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Research Article
Evaluation of *Piper betle* L. Aqueous Extract on *Salmonella* sp. Isolates from Small Intestine of Quails

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Abstract

Background and Objective: *Piper betle* L. leaves (beetle leaves) is an example among medicinal plants that could inhibit the growth of *Salmonella*. This research was aimed to evaluate beetle leaves aqueous extracts ability to inhibit *Salmonella* sp. by *in vivo* and *in vitro* research. **Materials and Methods:** The research was consisted of three steps. Those steps were finding optimum concentration of extract supplementation, raising the quails and evaluating *Salmonella* sp. colonies in small intestine of quails which had been given treatment for six weeks. A completely randomized design of seven treatments and three replication was used in this study. The treatments were: P0 = commercial anti-stress supplementation since Day Old Quail (DOQ); P1 = 10% extract supplementation since DOQ; P2 = 20% extract supplementation since DOQ; P3 = 30% extract since DOQ; P4 = 40% extract supplementation since laying period; P5 = 10% extract supplementation since laying period; P6 = 20% extract supplementation since laying period; P7 = 30% extract supplementation since laying period. Data was analyzed with Duncan post hoc test if shows a significant difference ($p<0.05$) using SPSS as statistical analysis software. **Results:** According to the inhibition zone, the results show that right level supplementation of extracts to be used was 10% (2.5 mm), 20% (3.5 mm) and 30% (7 mm) which had been added in drinking water. Compared to control treatment (P0), addition of extracts (P1, P2, P3, P4, P5 and P6) could decrease the colonies of *Salmonella* sp. in small intestine of quail significantly ($p<0.05$). **Conclusion:** Beetle leaves aqueous extract supplementation could inhibit *Salmonella* contamination. Higher concentration of extract leads to higher inhibition zone. The best treatment to reduce *Salmonella* sp. was P6. Extracts supplementation are better given at laying period rather than DOQ period.

Key words: Natural medicine, herbal antibiotic, *Piper betle* L., *Salmonella* sp., phytogenic feed additive, coturnix coturnix japonica, agar well diffusion

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Salmonella infections in poultry could lead into human salmonellosis[1]. Most of *Salmonella* contamination come from contaminated food and water[2]. To prevent *Salmonella* contamination in poultry, farmers usually use Antibiotic Growth Promoters (AGP) to kill *Salmonella*[3]. Antibiotic Growth Promoters (AGP) utilization in poultry feed has been banned in some countries[4]. The ban was due to the residues in the animals body and cause bacteria in becoming resistant to certain antibiotics. The residue in livestock products are very dangerous for consumers because it can cause allergies and bacteria to certain drugs[5]. *Salmonella* has become a resistant bacteria to some antibiotics such as ampicillin, chloramphenicol and fluoroquinolones[6]. The use of AGP on animal feed is aimed to kill the bacteria which found in the digestive tract to increase the absorption of nutrients[7]. This shows that farmers still rely on the use of AGP to improve livestock productivity. So it is important to replace the use of these antibiotics.

The use of natural antibiotics is an efforts that can be done to replace the AGP[8]. Natural antibiotics usually are obtained from plant oil extracts and another secondary metabolites which could inhibit or kill bacteria[9]. Beetle leaves extract had an antimicrobial, anti-oxidative and anti-hemolytic effect[10]. Beetle leaves contain a lot of active substances that can inhibit some bacteria[11]. The content has been demonstrated in study that beetle leaves have the potential to be used as a natural antibiotic for poultry in some countries[11]. The extraction process is also important to ensure effectiveness in inhibiting bacteria beetle leaves. The ethanol extract of green beetle leaves is more effective than extracted with water solvent in inhibiting the growth of pathogenic bacteria[12]. Aqueous extractions method are easier to perform rather than the methanolic extraction methods. Most of the aqueous extraction methods didn't show an inhibition zone on *Salmonella* colonies. But a proper method of aqueous extraction methods could bring a better results compared to ethanolic extracts. Some aqueous extraction methods of plants showed a better result than ethanolic extraction methods[13].

This research was aimed to evaluate the ability of beetle leaves aqueous extract to inhibit *Salmonella* sp. and find its optimum levels to be used for certain purpose such as traditional medicine for human and feed supplements for livestock. Extraction storage time evaluation was required to be observed. Afterwards, *Salmonella* contaminations on small intestine of quails would be observed. This research probably the first research that had been done to observe the anti-*Salmonella* effect of beetle leaves aqueous extract both on *in vitro* and *in vivo* test.

MATERIALS AND METHODS

The test was completed with 210 heads of laying quails (*Coturnix coturnix japonica*) by the age of 6 weeks. The ration that has been used was commercial ration from Sinta Feedmill Corporation. Beetle leaves were obtained from Nagrak, Sukabumi, West Java, Indonesia. Extract concentration of 100% was obtained by mixing 100 g of water and 100 L of fresh leaves (1:1 (w/v)), chopping the mixture with blender, boiling the liquor at $\pm 90^{\circ}$ C in 15 min and squeezing it to get the extracts. In addition to get a lower concentration of the extracts, water was added to reduce the extracts concentration. Extract concentrations of 10, 20 and 30% was obtained by mixing 100, 200 and 300 mL extracts with 900, 800 and 700 mL of water. Inhibition zone tests were conducted in the Laboratory of Food Security, Faculty of Agriculture Technology, Bogor Agricultural University (2016). There were two methods to evaluate extracts ability to inhibit *Salmonella*. Agar well diffusion test was performed to measure the inhibition zone[14]. Agar dilution test was aimed to measure the durability of storage time within 1 month[14]. Agar well diffusion test and agar dilution test methods which have been conducted were similar with the methods explained by Balouiri *et al.*[14]. In addition to giving it to the animals, extracts concentration should had been given as low as possible due to quails palatability and production efficiency. To find the proper concentration, 100% concentration had been tested and reduced it gradually at a half of it into 50, 25, 12.5 and 6.25%. Afterwards, the test again was conducted to find low, medium and high (<3 mm, 3-6 mm, >6 mm) level of inhibition[15] to give it to the quails.

Experimental design: Completely randomized design of seven treatments and three replication was used in this study. The treatments were control treatment which has been given a commercial anti-stress (Vita Stress) in the drinking water and supplementation of three different concentration between 10, 20 and 30% of extracts in the drinking water on quails which had been given from Day Old Quail (DOQ) and laying period. The ration which had been given in this study was quails commercial diet from PT. Sinta Feedmill. The treatment details were P0 = Vita Stress supplementation since DOQ; P1 = 10% extracts supplementation since DOQ; P2 = 20% extracts supplementation since DOQ; P3 = 30% extracts supplementation since DOQ; P4 = 40% extracts supplementation since laying period; P5 = 10% extracts

Table 1: Micro nutrient contents of commercial anti-stress

Micro nutrients	Values
Vitamin A	6.000.000 IU
Vitamin D_3	1.200.000 IU
Vitamin E	2.500 IU
Vitamin K	3.000 mg
Vitamin B_1	2.000 mg
Vitamin B_2	3.000 mg
Vitamin B_6	1.000 mg
Vitamin B_{12}	2 µg
Vitamin C	20.000 mg
Nicotinic Acid	15.000 mg
Calcium-D-Pantothenate	5.000 mg
Electrolyte (Na, K, C, Mg)	750.000 mg
Carrier	1 kg

Vita stress composition from product catalogue of PT. Medion Indonesia

Table 2: Nutrient compositions of quails diet

Composition	Contents (%)
Ash	<6.5
Water	12
Protein	21-23
Fat	4-8
Crude fiber	<4
Ca	0.9-1.1
P	0.7-0.9

Sinta Feedmill BR-1 composition from product catalogue of PT. Sinta Feedmill

supplementation since laying period; P6 = 20% extracts supplementation since laying period; P7 = 30% extracts supplementation since laying period. Commercial anti-stress and diet contents are shown in Table 1 and 2.

RESULTS AND DISCUSSION

Active compounds of beetle extract: The active compounds of beetle extract that has been observed in this research are shown on Table 3. The extract contains high level of alkaloids, flavonoids and phenolic hydroquinones. Steroids, triterpenoids and saponins level were normal. Phenolics have an ability to inhibits microbe[16]. Some anti-salmonella component in beetle extracts is sterols[10]. Nevertheless, karvakrol and eugenol are components which also have an antimicrobial effects[17]. Tannins was found in extract components at low level[18,19]. The main essential oils content of beetle leaves is eugenol with 50.29% concentration from its essential oils[20]. Eugenol could distract by disrupting its cellular membrane[21].

Extraction methods from these researches are different from our research. Those methods using ethanol extract as a solvent while our research using a water as a solvent. Ethanolic extracts of beetle leaves are better rather than aqueous extract on inhibiting bacteria[22]. Those results could be caused of the ethanolic extracts comprise more active compound rather than aqueous extract. Results showed that a proper method using water as a solvent is as good as using ethanolic extraction methods.

Table 3: Chemical constituent of beetle leaves extract

Chemical constituents	Level
Alkaloids	+++
Flavonoids	+++
Phenolic hydroquinone	+++
Steroids	++
Triterpenoids	++
Tannins	+
Saponins	++

Laboratory of Chemistry, Faculty of Science IPB (2016), +++: High level, ++: Normal level, +: Low level

Table 4: Inhibition zone on 100, 50, 25, 12.5 and 6.25% concentration of extracts

Concentration (%)	Inhibition zone (mm)	Categories
100	14.0	High
50	9.0	High
25	4.5	Normal
12.5	2.5	Low
6.25	2.0	Low

Laboratory of Food Security, Faculty of Agriculture Technology, Bogor Agricultural University, Bogor, Indonesia (2016)

Beetle extract ability to inhibit *Salmonella* sp.: Extract concentration of 100% showed the highest level of inhibition zone on 14 mm. Lowest level of inhibition zone was on 6.25% extract concentration at 2 mm (Table 4). Ethanolic extract of beetle leaves showed a high inhibition zone (7 mm) on 100% concentration[23]. Another research proofed that the inhibition zone of aqueous extracts on beetle leaves (12 mm) is better than methanolic extracts (10 mm)[13]. Those researches support the result that proper methods on aqueous extraction were as good as using ethanolic extract. Because the inhibition zone on 100% concentration in our research has a bigger zone than the previous research[13,23]. Besides, different beetle leaves variations could have a different chemical composition which affects the ability to inhibit bacteria[13].

Extracts supplementation on quails have to consider several aspects that affect the determination of the concentration that would had been used in this research. High extracts concentration in drinking water of quails could lead in a high production cost. Besides, high concentration of extracts could also result in a bad palatability because of the high fibre contents which contain some anti-nutritive compounds[23,24]. Inhibition zone which represent low (<3 mm), medium (3-6 mm) and high (>6 mm) on inhibiting bacteria should be met[15]. Proper concentration was obtained by testing the inhibition zone in several levels that gradually lowered half of those levels (100, 50, 25, 12.5 and 6.25%) until

Table 5: Inhibition zone on 30, 20 and 10% concentrations of extracts

Concentration (%)	Inhibition zone (mm)	Categories
30	7.0	High
20	3.5	Normal
10	2.5	Low

Laboratory of Food Security, Faculty of Agriculture Technology, Bogor Agricultural University, Bogor, Indonesia (2016)

Table 6: *Salmonella* contaminations on fourth week (grower phase)

Treatment	Total colonies (CFU mL^{-1})
P0	3.86×10^{4c}
P1	2.64×10^{3b}
P2	8.18×10^{2b}
P3	6.00×10^{0a}

Laboratory of Bacteriology, Faculty of Veterinary, Bogor Agricultural University, Bogor, Indonesia (2016), P0: Basal diet+Vita stress, P1: Basal diet+10% extract, P2: Basal diet+20% extract, P3: Basal diet+30% extract. The numbers followed by the small alphabet in the same column indicates a significance level ($p<0.05$)

found its optimum levels. Results from Table 4 show that the optimum concentrations were expected to be obtained at the level of 10, 20 and 30%. Then inhibition zone test was carried back to discover the inhibition ability of those expected concentrations.

Inhibition zone that represent low, medium and high of the extracts are shown in Table 5. The results were 30, 20 and 10% concentration of extracts have 7 mm (high), 3.5 mm (medium) and 2.5 mm (low) that can be seen on Fig. 1. Proper extracts in concentration could give a better feed palatability on quails which could lead in higher feed intake[25]. It may be caused by its influence on increasing of digestion, secretion of digestive enzymes, has an antibacterial, antiviral and antioxidant effects[26].

The durability of extracts within one month storage of times had been performed. Agar dilution test was conducted to find the quality reductions of extracts. All levels of extracts (10, 20 and 30%) were able to inhibit colonies of bacteria until 0 CFU mL^{-1} from week 0 until week 4. However, control treatment shows that the colonies of bacteria are more than 300 CFU mL^{-1}. This means that extracts products were able to maintain its quality in 4 months of storage time.

Effect of beetle extract on *Salmonella* contamination in small intestine of quails: Results from Table 6 indicate that *Salmonella* colonies were reduced significantly ($p<0.05$) at higher levels of supplementations on grower phase. The highest contamination was on control treatment with 3.86×10^4 CFU mL^{-1} and significantly ($p<0.05$) different with T1, T2 and T3. T1 (2.64×10^3 CFU mL^{-1}) and T2 (8.18×10^2 CFU mL^{-1}) contaminations are significantly ($p<0.05$) different with T3 (6×10^0 CFU mL^{-1}). Result from Table 7 shows that the highest contamination was at control

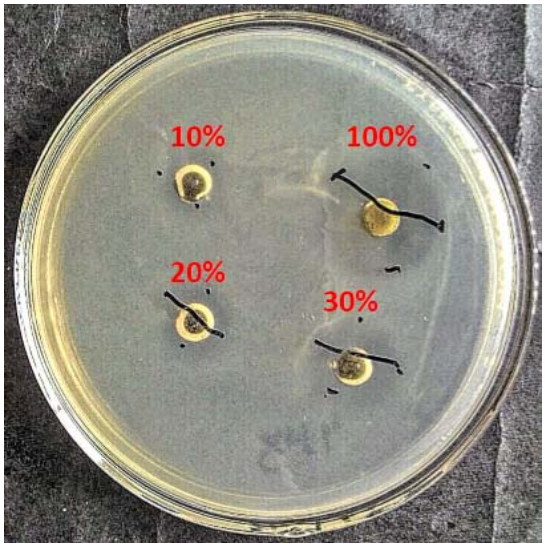

Fig. 1: Inhibition zone diameters from extracts with 10, 30, 20 and 10% of concentration

Table 7: *Salmonella* contaminations on 12 weeks (laying phase)

Treatments	Total colonies (CFU mL^{-1})
P0	$2.33\times10^{-4}\pm4.39\times10^{-3}$b
P1	$2.47\times10^{-3}\pm8.15\times10^{-2}$a
P2	$2.77\times10^{-3}\pm5.13\times10^{-2}$a
P3	$1.63\times10^{-3}\pm4.16\times10^{-2}$a
P4	$3.83\times10^{-3}\pm2.08\times10^{-2}$a
P5	$2.27\times10^{-3}\pm2.08\times10^{-2}$a
P6	$2.62\times10^{-2}\pm2.89\times10^{-1}$a

P0: Basal diet + Vita stress, P1: Basal diet+10% extract+DOQ, P2: Basal diet+20% extract+DOQ, P3: Basal diet+30% extract+DOQ, P4: Basal diet+10% extract+laying phase, P5: Basal diet+20% extract+laying phase, P6: Basal diet+30% extract+laying phase. The numbers followed by the small alphabet in the same column indicate a significance level ($p<0.05$)

treatment (2.33×10^{-4} CFU mL^{-1}). All treatments, except the control treatment have a lower *Salmonella* sp. colonies in small intestine of quails and significantly different ($p<0.05$) with the control treatment. At the starter phase, the highest *Salmonella* colony was on the control treatment (P0). Treatment of P1 and P2 have a significant effect ($p<0.05$) to decrease the number of *Salmonella* colonies when compared to P0. P1 and P2 treatment did not differ significantly ($p> 0.05$) in decreasing *Salmonella* colonies. Giving the highest concentration extract (P3) has the best ability to decrease the number of *Salmonella* colonies when compared with P0, P1 and P2 ($p<0.05$). At laying periods, most of the lowest *Salmonella* colony was found on extracts supplementations which had been given since laying phase treatments (P4, P5, P6). Results on P1, P2 and P3, the contamination was higher on the twelfth week rather than fourth week.

When quails had been given the extracts since DOQ, results have shown that *Salmonella* inhibiting ability was better on the grower period (fourth week) rather than laying periods (twelfth week). Besides, quails that had been given the extracts since laying periods had lower *Salmonella* contamination rather than given since DOQ. Bacteria resistances could be the reason that the amount of *Salmonella* was higher on longer supplementation of extracts. *Salmonella* is a resistant bacteria to multiple antibiotics[27]. *Salmonella* is the most resistant bacteria to herbal antibiotics[21].

The tests that had been conducted showed that all treatments could inhibit *Salmonella* contamination in small intestine. In overall, the best treatment to reduce *Salmonella* contamination in small intestine of laying quails are 10% concentration which had been given since laying periods (T5). The inhibiting ability of the T5 is not significantly different with the other treatments. Practically 20 and 30% concentrations will lead to a higher cost rather than 10%.

The dangerous mechanism of *Salmonella* is the probability to cross mucosa of the ileum which could harm the ileum from its macrophages ability and could enter the blood stream[28]. When *Salmonella* enter the blood stream, it causes a gastroenteritis and salmonellosis due to severe damage in epithelial cells[29]. This study concluded that beetle leaves extract supplementation could reduce those problems in poultry. Besides, utilizations of this extracts could be considered to be used in human as a medicine. This research was observed on quails as a role model of animals which have a similar physiology with human as the mono gastric. Further study will be needed to observe beetle extract application on human food and medicine.

CONCLUSION

Extract of beetle leaves has the ability to inhibit *Salmonella* sp. on *in vitro* observation. Appropriate extracts supplementation of beetle leaves on quails have been found that 10, 20 and 30% could be used safely in quails. The best concentration of extracts to give on quails was 10% concentration. The 10% beetle leaves extract supplementation could inhibit *Salmonella* in small intestine of quails. Besides, 10% extract supplementation has a lower production cost and higher efficiency on farm management.

SIGNIFICANCE STATEMENT

Plants substances could be used to replace the synthetic antibiotics. *Piper bettle* L. is a potential medicinal plant which has not been observed on many occasions especially on *in vitro* observations. This study showed that beetle leaves have an ability to inhibit bacteria such as *Salmonella* sp. on *in vitro* test. These results could lead into further research to observe the ability of beetle leaves to inhibit the other bacteria on *in vitro* observations. These results have a good impact on giving a more options in medicine plants that could be used as natural antibiotics.

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