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Isolation and Identification of Antimicrobial Substance from *Canavalia gladiata*

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Abstract Novel antimicrobial substance was isolated from seed coat of *Canavalia gladiata* by extraction with 75% methanol. Isolation and purification were conducted with solvent fractionation and chromatography on silica gel and sephadex LH-20 columns. Each fraction of antimicrobial activity was tested by paper disc method. Single compound was obtained from the 4th fraction of sephadex LH-20 column chromatography using chloroform/methanol (1:4, v/v), and identified as 3,4,5-trihydroxybenzoic acid methyl ester (methyl gallate) based on HPLC, GC/MS, FT-IR, ^{1}H NMR, and ^{13}C NMR analyses. This is the first report describing the presence of methyl gallate in *C. gladiata.*

Keywords: *Canavalia gladiata*, antimicorbial, 3,4,5-trihydroxybenzoic acid methyl ester, methyl gallate

Introduction

Canavalia gladiata (sword bean) is known mainly as a cultivated species derived by selection from *C. virosa*, a wild species occurring principally in Africa. It has been cultivated widely in South and Southeast Asia, especially in India, Sri Lanka, and Burma. Although a minor vegetable crop, it is widely spread in the tropical regions and is mostly cultivated for local consumption. *C. gladiata* contains growth-inhibiting protein substances, i.e. canavaline, concanvalin A (Con A), amino acid, canavanine, and *C. gladiata* agglutinin (CGA), among which CGA and Con A have amino acids similar in structures. The best-known substance of *C. gladiata* is Con A, a lectin that has been reported to interfere with the nutrient absorption. Con A has been well characterized and shown to possess some biological activities. Canavaline is characterized as a thermostable and poisonous alkaline amino acid, which is a structural analogue of arginine. It is also present in seeds at more than 3% of dry matter (1-4). Although antimicrobial activity has been detected in *C. gladiata*, the agent responsible has not yet been isolated. The aim of this work was to investigate the possibility of using this substance as a natural preservative due to the increasing demand of consumers for 'natural food' with no chemical (manmade) preservatives added. Therefore, this study focused on the optimal method for the extraction of antimicrobial substance from *C. gladiata* using various solvents, as well as on the isolation, purification, and identification of this substance.

Materials and Methods

Materials *C. gladiata* cultivated in Jincheon-kun, Korea was collected and dried for sample preparation. The seeds were sorted by colors, red (red sword bean) and white (white sword bean). Seed coat, cotyledon, and seed pod of red and white sword beans were separated and crushed with a homogenizer (SMT Co, Tokyo, Japan) and passed through a 10-mesh sieve for extraction. The crushed samples were stored at -80°C in a deep freezer.

Optimization of extraction procedures Different solvents, i.e. methanol, ethanol, and water, were tested to determine the most effective solvent for the extraction of active antimicrobial substance from sword beans. Extracts were then evaluated for their antimicrobial activities by a paper disc method (8 mm, Toyo Roshi Kaisha, Ltd.) (5-6). The solvents were also mixed at different ratios (25, 50, 75, and 100%), and subjected to different extraction temperatures (25, 50, and 80°C) and extraction times (3, 6, 9, 12, and 24 hr). Three parts of seed coat, cotyledon, and seed pod were extracted. Extraction conditions were determined after the extracts had been tested for antimicrobial activities. Each sample was filtered through Whatman No.2 filter paper (Whatman, Maidstone, England). The filtrate was collected and condensed using a rotary evaporator (Rotavapor, Buchi, Switzerland) at 40°C.

Solvent fractionation of antimicrobial substance The concentrated extracts (100 mL) were fractionated using *n*-hexane (100 mL×2), chloroform (100 mL×2), ethyl acetate (100 mL×2), and water successively with a separating funnel. Antimicrobial activity of each solvent fraction was measured using the paper disc method.

Isolation and purification of antimicrobial substance Ethyl acetate fractions containing the antimicrobial activity substances were separated on a silica gel column (Kieselge 60, 70-230 mesh, Merck & Co., Inc, Darmstadt, Germany) and eluted with a linear gradient of methanol in chloroform (100% chloroform→100% methanol). Subsequently, 3 mL each fraction was collected, and the antimicrobial activity was measured using the paper disc method. The active fraction was reapplied onto a silica gel column (Kieselge 60, 70-230 mesh, Merck, Darmstadt, Germany), packed, and

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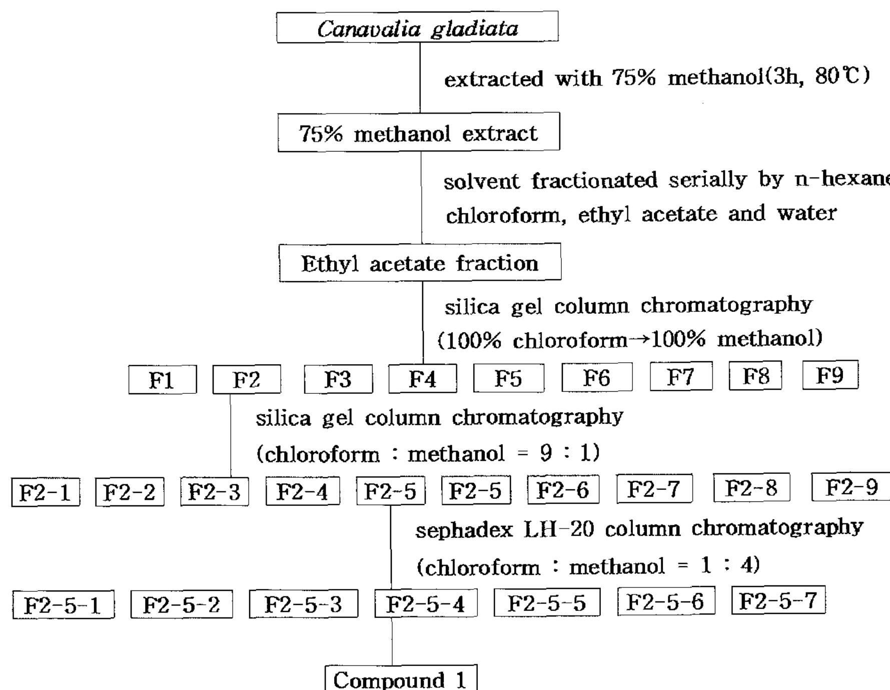

Fig. 1. Schematic procedure for the extraction, isolation and purification of antimicrobial substances from *Canavalia gladiata*.

eluted with chloroform-methanol (9:1, v/v). Active fractions from the silica gel column were consecutively subjected to chromatography equipped with a Sephadex LH-20 column and eluted with methanol-chloroform (4:1, v/v). The schematic procedure for the extraction, isolation and purification of antimicrobial substance from *C. gladiata* is shown in the Fig. 1.

Identification of antimicrobial substance High performance liquid chromatography (HPLC) was used to confirm the purity of isolated antimicrobial substance. HPLC analysis was performed on an HPLC system (Thermo Separation Products, France) equipped with a UV-1000 detector and AS-1000 autosampler. A phenomenex lichrosorb 5RP-18 column (250×4.6 mm) with mobile phase of MeOH:Water (4:6, v/v) was used at 0.8 mL/min with UV detector at 273 nm. The active fraction was tested with UV/VIS spectrum using a spectrophotometer (DU 650, Beckman, USA) at the range of 200 to 800 nm. FT-IR spectrum was recorded on an FT-IR spectrometer (IFS-66/FRA106S, Brucker, Germany). GC/MS spectrum was recorded on a GC/MS spectrometry (GC 8000/MD 800, Fisons Instruments) under the following GC conditions: column, HP-5 capillary; carrier gas, He; flow rate, 1.0 mL/min; oven temperature, 50°C(5 min)→5°C/min→180°C(5 min)→1°C/min→230°C; ionization mode, EI (70 eV). The active fraction was dissolved in methanol-d_4 (Aldrich). ^{1}H-NMR spectrum was recorded on an NMR Spectrometer (DPX 300, Brucker, Germany) operated at 300 MHz. ^{13}C-NMR spectrum was recorded on an NMR Spectrometer (DPX 300, Brucker) operated at 75 MHz.

Microorganism and bioassay Eight microorganism strains were used for antimicrobial activity test. The media used to culture the test microorganisms were Mueller Hinton broth (Difco, Detroit, MI, USA) for *Bacillus subtilis* ATCC 14893, *B. cereus* ATCC 6633, *Micrococcus luteus* ATCC 9341, *Staphylococcus aureus* ATCC 25923, *E. coli* ATCC 27662, *Pseudomonas aeruginosa* ATCC 9027, and *Salmonella typhimurium* ATCC 14028, and Tryptic soy broth (Difco) for *Listeria monocytogenes* ATCC 19111. Antimicrobial activity was measured using the paper disc (8 mm, Toyo Roshi Kaisha, Ltd. Japan) method (5-6). To measure the antimicrobial activity, a paper disc treated with each sample was placed on an agar plate. All samples (30 μL each) were tested at 1 mg/mL. The diameter of inhibited zone was measured in mm after 24 hr of incubation at 37°C.

Results and Discussion

Optimization of antimicrobial activity extraction The seeds of *C. gladiata* (red and white sword beans) were extracted with ethanol, methanol, and water. Each extract was tested to determine its antimicrobial activity against each of the eight microorganisms. Methanol extract from red sword bean showed the highest growth inhibitory activity against the tested microorganisms (Table 1). The sample was further extracted with water-methanol (v/v) at the ratios of 0:100, 25:75, 50:50, and 75:25. Each extract was also tested to determine its antimicrobial activity against each microorganism. When various strength of methanol was used, i.e. at the ratios of 0:100, 25:75, 50:50, and 75:25 water to methanol, the extract treated with the water-methanol mixture of 25:75 (v/v) showed the highest growth inhibitory activity against all tested microorganisms (Table 2). To determine the maximum extraction condition, 75% methanol extract was tested at various extraction temperatures (25, 50, and 80°C). The methanol extract prepared at 80°C showed the highest growth inhibitory

Table 1. Antimicrobial activity of several solvent extracts from *Canavalia gladiata*

Microorganism	Red sword bean			White sword bean		
	E	M	W	E	M	W
B. subtilis	13	12	11	-	10	-
B. cereus	10	13	11	-	-	-
M. luteus	-	11	-	-	-	-
L. monocytogenes	14	17	17	15	16	16
S. aureus	-	14	-	-	-	12
S. typhimurium	13	11	-	10	10	-
E. coli	10	15	-	-	-	-
P. aeruginosa	12	13	12	11	11	10

E=eathanol, M=methanol, W=water
Number denotes the inhibiting diameter (mm) of clear zone on the agar plate

Table 2. Antimicrobial activity of the extracts obtained at various methanol concentrations

Microorganism	Ratio of water-methanol(v/v)			
	0:100	25:75	50:50	75:25
B. subtilis	12	22	22	14
B. cereus	15	15	14	10
M. luteus	11	11	-	-
L. monocytogenes	10	11	-	-
S. aureus	12	17	17	14
S. typhimurium	11	15	12	11
E. coli	18	24	21	18
P. aeruginosa	12	19	16	13

Number denotes the inhibiting diameter (mm) of clear zone on the agar plate

Table 3. Antimicrobial activity of *Canavalia gladiata* fractions at different extraction temperature

Microorganism	Temperature (°C)		
	25	50	80
B. subtilis	22	22	23
B. cereus	15	14	15
M. luteus	11	11	11
L. monocytogenes	11	12	12
S. aureus	17	17	18
S. typhimurium	15	16	15
E. coli	24	24	24
P. aeruginosa	19	19	19

Number denotes the inhibiting diameter (mm) of clear zone on the agar plate

activity (Table 3). To optimize the duration of extraction, 75% methanol extract was also tested for 3, 6, 9, 12, and 24 hr at 80°C (Table 4). The duration of extraction time did not affect the extraction of the antimicrobial substance from sword beans; the antimicrobial activity remained similar during the extraction period of 3 to 24 hr. Therefore, 75% methanol for 3 hr at 80°C was determined to be the most optimum condition for the extraction of antimicrobial substance from sword beans.

Sword beans were separated into three parts of seed coat, cotyledon, and seed pod to examine the antimicrobial activity of each part. Among the three parts, seed coat

Table 4. Antimicrobial activity of 75% methanol extract at different extraction times

Microorganism	Extraction time (hr)				
	3	6	9	12	24
B. subtilis	22	22	23	22	22
B. cereus	15	14	15	15	15
M. luteus	11	11	11	11	12
L. monocytogenes	11	12	12	11	11
S. aureus	17	17	18	19	17
S. typhimurium	15	16	15	15	15
E. coli	24	24	24	25	24
P. aeruginosa	19	19	19	19	19

Number denotes the inhibiting diameter (mm) of clear zone on the agar plate

Table 5. Comparison of antimicrobial activity in different parts of *Canavalia gladiata*

Microorganism	Red sword bean			White sword bean		
	SC	C	SP	SC	C	SP
B. subtilis	14	13	-	-	12	-
B. cereus	19	-	-	-	-	-
M. luteus	14	-	-	-	-	-
L. monocytogenes	15	20	-	-	15	-
S. aureus	14	-	-	-	-	-
S. typhimurium	15	-	-	-	-	-
E. coli	28	-	-	-	-	-
P. aeruginosa	19	-	-	-	-	-

SC=seed coat, C=cotyledon, SP=seed pod
Number denotes the inhibiting diameter (mm) of clear zone on the agar plate

showed the highest antimicrobial activity against all tested microorganisms (Table 5). Therefore, the extract prepared from the red sword bean seed coat was used for further studies on the isolation and characterization of antimicrobial substance from *C. gladiata*.

Isolation and purification of antimicrobial substance The 75% methanol extracts were fractionated by *n*-hexane, chloroform, ethyl acetate, and aqueous phases successively in separating funnels. Each fraction was tested for antimicrobial activity against all eight microorganisms. The ethyl acetate fraction showed the highest growth inhibition against all tested microorganisms (Table 6). When the active fraction (ethyl acetate fraction) was chromatographed on a Silica gel column (Kieselgel 60, 70-230 mesh, Merck, Germany) and eluted with a linear gradient of methanol in chloroform (100% chloroform→100% methanol), the Silica gel chromatography gave three active fractions of F2, F3, and F5 (Table 7). Among the three active fractions, F2 fraction showed higher antimicrobial effect against all eight microorganisms than F3 and F5. Therefore, F2 was chosen for further analysis for the characterization and isolation of antimicrobial substance in sword bean.

When the F2 fraction was subjected to a Silica gel column, which was packed and eluted with chloroform-methanol (9:1, v/v), the chromatography gave four active fractions, F2-3, F2-4, F2-5, and F2-6 (Table 8). Antimicrobial effects of F2 fraction on *L. monocytogenes* and *M. luteus*

Table 6. Antimicrobial activities at different solvent fractions of 75% methanol extracts of *Canavailia gladiata*

Microorganism	Solvent fraction			
	n-Hexane	Chloroform	Ethyl acetate	Water
B. subtilis	-	-	16	16
B. cereus	-	-	19	16
M. luteus	-	-	19	-
L. monocytogenes	-	-	10	-
S. aureus	-	-	14	13
S. typhimurium	-	-	15	17
E. coli	-	-	20	14
P. aeruginosa	-	-	25	21

Number denotes the inhibiting diameter (mm) of clear zone on the agar plate

were 11 and 13 mm, respectively (Table 7). On the other hand, F2-3 to F2-6 fractions showed no antimicrobial effects on *L. monocytogenes* and 10 mm on *M. Luteus*. These results suggested that antimicrobial materials of F2 fraction was reduced or separated during chromatography. F2-5 subfraction had the highest antimicrobial activity against all microorganisms tested except *B. subtilis* (Table 8), followed by F2-4, F2-3, and F2-6. Therefore, F2-5 was chosen and further characterized for antimicrobial activity in sword bean.

When F2-5 was applied onto Sephadex LH-20 column and eluted with chloroform-methanol (1:4, v/v), Sephadex LH-20 column chromatography gave one active fraction, F2-5-4. The outstanding antimicrobial efficacy of F2-5-4 fraction against *S. aureus* is shown in Table 9. The antimicrobial efficacy of F2-5-4 fraction increased to 17

Table 7. Antimicrobial activities of subfractions obtained with silica gel column chromatography of ethyl acetate fraction

Microorganism	Diameter of clear zone (mm)								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
B. subtilis	-	22	11	-	9	-	-	-	-
B. cereus	-	18	11	-	-	-	-	-	-
M. luteus	-	13	-	-	-	-	-	-	-
L. monocytogenes	-	11	-	-	-	-	-	-	-
S. aureus	-	16	10	-	9	-	-	-	-
S. typhimurium	-	14	13	-	-	-	-	-	-
E. coli	-	14	13	-	12	-	-	-	-
P. aeruginosa	-	24	15	9	10	-	-	-	-

Number denotes the inhibiting diameter (mm) of clear zone on the agar plate

Table 8. Antimicrobial activities of subfractions (silica gel column chromatography) of F2 fraction

Microorganism	Diameter of clear zone (mm)								
	F2-1	F2-2	F2-3	F2-4	F2-5	F2-6	F2-7	F2-8	F2-9
B. subtilis	-	-	18	22	20	18	-	-	-
B. cereus	-	-	12	15	22	12	-	-	-
M. luteus	-	-	-	-	10	-	-	-	-
L. monocytogenes monocytogenes	-	-	-	-	-	-	-	-	-
S. aureus	-	-	10	10	12	11	-	-	-
S. typhimurium	-	-	12	15	22	12	-	-	-
E. coli	-	-	18	23	23	18	-	-	-
P. aeruginosa	-	-	12	16	20	12	-	-	-

Number denotes the inhibiting diameter (mm) of clear zone on the agar plate

Table 9. Antimicrobial activities of subfractions obtained with Sephadex LH-20 column chromatography of F2-5 fraction

Microorganism	Diameter of clear zone (mm)						
	F2-5-1	F2-5-2	F2-5-3	F2-5-4	F2-5-5	F2-5-6	F2-5-7
B. subtilis	-	-	-	15	-	-	-
B. cereus	-	13	14	24	12	-	-
M. luteus	-	-	-	13	19	-	-
L. monocytogenes	-	-	-	-	-	-	-
S. aureus	-	-	-	29	-	-	-
S. typhimurium	-	-	-	29	-	-	-
E. coli	-	-	-	30	-	-	-
P. aeruginosa	-	-	-	23	-	-	-

Number denotes the inhibiting diameter (mm) of clear zone on the agar plate

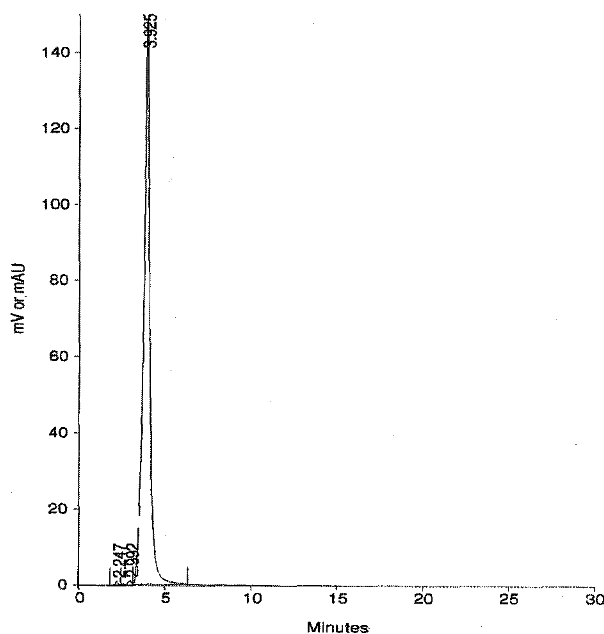

Fig. 2. HPLC chromatogram of purified antimicrobial compound from the seed coat of *Canavalia gladiata*.

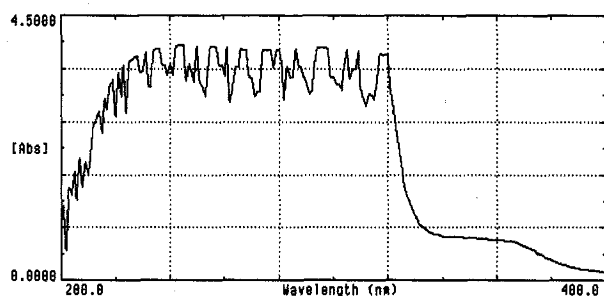

Fig. 3. UV spectrum of the purified antimicrobial compound from seed coat of *Canavalia gladiata*.

mm as compared with F2-5 (Table 8). These results suggest that the isolated substance has selectiveness against *S. aureus*. This fraction was designated as RSCC 1.

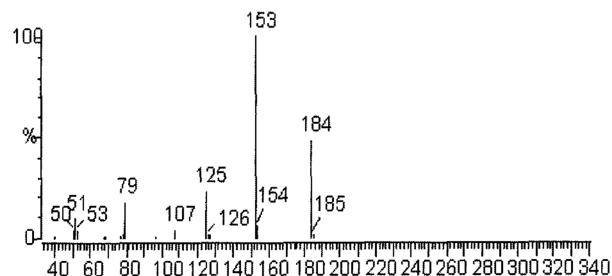

Fig. 5. EI-MS spectrum of the purified antimicrobial compound from seed coat of *Canavalia gladiata*.

Identification of the purified antimicrobial component

Purity of the isolated antimicrobial component was evaluated by HPLC. A single peak was found (Fig. 2), an indication that the component was purified to homogeneity. Fig. 3 shows absorption spectrum of RSCC 1. Absorption was observed at 230-320 nm for the purified compound with the maximum absorption shown at 273 nm. Fig. 4 shows the FT-IR spectrum of the purified RSCC 1. FT-IR spectrum revealed the presence of 3360 cm^{-1} (OH), 1695 cm^{-1} (C=O), 1444-1617 cm^{-1} (aromatic), and 1212 cm^{-1} (C-O). RSCC 1 was also analyzed on GC/MS. Molecular ion peak [M^+] of RSCC 1 was shown at 184 m/z on EI-MS spectrum (Fig. 5), indicating the determined molecular weight was 184. ^{1}H-NMR analysis showed high familiarity for structure elucidation. Fig. 6 shows ^{1}H-NMR spectrum of RSCC 1 (methanol-d_4, 300 MHz), in which the presence of a methyl ester group, protons of benzene ring, and protons of hydroxy group were observed at δ3.81 (3H, s, $COOCH_3$), δ7.03 (2H, s, H-2, 6), and δ4.89 (3H, s, OH), respectively. Fig. 7 shows ^{13}C-NMR spectrum of RSCC 1 (methanol-d_4, 75 MHz) with eight carbon atoms observed. The signal of δ169.06 ppm indicated an ester group. The signals of δ110.07, δ121.46, δ139.78, and δ146.51 ppm were attributed to the carbons of a benzene ring, and that of δ52.31 ppm to the carbons of a methyl group. Therefore, the purified antimicrobial substance from the seed of *C. gladiata* was identified as 3,4,5-trihydroxybenzoic acid methyl ester (methyl gallate) based on the experimental

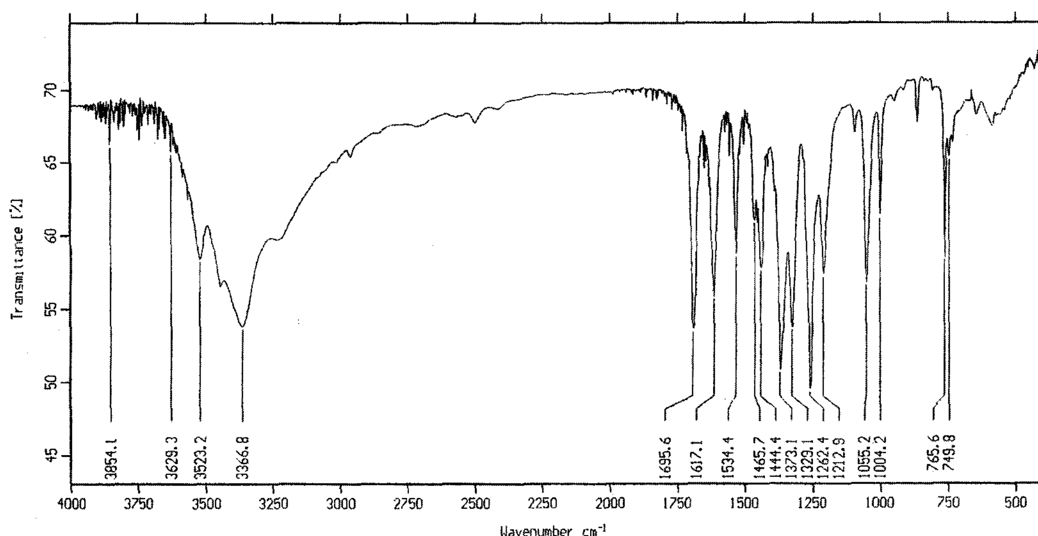

Fig. 4. FT-IR spectrum of the purified antimicrobial compound from seed coat of *Canavalia gladiata*.

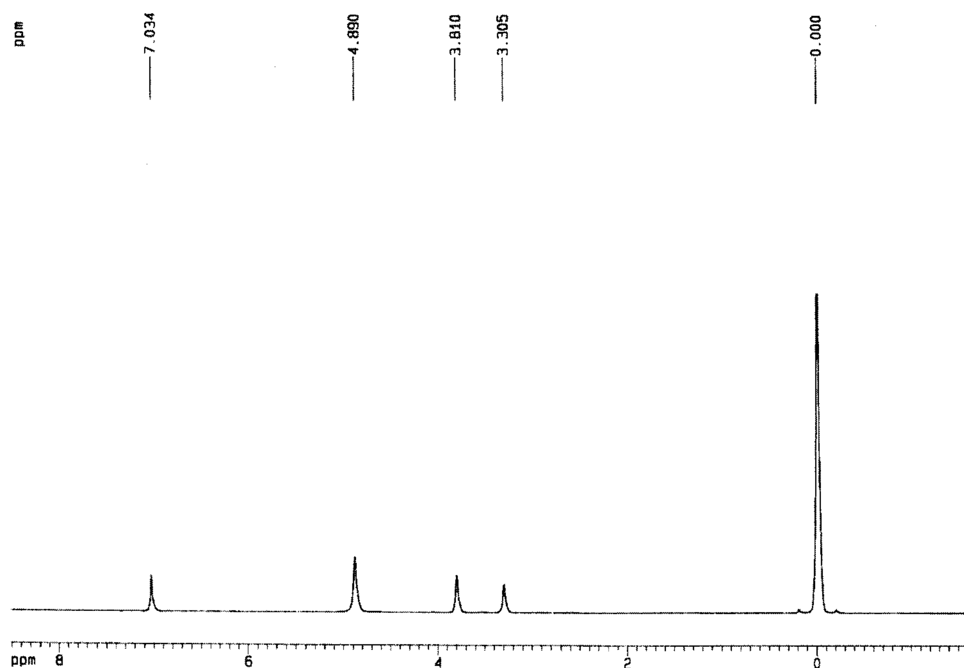

Fig. 6. ^{1}H-NMR spectrum of the purified antimicrobial compound from seed coat of *Canavalia gladiata* (Methanol-d$_4$, 300 MHz, δ3.81 (3H, s, COOCH$_3$), δ7.03 (2H, s, H-2,6), and δ4.89 (3H, s, OH)).

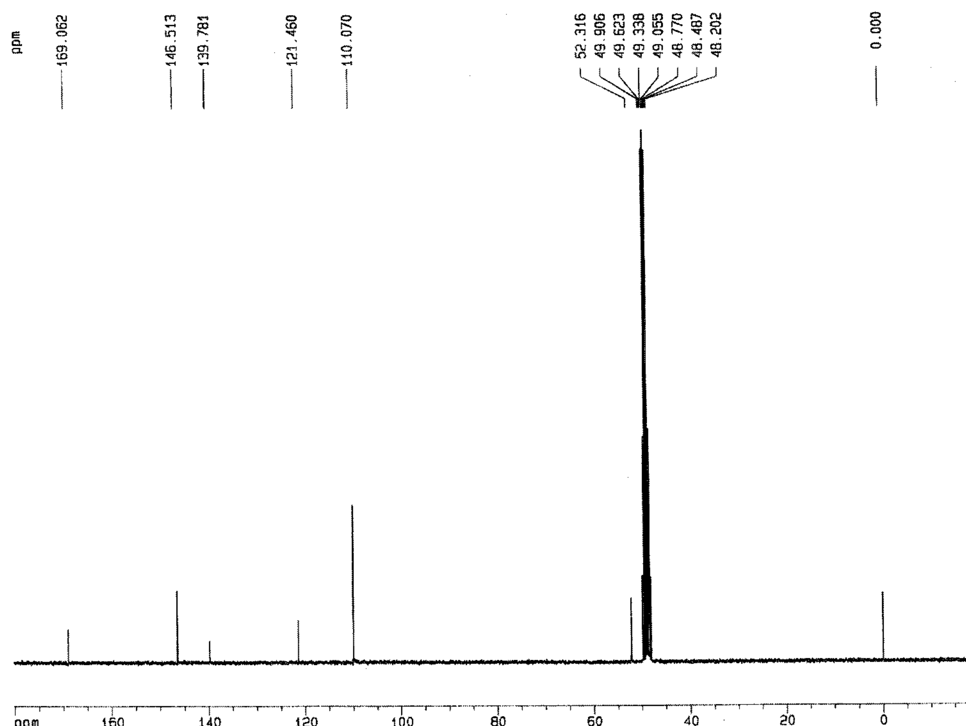

Fig. 7. ^{13}C-NMR spectrum of the purified antimicrobial compound from seed coat of *Canavalia gladiata* (Methanol-d$_4$, 75 MHz, δ52.31 (OCH$_3$), δ110.07 (C-3,5), δ121.46 (C-4), δ139.78 (C-1), δ146.51 (C-2,6), and δ169.06 (C=O)).

results described above. The structure of the inhibitory compound is shown in Fig. 8.

3,4,5-trihydroxybenzoic acid methyl ester was isolated from *Galla rhois* (7), Acer Ginnala Max (8), *Cuscuta chinensis* (9), and Chestnut galls (10) and identified. It has been reported to have biological activities, such as inhibition of PhIP mutagenicity (11), antitumor activity (12), and protectors (13). Sharm *et al.* (14) reported the isolation method of this compound using thin-layer chromatography (TLC). In addition, the isolated methyl gallate from *Rhus glabra* has a strong antimicrobial activity against eleven microorganisms, including Gram-positive and -negative bacteria, with *Pseudomonas aeruginosa* showing the highest activity at 2.5 μg/mL (15). Comparison of the methyl gallates extracted

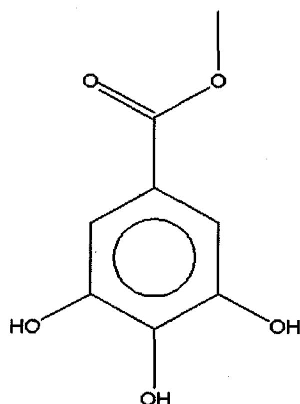

Fig. 8. Structure of the purified antimicrobial compound from seed coat of *Canavalia gladiata*. The compound was identified as 3,4,5-trihydroxybenzoic acid methyl ester also known as methyl gallate.

from *C. gladiata* with the authentic standard compound (Aldrich Chem. Co) as well as the *R. glabra* extract using the spectroscopic data (15) revealed no differences among the three sources.

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