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Effect of Acidified Ethanol on Antioxidant Properties of *Morinda citrifolia* Leaf Extract and Its Catechin Derivatives

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Abstract

This study was conducted to investigate the effect of ethanol acidification on the antioxidant properties of *Morinda citrifolia* leaf (MCL) extract and its catechin derivatives. Four different ethanol (100%, 99.5%, 70%, 50%) with or without 0.5% acetic acid were used for extraction. The antioxidant profile was studied with DPPH radical scavenging activity, FRAP and TPC. The quantification of catechins in MCL was performed using HPLC, and the identification of catechins derivatives was performed with UPLC-TWIMS-QTOF. The results showed that an extraction solvent composed of 70% ethanol: 29.5% water: 0.5 % acetic acid exhibited the highest DPPH percentage of inhibition (86.12±2.96%) and highest TPC value with 97.80±0.25 mg GAE/g extract, while 100% ethanol acidified with 0.5% acetic acid showed highest FRAP antioxidant power with 1.31±0.05mg FSE/g extract. All eight types of catechins were identified in MCL and the most total catechins were quantified in 70% ethanol: 29.5% water: 0.5 % acetic acid at 153.57mg/g. The catechin derivatives identified included epigallocatechin-3-O-gallate (EGCG), epigallocatechin (4β, 8)-gallocatechin, gallocatechin (4α→8)-epicatechin, catechin-3-O-gallate (CG) and epigallocatechin (EGC). The results suggest that acidification improves the extraction of polyphenols as well as catechin content.

Article History

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Keywords

Acidification;
Antioxidant;
Catechin;
Morinda citrifolia;
UPLC-TWIMS-QTOF.

Introduction

Oxidative stress research has attracted interest in the past few decades as it is the root cause of several diseases such as cancer, atherosclerosis and brain dysfunction.[1] Intake of exogenous antioxidants is a promising way to counter the undesirable effects of reactive oxygen species (ROS), thus reducing oxidative damage.[2] Plants' secondary

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metabolites have numerous functions. Even when they are not involved in crucial functions such as UV protection, anti-pathogen and anti-herbivore activities, pigmentation facilitates pollination and enhances plants' health and survivability.[3] Catechins, also known as flavan-3-ols, are a subgroup of flavonoids classified under polyphenols which are abundant in vegetables and plants.[4]

Morinda citrifolia (*M. citrifolia*) or noni originates in tropical Asia or Polyneisa.[5] Every single part of *M. citrifolia*, including the roots, barks, stems, leaves and fruit, has been used in traditional medicine to treat diseases such as diabetes, hypertension, cardiopathy and arteriosclerosis. *M. citrifolia* is a valuable plant which contains more than 150 nutraceuticals, vitamins, minerals, micro and macro nutrients that assist the body in many ways, from the cellular to organ level.[6] *M. citrifolia* leaf (MCL) contains a considerable amount of antioxidant which is comparable to green tea.[7,8] In addition, the antioxidant properties of MCL have been found to be comparable to natural antioxidants (α-tocopherol) and artificial antioxidants (BHT) in certain assays.[5] Catechins and epicatechins have been found in M. citrofolia extract.[9] Researchers have described noni tea's beneficial effects in terms of anti-inflammation, antioxidation, anti-allergy and anti-obesity, primarily due to the high catechin derivatives content.[10,11]

Catechins are highly polar and structural stabilized in polar solvents. Thus, researchers have suggested that polar solvents such as water, ethanol, methanol, DMF and acetone should be used.[12,13] Several studies have reported aqueous ethanol provides a higher yield of catechins compared to absolute organic solvents and water per se.[14,15,16] There have been few studies on the extraction of catechins from MCL. One study used 50% ethanol with solid-liquid extraction.[17] In addition, catechins in MCL have been reported to be extracted with absolute ethanol by solid-liquid extraction, microwave-assisted extraction, ultrasound-assisted extraction and supercritical fluid extraction.[18] Water and ethanol are among the most popular solvents used in extraction due to their low toxicity and high extraction yield. In addition, water and ethanol are Generally Recognized as Safe (GRAS) solvents. There is an increasing trend of utilizing GRAS in the food industry (Herrero *et al*., 2005).[19] By modulating the ratio of water and ethanol in the extraction solvent, different polarities can be achieved to improve the extraction yield.[20] The acetic acid used in the present study is widely recognized as a GRAS acid.[21] The addition of acid in an extraction solvent is known to improve polyphenols extraction in several ways. Acid can improve stability of some phenolic compounds such as anthocyanins and catechins.[22,13] In addition, polyphenols that are initially part of polymers or bound to the cell wall constituents leach more readily in acidic medium through hydrolysis, as reported in a study of hydroxycinnamic acid and procyanidins.[23,24] Furthermore, acid could facilitate the disintegration of the cell walls, thus improving the solubilization and diffusion of polyphenols from the plant matrix.[25] The selection of extraction solvents and conditions is crucial in terms of total phenolic compounds, total flavonoids, and antioxidant activity, due to their great influence on extract yield and composition.

In addition to this, research on the impacts of solvent choice on the extraction of active components from MCL is lacking. The aim of this research was therefore to establish the effects of acidification and different concentrations of ethanol on the biological activity and content of bioactive catechins in MCL.

Material and Methods

Plant Materials

Fresh *Morinda citrifolia* L. leaves (MCL) (DINO 04-1425) were obtained from MARDI (Jerangau Station), Terengganu, Malaysia. The samples were washed with running tap water, separated and air dried on the surface before being cut into pieces and dried using an oven at 40°C. The dried samples were then ground up to 0.5mm for approximately 2-3 min using a grinder before any further processing.

Extraction

The extraction of MCL was conducted following the process of Chang *et al*.[26] with several changes. 10g samples were combined with 100ml of solvent in a conical flask (Table 1). The mixture was left for 24h in an incubator shaker at 25°C, 175 rpm. The extracts were then filtered through Whatman filter paper (no.1) and another 100ml fresh solvent was added to residue mixture and incubated for 24h for re-extraction. Then, all the supernatants were pooled and the solvent was removed with a rotary evaporator under vacuum at 40°C. The acidified ethanol had a pH range of pH 3.51-3.76 while the

non-acidified ethanol had a pH of 7.5 -7.85. The yield (dry weight) of extraction was calculated using the following equation:

$$Yield\ (\%) = \frac{Weight\ of\ concentrated\ MCL\ liquid\ crude\ extract\ (g)}{Weight\ of\ MCL\ powder\ (g)} x\ 100$$

Table 1: Extraction solvent composition utilized in MCL extraction

Acidified ethanol (%)		Non-acidified ethanol (%)	
A=Ethanol: acetic acid (99.5:0.5, v/v)	pH 3.53	D=Ethanol (100, v/v)	pH 7.58
B=Ethanol: water: acetic acid (70:29.5:0.5, v/v)	pH 3.76	E=Ethanol: water (70:30, v/v)	pH 7.85
C=Ethanol: water: acetic acid (50:49.5:0.5, v/v)	pH 3.51	F=Ethanol: water (50:50, v/v)	pH 7.5

Antioxidant Assay

2, 2-ddiphenyl-2-picrylhydrazyl (DPPH) Assay

DPPH was performed using a method adapted from Re *et al*.[27] The diluted working solutions of the extracts were prepared in methanol. 0.1 mM of DPPH was prepared in methanol and 2ml of this solution was mixed with 1ml of sample solution and BHT as standard. These solutions were kept in dark condition for 30min and then measured at 518nm. The results were reported in terms of percentage of inhibition.

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP test was conducted using the Benzie and Strain[28] methods with some modification. Combination of 2.5ml of 10mM 2,4,6-tri (2-pyridyl-s-triazine) (TPTZ), 25ml of 300mM (pH 3.6) sodium acetate buffer and 2.5ml of 20mM iron (III) chloride anhydrous was prepared for FRAP reagent. Ferrous sulphate was used as a standard antioxidant. 0.5ml standard and sample was added to 1ml of FRAP reagent and kept for 30min at room temperature. The mixture was then measured at 593nm using a spectrophotometer. The results were expressed in terms of mg ferrous sulphate equivalent (FSE)/g extract.

Total Phenolic Content (TPC) Assay

TPC was measured using Folin-Ciocalteu's reagent.[29] An amount of 0.25ml water was added to 0.25ml MCL extract. Then, the mixture was left standing at room temperature for 30min. The absorbance of the mixture was measured via a spectrophotometer at 570nm. Standard gallic acid was used. The results were reported in terms of mg of gallic acid equivalent (GAE)/g extract.

Quantification and Identification of Catechins in *Morinda Ctrifolia* Leaf Extract

HPLC analysis for eight catechin standards and MCL extracts were conducted using a Shimadzu HPLC system. The eight catechin standards include catechin (C), epicatechin (EC), gallocatechin (GC), epigallocatechin (EGC), catechingallate (CG), epicatechingallate (ECG), gallocatechingallate (GCG) and epigallocatechin gallate (EGCG). The HPLC is 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. An AGILENT 690970-902 Poroshell 120, EC-C18, 4.6x250mm, 4µm was used. HPLC conditions were modified from Theppakorn *et al*.[4] while the mobile phase was composed of water and acetonitrile (87:13) with 1ml/min flow rate. The column oven was set to 30°C and the wavelength detector was set to 210nm. Injection volume for both standards and sample were set at 20µl. The catechin quantification of sample was based on the peak area of the standards using an external calibration method. Total catechins is the summation of all eight catechins.

UPLC-TWIMS-QTOF Condition for Catechin Analysis

The MCL sample with the highest total catechin was selected for further catechin derivative identification. Chromatographic analysis was carried out using Waters Acquity I-Class UPLC system (Waters Corporation, Milford, MA, USA) consisting of a

column oven, sampler manager FTN and I-class binary solvent manager. Separation was achieved by chromatography using Waters Acquity UPLC HSS T3 (2.1x 100mm, 1.8µm) 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 follows: 1% B (0-0.5 min), 35% B (0.5-16 min), 100% B (16-18 min), 1% B (18-20 min). The flow rate was that of 0.6ml/min and injection volume was 5µL. The temperatures of the column and sample were maintained at 40°C and 15°C, respectively. Mass spectrometry was conducted on a mass spectrometer Waters Vion™ IMS-QT (Waters Corporation, Milford, MA, USA). Ionization was achieved in the positive mode using electrospray (ESI+). At 550°C, the desolvation gas was set at 800 L/h, the cone gas to 50L/h, the source temperature at 120°C, and capillary voltage to 1.5V. Vion Data were acquired in the high definition MS^E ($HDMS^E$) with full scan in mass range 100-1000m/z and scan time 0.2s. In $HDMS^E$, the MS / MS acquisition mode was programmed with two different scan functions. One scan function was set to 4eV (electronvolt) low-energy-collision-induced dissociation (CID) in the trap cell, while the other scan function was set to 10eV to 45eV in the transfer cell at high CID ramping. The ion mobility separation (IMS) was done with a travelling wave (TWIMS). Instrument control and data processing was performed with Waters UNIFI software version 1.8.

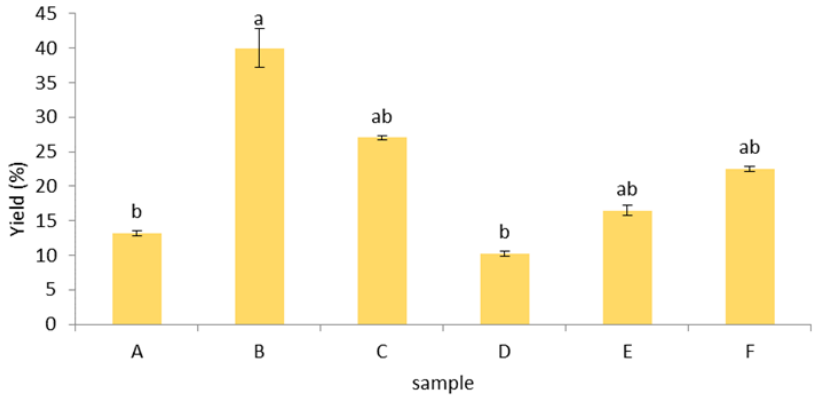
Statistical Analysis

All data were subjected to ANOVA one way, followed by a Tukey test at 5% significance level ($p<0.05\%$) using SPSS software.

Results and Discussion

Extraction Yield

The first step towards the utilization of phytochemicals is the extraction of bioactive compounds from plant materials in preparation of dietary supplements or nutraceuticals, pharmaceutical products and also in food ingredients.[30] As shown in Figure 1, highest yield (40%) of extraction seen in sample B which extracted with ethanol: water: acetic acid (70: 29.5: 0.5, v/v) and is significantly ($P<0.05$) higher than samples A and D. In contrast, sample D extracted with 100% ethanol showed lowest extraction efficiency at only 10% yield. This shows that the inclusion of water improved the extraction yield.



A: Absolute ethanol acidified with 0.5% acetic acid, B: 70% ethanol acidified with 0.5% acetic acid, C: 50% ethanol acidified with 0.5% acetic acid, D: Absolute ethanol, E: 70% ethanol, F: 50% ethanol
Values with different letters (a-b) differ significantly ($p<0.05$ with $n=3$) between the sample. Data represent in mean ± standard deviation

Fig.1: Extraction yield of MCL extracted with different solvent compositions

The mixture of ethanol and water gave better extraction yields than pure ethanol. Similar observations were reported by Do *et al.*,[31] who found that the yield of pure solvent is less than yield of aqueous solvents. Single solvent cannot fully extract all the compounds from plant material; therefore,

numerous solvents of different polarities need to be used to extract different phenolic compounds from plants with a higher grade of accuracy. [32]

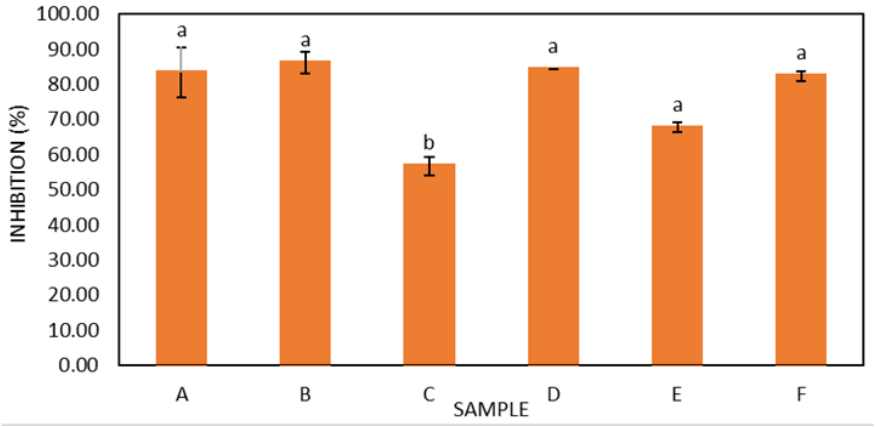
In the context of ethanol concentration, sample B gave significantly (P<0.05) higher yield compared to samples A and D, but no significant difference (P>0.05) compared to samples C, E and F. The difference in the extraction solvent is that sample B used 70% ethanol, while sample C used 50% ethanol. These findings are in line with those of Thoo *et al*.,[33] who found that higher ethanol concentration at a lower extraction temperature is advisable to increase the extraction of total flavonoid compounds.

The presence of acid has a positive effect of the extraction yield. This trend is in agreement with Magwaza *et al*.,[34] who stated acidic aqueous methanol is suitable for extracting phenolic acids and flavones. Furthermore, the addition of a small amount of acid (0.5% acetic acid) was able to increase the polyphenol yield. This trend was in line with Chirinos *et al*.,[25] as they reported higher polyphenol extraction ay 90% methanol acidified with 0.01% HCl (pH 3.08) over 0.005% HCl (pH 5.00). The concentration and pH used in the present study of 0.5% acetic acid with pH 3.51-3.76 are comparable to those used in a study by Chirinos *et al*.,[25] Adding an acid to the extraction solvent has multiple benefits in extracting polyphenols, as they can hydrolyse the polyphenols that were originally bound to polymers or cell walls and disintegrate the cell wall, freeing polyphenol. This facilitates the leaching of polyphenols into the extraction solvent.[23,24,25] Furthermore, the acid might have a stabilizing effect on the polyphenols throughout the extraction process.[22] Thus, higher extracted yields are observed in acidified ethanol.

Antioxidant Profile

2,2-ddiphenyl-2-picrylhydrazyl (DPPH) Assay

Figure 2 shows the free radical scavenging activity (DPPH) of different extraction solvents from MCL. Sample B showed highest percentage of inhibition (86.12±2.96%) but was not significantly different than the other samples, except for sample C. These findings are supported by Thoo *et al*.[33] where higher ethanol concentration give better yield of antioxidant in acidified ethanol but not in the case of non-acidified ethanol. The DPPH inhibition percentage of MCL was comparable to camphor leaf, a type of Chinese herb extracted with 96% ethanol (87% DPPH inhibition).[35]

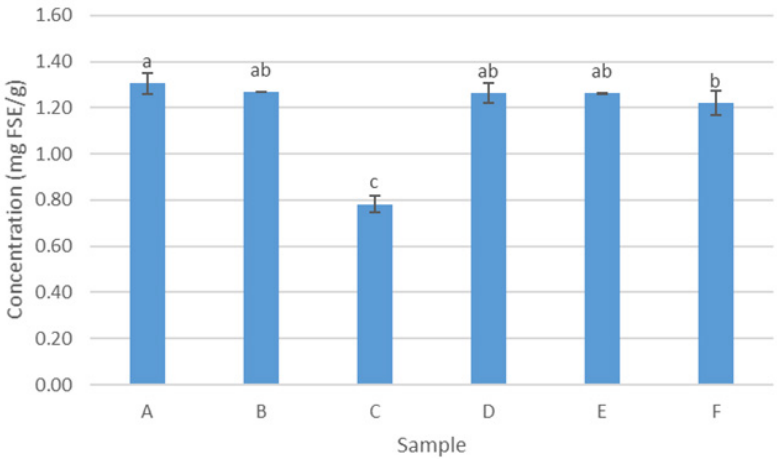


A: Absolute ethanol acidified with 0.5% acetic acid, B: 70% ethanol acidified with 0.5% acetic acid, C: 50% ethanol acidified with 0.5% acetic acid, D: Absolute ethanol, E: 70% ethanol, F: 50% ethanol
Values with different letters (a-b) differ significantly (p<0.05 with n=3) between the sample. Data represent in mean ± standard deviation

Fig.2: DPPH free radical percentage of MCL extracted with different solvent compositions

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay results for MCL from different solvent extraction is shown in Figure 3. There are no significant differences between absolute ethanol and 70% ethanol in both acidified and non-acidified ethanol. This was in contrast to results from Bhullar and Rupasinghe[36] for partridgeberry, where 70% ethanol showed significantly higher FRAP value than pure ethanol.



A: Absolute ethanol acidified with 0.5% acetic acid, B: 70% ethanol acidified with 0.5% acetic acid, C: 50% ethanol acidified with 0.5% acetic acid, D: Absolute ethanol, E: 70% ethanol, F: 50% ethanol
Values with different letters (a-c) differ significantly ($p<0.05$ with n=3) between the sample. Data represent in mean ± standard deviation

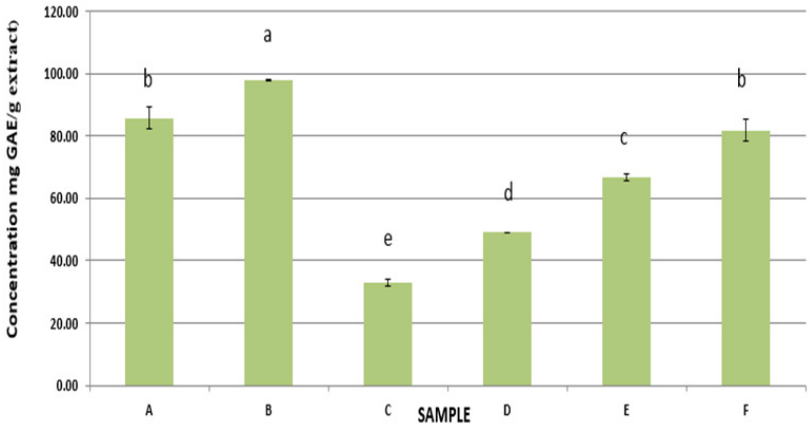
Fig.3: FRAP value of MCL extracted with different solvent compositions

Total phenolic content. TPC results from different solvent extraction of MCL are shown in Figure 4. Sample B shown significantly ($P<0.05$) higher phenolic content (97.80±0.25 mg GAE/g extract) compared to the other samples. TPC of ethanolic MCL extract is higher than 60% ethanolic extract of medicinal herb, *Vernonia cinerea* with TPC reported 53.96±1.45 mg GAE/g extract.[37] In addition, TPC of MCL dry powder (39.12 mg GAE/ g dw, after conversion with extraction yield) is also slightly higher than 70% ethanol Moringa stenopetala extract (33.6 mg of GAE/ dw) when the leaves were previously dried with oven drying at 50°C.[38]

The recovery of phenolic compounds dependant on the form of solvent used, its polarity index (PI), and the solubility of phenolic compounds in the solvent extraction.[39] TPC of ethanolic MCL extracts were higher than MCL water extract (42.66±8.870 mg GAE/mg extract) as reported by Chong *et al*.[40] Kopjar *et al*.[41] reported that acidified methanol extracts of pulverized yellow tea leaves exhibit the highest TPC. Similarly, a study of finger millet by Chethan and Malleshi[42] found the solvents acidified with 1% HCl (water, acetone, propanol, ethanol, and methanol) gave higher polyphenol extraction yield compared to the non-acidified solvents. The findings show that the finger millet polyphenol was more stable under acidic conditions in line with the present study, in which the acidified extraction solvents in samples A and B showed higher TPC values.

In acidified ethanol, 70% ethanol showed significantly higher TPC than pure and 50% ethanol. These results are similar to those of a study by Chirinos *et al*.,[25] who found that water proportion more than 50% showed reduction in the TPC, TFC and ORAC. The addition of acid had beneficial effect in the polyphenol profile, as described by Pompeu *et al*.,[43] The authors concluded that a low concentration of

acid is required to rupture the cell walls of the plant matrix to facilitate the polyphenol leaching process, while the concentration of acid has no significant effect on TPC.



A: Absolute ethanol acidified with 0.5% acetic acid, B: 70% ethanol acidified with 0.5% acetic acid, C: 50% ethanol acidified with 0.5% acetic acid, D: Absolute ethanol, E: 70% ethanol, F: 50% ethanol
Values with different letters (a-e) differ significantly ($p<0.05$ with n=3) between the sample. Data represent in mean±standard deviation

Fig.4: TPC value of MCL extracted with different solvent compositions

HPLC Analysis on MCL Catechins

Table 2 shows total catechin contents of different MCLs extracted with different solvent compositions. Sample B had the highest total catechins at 153.57mg /g, while sample D had the lowest total catechins at 70.65mg/g. The total catechins of MCL were close to the findings of Friedman *et al*.,[44] who found that green tea extracted with boiling water for 5 min contained seven catechins (except GC) at 4.4-100.0 mg/g dw.

Table 2: Total catechin contents of different MCL extracted with different solvent compositions

Sample	Individual catechins (mg/g extract)								Total Catechin (mg/g extract)
	GC	EGC	C	EC	EGCG	GCG	ECG	CG	
A	28.844	4.401	5.198	2.211	15.867	10.297	31.458	2.662	100.94
B	47.401	14.256	4.598	1.252	5.962	nd	33.571	46.529	153.57
C	32.775	12.213	3.850	1.383	1.551	nd	5.396	30.244	87.41
D	13.572	2.570	2.623	4.059	3.219	2.407	14.598	27.601	70.65
E	nd	69.002	22.373	4.196	2.839	2.950	1.304	13.513	116.18
F	nd	39.105	2.730	3.407	3.403	nd	6.208	33.869	88.72

A: Absolute ethanol acidified with 0.5% acetic acid, B: 70% ethanol acidified with 0.5% acetic acid, C: 50% ethanol acidified with 0.5% acetic acid, D: Absolute ethanol, E: 70% ethanol, F: 50% ethanol

*GC(Gallocatechin), EGC(Epigallocatechin), C(Catechin), EC(Epicatechin), EGCG(Epigallocatechin), GCG(Gallocatechingallate), ECG (Epicatechingallate), CG (Catechingallate).
**nd-not detected

There have been few studies on the extraction of catechins from MCL. Lim *et al*.[17] reported 3.14% EC with 50% ethanolic MCL extract, while Pak-Dek *et al*.[18] found 63.46 ± 17.8 mg/g extract of C and 23.08 ± 11.7 mg/g extract of EC in ethanolic MCL extract. The present study found a higher amount of catechins in MCL extract, which may be due to the extra catechins analysed.

70% ethanol in both acidified and non-acidified ethanol gave better catechins recovery than either pure ethanol or 50% ethanol, respectively. This shows the proportion of ethanol and water has affected the catechin extraction. These findings are in line with Escribano-Bailón and Santos-Buelga,[22] who reported a minimum of 70% methanol is needed to inactivate polyphenol oxidases to facilitate the maximum recovery of monomeric flavan-3-ols. Acidified ethanol in pH range of 3.51-3.76 resulted in higher catechins recovery than non-acidified ethanol in a pH range of 7.5-7.85. Low pH extraction solvent can prevent oxidation of polyphenols, which may improve the stability of catechins.[45] Previous findings have shown that catechins were stable at a pH level of less than 4, with stability declining when pH increased from pH 4 to 8.[46] However, the stability of catechins varies with different types of acid. For instance, ascorbic acid has been shown to significantly improve stability of catechins, while the effect of citric acid on stability of catechins is minimal.[47]

Identification of Catechins in MCL Ethanoic Extract

Figure 5 shows the identified catechin derivatives with the closely related isomers. The derivatives with the lowest mass error are believed to be the actual compound identified, based on Waters in-house database MS/MS. The identified catechin derivatives include epigallocatechin-3-O-gallate (EGCG), epigallocatechin (4β, 8)-gallocatechin, gallocatechin (4α→8)-epicatechin, catechin-3-O-gallate (CG) andepigallocatechin (EGC). Even though eight catechins were quantified in HPLC, yet only four catechins had been identified based on m/z values. This might be due to the instrument settings and data processing parameters having yet to be optimized in order for the successful detection of all the major catechins.[47,48]

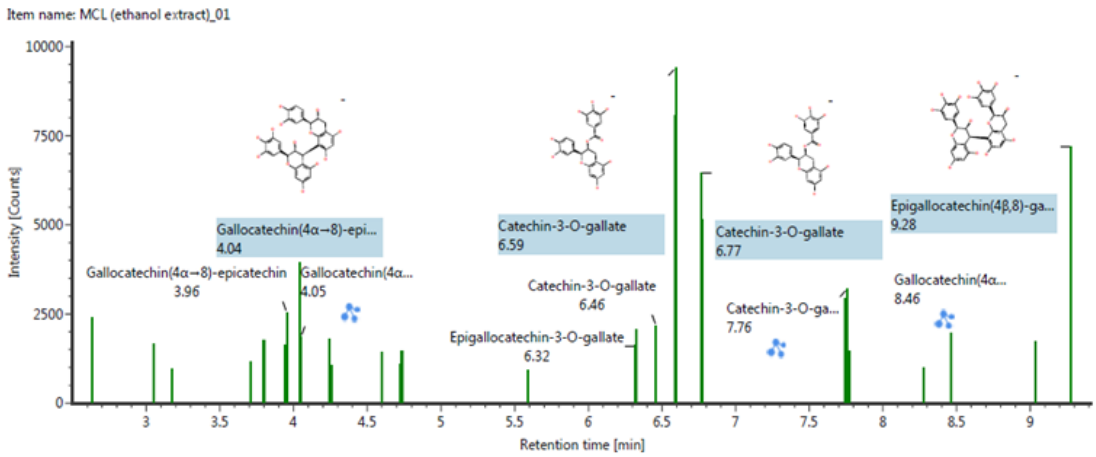


Fig.5: Identified catechins derivatives in MCL

Epigallocatechin(4β→8)-gallocatechin and gallocatechin(4α→8)-epicatechin are oligomers (dimers) made up from flavan-3-ols that belongs to proanthocyanidins.[49] Proanthocyanidins can be further sub-classified depending on the monomers. Epigallocatechin(4β→8)-gallocatechin and gallocatechin(4α→8)-epicatechin are classified as prodelphinidins, as the monomers are from gallocatechins.[50] Epigallocatechin(4β→8)-gallocatechin is also known as prodelphinidin B9, while gallocatechin(4α→8)-epicatechin is also known as prodelphinidins B4.[51] Proanthocyanidins had been reported to have high antioxidant activity, with some showing greater potency than

L-ascorbic acid.[52,53,54,55,56] Prodelphinidin B4 has been shown to possess antitumor effect on PC-3 prostate cancer cel.[57] The dimeric prodelphinidins also demonstrated higher scavenging free radical activity than monomer, which might be due to the higher number of hydroxyls.[58] Prodelphinidin B9 was shown to be significantly more potent in scavenging DPPH radical than vitamin C and Trolox. In addition, Theisen and Muller[59] found that prodelphinidin B9 exhibits anti-influenza virus activity 4 to 13-fold greater than its monomer counterparts.

Several compounds identified as the same compounds were assumed to be the closely related isomers, similar to a study by Yassin *et al*.,[60] There are six types of EGCG, three types of epigallocatechin (4β, 8)-gallocatechin, twelve types of gallocatechin(4α→8)-epicatechin, and ten types of CG identified in MCL extract. Thus, these closely related isomers might contribute to nutraceutical benefits yet to be elucidated.

Conclusion

An extraction solvent composed of 70% ethanol: 29.5% water: 0.5 % acetic acid (sample B) may be the solvent of choice for extracting catechins and polyphenols from MCL. Sample B showed the highest DPPH percentage of inhibition (86.12±2.96%), TPC value (97.80±0.25 mg GAE/g extract) and total catechins (153.57mg /g extract). All eight catechins were identified in MCL and catechin derivatives detected with UPLC-TWIMS-QTOF, including epigallocatechin-3-O-gallate (EGCG), epigallocatechin(4β,8)-gallocatechin, gallocatechin(4α→8)-epicatechin, catechin-3-O-gallate (CG) and epigallocatechin (EGC).

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Conflict of Interest

The authors declare that there is no conflict of interest in conducting this study.

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