetic activity nor the oral toxicity of the plant fruit-extracts. This paper aims to evaluate the hypoglycemic effect of *C. grandis* fruits extract *in vivo,* and assess the acute oral toxicity, and sub-acute oral toxicity. The antioxidant activity and the α-glycosidase inhibitor effect were also evaluated.

Methods: The extract was obtained by maceration of the fruit pulp with 70% hydroalcoholic solution (1:2, m:v). The extractive solution was concentrated in a vacuum rotary evaporator, up to a drug: solvent ratio of 2:1 (g/ml). Soluble solids, relative density, refractive index, pH, total phenolics, and flavonoids were determined. A preliminary phytochemical screening was made, followed by the quantitation of volatiles by GC/MS. The acute and sub-acute oral toxicity was evaluated in Sprague Dawley rats, by using biochemical and hematological parameters. The radical scavenging activity (DPPH·, ABTS·) and α-glycosidase inhibitory effect were tested. The hypoglycemic effect was assessed in alloxan-induced diabetic rats.

Results: The extract of *C. grandis* contains alkaloids, coumarins, flavonoids, free amino acids, amines, phenols, tannins, reduced sugars, resins, saponins, steroids, and triterpenes, plus 38 volatile compounds, being linalool the most abundant (1,66%). The extract exhibited an LD_{50} > 2000 mg/kg, and after a continuous administration (1000 mg/kg, 28-days), the hematological and biochemical parameters were normal. The extract showed hypoglycemic effect, being the dose 200 mg/kg no statistically different from glibenclamide at 25 mg/kg. Good antioxidant activity and a potent α-glycosidase inhibitory effect were also observed.

Conclusion: *C. grandis* extract is an excellent hypoglycemic and non-toxic plant product. The hypoglycemic mechanism could be associated with the antioxidant effect and with the α-glycosidase inhibition. Up to the best of our knowledge, this is the first report on the hypoglycemic effect *in vivo* of *C. grandis* fruits extract.

1. Introduction

Diabetes mellitus is a metabolic disease widespread all over the world, characterized by excess in blood glucose and changes in the carbohydrate metabolism. The global prevalence of diabetes among adults over 18 years in 2014 was 4%, and it was estimated at 5.22% (366 million of people) for 2030 [1]. Despite the progress of the diabetes management, it is still not possible to avoid its lethal consequences. However, antidiabetic drugs treatments are reduced [1,2]. For this reason, many people use medicinal plants for lowering the

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blood glucose levels.

Various species of the genus Cassia are used by diverse populations to treat diabetes [3,4], the hypoglycemic effect of some species has been confirmed *in vivo* [5–8]. Inhabitants of Central America, the Caribbean, and South America use the fruit pulp of *Cassia grandis* Lf for digestive disorders, anemia [9], and diabetes [10,11]. The species *Cassia grandis* (Cañandonga, Cassia Rosa, Cassiagrande), belongs to Fabaceae family, subfamily Cesalpinaceae. The stem bark extract of *Cassia grandis* reduces the blood glucose in normal rats and diabetic rats [12]; however, removing the stem bark of plants can cause severe damages. Contrarily, the fruits are harvested twice a year, producing a considerable amount of pulp. The fruit pulp has a characteristic smell and strong sweet flavor. Additionally, the ripe fruit contains phenols, flavonoids, coumarins, terpenes, reducing sugars, amino acid, amines, saponins, and glycosides [13]. Some of these metabolites could explain the ethnobotanical use of this plant fruit.

The hydroalcoholic extract of *C. grandis* fruits is non-genotoxic in mice [14]. Nonetheless, there is neither acute oral toxicity nor subacute oral toxicity studies of the *C. grandis* fruit extract (CgE). In the same way, the hypoglycemic effect was never assayed *in vivo*. Thus, this paper aims to evaluate the hypoglycemic potential of the CgE in alloxan-induced diabetic rats and assess the acute and 28-day sub-acute oral toxicity in Sprague-Dawley rats. The antioxidant activity and the α-glycosidase inhibitory activity were also evaluated. Additionally, the extract was characterized, using spectrophotometric techniques, and GC/MS, to obtain a vegetal product that could be used in pharmaceutical preparations.

2. Materials and methods

2.1. Plant material

Fruits of *C. grandis* L. f were collected in El Caney (20.0569 N; −75.7719 W), Cuba, in April 2016. A sample of the species was identified by Félix Acosta Cantillo and was deposited in the Eastern Center of Ecosystems and Biodiversity herbarium (BIOECO), in Santiago de Cuba, Cuba, with voucher No.1965.

2.2. Extract preparation

The ripe fruit pulp (1 kg) was macerated with 2 l of 70% hydroalcoholic solution for 72 h, at room temperature [15]. After that, the extract was filtered at vacuum and concentrated using a rotary evaporator at 40 °C (KIKA WERKE GMBH & Co. Germany). The final drug/solvent ratio was 2:1 (w/v) [16]. CgE was poured into an amber flask and stored protected from light, at 25 ± 2 °C.

2.3. Phytochemical evaluation

A preliminary phytochemical screening was made, to detect the presence of metabolites such as mucilage (polysaccharide), saponins, alkaloids, triterpenes, steroids, quinones, coumarins, resins, essential oils, reducing sugars, phenols and tannins, flavonoids, free amino acids, amines, and glycosides [16].

Total solids, relative density, and the refractive index of the CgE were determined [17]. The pH was directly measured using a pH-meter (Tecnopon, Brazil) previously calibrated with buffer solutions (pH 4 and 7, Alphatec, Brazil). Total phenolics as pyrogallic acid and Total flavonoids as rutin were spectrophotometrically determined [18]. All the measures were made in triplicate.

2.4. Gas chromatography/mass spectrometry analysis

Three successive extractions from 100 ml of CgE were performed, using a separatory funnel, and ethyl ether as solvent (15 mL). The extracts were pooled and dried for 12 h over anhydrous sodium sulfate. After that, the ethereal extract was concentrated to 10 ml under a nitrogen stream. The volatile composition was performed by GC/MS (SHIMADZU GCMS-QP500, Japan). A DB-5MS capillary column (Agilent Technologies, USA) of 30 m × 0.32 m and 0.25 mm thick film was used. The temperature was programmed at 40 °C for 3 min, with an increase of 5 °C/min up to 250 °C. In the end, the temperature was kept constant at 250 °C. The mass spectra were recorded over 60–260 AMU range, with ionization energy of 70 eV. The injection volume was 10 μL with a split ratio of 100:1. Helium was used as the carrier gas at a flow rate of 0.5 mL/min. Both, the injector and detector temperatures were kept at 280 °C. The constituents were identified by comparing the retention Kovac index and the mass spectrum with the indexes and reference recorded in the FFNSC 1.3 library and NIST database. The percentage composition was calculated using the peak normalization method. Results were expressed in g/100 ml of extract.

2.5. Evaluation of acute oral toxicity

Female Sprague-Dawley rats, nulliparous of 10 weeks old, with body weight between 150–200 g, were used. Animals were supplied by the National Center for Production of Laboratories Animal (CENPALAB), Havana City. They were randomly grouped (Two groups, 5 animals each) and marked to allow the individual identification. Before the administration, animals were acclimatized for seven days at a controlled temperature of 23 ± 2 °C, relative humidity of 60 ± 10% and a 12/12 h light/dark cycle [19]. Animals had free access to food (standard chow CMO-1000) and distilled water. All the tests were conducted agreeing to the Good Laboratory Practice. The ethical considerations established by the Ethics Committee of the Toxicology and Biomedicine Center (TOXIMED), Medical University of Santiago de Cuba, Cuba, were taken into account.

The first day, after a fasted period of 12 h, the animals were weighed. After that, the Group I (Control) received distilled water (2 mL) and the Group II received 2000 mg/kg [20] of CgE in one dose, dissolved in distilled water (up to 2 mL). An intragastric cannula (Vygón, France) was used for administration. Three hours after, the food and water access were restored.

2.5.1. Gross observation, food and water intake, and relative organ weight

The animal's behavior was recorded the first 4 h after the administration, and occasionally along the rest of the day. The record was made twice a day for the next 14 days. Clinical observations were made to evaluate the physical condition and the nasal-ocular mucosa. Particular attention was paid to possible changes in the skin and coat, the respiratory rhythm, nervousness and possible death occurrence. Daily, the food and water intake were recorded [19,20]. The day 14, animals were euthanized using a ketamine overdose (ip) and subjected to a gross necropsy for detecting possible pathological changes. The kidney, lung, liver, pancreas, vessel, spleen, stomach, intestines, and heart were removed, rinsed with distilled water, dried with filter paper, and weighed. Macroscopic observations were made for detecting morphological changes/and or anatomical alterations. The relative organ weight (ROW) in percent, was calculated (Organ weight ×100/ Animal body weight).

2.6. Evaluation of 28-dayssub-acute oral toxicity

Female Sprague-Dawley rats, 14 weeks old, body weight 225–265 g were used. The Pharmaceutical Research Laboratory, Federal University of Amapá, Brazil, supplied the animals. They were acclimatized for a week at controlled temperature (22 ± 3 °C), relative humidity (60 ± 10%), and a 12/12 h light-dark cycle. The feed was standard chow (NUVILAB MCP 689) and distilled water, *ad-libitum*. Ethical considerations established by the Ethics Committee of the Federal University of Amapá (Protocol Number 012/2017), were taken into account.

The methodology described in OECD 407 [20] was used. Two experimental groups (5 animals each) were randomly formed. The control group (Group I) received only distilled water through the experiment. The Group II received CgE in a dose of 1000 mg/kg, daily for 28 days. Animals were weighted every nine days.

Clinical observations were made daily during the assay for detecting changes in the physical condition, mucosa, skin and coat, the respiratory rhythm, and for a possible death occurrence. The food and water intake were recorded daily. The day 29th, after a fasting period of 12 h, the animals were anesthetized with ethyl ether, and blood samples were collected from the orbital plexus for biochemical and hematological studies [20]. The blood was collected into assay tubes with and without heparin. Hemoglobin, hematocrit, platelet count, red and white blood cell count were assessed using an automatic hematology analyzer (Mytic, Switzerland).

For biochemical analysis, the blood was centrifuged (3500 rpm) at 4 °C, for 20 min (Sigma, USA), and the serum was separated. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, alkaline phosphatase (ALP) and total protein (TP) were determined using a standard analytical kit (Sigma-Aldrich®, USA). It was also evaluated the serum cholesterol and triglycerides using the methodology described by Allain et al. [21]. Blood glucose levels were assessed using a commercial glucometer (Roche, Germany).

Animals were sacrificed by cervical dislocation after ether anesthesia. The internal organs (kidney, lung, liver, pancreas, vessel, and heart) were observed macroscopically and microscopically. For each organ, the relative weight (in percent) was calculated [22].

2.7. Hypoglycemic activity

Sprague-Dawley rats supplied by the Pharmaceutical Research Laboratory, Federal University of Amapá, were used. Animals were acclimatized, during a week, under controlled temperature 22 ± 3 °C, relative humidity 60 ± 10%, and 12/12 h light-dark cycle. Animals were fed with standard chow NUVILAB MCP 689 and distilled water, *ad-libitum*. The experiment was carried out in agreement with the Ethics Committee for the Use of Laboratory Animals (CEUA). Federal University of Amapá (UNIFAP), Macapá-AP, Brazil, protocol number 012/2017.

Diabetes was induced with Alloxan (150 mg/kg, ip, every 72 h), completing three administrations [23]. After seven days, the blood glucose was determined using a standard glucometer (Roche, Germany). Normal blood glucose in fasted mice is between 80–110 mg/100 ml [24,25]. Animals with blood glucose above 250 mg/100 m l were included in the experiment. Five experimental groups (5 animals each) were randomly formed.

Group (I), healthy animals, received one mL of distilled water along the assay.

Group II: Diabetic rats, without treatment, received one mL of distilled water along the assay.

Group III, Diabetic rats, received daily, glibenclamide 25 mg/kg

Group IV, Diabetic rats, received daily, 100 mg/kg of CgE

Group V, Diabetic rats, received daily, 200 mg/kg of CgE

The treatments were administered orally, using an intragastric cannula. The animals have free access to food and water along the experiment. The blood glucose level was determined the days 0, 5, 10, 15, 20, and 25 using a standard glucometer (Roche, Germany).

2.8. DPPH· radical scavenging

The radical scavenging activity was evaluated spectrophotometrically using the DPPH· (1,1-diphenyl-2-picrylhydrazyl) radical assay [26]. Solutions of CgE (30 µL) of different concentrations (0.013, 0.026, 0.052, 0.103, 0.206, 0.412, 0.825, 1.650 µg/ml) in distilled water were incubated in the dark, at room temperature, for 30 min, with 270 µl of DPPH· solution (0.05%). Solutions of gallic acid (30 µL, 1 mg/mL) and ascorbic acid (30 µL, 1 mg/mL) were used as positive controls (reference antioxidants). A mixture of DMSO (270 µL) plus CgE (30 µL) was used as a negative control. The absorbance diminution was measured at 492 nm using a microplate detector (DTX 800, Beckman, UK). The scavenging activity as a percent of inhibition was calculated:

$$\% \text{ inhibition} = 100 - [\text{Abs}_{a/p}/\text{Abs}_{C}] \times 100 \quad (1)$$

Where: $\text{Abs}_{a/p}$ = (Absorbance of the Sample – Absorbance of the blank)

Abs_{C} = (Absorbance of the negative control – Absorbance of the Blank)

The experiment was made in triplicate, and the results were expressed as the means ± standard deviation. The IC_{50} concentration was determined by Probit analysis using the GraphPad Software (CA, USA), at a significance level of 0.05.

2.9. ABTS· assay

The assay was made using the Shanty's method [27] with some modifications. The working solution was prepared by mixing an ABTS· (2-2′-azino-di-(3-ethylbenzthiazoline sulfonic acid) stock solution (7 mM) and a potassium persulfate solution (2.4 mM) in a ratio 1:1. The mixture was incubated (14 h) at room temperature, in the dark. After that, the mixture was diluted with distilled water (1:5). For the assay, 30 µl of the CgE of different concentrations (0.013, 0.026, 0.052, 0.103, 0.206, 0.412, 0.825, 1.650 µg/ml) was mixed with 270 µl of the working solution. The mixture was incubated for 15 min, in the dark, at room temperature. The blank was composed of water (270 u L) plus 30 µl of the CgE solution. Gallic acid (1 mg/mL) and ascorbic acid (1 mg/mL) were positive controls, while a mixture of DMSO (270 µL) plus CgE (30 µL) was used as a negative control. The absorbance was measured at 620 nm in a microplate detector (DTX 800, Beckman, UK). All tests were performed in triplicate (n = 3). The results were expressed as percent of inhibition and were calculated as:

$$\% \text{ inhibition} = 100 - [\text{Abs}_{a/p}/\text{Abs}_{C}] \times 100 \quad (2)$$

Where: $\text{Abs}_{a/p}$ = (Absorbance of the Sample – Absorbance of the blank)

Abs_{C} = (Absorbance of the negative control – Absorbance of the Blank)

The IC_{50} concentration was determined by Probit analysis using the GraphPad Software (CA, USA), at 0.05 level of significance.

2.10. Murine α-glycosidase inhibitory activity

The assay was made according to Andrade and coworkers [28], with slight modifications. The release of 4-nitrophenol from 4-nitrophenyl α-d-glucopyranoside (4-NPGP) was determined. Three mg of rat intestinal acetone powder (Sigma, USA) was homogenized with 1 mL of phosphate buffer (pH 6.9, 0.1 M). The mixture was vortexed for 1 min and centrifuged at 13,000 rpm, for 10 min. The supernatant enzyme solution was immediately used. A solution 5 mg/mL of 4-NPGP in sodium phosphate buffer pH 6.9 was also prepared. In the microplate wells, was placed 30 µL of standard solution (Acarbose, 100 µg/mL), the control (DMSO) and the CgE solutions of 2.5, 5, 10, 15, 20, 25, 30 and 33 ug/mL of concentration, with 170 u l of the enzymatic solution. Microplates were incubated for 5 min at 37 °C, in the dark. After that, 100 µL of 4-NPGP was added to each well and incubated for additional 20 min and/or the control absorbance at 405 nm was equal to 1.000. A microplate reader (DTX 800, Beckman, UK) was used. Samples with inhibition ≥50% were used for estimating the IC_{50} value. The analysis was performed in triplicate, and the percent inhibition (% I) was calculated using the formula:

$$\% \text{ I} = 100 - [\text{Abs}_{a/p} / \text{Abs}_{C}] \times 100.$$

Where: $\text{Abs}_{a/p}$ = (Absorbance of the Sample – Absorbance of the blank)

Table 1
Metabolites identified in the extract of *Cassia grandis* Lf fruits.

Metabolites	Assay	Positive result	Occurrence
Alkaloids	2 m l CgE + few drops of Hager's reagent	Yellow precipitate	+
Coumarins	2 m l CgE + 3 m l NaOH (10%)	Yellow coloration	+
Flavonoids	1 m l CgE + 1 m l Pb $(OAc)_4$ (10%)	Yellow coloration	+ +
Amino acids/amines	1 m l CgE + 5 drops of ninhydrin 10%	Blue coloration	+
Phenols and tannins	2 m l CgE + 2 m l H_2O + 3 drop $FeCl_3$ (5%)	Brown coloration	+ + +
Reduced sugars	3 m l CgE + 1 ml $CuSO_4$ alkaline + heat	Red precipitate	+
Resins	1 m l CgE + 3 drops of acetic anhydride + H_2SO_4	Yellow coloration	+
Saponins	5 m l CgE + 5 m l H_2O + heat	Foam formation	+
Steroids	2 m l CgE + 2 m l $CHCl_3$ + 2 m l H_2SO_4 (conc.)	Reddish brown ring	+
Triterpenes	2 m l CgE + 2 m l $(CH_3CO)_2O$ + 2-3 drops conc.	Red precipitate	+

Positive results expressed as high (+ + +), moderate (+ +), and low (+) amounts of phytochemicals, according to the intensity of color and/or precipitated.

Abs $_C$ = (Absorbance of the negative control – Absorbance of the Blank)

The IC_{50} concentration was determined by Probit analysis using the GraphPad Software (CA, USA), at a level of significance of 0.05.

2.11. Statistical analysis

Stat Graphics Centurion XV.I (Stat-Ease Co. MA, USA) was used for data analysis. The mean and standard deviation were reported. Comparison between two groups was made using a t-test for unpaired samples. For comparing more than two groups, a One-way ANOVA was made; followed by Tukey's HSD test. Statistically significant differences were considered at $p < 0.05$.

3. Results

3.1. Extract characterization

Cassia grandis extract appears as a dark brown liquid, with a pungent smell. The phytochemical screening of the extract is shown in Table 1. CgE contains 8.67 ± 0.83% of soluble solids, a relative density of 1.365 ± 0.087 g/ml (25 °C), refractive index 1.392 ± 0.037 (25 °C), pH 5.8 ± 0.5, 4.25 ± 1.25% of flavonoids as rutin, and 6.58 ± 0.88% of polyphenols as pyrogallic acid.

3.2. CgE volatiles composition

The GC/MS analysis detected 38 volatiles compounds (Table 2). The 99.49% of the curve area was quantitatively determined. The most abundant volatiles presents were linalool (1.66%), isovaleric acid (0.84%), eugenol (0.67%), β-caryophyllene (0.63%), 2,3-pentadione (0.62%), camphor (0.52%), sabinene (0.51%), isovarelalehyde (0.44%), benzaldehyde (0.44%), benzyl salicylate (0.43%), isoamyl alcohol (0.33%), cinnamic acid (0.32%), β-elemene (0.31%), dimethyl trisulfide (0.30%), tetradecanoic acid (0.27%), dimethyl disulfide (0.24%), docecanoic acid (0.23%) and β-eudesmol (0.23), by this order.

3.3. Acute oral toxicity

During the test, animals do not show signs of toxicity. The oral, ocular, and nasal membranes preserved a normal aspect. No changes in the skin and coat were detected, and the somatomotor activity and behavior were normal. The experiment finished with 100% survival. Fig. 1 shows the body weight (Fig. 1A) and the water and food intake (Fig. 1B) along the study. In the Group I, the daily water and food intake, per animal, was 35.47 ± 0.93 ml and 25.12 ± 1.19 g, respectively. In the Group II was 36.53 ± 0.93 ml of water, and 24.98 ± 2.37 g of food. No significant difference was observed in water intake ($F = 0.37$; $p = 0.6378$) and food ($F = 0.32$; $p = 0.7327$) between the groups. In the same way, the body weight of both groups did not show statistical differences in the time ($F = 0.002$, $p = 0.9600$).

Table 2
Volatiles present in *Cassia grandis* fruit extract.

No.	Compound	Formula	Rt	RI	g/100 m L
1	3-methylbutanal	$C_5H_{10}O$	4.2006	651	0.436
2	1-penten-3-ol (E)	$C_5H_{10}O$	4.9429	671	0.005
3	2,3-pentanedione	$C_5H_9NO_2$	6.7614	702	0.623
4	Isoamyl alcohol	$C_5H_{12}O$	8.0047	745	0.433
5	Dimethyldisulfide	$C_2H_6S_2$	8.5410	748	0.436
6	Ethyl isobutyrate	$C_6H_{12}O_2$	10.2912	759	0.005
7	Isobutyric acid	$C_4H_8O_2$	10.3351	788	0.003
8	Hexanal	$C_6H_{12}O$	11.1028	805	t
9	2-furfural	$C_5H_4O_2$	11.8931	839	t
10	Isovaleric acid	$C_5H_{10}O_2$	13.3310	892	0.345
11	Benzaldehyde	C_7H_6O	14.0105	964	0.436
12	Dimethyl trisulfide	$C_2H_6S_3$	16.8217	972	0.305
13	Sabinene	$C_{10}H_{16}$	18.0874	978	0.509
14	Myrcene	$C_{10}H_{16}$	18.9276	994	0.326
15	α-terpinene	$C_{10}H_{16}$	20.2980	1021	0.024
16	p-cymene	$C_{10}H_{14}$	21.9127	1029	0.033
17	Linalool	$C_{10}H_{18}O$	23.4831	1099	1.662
18	Camphor	$C_{10}H_{16}O$	24.0584	1149	0.525
19	2-methoxy-1-phenyletanone	$C_9H_{10}O_2$	26.1021	1176	0.068
20	α -terpineol	$C_{10}H_{18}O$	29.2181	1192	0.169
21	Geraniol	$C_{10}H_{18}O$	30.0311	1257	0.127
22	Geranial	$C_{10}H_{16}O$	30.9107	1269	0.095
23	n-undecanal	$C_{11}H_{22}O$	32.1401	1313	0.069
24	4-methoxy-4-vynilphenol	$C_9H_{10}O_2$	33.1246	1317	0.024
25	Eugenol	$C_{10}H_{12}O_2$	34.0216	1362	0.635
26	α-copaene	$C_{15}H_{24}$	36.4213	1381	0.017
27	Methyl cinnamate	$C_{10}H_{10}O_2$	38.0470	1385	t
28	β-elemene	$C_{15}H_{24}$	39.7701	1394	0.315
29	Methyl eugenol	$C_{11}H_{14}O_2$	40.0007	1410	t
30	β-caryophyllene	$C_{15}H_{24}$	42.0140	1421	0.668
31	Cinnamic acid	$C_9H_8O_2$	43.4637	1459	0.325
32	Dodecanoic acid	$C_{12}H_{24}O_2$	44.2020	1570	0.235
33	β-eudesmol	$C_{15}H_{26}O$	46.3319	1654	0.233
34	Myristic acid	$C_{14}H_{28}O_2$	47.9450	1780	0.267
35	Benzyl salicylate	$C_{14}H_{12}O_3$	48.8808	1867	0.426
36	Palmitic acid	$C_{16}H_{32}O_2$	49.9090	1993	0.085
37	Ethyl hexadecanoate	$C_{18}H_{36}O_2$	51.0209	1998	0.037
38	Isopropyl hexadecanoate	$C_{19}H_{38}O_2$	53.2031	2004	0.048

Rt, retention time; RI, retention index; t, trace < 0.01%.

3.4. Anatomopathological examination

No changes in the organs' cytoarchitecture that could be attributed to toxic processes were observed. The anatomy and color of kidney, pancreas, heart, lungs, liver, were normal without any macroscopic alterations. No statistical difference was observed between the relative organ weights of both experimental groups ($p > 0.05$, Table 3).

3.5. Sub-acute toxicity

Neither clinical nor behavioral signs, which could be associated with systemic toxic effects, were observed. There were no changes in animals' skin and fur. Oral, ocular, and nasal membranes maintained a

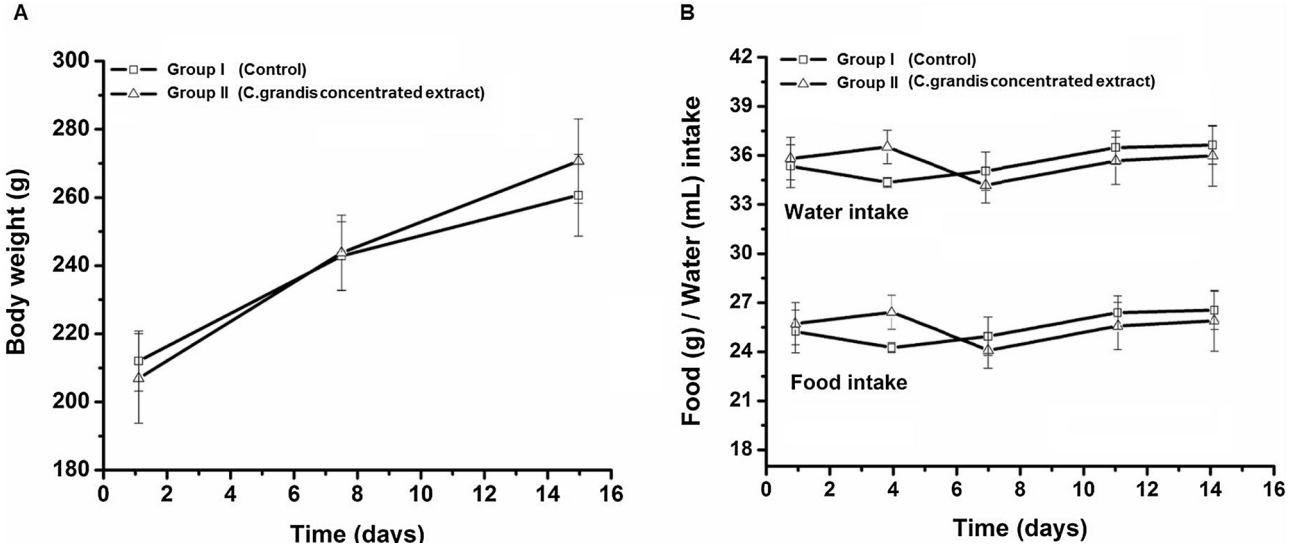

Fig. 1. Body weight (A), and food and water intake (B) of experimental groups during the acute oral toxicity evaluation.

Table 3
Relative organ weights of both experimental groups, in the acute oral toxicity evaluation.

Organ	Group I (%)	Group II (%)	(*t*-test; p-value)
Kidney	0.77 ± 0.15	0.80 ± 0.04	−0.8356; 0.4503
Pancreas	0.35 ± 0.14	0.33 ± 0.06	0.6363; 0.5591
Heart	0.32 ± 0.11	0.37 ± 0.16	0.2933; 0.4357
Lungs	0.55 ± 0.12	0.58 ± 0.09	1.5514; 0,9842
Liver	4.04 ± 0.35	4.33 ± 0.23	2.5683; 0.6314

Group I, control group; Group II, extract *of C. grandis*. n = 5; statistical difference at $p < 0.05$.

normal humidity and color during the experiment. The essay concluded with a 100% survival. Group I intake 178.45 ± 3.61 ml of water (35.69 mL/animal), and 91.33 ± 5.35 g of chow (18.27 g/animal). Group II intake 175.15 ± 4.25 ml of water (35.03 mL/animal), and 89.05 ± 3.84 g of chow (17.81 g/animal). There was no statistical differences between the water intake (F = 2.82, $p = 0.1366$) and food (F = 3.29, $p = 0.1086$) of the groups during the study.

3.6. Relatives' organ weight

Table 4 shows the relative weights of internal organs, in the sub-acute toxicity evaluation. In all cases, the relative weight of the same organ in both groups was not statistically different ($p > 0.05$, Table 4).

3.7. Hematological and biochemical evaluation

Table 5 shows the hematological parameters evaluated for assessing the sub-acute oral toxicity. In both groups, the hematological values were normal and showed no statistical differences (p-value > 0.05, Table 5).

Table 6 exhibits the biochemical markers evaluated assessing the sub-acute oral toxicity. In both groups, the biochemical parameters were normal and showed no statistical differences (*p*-value > 0.05, Table 6).

3.8. Hypoglycemic activity

In Fig. 3 is shown the effect of the experimental treatments in blood glucose levels of the five experimental groups.

Table 4
Relative organ weights of both experimental groups after 28-days, in the sub-acute oral toxicity evaluation.

Organ	Group I (%)	Group II (%)	(*t*-test; p-value)
Kidney	0.71 ± 0.02	0.72 ± 0.02	2.26; 0.1640
Pancreas	0.36 ± 0.04	0.35 ± 0.06	1.58; 0.0985
Heart	0.31 ± 0.03	0.30 ± 0.02	2.19; 0.0780
Lungs	0.53 ± 0.02	0.48 ± 0.04	2.78; 0,1018
Liver	3.88 ± 0.35	4.00 ± 0.23	6.56; 0.1249
Spleen	0.28 ± 0.02	0.28 ± 0.01	1.75; 0.1728

Group I, control group; Group II, extract *of C. grandis*. ROW, relative organ weight; n = 5; statistical difference for $p < 0.05$.

Table 5
Hematological parameters of animals in the sub-acute toxicity evaluation of the *C. grandis* L f. fruit extract.

Parameter	Group I	Group II	t-test; p-value
Hemoglobin (g/100 m L)	13.25 ± 0.47	13.19 ± 0.33	0.56; 0.6004
Hematocrit (%)	39.28 ± 1.08	37.89 ± 1.25	0,86; 0.5968
Red Blood Cell (10^6 µL^{-1})	7.15 ± 0.36	7.85 ± 0.52	1,02; 0.1247
White Blood Cell (10^3 µL^{-1})	5.84 ± 0.53	6.07 ± 0.52	1.09; 0,1896
Platelet (10^3 µL^{-1})	823.88 ± 85.53	902.19 ± 75.73	4.36; 0,7823

Results expressed as the mean ± SD; n = 5; statistical differences for $p < 0.05$; I, control group; II, extract of *C. grandis*.

Table 6
Serum biochemical parameters of both experimental groups in the sub-acute oral toxicity evaluation.

Parameter	Group I	Group II	t-test; p-value
ALT (U/L^{-1})	35.26 ± 0.92	56.96 ± 1.95	1.45; 0,0952
AST (U/L^{-1})	22.85 ± 2.33	27.12 ± 3.42	2.35; 0,1279
ALP (U/L^{-1})	185.88 ± 3.74	190.01 ± 13.99	3.27; 0,0951
TP (mg/100 mL)	7.22 ± 0.33	7.48 ± 0.69	0.96; 0.2660
Creatinine (mg/100 mL)	5.75 ± 0.49	5.99 ± 0.82	0.96; 0,2594
Urea (mg/100 mL)	26.54 ± 0.55	28.09 ± 1.22	1.21; 0,1451
Triglycerides (mg/100 mL)	46.55 ± 1.32	45.26 ± 2.57	2.36; 0.4782
Cholesterol (mg/100 mL)	96.44 ± 3.48	93.10 ± 3.29	3.12; 0,5268
Blood glucose (mg/100 mL)	104.66 ± 2.55	105.00 ± 247	2.20; 0,6749

Results are expressed as the mean ± SD; n = 5; p < 0.05 is significant; I, Control group; II, concentrated extract of *C. grandis*. ALT, alanine amino-transferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase.

Table 7
IC_{50} values of *Cassia grandis* extract, gallic acid, and ascorbic acid (as reference antioxidants) in the DPPH· and ABTS· radical scavenging assay, and the murine α-glycosidase inhibitory activity.

Substance	IC_{50} (μg/mL)		
	DPPH·	ABTS·	α-glucosidase
Extract of *Cassia grandis*	1.30 ± 0.04	0.72 ± 0.04	32.30 ± 1.30
Gallic acid[a]	2.90 ± 0.06	2.13 ± 0.06	–
Ascorbic acid[a]	2.23 ± 0.12	2.98 ± 0.16	–
Acarbose[a]	–	–	62.30 ± 1.30

[a] Reference substance.

The control Group (I) showed blood glucose levels between 90–100 mg/dL along the study. The Group II showed a progressive increase in the blood glucose. Contrarily, Group III (Glibenclamide), Group IV (100 mg/kg of CgE), and Group V (200 mg/kg of CgE) showed a progressive blood glucose reduction. The ANOVA test showed statistical differences between the blood glucose level of the experimental groups ($F = 192.54$; $p = 0.0000$). The Tukey's HSD test showed that the Group I, Group II, and Group IV were statistically different between them and different from the groups III and V, which were statistically equal.

3.9. Radical scavenging activity and α-glycosidase inhibition

Table 7 presents results of the antioxidant activity (DPPH· and ABTS·) and the assay for the α-glycosidase inhibitory activity of the extract of *C. grandis*.

4. Discussion

Cassia grandis extract appears as a viscous liquid, with a pungent aroma and a characteristic sweet taste. The qualitative screening revealed the presence of alkaloids, coumarins, flavonoids, free amino acids and amines, phenols and tannins, reduced sugars, resins, saponins, steroids, and triterpenes (Table 1). A similar composition was informed on the lyophilized fruit pulp [13,29]. Among natural compounds, saponins, triterpenes, and steroids differ structurally by the presence of saccharide units linked by a glycosidic bond.They are relatively abundant in the Kingdom Plantae. Reducing sugars were also detected. These, are carbohydrates that are oxidized causing the reduction of other substances without having to be hydrolyzed first. They are produced by the primary metabolism of plants and are practically the same in all plant species.

It was quantitated the flavonoids content (6.58 ± 0.88%) and phenolics (8.67 ± 0.83%) in CgE. Currently, is recognized that polyphenols (i.e. tannins, flavonoid, anthocyanins, coumarins etc.) are beneficial for treatments of various characteristics of type 2 diabetes [30]. It was also reported that flavonoids from vegetal extracts improve the lipid profile, protecting human being from diseases like obesity [31]. Polyphenols and flavonoids possess a potent antioxidant activity, protecting the body against free radicals and other oxidative species. In type 2 diabetes, there is an increase in pro-oxidative compounds (reactive oxygen species, malondialdehyde, and lipid peroxides etc. [30]. Thus, flavonoids and phenols have an enormous potential to reduce the risk associated with diabetes. The high content of polyphenols, flavonoids, and coumarins in CgE, could be responsible for the antidiabetic effect reported by native populations from Central and South America.

The pH of CgE was 5.8, probably, due to the presence of weak acids as phenols, flavonoids, coumarins, and fatty acids. CgE contains 8.67 ± 0.83% of soluble solids, indicating an adequate extractive process. In addition, the extract has a relative density of 1.365 ± 0.087 g/ml and a refractive index of 1.392 ± 0.037. These properties, together with the values of polyphenols, flavonoids, and pH, will allow establishing the quality parameters for the standardization of the CgE as an active substance.

Volatile compounds identified in CgE (Table 2) agree with those reported previously for the fruit pulp [30]. In this work only 38 volatiles were determined, because of the solvent used for the extract preparation (70% ethanol) do not favor the extraction of non-polar compounds, nonetheless, the main constituent was linalool, the same compound reported previously [32]. Other volatiles present in CgE matching with the compounds previously reported were 3-methyl butyl aldehyde (0.44%), dimethyl disulfide (0.24%), and dimethyl trisulfide (0.30%). These substances provide the pungent and unpleasant smell of the fruits and their extracts.

Metabolites present in the CgE have been related as strong antioxidants, with an important free radical scavenging activity [33]. In diabetic patients, lipid peroxidation increases aggravating chronic complications related to oxidative stress. The diversity of antioxidant compounds present in CgE could explain the ethnobotanical use of this fruit for the diabetes treatment. The extract of *Cassia grandis* prepared and characterized here, would be used for the preparation of pharmaceuticals as capsules, tablets, and nanoparticles, which could mask the taste and unpleasant smell of the fruit pulp extracts.

Medicinal plants can produce toxic effects that have to be evaluated to ensure the safe use [34]. Toxicological studies allow establishing the dosage regimens and therapeutic window of drugs [22]. Within the toxicity tests performed during drug development, the acute toxicity (high single dose) and sub-acute toxicity (repeated dose) are crucial. These assays, permit evaluating the toxic effect of drugs in target organs like liver and kidneys [22]. They also allow demonstrating a dose-response relationship and, frequently, predict possible mechanisms of toxic action [21]. On the other side, the evaluation of acute oral toxicity is the basis of substance classifications within the "acute toxicity class". This test is an essential stage in the establishment of dosing regimens [22].

Chemical toxins can produce behavioral modifications of animals. The change depends on the affected organ, exposure time, amount of substance absorbed, age, and the animals health status [21,24]. Along the study, animals maintained a normal behavior with 100% survival. Both experimental groups uptake water and food normally (Fig. 1B) and gained body weight (Fig. 1A). Internal organs of animals in both groups presented a healthy appearance and color. No statistical differences were observed between the relative organ weights of both experimental groups (Table 3). This evidence suggests that CgE did not produce toxic effects on experimental animals when was administered in a unique dose up to 2000 mg/kg [21,33–35]. The CgE was framed as "Unclassified" substance within the scale of toxicity classes, with $LD_{50} > 2000$ mg/kg [21].

During the drug development process, it is not only important to assess the toxicity in a unique high dose (Acute toxicity), it is also important assessing the drug effects administered for a long period (Sub-acute toxicity), searching for information about the risks arising from the repeated use [22]. Neither clinical nor behavioral changes that could be associated with toxic effects were observed. Animals of both experimental groups gained body weight normally (Fig. 2). Several toxicological studies point to body weight as the most sensitive variable to toxic effects caused by chemicals [22,35–37]. The internal organs of animals of both groups preserved a normal cytoarchitecture, appearance, and color, indicating the absence of toxic effects. No statistical differences between the relative organ weights of both experimental groups were observed (p-values > 0.05, Table 3). These findings suggest that CgE does not affect the organs external anatomy.

Toxins can also affect the hematological and biochemical parameters. The hematological system reacts with high sensitivity to toxic substances [22,38]. The hematological parameters in both experimental groups were within the normal values (Table 5) [39], without significant differences. This result suggests that CgE does not affect the hematological system.

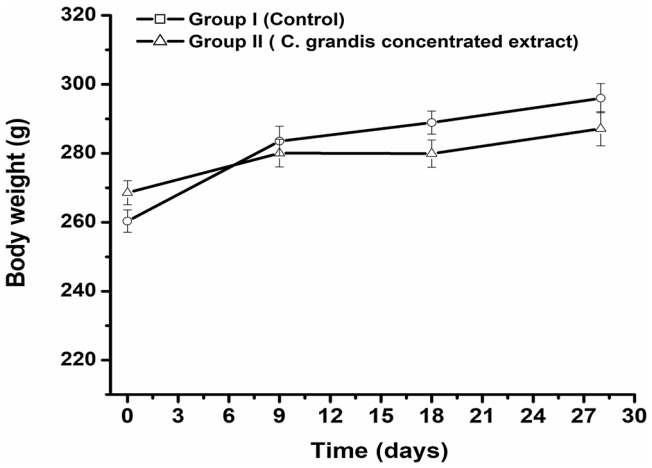

Fig. 2. Body weight behavior of both experimental groups during the sub-acute toxicity evaluation.

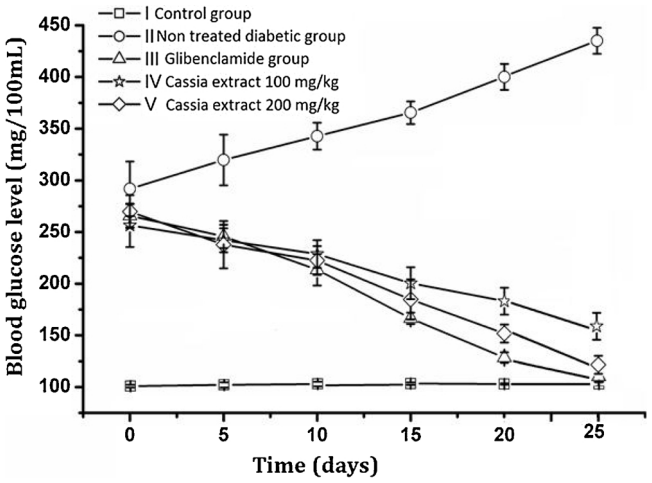

Fig. 3. Effect of the experimental treatments in blood glucose levels of the five experimental groups.

Biochemical parameters can be used for monitoring the functionality of internal organs, especially, those organs called "target organs of toxic processes" as liver and kidney. In this assay, it was evaluated the liver functionality (ALT, AST, ALP, TP, cholesterol, and triglycerides), kidneys (creatinine, urea) and pancreas (Blood glucose level). Biochemical parameters evaluated in both experimental groups (Table 6) were within the normal values [40,41]. No statistical differences were found between the biochemical parameters of both experimental groups ($p > 0.05$, Table 6).

Liver and kidneys are the principal's detoxifier organs in the body and the most affected because of the toxic effect of chemicals. Normal levels of ALT, AST, ALP, TP, cholesterol, and triglycerides indicate a normal liver functionality after 28-days receiving 1000 mg/kg of CgE. In the same way, normal levels of urea and creatinine suggest a normal kidneys functionality [39,40]. On the other side, blood glucose levels kept within the normal values suggesting a normal pancreatic function. Since in both experimental groups after 28-days of oral administration of CgE (1000 mg/kg), the anatomy of internal organs was normal, the hematological and biochemical parameters were within the normal values, it could be suggested that the CgE does not cause systemic toxic effects after a long-term administration.

The pancreas is an organ of the digestive system with exocrine and endocrine function [39]. The endocrine function is responsible for preserving the blood glucose equilibrium. The α-cells produce glucagon helping to increase the blood glucose level when it is too low (stimuli glycogenesis). The β-cells produce insulin, helping to decrease the blood glucose when it is too high, stimulating glycogenolysis [41]. In diabetic patients, increase the blood glucose levels due to a decrease in the insulin secretion. This increase occurs due to a partial or a total destruction of β-cells (in the Islets of Langerhans), produced by the Alloxan [42]. In this condition, it is necessary to use drugs as glibenclamide (Hypoglycemic standard oral drug) for reducing the blood glucose level.

The normal blood glucose in rats is between 80–130 mg/dL [38]. In this work, the control (Group I) showed normal glucose levels along the experiment (Fig. 3). In the Group II (untreated diabetics), an increase in blood glucose levels was observed, produced by the Alloxan toxic effect [43,44]. The CgE reduced the animal's blood glucose to normal levels in both doses (Group IV, 100 mg/kg and Group V, 200 mg/kg). The hypoglycemic effect of 200 mg/kg was not statistically different from glibenclamide at 25 mg/kg. The effect was similar to the stem bark extract [12], however, using ripe fruit for extract preparation is feasible for keeping the plant health, and besides the volume of pulp available in harvested seasons is enormous. This finding suggests the high hypoglycemic potential and feasibility of the extract of *C. grandis* L f fruits.

The DPPH· assay is used for assessing the free radical scavenging potential of antioxidant substances. It is a standard method for the evaluation of antioxidant properties. The IC_{50} of CgE in the DPPH· assay (1.30 μg/mL) was minor than the gallic acid (2.90 μg/mL), and ascorbic acid (2.23 μg/mL). Against the ABTS· radical, the IC_{50} value of CgE was still minor (0.75 μg/mL; Table 7). CgE exhibited a potent antioxidant capacity. Despite that, there is no report on the antioxidant capacity of *C.grandis* fruits and the extracts. Several studies reported the high correlation between the antioxidant capacity and the polyphenols content on natural extracts [45]. The content of phenols, flavonoids, and coumarins in CgE explain the potent antioxidant activity observed.

Alpha-glucosidase inhibitors (i.e. Acarbose) are drugs that help in the diabetes control, making slow the increase of blood glucose, improving the use of insulin [46]. CgE exhibited a potent α-glycosidase inhibitor effect with IC_{50} of 32.30 ± 1.30 μg/ml (Table 7). The effect was far superior to the effect of Acarbose, the reference drug (Table 7). Compounds present in CgE could compete with carbohydrates in the bloodstream, retarding the bonding of these to the α-glycosidase, producing a potent enzyme inhibition [47].

In diabetic patients, there is an increase in the reactive oxygen species. In this condition, the insulin secretion decrease, and as a protein, could be chemically oxidized [48,49]. In this work, CgE exhibited a potent antioxidant activity and an important α-glycosidase inhibitory effect. Due to the richness of antioxidant compounds of the CgE, is probably that the hypoglycemic effect occurs through an antioxidant mechanism, preventing the islets of Langerhans destruction, keeping the β-cells integrity and the insulin secretion. The antioxidant effect associated with the slow increase of the blood glucose (effect α-glucosidase inhibitor) could maintain the pancreas functionality and adequate blood glucose levels; these could be the possible hypoglycemic mechanisms of *Cassia grandis* L f extract. Results obtained in this work suggest that CgE could be a useful product for the diabetes management.

5. Conclusions

The fruit pulp of *Cassia grandis* has been used for centuries as antianemic, and for maintaining the normal blood glucose. Few studies have been made about the possible pharmacological utility of this plant fruit. In this work, it was confirmed the hypoglycemic activity of the *C. grandis* fruit in Sprague-Dawley diabetic rats. It was also established the non-toxicity (acute and sub-acute) of this extract by the oral route, resulting innocuous in a rat model. The results presented in this work, suggest that CgE could be a useful product for the diabetes management. The mechanism of action of this herbal product could be associated with a potent antioxidant activity and the inhibition of the α-

glycosidase enzyme. As far as we know, this is the first report on the hypoglycemic effect *in vivo* of *C grandis* Lf fruits extract.

Authors' contributions

ALP, EPZ, HK, and HC made all the clinical experiments. ALP and TPS made the physicochemical characterization of the extract. JCTC and ESL made the proofreading of the manuscript. JRRA designed and coordinated all the experiments, wrote and translated the article first version. All authors have read and approved the final version of the article.

Conflict of interest

The authors declare that there are no conflicts of interest.

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