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Effects of Drinking Water Supplementations with Nanoencapsulated Lime (*Citrus aurantifolia*) Leaf Extract on Broiler Chicken's Blood Antioxidant Profiles and Meat Proximate Compositions

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ABSTRACT
Objective of this study was to investigate the effect of nanoencapsulated lime leaf extract (LLE) on blood antioxidant profiles and meat proximate compounds of broiler chickens. Ionic gelation method was used to encapsulate nanoparticle of LLE using chitosan and sodium tripolyphosphate. A total number of 192 male New Lohmann broiler chickens was kept in semi-closed house with same environmental condition for 35 days. The extract was supplemented through drinking water. Birds in current study were fed a same basal diet with one of the following six treatments: drinking water only (T_0; Negative control), drinking water with 50 ppm Tetracycline (T_1), or drinking water with 0.015% v/v LLE (T_2), 0.030% v/v LLE (T_3), 0.015% v/v nanocansulated LLE (T_4), 0.030% v/v nanocapsulated LLE (T_5). Results showed that group T_4 or T_5 had the highest level of Catalase ($P<0.01$) as well as Glutathione peroxidase ($P<0.01$) enzymes in the blood serum, when compared to other treatments. Meat proximate analyses showed that drinking water supplementations with LLE or nanoencapsulated LLE did not affect meat chemical compositions. In conclusion, nanoencaosulated lime leaf extract would be beneficial as a natural antioxidant as the low dose of the extract increased the levels of two enzymes that closely related to the antioxidation process in the blood of broiler chickens.

Keywords: *Broiler, Citrus aurantifolia, Meat quality, Nanoencapsulated*

1. INTRODUCTION

Diet plays a vital role in maintaining animal health, productivity, and reproductive performance of farm animals and poultry. Among those dietary factors, natural antioxidants have special importance in the maintenance of growth performance, reproduction, immune-competence in poultry production [1], and having no side effects after replacing synthetic antioxidants [2]. Therefore, natural antioxidants studies have been targeted to find the sources of potentially safe, effective, and affordable antioxidants, including natural antioxidant from fruit [3].

Lime is a polyembryonic plant cultivated in many countries and grows in hot subtropical or tropical regions. The plant belongs to the Kingdom Plantae, Phylum Magnoliophyta, Class Magnoliopsida, Order Sapindales, Family Rutaceae, Genus Citrus, and Species *Citrus aurantifolia* [4]. Citrus fruits have received attention not only for their nutritional properties, but also their medicinal values. Some of their species have variety of health properties, including antibacterial, antiviral, antioxidant, antifungal, analgesic, and anti-inflammatory activities [5]. Moreover, the leaves are believed to possess a powerful antioxidant activity against various oxidative systems [6]. Despite of its health-related properties, *Citrus aurantifolia* is highly associated with the high amount of bioactive compounds, such as phenol, flavonoid, carotenoid, vitamin, and minerals which make lime leaves become one of effective natural antioxidant resources [7]. However, in order to deliver those compounds to particular sites-of-action and increase utilization efficiency of those compounds, nanoencapsulation technology can be applied [8] since

most of bioactive compounds are hydrophobic and easily degraded in presence of light, air, or high temperature [9].

Nanoencapsulation is defined as a technology to encapsulate substances in tiny size and describes to natural bioactive packing at nanoscale (10 to 1,000 nm) range which has the capability to improve bioavailability, enhance controlled release, and allow precision targeting of the bioactive substances [10] by protecting those compounds from unpleasant environmental conditions, eradication of incompatibilities and solubilization until reach the site-of-action [11]. Accordingly, nanoencapsulation technologies provide possible solutions to improve the effectiveness of those bioactive compounds including antioxidant [12].

Therefore, objective of this study was to investigate the effects of drinking water supplementations with lime leaf extract (LLE), non-nanocapsulated or nanocapsulated, as natural free radical scavenger, on the blood antioxidant enzymes and some proximate contents in the meat of broiler chickens.

2. MATERIALS AND METHOD

2.1. Chemical, Reagents, and Instrument

Methanol, ethanol 96%, acetic acid 1%, chitosan powder, sodium tripolyphosphate (NaTPP), polysorbate 80 (Tween 80), aquades, petroleum ether, filtering paper, nitrogen-free weighing paper water bath, beaker glass, micropipette, magnetic stirrer (RO 15, IKA, Staufen, Germany), water bath, furnace (F48025-80, ThermoFisher Scientific, Massachusetts, USA), oven (OV-12A, Blue M Electric Company, Illinois, USA), Kjel-Foss Automatic (16210, Foss Food Technology Corp., Hillerød, Denmark), and grinder (KBB-250GL, Kirin, South Jakarta, Indonesia) were used in this study.

2.2. Lime Leaf Extraction

Fresh lime leaves were collected from Sriharjo village, Imogiri sub-district, Bantul district, Yogyakarta city, Indonesia. The leaves were then air dried under shade at room temperature for approximately 3 days and ground to pass a 1 millimeter sieve using grinder. Maceration method was applied to extract by soaking 100 g of dry lime leaves in 500 mL of methanol for 3 days before filtering. After 3 days of maceration, the solvent containing extract was filtered by using filtering paper, and thenincubated using water bath at 45°C until methanol was completely evaporated and the extract became semi-solid. Afterward, the extract was diluted into 1% solution by adding 0.2 g of extract into a beaker glass, and adding ethanol 96% 1 mL and 0.2 mL of Tween 80 into the glass. The solution was shaken until it was completely diffused, then covered and chilled inside 5°C refrigerator for approximately 10 minutes. The solution was adjusted to reach 20 mL using aquades in order to make 1% solution of LLE. This 1% LLE was further used in this experiment and nanoencapsulated.

2.3. Nanoencapsulation

To nanoencapsulate LLE, reagent chitosan 0.1% and NaTTP 0.1% were required. Chitosan 0.1% was made by dissolving 0.1 g of fine chitosan powder in 100 mL of acetic acid 1% (v/v) using magnetic stirrer for 1 hour with 700 rpm speed, and NaTTP 0.1% solution was created by simply dissolved 0.01 g of fine NaTTP in 10 mL aquades using magnetic stirrer for 1 hour at 700 rpm.

Ionic gelation method was used in this study to create nanoencapsulated LLE. Firstly, 10 mL of 1% LLE was put it into beaker glass, then added 50 mL of reagent chitosan. The solution then was stirred for 30 minutes with magnetic stirrer using 600 rpm. Upon stirring, 0.769 of reagent NaTPP was added into the solution using micropipette, then keep stirring with 600 rpm for 1 more hour.

2.4. Experimental Design

Table 1. Feed formulation

Raw Material	Proportion (%)
Corn	55.00
Soybean meal	30.30
Meat bone meal	6.00
Rice bran	2.00
Cooking oil	5.00
Top mix[1]	0.25
L-Lysine HCl	0.10
DL-Methionine	0.15
Limestone	1.00
Salt	0.25
Nutrient content	**Amount (%)**
Crude protein	22.01
Metabolic Energy (kcal/kg)	3113.12
Fat	8.24
Fiber	3.55
Calcium	1.10
Phosphorus	0.64
Lysine	1.18
Methionine	0.45
Threonine	0.78

[1] Top mix mineral-vitamin contains: Ca = 32,5%; P = 1,0%; Fe = 0,6g, Mn = 4g; Iod = 0,075g; Zn = 3,75g; Vitamin B_{12} = 0,5mg; Vitamin A = 300.000 IU; Vitamin D_3 = 50.000 IU.

The corn-soy bean meal basal diet (Table 1) was formulated to meet nutrient requirement as recommended by Hartadi *et al*. (2005) [13].

A total of number 192 one-day-old New Lohmann broiler chicks were divided into six treatments and 4 replications in a completely randomized design. Each birds received one of the following treatments: drinking water only (negative control; T_0), drinking water + 50 ppm of tetracycline (positive control; T_1), drinking water + 0,015% v/v LLE (T_2), drinking water + 0,030% v/v LLE (T_3), drinking water + 0,015% v/v nanocapsulated LLE (T_4), or drinking water + 0,030% v/v nanocapsulated LLE (T_5). The basal diet and drinking water were supplied for *ad libitum* consumption.

2.5. Antioxidant Assessment in Broiler Blood

At day 35, blood samples were extracted from the jugular vein and sent quickly to The Integrated Research and Testing Laboratory, Universitas Gadjah Mada for antioxidant profile assessment. Catalase (CAT) and Glutathione peroxidase (GPx) contents were measured using enzyme-linked immunosorbent assay (ELISA, Wuhan Fine Biotech Co., Ltd., China).

2.6. Meat Proximate Analysis

Dry matter and organic matter of the meat were analysed using method described by Horwitz and Latimer [14]. Ceramic alumina crucible cups were properly weighed and afterward 1g chicken meat samples were placed into the cups. The samples were oven dried using temperature of 105°C for 24 hours. Dry matter of the samples were calculated using the following equation:

$$\% \text{ Dry Matter} = \frac{\text{WDC - WC}}{\text{WS}} \times 100$$

WDC = Weight dry matter and crucible
WC = Weight crucible
WS = Weight sample

For assessing organic matter, the above samples, then, incinerated using muffle furnace with the temperature of 550°C for 2 hours. After that, the samples were cooled down overnight and dried again in 105°C oven for 2 hours. Organic matter of the samples were calculated using the following equation:

$$\% \text{ Dry Matter} = \frac{\text{WOC - WC}}{\text{WS}} \times 100$$

WOC = Weight organic matter and crucible

For fat analysis, Soxhlet method described by Nielsen [15] was used to remove fat from the samples. Initially, 0.7g of fresh meat samples were taken and packed inside nitrogen-free weighing papers, then were oven dried overnight in 105°C temperature. After dried, the samples were placed into thimble of Soxhlet extractor. The thimble was filled with petroleum ether through condenser until the samples were completely soaked. Boiling flask of the Soxhlet extractor was also filled with approximately 50 mL of petroleum ether, then was attached to thimble and heated using water bath with temperature of 80°C for 16 hours. The fat-removed samples in thimble was taken out and air dried until petroleum ether was completely evaporated. The samples were then redried overnight using 105°C oven. The fat content was calculated using below equation:

$$\% \text{ Fat} = \frac{(W_1 - W_2) - \text{Weight weighing paper}}{\text{Weight sample}} \times 100$$

W_1 = Weight dry matter with weighing paper
W_2 = Weight fat-removed dry matter with weighing paper

Automated Kjeldahl method, as described by Horwitz and Latimer [14], was also used to analyze crude protein content in broiler breast meat.

2.7. Statistical Analysis

All collected data were subjected to One way ANOVA in Statistical Package for the Social Sciences (SPSS) version 21.0.

3. RESULT AND DISCUSSION

3.1. Blood Antioxidant Profiles

Table 2. CAT and GPx profiles of broiler's blood

Treatment	CAT	GPx
T_0	503.07^{c}	3.66^{c}
T_1	510.63^{c}	8.56^{b}
T_2	689.29^{abc}	10.41^{ab}
T_3	759.48^{ab}	12.15^{ab}
T_4	604.47^{bc}	15.44^{a}
T_5	839.34^{a}	12.52^{ab}
SEM	32.818	0.977
P-value	0.007	0.004

Results in current experiment (Table 2) indicated that the amount of CAT in the blood was escalated remarkably in group T5 ($P<0.01$), while GPx was drastically increased in group T4 ($P<0.01$) following nanocapsulated LLE supplementations. This finding was in line with the findings of Zhou *et al*. [16] that reported supplementations of flavonoid baicalein, extracted from *Scutellaria baicalensis's* root, increased the profile of CAT and GPx in the blood serum of broiler chickens. Daramola [17] also noticed that supplementation of bitter leaf meal and *Moringa oleifera* leaf meal escalated the quantity of CAT and GPx in blood serum. According to

Lee *et al*. [17] study, dietary phytochemicals extracted from various vegetables, fruits, spices, and herbal medicines activated Nuclear factor erythroid 2-related factor 2 (Nrf2) to produce antioxidant. Therefore, the use of phytochemical had positive effect on antioxidant profiles in broiler's blood [18].

3.2. Proximate Compositions of Broiler Meat

As shown in Table 3, LLE supplementations did not influence the percentage of carcass as well as the contents of dry matter and fat in the meat, except the content of organic matter. The groups T_3 and T_4 provided the highest organic matter content ($P<0.01$), followed by T_0, T_2. The groups T_5 and T_1 had the lowest organic matter content among other groups. Similar study by Tashla *et al*. [19] showed that dietary medicinal plants supplementations did not influence dry matter, but reduced fat content of the meat of broiler chickens. Kristina *et al*. [20] also showed that dietary supplementations of phenolic compounds, such as thymol, tannic acid, and gallic acid did not affect dry matter and ash contents, but decreased the protein and fat contents of chicken breast meat. With reference to a review conducted by Baracho *et al*. [21], the factors ranging from age, gender, nutrition, management, bird density, harvesting method, environmental condition, and handling, might results in varieties of meat quality, and this statement agreed with the data of carcass, dry matter and fat only [21]. Although the analysis methods used in current study were the same with other studies, the reason that caused different result in the organic matter content has still been remain unknown.

Table 3. Proximate compositions in broiler meat

Treatment	Dry matter (%)	Organic matter (%)	Fat (%)	Protein (%)
T_0	26.73	1.43^{ab}	1.10	22.30
T_1	26.01	1.33^{b}	1.28	23.83
T_2	26.01	1.47^{ab}	1.27	21.57
T_3	25.86	1.63^{a}	1.32	22.86
T_4	26.12	1.66^{a}	1.32	22.98
T_5	25.85	1.53^{ab}	1.29	21.00
SEM	0.133	0.034	0.056	0.319
P-value	0.180	0.045	0.907	0.086

4. CONCLUSION

Nanoencapsulated LLE could be used as a natural antioxidant due to the beneficial effects on improving blood catalase and gluthathione levels, two enzymes that closely related to the antioxidation process in blood of broiler chickens.

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