

Antioxidant and Antimicrobial Activities of Phenolic Components and Organic Acids from *Camellia Oleifera* Cake

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Abstract: There is a great interest in finding antioxidants and products with antimicrobial activities from natural sources. The aim of this study was to obtain and identify the phenolic components and organic acids from *Camellia oleifera* cake. In addition, their antioxidant and antimicrobial activities were also investigated. The purity of phenolic components and organic acids obtained were $94.1 \pm 0.5\%$ w/w and $96.0 \pm 0.3\%$ w/w, respectively. There are 15 phenolic components have been detected and identified by HPLC-ESI-MS. Oxalic, citric, acetic, malic, and succinic acids are found to be major organic acids. The phenolic components and organic acids both had good antioxidant capacity, evaluated by 4 antioxidant activity assays (hydroxyl radical scavenging, superoxide radical scavenging, 1,1-diphenyl-2-picryl-hydrazyl radical scavenging and lipid peroxidation inhibition). In addition, the phenolic components and organic acids exhibited significant inhibitory activity against bacteria *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, and fungi *Mucor racemosus*, *Aspergillus oryzae*, *Rhizopus stolonifer*. The phenolic components and organic acids from *C. oleifera* cake both showed good antioxidant capacity and exhibited antimicrobial activities. These results may be useful for the future use of phenolic components and organic acids from *C. oleifera* cake.

Keywords: *Camellia oleifera* cake; phenolic component; organic acid; antioxidant capacity; antimicrobial activity

1. Introduction

Currently, more and more natural products have been found to have many benefits for the human body [1,2] and there is a great interest in finding antioxidants from natural sources to prevent oxidative stress and minimize oxidative damage to living cells [3]. Oxidative damage is involved in the pathogenesis of various diseases such as atherosclerosis, cancer, diabetes mellitus and reperfusion disorder [4]. Antioxidants have been widely studied because of their applications in the food industry as food preservatives and in medication as treatments against diseases that are caused by oxidative stress [5]. Accordingly, considerable attention has been paid to the evaluation of the antioxidant activity of naturally occurring substances in recent years [6,7]. In addition, there also has been considerable interest in natural products from plants with antimicrobial activities for controlling toxin-producing microorganisms because the use of antibiotics is no longer desirable due to concerns about bacterial resistance [8]. Increased awareness of the potential problems associated with antibiotics stimulates research efforts to carry out novel approaches to the development of new antimicrobial compounds.

Polyphenols, the secondary metabolites that have an important role in reducing oxidative stress, are reported to exhibit antioxidant activities [9,10] and act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelating agents [11]. Extracts of herbs and plant materials rich in phenolics are increasingly of interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food. There are many

examples [12]. Phenolic compounds are also reported to have antibacterial properties [13,14]. In addition, organic acids are widely distributed in fruits, vegetables, herbs and plants. The content of organic acids in food could influence their nutrition, antioxidant and antimicrobial properties [15].

The seeds of *Camellia oleifera* contain numerous valuable compounds and nutritious oils, which are frequently used in oriental countries, especially in Southeast Asia. After the oil refining process, defatted seeds are always used as a detergent or organic fertilizer with low economic value. The remainder of seeds after oil extract is called the *C. oleifera* cake. There are several million tons of *C. oleifera* cakes that are discarded every year in China [8]. However, the *C. oleifera* cake contains large amounts of active compounds. Hence, the extraction of phenolic compounds and organic acids from *C. oleifera* cake will be valuable.

To the authors' knowledge, there are limited published reports on phenolic components of *C. oleifera* cake and their activities. Similarly, few researches have been done to understand the organic acid compositions of *C. oleifera* cake. Due to the lack of such information, the phenolic components and organic acids of *C. oleifera* cake along with their antioxidant and antimicrobial potentials were investigated.

2. Experimental

2.1. Materials and reagents

The seeds of *C. oleifera* were collected in Yichun County and the cakes were

made after expression in Meiling County both from the Jiangxi Province in China. The dried cakes were battered and crushed to small pieces by a pulverizer (GC-ZN-08, Xihua Corp. Beijing, China), and then grounded by a grinder (YD-313, Youji Corp. Shanghai, China) to fine powders. The powders were then packed in a polyethylene (PE) pail and stored at room temperature (15 ± 3 °C) before use.

Staphylococcus aureus (1.128), *Escherichia coli* (1.1369), *Bacillus subtilis* (1.1630), fungi *Mucor racemosus* (3.3446), *Aspergillus oryzae* (3.5232), and *Rhizopus stolonifer* (3.4110), which were used for antimicrobial sensitivity test, were obtained from Institute of Microbiology Chinese Academy of Sciences. All the reagents used were of analytical grade and obtained from Shanghai Chemicals and Reagents Co. (Shanghai, China).

2.2. Preparation of phenolic components and organic acids

The powders of *C. oleifera* cake (500 g) were defatted with petroleum ether in a round-bottom flask for 3 h. After that, the organic solvent was volatilized using a rotary evaporator (RE-52A, Yarong Bio-instrument Co., Shanghai, China), and the defatted powders were obtained.

2.2.1. Extraction and purification of phenolic component

Extraction and purification procedure of phenolic components was performed as follow: the defatted powders were extracted with 500 mL L⁻¹ methanol [matters to solvent ratio (g mL⁻¹) = 1:15] in a 50 °C water bath for 2 h. The mixtures were filtered using Whatman NO.1 filter paper. The filtrates were then concentrated by a rotary evaporator at 55 °C. The concentrated filtrates were passed through AB-8

macro-reticular resin column (80 x 20 mm, Sigma-Aldrich, Shanghai, China) and eluted with 850, 700, 500, and 300 mL L⁻¹ ethanol, successively according to the method of Singleton & Rossi (1965) [16]. The 300 mL L⁻¹ ethanol eluate, which was found to contain the primary phenolic components, was considered as the phenolic component-rich fraction. This fraction was then evaporated to dryness by a rotary evaporator at 55 °C. A minimum volume of distilled water was added to this fraction and the resulting solution was freeze-dried in a Freezone 12Plus lyophilizer (Labconco, USA). The procedure above was repeated several times to obtain sufficient sample.

2.2.2. Extraction and purification of organic acids

The defatted powders were extracted with deionized water [matters to solvent ratio (g mL⁻¹) = 1:10] in a 70 °C water bath for 2 h. After that, the mixtures were filtered using Whatman NO.1 filter paper. The filtrates were evaporated by a rotary evaporator at 55 °C. Then, the concentrated filtrates were passed through HPD100 macro-reticular resin column (80 x 20 mm, Sigma-Aldrich, Shanghai, China) and eluted with distilled water and 950 mL L⁻¹ ethanol in H₂O, successively. The mL L⁻¹ ethanol eluate was evaporated to dryness by a rotary evaporator at 55 °C. A minimum volume of distilled water was added to this dried eluate, and then the resulting solution was freeze-dried in the Freezone 12Plus lyophilizer. The procedure above was repeated several times to obtain sufficient sample.

The obtained phenolic components and organic acids were then re-dissolved in distilled water to measure their total phenolic content, total organic acids content,

antioxidant capacity, and antimicrobial activities.

2.3. Phenolic determination

2.3.1. Total phenolic content

Total phenolic content was analyzed using the Folin–Ciocalteu reagent according to the method described by Singleton & Rossi (1965) [16] with some modifications. 0.2 mL of the sample (1 g L⁻¹ dissolved in distilled water) were added to a conical flask and mixed with 1 mL of Folin–Ciocalteu reagent and 46 mL of distilled water. The mixture was allowed to react for 3 min, and 3 mL of aqueous solution of Na₂CO₃ (75 g L⁻¹) was added. Then the mixture was shaken vigorously. At the end of incubation for 2 h with intermittent shaking at room temperature, the absorbance of each mixture was measured at 760 nm on a TU-1900 spectrophotometer (PGeneral). The same procedure was also applied to the standard solutions of gallic acid. Total phenolic content was expressed as micrograms of gallic acid equivalents per kilogram of dry sample. The total phenolic content determination was repeated three times.

2.3.2. Identification of phenolic compounds

The phenolic compounds were analyzed using an Agilent 1200 HPLC–MS system containing of a surveyor auto-sampling system, interfaced to a 6430 ion-trap mass spectrometer via an electrospray ion source. HPLC was carried out on the liquid chromatograph system, equipped with a quaternary pump, a column temperature controller, and a photodiode array (PDA) detector. The analytical column temperature was kept at 35 °C. The sample was separated with a RP-C18 column (4.6 mm x 250

mm, 5 μm) using 10 mL L⁻¹ acetic acid in water (eluent A) and 5 mL L⁻¹ acetic acid in water and acetonitrile (50:50, v/v; eluent B) under gradient conditions [from 10 to 25% B (15 min), 25 to 35% B (15 min), 35 to 40% B (5 min), 40 to 50% B (10 min), 50 to 100% B (25min), 100 to 10% B (10 min)]. The mobile phase was at a flow rate of 0.3 mL min⁻¹ within 80 min and the detector was employed to set at a wavelength of 280 nm. The injection volume was 20 μL . Peaks were assigned by their retention time. Source settings used for the ionization of phenolic compounds were: nebulizer gas pressure of 45.00 psi; dry gas flow rate of 11.00 L min⁻¹; electrospray voltage of the ion source of 3000 V; capillary temperature of 350 °C; fragmentor of 120.0 V. Nitrogen (>99.99%) and He (>99.99%) were used as sheath and damping gas, respectively.

2.4. Determination of organic acids

Analysis was performed on a Waters liquid chromatograph system (UK6 injector and 515 HPLC pump, Waters, Milford, MA, USA), equipped with a Waters C18 column (4.6 mm x 250 mm, 5 μm , Waters, Milford, MA, USA), a guard column (10 x 4 mm, Waters, Milford, MA, USA), and a Waters 2996 PDA detector connected to a Waters software. A constant elution system consisted of phosphate buffer solution (20 mmol L⁻¹ KH₂PO₄, pH = 2.7) (A) and methanol (B). The separation was achieved using the following program: 0–30 min, 5% B. The flow rate was 0.5 mL min⁻¹ and the system was operated at room temperature when the sample solution (10 μL) was injected in each run. The PDA detector was set at a wavelength of 214 nm.

2.5. Antioxidant activity assays

2.5.1. Hydroxyl radical-scavenging activity

The hydroxyl radical scavenging ability was measured using a modified method of Halliwell et al [17]. A reaction mixture was prepared by adding 0.1 mL EDTA (1 mM), 0.01 mL of FeCl₃ (10 mM), 0.1 mL of H₂O₂ (10 mM), 0.36 mL of deoxyribose (10 mM), 1.0 ml of samples (0.1–10 g L⁻¹) dissolved in distilled water, 0.33 mL of phosphate buffer (50 mM, pH 7.4) and 0.1 mL of ascorbic acid (1 mM) in sequence. The mixture was then incubated at 37 °C for 1 h. 1.0 mL of the incubated mixture was mixed with 1.0 mL of 100 mL L⁻¹ trichloroacetic acid and 1.0 mL of 5 mL L⁻¹ thiobarbituric acid (in 25 mM NaOH containing 250 μL L⁻¹ butylated hydroxyl anisole) to develop the pink chromogen measured at 532 nm. The hydroxyl radical-scavenging activity of the samples was reported as the percentage of inhibition of deoxyribose degradation and was calculated according to the following equation:

$$\% \text{ inhibition} = (A_0 - A_t) / A_0 \times 100 \quad (1)$$

where A_0 was the absorbance of the control (blank, without samples) and A_t was the absorbance in the presence of the samples. All of the tests were carried out in triplicate and IC₅₀ values were expressed as means ± standard deviation (SD). Ascorbic acid was used as a positive control.

2.5.2. Superoxide radical-scavenging activity

This activity was measured using nitro blue tetrazolium (NBT) reagent as described by Sabu & Kuttan [18]. The method is based on generation of superoxide

radical O_2^- by auto-oxidation of hydroxylamine hydrochloride in the presence of NBT, which gets reduced to nitrite. Nitrite in the presence of EDTA gives a color that was measured at 560 nm. Test solutions of sample ($0.1-10 \text{ g L}^{-1}$) were taken in a test tube. To this, reaction mixture consisting of 1 mL of (50 mM) sodium carbonate, 0.4 mL of (24 mM) NBT and 0.2 mL of 0.1 mM EDTA solutions were added to the test tube and immediate reading was taken at 560 nm. 0.4 mL of (1 mM) of hydroxylamine hydrochloride was added to initiate the reaction. The reaction mixture was then incubated at 25°C for 15 min and the reduction of NBT was measured at 560 nm. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. All the samples were treated in the similar manner, absorbance was recorded and the percentage of inhibition was calculated according to the following equation:

$$\% \text{ inhibition} = (A_0 - A_t) / A_0 \times 100 \quad (2)$$

where A_0 was the absorbance of the control (blank, without samples) and A_t was the absorbance in the presence of the samples. All the tests were performed in triplicate and IC_{50} values were obtained. Ascorbic acid was used as a positive control.

2.5.3. 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity

DPPH radical scavenging activity of samples was measured according to our published method [19]. The 0.2 mM solution of DPPH in 950 mL L^{-1} ethanol was prepared daily before UV measurements were taken. One milliliter of the samples of different quantities (0.1-10 mg) in water was thoroughly mixed with 2 mL of freshly prepared DPPH and 2 mL of 950 mL L^{-1} ethanol. The mixture was shaken vigorously

and left to stand for 30 min in the dark. The absorbance of the supernatant obtained after centrifugation was then measured at 517 nm.

The DPPH radical scavenging ability was calculated using the following equation:

$$I\% = [1 - (A_i - A_j) / A_c] \times 100\% \quad (3)$$

where A_c is the absorbance of DPPH solution without sample (2 mL DPPH + 3 mL of 950 mL L⁻¹ ethanol); A_i is the absorbance of the test sample mixed with DPPH solution (1 mL sample + 2 mL DPPH + 2 mL of 950 mL L⁻¹ ethanol) and A_j is the absorbance of the sample without DPPH solution (1 mL sample + 4 mL of 950 mL L⁻¹ ethanol). All the tests were performed in triplicate and IC₅₀ values were obtained. Ascorbic acid was used as a positive control.

2.5.4. Inhibition of lipid peroxidation

This test was conducted using the method of Zhang et al [20] with some modifications. Briefly, an equal volume of egg yolk was added to 0.1 mol L⁻¹ phosphate buffered saline (PBS, pH 7.45). The mixture was stirred magnetically for 10 min and then diluted with 24 volumes of PBS. The yolk homogenate (1 mL), samples (0.5 mL, 0.1-10 g L⁻¹), PBS (1 mL) and 25 mmol L⁻¹ FeSO₄ (1 mL) were mixed in a tube and shaken at 37 °C for 15 min. The reaction was stopped by the addition of trichloroacetic acid and the mixture was centrifuged. Then 1 mL of 8 g L⁻¹ thiobarbituric acid solution was added to 3 mL of the supernatant. This solution was heated at 10 °C for 10 min, after which its absorbance at 532 nm was measured. The ability to inhibit lipid peroxidation was calculated as follows:

$$\% \text{ inhibition} = [(B_0 - B)/B_0] \times 100 \quad (4)$$

where B_0 is the absorbance of the control and B is the absorbance in the presence of samples. All the tests were performed in triplicate and IC_{50} values were obtained. Ascorbic acid was used as a positive control.

2.6. Antimicrobial activity assay

The activities of different samples were tested against bacteria *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, and fungi *Mucor racemosus*, *Aspergillus oryzae*, *Rhizopus stolonifera*. The bacteria and fungi were grown and maintained on nutrient agar medium slants and potato medium slants respectively when they were obtained from the Institute of Microbiology Chinese Academy of Sciences. Before inoculation, the liquid nutrient medium for bacteria and potato mediums for fungi were autoclaved at 121 °C in vertical heating pressure steam sterilizer (LDZX-50KB, Shenan Medical Instrument Works Co., Shanghai, China) for 20 min. After that, a loop of each microorganism was inoculated into cool liquid medium. They were then stored under aerobic conditions. The three bacteria were cultivated 24 h at 37 °C, while the three fungi were cultivated 48 h at 30 °C which was further adjusted to obtain a suitable turbidity for further use [21]. After cultivation, average counts of bacteria *Staphylococcus aureus*, *Escherichia coli* and *Bacillus subtilis* were 3.5×10^6 , 8.6×10^5 , 5.4×10^6 colony forming units (cfu) mL^{-1} , and for fungi *Mucor racemosus*, *Aspergillus oryzae*, and *Rhizopus stolonifer* were 1.5×10^6 , 7.4×10^6 , and 2.9×10^6 (cfu mL^{-1}), respectively. CfU mL^{-1} density of cultures was confirmed after distribution into assay plates by the following procedure. All

bacterial and fungi stock cultures were serially diluted 10-fold in a series of tubes, and plated by inoculating 100 μL onto medium plates which were incubated, and then colonies were counted to calculate density of the bacterial and fungi stock cultures.

2.6.1. Determination of inhibitory effect

The inhibition zone method was firstly employed to determine the antimicrobial activity of the samples [22]. Twenty milliliters of medium were poured into each Petri dish. After the medium had solidified, 100 μL of suspension of the test microorganisms were smeared onto it. The wells were made, and the samples (100 or 200 mg L^{-1}) were added to these wells (20 μL) and the same volume (20 μL) of gentamicin (1 g L^{-1}) was used as a positive control. Deionized water was used as negative control. The inoculated plates were incubated and the diameter (mm) of the inhibition zone expressed as an average of the maximum diameter in three different directions was measured after the bacteria were cultivated 24 h at 37 $^{\circ}\text{C}$ and the fungi were cultivated 48 h at 30 $^{\circ}\text{C}$.

2.6.2. Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was determined by liquid medium dilution method [23]. Two-fold serial dilutions of the samples with appropriate antibiotic control were prepared in liquid nutrient medium for bacteria and potato mediums for fungi [24]. For liquid medium dilution test, 100 μL of stock cultures of bacteria or fungi were added to each tube (containing two-fold serial dilutions of sample at a final concentration of 7.8–4000 mg L^{-1} for bacteria and fungi) and

incubated either at 37 °C for bacteria or at 30 °C for fungi for 24 h or 48 h. After the cultivation period, the turbidity was taken as the indication of growth and the lowest concentration which remained clear after microscopic evaluation was thus taken as the MIC, which was recorded as the mean concentration of triplicates.

2.7. Statistical analysis

All the experiments were done in triplicate. Statistical analysis was carried out using SPSS (version 16.0, Chicago, United States). The results were expressed as mean \pm standard deviations and compared using the Tukey test at 5% confidence level.

3. Results and discussion

3.1. Phenolic components and organic acids

Phenolics are aromatic secondary plant metabolites, and are widely spread throughout the plant kingdom [25]. The purity of phenolic components obtained from *C. oleifera* cake is $94.1 \pm 0.5\%$ w/w. Figure 1 showed the HPLC chromatogram of the phenolic components. Under the LC–MS, the MS spectra of phenolic components from *C. oleifera* cake were acquired in the positive ion mode and Table 1 presented their most possible identification. Among the phenolics, there are 15 phenolic components have been detected (Figure 1, Table 1). Identification were aided by comparison with reference standards where available and by correlation with previous literature reports. The detected phenolic components are pelargonidin-diglucoside, gallotannin, ellagitannin, cyanidin-glucoside, pelargonidin-glucoside, catechin,

p-coumaroyl-glucoside, *p*-coumaroyl-ester, *p*-coumaroyl-glucoside, quercetin-rutinoside, ellagic acid, quercetin-glucoside, quercetin-glucuronide, methyl-ellagic acid-pentose, and kaempferol-glucuronide.

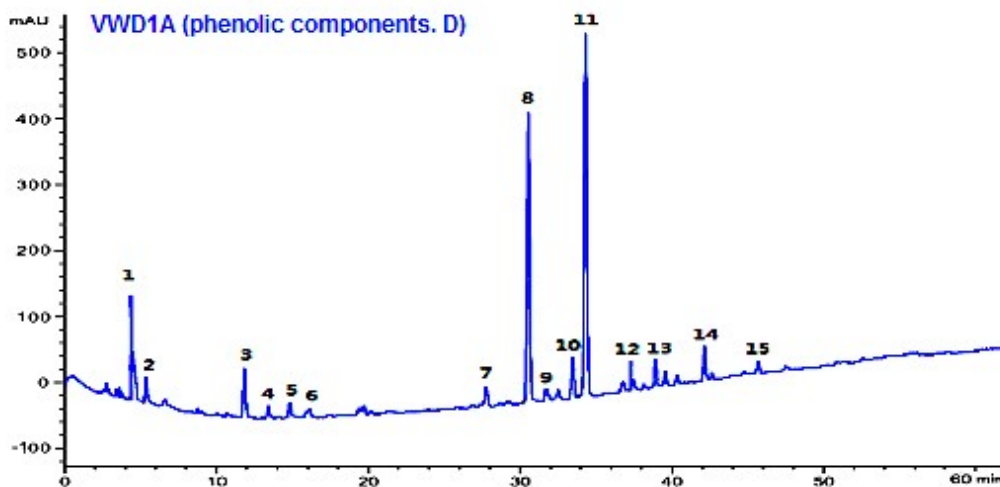


Figure 1. The HPLC chromatogram of the phenolic components.

Table 1. Identification of phenolic compounds from *C. oleifera* cake using their HPLC-DAD, LC-MS and LC-MSⁿ data.

Peak ^a	t _R (min)	Mw	Tentative ID
1	4.25	594	Plg-diglucoside
2	5.32	784	Gallotannin
3	11.89	640	Ellagitannin
4	13.64	448	Cyanidin-glucoside
5	14.58	432	Plg-glucoside
6	16.03	290	Catechin
7	27.76	325	<i>p</i> -coumaroyl-glucoside
8	30.29	356	<i>p</i> -coumaroyl-ester
9	31.82	326	<i>p</i> -coumaroyl-glucoside
10	31.74	610	Quercetin-rutinoside
11	34.20	302	Ellagic acid
12	37.41	464	Quercetin-glucoside
13	39.03	478	Quercetin-glucuronide
14	42.10	448	Methyl-ellagic acid -pentose
15	45.88	462	Kaempferol-glucuronide

^a Peak numbers refer to HPLC chromatograms in Figure 1.

Organic acids are important for their contribution to sensory attributes and nutrition value in fruits and plants [26]. The purity of organic acids obtained from *C. oleifera* cake is $96.0 \pm 0.3\%$ w/w. Oxalic, citric, acetic, malic and succinic acids are found to be major organic acids in the *C. oleifera* cake (Figure 2).

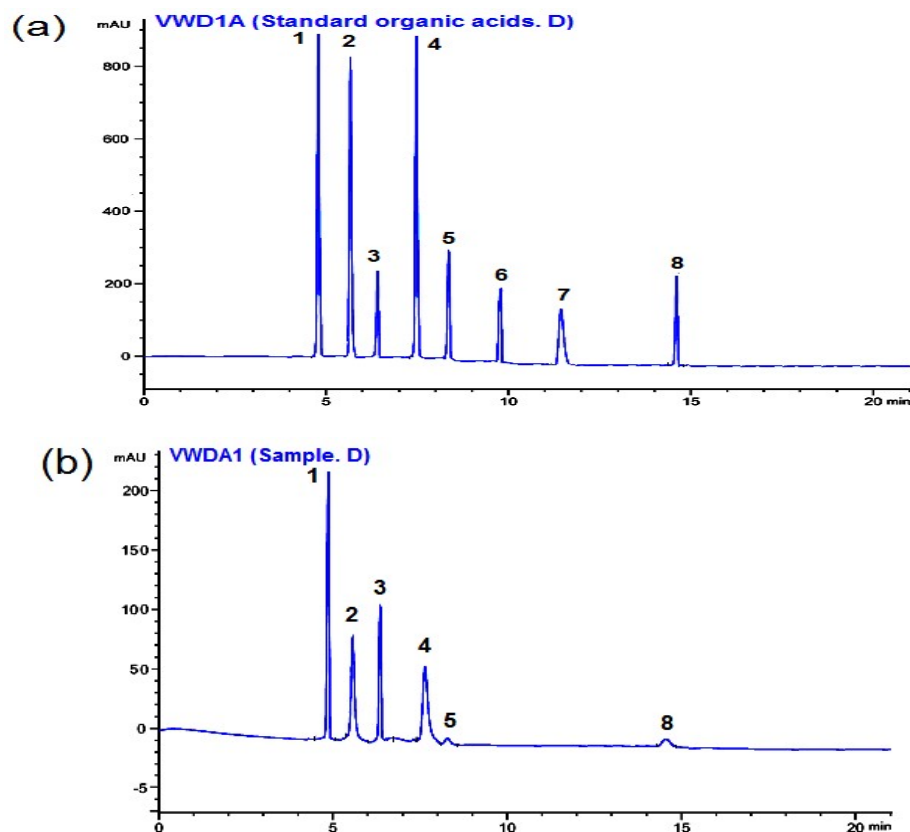


Figure 2. Liquid chromatograms of standards and samples. (a)standard organic acids; (b)sample.

3.2. Antioxidant activities

The importance of antioxidant constituents from plant materials is raising interest among scientists, food manufacturers, and consumers, as the trend of the future is moving towards functional food with specific beneficial effects [27]. The

phenolic compounds have been reported to have multiple biological effects, including antioxidant activity [28] reported that phenolic compounds in plants possess antioxidant activity, and may help protect cells against the oxidative damage caused by free radicals. The function of organic acids, in the reactive oxygen species scavenging, has also been demonstrated by other authors [29]. In this study, antioxidant activity of phenolic compounds and organic acids were evaluated using four different assays. The IC₅₀ values of different samples were summarized in Table 2.

As shown in Table 2, phenolic components (IC₅₀ = 184 ± 2 mg L⁻¹) had more scavenging power for hydroxyl radical than organic acids (IC₅₀ = 594 ± 3 mg L⁻¹) and ascorbic acid (IC₅₀ = 220 ± 3 mg L⁻¹, *p* < 0.05).

Table 2. Half-inhibition (IC₅₀) values of antioxidant activities of phenolic components and organic acids measured using hydroxyl radical-scavenging, superoxide radical-scavenging, DPPH radical scavenging and inhibition of lipid peroxidation assays.

Sample	IC ₅₀ (mg L ⁻¹) ^a			
	Hydroxyl radical-scavenging	Superoxide radical-scavenging	DPPH radical scavenging	Lipid peroxidation inhibition
Phenolic components	184 ± 2 a ^b	221 ± 2 a	103 ± 8 a	203 ± 8 a
Organic acids	594 ± 3 b	671 ± 3 b	184 ± 5 b	376 ± 8 b
Ascorbic acid ^c	220 ± 3 c	304 ± 5 c	205 ± 3 c	440 ± 3 c

^a The IC₅₀ value is expressed as mg L⁻¹ and represents the concentration of sample that is required for 50% inhibition of hydroxyl radical, superoxide radical, DPPH radical, and lipid peroxidation. A lower IC₅₀ value indicates a higher antioxidant activity. Each value in the table was obtained by calculating the average of three determinations ± standard deviation.

^b Mean values in the same column with different letters are significantly different (Tukey test, *p* < 0.05).

° Ascorbic acid was used as a positive control.

Superoxide anion plays an important role in the formation of reactive oxygen species such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induces oxidative damage in lipids, protein, and DNA [31]. In this study, the IC₅₀ value for superoxide radical scavenging ability of phenolic components ($304 \pm 5 \text{ mg L}^{-1}$, Table 2) was higher than organic acids ($671 \pm 3 \text{ mg L}^{-1}$) and ascorbic acid (IC₅₀ = $304 \pm 5 \text{ mg L}^{-1}$, $p < 0.05$).

The model of scavenging the stable DPPH radical is a widely used method of evaluating the free radical scavenging ability of natural compounds. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen-donating ability [32]. The DPPH scavenging activity of the phenolic components expressed in the term of IC₅₀ was $103 \pm 8 \text{ mg L}^{-1}$ (Table 2), with the higher DPPH scavenging power than that of organic acids ($184 \pm 5 \text{ mg L}^{-1}$) and ascorbic acid ($205 \pm 3 \text{ mg L}^{-1}$) ($p < 0.05$).

Lipid peroxidation (oxidative degradation of polyunsaturated fatty acid in the cell membranes) generates a number of degradation products, such as malondialdehyde (MDA), which is found to cause cell membrane destruction and cell damage, leading to liver injury, atherosclerosis, kidney damage, aging, and susceptibility to cancer [33]. The IC₅₀ value (mg L^{-1}) showed the lipid peroxidation inhibitory capacity of phenolic components (IC₅₀ = $203 \pm 8 \text{ mg L}^{-1}$) was stronger than organic acids (IC₅₀ = $376 \pm 8 \text{ mg L}^{-1}$) and ascorbic acid (IC₅₀ = $440 \pm 3 \text{ mg L}^{-1}$, $p < 0.05$).

There are 15 phenolic components have been detected in *C. oleifera* cake

(Figure 1, Table 1). It could be seen from the results above that these phenolic compounds in *C. oleifera* cake showed good antioxidant capacity for hydroxyl radical scavenging, superoxide radical scavenging, DPPH scavenging and lipid peroxidation inhibition. It was also reported that phenolic compounds have good radical-scavenging ability [34] and are also potent metal chelators and lipid peroxidation inhibitors [35], which was consistent with our results. In addition, different phenolic compounds may have various antioxidant capacities [36]. We determined the inhibition of lipid oxidation of the phenolic compounds and found that catechin, quercetin and ellagic acid could have different antioxidant capacities, in a linoleic acid system. Meanwhile, the free radical-scavenging activity of them was also different. Thus, the phenolic compounds that we found in *C. oleifera* cake may also have different antioxidant capacities.

Other molecules, such as organic acids, are also present in a great amount in plants, fruits or vegetables. Previous studies attributed a direct action on free radicals scavenging and other antioxidant capacity to some organic acids [37]. Besides, an effective antioxidant action of organic acids is well-known as their chelating action, which could inactivate reducing cations. In this study, oxalic, citric, acetic, malic and succinic acids are found to be major organic acids in the *C. oleifera* cake (Figure 2). It was reported that citric acid and other hydroxycarboxylic acids such as tartaric, malic and isocitric acids also have antioxidant activity, for example, hydroxycarboxylic acids act as metal scavengers, and stimulate the decomposition of hydroperoxides by pathways that do not result in the formation of free radicals [38].

These results was consistent ours. In addition, some other works confirmed that organic acids possess the biological activity both in reducing reactive oxygen species and in enhancing phenolics bio-availability [39]. Thus, it could be supposed that the organic acids in *C. oleifera* cake might also enhance the antioxidant activity of the phenolic compounds in *C. oleifera* cake.

3.3. Antimicrobial activities

Plants can possess antimicrobial natural products to protect themselves from microbial infection and deterioration [40]. There are growing interests in using natural antimicrobial compounds, especially extracted from plants, for the preservation of foods. In addition, there are more consumers who tend to question the safety of synthetic additives and would prefer natural foodstuffs [41]. Antimicrobial activities of some spices in plant leaves, flowers, stems, roots, or fruits have been reported by scientists. The phenolic compounds and organic acids are very important antimicrobial compounds in plants [42].

Salmonella spp. is one of the major foodborne pathogens, and the widespread increase in the number of cases of salmonella infection in recent years has major social and economic consequences. *Escherichia coli* strains are also associated with adverse gastrointestinal incidents [43]. The inhibition zone diameters of the phenolic components and organic acids from *C. oleifera* cake are shown in Figure 3, and the MICs are shown in Table 3. As shown in Figure 3, there is significant difference in the diameter of inhibition zone for phenolic components and organic acids (100 or 200 mg L⁻¹) compared with deionized water (negative control, $p < 0.05$). It was indicated

from Figure 3 that phenolic components and organic acids (100 or 200 mg L⁻¹) could inhibit these three bacteria, and their inhibiting capacity was sometimes better than gentamicin (positive control, 1 g L⁻¹).

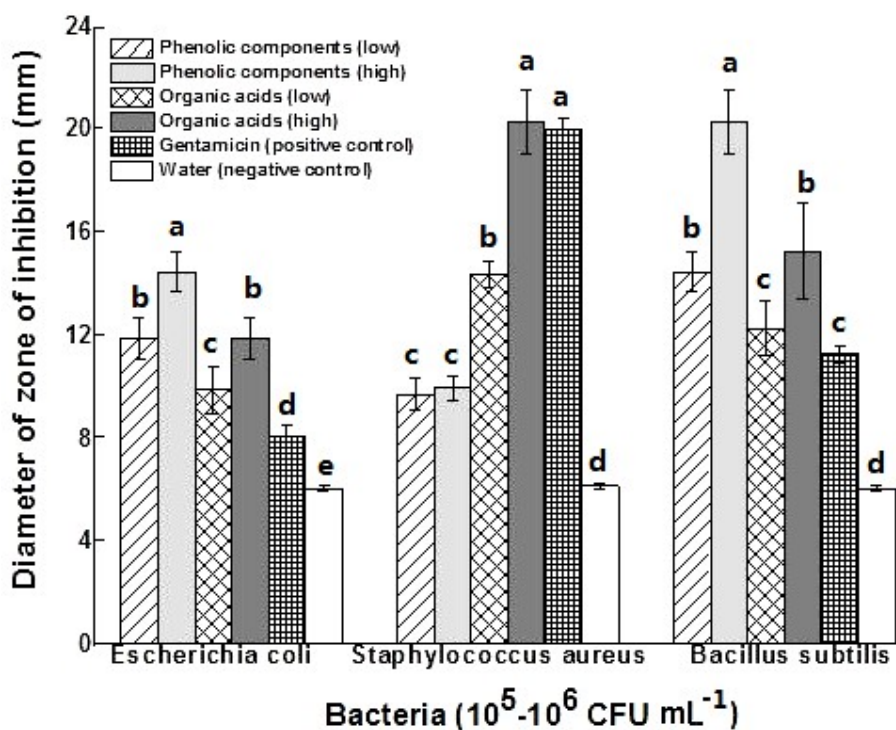


Figure 3. Diameter of zone of inhibition.

Table 3. MICs ^a of antimicrobial activity for phenolic components and organic acids from *C. oleifera* cake against bacteria and fungi.

Microorganisms	MIC (mg L ⁻¹)	
	Phenolic components	Organic acids
<i>Escherichia coli</i>	125	250
<i>Staphylococcus aureus</i>	250	63
<i>Bacillus subtilis</i>	63	125
<i>Rhizopus stolonifer</i>	500	1000
<i>Aspergillus oryzae</i>	1000	500
<i>Mucor racemosus</i>	2000	1000

^a The lowest concentration which the liquid medium remained clear after microscopic evaluation was taken as the minimum inhibitory concentration (MIC). It was recorded as the mean

concentration of triplicates.

Inhibition zones of the phenolic components and organic acids for each assay on test bacteria showed an opposite relationship with MIC (Table 3). A lower MIC indicates a stronger antimicrobial activity. As shown in Table 3, MIC was at 125, 250 and 63 mg L⁻¹ for phenolic components against bacteria *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*, while it was 250, 63 and 125 mg L⁻¹ for organic acids against the same bacteria. When against fungi, *Rhizopus stolonifer* was affected by the phenolic components and organic acids as the MICs were 500 and 1000 mg L⁻¹. In addition, phenolic components and organic acids could inhibit *Aspergillus oryzae* with MIC level of 1000 and 500 mg L⁻¹. Furthermore, MIC was 2000 and 1000 mg L⁻¹ for phenolic components and organic acids, respectively against fungi *Mucor racemosus*. These results suggested that the phenolic components and organic acids had lower inhibitory activity against fungi than bacteria tested.

It is important to find food additives and preservatives for preventing food deterioration and extending the shelf life of perishable food ingredients. Many phenolic compounds are known to possess antimicrobial properties. In this work, there are 15 phenolic components found in *C. oleifera* cake (Figure 1, Table 1). It was also reported that ellagitannins could be one of the main phenolic components causing the inhibition against *Salmonella* [35], which was also one of our detected phenolic components in *C. oleifera* cake. In addition, different bacterial species exhibit different sensitivities towards phenolics, and the variations may come from the cell surface structures between Gram-negative and Gram-positive bacteria. In particular,

the outer membrane of Gram-negative bacteria could be a preventive barrier against hydrophobic compounds [44]. However, sometimes phenolics also showed activity against Gram-negative bacteria [35], which was consistent with our results. Helander et al. (1998) found some phenolic compounds could inhibit *E. coli* and *Salmonella*, and the inhibitory mechanism involved the disruptive action of these compounds on the outer membrane [44]. The antimicrobial activity of phenolics may also be due to the glycosides in phenolics [45]. Thus, the antimicrobial activities of the phenolic compounds from *C. oleifera* cake may also result from their glycosides.

Specific organic acids have also been advised to control microbial contamination and dissemination of foodborne pathogens in food production and processing [46]. In this study, oxalic, citric, acetic, malic and succinic acids are found to be major organic acids in the *C. oleifera* cake (Figure 2). The antibacterial mechanisms for organic acids may depend on physiological status of the organism or the physicochemical characteristics of the external environment [47]. Other toxicity mechanisms have also been proposed that organic acids may possess membrane uncoupling capacity [48]. Furthermore, undissociated acids are considered to be the active form, because they can freely diffuse through the cell membrane and enter the cytoplasm [49]. However, the toxic mechanisms of organic acids (either directly or indirectly) for foodborne pathogens have not been fully elucidated. This is in part due to complexities involved in organic acids, and factors such as chain length, side chain composition, and hydrophobicity could affect the antimicrobial activity of organic acids [50]. This could be investigated in future study.

4. Conclusions

Phenolic components and organic acids were obtained and identified from *C. oleifera* cake. The phenolic components and organic acids both showed good antioxidant capacity and exhibited significant inhibitory activity against bacteria *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, and fungi *Mucor racemosus*, *Aspergillus oryzae*, *Rhizopus stolonifer*. These results could provide useful information for the future use of phenolic components and organic acids from *C. oleifera* cake.

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