

A COMPARATIVE STUDY OF ANTIBACTERIAL, ANTIOXIDANT ACTIVITY AND TOTAL CONTENT OF PHENOLIC COMPOUNDS FROM A COMBINATION OF CLOVE AND CINNAMON ESSENTIAL OILS

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ABSTRACT

Objective: This study aimed to identify the right combination of Clove Flower Essential Oil (CFEO) and Cinnamon Essential Oil (CEO) that has the potential to be used as a medicine for diabetic wounds.

Methods: Antibacterial activity against *Staphylococcus aureus* was observed using the paper disc method. Antioxidant activity was examined the DPPH (2,2-Diphenyl-1-Picrylhydrazyl acid) method. Phenol content was tested using the Folin Ciocalteu method.

Results: The results showed that the antibacterial activity of CFEO was lower than that of CEO in a single form. The combination of CFEO: CEO was made with varied concentrations of 1.25; 2.5; and 5%. Each concentration has the following ratios (1:1), (1:2), (1:3), (2:1), and (3:1). The highest antibacterial activity was found at a concentration of 5% combination (1:3) with an average inhibition zone diameter of 20.61 ± 1.07 . The minimum inhibitory concentrations of CFEO, CEO, and the combination of CFEO: CEO (1:3) against *Staphylococcus aureus* bacteria were 0.078%, 0.046%, and (0.0195:0.0935%). The antioxidant activity of the CFEO: CEO (3:1) combination showed the highest antioxidant activity with an IC₅₀ of 42,706 ppm. Single CFEO showed had a higher phenol content of 548.065 mgGAE/g compared to single CEO. The combination CFEO: CEO (3:1) showed the highest phenol content of 262,473 mgGAE/g compared to combination (1:1) and (1:3).

Conclusion: Based on the results, the combination of CFEO and CEO (3:1) has the highest antibacterial activity, antioxidant and phenol content. Therefore, it can be proposed as an active ingredient for diabetic wound preparation.

Keywords: Combination, *Syzygium aromaticum*, *Cinnamomum burmanii*, Antibacterial, Antioxidant

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INTRODUCTION

Diabetic gangrene is a pavement led by infection due to the appearance of bacteria or wound inflammation [1]. The incidence rate in Indonesia is around 15%, with an amputation rate of 30% [2]. The dominant bacteria that infect wounds in diabetic gangrene inpatients are *Pseudomonas*, *Staphylococcus*, *Klebsiella*, *Bacillus*, and *E. coli* [3]. In chronic wounds such as diabetic gangrene, the wound-healing process can be hampered or even fail due to the presence of a biofilm layer of microbial colonies [4] and the presence of resistance to antimicrobials worsens the situation in diabetic wounds.

An alternative solution can be developed by using natural ingredients which have antibacterial potentials and have been proven to help wound healing by accelerating cell regeneration. They are relatively safer compared to synthetic drugs [5]. These natural ingredients have also been a focus for developing medicines from natural ingredients. Natural resources in Indonesia that have been developed as antibacterials are essential oils, including clove flower and Cinnamon Essential Oils (CEO), which can help in cell regeneration and wound healing [7-9].

Several essential oils have been showed antimicrobial and antioxidant activity. One of the most significant components of Clove Flower Essential Oil (CFEO) is eugenol [7]. CFEO contains eugenol acetate, β -Caryophyllene [8], nitrogen, sulfur, halogens, phenols, aldehydes, ketones, and anthraquinones [9]. The most significant component of cinnamon (*Cinnamomum burmanii*) essential oil is cinnamaldehyde, which has been reported to have anti-biofilm activity [10]. CEO showed contains some major compounds including p-coumaric, ferulic acids, linalool, eugenyl acetate, and cinnamyl acetate [11]. The results showed that the eugenol compound had biofilm inhibitory activity against MRSA strains [12]. Cinnamaldehyde also inhibit *Pseudomonas aeruginosa* biofilms [13]. Trans-CNMA and 4-nitroCNMA also potentially inhibit biofilms from *Escherichia coli* and *S. aureus* [14]. The

antioxidant activity of clove and CEO has been widely reported, and phenolic compounds in essential oils play an important role in antioxidant activity [15]. Essential oils' antioxidant activity aids healing by reducing tissue damage and preserving oxygen and blood supply at the affected site [16].

The large number of chemical contents in the essential oils of clove and cinnamon flowers allows the existing compounds to synergize with each other and inhibit the activity of the compounds found in each of these plants. Therefore, it is essential to prove the relationship between the two plants by scientifically proving their efficacy. Based on the above problems, this study aims to test the antibacterial activity, antioxidants, and phenolic content of each clove and CEO and compare it with the combination of clove and CEO in the ratio (1:1), (1:3), (3:1).

MATERIALS AND METHODS

Material

The oil sample was collected from Center for Essential Oil Studies of the Indonesian Islamic University of Yogyakarta. CEO obtained from PT. Lansida Herbal Technology. The instruments used in this research were Iwaki Pyrex glass-ware, GC-MS instrument, UV-Vis Spectrophotometer (UV, 1800, Shimadzu, Japan), biological safety cabinet (Monmouth), Incubator (Binder), and autoclave (Shenam)

Gas-Mass Spectrometry (GC-MS) analysis

GC-MS analysis of essential oils was carried out on the ISQD1702517 operating system, HP-5MS UI capillary column (30 x 0.25 mm, film thickness 0.25 m) with temperature from 50 °C to 200 °C at a speed of 6 °C/min and from 200 °C to 280 °C, the speed was 30 °C/min. The process was carried out for 10 min at a temperature of 280 °C with helium as the carrier gas at a flow rate of 1 ml/min. The identification process using the GC-MS tool produced several

bioactive compounds, which can be seen from the peak of the chromatogram as identification of chromatography and mass spectrometry (MS) data seen from the mass spectrum with the molecular weight of each bioactive compound [17].

Total phenolic content assay

Total Phenolic Content (TPC) assay of CFEO of CEO was using the Folin-Ciocalteu method [18]. Singly or combined of CFEO and CEO (1:1, 1:3, 3:1) were redissolved with ethanol, 1 ml solution and 5 ml of 7.5% folin-ciocalteu LP in water were placed in containers. Incubated for 8 min, added 4 ml of 1% NaOH, and then of incubation for 1 min. Absorption was measured at 713.5 nm, and the percentage of TPC as gallic acid was calculated using a specific formula followed the previous method [18].

Antioxidant activity assay

Antioxidant activity assay of singly or combined of CFEO and CEO (1:1, 1:3, 3:1) was using the 0.1 mmol of DPPH. The free-radical was read at 517 nm using UV spectrophotometer (1800, Japan). Gallic acid was using for positive control. Antioxidant Activity was calculated using presented as mean±SD followed the previous method.

Combination index antioxidant activity assay

Combination index of antioxidant interaction of CFEO and CEO was analyzed based on the 50% free radical inhibition concentration (IC₅₀). Classical isobolograms of combination index (CI) equations were used to analyze the data calculated using previous method [20, 22].

Antibacterial activity assay

Antibacterial activity assay was using the disk diffusion method using 6 mm paper disc on *Staphylococcus aureus* ATCC 25923 strain [23]. The strain obtained from Yogyakarta Health Laboratory (LABKES). Singly doses (1.25%, 2.5%, and 5% in acetone) or

combined (1:1, 1:2, 1:3, 2:1, 3:1) of CFEO and CEO were using for assay. Mueller-Hinton agar (MHA) was using for medium and Gentamicin sulfate for positive control. The inhibition growth zone was measuring followed the previous method [23].

Minimum inhibitory concentration (MIC) assay

MIC assay was determined using the microdilution method on *Staphylococcus aureus* ATCC 25923 strain using BHI medium. 50 µl of singly or combined of CFEO and CEO and 50 µl BHI was added to 96-well plate. Multilevel dilutions were conducted for get concentration variations in a row from high to low of CFEO and CEO. 100 µl of bacterial suspension (5 x 10⁵ CFU/ml) was added to each 96-well. Gentamicin sulfate was using for positive control. The MIC was interpreted by comparing the turbidity to the bacterial control [19].

Minimum bactericidal concentration (MBC) assay

The MBC assay of singly or combined of CFEO and CEO was measuring followed the previous method [25]. 100 µl of culture from each microbroth assay well was subcultured on MHA plates anaerobically for 24 h at 37 °C. The lowest concentration that does not show bacterial growth is considered MBC [25].

RESULTS AND DISCUSSION

GC-MS analysis results

The chemical content of CFEO and CEO by GC-MS analysis can be seen in fig. 1A and 1B, respectively.

TPC results

The results of TPC showed of singly or combined of CFEO and CEO is given in tables 1. Combined of CFEO and CEO showed highest phenolic content, which ratio of 3:1 showed highest phenolic content.

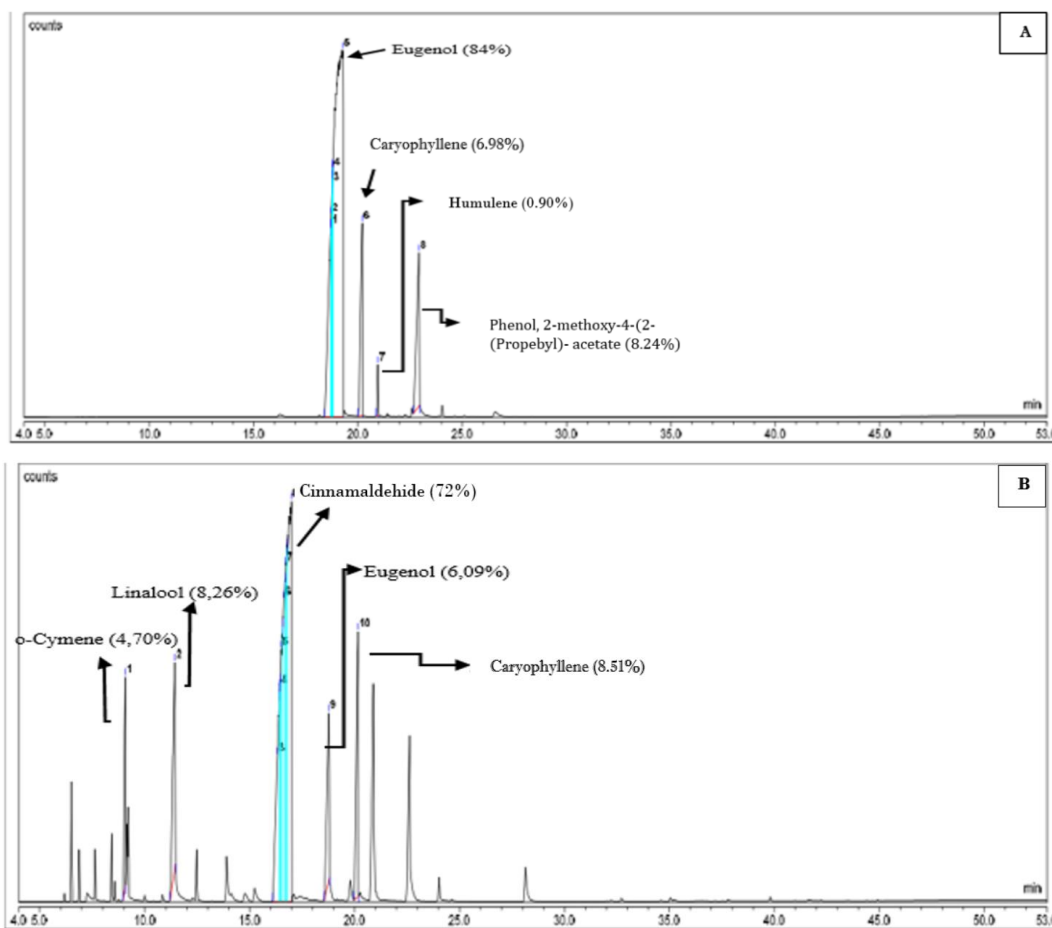


Fig. 1: Chromatogram of CFEO (A) and CEO (B)

Table 1: Total phenolic content of singly or combined of CFEO^a and CEO^b

No	Sample	TPC (mg GAE/g of extract)
1.	CFEO	548.0654
2.	CEO	92.2296
3.	CFEO+CEO (1:1)	222.848
4.	CFEO+CEO (1:3)	153.0311
5.	CFEO+CEO (3:1)	262.4738

Clove Flower Essential Oil (CFEO), Cinnamon Essential Oils (CEO)

Table 2: Antioxidant index (CI) values singly or combined of CFEO^a and CEO^b by DPPH. Result are mean±of triplicate experiments, *p<0.05 compared to the IC₅₀ values of individual components

Treatment	IC ₅₀ (µg/ml)	CI=(D) ₁ /(Dx) ₁	CI=(D) ₂ /(Dx) ₂	CI=CI ₁ +CI ₂	Remark
Galic Acid	11.118±0.85	-	-	-	-
CEO	491.393±7.16	-	-	-	-
CFEO	36.005±0.21	-	-	-	-
CFEO+CEO (1:1)	91.200±1.71*	0.18	2.53	2.71	ANT
CFEO+CEO (1:3)	71.701±0.29*	0.14	1.99	2.13	ANT
CFEO+CEO (3:1)	42.706±1.42*	0.08	1.18	1.27	ANT

Clove Flower Essential Oil (CFEO), Cinnamon Essential Oils (CEO)

Table 3: Diameter of inhibition zone of singly or combined of CFEO^a and CEO^b

Sample	Diameter of inhibition zone (mm)		
	1.25%	2.5%	5%
Aceton (-)	0.00±0.00	0.00±0.00	0.00±0.00
Gentamicin (+)	18.37±0.25	18.33±0.25	18.37±0.25
CFEO	0.00±0.00	3.19±0.07	5.87±0.31
CEO	7.29±0.52	10.71±0.63	17.03±0.94
CFEO+CEO (1:1)	3.05±0.37	6.25±0.87	11.29±0.22
CFEO+CEO (1:2)	5.06±0.46	11.04±0.19	17.08±0.97
CFEO+CEO (1:3)	6.37±0.50	16.91±1.10	20.61±1.07
CFEO+CEO (2:1)	2.92±0.48	5.36±0.76	11.12±0.33
CFEO+CEO (3:1)	1.90±0.46	5.72±1.51	9.82±1.46

Clove flower essential oil (CFEO), Cinnamon essential oils (CEO)

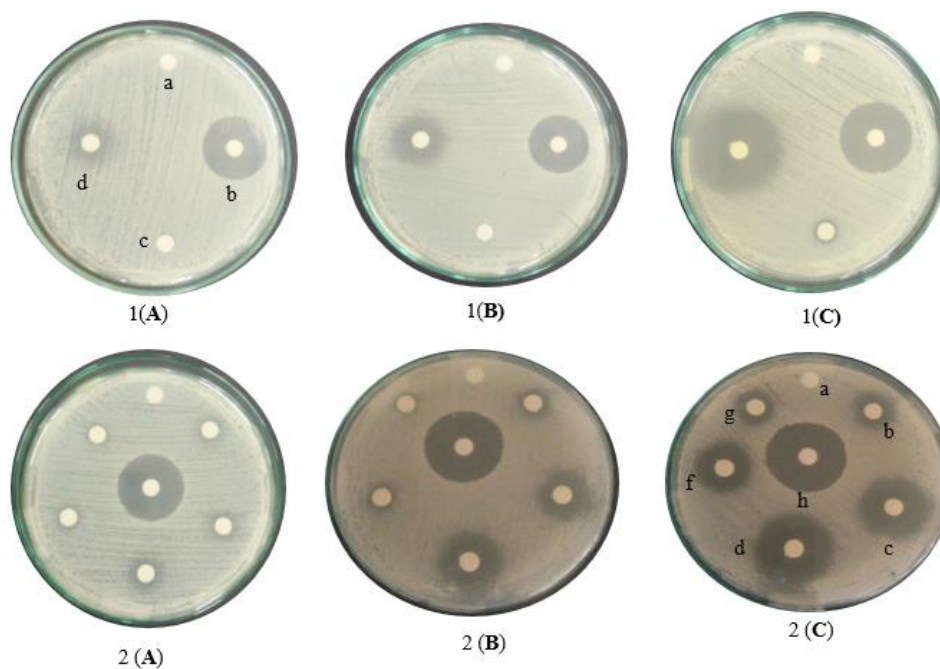


Fig. 2: Antibacterial activity of disk diffusion shows the activity of 1(A), which is a single essential oil with a concentration of 1.25%, 1(B) 2.5% concentration, and 1(C) 5% concentration. a= control (-); b=control (+); c=CFEO; d=CEO. 2(A)= Combination CFEO+CEO concentration 1.25%; 2(B)=Combination CFEO+CEO concentration 2.5%; and 2(C)= CFEO+CEO combination with 5% concentration. a=control (-); b= CFEO+CEO (1:1); c= CFEO+CEO (1:2); d= CFEO+CEO (1:3); f= CFEO+CEO (2:1); g= CFEO+CEO (3:1); h= control (+)

Table 4: MIC^a and MBC^b values of essential oils against *S. aureus* ATCC 25923

Bacterial	CFEO ^c		CEO ^d		CFEO ^c +CEO ^d	
	MIC ^a (mg/ml)	MBC ^b (mg/ml)	MIC ^a (mg/ml)	MBC ^b (mg/ml)	MIC ^a (mg/ml)	MBC ^b (mg/ml)
Staphylococcus aureus ATCC 25923	0.078	0.078	0.0467	0.0467	0.0195	0.0935

^aMinimum Inhibitory Concentration (MIC), ^bMinimum Bactericidal Concentration (MBC), ^cClove Flower Essential Oil (CFEO), ^dCinnamon Essential Oils (CEO)

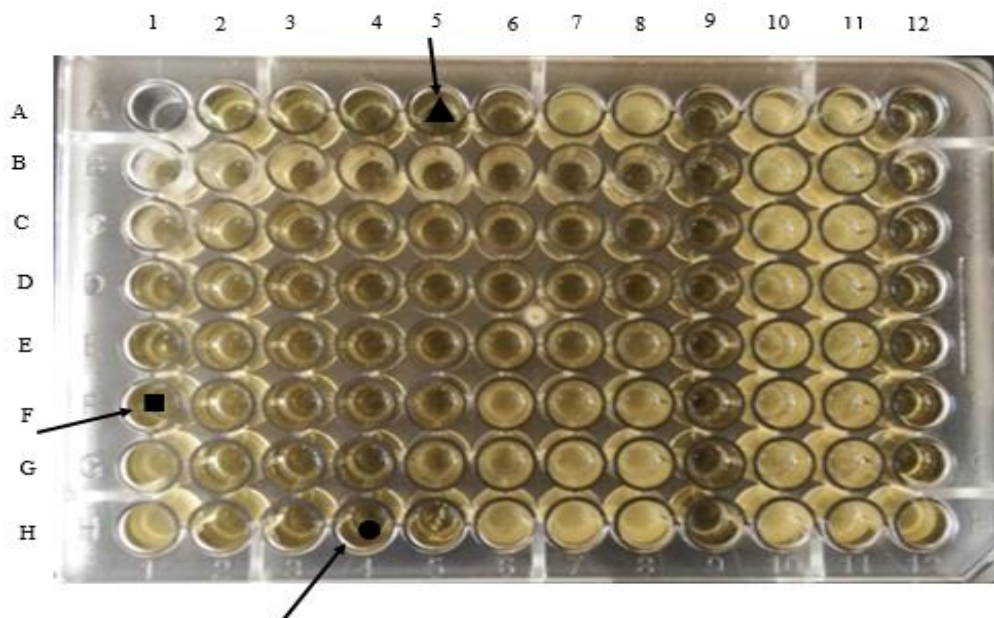


Fig. 7: Determination of MIC of CFEO and CEO alone and combined against *S. aureus* ATCC 25923 after incubation. Numbers 1-8 are single CEOs with a concentration range of 0.375%-0.0058%. The letters A-H contain a single CFEO with a concentration range of 1.25%-0.0195%. Well number 5 in column A with a ▲ sign is the MIC and MBC of a single CEO with a concentration of 0.0467%. Well number 1 column letter F with a ■ is the MIC and MBC of a single CFEO with a concentration of 0.078%. Then well number 4 column H with a ● sign is the MIC and MBC of the combination of CFEO and CEO, namely 0.0195% and 0.0935%. Well, number 9 is media control (BHI), 10 is solvent control (acetone), 11 is bacterial control, and 12 is positive control

Antioxidant activity results

The IC₅₀ values results of the antioxidant activity of single and combination of CFEO and CEO showed on table 2.

The IC₅₀ value is the concentration of the test sample that is able to reduce 50% of DPPH as free radicals and is used as a parameter to determine the antioxidant activity of the test sample. Table 2, showed that CFEO very strong antioxidant. Combined of CFEO+CEO (3:1) showed strong antioxidant [26].

Antibacterial activity results

Antibacterial activity resulted using the disk diffusion method and the microdilution methods were showed in table 3 with fig. 2 and table 4 with fig. 3, respectively. The zone of inhibition was calculated in determining the disk diffusion method and IC₅₀ was calculated in determining the disk microdilution method.

DISCUSSION

According to GC-MS results (fig. 2), compound components of CEO include o-Cymene, Linalool, Cinnamaldehyde (72% predominant), eugenol, and caryophyllene [27]. Other essential components such as eugenol, eugenol acetate, and phenol are compounds that play a role in antioxidant activity. Cinnamaldehyde inhibits and kills *S. aureus* and *S. epidermidis* (MIC: 0.25-0.50 mg/ml) and shows antifungal properties [10]. It targets proteins like thymidylate kinase impacting bacterial DNA biosynthesis. The cinnamaldehyde compound has also been reported to have antifungal activity [28]. Cinnamaldehyde disrupts microbial cell membranes, affecting

permeability and leading to cell damage. Terpenes in essential oils exhibit bactericidal effects by disrupting cell wall permeability [28]. *Thymidylate kinase (TMK)* is an essential enzyme in bacterial DNA biosynthesis and is a new target for developing antibacterial agents [29]. Cinnamaldehyde disrupts microbial cell membranes, affecting permeability and leading to cell damage [14, 13]. Terpenes in essential oils exhibit bactericidal effects by disrupting cell wall permeability [30]. Essential oil components with phenol and alcohol groups can be toxic to microbes. CFEO contains eugenol (84%) and caryophyllene (6.98%); the same results also show that the main compounds in the essential oil cinnamon are eugenol [31]. Eugenol, with antibacterial properties, alters membrane structure, affects ion transport, and inhibits bacterial enzymes, influencing bacterial growth [32].

According on table 3, CEO exhibits superior antibacterial activity compared to CFEO due to its higher concentration of active compounds. Concentrated essential oils can damage to cell membranes, inhibit ATPase, and form biofilms [33]. The main compound in CFEO, also found in CEO, has antibacterial activity by penetrating cell membranes and destroying cytoplasmic membranes. Abnormalities in the cell membrane and enzymes found in the cell membrane cause changes in protein conformation, inhibiting ATPase activity [33].

Both oils contain eugenol, the main compound component in CFEO (83.89%), with antibacterial properties that disrupt cell membranes and enzymes [32]. Eugenol and cinnamaldehyde show potential as antimalarial drugs due to isothiocyanate content [34]. Cinnamaldehyde displays stronger antibacterial activity than

eugenol, evident in larger zones of inhibition with CEO. A combination of both oils (CFEO: CEO-1:3) demonstrates high antibacterial activity. Eugenol and cinnamaldehyde can affect bacterial proteins, inhibiting ATPase and disrupting ATP-dependent processes. The combination of 2.5% and 5% CFEO and CEO, individually and combined, shows antibacterial activity.

In the antioxidant activity test, single-form CFEO displays potent antioxidant activity (IC₅₀: 36,005±0.21 µg/ml), indicating its strong antioxidant properties because the lower IC₅₀ value showed better antioxidant activity [35]. The 3:1 ratio combination of CFEO and CEO exhibits the highest antioxidant activity compared to other ratios. CFEO's high phenolic content, attributed to more hydroxyl groups, enhances radical scavenging activity [36]. Phenolic compounds safeguard the body against free radicals due to their antioxidant capacities [37].

CONCLUSION

Based on the results of research that has been carried out, it shows that CFEO and CEO in single or combined form have antibacterial, antioxidant activity and contain phenolic compounds. The antibacterial activity of CEO in single form at a concentration of 5% showed the highest activity with an average diameter of the inhibition zone of 17.03 mm±0.94. The combination of both CFEO: CEO with a ratio of (1:3) at a concentration of 5% showed the highest antibacterial activity with an inhibition zone diameter of 20.61 mm±1.07. The antioxidant activity of a single CFEO showed the highest activity with an IC₅₀ of 36,005 µg/ml±0.21. The highest phenolic content was found in a single CFEO at 548.0654 mg GAE/g of extract. The combination of the two shows the highest antioxidant activity seen in the ratio CFEO: CEO (3:1) with an IC₅₀ of 42.706 µg/ml±1.42 with the highest phenolic content of 262.4738 mg GAE/g of extract. The results obtained can certainly be used as a reference for further development.

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AUTHORS CONTRIBUTIONS

All authors have contributed equally as Researchers and Authors. Meta Safitri, Nanik Sulistyani, and Iis Wahyuningsih, as Correspondence author. Diana Sylvia, and Arini Aprilliani as Academic Editor.

CONFLICT OF INTERESTS

Declared none

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