

Original Article

# Effects of pomegranate rind (*Punica granatum* Linn.) and guava leaf extract (*Psidium guajava* Linn.) for inhibition of multidrug resistant *Escherichia coli* recovered from diarrhoeal piglets

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## Abstract

The resistance of *Escherichia coli* to antimicrobial drugs is widespread in Thailand and globally. The antimicrobial properties of some traditional plants have been studied as potential replacements of modern antibacterial agents. The objective of this study was to determine the antimicrobial resistance of *E. coli* isolated from diarrhoeal piglets and the efficacy of pomegranate rind and guava leaf extract to inhibit the growth of *E. coli*. Fifty isolates of *E. coli* from piglets with diarrhea were collected from swine farms in Chiang Mai province. The resistance to 11 antimicrobial drugs was tested using the broth micro-dilution method. All isolates were multidrug resistant. The most frequent were novobiocin, streptomycin, sulfamethoxazole, tetracyclin, and tiamulin (100 %). Followed by resistance to amoxicillin (98%), oxytetracyclin (96%), nalidixic acid (82%), gentamicin (56%), and colistin sulphate (46%). Pomegranate rind, guava leaf extract using the Soxhlet extraction method, and 95% ethanol as solvent were tested for their ability to inhibit the growth of drug resistant *E. coli* using the agar dilution method. Pomegranate rind and guava leaf extract exhibited antibacterial activity against all *E. coli* isolates with an average minimum inhibitory concentration of 5.94 mg/mL and 9.44 mg/mL, respectively. The results demonstrate the possibility of using herbal extracts in the prevention and treatment of high level antimicrobial-resistant *E. coli* in pigs.

**Keywords:** *E. coli*, pomegranate rind, guava leaves, antimicrobial resistance, piglet

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## 1. Introduction

*Escherichia coli* is a gram negative, flagellated, non-spore forming aerobic bacillus of the Enterobacteriaceae family. It is approximately  $1 \times 3 \mu\text{m}$  in size (Reshes, Vanou nou, Fishov & Feingold, 2008). There are many type of *E. coli*, each of which might possess many virulence factors. Depending on the serotype, the growth of the bacteria in the gastrointestinal tract and the production of toxins can cause disease in both human and animals. Generally, *E. coli* are not

harmful to human health but are part of the healthful bacterial flora in the human gut. However, some types can cause illness in humans, including diarrhea, abdominal pain, fever, and sometimes vomiting. A shiga toxin-producing *E. coli* cause of foodborne outbreak which can lead to urinary tract infections, respiratory illness, pneumonia, and other illnesses like meningitis. In piglets, *E. coli* infections are normal with general clinical sign of diarrhea. Recovered piglets have retarded growth and are easily infected with other pathogens, leading to an increase in the use of antibiotics for control, treatment and growth promoting in farm, resulting in the antibiotic resistance of many bacteria including *E. coli*. Resistance to tetracyclin, sulfa-trimetroprim, ampicillin, tylosin, penicillin, spectinomycin, lincomycin, and gentamicin has been reported in *E. coli* (Choi *et al.*, 2002; Stannarius *et al.*,

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2009; Van den Bogaard, London & Stobberingh, 2000). In Thailand, *E. coli* resistant to aminoglycosides and extended-spectrum beta-lactamase (ESBL) were significantly increased in the nursery and growing periods in pig farm (Lugsomya *et al.*, 2018). Antibiotic resistance in pig farms affects the health of the animals and the treatment of disease as well as human health. Resistance might be spread to humans via the food chain and affect human health, resulting in antibiotics use in the general treatment of patients being not effective. Higher doses of medication and more expensive medications might be required. In addition, *E. coli* resistance to antibiotics has the potential to transfer to other microorganisms (Hammerum & Heuer, 2009). Hence, the use of antibiotics in animals is a public health concern and creates a need for more surveillance.

Since, antibiotic use as a growth promoter in animals is prohibited. An alternative is to use herbs for replacement. Thai herbal extracts have been studied for their ability to inhibit the growth of diarrhea-causing pathogens in pigs. For example, galangal, golden flowers, and ginger can inhibit the growth of *E. coli*. (Chanchay & Jantan, 2016; Yamsakul *et al.*, 2004). In addition, pomegranate extracts inhibit the growth of Gram-negative bacterial pathogens including *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella boydii*, and *Salmonella London* (Sirirak, Supavita & Panthong, 2005). Guava leaf extract contains tannin and flavonoids which inhibit the growth of *Salmonella* spp. (Yaitummasarn, 2002).

The application of herbs to replace antibiotics can reduce antibiotic use, reduce costs, and reduce the occurrence of antibiotic resistant bacteria on a farm. It also reduces the drug residue in meat which will affect the consumer. Even many studies focused in effectiveness of herbal on bacterial growth, but only few study investigated the efficiency against resistance bacteria. Therefore, this study investigated the efficacy of pomegranate extracts and guava leaf extract to inhibit the growth of resistance *E. coli* isolated from diarrheal piglet. Because of both herbals are indigenous plant and easily to collect in Thailand. This may be the possibility of using local herbal extracts to replace antibiotics in the development of an organic pig farm was evaluated.

## 2. Materials and Methods

### 2.1 Sample collection

Stool samples were collected from suckling piglets with diarrhea aged 7 to 21 days from 13 farms in five districts in Chiang Mai. Samples were collected four times per month. Each time of collection 2-3 specimens were collected and kept in plastic bag at 4 °C for the re-isolation of *E. coli*.

### 2.2 *E. coli* isolation and identification

One gram of stool was placed into lauryl sulphate broth and incubated at 37 °C for 18–24 hours. The solutions were transferred to MacConkey agar (MAC) and Eosin Methylene-blue Lactose Sucrose agar (EMB) and then incubated at 37 °C for 18–24 hours. The pre-sumptive colonies were tested for the presence of *E. coli* using a biochemical assay that tested positive for the Indole test, methyl red reaction, and mobile, and negative for the citrate and

Voges-Prokauer reactions when *E. coli* was present. Confirmed isolates were stored in tryptic soy broth +20% glycerol at -20 °C for further testing.

### 2.3 Antimicrobial susceptibility testing

The antimicrobial susceptibility test used the broth microdilution method according to the National Committee for Clinical Laboratory Standard (CLSI, 2018). Eleven types of antibiotic were used, namely amoxicillin, colistin sulphate, enrofloxacin, gentamicin, nalidixic acid, novobiocin, oxytetracyclin, streptomycin, sulfamethoxazole, tetracyclin, and tiamulin. Those drugs were dissolved and made two-fold dilution to concentrations of 5,120 µg/ml. The minimum inhibitory concentration (MIC) was then made in 96 well plates using Muller Hinton Broth (MHB) (Himedia / India) from a concentration of 256 µg/mL to 0.125 µg/mL. Each *E. coli* isolate was added to a well at a final concentration of  $5 \times 10^5$  CFU/mL then incubated at 37 °C for 18–24 hours. The turbidity of the culture media was observed and the lowest dilution which inhibited growth (not turbid) was recorded. Antibiotic resistance was interpreted according to CLSI (2008) and BSAC (2011).

### 2.4 Herbal extracts preparation

Two types of herbs, ripe pomegranate peel and guava leaves (not too soft or too old and before flowering), were washed and dried. They were then baked in an oven, at 40–50 °C for 24 hours. They were subsequently mashed, weighed, put into a thimble, and mixed in a Soxhlet extractor (Xiemen ollital Technology C., Ltd.) and extracted with 95% ethanol continuously (Bicking, 2000). The extract was dissolved in alcohol then evaporated in a rotary evaporator (IKA, Germany), weighed, and kept at 4 °C in a glass bottle with the lid closed.

### 2.5 Herbal extracts efficacy testing

The minimal inhibition concentration (MIC) was determined using the agar dilution method. Two fold dilutions of extract were prepared using one portion of the extract and three portions of sterile distilled water. The dilutions were mixed with Muller Hinton Agar (MHA) (Difco, BBL / USA) in a ratio of 1:10 then poured onto MHA plate. Concentrations of herbs from 25 mg/mL to 1.56 mg/mL were prepared. The media were allowed to become solid and separated into five parts. Each strain of *E. coli* was adjusted to Standard Mcfarland No. 0.5 ( $10^8$  CFU/mL) and diluted 10 times with 0.9% normal saline to obtain a concentration of  $10^7$  CFU/mL. A 2 µL micropipette was used to suck up the dilution and place four drops on each part of agar. The concentration of bacteria in each drop was  $10^4$  CFU/mL. The plates were incubated at 37 °C for 18–24 hours. Two replicates were tested. The lowest concentration of the extract that represented the MIC was compared to the controlled plate (95% ethanol 0.5 mL + distilled water 1.5 mL).

### 2.6 Statistics analysis

Data were analyzed by using Microsoft excel as percentage and further by descriptive analysis.

### 3. Results and Discussion

Bacterial isolation from diarrheal piglets got 50 isolate of *E. coli*. The piglets were infected as a result of poor sanitation conditions in the pens or from an infected sow or sow with mastitis. Negative samples were found in the farm which used antibiotics before sampling. All isolates of *E. coli* were multidrug resistant (MDR) (100%). The most frequent resistance was to novobiocin, streptomycin, sulfamethoxazole, tetracyclin, and tiamulin (100%), followed by amoxicillin (98%), oxytetracycline (96%), nalidixic acid (82%), gentamicin (56%), enrofloxacin (54%), and Colistin sulphate (46%), respectively. The most susceptible drugs were colistin sulphate (54%), gentamycin (38%), enrofloxacin (36%), and nalidixic acid (18%) (Table 1).

Extracts of pomegranate rind and guava leaves by using 95 % ethanol was a brown color of viscous liquid and green with dark green sticky liquid, respectively (Figure 1). Concentrations of herbs were prepared by two-fold dilution from the original concentration (25 mg/mL.) into 12.50, 6.25, 3.125 and 1.63 mg/mL. The results showed the percentage of inhibition by pomegranate extract were 8% at 12.50 mg/mL, 66% at 6.25 mg/mL, and 26% at 3.125 mg/mL. While, the percentage of inhibition by guava leaf extract were 56% at 12.50 mg/mL, 34% at 6.25 mg/mL and 10% at 3.125 mg/mL. The highest percentages of *E. coli* inhibition with pomegranate extract was achieved with 6.25 mg/mL concentration and by guava leaf extract with a concentration of 12.50 mg/mL

(Table 2). The average of MIC of pomegranate extract and guava leaf extract on growth of *E. coli* were 5.94 and 9.44 mg/mL. Mean that at the lower concentrations, pomegranate extract was more effective than guava leaf extracts against the growth of *E. coli*.

*E. coli* are common bacteria in suckling piglets. The resistance is usually due to the swine having been treated with antibiotics for a variety of diseases. In this study, *E. coli* isolates were resistant to multiple antibiotics (multidrug resistant). Twenty-five strains were resistant to ten antibiotics;



Figure 1. Comparison of dried pomegranate rind and its extract (a, b), and dried guava leaves and their extract (c, d).

Table 1. Interpretation of antibiotic susceptibility of *E. coli* isolated from piglets.

Antibiotic	No. of isolates					
	Susceptible		Intermediate		Resistant	
	(n=50)	%	(n=50)	%	(n=50)	%
Amoxicillin	1	2	0	0	49	98
Colistin sulfate	27	54	0	0	23	46
Enrofloxacin	18	36	5	10	27	54
Gentamycin	19	38	3	6	28	56
Nalidixic acid	9	18	0	0	41	82
Novobiocin	0	0	0	0	50	100
Oxytetracyclin	2	4	0	0	48	96
Streptomycin	0	0	0	0	50	100
Sulfamethoxazole	0	0	0	0	50	100
Tetracyclin	0	0	0	0	50	100
Tiamulin	0	0	0	0	50	100

Table 2. MIC of pomegranate rind and guava leaf extract for inhibition of *E. coli* isolated from piglets.

Extract	Concentration (mg/mL)	No. of isolates	Percentage of inhibition (%)
pomegranate rind	12.5	4	8
	6.25	33	66
	3.125	13	26
	1.63	0	0
	Total	50	100
guava leaf extract	12.5	28	56
	6.25	17	34
	3.125	5	10
	1.63	0	0
	Total	50	100

resistance to colistin sulphate was the least common (twenty-three strains). The different proportions of drug resistance might be due to antibiotic use on the farm and the mechanism of drug resistance. *E. coli* from pigs easily develops resistance to many drugs, such as penicillin and tetracycline (Choi *et al.*, 2002; Stannarius *et al.*, 2009; Van den Bogaard *et al.*, 2000). Drug resistance problems are caused by the misuse of antibiotics, such as use to accelerate growth, as well as inappropriate dose, route of administration, and duration (Alan, Robin, & Liamthong, 2007). This might be due to a lack of knowledge of rational drug use or no veterinary supervision, resulting in unreasonable and frequent drug use. Data collection on antibiotic use found that *E. coli* from farms that used multiple antibiotics was more resistant than that from farms that used fewer antibiotics. In addition to the problem of antibiotic resistance on the farm, there are public health concerns about the drug residue in animal products having a negative impact on consumer health. Such exposure by those who are allergic to a certain antibiotic could be life-threatening. Therefore, local herbs are currently being studied in-depth to identify their properties that could be used instead of drugs. A previous study showed that the metabolic extract of *Psidium guajava* Linn exhibited antibacterial activity against *E. coli* with a MIC 0.78 µg/mL, and a minimum bactericidal concentration (MBC) of 50 µg/mL (Dhiman, Nanda, Ahmad & Narasimhan, 2011). In addition, Kanbutra *et al.* (2003) found that the alcoholic extracts of guava leaf (MIC=2.0, MBC=8.0 mg/mL), guava green fruit (MIC=7.3, MBC=29.1 mg/mL), roselle calyx (MIC=4.7, MBC=9.4 mg/mL), "Koon" leaf (MIC=6.8, MBC=27.0 mg/mL), and "Koon" stem bark (MIC=2.8 mg/mL) showed high antibacterial activity and seemed to be the choices of Thai herbs for anti-*E. coli* effects.

In this study, it was found that the MIC of the pomegranate extract for the 50 *E. coli* serotypes was lower than the MIC of the guava leaf extract. This indicates that the pomegranate extract was more effective than the guava leaf extract at inhibiting the growth of *E. coli*. However, the research of Masadeh *et al.* (2013) found that guava leaf extract could inhibit *E. coli* with an average MIC of 153 µg/mL, a lower concentration than in this study (9.44 mg/mL). The hot-water extract of the pomegranate peels was the most potent with the minimal inhibitory concentration of 207 mg/ml against *E. coli* (Nuamsetti, Dechayuenyong & Tantipai bulvut, 2012). Many studies demonstrated that guava leaf extract could inhibit the growth of *E. coli* with different MIC values (Dhiman *et al.*, 2011; Kanbutra *et al.*, 2003; Masadeh *et al.*, 2013). This might be due to the different active compounds in each of the herbs. However, the active ingredient in pomegranate rind and guava leaf that can be used to control bacterial resistance to antibiotics might be a group of tannins. Tannin antimicrobial activity includes the inhibition of extracellular microbial enzymes, the deprivation of the substrates required for microbial growth, and direct action on microbial metabolism through the inhibition of oxidative phosphorylation (Scalbert, 1991). Factors that affect the MIC value may include the cropping areas of the herbs, the season, extract, and extraction methods. Previous study by Netshiluvhi & Eloff (2016) indicated that the factors such as genetic, edaphic, microclimate, herbivory and pathogens could also have had an effect to antibacterial activity of plant extract.

The results showed that the growth of multidrug resistant *E. coli* could be inhibited by both herbal extracts but pomegranate extract are more effective than guava leaf extract. The limitation of this study was the small number of *E. coli* isolate and lack of knowledge on exactly mechanism on *E. coli* inhibition. However, further studies on the amount that should be used, mechanism of two herbs against *E. coli* and the toxicity and side effects should be performed. Although, this study gave the basic data on herbal efficiency against antibiotic resistance *E. coli* but it may encourage farmers to consider in herbal use instead of antibiotics use.

#### 4. Conclusions

Fifty *E. coli* samples were isolated from one hundred stool samples from piglets at the breastfeeding stage (50%). All the isolates were resistant to antibiotics. The antibiotic drugs that had a high resistance were: novobiocin, streptomycin, sulfamethoxazole, tetracyclin, and tiamulin (100%), followed by amoxicillin (98%), oxytetracyclin (96%), nalidixic acid (82%), gentamicin (56%), enrofloxacin (54%), and colistin sulphate (46%), respectively. Guava leaf extract and pomegranate rind extract could inhibit the growth of all the *E. coli* strains that were resistant to antibiotics. The amount of pomegranate rind extract required to inhibit *E. coli* was less than of guava leaf extract. The results of this study suggest that both herbal extract should be trialed for use in *E. coli*-infected piglets.

#### Welfare statement

This study was performed under field conditions, in which the animals were kept in housing that was adequate and were humanely cared for.

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