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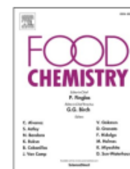

Metabolite profiles and antidiabetic activity of the green beans of Luwak (civet) coffees

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ABSTRACT

Metabolite profiles of green beans (the caged and the wild) Luwak (civet) coffees were evaluated by NMR techniques combined with chemometrics. The bioactivities of the green coffee beans were examined with antioxidant tests and an alpha-glucosidase inhibitory assay. Both are *invitro* tests related to the antidiabetic properties. Our results showed the civet coffees possessed unique metabolomes and were different from the regular arabica coffee. Both civet coffees were characterized by higher concentrations of alanine, citrate, lactate, malate, and trigonelline. Lactate and lipids were found as the most important compounds discriminating the caged civet coffee from the wild civet coffee. Bioactivity assays exhibited the antidiabetic activities of the civet coffees were better than the activity of the regular coffee. These results suggested that the civet coffees are promising functional foods reducing the diabetes risk. It is the first report evaluating metabolite profiles of both civet coffees using ^{1}H NMR-based metabolomics.

1. Introduction

Coffee, together with tea, is the most consumed beverage in the world. The most appreciated and prestigious coffee product is the Luwak (civet) coffee (Jumhawan et al., 2013; Marcone, 2004). This coffee is produced from coffee cherries that have been fermented in the digestive system of the Asian palm civet, *Paradoxurus hermaphroditus* (Marcone, 2004). The fermentation process leads to the civet coffee possesses a unique and different flavor from the regular coffees. It indicates a change in the metabolite profile of the coffee beans after the fermentation in the civet digestive system.

Metabolomics analysis of Luwak (civet) coffee had been performed with various instruments including UV–VIS spectroscopy (Suhandy & Yulia, 2017), fluorescent spectroscopy (Suhandy & Yulia, 2018), and GC–MS (Jumhawan et al., 2013, 2016). Data reported by Suhandy and Yulia (2017) showed that UV–VIS spectroscopy-based metabolomics had succeeded in differentiating the metabolite content of the civet coffee from its regular coffee based on the UV absorption patterns. This method was fast, sensitive, and low cost. However, this method could not clearly describe the metabolite profile contained in the civet coffee, since it is limited to the compounds having chromophore groups. Meanwhile, fluorescent-spectroscopy-based metabolomics was reported to successfully differentiate the emission-excitation patterns in the civet coffee spectra compared to other coffee samples (Suhandy and Yulia, 2018). Metabolomic analysis based on GC–MS data has advantage of being able to describe the profile of volatile compounds and acid compounds contained in the civet coffee. Some compounds were successfully identified by this method in the civet coffee samples, including glycolic acid, malic acid, pyroglutamic acid, citric acid, quinic acid, inositol, caffeic acid, sucrose, and chlorogenic acid (Jumhawan et al., 2016). However, the sample preparation in this method requires derivatization in advance to increase the volatility of the metabolites.

The other metabolomics method that is often used to analyze the metabolite profile of food is NMR-based metabolomics. This method has been successfully applied to study metabolomes of various foods without separation, including vinegar (Caligiani et al., 2007) and coffee (Wei et al., 2010). The use of NMR for metabolite analysis has several advantages, not only easy sample preparation, not destructive sample, broader metabolite profile (not only dependent on volatile metabolites), but also can be used for absolute quantification (Burgess et al., 2014). To the best of our knowledge, the application of the NMR-based metabolomics on the civet coffee analysis has not been reported before.

Coffee was reported to have antidiabetic activity (Ong et al., 2013). The coffee action mechanism as antidiabetic was reported through

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various ways, including reducing body weight (Cho et al., 2010), increasing expression of GLUT 4 (glucose transporter 4) (Ong et al., 2013), preventing glycation between glucose and protein (Bhattacherjee & Datta, 2015), inhibiting the enzyme alpha-glucosidase (Alongi & Anese, 2018) as well as antioxidant mechanism (Rani et al., 2018). The antidiabetic activity of coffee is inseparable from the secondary metabolites contained therein including chlorogenic acid (Hunyadi et al., 2012), trigonelline (Yoshinari & Igarashi, 2010), and malic acid (Gou et al., 2014). After the fermentation process in the civet digestive system, the concentrations of the active antidiabetic compounds in the coffee beans are predicted changes. However, our literature study showed that there is no work reporting the antidiabetic activity of the coffee beans after fermented by the civet yet.

This study aims to comprehensively examine the metabolome profile of the green beans of the Asian palm civet coffee using ^{1}H NMR-based metabolomics. The coffee samples used in this work consisted of the wild and the caged civet coffees, and the regular arabica coffee as well. Partial least square discriminant analysis (PLSDA) was applied to classify the metabolomes of the green beans of coffee samples. S-plots of this model were further analyzed to reveal the characteristic metabolites for each coffee sample. ^{1}H NMR quantitative analysis was performed for determining the concentration of some identified metabolites. Furthermore, the antidiabetic activity of the green beans of the civet coffee was examined with alpha-glucosidase inhibition and antioxidant assays.

2. Materials and methods

2.1. Materials

The coffee samples used in this research were *Coffea arabica* var. sigararutang, supplied by Rahayu Farmer Group, Pangalengan, Bandung, West Java. This coffee variety was certified by the Directorate General of Plantation, Ministry of Agriculture, Republic of Indonesia, under license No. 65/Kpts/Sr.120/2/2014. The coffee samples consisted of three types of green coffee beans including the wild civet coffee, the caged civet coffee, and the regular coffee. All green beans of the civet coffees were originated from *C. arabica* var. sigararutang. The regular coffee was the green beans of *C. arabica* var. sigararutang processed with the wet method. All specimens were deposited in Organic Chemistry Laboratory, Bandung Institute of Technology, with voucher numbers: the wild civet coffee, BPM-1WC; the caged civet coffee, BPM-1CC; the regular coffee, BPM-1FW. Deuterium oxide (D_2O, CID: 24602), methanol (CH_3OH, CID: 887), ferric chloride ($Cl_3FeH_{14}O_7$, CID: 154213), Ethanol (CH_3CH_2OH, CID: 702), sodium acetate trihydrate ($C_2H_9NaO_5$, CID: 23665404), acetic acid ($C_2H_4O_2$, CID: 176) and 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid sodium salt (TSP, CID: 23688921) were purchased from Merck (Darmstadt, Germany). Ascorbic acid ($C_6H_8O_6$, CID: 54670067), 1,1-diphenyl-2-picryl hydrazyl (DPPH, CID: 74358), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salts (ABTS, CID: 16240279), Potassium persulfate ($K_2O_8S_2$, CID: 24412), 2,4,6 tripyridyl-s-triazine (TPTZ, CID: 77258), alpha-glucosidase from *Saccharomyces cerevisiae* (SID: 318693955), and *p*-nitrophenyl-alpha-D-glucopyranoside (PNPG, CID: 92969) were bought from Sigma-Aldrich (St. Louis, USA). Acarbose (CID: 41774) was obtained from TCI (Tokyo, Japan).

2.2. Sample preparation for NMR analyses

All green coffee beans samples were ground into a powder with a coffee grinder 600 N (Yang Chia Machine Work, Taiwan). The coffee powder was extracted according to Wei et al. (2010) with slight modifications. 200 mg of green beans powder was poured into the plastic tube and mixed with 1 mL D_2O which contains TSP 1.0 mM in phosphate buffer pH 6.0. Then, the samples were homogenized, sonicated for 10 min, incubated at 95 °C for 30 min. Afterward, the samples were chilled with water flow and centrifuged using an MC-12 microcentrifuge (Benchmark, New Jersey, United States) at 12.000 rpm for 6 min. The supernatant was transferred into a new tube and mixed with 100 μL of D_2O containing phosphate buffer (pH 6.0). Finally, it was homogenized and moved into an NMR tube.

2.3. NMR spectroscopic analysis.

^{1}H NMR Spectra were recorded with NMR Variant Unity INOVA-500 Spectrometer (Agilent Technologies, Santa Clara, United States). The measurement was performed using the presaturation program with an acquisition time of 2.72 s, the delay time of 2.00 s, the number of data points of 64 K, spectra width of 8012 Hz, and the number of scans of 128. NMR FID data were further processed using ACD / Labs12.0 software (Toronto, Canada). The characteristic signals of the identified metabolites in the ^{1}H NMR spectra were further confirmed by 2D NMR experiments, including COSY, TOCSY, and J-Resolved using 4 scans and each increment of scans was 512, 256, and 128, respectively.

2.4. Quantitative ^{1}H NMR analysis

The metabolite quantification in the samples was performed according to a reported method Burgess et al. (2014). The calculation of the metabolite concentration was based on the comparison of the integration of the corresponding signal of the target metabolites to the integral of TSP signal as an internal standard. The signal of the TSP (1 mM) was selected as the internal standard since it has a singlet peak and does not overlap with other signals. The metabolite's concentrations are expressed as mean ± standard deviation for six biological replicates. The differences among groups were evaluated by one-way analysis of variance (ANOVA) and Duncan's multiple range tests with Microsoft Excel (Microsoft Office 365). A level of $p < 0.05$ was considered statistically significant.

2.5. Multivariate statistical analysis

All ^{1}H NMR spectra of green coffee beans were aligned and bucketed with a width of 0.04 ppm throughout the range of 0.50–10.00 ppm using ACD / Lab 12.0. Buckets 4.75–5.29 were removed because they were water signal areas. This clean data was normalized to the total area of the bucket to avoid bias due to the measurement. Furthermore, the data was analyzed by SIMCA version 12.0 (Umetrics, Umea, Sweden) for multivariate data analysis. Pareto scaling was applied in the analysis. Partial least square discriminant analysis (PLSDA) was performed as a primary model in this multivariate data analysis. In PSLDA, the variable X (predictor) represents the data in the NMR spectrum while the Y (response) variable represents the test group data. The resulting model validation was carried out with a 200-permutation test by looking at the value of R2Y exhibiting the accuracy of the model and the value of Q2 indicating the accuracy of the prediction.

2.6. Sample preparation for bioactivity assays

All coffee samples were extracted using solid–liquid extraction with deionized water (Barnstead Easypure II UF Deionization System, Thermofischer Scientific,United States). 5 g of the green bean powder was extracted using 40 mL of the deionized water. The ratio between the powder and the water was 1: 8 (w / w). Samples were homogenized and sonicated for 20 min. Afterward, it was incubated for 30 min at 90 °C, cooled on the water for 10 min, and then centrifuged at 12.000 rpm for 6 min. The obtained supernatant was dried with a freeze dryer machine (Bench Top Freeze Dryer FDB-5503, Operon, Korean).

2.7. Bioactivity assays

2.7.1. Antioxidant assay

2.7.1.1. DPPH radical scavenging activity. The DPPH radical ($DPPH^{\bullet}$) scavenging assay was applied to determine the antioxidant activity of the coffee samples. This method was performed according to Mahdi-Pour et al. (2012). Two mL of the coffee sample was mixed with 2 mL of 0.004% $DPPH^{\bullet}$ solution (in methanol). The mixture was vortexed, incubated in a dark room at 27 °C for 30 min and then the absorbance was recorded at the wavelength of 522 nm by a spectrophotometer UV–Vis (Halo DB-20 s UV–Vis Double Beam Spectrophotometer, Dynamica, United Kingdom). The triplicate measurements were performed for each coffee sample and ascorbic acid was used as the positive control. $DPPH^{\bullet}$ scavenging effect was calculated using the following equation:

$$\%\ DPPH\ radical\ scavenging\ activity = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100\%$$

where, $A_{control}$ was the absorbance of the control (without coffee sample), A_{sample} was the absorbance of the coffee, and methanol was used as a blank. The IC_{50} value was calculated from the linear regression between the percentage of $DPPH^{\bullet}$ scavenging activity and the sample concentration.

2.7.1.2. ABTS radical scavenging activity. The ABTS radical cation ($ABTS^{\bullet+}$) scavenging assay was carried out according to a previous method (Youn et al., 2019). $ABTS^{\bullet+}$ was produced by reacting 7 mM ABTS stock solution with 140 mM potassium persulfate. After 16 h in the dark at room temperature, 250 µL of the mixture solution was diluted in 22 mL ethanol. The sample was mixed with the $ABTS^{\bullet+}$ solution for 3 min and then the absorbance was recorded at the wavelength of 734 nm by a spectrophotometer UV–Vis (Halo DB-20 s UV–Vis Double Beam Spectrophotometer, Dynamica, United Kingdom). The $ABTS^{\bullet+}$ scavenging activity was calculated using the following equation:

$$\%\ ABTS\ radical\ cation\ scavenging\ activity = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100\%$$

where, $A_{control}$ was the absorbance of the control (the mixture of $ABTS^{\bullet+}$ solution and ethanol) and A_{sample} was the absorbance of the coffee samples. Ascorbic acid was used as a positive control. The triplicate measurements were performed for each coffee sample and positive control. The IC_{50} value was calculated from the linear regression between the percentage of $ABTS^{\bullet+}$ scavenging activity and the sample concentration.

2.7.1.3. Ferric-reducing antioxidant power (FRAP) assay. The FRAP assay was performed according to a previous method (Benzie & Strain, 1996). The FRAP reagent was prepared by mixing 10 mL of 300 mM sodium acetate buffer (pH 3.6), 2.5 mL of 2,4,6-tripyridyltriazine (TPTZ) solution and 2.5 mL of $FeCl_3{\cdot}6H_2O$ solution and then warmed (preheated) at 37 °C before used. The sample was mixed with the FRAP reagent and then incubated at 37 °C for 10 min in the dark. The absorbance was determined at the wavelength of 593 nm by a spectrophotometer UV–Vis (Halo DB-20 s UV–Vis Double Beam Spectrophotometer, Dynamica, United Kingdom). Antioxidant activity was calculated using the following equation:

$$\%\ Antioxidant\ capacity = (1 - Ts) \times 100\%$$

$$As = -\log Ts$$

where, As was the absorbance of the FRAP solution after the addition of the coffee sample. Ascorbic acid was used as a positive control. The EC_{50} value was calculated from the linear regression between the percentage of the antioxidant capacity and the sample concentration. The EC_{50} of FRAP capacity is the concentration of sample exhibiting 50% of FRAP capacity.

2.7.2. Alpha-glucosidase inhibition assay

The inhibitory activity of coffee sample against alpha-glucosidase was assessed spectrophotometrically (xMark™ Microplate Absorbance Spectrophotometer, Bio-Rad, United State), following the method published by Alongi & Anese (2018) with modifications. The sample was dissolved in a solution of methanol–water (1:9). 3,5 µL of the coffee extract sample was added into 200 µL of phosphate buffer pH 7.0 containing 10 µL of glucosidase 1 U/mL and then incubated at 37 °C for 10 min. Afterward, the sample was mixed with 35 µL of PNPG 5 mM (dissolved in phosphate buffer pH 7.0), incubated at 37 °C for 15 min, and then measured with a UV spectrometer at the wavelength of 405 nm. The sample without the coffee extract was used as the control. Meanwhile, the sample without alpha-glucosidase enzyme was used as the blank. The percentage of alpha-glucosidase inhibition was calculated by the following equation:

$$Percentage\ of\ Inhibition = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100\%$$

where, $A_{control}$ was the absorbance of the control and A_{sample} was the absorbance of the coffee sample. IC_{50} value calculation was carried out by varying the concentration of the sample within the range of 0.25–2.00 mg/mL. The IC_{50} value was calculated from the linear regression between the inhibition percentage and the sample concentration.

The Michaelis-Menten kinetic parameters were determined by varying the concentration of PNPG as a substrate in the range 0.34–2.39 mM. The V_{max} and K_M in absence and V_{max} and K_M values in the presence of inhibitor (I) as well as the mode of inhibition were calculated from a multiple reciprocal plot (Lineweaver-Burk). The value of Ki (inhibition constant) is determined by the following equation:

for competitive inhibition $ki = \frac{K_M(I)}{K_M' - K_M}$

for noncompetitive inhibiton $ki = \frac{Vmax'(I)}{Vmax - Vmax'}$

for mixed type inhibition $ki = \frac{Vmax X K_M X(I)}{Vmax K_M' - Vmax' K_M}, ki' = \frac{Vmax(I)}{Vmax - Vmax'}$

2.7.3. Statistical analysis

The biological activity data were presented as the mean ± standard deviation (SD) for triplicate determinations. All Statistical analyses were performed using Duncan's multiple range test one-way analysis of variance (ANOVA) with Microsoft Excel (Microsoft Office 365). Correlation between the concentration of the quantified metabolites (5-CQA, caffeine, malic acid and trigonelline) and the biological activities was tested with Pearson correlation coefficient (r) using Microsoft Excel (Microsoft Office 365). The p value < 0.05 is considered statistically significant. The value of $-1 \leq r < 0$ is considered having a positive correlation since the lower IC_{50} and EC_{50} values, the higher the antioxidant activity.

3. Results and discussion

3.1. Metabolite identification

Metabolites identification in the samples of green coffee beans was conducted by detecting their characteristic signals in the ^1H NMR spectra as depicted in Fig. 1. These signals were verified with 2D NMR spectra analysis, including COSY, TOCSY, and J-resolved. The COSY signal correlation of some identified metabolites as described in Fig. 2. The result of the identification was further confirmed by comparing the detected characteristic signals with their corresponding reference spectra obtained from metabolite database (www.hmdb.ca) and the data in the literature (Arana et al., 2015; Wei et al., 2010).

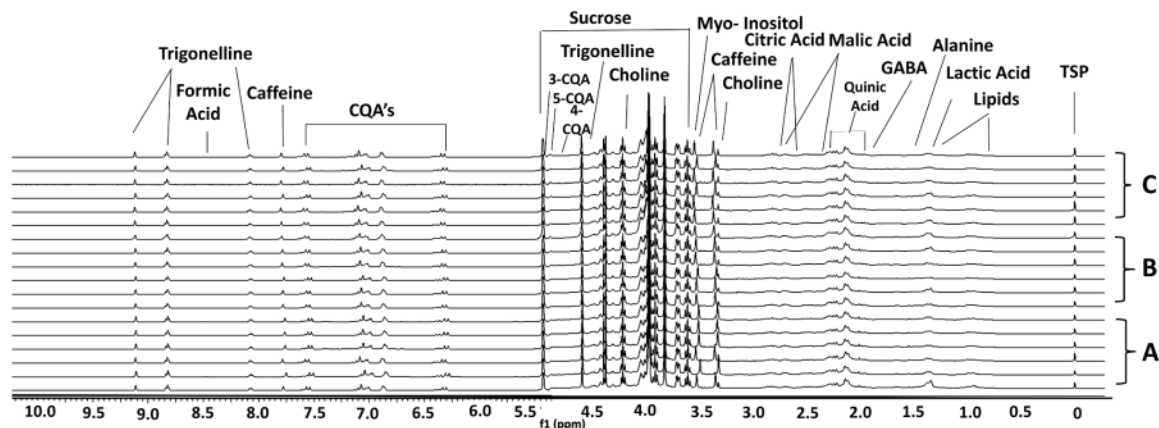

Fig. 1. ^{1}H NMR spectrum of the green bean extracts of the caged civet coffee (A), the wild civet coffee (B) and regular arabica coffee (C).

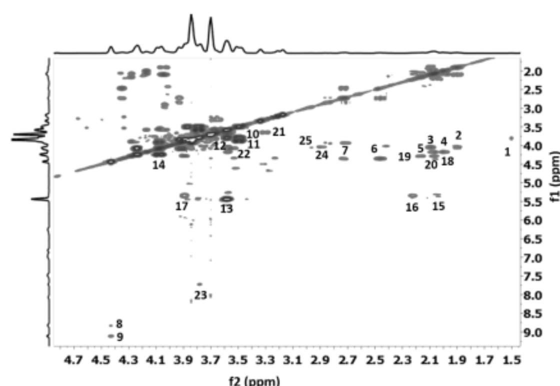

Fig. 2. The signal correlations of identified metabolites in the COSY spectrum. 1. H2/H3 of Alanine; 2. H2a/H3 of quinic Acid; 3. H2e/H3 of quinic Acid; 4. H6a/H5 of quinic Acid; 5. H6e/H5 of quinic Acid; 6. H3/H2 of malic Acid; 7. H3/H2 of malic Acid; 8. NCH_3/H4 of trigonelline; 9. NCH_3/H2 of trigonelline; 10. H4/H3 of sucrose; 11. H4/H5 of sucrose; 12. H2/H3 of sucrose; 13. H2/H1 of Sucrose; 14. H4′/H3′ of sucrose; 15. H13/H10 of 5-CQA; 16. H15/H10 of 5-CQA; 17. H11/H10 of 5-CQA; 18. H13/H10 of 3-CQA; 19. H15/H10 of 3-CQA; 20. H13/H12 of 4-CQA; 21. H2/H1 of myo-Inositol; 22. H5/H4 of myoinositol; 23. N7CH_3/H8 of caffeine; 24. H3/H2 of asparagine; 25. H3/H2 of asparagine.

In total, 17 metabolites were identified in the civet coffees (wild civet and caged civet) and regular arabica coffee, including alanine, quinic acid, citric acid, malic acid, caffeine, trigonelline, sucrose, 5-CQA, 4-CQA, 3-CQA, choline, myo-inositol, GABA, lactic acid, formic acid, lipids, and asparagine. These results complement the scientific data regarding the metabolite profile of civet coffee previously reported by Jumhawan et al., (2013). For instance, in this work, amino acids including alanine, asparagine and GABA which could not be identified with the GCMS method, were successfully detected in the civet coffees.

The signals of major compounds in the green coffee beans, including sucrose, caffeine, chlorogenic acid, and trigonelline, were detected in the ^{1}H NMR spectra as described in Fig. 1. The signals belong to the glucose part of the sucrose was detected at δ 5.43, 3.58, 3.79, 3.49, 3.88, and 3.84 ppm. Meanwhile, the signals that correlate to the fructose part of the sucrose, were detected at δ 3.70, 4.23, 4.08, 3.84, 3.90 ppm. Four singlet caffeine signals were detected clearly in the spectra. Three signals at δ 3.17, 3.33, and 3.88 ppm were assigned as 3 *N*-methyl groups of caffeine, while a singlet signal at δ 7.72 ppm was identified as the aromatic proton of caffeine. In the lower field spectra region, four proton signals at δ 8.06, 8.80, 8.82, and 9.10 ppm were assigned as the pyridine ring protons of trigonelline. Meanwhile, the *N*-methyl signal of trigonelline was detected at δ 4.43 ppm as a singlet signal. The signals of chlorogenic acids, other major metabolites, were detected in the ^{1}H NMR spectra as depicted in Fig. 1.

A doublet signal at δ 1.49 ppm was assigned as a methyl signal of alanine. This signal was correlated to the signal at δ 3.81 ppm belong to the proton signal of C-tersier of alanine (Fig. 2). Other amino acids detected in the spectra were gamma-aminobutyric acid (GABA) at δ 1.90, 2.32, and 3.02 ppm and asparagine at δ 2.89 and 2.96 ppm. Other acidic compounds detected in the NMR spectra of the green coffee beans were citric acid, formic acid, lactic acid, malic acid, and quinic acid. Other identified metabolites in the spectra were choline, lipids (regions at δ 0.94 and 1.27 ppm), and myoinositol. The detailed information of the chemical shifts of the detected metabolites was presented in Table 1.

3.2. Quantitative analysis

The metabolite identification in the ^{1}H NMR spectra showed that the metabolites in the (wild and caged) civet coffees and the regular coffee were similar qualitatively. Naturally, the civet consumes the coffee fruit in the night and secreted it in the next morning. Thus, the coffees are fermented in the civet digestive system for about 12 h. This fermentation duration probably could not change the type of metabolites present in the green coffee beans. However, it possibly altered the metabolite concentration in the green coffee beans as revealed by the quantitative analysis.

Some detected compounds in the green coffee beans, including alanine, caffeine, choline, malic acid, sucrose, trigonelline, and 5-caffeoylquinic acid (5-CQA) were further investigated with quantitative ^{1}H NMR analysis (Table 2). The concentrations of quantified compounds in the green coffee beans of the caged civet were compared with those in the green coffee beans of the wild civet. The concentrations of caffeine and malic acid were higher in the wild civet than in the caged civet coffee. Meanwhile, the opposite case found when comparing the other quantified compounds. Concentrations of alanine, sucrose and trigonelline were higher in the caged civet coffee than in the wild civet coffee. The sucrose level was higher in the caged civet coffee (35.10 mM $\pm$ 0.17) than in the wild civet coffee (31.23 mM $\pm$ 0.27). It is probably caused by the inhibition of several digestive enzymes in the caged civet due to the high number of reactive oxygen species (ROS) (Isnaini et al., 2018). The high number of ROS in the caged civet could be stimulated by the changes in the shelter and in the feeding patterns (Keeling & Jensen, 2009). Furthermore, the inhibition of several digestive enzymes probably induced the lower concentrations of malic acid and citric acid

Table 1
^{1}H NMR signals of the identified compounds in all coffee samples. RA: regular arabica coffee; CC: caged civet coffee; WC: wild civet coffee. +: detected.

Compounds	Chemical Shift (ppm) and Coupling Constants (Hz)	RA	CC	WC
1. Alanine	δ 1,49* (H-3, d, J = 8 Hz), δ 3,81 (H-2, q)	+	+	+
2. Quinic Acid	δ 1,89 (H-2a, dd), δ 2,07 (H-2e/H-6e, dd), δ 4,03 (H-3, dd), δ 3,55 (H-4, dd), δ 4,15* (H-5, dd), δ 1,98 (H-6a, dd)	+	+	+
3. Citric Acid	δ 2,58 (H-3, d, J = 20,30 Hz), δ 2,72 (H-3, d)	+	+	+
4. Malic Acid	δ 2,46 (H-3, dd), δ 2,71 (H-3, dd), δ 4,33 (H-2, dd, J = 16.50 Hz, J = 6.50 Hz)	+	+	+
5. Caffeine	δ 3,17 ($N1CH_3$, s), δ 3,33 ($N3CH_3$, s), δ 3,78 ($N7CH_3$, s), δ 7,72* (H-8, s)	+	+	+
6. Trigonelline	δ 4,43 (NCH_3, s), δ 8,06 (H-5, t), δ 8,82 (H-4/H-6, m), δ 9,10* (H-2, s)	+	+	+
7. Sucrose	δ 3,49 (H-4, t, J = 9,51 Hz), δ 3,58 (H-2, dd, J = 10,57 Hz, J = 5,03 Hz), δ 3,70 (H-1′, s), δ 3,79 (H-3, t, J = 9,51 Hz), δ 3,84 (H-6′/H-6, m), δ 3,88 (H-5, dd), δ 3,90 (H-5′, dd), δ 4,08 (H-4′, t, J = 9,51 Hz), δ 4,23 (H-3′, d, J = 9,27 Hz), δ 5,43* (H-1, d, J = 4,10 Hz)	+	+	+
8. 5-CQA	δ 2,07 (2H-13, m), δ 2,20 (2H-15, m), δ 3,90 (H-11, dd, J = 9,9 Hz, J = 4,5 Hz), δ 4,24 (H-12, d), δ 5,33 (H-10, m), δ 6,31* (H-1, d, J = 16,04 Hz), δ 7,55 (H-2, d, J = 16,04 Hz), δ 6,91 (H-4, dd, J = 8,86 Hz, J = 3,51 Hz), δ 6,81 (H-5, d, J = 8,68 Hz), δ 7,48 (H-8, d)	+	+	+
9. 4-CQA	δ 2,07 (2H-13, m), δ 2,20 (2H-15, m), δ 4,32 (H-10, m), δ 4,36 (H-12, m), δ 4,92 (H-11, dd, J = 9,1 Hz, J = 2 Hz), δ 6,37 (H-1, d, J = 16,04 Hz), δ 7,55 (H-2, d, J = 16,04 Hz), δ 6,81 (H-5, d, J = 8,68 Hz), δ 6,91 (H-4, dd, J = 8,86 Hz, J = 3,51 Hz), δ 7,48 (H-8, d)	+	+	+
10. 3-CQA	δ 2,07 (2H-13, m), δ 2,20 (2H-15, m), δ 4,19 (H-10, dd, J = 6,9 Hz, J = 2 Hz), δ 5,40 (H-12, m), δ 3,72 (H-11, m), δ 6,40 (H-1, d, J = 16,04 Hz), δ 7,55 (H-2, d, J = 16,04 Hz), δ 6,81 (H-5, d, J = 8,68 Hz), δ 6,91 (H-4, dd, J = 8,86 Hz, J = 3,51 Hz), δ 7,48 (H-8, d)	+	+	+
11. Choline	δ 3,21* ($N(CH_3)_3$, s), δ 3,49 (H-1, t, J = 9,51 Hz), δ 4,08 (H-2, t, J = 9,51 Hz)	+	+	+
12. Myo-Inositol	δ 3,28 (H-2, t, J = 9,59 Hz), δ 3,54 (H-5, t), δ 3,62 (H-1/H-3, dd, J = 11,45 Hz, J = 4,36 Hz), δ 4,02 (H-4, t, J = 9,59 Hz)	+	+	+
13. GABA	δ 1,90 (2H-2, t, J = 12 Hz), δ 2,32 2(H-3, m), δ 3,02*(2H-4, t)	+	+	+
14. Lactic Acid	δ 1,31* (3H-3, d, J = 3,92 Hz), δ 4,17 (H-2, d, J = 3,3 Hz)	+	+	+
15. Formic Acid	δ 8.47 (H-1, s)	+	+	+
16. Lipids	δ 0.94 (–CH_2-CH_3, br), δ 1.34 ((–CH_2-)n, br)			
17. Asparagine	δ 2.89* (H-3, dd), δ 2.96 (H-3, dd), δ 4.04 (H-2, dd)			

*The signal was used for the quantification analysis.

Table 2
Concentrations of the quantified metabolites in the coffee samples.

Metabolites	Concentration (in mM)			p value[2)]
	Regular Arabica Coffee[1)]	Caged Civet Coffee[1)]	Wild Civet Coffee[1)]	
Alanine δ 1.49 (δ 1.48–1.51)	1.60 ± 0.02^{a}	2.58 ± 0.04^{c}	2.20 ± 0.05^{b}	3.00E-09
Asparagine δ 2.89 (2.88–2.91)	2.85 ± 0.04^{a}	4.77 ± 0.05^{bc}	4.26 ± 0.05^{b}	2.99E-05
GABA δ 3.02 (δ 3.00–3.03)	1.45 ± 0.04^{a}	1.94 ± 0.03^{b}	2.45 ± 0.07^{c}	2.19E-05
Malic Acid δ 4.33 (δ 4.30–4.33)	8.70 ± 0.08^{a}	9.06 ± 0.03^{ab}	10.53 ± 0.04^{c}	1.74E-07
5-CQA δ 6.31 (δ 6.29–6.36)	16.6 ± 0.07^{c}	14.31 ± 0.03^{a}	15.40 ± 0.05^{b}	9.25E-08
Quinic Acid δ 4.15 (δ 4.14–4.17)	8.61 ± 0.03^{a}	11.81 ± 0.04^{c}	10.32 ± 0.04^{ab}	2.43E-09
Choline δ 3.21 (δ 3.19–3.22)	0.82 ± 0.09^{a}	0.98 ± 0.04^{b}	1.00 ± 0.11^{bc}	1.24E-04
Sucrose δ 5.41 (δ 5.39–5.44)	38.66 ± 0.16^{c}	35.10 ± 0.17^{b}	31.23 ± 0.27^{a}	5.99E-05
Caffeine δ 7.78 (δ 7.77–7.79)	10.00 ± 0.08^{c}	6.63 ± 0.08^{a}	7.82 ± 0.04^{ab}	8.29E-07
Trigonelline (δ 9.10–9.14)	6.87 ± 0.07^{a}	10.11 ± 0.06^{c}	8.53 ± 0.08^{b}	5.48E-06

[1)]Values are given as mean ± standard deviation (SD). The SD value (n = 3) < 0.5 indicates a good reproducibility.[2)]Ascorbic Acid is the positive control. [3)]Values are derived from one-way Anova analysis. p value < 0.05 means the significantly different. [a-d]Mean values followed by the different superscripts within the same row express the significant difference computed by Duncan multiple range test.

(metabolism products of sucrose) in the caged civet coffee as compared with their amounts in the wild civet coffee. Concentrations of other quantified metabolites in both coffee samples slightly vary as described in Table 2.

The quantified compounds in the green beans of both civet coffees (the caged and wild civets) were compared with those in the green beans of regular arabica coffees. Concentrations of alanine, asparagine, malic acid, and trigonelline were higher in the civet coffees than in the regular coffee sample as shown in Table 2. The higher amounts of alanine and asparagine in the civet coffees were probably caused by the protein degradation of the coffee fruits in the civet digestive system yielding amino acids (including alanine and asparagine) as reported in the previous work (Marcone, 2004). The higher concentration of trigonelline in the civet coffees could be caused by the differences in the post-harvest processing methods. The peeling process of the civet coffees was occurred in the civet digestive system yielding the green coffee beans that further cleaned with water for eliminating the dirt. Whereas the green beans of the regular coffee were processed with the wet method involving the soaking process in the water for about 12 h for removing the mucus. This soaking process possibly dissolved trigonelline, resulting in lower amount in the regular coffee than in the civet coffees.

Table 2 showed the concentrations of 5-CQA, caffeine, and sucrose were lower in the civet coffee than in the regular coffee samples. 5-CQA was selected representing other chlorogenic acids since it had non-overlapped signals. It was reported that this compound could be metabolized by bacteria in the large intestine cecum of Wistar rats (Lafay et al., 2006). Thus, it seems reasonable to assume that some 5-CQA of coffee fruits were also metabolized by bacteria in the civet digestive system, resulting in lower amount in the civet coffee than in the regular coffee. The lower caffeine content in the civet coffees was probably induced by the absorption and the degradation of caffeine into paraxanthine and methylxanthine in the small intestine of the civet as reported in the literature (Higdon and Frei, 2007). The lower concentrations of sucrose in the civet coffees possibly were caused by the breakdown of sucrose in the civet digestive system into other metabolites, including malic acid and citric acid (Geromel et al., 2006). Furthermore, it was reinforced by the higher levels of malic acid and citric acid in the civet coffees compared to their concentrations in the regular arabica coffee.

3.3. Multivariate data analysis

For obtaining a better analysis of differences in the levels of metabolites in all samples, the data set extracted from ^{1}H NMR spectra were further evaluated by multivariate data analysis. PLSDA was applied as a primary model for analyzing the metabolite profiles of green beans of all coffee samples. PLSDA is a supervised analysis approach with the nature of partial squared regression with good discriminatory power resulting the discriminant model for the analyzed groups (Ballabio & Consonni, 2013). The resulting PLSDA model had 0.64 (R2X) and 0.972 (R2Y) of total variations. Meanwhile, the value of Q2 was 0.947, showing a good

predictive level of the obtained PLSDA model.

By combining the first (44.6%) and the second (19.4%) components, the score plot of the PLSDA model resulted in a clear definition of three well-separated clusters corresponding to their green coffee bean types as depicted in Fig. 3A. This indicated that each coffee sample had a unique metabolite profile. The compounds contributing to the differentiation were identified by analyzing the loading plot. Based on the loading plot of the first component (Fig. 3B), sucrose (buckets at δ 3.67, 3.86, 3.96, and 4.19 ppm), chlorogenic acids (buckets at δ 2.02, 4.25, 5.29 and 5.31 ppm), caffeine (bucket at δ 7.72 ppm), lactic acid (buckets at δ 1.34 and 4.13 ppm), trigonelline (buckets at δ 4.41, 8.80 and 9.09 ppm), malic acid (bucket at δ 2.46 ppm) and citric acid (buckets at δ 2.58 and 2.71 ppm) were found as contributing metabolites in the classification of green coffee beans. This PLSDA model was further validated with 200 permutation tests (Fig. 3C). It resulted the regressions of Q2 lines intersected the y-axis at points below zero [Q2 = (0.0,0.218); R2 = (0.0, −0.303)], confirming the validity of the model.

Three models of two-classes PLSDA were generated for acquiring a better assessment of the discriminant metabolites for each green coffee bean sample. The S-plots of these models were investigated to reveal the most important buckets in the discriminations. S-Plot illustrates the magnitude of the correlation between intensity (p[1]) and reliability or consistency (p(corr)1) of a variable to contribute to the differentiating compounds in the two compared groups. Cutoff values for p [1] $\geq$ 0.05 and p (corr) $\geq$ 0.5 were used (Wei et al., 2012).

The first model was generated to discriminate the wild civet coffee from the regular coffee. This model resulted in R2X of 77.9%, R2Y of 99.7% and Q2 of 99.3%. The corresponding S-plot (Fig. 4A) analysis revealed the wild civet coffee was characterized with lactic acid (buckets at δ 1.34 and 413 ppm), citric acid (bucket at δ 2.58 ppm), malic acid (buckets at δ 2.46 and 2.71 ppm), and lipids (bucket at δ 1.28 ppm). Meanwhile, the regular coffee was identified with 5-CQA (buckets at δ 2.02 and 4.25 ppm), caffeine (bucket at δ 7.72 ppm), and sucrose (buckets at δ 3.67, 3.86 and 3.96).

The second model, obtained from the green beans of the caged civet coffee and the regular coffee, possessed R2X of 66.1%, R2Y of 99.6% and Q2 of 98.6%. In the corresponding S-plot (Fig. 4B), the sample of wild civet coffee was characterized with lactic acid (buckets at δ 1.34 and 413 ppm), malic acid (bucket at δ 2.46 ppm), trigonelline (bucket at δ 4.41 ppm) and lipids (bucket at δ 1.28 ppm). Meanwhile, the discriminant metabolites of the regular coffee sample were 5-CQA, (buckets at δ 2.02 and 4.25 ppm), caffeine (bucket at δ 7.72 ppm) and sucrose (buckets at δ 3.86 and 3.96).

The last model (Fig. 4C) comparing the green bean of wild civet coffee with the green bean of caged civet coffee, had R2X of 70.7%, R2Y of 99.5% and Q2 of 94.7%. In this S-plot, caffeine (bucket at δ 7.72 ppm), citric acid (bucket at 2.58 ppm) and malic acid (buckets at δ 2.46 and 2.71 ppm) were responsible compounds for the sample of wild civet coffee. Meanwhile, lactic acid (buckets at δ 1.34 and 413 ppm), trigonelline (bucket at δ 4.41 ppm) and lipids (bucket at δ 1.28 ppm) were found as the discriminant compounds for the caged civet coffee sample.

Based on the two-classes PLSDA models, citric acid and malic acid were the most characteristic compounds for the green bean of wild civet coffee since both always contributed to the corresponding discriminations. Malic acid as the characteristic compound in this sample was in accordance with the previous quantitative analysis. Meanwhile, lactic acid, trigonelline, and lipids were the most important marker for the caged civet coffee. These compounds were found as the discriminant metabolites of the caged civet coffee in the corresponding S-plots. Trigonelline as the marker of the caged civet coffee confirmed the result of the quantitative analysis explained before. The digestive system of the caged civet contained more lactic acid bacteria, compared to that of the wild civet coffee (Muzaifa et al., 2018). It possibly led to the higher concentrations of lactic acid and lipids in the caged civet coffee compared to the counterpart. According to the corresponding s-plot, regular arabica coffee was characterized by sucrose, caffeine, and 5-

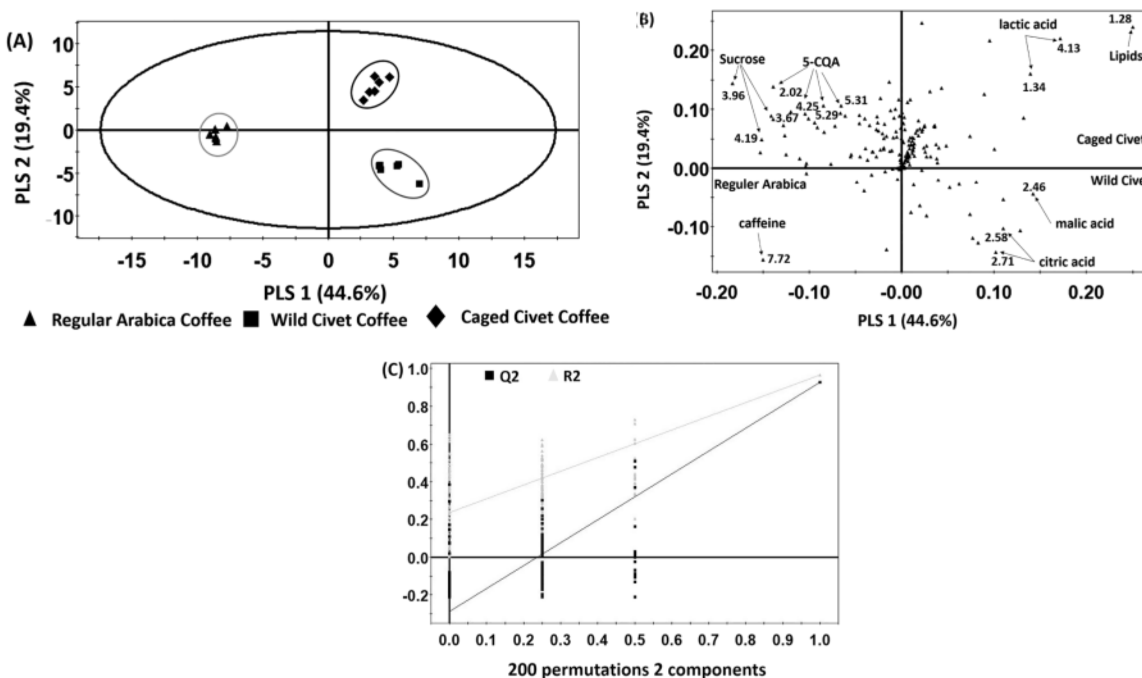

Fig. 3. The plots of the PLSDA model computed by considering all green coffee bean samples. The score plot (A) showed a good separation within the three test groups. The loading plot (B) reveals the compounds contributing on the discrimination, including malic acid, citric acid, lactic acid, lipids, trigonelline, sucrose, caffeine and 5-CQA. The permutation test plot (C) confirmed the validation of the model.

Fig. 4. S-plots generated from the two-classes PLSDA models. Cutoff values for $|p| \geq 0.05$ and $|p(corr)| \geq 0.5$, respectively. (A) Regular arabica coffee and the wild civet coffee. (B) Regular arabica coffee and the caged civet coffee. (C) The wild civet coffee and the caged civet coffee.

CQA. Thus, it verified the results of the quantitative analysis described before.

During the coffee beans in the digestive system of the civet, gastric juices and digestive enzymes of the civet penetrated into the beans resulting in the modification of the micro-structural properties of the beans and degradation of the storage proteins (Marcone, 2004). This penetration probably metabolized and degraded the coffee metabolites yielding the change of their concentrations. For instance, some sucrose in the coffee bean possibly was degraded by the gastric juices and (or) digestive enzymes resulting in organic acids, including citric acid and malic acid. It was verified by the higher levels of citric and malic acids, and the lower sucrose concentration in the civet coffees compared in the regular coffees (Table 2). Moreover, the protein degradation in the coffee beans possibly was confirmed by the higher concentration of alanine in the civet coffees than in the regular coffees. Therefore, the penetration of gastric juices and digestive enzymes into the coffee beans possibly altered the metabolite profiles as documented in the multivariate data analysis.

3.4. Antidiabetic activity

Coffee was reported to have antidiabetic activity (Ong et al., 2013). In this work, antidiabetic assay of the coffee samples was carried out to evaluate the correlation of the metabolite profile with the antidiabetic property. The antidiabetic activity test was performed using antioxidant tests and alpha-glucosidase inhibition assay. Antioxidant assay was selected as one of antidiabetic activity tests since coffee has ability to reduce the free radicals stimulating the chronic diabetes complications (Bajaj & Khan, 2014). Moreover, some characteristic compounds involved in the differentiation of coffee samples, were reported having antioxidant activity, including malic acid (Brittain, 2001), chlorogenic acid (Hunyadi et al., 2012), caffeine (León-Carmona & Galano, 2011), and trigonelline (Yoshinari & Igarashi, 2010). Meanwhile, alpha-glucosidase inhibitory assay was elected since the inhibition of this enzyme prevents the formation of sugars from the carbohydrate digestion process, thus decrease the diabetic risk the (Abbasi et al., 2019).

3.4.1. Antioxidant activity

The antioxidant activity of regular arabica coffee and both civet coffees were examined with the tests of $DPPH^{\bullet}$, $ABTS^{\bullet+}$, and FRAP. The IC_{50} value of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ tests, indicates the sample concentration required to inhibit 50% of the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$. Meanwhile, the EC_{50} value of the FRAP capacity test, indicates the sample concentration required to exhibit 50% of the FRAP capacity. The results showed that the highest antioxidant activity was shown by the green beans of the wild civet coffee (Table S1.1-S1.3). The IC_{50} values of this sample were 30.90 ± 0.08 mg/L ($DPPH^{\bullet}$ test, p value < 0.5) and 37.43 ± 0.15 mg/L ($ABTS^{\bullet+}$ test, p value < 0.5). Meanwhile, its EC_{50} value in the FRAP assay was 30.67 ± 0.13 mg/L. The green beans of the caged civet coffee had better antioxidant activity than the antioxidant activity of the regular arabica coffee. For instance, in the $ABTS^{\bullet+}$ test, the caged civet coffee had IC_{50} value of 44 ± 0.26 mg/L, while the regular coffee possessed IC_{50} value of 49.61 ± 0.36 mg/L. The IC_{50} plots of all coffee samples and ascorbic acid were depicted in Fig. S1A–S1C.

The correlation of the antioxidant activity with the quantified metabolites, including malic acid, trigonelline, 5-CQA and caffeine were evaluated with the Pearson correlation coefficient method. This statistical analysis result was presented in Table S2. Among the tested metabolites, malic acid was found as the most positively correlated compound with the antioxidant activity ($r \geq -0.921$, $p < 0.05$).

Trigonelline levels in the coffee samples possessed a positive correlation insignificantly with the antioxidant activity ($r \geq -0.255$, $p > 0.05$). Meanwhile, the concentrations of other tested metabolites, caffeine ($r \geq 0.687$, $p < 0.05$) and 5-CQA ($r \geq 0.587$, $p < 0.05$), had weak correlations with the antioxidant activity.

Malic acid as the most positively correlated compounds with the antioxidant activity was confirmed by the result of the quantitative analysis showing the wild civet coffee (the highest antioxidant activity), containing the most malic acid. Moreover, PLSDA analysis revealed malic acid as the characteristic compound for the wild civet coffee as well. Thus, this Pearson test result was in accordance with the results of the quantitative analysis and the multivariate data analysis. Interestingly, malic acid was also reported to have synergistic effect on the antioxidant activity (Quiroga et al., 2019, Keykhosravy et al., 2015). Malic acid had a great effect increasing the α-terpinene antioxidant activity (Quiroga et al., 2019). This compound also effectively maintained high level of antioxidant properties of *Agaricus bisporus* (Keykhosravy et al., 2015). Thus, this synergistic property probably led to the positive correlation of malic acid with the antioxidant activity of the coffee samples. Possibly, the higher concentration of malic acid, the greater synergistic effect on the antioxidant activity of the coffee samples. However, it still requires further investigation to study in detail the interactions between malic acid with the other metabolites responsible for the antioxidant activity of the coffees.

Based on PLSDA loading plot data, civet coffee had lower chlorogenic acid and caffeine levels and higher levels of malic acid. This metabolite profile change provided benefits to the resulting antioxidant activity (as depicted by lower IC_{50} values and reducing power). These results illustrated the possibility of civet coffee as a better antidiabetic compared to the regular arabica coffee. Provision of antioxidant supplements or functional foods containing antioxidants has the potential to improve endothelial dysfunction in T2DM (type 2 diabetes mellitus) so that it can reduce micro and macro-vascular complications due to diabetes mellitus (Bajaj & Khan, 2014).

3.4.2. Alpha-glucosidase inhibitory activity

The green beans of coffee samples were evaluated with the alpha-glucosidase inhibitory assay, an enzymatic *invitro* test. The inhibition of the alpha-glucosidase prevents the formation of sugars from the carbohydrate digestion process, thus decreasing the diabetic risk (Moein, 2019). The assay results revealed that the green beans of the wild civet coffee possessed the highest alpha-glucosidase inhibitory activity ($IC_{50} = 3.94 \pm 0.04$ mg/mL) as shown in Table S3. Meanwhile, the caged civet coffee ($IC_{50} = 4.77 \pm 0.13$ mg/mL) had better alpha-glucosidase inhibitory activity than the regular arabica coffee ($IC_{50} = 5.39 \pm 0.27$ mg/mL). According to the literature study, chlorogenic acid, malic acid, and trigonelline in coffee had inhibitory activity against the alpha-glucosidase (de Sales et al., 2012; Gou et al., 2014; Yoshinari & Igarashi, 2010). However, the alpha glucosidase inhibitory activity of chlorogenic acid was lower than the activities of malic acid and trigonelline (Gou et al., 2014; Oboh et al., 2015).

The Pearson correlation coefficient test was carried out to evaluate the correlation of some quantified metabolites with the alpha glucosidase inhibitory activity of coffee samples. The results showed the concentrations of trigonelline possessed an insignificant positive correlation with the inhibitory activity ($r = -0.659$, $p > 0.05$). The levels of 5-CQA ($r = 0.617$, $p < 0.05$) and caffeine ($r = 0.714$, $p < 0.05$) exhibited weak correlations with the alpha glucosidase inhibitory activity. Meanwhile, malic acid possessed the best positive correlation with the alpha glucosidase inhibitory activity significantly ($r = -0.976$, $p < 0.05$). Interestingly, this result was accordance with the quantitative analysis and PLSDA model evaluation. The wild civet coffee possessing the highest alpha glucosidase inhibitory activity containing the most malic acid. Moreover, malic acid was also found as the characteristic compound for the wild civet coffee in the PLSDA model analysis.

The inhibition kinetic of alpha-glucosidase was observed on the wild civet coffee samples having the highest inhibitory activity. The values of kinetic parameters, including the Michaelis-Menten constant (K_M : without sample; K_M* : with sample) and maximum reaction rate (V_{max} : without sample; V_{max}* : with sample), were calculated based on the double reciprocal Lineweaver-Burk plot as presented in Fig. S2. The regression data ($R^2 \geq 0.97$, $p < 0.05$) showed convergent lines intersecting the y-axis and the x-axis, as depicted in Fig. S2. The results showed that the wild civet coffee in the solution decreased the V_{max}* and increased the K_{M*} indicating the mixed inhibitory mechanism (Table S4). A mixed inhibitor binds exclusively to the enzyme-substrate complex yielding an inactive enzyme-substrate-inhibitor complex (Ahmed et al., 2014). This result was accordance with the previous work reporting the mechanism of the mixed inhibition in coffee samples (Alongi & Anese, 2018). Malic acid was reported to possess a non-competitive inhibitory mechanism (Gou et al., 2014). It was different from the coffee samples that have a mixed inhibition mechanism. However, not only malic acid contributed to the alpha-glucosidase inhibitory activity of the coffee samples. Trigonelline, 5-CQA and caffeine contributed to the activity but unfortunately, to the best of our knowledge, there is no scientific report regarding their inhibition mechanisms.

This study is the first scientific report on the antidiabetic activity of the civet coffees, both wild and caged civets. Although this study results were still far from correlating the effects of the civet coffee consumption on diabetes, however the changes in the metabolite profile of coffee after fermented by civet showed a positive effect on antioxidant activity and inhibition of alpha-glucosidase enzymes. Both were *invitro* tests related to type 2 diabetes mellitus. *In-vivo* validation of this activity would open up the possibility to claim the use of civet coffee as an antidiabetic functional food.

4. Conclusions

PLSDA model were successfully classified the green coffee beans based on their coffee types. Our results confirmed the fermentation in the civet digestive system leads to the change on metabolomic profiles of the green beans of the civet coffees. Compared to the regular coffee, the green bean of the civet coffees had higher concentrations of lactic acid, citric acid, malic acid and trigonelline, and had lower contents of caffeine, sucrose and chlorogenic acids. Meanwhile, lactic acid and lipids were identified as the most contributing compounds in discriminating the caged civet coffee from the wild civet coffee. Interestingly, the assay results indicated that the change on metabolite profiles of both civet coffees possessed a positive effect with their antidiabetic activities.

CRediT authorship contribution statement

Lizma Febrina: Methodology, Investigation, Data curation, Writing - original draft. **Nizar Happyana:** Conceptualization, Methodology, Investigation, Data curation, Writing - original draft, Writing - review & editing, Supervision, Visualization, Funding acquisition. **Yana Maolana Syah:** Data curation, Supervision, Visualization.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.129496.

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