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Title: Cholesterol-lowering effects of piceatannol, a stilbene from wine, using untargeted metabolomics

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Abbreviations: HFD, high fat diet; TG, triacylglycerols; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; PCT, piceatannol; GC-MS, gas chromatography-mass spectrometry; UPLC-QTOF-MS, ultra-performance liquid chromatography quadrupole time of flight-mass spectrometry; lysoPC, lysophosphatidylcholine; NEFA, non-esterified fatty acid; EFA, esterified fatty acid; QC, quality control.

Chemical compounds studied in this article: Piceatannol (PubChem CID: 667639).

Abstract

This study aims at examining the hypolipidemic effect of piceatannol on high fat diet (HFD)-induced hypercholesterolemic Sprague-Dawley rats and serum metabolite changes. Piceatannol supplement significantly lowered the total cholesterols, low density lipoprotein cholesterol levels and the atherogenic index as compared to the HFD model which only have increased dietary cholesterol intake. Using untargeted mass spectrometry-based metabolomic platforms, multivariate statistics revealed that HFD significantly perturbed fatty acids, lysophospholipids, bile acids and conjugated bile acids. Reduced CYP7A1 protein expression and increase in glycocholate and taurodeoxycholate after piceatannol treatment suggested the conjugated bile acid might contribute to the cholesterol-lowering effect. For lipid profiles, lysoPC (20:2) and lysoPC (20:0) were decreased while the ratio of esterified arachidonic acid to esterified dihomo-γ linoleic acid was up-regulated for rats after piceatannol supplement. These results indicated that the therapeutic effect of piceatannol is associated with bile acid and fatty acid metabolisms and reduced absorption of dietary cholesterols.

1. Introduction

Stilbene is a class of compounds available in some foodstuffs of plant origin (Neveu et al., 2010) and the consumption of these phytochemicals has been associated with a variety of healthy benefits (Shahidi & Ambigaipalan, 2015). For instance, stilbenes in red wine, such as resveratrol, received a lot of attentions and was considered to contribute, in part, to the 'French paradox', an observation of relative low rate of cardiovascular diseases (CVDs) in France despite the relative high intake of saturated fat in diet (Catalgol, Batirel, Taga, & Ozer, 2012). CVDs, including ischemic heart disease and stroke which were the leading causes of global death from 2002 to 2012 (Nelson, 2013; World Health Organization, 2014), have produced immense health and economic burdens globally (Fox et al., 2015; Kim & Boye, 2009; Mozaffarian et al., 2015; Xi, Liu, Hao, Dong, & Mi, 2014). These phytochemicals are considered to be safe, as evidenced through the extensive human consumption (Sham et al., 2014) and very good candidates for developing functional foods which protect us from CVDs. Thus, the understanding of the health benefits of these stilbenes would certainly improve the quality of our life, particularly when we are heading to an aging society.

Piceatannol, a metabolite of resveratrol (Kim, Ahn, Jung, Pan, & Yun, 2009; Piver et al., 2004; Potter et al., 2002; Zhu et al., 2003), is also a stilbene naturally available from many human fruits and plants, such as grapes (Flamini, De Rosso, & Bavaresco, 2015), passion fruit (Matsui et al., 2010), the root of peanut, traditional medicines such as root and rhizome of *Polygonum cuspidatum* (Lin, Lien, Cheng, & Ku, 2007) and rhizome of *Rheum emodi* (Chai, Wang, Li, Liu, & Xu, 2012) in the free form or as piceatannol glucosides. It is also common in functional drinks such as red wine (Tassoni, Tango, & Ferri, 2014; Viñas, Campillo, Martínez-Castillo, & Hernández-Córdoba, 2009) and tea (Viñas, Martínez-Castillo, Campillo, & Hernández-Córdoba, 2011). The content of piceatannol, *trans*-resveratrol and *cis*-resveratrol in red wine were 7.7-14 ng/mL, 32-41 ng/mL and 4.4-37 ng/mL (Viñas et al., 2009) respectively. Piceatannol, like many stilbenes, exhibiting health benefits in anti-inflammatory effect (Choo, Yeo, Ho, Tanaka, & Lin, 2014), and improvement of endothelial function (Frombaum et al., 2011; Kinoshita et al., 2013). It is also slightly more soluble and bioavailable than resveratrol (Chen, Yeo, Elhennawy, & Lin, 2016; Roupe, Yanez, Teng, & Davies, 2006). Moreover, piceatannol is more potent than resveratrol in terms of antioxidant activity (Ovesná et al., 2006; Rüweler, Gülden, Maser, Murias, & Seibert, 2009). There are many evidences suggesting that piceatannol would be a promising agent for treating CVDs (Tang & Chan, 2014). Since long-term supplementation of *trans*-resveratrol

improved energy metabolism and metabolic profile on obese human subject (Timmers et al., 2011), piceatannol or piceatannol-rich food may also be dietary supplement. Given the wide range of biological activities reported and various sources in functional foods, the exact detail mechanisms underlying the protective effects of piceatannol are largely unknown.

Metabolomics is a newly emerging field in advanced and specialized analytical biochemistry under evolution in the past 15 years (Courant, Antignac, Dervilly-Pinel, & Le Bizec, 2014; Oliver, Winson, Kell, & Baganz, 1998). By profiling the endogenous metabolites, the data gives an overview of the metabolic status and global biochemical events associated with a cell or biological system (Metabolomics Society, 2016). Many metabolomics studies aim at discovering the biological pathways associated to disease pathogenesis and identifying the biological actions of treatment (Meikle, Wong, Barlow, & Kingwell, 2014) through determination of the relative contents of the endogenous metabolites using high resolution nuclear magnetic resonance (Song et al., 2013), and mass spectrometry (Zhu et al., 2013). These methods have been applied successfully in understanding the health benefits of fruits (Liu, Tayyari, Khoo, & Gu, 2015), vegetables (González-Peña et al., 2015) and phytochemicals (Gu et al., 2010), multi supplementation of antioxidant plus phytochemicals (Jang et al., 2016), as well as understanding the treatments of metabolic syndromes and CVDs using traditional Chinese medicine formulation (Guo et al., 2014; Liu et al., 2014). All these suggest that metabolomics is a promising way in understanding the detailed pathophysiological changes in the development and treatment of metabolic diseases.

In the present work, combination of ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) and gas chromatography-mass spectrometry (GC-MS) were used to examine the metabolite changes in the serum samples from rats to investigate the therapeutic effect of piceatannol on HFD-induced hypercholesterolemic rats. Hyperlipidemia is one of the major controllable risk factors for CVDs (Navar-Boggan et al., 2015; Nelson, 2013). It is characterized by high levels of one or more lipids and/or lipoproteins including atherogenic free fatty acids, triacylglycerols (TG), small dense low density lipoprotein cholesterol (LDL-C), and apolipoprotein B, and/or low level in high density lipoprotein cholesterol (HDL-C) in blood (Athyros, Tziomalos, Karagiannis, & Mikhailidis, 2011; Durrington, 2003; Shah, Kaul, Nilsson, & Cercek, 2001; Sham et al., 2014). GC-MS results indicated that piceatannol recovered the circulating esterified fatty acid profiles while over 20 biomarkers screened from UPLC-MS showed that piceatannol interrupted the glycerophospholipid metabolism, and especially bile acid metabolism.

To the best of our knowledge, this is the first study demonstrating piceatannol supplement lowered circulating cholesterol levels, which is elevated due to HFD, by regulating the bile acid biosynthesis pathway. We hope that this knowledge will lead to future research to develop new functional foods for CVDs and provide support of consumption of piceatannol-rich food.

2. Materials and methods

2.1 Chemicals

HPLC-graded acetonitrile, methanol, analytical reagent-graded concentrated sulphuric acid (>95% purity) and potassium hydroxide were obtained from Fisher Scientific (Hampton, NH, USA) while HPLC-graded n-hexane was purchased from Duksan (ANSAN-SI, South Korea). Formic acid, oleic acid, cis-10-nonadecenoic acid and their methyl esters, glycerol 1-oleate, Supelco 37 Component FAME Mix, hyodeoxycholic acid, ursodeoxycholic acid, sodium chenodeoxycholate, sodium tauroursodeoxycholate and sodium taurochenodeoxycholate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cholesterol 7α-hydroxylase (CYP7A1), deoxycholic acid and taurodeoxycholic acid were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Glycoursodeoxycholic acid and ursodeoxycholic acid were purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). Cholic acid, taurocholic acid, glycohyodeoxycholic acid, sodium glycochenodeoxycholate, taurohyodeoxycholic acid, glycodeoxycholic acid were purchased from Steraloids (Newport, RI, USA). Sodium glycocholate hydrate was obtained from Acros Organics (Morris Plains, NJ, USA). Simvastatin 20 mg tablets (10% w/w, confirmed by HPLC) and piceatannol (98%, confirmed by HPLC) were purchased from Merck Sharp & Dohme (Hangzhou, China) and Nanjing Zelang Medical Technology Co. Ltd. (Nanjing, China) respectively. Water was purified in-house using a Milli-Q Advantage A10 water purification system (Millipore, Bedford, MA, USA).

2.2 Animal studies

Male Sprague-Dawley rats of 3 months old with body weight of about 300 g were purchased from the Centralized Animal Facilities of The Hong Kong Polytechnic University (Hong Kong, China). Rats were housed under a temperature-controlled room (25 ± 2 ℃) with a regular 12 h/12 h light/dark cycle with free access to water and rat chow. After acclimation for a week in the laboratory environment, the rats were randomly divided into four experimental groups (each with $n = 8$): (i) the normal control group, (ii) HFD model group, (iii) the simvastatin (3mg/kg bw/day) treated group and

(iv) piceatannol (100 mg/kg bw/day) treated group. The control group was fed with normal rat chow [protein (~14%), fat (~10%), and carbohydrate (~76%)] while all the other groups were fed with HFD which was made by addition of 1% cholic acid, 2% pure cholesterol and 5.5% peanut oil to the normal rat chow. The diets were bought from Guangdong Medical Laboratory Animal Center (Guangzhou, China). The rats were administrated with distilled water or their corresponding drugs by oral gavages once every morning for four weeks. During the whole experiment period, the body weight of individual animals was monitored daily. At the end of experimental period, the rats were fasted overnight and then sacrificed by carbon dioxide asphyxiation, followed by the collection of blood and tissues for further analysis. The experimental protocol was conducted under the animal license released by the Department of Health, the Government of the Hong Kong Special Administrative Region and the Animal Subjects Ethics Sub-committee of The Hong Kong Polytechnic University (ASESE no. 05/21). All procedures were complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and the principles outlined in the Declaration of Helsinki.

2.3 Collection of serum samples

Immediately after the rats were sacrificed, blood was collected with centrifuge tubes by cardiac puncture. The blood was stood at room temperature for 30 min, then centrifuged at 3900 ×*g* and 4 ℃ for 15 min. Each supernatant (serum) was aliquoted and stored at -80 ℃ before analysis.

2.4 Analysis of lipid levels in serum samples

Total serum cholesterols, TG, HDL-C and LDL-C were measured by using the Nanjing KeyGEN Biotech's kits (Nanjing, China).

2.5 Metabolomic analysis

2.5.1 Quality control sample preparation method

An aliquot of 20 μL of each serum sample were pooled, vortexed and aliquoted to provide a quality control (QC) sample, and kept at -80 ℃ until use. For each analytical batch, QC samples went through the GC-MS and UPLC-MS extraction protocols as described below similar to all other samples. Before the start of the chemical analysis, five repeated injections of the same QC sample were used to verify the working condition the instruments. Afterwards, a QC sample was injected to monitor the stability of the instruments after every five-sample runs.

2.5.2 GC-MS sample preparation method

The methods reported by Sánchez-Avila *et al* and Yi *et al* (Sánchez-Avila, Mata-Granados, Ruiz-Jiménez, & Luque de Castro, 2009; Yi, He, Liang, Yuan, & Chau, 2006) were used as a starting point for the method development. A two-step methylation of esterified fatty acids (EFAs) and non-esterified fatty acids (NEFAs) fractions in each serum without a protein-removal step was applied. 200 μL serum aliquot was spiked with 100 μL internal standards (a mixture of 125 μM cis-10-nonadecenoic acid and 125 μM of cis-10-nonadecenoic acid methyl ester dissolved in methanol). In the first step, 2 mL of 0.4 M methanolic potassium hydroxide was added to the serum aliquot, vortexed for 30 s and stood at room temperature for 10 min. Then, 2 mL n-hexane was added, vortexed for 30 s twice and the upper layer of hexane phase was isolated. The hexane extraction was done twice and combined to isolate EFA methyl esters. In the second step, 2 mL 10% methanolic sulphuric acid was added to the serum phase and vortexed for 1 min. The mixture was incubated in a water bath at 70 ℃ for 30 min. Isolation of NEFA methyl esters by hexane extraction was the same as EFA methyl esters. The two hexane phases of EFA and NEFA methyl esters were dried in a rotational vacuum concentrator (RVC 2-25, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The dried sample was finally reconstituted with 200 μL n-hexane before GC-MS analysis.

2.5.3 GC-MS condition

The GC-MS system used was an Agilent 6890N GC/5975C VL MSD system equipped with an Agilent 7683 Automatic Liquid Sampler (Agilent technologies, Inc., Santa Clara, CA, USA). The column was a DB-WAX column (30 m x 0.25 mm i.d., 0.25 μm; Agilent J&W Scientific, Folsom, CA, USA). The inlet temperature of the GC was kept at 240 ℃. Helium (≥ 99. 999%) was used as carrier gas with a constant linear velocity of 1.0 mL/min. 1.0 μL of samples was injected in splitless mode. The temperature program that was optimized for GC was as follows: the initial oven temperature as 70 ℃, held for 1 min; 20℃/min to 170℃; 9℃/min to 190℃; 2℃/min to 220℃; 4℃/min to 230℃; 230℃ held for 7.5 min. The MS conditions were as follows: electron impact mode at ionization energy of 70 eV; ion source temperature, 230℃; full scan mode in m/z range 35 - 550 with 0.3 s/scan velocity. The solvent delay was 3 min.

2.5.4 UPLC-MS sample preparation method

100 μL serum from each sample were mixed with cold 300 μL methanol and then vortexed for 30 s. The mixture were cooled at ice water bath for one hour prior to centrifugation at 18790 ×*g* at 4℃ for 20 min. 340 μL supernatant were dried with nitrogen gas and reconstituted in 100 μL 70% methanol in water followed by

centrifugation at 18790 ×*g* and 4℃ for 10 min. The supernatant was obtained for subsequent UPLC-QTOF-MS analysis.

2.5.5 UPLC-QTOF-MS condition

A 3 μL aliquot was injected into a Waters ACQUITY UPLC system. The separation was performed on a Waters ACQUITY UPLC HSS T3 column (2.1 mm × 50 mm, 1.8 μm) with HSS T3 pre-column (2.1 mm × 5 mm, 1.8 μm, Waters Corporation, Milford, MA). The mobile phase consisted of combinations of A (0.1% formic acid in water, v/v) and B (0.1% formic acid in acetonitrile, v/v) at a flow rate of 0.3 ml/min with elution gradient as follows: 0-1.5 min, 5% B; 2 min, 35% B; 4 min, 50% B; 9 min, 55% B; 12-17 min, 95% B. A 3-min post-run time was set to fully equilibrate the column. Column and sample chamber temperature were 35℃ and 5℃ respectively.

MS was performed on a Waters SYNAPT G2 Q-IM-TOF HDMS system (Waters, Milford, USA) operating in an electrospray ion source (ESI) in both positive and negative modes. Nitrogen was used as nebulisation and cone gas. The nebulisation gas was set to 600 L/h at a temperature of 300 ℃, and the cone gas was set to 40 L/h. The source temperature was set at 120 ℃. The capillary voltages in positive and negative ion modes were 3.0 kV and 2.5 kV, respectively. The sampling and extraction cone voltages were 40 V and 4.0 V, respectively. The data acquisition rate was set to 1 s with a 0.024 s interscan delay. Data between m/z 50 to 1000 were recorded in the centroid mode. For accurate mass acquisition, a lock-mass of leucine enkephalin at a concentration of 10 ng/mL was used via a lock spray interface at a flow rate of 4 uL/min and 30 eV trap collision energy, 2.5 kV capillary energy, 40 V cone voltage, monitoring for positive ion mode ($[M+H]^+$: 556.2771) and negative ion mode ($[M-H]^-$: 554.2615) to ensure accuracy during the MS analysis. MS/MS analysis was carried out to study the structure of potential biomarkers. In this section, argon was employed as collision gas and the collision energy was set between 5 to 50 eV according to the situation.

2.5.6 Data processing and analysis

For LC-MS data, the peak finding, alignment, and filtering of the raw data were carried out with the MarkerLynx application manager Version 4.1 SCN 901 (Waters, Milford, USA). The parameters were used: m/z range 50-1000, mass tolerance 0.02 Da, intensity threshold 500 counts, retention time tolerance 0.2 min; apex track peak parameter was calculated automatically; no smoothing was applied and isotopic peaks were excluded for processing. Quality screening was done by filtering out metabolites which did not exist in 80% of each data set to reduce the contribution of unstable peaks and eliminate noise from the dataset. MarkerLynx built a matrix of exact mass, retention time and intensity pairs. Peak area was normalized to the total peak area of each sample. The matrix was statistically analysed by the built-in Extended Statistical tool (EZinfo v2.0 software, Umetrics AB).

The data were scaled to unit variance for Principal Component Analysis (PCA), to give an overview of the repeatability of QC samples. The QC samples with high repeatability should cluster together in both ESI modes in the score plot of PCA. The samples excluding QC samples were pareto-scaled for Partial Least Squares-Discriminant Analysis (PLS-DA) and Orthogonal Partial Least Squares - Discriminant Analysis (OPLS-DA). Potential markers of interest were extracted from the S-plots of OPLS-DA based on their contribution to the variation and correlation in the data set. The markers were identified by LC-MS/MS and matched with the METLIN (http://metlin.scripps.edu), the KEGG (www.kegg.com/) and the Human Metabolome Databases (www.hmdb.ca) and/or confirmed by authentic standards based on retention times, mass spectra and accurate masses.

For GC-MS data, peak area was integrated by Agilent Chemstation (GC6890 MSD Chemstation E.02.02.1431, Agilent) and then normalized by the spiked internal standard in each sample. Peak identification was carried out by comparing the retention time of authentic standards and the mass spectrum with NIST11 library. Multivariate statistical analysis with the same scaling procedure as LC-MS was also performed by the EZinfo software.

2.6 Western Blot Immunoreactivity Assay

CYP7A1 protein levels were quantified using immunoblotting procedures. Homogenized liver samples were lysed with lysis buffer and kept in ice for 20 min. The mixture was centrifuged at 8265 ×*g* for 15 min. The supernatant was collected and assayed for protein contents using Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Protein extracts (80 μg) were applied to 7.5% SDS polyacrylamide gels and

transferred to polyvinylidene difluoride membranes. The membranes were blotted for 1 h with 5% non-fat dry and then incubated with CYP7A1 antibody at 4 ℃ overnight. After three washes in Tris-buffered saline containing 0.1% Tween 20, the membranes were incubated with horseradish peroxidase-linked secondary antibodies for 1 h. Proteins were detected with the chemiluminescence (ECL) detection system (Amersham Biosciences, Little Chalfont, UK) and visualized with Fujifilm autoradiographic films. Densitometric analysis of optical densities was performed using software (Alpha Ease FC, San Leandro, CA).

2.7 Statistical analysis

Statistical analyses were performed using SPSS PASW Statistics 18 (Chicago, IL, USA). After filtering out outliers (1.5 times of the interquartile range), statistical differences were analysed at a univariate level by one-way analysis of variance (ANOVA); least significance difference (LSD) *post-hoc* test was used with assumption of equal variances. The correlation analysis was performed using linear regression and the correlation coefficient (r) was determined performed using Graphpad Prism for Windows 5.01 (GraphPad Software, San Diego, CA, USA). A p value of 0.05 was regarded as statistically significant.

3. Results and Discussion

3.1 Effect on serum lipid profiles

The lipid profiles of total serum cholesterols, TG, HDL-C and LDL-C from various rat groups are shown in Fig. 1. The HFD significantly increased the total cholesterol (4.32 ± 0.43 mmol/L) and LDL-C (0.60 ± 0.08 mmol/L) of serum compared with normal control (1.88 ± 0.09 mmol/L, 0.19 ± 0.01 mmol/L respectively) while HDL-C and TG showed no significant changes in all groups. The atherogenic index [(total serum cholesterol - HDL-C) / HDL-C] is a parameter to measure the risk of coronary heart disease. Administration of either simvastatin (0.83 ± 0.14 mmol/L) or piceatannol (0.89 ± 0.12 mmol/L) could significantly decrease the atherogenic indexes compared to HFD group (1.54 ± 0.25 mmol/L), suggesting that piceatannol possessed protective potential in atherosclerosis.

3.2 Reliability of the metabolomics platforms

The stabilities of UPLC-MS and GC-MS detections were assessed by inter-day measurement of the pooled QC sample injections and 37 FAME standards mixture, respectively. 10 metabolites including both positive and negative modes from QC samples and 16 detected fatty acid methyl esters from FAME mixture were selected for comparison (supplementary material Table S1). PCA score plot was used to evaluate

the stability of the analytical instruments and Fig. 2A showed the QC sample injections of inter-day experiments were clustered together in PCA score plots. The coefficient of variation (CV) of 10 analytes in UPLC-MS was less than 22% while the CV of 16 analytes in GC-MS was less than 7%. Results indicated high reproducibility was achieved across the runs. This ensured the changes among the different groups observed from the statistical analysis were biologically related.

Additionally, peak retention time reproducibility was also evaluated throughout the study to calculate the CV and the results showed that the CV values of all the retention time were less than 0.5%. Besides, recoveries of transesterification and esterification were also examined by spiking a known amount of analytes before extraction to serum samples. 50%, 100% and 200% glycerol 1-oleate and oleic acid relative to the original amount present in a blank serum were spiked into the blank serum and the results of their recoveries were in the range of 100.05 to 113.21%.

Good recovery, high retention time and analytical reproducibility of the metabolite detection demonstrated that the presented methodologies have the reliability and robustness as required by a metabolic profiling study.

3.3 Effect of piceatannol to HFD

In the data collected from UPLC-MS, MarkerLynx peak extraction obtained 4904 and 1976 peaks in QC samples under positive and negative modes, respectively. After quality screening, 2491 and 878 metabolite features were detected at positive and negative modes, respectively. Free fatty acids, lysoPCs, one lysoPE were tentatively identified by accurate mass measurement, MS fragmentation, retention time matching with database search and literatures. Bile acids and fatty acid methyl esters were identified by matching with standard compounds.

Metabolite changes in the blood serum after four- week HFD intake were investigated using OPLS-DA and Fig. 2B showed clear separation between the normal control and the HFD groups. Metabolites with significant contribution to the differentiation of two groups by OPLS-DA are usually identified based on their Variable Importance in the Projection (VIP) values (threshold of VIP $\geq$ 1) and the location in S-loading plot. The higher the VIP values, the greater importance of the variable for the separation of two classes. As for the S-plot, variables located in the lower left corner [lower p (corr) and p (loading)] and upper right corner [higher p (corr) and p (loading)] are the most likely putative markers due to their high contribution and correlation/reliability. According to the results obtained by VIP value and S-loading plot (the highlighted features in the red

square in Fig. 2C had VIP values ≥ 1.5) followed by validation with one-way ANOVA ($p < 0.05$), over 20 endogenous metabolites were highlighted and these metabolites belonged to three main classes: fatty acids, lysophospholipids, bile acids and their conjugated products. The fold changes for these metabolites were listed in Table 1 and more details could be found in a heatmap in the Fig. S3 of the supplementary materials. The above model provides a base for studying the role of piceatannol in lowering the blood cholesterol level in HFD rats.

To study the effect on piceatannol, PLS-DA was further carried out to examine the difference of metabolite profiles among the normal control, HFD and piceatannol groups. As shown in Fig. 2D, the results of PLS-DA score plot showed a clear distinction of the normal control, HFD and piceatannol groups. Significant differences, based on the results of loading plot (Fig. 2E, $p < 0.05$), were observed in the following classes of metabolites including lysophospholipids, fatty acids and bile acids.

3.4 Serum metabolites change of lysophospholipids after piceatannol treatment

Our result showed that five lysoPCs (C18:1, C18:2, C20:0, 20:2 and 20:3) and lysoPE (C20:1) were increased in HFD models but restored in piceatannol-treated HFD models. Lysophospholipids not only play a structural role as the composition of cell membrane, but also involve in many cellular functions such as activating cell, acting as messengers in signal transmission within the cell and synthesis of precursors of prostaglandins. In recent years, observations of the up-regulation of lysophospholipids, especially lysoPC derived from hydrolysis of phosphatidylcholines, have been reported in those hyperlipidemias-related studies including the rabbit model of atherosclerosis (Liu et al., 2014), HFD-induced hyperlipidemic rats (Wu et al., 2014), and HFD-fed Ossabaw pig (Hanhineva et al., 2013). Moreover, Frostegård's team (Frostegård, 2010) also pointed out the toxicity of a high concentration of lysoPCs from the increased phosphorylcholine might cause the autoimmune response and inflammation observed in many hyperlipidemias-related diseases.

3.5 Serum metabolites change of fatty acids after piceatannol treatment

The OPLS-DA score plots (Fig. 2B) from the data of LC-MS and GC-MS indicated that both saturated and unsaturated free and esterified fatty acids were disturbed by HFD. There were lower circulating levels of free palmitic acid, stearic acid, free and esterified arachidonic acid as well as free and esterified DHA in HFD group compared to normal control group ($p < 0.05$, Table 1, VIP > 1.5). Even though piceatannol could not restore the above fatty acid levels, it significantly down-regulated the esterified oleic acid and

linoleic acid levels sourced according to GC-MS results restored them to the normal level ($p < 0.05$, VIP > 1.5).

To further examine the fatty acid GC-MS results, the circulating fatty acid product to its precursor ratio was evaluated, which is commonly employed as an estimation of hepatic enzyme activities and as an indicator of endogenous fatty acid metabolism (Matthan et al., 2014). As shown in Fig 3, the ratio of esterified arachidonic acid (EC20:4n6c) to esterified dihomo-γ linoleic acid (EC20:3n6c) was significantly upregulated after the piceatannol treatment ($p < 0.01$) compared to that of the HFD model. Matthan *et al* reported that this ratio is positively correlated to the activity of Δ5-desaturase (20:4n6c / 20:3n6c) (Matthan et al., 2014). Our results revealed the same observation from the human study which concluded that higher activities of Δ5-desaturase is positively associated with lower coronary heart disease (CHD) risk (Matthan et al., 2014). This elevated activity of Δ5-desaturase facilitated the conversion of dihomo-γ linoleic acid to arachidonic acid, thus we observed a significant reduction of dihomo-γ linoleic acid by 20.8% in piceatannol group ($p < 0.05$) compared to HFD model. However, the free arachidonic acid level shows no significant difference between the piceatannol-treated and HFD groups ($p < 0.01$). This suggested that the extra arachidonic acid generated could probably be further converted to pro-inflammatory mediators by the cyclooxygenase 1, 2 isoenzymes (COX-1, COX-2) and 5- and 15-lipoxygenase (5-LOX, 15-LOX) (Calder, 2006) or other long-chain ω6 fatty acids (Simopoulos, 2008). Since piceatannol is an inhibitor of 5-LOX (Kutil et al., 2014) and COX-2 (Murias et al., 2004), we would anticipate that the arachidonic acid was probably converted to long chain fatty acids in the piceatannol-treated group. Moreover, a non-alcoholic human fatty liver study (Kotronen et al., 2009) demonstrated that liver fat content was inversely correlated to hepatic elongase activity index (C18:0 / C16:0) and positively correlated to stearoyl-CoA desaturase 1 activity index (C18:1n9c / C18:0). Piceatannol treatment unchanged the elongase activity. Yet, it restored the stearoyl-CoA desaturase activity from HFD, as evidenced by lower ratio of EC18:1n9c to EC18:0 ($p < 0.01$) than HFD group, thus limiting the major substrates (monounsaturated and polyunsaturated fatty acids) for synthesis of triacylglycerols and other lipids (Ntambi & Miyazaki, 2004).

3.6 Serum metabolites change of bile acids after piceatannol treatment

Bile acids and their metabolites were significantly affected by HFD as well as the piceatannol treatment. The fold changes are shown in Table 1. Fig. 4 shows the proposed pathway of bile acid biosynthesis and indicates the bile acids affected by HFD and piceatannol treatment.

For primary bile acids, HFD group had a lower level of cholate ($p < 0.05$). HFD induced the increase in secondary (deoxycholate and hyodeoxycholate / ursodeoxycholate) and conjugated (glycochenodeoxycholate, glycodeoxycholate and taurodeoxycholate) bile acids ($p < 0.05$). Our findings are consistent with the previous reports that dietary cholesterol increased bile acid pool size and faecal bile acid excretion (Guo et al., 2011; Li et al., 2012; Tiemann et al., 2004).

Piceatannol supplement to HFD rats resulted in significant decreases in two primary (cholate and chenodeoxycholate) and two secondary bile acids (deoxycholate and hyodeoxycholate / ursodeoxycholate) ($p < 0.05$). In addition, piceatannol supplement raised eight conjugated bile acids (glycocholate, glycodeoxycholate, glycohyodeoxycholate/glycoursodeoxycholate, glycochenodeoxycholate, taurodeoxycholate, taurohyodeoxycholate/tauroursodeoxycholate, taurochenodeoxycholate) ($p < 0.05$).

Regarding the mechanism of bile acid disturbance, CYP7A1, the rate-limiting enzyme in the classical pathway of bile acid biosynthesis (Chiang, 2009; Dawson, 2016), was thought to be up-regulated by piceatannol because of a similar activity in resveratrol reported in literatures. Resveratrol has been shown to up-regulate CYP7A1 which affects the bile acid synthetic pathway (Chen, Wang, Ma, & Zhai, 2012; Miura, Miura, & Yagasaki, 2003; Zhu, Luo, & Jin, 2008) and enhances the excretion of fecal bile acids (Zhu et al., 2008) to lower cholesterol. However, our result (Fig. 5) showed that the CYP7A1 protein expression levels in the liver HFD group and simvastatin-treated group were lower than that of the normal control group ($p < 0.001$). It is probably because in the HFD and simvastatin groups, this study's high cholesterol diet (2% cholesterols) might impair the classical pathway of bile acid synthesis which is originally responsible for cholesterol elimination (Zinkhan et al., 2014) and so did the CYP7A1 protein expression. The increased levels of secondary and conjugated bile acids in HFD group might be the results of increased stimulation of dietary absorbed cholesterols to the receptors of alternative (acidic) pathway for bile acid synthesis but the elimination rate was not high enough to cope with the influx of cholesterol from diet. The CYP7A1 protein expression of piceatannol-treated group was even further lower than that of the HFD group ($p < 0.01$). This indicated that piceatannol might not rely on CYP7A1 action in removal of cholesterol.

As for the cholesterol-lowering effect of piceatannol, it might be contributed by its inhibition of dietary cholesterol absorption in the small intestine in aid of binding effects of piceatannol and conjugated bile acids. (Montilla, Mu, Bujalance, Mu, &

Tepartment, 2004). Scientists found that there was a region-specific interaction of polyphenols in tea (Ogawa, Hirose, Nagaoka, & Yanase, 2016) and grapes (Ngamukote, Mäkynen, Thilawech, & Adisakwattana, 2011) with conjugated bile acids to decrease the solubility of cholesterol in bile acid micelle. Milica Atanackovi´c also demonstrated that there was formation of hydrogen bonds between resveratrol's hydroxyl groups and keto groups of bile acids (Atanacković, Posa, Heinle, Gojković-Bukarica, & Cvejić, 2009). These binding effects in the micelle might result in delayed cholesterol absorption. Since piceatannol is a polyphenol, it might also interact with the conjugated bile acids and lowered the solubility of cholesterol micelles. It was found that polyphenols found in the red wine probably inhibited the absorption of exogenous cholesterol and lipids in the small intestine, resulting in an increase in the cholesterol sulphate content in feces (Jimeez-Giro et al., 2014). Thus, the conjugated bile acids in piceatannol group were higher than that of the other groups' results in lowering the transfer of cholesterol from intestine to the hepatocyte. That might reduce the stimulation to CYP7A1 receptor and resulted in a lower protein expression in piceatannol group with respect to the HFD group. Examination of the relationship between total cholesterols and conjugated bile acids across different groups was shown in Fig. 6 and a significant positive correlation was revealed between the normal control and piceatannol groups ($r = 0.5620$, $p < 0.05$).

It is well known that primary bile acids secreted into intestinal lumen are metabolized to secondary bile acids by intestinal microflora (Qi et al., 2015). Our result of deoxycholate reduction in the piceatannol group are consistent with a previous study of dietary supplement of the some phytochemicals in the same polyphenol class as piceatannol, which reduced faecal deoxycholic acid of the HFD-induced rats (Han et al., 2009). Piceatannol might interfere the biotransformation of secondary bile acids. This could be found hints from the study of Hijona *et al.* in 2016 that piceatannol supplement altered the abundance of several species of the *Firmicutes* and *Bacteroidetes* phyla, but did not affect the gut microbial composition of the genetically obese Zucker rat model taking normal diet (Hijona et al., 2016). Another report shows that resveratrol improved the HFD-induced gut microbial imbalance (Qiao et al., 2014). We speculate that piceatannol supplement to HFD rats improved the HFD-induced hypercholesterolemia by modifying the gut microbial composition as well. Modification of gut microbial composition because of piceatannol or other stilbenes supplements may play an important role in the health benefits of these plant polyphenols. The present study provides some evidences of this and further study is certainly necessary to provide a better picture on the role of gut microbiota in improving the health of the host through the consumptions of phytochemicals.

4. Conclusion

The results acquired in this study support the cardioprotective and hypolipidemic effects of piceatannol. Untargeted metabolomic approach was employed to characterize the serum metabolic profile of HFD rats receiving piceatannol supplement to provide more detail understanding and insights into the underlying mechanisms of piceatannol in regulating the atherogenic index. Significant metabolite changes in fatty acids, have been observed suggesting the regulation of fatty acid pathways by up-regulating Δ5-desaturase and down-regulating stearoyl-CoA desaturase activities. Lower CYP7A1 protein expression than HFD group and rise in conjugated bile acids in the piceatannol treatment group suggested the reduction in cholesterol absorption in the intestine and the transfer to hepatocyte might be caused by the binding effect of piceatannol to cholesterol and bile acids in bile acid micelles. The observation of lower secondary bile acid in the piceatannol treatment group gave hints to the health benefits that may relate to the modification of gut microbial composition. These conclusions may also apply to other plant polyphenols. The data suggested new directions in understanding the health benefits of these phytochemicals.

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Conflict of interest Tung-Ting Sham, Meng-Heng Li, Chi-On Chan, Huan Zhang, Shun-Wan Chan and Daniel Kam-Wah Mok declare that they have no conflict of interest.

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List of table:

One-way ANOVA, LSD: *p < 0.05, **p < 0.01, ***p < 0.001. n = 5-8.

List of figures:

Data are expressed as means ± SEM, n = 6-8. One way-ANOVA, LSD: #p < 0.05, ##p < 0.01 and ###p < 0.001 represent significant differences when compared with the normal control group. *p < 0.05, **p < 0.01 and ***p < 0.001 represent significant differences when compared with the HFD model group.

Data are expressed as means ± SEM, n = 6-8. One-way ANOVA, LSD: ##p <0.01 and ###p < 0.001 represent significant differences when compared with the normal control group. **p < 0.01 represents significant difference when compared with the HFD model group.

⇧, ⇩: increase or decrease in piceatannol-treated group compared to HFD group. ↑, ↓: increase or decrease in HFD model group compared to normal control group. ns: no significant difference. †: not detected. Dotted line: skipped pathway. One way-ANOVA, *p <0.05.

The expression level of each protein was normalized to that of the β-actin protein in each sample. Data are expressed as means ± SEM, n = 8. One way-ANOVA, LSD: ###p < 0.001 represents significant differences when compared with the normal control group. **p < 0.01 represents significant difference when compared with the HFD group.

Total sum of conjugated bile acids: peak areas of taurohyodeoxycholate/tauroursodeoxycholate, taurocholate, glycocholate, glycohyodeoxycholate/glycoursodeoxycholate, taurochenodeoxycholate, taurodeoxycholate, glycochenodeoxycholate glycodeoxycholate acquired at negative ESI mode that had significant difference between piceatannol group and HFD model.