Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Original article http://dx.doi.org/10.1016/j.apjtb.2017.07.019

# Antibacterial activity of naringenin-rich fraction of pigeon pea leaves toward *Salmonella thypi*

Sarifa Agus[1], Suminar Setiati Achmadi[1*], Nisa Rachmania Mubarik[2]

[1]*Department of Chemistry, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Kampus Dramaga, Bogor 16680, Indonesia*

[2]*Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Kampus Dramaga, Bogor 16680, Indonesia*

ARTICLE INFO

*Article history:*
Received 15 Nov 2016
Received in revised form 12 Dec 2016
Accepted 26 Jul 2017
Available online 4 Aug 2017

*Keywords:*
Antibacterial activity
*Cajanus cajan*
Flavanones
Naringenin
*Salmonella thypi*
Typhoid

ABSTRACT

**Objective:** To identify bioactive compound in pigeon pea leaves (*Cajanus cajan*) that inhibits *Salmonella thypi* (*S. thypi*).
**Methods:** The leaf sample was powdered and macerated with methanol and fractioned by liquid–liquid extraction using ethyl acetate. The fraction was chromatographed and the isolates were identified for major component with liquid chromatography-mass spectrometry and the antibacterial activity was tested against *S. thypi* by Kirby–Bauer method.
**Results:** Subfraction 1 from the ethyl acetate fraction formed a yellowish solid with *m/z* 272, identified as naringenin. The naringenin-rich fraction shows fairly well inhibitory toward *S. thypi* in comparison with chloramphenicol.
**Conclusions:** Naringenin shows antibacterial activity and can be developed to treat typhoid.

## 1. Introduction

Pigeon pea (*Cajanus cajan*) grows well in tropical and subtropical regions such as Indonesia. This species ranked sixth in the utilization of their products compared to other leguminous plants [1]. The pea has countless benefits, namely as food ingredient [2] and as an alternative protein source for people dieters [3]. In addition, the peas were also traditionally used as medicinal plants.

Pigeon pea is used to treat diabetes [4,5], jaundice [6], appendicitis, fever [7], heartburn, constipation, analgesic, and to kill parasites [8]. Compounds that have been identified from the leaves of pigeon pea are luteolin [9], cajanus lactone, pinostrobin chalcone, longistylin A, longstylin C [10], cajaninstilbene acid [10,11], and pinostrobin [11–13]. Chemical and pharmacological studies indicate a major component in pigeon pea leaves that have potential benefits to human health are classified into two groups, namely flavonoids and stilbene [9,12].

Besides, pigeon pea leaves were also believed to cure typhoid. It is based on the knowledge of local people in Bone Regency, South Sulawesi who use the leaves to treat typhoid fever by boiling the leaves and then drink it as tea. The antibacterial effects of extracts of the leaves against some pathogenic bacteria have been tested and reported that the extract could inhibit the proliferation of bacteria *Salmonella thypi* (*S. thypi*) [14].

Typhoid is an infectious disease caused by Gram-negative bacteria *S. thypi* [15]. The disease is still a burden for some developing countries, particularly in areas with poor sanitation and inadequate health facilities for early diagnosis [16]. Some regions become the endemic area of typhoid, including South Asia, Southeast Asia, and southern regions of Africa. Indonesia is the third country of typhoid sufferer after Pakistan and India [17]; typhoid disease is in the top 10 inpatients in hospitals with 274 people died of typhoid in the last 5 years [18].

*Corresponding author: Suminar Setiati Achmadi, Department of Chemistry, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Kampus Dramaga, Bogor 16680, Indonesia.
Tel: +62 8161857886
E-mail: ssachmadi@cbn.net.id (S.S. Achmadi).

Peer review under responsibility of Hainan Medical University. The journal implements double-blind peer review practiced by specially invited international editorial board members.

## 2. Materials and methods

### 2.1. Sample preparation

Pigeon pea leaves were collected from Bone Regency, South Sulawesi, in October 2015 and the specimen was verified in the Bogoriense Herbarium Laboratory, LIPI Bogor, Indonesia, as a leguminous *Cajanus cajan* (L.) Millsp. The sample was dried at room temperature for 5 days and was pulverized to 60-mesh size [2].

### 2.2. Tested bacteria

*S. thypi*, *Staphylococcus aureus* (*S. aureus*), and *Escherichia coli* (*E. coli*) EPEC K1.1 used in this study were obtained from the Microbiology Laboratory, Department of Biology and IPB Culture Collection, Bogor Agricultural University, Indonesia. All three bacteria are pathogenic to humans. *S. aureus* and *E. coli* EPEC K1.1 were used as a comparison to *S. thypi*. *S. aureus* represented Gram-positive, while *E. coli* EPEC K1.1 represented Gram-negative bacteria other than *S. thypi*.

Chloramphenicol served as positive control in inhibiting the growth of pathogenic bacteria [19]. Chloramphenicol is a broad-spectrum bacteriostatic that is able to inhibit Gram-negative and Gram-positive both anaerobic and aerobic bacteria by disrupting the protein synthesis.

### 2.3. Sample extraction

The sample (800 g) was macerated in methanol for 24 h (three replications) and filtered to separate the residue from the filtrate. The filtrate was concentrated using a rotary evaporator to obtain a thick methanol extract. Phytochemical screening was employed to the methanol extract [20].

### 2.4. Antibacterial test of the methanol extract

The methanol extracts (28.4 g) was partitioned using a mixture of *n*-hexane and ethyl acetate. The *n*-hexane, the ethyl acetate, and the methanol extracts were tested separately for its bioactivity against the three pathogenic bacteria. The testing method used was agar diffusion (Kirby–Bauer) method with nutrient agar (NA) and nutrient broth (NB) as culture media. The concentration of each fraction and of the positive control (chloramphenicol) was 1 000 ppm and 100 ppm, respectively.

### 2.5. Flavonoid isolation

Ethyl acetate fraction that was identified as active against bacteria was further fractionated through gravity column chromatography. The stationary phase used was G60 silica (Merck) and the mobile phase was in the form of mixtures of *n*-hexane and ethyl acetate in increasing polarity. The subfractions were monitored on TLC plates using *n*-hexane: ethyl acetate (14: 6) mixture. Subfractions with a similar pattern on the TLC plate were combined and evaporated at room temperature. The combined subfractions which form solids were purified.

The solids were recrystallized using *n*-hexane and were dissolved in ethyl acetate. The purity of the isolate was checked by two-dimensional TLC. A major compound of the isolate was identified using LC-MS spectroscopy.

### 2.6. Antibacterial test of subfraction of the ethyl acetate fraction

The inhibition index of the ethyl acetate subfraction was determined using the disc agar diffusion method (Kirby–Bauer). The media consisted of a solid medium (NA) and a liquid medium (NB). The tested bacteria was firstly inoculated into NB and incubated for 24 h at 37 °C. A number of paper discs were dipped into the test sample which was dissolved in dimethyl sulfoxide (DMSO) thus obtained sample concentration in the paper disc were 200, 400, 600, 800, and 1 000 ppm. Chloramphenicol as a positive control and DMSO as a negative control each in 100 ppm was also dripped on other paper discs. Solid medium was poured into petri dishes as the first layer and the second layer-containing bacterial inoculant 1% (v/v) -was added in the form of semi-solid media. The paper discs were laid on the semi-solid layer and the petri dishes were incubated at 37 °C for 24 h and the inhibition index was calculated. Antimicrobial activity is categorized as high sensitivity level if the diameter of the zone inhibition is > 12 mm, moderate sensitivity level was given if the inhibition zone diameter of about (9–12) mm. Category level low sensitivity, when diameters ranging from 6 to 9 mm and resistant if < 6 mm (it has no inhibitory zone) [21].

## 3. Results

### 3.1. Isolation of naringenin-rich fraction

The yield of methanol crude extract from the maceration was 20.30%. The crude extract positively contains flavonoids, phenolics, and steroids after phytochemical qualitative tests. Further partitioning of the crude extract gave *n*-hexane fraction (45.95%), ethyl acetate fraction (18.05%), and some residual methanol fraction (22.43%). The ethyl acetate fraction was the one which able to inhibit the growth of all bacteria under observation (Table 1).

Purification of the ethyl acetate fraction was chromatographed to yield 70 subfractions. The subfraction 1 formed a yellowish solid and based on the 1-dimensional and 2-dimensional elution on TLC plate exhibited 2 spots, *i.e.* a yellow stain with *Rf* 0.8 and a bluish-green stain with *Rf* 0.725. Identification of the main component of the subfraction 1 based on LC-MS

**Table 1**
Inhibition index of fractions of the liquid–liquid extraction.

| Bacteria | Gram type | Inhibition index (mm) | | | | Naringenin-rich fraction | Chloramphenicol |
|---|---|---|---|---|---|---|---|
| | | *n*-Hexane | Ethyl acetate | Methanol | Chloramphenicol | | |
| *S. aureus* | Positive | 10.5 | 18.5 | 8.0 | 15.5 | 12.5 | 22.5 |
| *S. thypi* | Negative | – | 14.0 | 8.5 | 14.0 | 11.0 | 22.5 |
| *E. coli* EPEC K1.1 | Negative | – | 8.5 | – | 11.0 | 9.5 | 12.0 |

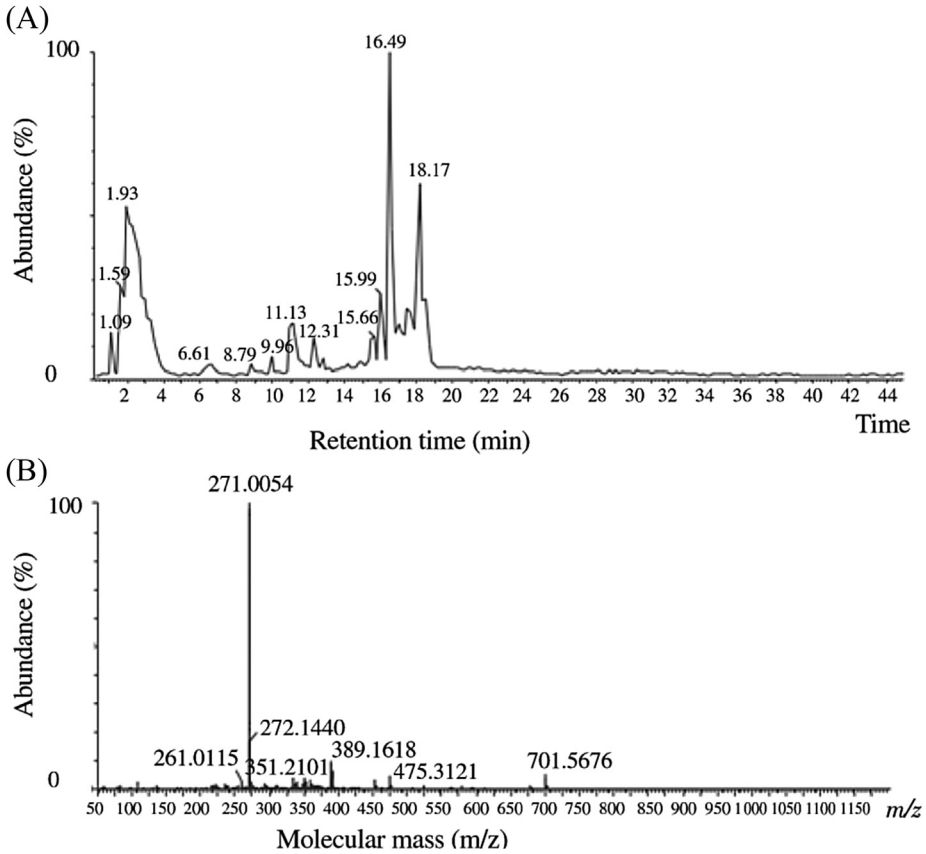


**Figure 1.** Major compound identification of subfraction by LC-MS.
(A) Chromatogram of subfraction; (B) Mass spectrum of subfraction on retention time 16.492 min.

spectra showed a compound with *m/z* 272 by stable fragmentation at *m/z* 271 which is evidenced by the abundance reached 100% at a retention time of 16 min (Figure 1).

### *3.2. Antibacterial activity of naringenin-rich fraction*

The naringenin-rich fraction was tested for its antibacterial activity against the three bacteria using Kirby–Bauer method. In this test, naringenin-rich fraction showed a good antibacterial activity against all tested bacteria (Table 1). The highest inhibition zone was shown by *S. aureus*, *i.e.* 11 mm. For comparison, the inhibition zone exhibited by chloramphenicol against *S. thypi* was twice as high.

## 4. Discussion

In this study, the methanol extracts turn to contain phenolics, flavonoids, and steroids. In fact, flavonoids are mostly found in pigeon pea leaves [22]. The same authors also report that flavonoids are well known secondary metabolite effective in the application of some medical treatments. The ethyl acetate fraction, which exhibited the most active toward the tested bacteria as compared to *n*-hexane and the residual methanol fractions in our observation, was also confirmed the likely presence of flavonoids in this particular fraction [10]. A semi-polar compound is able to inhibit the growth of bacteria for the bacterial cell membrane is not absolutely hydrophobic nor absolutely hydrophilic [23].

Our purified subfraction that stained the TLC plate as yellowish-green, bluish-green, or green is likely to contain flavonoids of flavanones types [24]. The subfraction 1 appeared at *m/z* 271 was believed to be naringenin. There was also a fragmentation pattern at *m/z* 177, 151, 119, 107, 93, and 83 [25]. Naringenin has been reported in the leaves pigeon pea [26]. The new finding of our study is a proof of antibacterial activity toward *S. thypi*.

Based on the results of antibacterial tests, naringenin-rich fraction with a concentration of 1 000 ppm was positively inhibited the bacterial growth. The inhibition was observed in accordance with the formation of a clear zone around the paper disc. The clear zone was formed by the active compound contained in paper disc that diffuses into the agar medium containing the bacteria and inhibited their growth. The bioactive compounds inhibit the synthesis of cell wall, nucleic acid, and protein, disturb the metabolism of bacteria or change the cell membrane permeability [27]. In this study, we verify that pigeon pea leaves contain naringenin, flavanones that is effective to inhibit the growth of *S. thypi, S. aureus*, and *E. coli* EPEC K1.1. This confirms the traditional use of the plant in the treatment of typhoid disease.

The naringenin-rich fraction showed the highest inhibition toward *S. aureus* among the three bacteria. *S. aureus* is a Gram-positive that has simple cell wall structure, single-layered with low lipid content, and enables the bioactive compounds to enter the cells. On the other hand, *S. thypi* and *E. coli* EPEC K1.1, are Gram-negative bacteria with more complex cells, three-layer lipoprotein consisting of an outer layer, a middle layer of lipopolysaccharide which acts as a barrier to antibacterial bioactive material, and a coat of peptidoglycan with high lipid content and thus more difficult to be destroyed. This proves that the

flavanones impede the bacterial proliferation by inhibiting cell wall synthesis. Flavanones has been previously reported as antibacterial [28,29]. In comparison, the inhibition of the subfraction 1 is half of that of the chloramphenicol against *S. thypi*. It can be presumed that the naringenin-rich fraction has inhibitory fairly well because with a concentration of 1 000 ppm, the fraction has inhibitory half of chloramphenicol inhibition at the concentration of 100 ppm. The naringenin-rich fraction inhibition is lower than that of chloramphenicol, as a pure compound, with a specific inhibitory mechanism. The fraction also has good antibacterial toward *E. coli* and EPEC K1.1. This particular strain produces an extracellular protease that degrades mucin so it can be attached to intestinal epithelial cells and cause diarrhea in the host [30].

The conclusion is that the compound suspected to be naringenin-a flavanone has a good antibacterial capability that could inhibit the growth of *S. thypi*, S. *aureus*, and *E. coli* EPEC K1.1, and have an indication to be able to treat typhoid.

## Conflict of interest statement

We declare that we have no conflicts of interest.

## References


[1] Fu Y, Zu Y, Liu W, Efferth T, Zhang N, Liu X, et al. Optimization of luteolin separation from pigeon pea [*Cajanus cajan* (L.) Millsp.] leaves by macroporous resins. *J Chromatogr A* 2006; **1137**(2): 145-52.

[2] Okpala LC, Okoli EC. Development of cookies made with cocoyam, fermented sorghum and germinated pigeon pea flour blends using response surface methodology. *J Food Sci Technol* 2014; **51**(10): 2671-7.

[3] Mahitha B, Archana P, Ebrahimzadeh MH, Srikanth K, Rajinikanth M, Ramaswamy N. *In vitro* antioxidant and pharmacognostic studies of leaf extracts of *Cajanus cajan* (L.) Millsp. *Indian J Pharm Sci* 2015; **77**(2): 170-7.

[4] Nahar L, Nasrin F, Zahan R, Haque A, Haque E, Mosaddik A. Comparative study of antidiabetic activity of *Cajanus cajan* and *Tamarindus indica* in alloxan-induced diabetic mice with a reference to *in vitro* antioxidant activity. *Pharmacog Res* 2014; **6**(2): 180-7.

[5] Uchegbu NN, Ishiwu CN. Germinated pigeon pea (*Cajanus cajan*): a novel diet for lowering oxidative stress and hyperglycemia. *Food Sci Nutr* 2016; **4**(5): 772-7.

[6] Chander MPP, Kartick C, Vijayachari P. Ethnomedicinal knowledge among Karens of Andaman & Nicobar Islands India. *J Ethnopharm* 2015; **162**: 127-33.

[7] Kichu M, Malewska T, Akter K, Imchen I, Harrington D, Kohen J, et al. An ethnobotanical study of medicinal plants of Chungtia village, Nagaland, India. *J Ethnopharm* 2015; **166**: 5-17.

[8] Pal D, Mishra P, Sachan N, Ghosh AK. Biological activities and medicinal properties of *Cajanus cajan* (L) Millsp. *J Adv Pharm Tech Res* 2011; **2**(4): 207-14.

[9] Fu YJ, Liu W, Zu YG, Tong MH, Li SM, Yan MM, et al. Enzyme assisted extraction of luteolin and apigenin from pigeon pea [*Cajanus cajan* (L.) Millsp.] leaves. *Food Chem* 2008; **111**(2): 508-12.

[10] Patel NK, Bhutani KK. Pinostrobin and cajanus lactone isolated from *Cajanus cajan* (L.) leaves inhibits TNF-α and IL-1β production: *in vitro* and *in vivo* experimentation. *Phytomedicine* 2014; **21**(7): 946-53.

[11] Kong Y, Zu YG, Fu YJ, Liu W, Chang FR, Li J. Optimization of microwave-assisted extraction of cajaninstilbene acid and pinostrobin from pigeon pea leaves followed by RP-HPLC-DAD determination. *J Food Compos Anal* 2010; **23**(4): 382-8.

[12] Luo QF, Sun L, Si JY, Chen DH. Hypocholesterolemic effect of stilbenes containing extract fraction from *Cajanus cajan* on diet induced hypercholesterolemia in mice. *Phytomedicine* 2008; **15**(11): 932-9.

[13] Nicholson RA, David LS, Le Pan R, Liu XM. Pinostrobin from *Cajanus cajan* (L.) Millsp. inhibits sodium channel-activated depolarization of mouse brain synaptoneurosomes. *Fitoterapia* 2010; **81**(7): 826-9.

[14] Okigbo RN, Omodamiro OD. Antimicrobial effect of leaf extracts of pigeon pea (*Cajanus cajan* (L.) Millsp.) on some human pathogens. *J Herbs Spices Med Plants* 2006; **12**(2): 117-27.

[15] Galán JE. Typhoid toxin provides a window into typhoid fever and the biology of *Salmonella typhi*. *Proc Natl Acad Sci USA* 2016; **113**(23): 6338-44.

[16] Tala DS, Gatsing D, Fodouop SPC, Fokunang C, Kengni F, Djimeli MN. *In vivo* anti-salmonella activity of aqueous extract of *Euphorbia prostrate* Aiton (Euphorbiaceae) and its toxicological evaluation. *Asian Pac J Trop Biomed* 2015; **5**(4): 310-8.

[17] Ochiai RL, Acosta CJ, Danovaro-Holliday MC, Baiqing D, Bhattacharya SK, Agtini MD, et al. A study of typhoid fever in five Asian countries: disease burden and implications for controls. *Bull World Health Organ* 2008; **86**(4): 260-8.

[18] Ministry of Health Republic of Indonesia. *Indonesia health profile 2010*. Jakarta: Ministry of Health Republic of Indonesia; 2010. [Online]. Available from: http://www.depkes.go.id/resources/download/pusdatin/profil-kesehatan-indonesia/profil-kesehatan-indonesia-2010.pdf [Accessed on 13th July, 2015]

[19] Prasad ER, Merzendorfer H, Madhurarekha C, Dutta-Gupta A, Padmasree K. Bowman-Birk proteinase inhibitor from *Cajanus cajan* seeds: purification, characterization, and insecticidal properties. *J Agric Food Chem* 2010; **58**(5): 2838-47.

[20] Harborne JB. *Phytochemical methods*. 2nd ed. London: Chapman and Hall; 1987.

[21] Pan X, Chen F, Wu T, Tang H, Zhao Z. The acid bile tolerance and antimicrobial property of *Lactobacillus acidophilus* NIT. *Food Control* 2009; **20**: 598-602.

[22] Nix A, Paul CA, Colgrave M. The flavonoid profile of pigeon pea, *Cajanus cajan*: a review. *Springer Plus* 2015; **4**(125): 1-6.

[23] Kanazawa A, Ikeda T, Endo T. A novel approach to mode of action of cationic biocides morfological effect on bacterial activity. *J Appl Bacteriol* 1995; **78**: 55-60.

[24] Markham KR. *Techniques of flavonoid identification*. London: Academic Press; 1988.

[25] Sanchez-Rabaneda F, Jauregui O, Casals I, Andres-Lacueva C, Izquierdo-Pulido M, Lamuela-Raventos RM. Liquid chromatographic/electrospray ionization tandem mass spectrometric study of the phenolic composition of cocoa (*Theobroma cacao*). *J Mass Spectrom* 2003; **38**(1): 35-42.

[26] Wei ZF, Luo M, Zhao CJ, Li CY, Gu CB, Wang W, et al. UV-induced changes of active components and antioxidant activity in postharvest pigeon pea [*Cajanus cajan* (L.) Millsp.] leaves. *J Agric Food Chem* 2013; **61**(6): 1165-71.

[27] Prescott LM, Harley JP, Klein DA. *Microbiology*. 6th ed. New York: McGraw-Hill; 2005.

[28] Rauha JP, Remes S, Heinonen M, Hopia A, Kähkönen M, Kujala T, et al. Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *Int J Food Microb* 2000; **56**(1): 3-12.

[29] Li Y, Zhao J, Gao K. Activity of flavanones isolated from rhododendron Hainanense against plant pathogenic fungi. *Nat Prod Commun* 2016; **11**(5): 611-2.

[30] Budiarti S, Mubarik NR. Extracellular protease activity of Enteropathogenic *Escherichia coli* on mucin substrate. *HAYATI J Biosci* 2007; **4**(1): 36-8.