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RESEARCH ARTICLE

Antimicrobial activity of *Phyllantus niruri* extract on Avian pathogenic *Escherichia coli* Isolated from Chicken with Colibacillosis symptoms

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ABSTRACT:

Objective: In this study, we evaluated the antimicrobial properties of methanol extracts of *Phyllantus niruri* on avian pathogenic *Escherichia coli* associated with avian colibacillosis isolated from areas of East Java, Indonesia. We compared the activity of the extracts with oxytetracyclin, gentamycin, enrofloxacin and cyprofloxasin. **Materials and Methods:** This research used 60 samples taken from the infundibulum of layer chickens from 10 districts in East Java, Indonesia (Sidoarjo, Mojokerto, Kediri, Blitar, Bojonegoro, Jombang, Lamongan, Tuban, Jember and Banyuwangi) with 6 samples from each district. **Results:** The results showed that 6 samples collected from Sidoarjo, Blitar, Bojonegoro, Jombang, Tuban, and Jember were *E. coli* positive and 4 samples from Lamongan, Mojokerto, Kediri, and Banyuwangi were negative. Polymerase Chain Reaction showed that seven isolates of *E. coli* were positive for the presence of the *yaiO* gene using a 115 base pairs amplified fragment. An MBC test showed that 25 and 50 mg/ml concentrations of *P. niruri* extract were lethal against *E. coli*. Cyprofloxacin also affected *E. coli*, but these strains were resistant to oxytetracycline, gentamycin and enrofloxacin at the same dose. **Conclusion:** This study indicated that the *P. niruri* extract has antibacterial activity and has the potential to be used as a source for a new broad-spectrum oral antibiotic.

KEYWORDS: Antimicrobial activity, *Escherichia coli*, Minimum Bactericidal Concentration, Minimum Inhibitory Concentration, *Phyllantus niruri* extract.

INTRODUCTION:

In recent years, commercial poultry farms have grown in size and the poultry themselves have undergone dramatic improvements in growth, feed efficiency and production. However, a major problem affecting the growth of the poultry industry in Indonesia is the occurrence of disease outbreaks. Some regions have reported a dramatic increase in the incidence of infectious disease outbreaks during this time of rapid expansion.

Amongst these infections, *E. coli* infection is quite common and causes a large number of disease conditions such as pericarditis, perihepatitis, airsacculitis, peritonitis, salpingitis, panophthalmitis, omphalitis, cellulitis, colisepticemia, coligranuloma, and swollen-head syndrome[1].

Colibacillosis is one of the main causes of economic losses in the poultry industry worldwide[2,3]. Though E. coli is a commensal resident in the intestinal tract of poultry, it often turns pathogenic under certain adverse conditions such as poor ventilation, overcrowding, and immunosuppression[4].

The use of antibiotics has led to success in limiting most of the prevalent bacterial diseases that have affected man and animals to epidemic proportions. The World Health Organization (WHO) has recommended development and use of environment-friendly alternative methods to control diseases in poultry and other food-producing animals such as extract of the widespread tropical plant *Phillantus niruri*[5].

P. niruri and the related species *P. amarus* primarily contain active constituents including phyllantine and hypohyllantine, as well as alkaloids and bioflavonoids like quercetin. Reports have shown that their functions are diverse, including the provision of strength to plants, the attraction of insects for pollination and defense against predators, provision of colors, while some are waste products. The discovery of phytochemicals such as tannins, saponin, flavonoids, alkaloids, glycosides from *P. niruri* and *P. amarus* extracts has provided some justification for many medicinal uses of the plant. Most uses of *P. niruri* and *P. amarus* have used the whole plant as it is a small herb[6].

This research was aimed at evaluating the antimicrobial properties of phenolic extracts of *P. niruri* on avian pathogenic *E. coli* associated with avian colibacillosis in several districts in East Java, Indonesia, compared to the effects of the antibiotics oxytetracycline, gentamycin, enrofloxacin and ciprofloxacin.

MATERIALS AND METHODS:

Isolate of *E. coli*:

A total of 60 infundibula from chickens of various ages that were suspected and showed clinical signs of colibacillosis were tested. The 60 samples were collected from ten districts in East Java, i.e., Mojokerto (E1), Sidoarjo (E2), Tuban (E3), Jember (E4), Bojonegoro (E5), Jombang (E6), Blitar (E7), Kediri (E8), Lamongan (E9) and Banyuwangi (E10), six from each district.

Isolation of bacteria was carried from the "Serial Dilution Method":

A total of 10g of each infundibulum sample was placed into a 100ml beaker and noted as 10^{-1} dilutions. From the 10^{-1} dilutions, 1 ml of water was taken using a sterile pipette and added to 9ml distilled water and noted as a 10^{-2} dilution. This process was continued up to a 10^{-6} dilution. Then 1ml of sample was taken from 10^{-6} dilution, and the sample was spread on a Petri plate with EMBA media to isolate E. coli. The plate was incubated overnight at 37ºC, and this method allowed for the isolation of colonies developed from a single cell[7].

Identification and characterization of the cultures were done by morphological (colony color, shape, and size) and biochemical tests. The observed characteristics were compared with Bergey's Manual of Determinative Bacteriology for proper identification of the organisms[8].

Polymerase Chain Reaction (PCR):

An orphan gene, *yaiO*, was selected as the target for the specific identification of E. coli. We used F: 5′ TGATTTCCGTGCGTCTGAATG 3′ 115 R: 5′ ATGCTGCCGTAGCGTGTTTC 3′ as the primer, with a product size of 115 bp.

All DNA samples for PCR were adjusted to 50µl with RNase-free water and contained 1µl of 10mM dNTP mix, 125nM of each required oligonucleotide primer, 1.25 U of DNA polymerase (iTaq, Bio-Rad), 30ng of template DNA and 1X PCR reaction buffer (20mM Tris–HCl pH 8.4, 50 mM KCl). To optimize the multiplex amplification, the concentration of $MgCl_2$ and the annealing and extension temperatures were varied (data not being shown). The best results were achieved under the following conditions: 1.5mM MgCl2, initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30s, primer annealing at 58°C for 30s, primer extension at 72°C for 1 min, and a final extension at 72°C for 10 min. In every assay, a buffer control, to which no DNA template was added, was used as a negative control. To evaluate its reproducibility, all multiplex PCRs were performed four times, twice on an iCycler iQ system and twice with a Verity-96 Well Thermal Cycler.

Additionally, amplifications with lacZB-uidA were carried out as described elsewhere. Briefly, the thermocycling conditions were as follows: initial denaturation at 94°C for 10 min. Followed by 44 cycles of denaturation at 94°C for 1 min, primer annealing at various temperatures (2 cycles at 62°C, 2 cycles at 61°C, 2 cycles at 60°C, 2 cycles at 59°C and 36 cycles at 58 °C) for 1 min, primer extension at 72°C for 1 min, and a final extension at 72°C for 10 min[9].

Inoculum preparation:

The bacterial slants were incubated overnight at 37ºC. A one-McFarland density bacterial culture was adjusted in normal saline using a densitometer to achieve the final concentration of 108CFU/ml of each test organism individually. This was used as an adjusted inoculum for all the further studies[10].

Extraction of *P. niruri* :

Meniran (*P. niruri*) was harvested at maturity in the Malang region. A total of 1000g of the plant powder of P. niruri were macerated for 24 hours at room temperature, in 1000ml of 96% methanolic solvent, filtered on Whatman paper, and the extraction was repeated several times with renewal of the solvent. The solvent was removed from the filtrate by rotary evaporation. The extracts were dried and stored until later use[11].

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC):

The minimum inhibitory concentration (MIC) of the active extract was evaluated by a tube dilution method. A stock solution of 100% *P. niruri* concentrate was made by dissolving 10g of *P. niruri* extract into 10ml of nutrient broth. Then, to make a solution of *P. niruri* extract with a concentration of 50%, 5ml of 100% solution was taken and added to 5ml of nutrient broth in a test tube with a capacity of 10ml. Concentrations of 25%, 12.5%, 6.25%, 3,125% and 1.56% were carried out in a similar manner.

To each tube, 1.5 x 108 CFU/ml of the E. coli cultures were added. The suspension was incubated for 24 hours at 37°C[11]. After overnight incubation at 37ºC, the tubes were examined for turbidity, indicating growth of the microorganisms. The lowest solution of the extract that inhibited the growth of the organism as detected by the lack of visual turbidity (matching the negative growth control) was designated the minimum inhibitory concentration.

Minimum bactericidal concentration (MBC) was determined by taking the solution from each MIC tube and plated on EMBA (Eosin Methylene Blue Agar) media, which was then incubated for 24 hours at 37°C to observe the growth of *E. coli*. If there was no *E. coli* growth in EMBA media, its meant that specific concentrations of *P. niruri* extract or the antibiotics (gentamycin, ciprofloxacin, oxytetracycline and enrofloxacin) could kill *E. coli*[12], each antitoxin used was obtained from local pharmacy store (Tithebarn, UK). All of the medicines were employed at 0.5ml/1L concentration against *E. coli*[13].

RESULTS:

***E. coli* identification:**

At the end of the antimicrobial tests, the result showed that six samples collected from Sidoarjo, Blitar, Bojonegoro, Jombang, Tuban, and Jember were E. coli positive and four samples from Lamongan, Mojokerto, Kediri, and Banyuwangi were negative E. coli colonies. The positive colonies on EMBA media showed metallic green color with steady growth, with circular colony shape, rough surface, low convex elevation, and erose edges. In the EMBA medium, most of the E. coli colonies had a distinctive green sheen. Other lactose fermenters produced larger mucoid colonies, often purple only in the middle. Fast lactose fermentation and strong acid production and the rapid reduction of EMBA medium pH were important factors in the formation of green metal sheen which was observed with E. coli as shown in Table 1.

Table 1. Properties used to identify of *E. Coli.*

Variable	Result
Colony	Round shape with green metallic color
Gram staining	Bacillus with red color
Sulfide Indole Motility (SIM)	Motile (+) Indol (+) H_2S (-)
Simmons' Citrate Agar (SCA)	Negative (-)
Urea Agar	Negative (-)
Triple Sugar Iron Agar (TSIA): H_2S Gas	Acid/Acid Negative Positive
Glucose Lactose Sucrose Mannitol Maltose	Positive, Gas positive Positive, Gas positive Positive, Gas positive Positive, Gas positive Positive, Gas positive

The results of the PCR showed the presence of bands in samples E2 (Sidoarjo), E3 (Tuban), E4 (Jember), E5 (Bojonegoro), E6 (Jombang), and E7 (Blitar) of the 115 bp product. The appearance of this band indicated that all samples were positive for the presence of the *yaiO* gene. Thus, if a bacterium showed a positive result on PCR examination using this *yaiO* gene, it could be ascertained that the bacterium was *E. coli*. The *yaiO* protein is found in the outer membrane (outer membrane) in the capsule and is found in almost all E. coli (Figure 1).

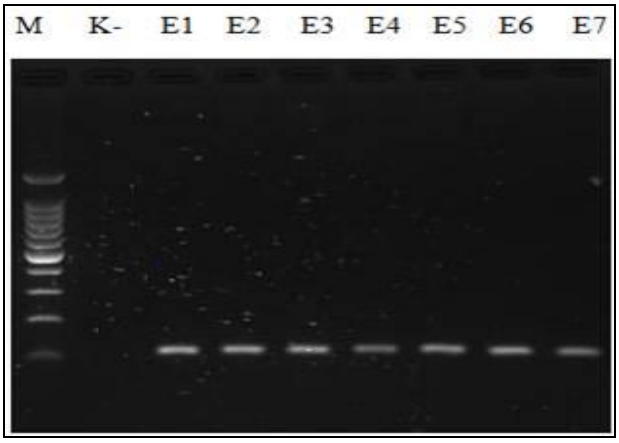

Figure 1. *yaiO* gene with product 115 bo by Polymerase Chain Reaction. M: Marker DNA 100 bp, K-: Negative control, E1: Positive control, E2: Sidoarjo, E3: Tuban, E4: Jember, E5: Bojonegoro, E6: Jombang, E7: Blitar

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Test:

Observation of MIC from *P. niruri* extract was done by observing the turbidity of the suspension at each concentration. If there were an activity inhibiting the growth of *E. coli* at a specific frequency, the suspension on the tube would look clear.

Table 2. Minimum inhibibitory concentration of *P. niruri* and antibiotics.

Replication	***P. niruri* (mg/ml)**						**Antibiotic (0.5ml/L)**			
	1.56	**3.125**	**6.25**	**12.5**	**25**	**50**	**Oxy**	**Genta**	**Cypro**	**Enro**
1	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Rather cloudy	Rather cloudy	Rather cloudy	Rather cloudy
2	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Rather cloudy	Rather cloudy	Rather cloudy	Rather cloudy
3	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Rather cloudy	Rather cloudy	Rather cloudy	Rather cloudy
4	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Rather cloudy	Rather cloudy	Rather cloudy	Rather cloudy

Table 3. Minimum bactericidal concentration of *P. niruri* and antibiotics.

Replication	***P. niruri* (mg/ml)**						**Antibiotic (0.5ml/L)**			
	1.56	3.125	6.25	12.5	25	50	Oxy	Genta	Cypro	Enro
1.	+	+	+	+	-	-	+	+	-	+
2.	+	+	+	+	-	-	+	+	-	+
3.	+	+	+	+	-	-	+	+	-	+
4.	+	+	+	+	-	-	+	+	-	+

+ : presence; - : absence

In this study, we could not observe the bacterial growth inhibition process, because in all levels *P. niruri* suspension in the tube looked cloudy (Table 2).

The observations of MBC showed that the killing power of *P. niruri* extracts started at a concentration of 25 to 50 mg/ml, and only ciprofloxacin, could kill *E. coli* as well. This was not seen in other antibiotics (Table 3).

The result showed that a methanol extract of the plant leaves had antibacterial activity on *E. coli*: at 25 mg/ml and 50 mg/ml of the plant extract, the organisms tested showed susceptibility equivalent to ciprofloxacin.

DISCUSSION:

The higher activity of the methanol extracts may be due to the higher solubility of the active compounds in these solvents. Methanol had a higher power to extract the active antibacterial compounds in the plant, which exhibited higher activity with higher zones of inhibition. The aqueous extracts had little action against the test organisms. Cold water extract showed the least business with minimal movement in E. coli (data not shown). This result was supported by another research study[14,15,16].

The antibacterial activity of various plant species has been compared to that of standard antibiotics[17]. The antimicrobial activity of the extracts of Phyllanthus spp. Might be due to the presence of lignans; phyllanthin and hypophylanthin, flavonoids, triterpenoids, glycosides, and tannins, in the plant extract[18]. Phytochemical constituents like flavonoids were known to prevent gastric ulcers due to the astringent and antimicrobial properties, which appeared to be responsible for the gastro-protective activity. P-cymene, a monoterpenoid, had also tested for antimicrobial properties using the paper disc diffusion method, in which it revealed an excellent antimicrobial activity[19]. More importantly, there had been no side-effects or toxicity reports for many years on this plant[20]. Although there had been extensive research on this plant, further research needs to be done especially towards the mechanism of biological activity of phytochemicals from this plant.

Many factors have been found to influence the active principles present in plants. These included the age of the plant, the extracting solvent, method of extraction and time of harvesting of plant materials. These could affect the results of the experiment[21].

As the conclusion, the result of this study showed that *E. coli* had resistance to gentamycin, oxytetracycline and ciprofloxacin. But, this study indicated that the *P. Niruri* had antibacterial activity and the potential to be used as a source for new broad-spectrum oral antibiotic Periodic monitoring of antimicrobial susceptibility in the chicken's farm was recommended.

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CONFLICT OF INTEREST:

All authors declare no conflict of interest of this study.

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