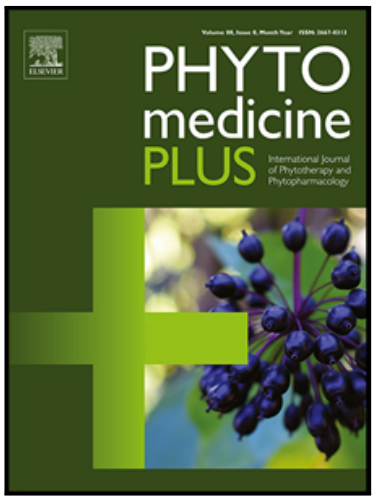

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Highlights

- *Phyllanthus niruri* is used in traditional medicine to treat diabetes
- *P. niruri* extract has inhibitory effect on α-glucosidases of diverse origin
- Inhibition of α-glucosidase relates to anti-hyperglycaemic effect
- Binary solvent mixtures are effective to extract anti-hyperglycaemic compounds
- *In vivo* anti-hyperglycaemic potential was probed by oral starch overload model

Extraction of Anti-hyperglycaemic Bioactive Compounds from *Phyllanthus niruri* L. through Solvent Mixture Design*: in vitro* and *in vivo* Evaluation

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Abstract

Background: Species within *Phyllanthus* genus exhibit a diverse range of experimentally validated pharmacological activities. Notably, *Phyllanthus niruri* Linneo (Phyllanthaceae), commonly referred to as stonebreaker, is a native Central and South American species with a rich history of use in traditional ethnomedicine for treating gastrointestinal and renal disorders.

Purpose: the aim of this study was to investigate the application of various solvent mixtures to optimize the extraction of anti-hyperglycemic secondary metabolites from *P. niruri*.

Methods: to optimize the extraction of anti-hyperglycemic compounds from *P. niruri*, a solvent mixture design strategy was employed. The optimized extracts were subjected to chemical characterization using spectrophotometric methods and HPLC-DAD analysis. Also, the influence of edaphoclimatic conditions on the composition of extracts was investigated. The anti-hyperglycaemic potential of the extracts was evaluated through both *in vitro* and *in vivo* assays. The *in vitro* studies included enzyme inhibition assays targeting α-glucosidase from two sources (yeast and pig). For *in vivo* studies, a rat model with oral starch overload was used to confirm the anti-hyperglycaemic activity of the extracts.

Results: an optimal solvent mixture (70% methanol and 30% ethyl acetate) was found as effective for extracting compounds with anti-hyperglycemic potential from *P. niruri*. All the extracts obtained using this solvent inhibited both, yeast and pig α-glucosidase, with greater selectivity for inhibiting the yeast-derived enzyme. Extracts from aerial parts (AP) exhibited the strongest inhibitory activity. These extracts contained phenolic compounds, specifically flavonoids, but no condensed tannins were detected. Chromatographic analysis of profile revealed a major peak consistent with corilagin. *In vivo* studies using a dose of 500 mg kg^{-1} demonstrated that this extract significantly reduced blood sugar levels after starch overload in rats.

Conclusion: this study demonstrates the importance of selecting appropriate solvent mixtures to extract bioactive compounds with anti-hyperglycemic properties from *P. niruri*. Our findings support the traditional use of this plant as a anti-hyperglycaemic agent in ethnomedicine.

Keywords: anti-hyperglycaemic, mixture design, starch overload, diabetes

Abbreviations: **CTC**, Condensed Tannin Content; **GOD/POD**, Glucose Oxidase and Glucose Peroxidase; **HPLC-DAD**, High Performance Liquid Chromatography – Diode Array Detector; **IAG**, intestinal α-glucosidase; **pNPG**, p-nitrophenyl-α-D-Glucopyranoside; **PnE**, *Phyllanthus niruri* Extract; **T1D**, Type 1 Diabetes; **T2D**, Type 2 Diabetes; **TPC**, Total Phenolic Content; **TFC**, Total Flavonoid Content; **LC_{50}**,Lethal concentration 50.

1. Introduction

Diabetes, a chronic and non-communicable disease, has reached global prevalence. According to the International Diabetes Federation (2019), a staggering 95% of all diabetes cases worldwide are of type 2 (T2D). This form of diabetes is characterized by high blood glucose levels (hyperglycaemia) and insulin resistance (Blaak et al., 2012)

The discovery of metformin, a diabetes medication derived from the *Galega officinalis*, , highlighted the potential of plants in treating metabolic diseases (Bailey, 2017). This has led to a renewed interest in exploring plant-based therapies as innovative approaches to prevent and manage T2D and other conditions (Kumar et al., 2012; Raj et al., 2016)**.** Numerous recent studies (Borges et al., 2021; Gomes et al., 2021; Oliveira et al., 2022; Srinuanchai et al., 2021)) have focused on this idea. These studies investigate the anti-hyperglycemic activity of various plant species by examining their ability to inhibit intestinal α-glucosidase (IAG). IAG is an enzyme responsible for the digestion and absorption of carbohydrates. Inhibition of this enzyme has become a particularly relevant strategy for managing blood sugar levels in T2D patients. Studies have shown that decreasing or delaying postprandial hyperglycaemia might be highly effective in maintaining overall metabolic health (Dirir et al., 2022; Hossain et al., 2020)**.**

Species within *Phyllanthus* genus possess numerous experimentally verified pharmacological activities, including antioxidant (Hadzri et al., 2014; Harish and Shivanandappa, 2006; Sarkar et al., 2009), anti-inflammatory, (Obidike et al., 2010), hepatoprotective (Harish and Shivanandappa, 2006) and hypolipidemic (Khanna et al., 2002; Rani and Kumar, 2015) among others. These properties are attributed to diverse bioactive constituents, particularly flavonoids, tannins and lignans (Bagalkotkar et al., 2010; Kaur et al., 2017).

Phyllanthus niruri Linneo (Phyllanthaceae), also known as 'stonebreaker', is native to Central and South America. It holds a significant role in local ethnomedicine as anti-hyperglycaemic, diuretic, antimicrobial and purgative (Ariza et al., 2009; Najari Beidokhti et al., 2017). Previous research has documented the hypoglycemic potential of both ethanolic and hot water leaf extracts (Najari Beidokhti et al., 2017). Extracts prepared with methanol (Obidike et al., 2010) or ethanol (Okoli et al., 2011) have also been reported to exhibit this property**.** Notably, methanolic extract also improved the lipid profile of streptozotocin-induced diabetic rats (Rani and Kumar, 2015).

The type of solvents and extraction methods could significantly impact the chemical diversity of extracts, thus affecting their bioactivity. This highlights the importance of experimental design when validating the properties of natural products (Tian et al., 2021). Solvent mixtures with varying polarities are often more effective than pure solvents in extracting diverse groups

of bioactive compounds (Markom et al., 2007; Okoli et al., 2011). Furthermore, seasonal and geographical variations could influence the concentration of secondary metabolites, ultimately affecting the biological activity of the plant material (Mediani et al., 2015; Sheoran et al., 2019).

Regarding metabolism related studies, results could also be influenced by the enzyme source used for *in vitro* assays and/or the animal model and protocols employed for *in vivo* studies. For instance, research shows that some inhibitors, both synthetic and natural, exhibit variable α-glucosidase inhibitory effects depending on the enzyme's origin, such as yeast, rat, rabbit, or pig small intestine (Oki et al., 1999; Önal et al., 2005).

Therefore, to adequately assess the anti-hyperglycaemic potential of natural products, it is necessary to design *in vitro* and *in vivo* assays taking these considerations into account. To the best of our knowledge, there is a lack of research simultaneously addressing extraction optimization of anti-hyperglycemic compounds, their differential effects on diverse enzymes, and *in vivo* responses during oral starch overload tests. In order to extent the knowledge of a native species, the present work aims to evaluate the effect of solvent mixtures on extracting anti-hyperglycemic compounds from *P. niruri*. Phytochemical determinations, in *vitro* and *in vivo* experiments were carried out to better assess the characteristics and effect of the extracts. Our findings aim to contribute to the growing interest in natural products as potential complementary therapies for diabetes and other metabolic disorders.

2. Materials and Methods

2.1 Chemicals and Standards

Saccharomyces cerevisiae Type I α-glucosidase, p-nitrophenyl-α-D-Glucopyranoside (pNPG) and Acarbose were obtained from Sigma Aldrich (USA and Germany). Methanol, ethyl acetate, Na_2CO_3, Folin-Ciocalteu reagent from Cicarelli (Argentina), Na_2HPO_4, NaH_2PO_4, NaOH, $AlCl_3$ and $NaNO_2$ from Biopack (Argentina) and glucose oxidase/peroxidase (GOD/POD) kit from Wiener Laboratories (Argentina).

2.2 Plant Material

In order to evaluate possible seasonal and geographical variations, complete specimens (Aerial Parts and Root) of *Phyllanthus niruri* were collected during summer (III) and spring (II) in two localities of Corrientes, Argentina: Capital (27°28’09.1”S 58°47’45.1”W) referred as (C) and Laguna Brava (27’28'4”.6"S 58’43'3”.0"W) referred as (LB) and from one locality of Misiones, Argentina (-26’59'5”.3"S 54’28'5”.8"W) (M). The species were identified by Dr. Walter Medina in the Institute of Botany of the Northeast (IBONE / UNNE) and one voucher specimen was

deposited as a reference at the Herbarium of the IBONE Torres A.M. 26 (CTES). The plant material was air dried for 48h at room temperature (25°C) and sheltered from the sun. The material was separated into Aerial Parts (AP) and Roots (R), ground and sieved using a sieve (mesh size n° 12).

2.3 Extraction

Extraction of bioactive compounds with inhibitory effect of yeast α-glucosidase activity was carried out using a simplex lattice ternary mixture design. The Design-Expert® 11 software, indicated 12 mixtures with different proportions of three solvents (Water-Methanol-Ethyl Acetate) as indicated in Table 1. Based on preliminary tests, the solid : solvent ratio remained constant at (1 : 10). The solid : solvent mixtures were macerated with constant stirring for 48 h using AP - M- III (this material was selected since it was the most abundant). Subsequently, the mixtures were filtered and dried in a Büchi R-124 rotary evaporator under reduced pressure. The inhibition potential on yeast α-glucosidase activity (as further described) was assayed for each extract.

2.4 Optimization and Validation

To evaluate the impact of solvent selection on yeast α-glucosidase inhibition and identify the optimal extraction conditions, the inhibition data was fitted to various mathematical models (linear, quadratic, cubic, etc.). Subsequently, numerical optimization was performed using the Desirability (D) function as a measure of effectiveness, with maximization of inhibition potential as the primary objective.

For validation purposes, AP-M-III was extracted using the optimized solvent mixture and the results were statistically analyzed. This optimized extraction procedure was then employed for all subsequent extracts evaluated in the study. To minimize waste and conserve the *P. niruri* species, the optimized conditions established for aerial parts were also applied to root extractions.

2.5 Phytochemical characterization of extracts

2.5.1 Total phenolic content

Total phenolic content (TPC) was determined following the technique described previously by Bobo-García et al. (2015) using 96-well flat-bottom microplates. Briefly 20 µL of extract were mixed with 100 µL of diluted (1:4) Folin-Ciocalteu reagent. The mixture was left to stand for 4 min, then 75 µL of sodium carbonate solution 10% were added. After incubating for 30 min at 40 °C, absorbance was determined at 750 nm using a microplate reader Multiskan Sky

ThermoScientific. Results were expressed as µg gallic acid equivalents (GAE) mg *P. ninuri* extract (PnE) $^{-1}$. Determinations were performed in triplicate (n = 3).

2.5.2 Total flavonoid content

Total flavonoid content (TFC) was determined following the technique previously described by Herald et al. (2012) with some modifications. Each of the extracts was mixed with 5% $NaNO_2$ in a 96-well flat-bottom microplate. After 5 min, 2% $AlCl_3$ and 4% NaOH were added. The reaction was read at 470nm. Results were expressed as µg Quercetin equivalent (QE) mg PnE $^{-1}$. Determinations were performed in triplicate (n = 3).

2.5.3 Total condensed tannins

Condensed tannin content (CTC) was determined using the vanillin-HCl spectrophotometric method described by Herald et al. (2014). In 96-well flat bottom microplates, 30uL of each of the extracts were mixed with 150 uL of vanillin reagent, the reaction was incubated for 20 min at 30°C and the absorbance was read at 500nm. A calibration curve was made using catechin as standard (0.025-1 mg mL^{-1}). Results were expressed as µg Catechin equivalent (CE) mg PnE^{-1}. Determinations were performed in triplicate (n = 3).

2.5.4 High performance liquid chromatography

The chromatographic experiment was performed using a Shimadzu LC-10/20AT HPLC (Tokyo, Japan) following the method previously described by Fyhrquist et al. (2020) with slight modifications. The mobile phase consisted of 0.1% (v/v) formic acid (Phase A) and acetonitrile (Phase B). The flow rate was 1.1 mL min^{-1} and the elution gradient profile was as follows: 0-15 min 2% –; 15 - 35 min 15% –; 35 - 45 min 35% B; 45-55min 100% B followed by washing and reconditioning of the column. A Hypersil ODS column of 250 × 4.6 mm and 5 µm particle size (Thermo Scientific, Whatman, MA, USA) was used to separate the compounds and UV spectrum was evaluated with a Diode Array Detector (DAD) Shimadzu UV-Visible detector SPD-20 MA (Tokyo, Japan). The extract obtained under optimized conditions (1 mg) was dissolved in 100 uL of DMSO and 900 uL of methanol. This solution was filtered through a 0.45 µm Nylon membrane before injection. The elution was monitored at 254, 280 and 320 nm.

2.6 Anti-hyperglycaemic potential

In order to gain insights into the anti-hyperglycaemic potential of *P. niruri* extracts, the inhibition of α-glucosidase activity from two sources (yeast and pig) was evaluated.

2.6.1 *In vitro* assays

2.6.1.1 Inhibition of α-glucosidase activity from *S. cerevisiae*

Inhibition of α-glucosidase activity was determined using a previously described method by Najari Beidokhti et al. (2017) with some modifications. Briefly, 100 uL of each extract (5 ug mL^{-1}) dissolved in 10% methanol and 100 mM Sodium phosphate buffer pH 7.5 were incubated with 20 uL of 5 mM pNPG at 37 °C for 5 min. With the addition of 20 uL of enzyme (0.01 UI mL^{-1}) the reaction started. The reaction mixture was incubated at 37°C for 30 min, then stopped with 50 uL of 2% Na_2CO_3 added to each well. Final absorbance was determined at 405 nm in a microplate reader Acarbose (1 mg mL^{-1}) was used as positive inhibition control. For each extract, a blank was performed, replacing pNPG with 20 uL of 100 mM sodium phosphate buffer pH=7.5. A decrease in the release of p-nitrophenol (yellow) from pNPG by the enzyme was indicative of the inactivation of α-glucosidase activity. Determinations were performed in quadruplicate. The % inhibition was calculated using equation (1).

$$\%Inhibition = \left(\frac{Ae-As}{Ae}\right) \times 100 \qquad (1)$$

where Ae = Absorbance of the enzyme affected by reagent blank, As = Absorbance of sample (extract or acarbose). The final results were expressed as a ratio in relation to the positive inhibition control using the following equation (2).

$$Ratio = \frac{\%inhibition/ugExtract}{\%inhibition/ugAcarbose} \qquad (2)$$

2.6.1.2 Inhibition of pig intestine α-glucosidase activity

Pig α-glucosidase was obtained according to Kato-Schwartz et al. (2020) with some modifications. Jejunum, ileum and duodenum of the small intestine of one pig (80 days old) were removed and washed inside and out with 1% KCl at 4 °C. The intestinal mucosa was obtained by scraping with glass slides and homogenized with 4 volumes of cold 20 mM Potassium Phosphate Buffer pH 6. The homogenate was centrifuged at 10000 g or 10 min twice. The supernatant was lyophilized and stored at -20 °C and referred to as enzyme extract. At the time of use, the powder (10 mg) was dissolved in 1 mL of 100 mM Sodium phosphate Buffer pH=7.5.

Inhibition of α-glucosidase was determined using the previously described method by García et al. (2017) with some modifications. Maltose (5 mg mL^{-1}) dissolved in 50 mM phosphate buffer pH=7.5 was used as substrate. Each extract (5 mg mL^{-1}) was incubated with 50 uL of enzyme

extract (10 mg mL^{-1}) for 10 min at 37 °C, then 200 uL of maltose were added and left to react for 20 min at 37 °C. The reaction was stopped by heating at 100 °C during 5 min. The reaction mixture was centrifuged at 2680 g for 5 min and 6 uL of the supernatant were mixed with 200 uL of GOD/POD reagent. The reaction was incubated for 5 min at 37°C and the amount of glucose released from maltose was determined at 505 nm. A calibration curve with anhydrous glucose as standard (0.5 - 8 mg mL^{-1}) was used. Results were expressed as presented in equation 3.

$$\%Inhibition = \left(\frac{Enz_{Glc} - S_{Glc}}{Enz_{Glc}}\right) \times 100 \quad (3)$$

where Enz_{Glc}= Glucose generated by the enzyme and S_{Glc} = Glucose generated by the enzyme when incubated with the extract. The final results were expressed as a ratio in relation to the positive inhibition control using equation (2).

2.6.2 *In vivo* assay

2.6.2.1 Acute toxicity test in *Artemia salina* Leach

The acute toxicity test in *Artemia salina* L. was carried out following the technique described by Zhu et al. (2017). Commercial dehydrated cysts of *A. salina* (1 g) were incubated in 1 L of artificial seawater at 28 °C, with continuous aeration and light regime of 1300 lx. After 24 h the separation of the nauplii (stage I larva) was made based on their phototactic migration. The test was performed in triplicate by introducing ten nauplii into each well of 96-well plates containing 100 µl of artificial seawater with *P. niruri* extract suspensions (0,5; 2,5 and 5 mg mL^{-1}). All plates were incubated at 28°C on a 16 : 8 h light/dark cycle. During the exposure, the larvae analyzed were not fed. After 24 h, the number of dead (completely immobile) larvae was counted under Leica EZ4E binocular magnifying glass with 35X graduated magnification and the mortality of each treatment was calculated and expressed as LC_{50} using the statistical method of Probits.

2.6.2.2 Oral starch overload in *rats*

Male Wistar rats (n=24) weighing 250 ± 17 g were kept in acclimation conditions for 48 h with free access to food and water and 12 h : 12 h light/darkness cycle. The experiments were carried out using 6 animals per group. All animal experiments were in compliance with the Committee for the Care and Use of Laboratory Animals of the Faculty of Medicine (Resolution No. 0013 CICUAL-MED-UNNE/22).

In vivo inhibition of α-glucosidase was carried out following the procedure previously reported by Pereira et al. (2015) with some modifications. Rats were fasted for 14 h prior to the test with free access to water. The most active extract obtained under optimized conditions (AP-C-II) was dissolved in an aqueous solution of 10% ethanol and Tween 80% and orally administered in two dose levels (500 mg kg^{-1}) and (200 mg kg^{-1}), after 20 min of ingestion an oral starch overload (2 g kg^{-1}) was administered. Blood samples were collected by tail puncture and blood glucose was determined with an *ACCU-CHEK* guide (Roche®) automatic glucometer immediately after extract administration (t0) and after 30 (t1), 60 (t2), 90 (t3), 120 (t4) and 150 (t5) minutes of starch overload. Control groups were administered with 15 mg kg^{-1} acarbose (+ control) or distilled water (- control) prepared in the same conditions as extract prior to starch overload. Results were expressed as blood glucose mg dL^{-1}.

2.7 Statistical analysis

Three independent experiments were performed and results are expressed as mean ± standard deviation. The results were statistically analyzed using analysis of variance (ANOVA) (α = 0.05). The differences among means were tested for statistical significance using a multiple-range least significant difference (LSD) with Info-Stat Statistical Software 2015 (Córdoba, Argentina).

3 Results and Discussion

3.1 Solvent extraction optimization

The result from optimization experiment for solvent mixtures aimed at maximizing α-glucosidase inhibition fit to a quadratic model (p-value = 0.0228). The final predicted equation was % Inhibition α-glucosidase = (+233.36 A + 1.27 B + 25.15 C -38.39 AB + 396.30 AC + 223.73 BC), where A=methanol, B=water and C=ethyl acetate.

The predicted model was mainly influenced by the AC term, which corresponds to the content of A=methanol and C=ethyl acetate in the extraction mixture. These results indicate that extracts prepared with binary mixtures exhibited higher inhibition potential than those obtained with pure solvents. Traditionally, plant extractions have relied on single solvents (Najari Beidokhti et al., 2017; Oki et al., 1999; Sheoran et al., 2019), however, our findings highlight the potential benefits of exploring binary solvent mixtures to optimize bioactive compound extraction in coincidence with the results of Markom et al. (2007).

Table 1 summarizes the relative solvent proportions for each mixture and the corresponding α-glucosidase inhibition results expressed as both percentage (%) and ratio. Based on these

findings, numerical optimization was performed using the desirability (D) function. As shown in Fig. 1 the optimization process revealed that binary mixtures of methanol and ethyl acetate at a 70 : 30 ratio yielded the highest inhibition rates, achieving a desirability value of D=0.976. Najari Beidokhti et al. (2017) have reported that *P. niruri* extracts prepared with hot water or ethanol exhibited α-glucosidase inhibitory effect with IC50 values of 3.7 ± 1.1 and 6.3 ± 4.8 μg mL^{-1}, respectively. Extracts obtained with 80% ethanol were also reported to have α -glucosidase inhibitory activity with an IC50 of 4.27 μg mL^{-1} (Mediani et al., 2015). These results showed that the metabolites that confer the anti-hyperglycaemic property to this species are mostly extracted with polar solvents.

Following the establishment of optimal extraction conditions, the experimental and predicted values of α-glucosidase inhibition were compared to validate the accuracy of the model. The validation results were in agreement with the predicted values. Notably, the optimized extract exhibited an 87% inhibition of α-glucosidase from *S. cerevisiae* which was equivalent to an inhibition potential 255 times higher than acarbose (Table 2). It is important to note that the enzyme inhibition assays in this work employed extract concentrations of 5 μg mL^{-1} to achieve the observed 87% inhibition. Our results are in agreement with those reported by other researchers (Mediani et al., 2015; Najari Beidokhti et al., 2017), highlighting the effectiveness of solvent mixture optimization in enhancing the extraction of bioactive metabolites. The optimization strategy translates in higher α-glucosidase inhibition ratios with lower concentration of extract. Furthermore, the observed inhibition falls within the predicted interval with a 95% confidence level, and the experimental value exhibits a strong correlation with the predicted mean. These findings collectively support the adequacy of our experimental design.

Markom et al. (2007) ddemonstrated that Soxhlet technique increased the extraction of ellagic and gallic acids from *P. niruri*, moreover, the extraction of these compounds was enhanced with the polarity of the solvent. This is in agreement with our results, highlighting the importance of optimization step, especially regarding solvent selection.

Figure 2a shows the HPLC-DAD chromatogram of the optimized extract obtained from Aerial Parts of *P.niruri* (AP-M-III). A major peak was observed at 23 min, this compound had λ_{max} = 219 nm and 270 nm (Fig. 2b), with a peak purity index of 0.67 and single-point threshold of 0.98 indicating no co-elution. The peak profile and UV spectrum of this peak was similar to those reported by Kochumadhavan et al. (2019) who evaluated the hydroalcoholic extract of *Phyllanthus* species. Fyhrquist et al. (2020) identified the gallotannin corilagin from the butanol extract of *Combretum psidioides* stem bark, whose absorption spectrum matches that shown in image 1b corresponding to the major peak of our optimised extract. Moreover,

Kochumadhavan et al. (2019) using NMR and HPLC identified corilagin in hydroalcoholic extracts of *Phyllanthus* species, showing an UV spectrum similar to the reported in this work. Based on the aforementioned data, corilagin, a hydrolyzable tannin undetectable by vanillin-HCl method, was tentatively assigned as the primary bioactive compound in the optimized extract (PnE).

3.2 Phytochemical characterization

Table 3 summarizes the quantitative analysis of major constituents in extracts obtained using the optimized solvent mixture from *P. niruri* specimens. Notably, condensed tannins were exclusively detected in root extracts, with R-LB-II exhibiting the highest content followed by R-M-III. These findings are in coincidence with the results of Sri Kosnayani et al. (2019) who did not find condensed tannins in aerial parts of *P. niruri*. However, they showed enrichment of hydrolysable and complex tannins in extracts obtained with polar solvents. Additionally, Najari Beidokhti et al. (2017) attributed the hypoglycaemic potential to hydrolysable tannins; particularly corilagin and repandusinic acid isolated from aqueous and ethanol extracts of aerial parts of *P niruri.*

Aerial parts (AP-LB-II) exhibited the highest total phenolic content (TPC) at 192.24 ± 9.03 ug GA mg PnE^{-1}, while root extracts R-C-II and R-LB-III showed the lowest values. Notably, significant geographical variations in TPC were observed, highlighting the influence of edaphoclimatic factors (origin and collection time) on the chemical profile of the extracts. As reported by Bagalkotkar et al. (2010), *P. niruri* leaves, stems, and roots harbor various bioactive components, including lignans, tannins, flavonoids, alkaloids, saponins, and coumarins.

Extracts exhibited significant variations in flavonoid content. The highest content (460.53 ± 26.27 µg QE mg^{-1} PnE^{-1}) was found in R-M-III, while R-LB-II showed the lowest (38.68 ± 4.22 µg QE mg^{-1} PnE^{-1}). This suggests a notable influence of edaphoclimatic factors on flavonoid levels. Interestingly, TFC was consistently higher in summer samples (season III). In contrast, flavonoid content was generally greater in aerial parts (AP) compared to roots (R), with the exception of Misiones (M) specimens. This pattern suggests that both geographic origin and plant part are significant variables for flavonoid content. In line with our findings, previous studies by Bagalkotkar et al. (2010) and Rusmana et al. (2017) reported similar variations.

The relative content of analyzed phenolic compounds exhibited significant variations across organs, seasons, and geographical origin. These variations could potentially impact the efficiency of extracting bioactive components. Therefore, in depth characterization of the

starting plant material is of particular importance for achieving standardized extracts and ensuring consistent quality in plant-derived products.

3.3 Anti-hyperglycaemic potential

3.3.1 *In vitro* assays

To assess the anti-hyperglycaemic potential of the extracts, their inhibitory activity against α-glucosidase from both yeast and porcine sources was evaluated. This selection aligns with prior reports highlighting the potential variability (mixed results) in the inhibition potential of plant extracts depending on the α-glucosidase source (Oki et al., 1999; Zhu et al., 2020). α-glucosidases from different origins exhibit structural and functional variations that could influence inhibitor interaction and efficacy. Additionally, some inhibitors may possess greater specificity towards α-glucosidase from a particular source due to subtle differences in active site conformation. By employing two enzyme sources, we ensured cross-validation of the results, minimizing the possibility of source-specific effects and strengthening the robustness of our findings.

Table 4 summarizes the α-glucosidase inhibition ratios relative to acarbose for the tested enzymes. Statistical analysis revealed no significant differences ($p > 0.05$) in yeast α-glucosidase inhibition among extracts from aerial parts, regardless of season or geographical origin. In contrast, root extract inhibition potentials exhibited significant variations based on both geographical origin and season. Notably, aerial parts generally had higher rates of inhibition for yeast α-glucosidase than roots, with the exception of R-LB-II, which exhibited an inhibition ratio similar to AP-LB-II. Furthermore, LB-II extracts (from both aerial parts and roots) demonstrated the strongest inhibition potential against yeast α-glucosidase. These observed differences in inhibition potential could be attributed to variations in the bioactive profiles of plant organs and seasonal fluctuations in phytometabolites' concentrations, as previously reported by other authors (Mediani et al., 2015; Sheoran et al., 2019).

Pig α-glucosidase inhibition results diverged from those observed with the yeast enzyme, with most extracts exhibiting inhibition ratios below 1 compared to acarbose. These results highlight the importance of employing multiple enzyme sources when evaluating anti-hyperglycaemic potential to minimize misinterpretations, as reported by Oki et al. (1999) and Oliveira et al. (2022). Although the inhibition potential on pig α-glucosidase was below expected for all the extracts, the results obtained in this work could be useful for future studies. Moreover, it should be considered that these results are referred to or compared to a potent inhibitor as acarbose. This highlights the importance of further investigation using

alternative assays (i.e *in silico* studies, alternative inhibitors, etc.) to comprehensively assess the potential anti-hyperglycemic properties of these extracts against pig α-glucosidase.

Among the extracts, AP-C-II, AP-C-III, AP-M-III, and R-LB-II had the highest inhibitory activity against yeast α-glucosidase. However, AP-C-II was chosen to perform the *in vivo* tests, due to its abundance and comparable inhibitory effect to the other extracts. Our results illustrate the importance of adequate selection and design of *in vitro* bioactivity screening assays. The observed discrepancies between microbial and mammalian α-glucosidase inhibition might be a consequence of the challenges in replicating optimal enzyme activity conditions *in vitro*, such as membrane attachment and interactions with other biomolecules.

3.3.2 *In vivo* assay

3.3.2.1 Acute toxicity

Ethical considerations necessitate the exploration of alternative models to replace laboratory animals in toxicological testing. The *Artemia salina* Leach bioassay, employing the Meyer toxicity index and Clarkson toxicity criterion, classifies extracts with LC_{50} > 1000 µg mL^{-1} as non-toxic (Meyer et al., 1982). This is in coincidence with findings reported by Hamidi et al. (2014), who found a correlation between LC_{50} values in *A. salina* (>1000 µg mL^{-1}) and LD_{50} values for acute toxicity of methanolic *Mentha spicata* extract in rats (>5000 mg kg^{-1}). Based on this established correlation, the LC_{50} value of 2244.4 µg mL^{-1} PnE for *A. salina* assay obtained in our work, we tentatively extrapolated a potential lethal dose in rats to exceed 5000 mg kg^{-1}. Therefore, to ensure safety in our *in vivo* studies, we employed significantly lower doses (500 and 200 mg kg^{-1}).

3.3.2.2 Anti-hyperglycaemic activity

The process of converting dietary complex carbohydrates into glucose is primarily carried out by α-glucosidases. Hence, their inhibition represents a valuable strategy for postprandial hyperglycaemia management (Chiba, 1997; International Diabetes Federation, 2019; Pereira et al., 2015).

The results obtained for the *in vivo* inhibition of starch degradation and absorption are presented in Figure 3. At a dose of 500 mg kg^{-1}, *P. nir*uri extract (AP-C-II) significantly reduced ($p < 0.05$) postprandial glycaemia compared to control, with the effect beginning at 90 minutes and persisting until the end of the experiment. Acarbose, the positive control, exhibited a faster time of response (30 minutes). The delay in the effect of AP-C-II might be attributed to its complexity and composition. Unlike commercial acarbose, AP-C-II comprises an extract

prepared from aerial parts, containing various compounds (fiber, chlorophylls, terpenes, etc.) that could potentially hinder the rapid action of the inhibitors on α-glucosidase. Additionally, fiber and other components may delay digestive processes.

Supporting our findings, Okoli et al. 2011 reported a similar reduction in blood glucose levels in diabetic and non-diabetic rats following administration of a *P. niruri* methanolic extract. It is noteworthy mention; that our study employed starch, a direct enzyme substrate, to assess inhibitory effects, and further evaluated the impact on the absorption of enzymatic products by measuring blood glucose. This starch overload test provides a more comprehensive perspective on the extract's impact on both degradation and absorption. Starch-rich meals lead to significant postprandial hyperglycaemia, therefore, mitigating this rise is crucial for preventing and treating type 2 diabetes, cardiovascular disease, and obesity (Blaak et al., 2012). The extract obtained using the optimized solvent mixture (AP-C-II) demonstrated promising efficacy in managing hyperglycaemia after *in vivo* starch overload, highlighting its potential for future developments.

4. Conclusions

This study comprehensively highlights the importance of experimental design when utilizing solvent mixtures for extracting bioactive anti-hyperglycemic compounds from *P. niruri*. Our results provide a systematic analysis of the influence of plant organs, geographical origin, and season on the extract activity. Interestingly, the most active extracts exhibited low condensed tannin content, while HPLC-DAD analysis tentatively identified corilagin, a hydrolyzable tannin, as the potential anti-hyperglycaemic compound. *In vitro* studies revealed a significant inhibitory effect on yeast α-glucosidase. To complement these findings, *in vivo* studies demonstrated that the *P. niruri* extract had an inhibitory effect comparable to acarbose. These results reveal the importance of appropriate selection of extraction techniques and adequate design of both *in vitro* and *in vivo* assays for accurate characterization of the anti-hyperglycaemic potential of natural product extracts. Future studies will focus on elucidating the underlying molecular and cellular mechanisms responsible for the anti-hyperglycaemic effects of *P. niruri* extracts.

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Figure and table legends

Table 1. Solvent mixture design and α-glucosidase inhibition for each of the extracts.

Nd: not detected. Solvent proportions and results for α-glucosidase inhibition, values are presented as the mean ± standard deviation (n=4). The ratio values represent the inhibition of each extract referred to the positive control (acarbose).

Figure 1. Contour plot showing desirability (D) function for numerical optimization of α – glucosidase inhibition using solvent mixtures.

Table2. Verification of optimal predicted extraction conditions.

Figure 2.a) Chromatogram of *P. niruri* aerial parts extract optimized showing the major peaks. b) Absorption spectra of the peak at 23min.

Table 3. Major chemical constituents of *P. niruri* extracts obtained with an optimized mixture solvent. The content of total phenolic compounds (TPC), flavonoids (TFC) and condensed tannins (CTC) are expressed in µg equivalents of Gallic Acid (GAE), Quercetin (Q) and Catechin (C) respectively per mg of extract (PnE). The values are presented as the mean ± standard deviation (n=4). Values followed by different capital letters within a column indicate statistically significant differences ($p < 0.05$) between extracts obtained from different plant organs (AP,R) and geographical origin (M,C,LB). Values followed by different lowercase letters within a column indicate statistically significant differences ($p < 0.05$) between extracts obtained from different plant organs (AP, R) for the same geographical origin in different seasons (II, III).

Table 4. Inhibition of enzymatic activity for α-glucosidase of different origin by *P. niruri.* extracts. Extracts: aerial parts (AP) and roots (R) of *P.niruri for* summer (III) and spring (II)

seasons, from Corrientes Capital (C), Misiones (M) and Laguna Brava (LB). The value represents a ratio of inhibition for each extract referred to the positive control (acarbose).

The values are presented as the mean ± standard deviation (n=4). Values followed by different capital letters within a column indicate statistically significant differences ($p < 0.05$) between extracts obtained from different plant organs (AP,R) and geographical origin (M,C,LB). Values followed by different lowercase letters within a column indicate statistically significant differences ($p < 0.05$) between extracts obtained from different plant organs (AP, R) for the same geographical origin in different seasons (II, III).

Figure 3. Effect of *P. niruri* extract (AP-C-II) on the oral starch overload test

Figures and tables

Table 1

Extract	Solvent mixture (mL)			% Inhibition α-glucosidase	Ratio
	Methanol	Water	Ethyl acetate		
1	1.66	1.66	6.66	48.36 ± 3.43	186.00 ± 13.20
2	0	5	5	14.08 ± 2.48	54.16 ± 9.53
3	10	0	0	68.21 ± 3.29	262.33 ± 12.67
4	0	0	10	Nd	Nd
5	5	0	5	76.31 ± 2.78	244.60 ± 8.91
6	0	10	0	Nd	Nd
7	10	0	0	54.48 ± 5.29	232.82 ± 22.60
8	3.33	3.33	3.33	39.90 ± 4.54	153.45 ± 17.46
9	6.66	1.66	1.66	31.02 ± 8.14	132.29 ± 0.32
10	0	10	0	Nd	Nd
11	1.66	6.66	1.66	14.48 ± 1.29	46.41 ± 4.13
12	5	5	0	41.78 ± 6.13	142.26 ± 19.05
ACARBOSE	-	-	-	52.28 ± 6.96	1.00 ± 0.00

Figure 1

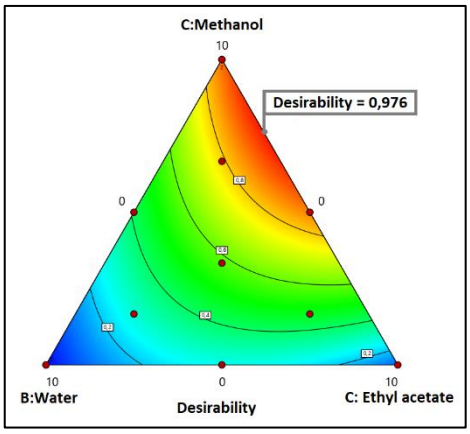


Table 2

Analysis	Predicted Mean	Predicted Median	Std Dev	n	SE Pred	95% PI low	Data Mean	95% PI high
%Inhibition α-glucosidase	254.12	254.12	53.08	2	66.44	91.54	255.20	416.70

Figure 2

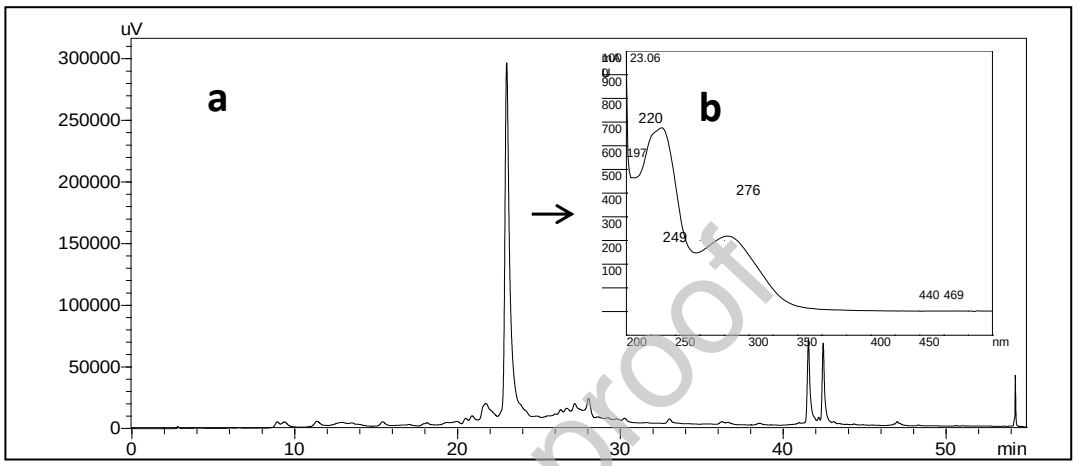


Table 3

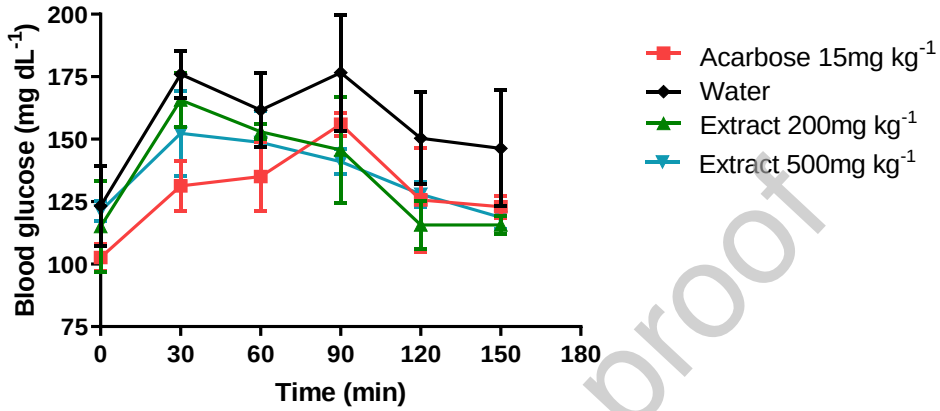
Extract	CTC (ug CE mg PnE^{-1})	TPC (ug GAE mg PnE^{-1})	TFC (ug QE mg PnE^{-1})
AP-M-III	ND	129.19 ± 5.89 ^{AB}	135.19 ± 9.90 ^{A}
R-M-III	102.08 ± 14.26 ^{AB}	155.56 ± 6.32 ^{A}	460.53 ± 26.27 ^{B}
AP-LB-III	ND	146.61 ± 9.21 ^{B,b}	190.90 ± 14.61 ^{B,a}
R-LB-III	86.02 ± 3.45 $^{B, b}$	80.17 ± 8.70 ^{A,b}	113.64 ± 12.34 ^{C,b}
AP-LB-II	ND	192.24 ± 9.03 ^{A,a}	185.71 ± 18.60 ^{B,a}
R-LB-II	180.09 ± 12.13 ^{B,a}	139.19 ± 8.52 ^{A,a}	38.68 ± 4.22 ^{C,a}
AP-C-II	ND	168.12 ± 6.13 ^{A,a}	134.22 ± 14.42 ^{A,a}
R-C-II	71.89 ± 4.41 ^{A,a}	80.51 ± 2.53 ^{A,a}	85.08 ± 3.23 ^{A,a}
AP-C-III	ND	147.43 ± 4.61 ^{A,b}	97.98 ± 4.91 ^{A,a}
R-C-III	85.42 ± 9.83 ^{A,b}	95.00 ± 8.01 ^{A,b}	143.83 ±4.39 ^{A,b}

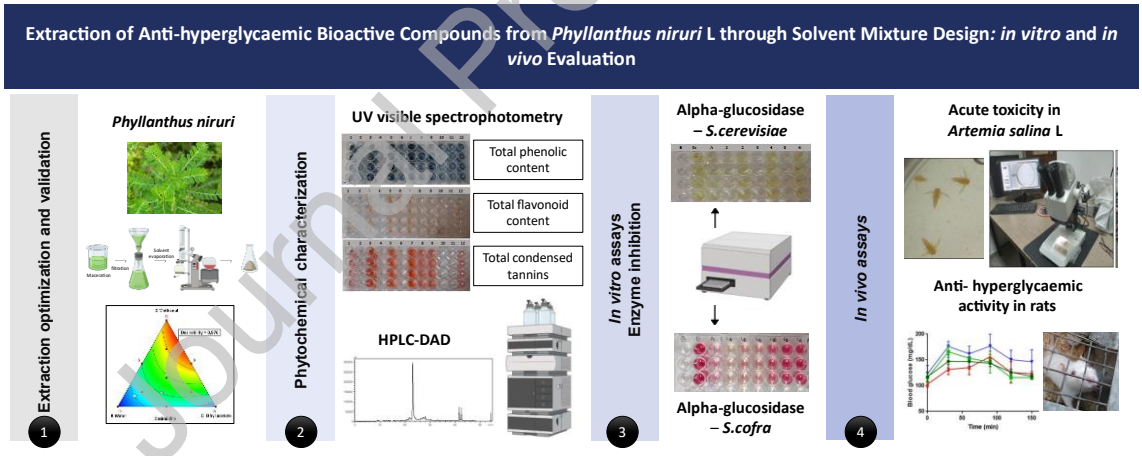
Table 4

Extract	α-glucosidase *(S. cerevisiae)*	α-glucosidase *(Pig)*
AP-M-III	228.08 ± 15,30 ^{B}	0.059 ± 0.006 ^{A}
R-M-III	156.51 ± 9.99 ^{A}	ND
AP-LB-III	196.39 ± 14,62 ^{A,a}	0.044 ± 0.003 ^{A,B,a}
R-LB-III	111.28 ± 11.43 ^{A,a}	ND

AP-LB-II	205.14 ± 15.06 ^{A,a}	0.074 ± 0.005 ^{A,B,b}
R-LB-II	226.00 ± 11.20 ^{A,b}	ND
AP-C-II	226.20 ± 9.07 ^{B,b}	0.080 ± 0.004^{B,b}
R-C-II	63.91 ± 2.81 ^{A,b}	0.030 ± 0.003 ^{B,b}
AP-C-III	222.33 ± 12.60 ^{B,a}	0.069 ± 0.003^{B,a}
R-C-III	109.75 ± 11.43 ^{A,a}	ND

Figure 3





Graphical abstract

Author contributions

Ana Melissa Gonzalez Miragliotta: conceptualization, investigation, methodology, data curation, visualization, performed experiments, writing original draft. **Gonzalo Adrián Ojeda:** investigation, methodology, data curation, visualization, writing original draft. **Romina Belen Gonzalez:** investigation, performed experiments, methodology. **Estela Rosa Jara:** performed experiments, methodology**. Gladys Pamela Teibler:** investigation, performed experiments, methodology. **Nélida María Peruchena:** supervision, resources, writing - review & editing. **Ana María Torres:** investigation, data curation, visualization, writing original draft, supervised experiments, corrected original draft.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: