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RESEARCH ARTICLE

Chemical composition and antibacterial activities of the ethanol extracts from the leaves and tubers of *Amorphophallus pusillus*

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ABSTRACT

Amorphophallus pusillus is a rare species which is found only in Binh Chau-Phuoc Buu Nature Reserve. In this study, we determined 7 flavonoid compounds in *A. pusillus* tubers and leaves, including of vitexin, orientin, vitexin 2"-*O*-glucoside, cyanidin 3-*O*-rutinoside, pelargonidin 3-*O*-glucoside, schaftoside, and peonidin 3-*O*-rutinoside via liquid chromatography-mass spectrometry (LC/MS). Furthermore, we have proved the antibacterial activities of ethanol extracts obtained from *A. pusillus* leaves and tubers for the first time. The data revealed that ethanol extracts could inhibit the growth of 6 tested microorganisms, such as *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Salmonella typhimurium* and *Staphylococcus aureus*. These data suggested the potential application of ethanol extracts isolated from this species as natural antimicrobial agents for treatment of infection caused by bacteria, especially in dermatologic and enteric infection.

Introduction

Amorphophallus is a large genus of the Araceae family which comprises of 200 species over the world with 30 species in Vietnam (1-4). The application of several species of this genus in food industry and medicine has been documented. For example, the root of *A. paeoniifolius* contains high concentration of essential minerals, starch, carbohydrate, which suggests the potential application of this species in food industry (5). Furthermore, analgesic, anti-helminthic and antimicrobial activities of *A. paeoniifolius* extracts also have been recorded (5). Accordingly, six plant species of *Amorphophallus* genus, including *A. krausei*, *A. yunnanensis*, *A. yuloensis*, *A. paeoniifolius*, *A. konjac* and *A. corrugatus* recorded in Northern mountain provinces of Vietnam, contain glucomannan, a plant component with many health benefits, such as treatment for constipation, improving of type 2 diabetes, lowering blood cholesterol and sugar (4). Recently, it has been revealed that methanol extract

from *A. lanceolatus* tuber contained 12 chemical compounds and could hinder the growth of 6 bacterial strains (6).

Amorphophallus pusillus was described by Hetterschied and Serebryanyi from Xuyen Moc District, Ba Ria-Vung Tau Province, Vietnam (7). This is an endemic species of Vietnam till date, *A. pusillus* has only been mentioned. In some studies on the genus *Amorphophallus* and flora Vietnam, but its chemical composition and bioactivities have not been studied yet. The previous studies about the antibacterial activity of other members of *Amorphophallus*, including *A. paeoniifolius* and *A. lanceolatus*, give a possibility that *A. pusillus* extract has the bacterial activity (5, 6). In this study, we elucidated the chemical composition via liquid chromatography-mass spectrometry (LC/MS) and proved antibacterial activities of ethanol extracts of *A. pusillus* leaves and tubers, which provides the information for further application and improves the value of *A. pusillus*.

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Materials and Methods

Plant material

Specimens of *A. pusillus* (1 kg) were collected from Binh Chau-Phuoc Buu Nature Reserve, Bung Rieng Ward, Xuyen Moc District, Ba Ria-Vung Tau Province, location of about 10°32'55"N; 107°29'12"E, May 17, 2019, 31 m in elevation (Fig. 1). The plant was identified by Dr. Hong Thien Van (Department of Biotechnology, Institute of Biotechnology and Food-technology, Industrial University of Ho Chi Minh City, Vietnam) and a voucher specimen of this material was deposited at the Herbarium of Binh Chau-Phuoc Buu Nature Reserve (H.T. Van 202).

Microorganisms

The antibacterial activity of the extract was evaluated using six bacterial strains, including two Gram-positive bacteria (*Bacillus cereus* (ATCC 11774) and *Staphylococcus aureus* (ATCC 25923), and four Gram-negative bacteria (*Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella enteritidis* (ATCC 13976), *Salmonella typhimurium* (ATCC 13311)). All of bacterial strains were provided from the microbiology collection, Department of Biotechnology, Institute of Food and Biotechnology, Industrial University of Ho Chi Minh city, Vietnam. The strains were stored in 20% glycerol solution at -20°C and subsequently re-activated in Luria-Bertani broth at 37°C for 24 h before the experiments.

Extraction procedure

The ethanol extracts of *A. pusillus* leaves and tubers were prepared (6). Briefly, 500 g of fresh leaves or sliced tubers of *A. pusillus* were moderately dried at 50°C for 1-2 days until constant weight and milled to

dried powder by electric blender (HR- 2068, Philips, China). 100 g of the resulting powder was macerated in 1000 ml of 99% ethanol for 7 days at room temperature and filtered through filter paper to collect the extract. The extract was then condensed under vacuum pressure at 60°C to obtain the brown medicinal paste. To ensure the absolute absence of ethanol in the paste, sublimation dryer was utilized. The obtained extracts were stored at 4°C until further use.

Liquid chromatography mass spectrometry (LC/MS)

For identification of the components in the extract, LC-MS analysis was performed at the Central Laboratory for Analysis, University of Science, Vietnam National University of Ho Chi Minh City. Aliquots of ethanol extracts were injected into HPLC Agilent 1200 infinity liquid chromatography system (Agilent Technologies, CA, USA) coupled with

Table 1. Gradient elution program for the chromatographic separation

Time (min)	Solvent A*	Solvent B*
0	90	10
15	0	100
30	0	100
31	90	10
40	90	10

(*): presented as the percentage of volume of mobile phase
Solvent A: Deionized water with 0.1% formic acid
Solvent B: Acetonitrile with 0.1% formic acid

MicroTOF-QII mass spectrometer (Bruker Daltonics, Germany). The components were separated on ACE3-C₁₈ analytical column (4.6 ×150 mm, 3.5 μm) as stationary phase and the temperature was kept at 40°C. The mobile phase used consisted of deionized

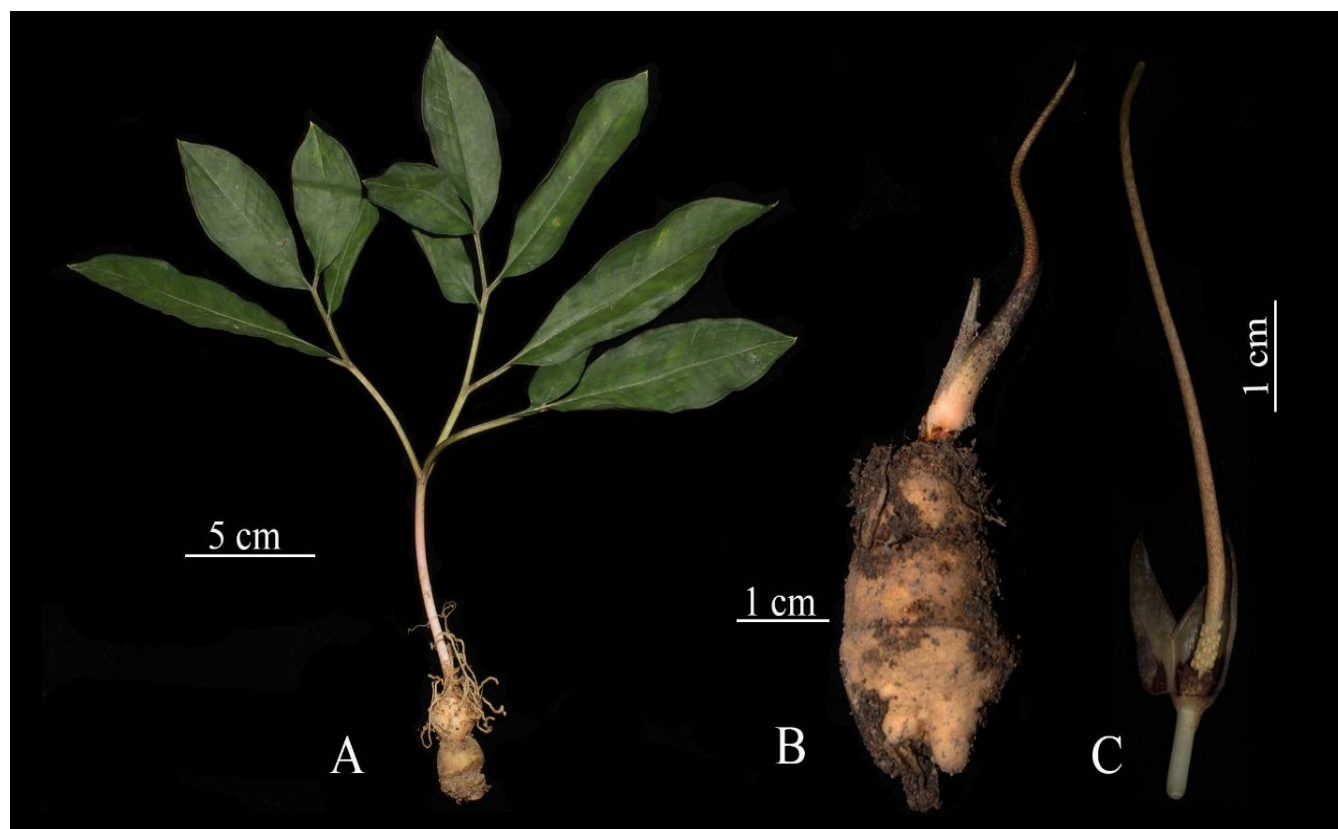


Fig. 1. *Amorphophallus pusillus*. A. Tuber and leaf, B. Tuber and spathe, C. Spadix

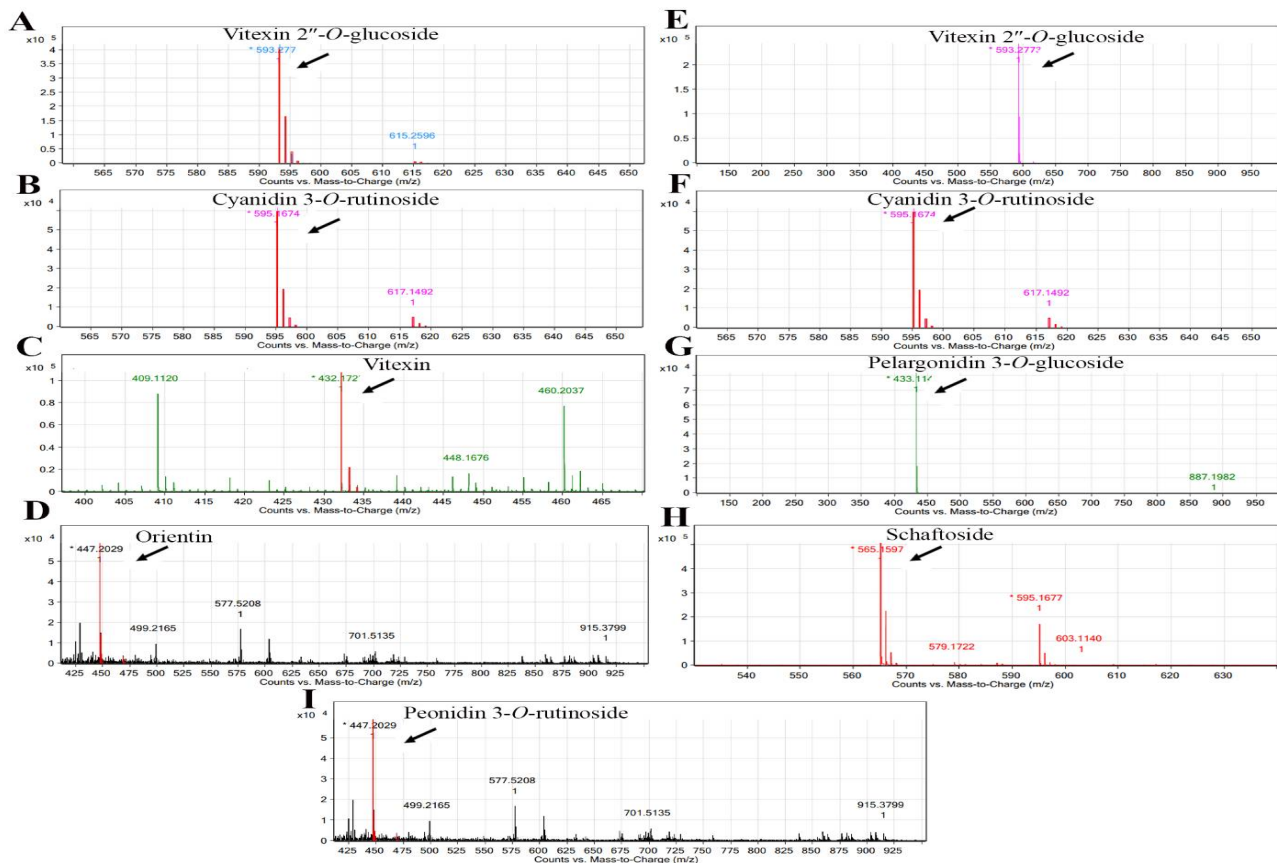


Fig. 2. Mass spectrometry diagrams of 7 compounds of ethanol extracts of *A. pusillus* leaves and tubers Note: A, B, C, D are diagrams of compounds found in tubers; while E, F, G, H, I are diagrams of compounds found in leaves

water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B), and the flow rate was set at 0.3mL/min. The gradient elution program for chromatographic separation was summarized in Table 1. In mass spectrometer, the extract was then ionized using electrospray

Table 2. Phytochemical composition of ethanol extracts of *A. pusillus* tubers

Compounds	RT	m/z	Bioactivity	Ref.
Vitexin	1.458	432	cardio-protective, anticancer, anti-inflammation, antioxidant, and anti-diabetic effects	(11)
Orientin	6.907	447	Anti-adipogenesis, antibacterial, antiviral, neuroprotective effects	(10)
Vitexin 2''-O-glucoside	10.994	593		
Cyanidin 3-O-rutinoside	3.628	595	Anti-leukemic activity	(15)

Table 3. Phytochemical composition of ethanol extracts of *A. pusillus* leaves

Compounds	RT	m/z	Bioactivity	Ref.
Pelargonidin 3-O-glucoside	3.867	433	Anti-inflammatory effect	(14)
Schaftoside	3.773	565	Antioxidant and antinematodal drug	(23)
Vitexin 2''-O-glucoside	11.005	593		
Cyanidin 3-O-rutinoside	3.657	595	Anti-leukemic activity	(15)
Peonidin 3-O-rutinoside	9.514	609		

ionization source (ESI) at positive mode and the mass spectra data were recorded on mode for a mass

range 50-2000 m/z. Data Analysis software (Bruker, Germany) was used for data analysis.

Antibacterial activities

Evaluation of the antibacterial activity of ethanol extracts of *A. pusillus* leaves and tubers was carried out (8). The bacteria were inoculated into LB Broth for growing until the suspension reached the turbidity of 0.5 McFarland standards. 100 μ l of suspension were then spread on the surface of Mueller Hinton plate, and the sterilized 6 mm diameter discs were subsequently placed on. 20 μ l of ethanol extract were put onto the paper disc and the plates were maintained at 4°C for 2 hrs for extract diffusion. The plates were incubated at 37°C for 24 hrs, and the diameter of the zone of inhibition of extracts against tested bacteria was measured. Sterilize distilled water and Gentamycin antibiotic discs (Nam Khoa BioTek, Vietnam) were used as negative and positive controls respectively.

The experiments of antibacterial activities were repeated in triplicate. The average and standard deviation were calculated as per Excel 2010. The data obtained from experiments were presented as mean \pm standard deviation (SD).

Results and Discussion

Ethanol extract composition

Based on comparison of mass of compounds (m/z) which were identified in *A. pusillus* with other compounds in *Amorphophallus titanum*, other

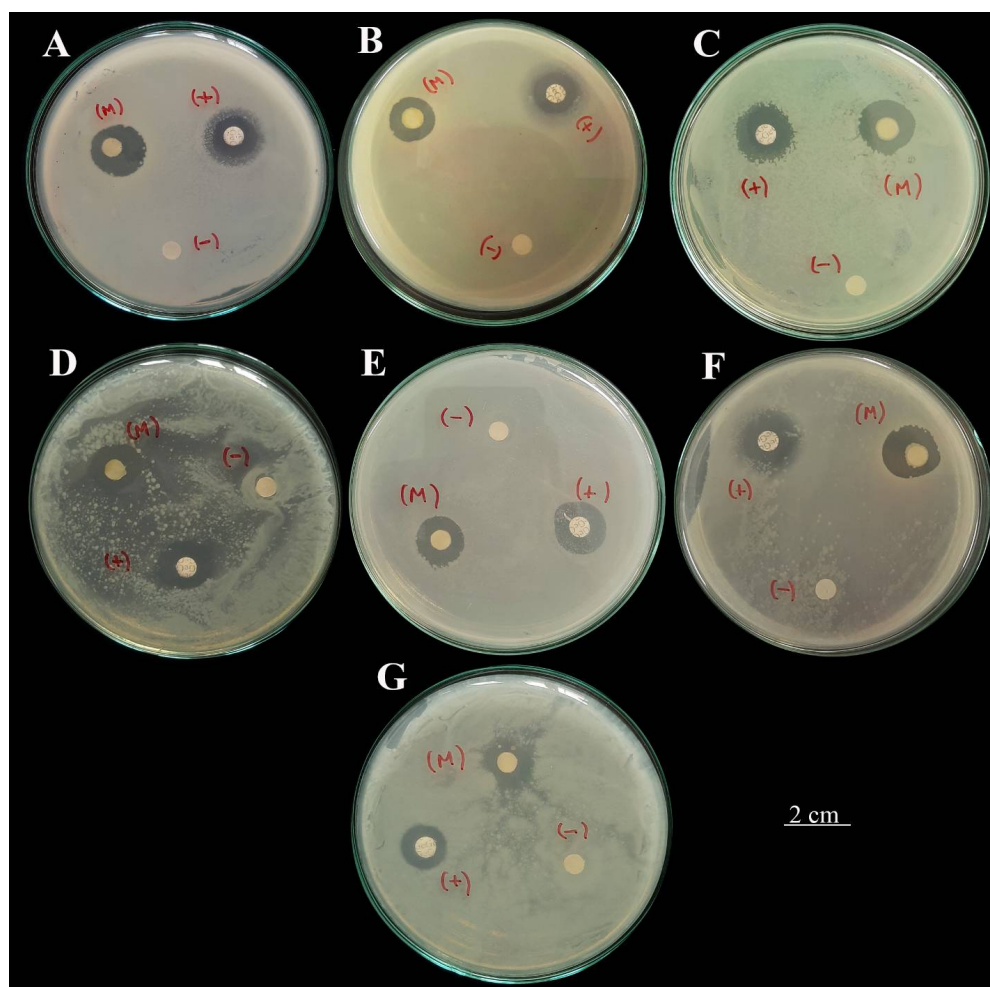


Fig. 3. Antibacterial activity of ethanol extracts from tubers and leaves of *A. pusillus* against test bacteria. A-F: leaves. A: *B. cereus*. B: *E. coli*. C: *P. aeruginosa*. D: *S. typhimurium*. E: *S. enteritidis*. F: *S. aureus*; G: *S. typhimurium* (tuber). (-) Negative control with sterilized distilled water, (+) Positive control with discs containing gentamycin, (M) sample of ethanol extract

member of *Amorphophallus* (9), we determined 7 compounds such as vitexin, orientin, vitexin 2''-O-glucoside, cyanidin 3-O-rutinoside, pelargonidin 3-O-glucoside, schaftoside and peonidin 3-O-rutinoside, which belong to flavonoid class in ethanol extracts of *A. pusillus* leaves and tubers. Among them, there were 3 anthocyanin compounds including pelargonidin 3-O-glucoside, cyanidin 3-O-rutinoside, peonidin 3-O-rutinoside, and 4 other flavonoids including vitexin, vitexin 2''-O-glucoside, orientin and schaftoside. Of note, vitexin 2''-O-glucoside and cyanidin 3-O-rutinoside existed in tubers and leaves of *A. pusillus* whereas vitexin and orientin were only found in tubers. On the other hand, pelargonidin 3-O-glucoside, peonidin 3-O-rutinoside and schaftoside were presented in leaves (Fig. 2 and Table 2, 3).

Flavonoid is a class of polyphenols with over 8000 compounds which characterizes by C6-C3-C6

skeleton. Flavonoids are not only well-known antioxidants but also have bioactive compounds which exhibit several beneficial effects such as anti-inflammation, anticancer and protection of cardiovascular illness (10). All of seven compounds which we identified in ethanol extracts of *A. pusillus* leaves and tubers are flavonoids. This finding suggests this species as a potential resource for extraction of bioactive compounds and further application in medicine. For example, vitexin, a constituent in *A. pusillus* tuber, is an apigenin flavone glycoside exhibiting several bioactivities such as cardio-protective, anticancer, anti-inflammation, antioxidant, anti-nociceptive, anticonvulsant, and anti-diabetic effects (11). It was also proved protective effect of vitexin in murine model of myocardial ischemia/reperfusion injury and suggested that the protective effect related to its potential for up-regulating Bcl-2 expression and down-regulating Bax and NF- κ B p65 (12). Moreover, vitexin can diminish acute lung injury induced by lipopolysaccharide via enhancing the nuclear factor erythroid-2-related factor 2 (Nrf2) and activity of heme oxygenase (HO)-1 (13). Furthermore, orientin, another bioactive compound found in *A. pusillus* tuber, possesses a variety of beneficial effects such as anti-adipogenesis, anti-inflammation, antioxidant, anti-aging, antibacterial, antiviral, neuroprotective and antinociceptive (10). On the other hand,

Table 4. The inhibition zone of ethanol extract of *A. pusillus* leaves and tubers against five tested bacteria

Tested bacteria	Growth inhibition zone (mm)	
	Leaves	Tubers
<i>Bacillus cereus</i>	14.7 ± 2.1	-
<i>Escherichia coli</i>	11.7 ± 0.6	-
<i>Pseudomonas aeruginosa</i>	15.7 ± 0.6	-
<i>Salmonella typhimurium</i>	16.2 ± 0.8	15.2 ± 1.8
<i>Salmonella enteritidis</i>	12.3 ± 1.5	-
<i>Staphylococcus aureus</i>	14.0 ± 0.9	-

pelargonidin 3-*O*-glucoside, bioactive compound found in *A. pusillus* leaves has anti-inflammatory effect via increasing the interleukin 10 (IL-1), the predominant anti-inflammatory cytokine which is related to inhibition of pro-inflammatory cytokines, chemokines, macrophage activations, and may be suppress atherosclerosis (14). Furthermore, cyanidin-3-rutinoside, the anthocyanin found in both tubers and leaves of *A. pusillus*, selectively induces apoptosis in leukemic HL-60 cells via peroxides accumulation and altering the mitochondrial pathway (15). The presence of these bioactive compounds in *A. pusillus* has proposed a promising application of this species in medicine.

Antibacterial activity

Antibacterial activities of ethanol extracts of *A. pusillus* tubers and leaves were determined via diameter of the zone of inhibition against tested bacteria (Fig. 3 and Table 4). The results showed that ethanol extract of *A. pusillus* leaves could inhibit 6 tested bacterial strains, including *B. cereus*, *S. aureus*, *E. coli*, *P. aeruginosa*, *S. enteritidis* and *S. typhimurium* whereas that of *A. pusillus* tubers only hindered the growth of *S. typhimurium*. Among 6 bacterial strains, ethanol extract of *A. pusillus* leaves showed the strongest antibacterial activity against *S. typhimurium* (16.2 ± 0.8 mm), followed by *P. aeruginosa* (15.7 ± 0.6 mm), *B. cereus* (14.7 ± 2.1 mm), *S. aureus* (14.0 ± 0.9 mm), *S. enteritidis* (12.3 ± 1.5 mm), and *E. coli* with diameter of the zone of inhibition about 11.7 ± 0.6 mm. Otherwise, the ethanol extract of *A. pusillus* tubers exhibited a strong antibacterial activity against *S. typhimurium* with 15.2 ± 1.8 mm. In summary, the ethanol extract of *A. pusillus* tubers and leaves could inhibit all 6 tested bacteria.

These results are in line with previous studies which demonstrate the antibacterial activity of several species of *Amorphophallus*. For example, amblyone, a triterpenoid found in *A. campanulatus*, could hinder the growth of 10 bacterial strains including *Streptococcus β-haemolyticus*, *Bacillus subtilis*, *Bacillus megaterium*, *S. aureus*, *Shigella dysenteriae*, *Shigella sonnei*, *Shigella flexneri*, *P. aeruginosa*, *Salmonella typhi* and *Escherichia coli* (16). In previous study, various extracts of *A. paeoniifolius* exhibited antibacterial activity against *B. subtilis*, *S. aureus*, *Klebsilla pneumonia*, and *P. aeruginosa* (17). Furthermore, acetone and ethanol extracts of *A. konkanensis* also inhibited *B. subtilis*, *M. aureus*, *P. aeruginosa*, *E. coli*, *S. typhi*, and *K. pneumoniae* (18). Recently, it has been proved that ethanol extract of *A. lanceolatus* tuber could impede the growth of 6 bacteria, including *B. cereus*, *S. aureus*, *E. coli*, *P. aeruginosa*, *S. enteritidis* and *S. typhimurium* (6).

All of 7 compounds in this study belong to flavonoids, which exhibit a strong antibacterial effect against pathogenic microorganisms through the inhibition of nucleic acid synthesis, inhibition of cytoplasmic membrane function and energy metabolism altering the biofilm formation and membrane permeability (19). Therefore, the presence of these flavonoid compounds in *A. pusillus* tubers and leaves proposes an explanation for antibacterial activities of those resources. Furthermore, the

difference of antibacterial activities between the tubers and leaves of *A. pusillus* may be owing to differences in chemical composition. Vitexin, the bioactive compound found only *A. pusillus* tubers, is suggested as an antibacterial agent against *Enterobacter cloacae*, *Proteus mirabilis* and *E. coli* (20). In the previous study, some bacteria such as *S. aureus*, *Staphylococcus cohnii*, *K. pneumonia* were proved to be susceptible to orientin, other flavonoid in *A. pusillus* tubers (21). On the other hand, *A. pusillus* leaves contain 2 anthocyanin compounds, pelargonidin 3-*O*-glucoside and peonidin 3-*O*-rutinoside, which are not found in tuber. Some anthocyanins may underlie the antimicrobial effect of several natural plants (22). These results suggests the potential application of ethanol extracts isolated from this species as natural antimicrobial compounds for the treatment of infection caused by bacteria, especially in dermatologic and enteric infections.

Conclusion

In this study, chemical compositions of *A. pusillus* leaves and tubers were elucidated with 7 flavonoid compounds, including vitexin, orientin, vitexin 2"-*O*-glucoside, cyanidin 3-*O*-rutinoside, pelargonidin 3-*O*-glucoside, schaftoside and peonidin 3-*O*-rutinoside in. Furthermore, ethanol extracts of *A. pusillus* leaves and tubers exhibited antibacterial effect against six tested bacteria such as *B. cereus*, *E. coli*, *P. aeruginosa*, *S. enteritidis*, *S. typhimurium* and *S. aureus*.

Authors' contribution

Hong Thien Van and Gia Buu Tran conceived and designed the study. Hong Thien Van, Hong Hai My Nguyen, Nguyen Tuong An Huynh, Van Son Le, Gia Buu Tran performed experiments and handled the research data. Hong Thien Van, Hong Hai My Nguyen, and Nguyen Tuong An Huynh conducted data analysis and interpreted the results. Hong Thien Van drafted the manuscript. Gia Buu Tran revised the manuscript. All authors read and approved the final manuscript.

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Completing interest

The authors declare there is no conflict of interests.

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