Full Length Research Paper

Fumaric acid, an antibacterial component of *Aloe vera* L.

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The acetoacetate fraction partitioned from *Aloe vera* L. showed an effective antibacterial activity against four clinically frequent pathogenic bacteria. Through a series of chromatographic methods and activity assays, one compound was obtained and it has potent antibacterial activity. Based on the data of mass spectrometry, ^{1}H-NMR, ^{13}C-NMR and IR spectra, this compound was determined to be fumaric acid. This study concluded that fumaric acid is one of the antibacterial components in *A. vera*.

Key words: Fumaric acid, *Aloe vera*, antibacterial activity.

INTRODUCTION

Aloe is a large family containing 300 different species with its origin in Africa, but it is recognized as the "true *Aloe vera*" for its widespread use and purported healing powers (Paes-Leme et al., 2005). *A. vera* has been used for traditional medical purposes for thousands of years in the African continent, the western Indian Ocean Islands and Arabian Peninsula (Grace et al., 2008). The plant is made of elongated and pointed leaves, which contains two separate juice materials, a yellow latex (exudate) and a transparent mucilaginous gel (Chow et al., 2005). The inner gel from *A. vera* is generally viewed as the source of biological activity attributed to the plant.

As a traditional medicine, *A. vera* has various bio-activities of assisting in the healing of wounds, burns, eczema, psoriasis, tumors and infectious diseases (Beppu et al., 2004; Rosca-Casian et al., 2007). These therapeutic efficacies are attributed to the active components of *A. vera*. Acetylated glucomannan, which make up the majority of the mucilaginous *A. vera* gel, have been reported to affect wound closure in chronic wounds (Jensen et al., 1998), aphthous ulcers (Abebe,

2003) and reduction of dry socket associated with third-molar extraction sites (Poor et al., 2002). The refined polysaccharide has been shown to act as an immune-stimulant, displaying adjuvant activity on specific antibody production (t'Hart et al., 1989) and healing burns, ulcers and other wounds of the skin and gastrointestinal lining through enhancing the release of interleukin-1, interleukin-6, tumor necrosis factor and interferon (Yates et al., 1992; Peng et al., 1991). The other active components of *A. vera*, used for the treat-ment of a variety of ailments, concretely include barbaloin, isobarbaloin, aloesin, isoaloeresin D, aloeresin E, tannins, sterols, organic acids, enzymes, saponins, vitamins and minerals (Rosca-Casian et al., 2007; Ni et al., 2004).

Widespread antimicrobial use cause significant increa-ses in resistant bacteria. Due to the inefficacy of the frequently used antimicrobial, the new antibacterial agents are needed to be found. Medicinal plants that are commonly used may be good sources for safe and effective antibacterial agents (Sato et al., 2000). It has been known that Aloe is also a good antibacterial agent against a multitude of bacteria. Crude extract of *A. vera* leaf had antibacterial effects against clinically isolated bacterial pathogens (Pandey and Mishra 2010) and so does *A. vera* juice (Alemdar and Agaoglu, 2009). However, there were reports that different portions of *A. vera* had disparate antibacterial activities. Agarry et al. (2005) found that, the leaf possessed inhibitory effects on

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both *Pseudomonas aeruginosa* and *Candida albicans*, while the gel only inhibited the growth of *Trichophyton mentagraphytes*. In addition, the upper stem, root and bark of the South African aloe tree also had different antibacterial bio-activities (Ndhlala et al., 2009).

Although, the potent antibacterial activity of *A. vera* is demonstrated, to the knowledge of this study, the exact components producing the bioactivity of *A. vera* are still unclear. The purpose of this study, was to screen and identify the antibacterial components in the extract of *A. vera* using chromatographic methods linking antibacterial activity assay, and further to assess the activities of the components against four clinically frequent pathogenic bacteria.

MATERIALS AND METHODS

Plant

Fresh *A. vera* was harvested from Jiangsu Province in China. This plant was identified and authenticated by Prof. Y. L. Hu at Nanjing Agricultural University. A voucher specimen was stored at Department of Clinical Veterinary Medicine, College of Animal Science and Veterinary Medicine, Jilin University, Jilin, People's Republic of China. The plant was dried in the sun for about half-month period, finely ground and prepared for extraction.

Preparation of the extracts and solvent fractionation

Approximately 500 g dried plant material was extracted with 5 L of 95% ethyl alcohol (EtOH) for 24 h at room temperature. The extract was filtered through Whatman No. 1 filter paper and evaporated to 200 ml at 45°C under reduced pressure with a rotary vacuum evaporator, suspended in H_2O, and partitioned with chloroform ($CHCl_3$) and acetoacetate (EtOAc), successively. The solvent of each fraction was removed, each fraction extract was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 0.1 g/ml and the antibacterial activity of each fraction was determined. This procedure was performed at each purification step as follows:

Microorganisms and culture

Tested microorganisms were frequent pathogenic bacteria, including *Staphylococcus aureus* (CVCC 2261), *Streptococcus* (CVCC 2350), *Escherichia coli* (CEC 20060738) and *Salmonella* (CVCC 2139). All strains were purchased from China Veterinary Culture Collection Center. An inoculum of each strain was suspended in 5 ml of tryptone soya broth (TSB) and incubated at 37°C for 24 h. The incubated cultures were diluted 10 times with TSB and the bacterial suspension concentration was adjusted to 10^8 colony forming units (CFU/ml) before use.

Antibacterial activity assay

The hole-plate diffusion method described by Brantner et al. (1996) was used to assay the antibacterial activity of each fraction extract of *A. vera* at each purification step. Twenty millilitres of Mueller-Hinton agar were poured into Petri dishes, and then 0.1 ml of the appropriate bacterial suspension was inoculated on the agar and uniformly spread on the entire surface of the dish. 6 mm holes were made and filled up with 0.05 ml of each fraction extract. After the

inoculated plates were incubated at 37°C for 24 h, the anti-bacterial activity was detected. The DMSO solvent without extract was used as control.

The minimum inhibitory concentrations (MIC) and the minimum bactericidal concentrations (MBC) were used to detect the antibacterial activity of the final antibacterial components obtained from *A. vera* with the agar dilution method (Choi et al., 2009). MIC and MBC values were taken as the lowest concentration of the antibacterial components that completely inhibited bacterial growth and killed bacterium after 24 h of incubation at 37°C. Ofloxacin was used as the reference, and appropriate controls with no extract and solvent were used.

Isolation and purification of antibacterial fraction

Antibacterial fraction was isolated and purified with a series of chromatographic methods. The active fraction extract was subjected to a column chromatography on activated silica gel 60 (70 - 230 mesh, Merck, Darmstadt, Germany) with a serial concentration gradient of $CHCl_3$-MeOH (15:1, 10:1, 10:2, 10:3 and 2:1, v/v), followed by MeOH. Six fractions were collected and assayed for antibacterial activity. The antibacterial fraction was subjected to the second silica gel column chromatography with mobile phase $CHCl_3$-acetone (10:1, 10:2 and 10:3, v/v) and a total of 18 fractions were collected. After assaying the antibacterial activity, active fractions were combined, subsequently subjected to gel filtration (Sephadex LH – 20), and eluted with MeOH, producing a total of 54 fractions. Then, the active fraction was subjected to a column chromatography on octadecylsilane chemically bonded silica (ODS) using MeOH-H_2O (75:25, v/v) as the mobile phase to give the compound 1 (100 mg). The analysis of the compound 1 was completed using mass spectrometry, ^{1}H-NMR, ^{13}C-NMR and IR spectra. Finally, the antibacterial activity of the compound 1 including MIC and MBC were determined.

RESULTS AND DISCUSSION

In the preliminary experiments of this study, the antibacterial effectiveness of many Chinese herbal medicines had been determined which were prescribed for the treatment of avian colibacillosis in China. Some of these herbs, especially *A. vera*, had the antibacterial activity against avian pathogenic *E. coli* (data not shown). In this study, to further research and obtain the active components of *A. vera*, three fractions of ethanol extract of *A. vera* (41.84 g) were partitioned successively, including the $CHCl_3$ fraction (0.47 g), the EtOAc fraction (1.66 g) and the aqueous fraction (22.28 g). All the fractions were assayed for the antibacterial activity (Table 1). The EtOAc fraction inhibited the growth of all the tested bacteria, but the $CHCl_3$ fraction and the aqueous fraction did not show any inhibitory effect. In addition, the EtOAc fraction showed a higher inhibitory activity against gram-positive bacteria compared with gram-negative bacteria. Ferro et al. (2003) reported that *A. vera*, consisted of inner gel, which significantly inhibited *Shigella flexneri*, a Gram-negative bacteria. Further research demonstrated that effective growth inhibition, up to 24 h, was achieved with concentrations of more than 100 mg of *A. vera* per ml for *S. flexneri*, while it was just 25 mg of *A. vera* per ml for *Streptococcus pyogenes* (a

Table 1. Antibacterial activities of the CHCl₃ fraction, the EtOAc fraction and the aqueous fraction from the 95% ethanol extraction of *A. vera*

Bacteria	Inhibitory zone (mm)			
	CHCl₃ fraction	EtOAc fraction	Aqueous fraction	Solvent control
Staphylococcus aureus [a]	-	18	-	-
Streptococcus [a]	-	19	-	-
Escherichia coli [b]	-	10	-	-
Salmonella [b]	-	10	-	-

[a] Gram-positive bacterium; [b] Gram-negative bacterium; - the inhibitory zone was not observed.
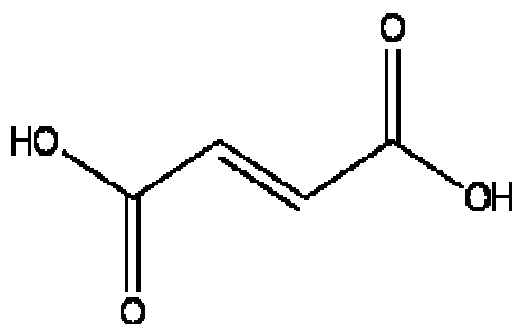
Figure 1. Structure of the compound 1.

Table 2. Mass spectrometry data of the compound 1.

m/z	Molecular ion
116	M^+
98	$[M - H_2O]^+$
81	$[M - OH - H_2O]^+$
53	$[M - CO - OH - H_2O]^+$

Table 3. NMR data of the compound 1 in DMSO.

Chemical group	δ_H^a (ppm)	δ_C^b (ppm)
- CH = CH-	6.63	134.01
- COOH	13.07	165.99

[a] ¹H-NMR, 400 MHz; [b] ¹³C-NMR, 125 MHz.

Table 4. IR spectra data of the compound 1.

Chemical group	Absorption bands (cm^{-1})
O - H	2500 ~ 3000
C = O	1600 ~ 1750
C = C	900 ~ 940

could more effectively inhibit Gram-positive bacteria than antibacterial activities of the fractions in each purification step. Finally, the compound 1 (Figure 1) was isolated and confirmed to have antibacterial activity. The results of mass spectrometry (Table 2), ¹H-NMR, ¹³C-NMR (Table 3) and IR spectra (Table 4) showed good concordance with the spectrum data of fumaric acid reported by Okamura et al. (1997).

The compound 1 (fumaric acid) displayed a potent antibacterial activity (Table 5). It showed the highest inhibitory activity against *S. aureus* and *Streptococcus* with a MIC value of 50 µg/ml and against *E. coli* and *Salmonella* with a MIC value of 50 µg/ml. Also, it showed the highest bactericidal activity against *S. aureus* and *Streptococcus* with a MBC value of 100 µg/ml and respectively against two Gram-negative bacteria (*E. coli* and *Salmonella*) with MBC value of 1560 and 780 µg/ml. Consistent with the activity of the EtOAc fraction, fumaric acid also possessed a higher inhibitory activity against Gram-positive bacteria, which was similar to the antibacterial activity of aloe extracts reported by Pandey and Mishra (2010). The effect of fumaric acid might be attributed to the structural differences between the two organisms. In addition, because of the physico-chemical property of the fumaric acid, it may exist in ethanol extract more than aqueous extract from *A. vera*. Therefore, Pandey and Mishra (2010) found that there was no inhibitory effect of aqueous extract on the Gram-negative bacteria, and weak inhibitory effect on Gram-positive bacteria.

Fumaric acid belongs to organic acid, which has been reported as an effective antibacterial agent. Molatová et al. (2010) demonstrated that fumaric acid had clear antimicrobial activity to *Campylobacter jejun*. Kim et al. (2001) reported that fumaric acid isolated from *Camellia japonica* L. produced an inhibitory activity against the pathogens *Salmonella typhimurium*, *E. coli*, *Listeria*

gram-positive bacteria). It was indicated that *A. vera* Gram-negative bacteria, this was in correlation with the results of this study. Unfortunately, substances responsible for this effect was not studied in their research. According to earlier studies, antibacterial components of *A. vera* existed in the EtOAc fraction, and further purification of this portion was performed.

The silica gel adsorption column chromatography, Sephadex LH-20 column chromatography and ODS column chromatography were successively used to purify the active EtOAc fraction, guided by the results of

Table 5. Antibacterial activity of the compound 1 isolated from *A. vera*.

Microorganism	Compound 1 (μg/ml)		Ofloxacin[c] (μg/ml)	
	MIC	MBC	MIC	MBC
Staphylococcus aureus [a]	50	100	2	2
Streptococcus [a]	50	100	2	4
Escherichia coli [b]	780	1560	2	4
Salmonella [b]	780	780	2	4

[a] Gram-positive bacterium; [b] Gram-negative bacterium; [c] positive control. MIC, minimum inhibitory concentrations and MBC, minimum bactericidal concentrations.

monocytogenes and *S. aureus*. In addition, other organic acid or its derivatives such as syringic acid, 2-amino-benzoic acid (Kong et al., 2008) and beilschmiedic acids C (Chouna et al., 2009) found in some plants had greater antibacterial activity against various bacteria. In this research, although fumaric acid that was obtained showed potent antibacterial activity, unfortunately, the bioactivity of fumaric acid was lower than that of ofloxacin which could inhibit both Gram-positive and negative bacteria at lower concentrations.

Fumaric acid is one of the important compounds in a lot of fruits and herbs, and it is a main substance in the tricarboxylic acid (TCA) cycle and has a low molecular weight (Cunha et al., 2001). It also exists in *A. vera* , but the quantity of this organic acid is very low in this plant (Bozzi et al., 2007). Fumaric acid, especially its derivatives such as fumaric acid esters, is extensively applied in the treatment of multiple sclerosis (Moharregh-Khiabani et al., 2009), psoriasis (Roll et al., 2007) and granuloma annulare (Kreuter et al., 2002). But, as an antibacterial agent, fumaric acid is mostly used for food preservative (Comes et al., 2002). Although, it is reported that fumaric acid has a strong antibacterial activity as a food preservative (Comes et al., 2002; Liao et al., 2008), to the knowledge of this study, this is the first report on fumaric acid as an antibacterial component in *A. vera*.

Conclusion

In conclusion, this study demonstrated that the aceto-acetate fraction partitioned from *A. vera* showed an effective antibacterial activity against four clinically frequent pathogenic bacteria. Through a series of chromatographic methods and activity assays, fumaric acid was obtained and identified. It inhibited all the tested bacteria with the MIC and MBC values of 50 to 780 and 100 to 1560 μg/ml, respectively. It was indicated that fumaric acid is one of the antibacterial components in *A. vera*. These findings may contribute to future research on screening other antibacterial components in *A. vera* and provide a basis for application of *A. vera* in treatment of infectious deseases.

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