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Evaluation of antioxidant and hypoglycemic activities and metabolite profiles of the fractionated extract of the *Crescentia cujete* Linn. fruit

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ABSTRACT

Diabetes mellitus (DM) is a prevalent disease that is the sixth leading cause of death in the Philippines. The fruit juice of *Crescentia cujete* Linn, or "miracle fruit", is utilized by locals from Agusan del Sur, Zamboanga del Sur, and Zamboanga Sibugay in the Philippines as an anti-diabetic and antioxidant treatment alternative or additive due to the high costs of their current prescriptions. Phytochemical screening of the fruit extract indicated the presence of alkaloids and reducing sugars with trace amounts of saponins, volatile oil, tannins, and polyphenols. Hypoglycemic and antioxidant assays were utilized to determine the potential activity. Alpha-glucosidase inhibition at 10 ppm of the fruit exhibited low enzyme inhibition activity. Methanol/H_2O solvent fraction showed notable antioxidant activity in the 2,2-Diphenyl-1-PicrylHydrazyl (DPPH) assay, averaging 55.69% inhibition. For more comprehensive bioactivity comparison of *C. cujete*, the half-maximal inhibitory concentration (IC_{50}) of α-glucosidase, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) should be identified and various concentration screening protocols for the assays should be conducted to help identify the effective enzyme inhibitory effects of the sample. Mass Spectral Library included 1-Stearoyl-2-hydroxy-sn-glycero-3-phosphocholine, 15(S)-15-Methyl Prostaglandin E1, and 5,6-Dihydroxy-8Z,11Z,14Z-eicosatrienoic acid.

Keywords: 2,2-diphenyl-1-picrylhydrazyl, α-glucosidase, GNPS, miracle fruit, phytochemical screening

INTRODUCTION

Crescentia cujete Linn is locally known as "calabas" or "kalabash" (Lim 2012), earning the name "miracle fruit" from its reputation of being a 'miracle cure' (Rellin et al. 2018). Traditional herbal medicine remains popular in the country, and native traditional healers from Agusan del Sur (Arquion et al. 2015), Zamboanga del Sur (Morilla and Demayo 2019), and Zamboanga Sibugay (De Guzman et al. 2020), Philippines are utilizing the fruit, leaf, and bark decoction of *C. cujete* to treat various ailments like diabetes due to high costs of the current medications available in the market (Glaudson et al. 2016; Gonzales et al. 2022). The effect was attributed to the phytochemicals present in the fruit, such as cyanhydric

acid, iridoids, pectins, and citric acid (Tupas et al. 2018). *Crescentia cujete* possesses significant free radical scavenging properties compared with stem bark and a clear correlation exists between the antioxidant activity and phenolic content (Das et al. 2014). Local studies presented by the Department of Science and Technology - Philippine Council for Health Research and Development (DOST-PCHRD) helped establish the status and demand of the sought-after miracle fruit for its anti-cancer, anti-tumor, and anti-diabetic properties. The widespread popularity of use and demand as an alternative herbal cure and cultivation has increased throughout the Philippines in recent years, and people are taking the opportunity to propagate the tree throughout the country (Arquion et al. 2015; Morilla and Demayo 2019; De Guzman et al. 2020; Glaudson et al. 2016; Gonzales et al. 2022). Despite its popularity as a traditional medicine, there is still a limited amount of research done pertaining to its medicinal use as an anti-diabetic and antioxidant (Glaudson et al. 2016; Gonzales et al. 2022). The study aims to fill the gap of knowledge and recognize the hypoglycemic and antioxidant potentials of the calabash fruit by conducting constituent description and metabolite profiling. The study used molecular networking to putatively identify active compounds present in the fractionated extracts of *C. cujete*.

METHODS

Sample Preparation and Extraction

Fruits of *C. cujete* Linn. were identified and authenticated by the University of Santo Tomas - Research Center for the Natural Sciences & Applied Sciences (RCNAS) Herbarium. The whole fruit was scraped, the seeds removed and the juice was filtered out. The scraped flesh fruit was dried in the hot air oven at 50°C temperature then it was ground into a coarsely powdered fruit sample. The powdered sample was soaked in 70% methanol for 4 hours, transferred to the percolator and the remaining menstruum was added and was macerated for 24 hours. The eluted extract was collected and kept in a 2-8°C cold storage for further fractionation. The percolation process (Mukherjee 2019) was repeated thrice to collect enough crude extract of approximately 3,950 ml.

Phytochemical Screening

Qualitative phytochemical screening of the sample was performed to identify the chemical constituents present in the extract: alkaloids, saponins, steroids, tannins, flavonoids, reducing sugars, and volatile oils. Each test was done in triplicates (Billacura and Laciapag 2017).

Test for alkaloids (Dragendroff's Test). Two milliliters of 2M HCl were added to the 1 ml crude extract and placed in boiling water for 5 min. A 0.30 g of NaCl was added to the sample after it cooled down and filtered. It was washed with 2 ml of HCl and then a sufficient amount of HCl was added to make it 5 ml, then 2-3 drops of Dragendorff's reagent were added to the sample. The presence of alkaloids can be identified if there is formation of precipitate or turbidity.

Test for saponins (Foam Test). One milliliter of crude extract was vigorously shaken with 5 ml distilled water. The persistent appearance of honeycomb froth above the surface indicates the presence of saponins.

Test for steroids (Liebermann Burchard's Test). Two milliliters of chloroform were added to the 1 ml crude extract. A few drops of concentrated sulfuric acid were added to form layers. The appearance of reddish-brown color indicates the presence of steroids.

Test for tannins (Ferric Chloride Test). Fifteen milliliters of hot distilled water were added to 1 ml crude extract. It was cooled down and decanted into another test tube. Three drops of 10% NaCl solution were added and then filtered. It was divided into three separate test tubes with their corresponding labels: (1) test tube A, (2) test tube B, and (3) test tube C. Test tube A was the negative control. Three drops of gelatin salt reagent were added to test tube B. Three drops of 1% $FeCl_3$ were added to test tube C. The appearance of a dark-blue color indicates the presence of hydrolyzable tannins, while the brownish-green color indicates the presence of condensed tannins.

Test for flavonoids (Alkaline reagent Test). Half milliliter of crude extract was treated with 1 ml 0.1 N NaOH solution and observed for yellow color that indicates the presence of flavonoids.

Test for reducing sugars (Fehling's Test). One milliliter of crude extract was dissolved in 3 ml of distilled water. A few drops of Fehling's A and B were added to the solution, and then mixed. It was then placed in a boiling water bath for 1-5 min and cooled down. The appearance of a red-brown precipitate indicates the presence of a reducing compound.

Test for volatile oils. One milliliter of crude extract was dissolved in 90% ethanol. A few drops of 1% $FeCl_3$ were added. The appearance of green color indicates the presence of volatile oils.

Fractionation

The crude extract was partitioned using a modified Kupchan liquid-liquid partitioning method (Abdel-Mageed et al. 2020) with five different solvents: water, sec-butanol, methanol:water, dichloromethane (DCM), and hexane (Figure 1).

α-Glucosidase Inhibition Assay

An optimized α-glucosidase assay was performed using the fractionated extracts (Naing et al. 2019). Working stock solutions of the substrate and α-glucosidase enzyme were prepared for plating.

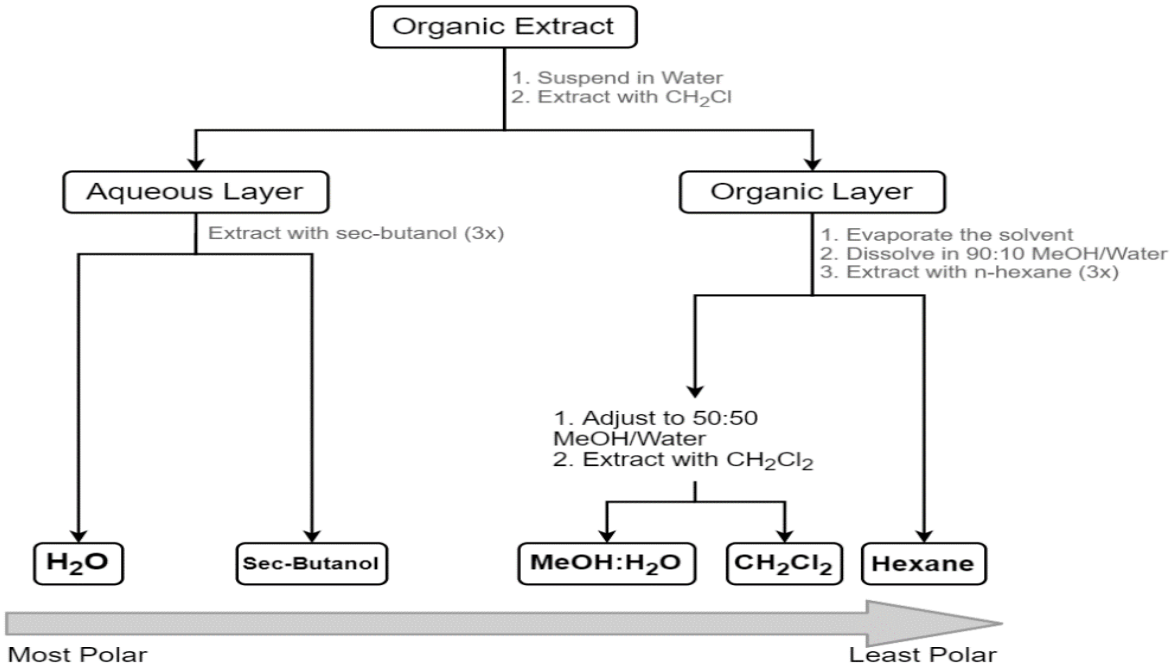

Figure 1. Schematic diagram of the fractionation method.

Samples were dissolved using 100% AR-grade dimethyl sulfoxide (DMSO) to produce a 10,000 ppm mother stock solution, from which a 300 ppm working stock solution was prepared using the same solvent. Sample solutions were homogenized using a vortex mixer, sonicated, and centrifuged. A 10 µl aliquot from the sample working stock solution was dispensed into designated wells to achieve an effective screening concentration of 10 ppm. Phosphate-buffered saline (PBS) (190 µl) and the enzyme working stock solution (50 µl) were subsequently added to each sample well. Meanwhile, 200 µl of a 1,500-ppm acarbose solution was placed in the wells for the positive control and 200 µl of 5% DMSO in PBS for the negative control wells. Both control wells were also dispensed with 50 µl enzyme working stock solution.

The effective concentration of acarbose was 1,000 ppm, while the effective concentration of Dimethylsulfoxide (DMSO) in the sample, positive control, and negative control wells was 3.33%. One trial of the screening concentration with three replicates was employed in the assay. After all reagents were plated except the substrate, the 96-well quartz plate was incubated for 10 min at 37°C. Afterwards, 50 µL of the substrate working stock solution was added to each well to initiate the enzymatic reaction. The assay is dependent on the liberation of p-nitrophenol by enzymatic hydrolysis of the substrate. This is correlated with the activity of the sample when compared with the negative control. The absorbance of the liberated p-nitrophenol was measured at 405 nm every 30 seconds for 30 min using a Multiskan Go® UV/Vis Spectrophotometer.

A sample is considered active if the % inhibition is greater than or equal to 50% and if there is a statistically significant mean difference of the slopes between the sample and the negative control at $P < 0.05$. Kolmogorov-Smirnov Test, Levene's test, Brown-Forsythe Test, Welch Test, and One-Way ANOVAwere employed to analyze the data statistically using Stata software version 13. The inhibitory activities of the samples and the positive standard (acarbose) were determined based on the average slope of the replicates using the equation 1:

***In vitro* antioxidant screening.** The *in vitro* free radical scavenging activity of the fractionated extracts was evaluated using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay through a modified method (Tai et al. 2011; Clarke et al. 2013). The 20 µl of the sample diluted appropriately in DMSO was mixed with 180 µl of DPPH in methanol using a 96-well plate. The plate was kept in the dark for 15 min after the absorbance of the solution was measured at 540 nm in a plate reader. Dimethyl sulfoxide served as a blank, and Ascorbic Acid served as the standard. The assay was done in one trial of the screening concentration with three replicates. The sample was tested at a single concentration of 4 mg/mL to determine the antioxidant activity. The data was reported as percent DPPH scavenging effect using the equation 2 (Valko et al. 2007; Shekhar and Anju 2014):

$$\%\ Inhibitory\ Activity = \frac{Slope_{uninhibited} - Slope_{inhibited}}{Slope_{uninhibited}}\ x\ 100 \quad (1)$$

$$DPPH\ scavenging\ effect\ (\%) = \frac{Absorbance\ of\ DPPH - Absorbance\ of\ sample}{Absorbance\ of\ DPPH}\ x\ 100 \quad (2)$$

Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS) Analysis

Untargeted LC-MS/MS metabolite profiling of the fractionated extracts was performed using a Waters Acquity UPLC® H-Class System with a Xevo® G2-XS Quadrupole Time-of-Flight (QToF) High-Resolution Mass Spectrometer.

Untargeted Metabolite Profiling

Molecular networking of compounds was done by the analysis of the tandem MS spectra through the Global Natural Products Social (GNPS) Molecular Networking Platform, utilizing the fragmentation pattern of the compounds in fractions of water, methanol/water, sec-butanol, and dichloromethane extracts. Molecular networking was done by checking fragmentations of compounds using MZmine (ver. 3.2.8) and then the molecular formula of compounds was predicted based on their monoisotopic mass using the ChemSpider, COCONUT, and PubChem databases.

Molecular Networking

From the MS/MS spectra generated from the Data-Dependent Acquisition (DDA) mode, the detected metabolites were putatively identified through GNPS molecular network using the online workflow (https://ccms-ucsd.github.io/GNPSDocumentation/) on the GNPS website (http://gnps.ucsd.edu). Edges were filtered to have a cosine score above 0.7 and more than six matched peaks. The result network was visualized by using Cytoscape (version 3.10.3).

RESULTS

Phytochemical Screening

The crude extract exhibited the presence of secondary metabolites including alkaloids and reducing sugars (Table 1). Traces of flavonoids, saponins, volatile oils, tannins, and polyphenols were also present.

α-Glucosidase Inhibition Assay

All fractions of the extract exhibited 7.44% (hexane), 0.85% (DCM), 1.94% (MeOH/H_2O), 1.83% (sec-butanol), 0.70% (water), and 1.26% (crude) inhibition activity against α-glucosidase (Table 2).

Table 1. Phytochemical constituents in the crude methanolic extract of oven-dried *Crescentia cujete* fruit. Legend: ++ (present) , + (trace), - (absent).

Phytochemical Screening	Result	Indication
Alkaloids	Presence of turbidity	(++)
Flavonoids	No yellow coloration	(-)
Reducing Sugar	Precipitation was observed	(++)
Saponins	Persistent froth was observed	(+)
Steroids	No reddish-brown coloration	(-)
Tannins and Polyphenols	Brownish-green coloration detected	(+)
Volatile oil	Green coloration detected	(+)

Table 2. Percentage Inhibition of α-Glucosidase Activity of Fractionated *Crescentia cujete* fruit extracts.

Sample No	Sample Code	% Inhibition ± SD
1	Hexane	7.44 ± 0.56
2	DCM	0.85 ± 0.48
3	MeOH/H_2O	1.94 ± 0.38
4	Sec-Butanol	1.83 ± 0.37
5	H_2O	0.70 ± 0.51
6	Crude	1.26 ± 1.32
Acarbose		90.79 ± 0.55

2 , 2 - diphenyl -1- picrylhydrazyl (DPPH) Antioxidant Assay

All fractions of the extract exhibited 55.69% (MeOH/H_2O), 20.81% (water), 19.44% (crude), 16.27% (hexane), 26.64% (DCM), and 16.10% (sec-butanol) inhibition activity against DPPH (Figure 2).

Dereplication of Compounds

From the MS/MS spectra generated from the DDA mode, the detected metabolites were putatively identified through GNPS molecular networking and visualized using Cytoscape (Figure 3). Three compounds were returned with spectral similarity in the NIST14 database (Table 3). Cosine scores have values ranging from 0 to 1, with 1 pertaining to absolute similarity (Wang et al. 2016). In both DCM and sec-butanol fractions, 1-Stearoyl-2-hydroxy-sn-

glycero-3-phosphocholine is present with a cosine score of 0.97 (Figure 4). Only in the DCM fraction, 15(S)-15-Methyl Prostaglandin E1 is present but with low similarity to the NIST 14 spectral match with a cosine score of 0.74 (Figure 5). In DCM and MeOH:H_2O fractions, 5,6-Dihydroxy-8Z,11Z,14Z-eicosatrienoic acid is present and with the lowest similarity with only a cosine score of 0.51 (Figure 6).

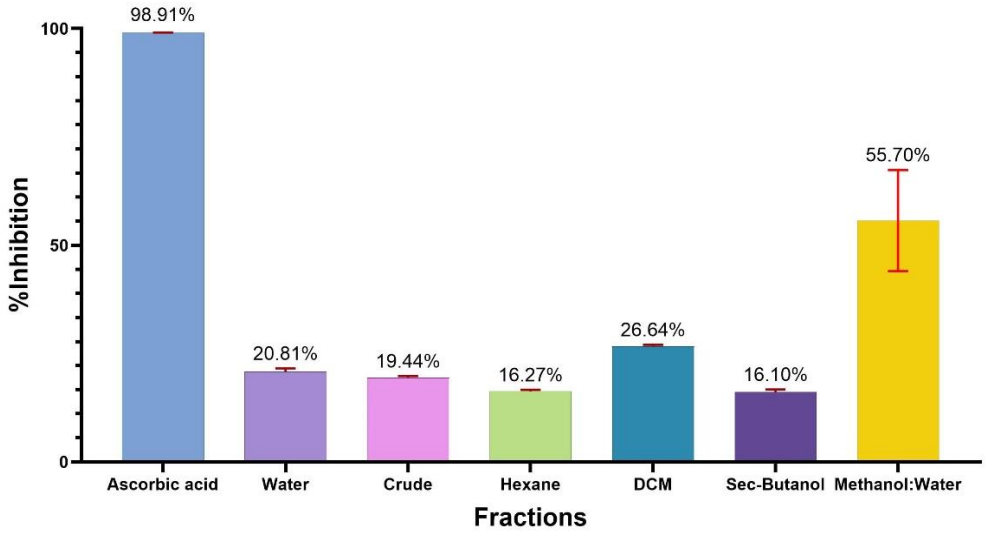

Figure 2. Percentage Inhibition of DPPH (free radical) treated with Fractionated *Crescentia cujete* fruit extracts + DMSO measured after 15 minutes at 540 nm absorbance.

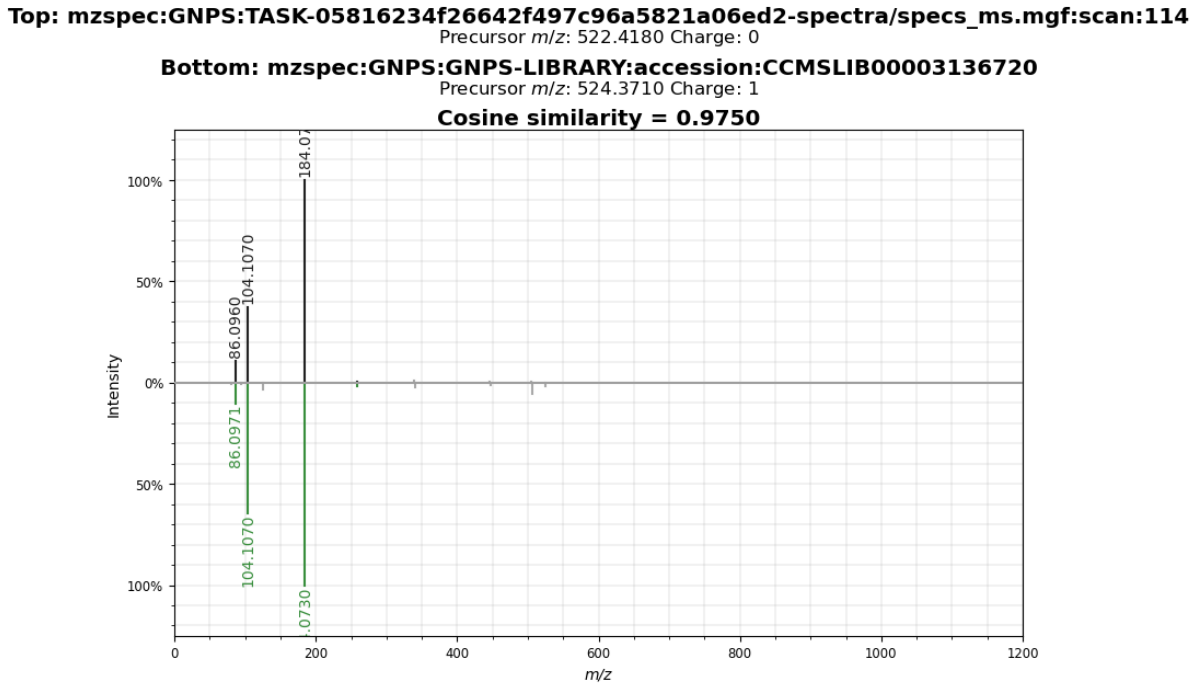

Figure 3. Mirror match comparison of MS/MS profiles of the sample at the top (precursor m/z at 522.4180) and the reference 1-Stearoyl-2-hydroxy-sn-glycero-3-phosphocholine at the bottom (precursor m/z at 524.3710). The cosine score between the sample and the GNPS reference spectrum is 0.97.

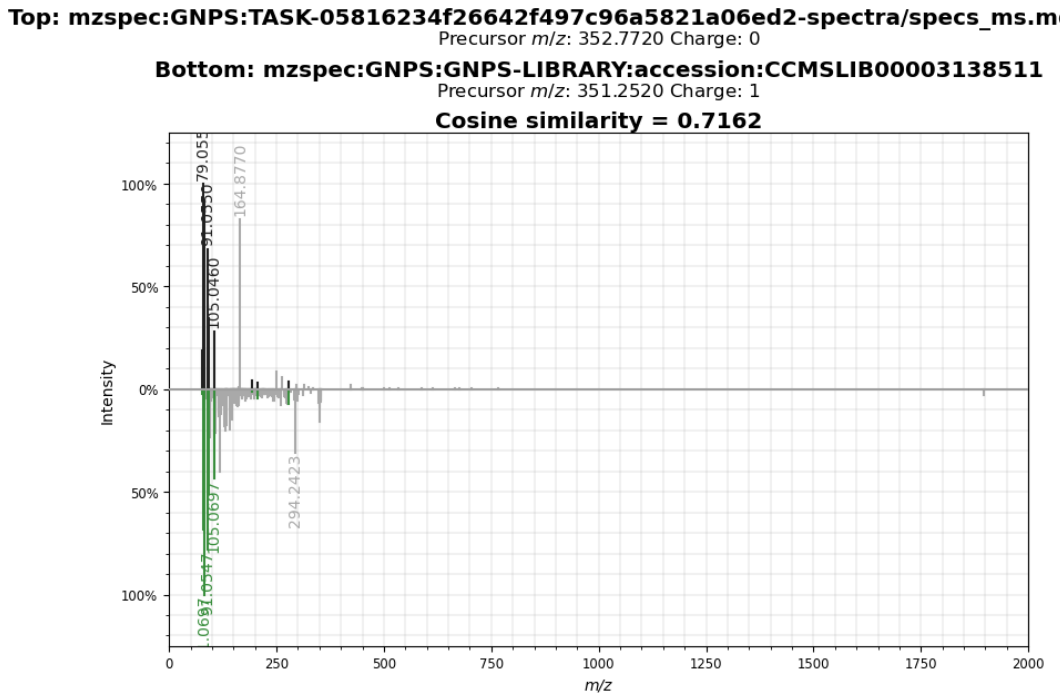

Figure 4. Mirror match comparison of MS/MS profiles of the sample at the top (precursor m/z at 352.7720) and the reference 15(S)-15-Methylprostaglandin E1 at the bottom (precursor m/z at 351.2520). The cosine score between the sample and the GNPS reference spectrum is 0.71.

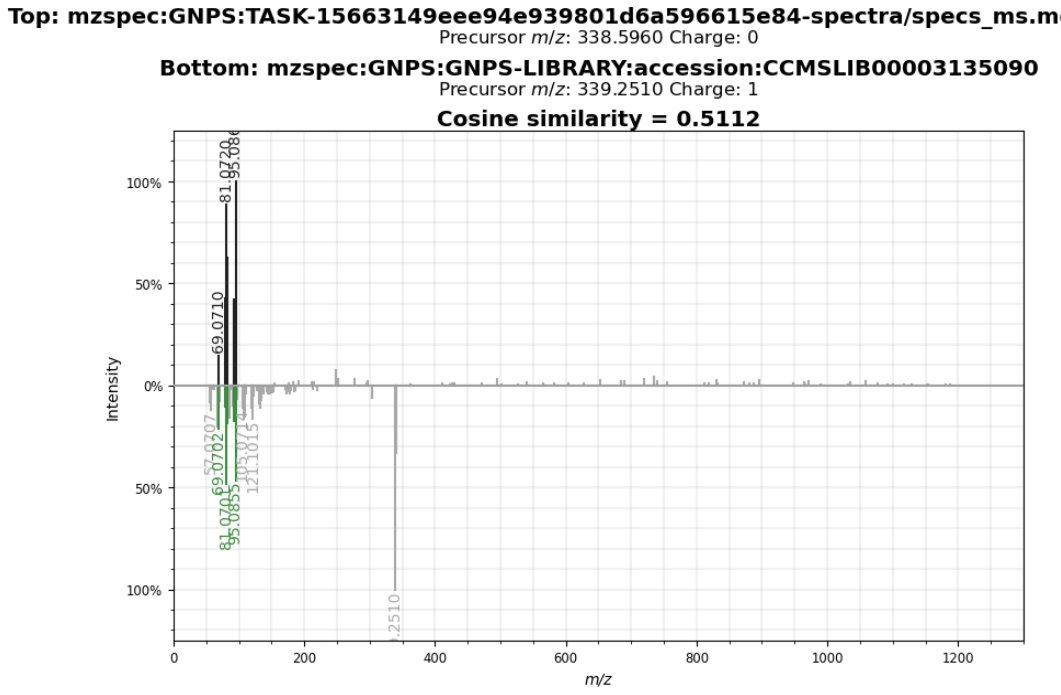

Figure 5. Mirror match comparison of MS/MS profiles of the sample at the top (precursor m/z at 338.5960) and the reference 5,6-Dihydroxy-8Z,11Z,14Z-eicosatrienoic acid at the bottom (precursor m/z at 339.2510). The cosine score between the sample and the GNPS reference spectrum is 0.51.

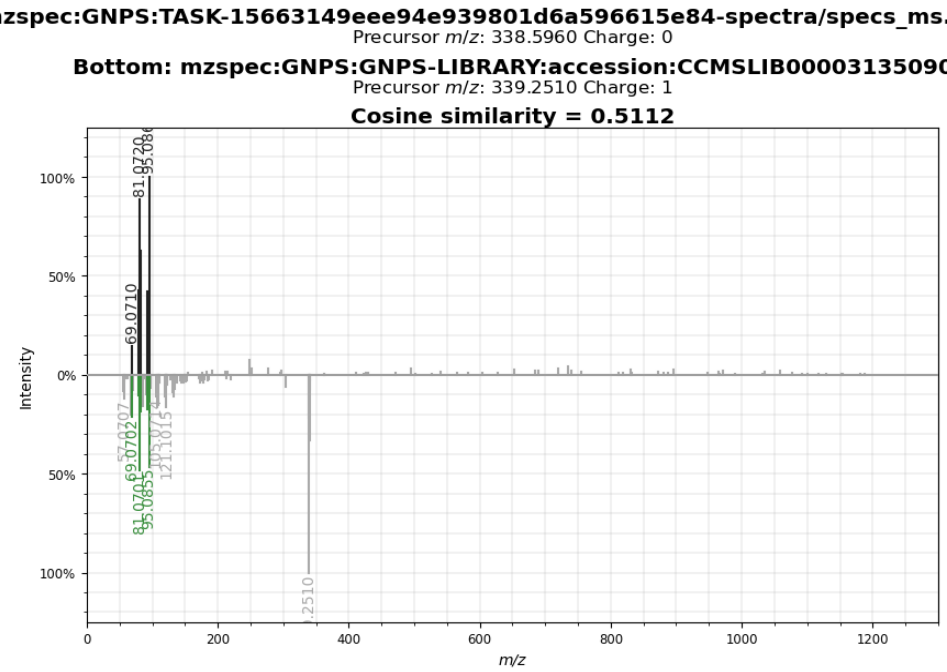

Figure 6. Mirror match comparison of MS/MS profiles of the sample at the top (precursor m/z at 352.7720) and the reference 5,6-Dihydroxy-8Z,11Z,14Z-eicosatrienoic acid at the bottom (precursor m/z at 351.2520). The cosine score between the sample and the GNPS reference spectrum is 0.71

Untargeted Metabolite Profiling

A molecular network was created using the online workflow (https://ccms-ucsd.github.io/GNPSDocumentation/) on the GNPS website (http://gnps.ucsd.edu). The data was filtered by removing all MS/MS fragment ions within +/- 17 Da of the precursor m/z. MS/MS spectra were window filtered by choosing only the top 6 fragment ions in the +/- 50 Da window throughout the spectrum. The precursor ion mass tolerance was set to 2.0 Da and a MS/MS fragment ion tolerance of 0.5 Da. A network was then created where edges were filtered to have a cosine score above 0.7 and more than 6 matched peaks. Further, edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top 10 most similar nodes. Finally, the maximum size of a molecular family was set to 100, and the lowest scoring edges were removed from molecular families until the molecular family size was below this threshold. The spectra in the network were then searched against GNPS' spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.7 and at least 6 matched peaks. The GNPS online workflow result can be found in this URL: https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=195e8cd40a344097a8c021f8c191f40f

Table 3 shows the three compounds that returned with spectral similarity in the NIST14 database. 1-Stearoyl-2-hydroxy-sn-glycero-3-phosphocholine, 15(S)-15-Methyl Prostaglandin, and 5,6-Dihydroxy-8Z,11Z,14Z-eicosatrienoic acid.

1-Stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (18:0 Lyso PC) is a saturated lysophosphatidylcholine, has emerged as a potent anti-diabetic candidate due to its role as a significant dipeptidyl peptidase-4 (DPP-IV) inhibitor. By enhancing pancreatic insulin secretion and suppressing glucagon production, 18:0 Lyso PC improves glycemic control, with additional protective effects against obesity phenotypes in high-fat diet models (Han et al. 2021; Gilbert and Pratley 2020).

15(S)-15-Methyl Prostaglandin E1 (15-methyl PGE1) is a synthetic analog of Prostaglandin E1, modified with a methyl group in the S-configuration at carbon-15. This alteration enhances its metabolic stability and prolongs its half-life by preventing rapid inactivation. It activates prostaglandin receptors, eliciting biological effects such as vasodilation, reduced clot formation, and cytoprotection (Kushwaha et al. 2023). Clinically, it is used for labor induction due to its uterotonic properties (Bakker et al. 2017) and in gastroenterology for its role in mucosal protection against peptic ulcers.

5,6-Dihydroxy-8Z,11Z,14Z-eicosatrienoic acid (5,6-DHET) is a bioactive lipid derived from eicosatrienoic acid (20:3) and belongs to a specialized

class of lipid mediators. Despite its biological significance, research specifically focusing on 5,6-DHET is limited. One study highlights reduced plasma levels of 5,6-DHET in diabetic nephropathy (DN) patients compared to those with T2DM without nephropathy. This finding suggests a protective role for 5,6-DHET in preventing DN, with potential applications as a biomarker for early detection and progression of DN in T2DM patients (Peng et al. 2021). The therapeutic potential of interventions aimed at restoring or mimicking 5,6-DHET activity to mitigate DN warrants further exploration.

DISCUSSION

Phytochemical Screening

A definitive relationship between the secondary metabolites and the fruit's biological activity is difficult to establish due to a variety of phytochemicals with similar chemical structures (Saxena et al. 2013). The presence of tannins, polyphenols, and reducing sugars showed that the fruit has a potentially good source of antioxidant and hypoglycemic properties (Billacura and Laciapag 2017). The presence of alkaloids and saponins showed that *C. cujete* has potential antibacterial effect (Billacura and Laciapag 2017). Saponins may also exhibit an anti-inflammatory effect (Saxena et al. 2013).

α-Glucosidase Inhibition Assay

The inhibiting activity against α-glucosidase reduces glucose absorption by slowing down digestion of carbohydrates thereby controlling blood sugar levels (Feng et al. 2022). None of the six samples analyzed exhibited significant inhibitory activity against α-glucosidase (Table 2). A higher concentration screening may elicit higher inhibitory activity.

Antioxidant Assay

Antioxidants inhibit cellular damage through their free radical scavenging property (Lobo et al. 2010). The MeOH:H_2O fraction exhibited potential antioxidant activity (Figure 2). The polarity of the solvent, along with compounds such as flavonols, alkaloids, polyphenols, and saponins may have contributed to the observed action (Tai et al. 2011).

Dereplication of Compounds and Untargeted Metabolite Profiling

The GNPS spectral library is composed of MS/MS spectra coming from community deposits and aggregations from different sets of collections of libraries, while natural products and other pertinent databases with an extensive collection of data have expensive access. As a result, the scope of the search was primarily limited to the available library collections and publicly accessible databases. A more exhaustive metabolite profiling and dereplication of compounds would be possible if given the opportunity of resources. Further solvent fractionation of the crude extract using MeOH/H_2O improved the antioxidant activity by an average of 55.69%. However, no significant α-glucosidase inhibitory activity can be inferred from a single 10 ppm concentration screening of the same fractionated extracts.

To provide a more comprehensive comparison of the bioactivity of *C. cujete*, the half-maximal inhibitory concentration (IC_{50}) of the α-glucosidase, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) should be identified and various concentration screening protocols for the assays should be conducted to help identify the effective enzyme inhibitory effects of the sample. Further, isolation of the bioactive fractions could distinctly determine and identify the secondary constituents present in the sample. Nuclear magnetic resonance (NMR) spectroscopy could be performed for better identification, analysis, and structure elucidation of organic compounds.

The identification of 18:0 Lyso PC in *C. cujete* fruit highlights its potential as a natural product for Type 2 diabetes mellitus (T2DM) treatment by targeting DPP-IV, a key enzyme in T2DM management (Ambhore et al. 2023; Shaikh et al. 2021). Further investigation into its bioactivity, such as determining the IC_{50} values of the fruit extract, could validate its therapeutic efficacy and pave the way for developing cost-effective, plant-derived anti-diabetic agents.

The detection of 15-methyl PGE1 in *C. cujete* extracts may partially explain its reputed "miracle cure" properties, including potential vasoprotective and cytoprotective effects. However, the presence of this compound raises concerns about potential risks for pregnant women, as uterotonic effects could pose a threat. With a moderate cosine score of 0.71 from spectral matching, its identification remains tentative, necessitating advanced techniques such as nuclear magnetic resonance (NMR) for structural validation and bioactivity confirmation.

The fruit of *C. cujete* presents a potential natural source of 5,6-DHET. However, the spectral data in this study indicate low cosine scores in the compound's spectral match, meaning its presence in the fruit is not yet reliably confirmed. Further research employing more precise analytical methods is essential to validate *C. cujete* as a viable source of 5,6-DHET for therapeutic applications.

FUNDING

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ETHICAL CONSIDERATIONS

This research adheres to the highest level of ethical standards. All protocols are approved by the College Research Ethics Committee in accordance with its existing rules and guidelines.

DECLARATION OF COMPETING INTEREST

The authors declare that there are no competing interests to any authors.

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