ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 11, Issue 1, 2018

Online - 2455-3891
Print - 0974-2441

Research Article

FLAVONOIDS FROM ENDOPHYTIC BACTERIA OF *COSMOS CAUDATUS* KUNTH. LEAF AS ANTICANCER AND ANTIMICROBIAL

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Received: 14 August 2017, Revised and Accepted: 10 October 2017

ABSTRACT

Objective: The present study aimed to isolate and examine the characteristics of endophytic bacteria from kenikir (*Cosmos caudatus* Kunth.) leaves, to determine the endophytic bacteria capable of producing flavonoids, and to test their potency as anticancer and antimicrobial.

Methods: The isolation of endophytic bacteria from *C. caudatus* Kunth. leaf was conducted by technical surface sterilization. Each of these isolates was produced from the insulation and then cultured on a liquid medium of 0.1% soluble starch, 0.5% peptone, and 0.15% yeast extract with a pH of 7 which have been incubated in room temperature for 5 days with agitation of 120 rpm and extracted with ethyl acetate solvent. The extract was then tested for flavonoid content using thin-layer chromatography method. The anticancer ability of flavonoids was tested by brine shrimp lethality test (BSLT) method, while that for antimicrobial, the test used paper disc method.

Results: A total of 15 isolates of endophytic bacteria were successfully isolated from the kenikir leaves, but only 4 isolates produced flavonoids, among others PKM 1 (*Serratia* sp.), PKM 2 (*Neisseria* sp.), PKM 17 (*Acinetobacter* sp.), and PKM 19 (*Yersinia* sp.). BSLT test results showed that the flavonoids cause mortality in *Artemia salina* leach with LC_{50} as much as 16.736 in PKM 17, 17.267 in PKM 2, 18.672 in PKM 1, and 23.411 in PKM 19. The flavonoids also inhibited the growth of pathogens in human-based antimicrobial test results.

Conclusion: Flavonoids produced by four endophytic bacterial isolates from kenikir leaves have great potential as anticancer and antimicrobial.

Keywords: *Cosmos caudatus*, Leaf, Endophytic bacteria, Flavonoids, Anticancer, Antimicrobial.

 DOI: http://dx.doi.org/10.22159/ajpcr.2018.v11i1.21987

INTRODUCTION

Cosmos caudatus Kunth. known as kenikir (Indonesia) or ulam raja (Malaysia) is an edible vegetable. The nutritious kenikir leaves are usually used as an appetite enhancing herb, a bone-strengthening agent, a weak stomach medication, and an insect repellent [1]. Kenikir leaves contain high antioxidant compound equal to 70 mg/L [2]. Methanol leaf extract of kenikir contains flavonoids and quercetin glycosides potential as cancer drugs [3]. Studies involving kenikir leaves as cancer drugs have been reported to be able to induce apoptosis of colon cancer cells [4], the leukemia cancer cells [5], and breast cancer cells [1].

Aside from being an anticancer, kenikir leaves also proved to be antimicrobial. Extract hexane, diethyl ether, and ethanol leaves of *C. caudatus* Kunth. were reported to inhibit the growth of Gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus* and Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* in which the minimum inhibitory concentrations (MIC) value ranges for 6.325 mg/ml [6]. This plant is also reported to possess anti-QS activity against *Chromobacterium violaceum* with DIZ of 21 mm and MIC of 31 mg/ml [7]. Sequential leaf extracts of *C. caudatus* Kunth. screened for antifungal activity using cup method showed that the ethyl acetate extract was the most effective agent in inhibiting fungal growth and spore germination [8].

Kenikir leaves contain phenolic compounds of caffeoylquinic acids, quercetin glycosides, catechins, and proanthocyanidins. Caffeoylquinic acids consist of chlorogenic, neochlorogenic, and cryptochlorogenic [9]. Quercetin glycosides include arabinofuranoside, glucoside, rhamnoside, and rutinososide [10,11]. Quercetin is the most dominant flavonoid compound in *C. caudatus* Kunth. leaf (51%) [12-14].

Quercetin could act as an antioxidant [15]. Quercetin has extraordinary antioxidant properties caused by its pharmacophores B ring, and the group $ONOO^-$ makes quercetin actable as a strong inhibitor of lipid peroxidation. In addition to the antioxidant properties, quercetin may also increase glutathione concentrations potentially inhibiting free radical formation [16]. Quercetin is able to regulate the cycle of cancer cells by binding to multiple targets, for example, by blocking the cell cycle at the G2/M phase or G1/S transition and also capable of interfering with MMP, thus triggering the release of cytochrome c in the cytoplasm and the activation of caspase-3 and caspase-7 [17]. Quercetin also inhibits the growth of *Staphylococcus aureus, Clostridium botulinum*, *Actinobacillus actinomycetemcomitans,* and *Porphyromonas gingivalis* [18].

Endophytic microbes are bacterial microorganisms (including actinomycetes) or fungi that spend all or part of their life cycle to live in plant tissues. Endophytic bacteria will not affect or disinfect the host cells [19,20]. Each plant tissue possesses endophytic bacteria in it. Endophytic bacteria living in plants can produce secondary metabolite of compounds that resemble or are similar to their hosts and are suspected as a result of coevolution and genetic recombination of the host plants to the microorganism [21]. Therefore, endophytic bacteria in kenikir are expected to produce secondary metabolites similar to kenikir. In addition, the existence of life cycles and the ability of bacteria in multiplying quickly make endophytic bacteria grow and produce secondary metabolites faster as well. In conclusion, endophytic bacteria have the potential to be the vast producer of anticancer and antimicrobial compounds because of its relatively fast growth and large amount. This study aimed to isolate and examine the characteristics of endophytic bacteria from kenikir (*C. caudatus* Kunth.) leaves, to determine the endophytic bacteria capable of producing flavonoids, as well as to test its potential as anticancer and antimicrobial.

METHODS

Isolation of endophytic bacteria

Isolation of endophytic bacteria from *C. caudatus* Kunth. leaf was carried out by technical surface sterilization. The obtained leaf samples of *C. caudatus* Kunth. were cleared from the dirt and weighed about 1 g of its wet weight. The surface of the leaf tissue was sterilized by soaking the leaves in 70% alcohol for 1 min, and then, NaOCL 3+0.05% Tween 20 for 3 min. The last step was rinsing in a sterile aquadest for 3 times. Subsequently, the sample was mashed using a sterile mortar until smooth, and its dilution was performed at 10^{-1}. The suspension at 10^{-1} dilution was inoculated on Tryptic Soybean Agar (TSA) medium and incubated for 24 h at room temperature. As a control, the sterilized kenikir leaves that have not been mashed were streaked on TSA medium [22]. The identification of endophytic bacteria applied the Manual for Identification of Medical Bacteria [23].

Secondary metabolism extraction of endophytic bacteria

Endophytic bacteria, grown earlier in Erlenmeyer containing sterile liquid medium with composition: (0.1% soluble starch, 0.5% peptone and 0.15% yeast extract with pH of 7), were incubated in room temperature for 5 days with agitation of 120 rpm. This culture was inserted in a sterile tube and then centrifuged at 10,000 rpm for 10 min to separate the pellets and supernatant. The supernatant was given amyl acetate of 1:1. This mixture was shaken for 15 min to form three layers. The upper layer was taken and evaporated to obtain the secondary metabolism of endophytic bacteria which was then used for testing its flavonoid content [24].

Thin-layer chromatography (TLC) test

The supernatant content of the aqueous culture of endophytic bacteria extraction was tested using TLC by heating KLT Silica gel F254 plate in the oven at 110°C for 15 min. 0.01 g of secondary metabolism extract and standard quercetin were dissolved in 0.5 acetic acids, and then, bottled in capillary pipe at 1 cm from the bottom edge of the plate, dried, and eluted with chloroform eluent:methanol (1:4). Observations were made on stain spots on the plate surface under visible light and ultra violet (UV) light at a wavelength of 365 nm [25,26].

Brine shrimp lethality test (BSLT)

Anticancer testing used BSLT method involving *Artemia salina* Leach as test subject. *A. salina* Leach larvae was hatched by soaking its eggs in seawater for 48 h in a vessel equipped with an aerator. Parts of seawater that did not contain larvae eggs were illuminated in order the hatched larvae moved toward the light and left its shell [27].

The test execution was carried out by equalizing the final volume of the extract flavonoids with the concentration of the treatment at 0 ppm, 1 ppm, 10 ppm, 100 ppm, and 1000 ppm diluted by first adding 5 ml of seawater into each test tube until the flavonoid extract was dissolved, and then, inserted 10 tails of shrimp larvae aged 48 h in each tube. The test tube was then placed under lighting for 24 h, and the number of dead shrimp larvae was counted. Larvae were considered dead if they did not exhibit any form of movement during several seconds of observation. Each concentration of treatment was replicated 5 times. LC_{50} values were determined by probit analysis LC_{50} [27,28].

Antimicrobial test

The flavonoid extracts resulted by endophytic bacteria isolates were tested on the antimicrobial capabilities on four human pathogenic bacteria *(E. coli, Salmonella typhi, Shigella dysenteriae,* and *Vibrio cholerae)* and three human pathogenic fungi (Aspergillus flavus, A. niger, and Candida albicans) by a paper disk method by putting paper discs soaked in the test solution on a solid medium inoculated with bacteria. After the incubation period of 24 h for bacteria and 72 h for fungi, the diameter of inhibition zone formed around the paper disc was measured [29]. As control group, chloramphenicol antibiotic was used for bacteria, and ketoconazole antibiotic was used for fungi.

RESULT AND DISCUSSION

Isolation of endophytic bacteria and TLC test

15 endophytic bacteria isolates were successfully collected from kenikir (*C. caudatus* Kunth.) leaves. The bacterial isolates were cultured and then extracted for their flavonoids. All isolates yielded yellow-to-bright yellow extracts. The flavonoids extracts were then tested using TLC. The flavonoid compound tested using TLC with non-reagent-visible-light yielded no color, whereas with reagent-visible-light yielded light yellow color. Using UV light 366 with or without reactants, the compounds showed a blue color [25,26]. Based on the observation, 4 positive flavonoid isolates were found as shown in Table 1.

One of the flavonoids in endophytic bacterial isolates of kenikir is predicted to be quercetin compound. The kenikir leaves produced several derived flavonoid compounds such as quercetin 3-O-glucoside, quercetin pentose, quercetin deoxyl-hexose, and catechin, while others are derivatives of phenolic acids [14].

Of the 4 endophytic bacteria, isolates proven to produce flavonoids were characterized using the Manual for the Identification of Medical Bacteria [23]. The identification results of the four bacteria are shown in Tables 2 and 3.

Based on the spread of the characteristics (Tables 2 and 3), PKM 1 refers to *Serratia* sp., PKM 2 refers to *Neisseria* sp., PKM 17 refers to *Acinetobacter* sp., and PKM 19 refers to *Yersinia* sp. The characteristics of *Serratia* sp. are motile, rod-shaped, facultative anaerobe, 0.5–0.8 µm in diameter, and 0.9–2 µm in length. This species can grow at a temperature of 540°C and is naturally found in soil, water, and plant surfaces. Some strains of *Serratia. marcescens* may produce prodigiosin pigments that are dark red to pink, depending on the age of the bacterial colony. The *Neisseria* sp. is aerobe, motile, rod-shaped, 0.1–3 µm diameter in colony length and yellow in color, Gram-negative, positive in catalase and oxidation, and H_2S production. The optimum temperature is 35–37°C. The *Acinetobacter* sp. is aerobes, motile, Gram-negative, rod-shaped, 0.1–0.3 µm in colony diameter, white and shiny in colony surface, and positive in catalase, oxidase, and H_2S production. The optimum growth temperature is 33-35°C. The *Yersinia* sp. is stem cell-shaped, Gram-negative, aerobe, motile, 0.5–0.8 µm in colony diameter, yellow and shiny on the colony surface, positive in oxidase, and negative H_2S production. The optimum growth temperature is 28–30°C.

BSLT

To find the potential of flavonoids yielded by the four endophytic bacteria, BSLT as the most widely used method to search for new anticancer compounds from plants was used. The BSLT method has been proven to have a correlation with anticancer activity. In addition, it is also easy to do, cheap, fast, and accurate enough [27]. BSLT test results are shown in Table 4.

The result of BSLT test showed that the flavonoids produced by four endophytic bacteria caused the death of *A. salina* leach larvae. The percentage of deaths increased along with the increasing concentrations of flavonoid compounds. The result of probit analysis using SPSS showed that the value of LC_{50} from PKM 1 flavonoid compound was 18.672 ppm, PKM 2 was 17.267 ppm, PKM 17 was 16.736 ppm, and PKM 19 was 23.411 ppm.

An extract shows toxicity activity if the extract can cause 50% mortality of the test subjects at concentrations <1000 ppm. Based on these statements, the flavonoid extract from four endophytic bacteria has potential as anticancer, antibacterial, and antifungal due to its toxicity [27].

Flavonoid compounds produced from endophytic bacteria from kenikir leaves are purported to be quercetin. Quercetin is a natural anticancer antioxidant proven both *in vitro* and *in vivo* testing. Numerous studies have shown that quercetin significantly inhibits breast, colon, prostate, ovarian, endometrial, and lung cancer.

Table 1: TLC results of endophytic bacteria

No	Bacterial isolate	Gram (+/−)	TLC test results				
			Visible light		UV 365 nm		Note
			Without reacting	With reacting	Without reacting	With reacting	
1	PKM 1	−	-	Bright Yellow	Blue	Blue	Flavonoid
2	PKM 2	−	-	Bright Yellow	Blue	Blue	Flavonoid
3	PKM 5	−	-	-	Blue	Blue	-
4	PKM 6	−	-	-	Blue	Blue	-
5	PKM 7	−	-	-	Blue	Blue	-
6	PKM 10	−	-	-	Blue	Blue	-
7	PKM 11	−	-	-	Blue	Blue	-
8	PKM 12	−	-	-	Blue	Blue	-
9	PKM 17	−	-	Bright Yellow	Blue	Blue	Flavonoid
10	PKM 19	−	-	Bright Yellow	Blue	Blue	Flavonoid
11	PKM 20	−	-	-	Blue	Blue	-
12	PKM 21	−	-	-	Blue	Blue	-
13	PKM 23	−	-	-	Blue	Blue	-
14	PKM 24	−	-	-	Blue	Blue	-
15	PKM 26	−	-	-	Blue	Blue	-

TLC: Thin-layer chromatography

Table 2: Morphology observation results of endophytic bacteria producing flavonoids

Bacteria isolate	Colony				Cell form
	Color	Form	Elevation	Edge	
PKM 1	Red	Circular	Raised	Entire	Basil
PKM 2	Yellow	Irreguler	Flat	Lobate	Basil
PKM 17	White	Circular	Flat	Entire	Basil
PKM 19	Yellow	Circular	Flat	Entire	Coccus

Table 3: Physiological observation results of endophytic bacteria producing flavonoids

Bacteria isolate	Physsiological observation results					Species
	Motility	O_2 requirement	Catalase	Oxidation	Glucose	
PKM 1	+	+	+	+	−	*Serratia* sp.
PKM 2	+	+	+	+	−	*Neisseria* sp.
PKM 17	+	+	+	−	−	*Acinetobacter* sp.
PKM 19	+	+	+	+	−	*Yersinia* sp.

Table 4: BSLT test results

Sample	Concentration (ppm)	Mortality (%)	LC_{50} (ppm)
PKM 1	0	0	18,672
	1	8.42	
	10	40	
	100	79.52	
	1000	97.41	
PKM 2	0	0	17,267
	1	9.57	
	10	41.33	
	100	80.72	
	1000	98.29	
PKM 17	0	0	16.736
	1	9.57	
	10	40	
	100	78.31	
	1000	97.39	
PKM 19	0	0	23.411
	1	9.57	
	10	33.77	
	100	75.31	
	1000	95.49	

BSLT: Brine shrimp lethality test

Some theories underpin the mechanism of flavonoids as anticancer. Flavonoids act as antioxidants through the mechanism of activation of cancer cell apoptosis pathway. The mechanism of cell apoptosis in this theory is due to DNA fragmentation beginning with the release of proximal chains of DNA by reactive oxygen compounds such as hydroxyl radicals. Another effect is flavonoids as inhibitors of cancer proliferation, one of them is by inhibiting the activity of protein kinase that inhibits signal transduction path from the membrane to core cell. Flavonoids inhibit the activity of tyrosine kinase receptors because the increased activity of tyrosine kinase receptors contributes to the growth of malignant cancer cells. Flavonoids also serve to reduce tumor resistance to chemotherapeutic agents [30].

Flavonoids can stimulate apoptosis through several mechanisms such as inhibition of topoisomerase I/II DNA activity, modulation of signaling pathways, decreased expression of Bcl-2 and Bcl-XL genes, enhanced expression of Bax and Bak, and endonuclease activation [4]. Quercetin has the ability to stimulate apoptosis of Caco-2 and HT-29 colon cancer cells and HL-60 leukemia cancer cells by stimulating the release of cytochrome c from mitochondria [5]. Quercetin also showed synergistic effects with *cisplatin in vitro* and *in vivo* through inhibition of protein kinase C [31].

Table 5: Antimicrobial activity of flavonoids from endophytic bacteria of *Cosmos caudatus* Kunth. Leaf

Tested microorganisms	Secondary metabolites from isolate				
	Diameter of inhibition zone (mm)				
	PKM 1	PKM 2	PKM 17	PKM 19	Control
Escherichia coli	9.88±1.13^{b}	8.30±0.88^{a}	10.26±0.10^{b}	6.82±0.65^{a}	12.40±1.11^{c}
Salmonella typhi	9.04±0.76^{b}	8.22±0.13^{ab}	9.28±0.85^{b}	7.21±0.82^{a}	10.72±1.11^{c}
Shigella dysenteriae	8.16±0.04^{b}	7.82±0.17^{b}	9.41±0.52^{c}	6.16±0.17^{a}	11.20±0.78^{d}
Vibrio cholerae	9.36±0.71^{bc}	8.12±0.62^{b}	10.24±1.03^{cd}	5.24±0.37^{a}	10.24±0.28^{d}
Aspergillus flavus	9.43±0.85^{bc}	7.42±0.79^{a}	8.74±0.09^{b}	6.54±0.75^{a}	10.32±0.44^{c}
Aspergillus niger	9.62±0.29^{c}	7.63±0.55^{b}	8.02±0.19^{b}	5.71±0.71^{a}	9.84±1.07^{c}
Candida albicans	9.34±0.59^{d}	7.42±0.22^{b}	8.23±0.32^{c}	5.82±0.35^{a}	10.12±0.04^{e}

Description: The data are mean of±SD, n=5. Means with different superscript letters in the same row indicate significant ($p<0.05$) based on LSD test 95%. LSD: Least significant difference

Antimicrobial test

Besides known as anticancer, kenikir is also antimicrobial. To ascertain whether the flavonoid produced by endophytic bacteria is antimicrobial, the antimicrobial test is done using paper disc method. The results of the measurement of the clear zone diameter can be seen in Table 5.

The results (Table 5) show that flavonoids from endophytic bacteria inhibit human microbial pathogens both bacteria and fungi. The best flavonoids inhibitory against bacteria are produced by PKM 17, while the best inhibitory power against the fungi is the flavonoid produced by PKM 1.

Flavonoids are bioactive molecules. Its biological activity is closely related to the molecular structure, i.e., their hydroxyl groups or phenolic ring. Phenolic compounds have the capacity to link with proteins and bacterial membrane to form complexes [32]. The flavonoid antibacterial mechanisms include inhibiting nucleic acid synthesis, disrupting cytoplasmic membrane function, and inhibiting energy metabolism [33] and affect peptidoglycan synthesis around a bacterial cell [34].

Quercetin, one type of flavonoids, proved to inhibit Gram-positive and Gram-negative bacteria through the inactivating extracellular proteins [35,36]. It has been demonstrated that the antibacterial mechanism of quercetin probably depended on disruption of the membrane and inactivation of extracellular proteins by forming irreversible complexes, but the exact mechanism remains unclear [37].

CONCLUSION

A total of 15 isolates of endophytic bacteria were successfully isolated from kenikir leaves, but only four produced flavonoids were found to have anticancer and antimicrobial activity.

AUTHORS CONTRIBUTION

Fiqih Ramadhan, has majorly performed the experiment in the laboratory, and data analysis Luluk Mukarramah, has has majorly performed the experiment in the laboratory Fikri Ainur Risma Hardiyanti Oktavia, has majorly performed the experiment in the laboratory Ria Yulian, has majorly performed the experiment in the laboratory Nurul Hilyatun Annisyah, has majorly performed the experiment in the laboratory Iis Nur Asyiah, has intellectual content, data analyis and responsible for the preparation of article.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article

ACKNOWLEDGMENTS

The authors would like to thank the Indonesian Ministry of Research, Technology and Higher Education for the financial support through PKM Research program in 2017, First Vice Rector (Academic and Student Affairs), and Dean of Faculty of Teacher Training and Education of University of Jember

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