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Research article

Catechin profile and hypolipidemic activity of *Morinda citrifolia* leaf water extract

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ABSTRACT

Cardiovascular diseases (CVDs) are silent killers and hyperlipidemia is a high-risk factor. *Morinda citrolia leaf* (MCL), which is commonly consumed by many cultural groups and has high level of catechins, might exert antihyperlipidemic properties. In this study, the catechins profile of MCL water extract was determined via HPLC and ultraperformance liquid chromatography-traveling wave ion mobility-quadrupole time of flight mass spectrometry (UPLC-TWIMS-QTOF). The major catechin in MCL and the most widely studied catechin with hypolipidemic activity, epigallocatechin gallate (EGCG), was studied in a cytotoxicity test on HepG2 cells prior the *in vitro* anti-hyperlipidemic assay. The total catechins of MCL reached 141.88 ± 5.04 mg/g, with catechin gallate (CG) (75.27 ± 8.49 mg/g) as the major catechin. Catechin derivatives that were identified include epigallocatechin-3-O-gallate (EGCG) with *m/z* 459.0912 $[M + H]^+$, epigallocatechin (EGC) with *m/z* 307.0818 $[M + H]^+$, CG with *m/z* 443.0976 $[M + H]^+$, epigallocatechin(4β→8)-gallocatechin with *m/z* 649.0951 $[M + K]^+$, and gallocatechin(4α→8)-epicatechin with *m/z* 633.1 $[M + K]^+$. Cell inhibitions of MCL, CG and EGCG were more than IC_{50} of 100 μg/ml. MCL increased LDL-c uptake up to 1.11 ± 0.03-fold, but this was insignificant relative to control. CG and EGCG significantly increased LDL-c uptake up to 1.37 ± 0.19-fold and 1.59 ± 0.19-fold, respectively. Thus, MCL with CG has shown potential for modulating hyperlipidemia.

1. Introduction

Morinda citrifolia is an evergreen shrub that has been naturalized in many tropical countries (Potterat and Hamburger, 2007). It is commonly known as noni in English, and in Malaysia it is called *mengkudu* (Chan--Blanco et al., 2006). *Morinda citrifolia* leaf (MCL) is consumed by many cultural groups. For this reason, it is listed in the WHO's food composition tables for East Asia and Islands of the Pacific (West et al., 2007).

MCL has been reported to contain oxalic acid, campesterol, stigmasterol, β-sitosterol, (+)-catechin, (-)-epicatechin, rutin, scopoletin, kaempferol and quercetin (West et al., 2007; Pak-Dek et al., 2008; Lim et al., 2016; Saraphanchotiwitthaya and Sripalakit, 2015). MCL has been used to treat helminthic infections, oxidative stress, open wounds, hyperlipidemia and as an anti-allergen (Mandukhail et al., 2010; Raj, 1975; Palu et al., 2010; Serafini et al., 2011; Murata et al., 2014; Nerurkar et al., 2015). Additionally, MCL offers several benefits which include antioxidation, liver-protection and anti-osteoporotic effects (Lim et al., 2016; Nayak et al., 2009; Shalan et al., 2017). Furthermore, MCL has shown negative results in systemic anaphylaxis tests. This indicates that MCL lacks allergenicity and has a low chance of causing sensitization in people with no previous exposure to MCL (West et al., 2007).

Catechins (flavan-3-ols) are the subgroup of flavonoids that belongs to polyphenol (de Pascual-Teresa et al., 2010). There are eight major type of catechins: (+)-catechin (C), (-)-epicatechin (EC), (-)-gallocatechin (GC) and (-)-epigallocatechin (EGC) are the non-esterified catechins; while (-)-catechin gallate (CG), (-)-gallocatechin gallate (GCG), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG) are the esterified catechins (Theppakorn, 2016). MCL has been reported to contain C and EC (Zin et al., 2006; Pak-Dek et al., 2008). LDL-c is one of the important surrogate risk factors in identifying the progression of cardiovascular diseases and remains the most commonly used measure in clinical trial and clinical care (Wadhera et al., 2016). EGCG is the most widely studied catechin and has been reported to offer hypolipidemic activity by alleviating LDL-c levels in epidemiological studies (Momose et al., 2016). The catechin profile in green tea has been widely studied while catechin profile in MCL was limited and the hypolipidemic action

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of the most abundant catechin in MCL is still in the dearth of research. The hypothesis of this work is that MCL water extract might contain another type of major catechin with hypolipidemic activity that could serve as an alternative to EGCG as potential anti-hyperlipidemic bio-active compound.

2. Materials and methods

2.1. Plant materials

Fresh MCL were obtained from MARDI (Jerangau Station), Terengganu, Malaysia with accession number DINO 04–1425.

2.2. Chemicals

Epicatechin (EC), gallocatechin (GC), catechin gallate (CG), gallocatechin gallate (GCG), epigallocatechin (EGC), epicatechin gallate (ECG) and catechin (C) were purchased from Chemfaces with purity >98%. Epigallocatechin gallate (EGCG) with guaranteed grade was purchased from Nacalai Tesque. A high-performance liquid chromatography (HPLC) grade acetonitrile was obtained from JT Baker. HepG2 cells (ATCC® HB-8065™) were obtained from Institute of Marine Biotechnology, Universiti Malaysia Terengganu. Alpha-minimum essential media (α-MEM) with SP grade, Penicillin-Streptomycin solution with SP grade and MTT powder with guaranteed reagent (GR) grade were obtained from Nacalai Tesque. Fetal bovine serum (FBS) was obtained from Tico Europe. MEM non-essential amino acids solution, trypsin ethylenediaminetetraacetic acid (EDTA), trypan blue stain, phosphate buffered saline (PBS) tablet, dimethyl sulfoxide (DSMO) with AR grade and BODIPY FL LDL were obtained from Thermo Fisher Scientific. Lipid depleted FBS was obtained from Biowest. Cholesterol powder with purity ≥99%, 25-hydroxylcholesterol with purity ≥98% was purchased from Sigma-Aldrich, while berberine chloride (BC) was purchased from Merck.

2.3. Sample preparation and extraction

The leaves were freeze dried, ground and sieved to obtain particle size of <1 mm adopted from Vuong et al. (2011). MCL powder was extracted with hot water extraction at water ratio of 1: 20 at 30 min and 80 °C (Chong et al., 2018). Hot water extraction has been selected as water is safe that did not leave any residue after extraction as compared to other generally recognized as safe (GRAS) solvent and water is the normal solvent to obtain benefits from herbs (Bergeron et al., 2005; Wong et al., 2006). The sample extracts were filtered through Whatman No. 40 filter paper and freeze dried.

2.4. HPLC analysis

HPLC analysis for catechin standards and samples was conducted on Shimadzu HPLC system equipped with LC-20AT series-type double plunger, DGU-20A5R online degassing unit, SPD-20A UV-Vis detector, SIL-20A autosampler and CTO-10ASVP Column Oven. The column used in the present study was the AGILENT 690970-902 (Poroshell 120, EC-C18, 4.6 × 250 mm, 4 μm). HPLC conditions were modified from Theppakorn et al. (2014). The mobile phase comprised of water: acetonitrile (89: 11) with 1.4 ml/min flow rate. The column oven was thermostated at 30 °C and detection wavelength was set at 210 nm with injection volume of 20 μl. Concentrations of catechins were quantified by their peak areas against standards.

The amount of catechin was quantified according to the formula adapted from Tee and Lim (1991) and Mustafa et al. (2010) as follows:

$$Flavonoid\ \ content\left(\frac{\mu g}{g}\right)=\frac{peak\ \ area\ \ of\ \ sample}{peak\ \ area\ \ of\ \ standard}\times amount\ \ of\ \ standard\ (\mu g)\times\frac{vol.make\ \ up\ (ml)}{injection\ \ vol.\ (ml)}\times\frac{1}{weight\ \ of\ \ sample(g)} \quad (1)$$

2.5. UPLC-TWIMS-QTOF analysis

Ultraperformance liquid chromatography-traveling wave ion mobility-quadrupole time of flight mass spectrometry (UPLC-TWIMS-QTOF) analysis was performed based on Waters Natural Products Application Solution with UNIFI adapted to document number 715004264 Rev. Chromatographic analysis was performed via a Waters Acquity I-Class UPLC system (Waters Corporation, Milford, MA, USA) consisting of a column oven, sample manager with flow through needle and I-class binary solvent manager. The chromatographic separation was done on Waters Acquity UPLC HSS T3 (2.1 × 100 mm, 1.8 μm) column. The mobile phase consisted of (A) 0.1% formic acid in ultrapure water and (B) 0.1% formic acid in acetonitrile. The linear gradient elution was set as follow: 1% B (0–0.5 min), 35% B (0.5–16 min), 100% B (16–18 min) and 1% B (18–20 min). Flow rate and injection volume were 0.6 ml/min and 5 μL, respectively. The column and sample temperature were maintained at 40 and 15 °C, respectively. Mass spectrometry was performed on a Waters Vion™ IMS-QTof mass spectrometer (Waters Corporation, Milford, MA, USA). Ionization was achieved with electrospray in the positive mode (ESI+). Desolvation gas was set to 800 L/h at 550 °C, cone gas was set to 50 L/h, source temperature set was to 120 °C and capillary voltage was set at 1.5 V. Vion data were acquired in the MS^E mode (MS^E is low energy (MS) and elevated energy (E) mode of acquisition). Two separate scan functions were programmed for the MS/MS acquisition mode. One scan function was set at low energy collision-induced dissociation (CID) of 4 eV (electronvolt) in the trap cell, while the other scan function was set at high CID ramping from 10 eV to 45 eV in the transfer cell. The mass spectrum acquisition mode selected was sensitivity mode (high definition MS with full scan in mass range 100–1000 m/z) and scan time 0.2 s. Ion mobility separation (IMS) was conducted with travelling wave (T-wave). Instrument control and data processing was performed with Waters UNIFI software version 1.8. MS scanning was performed using $HDMS^e$ (data-independent acquisition type in ion mobility). The compound identification was based on MS/MS fragment isotopic distribution and collisional cross section (CCS) value from Waters in house natural product database (Waters Traditional Medicine Library). The criteria for data comparison were adapted from a previous study, and the CCS value acceptable error, MS tolerance and MS/MS tolerance were set at 5%, 3 mDa and 10 mDa, respectively (Yang et al., 2018).

2.6. MTT assay

Prior to conducting LDL-c uptake cell culture experiment, cytotoxicity testing of MCL water extract, CG, EGCG (most widely studied catechin) and berberine chloride (BC), positive control of LDL-c uptake assay on HepG2 cells were determined with MTT assay adapted from Mosmann (1983). HepG2 cell is human hepatoma that commonly used in the studies of drug metabolism and hepatotoxicity (Donato et al., 2015). 100 μl of cell suspension with density of 8×10^3 was seeded into each well of 96 well plate and grown to confluency by incubating for 24 h at 37 °C at 5% CO_2 and 95% humidity.

After cell seeding, the medium was then aspirated and several concentrations of 100 μl MCL water extract in complete media were pipetted into a 96-well plate and further incubated for 72 h at 37 °C at 5% CO_2 and 95% humidity. The MTT stock solution was prepared by dissolving 5 mg/

ml PBS in the dark. 20 μl of MTT was added to each well and incubated for 4 h. The tetrazolium salts will be reduced by viable cells' mitochondria into insoluble formazan crystal during incubation. The formazan crystals formed were proportional to the number of existing viable cell. A volume of 120 μl solution was aspirated and added with 100 μl DMSO to lyse the cell membranes and solubilize the formazan crystals. MTT assays were repeated with CG, EGCG and BC. Optical density (OD) of the cell was measured at 570 nm. The cytotoxic effect was reported as IC_{50}, where IC_{50} was determined from the growth curve. Cell viability was calculated with the following formula:

$$Cell\ \ viability(\%) = \frac{(OD_{sample} - OD_{blank})}{(OD_{control} - OD_{blank})} \times 100\% \quad (2)$$

Where,

OD_{sample} is the absorbance of the samples
OD_{blank} is the absorbance of the blank (complete media)
$OD_{control}$ is the absorbance of the control wells (untreated cell with complete media).

2.7. LDL-c uptake assay

The IC_{50} value of MCL water extract, CG, EGCG and BC obtained with MTT assay were used to determine the dosages used in LDL-c uptake assay. HepG2 cell was used for both MTT and LDL-c uptake assays as this cell line has similar cholesterol biosynthesis pathway to the normal hepatocytes (Wilkening et al., 2003). Hypolipidemic assay that involved the *in-vitro* LDL-c uptake by HepG2 cells was adapted from Ji et al. (2012) and Yusof et al. (2016). A volume of 10 μg/ml cholesterol and 1 μg/ml 25-hydroxylcholesterol stock solution were prepared by dissolving into 2 mg/ml and 1 mg/ml DMSO, respectively. Cholesterol medium was prepared by adding 0.05% lipid depleted FBS, 10 μg/ml cholesterol and 1 μg/ml 25-hydroxylcholesterol to α-MEM medium. The DMSO in the medium concentration would be 0.6% that is below 1%, the cytotoxic concentration of DMSO towards HepG2 cells (Machana et al., 2011). HepG2 cells were seeded at 8.0×10^3 cells per well in 96 well plate for 24 h. MCL water extract sample preparation was done by serial dilution to concentrations of 0.5, 1, 2, 3 and 4 μg/ml with cholesterol medium. After cell seeding, the cells were incubated with MCL water extract for 16 h. Negative control was prepared from cells with a cholesterol medium only. After 16 h, media were aspirated and washed three times with PBS. BODIPY FL LDL label was mixed with α-MEM medium and 0.05% lipid depleted FBS to obtain 5 μg/ml BODIPY FL LDL. A volume of 100 μl 5 μg/ml BODIPY FL LDL was added to each well. The cells were then incubated for 4 h in dark in the incubator. After 4 h, the BODIPY mixtures were aspirated and the cells were washed three times with PBS to ensure BODIPY FL LDL do not adhere to the cell surface and the wells to avoid positive error. The 96-well plate was read with microplate reader using excitation and emission wavelength of 490 and 515 nm, respectively. Percentage of LDL-c uptake were calculated as follow:

$$\%\ \ of\ \ LDL-c\ \ \ uptake = \frac{(OD_{sample} - OD_{blank})}{(OD_{negative\ control} - OD_{blank})} \times 100\% \quad (3)$$

2.8. Statistical analysis

The data were analysed using SPSS version 20.0. The normality of the data was analysed by Shapiro-Wilk test and Levene test was used to analyse the homogeneity of variance. Data fulfilled assumptions of parametric test was analysed by One-way ANOVA with Tukey test as post-hoc test. Data not fulfilled assumptions of parametric test were proceed to non-parametric tests which include Kruskal-Wallis test and Mann-Whitney test.

3. Results and discussion

3.1. Catechin identification and quantification in MCL water extract by HPLC

The MCL water extract with extraction yield of 24.54 ± 2.52% was analysed for catechin content with HPLC, as shown in Table 1. Figure 1 shows the chromatograms of catechins standards and MCL water extract, respectively. The order of chromatographic elution found in the present study was GC > EGC > C > EC > EGCG > GCG > ECG > CG, which is in agreement with study of Theppakorn et al. (2014) but slightly different from a study by Rahim et al. (2014) in which the researchers found EGCG elute first before EC. It was found the total catechins present in MCL water extract was 141.88 ± 5.04 mg/g. The most abundant catechin was CG with 75.27 ± 8.49 mg/g followed by GC with 28.37 ± 1.77 mg/g while the EC presented the least with 3.28 ± 0.88 mg/g. In addition, GCG that identified in standard's chromatogram was not identified in the MCL water extract.

Vuong et al. (2011) extracted 1 g of green tea powder with 100 ml water at 80 °C for 30 min. The catechin contents were EGCG (55.7 ± 1.1 mg/g), EGC (26.5 ± 1.5 mg/g), ECG (12.7 ± 0.6 mg/g), EC (9.7 ± 0.4 mg/g), GCG (3.0 ± 0.8 mg/g), GC (10.9 ± 1.3 mg/g), CG (3.4 ± 0.1 mg/g) and C (6.5 ± 0.1 mg/g). The total catechins of MCL was slightly higher than green tea (128.4 mg/g) compared to Vuong et al. (2011). Rahim et al. (2014) extracted green tea in water: acetonitrile: methanol (83: 6: 11) with microwave assisted extraction for 6 min. They found that green tea contains all eight catechins. The most abundant catechin in green tea was EGCG (24.5 ± 0.16–36.15 ± 0.54 mg/g) followed by EGC (10.09 ± 0.22–18.5 ± 0.85 mg/g), while the least was CG (0.17 ± 0.04–0.31 ± 0.05 mg/g). It is interesting to note that total catechins were higher than the green tea (80.3–123.67 mg/g) in this study. In this study, focusing on CG, the most abundant catechin found in MCL water extract is very limited. Owing the higher total catechins content of MCL compared to green tea, these findings show that MCL may be a potential alternative source of catechins.

3.2. Presence of catechin in MCL by UPLC-TWIMS-QTOF

Figure 2 shows the chromatogram of identified catechins derivatives, while Table 2 shows the catechin derivatives with the closely related isomers identified in MCL water extract. Identified derivatives with the lowest mass error was regarded to be the actual compound identified based on Waters in-house database MS/MS. The catechin derivatives identified include epigallocatechin-3-O-gallate (EGCG) with *m/z* 459.0912 $[M + H]^+$, epigallocatechin (EGC) with *m/z* 307.0818 $[M + H]^+$, catechin-3-O-gallate (CG) with *m/z* 443.0976 $[M + H]^+$, epigallocatechin(4β→8)-gallocatechin with *m/z* 649.0951 $[M + K]^+$, and gallocatechin(4α→8)-epicatechin with *m/z* 633.1 $[M + K]^+$. The present study is supported by Jambocus et al. (2017), as the researchers reported the present of catechins in MCL with proton nuclear magnetic resonance (^{1}H NMR). In relation to CG quantified as the major catechin in previous section, the result of UPLC-TWIMS-QTOF further reaffirms the presence of CG in MCL water extract.

Epigallocatechin(4β→8)-gallocatechin and gallocatechin(4α→8)-epicatechin are oligomers (dimers) made up from flavan-3-ols classified under proanthocyanidins (Xie and Dixon, 2005). There are several subclasses of proanthocyanidins depending on their monomers. Epigallocatechin(4β→8)-gallocatechin and gallocatechin(4α→8)-epicatechin are classified as prodelphinidins as the monomers are from gallocatechins (Ou and Gu, 2013). Studies suggested that the health promoting effects of proanthocyanidins depend on the degree of polymerization, stereochemistry and number of phenolic hydroxyl groups (Hatano et al., 2002).

Despite seven major catechins having been quantified in HPLC, only three major catechins had been identified based on *m/z* values. There is no previous study been found to identify polyphenol compounds in MCL by using LCMS method. In a previous study using triple quadruple

Table 1. Composition of individual catechins and total catechins in MCL water extract.

Individual catechins (mg/g)								Total catechins (mg/g)
GC	EGC	C	EC	EGCG	GCG	ECG	CG	
28.37 ± 1.77	8.07 ± 0.12	11.94 ± 1.36	3.28 ± 0.88	2.17 ± 0.45	-	8.11 ± 0.13	75.27 ± 8.49	141.88 ± 5.04

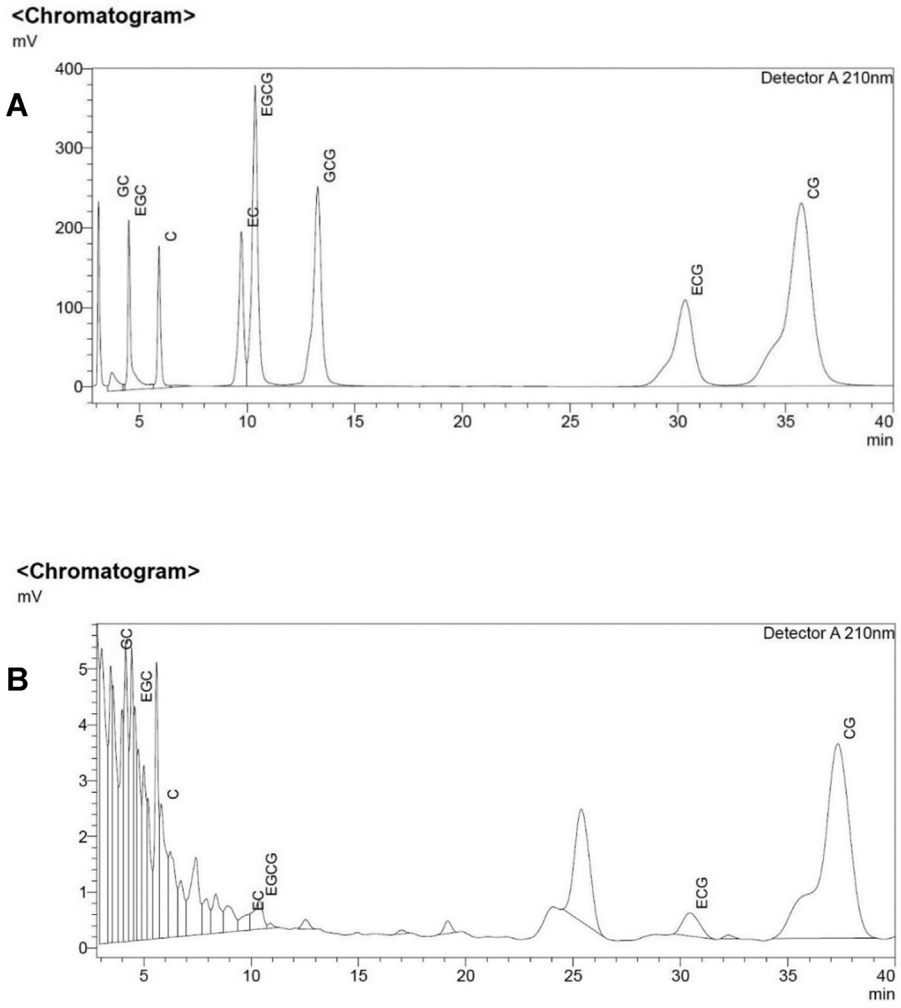

Figure 1. (A) Chromatographic separation of catechins standard with 10ppm (GC, EGC and C), 20 ppm EC, 50ppm (EGCG, GCG and ECG) and 100ppm (CG). (B) Chromatographic separation of MCL water extract.

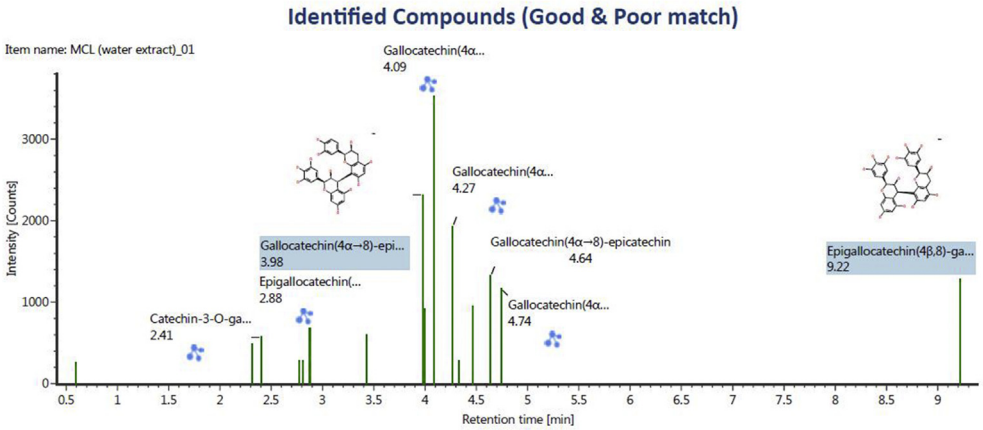

Figure 2. Identified catechins derivatives in MCL water extract.

Table 2. Catechin derivatives with closely related isomers identified in MCL water extract.

Precursor ions (*m/z*)	Observed neutral mass (Da)	Mass error (mDa)	RT (min)	DT (ms)	CCS (Å)	Product ions (*m/z*)	Adducts
Epigallocatechin-3-O-gallate							
459.0908	458.0835	-1.4	4.33	6.61	197.49	445.12159	+H
459.0917	458.0844	-0.5	4	6.66	198.8	147.04364	+H
Epigallocatechin							
307.0795	306.0722	-1.7	2.8	6.28	189.62	152.05653	+H
307.0795	306.0722	-1.7	2.78	5.53	170.59	-	+H
307.0818	306.0746	0.6	2.31	5.29	164.51	-	+H
Catechin-3-O-gallate							
443.0976	442.0904	0.4	2.41	6.31	189.89	-	+H
Epigallocatechin(4β→8)-gallocatechin							
633.1199	610.1307	-1.5	0.59	7.78	229.01	439.14266	+Na
649.0951	610.1319	-0.3	2.88	6.09	184.68	183.02876	+K
649.0947	610.1315	-0.7	2.88	4.9	155.42	-	+K
649.095	610.1319	-0.4	3.43	7.89	232.05	-	+K
649.0947	610.1316	-0.7	2.88	7.9	232.31	147.04378 591.15669	+K
649.0969	610.1337	1.5	9.22	8.3	243.57	319.08147	+K
Gallocatechin(4α→8)-epicatechin							
633.0989	594.1357	-1.6	4.47	7.83	230.49	-	+K
633.0997	594.1365	-0.8	4.26	5.78	176.9	147.04379	+K
633.1	594.1368	-0.5	4.74	7.89	232.04	441.07239	+K
633.0998	594.1366	-0.7	4.27	4.74	151.61	-	+K
633.0999	594.1368	-0.6	4.64	7.85	231.12	-	+K
633.0994	594.1363	-1.1	3.98	6.09	184.68	-	+K
633.0989	594.1357	-1.6	4.09	5.88	179.42	147.04364	+K
633.0997	594.1365	-0.8	4.27	7.84	230.62	148.04724	+K
633.0994	594.1363	-1.1	3.98	7.88	231.74	147.04364	+K
633.099	594.1359	-1.5	4.09	7.91	232.53	148.04712	+K

RT = retention time, DT = drift time, CCS = observed collisional cross section.

tandem LCMS, UPLC separation for catechin isomers needed to be optimized to ensure the best results. This is because catechin epimers have the same molar mass and consequently same *m/z* value for the parent $[M-H]^-$ ions as well as identical daughter ions. Thus, the catechin epimer separation solely depends on chromatographic separation (Bataglion et al., 2015). In addition, instrumental setting and data processing parameters of UPLC-TWIMS-QTOF have yet to be optimized in order for the successful detection of all major catechins (Righetti et al., 2018). This may be the reasons behind why only three major catechins were identified in the present study.

Several compounds identified as the same compounds were assumed to be the closely related isomers as similar to the study of Yassin et al.

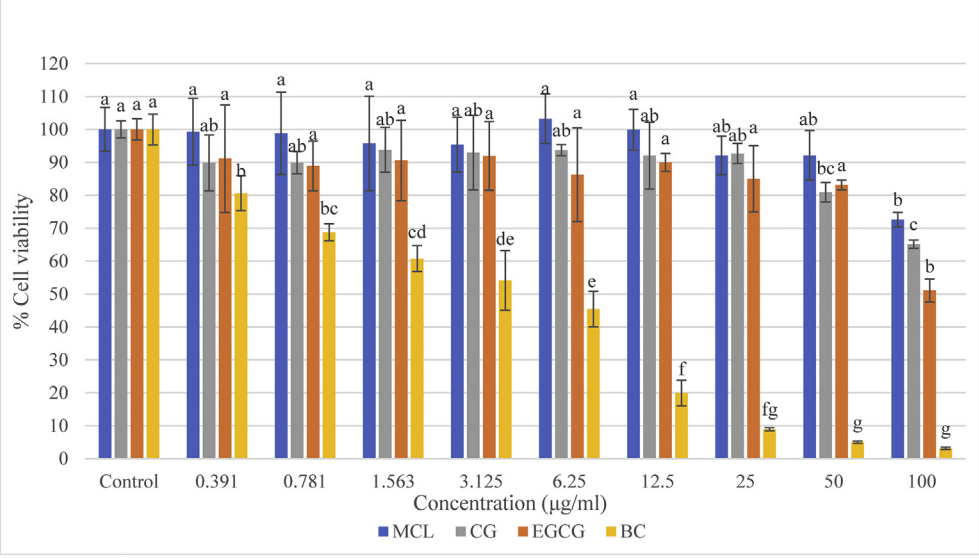

Figure 3. Cell viability of HepG2 cells after incubation with MCL, CG, EGCG and BC at different concentrations for 72 h. Values with different letter (a, b, c) are significantly different ($p < 0.05$ with $n = 3$) between sample concentrations. Data represent mean ± standard deviation.

(2014). There are two types of EGCG, three types of EGC, six types of epigallocatechin(4β,8)-gallocatechin and ten types of gallocatechin(4α→8)-epicatechin identified in MCL water extract. Due to the unique characteristics of CCS value, present study reported CCS value for each closely related isomer can contribute to future studies on the structure elucidation of the compounds (Lanucara et al., 2014). The result showed that there are numerous types of catechins that can be further investigated on their isomeric structures, respectively. In addition, these closely related catechin derivatives isomers show great potential for use in human health as common major catechins.

3.3. Cytotoxicity of MCL and catechins

The cell viability rates of HepG2 cells after incubation with MCL water extract, CG, EGCG and BC are shown in Figure 3. CG was tested due to it was the major catechin presents in MCL water extract, EGCG is the most bioactive and widely studied catechin while BC is the positive control that was used in hypolipidemic assay. The IC_{50} of MCL water extract, CG and EGCG on HepG2 cells were more than 100 μg/ml. MCL water extract, CG and EGCG can be classified as non-cytotoxic to HepG2 cell line, as the guidelines of the US National Cancer Institute plant screening program stated that crude extract and pure compound with IC_{50} less than 20 μg/ml and 4 μg/ml, respectively after incubation between 48 and 78 h are classified as cytotoxic (Boik, 2001). BC with IC_{50} of 4.77 μg/ml was close to the classification of 4 μg/ml to be cytotoxic to HepG2 cells.

MCL water has been was found to be non-cytotoxic based on previous studies. Thani et al. (2010) found the IC_{50} of MCL aqueous extract more than 600 μg/ml on human cervical carcinoma (HeLa), human breast cancer cells (MCF-7), HepG2 cells and African green monkey kidney cells (Vero), and more than 300 μg/ml on the subline of the ubiquitous KERATIN-forming tumour cell line HeLa (KB) cells after incubation for 48 h. In relation to animal studies, MCL water extract did not exert any acute, sub-acute or sub-chronic oral toxicity at doses of 2000, 200 and 20 mg/kg body weight, respectively (West et al., 2007).

The non-cytotoxic effects of EGCG are in line with a study by Khiewkamrop et al. (2018), who reported the IC_{50} of EGCG on HepG2 cells after 72 h incubation was 0.5 mM (equivalent to 229.1 μg/ml). The limitation of the present study was the exclusion of isolation for CG in MCL as the prolonged isolation protocol might destabilize the environmental sensitive CG which in turns limit the validity of the study. Cytotoxicity study on CG is in paucity of information. The present study found CG were non-cytotoxic. Babich et al. (2007) also reported the non-cytotoxic effect of CG on immortalized human gingival epithelioid (S-G), human tongue squamous carcinoma (CAL27), human salivary gland carcinoma (HSG) and normal human gingival fibroblasts (HGF-1) by NR assay. The NR50 of CG were 58 μM (25.66 μg/ml) for S-G cells, 62 μM (27.43 μg/ml) for CAL27 cells, 90 μM (39.81 μg/ml) for HSG cells and 132 μM (58.98 μg/ml) for HGF-1 fibroblasts after 72 h of incubation.

BC was close to the classification of being cytotoxic to HepG2 cells, showing cell inhibition in dose-dependent manner. The result was supported by previous study where Zhou et al. (2014) assessed cytotoxicity of BC on HepG2 cells (6×10^4 cell/well) with MTT assay. The result demonstrated 15 μM (5.58 μg/ml) BC is not cytotoxic to HepG2 cells for 24 h incubation with cell viability of more than 95%. In 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, BC significantly inhibited HepG2 cells (cell number of 5×10^3 cell/well) approximately 50 and 80% after 24 h incubation with 50 (18.59 μg/ml) and 100 μM (37.18 μg/ml), respectively. After 72 h incubation, concentrations of 20 (7.44 μg/ml), 50 and 100 μM significantly inhibited HepG2 cells reached approximately 40, 80 and 95%, respectively.

3.4. LDL-c uptake of MCL and catechins

Figure 4 shows the increase in LDL-c uptake of HepG2 cells relative to control. The doses of MCL, CG, EGCG and BC were tested up to 4 μg/ml in reference to the IC_{50} of BC in cytotoxicity test. MCL at concentration of 1, 2 and 3 μg/ml increased LDL-c uptake up to 1.11 ± 0.03-fold but the increment was not significant relative to control. As comparison, CG significantly increased LDL-c uptake at 3 μg/ml with 1.37 ± 0.19-fold. In addition, EGCG significantly increased LDL-c uptake at 4 μg/ml with 1.59 ± 0.19-fold. Furthermore, BC significantly elevated LDL-c uptake at 3 and 4 μg/ml with the highest at 3 μg/ml (1.77 ± 0.33-fold). One human study found after ingestion of 668 mg EGCG and 663 mg ECG, plasma catechins reached 1.3 μmol/L (0.596 μg/ml) EGCG and 3.1 μmol/L (1.37 μg/ml) ECG (Mazza et al., 2002). Thus, the catechins concentrations used in this present study were applicable to catechin plasma concentrations in the human body.

Plasma cholesterol is mostly removed via LDLR pathway. Elevated expression of LDLR reduces plasma cholesterol through an increase in LDL-c uptake in the liver (Brown and Goldstein, 2009). Thus, the increase in LDL-c uptake reflecting the lower plasma cholesterol. Pal et al. (2002) reported the consistency between LDLR binding activity done with colloidal gold-LDL conjugates, cellular LDLR protein by western blotting, LDLR mRNA by Southern blotting of reverse-transcribed and polymerase reaction-amplified complementary deoxyribonucleic acid (cDNA). Since the current method similar to LDLR binding activity, the result may be generalized into molecular biology aspects.

MCL, EGCG and CG showed lower LDL-c uptake than BC. BC is a novel anti-hyperlipidemic drug that reported to significantly reduced LDL-c by 20–25% in clinical trials (Cicero et al., 2007). All the three standard compounds significantly improved LDL-c uptake but MCL water extract show no significant increment. Based on the previous section, MCL water extract contains 75.27 ± 8.49 mg/g of CG and 2.17 ± 0.45 mg/g EGCG. Thus, CG significantly increased LDL-c uptake in HepG2 cells and was able to contribute to the effect of LDL-c uptake in MCL water extract. Furthermore, the insignificant of LDL-c uptake increment in MCL water extraction might be due to its low CG content (7.5%) as compared to the pure CG standard (>98%). In addition, the low level EGCG (0.2%) might be another reason for the insignificant LDL-c uptake effect. Despite present study found the MCL water extract showed no significant in LDL-c uptake, animal models reported MCL ethanolic extract significantly exert hypolipidemic effect via inhibition of HMG-CoA reductase in hyperlipidemic rats. MCL ethanolic extract significantly reduced total cholesterol, LDL-c, triglyceride TC/HDL ratio, atherogenic index, weight gain and visceral fat in rats (Jambocus et al., 2017). The reason for the insignificance of the result in the present study might be due to the low concentration of MCL water extract being unable to exert a significant effect on LDL-c uptake.

Both catechins used in this study significantly increased LDL-c uptake in HepG2 cells. The result was supported by Goto et al. (2012). The researchers performed microarray analysis and found that 25 μM EGCG increased 13 genes of sterol metabolism by > 1.5-fold in HepG2 cells with LDLR strongly up-regulated by 2.2-fold. In the same study, 10 μM and 25 μM EGCG increased mRNA expression of LDLR by 1.8 and 1.7-fold in HepG2 cells. *In vitro* study reported the binding activity of catechins towards LDL-c with the ability in the sequence of ECG > CG > EGCG while C, EC, EGC and GC showed low binding activity. Thus, catechins may be able to bind with LDL-c, reducing LDL-c oxidation and thus exert a cardioprotecting effect (Manach et al., 2004). On the other study, CG and EGCG reported to inhibit cholesterol solubilization thus reducing plasma cholesterol level (Kirana et al., 2005).

Kuhn et al. (2004) found the expression of p68/SREBP-2 and LDLR in HepG2 upregulated by catechins were in the sequence EGCG > ECG > EGC > EC. This study also suggested catechin with galloyl moiety exert more hypolipidemic activity compared to the counterparts. Thus, CG and

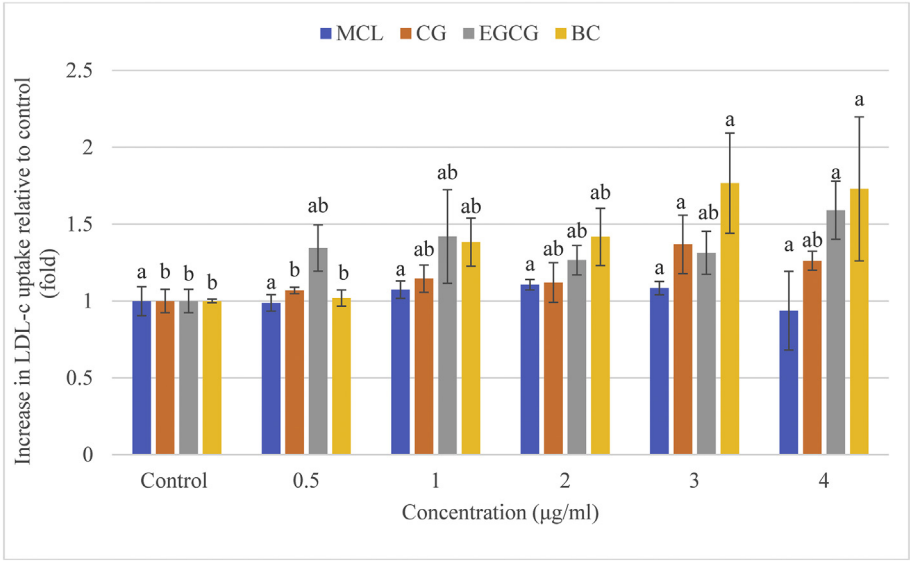

Figure 4. Increase in LDL-c uptake relative to control in HepG2 cells after incubation with MCL, CG, EGCG and berberine chloride (BC) at different concentrations. Values with different letter (a, b, c) are significantly different ($p < 0.05$ with $n = 3$) between sample concentrations. Data represent mean ± standard deviation.

EGCG with galloyl moiety explain the significant LDL-c uptake found in the present study.

Lee et al. (2008) investigated the effects of GCG and EGCG in LDLR expression with HepG2 cells using western blotting with a polyclonal anti-LDLR antibody. Both GCG and EGCG significantly increased LDLR protein expression with the effect of GCG (approximately 450% of control) being stronger than EGCG. Furthermore, the researchers investigated the effect of 50 µM (22.9 µg/ml) EGCG and GCG on LDLR activity of in HepG2 cells by BODIPY labeled LDL. GCG treatment significantly increased the LDLR activity approximately 2-fold compared to control. The GCG treatment also found to be significantly higher LDLR activity than epimer EGCG. This study suggested non-epimer catechins such as CG might possess greater hypolipidemic action than the epimer catechins counterparts. Thus, CG exerts hypolipidemic action via multiple mechanisms and might serve as a good candidate for a hypolipidemic drug.

4. Conclusion

The findings of the study show that MCL water extract contains high levels of catechins comparable to those found in green tea. Among individual catechin, CG is the most abundant in MCL water extract. UPLC-TWIMS-QTOF further affirms the presence of CG in MCL water extract and unveiled the presence prodelphinidins, which might have higher bioactivity than the monomer catechin counterparts. Thus, MCL water extract not only contains high amount of catechins but large variety of the monomeric and dimeric catechins. In addition, both MCL water and CG showed no cytotoxic effects on HepG2 cells. LDL-c uptake assays revealed the antihypertensive activity of MCL water extract and CG. Despite LDL-c uptake being lower compared to the commercial anti-hyperlipidemic drugs (BC) and the most bioactive catechins (EGCG), both MCL water extract and CG might become an additional option for the treatment of hyperlipidaemia due to its low cytotoxicity.

Declarations

Author contribution statement

Chong Kah Hui: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Nurul Iwanie Majid: Performed the experiments; Analyzed and interpreted the data.

Hayati Mohd Yusof: Conceived and designed the experiments; Analyzed and interpreted the data.

Khairi Mohd Zainol: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Habsah Mohamad: Contributed reagents, materials, analysis tools or data.

Zamzahaila Mohd Zin: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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