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Effect of Mangosteen Pericarp Meal and Vitamin E Supplements on the Performance, Blood Profiles, Antioxidant Enzyme and HSP 70 Gene Expression of Laying Hens in Tropical Environment

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Abstract: This research aims to study the effects of mangosteen pericarp meal (MPM) and vitamin E (VE) on the performance, blood profile, antioxidant enzyme and HSP 70 gene expression of laying hens in tropical environment when supplemented in their diets. In this study, 160 laying hens of Lohman strains were used; they were 24 weeks old and were observed for 11 weeks. They were arranged in a completely randomized design with four treatments and four replications (10 birds each). The treatments consisted of R0 (control diet), R1 (R0+1 g MPM/kg rations), R2 (R0+2 g MPM/kg rations) and R3 (R0+200 mg VE/kg rations). The data were analyzed using analysis of Variance (ANOVA); if there was any significant difference among the treatments, further tested was done using Duncan Multiple Range Test. The results showed that supplementation of MPM and VE in the diet of the hens significantly ($p < 0.01$) increased the activity of the antioxidant enzyme superoxide dismutase (SOD) and decreased the thiobarbituric acid reactive substances (TBARS) values. Supplementing the hens' diet with MPM and VE did not affect ($p > 0.05$) their performance, blood profile and HSP70 gene expression. It is concluded that supplementing the diets of the laying hens with MPM and VE increased the activity of SOD and decreased TBARS values of yolk.

Key words: HSP 70 gene, laying hens, mangosteen pericarp meal, performance, vitamin E

INTRODUCTION

Laying hens raised in tropical climate countries such as Indonesia may be exposed to heat stress. This heat stress specifically occurs in an environment where the temperature is above the thermoneutral zone. Heat stress may affect the performance, physiological and productivity of chickens (Mazzi *et al.*, 2003; Mashaly *et al.*, 2004; Gu *et al.*, 2008), leading to death (Noor and Seminar, 2009) and economical losses (St-Pierre *et al.*, 2003; Guerreiro *et al.*, 2004).

Heat stress at cellular level may induce oxidative stress of the body leading to excessive free radicals (Mujahid *et al.*, 2007). Free radicals may damage protein, nucleic acid (Black, 2004; Kern and Kehrer, 2005) and cause membrane lipid peroxidation (Yalcyn *et al.*, 2012). Naturally, chickens may counteract free radical by increasing the activity of antioxidant enzyme such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT). If chickens experience long heat stress, the activity of antioxidant enzyme will decrease due to the increase in mineral excretion (Zn, Cu and Se) and Vitamin (C and E), which plays the role of an antioxidant cofactor (Sahin and Kucuk, 2003). Noor and Seminar (2009) stated that if stress continues to increase and the body metabolism is unable to cope

with the stress level then the genetic pathway is used by activating heat shock protein 70 (HSP 70) gene. HSP 70 gene is activated to prevent protein from high temperature and degradation as well as permanent cell damage, which in turn can affect survival (Etches, 2008). The addition of antioxidant into diet is to increase the activity of antioxidant enzyme which acts to protect the body from oxidative damage caused by free radical received during heat contact. Most antioxidants used are synthetic. In these recent years, research regarding the utilization of natural antioxidant such as oregano, scullcap, rosemary, green tea and black tea has already been proven that they can reduce the effect of stress, increase antioxidant enzyme activity and reduce lipid peroxidation in birds (Uganbayar *et al.*, 2005; Florou-Paneri *et al.*, 2006; An *et al.*, 2010; Park *et al.*, 2015) and sheep (Fassah *et al.*, 2015).

Mangosteen pericarp meal (MPM) is one of the mangosteen (*Garcinia mangostana* L.) wastes, which has been processed. MPM contains active compounds such as xanthone and its derivatives (alpha-mangostin, gamma-mangostin, mangostino, mangostingon, 8-hydroxycudraxanthone G, cudraxanthone G, 8-deoxygartanin, garcimangosone B, garcinone D, garcinone E, gartanin, 1-isomangostin, smeathxanthone

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A and torophyllin A) (Jung *et al.*, 2006). These compounds have pharmacological functions including antioxidant (Moongkarndi *et al.*, 2004; Jung *et al.*, 2006). Vitamin E (VE) is one of the fat-soluble vitamins acting as an antioxidant (Kirunda *et al.*, 2001), which can counteract free radical (Florou-Paneri *et al.*, 2006). This leads to the improvement of the immune system by preventing involved cells such as lymphocytes, macrophages and plasma cells from oxidative damage, proliferation and macrophage phagocytosis (Gebremichael *et al.*, 1984). Several researches reported that adding VE supplement into hens' diet during exposure to heat can increase the egg quality and performance (Puthongsiriporn *et al.*, 2001; Ipek *et al.*, 2007; Irandoust *et al.*, 2012), reduce lipid peroxidation in yolk (Cherian *et al.*, 1996; Kirunda *et al.*, 2001) as well as serum and tissue (Jiang *et al.*, 2013).

Data on supplementing MPM in laying hens' diet are still limited. Therefore, this research aims to study the effect of MPM (natural antioxidant) and VE (synthetic antioxidant) on the performance, blood profile, antioxidant enzyme and HSP 70 gene expression on laying hens in tropical environment.

MATERIALS AND METHODS

Design, bird and diet: All procedures and methods used in this research had been approved by the Animal Ethic Commission of Bogor Agricultural University (IPB) No. 12-2014 IPB. 160 laying hens of Lohman strain; (Japfa Comfeed, Indonesia) 24 weeks old were used in this work. There were four feeding treatments: R0 (control), R1 (R0+1 g MPM/kg rations), R2 (R0+2 g MPM/kg rations) and R3 (R0+200 mg VE/kg rations), with four replications. Each replication used 10 chickens (2 hens/cage sized of 35 x 36 x 42 cm). Drinking water was provided *ad libitum* and feed was provided twice a day; that is, in the morning (07.00 am of Indonesian Western Time) and evening (05.00 pm of Indonesian Western Time). One week prior treatment was allocated for chickens' adaptation. This research was carried out for 11 weeks. The cage was equipped with lamp for 16L/8D.

Vitamin E (VE) used in this research was Vitamin E, alpha-tocopherol acetate (Interchemie, Netherlands). Mangosteen pericarp used was collected from smallholders' estate area in Leuwiliang, Bogor, West Java. Mangosteen pericarp was processed into meal using drying oven (LTE Scientific Swallow, UK) at temperature of 50°C and then milled using disk mill (Jiayu Electrical Machinery, Taiwan). The proximate analysis of MPM was conducted using AOAC method (1995). Meanwhile, analysis of saponin, tannin, antioxidant and alpha-mangostin was carried out using Hiai *et al.* (1976), Makkar *et al.* (1993), Salazar-Aranda *et al.* (2011) and Suvarnakuta *et al.* (2011) method, respectively. The nutrient content of MPM is shown in

Table 1: Chemical composition and nutritive value of mangosteen pericarp meal (MPM) as fed basis

Chemical composition	
Moisture (%)	12.43
Ash (%)	2.37
Crude protein (%)	4.37
Crude fat (%)	0.98
Crude fiber (%)	24.20
Beta-N (%)	60.25
Metabolizable energy (kcal/kg)	4676
Ca (%)	0.12
P (%)	0.02
Saponin (g/100 g)	8.24
Tannin (g/100 g)	32.49
Antioxidant (IC ₅₀ ppm)	11.15
Alpha-Mangostin (ppm)	40.63

Table 2: Ingredient and nutrient content of basal diets

Ingredient	(%)
Maize	55.00
Soybean meal	24.00
Fish meal	8.50
Coconut oil	3.00
CaCO ₃	8.50
Salt	0.20
DL-Methionine	0.30
Premix ^a	0.50
Nutrient content: ^b	
Metabolizable energy (kcal/kg)	2900.75
Crude protein (%)	17.19
Crude fiber (%)	2.18
Crude fat (%)	5.32
Ca (%)	3.83
Available P (%)	0.49
Methionine (%)	0.73
Lysine (%)	1.33
Cystine (%)	0.34
Tryptophan (%)	0.27
Threonine (%)	0.95
Arginine (%)	1.42
Na (%)	0.15
Zinc (mg/kg)	38.55

^aPremix provided (in mg/kg premix): vit A 500.000 IU; vit D 100.000 IU; vit E 150 mg; vit K 50mg; vit B1 50 mg; vit B2 250 mg; vit B12 250 mcg; niacinamide 375 mg; Ca-d-pantothenate 125 mg; folic acid 25 mg; choline chloride 5.000 mg; Glycine 3.750 mg; DL-methionine 5.000 mg; Mg sulfat 1.700 mg; Fe sulfat 1.250 mg; Mn sulfat 2.500 mg; Cu sulfat 25 mg; Zn sulfat 500 mg; K iodine 5 mg. ^bCalculated value

Table 1. Feed requirement of laying hens was determined using Leeson and Summers (2005)'s method. The Ingredient and nutrient content used in this research are as shown in Table 2.

Performance: Feed intake (g/bird/day) was recorded weekly at each replication by weighing the remaining diet. Feed conversion was calculated by dividing the feed intake with egg mass. Egg production (%) was calculated by dividing egg number with the birds on each day. Egg weight (g) was measured with digital weighing scale (Osuka-HWH®, Japan). Egg mass was calculated by multiplying the egg production with egg weight.

Antioxidant enzymes and lipid peroxidation: SOD enzyme activity of yolk was analyzed using Misra and Fridovich (1972). Lipid peroxidation of yolk was determined using Thiobarbituric Acid Reactive Substances (TBARS) by Rice-evans and Anthony method (1991). The activity of SOD and TBARS values was identified using spectrophotometer (Hitachi U-2001, Japan).

Blood profiles: Blood sampling was carried out at the end of the study and chickens were randomly selected from each treatment (one hen per replication). Blood was taken from jugular vein using a sterile syringe. Sample was then collected into tube containing Ethylenediaminetetra-acetic acid (EDTA), Analar® (BDH Laboratory Supplies Poole, England). Blood sampling analysis consisted of erythrocyte, leukocytes, heterophile, lymphocytes and manocytes using haemocytometer and microscope (Nikon YS 100, Japan), hemoglobin using complete reagent kit (Merckotest®) and hematocrit using Micro-Capillary Reader (USA).

RNA extraction and real time RT-PCR: RNA extraction and real time RT-PCR was carried out at the end of study by slaughtering the chicken (one hen per replication). The brain was taken out and then placed into tube (1,5 µl) containing 500 µl RNA later (RNA later® solution, USA). The total RNA was extracted using GeneJET RNA Purification Kit (Thermo Scientific, Lithuania, EU) and then stored at -20°C temperature. RNA was then transcribed into complementary DNA (cDNA) using Transcriptor Synthesis First Strand cDNA Kit (Thermo Scientific, Lithuania, EU) and PCR (Gene Amp PCR system 9700, Applied Biosystem, Singapore). Quantification of cDNA was analyzed using Nanodrop Spectrophotometers (Nanodrop Spectrophotometers 2000, Thermo Scientific, USA). cDNA was then used for quantitative real-time reverse transcription-polymerase chain reaction (qRT PCR) (Analytic Jena, AG qTower 4 kanal, Germany) and for RT PCR, SYBR GREEN select master kit was used (Applied biosystem, USA).

The primer used in this study was designed to see the HSP 70 gene expression cGAPDH: F-5'GTGTT ATCAT CTCAG CTCCC TCAG-3', R-5'GGTCA TAAGA CCCTC CAC AA TG-3', cHSP70: F-5'GACAA GAGTA CAGGG AAGGA GAAC-3', R-5'CTGGT CACTG ATCTT TCCCT TCAG-3' (Al-Zhgoul *et al.*, 2013). 10 µl reactant solution consisted of: 5 µl master mix, 0.25 µl forward primer, 0.25 µl reverse primer, 1 µl cDNA sample and 3,5 µl nuclease free water. Real time PCR condition used were; 95°C for 5 min, 40 cycle at 95°C for 10 sec, 60°C for 20 sec and 72°C for 30 sec. Data from PCR were analyzed using 2[delta-delta] CT method (Schmittgen and Livak, 2008).

Statistical analysis: Data were then statistically analyzed using analysis of Variance (ANOVA) by means of Statistical Package for the Social Sciences (IBM®SPSS® versi 21.0). Duncan Multiple Range Test was applied to determine the differences among treatments. Differences were considered significant at $p < 0.05$.

RESULTS

Laying performance: The effect of Mangosteen pericarp meal (MPM) and Vitamin E (VE) supplements on the performance of the laying hens is shown in Table 3. 1 g MPM/kg ration, 2 g MPM/kg ration and 200 mg VE/kg ration supplements did not influence ($p > 0.05$) feed intake, egg weight, egg production, egg mass and feed conversion.

Blood profiles: 1 g MPM/kg ration, 2 g MPM/kg ration and 200 mg VE/kg ration supplements did not influence ($p > 0.05$) the erythrocyte, hemoglobin, hematocrit, leukocytes, heterophile, lymphocytes, manocytes and H:L ratio compared to control (Table 4).

Antioxidant enzymes and lipid peroxidation: 1 g MPM/kg ration, 2 g MPM/kg ration and 200 mg VE/kg ration supplements increased ($p < 0.01$) the activity of SOD enzyme of yolk compared to control. 1 g MPM/kg ration, 2 g MPM/kg ration and 200 mg VE/kg ration reduce ($p < 0.01$) the TBARS values of yolk compared to control (Table 5).

Gene expression of heat shock protein 70: Gene expression of heat shock protein 70 (HSP 70) in laying hens was not influenced by 1 g MPM/kg ration, 2 g MPM/kg ration and 200 mg VE/kg ration (Table 6).

DISCUSSION

Mangosteen pericarp meal (MPM) consists of active compounds, namely: xanthone and its derivatives (alpha-mangostin, gamma-mangostin, mangostinon, mangostingon, 8-hydroxycudraxanthone G, cudraxanthone G, 8-deoxygartanin, garcimangosone B, garcinone D, garcinone E, gartanin, 1-isomangostin, smeathxanthone A and tovophyllin A) (Jung *et al.*, 2006), anthocyanins (Palapol *et al.*, 2009), saponin and tannin. These compounds have pharmacological properties such as: antioxidant (Moongkarndi *et al.*, 2004; Jung *et al.*, 2006), anti-tumor, anti-bacterial, anti-malaria (Pedraza-Chaverri *et al.*, 2008), anti-allergic (Chae *et al.*, 2012) and anti-cancer (Mizushima *et al.*, 2013). Vitamin E (VE) also acts as antioxidant (Kirunda *et al.*, 2001) preventing free-radical (Florou-Paneri *et al.*, 2006); it helps the immune system (Xiao *et al.*, 2011) by protecting the cells involved such as lymphocytes, macrophages and plasma cells against oxidative damage, proliferation and macrophage phagocytosis (Gebremichael *et al.*, 1984). Both of these

Table 3: Effect of supplementation mangosteen pericarp meal, vitamin E on laying hens performance

Parameters	Treatment			
	R0	R1	R2	R3
Feed intake (g/hen/day)	103.75±9.31	100.26±11.65	102.96±11.28	103.95±95
Egg weight (g)	53.13±1.77	52.25±1.99	51.94±1.35	52.25±1.30
Egg production (%)	86.65±9.29	85.15±7.61	85.40±7.98	84.99±9.07
Egg mass	46.41±5.19	44.17±5.21	44.04±4.32	44.75±5.06
Feed conversion	2.28±0.29	2.32±0.34	2.39±0.29	2.35±0.25

R0 (control feed), R1 (R0+1 g MPM/kg ration), R2 (R0+2 g MPM/kg ration), R3 (R0+200 mg VE/kg ration)

Table 4: Effect of supplementation mangosteen pericarp meal, vitamin E on blood profiles in laying hens

Parameters	Treatment			
	R0	R1	R2	R3
Erythrocytes (10 ⁶ /mm ³)	2.33±0.06	2.44±0.11	2.34±0.21	2.50±0.18
Hemoglobin (%)	10.75±0.73	11.52±1.15	10.58±0.83	12.47±2.28
Hematocrit (%)	21.74±1.42	21.88±2.17	22.50±1.92	21.82±3.11
Leukocytes (10 ³ /mm ³)	12.93±1.03	8.50±2.70	7.90±4.14	9.70±3.79
Heterophils (%)	32.67±13.20	34.00±7.07	32.25±9.74	33.75±4.99
Lymphocytes (%)	60.00±11.97	58.75±6.24	61.75±7.85	59.50±5.45
Monocytes (%)	6.00±1.15	6.00±1.00	4.25±0.50	5.50±1.29
H:L	0.61±0.45	0.59±0.18	0.54±0.23	0.58±0.14

R0 (control feed), R1 (R0+1 g MPM/kg ration), R2 (R0+2 g MPM/kg ration), R3 (R0+200 mg VE/kg ration)

Table 5: Effect of supplementation mangosteen pericarp meal, vitamin E on SOD and TBARS on yolk

Parameters	Treatment			
	R0	R1	R2	R3
SOD (unit/mg protein)	1.77±0.10 ^C	2.53±0.22 ^B	6.13±0.47 ^A	2.53±0.22 ^C
TBARS (mg/100 g)	0.98±0.09 ^A	0.63±0.06 ^B	0.50±0.02 ^C	0.57±0.09 ^{BC}

R0 (control feed), R1 (R0+1 g MPM/kg ration), R2 (R0+2 g MPM/kg ration), R3 (R0+200 mg VE/kg ration)

^{ABC} Means within the same row with different superscripts are significantly different (p<0.01)

Table 6: Effect of supplementation mangosteen pericarp meal, vitamin E on HSP 70 gene expression in the brains

Parameters	Treatment			
	R0	R1	R2	R3
HSP 70	0.99±0.24	0.88±0.06	1.00±0.37	1.00±0.29

R0 (control feed), R1 (R0+1 g MPM/kg ration), R2 (R0+2 g MPM/kg ration), R3 (R0+200 mg VE/kg ration)

materials may have benefit in improving and enhancing the birds' performance and chickens' product quality.

Feed intake, egg weight, egg production, egg mass and feed conversion were not influenced by MPM and VE supplements. These findings are in accordance with that of Florou-Paneri *et al.* (2006) who compared the effect of VE supplement on laying hens. VE requirement of laying hens is 50 IU/kg (Leeson and Summers, 2005). Although the excess supplementation of VE into feed is not formally reported, feed containing 400 mg alpha-tocopherol acetate/kg (Jiang *et al.*, 1994) and 600 mg/kg (Mori *et al.*, 2003) could reduce feed intake and egg production. This research showed that 1 g MPM/kg ration, 2 g MPM/kg ration and 200 mg VE/kg ration reduced the negative effect of heat stress and are safe on laying hens without reducing their productivity.

Animal blood profiles described the nutrient physiological status in accordance with the internal and external environment. Invisibility profile changes Hematology and blood leukocytes differentiation of the chicken on this research (Table 4), due to good nutrient intake so that erythrocytes, hemoglobin, hematocrit function in transporting nutrients, O₂, CO₂ and metabolic

waste in the normal range. Normal condition of erythrocytes, hemoglobin, hematocrit positively impacted the chickens' performance in tropical environment so that chickens are not experiencing stress. Stress indicators one of which can be seen by the ratio of H: L. The ratio of H: L normal chicken is 0.3-0.5 (Swenson, 1984). Some researchers previously reported that giving of vitamins and minerals that act as antioxidants in the ration can reduce the effects of heat stress on hematologi profile (Aengwanich and Chinrasri, 2003; Sahin *et al.*, 2003). TKM and vitamin E as an antioxidant activity, this research showed that 1 g MPM/kg ration, 2 g MPM/kg ration and 200 mg VE/kg ration reduced the negative effect of heatstress and are safe on laying hens without interfering the health of the chicken.

Research on improving the oxidative stability of egg and meat through the addition of antioxidant such as sesamol (Chen *et al.*, 1999), palm oil (Kang *et al.*, 2001), green tea (Rababah *et al.*, 2006) in the feed can reduce the TBARS values. This study showed that high antioxidant status in yolk was followed by decrease in TBARS values (Table 5). TBARS is the most frequently used test to determine lipid oxidation on a product

(Cherian *et al.*, 2002). The value of the TBARS indirectly reflected the level of lipid peroxidation and excess reactive oxygen species (ROS) in the body. The reduction of the TBARS values is associated with the supplementation of MPM and VE. Xanthone contained in MPM works by donating a hydrogen atom (H) derived from hydroxy group (OH) to the peroxy radicals (ROO^{*}) so that stable radical compounds (ROOH) are formed (Pedraza-Chaverri *et al.*, 2008). Jung *et al.* (2006) report that some xanthone derivatives such as alpha-mangostin can counteract the peroxy radical anion (ONOO⁻). VE works by donating a hydrogen atom which converts peroxy radical (ROO^{*}) to become less reactive radical tocopherols, making it unable to damage the fatty acid. Furthermore, VE also has a synergistic effect through enhancing the performance of antioxidant enzymes such as SOD, GSH-Px and CAT (Leeson and Summers, 2001).

Stress at the cellular level such as physical, chemical, biological (including thermal radiation) can stimulate the synthesis of HSP genes (Staib *et al.*, 2007; Sahin *et al.*, 2010). HSP is a protein that serves as the last defense to prevent the organism from stress. This research showed that the expression of HSP 70 gene did not influenced by 1 g MPM/kg ration, 2 g MPM/kg ration and 200 mg VE/kg ration. Gu *et al.* (2012) reported that HSP 70 could also increase the capacity of the antioxidant and inhibit the production of lipid peroxidation. Recently, researchers found that animal under stress may also experience small intestine mucosa, impaired digestion and absorption which reduces feed intake (Ryder *et al.*, 2004). Hao *et al.* (2012) reported that active HSP70 does not affect the intestinal morphology under heat stress; however, there is a strong positive correlation between HSP70 expression and the digestive enzyme activity. The expression of HSP70 significantly increases the amylase, lipase and trypsin activities under heat stress. Provision of alpha and gamma-mangostin to mouse could modulate the production of interleukin-6 (IL-6), prostaglandin D₂ (PGD₂) and leukotriene C₄ (LTC₄). Besides, alpha and gamma-mangostin can reduce the expression of cyclooxygenase-2 (COX-2) mRNA involved in the process of allergic diseases (Chae *et al.*, 2012). Other studies reported that alpha-mangostin potentially suppresses proliferation of colon cancer cells in humans. Cancer cell is suppressed by inhibiting the DNA topoisomerases (topos) metabolic enzyme that contributes to the replication, repair, recombination of DNA, and cell division (Mizushima *et al.*, 2013).

VE is found throughout the body, both in cell and cell membrane. At the cellular level, tocopherol is involved in the biosynthesis of DNA, possibly in the regulation of the pyrimidine into the structure of nucleic acid (Leeson and Summers, 2001). VE supplemented in broiler chicken ration reduced gene expression of sterol carrier protein 2 (SCP2) (Xiao *et al.*, 2011). SCP2 is a gene that may

facilitate hydroperoxide lipid among membranes and thus potentially spread the peroxidative damage in stress condition (Kriska *et al.*, 2006).

Each supplement treatment did not influence the gene expression of HSP 70. This could be because a dose of the treatment was not yet right for the adaptive laying hens living in tropical environment. Laying hens used in this research (final stock) had parent stock which had been produced in Indonesia long time ago (Poultry Division, 2005). Therefore, in order to determine the gene expression response, chickens with higher responsiveness to stress is needed.

Conclusion: Supplementing MPM and VE in the diet of the hens could increase their antioxidant enzyme activity and reduce the TBARS value of yolk. MPM and VE supplements did not influence the performance, blood profile and HSP 70 gene expression of the laying hens.

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