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Evaluation of *Medinilla speciosa* fruit as natural feed additive for poultry through inhibition, resistance and antioxidant capacity tests

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ABSTRACT

The study aimed to assess the antibacterial activity of encapsulated *Medinilla speciosa* extract against pathogenic bacteria *Escherichia coli* and *Salmonella*, pH and bile salt resistance and antioxidant capacity (total phenols, flavonoids and antioxidants) in vitro. Extraction was carried out by maceration method using 96% ethanol solvent, while freeze drying technique was done using freeze dryer. The main ingredients used were encapsulated *Medinilla speciosa* fruit extract (EMSFE) and maltodextrin solution (MS). The research design used a completely randomized design with 5 treatments and 4 replicates. The levels of encapsulated *Medinilla speciosa* fruit extract included concentrations L-=100 (MS), L+=100 (EMSFE), L1=25 (EMSFE): 75 (MS), L2 = 50 (EMSFE): 50 (MS) and L3 = 75 (EMSFE): 25 (MS). Parameters measured included *Escherichia coli* and *Salmonella* inhibition, pH and bile salt resistance and antioxidant capacity (total phenols, flavonoids and antioxidants). Data were processed using variance analysis at 5% real level, if significant effect, Duncan test was conducted at 5% level. The results showed that the antibacterial activity of encapsulated *Medinilla speciosa* extract had a significant effect ($p<0.05$) on *Escherichia coli* and *Salmonella* inhibition, pH and bile salt resistance and antioxidant capacity (total phenols, flavonoids and antioxidants) in vitro. It was concluded that *Medinilla speciosa* extract encapsulated with a concentration of 75%:25% (L3) is able to pass through exposure to pepsin-HCl and bile salts and has a high inhibition zone against pathogenic bacteria and a mixture of *Medinilla speciosa* extract and maltodextrin solution can be used as a natural feed additive for poultry.

Introduction

Public awareness about the importance of health for poultry is increasing, leading to wide implications in choosing feed additives. This has led to the development of research on natural feed additives that have healthful effects, improving nutrient absorption and poultry performance. Bioproducts sourced from herbal plants have the potential to improve gut physiological function by modifying gut microflora (Zhao *et al*., 2022). Herbal plants as phytobiotics have bioactive contents, such as total phenols, flavonoids, saponins, and tannins that provide benefits for the health of the body and can be used as a substitute for antibiotics to suppress the growth of pathogenic bacteria. Some bacteria are resistant to synthetic materials, Consequently, materials from nature (herbs) as *Medinilla speciosa* fruit is used.

Medinilla speciosa fruit has the potential to be a natural feed additive for poultry due to its ability to inhibit pathogenic bacteria, which can significantly reduce the risk of disease on poultry farms. Additionally, *M. speciosa* fruit extract is rich in bioactive compounds that improve feed quality and promote health and production in poultry. The fruit's antioxidant capacity plays an important role in neutralizing free radicals, thus protecting poultry from oxidative stress and improving their overall health (Surai, 2020). Furthermore, *M. speciosa* may exhibit phytobiotic properties that support gut health and digestion. To explore the role of *Medinilla speciosa* as a sustainable feed additive, it is essential to test its durability, inhibition, and antioxidant capacity, which would ultimately contribute to improved poultry health and productivity.

This study examined the novelty of encapsulated *Medinilla speciosa* fruit extract as an antibacterial agent against *Escherichia coli* and *Salmonella*, as well as its pH and bile salt resistance and antioxidant capacity. This study provided the latest information on antibacterial compounds in *M. speciosa* fruit and its potential for developing natural feed additives.

Materials and methods

Sample collection

Medinilla speciosa fruits were obtained from the Mount Muria region of Kudus Regency. The fruits used were of the same maturity level (purplish red). The drying process used an oven with a temperature of 50°C for 2 days (moisture content range 12-14%). The dried *Medinilla speciosa* fruit was pulverized using a grinder with a sieve size of 80 mesh. *Medinilla speciosa* fruit flour continued the extraction and encapsulation process.

Extraction and encapsulation procedure

Medinilla speciosa fruit encapsulation begins with extraction based on the modified method of Gouda *et al*. (2021). The fruit was dried in an oven at 50°C until dry and pulverized into flour. *Medinilla speciosa* fruit flour was dissolved using 96% ethanol in a ratio of 1:10 (b/v), stirred until homogeneous and filtered using Whatman 41 filter paper. The filtered solution was sonicated at 37°C and room temperature at a wavelength of 50 Hz for 30 min. Sonification was performed at 45°C to evaporate the ethanol to obtain a thick extract. The next process was encapsulation based on the modified method of Agusetyaningsih *et al*. (2022), which started with the preparation of a mixture of maltodextrin and distilled water in a ratio of 3:1 (b/v). The evaporation results were mixed with the dressing solution in a ratio of 1:3 (v/v) and then dried using the freeze-drying method at -50°C until a dry form was obtained. The encapsulated products were pulverized using a mortar and pestle. The test results of bioactive compounds of *Medinilla speciosa* fruit extracts of various maturity levels are listed in Table 1 and the fruit picture is shown in Fig. 1.

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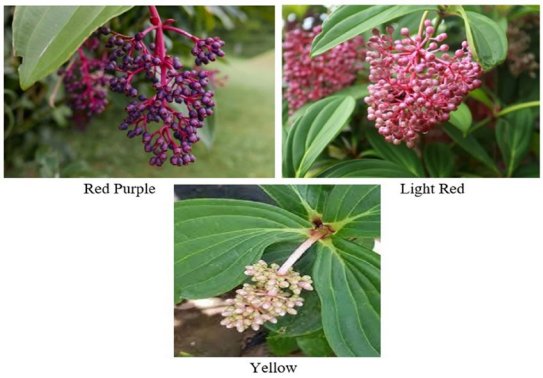

Fig. 1. *Medinilla speciosa* fruit ripeness colors.

Research design and treatments

The study was conducted using a completely randomized design with five treatments and four replications, resulting in 20 experimental units. The main ingredients used were encapsulated *Medinilla speciosa* fruit extract (EMSFE) and maltodextrin solution (MS). The concentration levels applied were as follows:

T+= 100% EMSFE
T-= 100% MS
T1= 25% (EMSFE): 75% (MS)
T2= 50% (EMSFE): 50% (MS)
T3= 75% (EMSFE): 25% (MS)

Inhibition test

The antibacterial activity test was conducted using the disc diffusion method (Kirby-Bauer Test) according to Park *et al.* (2017) which has been modified. *E. coli* and *Salmonella* bacterial isolates from chicken excreta were rejuvenated on Nutrient Agar media. The test bacterial isolates were then suspended in 0.9% NaCl solution and the turbidity was equalized with McFarland 0.5 solution (equivalent to $1.5x10^8$ CFU/mL). Each bacterial suspension was streaked evenly on the surface of the solidified MHA media using a sterile catton swab. Sterile paper disks were then immersed in 96% ethanol extract concentration of 1000 mg/mL for 1 minute. Then the Petri dish was incubated at 37°C for 48 hours.

Measurement of zone of inhibition

Measurement of inhibition zone refers to Priya *et al.* (2023) with observations made by measuring the diameter of the inhibition of bacterial growth around the filter paper using a caliper. At first, the vernier was placed on the surface of the Petri dish lid, just above the measured inhibition zone. The position of the beak of the vernier (fixed jaw and thrust jaw) was in line with the measured inhibition zone. Then slide the thrust jaw according to the diameter of the visible resistance zone. Furthermore, readings were taken on the main scale and the nonius scale on the vernier scale to determine the diameter of the resistance zone in millimeters (mm).

Resistance to pH testing

Measurement of pH resistance in vitro according to Wang, C. *et al.* (2017) which was modified. There were 40 test tubes with 20 each filled with pepsin-HCl at pH 2.5 and 20 tubes filled with pepsin-HCl at pH 5. Then, each tube was filled with pepsin-HCl as much as 8 ml with 2 g sample added. Then incubated at 37°C for 90 minutes.

Test for resistance to bile salts

The pH resistance was measured in vitro according to the modified method of Xu *et al.* (2016). Forty test tubes were used, 20 of which were filled with 1% bile salt and 20 with 5% bile salt. Then, 4 ml of bile salt solution was added to each tube by mixing 1 g of the sample with HCl pepsin solution. The tubes were then incubated at 37°C for one hour.

Antioxidant capacity testing

Total phenolics were measured by the Folin-Ciocalteu method according to Pedan *et al.* (2019) with modifications. 1 ml of *Medinilla speciosa* extract in bile solution was mixed with 4 ml of sodium carbonate solution (75 g/L) in a 10 ml volumetric flask, then homogenized. Folin-Ciocalteu reagent as much as 2 ml was put into the flask and homogenized. Next, after homogenization, distilled water was added to the reaction mixture up to the mark. The mixture was allowed to stand in a dark room at room temperature for 1 hour, then the absorbance was measured at a wavelength of 760 nm using a spectrophotometer.

Determination of total flavonoids was determined by spectrophotometer method according to Ramos *et al.* (2017) with some modifications. A 10 µm sample of encapsulated extract, 60 µL methanol, 10 µL aluminum chloride (10% w/v), 10 µL potassium acetate (1 M) and 120 µL distilled water were mixed homogeneously and incubated at room temperature for 30 min followed by absorbance measurement at 415 nm using a spectrophotometer. The total amount of flavonoids was expressed as quercentin equivalent (QE).

Antioxidant activity was determined using the DPPH assay according to Sirivibulkovit *et al.* (2018). The sample solution was 15 µL of reagent volume consisting of 0.095 mM DPPH. The absorbance was measured at a wavelength of 505 nm for 10 min.

Statistical analysis

Data were analyzed for variance at the 5% significance level, and if the treatment had a significant effect, Duncan's test was conducted at the 5% significance level to determine differences between treatments (Gaspersz, 2006).

Results

Bioactive content of Medinilla speciosa fruit at various ripeness levels

Based on the results of bioactive testing of *Medinilla speciosa* extracts listed in Table 1, the age of maturity in purple red color is higher than pink and yellow colors. Bioactive contents such as total phenols, flavonoids, antioxidant activity, tannins and saponins consistently increased and decreased according to the age of maturity.

Table 1. Bioactive contents (%) of *Medinilla speciosa* fruit extracts of different maturity levels.

Color	Antioxidant Activity	Total Phenol	Flavonoids	Tannins	Saponins
Red Purple	13.83	4.68	4.11	2.23	1.45
Light Red	7.45	2.45	1.85	1.33	0.72
Yellow	2.49	1.84	0.48	1.25	0.81

Test results at the Animal Nutrition and Feed Science Laboratory, Faculty of Animal and Agricultural Sciences, Universitas Diponegoro.

Zone of inhibition activity against pathogenic bacteria

Inhibition zone measurement on encapsulated *Medinilla speciosa* fruit extract significantly ($p<0.05$) affected the inhibition of *Escherichia coli* and *Salmonella* (Table 2). Concentrations L3 and L+ showed that inhibition against *Escherichia coli* was significantly ($p<0.05$) stronger than the other concentrations. Inhibition against *Salmonella* at L+ was significantly ($p<0.05$) higher than the other concentrations.

Total phenol assay against pepsin-HCL and bile salts

The results of total phenol testing on *Medinilla speciosa* extract encapsulated in pepsin-HCl solution are listed in Table 3. Total phenol in pepsin-HCl solution 2.5 showed that the concentration of L+ and L3 was significantly ($p<0.05$) higher than the other concentrations, while in pepsin-HCl solution 5 resulted in concentration L3 significantly ($p<0.05$) higher than the other concentrations. The results of total phenol to pepsin-HCl seen from pH 2.5 and 5 there is a difference, where total phenol at pH 2.5 is lower, while pH 5 has increased.

The results of total phenol testing on *Medinilla speciosa* extract encapsulated in pepsin-HCl solution followed by bile salt solution are listed in Table 3. Total phenol to 1% and 5% bile salt solution showed that L3 concentration was significantly ($p<0.05$) higher than the other concentrations. Bile salt solution with a concentration of 1% produced higher total phenols compared with 5% concentration.

Flavonoid assay against pepsin-HCL and bile salts

The results of total flavonoids testing on *Medinilla speciosa* extract encapsulated in pepsin-HCl solution are listed in Table 4. Total flavonoids in pepsin-HCl solution 2.5 showed that concentrations L+ and L3 were significantly ($p<0.05$) higher than the other concentrations, while in pepsin-HCl solution 5 resulted in concentration L3 significantly ($p<0.05$) higher than the other concentrations. The results of total flavonoids against pepsin-HCl were seen from pH 2.5 and 5 there is a difference, where the total flavonoids at pH 2.5 is lower, while pH 5 has increased.

The results of total flavonoids testing on *Medinilla speciosa* extract encapsulated in pepsin-HCl solution followed by bile salt solution are listed in Table 4. Total flavonoids against 1% and 5% bile salt solution showed that L3 concentration was significantly ($p<0.05$) higher than the other concentrations. Bile salt solution with 1% concentration produced higher total flavonoids compared to 5% concentration.

Antioxidant activity testing of pepsin-HCL and bile salts

The results of antioxidant activity testing on *Medinilla speciosa* extract encapsulated in pepsin-HCl solution are listed in Table 5. Antioxidant activity in pepsin-HCl solution 2.5 showed that L3 concentration was significantly ($p<0.05$) higher than the other concentrations, while in pepsin-HCl solution 5 resulted in L3 concentration significantly ($p<0.05$) higher than the other concentrations. The results of antioxidant activity against pepsin-HCl seen from pH 2.5 and 5 are different, where antioxidant activity at pH 2.5 is lower, while pH 5 has increased.

The results of antioxidant activity testing on *Medinilla speciosa* extract encapsulated in pepsin-HCl solution followed by bile salt solution are listed in Table 5. Antioxidant activity against 1% and 5% bile salt solution showed that concentration L3 was significantly ($p<0.05$) higher than the other concentrations. Bile salt solution with 1% concentration produced higher antioxidant activity compared to 5% concentration.

Table 2. Zone of inhibition activity.

Parameter	Inhibition Zone Diameter (mm)					P-value
	L+	L-	L1	L2	L3	
Escherichia coli	16.92±2.24^{a}	0.00±0.00^{d}	11.25±0.87^{c}	13.25±1.26^{b}	15.75±0.78^{a}	0
Salmonella	14.25±0.83^{a}	0.00±0.00^{e}	7.79±0.42^{d}	9.00±0.72^{c}	13.25±0.57^{b}	0

L+: 100% MSFE, L-: 100% MS, L1: 25% (MSFE):75% (MS), L2: 50% (MSFE):50% (MS), L3: 75% (MSFE):25% (MS), MS: maltodextrin solution, MSFE: *Medinilla speciosa* fruit extract

Table 3. Total phenolics in *Medinilla speciosa* fruit extract encapsulated in HCl liquid and followed by bile salt solution.

Parameter	Concentration					P-value
	L+	L-	L1	L2	L3	
pH Pepsin HCl						
2.5	2.13±0.22^{a}	0.00±0.00^{d}	1.27±0.15^{c}	1.83±0.16^{b}	2.26±0.28^{a}	0
5	3.06±0.29^{b}	0.00±0.00^{e}	1.66±0.07^{d}	2.77±0.09^{c}	4.04±0.15^{a}	0
Bile Salts						
1%	3.04±0.13^{b}	0.00±0.00^{e}	1.43±0.08^{d}	2.44±0.1^{c}	3.75±0.2^{a}	0
5%	1.81±0.23^{b}	0.00±0.00^{d}	1.18±0.11^{c}	1.37±0.1^{c}	2.56±0.4^{a}	0

L+: 100% EMSFE, L-: 100% MS, L1: 25% (MSFE):75% (MS), L2: 50% (MSFE):50% (MS), L3: 75% (MSFE):25% (MS), MS: maltodextrin solution, MSFE: *Medinilla speciosa* fruit extract.

Table 4. Total flavonoids in *Medinilla speciosa* fruit extract encapsulated in HCl liquid and followed by bile salt solution.

Parameter	Concentration					P-value
	L+	L-	L1	L2	L3	
pH Pepsin HCl						
2,5	1.98±0.13^{a}	0.00±0.00^{d}	0.87±0.24^{c}	1.25±0.16^{b}	2.18±0.13^{a}	0
5	2.59±0.15^{b}	0.00±0.00^{e}	1.26±0.08^{d}	1.59±0.1^{c}	3.68±0.14^{a}	0
Bile Salts						
1%	2.30±0.1^{b}	0.00±0.00^{e}	0.98±0.11^{d}	1.24±0.06^{c}	2.55±0.15^{a}	0
5%	1.75±0.06^{b}	0.00±0.00^{e}	0.84±0.06^{d}	1.21±0.07^{c}	1.99±0.12^{a}	0

L+: 100% EMSFE, L-: 100% MS, L1: 25% (EMSFE):75% (MS), L2: 50% (EMSFE):50% (MS), L3: 75% (EMSFE):25% (MS), MS: maltodextrin solution, EMSFE: encapsulated *Medinilla speciosa* fruit extract

Table 5. Antioxidant activity of *Medinilla speciosa* fruit extract encapsulated in HCl liquid and followed by bile salt solution.

Parameter	Concentration					P-value
	L+	L-	L1	L2	L3	
pH Pepsin HCl						
2,5	6.03±0.55^{b}	0.00±0.00^{d}	3.38±0.39^{c}	5.96±0.34^{b}	7.85±0.45^{a}	0
5	8.7±0.48^{b}	0.00±0.00^{d}	5.86±0.41^{c}	8.26±0.33^{b}	9.95±0.59^{a}	0
Bile Salts						
1%	7.44±0.11^{b}	0.00±0.00^{d}	4.51±0.24^{c}	7.22±0.37^{b}	8.73±0.32^{a}	0
5%	5.11±0.57^{c}	0.00±0.00^{e}	2.72±0.1^{d}	6.72±0.44^{b}	7.31±0.33^{a}	0

L+: 100% EMSFE, L-: 100% MS, L1: 25% (EMSFE):75% (MS), L2: 50% (EMSFE):50% (MS), L3: 75% (EMSFE):25% (MS), MS: maltodextrin solution, EMSFE: encapsulated *Medinilla speciosa* fruit extract

Discussion

Ripe fruits generally have higher flavonoid concentrations than unripe fruits due to biochemical changes that occur during ripening. These changes are influenced by gene expression, enzyme activity, and the synthesis of phenolic metabolites, all of which increase the flavonoid profile of the fruit. According to Wang Z. *et al*. (2017), as fruits ripen, especially from green to red, there is a significant increase in flavonoid levels. These flavonoids are responsible for the red, blue, and purple colors of the fruit. The expression of genes related to flavonoid biosynthesis, such as UFGT and MYBA, is much higher in ripe fruits, leading to increased flavonoid content (Medda *et al*., 2021). Different fruit varieties have different flavonoid profiles. For instance, red grapes have higher anthocyanin content than white varieties.

The relationship between tannin and saponin levels and fruit ripeness is characterized by significant fluctuations in concentration during ripening. Research shows that both compounds play an important role in the nutritional and sensory qualities of fruits, and their levels vary markedly at different stages of ripeness. Tannins increase markedly during the early stages of fruit development, peaking before decreasing as the fruit ripens. Tannin content increases approximately 90-fold during early cell enlargement, followed by an 8-fold decrease as ripening progresses (Chang *et al*., 2019). Fruits' antioxidant activity, which correlates with saponin content, is highest in unripe fruits (Nakitto *et al*., 2023).

Measurement of the formed inhibition zone showed that the antibacterial bioactivity of the encapsulated *Medinilla speciosa* extract against *Escherichia coli* was highest at 48-hour incubations at concentrations L3 and L+. During the 48-hour incubation period, the diameter of the inhibition zone increased at consecutive concentrations starting from L1, L2, and L3. In *Salmonella*, the L+ concentration showed a larger inhibition zone diameter compared to the other concentrations.

Encapsulated *Medinilla speciosa* extract is bacteriostatic because it is only able to inhibit the growth of *Escherichia coli* bacteria during the 48-hour incubation period. According to Zhang and Cheng (2022), that an antimicrobial is bacteriostatic if the antimicrobial compound is only able to inhibit bacterial growth if the administration of the compound continues and if it is stopped or exhausted, the growth and multiplication of bacteria will increase again which is characterized by a decrease in the diameter of the inhibition zone during the second incubation period. Conversely, it is bacteriocidal if the diameter of the inhibition zone increases during the second incubation period, this is because this compound is able to kill and stop the physiological activity of bacteria, even though the administration of the compound is stopped. Flavonoid compounds are thought to have a mechanism of action that denatures bacterial cell proteins and damages cell membranes beyond repair. Flavonoids are also lipophilic which will damage microbial membranes (Donadio *et al*., 2021). Flavonoids contain a phenol compound. The growth of *Escherichia coli* and *Salmonella* bacteria can be disrupted due to phenol compounds. Phenol is an alcohol that is acidic so it is also called carbolic acid. Phenol has the ability to denature proteins and damage cell membranes. Acidic conditions by the presence of phenol can affect the growth of pathogenic bacteria.

Assays of phenolics, flavonoids and antioxidant activities against pepsin-HCl and bile salts revealed significant interactions affecting the bioaccessibility and absorption of bioactive compounds. Inhibition of pepsin by bile salts, as shown in various studies, suggests that bile salts may modulate the digestive environment, potentially affecting the bioavailability of phenolic compounds. Encapsulation plays an important role in protecting phytobiotics and other agents from harsh acidic and bile salt conditions, enhancing LAB survival (Ishwari, 2024). This process involves enclosing the active agent in a protective matrix, which can protect the extract from degradation in the digestive tract. Encapsulation techniques vary, but they have the common goal of improving the stability and bioavailability of the encapsulated substances.

Phenol compounds naturally bind to sugars and during fermentation the complex components are degraded into free phenols with high antioxidant properties (Adebo and Meza, 2020). The results on exposure to pepsin HCl with pH 2.5 showed a decrease in the quality of bioactive levels, while pH 5 conditions of phenols, flavonoids and antioxidant activity were still high. This proves that the encapsulated *Medinilla speciosa* extract can pass through the proventriculus, or stomach acid, so that it can be utilized as an antibacterial agent. Encapsulation protects the active ingredients from gastric acid degradation and controls their release at the desired location, thereby increasing the extract's effectiveness (Bao *et al*., 2019).

The results of testing the resistance of encapsulated *Medinilla speciosa* to 1% bile salt concentration showed more bioactivity growth compared to 5% concentration. The amount of growth at a concentration of 1% is due to the bile salt concentration was not too high. Meanwhile, the 5% concentration decreased because the bile salt given was too much. The higher the concentration of bile salt, the lower the bioactive content. This is due to the ability of the encapsulated material to bile salt is not resistant to damage or degradation. According to Emzhik *et al*. (2024), which states that the concentration of bile salts 3-5% is a concentration that is high enough to reduce the quality of the encapsulated material.

Conclusion

Medinilla speciosa extract encapsulated with a concentration of 75%:25% (L3) is able to pass through exposure to pepsin-HCl and bile salts and has a high inhibition zone against pathogenic bacteria and a mixture of *Medinilla speciosa* extract and maltodextrin solution can be used as a natural feed additive for poultry.

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Conflict of interest

The authors have no conflict of interest to declare.

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