Evaluation of The Phytochemical Composition and Antimicrobial Properties of *Centella asiatica* Leaf Meal Extract as a Feed Additive Candidate for Poultry

ABSTRACT

The objective of this study was to assess the phytochemical composition of *Centella asiatica* leaf meal by analyzing the type of solvent used and the duration of the extraction process. In addition, it assessed the effectiveness of *Centella asiatica* leaf meal extract as a potential antibacterial agent when added to poultry feed. The study was conducted in two phases. The initial phase employed a fully randomized design featuring a 2 x 5 factorial design and three replications. Factor X denoted the solvent used, which included ordinary distilled water and distilled water heated to 100^{o}C. Factor Y signified the duration of the extraction process, ranging from 15 to 75 minutes in 15-minute increments. In the second stage the in vitro antibacterial test was carried out using the most effective extract of *Centella asiatica* leaf meal as determined in the first stage. The parameters consist of: total phenolic content, total flavonoid content, total tannin content, total antioxidant activity, Inhibition zone of *Escherichia coli* and *Salmonella sp*. The results showed a highly significant interaction ($p<0.05$) between the solvent type and extraction duration on the total phenolic content, flavonoid content, and antioxidant activity. However, the overall tannin concentration remained the same regardless of the solvent used or the duration of the extraction process. Moreover, the extract of *Centella asiatica* leaf meal extract with a concentration of 100% had a greater inhibition against *Escherichia coli* and *Salmonella sp.* bacteria compared to other concentrations. It was concluded that the best extraction method to produce phytochemical compounds from *Centella asiatica* leaf meal extract is heated distilled water solvent and 75 minutes

of extraction time. Furthermore, *Centella asiatica* leaf meal extract also has potential as an antibacterial agent candidate for Poultry.

Keywords: antibacterial, extraction, herbs, phytochemical, solvents

1. Introduction

Antibiotic Growth Promoters (AGPs) is an antibiotic compound added to animal feed to improve growth and feed efficiency and minimize pathogenic bacteria that can harm the digestive tract. In recent years, several countries have regulated and banned the use of AGPs, although subtherapeutic doses can be given to livestock, especially poultry. In Indonesia, the ban on the use of AGPs began in 2018 due to concerns that excessive use could lead to bacterial resistance and negatively impact human health. On the other hand, banning AGPs may reduce livestock performance and health.

In recent years, research has focused on identifying viable alternatives to AGPs in poultry, exploring diverse options, including herbal plants (1,2). Indonesia, as a tropical country, has a variety of plants (leaves, fruits, stems, and roots) that are rich in phytochemical compounds with anti-bacterial, antioxidant, anti-inflammatory, and anti-fungal properties. *Centella asiatica* leaf emerges as a promising medicinal plant with significant potential as an AGPs substitute among these botanical resources. These leaves contain various phytochemical compounds, including asiaticoside, asiatic acid, madecassic acid, madecassoside (3), alkaloids, flavonoids, phenolics, saponins, and tannins (4). *Centella asiatica* leaves are commonly fed in the form of meal to poultry (5) with positive effects. However, there is limited information on the use of *Centella aciatica* leaf extracts as a poultry feed additive. Hence, this study was undertaken as a preliminary

investigation to assess the phytochemical composition of Centella asiatica leaf extract, considering the solvent type and extraction duration. Additionally, the study aimed to examine its effectiveness as a potential antibacterial agent for poultry.

2. Material and Methods

2.1. Materials

Centella asiatica leaf meal was obtained from the Centre for Research and Development of Traditional Medicinal Plants, Ministry of Health of the Republic of Indonesia.

2.2. Extract preparation

The procedure of extracting the meal from *Centella asiatica* leaves was mentioned in the study conducted by Rusli et al. (2). *Centella asiatica* meal (10 g) mixed with regular distilled water and heated distilled water at 100ºC (100 ml). In addition, the extraction procedure was conducted using a hotplate (Thermo Scientific, USA) at a temperature of 50°C for 15, 30, 45, 60, and 75 minutes. Consequently, the obtained Centella asiatica leaf meal was allowed to cool down to the ambient temperature and then passed through a filter paper (Whatman No. 1, Cytiva, China). The extracted *Centella asiatica* leaf meal was stored in a refrigerator for further analysis.

2.3. Experimental design

The first stage used a complete randomized design with a 2 x 5 factorial design with three replications. Factor X (first factor) represented the type of solvent used, which included ordinary distilled water and heated distilled water. The initial phase employed a fully randomized design incorporating a 2 x 5 factorial design with three replications.

Factor X, the first factor, denoted the solvent type employed, encompassing regular and heated distilled water. Factor Y (second factor) represented the length of extraction time which ranged from 15, 30, 45, 60, and 75 minutes. The second stage consisted of in vitro antibacterial tests using the best *C. asiatica* leaf meal extract from the first stage. The study analyzed *C. asiatica* leaf meal extracts at the concentrations of 50%, 75%, and 100% (undiluted) and antibiotics (tetracycline) as controls.

2.4. Parameter observed

There were parameters observed in this study, namely; total phenolics content, total flavonoid content, total tannin content, total antioxidant activity, anti-bacterial testing of *Escherichia coli* and *Salmonella sp*. Each parameter analyzed used the following procedures.

2.4.1. Total phenolics content

In the examination of total phenolic content (TPC) using the Folin-Ciocalteu method (6), a 20 µl sample extract was combined with 120 µl of a 10% (v/v) folin-ciocalteu reagent in a microplate. Subsequently, it was let to remain undisturbed for a duration of 5 minutes at the ambient temperature. Following incubation, 80 µl of a 7.5% Na2CO3 solution was introduced into the mixture. The mixture was subsequently left undisturbed for 30 minutes at room temperature in the absence of light. Subsequently, the spectrophotometer (Shimadzu UV-1800, Japan) was used to detect the absorbance of the sample at a wavelength of 725 nm.

2.4.2. Total flavonoid content

The procedure for analyzing the total flavonoid content (TFC) was adapted from Chang et al. (7). The sample was diluted using methanol with a 1:10 g/ml ratio. Next, 1 milliliter of the sample was combined with 3 milliliters of methanol, 0.2 milliliters of 2%

aluminum chloride, 0.2 milliliters of 1 M glacial acetic acid, and 5.6 milliliters of distilled water. Subsequently, the combination was left undisturbed for 30 minutes, after which the absorbance was quantified using a Shimadzu UV-1800 spectrophotometer from Japan, specifically at a wavelength of 370 nm. Quercetin was used to construct a calibration curve.

2.4.3. Total tannin content

The total tannin content (TTC) analysis is conducted according to Kuentzel (8), which involves weighing a 1 g sample and subsequently dissolving it in 100 ml of distilled water. It was recently subjected to ultrasonic extraction for 15 minutes at ambient temperature. The precipitate was isolated using a centrifuge operating at 3000 revolutions per minute for 25 minutes, after which the solution was collected. A solution of 1 milliliter was mixed with 10 milliliters of distilled water to create a diluted solution. T specimen was subsequently examined at a wavelength of 278 nm utilizing a Shimadzu UV-1800 spectrophotometer manufactured in Japan.

2.4.4. Total antioxidant activity

Following the method described by Melia et al. (9), Total antioxidant activity (TAA) analysis involved combining 375 µl of 99% ethanol with 125 µl of a 0.02% DPPH solution in ethanol. Then, 500 µl of the sample was added in various quantities (25 µg/ml, 50 µg/ml, 75 µg/ml, 100 µg/ml, and 125 µg/ml) to serve as a source of free radicals. The solution was left at room temperature for 30 minutes, after which the absorbance was determined at a wavelength of 517 nm using a Shimadzu UV VIS-1800 spectrophotometer from Japan.

2.4.5. Anti-bacterial testing of *Escherichia coli* and *Salmonella sp*.

This stage used *Centella asiatica* leaf meal extract with the best yields obtained from the first stage. Anti-bacterial test was carried out according to Yuniza & Yuherman (10) by inoculating *Escherichia coli* (10^6 CFU/ml) and *Salmonella sp.* (10^6 CFU/ml) into nutrient agar (NA) and incubated overnight. Then, four holes with a diameter of 5 mm were filled with 20 µl of *Centella asiatica* leaf meal extract and antibiotics. The extract was then diluted with different concentrations (50%, 75%, and 100%) and incubated for 24 hours at 37°C with tetracycline antibiotic (0.002 g/ml) as a control. Then, it was incubated for 24 hours at 37°C. Then, the diameter of the clear zone formed was measured using ImageJ-ink software.

2.5. Data analysis

The first stage data were analyzed using analysis of variance. If significantly different results were found, Duncan's multiple range test was performed. Meanwhile, the second stage data were presented descriptively.

3. Results

3.1. Total phenolic content (TPC)

There was a very significantly different interaction ($P<0.01$) between the type of solvent and the length of extraction time for *Centella Asiatica leaf meal* (Table 1). The results showed that the highest TPC was found in the X2Y5 treatment (distilled water solvent heated at 100°C for 75 minutes).

3.2. Total flavonoid content (TFC)

The interaction of solvent type and extraction time significantly ($p<0.01$) affected the TFC of leaf Centella *asiatica* leaf meal extract (Table 2). The best TFC of *Centella asiatica* leaf meal extract was obtained in a solvent with heated distilled water and an extraction time of 60 minutes (X2Y4).

3.3. Total tannin content (TTC)

This study showed that there was no significant interaction ($P > 0.05$) between solvent type and extraction time on the TTC of *Centella asiatica* leaf meal extract (Table 3). The TTC value of *Centella asiatica* leaf meal extract obtained in this study ranged from 2.03 to 3.48%.

3..4. Total antioxidant activity (TAA)

A significant correlation was seen between the type of solvent and the duration of extraction ($P<0.01$) about the total antioxidant activity (TAA) of the Centella Asiatica leaf meal extract (Table 4). The treatment, including hot distilled water as a solvent (X2Y5), resulted in the greatest Total Antioxidant Activity (TAA) of 50.38% compared to the other treatments.

3.5. Antibacterial activity

The anti-bacterial activity of *Centella asiatica* leaf meal extract with different concentrations against *E. coli* and *Salmonella sp*. bacteria is illustrated in Figure 1. The results proved that *Centella asiatica* leaf meal extract at different concentrations has antibacterial properties.

4. Discussion

Indeed, TPC is influenced by factors such as solvent type and extraction time (11). The high TPC of *Centella asiatica* leaf in the X2Y5 treatment is because time is very important in solvent extraction for phenolic compounds, where compounds can be regulated by the equilibrium concentration for phenolic compounds achieved before the appropriate reduction. Similar to the previous study by Thoo et al. (11) reported that the optimal time to obtain phenolic compounds in *M. citrifolia* is 80 minutes; more than that time can reduce the yield of phenolic compounds. In addition to time, temperature also affected the flavonoid compounds obtained. X2 group with a water solvent preheated to a temperature of 100°C increased the yield of TPC. Increasing temperature favors the release of polyphenols bound in the sample by the degradation of cellular constituents of plant cells that cause cell membrane permeability (12). Kim et al. (3) stated that the higher the temperature, the higher the content of phytochemical compounds (*asiaticoside* and *asiatic* acid) obtained in *Centella asiatica* leaf.

A previous study reported that phenolic compounds can be used as natural feed additives in laying poultry (chickens, ducks, and quail), which improve performance and egg quality, extend egg storage time, and are antioxidants (13). Meanwhile, in broilers, phenolic compounds act as growth promoters. Their antioxidant and anti-inflammatory qualities are responsible for this effect. Phenolic chemicals stimulate growth by enhancing digestive enzyme secretion, reducing harmful bacteria in the digestive tract, or influencing the gut structure (14).

The difference in optimum time between TPC and TFC is thought to be caused by differences in the degree of phenolic polymerization, phenolic solubility, and phenolic interaction with other constituents (15). Similar results were also reported by Thoo et al.

(11) that there were differences in the optimum extraction time to obtain TPC and TFC of *M. citrifolia* extract. *Citrifolia.*

Flavonoids have a beneficial impact on various bodily systems in broilers, including the gastrointestinal tract, cardiovascular system, immunological system, lipid metabolism, insulin release, and antioxidant activity (16). Flavonoids in laying hens can alter the composition of fatty acids and decrease the amounts of cholesterol in eggs, enhancing their nutritional quality (17). Prihambodo et al. (16) discovered that flavonoids had a positive effect on the digestive tracts of chickens. Flavonoids contain antibacterial and antioxidant abilities which greatly enhance the health and function of the small intestine, particularly in nutrient absorption. These favorable benefits include increased villous density, crypt depth, and height. Higher villus enhances intestinal surface area and nutrient absorption, while deeper crypts can rapidly regenerate intestinal villus in response to inflammation caused by pathogenic bacteria.

Different results were reported by Rusli et al. (2) that total tannin content is influenced by solvent and extraction time; ordinary water extraction solvent with a 45-minute extraction time produces tannin of 1.01%, while solvents using heated distilled water for 15–75 minutes can remove tannin compounds in *Garcinia mangostana* leaf extract. Iylia & Harisun, (18) also reported that the total tannin content of *Quercus infectoria* leaf extract decreased when the extraction temperature reached 100ºC

Feeding tannin-containing feed ingredients to poultry has both positive and negative effects. Low doses of tannins (0.12%) have no adverse impact on amino acid digestibility in the ileum, growth of broiler chickens and carcasses (19). Instead, tannins can be efficacious as antioxidants, improve gut morphology (20), and enhance mucosal

immunity (21). However, excessive doses of tannins have a negative effect on lymphoid organs, amino acid digestibility, and growth in broilers (22). It was also reported that a diet containing 0.56% (20) tannins could reduce broiler chicken growth and feed efficiency. Decreased aminopeptidase, amino acid digestibility, and metabolic energy are closely related to reduced poultry performance due to high tannin levels.

Interestingly, in this study, the heated distilled water treatment and the longer time had a positive effect on antioxidant activity. Similar results were also reported by Thoo et al. (11), who found that the best antioxidant content of *M. citrifolia* was obtained with 65^{o}C extraction and 80 min. Perwiratami et al. (23) stated that there is a correlation between phenol value and antioxidant activity, where the higher the total phenol value, the higher the antioxidant activity, and vice versa. In this study, phenolic compounds in the X2Y5 treatment had a higher flavonoid value than the others, impacting the higher antioxidant activity.

Antioxidants play a crucial role in poultry rations. Antioxidants can protect body cells from oxidative damage, improve performance (24), boost the immune system (22) and reduce heat stress. In general, the mechanism of antioxidant activity of plant bioactive compounds in the bird's body includes directly scavenging reactive oxygen species by donating electrons, increasing the activation of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase, synergistic activity with other antioxidant substances (vitamins and minerals), and inhibiting pro-oxidants such as xanthine oxidase.

This can be explained by the size of the clear zone formed against *E. coli* and *Salmonella sp*. *E. coli* and *Salmonella sp.* are common pathogenic bacteria often found in poultry's digestive tractcan negatively affect poultry performance and health. The

Centella asiatica leaf meal extract used in this study is the best result based on the type of solvent used and extraction time (X2Y5). In this study, the ability of *Centella asiatica* leaf meal extract as an antibacterial agent proved to be lower than that of antibiotics (tetracycline), with inhibition zones of 9.94 mm (*E. coli*) and 11.75 mm (*Salmonella sp*). *Centella asiatica* leaf meal extract concentrations of 100% showed a greater inhibition zone compared to *Centella asiatica* leaf meal extract concentrations of 75% and 50%. An increase in *Centella asiatica* leaf meal extract concentration increased the concentration of antibacterial compounds that diffused in the agar medium, resulting in a larger inhibition zone. Conversely, the decrease in inhibition zone diameter was due to the reduced effectiveness of the antibacterial compound.

Phytochemical compounds attack bacteria by damaging cell membranes through reactions between phenolic compounds and cell wall phospholipids. As a result, the permeability of the cell membrane is disrupted, inhibiting mRNA function and bacterial development. Tannins are antibacterial because they inhibit microbial adhesins and enzymes, transport proteins for cell membranes, and form complexes with polysaccharides. Its lower molecular weight characteristics allow it to penetrate bacterial proteins easily (25).

In Conclusion, this study shows that the best extraction to produce phytochemical compounds from *Centella asiatica* leaves uses a heated distilled water solvent and a time of 75 minutes. *Centella asiatica* also has potential as an antibacterial agent candidate for Poultry.

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Conflict Of Interest

All authors declare that they have no conflicts of interest that could inappropriately influence this manuscript.

Ethical approval

Ethical approval was not required since all procedures were carried out in vitro study without using experimental animals.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

RKR: Conceptualization and design the experiment, , investigation, Writing-original draft, Visualization, supervision, editing, and finalization**. AD**: Data curation, methodology, formal analysis, Writing-original draft, and finalization**. CH**: Investigation, supervision, Review and editing, and finalization. **RK**: Review and editing, and finalization.

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Table 1. Effect of solvent type and extraction time on the total phenol content of *Centella asiatica* leaf meal (%)

Solvent (X)	Time (Y)					Average	*p-value*		
	Y1	Y2	Y3	Y4	Y5		X	Y	X*Y
X1	9.54±0.24^{c}	9.81±0.09^{c}	9.46±0.25^{c}	10.37±1.08^{c}	10.17±0.16^{c}	9.87±0.57			
X2	9.75±0.55^{c}	11.38±0.57^{b}	11.89±0.08^{b}	11.63±0.16^{b}	15.67±1.17^{a}	12.06±2.09	<0.0001	<0.0001	<0.0001
Average	9.65±0.40	10.60±0.93	10.68±1.34	11.00±0.97	12.92±3.10				

Note: X1= ordinary distilled water; X2= distilled water heated at 100 °C; Y1= 15 minute; Y2= 30 minute; Y3= 45 minute; Y4= 60 minute; Y5= 75 minute. Means in the same variable with different superscripts differ significantly (P<0.01).

Table 2. Effect of solvent type and extraction time on the total flavonoid content of *Centella asiatica* leaf meal (%)

Solvent	Time (Y)					Average	*p-value*		
(X)	Y1	Y2	Y3	Y4	Y5		X	Y	X*Y
X1	0.01±0.01^{c}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.01			
X2	0.00±0.00^{d}	0.02±0.00^{bc}	0.00±0.01^{d}	0.05±0.01^{a}	0.03±0.01^{b}	0.02±0.02	<0.0001	<0.0001	<0.0001
Average	0.01±0.01	0.01±0.01	0.00±0.00	0.02±0.03	0.01±0.01				

Note: X1= ordinary distilled water; X2= distilled water heated at 100 °C; Y1= 15 minute; Y2= 30 minute; Y3= 45 minute; Y4= 60 minute; Y5= 75 minute. Means in the same variable with different superscripts differ significantly (P<0.01).

Table 3. Effect of solvent type and extraction time on the total tannin content of *Centella asiatica* leaf meal (%)

Solvent	Time (Y)					Average	*p-value*		
(X)	Y1	Y2	Y3	Y4	Y5		X	Y	X*Y
X1	2.03±0.52	2.98±0.84	2.43±0.64	3.13±0.83	3.28±0.82	2.77±0.79			
X2	3.48±0.83	3.12±0.71	3.46±0.75	3.36±0.81	2.81±0.66	3.25±0.69	0.094	0.838	0.223
Average	2.75±1.01	3.05±0.70	2.94±0.84	3.25±0.74	3.04±0.71				

Note: X1= ordinary distilled water; X2= distilled water heated at 100 °C; Y1= 15 minute; Y2= 30 minute; Y3= 45 minute; Y4= 60 minute; Y5= 75 minute. ns = non significant

Tabel 4. Effect of solvent type and extraction time on the total antioxidant activity of *Centella asiatica* leaf meal (%)

Solvent	Time (Y)					Average	*p-value*		
(X)	Y1	Y2	Y3	Y4	Y5		X	Y	X*Y
X1	38.00±3.37^{de}	36.20±3.02^{e}	37.98±0.82^{de}	40.33±1.86^{cde}	35.72±0.72^{e}	36.74±2.54			
X2	41.37±5.67^{cde}	48.63±4.90^{ab}	45.30±1.24^{abc}	43.78±4.23^{bcd}	50.38±5.14^{a}	45.89±5.09	<0.0001	0.56	<0.0001
Average	39.68±4.56	42.42±7.72	41.64±4.12	42.05±3.48	43.05±8.68				

Note: X1= ordinary distilled water; X2= distilled water heated at 100 °C; Y1= 15 minute; Y2= 30 minute; Y3= 45 minute; Y4= 60 minute; Y5= 75 minute. Means in the same variable with different superscripts differ significantly (P<0.01).

Figure 1. Inhibition zone of *Centella asiatica* leaf meal against *Escherichia coli and Salmonella sp.*