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ORIGINAL ARTICLE

Tillandsia usneoides Protects RINm5F Cells from Streptozotocin-Induced Apoptosis and Stimulates Insulin Secretion

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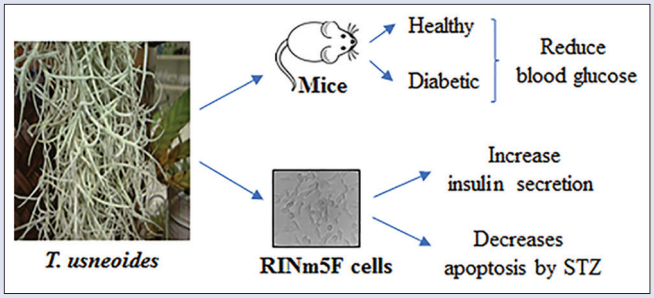
ABSTRACT

Background: *Tillandsia usneoides* (Bromeliaceous) is traditionally used in Mexico for diabetes treatment. Although a significant hypoglycemic effect has been reported, the participation of insulin in this action has not yet been explored. **Objectives:** The aim of this research was to determine the hypoglycemic effect of an aqueous extract from *T. usneoides* in normal and diabetic mice as well as to evaluate the participation of insulin in this effect using an *in vitro* model. **Materials and Methods:** Aqueous decoction of *T. usneoides* (250 mg/kg) was administered in healthy and diabetic mice and glycemia was measured. RINm5F cells were cultured with *T. usneoides* (0.1 and 1 μg/ml), and cell viability was measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, trypan blue, and apoptosis; secretion and expression of insulin were quantified by enzyme-linked immunosorbent assay and reverse transcription–polymerase chain reaction, respectively. **Results:** *T. usneoides* decreased blood glucose in healthy mice at 4 and 6 h (96 ± 11.2 and 68.2 ± 1.9 mg/dl, respectively) compared with time zero (138.5 ± 5.0 mg/dl, $P < 0.05$). In diabetic mice, aqueous extract significantly decreased glycemia at 6 h compared with time zero (212.7 ± 3.5 and 243 ± 5.3 and mg/dl, respectively). Besides, *T. usneoides* aqueous extract stimulated insulin secretion (20%, $P < 0.05$) without cause changes in insulin gene expression and protects RINm5F cells from streptozotocin-induced apoptosis. **Conclusion:** These results suggest that aqueous decoction of *T. usneoides* stimulates insulin secretion still in the absence of changes in the intracellular concentration of Ca^{2+} in RINm5F cells.

Key words: Ca^{2+}, insulin secretion, RINm5F cells, *Tillandsia usneoides*, Type 2 diabetes

SUMMARY

- *Tillandsia usneoides* is used for diabetes treatment, and a hypoglycemic effect has been reported, but it is still unknown if *Tillandsia usneoides* can stimulate insulin secretion. Hence, the aim of this research was to determine the hypoglycemic effect of *T. usneoides* in mice and insulin secretion in RINm5F cells. It was observed that *T usneoides* decreases glucose in healthy and diabetic mice, stimulates insulin secretion, and protects RINm5F cells from streptozotocin-induced apoptosis.



Abbreviations used: ANOVA: Analysis of variance; ATCC: American Type Culture Collection; cDNA: Complementary deoxyribonucleic acid; DNA: Deoxyribonucleic acid; ELISA: Enzyme-linked immunosorbent assay; HBSS: HANK'S Balanced Salt Solution; HMG: 3-hydroxy-3-methylglutaric acid; I.P: Intraperitoneal injection; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD: Optical density; PBS: Phosphate-buffered saline; RNA: Ribonucleic acid; RNAm: Messenger RNA; RNAse: Nuclease that catalyzes the degradation of RNA into smaller components; RT-PCR: Reverse transcription–polymerase chain reaction; FBS: Fetal bovine serum; STZ: Streptozotocin; WHO: World Health Organization.

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INTRODUCTION

Hyperglycemia is responsible for triggering the signaling pathways resulting in the development of late complications of diabetes; therefore, treatment should be targeted to correcting and/or keeping circulating glucose at low levels. Although the availability of drugs for the treatment and control of diabetes is varied, 80% of the world population uses also plants as medicinal remedy which, according to the World Health Organization (WHO),[1,2] is considered the first alternative treatment for several pathologies in underdeveloped countries.[3-5] Traditional herbal medicine may be used in addition to or as alternative pharmacological

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drugs for different reasons including availability, low cost,[5] and increased side effects of modern medicines.[6] Medicinal plants are known as treatment for several diseases, such as liver disorder, inflammatory disorder, hypertension, cardiovascular disease, and diabetes mellitus, among others.[6] There are more than 400 plants proposed for diabetes mellitus treatment worldwide; however, although the WHO[7] emphasized the importance of establishing the scientific bases that sustain the safety, effectiveness, and quality of medicinal plants,[8] only some have received scientific and medical evaluation.[9,10]

Tillandsia usneoides (Bromeliaceous), popularly known in Mexico as "heno," is an epiphytic plant commonly used plant in Mexico and Northern Louisiana (USA) for diabetes mellitus, indigestion, gastritis, and epilepsy, among others.[11-13] In previous studies, it was demonstrated that the aqueous and alcoholic extracts of *T. usneoides* produced significant hypoglycemia 24 h after administrated. Furthermore, it was observed that the aqueous extract inhibited the development of diabetes in alloxan-induced diabetes rats.[14] In chemical studies, 3-hydroxy-3-methylglutaric acid was suggested as the responsible compound of this effect.[15] However, it is yet unknown if this hypoglycemic effect implicates insulin secretion. The aim of this research was to determine the hypoglycemic effect of an aqueous extract from *T. usneoides* in normal and diabetic mice as well as to evaluate the participation of insulin using RINm5F cells.

MATERIALS AND METHODS

The entire plant of *T. usneoides* was bought in popular markets of Mexico City, and it was taxonomically identified in the herbarium of CMN-SXXI with the voucher number 15,992.

Obtaining of aqueous decoction

Thirty grams of the entire plant was taken, washed, and subjected to decoction in 1 L of water for 10 min. Aqueous phase was placed for 72 h inside a laminar flow hood with constant aeration, and once dried, it was separated, weighted, kept, and stored until its use (yield 1.63%).

Phytochemical screening

Polyphenol quantification

The total polyphenol content in the aqueous decoction was assessed by Folin–Ciocalteu method, as previously reported.[16] One sample of the aqueous extract (100 µl) at a concentration of 5.0 mg/ml was mixed with deionized water (500 µl), followed by the Folin–Ciocalteu reagent (250 µl). After 5 min, 1250 µl of a sodium carbonate solution (20%) was added. Then, deionized water was added to bring the volume up to 2.2 ml. After 2 h, the absorbance of the reactants at 725 nm was measured (Thermo Spectronic, BioMate 3). This measurement was compared to the standard curve (4.0–20.0 µg) of gallic acid (0.1 mg/ml, Sigma-Aldrich Chemical, St. Louis, MO, USA), and the result was expressed as grams of gallic acid/100 g of dry aqueous extract. A mixture of water and reagents was used as blank. This determination was done in triplicate, and the mean value and standard deviation were calculated.

Determination of saponins

A portion of decoction was dissolved in hot water, filtered, and then stirred vigorously for 30 s, and the foam quantity remained after 5 min was measured. Subsequently, 10 drops of olive oil were added to the previous dilution, and it was stirred again; the presence of saponins was indicated by the formation of an emulsion.[17]

Biologic assay

Animals

Thirty male *Mus musculus* CD-1 mice between 35 and 40 g were used, and they were divided into 5 groups (6 animals per group). They were maintained according to the NOM-062-ZOO-199 under light-dark automatic cycles 12 × 12 h at 22°C and controlled humidity (55°C ± 3°C), with water and food *ad libitum*. Twelve hours before, the experiment mice were fasted.

Response curve of treatment with Tillandsia usneoides

Five groups of mice comprising 5 male CD-1 mice each one were used. After 12 h of fasting glycemia was measured (t = 0), then, Group 1 (control) was treated with saline solution; Group 2 (positive control) with glibenclamide (10 mg/kg); and Groups 3, 4, and 5 with 100, 250, and 500 mg/kg with aqueous decoction of *T. usneoides* by intraperitoneal route (i.p.), respectively. Glycemia was measured at 120 (t = 1) and 240 min (t = 2) after treatment with a glucometer Roche® Accu-Chek Performa.

Hypoglycemic effect of Tillandsia usneoides in healthy and diabetic mice

After a 12-h fasting, mice were administered with streptozotocin (STZ) (135 mg/kg, i.p.) in 0.1 M citrate buffer pH 7.4. After 1 week, glucose was measured by tail vein puncture with a glucometer Roche® Accu-Chek Performa. Animals with glycemia ≥200 mg/dl were considered diabetic. Animals were divided into two groups: diabetic control (saline solution) and diabetic treated (250 mg/kg of decoction, i.p.). Four blood glucose measurements were made, at time 0 (before administering the treatment) and at 120, 240 and 360 minutes later.

RINm5F cells

Culture of RINm5F cells

Cell line RINm5F producer of insulin was acquired by American Type Culture Collection and was cultured in medium RPMI-1640 (GIBCO) supplemented with 10% fetal bovine serum, 1 mM of pyruvate, 1 mM of nonessential amino acids, 2 mM of L-glutamine, and 100 U/ml of gentamicin, with a humid atmosphere at 37°C and 5% of CO_2, to reach confluence (approximately 72 h) and reseeded for different treatments.

Cell functionality (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test)

Cell viability was measured with 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) test,[18] based on the capacity of intracellular dehydrogenases to reduce bromide from MTT to formazan. With this purpose, 5000 cells/well in 96 (Nunc)-well plates were seeded with supplemented RPMI-1640 medium. The next day, they were washed with PBS and aqueous decoction of *T. usneoides* was added to different concentrations in RPMI-1640 medium without serum (0.1, 0.5, 1, 2.5, 5, 10, 25, 50, and 100 µg/ml) and was incubated at 37°C and 5% of CO_2. After 24 h, the medium was removed and 0.1 mg/ml solution of MTT in pH 7.5 PBS was added, they were incubated for 3 more h, and 200 µl of HCl 0.04 M in 2-isopropanol was added to be dissolved in formazan. Optical density (OD) was registered at 570 nm in an enzyme-linked immunosorbent assay (ELISA) reader. Cell viability was determined dividing OD of the problem between the controls and multiplied by 100.[18]

Apoptosis in RINm5F cells induced by streptozotocin

RINm5F cells (1×10^6) were cultured in 25 cm^2 bottles when confluence was higher than 70%, they were washed with HBSS and the decoction of *T. usneoides* (1 µg / ml) in RPMI-1640 medium without serum was added. After 30 minutes STZ (10 mM, as apoptosis inducer) was added

and incubated at 37°C for 30 min. At the end of this time, this medium was removed, the cells were washed with HBSS and the decoction of *T. usneoides* (1 µg / ml) in RPMI-1640 medium without serum was added again. After 24 h, cells were scraped with PBS-EDTA 2 mM and washed with PBS by centrifugation. Extraction of apoptotic DNA fragments was performed by addition of lysis buffer (1% Igepal in 20 mM EDTA, 50 mM Tris-HCl, pH 7.5), after centrifugation at 1600 g, supernatant was recovered, and 1% SDS and RNAse A 5 µg/ml were added and left 2 h at 56°C. At the end, 2.5 µg/ml of K proteinase was added and incubated for 2 h at 37°C. DNA was precipitated with 10 M ammonium acetate and absolute ethanol (0.5:2.5) at −20°C. After centrifugation, the loading buffer was added and DNA was separated in 1% agarose gel.[19]

Total RNA extraction

Total RNA extraction of RINm5F cells was performed with Trizol, based on the Chomczynski method.[20] The cells (1×10^6) on 6-well plates (Nunc) were seeded, *T. usneoides* decoction (0.1 y 1 µg/ml). After 24 h, they were washed with PBS, 1 ml of Trizol was added, and they were lifted with sterile rubber gendarme. They were left at room temperature for 5 min. Then, 0.2 ml of chloroform was added, and after the centrifugation (11,700 g, 15 min at 4°C), aqueous phase was recollected; RNA was precipitated with isopropanol, left 15 min at room temperature, and centrifuged at 11,700 g, 10 min at 4°C. RNA was washed with 75% ethanol. Precipitate (RNA total) was vacuum dried and RNA button was resuspended in water free of RNases and quantified by spectrophotometry with an EPOCH® equipment.

cDNA synthesis and RNAm expression of insulin by polymerase chain reaction in real time

For the cDNA synthesis, the reverse transcriptase ImProm-II (Promega) was used with randomly initiators (random primers 0.5 µg/µl) in a thermal cycler Applied Biosystems GeneAmp PCR System 2700, following the manufacturer's instructions. With the following program: 42°C for 55 min, 70°C for 15 min, and finally at 4°C for 5 min.

cDNA amplification was performed with the kit "DNA Master PLUS SYBR Green 1" (Applied Biosystems) for insulin (sens: 5'-GGAGCGTGGA TTCTTCTACAC-3'; antisens: 5'-CAGTGCCAAGGTCTGAAGG-3') and β-actin (sens: 5'-TTCCATCCTCCAGAAACCAG-3'; antisens: 5'-CCCTCGAACTAAGGGGAAAG-3') as normalization gene in the Rotor-Gene (Qiagen) equipment.

Insulin secretion

RINm5F (1×10^6) cells were seeded on 6-well plates (Nunc). The next day, they were washed with Krebs-Ringer buffer (NaCl 115 mM, KCl 4.7 mM, CaCl 1.3 mM, KH_2PO_4 1.2 mM, MgSO 1.2 mM, $NaHCO_3$ 24 mM, HEPES 10 mM, BSA 1 g/ml, glucose 1.1 mM; pH 7.4) and were incubated 1 h in this medium. Later, Krebs-Ringer buffer was added with 5.5 mM glucose and *T. usneoides* decoction (0.1 and 1 µg/ml) and was incubated for 2 h.[21] Medium was recovered after 1 and 2 h, it was centrifugated at 2000 rpm, 5 min at 4°C, and the insulin concentration was quantified by ELISA with kit Rat Insulin 80-INSRT-E01 (ALPCO), following vendor instructions.

Ca^{2+} intracellular quantification

Intracellular Ca^{2+} was measured by the formation of complex Arsenazo III (acid 2,2' dibenzoarsenic 2 mM in Hepes 30 µM pH 7.4) – the intracellular free Ca^{2+},[22] using the standard curve of $CaCl_2$. After incubation of cells with *T. usneoides* decoction (0.1 and 1 µg/ml) in buffer Krebs-Ringer by 1 h, they were scraped with a gendarme in 1 ml of cold PBS and lysed (Sonics Vibra-cell VCX 750; 5 pulses of 30 seg) and centrifuged (12000 rpm, 20 min at 4°C). Ca^{2+} concentration was measured in the supernatant. Absorbance was registered at 627 nm, and the results were expressed as mmoles/L of free intracellular Ca^{2+}.

Statistical analysis

Each experiment was performed by triplicate in separate events. Statistical analysis of results was made by an analysis of variance, followed by the Duncan's multiple test with NCSS program and $P < 0.05$ significance level.

RESULTS

Phytochemical screening

T. usneoides aqueous decoction was positive for the analysis of polyphenolic (gallic acid content 1.98 ± 0.05 g/100 g) and saponins (+).

Hypoglycemic effect of different doses of *Tillandsia usneoides* in normal mice

Table 1 shows the results of the dose–response test by administration of *T. usneoides* decoction in normal mice. *T. usneoides* significantly decreased glycemia at 120 min with 100 and 250 mg/kg (63.8 and 49.6 mg/dl, respectively, compared with saline control (92.0 mg/dl). In contrast, 500 mg/kg did not decrease blood glucose. The dose of 250 mg/kg was selected for the next experiments.

Hypoglycemic effect of *Tillandsia usneoides* in diabetic mice

In diabetic animals, the administration of *T. usneoides* significantly decreased blood glucose until 6 h [Table 2].

Viability of RINm5F cells is not affected by the administration of *Tillandsia usneoides*

T. usneoides (0.1, 0.5, and 1 µg/ml) in RINm5F cells did not affect cell viability which remained close to control (98.5%, 95.8%, and 93.4%, respectively). This effect was corroborated by the measure of mitochondrial dehydrogenase activity (MTT test). The results showed that *T. usneoides* did not affect mitochondrial activity, remaining above 95%. Negative effects on viability of RINm5F cells were observed with concentrations of 10 µg/ml for the trypan blue test and 2.5 µg/ml for MTT [Figure 1]. Consequently, in RINm5F cell experiments, *T. usneoides* was used at concentrations of 0.1 and 1 µg/ml.

Tillandsia usneoides inhibits apoptosis in RINm5F cells treated with streptozotocin

T. usneoides inhibited DNA oligonucleosomal fragmentation a hallmark of apoptosis in RINm5F cells treated with STZ [Figure 2].

Effect of *Tillandsia usneoides* on insulin secretion

Table 1: Hypoglycemic effect of different doses of *Tillandsia usneoides* in normal mice

	Glucose (mg/dl)		
	T0	T120 min	T240 min
Saline solution	85.2±3.0	89.8±2.5	92±4.7
Glibenclamide (10 mg/kg)	95.6±4.0	65±5.4	64.4±3.0
T. usneoides (100 mg/kg)	91±5.8	82.2±4.7*	63.8±3.7*
T. usneoides (250 mg/kg)	84±3.4	104.2±2.2	49.6±6.2*
T. usneoides (500 mg/kg)	90±6.1	106±3.3	90±6.3

*$P<0.01$. *T. usneoides*: *Tillandsia usneoides*

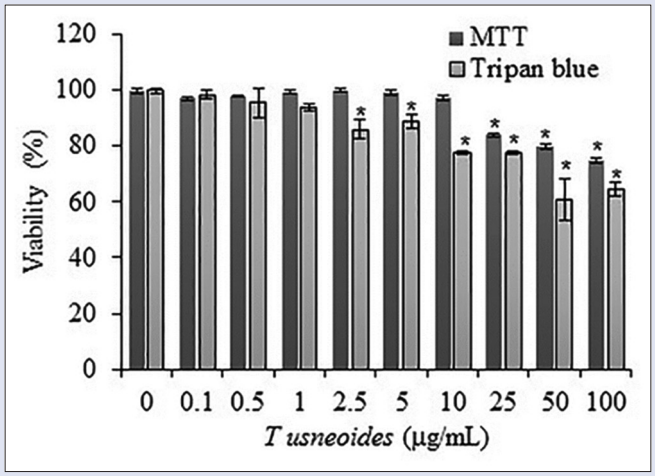

Figure 1: Viability percentage (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide and trypan blue exclusion) of RINm5F cells treated with aqueous decoction of *Tillandsia usneoides* (n = 20). *P < 0.05 versus control

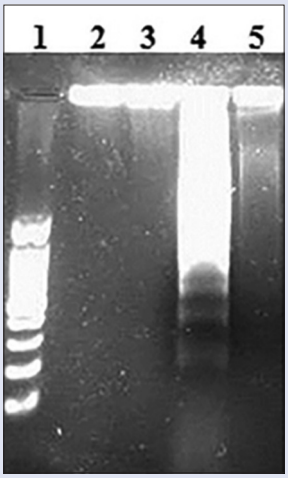

Figure 2: *Tillandsia usneoides* protects RINm5F cells from apoptosis induced by STZ. (1) MP; (2) Control; (3) *Tillandsia usneoides* (1 µg/ml), (4) STZ; (5) STZ + *Tillandsia usneoides* (1 µg/ml) (n = 5). STZ: Streptozotocin

Table 2: Hypoglycemic effect of *Tillandsia usneoides* in diabetic mice

	Glucose (mg/dl)			
	T0	T120 min	T240 min	T360 min
Saline solution	243.2±6.5	235.2±6.2	240.6±4.6	238.2±3.8
Glibenclamide	363±7.15	230±5	163±6.5	177±5.6
T. usneoides	243±5.3	283±6.2	241.7±3.2	212.7±3.5*

*P<0.05 versus T0. *T. usneoides*: *Tillandsia usneoides*

and intracellular Ca^{2+} concentration in RINm5F cells

Insulin secretion was significantly increased when RINm5F cells were incubated for 1 h with 5.5 mM of glucose and 0.1 or 1 µg/ml of *T. usneoides* (32.1% and 22.5%, respectively). This effect was also observed when incubation time was increased to 2 h, but it was significant only with 0.1 µg/ml of decoction [Figure 3a], with respect to control (P < 0.05). Regarding intracellular Ca^{2+} concentration, no significant changes were observed after the addition of *T. usneoides* aqueous decoction to the RINm5F cell culture [Figure 3b]. The addition of *T. usneoides* to RINm5F cell culture decreased 10 and 17% RNAm expression of insulin (0.1 and 1 µg/ml, respectively; P < 0.05) [Figure 3c].

DISCUSSION

This study evaluated the capacity of *T. usneoides* aqueous decoction to reduce serum glucose in healthy and diabetic CD1 mice; its effect over the insulin expression and secretion in RINm5F cells was also assessed. It was observed that after 4 h of decoction administration, glycemia is reduced in healthy and diabetic animals. In RINm5F cells, the addition of *T usneoides* to the culture stimulates the first stage of insulin secretion without affecting the expression and protects from the death induced by STZ.

It is known that *T. usneoides* is often used in traditional medicine against conditions such as bronchitis, diabetes and epilepsy, as well as astringent properties that are attributed to it.[11,23] In this study, we corroborated the capacity of *T. usneoides* to reduce glycemia in normal mice. We observed at 4 and 6 h after administration of aqueous decoction of *T. usneoides* (250 mg / kg weight), a blood glucose reduction of 30 and 50% respectively, versus the control. These data coincide with the reports in which the hypoglycemic activity in mice was corroborated.[14] Although the first effect is an increase of glycemia, this may be attributed to the high content of carbohydrates.[15] When data were compared to glibenclamide, the hypoglycemic effect of *T. usneoides* was maintained 2 h more than glibenclamide. This effect may be due to the *T. usneoides* decoction property to stimulate insulin secretion in healthy β-cells, as observed in RINm5F cell culture. In diabetic mice treated with *T. usneoides,* glycemia reduced 6 h after the treatment and in a smaller percentage (13%). These animals were treated with STZ to induce diabetes; consequently, they did not have enough β-cells for *T. usneoides* decoction to stimulate insulin secretion and incorporation of intracellular glucose. When the β-cell mass is reduced or the capacity to respond to changes in glucose concentration is lost, hyperglycemia occurs, a distinctive characteristic of diabetes. Patients with obesity, insulin resistance, and diabetes also have oxidative stress[24] and/or stress of the endoplasmic reticulum[25] that may induce the loss of β-cells by an increase of apoptosis rate.[26]

For *in vitro* studies, *T. usneoides* decoction effect over the viability of RINm5F cells by trypan blue exclusion and MTT was assessed. The addition of the extract to culture for 24 h decreases the viability of the RINm5F cells from 2.5 µg/ml [Figure 1]. Because even as at concentrations higher than 25 µg/ml viability of RINm5F cells is maintained above 80%, we also evaluated the capacity of *T. usneoides* to protect RINm5F cells from apoptosis induced by STZ. The results showed that the addition of *T. usneoides* decoction inhibits oligonucleosomal fragmentation of DNA a feature of apoptosis [Figure 2]. STZ is a structural analog of glucose entering to β-cells through GLUT2. Once inside, STZ is fragmented in glucose and methylnitrosourea, an alkylating agent that induces severe DNA damage, includes double and single-strand breaks and induces cell death due to apoptosis or necrosis.[27] Furthermore, the participation of oxygen reactive species in lesions to DNA has been proposed, so the antioxidant addition (Vitamin C and Vitamin E) inhibits DNA breaking induced by STZ.[28] Polyphenol content in *T. usneoides* decoction matches with previous reports.[29] These compounds are known by their antioxidant and inhibitor properties of free radical production,[30] so it is likely to inhibit apoptosis capturing free radicals generated by the treatment with STZ. The inhibition of apoptosis by the addition of medicinal plant extracts has been previously demonstrated and associated with the presence of polyphenolic compounds.[31,32]

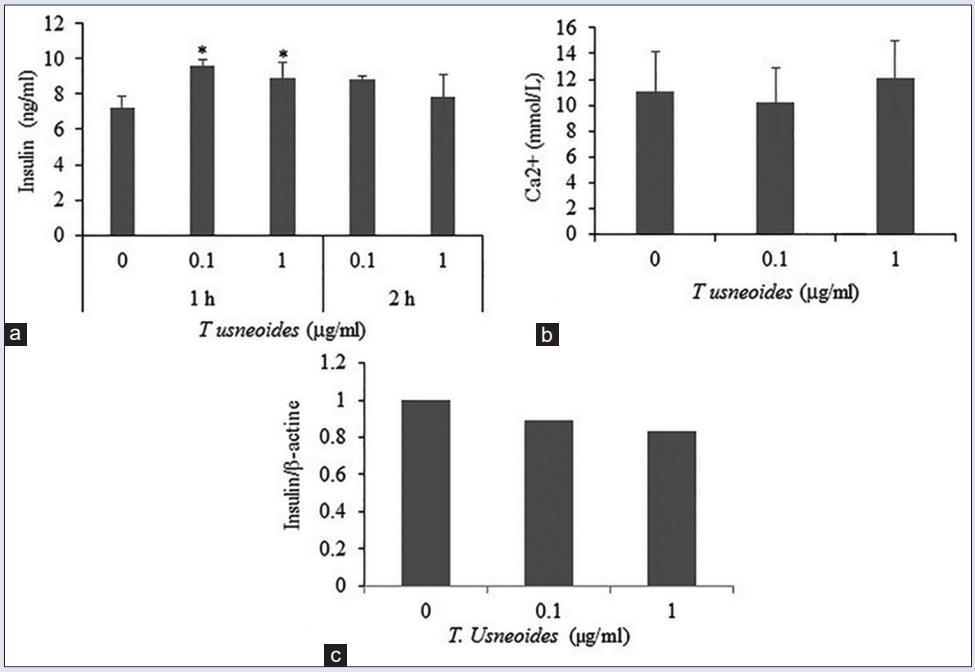

Figure 3: Insulin secretion (a) and Ca^{2+} intracellular concentration (b) in RINm5F cells cultivated with RPMI-1640, glucose 5.5 mM, and *Tillandsia usneoides* (0.1 and 1 µg/ml) for 2 h. Insulin expression in RINm5F cells cultivated with *Tillandsia usneoides* for 24 h (c) ($n = 3$). *$P < 0.05$

T. usneoides decoction stimulates the insulin secretion in RINm5F cells, without affecting the expression of this hormone or intracellular levels of Ca^{2+} [Figure 3b]. Although it was clear that insulin release stimulated by glucose requires the ATP increase, closure of K^{ATP} channels, and intracellular increase of Ca^{2+},[33] insulin secretion by permeability changes in cell membrane has been reported, regardless the Ca^{2+} concentration.[21,34] It is possible that the addition of *T. usneoides* decoction to the cell culture had modified membrane permeability and promoted insulin release of granules close to cell membrane. Besides that, polyphenols such as gallic acid have secretagogue properties of insulin,[31] increase the glucose capture, and reduce insulin resistance in rats with diabetes induced by high-fat diet.[35]

CONCLUSION

Based on these results, we may propose that since *T. usneoides* decoction stimulates the insulin secretion in RINm5F cells and reduces glycemia in normal and diabetic mice, *T. usneoides* may be used as coadjutant in the control of Type 2 diabetes.

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Conflicts of interest

There are no conflicts of interest.

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