Short Communication

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

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ABSTRACT

The objective of this study was to assess the phytochemical composition of Centella asiatica leaf meal by analyzing the type of solvent utilised and the duration of the extraction process. Furthermore, the study evaluated the efficacy of Centella asiatica leaf meal extract as a prospective antibacterial agent when incorporated into poultry feed. The study was conducted in two phases. The initial phase of the study employed a fully randomised design featuring a 2×5 factorial design and three replications. Factor X denoted the solvent utilised, which included ordinary distilled water and distilled water subjected to heating at 100°C. Factor Y was defined as the duration of the extraction process, ranging from 15 to 75 minutes in 15-minute increments. In the second stage of the experiment, the in vitro antibacterial test was conducted using the most effective extract of Centella asiatica leaf meal, as determined in the first stage of the experiment. The parameters of the study encompassed the total phenolic content, total flavonoid content, total tannin content, total antioxidant activity, and the inhibition zone of Escherichia coli and Salmonella sp. The findings revealed a highly significant interaction ($p < 0.05$) between the type of solvent and the duration of extraction on the total phenolic content, flavonoid content, and antioxidant activity. However, the overall tannin concentration remained constant, irrespective of the extraction solvent or the duration of the extraction process. Furthermore, the Centella asiatica leaf meal extract, at a concentration of 100%, exhibited a greater inhibitory effect against Escherichia coli and Salmonella sp. bacteria in comparison to other concentrations. Following a comprehensive review of the available literature, it was concluded that the most effective extraction method for producing phytochemical compounds from Centella asiatica leaf meal extract is that of heated distilled water solvent, with an extraction time of 75 minutes. In addition, Centella asiatica leaf meal extract has been identified as a promising antibacterial agent for use in poultry farming.

Keywords: Antibacterial, Extraction, Herbs, Phytochemical, Solvents.

1. Introduction

Antibiotic Growth Promoters (AGPs) are defined as antibiotic compounds that are added to animal feed with the intention of improving growth and feed efficiency, whilst minimising the presence of pathogenic bacteria that have the potential to cause harm to the digestive tract. In recent years, several countries have regulated and banned the use of AGPs, although subtherapeutic doses can be administered to livestock, especially poultry. In Indonesia, the prohibition on the utilisation of AGPs was initiated in 2018, owing to concerns that their excessive use might result in bacterial resistance and deleterious effects on human health. Conversely, the prohibition of AGPs has the potential to adversely impact 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, being a tropical country, is home to a plethora of plant species (leaves, fruits, stems, and roots) that are abundant in phytochemical compounds, which possess a variety of biological activities, including antibacterial, antioxidant, anti-inflammatory, and antifungal properties. Centella asiatica leaf has emerged as a promising medicinal plant with significant potential as an AGPs substitute among these botanical resources. These leaves contain a variety of phytochemical compounds, including asiaticoside, asiatic acid, madecassic acid, madecassoside (3), alkaloids, flavonoids, phenolics, saponins, and tannins (4). The administration of Centella asiatica leaves in the form of poultry meal has been demonstrated to engender favourable outcomes. However, there is limited information on the use of Centella aciatica leaf extracts as a poultry feed additive. The present study was conducted as a preliminary investigation to evaluate the phytochemical composition of Centella asiatica leaf extract, with consideration given to the solvent type and extraction duration. Moreover, the study sought to ascertain the effectiveness of the substance as a prospective antibacterial agent for poultry.

2. Materials and Methods

2.1. Materials

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

2.2. Extract Preparation

The extraction of the meal from Centella asiatica leaves was mentioned in the study conducted by Rusli et al. (2). Centella asiatica meal (10 g) was mixed with regular distilled water and heated distilled water at 100°C (100 ml). Furthermore, 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. Subsequently, the Centella asiatica leaf meal obtained was permitted to cool to ambient temperature, whereupon it was subjected to filtration through a Whatman No. 1 filter paper (Cytiva, China). The Centella asiatica leaf meal that had been extracted was then stored in a refrigerator for the purpose of further analysis.

2.3. Experimental Design

The initial stage employed a complete randomised design, incorporating a 2 x 5 factorial design, with three replicates. Factor X (hereafter referred to as the 'first factor') represented the type of solvent utilised, which included ordinary distilled water and heated distilled water. The initial phase of the study employed a fully randomised design incorporating a 2 x 5 factorial design, with three replications. Factor X, the first factor, denoted the solvent type employed, encompassing both regular and heated distilled water. Factor Y (second factor) was indicative of the length of the extraction time, which ranged from 15, 30, 45, 60, and 75 minutes. The second stage of the experiment involved the execution of in vitro antibacterial tests, utilising the most efficacious C. asiatica leaf meal extract from the initial stage of the study. The study analysed C. asiatica leaf meal extracts at concentrations of 50%, 75%, and 100% (undiluted) and antibiotics (tetracycline) as controls.

2.4. Parameter Observed

The present study set forth a series of research objectives, the achievement of which was dependent upon the successful observation of several predetermined parameters. These parameters included, but were not limited to, the following: total phenolic content, total flavonoid content, total tannin content, total antioxidant activity, and antibacterial testing of Escherichia coli and Salmonella sp. The following procedures were utilized for the analysis of each parameter.

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, the object was left undisturbed for a period of five minutes at the ambient temperature. Subsequent to the process of incubation, a solution containing 7.5% Na2CO3 was introduced into the mixture in a volume of 80 µl. Subsequently, the mixture was left undisturbed for 30 minutes at room temperature in the absence of light. Subsequently, the spectrophotometer

(Shimadzu UV-1800, Japan) was utilized to detect the sample's absorbance at a wavelength of 725 nm.

2.4.2. Total Flavonoid Content

The protocol for analyzing the total flavonoid content (TFC) was adapted from Chang et al. (7). The sample was then diluted using a solution of methanol at a ratio of 1:10 (g/ml). Subsequently, 1 milliliter of the sample was amalgamated 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 utilized 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 the weighing of a 1 g sample and its subsequent dissolution in 100 ml of distilled water. The specimen was recently subjected to ultrasonic extraction for a duration of 15 minutes at ambient temperature. The precipitate was isolated through the use of a centrifuge operating at 3,000 revolutions per minute for a period of 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. The specimen was subsequently examined at a wavelength of 278 nanometers (nm) using a Shimadzu UV-1800 spectrophotometer manufactured in Japan.

2.4.4. Total Antioxidant Activity

The method employed in this study was outlined by Melia et al. (9). The analysis of total antioxidant activity (TAA) entailed the combination of 375 µl of 99% ethanol with 125 µl of a 0.02% DPPH solution in ethanol. Subsequently, 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 nanometers using a Shimadzu UV VIS-1800 spectrophotometer from Japan.

2.4.5. Anti-Bacterial Testing of *Escherichia Coli* And *Salmonella Sp*.

In this stage, Centella asiatica leaf meal extract was utilized, with the optimal yields being derived from the preceding stage. The antibacterial test was conducted in accordance with the method outlined by Yuniza and Yuherman (10), which involved the inoculation of Escherichia coli (106 CFU/ml) and Salmonella sp. (106 CFU/ml) into nutrient agar (NA) and subsequent incubation for a duration of 24 hours. Subsequently, four holes with a diameter of 5 mm were filled with 20 µl of Centella asiatica leaf meal extract and antibiotics. The extract was subsequently diluted with varying concentrations (50%, 75%, and 100%) and incubated for a duration of 24 hours at a temperature of 37°C. During this incubation, the extract was exposed to tetracycline antibiotic (0.002 g/ml) as a control. Subsequently, the samples were subjected to an incubation period of 24 hours at a temperature of 37°C. Subsequently, the diameter of the clear zone formed was measured using ImageJ-ink software.

2.5. Data Analysis

The initial stage data were subjected to analysis of variance. In instances where substantial discrepancies were observed, the Duncan multiple range test was employed to ascertain statistical significance. Concurrently, the second stage data were presented in a descriptive manner.

3. Results

3.1. Total Phenolic Content (TPC)

There was a very significant 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 min).

3.2. Total Flavonoid Content (TFC)

As demonstrated in Table 2, the interaction of solvent type and extraction time had a significant impact on the TFC of leaf meal extract from Centella asiatica ($p < 0.01$). The optimal extraction of Centella asiatica leaf meal extract was achieved through the utilisation of a solvent comprising heated distilled water, with an extraction duration of 60 minutes (X2Y4).

3.3. Total Tannin Content (TTC)

The findings of this study demonstrated that there was no statistically significant interaction ($P > 0.05$) between the type of solvent and the 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 observed between the type of solvent and the duration of extraction ($P<0.01$) with respect to the total antioxidant activity (TAA) of the Centella Asiatica leaf meal extract (Table 4). The treatment involving the use of hot distilled water as a solvent (X2Y5) resulted in the highest Total Antioxidant Activity (TAA) of 50.38%, in comparison to the other treatments.

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	<0.0001	<0.0001	<0.0001
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			
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 (X)	Time (Y)					Average	*p-value*		
	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	<0.0001	<0.0001	<0.0001
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			
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 (X)	Time (Y)					Average	*p-value*		
	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	0.094	0.838	0.223
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			
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 (X)	Time (Y)					Average	*p-value*		
	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	<0.0001	0.56	<0.0001
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			
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).

3.5. Antibacterial Activity

The antibacterial activity of Centella asiatica leaf meal extract with different concentrations against E. coli and Salmonella sp. bacteria is illustrated in Figure 1. The results of the study demonstrated that Centella asiatica leaf meal extract, at varying concentrations, exhibited antibacterial properties.

4. Discussion

Indeed, TPC is influenced by factors such as solvent type and extraction time (11). The elevated TPC of Centella asiatica leaf in the X2Y5 treatment is attributable to the pivotal role of time in solvent extraction for phenolic compounds. In this process, the equilibrium concentration of phenolic compounds is a pivotal factor in achieving the desired reduction. This finding aligns with the conclusions of a previous study by Thoo et al. (11), which reported that the optimal time for the extraction of phenolic compounds in M. citrifolia is 80 minutes. It was also noted that an extraction duration exceeding this timeframe can result in a reduction in the yield of phenolic compounds. In addition to the factor of time, the temperature also exerted an influence on the flavonoid compounds obtained. The utilization of a water solvent, preheated to a temperature of 100°C, within the X2 group resulted in a notable augmentation in the yield of TPC. Increasing temperature has been shown to favor the release of polyphenols bound in the sample by the degradation of cellular constituents of plant cells, thereby causing cell membrane permeability (12). As posited by Kim et al. (3), an elevated temperature has been demonstrated to result in an increased content of phytochemical compounds (asiaticoside and asiatic acid) in Centella asiatica leaf. A preceding 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). Concurrently, in the context of broiler chickens, phenolic compounds have been demonstrated to function as growth promoters. The underlying mechanism of this effect is attributed to their antioxidant and anti-inflammatory properties. Phenolic chemicals have been demonstrated to stimulate growth through a variety of mechanisms, including the enhancement of digestive enzyme secretion, the reduction of harmful bacteria in the digestive tract, and the influence on gut structure (14). The discrepancy in the optimal time between TPC and TFC is hypothesised to be attributable to variations in the degree of phenolic polymerisation, phenolic solubility, and phenolic interaction with other constituents (15). Thoo et al. (11) also reported similar results, highlighting discrepancies in the optimum extraction time required to obtain TPC and TFC of M. citrifolia extract. Citrifolia. Flavonoids have been demonstrated to exert a beneficial impact on various physiological systems in broiler chickens, including the gastrointestinal tract, cardiovascular system, immune system, lipid metabolism, insulin release, and antioxidant activity (16). Flavonoids present in laying hens have been demonstrated to modify the composition of fatty acids and to reduce the levels of cholesterol in eggs, thereby enhancing their nutritional quality (17). As posited by Prihambodo et al. (16), flavonoids have been demonstrated to exert a favourable influence on the digestive tracts of chickens. Flavonoids have been demonstrated to possess antibacterial and antioxidant properties, thereby significantly enhancing the health and function of the small intestine, particularly with regard to nutrient absorption. The favourable outcomes of this process include increased villous density, crypt depth, and height. Higher villi enhance intestinal surface area and nutrient absorption,

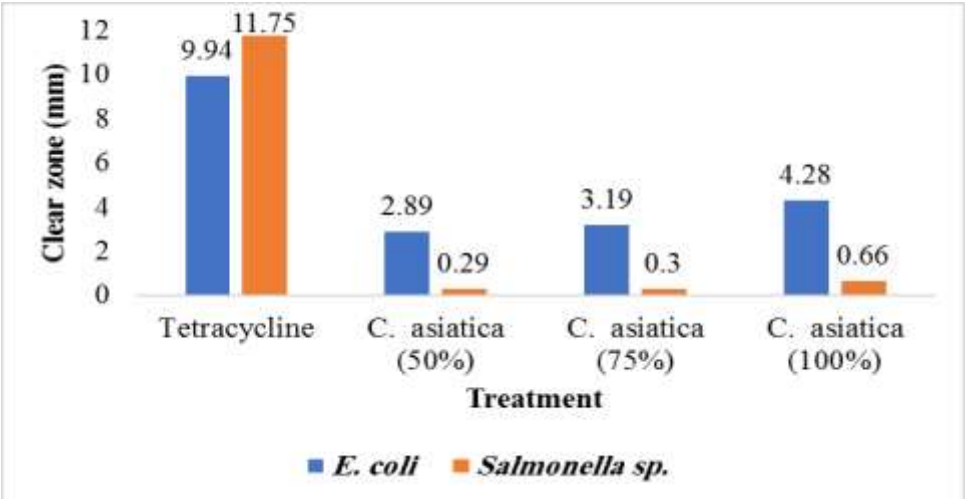

Figure 1. Inhibition zone of *Centella asiatica* leaf meal against *Escherichia coli and Salmonella sp.*

while deeper crypts can rapidly regenerate intestinal villi in response to inflammation caused by pathogenic bacteria. However, the findings of Rusli et al. (2) suggest that the total tannin content is influenced by both the solvent and the extraction time. Specifically, the use of ordinary water as the extraction solvent, in conjunction with a 45-minute extraction time, results in the production of tannin compounds equivalent to 1.01%. In contrast, the utilisation of heated distilled water for 15–75 minutes has been observed to be more effective in the removal of tannin compounds from Garcinia mangostana leaf extract. Ilya & Harisun (18) also reported that the total tannin content of Quercus infectoria leaf extract decreased when the extraction temperature reached 100°C. The effects of feeding tannin-containing feed ingredients to poultry have been shown to be both positive and negative. The impact of low doses of tannins (0.12%) on the ileum, growth of broiler chickens and carcasses has been examined. The results of the study demonstrated that low doses of tannins had no adverse impact on amino acid digestibility in the ileum, growth of broiler chickens and carcasses (19). In contrast, tannins have been demonstrated to possess antioxidant properties, to promote improvements in gut morphology (20), and to enhance mucosal immunity (21). However, it has been demonstrated that excessive doses of tannins have a detrimental effect on lymphoid organs, amino acid digestibility, and growth in broilers (22). It has been documented that a diet comprising 0.56% (20) tannins has the capacity to curtail the growth and feed efficiency of broiler chickens. Decreased aminopeptidase, amino acid digestibility, and metabolic energy have been demonstrated to be closely related to reduced poultry performance due to high tannin levels. The findings of this study suggest that the application of heated distilled water and an extended duration of treatment can yield a favourable outcome in terms of antioxidant activity. Consistent outcomes were also documented by Thoo et al. (11), who ascertained that the optimal antioxidant content of M. citrifolia was attained through extraction at 65°C for 80 minutes. Perwiratami et al. (23) stated that there is a correlation between phenol value and antioxidant activity. The researchers concluded that an increase in total phenol value is accompanied by an increase in antioxidant activity, and vice versa. In this study, the X2Y5 treatment was found to have higher phenolic compound concentrations than the other treatments, which impacted higher antioxidant activity. Antioxidants have been shown to play a crucial role in poultry rations. Antioxidants have been demonstrated to protect body cells from oxidative damage, improve performance (24), boost the immune system (22) and reduce heat stress. The antioxidant activity of plant bioactive compounds in the bird's body is generally understood to include the following mechanisms: direct scavenging of reactive oxygen species by donating electrons, increased activation of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase, synergistic activity with other antioxidant substances (vitamins and minerals), and inhibition of pro-oxidants such as xanthine oxidase. The formation of the clear zone can be attributed to the presence of E. coli and Salmonella sp. These bacteria, which are of a pathogenic nature, are frequently identified within the digestive tract of poultry. Their presence can have a detrimental effect on the performance and health of poultry. The Centella asiatica leaf meal extract utilised in this study has been shown to yield optimal results when considering the type of solvent employed and the extraction time (X2Y5). In this study, the antibacterial potential of Centella asiatica leaf meal extract was compared with that of the antibiotic tetracycline. The results demonstrated that the inhibition zones of Centella asiatica were smaller than those of tetracycline, measuring 9.94 mm for E. coli and 11.75 mm for Salmonella sp. The investigation revealed that Centella asiatica leaf meal extract concentrations of 100% exhibited a greater inhibition zone in comparison to those at 75% and 50% concentrations. An increase in the concentration of Centella asiatica leaf meal extract resulted in an increase in the concentration of antibacterial compounds diffused in the agar medium, resulting in a larger inhibition zone. In contrast, the diminution in the antibacterial compound's inhibition zone diameter can be attributed to its diminished effectiveness. Phytochemical compounds have been shown to attack bacteria by damaging cell membranes through reactions between phenolic compounds and cell wall phospholipids. Consequently, the permeability of the cell membrane is disrupted, thereby inhibiting mRNA function and bacterial development. Tannins have been demonstrated to possess antibacterial properties, which are attributable to several mechanisms. These include the inhibition of microbial adhesins and enzymes, the transportation of proteins for cell membranes, and the formation of complexes with polysaccharides. Its lower molecular weight characteristics facilitate facile penetration of bacterial proteins (25). In conclusion, the present study demonstrates that the most effective extraction method for the production of phytochemical compounds from Centella asiatica leaves involves the utilisation of a heated distilled water solvent, with a duration of 75 minutes. Centella asiatica also has potential as an antibacterial agent candidate for poultry.

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Authors' Contribution
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.

Ethics
Ethical approval was deemed unnecessary, as all procedures were conducted within the confines of an in vitro study, thereby eliminating the need for the use of experimental animals.

Conflict of Interest
All authors have declared that they have no conflicts of interest that could inappropriately influence this manuscript.

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

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