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α-Amylase *in vitro* inhibitors from the *n*-hexane extract of *Benincasa hispida* leaves of the Cucurbitaceae family

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Abstract

We explored the α-amylase- and α-glucosidase-inhibitory activities of *Benincasa hispida*, widely used in Asia as a food for its nutritional and pharmacological properties. *B. hispida* leaves were extracted with 70 % ethanol and subjected to successive solvent partitioning, yielding *n*-hexane, ethyl acetate, *n*-butanol, and aqueous extracts. Each extract was subjected to *in vitro* assays for α-amylase and α-glucosidase inhibition. The *n*-hexane extract was subjected to normal column chromatography, yielding an isolate specific against α-amylase. The isolate was subjected to ultraviolet-visible (UV-Vis) and ultra-high-performance liquid chromatography-elevated mass spectrometry (UHPLC-MS^E). The *n*-hexane extract showed the highest inhibition (98.68%) against α-amylase. The α-amylase EC_{50} inhibition of *the n*-hexane extract was 30.48 μg/mL, with a *p*-value of 0.05. The α-glucosidase inhibition of *n*-hexane extract was minimal at 11.2 %. The extract was subjected to normal-phase column chromatography, and fractions with similar thin-layer chromatography profiles were pooled and evaluated for *in vitro* inhibition of α-amylase and α-glucosidase. The isolate was dereplicated as cucurbitacin A in the *B. hispida* leaf extract. However, the presence of cucurbitacin C in an enol form was also detected. The EC_{50} of the isolate ranged from 226 μM to 299 μM at 95 % confidence level. These findings suggest that *B. hispida n*-hexane isolate containing cucurbitans A and C, in keto-enol form, is specific against α-amylase.

Keywords: LC-MS^E; α-amylase inhibitor; *Benincasa hispida*; mass spectrometry, cucurbitacin A; cucurbitacin C

Abbreviations

AGI	*α*-Glucosidase inhibitor
CE	Collision energy
DM	Diabetes mellitus
DPP-4	Dipeptidyl peptidase-4
GDM	Gestational diabetes
MF	Molecular formula
MS	Mass spectrometry
TLC	Thin-layer chromatography
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes Mellitus
PBS	Phosphate-buffered saline
UHPLC- MS^E	Ultra-high-performance liquid chromatography-mass spectrometry elevated energy
UHPLC	Ultra-high-performance liquid chromatography

Introduction

Diabetes mellitus (DM) is the eighth leading cause of death globally. DM has shown a 95 % increase in prevalence since 2000 [1] and is considered one of the fastest-growing global health emergencies [2]. It is a metabolic disorder with multiple aetiologies that occurs when the body cannot effectively use or produce enough insulin [3]. There are three main types: (a). type 1 diabetes mellitus (T1DM), (b). type 2 diabetes Mellitus (T2DM), and (c). gestational diabetes (GDM) [4]. Types 1 and 2 may develop due to loss or dysfunction of pancreatic islet cells [5]. T1DM results from autoimmune destruction of pancreatic β-cells [6], whereas T2DM is a progressive disorder characterised by insulin resistance and reduced insulin secretion. T2DM is more common than T1DM, accounting for approximately 96 % of all DM cases [7], and is associated with genetics, endocrine imbalance [8], sedentary lifestyle [9], infections [10], obesity [11], and ageing [12]. GDM is characterised by glucose intolerance during the third trimester, which can potentially lead to fetal overgrowth [13, 14]. Uncontrolled DM leads to complications like atherosclerosis, cardiovascular problems, cerebrovascular disease, peripheral vascular disease [15], nephropathy, neuropathy, and retinopathy [16]. DM can be managed through non-pharmacological treatments, *e.g.*, diet and exercise, as well as pharmacological treatments, including insulin and oral hypoglycemic agents [17].

α-Amylase is one of the most important digestive enzymes because it plays a major role in the breakdown of polysaccharides. The enzyme catalyses the α-1,4 glycosidic linkages of starch, amylose, amylopectin, glycogen, and other maltodextrins [18]. It shows a correlation between α-amylase and increased postprandial glucose levels; therefore, inhibiting this enzyme may prevent high postprandial blood glucose [19, 20]. Another essential enzyme located at the brush border of the small intestine is α-glucosidase, which is essential for the breakdown of carbohydrates into monosaccharides. This enzyme's inhibition effectively delays glucose absorption and averts high postprandial blood glucose levels. Inhibiting both enzymes may delay the progression of diabetes [19, 20].

Various synthetic medicines are commercially available, but side effects are commonly observed with long-term use [21]. Medications available for T1DM include insulin, whereas treatment for T2DM encompasses α-glucosidase inhibitors (AGIs), biguanides, dipeptidyl peptidase-4 inhibitors (DPP-4), glinides, gliflozins, sulfonylureas, and thiazolidinediones [22]. AGIs delay carbohydrate absorption, *i.e.,* acarbose and miglitol, but may cause flatulence and diarrhoea [23]. Postprandial hyperglycemia is a factor considered in the progression of T2DM. Decreasing postprandial hyperglycemia is a primary strategy for managing type 2 diabetes mellitus (T2DM). This method for controlling T2DM targets the enzymes α-amylase and α-glucosidase, which play a role in carbohydrate breakdown, thereby elevating postprandial glucose levels in patients with DM [24]. Inhibiting these enzymes can control postprandial hyperglycemia, thereby reducing the risk of developing diabetes [25]. AGIs have demonstrated a high effectiveness and safety and offer an advantage because they do not carry the risk of hypoglycemia [26]. In addition, AGIs reduce the risk of cardiovascular disease, decrease the progression of T2DM, and prevent weight gain [26]. Acarbose slows carbohydrate absorption and reduces postprandial blood glucose levels. The α-amylase and α-glucosidase enzymes are inhibited by binding to the carbohydrate-binding region, thereby interfering with their hydrolysis of carbohydrates into monosaccharides [27]. Additionally, acarbose has been reported to reduce triglycerides and increase postprandial insulin levels [28]. The causes of diabetes are well-recognised, and several hypoglycemic agents are already established. Nevertheless, the number of individuals with diabetes continues to increase [29]. Synthetic drugs carry side effects like hypoglycemia, dizziness, gastrointestinal discomfort, headache, nausea, and weight gain [19, 20]. Thus, researchers have widely studied plants and their secondary metabolites to lessen the side effects of synthetic drugs.

Numerous α-amylase inhibitors from plants, as safe and cost-effective alternatives, have been reviewed by Kashtoh and Baek [30]. The bioactive compounds responsible for the inhibitory activity of the plant extracts have been identified. The Cucurbitaceae family comprises a wide range of plants that are rich sources of bioactive compounds, including cucurbitacins, which exhibit diverse pharmacological activities, including anticancer, antidiabetic, anti-inflammatory, and antimicrobial [31]. Plants with hypoglycemic activity under Cucurbitaceae are *Benincasa hispida* (white pumpkin), *Coccinia indica* (ivy gourd), *Cucumis sativus* (cucumber), *Momordica charantia* (bitter gourd), and *Lucca acutangular* (ridge gourd) [32, 33]. As one of the most valuable plants in the Cucurbitaceae family [34], *B. hispida* exhibited a significant decrease in glucose tolerance curve and significant hypoglycemic activity [32]. This plant is widely used in Asia and has several documented nutritional and medicinal properties [35]. Researchers conducted studies on the fruit [36], leaves [37], seeds [38], and stems [21], all of which have been shown to lower blood glucose levels in test animals. A study has been conducted on *B. hispida* leaves to screen for hypoglycemic activity in diabetic mice [37].

Ultra-high-performance liquid chromatography-mass spectrometry elevated energy (UHPLC- MS^E) is a powerful analytical technique usually applied for identification and quantification [39]. When combined with mass spectrometry, this technique provides qualitative and quantitative data [39]. Combining UHPLC with MS provides various benefits, including increased selectivity, sensitivity, and throughput for analysing complex samples, including phytochemicals [40]. In addition, the speed of assay development and high specificity are some of the advantages of UHPLC-MS. Several studies [41] have utilised UHPLC- MS^E to characterise complex samples. One study isolated endophytic *Bacillus tequilensis* ALR-2 from the Aloe vera plant using UHPLC-MS^E to characterise bioactive compounds and determine their accurate masses; the method was considered superior for its efficiency and high-resolution molecular ion detection [41, 42]. The determination of seven taxoids and seven flavonoids was also established using UHPLC-MS^E, which showed a good performance that involved linearity, sensitivity, precision, and accuracy [42].

In our search for antidiabetic agents, we explored *Benincasa hispida* (Fig 1), a remarkable plant in the Cucurbitaceae family [34]. Hypoglycemic studies [37] have examined the plant parts. However, the potential target as an antidiabetic agent needs to be established. Hence, we study the potential hypoglycemic activity of the leaf extract of *B. hispida* using *in vitro* assays for α-amylase and α-glucosidase inhibition. Additionally, the extract with the highest inhibition was subjected to bioassay-directed purification using column chromatography and ultra-high-performance liquid chromatography with elevated-energy mass spectrometry (UHPLC-MS^E) to further characterise the secondary metabolites present in *B. hispida* leaves.

Results and discussion

Experimental design

This study (Fig 2) employed a bioassay-directed approach to evaluate the potential α-amylase- and α-glucosidase-inhibitory activities of *B. hispida* leaves through *in vitro* α-amylase and α-glucosidase assays, followed by the chemical characterisation of the bioactive compounds exhibiting the highest inhibitory activity. Initially, *B. hispida* leaves were extracted with 70 % ethanol. The ethanolic extract was then partitioned with *n*-hexane, ethyl acetate, and *n*-butanol to yield fractions of varying polarity. These fractions, including the aqueous extract, were

screened for their inhibitory activity using *in vitro* α-amylase and α-glucosidase assays. The *n*-hexane fraction was further subjected to normal column chromatography, analysed by thin-layer chromatography (TLC), and the active fractions were subjected to normal column chromatography, analysed by TLC, and the active *n*-hexane fraction was subjected to UHPLC-MS^{E}.

Solvent extraction, partitioning, and normal column chromatography

The dried leaves of *B. hispida* were subjected to exhaustive maceration in 70 % ethanol. The resulting extract was then concentrated using rotary evaporation under reduced pressure (*in vacuo*). The ethanolic crude extract was partitioned using *n*-hexane, ethyl acetate, *n*-butanol and water. All these extracts, including the ethanolic extract, were subjected to an *in vitro* enzyme inhibition assay for α-amylase and α-glucosidase. Extracts were concentrated *in vacuo* at 40 °C and tested in α-amylase and α-glucosidase inhibitory assays. The *n*-hexane extract with the highest hypoglycemic effect was subjected to normal-phase column chromatography and characterised by TLC and UHPLC-MS^{E}.

The *n*-hexane extract, which exhibited the highest α-amylase inhibition, was further purified by normal-phase column chromatography. The suitable solvent system, patterned on TLC, was *n*-hexane: ethyl acetate (8:2). After collecting 50 half-filled test tubes, the solvent system was changed to *n*-hexane: ethyl acetate (7:3), then (1:1). The fractions were collected in test tubes and analysed by TLC. The column chromatography fractions with similar profiles were pooled, resulting in 14 fractions. Of the 14 fractions, fraction 14 (4.6 mg) (Fig 3) with α-amylase inhibitory activities was pursued for UHPLC-MS^{E}.

***In vitro* enzyme inhibitory assays**

Screening

In the α-amylase (Fig 4A) and α-glucosidase inhibitory assays (Fig 4B), a screening was conducted in a 96-well plate. The expected positive result was a dark blue or violet color, indicating enzyme inhibition. Acarbose was used as the positive control. All test extracts, including ethanol, *n*-hexane, ethyl acetate, *n*-butanol, and aqueous, were dissolved in phosphate-buffered saline (PBS) and evaluated at concentrations ranging from 78.13 μg/mL, 156.25 μg/mL, 312.50 μg/mL, 625 μg/mL, 625 μg/mL, 1250 μg/mL, 2500 μg/mL, and 5000 μg/mL, via serial dilution, to final concentrations of 7.81 μg/mL, 15.62 μg/mL, 31.25 μg/mL, 62.5 μg/mL, 125 μg/mL, 250 μg/mL, and 500 μg/mL, respectively. The results showed a notable decrease in α-amylase activity, particularly in the *n*-hexane extract, indicating effective enzyme inhibition. Among the tested extracts, the *n*-hexane extract exhibited the highest inhibitory activity against α-amylase, achieving 98.68 % inhibition, indicating a strong potential for α-amylase inhibition. This activity was comparable to that of the positive control, acarbose.

For the alpha-amylase inhibitory activity assay, the expected positive result was a dark blue or violet colour, indicating enzyme inhibition. Acarbose was used as the positive control. All test extracts: ethanol, *n*-hexane, ethyl acetate, *n*-butanol, and aqueous, were dissolved in PBS and were evaluated at concentrations from 500 μg/mL to 78.13 μg/mL (Fig 4A). A notable decrease in the α-amylase activity, particularly in the *n*-hexane extract, suggests effective enzyme inhibition. The *n*-hexane extract exhibited the highest inhibition and was subsequently subjected to column chromatography, yielding 14 fractions. Fractions 14 exhibited the highest inhibition in *in vitro*

assays at 15 μg/mL and 30 μg/mL (Supplementary Information, Appendix 1), and were therefore characterised using UHPLC-MS^E.

The α-glucosidase inhibitory potential of *B. hispida* leaf extracts (70 % ethanol extract, *n*-hexane, ethyl acetate, and *n*-butanol) was assessed in a 96-well plate assay with acarbose as a positive control. The final concentrations tested were 10.42 μg/mL, 20.83 μg/mL, 41.67 μg/mL, 83.33 μg/mL, 166.67 μg/mL, 333.33 μg/mL, and 666.67 μg/mL. The positive control, acarbose, showed a dose-dependent inhibition, achieving 88.77 % at the highest concentration and 30.70 % at the lowest concentration. The *B. hispida* extracts showed a weak to minimal inhibitory effect, with the ethanolic extract demonstrating an inhibition of 23.10 % at 666.67 μg/mL. In contrast, the ethyl acetate, *n*-hexane, and *n*-butanol extracts showed very minimal inhibition (Fig 4B).

In the α-glucosidase inhibition assay, acarbose served as the positive control and exhibited a dose-dependent effect. Acarbose effectively inhibited α-glucosidase; however, the crude ethanolic and ethyl acetate extracts exhibited weak inhibitory activity. In addition, the *n*-hexane and *n*-butanol extracts showed no inhibitory activity, with inhibition values ranging from negative to approximately 1-5 % (Fig 4B).

α-Amylase and α-glucosidase inhibitory activities of the normal column chromatography fractions

The *n*-hexane extract exhibited the highest inhibitory activity against α-amylase (n=5), and was therefore selected for further purification. The α-amylase EC_{50} of the *n*-hexane extract was computed in GraphPad Prism 10 [43] to be 30.48 μg/mL, with a *p*-value of 0.05. The *n*-hexane extract was subjected to normal column chromatography to separate the compounds present. The collected column fractions were analysed by TLC to identify similar profiles. Fractions with similar TLC profiles were pooled, and 14 column chromatography fractions were collected and subjected to *in vitro* assays. Fraction 14 (Fig 4A), with the highest inhibition in the α-amylase assay, was subjected to α-amylase inhibitory activity assay at 15 μg/mL and 30 μg/mL (final well concentration), and to α-glucosidase inhibitory activity assay at 20 μg/mL and 40 μg/mL (Supplementary Information, Appendix 1). Fraction 14 inhibited α-amylase at both high and low doses, with 4.46 ± 1.48 % at 15 μg/mL and 9.18 ± 1.68 % at 30 μg/mL. The α-amylase inhibitory activity was comparable to the positive standard, acarbose, with a mean % inhibitory activity of 1.57 ± 2.93 % at 15 μg/mL, and 25.87± 5.86 % at 30 μg/mL. The aforementioned fraction exhibited a negative activity against α-glucosidase.

UHPLC-MS^E

UHPLC-MS^E analysis of the *n*-hexane extract of column-chromatography-active fraction 14 was dereplicated online based on fragmentation patterns at *m/z* values. Dereplication and literature search indicated the presence of a keto-enol mixture of cucurbitacins A and C (Figs 5 and 6). Cucurbitacins A and C were found in an *n*-hexane fraction 14 isolate (Fig 3A) of *B. hispida* leaves. Rice et al. [44] identified cucurbitacins A and C with R_f values of 0.347 and 0.394 on silica gel plates, using a fluorescent indicator developed in ethyl acetate: benzene (75:25). The spot observed in TLC (Fig 3B) was broad in the 8:2 *n*-hexane: ethyl acetate solvent system. At first, we hypothesised the presence of cucurbitacin C in the study. However, careful discrimination between cucurbitacins A and C, the presence of *m/z* 484 for cucurbitacin A in the observed chromatogram, and the absence of *m/z* 482

for cucurbitacin C confirmed that the isolated compound was cucurbitacin A. The peak difference between cucurbitacin A with *m/z* 484 and cucurbitacin C with *m/z* 482 was first observed by Rice et al. [44]. We did not observe *m/z* 482 in the observed chromatogram (Fig 6). However, we cannot totally eradicate the idea that there is a presence of cucurbitacin C due to the isomeric identity of both analytes (Fig 5). As observed in the TLC (Fig 2B), the R_f values of the cucurbitacin A spot coincided with those reported by Rice et al. [44], with an R_f of 0.347, while cucurbitacin C had an R_f of 0.394. Evidence of fragmentation was reported by Chen et al. [45] and Bhandari et al. [46]. A plausible fragmentation of cucurbitacin A, with the more stable compound as the product, was hypothetically proposed in Supplementary Information, Appendix 2.

EC_{50} of cucurbitacins A and C against α-amylase

The EC_{50} of the keto-enol cucurbitacins A and C isolate against α-amylase ranged from 226 μM to 299 μM (Table 1).

Summary and conclusion

The study indicates that *the B. hispida* leaves *n*-hexane fraction 14, containing cucurbitacins A and C, is a specific α-amylase inhibitor rather than an α-glucosidase inhibitor, inhibiting α-amylase and reducing carbohydrate absorption. In our study, cucurbitacins A and C are specific to α-amylase and negative to α-glucosidase. We identified the target enzyme and the compounds responsible for this activity in *n*-hexane isolate from *B. hispida* leaves. A keto-enol form of cucurbitacins A and C acting as specific α-amylase inhibitors could help researchers further study the enzyme as a target for future research.

Cucurbitacins are tetracyclic triterpenoid compounds integrated in plants from the Cucurbitaceae family with insulin-like activity by enhancing the utilisation of glucose and improving insulin sensitivity [47]. Cucurbitacin A exhibits anticancer activity by inhibiting ovarian cancer cell growth through suppression of the PI3K/Akt/mTOR signalling pathway. Cucurbitacin B from *Cucumis callosus* (Cucurbitaceae) has also been reported to exhibit antidiabetic and anticancer activities, and cucurbitacin C from *Cucumis sativus* is recognised as the bitter principle in cucumber plants [48]. Cucurbitacin D has reduced cancer cell proliferation and inhibited key signalling pathways–including STAT3 and Akt [49]. It also inhibits the proliferation of HepG2 cells and induces apoptosis by modulating the JAK/STAT3, PI3K/Akt/mTOR, and MAPK signalling pathways [50]. Additionally, cucurbitacin E has a diabetic effect by increasing glucose uptake in yeast cells [51]. Cucurbitacin I has exhibited analgesic, apoptotic, antimicrobial, and antipyretic properties [52]. The reported antidiabetic potential [49, 52, 53], along with other activities such as anti-tumour [54], antioxidant, anti-inflammatory, and anti-atherosclerotic [55], firmly establishes cucurbitacins as promising therapeutics for the management of metabolic and chronic diseases (Table 2).

This study presents the *Benincasa hispida* leaves, in which α-amylase and α-glucosidase inhibitory activities were concentrated in the *n*-hexane extract, and probable synergism was observed among *n*-hexane column chromatography fractions as the purification process succeeded. The UHPLC-MS^E characterised cucurbitacins A and C as the compounds associated with the observed α-amylase inhibitory activity, exhibiting

inhibitory potency comparable to acarbose. The results suggested that cucurbitacins A and C contribute to the selective α-amylase inhibition in *B. hispida*, which may be attributed to their *in vitro* hypoglycemic potential. At the same time, they exhibited no activity against α-glucosidase. Further research is recommended to quantify the molecular ratio of cucurbitacin A to C responsible for this activity.

The study, however, is limited to *in vitro* assays of α-amylase and α-glucosidase. The specificity of cucurbitacins A and C is limited only to the two enzymes tested. The study period could be too short to establish the impact of cucurbitacins A and C on diabetes mellitus and postprandial hyperglycemia. Thus, further studies on the aforementioned cucurbitacins are needed to establish the postprandial hyperglycemic effect. An *in vivo* study should also be performed as a further research to strengthen the α-amylase *in vitro* result. Although Arbotante and Arriola [37] tested the ethyl extract of *B. hispida* leaves *in vivo*, they did not disclose the compound present, as it requires further purification. We have isolated a TLC spot containing cucurbitans A and C. However, it was difficult to infer the mechanism of action of cucurbitacins A and C from this study, as the α-amylase has different routes. Clinical trials are needed to establish the safety and efficacy of human subjects. In conclusion, the *B. hispida* leaves *n*-hexane isolate contains cucurbitacins A and C, and has a specific α-amylase inhibitory activity as demonstrated by the α-amylase and α-glucosidase *in vitro* assays. Additionally, *in vivo* and clinical trials for cucurbitacins A and C should be considered as a future research to analyse long-term effects and mechanisms.

Experimental

General experiment procedure

The *B. hispida* ethanol leaf extract was subjected to solvent extraction and partitioning with *n*-hexane, ethyl acetate, and *n*-butanol, followed by bioassay-guided isolation using normal column chromatography, and then *in vitro* assays of α-amylase and α-glucosidase inhibition. The fractions exhibiting the highest hypoglycemic activity were analysed by UHPLC-MS^E at the Regional Research Center, University of the Philippines.

Plant materials

Mature leaves of *B. hispida* (Fig 1) were collected from San Juan Bautista, Betis, Guagua, Pampanga, Philippines (14°58’33.2"N 120°38'13.7"E, 14.975888, 120.637145). Fresh leaves were removed from the stem, wiped to remove dirt and foreign particles, and air-dried for 2 weeks. The plant sample was documented using the press method, submitted, and authenticated at the Institute of Biology, Jose Vera Santos Memorial Herbarium, College of Science, University of the Philippines Diliman. A voucher specimen for the plant was subsequently prepared and deposited at Angeles University Foundation, Angeles City, Philippines. A voucher specimen with accession number 7416 of *B. hispida* could be accessed at the Herbarium of King George V School, Hong Kong [56].

Extraction and isolation

The dried leaves, weighing 500 g, were pulverised, exhaustively macerated with 3 L of 70 % ethanol (3×) for 24 h, filtered, and concentrated *in vacuo* at 40 °C. Subsequently, solvent partitioning of the ethanolic extract was performed with *n*-hexane, ethyl acetate and *n*-butanol.

***In vitro* enzyme inhibitory assays**

α-Amylase

The α-amylase inhibition assay was adapted from Mogole et al. [57], Li et al. [58], and Bashary & Khatik [59], with modifications. The assay was performed using a 96-well U-bottom microplate (Greiner Bio-One®, Kremsmünster, Austria). Fifteen microliters (15 μL) of plant extract and 30 μL of α-amylase were added. The mixture was then incubated for 20 min at 37 °C. After incubation, 30 μL of a 1 % starch solution was added, and the mixture was incubated for an additional 20 min at 37 °C. The reaction was quenched by adding 60 μL of 0.1 M HCl, followed by approximately 15 μL of 1 % iodine solution. The reaction mixture was measured at 580 nm using a Rayto® 6100 microplate reader (Shenzhen, China). The percentage of inhibition was calculated using the following formula, *1*:

$$\% \text{ Inhibition} = 100 \times \left[1 - \frac{(AbsS - AbsC\,(-))}{AbsC\,(+) - AbsC\,(-)}\right] \quad (1)$$

Where, $AbsC(-)$ is the absorbance of the blank negative control

$AbsC\,(+)$ is the absorbance of the blank positive control

$AbsS$ is the absorbance of the plant extract

α-Glucosidase

A α-glucosidase inhibition assay was adapted from Rayar and Manivannan [60] with modifications. The reaction mixture, consisting of 20 μL of plant extracts at varying concentrations, 50 μL of phosphate buffer, and 10 μL of α-glucosidase, was placed in individual wells of a 96-well U-bottom plate (Greiner Bio-One®, Kremsmünster, Austria). The microplate was incubated at 37 °C for 15 min in the microplate reader. The sample plate was then removed after incubation, and 20 μL of 5 mM *p*-nitrophenyl glucopyranoside, a substrate, was added. The mixture was incubated at 37 °C for 20 min. The reaction was terminated by adding 50 μL of 0.1 M sodium carbonate. Phosphate-buffered saline (PBS) was used as a negative control, and acarbose was used as a positive control. The spectral changes were monitored by analysing the *p*-nitrophenol released from the substrate at 405 nm using a microplate reader. The assay was performed with n=5. The percentage of inhibition was calculated using the following formula, *2* and *3*:

$$\text{Corrected Abs} = [(\text{FinalAs} - \text{InitialAs} - (\text{FinalAsB-InitialAsB})] \quad (2)$$

$$\% \text{ Inhibition} = ([Abs(Control) - Abs(sample)]/AbsControl) \times 100 \quad (3)$$

Where: As = Absorbance of the sample; AsB = Absorbance of the sample background; Abs = Absorbance; AbsControl = Absorbance of control

Statistical analysis

The data obtained were expressed as Mean±SD, and the EC_{50} were statistically analysed using GraphPad Prism 10.6.1 [48] with n=5. The Log EC_{50} was estimated by least-squares nonlinear regression, with 95 % confidence intervals derived from profile likelihood.

Characterisation

Thin-layer and normal column chromatographies

For thin-layer chromatography (TLC), the test solution was spotted onto the silica gel plate using a micropipette. The applied spot was air-dried and developed into a glass chamber with a developing solvent. The developed chromatogram was observed at 254 nm and 366 nm. The retention factor (R_f) value of each spot was calculated using the formula, *4*:

$$Rf = \text{Distance travelled by the solute } (cm) / \text{Distance travelled by the solvent } (cm) \qquad (4)$$

For normal column chromatography, *n*-hexane extract was added to the silica gel column. The solvent system used in this procedure was *n*-hexane: ethyl acetate, with a ratio determined from TLC. The fractions were collected in a test tube until all bands eluted from the column. Similar fractions in TLC were pooled.

UHPLC-MS^E

n-Hexane extract and column chromatography active *n*-hexane fraction 14 were subjected to UHPLC-MS^E. Forty microliter (40 μL) of a 100 μg/mL extract was transferred to a small vial. The extracts were evaporated *in vacuo* at 40 °C, and then 40 μL of 10 % MeCN was added. The LC-MS and LC-MS/MS analyses were conducted at the Regional Research Centre, University of the Philippines in Visayas, using the UHPLC-MS^E technique, as described by Anas et al. [61] and Lacson et al. [62].

Chromatography

Chromatographic separations were performed on the C18 column (Waters Corp., Milford, USA) using Waters ACQUITY® I-Class System, eluted with a linear gradient from 10 % MeCN with 0.1 % formic acid to 100 % MeCN with 0.1 % formic acid over 10 min, and 10 % MeCN with 0.1 % formic acid at 10.01 min to 12 min, at a flow rate of 0.2 mL/min. The samples were diluted in 10 % MeCN, and a 2-μL aliquot of each sample was injected onto the column [61, 62].

Mass spectrometry

Elevated-mass spectrometry (MS^E) was performed on a Waters Xevo G2-XS QTof® (Waters MS Technologies, Manchester, UK) operated in positive-ion mode, following the method of Lacson et al. [62]. The spectrometer was calibrated according to the manufacturer's specifications. The leucine enkephalin preparation is a 50:50 mixture of ACN and water containing 0.1% formic acid. The sample was analysed with a source temperature of the mass spectrometer source temperature set to 100 °C, the desolvation temperature to 200 °C, the cone gas flow to 10 L/h, and the desolvation gas flow to 800 L/h. The parameters were set as follows: capillary voltage of 1 kV, cone voltage of 40 V, collision energy (CE) of 6 *eV* for MS, and 15-60 *eV* (ramp) for MS^E. Additionally, the acquisition range was set from *m/z* 100 to *m/z* 1500 in MS^E sensitivity mode. The chromatograms

were then acquired from Waters UNIFI Scientific Information System (Waters MS Technologies, Manchester, UK).

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Credit authorship statement

Conceptualization, A.P.G., A.R.J.A., and B.G.P.; methodology, A.P.G., A.R.J.A., and B.G.P.; software, A.P.G., and A.R.J.A.; validation, formal analysis, and investigation, A.P.G., and A.R.J.A.; data curation, A.P.G., and A.R.J.A.; writing—original draft preparation A.P.G., and A.R.J.A.; writing—review and editing, A.P.G., A.R.J.A., and B.G.P.; visualization, A.P.G., and A.R.J.A.; supervision, A.R.J.A., and B.G.P.; project administration, A.P.G.; funding acquisition, A.P.G. All authors have read and approved to the published version of the manuscript.

Declaration of competing interest

The authors declare no known conflicts of interest in this study.

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Figures



Fig. 1 *Benincasa hispida*.

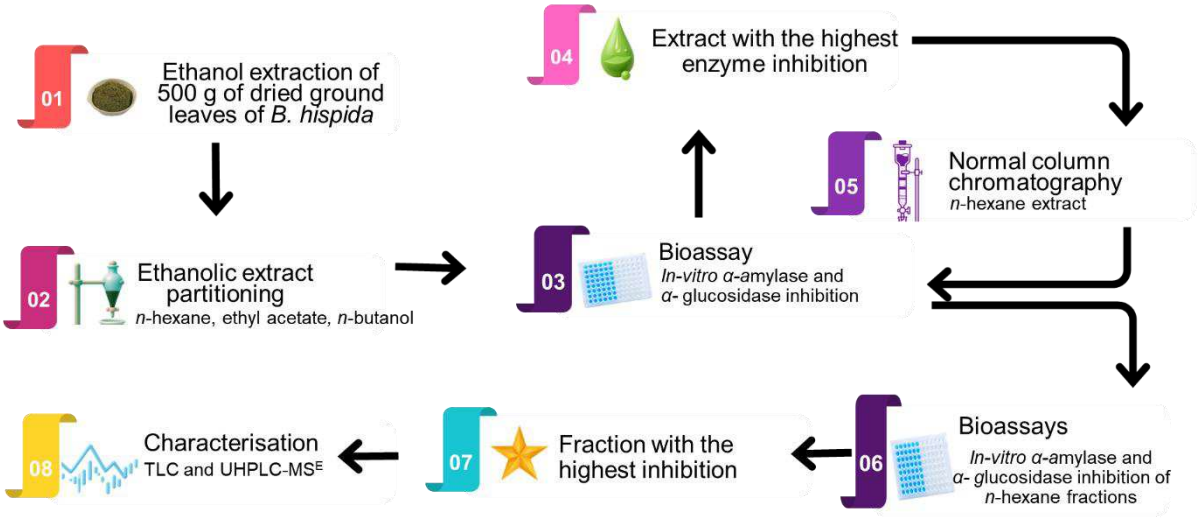


Fig. 2 Experimental design.

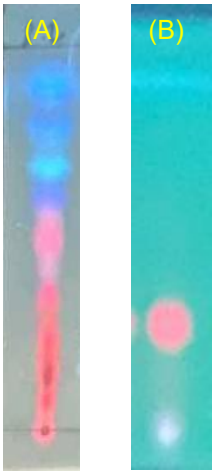
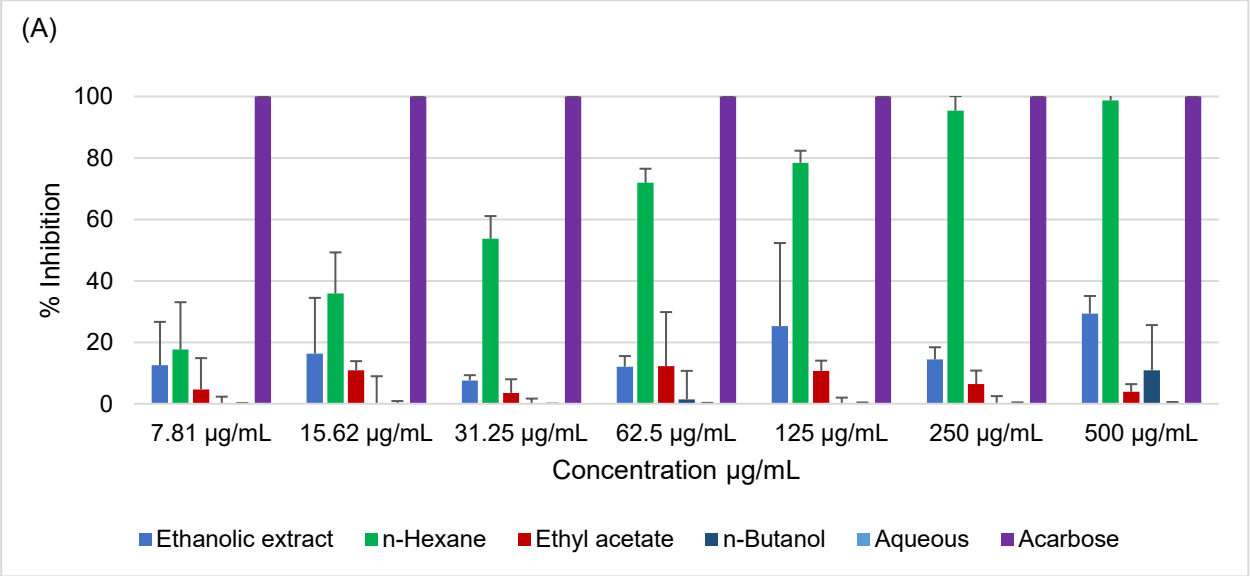


Fig. 3 TLC profiles of *Benincasa hispida* in 8:2 hexane: ethyl acetate. **(A)** *n*- Hexane extract, and **(B)** column chromatography *n*-hexane fraction14.



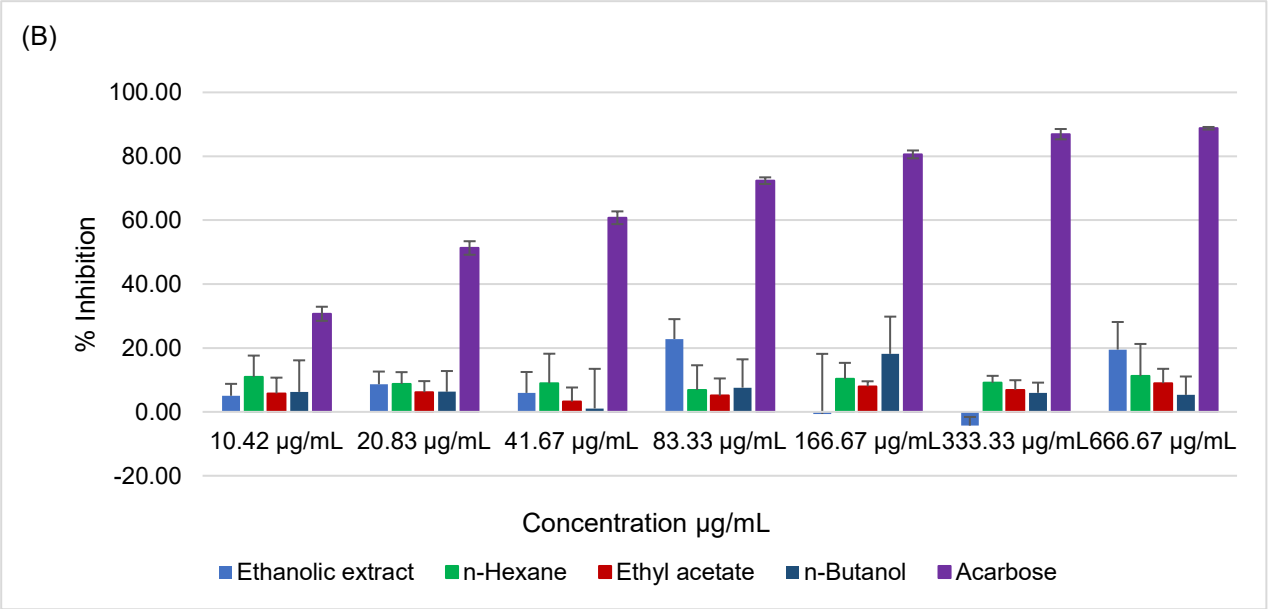


Fig. 4 Enzyme inhibition assay screening of *B. hispida* extracts. **(A)** α-Amylase and **(B)** α-Glucosidase, *n*=5.

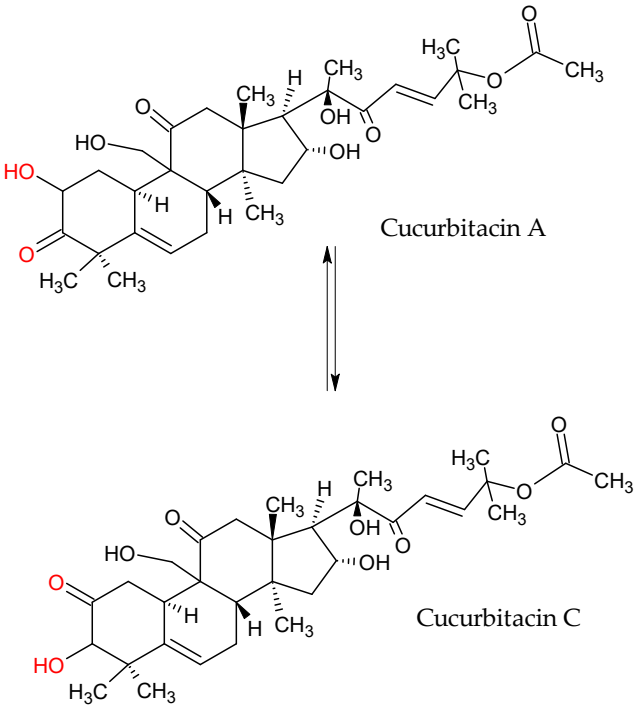
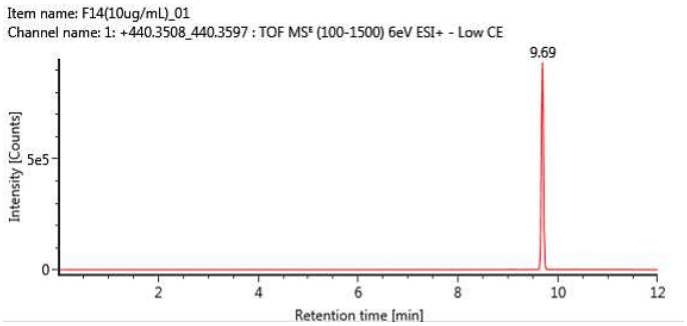
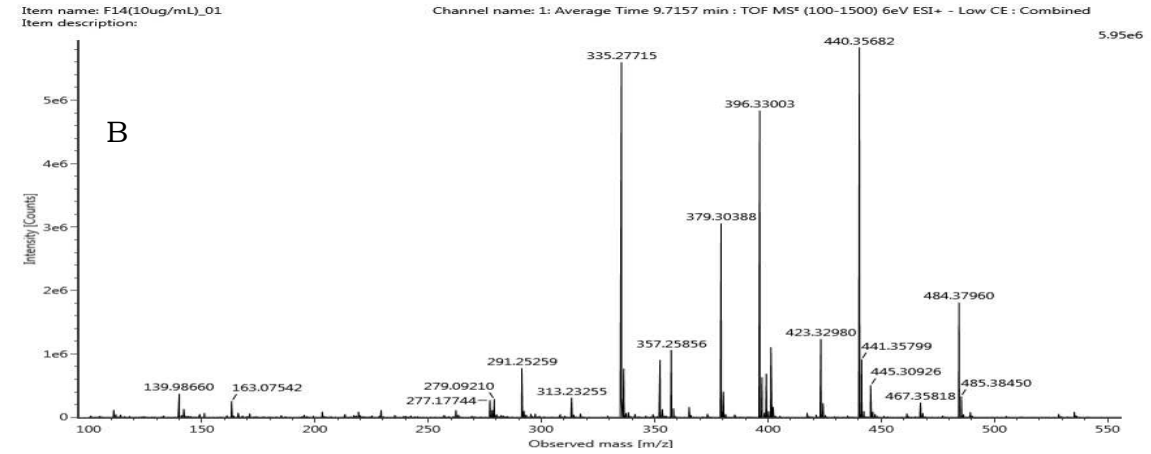


Fig. 5 Cucurbitacins detected from *B. hispida* leaves by UHPLC-MS^{E}.

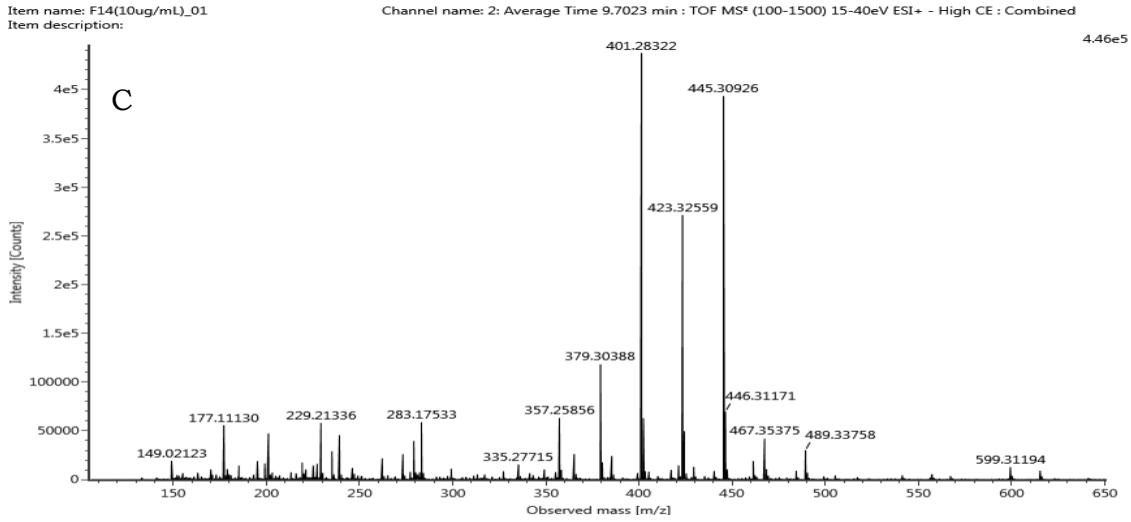
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679 **Fig. 6** UHPLC-MS^E of *n*-hexane fraction 14. **(A)** Low CE, EIC, **(B)** Low CE, t_R 9.7 and **(C)** high CE, t_R
680 9.7.

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Tables

Table 1 EC_{50} of cucurbitacins A and C mixture as α-amylase inhibitors.

Cucurbitacins	EC_{50} (μM)
A and C mixture	226.36 – 298.79

Note: At 95 % confidence level, p=0.05.

Table 2 Cucurbitacins and their pharmacological uses.

Cucurbitacin	*Source plant*	*Pharmacological use*	*Reference*
A	*Cucumis sativus,*	Anticancer	[48]
	Momordica charantia,		
	Momordica balsamina		
	Benincasa hispida	α-Amylase inhibitor	This study
B	*Cucumis callosus*	Antidiabetic, anticancer	[49]
C	*Cucumis sativus*	Anticancer	[48]
	Benincasa hispida	α-Amylase inhibitor	This study
D	*Ecballium elaterium*	Anticancer	[50]
E	*Momordica charantia*	Anti-oxidant	[32]
I	*Momordica charantia, M. balsamina*	Analgesic, anti-apoptosis, antidiabetic, anti-inflammatory, antimicrobial, antipyretic	[52]

α-Amylase *in vitro* inhibitors from the *n*-hexane extract of *Benincasa hispida* leaves of the Cucurbitaceae family

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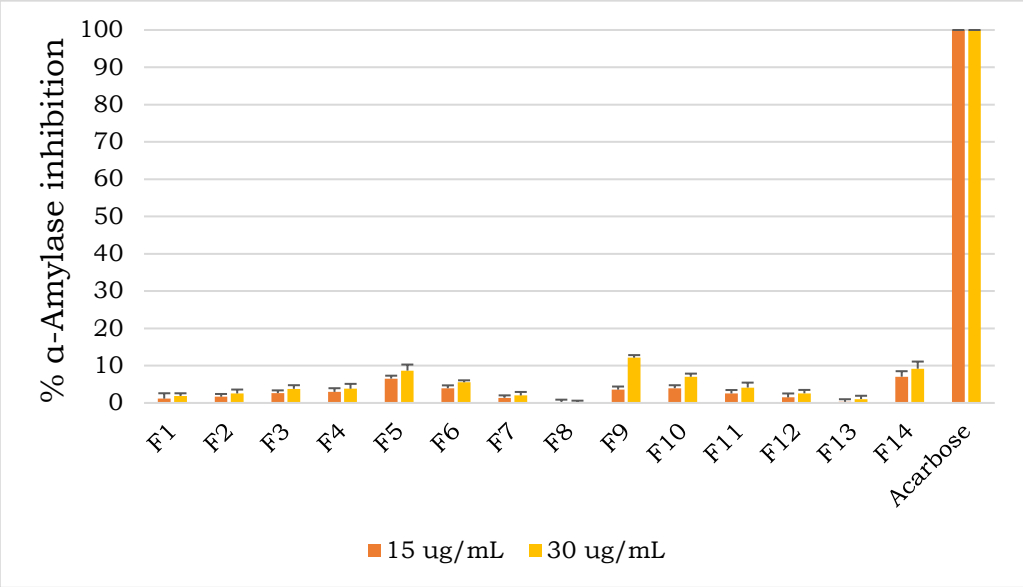
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Supplementary Information

Appendix 1 α-Amylase inhibition assay of *n*-hexane fractions and acarbose.



Appendix 2 Dereplication and proposed MS fragmentations of cucurbitacin A.

Cucurbitacin A from *n*-hexane fraction 14 UHPLC-MS^{E} chromatogram. The presence of extra hydrogen at the C3-position justifies the presence of cucurbitacin C, in which cucurbitacins A and C co-exist in keto-enol form, with cucurbitacin A as the dominant structure due to the presence of *m/z* 484 rather than *m/z* 482. A loss of acetate, dehydration, dehydroxylation, and hydrogenation exhibited an observed *m/z* 484 with molecular formula (MF) $C_{30}H_{44}O_5^+$. Successive dehydroxylations and hydrogenations of cucurbitacin A yield m/z 467 (MF $C_{30}H_{43}O_4^+$), *m/z* 440 (assigned to $C_{29}H_{43}O_3^+$), and *m/z* 423. An unassigned peak at *m/z* 445 might be due to a formic acid adduct and the loss of carbon dioxide ($CO_2\uparrow$), indicating a mass difference (Δm) of 22. The *m/z* 401 was due to the loss of water, acetic acid, and -CO(CH)$_2$C(CH$_3$)$_2$- moiety with MF $C_{24}H_{33}O_5^+$. Loss of hydrogen atoms showed an m/z of 396, with an MF of $C_{24}H_{28}O_5^+$. The fragmentations coincide with those reported by Chen et al. [45], and the fragmentation data support the presence of cucurbitacin C in the structure. The *m/z* 396 was adopted from Bhandari et al. [46] and corresponds to the tetracyclic triterpenoid backbone of cucurbitacins A and C.

