



Chemical Composition and Antibacterial Activities of Ethanol Extract of *Geodorum attenuatum* Griff. (Orchidaceae)

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Highlights

- This paper focuses on chemical compounds and antibacterial activity of *G. attenuatum* tuber.
- Liquid chromatography mass spectrometry and the antibacterial assay are applied.
- The specimen contained nine chemical compounds and could inhibit six tested bacteria.

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Abstract

Geodorum attenuatum has been found in Thailand, China, India and Vietnam, however, the phytochemical composition and antimicrobial activity of *G. attenuatum* have not been studied yet. In this study, the phytochemical composition of ethanol extract of *G. attenuatum* tuber was determined using liquid chromatography-mass spectrometry (LC-MS) and evaluated its antibacterial activity of ethanol extract via disk diffusion test. 9 compounds in ethanol extract of *G. attenuatum* tuber were identified, such as disaccharide, oxoheneicosanoic acid, shikimic acid, chlorogenic acid, 1-Oleoyl-2-acetyl-glycerol, azafrin, 5,6,7-trihydroxyfavone, 2,3-isopropylidene-1-oleoyl-glycerol and trans-docosanoyl ferulate. Moreover, it's been proved that the ethanol extract of *G. attenuatum* tuber could inhibit the growth of *B. cereus*, *E. coli*, *P. aeruginosa*, *S. enteritidis*, *S. typhimurium*, and *S. aureus*.

1. INTRODUCTION

Geodorum Jack., belonging to Orchidaceae family is a genus of only about 10 species occurring primarily in temperate regions of Asia, Australia and in the Southeast Pacific islands [1]. Several studies indicated the potential application of species of this genus in medicine. The phytochemical composition, antimicrobial and other bioactivities of the compounds extracted from several plants of *Geodorum* genus have been shown by many recent studies [2,3]. In Vietnam, six species of the *Rothmannia* genus were recorded such as *G. attenuatum*, *G. densiflorum*, *G. pulchellum*, *G. recurvum*, *G. siamense* and *G. citrinum* [4-7].

Geodorum attenuatum Griff. was described for the first time by Griffith [8]. To date, *G. attenuatum* has been found in Thailand, China, India and Vietnam [1,7,9]. In Vietnam, following Pham-Hoang (2000) [7], the species has been found in Hue, Gia Lai, Kon Tum, Tay Ninh Provinces and Hai Phong City. Recently, *G. attenuatum* was recorded in Binh Chau-Phuoc Buu Nature Reserve, Ba Ria-Vung Tau Province, Vietnam by Bui *et al.* (2019) [10], who was the second coauthor of this paper. Currently, the number of studies about this species is limited and its bioactivity is still unknown.

Accordingly, the research on chemical composition and bioactivity of *G. attenuatum* extract becomes the important step for wider application and value improvement of this species. In this study, the phytochemical composition and antibacterial activity of ethanol extract of *G. attenuatum* tuber were firstly determined, subsequently, provided more information for further application of this species.

2. MATERIALS AND METHOD

2.1. Plant Materials

Specimens of *G. attenuatum* were collected from Binh Chau-Phuoc Buu Nature Reserve, Bung Rieng ward, Xuyen Moc District, Ba Ria-Vung Tau Province, location of about 10°32'51"N; 107°31'35"E, July 4, 2018, 48 m in elevation (Figure 1).

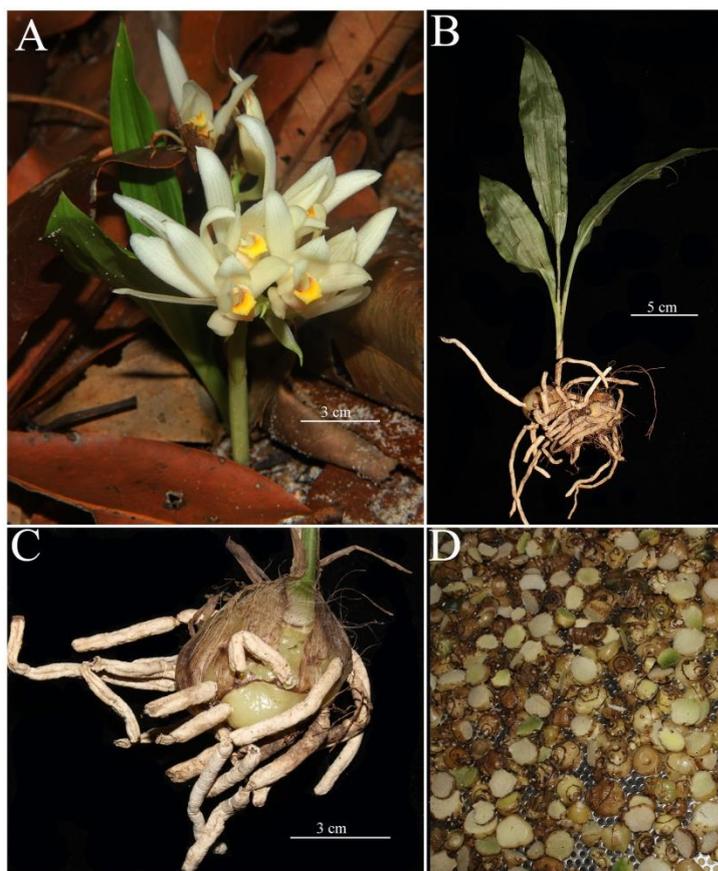


Figure 1. *Geodorum attenuatum*: A. Habitat; B. Leaf; C. Tuber; D. Dried tubers

2.2. Bacterial Strains

Six bacterial strains were used to determine the antibacterial activity of ethanol extract from *G. attenuatum*. Two Gram-positive bacteria, including *Bacillus cereus* (ATCC 11774), *Staphylococcus aureus* (ATCC 25923), and four Gram-negative bacteria, including *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella enteritidis* (ATCC 13976) and *Salmonella typhimurium* (ATCC 13311) were obtained from the microbiology collection, Department of Biotechnology, Institute of Food and Biotechnology, Industrial University of Ho Chi Minh city, Viet Nam. All bacterial strains were maintained at -20°C in 20% glycerol solution and inoculated into Luria-Bertani broth at 37°C for 24 h to be re-activated again before using in further experiments.

2.3. Extraction Procedure

Fresh sliced tubers were dried at a 50°C until constant weight and grounded into medicinal powder. 100g of the resulting powder was soaked in 1000 mL of 99% ethanol for 7 days at room temperature and filtered to collect the extract. The extract was then condensed under vacuum pressure at 60°C to obtain the brown medicinal paste, according to Altemimi *et al.* (2017) To ensure the absolute absence of ethanol in the paste, sublimation dryer was utilized [11].

2.4. Liquid Chromatography Mass Spectrometry (LC-MS)

The LC-MS analysis of extract was performed on HPLC Agilent 1200 infinity liquid chromatography system (Agilent Technologies, CA, USA) coupled with MicroTOF-QII mass spectrometer (Bruker Daltonics, Germany). In brief, aliquots of ethanol extract were injected to HPLC Agilent 1200 infinity liquid chromatography system and then the chromatographic separation was carried out using an ACE3-C₁₈ analytical column (4.6 ×150mm, 3.5 µm) at 40°C as a stationary phase. The mobile phase consisted of deionized water with 0.1% formic acid as solvent A and acetonitrile with 0.1% formic acid as solvent B. The gradient elution program for the chromatographic separation (Table 1) was set as following conditions: 90 solvent A:10 solvent B (0-15min), 0 solvent A:100 solvent B (15-30 min), 90 solvent A:10 solvent B (31-40 min) at the flow rate of 0.3 mL/min. The mass spectrometer was implemented with electrospray ionization source (ESI) at positive mode and mass spectra data were recorded in a mass range 50-2000 m/z. Data analysis was performed using Data Analysis software (Bruker, Germany).

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

2.5. Antibacterial Activities

The antibacterial assay of ethanol extract of *G. attenuatum* tuber was conducted using the method detailedly described by Bauer *et al.* (1996) [12]. The bacteria were inoculated in LB Broth until a turbidity of 0.5 McFarland standards was reached. Subsequently, 100 µL bacterial suspensions was spread on sterile Mueller Hinton plate and a sterile 6mm diameter discs were put on the inoculated surface. 20 µL of the sample was added onto each disc and the plates were maintained at 4°C for 2 hours to allow extract diffusion into the medium. The plates were kept at 37°C for 24h and the antibacterial activity of sample was determined via the inhibition zone diameter of tested bacteria. Sterilize distilled water was used as negative control and Gentamycin antibiotic discs (supplied by Nam Khoa BioTek, Viet Nam) were used as positive control.

2.6. Data Analysis

The one-way analysis of variance (ANOVA) method was used to analyze the results. Fisher's least significant difference (LSD) procedure using Statgraphics software (Centurion XV) was the method to determine significant differences among the means from triplicate analyses at ($p < 0.05$). The data were expressed as a mean ± standard deviation (SD).

3. RESULTS AND DISCUSSIONS

3.1. Phytochemical Analysis

In this study, mass spectra of compounds which identified in the ethanol extract of *G. attenuatum* tuber were compared with previous studies about chemical composition of species of Orchidaceae family. As presented in Table 2 and Figure 2, 9 compounds in ethanol extract of *G. attenuatum* tuber were found, such as Disaccharide, Oxoheneicosanoic acid, Shikimic acid, Chlorogenic acid, 1-Oleoyl-2-acetyl-glycerol, Azafrin, 5,6,7-trihydroxyfavone, 2,3-isopropylidene-1-oleoyl-glycerol, 2,3-isopropylidene-1-oleoyl-glycerol and trans-Docosanoyl ferulate.

Disaccharide, a type of carbohydrate, has important roles in the human diets and many bioactivities. Genova *et al.* (2004) have proved disaccharides, such as sucrose, trehalose influenced the bending elasticity of SOPC (1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine) lipid membranes [13]. Shikimic acid was first isolated from fruits of aniseed (*Illicium anisatum*) by Eykman which is the known precursor of aromatic amino acids, L-phenylalanine, and L-tyrosine [14]. The shikimic acid biosynthesis pathways were operated in leaves or fruit of many plants and also in microorganisms, but not in humans or animals. Consequently, they are considered essential nutritive compounds in the human nutrition [15,16].

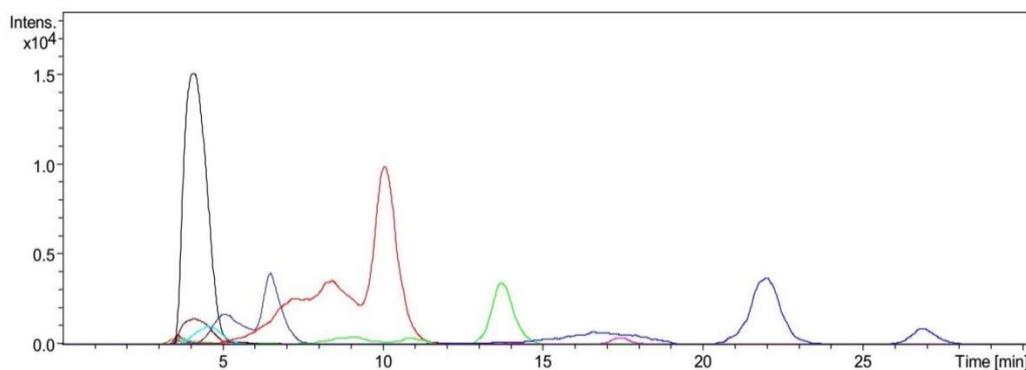


Figure 2. Chromatogram (LC-MS) of the ethanol extract of *G. attenuatum* tuber obtained by maceration

Table 2. Phytochemical composition of ethanol extract of *G. attenuatum* tuber

No.	Compounds	Formula	References
1	Disaccharide	C ₁₂ H ₂₂ O ₁₁	[17]
2	Oxoheneicosanoic acid	C ₂₁ H ₄₀ O ₃	[17]
3	Shikimic acid	C ₇ H ₁₀ O ₅	[17]
4	Chlorogenic acid	C ₁₆ H ₁₈ O ₉	[17]
5	Azafrin	C ₂₇ H ₃₈ O ₄	[17]
6	5,6,7-trihydroxyfavone	C ₁₅ H ₁₀ O ₅	[17]
7	1-Oleoyl-2-acetyl-glycerol	C ₂₃ H ₄₂ O ₅	[18]
8	2,3-isopropylidene-1-oleoyl-glycerol	C ₂₄ H ₄₄ O ₄	[18]
9	trans-Docosanoyl ferulate	C ₃₂ H ₅₄ O ₄	[19]

Chlorogenic acid, belonging to the hydroxycinnamic acid group, is one of the most available phenolic acid compounds in foods, such as coffee and tea. Many *in vivo* studies showed the role of chlorogenic acid as a nutraceutical for the prevention and treatment of metabolic syndrome and associated disorders [20,21]. For instance, chlorogenic acid has an effect on body weight, body fat, and obesity-related hormones of induced-obese imprinting control region mice. Azafrin - a natural carotenoid isolated from the root of

Centranthera grandiflora, a Chinese ethnomedicine, has been widely used to treat cardiovascular diseases in China. Azafrin has protected cardiomyocytes by reducing oxidative stress [22].

5,6,7-trihydroxyflavone, belonging to the flavones, was first isolated from *Scutellariae baicalensis*. Previous studies showed that 5,6,7-trihydroxyflavone has beneficial effects on health, such as anti-oxidant, anti-viral, anti-thrombotic, and anti-inflammatory effects [23]. Fu (2012) demonstrated that 5,6,7-trihydroxyflavone improved hyperglycemia, glucose tolerance, and blood insulin levels in high-fat diet diabetic mice [24]. Christina *et al.* (2005) showed the role of 1-Oleoyl-2-acetyl-glycerol in Vitro condition. For instance, it is used as an agonist for human polymorphonuclear leukocytes [25]. Moreover, 1-Oleoyl-2-acetyl-glycerol stimulates 5-Lipoxygenase activity, a compound that catalyzes the formation of corresponding hydroperoxides from polyunsaturated fatty acids [26].

3.2. Antibacterial Activity

Table 3. The inhibition zone of ethanol extract of *G. attenuatum* tuber against six tested bacteria. (-): No inhibition

Tested bacteria	Growth inhibition zone (mm)			
	Original extract	Diluted extract (1:2)	Diluted extract (1:4)	Diluted extract (1:6)
<i>S. aureus</i>	14.0 ± 1.0 ^b	8.0 ± 0.5 ^a	7.7 ± 0.3 ^a	8.2 ± 0.8 ^a
<i>B. cereus</i>	7.5 ± 0.5 ^a	7.7 ± 0.3 ^a	7.7 ± 0.3 ^a	7.5 ± 0.0 ^a
<i>S. enteritidis</i>	8.2 ± 0.3 ^b	8.0 ± 0.5 ^b	7.2 ± 0.3 ^a	-
<i>S. typhimurium</i>	8.2 ± 0.3 ^b	8.0 ± 0.5 ^b	8.0 ± 0.3 ^b	7.2 ± 0.3 ^a
<i>E. coli</i>	8.2 ± 0.3 ^b	7.9 ± 0.3 ^{ab}	7.5 ± 0.5 ^a	7.9 ± 0.3 ^{ab}
<i>P. aeruginosa</i>	8.2 ± 0.3 ^a	8.2 ± 0.8 ^a	8.0 ± 0.5 ^a	-

Different lowercase letters in the same row denote significant differences between various extracts ($p < 0.05$).

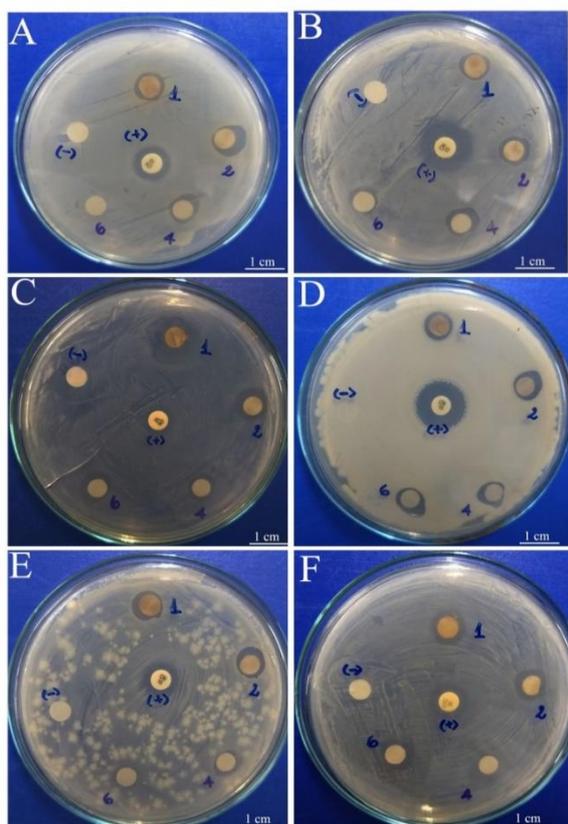


Figure 3. Antibacterial activity of ethanol extract of *G. attenuatum* tuber against tested bacteria. *P. aeruginosa* (A), *S. typhimurium* (B), *S. aureus* (C), *B. cereus* (D), *S. enteritidis* (E), *E. coli* (F). The paper

discs numbered as 1, 2, 4, 6 were loaded with original extract, two-fold four-fold, and six-fold diluted extracts, respectively. (-) Negative control with sterilized distilled water, (+) Positive control with discs containing gentamicin

Data stated in Table 3 and Figure 3 showed that the ethanol extract of *G. attenuatum* tuber was able to resist against six bacteria studied. In general, zones of inhibition were completely transparent in the six bacteria, such as *B. cereus*, *E. coli*, *P. aeruginosa*, *S. enteritidis*, *S. typhi*, *S. aureus*. However, diameters of inhibition zones were various in different bacterial strains and dilution factors. Among six tested bacteria strains, the original ethanol extract showed the highest inhibitory effect on *Staphylococcus aureus* (14 mm), followed by *E. coli*, *S. typhimurium*, *S. enteritidis* *P. aeruginosa* (8.2 mm) and *B. cereus* (7.5 mm). All six tested bacteria strains, the increase of dilution ratio of the extract as 1:2, 1:4 or 1:6 resulted in the decrease of antibacterial and in case of *Salmonella enteritidis* and *Pseudomonas aeruginosa*, no inhibitory effect was observed in diluted extract (1:6).

As aforesaid in the introduction, this is the first research showing the phytochemical composition and the antibacterial activity of ethanolic extract from the tuber of *G. attenuatum*. However, certain previous studies showed the antibacterial and antifungal activities of different extract from species belonging to genus *Geodorum*. For instance, Akter et al. (2010) proved the water, methanol and petroleum ether extracts from the pseudobulb of *G. densiflorum* were able to resist against 5 Gram-positive bacteria, 8 Gram-negative bacteria and 3 fungal strains studied [27]. In another study, Keerthiga and Anand (2014) demonstrated that various extracts of *G. densiflorum* was able resist against 6 bacterial strains, including *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumonia* [2]. Similarly, Keerthiga and Anand (2015) showed that water, ethanol and chloroform extracts of *G. densiflorum* were resistant to 4 fungal strains, such as *Penicillium chrysogenum*, *Aspergillus niger*, *Rhizoctonia solani* and *Colletotrichum falcatum* [3].

4. CONCLUSION

In this study, 9 compounds were firstly identified in ethanol extract of *G. attenuatum* tuber by LC-MS method. The antimicrobial activity of the ethanol extract against six bacterial strains, including *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, and *Salmonella typhimurium* was demonstrated. The result of this study might be a platform for further application of *G. attenuatum*.

CONFLICTS OF INTEREST

No conflict of interest was declared by the authors.

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