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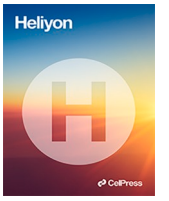

Research article

Glucosidase inhibition and compound identification of stingless bee honey and preserved fruits of *Citrus japonica*

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ABSTRACT

Food preservation has many benefits, such as increasing shelf life, retaining nutritional values and biological activities. In the current study, total phenolic content (TCP), antioxidant and anti-glucosidase activities, and kinetic of glucose inhibition of stingless bee honey, honey mixed with fruits, and extracts of *Citrus japonica* were evaluated by measuring color of a reaction using a spectrophotometer. The result showed that high TPC was found in ethanol extract of *C. japonica* leaves and fruits (26.79 ± 6.94 and 12.79 ± 0.87 mg of gallic acid per g extract), while stingless bee honey revealed the highest antioxidant activity ($1/EC_{50}$ = 0.2921) and honey mixed with fruits revealed the strongest anti-glucosidase activity ($1/EC_{50}$ = 1.8181), significantly (P-value <0.05). Kinetic of glucosidase inhibition of honey were found as uncompetitive and mixed competitive inhibition, while the honey mixed with fruits showed mixed competitive inhibition. The FTIR and GC-MS analysis demonstrated the presence of several bioactive compounds. Very strong positive relationship between total phenolic content with GC-MS data was found (r = 0.926, P-value < 0.05**).** This knowledge confirmed that stingless bee honey and honey mixed with fruits had greater anti-diabatic potential in comparison with the extracts of *C. japonica* leaves and fruits.

1. Introduction

Organic food contains many essential nutrients for human well-being. Thus, food preservation is a major factor to increase shelf life, retain nutritional values and biological activities. In recently, natural food preservatives (i.e. phytochemicals, organic acids, and essential oils) are increasingly popular to promote food safety and shelf life [1,2]. The natural preservatives are safe for human health, friendly to environment, low cost, and suitable for the current health situations. Nowadays, several factors, such as culture, social economy and lifestyle changes (i.e. eating habits and exercise) affects to the development of non-communicable diseases, espaically type 2 diabates. In 2021, more than 10.5 % of adult population worldwide have diabetes [3]. Diabetes can effect on metabolism of organs (i.e. pancreatic beta cells, liver, and skeletal muscles) and immune system of human body [4]. Moreover, the diabetic patients have to confront risks of both short- and long-term complications such as infectious disease and cardiovascular disorders [5,6]. Therefore, the management of long-term hyperglycaemia is important for the prevention and treatment of type 2 diabetes mellitus.

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Inhibition of enzyme responsible for carbohydrate hydrolyzation is an effective method to reduce hyperglycemia [7]. Alpha-glucosidase is an enzyme responsible for hydrolyzing 1,4-α glycosidic bonds of starch and disaccharides into glucose, which lead to increase blood glucose after meal. Nowadays, some research effort to search natural α-glucosidase inhibitors for application as therapeutic agents for type 2 diabetes mellitus. Interestingly, several bioactive compounds with alpha-glucosidase inhibitory activity (i.e. terpenes, alkaloids, quinines, flavonoids, phenols, phenylpropanoids and sterides) were rich in medicinal plants, which can be clinically improved for preventing and treating type 2 diabetes mellitus [8].

Furthermore, several reports show the health-promoting properties of honey, which is one of the natural products obtained from plant nectar and modified by different types of bees. Among these, stingless bees are one of eusocial bees that can produce honey which is considered as functional food for human health improvement for a long time. Beekeeping of stingless bees, especially *Tetragonula* species (i.e. *Tetragonula laeviceps* and *Tetragonula pagnedi*) were popularly kept for commercial enclosures or hive box by farmers in Thailand [9]. Bioactive compounds and pharmaceutical activities of stingless bee honey vary in different floral sources, geographical areas and stingless bee species [10]. It has been reported that stingless bee honey demonstrates several medical properties i.e. anti-inflammatory, antimicrobial, antioxidant and hypolipidemic properties [10]. Interestingly, trehalose, which is a low glycemic index sugar and is beneficial for patients with diabetes, is discovered as a major component of stingless bee honey [11]. Although several studies on stingless bee honey investigate the biological activities and bioactive compounds of stingless bee honey, the determination of anti-diabetic activity and bioactive compounds in the honey and honey fermented with fresh Citrus fruits was unknown.

Citrus japonica Thunb. or known as kumquat fruits, is an orange sour fruit tree in the Citrus genus, and widespread in South Asia and Asia–Pacific regions [12]. It has been reported that kumquat fruit peel has a high level of phenolic compounds (i.e. *p*-hydroxybenzoic acid,vanillic, protocatechuic, chlorogenic and sinapic acids) and flavonoids (apigenin 7-glucoside flavonoid), volatiles (i.e. monoterpenoid limonene, (D-germacrene, α-myrcene, α-pinene, bicyclogermacrene and sabinene), antioxidant activity and antimicrobial activity [13]. Moreover, it has been reported that essential oil and organic extract from the peel and kernel parts of *Citrus japonica* Thunb. have limonene and germacrene D, dodecanol-1 and linolenic acid, and both oils from different parts of *C. japonica* show antioxidant activity [14].

However, analyses of bioactive compounds and medicinal properties of stingless bee honey and *C. japonica* as natural preservative are lacking. Therefore, the natural food preservatives are the major goals of the current study were to macerate *Citrus japonica* fruits in stingless bee honey and determine the kinetics of glucosidase inhibition, total phenolic content (TPC), and antioxidant activity in comparison with those of stingless bee honey and extracts of *C. japonica* fruits and leaves. Moreover, Gas Chromatograph - Mass Spectrometer (GC-MS), Fourier transform infrared (FTIR) spectroscopy and multivariate analysis were used to determine the presence of active agents. This knowledge is useful for the improvement of food preservatives accomplished by high biological activities and bioactive compounds.

2. Materials and methods

2.1. Chemicals

Alpha-glucosidase from *Saccharomyces cerevisiae*, acarbose, 4-nitrophenyl-α-D- glucopyranoside, 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS), gallic acid, L-glutathione (reduced), absolute ethanol, potassium persulfate, sodium carbonate and Folin–Ciocalteu's phenol reagent were purchased from Sigma-Aldrich (USA).

2.2. Sampling

Honey samples produced from stingless bees *Tetragonula pagdeni* were collected from Chanthaburi province in Thailand (N = 3; sample codes were provided as TetraH1, TetraH2 and TetraH3) in November 2021 and Songkhla province in May 2022 (N = 1; TetraH0). Fruits and leaves of *Citrus japonica* Thunb (CTEF and CTEL sample codes) were collected from Pathum Thani province in June 2022.

2.3. Sample preparation

Stingless bee honey was diluted at 17 % with deionized water to determine total phenolic content, anti-glucosidase activity, and antioxidant activity. Fruits and leaves of *Citrus japonica* Thunb. were cleaned with water and left at room temperature for 2 h. Leaves of *C. japonica* Thunb. were dried at 60 °C for 48 h and mashed by a homogenizer, while fresh fruits of *C. japonica* Thunb. were sliced thinly. The sliced fruits (100 g) was preserved by mixing with stingless bee honey (TetraH0, 100 ml) in a sterile bottle, sealed, and left at room temperature for 10 days. The sliced fruits in the honey were done in triplicate (code names: FHO1, FHO2 and FHO3). The dried powder leaves and fresh sliced fruits of *C. japonica* Thunb. were extracted with absolute ethanol solvent in a ratio of 1 g per 25 ml-solvent for 45 °C for 48 h, and then were concentrated by rotary evaporation (IKAa RV10) at 45 °C for 20 min. Each sample was extracted in duplicate.

2.4. Total phenolic content

Total phenolic content was estimated by Folin-Ciocalteu method. Each sample (300 μl) of the diluted stingless bee honey, honey

mixed with fruits, and extracts of *C. japonica* fruits and leaves was reacted with Folin-Ciocalteu reagent (1.5 ml) at room temperature for 5 min, followed by adding 1.2 ml of 7.5 % w/v sodium carbonate, and incubated at room temperature for 30 min. The absorbance was determined at 765 nm wavelength and changed to total phenolic content by comparing with a standard curve of gallic acid [15]. The total phenolic content of extracts of *C. japonica* fruits and leaves was expressed as mg of gallic acid/g extract, while stingless bee honey and honey mixed with fresh fruits was expressed as mg of gallic acid per g sample.

2.5. Antioxidant activity

The diluted stingless bee honey, honey mixed with fruits, and extracts of *C. japonica* fruits and leaves were evaluated for antioxidant activity. Antioxidant activity was estimated with reaction between diluted ABTS radical cation (3.9 ml) with each extract (20 μl) in the dark at room temperature for 6 min. Briefly, the ABTS radical cation was prepared by mixing 7 mM ABTS solution (10 ml) and 140 mM potassium persulfate (179 μl) in the dark at room temperature overnight. After that, the ABTS radical cation was diluted at 0.700 ± 0.050 absorbance of 734 nm before use. The percentage of the ABTS radical scavenging capacity was calculated by using the below formula (Thummajistakul et al., 2019).

$$\%\ \text{ABTS radical scavenging capacity} = [\ (OD_{ABTS} - OD_{sample})/\ OD_{ABTS}] \text{ x } 100$$

OD_{ABTS} was the absorbance of the diluted ABTS radical cation.
OD_{sample} was the absorbance of sample reacted with the diluted ABTS radical cation.

The percentage of ABTS radical scavenging capacity was used to calculate effective concentration at 50 % (EC_{50}), which is sample concentration for 50 % ABTS radical scavenging.

2.6. Anti-glucosidase activity

Each sample (100 μl) of the diluted stingless bee honey, honey mixed with fruits, and extracts of *C. japonica* fruits and leaves were added with 3 mM glutathione (25 μl), 0.1 M potassium phosphate buffer pH 6.8 (250 μl) and 0.3 Unit/ml of alpha-glucosidase enzyme (50 μl), and then incubated at 37 ^{O}C for 15 min. After that, 10 mM 4-nitrophenyl-α-D- glucopyranoside (PNPG) (50 μl) was added and then incubated at 37 ^{O}C for 15 min. Finally, 0.1 M sodium carbonate (400 μl) was added, and its absorbance was detected at 400 nm [15]. Acarbose (1 mg/ml) was used as a positive control. The percentage of glucosidase inhibition was estimated by the below equation.

$$\%\ \text{inhibition} = \frac{(OD_{water} - OD_{blank\_water}) - \left(OD_{sample} - OD_{blank\_sample}\right)}{(OD_{water} - OD_{blank\_water})} \text{ x } 100$$

OD_{water} and OD_{sample} was the reaction absorbance of water and sample mixed with an enzyme
OD_{blank_water} and OD_{blank_sample} was the reaction absorbance of water and sample without enzyme

The percentage of glucosidase inhibition was translated to effective concentration at 50 % (EC_{50}), which is sample concentration for 50 % glucosidase inhibition.

2.7. Kinetics of alpha-glucosidase inhibition

A sample with high alpha-glucosidase activity was selected to study inhibition kinetic by the Lineweaver–Burk method. Anti-glucosidase activity was performed by following above method at different PNPG concentrations (substrate). The 1/V (O.D.450/min)$^{-1}$ and 1/[substrate] were used to generate the Lineweaver–Burk graph. K_m (Michaelis–Menten constant) and V_{max} (maximum velocity) were then calculated.

2.8. Fourier transform infrared spectrophotometer (FTIR)

Each sample of stingless bee honey, honey mixed with fruits, and extracts of *C. japonica* fruits and leaves were loaded in FTIR crystal. under wave number range from 550 to 4000 cm^{-1} with a resolution of 4 cm^{-1}. The FTIR spectra of each sample were compared with functional groups of references (Hemmalakshmia et al., 2017; sahayaraj et al., 2015; Hari et al., 2020; Hayat et al., 2020; Caunii et al., 2012; Topalăa et al., 2017).

2.9. Gas chromatograph - mass spectrometer (GC-MS)

For sample preparation, each sample was extracted with absolute ethanol solvent and sieved through a filter membrane prior to being subjected to GC-MS analysis using a Shimadzu gas chromatograph performed on a DB-5MS capillary column (30 m × 0.25 mm × 0.25 μm) with a Shimadzu mass spectrometer (Shimadzu, Kyoto, Japan) following the method of Thummajitsakul et al. [16]. Briefly, the oven program of the GC-MS analysis were performed under 40 °C for 1 min, 40–150 °C at 8 °C/min, 150–200 °C at 15 °C/min.

Injector temperature, transfer temperature and ion source temperature were held at 250 °C, 250 °C, and 200 °C, respectively. Helium was applied as the carrier gas using a flow rate at 1 mL/min. The mass spectrum peaks of phytochemicals in each sample were compared with data system libraries at 90 % similarity index (NIST14).

2.10. Statistical analysis

Descriptive and inferential statistics were determined using PSPP program version 0.10.5 [17]. Total phenolic content, antioxidant activity and anti-glucosidase activity were performed using descriptive statistics namely percentage, mean and SD, while one-way ANOVA was used to express differentiation of total phenolic content, antioxidant activity and anti-glucosidase activity among sample groups, and Pearson correlation coefficients was used to detect correlation between all variables. Cluster analysis and principal component analysis (PCA) in the paleontological statistic program (version 3.16) were used to express the similarity and relationships of all samples [18]. Moreover, a direct relationship among total phenolic content, antioxidant activity and anti-glucosidase activity, FTIR data and GC-MS data was performed by the partial least squares structural equation modeling (PLS-SEM) implemented in SmartPLS version 3 [19].

3. Results and discussion

3.1. Total phenolic content, anti-glucosidase and antioxidant activities

Nowadays, several factors (i.e. environment and life styles) are associated with an increased risk of type 2 diabetic diseases. Thus, surveying medicinal plants as natural sources of antioxidants and enzyme inhibitors to prevent deterioration of the human body is increasingly awareness. In the current study, the result showed that stingless bee honey, honey mixed with fruits of *C. japonica*, and ethanol extracts of *C. japonica* leaves and fruits had high total phenolic content, anti-glucosidase and antioxidant activities (Table 1). The highest level of total phenolic content was found in the leaf ethanol extract of *C. japonica* (26.79 ± 6.94 mg of gallic acid per g extract), followed by fruit ethanol extract of *C. japonica* (12.79 ± 0.87 mg of gallic acid per g extract). Interestingly, honey mixed with fruits of *C. japonica* showed greater total phenolic content (2.25 ± 0.21 to 3.17 ± 0.37 mg of gallic acid per g sample) and anti-glucosidase activity ($1/EC_{50}$ = 1.2930 to 1.818) than that of honey (0.59 ± 0.30 to 1.86 ± 0.20 mg of gallic acid per g sample and $1/EC_{50}$ = 1.0361 to 1.3323, respectively). However, the highest antioxidant activity was found in honey ($1/EC_{50}$ = 0.0776 to 0.2921), followed by honey mixed with fruits of *C. japonica* ($1/EC_{50}$ = 0.0415 to 0.1250), leaf ethanol extract of *C. japonica* ($1/EC_{50}$ = 0.0038)

Table 1
Total phenolic content, anti-glucosidase and antioxidant activities of honey, fruit and leaf ethanol extracts from *C. japonica* Thunb. and honey mixed with fruits of *C. japonica*.

Sample groups	Sample codes	Total phenolic content (mg of gallic/g extract)[b]	Antioxidant activity		Anti-glucosidase activity	
			EC_{50} (mg/ml)	$1/EC_{50}$	EC_{50} (mg/ml)	$1/EC_{50}$
Honey from stingless bees	TetraH0	0.94 ± 0.26	13.12 ± 2.23	0.0776 ± 0.0125	0.97 ± 0.07	1.0361 ± 0.0719
	TetraH1	0.97 ± 0.55	3.47 ± 0.50	0.2921 ± 0.0448	0.75 ± 0.02	1.3323 ± 0.0273
	TetraH2	1.86 ± 0.20	5.78 ± 0.62	0.1744 ± 0.0198	0.83 ± 0.09	1.2050 ± 0.1231
	TetraH3	0.59 ± 0.30	7.91 ± 2.05	0.1317 ± 0.0303	0.86 ± 0.06	1.1593 ± 0.0827
honey mixed with fruits of *C. japonica*	FHO1	2.25 ± 0.21	15.56 ± 8.51	0.0756 ± 0.0413	0.55 ± 0.06	1.8181 ± 0.1867
	FHO2	2.39 ± 0.10	24.18 ± 2.12	0.0415 ± 0.0036	0.61 ± 0.08	1.6625 ± 0.2165
	FHO3	3.17 ± 0.37	8.15 ± 1.41	0.1250 ± 0.0200	0.77 ± 0.02	1.2930 ± 0.0406
Ethanol extract of *C. japonica* fruits	CJEF (0.5 g/ml)	12.79 ± 0.87	384.36 ± 41.67	0.0026 ± 0.0003	264.80 ± 41.75	0.0038 ± 0.0006
Ethanol extract of *C. japonica* leaves	CJEL (0.1 g/ml)	26.79 ± 6.94	272.53 ± 60.83	0.0038 ± 0.0008	2697.10 ± 588.52	0.0004 ± 0.0001
Acarbose	–	–	–	–	1.31 ± 0.09	0.7678 ± 0.0521
	F (P-value)	**52.57 (0.000)[a]**		**58.87 (0.000)[a]**		**18.50 (0.000)[a]**

Note.
[a] Differentiation of total phenolic content, anti-glucosidase activity and antioxidant activity among samples of stingless bee honey and honey mixed with fruits of *C. japonica*, and ethanol extracts of *C. japonica* leaves and fruits was detected using one-way ANOVA at a significant level of P-value less than 0.005.
[b] Total phenolic content of extracts of *C. japonica* fruits and leaves was expressed as mg of gallic acid/g extract, while stingless bee honey and honey mixed with fruits was expressed as mg of gallic acid/g sample.

and the fruit ethanol extract (1/EC50 = 0.0026). It impiled that non phenolic compounds (i.e. bioactive peptides, non-strach polysaccharides, lipid and vitamins) in stingless bee honey involve the presence of antioxidant and anti-glucosidase potentials.

3.2. Kinetics of glucosidase inhibition

The glucosidase inhibitory kinetics were further performed by Lineweaver–Burk plots. Honey samples from different region origins (TetraH0 and Tetra H1), and honey mixed with fruits of *C. japonica* (FHO1) were selected to study kinetics due to they showed the highest anti-glucosidase activity. The result showed that TetraH1 had uncompetitive inhibition providing V_{max} and K_m decreased in comparison with those of control (V_{max} = 0.0019 O.D.$_{450}$/min and K_m = 0.62) (Table 2 and Fig. 1A), while TetraH0 and FHO1 showed mixed competitive inhibition providing V_{max} and Km increased in comparison with those of control (Fig. 1B and C). It indicated that inhibitors in TetraH1 bound to the enzyme-substrate complex, while inhibitors in TetraH0 and FHO1 bound to the free enzyme. Honey samples from different region origins provided different types of glucosidase inhibition, while the honey mixed with fruits was obtained from mixing of TetraH0 and the sliced fresh fruits revealed the same inhibitory type of TetraH0. This may be the result of the presence of different bioactive compounds in the samples. Therefore, FTIR and GC-MS analysis were used to identify bioactive compounds in the samples.

3.3. FTIR analysis

The FTIR tool was employed in this study for determining the functional groups of stingless bee honey, ethanol extracts from *C. japonica* leaves and fruits, and honey mixed with fruits. The FTIR result showed that 12 wavenumber ranges were found, associated with functional groups, namely alcohol, phenol, hydroxyl, fatty acid, lipid, protein, carbonyl, aldehyde, ketone, aromatic, alkane, amine, sulfonic, sulfate, acid, alkyl halide, ether, carboxylic acid, ester, phosphate ion, and C–H groups (Table 3). Stingless bee honey consisted of the most FTIR peaks, followed by the leaf and fruit ethanol extracts of *C. japonica* and honey mixed with fruits. Two specific wavenumber ranges were found in the leaf ethanol extract: 2854.1–2854.22 cm^{-1}, corresponding with fatty acids, lipids and proteins, and 1516.41–1516.33 cm^{-1}, corresponding with aromatic and alkane groups. One specific wavenumber range was found in the leaf and fruit ethanol extracts namely 1714.6–1733.04 cm^{-1}, corresponding with carbonyl and aldehyde groups. The functional groups found in each sample may be associated with bioactive compounds, which can act as antioxidants and glucosidase inhibitors.

3.4. GC-MS analysis

For the GC-MS result, the GC-MS chromatographs of each sample indicated the presence of several bioactive agents. The molecular formula, molecular weight, compound groups, and biological activities of each agent are shown in Table 5. It has been reported by de Lima Morais da Silva et al. (2017) that honey samples from different regions have their own differential chemical components [20]. In the current study, all honey samples consisted of several compounds that showed many pharmaceutical properties corresponding to the previous reports, i.e. antineoplastic activity, antiviral activity, antifungal activity, herbicidal activity, antibacterial activity, and antioxidant activity [21–24]. A total of fifteen different bioactive agents found in honey samples were recognized as various chemicals such as nitro, amine, amide, aldehyde, heterocyclic, alkane, isothiocyanate, ketone, nitrogenous, carbonyl, alcohol, organosilicon, phenyl, carbonyl, and anhydro sugar compounds. Of these, 5-hydroxymethylfurfural is one of the major components found in honey samples. This is in agreement with the report of Shapla et al. [25] that 5-hydroxymethylfurfural is an indicator of honey quality which possesses antioxidant, anti-allergic, anti-inflammatory, anti-hypoxic, anti-sickling, and anti-hyperuricemic properties. However, there were several bioactive compounds in honey samples that have not been reported.

For honey mixed with fruits of *C. japonica* Thunb, it possessed several bioactive compounds that have been reported about the presence of biological activities, namely anti-tyrosinase, antimicrobial activities, antioxidant, anti-proliferative, fungicidal, bacteriostatic, anti-cancer, and anti-metabolic syndrome properties [23,26–33]. However, n-butyl nitrite in the fermented honey has been reported to it that cause vasodilatation and initiate tumors [34]. The honey mixed with fruits showed more bioactive compounds than the honey samples due to the fact that they contained *C. japonica* fresh fruits.

Ethanol extract from *C. japonica* fruits possessed several compounds with biological activities, namely anti-tyrosinase, antimicrobial, antioxidant, anti-proliferative, antipyretic, analgesic, anti-inflammatory, flavoring, prolyl 4-hydroxylase inhibitory, and depigmenting properties [23,26,27,35–41]. Interestingly, the fruit ethanol extract consisted of major compounds with biological activities, namely diterpene and terpene alcohol, namely neophytadiene and 3,7,11,15-Tetramethyl-2-hexadecen-1-ol, respectively.

In similar, several bioactive compounds with biological activities were found in the leaf extract namely, acetic acid, 3-carene, 2-ethyl-1-hexanol, phenylethyl alcohol, alpha-terpineol, 1-nonanol, 2-hydroxy-1,8-cineol, 1-decanol, alpha-terpinyl acetate, δ-elemene,

Table 2
Kinetics of glucosidase inhibition.

Sample	Equation	V_{max} (O.D.$_{450}$/min)	K_m (mM)	Inhibition type
Control	y = 75.238x + 75.646, R^2 = 0.9631	0.0132	0.99	No inhibition
TeraH1	y = 330.36x + 536.78, R^2 = 0.6413	0.0019	0.62	uncompetition
TetraH0	y = 860.54x + 265.16, R^2 = 0.9588	0.0038	3.25	Mixed competition
FHO1	y = 536.34x + 226.54 R^2 = 0.9591	0.0044	2.37	Mixed competition

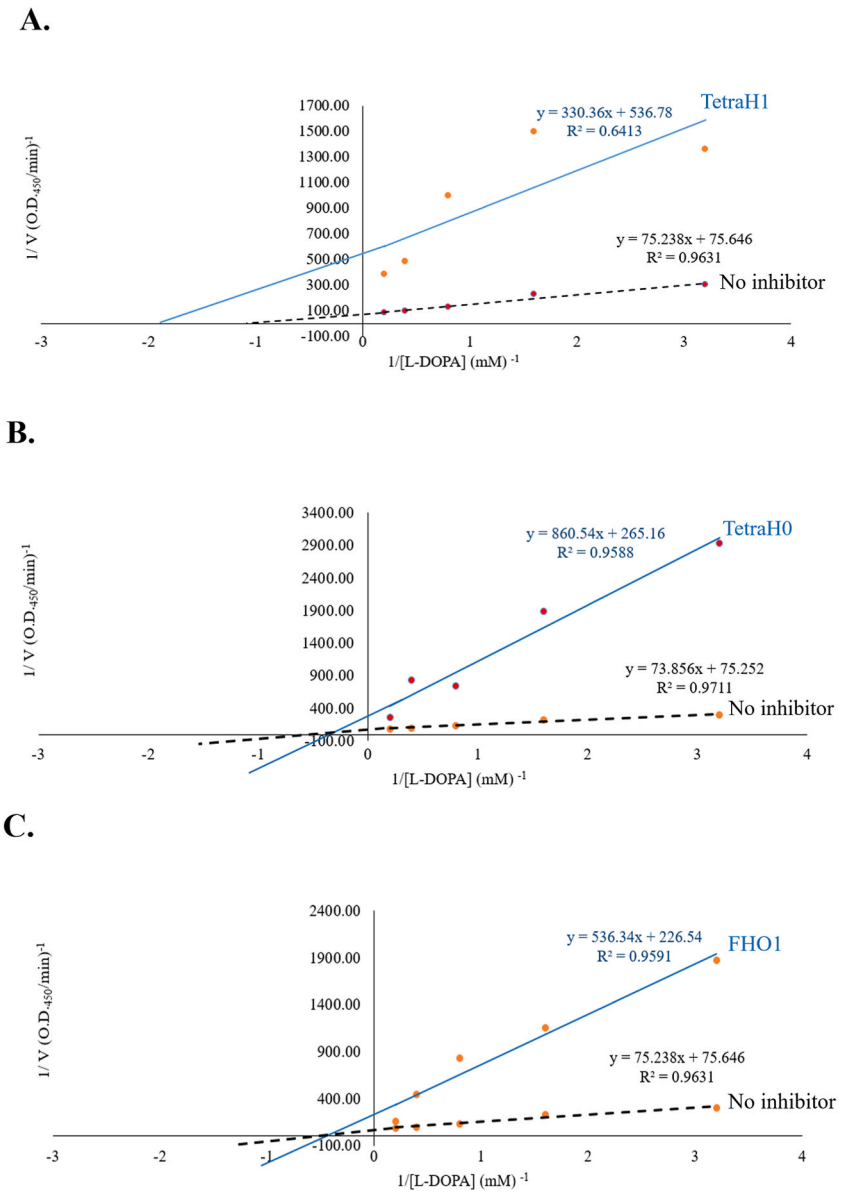

Fig. 1. Kinetics of glucosidase inhibition. A). Lineweaver–Burk plot of TetraH1 B). Lineweaver–Burk plot of TetraH0 and C). Lineweaver–Burk plot of FHO1.

caryophyllene, geraniol acetate, germacrene D, and farnesyl alcohol. The result also indicated that the leaf ethanol extracts consisted of the most amount of monoterpene, monoterpenoid, sesquiterpene, sesquiterpenoid, and sesquiterpene alcohol, which had wide biological activities. Corresponding with the previous report, essential oil from the peel and kernels of *C. japonica* Thunb consists of high limonene and germacrene D that show antioxidant activity [14]. Essential oil consists of monoterpenes and sesquiterpenes that can be oxidized to alcohol, aldehyde, ketone, and ether compounds [58].

Antioxidant activity found in the leaf and fruit ethanol extracts of *C. japonica* may occur from the presence of some bioactive compounds with antioxidant activity, namely 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-Pyran-4-one, 5-hydroxymethylfurfural and neophytadiene in the fruit ethanol extract, and alpha-terpineol and caryophyllene in the leaf ethanol extract. However, it has not been reported about the bioactive compounds with anti-glucosidase activity of *C. japonica* and stingless bee honey. Interestingly, this current study indicated that the highest value of anti-glucosidase activity was found in honey mixed with fruits and honey samples. It implied that some bioactive compounds in the fruit ethanol extract and honey fermented with the sliced fresh fruits, and honey samples showed anti-glucosidase activity. For example, it has been reported that neophytadiene is a major component in Morinda plant, which has high glucosidase inhibitory potential [59]. However, some components had no biological activities reported in any studies. This implies that they may be new compounds that have not been previously identified in plants or stingless bee honey.

Table 3
Twelve wavenumber ranges of FTIR peaks and functional groups found in stingless bee honeys, ethanol extracts from *C. japonica* fruit and leaves, and honey mixed with fruits.

Peak	Wavenumber ranges (cm^{-1})	Predicted function groups
1	3270.99–3390.36	alcohol, phenol, hydroxyl compound
2	2924.98–2934.94	saturated aliphatic compound-lipid
3	2854.1–2854.22	fatty acid, lipid, protein
4	1714.6–1733.04	carbonyl, aldehyde
5	1632.89–1643.81	ketone
6	1516.41–1516.33	aromatic and alkane
7	1376.52–1455.92	alkane, amine, aromatic, sulfonic, sulfate
8	1343.46–1376.92	phenol or tertiary alcohol
9	1223.18–1258.64	acid
10	1024.5–1171.94	alkyl halide, amine, ether, carboxylic acid, ester
11	1024.5–1055.72	phosphate ion
12	555.19–919.06	C–H group

Table 4
Pearson correlation coefficients and significance for all variables.

	TPC	Antioxidant activity	Anti-glucosidase activity	FTIR
Antioxidant activity	−0.639 (P-value = 0.034)*			
Anti-glucosidase activity	−0.831 (P-value = 0.002)*	0.602 (P-value = 0.050)		
FTIR	0.064 (P-value = 0.852)	0.428 (P-value = 0.189)	−0.084 (P-value = 0.805)	
GC-MS	0.926 (P-value = 0.000)*	−0.752 (P-value = 0.008)*	−0.713 (P-value = 0.014)*	−0.216 (P-value = 0.523)

Moreover, the direct relationship between independent and dependent variables was expressed by partial least squares structural equation modeling (PLS-SEM). The GC-MS and FTIR data were used in this analysis by using binary data, which was scored or without a peak of 1 or 0. The result showed that a very strong positive relationship between total phenolic content and GC-MS and FTIR data was found ($\beta = 0.926$ and 1.849, P-value <0.05), while a negative relationship between GC-MS and FTIR data was detected significantly ($\beta = -1.928$, P-value <0.05) (Fig. 2). It indicated that GC-MS and FTIR analysis can detect phenolic compounds.

In addition, Pearson correlation coefficients were measured for all variables. The result showed that a very strong positive relationship between total phenolic content and GC-MS data was found ($r = 0.926$, P-value <0.05). Moreover, negative relationships between total phenolic content and antioxidant activity, between total phenolic content and anti-glucosidase activity, between GC-MS data and antioxidant activity, and between GC-MS data and anti-glucosidase activity ($r = -0.639$, -0.831, -0.752, and -0.713, respectively, P-value <0.05) (Table 4). This result confirmed that GC-MS analysis can detect the presence of total phenolics better than FTIR analysis that was tool for identifying specific functional groups of compounds. Each compound has many different types of functional groups.

Moreover, it confrimed that antioxidant and antiglucosidase potentials of the stingless bees honey and honey mixed with fruits of *C. japonica* may occur from several non-phenolic compounds. In the previous report, it indicates that non-phenolic compounds (i.e., vitamins, organic acids, bioactive peptides, nonstarch polysaccharides, and lipids) can act as antioxidants and glucosidase inhibitors. For example, nonstarch polysaccharide shows noncompetitive inhibition for α-glucosidase, and mixed competitive inhibition for α-amylase by binding the enzyme [60,61]. It is similar to the result of glucosidase inhibitory kinetics that stingless bees honey and honey mixed with fruits of *C. japonica* showed uncompetitive and mixed competitive inhibition.

Moreover, carotenoids, which are pigments in the peel and pulp of citrus fruits, have been reported as antioxidants [62]. Furthermore, it has been reported that organic acids (acetic, citric, lactic, malic, succinic, and tartaric) have potential to inhibit α-amylase and α-glucosidase enzymes [63]. Functional groups involving anti-glucosidase activity relate to the presence of many hydroxyl groups in compounds [64]. However, phenolic compounds can act as antioxidants by a transferring hydrogen atom, a single electron, and chelating transition metals [65]. Functional groups of phenolic compounds related to antioxidant activity were methoxy, phenolic hydroxyl, and carboxylic acid groups [66]. However, isolation of bioactive componds with anti-glucosidase activity in each smaple type and molecular docking about the inhibitory activity of the compounds against alpha-glucosidase enzyme should be further studied.

The principal component analysis (PCA) and cluster analysis of total phenolic content, antioxidant and anti-glucosidase activity, FTIR, and GC-MS data can distinguish sample types into 4 groups: namely ethanol extract of *C. japonica* leaves, ethanol extract of *C. japonica* fruits, honey mixed with fruits, and stingless bee honey. The PCA showed two principal components, namely PC1 (62.30 % of all variances) and PC2 (31.80 % of all variances) (Fig. 3A). The PCA result showed positive relationship between total phenolic content and GC-MS data, which indicated the presence of many phytochemicals in ethanol extracts of *C. japonica* leaves and fruits. Additionally, honey samples from stingless bees showed the highest antioxidant activity, and honey mixed with fruits showed the highest anti-glucosidase activity. Therefore, FTIR and GC-MS analysis combined with PCA and cluster analysis can be used to separate sample types (Fig. 3B).

Table 5
Phytochemicals and their activities found in stingless bee honeys, fruit an leaf extracts from *C. japonica*, and honey mixed with fruits of *C. japonica* using gas chromatography-mass spectrometry.

Sample codes	Compound name	Formula	M. W.	Compound group	Activities
TetraH0	Nitro- methane	CH_3NO_2	61	Nitro	No activity reported
	Trifluoroguanidine	$CH_2F_3N_3$	113	Amine	No activity reported
	Formic acid hydrazide	CH_4N_2O	60	Aldehyde and amide	No activity reported
	2-Ethyl-oxetane	$C_5H_{10}O$	86	Heterocyclic	Antineoplastic, antiviral, and antifungal activities [22]
	2-Isothiocyanatobutane	C_5H_9NS	115	Alkane and isothiocyanate	No activity reported
	1,3-Dichloroacetone	$C_3H_4Cl_2O$	126	Ketone	No activity reported
	Urea	CH_4N_2O	60	Nitrogenous, carbonyl, amine	Herbicidal activity [21]
TetraH1	p-Dioxane-2,3-diol	$C_4H_8O_4$	120	Heterocyclic and alcohol	No activity reported
	Trimethylsilyl fluoride	C_3H_9FSi	92	Organosilicon	No activity reported
	Dimethyl-silanediol	$C_2H_8O_2Si$	92	Alcohol	No activity reported
	Methoxy-phenyl-oxime	$C_8H_9NO_2$	151	Phenyl	Antibacterial activity [24]
	5-Hydroxymethylfurfural	$C_6H_6O_3$	126	Heterocyclic and Carbonyl	Anti-allergic, anti-inflammatory, anti-hypoxic, anti-sickling, anti-hyperuricemic, antioxidant and antiproliferative activities [25,26]
TetraH2	Trimethylsilyl fluoride	C_3H_9FSi	92	Organosilicon	No activity reported
	5-Hydroxymethylfurfural	$C_6H_6O_3$	126	Heterocyclic and Carbonyl	Anti-allergic, anti-inflammatory, anti-hypoxic, anti-sickling, anti-hyperuricemic, antioxidant and antiproliferative activities [25,26]
TetraH3	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144	Ketone	Antioxidant activity [23]
	Levoglucosan	$C_6H_{10}O_5$	162	Anhydro sugar	No activity reported
	Silanediol, dimethyl-	$C_2H_8O_2Si$	92	Alcohol	No activity reported
	5-Hydroxymethylfurfural	$C_6H_6O_3$	126	Heterocyclic and Carbonyl	Anti-allergic, anti-inflammatory, anti-hypoxic, anti-sickling, anti-hyperuricemic, antioxidant and antiproliferative activities [25,26]
FHO2	Furfural	$C_5H_4O_2$	96	Heterocyclic and Carbonyl	Anti-tyrosinase and antimicrobial activities [27]
	2-Furanmethanol	$C_5H_6O_2$	98	Heterocyclic and alcohol	Antioxidant activity [28]
	Benzeneacetaldehyde	C_8H_8O	120	Aromatic and Aldehyde	Antibacterial and antioxidant activities [29]
	2,4(1H,3H)-Pyrimidinedione, 5-hydroxy-	$C_4H_4N_2O_3$	128	Pyrimidinedione derivative	No activity reported
	Maltol	$C_5H_6O_3$	126	Sugar derivative and hydroxypyrone	Antioxidant activity [30]
	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144	Ketone	Antioxidant activity [23]
	5-Hydroxymethylfurfural	$C_6H_6O_3$	126	Carbonyl	Antioxidant and anti-proliferative activities [26]
	2,3-dihydro-3-(1-methylpropyl) furan	$C_8H_{14}O$	126	Furan	No activity reported
	Levoglucosan	$C_6H_{10}O_5$	162	Anhydro sugar	No activity reported
	3,4-Altrosan	$C_6H_{10}O_5$	162	Anhydro sugar	Fungicidal and bacteriostatic properties [31]
	D-Allose	$C_6H_{12}O_6$	180	Aldohexose sugar	Anti-cancer effects, anti-metabolic syndrome effects [32]
	Nonanoic acid	$C_9H_{18}O_2$	158	Fatty acid	Antifungal activity [33]
	n-Butyl nitrite	$C_4H_9NO_2$	103	Nitrite	Leading vasodilatation and initiate tumors [34]
CTEF	Furfural	$C_5H_4O_2$	96	Heterocyclic and Carbonyl	Anti-tyrosinase and antimicrobial activities [27]
	Maleic anhydride	$C_4H_2O_3$	98	Acid anhydride	Antimicrobial activity [35]
	Ethanone, 1-(2-furanyl)-	$C_6H_6O_2$	110	Ketone	No activity reported
	2,5-Furandione, dihydro-3-methylene-	$C_5H_4O_3$	112	Ketone	No activity reported
	Uracil	$C_4H_4N_2O_2$	112	Pyrimidine bases	No activity reported
	2-Furancarboxaldehyde, 5-methyl-	$C_6H_6O_2$	110	Aldehyde	Flavoring activity (Duke,2014)
	2,5-Furandione, 3-methyl-	$C_5H_4O_3$	112	Ketone	No activity reported
	dl-3,4-Dehydroproline methyl ester	$C_6H_9NO_2$	127	Ester	No activity reported
	3-Butyn-2-amine, 2-methyl-	C_5H_9N	83	Amine	No activity reported
	3,4Dehydro-DL-proline	$C_5H_7NO_2$	113	Amino acid derivative	prolyl 4-hydroxylase inhibitory activity [36]
	1,2-Propadiene-1,3-dione	C_3O_2	68	Dicarbonyl complex	No activity reported
	dl-3,4-Dehydroproline methyl ester	$C_6H_9NO_2$	127	Ester	No activity reported

(*continued on next page*)

Table 5 (*continued*)

Sample codes	Compound name	Formula	M. W.	Compound group	Activities
	(+)-3,4-Dehydroproline amide	$C_5H_8N_2O$	112	Alpha amino acid amides	No activity reported
	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144	Ketone	Antioxidant activity [23]
	5-Hydroxymethylfurfural	$C_6H_6O_3$	126	Carbonyl	Antioxidant and anti-proliferative activities [26]
	Hydroquinone	$C_6H_6O_2$	110	Phenol	Depigmenting, antimicrobial activities [37,38]
	Neophytadiene	$C_{20}H_{38}$	278	Diterpene	Antipyretic, analgesic, and anti-inflammatory, antimicrobial, antioxidant activities (Duke,2014)
	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296	Terpene alcohol	Antimicrobial and anti-inflammatory activities [39–41]
CTEL	Acetic acid	$C_2H_4O_2$	60	Acid	Antibacterial activity [42]
	Glyceraldehyde	$C_3H_6O_3$	90	Triose monosaccharide and aldehyde	No activity reported
	Phenylglyoxal	$C_8H_6O_2$	134	Phenol, aldehyde and ketone	No activity reported
	1,2,3,4-Diepoxybutane	$C_4H_6O_2$	86	Cycloalkane	No activity reported
	2-Propenoic acid, ethenyl ester	$C_5H_6O_2$	98	Acid and ester	No activity reported
	cis-3-1-Hexenol	$C_6H_{12}O$	100	Alcohol	No activity reported
	1-Hexanol	$C_6H_{14}O$	102	Fatty alcohol	No activity reported
	Cyclohexylmethylsilane	$C_7H_{16}Si$	128	Cyclohexane	No activity reported
	1,1-Diethoxypentane	$C_9H_{20}O_2$	160	Acetals	No activity reported
	Hexanoic acid	$C_6H_{12}O_2$	116	Acid	No activity reported
	3-Carene	$C_{10}H_{16}$	136	Monoterpenes	Antimicrobial activity [43]
	2-Hexenoic acid	$C_6H_{10}O_2$	114	Acid	No activity reported
	2-Ethyl-1-hexanol	$C_8H_{18}O$	130	Alcohol	Growth inhibition, apoptosis, autophagy, caspase activation, DNA fragmentation, and cell cycle arrest [44]
	γ-Methyl-γ-caprolactone	$C_6H_{10}O_2$	114	Sesquiterpenes	No activity reported
	Phenylethyl alcohol	$C_8H_{10}O$	122	Phenol and Alcohol	Antibacterial activity [45]
	2,6-Dimethyl-1,3,5,7-octatetraene, E,E−	$C_{10}H_{14}$	134	Monoterpene	No activity reported
	Alpha-Terpineol	$C_{10}H_{18}O$	154	Monoterpene	Antibacterial, candiovascular antihypertensive, antioxidant, anticancer, anti-nociceptive, antiulcer, anticonvulsant sedative, anti-bronchitis, skin penetration enhancing, and insecticidal activities [46,47]
	1-Nonanol	$C_9H_{20}O$	144	Fatty alcohol	Antifungal activity [48]
	2,7-Dimethyl-1,7-octadien-3-amine	$C_{10}H_{19}N$	153	Monoterpene	No activity reported
	2-hydroxy-1,8-cineol	$C_{10}H_{18}O_2$	170	Monoterpene	Antioxidant activity [49]
	3-ethyl-4-methylmaleimide	$C_7H_9NO_2$	139	Sesquiterpenoids	No activity reported
	Linalool formate	$C_{11}H_{18}O_2$	182	Monoterpenoid	No activity reported
	1-Decanol	$C_{10}H_{22}O$	158	Fatty alcohol	Antibacterial activity [50]
	alpha-Terpinyl acetate	$C_{12}H_{20}O_2$	196	Monoterpenoid	Antimicrobial and anticholinesterase activities ([51,52]
	δ-Elemene	$C_{15}H_{24}$	204	Sesquiterpenoids	Antioxidant activity [53]
	Caryophyllene	$C_{15}H_{24}$	204	Sesquiterpenoid	Anticancer, antioxidant and antimicrobial properties [54]
	Geraniol acetate	$C_{12}H_{20}O_2$	196	Acyclic monoterpene ester	Antinociceptive activity [55]
	Germacrene D	$C_{15}H_{24}$	204	Sesquiterpenoid	Antioxidant activity [56]
	α-Selinene	$C_{15}H_{24}$	204	Sesquiterpenoid	No activity reported
	Farnesyl Alcohol	$C_{15}H_{26}O$	222	Sesquiterpene alcohol	Anti-proliferative activity [57]

4. Conclusion

This study showed the highest level of total phenolic content was found in leaf and fruit ethanol extracts from *C. Japonica*. Honey from stingless bees showed the highest antioxidant activity, while honey mixed with fruits showed the highest anti-glucosidase activity. The kinetics of glucosidase inhibition of stingless bee honey were found as uncompetitive and mixed competitive inhibition, while honey mixed with fruits showed mixed competitive inhibition. The FTIR result showed the most number of functional groups in stingless bee honey, followed by the ethanol extracts of *C. japonica* leaves and fruits, and honey mixed with fruits. The GC-MS analysis showed the presence of several bioactive compounds in the fruit and leaf ethanol extracts, honey mixed with fruits, and stingless bee honey. The principal component analysis (PCA) and cluster analysis of total phenolic content, antioxidant and anti-glucosidase activities, FTIR and GC-MS data can distinguish sample types, and a very strong positive relationship between total phenolic content with GC-MS and FTIR data was found. From this study, it was indicated that *C. japonica* fruits and leaves, stingless bee honey, and honey mixed with fruits can be used as novel sources of bioactive compounds. However, the research limitations are a lack of quantitative analysis of the effectiveness of the bioactive compounds on animal model. Therefore, an in vivo animal model should be further performed to approve the medical potentials of each bioactive compound of stingless bee honey and preserved fruits of *C. Japonica*.

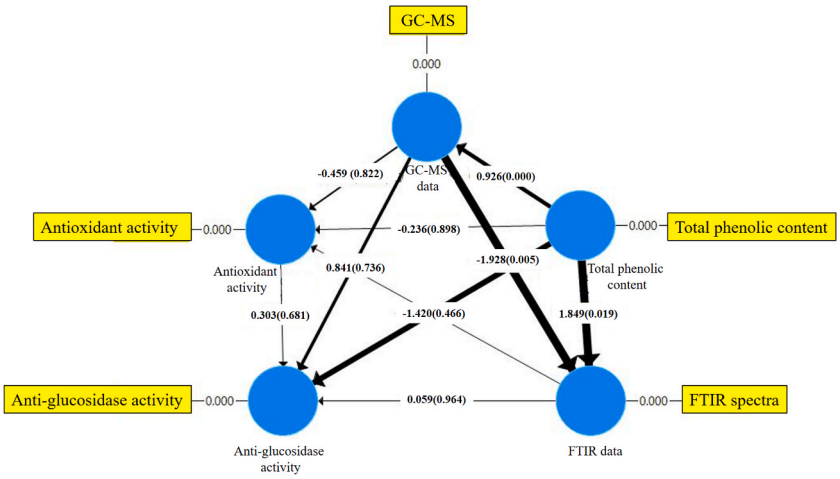

Fig. 2. Direct relationship from partial least squares structural equation modelling (PLS-SEM). Path coefficients (P-values) were expressed in each arrow (Bootstrapping = 10,000).

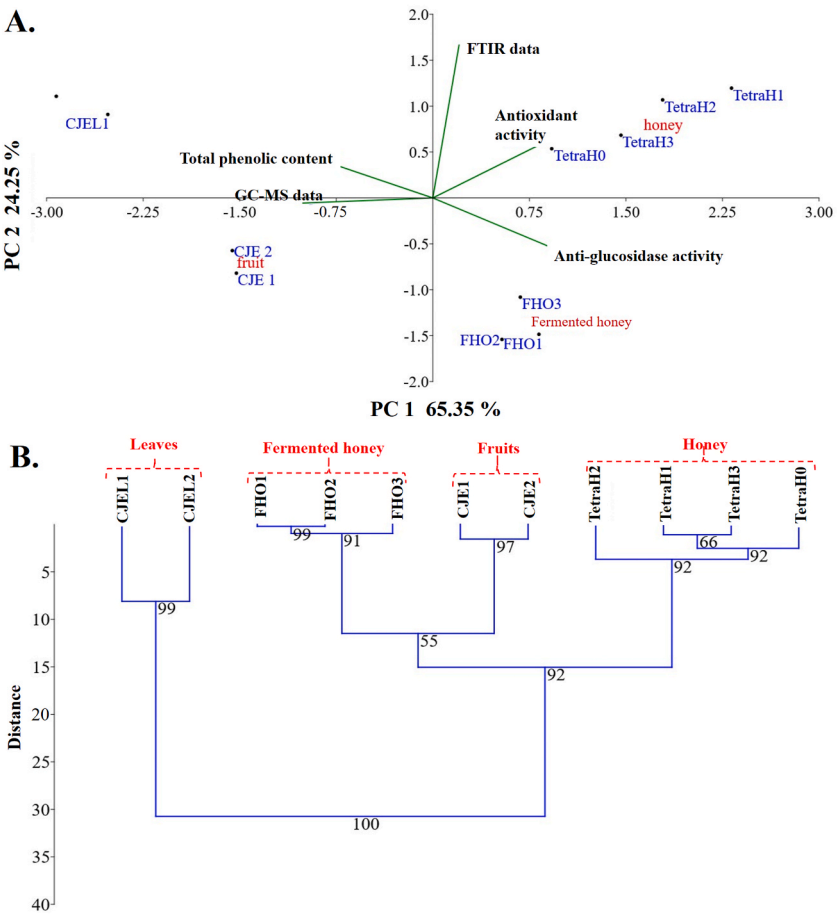

Fig. 3. PCA and cluster analysis (Paired group, UPGMA) based on total phenolic content, antioxidant activity, anti-glucosidase activity, FTIR and GC-MS data (Bootstrapping = 10,000).

CRediT authorship contribution statement

Sirikul Thummajitsakul: Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Tipwan Suppasat:** Resources. **Kun Silprasit:** Methodology.

Declaration of competing interest

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.

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