H NMR-Based Metabolomics Approach Revealing Metabolite Variation of Black Turmeric (Curcuma caesia) Extracts and Correlation with Its Antioxidant and α-Glucosidase Inhibitory Activities

Running head: Bioactivities and Metabolomic Analysis of Black Turmeric (Curcuma caesia) Extracts

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SUMMARY

Research background. Curcuma species (Zingiberaceae) are well known medicinal herbs in India and Southeast Asia. Despite various findings reporting their beneficial biological activities, very little information has been recorded on the Curcuma caesia. Thus, this study aims to determine the phenolic content, antioxidant and α-glucosidase inhibitory activity of both rhizome and leaves of C. caesia.

Experimental approach. Rhizome and leaves of C. caesia were dried with oven (OD) and freeze (FD) drying methods, and extracted with different ethanol/water ratios of 0/100, 50/50, 80/20 and 100/0. The bioactivities of C. caesia extracts were evaluated using in-vitro tests; total phenolic content (TPC), antioxidant (DPPH and FRAP) and α-glucosidase inhibitory activity. Proton nuclear
magnetic resonance (\(^1\)H NMR)-based metabolomics approach was employed to differentiate the most active extracts based on their metabolite profiles and correlation with bioactivities.

**Results and conclusions.** The 100/0 FD rhizome extract was observed to have potent TPC, FRAP and \(\alpha\)-glucosidase inhibitory activity with values of \((45.38\pm2.10)\) mg GAE/g extract, \((147.73\pm8.26)\) mg TAE/g extract and \((265.46\pm38.62)\) \(\mu\)g/mL (IC\(_{50}\)), respectively. Meanwhile, for DPPH scavenging activity, the 80/20 and 100/0 extracts of FD rhizome showed the highest activity with no significant difference between them. Hence, the FD rhizome extracts were selected for further metabolomics analysis. Principal component analysis (PCA) showed clear discrimination among the different extracts. Partial least square (PLS) analysis showed positive correlations between the metabolites, including xanthorrhizol derivative, \(l\)-hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)-(6E)-6-heptene-3,4-dione, valine, luteolin, zedoardiol, \(\beta\)-turmerone, selina-4(15),7(11)-dien-8-one, zedoalactone B and germacrone, with the antioxidant and \(\alpha\)-glucosidase inhibition activities. Meanwhile, curdione and 1-(4-hydroxy-3,5-dimethoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-(lE,6E)-1,6-heptadiene3,4-dione were correlated with \(\alpha\)-glucosidase inhibitory activity.

**Novelty and scientific contribution.** *C. caesia* rhizome and leaves extracts contained phenolic compounds and had various antioxidant and \(\alpha\)-glucosidase inhibitory capacities. These findings strongly suggest that the rhizomes of *C. caesia* are an invaluable natural source of active ingredients for applications in pharmaceutical and food industries.

**Keywords:** antioxidant activity; \(\alpha\)-glucosidase inhibition; *Curcuma caesia*; extraction; metabolomics

**INTRODUCTION**

Plants have been exploited for various purposes including in treating health problems. According to WHO, more than 80% of the world's population uses herbal plants for combating diseases. Zingiberaceae family comprises more than 80 different species of rhizomatous herbs, including *Curcuma caesia*. It is known as black turmeric and is mostly native to tropical South Asia. The plant is used traditionally to treat various ailments including asthma, bronchitis, cough, cancer, epilepsy, fever, wounds, leucoderma, pneumonia, cold, piles, tumour, toothache, vomiting and gout (1,2). Several studies conducted on *C. caesia* revealed its bioactivities, such as anti-bacterial, antimicrobial, anti-fungal (3), anti-tumor, anti-ulcer (4), anti-cancer, anti-thrombolytic (5), antioxidant, anti-inflammatory, and analgesic agents (6,7). Moreover, this plant is discovered to contain steroids, phenols, alkaloids, flavonoids, and tannins (8) as well as essential oils (9,10). These metabolites might be correlated with the medicinal properties of this plant. Several studies have been performed on the bioactivities of *C. caesia*, however findings on the metabolites responsible for their pharmacological activities are still scarce.
Metabolomics has emerged as an advanced technology tool, which involves quantitative and qualitative assessments of small molecules known as metabolites. It has diverse applications in various science fields, including human diseases, pharmacology, drug discovery, toxicology, nutrition, and crop improvement using samples ranged from plant and food to human and animal biofluids or tissues (11). In metabolomics studies, metabolic profiles can be acquired through high-throughput metabolic analysis using technologies, such as liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) followed by multivariate data analysis (MVDA) for data mining (12). Among the various analytical platforms, proton NMR (\(^1\)H NMR) is a popular choice due to its durability, repeatability, non-destructive nature, and easy sample preparation. In addition, the large number of compounds that have been identified lead to the emergence of \(^1\)H NMR as a primary analytical tool in metabolomic studies (13). In the study of medicinal plants, the combination of \(^1\)H NMR and MVDA has been used to determine the phytochemical changes of plant extracts resulted from different processing steps and their relationship with the studied bioactivity (14,15).

Plant metabolites are made up of non-polar and polar compounds that play essential roles in preventing oxidative stress and acting as free radical scavengers which responsible for the development of degenerative diseases such as cancers and diabetics (16). For the development of plant (herb)-based nutraceutical products, these valuable compounds should be extracted efficiently. Factors such as drying techniques, extraction method, parameters during extraction such as solvent, temperature and time, particle size of raw materials and solid-to-liquid ratio are among those affecting the extraction efficiency (17). Efficacy of extraction affects the amount and types of extracted metabolites from the plant matrix, which reflects the plant extract’s bioactivities. Therefore, the optimizing of the combination of drying techniques and extraction solvents is needed to ensure maximum bioactive metabolites were extracted from the samples.

In this research, the antioxidant and α-glucosidase inhibition activities of rhizomes and leaves of *C. caesia* dried with different drying techniques freeze drying (FD) and oven drying (OD) and extracted with different ethanol to water ratios (0/100, 50/50, 80/20, 100/0) were measured. The most potential and promising extract in terms of bioactivity were then analyzed using \(^1\)H NMR-based metabolomic approach for the chemical profiles. In addition, the correlations between metabolites and the bioactivities (DPPH, FRAP and α-glucosidase) were also determined by applying different MVDA tools. Thus, the outcome of this study may enhance the potential value of *C. caesia* and provide useful information for further research study.

**MATERIALS AND METHODS**

*Chemicals*
All chemicals used in this research are of analytical grade. The chemical used were Folin-Ciocalteau phenol reagent (Merck, Darmstadt, Germany), sodium carbonate (Na₂CO₃), gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ethanol, methanol, ascorbic acid, butylated hydroxyanisole (BHA), ferric chloride hexahydrate (FeCl₃.6H₂O) (Merck, Darmstadt, Germany), 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ), hydrochloric acid (HCl), sodium acetic trihydrate, glacial acetic acid (Merck, Darmstadt, Germany), Trolox, α-glucosidase (Megazyme, Sydney, Australia), p-nitrophenyl-α-glucopyranoside (p-NPG substrate), sodium phosphate dibasic, sodium phosphate monobasic, glycine, and quercetin. For NMR, chemicals used were potassium phosphate monobasic (KH₂PO₄), deuterated methanol-d₄ (CD₃OD), deuterium oxide (D₂O), sodium deuteroxide (NaOD) (Cambridge Isotope Laboratories, Tewksbury, USA), 3-(trimethylsilyl)propionic acid-d₄ sodium salt (TSP) (Acros Organic, Geel, Belgium). All other non-stated source of chemicals was from Sigma-Aldrich (St. Louis, MO, USA).

Plant materials

Plant samples (Fig. 1) were collected from Seri Subuh Herbal Park located in Kuala Pilah, Negeri Sembilan, Malaysia. The plant identification and certification was performed by botanists from the Herbarium research unit, Universiti Kebangsaan Malaysia (UKM) with specimen number of UKMB40386.

Drying process

After collection, samples were rinsed with water to remove debris. The rhizome and leaf samples were labelled and separated into two groups, which were FD and OD. The FD sample was prepared by subjecting it to freezing (HVF-301S Hesstar, Selangor, Malaysia) at −20 °C for 24 h and then lyophilization in a freeze dryer (Freeze Dryer Alpha 1-2 LD Plus, Osterode, Germany). Meanwhile for OD, sample was placed in the laboratory oven at 40 °C (UM400, Memmert Universal Ovens, Schwabach, Germany) under forced-air ventilation. The grinding was for the dried samples to a fine powder using a laboratory grinder (Waring Blender 7011S, Torrington, USA) and sieving was done using a 300 µm siever (mesh size: No. 50).

Sample extraction

Sample extraction was performed with some modifications (18). Ethanol/water in the ratio of 100/0, 80/20, 50/50, and 0/100 were used as extraction solvent. The dried powder (20 g) of each rhizome and leaf were immersed in 200 mL of solvent and left for 72 h in an incubator shaker (Ika Control Cooled Incubator Shaker KS4000, Wilmington, NC, USA) at ambient temperature 25 °C. Next, the filtration was performed using Whatman filter paper No. 1 and this step was repeated three times
for every 24 h. The filtered solvent was then combined before being removed using a rotary evaporator at 40 °C. The resulting viscose samples were frozen at -20 °C overnight and dried in a freezer dryer to remove excess water. All of the extracts were dissolved into the concentration required for the use of chemical analysis.

**Total phenolic content assay (TPC)**

The TPC of *C. caesia* extracts were determined by using method in the previous literature with slight modification (18). Each sample (20 µL) with concentration 5000 µg/mL was transferred into a 96-well microplate, followed by 100 µL Folin Ciocalteu reagent and let to rest for 8 min. Later, 80 µL of 7.0 % Na₂CO₃ solution was added into the mixture. The resulting mixture was shaken before incubated in the dark for 60 min at room temperature. The absorption readings were taken using the spectrophotometer (Epoch™ Microplate Spectrophotometer, Biotek Instrument, Santa Clara, USA) at 725 nm when complex blue was produced. The same procedure was repeated for the standard gallic acid solution and the calibration curve was obtained as follows:

\[ y=0.0057x+0.147, \quad R^2=0.9982 \]  

Based on the absorbance readings, the TPC of the sample was calculated from the calibration curve and results are expressed as milligrams gallic acid equivalence per gram extract (mg GAE/g extract).

**DPPH radical scavenging assay**

DPPH radical scavenging assay was examined and modified from the method described in the literature (18). Briefly, 250 µL of methanolic 0.1 mM DPPH solution was added to 50 µL sample extracts (78.13–5000 µg/mL) in a 96-well microplate. The mixtures were allowed to be in the dark for 30 min at room temperature. After 30 min, the absorption readings were determined at wavelength 517 nm by Epoch™ Microplate Spectrophotometer, Biotek Instrument (Santa Clara, USA) against a blank containing 250 µL of methanol. The percentage of DPPH scavenging activity was evaluated using the formula:

\[ \text{Scavenging activity}=\left(\frac{A_b-A_s}{A_b}\right)\times 100 \]  

where \( A_b \) is the blank absorbance and \( A_s \) is the sample absorbance. The procedure was repeated by replacing the sample with ascorbic acid (AA) and butylated hydroxyanisole (BHA) as positive controls. The expression of results was as IC₅₀ in µg/mL, which was obtained through linear regression analysis of the standard curve of the samples. DPPH IC₅₀ is the concentration of sample substance that achieve 50 % scavenging activity of DPPH free radicals.

**Ferric reducing assay (FRAP)**
Determination of the ferric reducing activity of sample extract performed referring to the literature (18). Three reagents namely 20 mM FeCl$_3$·6H$_2$O solution, 10 mM 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) solution in 40 mM HCl and acetate buffer solution (CH$_3$COONa) were prepared separately. Acetate buffer solution, TPTZ solution, and FeCl$_3$·6H$_2$O solution were mixed to produce fresh FRAP solution at a volume ratio of 10:1:1. The sample extracts (0.5 mL) were added with 1.95 mL of fresh FRAP solution. The mixture was then incubated for 30 min in the dark before transferred into a 96-well microplate. The absorbance was read at 593 nm wavelength using an EpochTM Microplate Spectrophotometer, Biotek Instrument (Santa Clara, USA). The same procedure was repeated for the standard Trolox solution and the calibration curve was obtained as follows:

$$y=0.0023x+0.1862, \ R^2=0.9997$$

The results are reported as milligrams of Trolox equivalence per gram extract (TAE/g extract).

$\alpha$-Glucosidase inhibition assay

The $\alpha$-glucosidase inhibitory activity was performed based on the method described by previous study with slight modifications (18). The positive control was quercetin based on its significant activity. The substrate solution ($\rho$-NPG) was prepared in 50 mM phosphate buffer (pH=6.5). As for enzyme stock solution, 15 μL of $\alpha$-glucosidase was added to 4.985 mL of the phosphate buffer and kept in ice-cold water prior used. For this assay, 100 μL of 30 mM buffer solution and 15 μL of $\alpha$-glucosidase (3 U/mL) together with sample extract were pre-incubated in 96-well plates at 25 °C for 5 min. Then, 75 μL of p-NPG substrate was added and the reaction mixture was incubated for 15 min at 25 °C. The reaction was stopped by adding 50 μL of 2M glycine (pH=10) to the mixture. Blank samples were prepared using the same method as extract samples. However, the enzyme and substrate solution used in the experimental sample were replaced with (50 μL) 30 mM buffer phosphate solution while glycine was replaced with (50 μL) distilled water. Sample extracts tested were in the range of 78.13–5000 µg/mL. Absorbance readings were taken using the EpochTM Microplate Spectrophotometer, Biotek Instrument (Santa Clara, USA) at 405 nm. The percentage of $\alpha$-glucosidase inhibition activity was determined by using the formula:

$$\text{Inhibition activity} = \left( \frac{\Delta A_{t} - \Delta A_{s}}{\Delta A_{t}} \right) \times 100$$

where $\Delta A_t$ is the difference between a negative control (with an enzyme) and blank (without an enzyme), meanwhile $\Delta A_s$ is the difference in absorption between sample (with enzyme) with blank (without enzyme). Results are expressed as IC$_{50}$ (µg/mL), which imply the concentration of the sample required to achieve 50 % enzyme inhibition.

$^1$H NMR analysis for metabolomics
The ¹H NMR analysis was done according to the previous methods (19,20). A 20 mg freeze-dried C. caesia extract was put into a 2 mL microcentrifuge tube, and dissolved with a total 0.75 mL of 1:1 mixture of methanol-d₄ and KH₂PO₄ (pH 6.0) buffer in D₂O containing 0.1 % TSP. The mixture was vortexed for 1 min and followed by ultrasonication for 15 min at room temperature. Then, the mixture was centrifuged at 13 000 rpm for 10 min. After that, a clear supernatant volume of 600 µL was transferred into NMR tubes before subjecting to ¹H NMR analysis. The ¹H NMR analysis was performed using a 500 MHz Varian INOVA NMR spectrometer (Varian Inc., California, USA), run at a frequency of 499.887 MHz at room temperature. Each ¹H NMR spectrum contained 64 scans, 20 ppm width, and 3.53 min acquisition time. To enhance the identification of metabolites, 2D NMR J-resolved was implemented. The J-resolved spectra were analyzed in 50 min and 18 s acquisition time with 8 scans per 128 increments for the axis of the spin-spin coupling constant with spectral widths of 66 Hz and 8 K for the chemical shift axis with spectral widths of 5000 Hz. The relaxation time delay was 1.5 s. Phasing and baseline correction was done using the Chenomx v. 5.1 software (21). The spectral were binned into a 0.04 ppm width and regions containing water (δ 4.70–5.10) and residual methanol (δ 3.26–3.35) were excluded from all spectra.

Statistical analysis

Each analysis was performed with six replicates (N=6). Data are expressed in mean±standard deviation. All data analyses were evaluated using one-way and two-way ANOVA with Tukey's comparison in Minitab v. 17 software (22). Significant differences between the samples were considered at p<0.05. As for NMR, after binning all the spectra, principal component analysis (PCA) and partial least square (PLS) were performed for multivariate data analysis (MVDA) by SIMCA-P v. 13 software (23). The selected scaling method was Pareto to reduce the effect of noise.

RESULTS AND DISCUSSION

Effects of drying methods and extraction solvent on total phenolic content (TPC)

The TPC of C. caesia rhizome and leave extracts acquired from the various groupings of drying methods and ethanol/water ratios are shown in Table 1. Based on the two-way ANOVA results, drying techniques (p=0.024) and different solvent ratios (p=0.000) had a significant effect (p<0.05) on the phenolic content in C. caesia rhizome extract. For the leaf extract, the drying factor (p=0.302) did not affect the TPC instead the solvent ratio (p=0.000) had a significant effect.

The TPC value of C. caesia extracts ranged from (16.21±1.47) to (45.38±2.10) mg GAE/g sample extract. Rhizome FD 100/0 revealed significantly (p<0.05) highest phenolic content compared to the other extracts. For the effect of the drying method, different trends were observed in the samples
extracted with different solvents. FD was seen to have better ability in the preservation of phenolic compounds than OD, on rhizome extract with a higher ethanol ratio which were 100/0 and 80/20. In contrast, OD samples showed higher TPC especially 50/50 or 0/100 extract that is water as the solvent. However, the combination of the two factors in this study, FD with ethanol ratio of 100/0 and 80/20 produced significantly higher TPC values than OD combined with 50/50 and 0/100, with the highest overall TPC (p<0.05) is by rhizome extract of PSB 100/0. These results are in line with literature report which recommend a low-temperature drying technique to retain the active ingredient in plants (24). The mechanism of FD in sublimation drying and freezing temperatures (-80 ºC) has inhibited the enzyme and microbial activity, thereby preventing biochemical reactions that can alter organoleptic properties which is associated by molecular structures of the compounds and their concentrations in plant samples (25).

Besides the drying effect, the choice of extraction solvent is important regarding the specific nature of the targeted bioactive compounds. According to Do et al. (26), ethanol is an efficient solvent in extracting polyphenols, and it is recognized as safe for human use. However, water solvents are the opposite due to polyphenol compounds that are more soluble in organic solvents whose polarity is lower than that of water (27). Moreover, other studies have also stated that solvents with lower viscosity and density levels are able to perform better absorption into plant cavities to produce bioactive compounds more easily (27,28). The density of ethanol (0.789 g/cm³) is also lower than that of water (1.0 g/cm³), this makes ethanol more effective in the extraction process of phenol compounds (18).

The results of this research found that TPC increased with increasing ethanol ratio, except for FD leaf extract where 80/20 and 50/50 extracts contained higher TPC (p<0.05) than 100/0 extract. Meanwhile, aqueous extracts (0/100) from all samples showed lower TPC values (p<0.05) compared to 100/0 and 80/20 extracts. A study by Sajak et al. (29) on Ipomoea aquatica extract also observed higher TPC values in extracts with higher ethanol percentage (50–100 %) compared to aqueous extracts. This may be because water solvents have higher potential to extract large macromolecules such as inactive organic acids, proteins and carbohydrates but are less effective in the extracting phenolic compounds that contribute to the antioxidant activity of the extract (30). In fact, ethanol is also capable to inactivate enzymes such as polyphenol oxidase that is released into the solvent when plant cell wall breakage during the extraction process (31). Thus, more phenolic compounds can be retained in ethanol solvent than in aqueous solvent. Overall, the results of the study indicate that the combination of FD and 100/0 is a good choice for preservation and extraction of high TPC from C. caesia.

Effect of drying methods and extraction solvent on the antioxidant activity
The ability of plant to act as an antioxidant agent is influenced by several factors, which largely depends on the composition of the extract and the analytical test system. The use of only one method cannot describe the antioxidant activity of an extract due to the diversity of molecules and compounds that act as antioxidants have different mechanisms (32). Thus, the present study conducted two antioxidant assays; DPPH free radical scavenging and ferric reducing antioxidant power (FRAP) assay. Again, the results of two-way ANOVA showed that the drying technique (p=0.000) and the different solvent ratio (p=0.000) had a significant effect (p<0.001) on the antioxidant activity in C. caesia rhizome extract. However, the drying technique (p=0.716) did not have an effect on the DPPH activity of C. caesia leaf extract, but was effective in FRAP activity (p=0.001). Meanwhile, the solvent ratio remained significant (p=0.000) on both antioxidant activities.

The antioxidant activity by C. caesia extract is as presented in Table 1. DPPH is a stable free radical and is often be used in the antioxidant analysis of plant extracts as well as food extracts (33). The scavenging activity of DPPH is expressed in IC\textsubscript{50} readings (µg/mL), which is lower values reflect stronger scavenging activity by the extract. From the result, different trends were observed in the combinations of drying methods and extraction solvents. None of extracts had a lower IC\textsubscript{50} compared with the positive control, ascorbic acid (25.26±1.24 µg/mL) and BHA (44.42±4.47 µg/mL). This is expected because among abundance of compounds in the extract, there might have compounds that exhibit antagonistic effect with other compounds that possess antioxidant properties. In contrast, the positive controls used were pure active compounds.

In terms of drying method, a similar trend with TPC was observed in which FD extract showed better DPPH scavenging activity with lowest IC\textsubscript{50} values detected by FD rhizomes 100/0 and 80/20 with (539.89±58.04) µg/mL and (484.02±63.80) µg/mL respectively. However, OD rhizome extract showed the opposite trend to TPC, with OD 50/50 extract showing significantly higher scavenging activity than OD 100/0. This condition can be explained by the potential presence of other non-phenolic components in the extract that act to accelerate the process of hydrogen transfer to DPPH radicals. According to previous record, biologically active water-soluble protein, known as turmerin have been reported from C. longa (34) with significant antioxidant activity. Besides, Angel et al. (35) found that extracted proteins from aqueous C. caesia rhizome exhibited high antioxidant activity and was comparable to C. zedoaria.

Meanwhile for FRAP assay, ferric reducing power by C. caesia extracts was in the range of 20.94 – 147.73 mg TAE/g extract. Again, the results obtained illustrate a similar trend with TPC when FD rhizomes with higher ethanol ratios showed better ferric reducing activity compared to OD extract. This finding can be explained by two possibilities; (1) the presence of phenolic compounds as major metabolites contributing to the antioxidant activity of the extract, (2) the effect of applied heat during the drying process (OD) has resulted in a decrease of the amount of metabolites beneficial to the
antioxidant activity of *C. caesia* rhizomes due to heat-sensitive metabolites being degraded. Meanwhile, the sublimation process in the FD method leads to the formation of ice crystals in the plant matrix, which in turn creates compressive forces and helps to break the plant cell wall (36), thus the metabolites can be extracted more easily from the plant matrix.

Overall, 0/100 extract showed the lowest FRAP activity and these results were seen to be consistent in all the tests that were conducted. The ethanol content in the extraction solvent helps in the extraction of a large number of antioxidant compounds. On the other hand, the low antioxidant activity shown by the aqueous extract can be explained due to the low solubility of antioxidant compounds in aqueous solvents. Sim *et al.* (37) also reported ethanol as the most effective extraction solvent for polyphenolic compounds influencing the antioxidant, antimicrobial, and anti-tyrosinase activities of *Hibiscus cannabinus* L. leaves compared to water solvents. These findings are also agreed by other researchers that ethanol solvent is more effective organic solvent in phenolic antioxidant (38,39). Therefore, it is very crucial to choose the right drying technique and solvent extraction for optimum outcome.

**Effect of drying methods and solvent on α-glucosidase inhibitory activity**

The α-glucosidase inhibitory activity is aimed at detecting the potential of the extract as an anti-diabetic agent. Based on the previous study, *C. caesia* extract was able to inhibit α-glucosidase activity (40). However, information related to the effect of drying techniques and solvent ratios on this bioactivity has not yet been clearly identified. In this study, extracts of rhizomes and leaf of *C. caesia* from the drying method (p=0.000) and different solvent ratios (p=0.000) were found to possess a significant effect (p<0.001) on the inhibitory activity of α-glucosidase enzyme. The results of α-glucosidase inhibition of leaf extract and rhizome of *C. caesia* are showed in Table 2. As can be observed, at the concentration of 625 µg/mL, the extract showed inhibitory activity between mild to good inhibitory activity with percentage value of 2.96–76.02 %. FD rhizome extract gave higher inhibitory activity (p<0.05) compared to OD, except for aqueous extract (0/100). Meanwhile, no significant differences (p>0.05) were noticed on the drying techniques and solvents used on the leaf extract except for higher inhibitory activity by FD 100/0. Again, the weakest inhibitory activity (p<0.05) among all extracts was the aqueous extract (0/100).

The IC50 value is the concentration of the extract that can inhibit 50 % of the enzyme α-glucosidase activity, which a lower IC50 value means the extract is more potent. The IC50 value of the extract was determined and compared with quercetin (IC50=(42.36±3.63) µg/mL). IC50 values were measured only in 100/0, 80/20 and 50/50 extracts as the rest of the extracts did not achieve 50% inhibition activity at the observed concentration. The lowest IC50 value was shown by the rhizome of FD 100/0 (IC50=(265.46±38.62) µg/mL) without significant differences with the rhizome of FD 80/20
(IC₅₀=(519.99±49.29) µg/mL). This result is probably because of the presence of a large number of polyphenol compounds that have been successfully extracted from the plant matrix. Other studies have also reported the ability of polyphenols in inhibiting the activity of carbohydrate digestive enzymes; α-amylase and α-glucosidase (41,42). The presence of hydroxyl and galols groups in the molecular structure of polyphenols contributes to the formation of hydrogen bonds and hydrophobic associations between polyphenols and enzymes thus assist in inhibiting enzyme activity and control postprandial hyperglycemia in T2DM diabetic patients.

Moreover, previous study by Majumder et al. (40) also reported the inhibition against α-glucosidase by methanolic extract of C. caesia rhizome but with lower IC₅₀ ((95.40±9.74) µg/mL) value. This difference might be due to several factors that influenced the extraction and bioactivities of the sample extracts including different geographical origin, preparation process, extraction method, drying technique, type as well as concentration of extraction solvent used (43). The present study found that rhizome C. caesia was more active compared to the leaf. The combination of FD and higher ethanol solvent ratios of both 100/0 and 80/20 in C. caesia rhizome extraction exhibited good results throughout the conducted tests. By considering this outcome, rhizome FD C. caesia extract extracted with the different ethanol/water ratio (0/100, 50/50, 80/20, and 100/0) were selected for further study using principal component analysis to identify the metabolite differences and find out the correlation between the bioactivities and metabolites using ¹H NMR-based metabolomics approach.

Metabolite identification in C. caesia rhizome

The metabolite identification was performed based on spectra from the 1D and 2D NMR (Fig. S1). The ¹H NMR spectra of FD C. caesia rhizome are shown in Fig. 2, which represents the signal of metabolites present in the extract including primary and secondary metabolites. In general, the ¹H NMR spectra of plant sample extract represents the signals of metabolites that are divided into three regions; namely aliphatic region (δ 0.5 - 3.0), carbohydrate region (δ 3.0 - 5.5), and aromatic region (δ 5.5 - 9.0).

A total of 27 metabolites were identified from this plant extract and their characteristic ¹H NMR signals were tabulated in Table 3. The identified metabolites were sucrose, glucose, amadannulen, curdione, xanthorrhizol derivative, germacrone, β-turmerone, curcuzederone, l-hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)-(6E)-6-heptene-3,4-dione, 1-(4-hydroxy-3,5-dimethoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-(IE,6E)-1,6-heptadiene3,4-dione, 13-hydroxygermacrone, xanthorrhizol, luteolin, threonine, valine, choline, formic acid, selina-4(15),7(11)-dien-8-one, zedoardiol, zedoalactone A, zedoalactone B, curzerene, zerumin B, gallic acid, demethoxycurcumin and curcumin. The detection of these metabolites was done by comparing the NMR chemical shifts and coupling constants with other research findings as well as 2D NMR experiments. The signal
differences, especially in the carbohydrate and aromatic regions were seen in all extracts. However, compared to other extracts, the $^1$H NMR signals in these regions were less prominent in 0/100 extract. Therefore, this section was given more attention as they are likely to act as a differentiating factor between the extracts.

Metabolites of various classes were indicated by the $^1$H NMR signals, including amino acids, organic acids, phenolic, carbohydrates, and terpenoids. Amino acids such as threonine ($\delta$ 1.33 d, $J=6.75$ Hz, $\delta$ 3.50 d, $J=4.0$ Hz) and valine ($\delta$ 1.03 d, $J=6.95$ Hz, $\delta$ 1.13 d, $J=6.05$ Hz) were successfully identified (44,45). Meanwhile, phenolic compounds detected in aromatic region were curcumin, 1-(4-hydroxy-3,5-dimethoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-(1E, 6E)-1,6-heptadiene3,4-dione and demethoxycurcumin, 1-hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)-(6E)-6-heptene-3,4-dione. Besides, in the same region there were also signals for zedoalactone B, luteolin, xanthorrhizol derivative, gallic acid and formic acid ($\delta$6.07s, $\delta$6.52s, $\delta$6.66 (d, $J=7.65$Hz), $\delta$7.15s, $\delta$8.47s) (14,29). Furthermore, metabolites consisting of sugars such as sucrose, $\alpha$-glucose, and $\beta$-glucose were identified based on signals from the carbohydrate range ($\delta$ 3.0 - 5.5) (46).

Other than that, metabolites from the terpenes group most commonly found as constituents in essential oils were also present in the extracts. Zerumin B was among those detected with two singlet chemical shifts $\delta0.68s$ and $\delta2.44s$. Sesquiterpenes such as curcuzederone, curdione, curzerene, germacrone and selina-4(15),7(11)-dien-8-one were successfully found. Two singlets at $\delta1.44s$ and $\delta1.61s$ were assigned to 13-hydroxygermacrone. Signals at $\delta1.88s$ and $\delta2.00s$ were matched with xanthorrhizol, while the three singlet signals $\delta1.10$, $\delta1.15$, $\delta2.19$, and one doublet ($\delta6.66$, $J=7.65$Hz) referred to the xanthorrhizol derivative. Choline was also identified at two chemical shifts $\delta3.22s$ and $\delta3.32s$.

**Principal component analysis (PCA) for C. caesia rhizome extract**

The comparison of the metabolite profile of *C. caesia* extract extracted with different ethanol/water ratios was assessed using the MVDA method as the PCA model. This method helps to identify differences or similarities between samples with the help of score plots. Fig. 3a shows a clear separation between extracts in the PCA score plot without any significant outliers. The total variance of the model was 80.6 %, for which PC1 contributed by 69.0 % and PC2 with 11.6 %. This model is considered a good model because its goodness of fit, R2X (cum) and Q2(cum)>0.5, as well as the difference between these two, was <0.3, showing the uniformity of each extract in contributing to the separation of each group (29).

As shown in Fig. 3a, four clusters can be seen representing each extract, 0/100, 50/50, 80/20 and 100/0. Based on the score plot, the 100/0 extract was separate from the others by PC2, meanwhile the 80/20 extract was separated from other samples by PC1. Through the score plot,
separate samples form clusters based on differences in ethanol and water solvent ratios, indicating the possibility of metabolic changes due to the solvent ratios used. Meanwhile, the loading plot (Fig. 3b) completes the PCA model by showing phytochemical metabolites that contribute to cluster separation between extracts. The identification of metabolites allows compounds that contribute to the separation between study samples and compounds commonly found in samples to be identified.

According to the loading plot (Fig. 3b), most of the metabolites were concentrated in the central and right quadrant, indicating higher metabolites constituents in extracts with more ethanol ratio (100/0 and 80/20). However, 13-hydroxygermacrone (5), amadannulen (11), α-glucose (23), and curzerene (26) were seen as the compounds that separate 80/20 extract from other extracts (0/100, 50/50, and 100/0). Based on the relative quantification of the metabolites in the extracts (Fig. 4), metabolite phenolic 1-(4-hydroxy-3,5-dimethoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-(1E, 6E)-1,6-heptadiene3,4-dione (4) and sesquiterpene curdione (9) were highest in 100/0 extract. These are the compounds suspected to be responsible for the higher antioxidant and antidiabetic activities in the 100/0 extracts compared to others. Interestingly, the demethoxycurcumin (2), β-turmerone (6), valine (7), germacrone (12), xanthorrhizol derivative (13), luteolin (17), zedoarol (18), selina-4 (15), 7(11)-dien-8-one (21) and xanthorrhizol (27) were found highest in both 100/0 and 80/20 extracts. The result showed both of the extracts contained almost similar bioactive compounds with various concentrations, hence less "discriminating power" that highlights the compound properties of the extract. The result can be explained due extraction solvent greatly affect the quantity extracted compounds in the extract. The difference in distribution of the compounds could be attributed to the nature of the metabolites and their solubility in the extraction solvents.

**Correlation between in-vitro bioassay and metabolites**

Correlation was performed with the help of PLS as an MVDA-supervised multivariate model. The PLS helps in determining the relationship between bioactivities of sample with the phytochemicals that are contained in it. In this study, PLS was used to determine the correlation between bioactivities (antioxidants and α-glucosidase inhibitory activity) of *C. caesia* extract with the identified metabolites. PLS separates the data into 2 blocks, namely block X (predictor variable; 1H NMR metabolite signal) and also block Y (reaction variable; bioactivity). Results that are expressed in IC₅₀ readings give clearer information in determining the effectiveness level of extracts. However, the IC₅₀ values in this study was unable to be used due to insufficient data by one of the samples, which was at the unidentifiable reading range. Therefore, bioactivities data of the extracts used were the inhibitory percentage (%) for DPPH and α-glucosidase, while mg Trolox equivalent (mg TAE/g extract) for FRAP.
The PLS biplot is the result of the combination of score plot and loading plot. The model validation was executed by permutation test with 100 permutations, to evaluate its goodness of fit and predictive power. It was also validated by evaluation the regression of the observed and predicted values (47). The comparison of the R2 and Q2 of the original model with those, where the Y variable has been permuted randomly, which provides an unbiased validity and degree of overfitting estimation of the PLS model (48). In this study, all PLS models showed the validity with R2Y intercept <0.3–0.4 and Q2Y intercept <0.05 (Fig. S2), suggesting the models showed the criteria of excellent validation and they were safe from over-fitting. The CV ANOVA results also revealed that all models were significant (p<0.05).

From the result in Fig. 5, the PLS biplot showed the same pattern as PCA, whereby four clusters were clearly observed separating the extracts. The 100/0 extract cluster was seen separated from the other extract clusters which was located in the upper right quadrant of the plot, and projected close to the bioactivities (α-glucosidase, DPPH, FRAP), thus suggesting strong correlation between them. The position of the 80/20 extract cluster was also close to the antioxidant activity of DPPH and FRAP, suggesting their strong activities shown in the bioassay tests conducted when compared to the 50/50 and 0/100 extracts which were located in the lower-left quadrant of the plot. The position of 50/50 and 0/100 extracts was far from bioactivities, indicating a very low correlation with the reaction variables. This finding is in accordance with the bioassay results where the 100/0 extract showed highest antioxidant and anti-diabetic activities, while 0/100 extract was the lowest in the conducted tests. As for 80/20 extract, although it is located on the same right region quadrant and close to the antioxidant activities, it only had no significant difference (p>0.05) with 100/0 extract in DPPH scavenging activity. This indicates that these two extracts have similar levels of efficiency for the extraction of metabolite compounds actively involved in the radical scavenging activity of DPPH. However, for ferric reducing activity (FRAP) and α-glucosidase inhibition, it was found that 100/0 extract gave higher activity (p<0.05) compared to 80/20, 50/50 and 0/100 extract. These results are also supported by previous studies that reported 100 % ethanol extracts produced better bioassay activities and contained more metabolites than aqueous extracts (29,47,49).

Metabolites that had major contributions to the bioactivities of the extract in PLS model were further evaluated through their variable important in projections (VIP) value, which separate the extract clusters with higher metabolites and bioactivity than the others. VIP values higher than 0.7 are usually suggested as the key signals that affected PLS model projection (20). According to the PLS biplot, several metabolites were found to be responsible for the antioxidant and antidiabetic activities of the C. caesia extracts. They were xanthorrhizol derivative (VIP=6.40), zedoalactone B (VIP=4.86), xanthorrhizol (VIP=3.68), selina-4(15),7(11)-dien-8-one (VIP=3.32), valine (VIP=3.12), zedoalactone A (VIP=2.63), 13-hydroxygermacrone (VIP=2.14), curdione (VIP=1.99), zedoardiol (VIP=1.95),
luteolin (VIP=1.76), demethoxycurcumin (VIP=1.75), l-hydroxy-1,7-bis (4-hydroxy-3-methoxyphenyl) - (6E)-6-heptene-3,4-dione (VIP=1.24), β-turmerone (VIP=1.05) and 1-(4-hydroxy-3,5-dimethoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-(E,6E)-1,6-heptadiene3,4-dioneVIP=0.73). All these metabolites had greater impact on the PLS model compared to other metabolites. Overall, high phenolic content in 100/0 extract may be attributed for the examined biological activities. This also supported by other findings that reported antioxidant and antidiabetic activities of plant extracts are mainly due to their phenolics content (50,51). Moreover, xanthorrhizol (52), luteolin (53) and demethoxycurcumin (54) has been reported previously to possessed antioxidant and anti-diabetic activities.

Based on the overall result, it can be suggested that both 100/0 and 80/20 extracts have same antioxidant potential by sharing almost similar metabolites content. Complete metabolomic identification of the extracts may increase the possibility to differentiate between the extracts. However, it is impossible to identify the whole metabolome of the crude extract due to the abundance of metabolites contained in it. Meanwhile for antidiabetic activity, phenolic metabolite 1-(4-hydroxy-3,5-dimethoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-(E,6E)-1,6-heptadiene3,4-dione (4) and sesquiterpene curdione (9) which have strong correlation with α-glucosidase activity (Fig. 4), were significantly (p<0.05) the highest in 100/0 extract compared to the rest. This also supported by the observed in-vitro test result where the 100/0 extract exhibited the lowest IC50 values value compared to the other extracts (Table 2). Thus, suggesting 100/0 C. caesia extract as a potential sample with the effectiveness in inhibiting the α-glucosidase enzyme activity compared to the rest.

CONCLUSIONS

In conclusion, the present study demonstrated that C. caesia leaves and rhizome that were dried (by freeze and oven drying method) and extracted with ethanol/water ratios (0/100, 50/50, 80/20, and 100/0) had variations in their TPC, DPPH radical scavenging, FRAP reducing and α-glucosidase inhibition activity. Based on the in-vitro tests, freeze-dried 100/0 C. caesia rhizome extract presented higher TPC, FRAP reducing activity and α-glucosidase inhibition capacity compared to the rest. Meanwhile, for DPPH assay, freeze-dried 100/0 and 80/20 C. caesia rhizome extracts exhibited better results without any significant difference between them. From the bioassay data, it is suggested that FD C. caesia rhizome extracts contain higher TPC and better antioxidant and anti-diabetic activities. Therefore, the FD rhizome extracts were further evaluated using 1H NMR metabolomics for metabolites profiling and identification of metabolites responsible for variation between the extracts. The most active (FD 100/0) and the second most active (FD 80/20) extracts in antioxidant activity contained a large amount of phenolic and sesquiterpenes compounds, however, FD 100/0 showed better α-glucosidase inhibition activity with higher content of metabolite 1-(4-hydroxy-3,5-
dimethoxyphenyl) -7- (4-hydroxy-3-methoxyphenyl) - (IE, 6E)-1,6-heptadiene3,4-dione and curdione. Despite the valuable information provided by this study, further investigation is required to assess their safety and long terms pharmaceutical benefits in the in-vivo model to help as a guide for the development of pharmacological products or functional foods.

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CONFLICT OF INTEREST

The authors declared that there is no conflict of interest to disclose.

SUPPLEMENTARY MATERIALS

All supplementary materials are available at: www.ftb.com.hr.

AUTHORS' CONTRIBUTION

Abdul Rahman, H. designed the experiment. Ibrahim, N. N. A. performed the experiments, analysed the data and wrote the manuscript. Mediani, A. Sajak, A. A. B., Lee, S. Y. provided suggestions and assistance for data analysis and manuscript revision. Kamal, N. Shaari, K. contributed to the check and revised the whole manuscript.

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https://doi.org/10.1007/s12272-014-0351-3

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Table 1. Determination of total phenolic content (TPC) and antioxidant activity (DPPH dan FRAP) of black turmeric (C. caesia) extracts

<table>
<thead>
<tr>
<th>Plant part</th>
<th>Drying techniques</th>
<th>Ethanol/water ratios</th>
<th>TPC</th>
<th>DPPH</th>
<th>FRAP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>GAE/(mg/g)</td>
<td>IC$_{50}$/µg/mL</td>
<td>TAE/(mg/g)</td>
</tr>
<tr>
<td>Rhizome</td>
<td>FD</td>
<td>100/0</td>
<td>(45.38±2.10)$^a$</td>
<td>(539.89±58.04)$^{gh}$</td>
<td>(147.73±8.26)$^a$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80/20</td>
<td>(34.08±3.15)$^{bc}$</td>
<td>(484.02±63.80)$^{h}$</td>
<td>(96.82±2.34)$^b$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50/50</td>
<td>(18.37±1.46)$^{hi}$</td>
<td>(1353.17±167.28)$^c$</td>
<td>(39.10±1.03)$^{gh}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0/100</td>
<td>(16.21±1.47)$^i$</td>
<td>(2499.64±297.86)$^a$</td>
<td>(29.33±0.72)$^{ij}$</td>
</tr>
<tr>
<td>OD</td>
<td></td>
<td>100/0</td>
<td>(37.30±1.97)$^b$</td>
<td>(1376.30±92.62)$^c$</td>
<td>(55.50±1.59)$^d$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80/20</td>
<td>(29.88±0.98)$^{de}$</td>
<td>(1234.98±53.20)$^{cd}$</td>
<td>(55.27±1.24)$^{de}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50/50</td>
<td>(22.06±0.50)$^g$</td>
<td>(990.24±32.07)$^{de}$</td>
<td>(33.21±1.89)$^{hi}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0/100</td>
<td>(19.59±0.90)$^{gh}$</td>
<td>(1203.71±125.33)$^{cd}$</td>
<td>(26.47±0.86)$^k$</td>
</tr>
<tr>
<td>Leaves</td>
<td>FD</td>
<td>100/0</td>
<td>(30.55±1.22)$^{de}$</td>
<td>(1132.95±33.13)$^{cd}$</td>
<td>(70.50±3.79)$^c$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80/20</td>
<td>(34.18±0.56)$^{bc}$</td>
<td>(780.61±27.44)$^{ef}$</td>
<td>(57.54±2.16)$^d$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50/50</td>
<td>(35.30±1.38)$^b$</td>
<td>(660.71±26.69)$^{gh}$</td>
<td>(57.41±1.63)$^d$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0/100</td>
<td>(17.48±0.36)$^{hi}$</td>
<td>(2695.67±300.85)$^a$</td>
<td>(20.94±1.22)$^a$</td>
</tr>
<tr>
<td>OD</td>
<td></td>
<td>100/0</td>
<td>(30.24±2.08)$^{de}$</td>
<td>(1645.52±18.40)$^b$</td>
<td>(49.40±0.72)$^{ef}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80/20</td>
<td>(31.90±1.07)$^{cd}$</td>
<td>(646.11±22.64)$^{gh}$</td>
<td>(59.86±1.77)$^d$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50/50</td>
<td>(27.55±0.58)$^{ef}$</td>
<td>(864.49±116.35)$^{ef}$</td>
<td>(44.18±0.86)$^d$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0/100</td>
<td>(26.26±1.18)$^f$</td>
<td>(2694.26±63.95)$^a$</td>
<td>(44.51±1.23)$^g$</td>
</tr>
<tr>
<td>AA</td>
<td>ND</td>
<td></td>
<td>(25.26±1.24)$^i$</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>BHA</td>
<td>ND</td>
<td></td>
<td>(44.42±4.47)$^j$</td>
<td></td>
<td>ND</td>
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</table>

Data were expressed as mean±standard deviation and analyzed using one-way ANOVA, Minitab version 17.0. All tests were performed in six replicates (N=6). Values with different alphabet showed significant differences (p<0.05) between sample extracts.
Table 2. Determination of anti-diabetic activity (in-vitro) $\alpha$-glucosidase activity in black turmeric (C. caesia) extract

<table>
<thead>
<tr>
<th>Plant part</th>
<th>Drying techniques</th>
<th>Ethanol/water ratios</th>
<th>Inhibition/% at 625 $\mu$g/mL</th>
<th>IC$_{50}$/($\mu$g/mL)</th>
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</thead>
<tbody>
<tr>
<td>Rhizome</td>
<td>FD</td>
<td>100/0</td>
<td>(76.02±4.80)$^a$</td>
<td>(265.46±38.62)$^{ab}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80/20</td>
<td>(62.48±8.85)$^b$</td>
<td>(519.99±49.29)$^{cd}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50/50</td>
<td>(41.08±2.66)$^c$</td>
<td>(831.36±46.13)$^c$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0/100</td>
<td>(8.62±0.41)$^{ghi}$</td>
<td>ND</td>
</tr>
<tr>
<td>OD</td>
<td>100/0</td>
<td>(28.86±5.52)$^d$</td>
<td>(1748.29±277.97)$^b$</td>
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</tr>
<tr>
<td></td>
<td>80/20</td>
<td>(17.57±1.47)$^{ef}$</td>
<td>(2200.75±93.95)$^b$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50/50</td>
<td>(13.40±1.97)$^{efg}$</td>
<td>(4498.19±620.40)$^a$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0/100</td>
<td>(2.96±0.58)$^i$</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Leaves</td>
<td>FD</td>
<td>100/0</td>
<td>(19.17±0.57)$^e$</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80/20</td>
<td>(10.84±0.88)$^g$</td>
<td>ND</td>
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<tr>
<td></td>
<td></td>
<td>50/50</td>
<td>(13.70±0.62)$^{efg}$</td>
<td>ND</td>
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<td></td>
<td></td>
<td>0/100</td>
<td>(9.46±1.28)$^{hi}$</td>
<td>ND</td>
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<td>OD</td>
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<td>(8.75±0.62)$^{ghi}$</td>
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<td></td>
<td>80/20</td>
<td>(8.73±0.60)$^{ghi}$</td>
<td>ND</td>
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<td></td>
<td>50/50</td>
<td>(12.57±0.54)$^g$</td>
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<tr>
<td></td>
<td>0/100</td>
<td>(4.43±0.29)$^{hi}$</td>
<td>ND</td>
<td></td>
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<tr>
<td>Quercetin</td>
<td>ND</td>
<td>ND</td>
<td>(42.36±3.63)$^e$</td>
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</table>

Data were expressed as mean±standard deviation and analyzed using one-way ANOVA, Minitab 17th version. All tests were performed in six replicates ($N=6$). Values with different alphabet showed significant differences ($p<0.05$) between sample extracts.
Fig. 1. Leaf (left) and rhizome (right) of C. caesia
Fig. 2. $^1$H NMR spectra of 0/100, 50/50, 80/20 and 100/0 FD C. caesia extracts (CD$_3$OD/D$_2$O, 500 MHz)
Table 3. Identification of $^1$H NMR metabolites in freeze dried rhizome *C. caesia* extract

<table>
<thead>
<tr>
<th>No.</th>
<th>ID</th>
<th>Metabolite</th>
<th>$^1$H NMR Signals</th>
<th>0/100</th>
<th>50/50</th>
<th>80/20</th>
<th>100/0</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>Curcumin</td>
<td>δ 3.89 s, δ 3.90 s, δ 7.26 s</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>Demethoxycurcumin</td>
<td>δ 3.94 s, δ 3.95 s, δ 5.90 s</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>3</td>
<td></td>
<td>1-Hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)-(6E)-6-heptene-3,4-dione</td>
<td>δ 7.04 (d, J=7.85 Hz)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>1-(4-Hydroxy-3,5-dimethoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-(1E,6E)-1,6-heptadiene3,4-dione</td>
<td>δ 6.81 s</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>13-Hydroxygermacrone</td>
<td>δ 1.44 s, δ 1.61 s</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>β-turmerone</td>
<td>δ 1.80 s, δ 2.05 s, δ 2.07 s</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>Valine</td>
<td>δ 1.03 (d, J=6.95 Hz), δ 1.13 (d, J=6.05 Hz)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>Choline</td>
<td>δ 3.22 s, δ 3.32 s</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>Curdione</td>
<td>δ 0.88 (d, J=7.55 Hz)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>Curcuzederone</td>
<td>δ 1.16 s, δ 1.34 s, δ 3.76 s</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>11</td>
<td></td>
<td>Amadannulen</td>
<td>δ 0.85 s, δ 3.80 s, δ 3.81 s</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
<td>12</td>
<td></td>
<td>Germacrone</td>
<td>δ 1.61 s, δ 1.74 s</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
<td>13</td>
<td></td>
<td>Xanthorrhizol derivative</td>
<td>δ 1.10 s, δ 1.15 s, δ 2.19 s, δ 6.66 (d, J=7.65 Hz), δ 6.66 (d, J=7.75 Hz)</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>14</td>
<td></td>
<td>Threonine</td>
<td>δ 1.33 (d, J=6.75 Hz), δ 3.50 (d, J=4.0 Hz)</td>
<td>+</td>
<td>+</td>
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<tr>
<td>15</td>
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<td>Formic acid</td>
<td>δ 8.47 s</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>16</td>
<td></td>
<td>Gallic acid</td>
<td>δ 7.15 s</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>17</td>
<td></td>
<td>Luteolin</td>
<td>δ 6.52 s</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>18</td>
<td></td>
<td>Zedoardiol</td>
<td>δ 1.41 s, δ 1.71 s, δ 1.98 s</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>19</td>
<td></td>
<td>Zedoalactone A</td>
<td>δ 1.56 s</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>20</td>
<td></td>
<td>Zedoalactone B</td>
<td>δ 1.75 s, δ 1.90 s, δ 6.07 s</td>
<td>+</td>
<td>+</td>
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<td>21</td>
<td></td>
<td>Selina-4(15),7(11)-dien-8-one</td>
<td>δ 1.68 s, δ 1.95 s, δ 1.97 s</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>22</td>
<td></td>
<td>Sucrose</td>
<td>δ 3.65 s, δ 4.18 (d, J=8.7 Hz), δ 5.41 (d, J=3.65 Hz)</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>23</td>
<td></td>
<td>α-glucose</td>
<td>δ 3.43 (t, J=9.7, 9.4 Hz), δ 3.44 (t, J=9.5, 9.45 Hz), δ 5.19 (d, J=3.7 Hz)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>24</td>
<td></td>
<td>β-glucose</td>
<td>δ 4.59 (d, J=7.9 Hz)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>25</td>
<td></td>
<td>Zerumin B</td>
<td>δ 0.68 s, δ 2.44 s</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
<td>26</td>
<td></td>
<td>Curzerene</td>
<td>δ 1.81 s</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>27</td>
<td></td>
<td>Xanthorrhizol</td>
<td>δ 1.87 s, δ 1.88 s, δ 2.00 s</td>
<td>+</td>
<td>+</td>
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Fig. 3. Score plot (a) and loading plot (b) FD rhizome C. caesia extract. 1, curcumin; 2, demethoxycurcumin; 3, l-Hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)-(6E)-6-heptene-3,4-dione; 4, 1-(4-Hydroxy-3,5-dimethoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-(IE,6E)-1,6-heptadiene3,4-dione; 5, 13-hydroxygermacrone; 6, β-turmerone; 7, valine; 8, choline; 9, curdione; 10, curcuzederone; 11, amadannulen; 12, germacrone; 13, xanthorrhizol derivative; 14, threonine; 15, formic acid; 16, gallic acid; 17, luteolin; 18, zedoardiol; 19, zedoalactone A; 20, zedoalactone B; 21, selina-4(15),7(11)-dien-8-one; 22, sucrose; 23, α-glucose; 24, β-glucose; 25, zerumin B; 26, curzerene; 27, xanthorrhizol
Fig. 4. Relative quantification of FD rhizome C. caesia metabolites extracted with solvents ethanol/water 0/100, 50/50, 80/20, and 100/0. The letters (a, b, c, d) show significant differences (p<0.05) as a result of Minitab 17th statistical analysis. (Refer to Table 3 for metabolite identification)
Fig. 5. PLS bi-plots showing correlations between the FD rhizome *C. caesia* extracted with solvents ethanol: water 0/100, 50/50, 80/20, and 100/0 as well as bioactivity (see Table 3 for metabolite identification)
SUPPLEMENTARY MATERIAL
Fig. S1. 2D J-resolved spectra of the FD C. caesia rhizome extract in the region δ 1.0 to 8.5. The observed signal as follows: 6, β-turmerone; 7, valine; 8, choline; 11, amadannulen; 13, xanthorrhizol derivative; 14, threonine; 15, formic acid; 17, luteolin; 21, selina-4(15),7(11)-dien-8-one; 22, sucrose and 24, β-glucose
Fig. S2. Validation by the permutation test of bioassay DPPH (a), α-glucosidase (b) and FRAP (c) using 100 permutations for the PLS model