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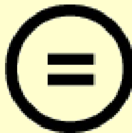

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Master's Thesis of Science in Agriculture

Study for the Bio-functionality and Application of Coffee Leaf and Coffee Husk

커피 잎과 커피 껍질에 대한 생물학적 기능성과
응용 방법에 대한 연구

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Abstract

Recently, the coffee leaves and coffee husk have attracted a lot of interest in coffee industry. Cascara known as a coffee cherry husk is one of the major solid residues from the processing of coffee. Caffeine, tannin and polyphenols are contained in cascara causing serious environmental pollution if discarded without special pretreatment. Thus, there are many attempts focusing on the reducing the industrial by-products and uses of agricultural by-products. In this study, enzymatic glycosylation using dextransucrases from *Leuconostoc mesenteroides* B-512F/KM and B-1299CB4 synthesized cascara oligosaccharides (CaOS). The degree of polymerization (DP) of oligosaccharides was confirmed by MALDI-TOF MS analysis. More than nine DP oligosaccharides were synthesized. Novel CaOS have 49.1% lower calories than commercial oligosaccharides. CaOS reduced 63% of the formation of insoluble glucan by mutansucrase with 60% of sucrose. Biofunctional characteristics CaOS were analyzed by using UPLC-MS such as the total phenolic, flavonoid, condensed tannin contents. CaOS had total phenolic, flavonoid and condensed tannin, with 11.43±2.8, 1.7±0.08 and 0.24±0.02 mg/g respectively. The DPPH radical scavenging activities, oxygen radical absorbance capacity (ORAC), ferric reducing antioxidant power assay (FRAP) analyses of CaOS were determined. CaOS had DPPH radical scavenging activity, ORAC and FRAP, with 20.3±1.2 mg TE/g, 1224±35 μmol TE/g, 393±3.1 mmol FE(Ⅱ)/g respectively.

Coffee leaves were manufactured through the process of green and black tea. Bio-functionality compounds in coffee leaves and coffee leaf teas were analyzed by using UPLC-MS. The total phenolic, flavonoid and condensed tannin contents in coffee leaves and coffee leaf teas were analyzed. DPPH radical scavenging activities, ORAC, FRAP in coffee leaves and coffee leaf teas were also analyzed. In addition, the α-glucosidase inhibitory activity were further investigated. Thus, young leaves had higher functional compounds, antioxidant properties and α-glucosidase inhibitory activity. In case of black tea, however, because of it oxidation during manufacturing black tea, its functional compounds and antioxidants capability were decreased. The re-uses of coffee husks and leaves would give added values in coffee production, higher incomes for coffee farmers, and potential solutions for the environmental problem in coffee production system.

Keywords : Coffee husk, Cascara, Dextransucrase, Oligosaccharides, Coffee leaf, Tea, Antioxidant, Insoluble glucan

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Chapter 1. Synthesis and Biological Characterization of Low-calorie Cascara Syrup

Introduction

Coffee is one of the most widely consumed beverages in the world. There are around sixty tropical countries who produce coffee and some of them have it as a major agricultural export [1]. In 2018, 168 million tons of coffee were produced. The consumption and production of coffee have been increasing every year. However, coffee production generates a lot of coffee by-products such as cascara. Cascara is known as a coffee cherry husk, one of the major solid residues from the processing of coffee. Caffeine, tannin and polyphenols contained in cascara cause serious environmental pollution if discarded without special pretreatment [2]. This case has become a big issue, especially in the coffee belt countries [3]. To solve those problems, many industries have a focus on reducing the environmental impact of industrial by-products and restoring the environment by using agricultural by-products and food waste for further application. Besides that, many researchers are working on it and using it to develop many application substitutes [4]. Cascara is the main solid residue from coffee processing, which is currently less profitable, and there is a

need to solve environmental problems with proper treatment. Thus, in accordance with sustainable development, innovative techniques and applications are needed to make the use of these residues become profitable and appropriate [5]. Previous studies on cascara have shown that it contains some bioactive compounds, such as caffeine, chlorogenic acid, gallic acid and protocatechuic acid [6].

In particular, caffeine, which is major compounds in cascara, effectively cleans the radical at a high rate of response compared to other efficient ·OH radical scavengers [7]. In addition, chlorogenic acid acts as an antioxidant and can scavenge reactive oxygen species, leading to the downregulation of nuclear factors- κB, resulting in various effects such as anti-inflammatory and cancer cell apoptosis [8]. It has been shown that cascara is a potential source of antioxidants and polyphenol compounds that should not be discarded. Cascara also has a delicious and sweet flavor because of its high total sugar content [2]. Therefore, in order to develop functional food from discarded cascara, glucooligosaccharides were synthesized to make cascara oligosaccharides. Glucooligosaccharides can be synthesized by using dextransucrase in a high concentration of sucrose, with or without the presence of acceptor. The degree of polymerization (DP) of produced CaOS from a minimum of 2 to a maximum of 11. The purpose of this study is to optimize the synthesis of the CaOS by using dextransucrase and analyze its properties.

Review of Literature

1. Cascara

Coffee cherry consists of a smooth and tough outer skin, usually green in unripe fruits and turns deep red when ripe [9]. Cascara is known as a coffee cherry husk, one of the major solid residues from the processing of coffee. However, caffeine, tannin and polyphenols content in cascara cause serious environmental pollution if discarded without special pretreatment [10]. Cascara contains about 50% of carbohydrates, 10% protein, 20% fibers, 2.5% fat, and 1.3% caffeine. Besides that, it contains other phenolic compounds. Cascara has four major classes of phenolic compounds which are identified as flavan-3-ols (monomers and procyanidins), hydroxycinnamic acids, flavonols, and anthocyanidins with chlorogenic acid as the main phenolic compound [11,12]. For this reason, it has been found that the cascara is a potential source of antioxidants and phenolic compounds that should not be wasted. Because cascara contains similar compounds like coffee beans, it can be used as food.

2. *Leuconostoc mesenteroides* B-512FM/K and B-1299CB4

Leuconostoc mesenteroides produced dextran which is composed of D-glucose units [13]. Dextrans are defined as polysaccharides of glucose that feature a substantial number of consecutive α(1,6)-linkages in their major chains, usually more than 50% of the total linkages [14]. Dextran combines various structure, which depends on the strain of *L. mesenteroides*. There are various strains of *L. mesenteroides* including B-512, B-1299 and B-1355. *L. mesenteroides* B-512F dextran is the classical dextran containing a 95% of consecutive α-(1,6)-linkages and 5% of α-(1,3)-branch linkages. *L. mesenteroides* B-1299 produces an L- and an S-dextran with α-(1,6)-linked main chains with single α-(1,2)- and α-(1,3)- linked glucosyl residues. *L. meseteroides* B-1355 produces a similar structure glucan as B-512F dextran and alternan with alternating sequence of 50% of α-(1,6)- and 50% of α -(1,3)-linkages [15]. The *Leuconostoc* specises require sucrose as an inducer in the culture to express glucansucrase. However, this strains had the low yield and impurity problems due to the sucrose inducer that is required to make glucans in the culture supernatant. To solve these problems, the mutans, chemical mutation process using ethly methane sulfonate, *L. mesenteroides* B-512 FM/K, B-1299CB4 and B-1355CF10 are developed as constitutive mutants to solve these problems [16]. The mutation strain had been developed to overcome problems of the original commercial strain. Especially, The *L.*

mesenteroides B-512FMCM produced more than 1000 times enzyme than B-512 [17]. Also, the optimized purification methods, the purified dextransucrase showed the highest specific activity for dextransucrase [18].

3. Glycosylation and acceptor reaction

Dextransucrase has several mechanism according to its characteristics. When sugar exist, extracellular glucosyltransferases hydrolyze sucrose and then transfer the glucose unit from sucrose to the acceptor to produce accpetor-oligosaccharides. This reaction called "acceptor reaction" [19]. The glucosyl residue from sucrose is converted from the synthesis of dextran and passed on to the free hydroxyl group of the sugars, called acceptors. Many sugars act as acceptors have classified them according to their capacity to divert glucosyl residues from dextran to form oligosaccharides and to their effect on the rate of reaction. (**Figure 1**) [20,21].

The dextransucrase B-512F produces a dextran through the formation of glucosyl intermediate [22]. The glucosyl units are transferred as the dextran chain at the reducing end. This elongation is terminated by acceptor reaction.

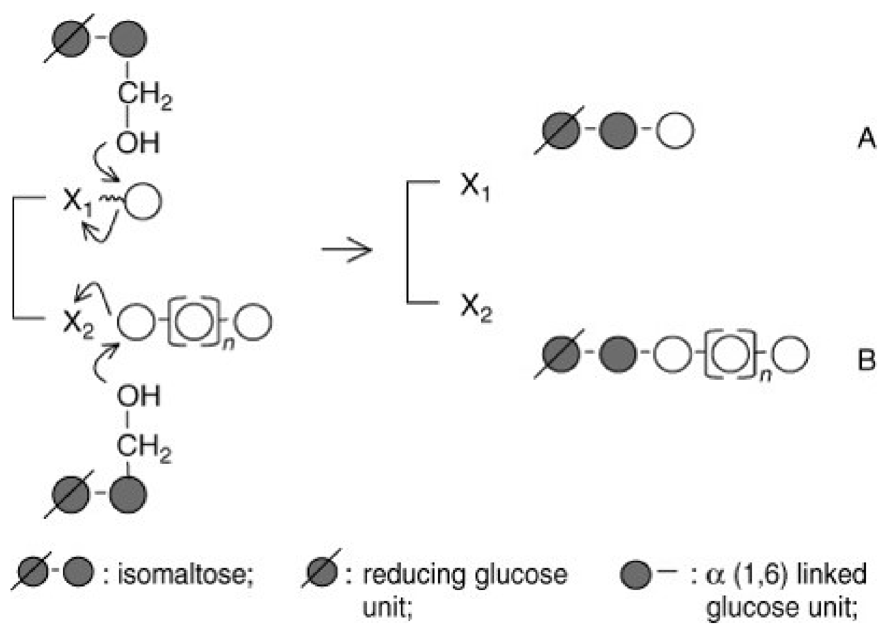

Figure 1. Acceptor reaction mechanism of acceptor reaction of B-512F dextransucrase [19]

4. Oligosaccharides

Oligosaccharide is a carbohydrate that has a degree of polymerization ranged from 2 to 10 monosaccharide units. There are many kinds of oligosaccharides depending on the number of conjugations or the method of coupling of monosaccharides, binds of monosaccharides and they have various properties and physiological functions. Functional oligosaccharides have been found to be effective in controlling blood glucose in diabetics and serum lipids in hyperlipidemics, enhancing immunity, facilitating mineral absorption, source of antioxidant, antibiotic substitutes, and patients with diabetes and hyperlipidemia [23]. Oligosaccharide synthesis is important to the food, pharmaceutical as biomass cells, or cosmetics as stabilizers, or prebiotics compounds [24]. In the non-digestible oligosaccharides, anomeric C atoms (C_1 or C_2) of the monosaccharide units have a composition that makes their osidic bonds non-susceptible to the hydrolytic activity of human digestive enzymes [25]. Oligosaccharides have been reported that they could promote the absorption of polyphenols [26]. Some previous studies showed that oligosaccharides enhanced the bioavailability of other compounds such as flavonoid or isoflavone [27]. Oligosaccharide also prevents the formation of insoluble glucan which attached to the tooth surface and form dental calculus. This is because mutansucrase, an enzyme of the cavity, reacts with oligosaccharide to form a water-soluble dextran [28].

Materials and Methods

1. Enzymatic preparation

Dextransucrase of B-512FM/K and Dextransucrase of B-1299CB4 were prepared by culturing the *Leuconostoc mesenteroides* B-512FM/K and 1299CB4 strain in LWG media at 28 °C and purified as previously described [24]. First, Dextransucrase was prepared by culturing *L. mesenteroides* B-512FMCM, a dextransucrase producing constitutive mutant obtained by mutating *L. mesenteroides* B-512FMC using vacuum ultraviolet radiation, in glucose medium and purifying as described previously [24]. Second, Kang et al. have reported the expression of dextransucrase from *L. mesenteroides* B-1299CB4 in *E. coli* BL21(DE3)pLysS at low temperature could be induced by isopropyl β- D –1-thiogalactopyranoside (IPTG). And then, fermentation for dextransucrase B-1299CB4 production was conducted in a 19 L bioreactor (Bioengineering Inc., USA) and purified as described previously [29]. It was sterilized by 0.2 ㎛ disposable filter. Enzyme activity was confirmed by incubating dextransucrase at 28 °C with 100 mM sucrose as a substrate in 20 mM sodium acetate buffer (pH 5.2) for different reaction times. The reacted samples were spotted on a silica gel 60 F_{254} TLC plate (Darmstadt Inc., Germany). The amount of fructose released by using the AlphaEaseFC 4.0 Image Program, was analyzed with a fructose standard.

2. Preparation of cascara

Cascara were purchased from a company, Len's coffee (Nicaragua). Cascara (10 g) was extracted for 30 min with 100 mL of hot water (60 °C) at room temperature. After extraction, the sample was centrifuged at 8,000 × *g* for 15 min and obtained the supernatant. Cascara extract was freeze-dried at 0 °C under 10 Pa for 3 days (Rikakikai Inc., Japan) and ground (Hanil Inc., Korea) to obtain homogeneous fine powder.

3. Synthesis of cascara oligosaccharides (CaOS)

CaOS were synthesized by using dextransucrase B-512F/KM and B-1299CB4. Enzyme reaction was carried out by adding 60% (w/v) sucrose and 10% (w/v) cascara extract powder. In this reaction condition, final concentration of two enzymes were 6 U/mL and 4 U/mL respectively. The reaction time was determined at the time that sucrose was almost consumed. CaOS were synthesized in incubator at 28 °C. The reaction was terminated by heating at 60 °C for 10 min. The end of reaction was analyzed by Silica gel 60 F_{254} TLC plate with nitromethane:1-propanol:water (2:5:1.5, v/v/v). Standard was sucrose, glucose and fructose. Solvents were evaporated and the spot was visualized by developing TLC plate in the developing solution, containing 0.5% (w/v) N-(1-napthyl) ethylenediamine dihydrochloride in methanol and 5% (w/v) sulfuric acid, followed by heating at 125 °C in oven [30].

4. TLC and MALDI-TOF MS analysis

In order to confirm DP of produced components, TLC(Thin Layer Chromatography) analysis and MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry) analysis were carried out. After synthesis of CaOS, the reaction mixture was spotted onto a TLC silica gel plate and developed using a solvent mixture of nitromethane:1-propanol:water (2:5:1.5, v/v/v). The standard material was 100 mM of sucrose, glucose, fructose and 10 μg of cascara extract. Solvents were evaporated and the spot was visualized by developing the TLC plate in the solution containing 0.5% (w/v) N-(1-napthyl) ethylenediamine dihydrochloride in methanol and 5% sulfuric acid, following by heating at 125 °C. The synthesized CaOS were analyzed by using MALDI-TOF MS. The polymer was removed using 70% ethanol. Mass spectra was confirmed by using a Voyager DESTR MALDI-TOF mass spectrometer (Applied biosystem Inc., USA). The mass spectra were obtained by delayed extraction (average of 75 laser shots) with a 65 kV acceleration voltage in the positive reflector mode.

5. Analysis of functional compounds

5.1. Compounds analysis of using UPLC-MS

Final CaOS was diluted with methanol and filtered by a 0.2 μm membrane syringe filter. 1 μl of final sample was injected into UPLC/MS (Waters Inc., USA) and Waters Acquity UPLC Beh C18 (1.7 μm, 2.1 × 150 mm) column was used to analyze phenolic contents in the sample. Mobile phase was prepared with solvent A (100% acetonitrile with 0.1% formic acid) and solvent B (100% triple distilled water with 0.1% formic acid). Waters QDa detector was used to quantify caffeine, chlorogenic acid, gallic acid, p-coumaric acid, and vanillic acid. Conditions for phenolic contents were set as electrospray ionization (ESI) positive, 1.3 kV of capillary energy, and 10 V of cone voltage (**Table 1**). The full scan of ions was ranged from 190 to 800 m/z in the positive and negative ion modes. Calibration curve for sample was set from 0.02 to 2.0 μg/mL (r^2 > 0.99). The concentration range of trigonelline was 1 to 100 μg/mL (r^2 > 0.99). The following elution gradient was applied for analysis using Waters Acquity UPLC Beh C18 (1.7 μm, 2.1 × 150 mm) column. The initial elution gradient was 5% A, and increased to 10% A at 1 min, 15% A at 2 min, 23% A at 10 min, 50% A at 12 min, 100% A at 15 min, back to initial gradient at 16 min and maintained until 25 min for equilibrium step (**Table 2**). Trigonelline was analyzed using Waters PDA detector (Waters Inc., USA) with the same UPLC machine Waters Acquity

UPLC Beh HILIC (1.7 μm, 2.1 × 100 mm) column was used. Detection wavelength for trigonelline was set 265 nm. Mobile phase for trigonelline analysis was progressed with solvent A (100% acetonitrile with 0.1% formic acid) and solvent B (100% triple distilled water with 0.1% formic acid and 15 mM ammonium formate). The elution gradient for trigonelline was 90% A and 10% B isocratically for 10 min.

Table 1. LC-QDa mass condition of standard samples

Standard	Molecular mass	Electrospray ionization	Cone Voltage (CV)
Chlorogenic acid	355.00	Positive	5
Caffeine	195.17	Positive	10
Gallic acid	170.12	Positive	10
p-coumaric acid	165.13	Positive	10
Vanillic acid	168.15	Positive	10

Table 2. Mobile phase flow gradient for phenolic compounds in UPLC-MS analysis

Time (min)	**Flow (mL/min)**	**A (%)**	**B (%)**
Initial	0.30	5.0	95.0
0.50	0.30	10.0	90.0
1.0	0.30	10.0	90.0
2.0	0.30	10.0	90.0
2.1	0.30	15.0	85.0
4.0	0.30	15.0	85.0
10.0	0.30	23.0	77.0
12.0	0.30	50.0	50.0
15.0	0.30	60.0	40.0
15.1	0.30	100.0	0.0
16.0	0.30	100.0	0.0
16.1	0.30	5.0	95.0
25.0	0.30	5.0	95.0

5.2. Total compounds analysis

5.2.1. Total phenolic content (TPC)

TPC was determined using Folin-Ciocalteu method as described previously with some modifications [31]. Briefly, 120 μL of extract was mixed thoroughly with 15 μL of Folin–Ciocalteu reagent and shaked for 3 min. 15 μL of 10% (w/v) sodium carbonate was added to the mixture and allowed to react in the dark for 30 min. The mixture was allowed to stand for a further 30 min in the dark, and absorbance was measured at 760 nm. TPC was calculated from the calibration curve, and the results were expressed as gallic acid equivalent (mg GAE/g cascara extract).

5.2.2. Total flavonoid content (TFC)

TFC was determined using aluminium chloride colorimetric method as described previously with some modifications [32]. Briefly, sample was mixed with 60 μl of methanol, 4 μl of 10% aluminium chloride, 4 μl of 1 M potassium acetate and 112 μl of distilled water. The mixture was incubated at room temperature for 30 min. Absorbance was measured at 415 nm with a SpectraMax M3 (Molecular devices Inc., USA). Total flavonoid content was calculated from the calibration curve, and the results were expressed as quercetin equivalent (mg QE/g cascara extract).

5.2.3. Total condensed tannin content (TCT)

TCT was determined using Broadhurst method with some modifications [33] with catechin as standard. Briefly, 18 μl of sample was thoroughly mixed with 122 μl of 4% vanillin solution for 3 min, followed by the addition of 60 μl of concentrated hydrochloric acid. The mixture was incubated at room temperature for 15 min. Absorbance was measured at 500 nm with a SpectraMax M3 (Molecular devices Inc., USA). Total condensed tannin content was calculated from the calibration curve, and the results were expressed as catechin equivalent (mg CE/g cascara extract).

6. Functionality of CaOS

6.1. Antioxidant activity

The antioxidant activity was determined using the oxygen radical absorbance capacity (ORAC), trolox equivalent antioxidant capacity (TEAC) using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method and ferric reducing/antioxidant power (FRAP). All experiment was analyzed in triplicate. Absorbance and fluorescence measurements were performed using a SpectraMax M3 (Molecular devices Inc., USA). The measurement of absorbance was carried out using transparent microplate. And black microplates were used to determine fluorescence measurements.

6.1.1. DPPH radical scavenging activity

The antioxidant activity was evaluated by measuring the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity [34]. Briefly, sample was mixed with 100 mM DPPH in methanol. The mixture was incubated for 30 min at room temperature. The absorbance of each mixture was recorded at 517 nm using SpectraMax microplate reader (Molecular Devices Inc., USA). A blank replaced sample with distilled water. DPPH radical scavenging activity was calculated from the calibration curve, and the results were expressed as trolox equivalent (mg TE/g cascara extract).

6.1.2. Oxygen radical absorbance capacity (ORAC)

The antioxidant capacity was measured using ORAC assay with some modification [35]. Briefly, Fluorescein and 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH) were dissolved in potassium phosphate buffer (pH 7.4). 10 μl of sample was thoroughly mixed with 90 μl of 25 nM of fluorescein was incubated at 37 °C for 10 min. Then, 100 μl of AAPH solution (25 mM) was added. Fluorescence was measured at excitation and emission wavelengths of 485 and 538 nm with a SpectraMax M3 (Molecular devices Inc., USA) every 3 min for 2 h. Trolox (5-10 Mm) was used as standard. A blank replaced sample with distilled water. It was calculated by integrating the fluorescence curve for each sample. ORAC value was calculated as μM, and the results were expressed as trolox equivalent (μM TE/g cascara extract).

6.1.3. Ferric reducing antioxidant power assay (FRAP)

The antioxidant activity was measured using ferric reducing/antioxidant power (FRAP) assay [36]. Briefly, 0.01 M TPTZ (2, 4, 6-tripyridyl-s-triazine) solubilized in 0.04 M HCl, 0.3 M acetate buffer (pH 3.6) and 0.02 M $FeCl_3.6H_2O$ were thoroughly mixed in a 10:1:1 (v:v:v) ratio just before experiment (FRAP working solution). Then, samples were thoroughly mixed with FRAP working solution, followed by the addition of distilled water in a 1:30:3 (v:v:v) ratio.

Absorbance was measured at 593 nm with a SpectraMax M3 (Molecular devices Inc., USA). $FeSO_4.7H_2O$ (10-2500 μM) was used as standard. FRAP value was calculated from standard calibration curve, and the results were expressed as $FeSO_4.7H_2O$ equivalent (mM $FeSO_4$/g cascara extract).

6.2. Inhibition assay of insoluble glucan formation from mutansucrase

Mutansucrase was prepared by *Streptococcus. mutans* in brain heart infusion (BHI) broth containing 2% sucrose as seed culture and then subculturing them in the BHI broth containing 0.5% (w/v) glucose as main culture at 37 °C in shaking incubator for 8 h as described previously [37]. After fermentation, cells were separated from the broth by centrifugation at 8,000 × *g* for 20 min. The culture was concentrated to 300 mL using hollow fiber membrane (Millipore Inc., USA) with 20 mM sodium phosphate buffer (pH 6.8). It was pooled and stored at –20 °C until further study. There was distilled water, sucrose and mutansucrase in the positive control. In negative control, distilled water and mutansucrase was added. In each sample, 100 mM of sucrose, 20 mM of sodium phosphate, mutansucrase and each sample were mixed. After incubation at 37 °C for 3 h, the water insoluble glucan synthesized in each enzyme reaction was collection by centrifugation at 8,000 × *g* for 10 min, redissolved in 1 M NaOH, and spotted on a Silica gel 60 F_{254} TLC (Darmstadt Inc., Germany). The amount of insoluble glucan was quantitatively determined on a TLC plate using the AlphaEaseFC 4.0 Image Program. As inhibitor to prevent from forming plague, CaOS, oligosaccharides and cascara water extracts were tested. At the final concentration of 1%, 5%, 8% and 12.5% (w/v) of each sample, and showed inhibition effect against forming insoluble glucan comparing to positive control.

7. Calorie analysis

Calorie of the CaOS was determined by using AOAC official method 985.29 [38]. Briefly, enzymatic hydrolysis of CaOS was carried out with three kinds of enzymes: 7.5 U/mL α-amylase (Megazyme Inc., Ireland) in pH 6.9 at 90 ℃ for 30 min for hydrolysis, 33 U/mL amyloglucosidase (Megazyme Inc., Ireland) in pH 4.8 at 60 ℃ for 30 min and 500 U/mL invertase (Sigma Inc., USA) at 60 ℃ for 30 min for hydrolysis of sucrose. Glucose and fructose were quantified through the k-FRUGL kit, which can represent calories in sugar syrup, CaOS and commercial oligosaccharides (Ottugi Inc., Korea).

Results and Discussion

1. TLC and MALDL-TOF MS analysis

The molecular weights (MW) of CaOS components were detected by MALDI-TOF MS and TLC. After reaction was accomplished in the presence of dextransucrase B-512F/KM and B-1299CB4, the CaOS were detected by TLC (**Figure 2**) (A). DP is the number of monomeric units in a polymer or oligomer molecule. CaOS with a total of 9 DP were measured in MALDI-TOF MS analysis (B). All products can be described in this, $[n\text{-}M\text{-}(n\text{-}1)\cdot H_2O+K^+]$ and $[n\text{-}M\text{-}(n\text{-}1)\cdot H_2O+Na^+]$. “n” is natural number which is more than two. “M” is the molecular weight of glucose. “K” is the molecular weight of potassium.

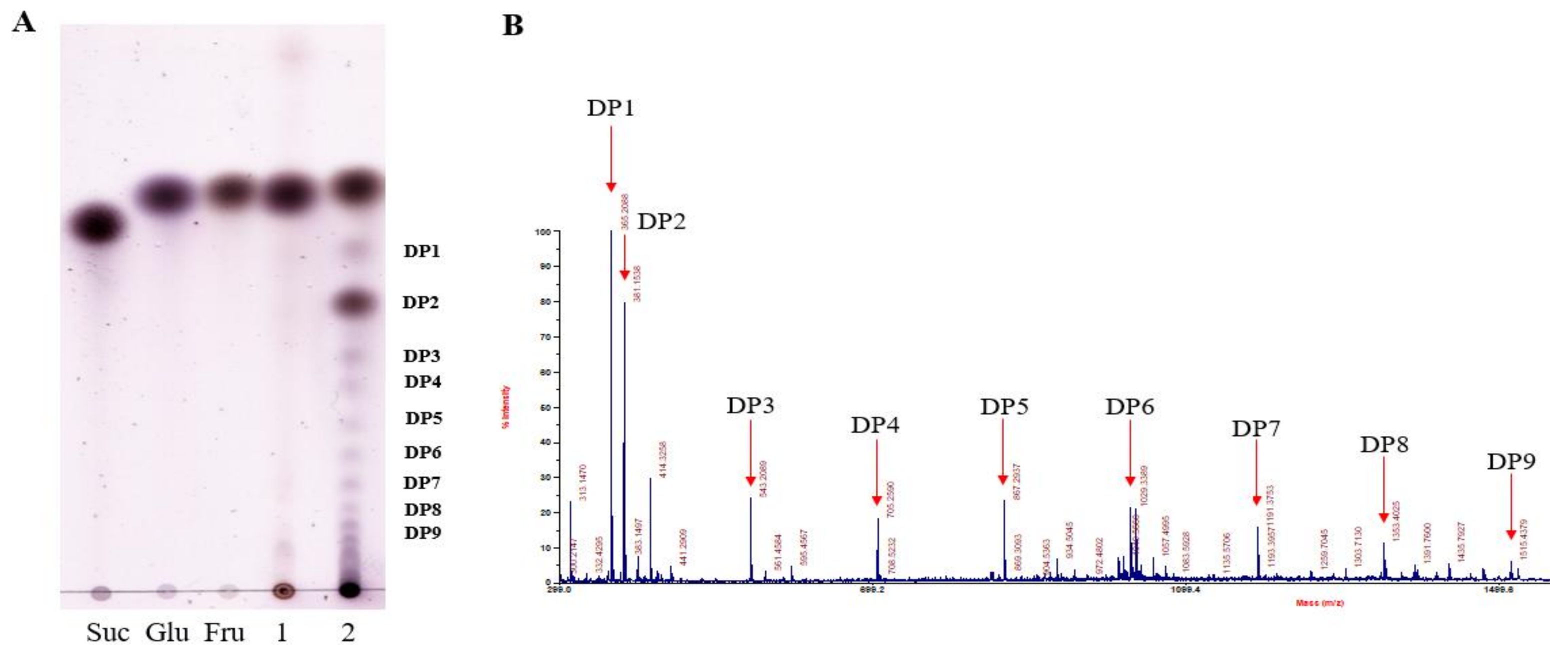

Figure 2. MW analysis of CaOS components using TLC (A) and MALDI-TOF MS (B). (A) Suc: Sucrose 100 Mm; Glu: Glucose 100 Mm; Fru. Fructose 100 Mm; Lane 1: before reaction; Lane 2: After reaction. (B) MALDI-TOF MS spectra of CaOS. Before analysis, polymer was removed by adding ethanol.

2. Functional compounds in CaOS

2.1. Compounds analysis using UPLC-MS

Chlorogenic acid, caffeine, gallic acid, trigonelline, p-coumaric acid, and vanillic acid content were quantified (**Figure 3**). In general, CaOS have chlorogenic acid, caffeine and trigonelline contents (**Table 3**). The most abundant polyphenol compounds were trigonelline, caffeine, and chlorogenic acid. In the previous study, coffee was reported to contain 1.82 mg/g of chlorogenic acid, 24.31 mg/g of trigonelline and 26.14 mg/g of caffeine [39]. From our results, CaOS have about 1.6 times higher amount of chlorogenic acid and 1.4 times lower in trigonelline, and 2.5 times lower in caffeine than coffee.

Table 3. Bioactive compounds of CaOS

Bioactive compounds (mg/g)	
Sample	**Cascara oligosaccharides**
Caffeine	10.7 ± 0.03
Chlorogenic acid	2.86 ± 0.04
Gallic acid	0.046 ± 0.01
Trigonelline	18.03 ± 0.3
p-coumaric acid	0.018 ± 0.01
Vanillic acid	0.017 ± 0.02

Results are expressed as means ± standard deviation of triplicate analysis;

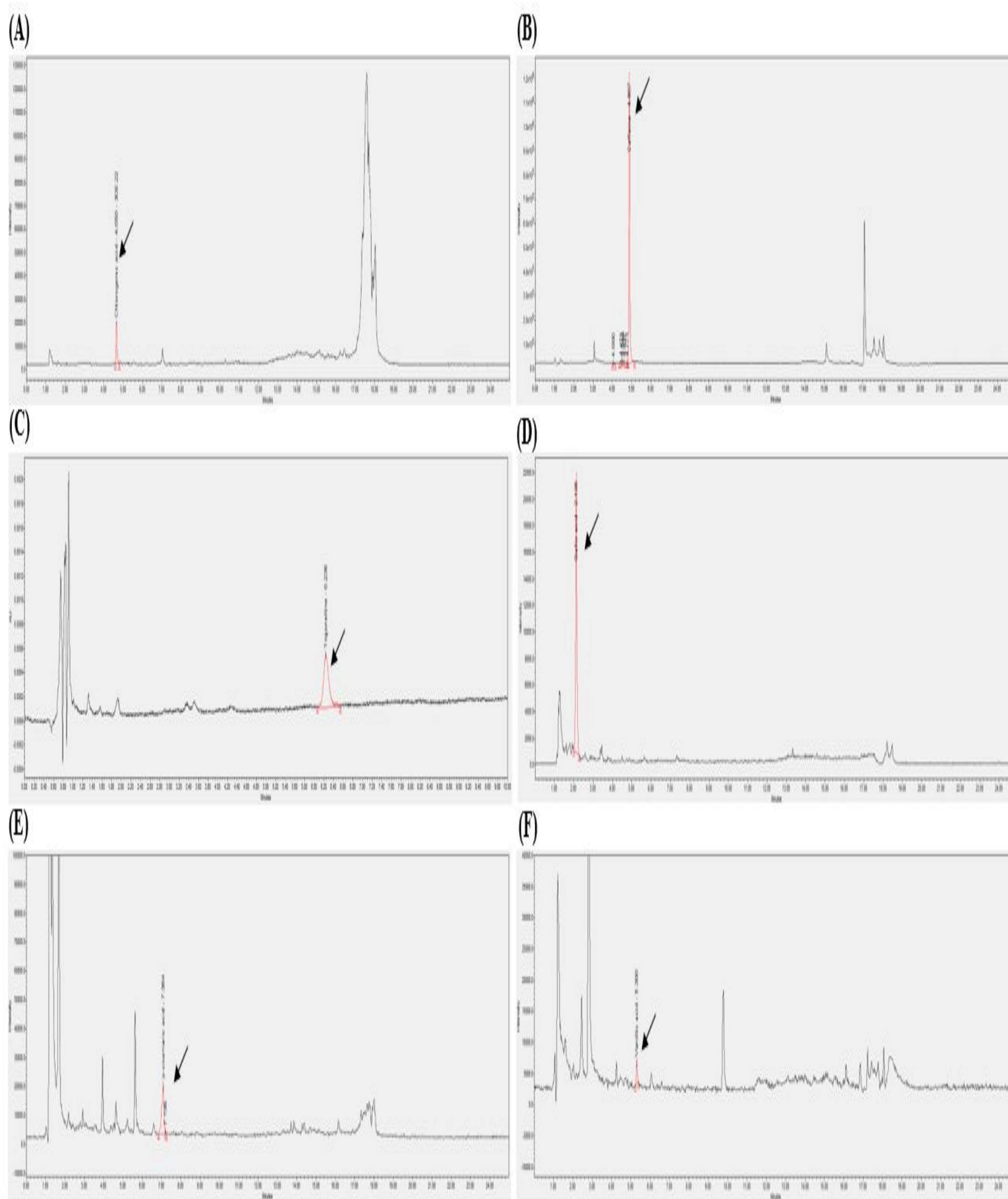

Figure 3. Chromatograms of CaOS by UPLC-MS.

(A) chlorogenic acid, (B) caffeine, (C) trigonelline, (D) gallic acid,

(E) p-coumaric acid, (F) vanillic acid

2. Functonal compounds in cascara oligosaccharides

2.2. Total functional compound contents analysis

Total phenolic, flavonoid and condensed tannin content were identified as 11.43, 1.7 and 0.24 mg/g, respectively (**Table 4**). According to previous studies, the cascara water extract was analyzed with a TPC of 13.9 mg/g, TFC of 1 mg/g and total tannin content of 0.19 mg/g. In addition, 70% ethanol extract of the cascara was analyzed as total phenol content of 14.1 mg/g, total flavonoid content of 1.08 mg/g and total tannin content of 0.29 mg/g. These results showed that CaOS have a total phenol content about 1.3 times lower than cascara extracts. In particular, compared to other studies, total phenolic content of coffee was 26.7 mg/g, which was 2.3 times higher than CaOS [40]. In addition, CaOS was found to have similar phenolic content to apple extract (11.6 mg/g) [41]. In this study, it was found that high levels of polyphenols were found in CaOS.

Table 4. Determination of total phenolic, flavonoid, condensed tannin content for CaOS

	Cascara oligosaccharides
Total phenolic contents (mg GAE[1]/g)	11.4 ± 2.8
Total flavonoid contents (mg QE[2]/g)	1.7 ± 0.08
Total condensed tannin contents (mg CE[3]/g)	0.24 ± 0.02

Results are expressed as means ± standard deviation of triplicate analysis;

[1]: Gallic acid equivalent, [2]: Quercetin equivalent, [3]: Catechin equivalent

3. Analysis of antioxidant capacity

The antioxidant activities of CaOS were analyzed using DPPH radical scavenging assay, ORAC and FRAP (**Table 5**). In general, CaOS had higher antioxidant activity compared to date syrup and maple syrup. According to another study, CaOS had an FRAP value of about 8 times higher than date syrup [42]. According to USDA Nutrient Database, it has also been confirmed that CaOS had ORAC values two times higher than that of maple syrup. Thus, the CaOS showed excellent antioxidant capability as potential functional syrup.

Table 5. Determination of DPPH radical scavenging activity, ORAC and FRAP value of CaOS

	Cascara oligosaccharides
DPPH (mg TE[1]/g)	20.3 ± 1.2
ORAC (μmol TE[1]/g)	1224 ± 35
FRAP (mmol FE(Ⅱ)[2]/g)	393 ± 3.1

Results are expressed as means ± standard deviation of triplicate analysis; DPPH; DPPH radical scavenging activity, ORAC; Oxygen radical absorbance capacity, FRAP; Ferric reducing antioxidant power assay.

[1]: Trolox equivalent, [2]: $FeSO_4$ equivalent

4. Inhibition of insoluble glucan formation by mutansucrase

S. mutans has the abilities to adhere to tooth surfaces and to form acids associated with the cariogenicity of *S. mutans*. Synthesis of insoluble glucan by *S. mutans* from sucrose is essential in the adherence process [43]. Mutan is one of the products that cause dental plaque and caries. As inhibitors to prevent from forming plaque, CaOS were tested. In this study, the production of insoluble glucan was found to decrease as the concentration of samples increased (**Figure 4**). At the final concentration of 125 mg/mL of sample, CaOS showed 63% inhibition rate against forming insoluble glucan comparing to positive control (**Figure 5**). CaOS can prevent the formation of insoluble glucans by acceptor reaction, or by lowering the amount of sucrose due to dextransucrase reaction [44]. Thus, CaOS appears to be a promising anti-caries agent that can inhibit dental plaque.

Figure 4. Effects of CaOS on the inhibition of insoluble glucan formation by mutansucrase Lane A: cascara water extracts, lane B: oligosaccharides without cascara, lane C: cascara oligosaccharides, Cont: positive control; Number (%): Concentration of samples.

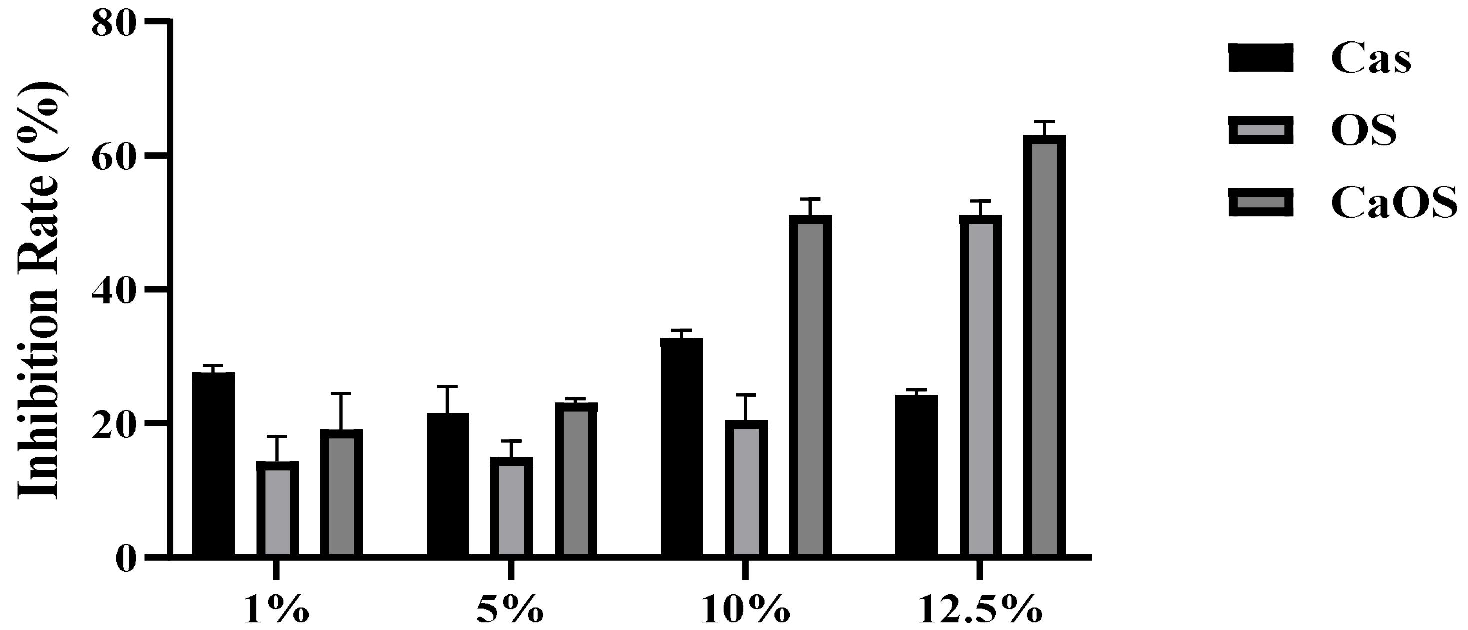

Figure 5. The inhibition rate for formation of insoluble glucan by mutansucrase from *S. mutans,* Cas: Cascara water extracts, OS: Oligosaccharides without cascara extracts, CaOS: Cascara oligosaccharides.

5. Calorie analysis

It is obvious that calorie intake are clearly associated with the development of obesity [45]. Dietary patterns that reduce energy intake in relation to weight loss [46]. By adding dextransucrase to sugar mixed cascara sample, the sucrose, glucose and fructose in sample were converted to oligosaccharides. As a result, CaOS were found that the total monosaccharides decreased by 49.1% compared to the sugar syrup. This results showed that CaOS have less calorie than sugar syrup by reducing monosaccharides (**Table 6**). CaOS have lower calories than commercial oligosaccharides. In the previous studies [28], when dextransucrase is treated to synthesize oligosaccharides, there is approximately 85% resistance to digestion enzymes. It is believed that CaOS contains lower monosaccharides after treating digestion enzymes. Because it is more resistant to digestion enzymes than commercial oligosaccharides.

Table 6. Estimated calorie analysis in CaOS

Sample	Glucose (g/L)	Fructose (g/L)	Total value (g/L)
Sugar syrup	244.2 ± 4.3	262.2 ± 33.6	506.3 ± 37.9
CaOS[1]	112.6 ± 9.1	119.7 ± 12.1	232.4 ± 21.2
Commercial oligosaccharides	199.7 ± 8.2	135.4 ± 24.0	335.1 ± 32.2

Results are expressed as means ± standard deviation of triplicate analysis;

[1]: Cascara oligosaccharides

Conclusion

CaOS was synthesized by using *L. mesenteroides* B-512FM/K and B-1299CB4 glucansucrase. CaOS has reduced calorie about 10 kcal of calories per intake. CaOS, thus, can be used to reduced oesity in today's sugar hyper ingestion problem. CaOS have lower caffeine than coffee (about 60 mg/g per intake), which provides about 10 mg of caffeine per intake. It also contains functional compounds such as chlorogenic acid and trigonelline. The total phenolic content was 11 mg/g.

Cascara showed anti-insoluble glucan forming activity. The combination of cascara and oligosaccharides showed high levels of insoluble glucan inhibition rate. By consuming this novel CaOS, prevetion of dental plaque and caries could be possible because of the reduction of insoluble glucan (mutan) formation. The CaOS can be used as functional syrup with higher antioxidant capacity compared to commercial syrups.

Chapter 2. A Study on the Utilization and Functionality of Coffee Leaf Tea

Introduction

High amount of coffee consumption leads it becomes one of the main agricultural export product in about 60 tropical and subtropical countries [47]. Besides as a lifestyle, the good and refreshing taste, also the pharmacological benefits of coffee are the reasons people consume coffee. Previous studies on coffee beans has shown that coffee beans contain some bioactive compounds, such as caffeine [48], chlorogenic acid [49], cafestol and kahweol [50], and trigonelline [51].

The harvest season of coffee bean depends on the region's climate and usually only can be harvested once a year in specific period (March through May, or November and December), meanwhile the coffee leaves can be harvested in the whole year. Furthermore, coffee leaves are known to have high quantity of feruloylquinic acid (FQA) [52] and mangiferin [53]. They also contain isomangiferin, epicatechin, procyanidin B, chlorogenic acid, isorhamnetin glucoside, rutin glucoside, and quercetin glucoside [54]. Mangiferin as the major constituent in *Mangifera indica L.* has attracted many interest due to

its various physiological activities like antioxidant, immunomodulatory, anti-inflammatory, and anti-microbial activities [55].

Coffee leaf has been traditionally consumed in many developing countries as beverage. In Ethiopia, Indonesia, India, and South Sudan, coffee leaves have been usually consumed as tea [56]. In general, there are three types of tea processing, which are un-fermented (green tea), semi-fermented (oolong tea), and full-fermented (black tea) [57]. The term of fermentation in tea processing is the oxidation process. The various tea processing methods have developed the different taste, color, and aroma because of the changes in phenolic and volatile compounds. The changes in those compounds composition also leads to the changes in its bioactive properties.

Many previous studies have shown the pharmacological benefits of coffee bean, such as dental caries prevention [51, 58], antidiabetic, parkinson disease prevention [59], etc. Coffee leaf has been believed to have many health benefits as coffee bean. Therefore, this study aimed to analyze the pharmacological effects of coffee leaf tea, especially focus on its anti-dental caries, antidiabetic, and antioxidant activities.

Review of Literature

1. Coffee leaf

Coffee leaves have a long history for use as ethnomedicine and tea by locals from countries where coffee plants grow. The coffee leaf has been consumed for hundreds of years around the world for various health applications and is known as a traditional tea under the name of 'kuti' in Ethiopia and 'kawa daun' in Indonesia. Especially in Indonesia, there are many Sumatrans who drink coffee leaf tea every day. This is because they believe that coffee leaves are more nutritious than coffee [60].

In fact, coffee leaf tea has a high level of antioxidant capability and healthy compounds, especially mangiferin. *Arabica* coffee leaves were found to contain the highest levels of mangiferin than *Robusta*, which was claimed to possess anti-inflammatory effects, reduce the risk of diabetes and blood cholesterol, and protect neurons in the brain [61].

Recently, a rich bioactive ingredient in coffee leaves has attracted attention to health benefits for humans. However, studies related to the effect of biological activity, application and treatment methods on the botanical composition and activity of coffee leaves are rare. In addition, various uses of coffee leaves were presented, including ethnomedicine, coffee leaf tea, packaging materials, tobacco substitutes,

personal hygiene products and animal feed. Thus, coffee leaves are a very promising resource in the food and industry, especially in the beverage industry [62].

A B

Figure 6. Coffee leaves (*Coffea arabica*) : (A) young leaves; (B) mature leaves [58]

2. Differences in the tea processing

The history of tea processing closely coincides with the preferred method in ancient chinese society. These changes in processing techniques are much more mechanized, but they are still used in modern tea processing. There are many ways to prepare the tea, but it is usually made with black, green, and oolong tea [63].

Tea is traditionally classified based on the degree or duration of oxidation or fermentation the leaves have experienced. Firstly, the black tea process goes through most of the stages. Once the leaves are picked, tea leaves wither for several hours. After the leaves are rolled, oils from the leaves are brought to the surface. These aromatic oils help in the oxidation process, which lasts for several hours. The final step is to put the leaves in an oven with a temperature of 200 °C. The color of the products will be brown or black color.

Secondly, the process of green tea is the shortest than other tea processes. Withering is done first, but this step can be omitted. It also skips the oxidation process by skipping leaves rolling step to break the membranes. After withering, the leaves are fired to prevent oxidation. The last step is to roll the leaves and dry them for the last shape. Green tea leaves usually remain green.

Lastly, the oolong tea process is similar to the black tea process. The Oolong tea also has withering and rolling steps. Instead of rolling, sometimes shaking is done to bruise the leaves. The oxidation time for

oolong tea is half of the black tea. Once the edge turns red-brown, while the center remains green, the oxidation process is stopped by firing. In the case of oolong tea, the leaves are heated at higher temperatures and stored for longer due to the lower resulting water content [64].

3. Mangiferin

Mangiferin is the major polyphenol compound in the leaves such as coffee leaves, fruits and stem barks of mango plant (*Mangifera indica*) [65]. The Mangiferin's xanthonoid structure (**Figure 7**) with C-glucosyl linkage and polyhydroxy component is believed to important for its free radical-scavenging ability that leads a potential anti-oxidant effect. Mangiferin have antioxidant capacity and the ability to regulate several major inflammatory pathways [66].

It was identified in coffee leaves and it is unique that it has anti-inflammatory, anti-diabetic, anti-hyperlipidemic, antioxidant, anti-microbial, and neuroprotective activities [67].

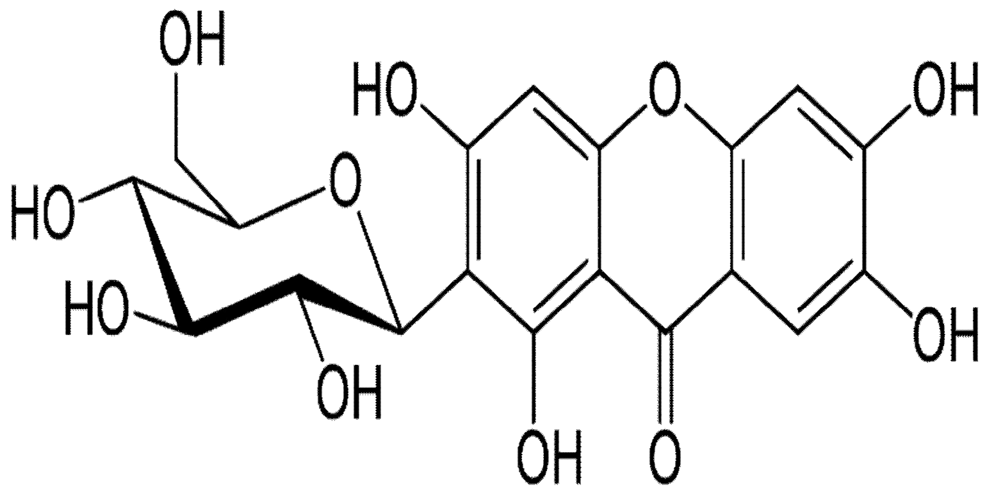

Figure 7. Structure of mangiferin

Materials and Methods

1. Preparation of coffee leaf tea

The tea processing was conducted in Dache Tea (Boseong, South Korea). The black tea and green tea processing methods were used in this study. Briefly, in the black tea processing, the coffee leaves were withered for 12 h, rolled until the texture became soft, and oxidized for 12 h. All the processes were conducted in 25 ºC, with humidity 83-85%. In the green tea processing, the coffee leaves were withered for 12 h and pan-fired three times in 150 ºC, 120 ºC, and 100 ºC to inactivate the enzymatic oxidation. All the processed coffee leaves were dried in 60 ºC. The coffee leaf teas were pulverized (Hanil Inc., Korea) to obtain homogeneous fine powder for further analysis. The sample types are young leaves (one to three months old), mature leaves (three to six months old), young leaf green tea, young leaf black tea, mature leaf green tea, mature leaf black tea and coffee leaf tea (Wize monkey Inc., Canada) as control.

The extraction was performed using water infusion and EtOH extraction method. In the water infusion, the powder (100 mg) were extracted in 10 mL hot water (95 ºC) for 30 min. In the EtOH extraction, the powder (100 mg) were extracted in 10 mL of 70% EtOH-water mixture under continuous shaking at 37 ºC, for 1 h. After

extraction, the samples were centrifuged at 8,000 × *g* and the extracts were collected and filtered using 0.2 µm disposable filter before analysis.

2. Analysis of functional compounds

2.1. Compounds analysis of using UPLC-MS

Final coffee leaf and coffee leaf tea was diluted with methanol and filtered by a 0.2 μm membrane syringe filter. 1 μl of final sample was injected into UPLC/MS (Waters Inc., USA) and Waters Acquity UPLC Beh C18 (1.7 μm, 2.1 × 150 mm) column was used to analyze phenolic contents in the sample. Mobile phase was progressed with solvent A (100% acetonitrile with 0.1% formic acid) and solvent B (100% triple distilled water with 0.1% formic acid). Waters QDa detector was used to quantify caffeine, chlorogenic acid, mangiferin, p-coumaric acid, catechin and rutin. Conditions for phenolic contents were set as electrospray ionization (ESI) positive, 1.3 kV of capillary energy, and 10 V of cone voltage (**Table 7**). The full scan of ions was ranged from 190 to 800 m/z in the positive and negative ion modes. Calibration curve for sample was set from 0.02 to 2.0 μg/mL (r^2 > 0.99). The following elution gradient was applied for analysis using Waters Acquity UPLC Beh C18 (1.7 μm, 2.1 × 150 mm) column. The initial elution gradient was 5% A, and increased to 10% A at 1 min, 15% A at 2 min, 23% A at 10 min, 50% A at 12 min, 100% A at 15 min, back to initial gradient at 16 min and maintained until 25 min for equilibrium step (**Table 2**).

Table 7. LC-QDa mass condition of standard sample

Time (min)	Flow (mL/min)	Electrospray ionization	Cone Voltage (CV)
Chlorogenic acid	355.00	Positive	5
Caffeine	195.17	Positive	10
Mangiferin	423.50	Positive	10
p-coumaric acid	165.13	Positive	10
Catechin	291.13	Positive	10
Rutin	609.00	Negative	20

2.2. Total compounds analysis

2.2.1. Total phenolic content (TPC)

TPC in coffee leaves and coffee leaf teas was determined using Folin-Ciocalteu reagent with gallic acid as standard [31]. Briefly, 120 μ L of extracts were mixed thoroughly with 15 μL of Folin–Ciocalteu reagent for 3 min in the dark, followed by the addition of 15 μL of 10% (w/v) sodium carbonate. The mixture was allowed to stand for a further 30 min in the dark, and absorbance was measured at 760 nm. TPC was calculated from the calibration curve, and the results were expressed as gallic acid equivalent (mg GAE/g leaf).

2.2.2. Total flavonoid content (TFC)

TFC was determined using aluminium chloride colorimetric method as described previously with some modifications [32]. Briefly, sample was mixed with 60 μl of methanol, 4 μl of 10% aluminium chloride, 4 μl of 1 M potassium acetate and 112 μl of distilled water. The mixture was incubated at room temperature for 30 min. Absorbance was measured at 415 nm with a SpectraMax M3 (Molecular devices Inc., USA). TFC was calculated from the calibration curve, and the results were expressed as quercetin equivalent (mg QE/g leaf).

2.2.3. Total condensed tannin content (TCT)

TCT was determined using Broadhurst method with some modifications [33] with catechin as standard. Briefly, 18 μl of sample was thoroughly mixed with 122 μl of 4% vanillin solution for 3 min, followed by the addition of 60 μl of concentrated hydrochloric acid. The mixture was incubated at room temperature for 15 min. Absorbance was measured at 500 nm with a SpectraMax M3 (Molecular devices Inc., USA). Total condensed tannin content was calculated from the calibration curve, and the results were expressed as catechin equivalent (mg CE/g leaf).

3. Bio-functionality of coffee leaf

3.1. Antioxidant capability

3.1.1. DPPH radical scavenging activity

The antioxidant activity was evaluated by measuring the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging according to a procedure with slight modification [68]. Briefly, each sample was mixed with 100 mM DPPH in methanol. The mixture was incubated for 30 min at room temperature. The absorbance of each mixture was recorded at 517 nm using SpectraMax microplate reader (Molecular Devices Inc., USA). Water and ethanol were used for the negative control. The relative radical scavenging activity was calculated as follows:

$$\text{DPPH radical scavenging activity (\%)} = [1-(A_1/A_0)] \times 100\%$$

where A_1 was the absorbance of sample and A_0 was the absorbance of control. The mean of duplicates IC_{50} (concentration for 50% inhibition) values of each sample was determined graphically.

3.1.2. Oxygen radical absorbance capacity (ORAC)

The antioxidant capacity was measured using oxygen radical absorbance capacity (ORAC) assay with some modification [35]. Briefly, Fluorescein and 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH) was dissolved in potassium phosphate buffer (pH 7.4). 10 μl of sample was thoroughly mixed with 90 μl of 25

nM of fluorescein was incubated at 37 °C for 10 min. Then, 100 µl of AAPH solution (25 mM) was added. Fluorescence was measured at excitation and emission wavelengths of 485 and 538 nm with a SpectraMax M3 (Molecular devices Inc., USA) every 3 min for 2 h. Trolox (5-10 Mm) was used as standard. A blank replaced sample with distilled water. It was calculated by integrating the fluorescence curve for each sample. ORAC value was calculated as µM, and the results were expressed as trolox equivalent (µM TE/10 g leaf).

3.1.3. Ferric reducing antioxidant power assay (FRAP)

The antioxidant activity was measured using ferric reducing/antioxidant power (FRAP) assay [36]. Briefly, 0.01M TPTZ (2, 4,6-tripyridyl-s-triazine) solubilized in 0.04 M HCl, 0.3 M acetate buffer (pH 3.6) and 0.02 M $FeCl_3.6H_2O$ were thoroughly mixed in a 10:1:1 (v:v:v) ratio just before experiment (FRAP working solution). Then, Samples were thoroughly mixed with FRAP working solution, followed by the addition of distilled water in a 1:30:3 (v:v:v) ratio. Absorbance was measured at 593 nm with a SpectraMax M3 (Molecular devices Inc., USA). $FeSO_4.7H_2O$ (10-2500 µM) was used as standard. FRAP value was calculated from standard calibration curve, and the results were expressed as $FeSO_4.7H_2O$ equivalent (mM $FeSO_4$/g cascara extract).

3.2. α-Glucosidase inhibitory activity

Alpha-glucosidase inhibitory activity was evaluated using a procedure reported with slight modification [69]. Briefly, α-glucosidase (E.C.3.2.1.20) solution was prepared by dissolving 1 mg of α-glucosidase in 10 mL of 50 mM potassium phosphate buffer (pH 6.8). Briefly, the enzyme solution (0.27 U/mL), coffee leaves and coffee leaf tea extracts, and potassium phosphate buffer (1 mM pH 6.8) were pre-incubated at 37 ^{o}C for 10 min. The p-nitrophenyl α-D-glucopyranoside (PNPG, Sigma) (1 mM) was added as a substrate and incubated further at 37 °C for 20 min. The absorbance was recorded at 405 nm at initial time (0 min) and final time (20 min). Control contained the same reaction mixture except the same volume of water and DMSO instead of samples. The inhibition (%) was calculated as follows:

$$\text{Inhibitory activity (\%)} = [1-(A_{1\ \text{initial-final}}/A_{0\ \text{initial-final}})] \times 100\%$$

where A_1 was the absorbance of sample and A_0 was the absorbance of control. The mean of duplicates IC_{50} (concentration for 50% inhibition) values of each sample was determined graphically.

3.3. Inhibition of insoluble glcan formation by mutansucrase

Briefly, 0.05 U/mL mutansucrase isolated from *S. mutans*, 20 mM sodium phosphate buffer with 0.2% sodium azide and 25 mg/mL sample were pre-incubated at 37 ^{o}C for 10 min [37]. After pre-incubation, 100 mM sucrose was added and the mixtures were incubated at 37 ^{o}C for 6 h. Control contained the same reaction mixture except the same volume of water instead of sample solution. After 6 h of incubation, the mixtures were incubated at 60 ^{o}C for 10 min to stop the reaction, and centrifuges at 8,000 × *g* for 15 min. The supernatant was separated for soluble glucan analysis, and the pellet was washed three times with distilled water. The insoluble glucans synthesized in the mixture were dissolved in 1 M NaOH and centrifuged at 8,000 × *g* for 15 min. The supernatant was used for analysis. The relative inhibition of the enzymes was determined using TLC by measuring the amount of insoluble glucan formation.

Results and Discussion

1. Functional compounds in coffee leaf tea

1.1. Compounds analysis using UPLC-MS

Chlorogenic acid, caffeine, mangiferin, catechin, p-coumaric acid, and rutin content of each sample were quantified. In general, both before and after tea processing, young leaves had higher chlorogenic acid, caffeine, mangiferin, catechin, and rutin content than mature leaves (**Table 8**). Young leaves before tea processing (Y-W and Y-E) had the most abundant bioactive compounds. Lower bioactive compounds content in mature leaves indicated the decrease of compounds during leaves expansion. Leaves undergo changes in the composition of secondary metabolites during development [70]. The leaves synthesized secondary metabolites continuously, but when the accumulation of this metabolites were used for the leaves development due to expansion, the actual concentration of metabolites has decreased.

Tea processing affected the concentration of chlorogenic acid, caffeine, mangiferin, catechin, p-coumaric acid, and rutin content in coffee leaves. All those compounds decreased after tea processing. In both water and EtOH-extracted samples, the content of those bioactive compounds in black tea was lower than green tea. It indicated that the oxidation process significantly affected the bioactive compounds content.

Table 8. Bioactive compounds of coffee leaves and coffee leaf tea

Sample	Chlorogenic acid	Caffeine	Mangiferin	Catechin	p-coumaric acid	Rutin
Y-W	40.4 ± 2.2	31.3 ± 0.2	12.0 ± 0.2	4.3 ± 0.04	0.01 ± 0.0003	0.07 ± 0.001
M-W	12.7 ± 0.6	7.8 ± 0.1	3.0 ± 0.02	1.2 ± 0.03	0.03 ± 0.0003	0.02 ± 0.0
YG-W	33.1 ± 2.4	15.7 ± 0.2	4.2 ± 0.1	1.8 ± 0.03	0.05 ± 0.001	0.04 ± 0.001
YB-W	8.9±0.4	10.0±0.0	1.4±0.0	0.19±0.002	0.07±0.003	N/D
MG-W	7.6 ± 0.3	7.6 ± 0.2	1.3 ± 0.03	0.8 ± 0.02	0.01 ± 0.0002	0.02 ± 0.002
MB-W	1.7±0.1	6.0±0.1	0.55±0.02	0.049±0.002	N/D	N/D
Control-W	2.0±0.1	5.0±0.1	1.1±0.0	N/D	0.049±0.002	1.2±0.02
Y-E	50.9 ± 1.1	42.7 ± 0.4	16.6 ± 0.1	5.6 ± 0.0	N/D	N/D
M-E	19.8 ± 1.0	11.5 ± 0.1	5.6 ± 0.0	1.05 ± 0.01	0.04 ± 0.001	N/D
YG-E	45.6 ± 2.5	23.9 ± 0.5	6.0 ± 0.2	4.6 ± 0.0	0.06 ± 0.004	N/D
YB-E	12.5 ± 0.6	17.4 ± 0.1	2.5 ± 0.0	0.4 ± 0.001	0.07 ± 0.003	N/D
MG-E	12.0 ± 0.6	11.4 ± 0.1	2.8 ± 0.1	1.1 ± 0.001	N/D	N/D
MB-E	2.7 ± 0.1	12.3 ± 0.1	1.8 ± 0.1	0.2 ± 0.002	N/D	N/D
Control-E	2.6±0.1	11.3±0.1	1.9±0.1	N/D	0.06±0.002	2.2±0.07

Results of coffee leaves and coffee leaf tea are expressed as means ± standard deviation of triplicate analysis;
N/D : Not detected
W is water extracted samples and E is 70% ethanol extracted samples.
Y: young leaves (one to three months old), M: mature leaves (three to six months old), YG: young leaf green tea, YB: young leaf black tea, MG: mature leaf green tea, MB: mature leaf black tea. Control: Coffee leaf tea (Wize monkey Inc., Canada).

1.2. Total functional compound content analysis

The Folin–Ciocalteu method measures the reduction of the reagent by phenolic compounds with the formation of a blue complex that can be measured at 760 nm, using gallic acid as a standard [71]. In general, before and after tea processing, the young leaves showed higher TPC value than mature leaves (**Table 9**).

In the water-extracted samples, the green tea processing did not affect the TPC value significantly. Meanwhile, the black tea processing decreased the TPC value of young leaf and mature leaf by 3 and 2.5 times, respectively. The EtOH-extracted samples showed different response from water-extracted samples. Using this extraction method, the green tea processing reduced the TPC value by 1.7 times, both in young and mature leaves. The black tea processing reduced the TPC value of young leaves significantly.

The TFC of coffee leaves and coffee leaf tea extracts were tested by using aluminium chloride colorimetric method. Flavonoid are a class of secondary plant phenolics with significant and they seem to display important anti-inflammatory, anti-allergic and anti-cancer activities [72]. In general, young leaf contained more flavonoid than mature leaf from 1.8 to 4.2 times. The flavonoid content were higher in green tea samples than black tea samples from 1.9 to 3.6 times.

The TCT of coffee leaves and coffee leaf tea extract were tested by using total condensed tannin content assay. The tannin content in

coffee leaf tea were found to be lower than flavonoid content. TCT were changed during tea processing. Green tea had a higher content of tannin than black tea from 1.9 to 6.3 times. The moisture decrease in during the green tea processing and high temperature inhibited the activity of polyphenol oxidase, which induced catechin oxidation [73]. The same as TFC, the condensed tannin content was higher in 70% ethanol extraction than water-extracted samples because it attracted more water-insoluble tannin compounds.

Table 9. Determination of total phenolic, flavonoid and condensed tannin content for coffee leaves and coffee leaf tea

Sample	TPC (mg GAE[1]/g leaf)	TFC (mg QE[2]/g leaf)	TCT (mg CE[3]/g leaf)
Y-W	69.2 ± 1.28	30.5 ± 1.18	0.73 ± 0.13
M-W	26.5 ± 0.37	7.3 ± 0.132	0.80 ± 0.16
YG-W	61.4 ± 2.39	20.9 ± 1.68	1.97 ± 0.04
YB-W	21.7 ± 0.50	7.5 ± 0.28	0.31 ± 0.10
MG-W	27.9 ± 1.54	5.4 ± 0.06	1.12 ± 2.06
MB-W	11.1 ± 0.06	2.2 ± 0.04	0.28 ± 0.04
Control-W	15.3 ± 0.14	6.5 ± 2.0	0.69 ± 0.07
Y-E	130.9 ± 0.08	38.2 ± 1.92	3.62 ± 0.20
M-E	58.5 ± 0.08	12.6 ± 0.49	2.57 ± 0.07
YG-E	78.9 ± 0.11	25.6 ± 1.04	0.26 ± 0.02
YB-E	41.6 ± 0.02	7.5 ± 0.26	0.49 ± 0.03
MG-E	33.7 ± 0.04	8.0 ± 0.37	1.48 ± 0.07
MB-E	49.2 ± 0.04	4.2 ± 0.26	0.41 ± 0.08
Control-E	18.7 ± 0.23	7.5 ± 0.8	0.91 ± 0.09

Results of coffee leaves and coffee leaf tea are expressed as means ± standard deviation of triplicate analysis; TPC; Total phenolic content, TFC; Total flavonoid content, TCT; Total condensed tannin content.

W is water extracted samples and E is 70% ethanol extracted samples.
Y: young leaves (one to three months old), M: mature leaves (three to six months old), YG: young leaf green tea, YB: young leaf black tea, MG: mature leaf green tea, MB: mature leaf black tea. Control: Coffee leaf tea (Wize monkey Inc., Canada).

[1]: Gallic acid equivalent, [2]: Quercetin equivalent, [3]: Catechin equivalent

2. Analysis of antioxidant activity

In this study, the antioxidant activities of coffee leaves and coffee leaf tea extracts were studied using DPPH radical scavenging activity, ORAC and FRAP assay. In general, the antioxidant activity of EtOH-extracted samples had higher antioxidant activity than water-extracted samples from 1.15 to 2.3 times in ORAC (**Table 9**). In both water and EtOH-extracted samples, the processing of mature and young coffee leaves into green tea higher antioxidant activity than black tea from 1.15 to 3 times in ORAC. According to other studies, green tea of *Camellia sinensis* has more health benefits than black tea in terms of antioxidant capacity [74]. All tested coffee leaf tea samples showed higher antioxidant activity compared to the commercial tea as control.

Table 10. Determination of DPPH radical scavenging activity, ORAC and FRAP for coffee leaves and coffee leaf tea

Sample	DPPH (IC_{50}[1] μg/g)	ORAC (TE[2] μmol/10g)	FRAP (μmol Fe(Ⅱ)[3]/g)
Y-W	317.39 ± 4.14	6162 ± 220	3.17 ± 0.11
M-W	427.69 ± 5.87	2925 ± 309	1.88 ± 0.07
YG-W	287.23 ± 4.40	7386 ± 201	5.17 ± 0.20
YB-W	586.38 ± 15.92	2396 ± 173	1.06 ± 0.03
MG-W	294.25 ± 4.22	2751 ± 183	1.42 ± 0.09
MB-W	670.71 ± 33.03	1606.± 295	1.01 ± 0.08
Control-W	728.05 ± 11.93	1928 ± 135	1.12 ± 0.07
Y-E	146.98 ± 3.09	7956 ± 981	5.53 ± 1.15
M-E	319.77 ± 21.79	3791 ± 308	2.83 ± 0.02
YG-E	264.02 ± 15.56	6903 ± 518	4.77 ± 0.29
YB-E	555.20 ± 15.56	1832 ± 299	1.01 ± 0.14
MG-E	280.53 ± 10.36	6189 ± 868	3.22 ± 0.07
MB-E	390.35 ± 21.26	4103 ± 346	1.74 ± 0.02
Control-E	702.56 ± 20.57	2605 ± 562	1.44 ± 0.05

Results of coffee leaves and coffee leaf tea are expressed as means ± standard deviation of triplicate analysis; DPPH; DPPH radical scavenging activity, ORAC; Oxygen radical absorbance capacity, FRAP; Ferric reducing antioxidant power assay.

W is water extracted samples and E is 70% ethanol extracted samples.
Y: young leaves (one to three months old), M: mature leaves (three to six months old), YG: young leaf green tea, YB: young leaf black tea, MG: mature leaf green tea, MB: mature leaf black tea. Control: Coffee leaf tea (Wize monkey Inc., Canada).

[1]: concentration for 50% inhibition, [2]: Trolox equivalent,
[3]: $FeSO_4$ equivalent

3. α-Glucosidase inhibitory activity

Alpha-glucosidase is a carbohydrate hydrolyzing enzymes that catalyze the breaking down of complex carbohydrates. The inhibition of α-glucosidase can delay the carbohydrate absorption and the control of hyperglycemia [75]. Therefore, the α-glucosidase inhibitors were developed for diabetes and obesity treatment. In this study, the inhibitory activity of coffee leaves and coffee leaf tea were determined. In the water-extracted samples, the processing of young leaf into green tea and black tea reduced their inhibitory activity 9.9 and 8.4 times, respectively (**Figure 8**).

The same as young leaf, the processing of mature leaf into tea also reduced its inhibitory activity. However, the reduces were not as high as young leaf (green tea and black tea, 2.7 and 3.3 times, respectively). The EtOH-extracted samples showed different result from water-extracted samples. Using this extraction method, the processing of young leaves into teas reduced their inhibitory activity both in green and black tea. However, the processing of mature leaves into teas increased their inhibitory activity (**Table 11**).

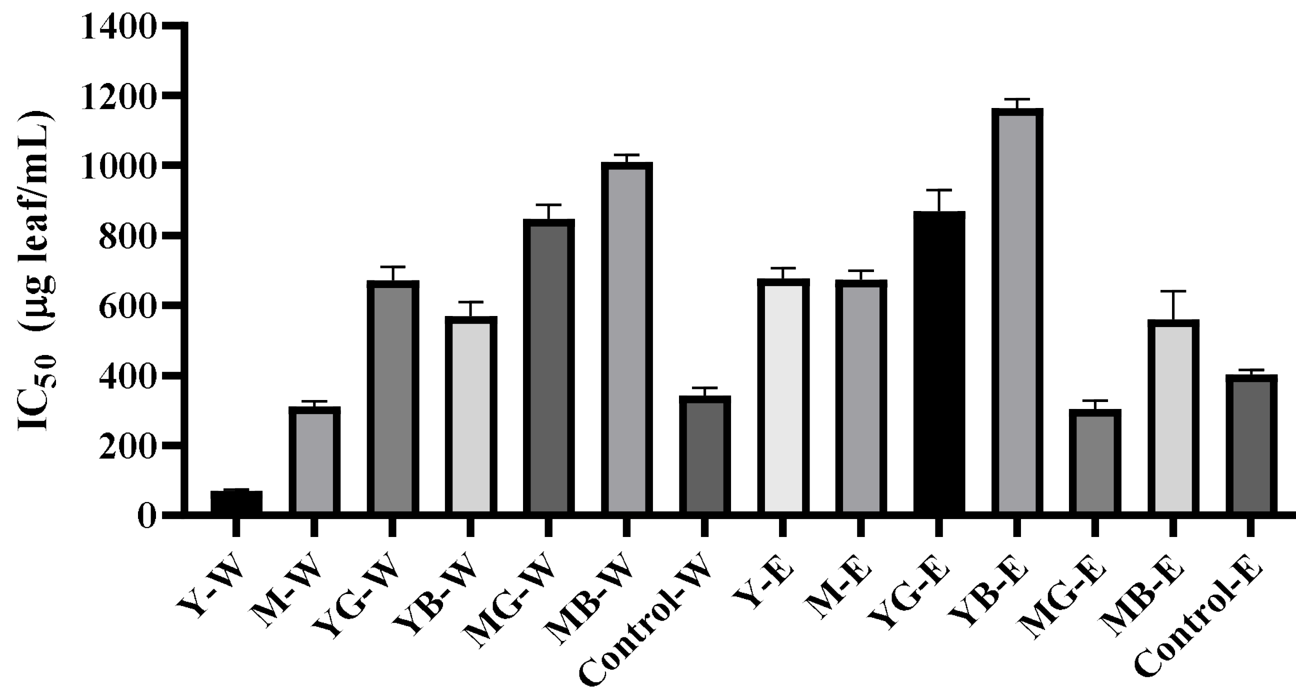

Figure 8. Inhibitory activity against α-glucosidase

W is water extracted samples and E is 70% ethanol extracted samples.
Y: young leaves (one to three months old), M: mature leaves (three to six months old), YG: young leaf green tea, YB: young leaf black tea, MG: mature leaf green tea, MB: mature leaf black tea. Control: Coffee leaf tea (Wize monkey Inc., Canada).

Table 11. Inhibitory activity against α-glucosidase

Sample	Inhibition of α-glucosidase (IC_{50}[1] μg/g)
Y-W	67.74 ± 5.19
M-W	310.28 ± 16.06
YG-W	671.00 ± 38.91
YB-W	568.77 ± 39.74
MG-W	846.65 ± 40.47
MB-W	1009.77 ± 20.96
Control-W	341.46 ± 21.74
Y-E	677.17 ± 29.20
M-E	672.84 ± 27.09
YG-E	868.69 ± 60.98
YB-E	1162.90 ± 26.87
MG-E	304.02 ± 23.75
MB-E	559.29 ± 80.89
Control-E	402.38 ± 13.69

Results of coffee leaves and coffee leaf tea are expressed as means ± standard deviation of triplicate analysis;

W is water extracted samples and E is 70% ethanol extracted samples.
Y: young leaves (one to three months old), M: mature leaves (three to six months old), YG: young leaf green tea, YB: young leaf black tea, MG: mature leaf green tea, MB: mature leaf black tea. Control: Coffee leaf tea (Wize monkey Inc., Canada).

[1]: concentration for 50% inhibition

4. Inhibition of insoluble glucan formation by mutansucrase

Coffee leaves and coffee leaf teas inhibited the synthesis of water insoluble glucans in the presence of sucrose compared to the control. In general, the EtOH-extracted samples showed higher inhibitory activity compared to the water-extracted samples. In the EtOH-extracted samples, there was no significant difference between the coffee leaf tea extracts and coffee leaves extracts. Meanwhile, in the water-extracted samples, the processing of black tea significantly reduced inhibition by up to 17% compared to green tea. (**Figure 9**). It was green tea that had inhibition activity of insoluble glucan formation in the water extraction, and it was found that this ability was reduced if processed with black tea.

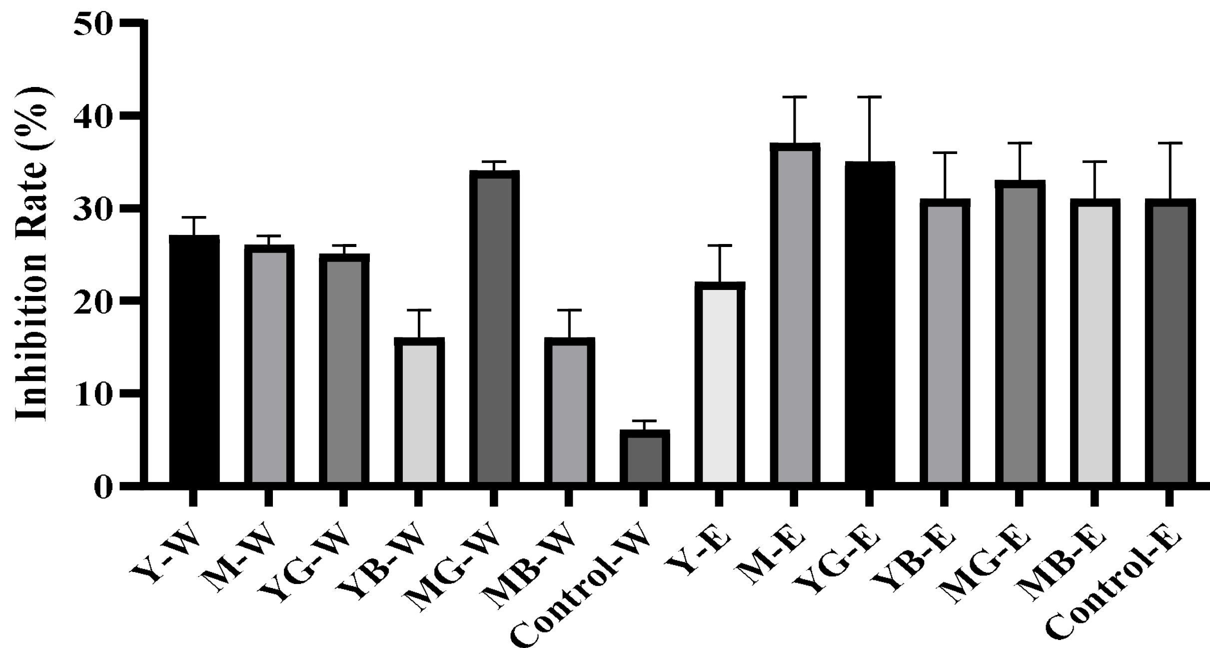

Figure 9. Effects of coffee leaves and coffee leaf tea on the inhibition of insoluble glucan formation by mutansucrase

-W is water extracted samples and E is 70% ethanol extracted samples.
Y: young leaves (one to three months old), M: mature leaves (three to six months old), YG: young leaf green tea, YB: young leaf black tea, MG: mature leaf green tea, MB: mature leaf black tea. Control: Coffee leaf tea (Wize monkey Inc., Canada).

Conclusion

Green tea and black tea were manufactured from young and mature leaves. I identified the potential anti-dental plaque, antidiabetic, and antioxidant activities of coffee leaves and coffee leaf tea. The prepared coffee leaf tea had a better ability than control (commercial coffee leaf tea). In the case of ethanol extraction, a higher amount of functional compounds were extracted compared to water extraction because ethanol has more polarity than water. In particular, most of the results showed that the functional compounds and antioxidant capacity of black tea were significantly reduced compared to green tea. *Camellia sinensis*, the tea we often eat, showed a similar result. The antioxidant activity of black tea was reduced by 1.5 times compared to green tea. In addition, it showed different results depending on the age of the leaf. Young coffee leaves showed higher content of functional compounds and higher antioxidant activities than mature coffee leaves. By making coffee leaf tea from coffee leaves, coffee farms offers potential job security for employees of coffee farms and an alternative production model for coffee-producing countries.

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Abstract in Korean

커피 체리 껍질로 알려진 카스카라는 커피콩을 생산하고 남는 주요 고체 잔류물 중 하나로, 3% 만이 비료 등으로 활용되고 있지만, 나머지 97% 는 매립지에 버려지고 있다. 카스카라가 함유하고 있는 카페인과 탄닌은 수질 오염을 일으키는 원인이 되며, 커피 벨트에 속해있는 국가가 해결해야 하는 문제 중 하나이다. 따라서 식품으로서의 카스카라의 적절한 활용은 이 문제에 대한 해결 방안을 제시해줄 것으로 생각된다. 본 연구에서는 *Leuconostoc mesenteroides* B-512F/KM과 B-1299CB4의 균주에서 얻어낸 덱스트란 수크라아제를 이용하여 고 중합도를 가지는 카스카라 올리고당을 합성하였다. 신규 제조한 카스카라 올리고당은 클로로겐산, 트리오넬린, 갈산과 같은 많은 생체 활성 물질을 함유하고 있었다. 열량은 1회 섭취 분량 당 10kcal로 꿀 혹은 메이플 시럽에 비해 2~3배 가량 낮은 값을 갖는 것으로 확인되었다. 또한, 치석을 구성하고 있는 불용성 글루칸 형성 연구에서 최고 농도 12.5%에서 약 60%의 억제능을 가진 것으로 확인되었다. 카스카라 올리고당의 항산화능은 TPC, ORAC, FRAP 및 DPPH 라디칼 소거능 방법을 통해 확인되었고, 시판되고 있는 여느 시럽 보다 높은 항산화능을 갖는 것으로 나타났다.

우리가 흔히 먹는 커피 콩의 경우 1년에 1번 수확을 하지만 수확 시기가 끝나면, 커피 농가에게 일정한 수입이 없는 시기가 된다. 그러나, 커피 잎의 경우 1년 내내 수확할 수 있다는 장점을 가지고 있으며, 예로부터 인도네시아, 아프리카 등의 나라에서 전통차로 섭취해오고 있다. 커피 잎은 또한 여러 생리 활성을 갖는 성분이 많이 함유되어 있고, 특히 폴리페놀의 일종인 망기페린이 특별한 성분으로서 포함되어 있다. 커피 잎을 새로운 차로 음용하는 것은 최근 소비자들의 관심을 받고 있지만, 가공 방법이나 잎의 연령이 최종 제품의 품질에 어떤 영향을 미치는지에 대해서는 알려진 바가 없다. 본 연구에서는, 커피 잎을 연령 별로 나누어 녹차와 홍차 법을 활용하여 커피 잎차를 제조한 후, 이가 함유한 성분과 기능성에 대한 연구를 수행하였다. 커피 잎에는 클로로겐산, 카페인, 망기페린, 카테킨, p-쿠마릭산, 루틴 등이 함유되어 있었고, 특히 잎의 연령이 성숙해질수

록 대부분의 성분이 감소하는 것을 확인하였다. 커피 잎차의 항산화능 측정으로는 TPC, ORAC, FRAP 및 DPPH 라디칼 소거능 방법을 사용하였으며, 잎의 연령이 성숙해질수록 항산화능이 낮아지는 것을 확인하였다. 잎의 연령뿐만 아니라, 차제법에 따라서도 결과가 유의미하게 달라지는 것을 확인 하였다. 홍차 제법을 이용해 제조된 커피잎 차는 녹차 제법을 이용한 커피잎 차에 비해 성분과 항산화능이 감소하였다. 커피 잎차는 치석을 구성하는 불용성 글루칸 형성 억제 능력이 있으며, 글리코사이드를 가수 분해 하여 당과 아글리콘의 생성 반응을 촉매하는 효소인 알파글루코시다제에도 억제 능력이 있는 것을 확인하였다.

카스카라와 커피잎의 식품소재로의 활용은 커피 산업에 더 많은 부가가치를 부여하고, 커피 비수확시기에 커피 농부들에게 수입을 줄 수 있으며 커피 생산 시스템의 환경 문제를 해결하는 데 도움이 될 것으로 보인다.

키워드 : 카스카라, 류코노스톡 메센테로이데스, 덱스트란수크라아제, 저열량 올리고당, 항산화제, 커피잎차, 망기페린, 녹차, 홍차, 항치석, 항당뇨