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ORIGINAL ARTICLE

The *in vitro* α-glucosidase and α-amylase inhibitory activity and *in vivo* postprandial antihyperglycemic activity of *Ficus nota* Blanco Merr. and *Ficus septica* Burm. F. leaf methanolic extracts

Kitz Paul D. Marco* and Gracia Fe B. Yu

Department of Biochemistry and Molecular Biology, College of Medicine, University of the Philippines Manila, Manila , Philippines

ABSTRACT

Background: One of the therapeutic strategies for type 2 diabetes mellitus involves suppressing postprandial hyperglycemia by inhibiting key enzymes in carbohydrate digestion, α-glucosidase and α-amylase. While such inhibitors are commercially available, some researchers have turned to plants for potentially cheaper and safer alternatives.
Objectives: The study aimed to investigate the *in vitro* α-glucosidase and α-amylase inhibitory activities of the leaf methanolic extracts of two native Philippine plants *Ficus nota* Blanco Merr. and *Ficus septica* Burm F, as well as their effects on postprandial blood glucose levels in a mouse model.
Methodology: The *in vitro* activities of the leaf methanolic extracts were evaluated against porcine pancreatic α-amylase and yeast α-glucosidase. The most active extract was partially purified into fractions by sequential solvent partitioning and subjected to *in vitro* testing. Postprandial antihyperglycemic activity was then assessed in normoglycemic ICR mice. Phytochemical analysis was also performed.
Results: The most active extract and fraction *in vitro* were FS-crude and FS-HexF, respectively, having significantly more potent α-glucosidase inhibitory activity than the commercial drug acarbose. FS-crude and FS-HexF exhibited strong inhibition of α-glucosidase and weak inhibition of α-amylase, which is considered favorable for novel inhibitors as it is hypothesized to reduce gastrointestinal adverse effects. However, FS-crude and FS-HexF did not significantly attenuate postprandial blood glucose levels in the oral starch tolerance test. Phytochemical analysis of FS-HexF putatively identified 6-gingerol as one of the possible bioactive components.
Conclusion: *F. septica* could be a potential source of glycoside inhibitors as it showed promising *in vitro* inhibition of α-amylase and α-glucosidase. While it did not exhibit significant postprandial antihyperglycemic activity in this study, more robust testing is recommended to make a definitive conclusion.

Introduction

Diabetes mellitus is one of the fastest growing global health emergencies of the 21st century and is a leading cause of morbidity and mortality [1]. In the Philippines, the prevalence of diabetes has been increasing since 2003 [2]. Type 2 diabetes mellitus (T2DM) is the predominant type, accounting for over 90% of cases worldwide [1].

Postprandial hyperglycemia in T2DM is a significant predictor of cardiovascular events and mortality [3]. Thus, one therapeutic approach for T2DM involves suppressing postprandial hyperglycemia through glycoside hydrolase inhibitors [4]. These agents inhibit key enzymes of carbohydrate digestion – intestinal α-glucosidase and pancreatic α-amylase – thereby resulting in delayed glucose absorption after a meal [5]. There are three commercially available anti-diabetic drugs that act mainly by competitively inhibiting these enzymes: acarbose, miglitol, and voglibose. Acarbose and voglibose are of microbial origin while miglitol is a derivative of the microbial compound deoxynojirimycin [6,7]. They are often prescribed as first-line treatment or as adjunct to other antidiabetics [8]. However, they have been associated with gastrointestinal adverse effects such as flatulence, abdominal pain, and diarrhea, which are proposed to be due to excessive inhibition of α-amylase [9,10]. Additionally, a meta-analysis suggests that α-glucosidase inhibitors may also increase the risk of liver enzyme elevation among T2DM patients [11].

There has been a continuing effort to discover effective glycoside hydrolase inhibitors with less adverse effects from microbial, marine, and plant sources. Hundreds of candidate compounds have been identified from preclinical studies as extensively reviewed elsewhere [12-14]. Of these, sugar mimics, marine bromophenols, chalcols, xanthones, imino and thiosugars, and cyclitols have shown the most promising *in vitro* and/or *in vivo* activity [15]. However, there has been very little progress in drug development. Quercetin, a flavonoid that is widely distributed in plants, has a wide range of antihyperglycemic pharmacologic activities including α-glucosidase inhibition [16]. It suppressed postprandial hyperglycemia in patients with T2DM but did not show significant improvements in fasting plasma glucose and HbA1c in a meta-analysis of randomized clinical trials [17,18]. Hence, the search for glycoside hydrolase inhibitors continues.

In the Philippines, plants have gained attention as a potential source of inhibitors due to its rich plant biodiversity and abundance of medicinal plants that have long been used for many ailments including diabetes [19]. *Ficus nota* Blanco Merr., locally known as *tibig*, is a native Philippine plant that is only found elsewhere in northern Borneo [20,21]. It has been reported to be traditionally used for diabetes among others [22]. *Ficus septica* Burm. F., locally known as *hauli*, is another native Philippine plant that is widely distributed throughout Asia, Australia, and the Pacific Islands [23]. It is traditionally used mainly for relief of headache and body pain [24-26]. Several plants of the same genus have shown α-glucosidase and α-amylase inhibition in foreign studies [27-30]. *F. nota* and *F. septica* leaf extracts have been found to have high antioxidant activity [31,32] which has been inconsistently correlated with alpha-amylase and alpha-glucosidase inhibitory activity [27,33]. However, the antidiabetic potential of *F. nota* and F. septica have not yet been directly investigated to the best of the authors' knowledge. This study investigates the *in vitro* inhibition of α-glucosidase and α-amylase by *F. nota* and *F. septica* methanolic extracts, determines their effects on postprandial blood glucose levels in a mouse model, and preliminarily characterizes the potential bioactive phytochemical constituents.

Corresponding author's email address:
kdmarco@up.edu.ph
Keywords: diabetes mellitus, alpha-glucosidase, alpha-amylase, *Ficus nota, Ficus septica*

Methodology

2.1 Reagents. α-Glucosidase from Saccharomyces cerevisiae, α-amylase from porcine pancreas, and p-nitrophenyl glucopyranoside were purchased from Sigma-Aldrich.

2.2 Plant Collection and Crude Extraction. *Ficus nota* and *Ficus septica* specimens were collected from Barangay Calapi, Motiong, Samar on December 2020. Voucher specimens were verified by Jay Torrefiel, a biologist in the University of the Philippines Visayas Tacloban College. The leaves were washed and air-dried indoors prior to crude extraction. Then, dried leaves were cut into small pieces and soaked in methanol overnight. The solvent was then removed and the extraction was repeated twice. The extracts were concentrated using a rotary evaporator and dried off on a water bath at 38 ºC.

2.3 Partial Purification. The most active crude extract was partially purified using solvent partitioning. Approximately 0.4 g of crude methanolic extract was dissolved in 25 mL 9:1 methanol:water. This was partitioned thrice with 25 mL hexane, yielding the hexane fraction (FS-HexF). The polar layer was diluted to 6:4 methanol:water and partitioned thrice with 25 mL of chloroform, yielding the chloroform (FS-ChF) and aqueous (FS-AqF) fractions.

2.4 Alpha-Amylase Inhibition. The assay protocol was adapted from Banerjee et al. (2017) with some modifications [23]. A 100-µL solution of the plant extract (final concentration of 10, 100, or 500 µg/L in 10% DMSO) was incubated with 200 µL of porcine pancreatic α-amylase solution (0.275 mg/mL) in 20 mM PBS (pH 6.9, 6.7 mM NaCl) at 37°C for 10 minutes. Then, 100 µL of 1% potato starch in 20 mM PBS was added and the reaction mixture was incubated at 37 °C for 10 minutes. The reaction was terminated with 200 µL of DNS reagent (1 g/100mL 3,5-dinitrosalicylic acid, 30 g/100mL sodium potassium tartrate, 0.4 M NaOH) and incubation in a boiling water bath for 5 minutes. The reaction mixtures were diluted 1:5 with distilled water prior to measuring their absorbance at 540 nm with a microplate reader. Acarbose was used as a positive control while 10% DMSO was used as a negative control. The extract, positive control, and negative control readings were blank corrected by replacing the enzyme with PBS. This test was conducted in three separate independent experiments with four replicates each. The enzyme inhibition was calculated as follows:

$$Inhibition\ (\%) = \frac{A_{540\ (neg\ control)} - A_{540\ (extract)}}{A_{540\ (neg\ control)}} \times 100$$

2.5 Alpha-Glucosidase Inhibition. Alpha-glucosidase inhibitory activities were determined according to Thanakosai & Phuwapraisirisan (2013) [24]. A reaction mixture of 40 µL of yeast α-glucosidase (0.1 U/mL in 0.1 M phosphate buffer, pH 6.9) and 10 µL of plant extract dissolved in DMSO (final well concentration of 10, 100, or 500 µg/L in 10% DMSO) was incubated at 37 ºC for 10 minutes. Then, 50 µL of the substrate p-nitrophenyl glucopyranoside (1 mM in 0.1 M phosphate buffer, pH 6.9) was added. The reaction was allowed to proceed for 20 minutes at 37 ºC and was terminated by adding 100 µL of 1 M sodium carbonate. The absorbance of each solution was measured at 405 nm with a microplate reader. Acarbose was used as a positive control while 10% DMSO was used as a negative control. Likewise, sample blanks were prepared for the extracts, positive control, and negative control. This test was conducted in three separate independent experiments with four replicates each. The enzyme inhibition was calculated with the following formula:

$$Inhibition\ (\%) = \frac{A_{405\ (neg\ control)} - A_{405\ (extract)}}{A_{405\ (neg\ control)}} \times 100$$

2.6 Experimental Animals. The study was conducted with the approval of the UP Manila Institutional Animal Care and Use Committee (UPM-IACUC). Six-week-old male ICR mice (27-32 g) were purchased from the Research Institute for Tropical Medicine. The mice were acclimatized for ten days in a light- (12 h on/12 h off cycle) and temperature-controlled room (25-28 ºC) with standard pellet diet and tap water available ad libitum.

2.7 Oral Starch Tolerance Test. To assess the effects of the extracts on postprandial blood glucose levels, the oral starch tolerance test (OSTT) was conducted according to Goto et al. (2012) with some modifications [25]. The mice were randomized into six treatment groups (n=6 per group). After an overnight fast, soluble potato starch (2 g/kg body weight) was administered with FS-Crude or FS-HexF (100 or 500 mg/kg) via oral gavage. Acarbose (10 mg/kg) was used as a positive control while palm oil was used as a vehicle for the extracts and control. Blood was obtained by pricking the tail vein at 0, 30, 60, 120, and 180 minutes after gavage. Blood glucose levels were measured at each time point using the Freestyle Freedom Lite Blood Glucose Meter (Abbott).

2.8 Phytochemical Analysis. Qualitative tests for proteins, alkaloids, glycosides, anthraquinones, coumarins, tannins, phenols, carbohydrates, cardiac glycosides, triterpenes and terpenoids, quinones, cyanins, flavonoids, saponins, amino acids, and reducing sugars was conducted according to Bueno and Yu (2021) [37].

2.9 UPLC-Q-ToF-MS. *Ficus septica* hexane fraction underwent profiling via ultra-high performance liquid chromatography - quadrupole time of flight mass spectrometry (UPLC-Q-ToF-MS). Briefly, the sample was dissolved in LCMS-grade methanol with <1% DMSO and subjected to ultra-high performance liquid chromatography (Waters UPLC Class I) on a C-18 column (ACQUITY HSS T3 C18, 1.8µm, 2.1 x 100 mm). A linear gradient solvent system comprised of solvent A (water + 0.1% formic acid) and solvent B (acetonitrile + 0.1% formic acid) was used starting with solvent A at 95% for 0.5 min, 95-1% for 9.5 min, and 1% for 5 min. The run was conducted at a flow rate of 0.5 mL/min at 40 ºC. The eluted compounds were detected via quadrupole time-of-flight mass spectroscopy (Waters Xevo G2-XS QToF) in negative ionization mode with leucine enkephalin as a reference for mass correction. Scan range was set to 50-1200 m/z, scan time was set to 0.150 s, and collision energy was set to a high energy ramp from 15-50 eV.

Accurate mass screening was carried out using UNIFI software (Waters UNIFI Scientific Information System v1.8.1.073). The mass spectra were subjected to library matching using the Waters Traditional Chinese Medicine Library. Annotation of the compounds was based on accurate mass match, isotopic ratio match, and precursor ion intensity counts.

2.9 Statistical Analysis. All statistical analyses were done in GraphPad Prism 7.0. The area under the curve (AUC) in the OSTT was also calculated setting the mean value at 0 min as baseline. *In vitro* inhibition, glucose levels at each time point in the OSTT, and AUC were subjected to one-way analysis of variance (ANOVA) and post hoc Tukey's multiple comparisons test. A p-value < 0.05 was considered statistically significant.

Results

The crude methanolic extracts of *F. nota* (FN) and *F. septica* (FS) leaves were extracted with similar percent yields of 3.92% and 4.14%, respectively (Table 1). These were then subjected to *in vitro* assays for inhibition of porcine pancreatic α-amylase and yeast α-glucosidase. Both extracts had similar levels of α-amylase inhibition at all concentrations tested (Fig. 1A). They were only able to weakly inhibit α-amylase compared to acarbose reaching only <20% inhibition at 500 mg/L. On the other hand, the α-glucosidase inhibition by *F. nota* crude extract was comparable to the positive control at 10 and 100 mg/L while that of *F. septica* was more potent than acarbose at all concentrations (Fig. 1B).

Since *F. septica* crude extract had significantly higher α-glucosidase inhibitory activity, it was selected for partial purification through solvent partitioning. This yielded three fractions – FS-HexF, FS-ChF, and FS-AqF. FS-HexF and FS-ChF inhibited α-amylase similarly but still significantly less compared to acarbose from 10 to 500 mg/L (Fig. 1C). Meanwhile FS-AqF did not exhibit α-amylase inhibitory activity. FS-HexF and FS-AqF virtually completely inhibited α-glucosidase at 500 mg/mL while FS-ChF did not show any inhibition (Fig 1D). The inhibitory activity of FS-AqF rapidly dropped at lower concentrations. Meanwhile, FS-HexF was able to maintain >50% inhibition even at 10 mg/mL and had significantly higher inhibitory activity than acarbose at all concentrations. Overall, FS-HexF was the most active fraction with the greatest α-glucosidase inhibition while retaining α-amylase inhibition.

Table 1. Crude extraction and fractionation yield.

Extract	Yield (g)	%Yield
FN-Crude	2.976	3.92%
FS-Crude	3.308	4.14%
FS-HexF	0.3258	24.54%
FS-ChF	0.1851	13.94%
FS-AqF	0.5657	42.61%

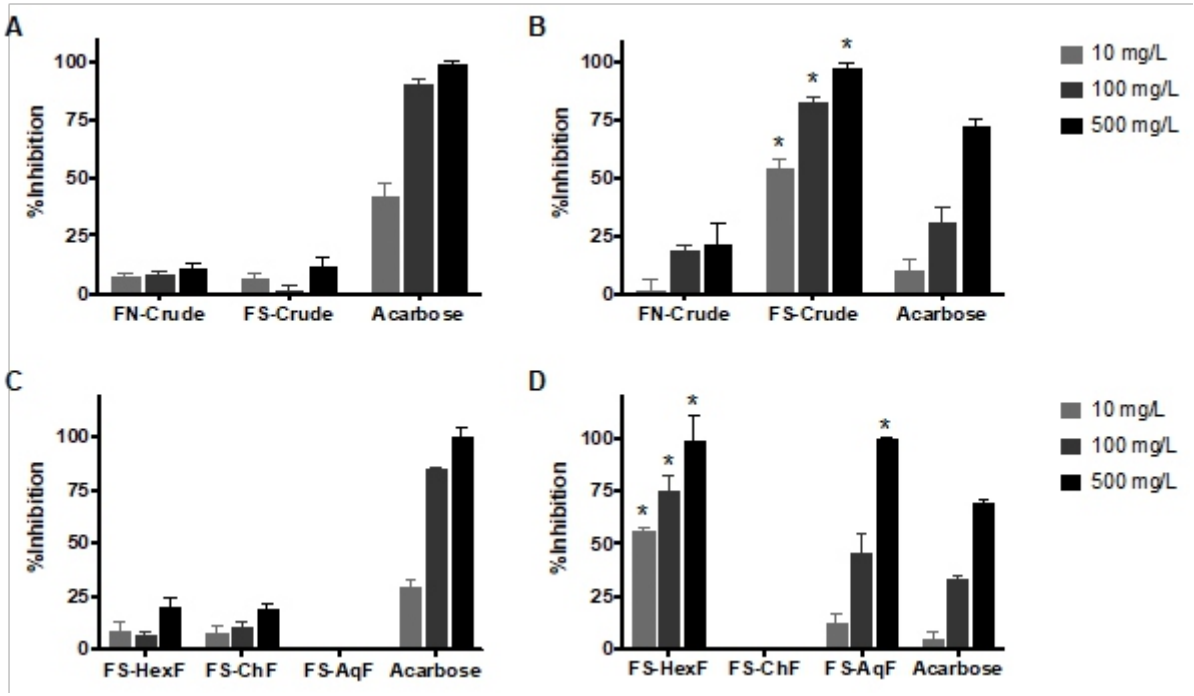

Figure 1. Porcine pancreatic α-amylase (A,C) and yeast α-glucosidase (B,D) inhibitory activities of *F. nota* and *F. septica* crude extracts (A-B) and *F. septica* partially purified fractions (C-D). Data is shown as mean ± SEM of three independent experiments. *p<0.05 compared to the equivalent concentration of acarbose based on Tukey's multiple comparisons test.

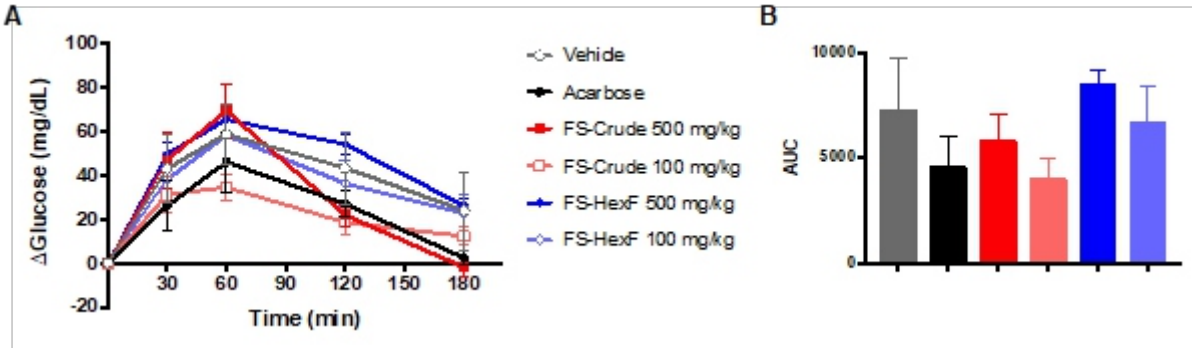

Figure 2. Baseline-corrected postprandial glucose levels (A) and corresponding area under the curve (B) in the oral starch tolerance test (n=6) of *F. septica* crude extract and hexane fraction. Data is shown as mean ± SEM. There is no significant difference (p>0.05) between all treatment groups according to Tukey's test for multiple comparisons.

The oral starch tolerance test was then conducted to determine whether the observed *in vitro* α-glucosidase and α-amylase inhibition by FS-Crude and FS-HexF would translate to lower postprandial blood glucose levels in male ICR mice. In all treatment groups, blood glucose rose after the starch load, peaked at 60 minutes, and gradually declined towards baseline (Fig. 2A). There was no significant difference in the change in glucose levels between the treatment groups at all time points. The glucose area under the curve (AUC) was also calculated for each treatment (Fig. 2B). The glucose AUC is an index of whole glucose excursion after the starch load and is used as a measure of postprandial antihyperglycemic activity [38]. There was also no significant difference in the glucose AUC between all treatment groups.

Table 2. Quantitative phytochemical analysis.

Phytochemical	FN-Crude	FS-Crude	FS-HexF
Proteins	-	-	-
Alkaloids	+	+	+
Glycosides	-	-	-
Anthraquinones	-	-	-
Coumarins	-	-	-
Tannins	+	+	+
Phenols	+	+	+
Carbohydrates	-	-	-
Cardiac glycosides	-	-	-
Triterpenes and terpenoids	-	-	+
Quinones	-	-	+
Cyanins	-	-	-
Flavonoids	-	-	-
Saponins	-	-	-
Amino acids	-	-	-
Reducing sugars	-	-	-

Table 2. Putatively identified components of FS-HexF using UPLC-Q-ToF-MS.

Component name	t_R (min)	Peak area	Observed m/z	Error (mDa)	Adducts
3-oxo-1,8-Cineole	3.33	4496	213.1166	3.3762	+HCOO
Sanleng acid	5.19	10852	329.2388	5.4905	-H
Acetylcorynoline	5.68	4029	454.1581	7.3244	-HCOO
6-O-Acetyl shanzhiside methyl ester	5.88	6691	447.1414	-9.3609	-H
Tetrahydrohelenalin	7.19	176213	265.1528	8.2825	-H
6-Gingerol	8.48	113607	293.1853	9.4606	-H

Qualitative phytochemical tests suggested the presence of alkaloids, tannins, and phenols in both crude extracts, while FS-HexF additionally tested positive for triterpenes and terpenoids and quinones (Table 2). FS-HexF was sent for UPLC-Q-ToF-MS profiling to potentially identify active compounds. Out of 24 candidate compounds, six were putatively identified by accurate matching of molecular mass and mass spectra with compounds deposited in the Waters Traditional Chinese Medicine Library (Table 3). None of the six compounds have been previously identified in *Ficus septica*.

Discussion

F. nota and *F. septica* crude extracts showed *in vitro* inhibition of yeast α-glucosidase and, to a lesser extent, porcine pancreatic α-amylase. Of the two, FS-Crude was the more active extract and was further fractionated. Both FS-Crude and its most active fraction FS-HexF potently inhibited α-glucosidase, at almost complete inhibition at 500 mg/L, while weakly inhibiting α-amylase compared to acarbose. α-Glucosidase is a superior target to α-amylase since it inhibits the final and key step of carbohydrate digestion. That is, unlike α-amylase inhibitors which can only inhibit the digestion of complex carbohydrates such as starch, α-glucosidase inhibitors can also delay the digestion of sucrose and other disaccharides [39]. Additionally, excessive inhibition of α-amylase results in undigested starch that becomes a substrate for colonic bacterial fermentation, which may lead to the common side effects of commercial glycosidase inhibitors (e.g., flatulence, diarrhea, abdominal pain, etc.) [10,40]. Thus, strong inhibition of α-glucosidase but only mild or weak inhibition of α-amylase is considered desirable [41,42], [43]. The *in vitro* inhibition profile of *F. septica* is therefore promising and may suggest decreased gastrointestinal side effects compared to acarbose. This hypothesis may be pursued in future studies.

The potent *in vitro* α-glucosidase inhibitory activity of FS-Crude and FS-HexF did not appear to translate well *in vivo*. Both extracts did not significantly decrease postprandial blood glucose levels compared to the negative control. However no significant difference was also observed for the positive control. Acarbose did decrease postprandial glucose levels and AUC compared to the negative control, though the difference was not statistically significant. This may be due to the large variability within treatment groups, especially in the negative control. Notably, FS-Crude at 100 mg/kg likewise decreased postprandial glucose levels, albeit not significantly, following a similar trend as acarbose. *F. septica* crude extract thus has the potential to have *in vivo* activity as well.

It should be noted that there was a significant difference in the baseline absolute blood glucose levels between the acarbose and the FS-HexF 500 mg/kg treatment groups, wherein the latter had lower baseline glucose levels (mean difference = 30.83 mg/dL, $p = 0.0062$). There was no significant difference in baseline blood glucose levels between all the other treatment groups. Lower baseline glucose levels may result in a greater increase in glucose levels after the same amount of starch load. Hence, the observed postprandial blood glucose levels with FS-HexF 500 mg/kg treatment may be overestimated relative to the positive control. However, this does not change the fact that FS-HexF at 500 mg/kg still did not significantly suppress postprandial hyperglycemia compared to the negative control.

This difference between the two groups at baseline and the generally large intra-treatment group variability may be partly due to limitations related to fasting. While food was withheld from all mice for the same amount of time, actual fasting periods may have differed since we cannot control their last feeding unlike with humans. Furthermore, the mice could have had varying levels of activity during the night hours wherein they are more active. This is thought to be the reason why overnight fasting resulted in greater variability in fasting blood glucose levels compared to daytime fasting in various mouse models [44]. The postprandial antihyperglycemic activity of *F. septica* crude extract and fractions may be further evaluated with more robust testing (e.g., with a larger sample size and using laboratory methods of glucose measurement) and with daytime rather than overnight fasting.

The lack of postprandial antihyperglycemic activity *in vivo* despite the promising *in vitro* results may be due to several factors. First, a limitation of the *in vitro* assays lies in the origin of enzymes used. Yeast α-glucosidase, which was used in the study, has different hydrolysis patterns compared to that of mammals [45]. Moreover, different inhibition profiles were observed for the same compounds on yeast and mammalian α-glucosidase [46-48]. In addition, compounds that were active *in vitro* may have undergone chemical modifications as they passed through the acidic stomach. The extracts may have also contained other components that can promote increased postprandial glucose levels, masking the effect of glycoside hydrolase inhibition. Alternatively, the active compounds may not have been able to effectively interact with the enzymes in the aqueous environment *in vivo* in the absence of a solubilizing agent (i.e., DMSO), especially with the nonpolar hexane fraction. In line with this, the postprandial antihyperglycemic activity of the *F. septica* aqueous fraction may also be evaluated in future studies as it also exhibited potent *in vitro* inhibition of α-glucosidase.

Qualitative phytochemical tests suggested the presence of alkaloids, tannins, and phenols in *F. nota* and *F. septica* crude leaf methanolic extracts. *F. nota* leaf ethanolic extract contained alkaloids, tannins, flavonoids, steroids, and anthraquinones in another study [49]. The presence of phenolics is also consistent with other reports that have quantified total phenolics in *F. nota* ethanolic leaf extract [50,51]. Alkaloids from *F. septica* leaves are known for their cytotoxicity in cancer cell lines [52-54]. Other studies have reported *F. septica* leaf methanolic extract containing terpenoids, flavonoids, and phenols and the ethanolic leaf extract containing quaternary bases, tannins, 2-deoxy sugars, and benzopyrone nucleus [55], [56]. Triterpenes, terpenoids, and quinones were enriched in FS-HexF upon fractionating the crude extract.

UPLC-Q-ToF-MS putatively identified six components of FS-HexF including 6-gingerol. 6-Gingerol is the principal pharmacologically active component of Zingiber officinale (ginger) with known anticancer, anti-inflammatory, and antioxidant properties [57]. Compared to acarbose, 6-gingerol showed similar inhibition of α-amylase and more potent inhibition of α-glucosidase *in vitro* [58]. Therefore, it is possibly one of the contributors to the observed *in vitro* activity of *F. septica* in this study. 6-Gingerol has also been shown to exert antidiabetic effects through multiple mechanisms [59-61]. Tetrahydrohelenalin, the most abundant among the putatively identified components, is a sesquiterpene lactone that has demonstrated potential antineoplastic properties but has not been investigated for potential anti-diabetic effects [62]. Likewise, the other identified components have no reported effects related to diabetes. It should be noted that the identification of the compounds is not necessarily correct using this method. The components were identified based on molecular mass, isotope ratio, and precursor ion spectra; however, several compounds may share the same mass and there was no manual curation and verification of the precursor ion spectra. In addition, the identification of compounds is limited by the library – it cannot identify novel compounds and is biased against compounds not typically found in traditional Chinese medicine. In this case, majority of the detected components of FS-HexF have not been identified. It is possible that the active components belong to these unidentified compounds and could even be novel.

Conclusions

F. nota and *F. septica* leaf methanolic extract exhibited *in vitro* α-glucosidase and α-amylase inhibitory activity. In particular, FS-Crude and its most active partially purified fraction, FS-HexF, strongly inhibited α-glucosidase while weakly inhibiting α-amylase compared to acarbose, which theoretically leads to less gastrointestinal side effects. Neither had significant postprandial antihyperglycemic activity in the oral starch tolerance test, although more robust testing is needed to confirm this finding. Because of the promising *in vitro* activity of *F. septica*, it can be a potential source of glycoside hydrolase inhibitors. Additionally, its crude extract had similar *in vitro* activity to its most active fraction, which may be beneficial for herbal drug development as fewer purification steps would be needed. Other crude extracts using nonpolar and aqueous solvents and using other plant parts (e.g., stem bark, fruit) may be investigated in future studies to maximize the potential of these plants as sources of inhibitors.

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