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Antimicrobial gallic acid from *Caesalpinia mimosoides* Lamk.

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Abstract

Different extracts of *Caesalpinia mimosoides* Lamk. (Leguminosae) were tested against eight human pathogenic bacteria and six fungal strains by the disc diffusion method. Among these extracts, the aqueous and the ethanolic extracts showed potent activity against some tested microorganisms. Subsequently, the latter extract was fractionated by means of Sephadex LH-20 column chromatography. A bioactive substance, responsible for the antimicrobial property was separated, and its structure was assigned as a known compound, gallic acid, by extensive chromatographic and spectroscopic analyses.

Keywords: *Caesalpinia mimosoides*; Antimicrobial activity; Gallic acid

1. Introduction

Caesalpinia mimosoides Lamk. (Leguminosae), local Thai name: Cha-Lueat, is a plant native to Thailand. It is an erect or climbing shrub that grows up to one meter tall with densely pocked thorns on all parts. The plant is distributed in old clearings, scrub areas, and mixed deciduous forests in northern and north-eastern Thailand. The young shoots and leaves are locally consumed as a fresh vegetable or appetizer (Jacquat, 1990; Smitinand, 1984). It has also been used as a carminative and to relieve dizziness and fainting (information from local herbal specialist).

The plant is known to contain monosaccharide from seed coat tissue (Anulov, Smirnova, & Belyaeva, 2000). In a previous investigation we screened the crude extracts of some indigenous plants in Thailand for their antioxidant activities and their chemical compositions, including vitamins, carotenoids, and phenolics (Chanwitheesuk, Teerawutgulrag, & Rakariyatham, 2005). This plant showed moderate antioxidant activity, and high tannin and total phenolics contents, which led us to examine it further for other biological activity. In this study, we present the antimicrobial property of various extracts of *C. mimosoides*, including aqueous, acetone, chloroform, and ethanolic extracts. Subsequently, the ethanolic extract was fractionated, and only the active substance in the fraction, which tested positively for antimicrobial activity, was further identified by the analyses of chromatographic and spectroscopic data.

2. Materials and methods

2.1. General

Column chromatography was performed on a Sephadex LH-20 column, using 95% ethanol as an eluent. TLC was carried out with a silica gel 60 F_{254} precoated alumina sheet (Merck). Spots were visualized under UV light (254 and 365 nm) or by spraying with Folin-Ciocalteu's reagent. UV–Vis absorption spectra were recorded in ethanol on a

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Perkin–Elmer Lambda 25 UV–Vis spectrometer. HPLC was performed using an HP 1100 series liquid chromatograph system comprising vacuum degasser, binary pump, auto-sampler, and variable wavelength detector. Fractions were analyzed on a C18 Hypersil ODS column (5 μm, 4.0 × 125 mm) with a mobile phase of (A) water–acetic acid (97:3 v/v) and (B) methanol (A:B, 80:20 v/v) at a flow rate of 0.5 ml/min. The detector recorded at 280 nm. IR spectra were recorded using KBr discs on a Nicolet 510 FT-IR spectrometer. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance-400 spectrometer (400 MHz), using acetone-d_6 as solvent and tetramethylsilane (TMS) as internal standard, and the chemical shifts reported in δ (ppm) units relative to TMS signal and coupling constants (J) in Hz. A complete attribution was performed on the basis of 2D-experiment (heteronuclear multiple bond correlation, HMBC). High resolution-mass spectrometry (electrospray) or HRMS (ESI) data were measured with a Micromass Instrument type QTOF 2 spectrometer.

2.2. Plant material

The plant *C. mimosoides* was bought from a local market in Chiang Mai, Thailand. It was identified by Asst. Prof. Paritat Trisonthi, Department of Biology, Faculty of Science, Chiang Mai University. A voucher specimen (No. CM-02) has been deposited at the Department of Chemistry, Faculty of Science, Chiang Mai University.

2.3. Microorganisms

The microorganisms used in the study consisted of eight strains of human pathogenic bacteria, one yeast, and five filamentous fungi (including two dermatophytic fungi). All the bacterial strains, yeast, and dermatophytic fungi, were obtained from the Department of Clinical Microbiology, Faculty of Associated Medical Sciences, Chiang Mai University. The plant pathogenic fungi were obtained from the Department of Plant Pathology, Faculty of Agriculture, Chiang Mai University. The bacteria were grown and maintained on nutrient agar slants, while yeast and fungi were on potato dextrose agar slants. The inoculated agar slants were incubated at 37 °C for bacteria and yeast, and at 30 °C for fungi.

2.4. Preparation of extracts

Dry plant materials (50 g) were extracted with 500 ml of various solvents (distilled water, acetone, chloroform, and ethanol) by shaking at 200 rpm for 3 × 30 min at room temperature. The crude extracts were filtered, and the filtrates were then evaporated under reduced pressure. The successive extractive values were 3.50 g in water, 3.28 g in acetone, 2.98 g in chloroform, and 4.80 g in ethanol. The extracts containing 250 mg/ml were prepared in the same solvent as for extraction, and used for investigation of antimicrobial activity.

2.5. Isolation and identification of active substance

Ten millilitres of crude ethanolic extract dissolved in ethanol (50 mg/ml) were applied onto a column packed with Sephadex LH-20 and eluted with 95% ethanol. The column dimension was 4 × 60 cm, and the flow rate was 1 ml/min. Ethanolic fractions (10 ml each) were collected in test tubes placed in a RadiFrac fraction collector, and their absorbances were read at 280 nm. Eluates were then pooled into fractions 1–8 (coded: FA–FH). The solvent of each separated fraction was evaporated under reduced pressure. Each fraction was then dissolved in ethanol at the concentration of 10 mg/ml.

All fractions obtained from the column were then investigated for antimicrobial activity. The active substance of the obtained fraction which exhibited antimicrobial activity was identified by extensive chromatographic and spectroscopic analyses.

2.6. Antimicrobial bioassay

Antimicrobial activity was determined by disc diffusion method (Bauer, Kirby, Sherris, & Truck, 1966), using bacterial cell suspension which equilibrated their concentration to a 0.5 McFarland standard or 10^5–10^6 spores/ml of yeast or fungal spore suspension. Each bacterial suspension (100 μl) was spread on a Mueller–Hinton agar plate, while yeast or fungal spore suspension was spread on a Sabouraud dextrose agar plate. Sterile paper discs, 6 mm diameter, were impregnated with 20 μl of each test sample. The discs were allowed to dry and then placed on the agar surface of each Petri dish, which had previously been inoculated with the test microorganisms. Discs with the solvents used for extraction were used as negative controls, while two standard antibiotics (10 μg streptomycin discs and 100 units nystatin discs) were used as positive controls. The plates were incubated at 37 °C overnight for bacteria and yeast, or at 30 °C for three days for fungi. After incubation, zones of inhibition appearing around the discs were measured and recorded. The values were averages of three measurements per disc, taken at three different directions in order to minimize errors. The inhibition zones were expressed as the means of four separation experiments.

Minimum inhibitory concentrations (MICs) were also investigated for the microbial strains which were determined as sensitive to the compounds in the disc diffusion assay. A broth macrodilution method was used, as previously described (Nakamura et al., 1999), with a slight modification. Serial twofold dilutions of the test compounds were prepared in 10% dimethylsulfoxide (DMSO), and 30 μl of each dilution were added to 3 ml of Mueller–Hinton broth or Sabouraud dextrose broth. These were inoculated with 30 μl of cultures of the test microbial strains. After incubation of the cultures at 37 °C for bacteria, or 30 °C for fungi (48 h), the MIC value was determined as the lowest concentration of the test compound that demonstrated no visible growth.

3. Results and discussion

The antimicrobial activity of the several extracts of *C. mimosoides*, at a concentration of 250 mg/ml, was determined by disc diffusion method as given in Table 1. A total of 14 microorganisms, which consisted of eight bacteria, one yeast, and five filamentous fungi, were tested. Standard antibiotics (streptomycin and nystatin) were used, and are also mentioned in Table 1. The solvents used for extraction were also used for dissolving the extracts, and all the solvent controls did not show any activity (data not shown). As shown in Table 1, the aqueous extract of *C. mimosoides* exhibited a potent activity against all of the test bacteria and dermatophytic fungi. The ethanolic extract also showed a high level of activity against the same microorganisms except *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The chloroform extract displayed the lower activity against some species (*Vibrio cholerae*, *Staphylococcus aureus*, and *S. epidermidis*), whereas, the acetone extract displayed a moderate activity against *V. cholerae*, Gram positive bacteria, and dermatophytic fungi.

To compare the sensitivity of the microorganisms to the different extracts, MIC values were determined for the known drugs and the extracts which showed antimicrobial activity (Table 2). There were substantial differences between the MICs of the different extracts. However, the bacterium *S. aureus* and the dermatophytes were the most sensitive strains to the extracts of *C. mimosoides*, especially the ethanolic extract.

As shown in Tables 1 and 2, the various extracts of *C. mimosoides* exhibited different levels of antimicrobial activity. The strong activity was observed in the aqueous and the ethanolic extracts. However, the aqueous fraction was analyzed and found not to contain any antimicrobial phenolic compounds which were the compounds of interest in this study. Instead, the aqueous extract was found to contain only alkaloids, which were not investigated any further. Moreover, the ethanolic extract gave a higher yield, and it has been reported that alcohol was better solvent for extraction of active antimicrobial substances (Ahmad, Mehmood, & Mohammad, 1998). Therefore, the isolation and identification of antimicrobial substance were studied in the ethanolic extract.

Since many plant phenolics have been found to be responsible for several biological properties, including antimicrobial properties (Panizzi, Caponi, Catalano, Cioni, & Morelli, 2002; Penna et al., 2001; Van der Watt & Pretorius, 2001), it was expected that the antimicrobial activity of this plant species would be related to its phenolic compounds.

The separation of antimicrobial substance was carried out by Sephadex LH-20 column chromatography. This method has been used extensively to fractionate several plant extracts (Amarowicz & Shahidi, 1996; Ma, Fukushi, Tahara, & Osawa, 2000; Tringali, Spatafora, & Longo, 2000). Sephadex LH-20 is probably one of the best stationary phases available for separation of phenolics because of the faster, yet satisfactory, separation of phenolics on the column (Wanasundara, Amarowicz, & Shahidi, 1994). A portion (10 ml) of the crude ethanolic extract was loaded onto a Sephadex LH-20 column. The elution profile of the extract is shown in Fig. 1. The chromatographic profile exhibited eight maxima at 280 nm, labelled FA–FH. The concentration of each fraction was adjusted to 10 mg/ml before assaying antimicrobial activity against a set of microbial strains. The antimicrobial activity was observed only in fraction D (FD) against *Salmonella typhi* and *S.*

Table 1
Antimicrobial activity of the extracts of *Caesalpinia mimosoides* (5 mg/disc) against microorganisms tested, based on disc diffusion method

Microorganism	Inhibition zone in diameter (mm)					
	Acetone extract	Aqueous extract	Chloroform extract	Ethanolic extract	Streptomycin	Nystatin
Gram negative bacteria						
Escherichia coli	–	12.75 ± 0.25	–	18.75 ± 0.50	16.00 ± 0.71	NT
Klebsiella pneumoniae	–	8.50 ± 0.25	–	–	16.50 ± 0.50	NT
Pseudomonas aeruginosa	–	14.25 ± 0.43	–	–	13.75 ± 0.43	NT
Salmonella typhi	–	25.25 ± 0.50	–	18.75 ± 0.71	17.00 ± 0.71	NT
Vibrio cholerae	11.25 ± 0.71	29.75 ± 0.71	8.25 ± 0.25	20.25 ± 0.83	20.75 ± 0.43	NT
Gram positive bacteria						
Enterococcus faecalis	10 ± 0.43	7.25 ± 0.25	–	12.50 ± 0.50	19.00 ± 0.71	NT
Staphylococcus aureus	14.50 ± 0.87	39 ± 0.83	11.50 ± 0.25	14.25 ± 0.50	16.25 ± 0.43	NT
Staphylococcus epidermidis	12.50 ± 0.50	35.25 ± 0.87	8 ± 0.25	15.25 ± 0.71	20.50 ± 0.50	NT
Yeast						
Candida albicans	–	–	–	–	NT	26.25 ± 0.43
Filamentous fungi						
Aspergillus sp.	–	–	–	–	NT	21.50 ± 0.50
Fusarium sp.	–	–	–	–	NT	16.00 ± 0.71
*Microsporum gypseum**	15.75 ± 0.83	42.50 ± 0.43	–	24.50 ± 0.87	NT	22.75 ± 0.43
Penicillium sp.	–	–	–	–	NT	18.25 ± 0.83
*Trichophyton rubrum**	11.50 ± 0.50	40.50 ± 0.50	–	13.75 ± 0.87	NT	18.50 ± 0.50

Values are means ± SD (mm)of four separate experiments; *: dermatophytic fungi; "–": no inhibition zone; NT: not tested.

Table 2
MIC values of the extracts of *Caesalpinia mimosoides*

Microorganism	MIC value					
	Acetone extract (mg/ml)	Aqueous extract (mg/ml)	Chloroform extract (mg/ml)	Ethanolic extract (mg/ml)	Streptomycin (μg/ml)	Nystatin (unit)
Gram negative bacteria						
Escherichia coli	NT	250	NT	125	1.25	NT
Klebsiella pneumoniae	NT	250	NT	NT	1.25	NT
Pseudomonas aeruginosa	NT	62.5	NT	NT	0.63	NT
Salmonella typhi	NT	62.5	NT	125	0.16	NT
Vibrio cholerae	62.5	31.3	250	31.3	0.16	NT
Gram positive bacteria						
Enterococcus faecalis	62.5	250	NT	62.5	0.63	NT
Staphylococcus aureus	62.5	15.6	250	7.81	0.16	NT
Staphylococcus epidermidis	62.5	31.3	250	62.5	0.63	NT
Yeast						
Candida albicans	NT	NT	NT	NT	NT	0.78
Filamentous fungi						
Aspergillus sp.	NT	NT	NT	NT	NT	6.25
Fusarium sp.	NT	NT	NT	NT	NT	12.5
*Microsporum gypseum**	125	62.5	NT	31.3	NT	0.78
Penicillium sp.	NT	NT	NT	NT	NT	6.25
*Trichophyton rubrum**	31.3	31.3	NT	15.6	NT	1.56

*: dermatophytic fungi; NT: not tested.

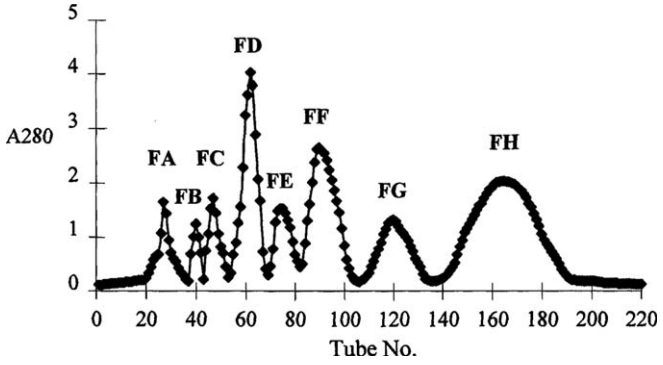

Fig. 1. Elution profile of Sephadex LH-20 chromatography of the ethanolic extract of *Caesalpinia mimosoides* (column size: 4 × 60 cm; sample volume: 10 ml (50 mg/ml); mobile phase: 95% ethanol; flow rate: 1 ml/min, fraction volume: 10 ml).

aureus, with the same small inhibition zone (7 mm). This result could be due to the low concentration of sample used. Minimum inhibitory concentration of FD was also determined against both bacteria. The bacterium *S. aureus* seems to be a sensitive strain to FD as it gave a MIC value of 1250 μg/ml, whereas *S. typhi* gave 2500 μg/ml.

In order to investigate the purity of the fraction FD, it was analyzed by TLC and HPLC techniques. The spot on the TLC chromatogram developed with hexane–ethyl acetate–acetic acid (2:1:0.3 v/v/v) produced a characteristic blue colour with Folin-Ciocalteu's reagent, indicating that the fraction FD contained a phenolic substance. The purity of FD was confirmed by HPLC as it exhibited only one peak on the chromatogram. Preliminary identification of FD was based on comparison of its R_f value and retention time with those of authentic compounds. Finally, the structure assignment of FD was based on its spectral data IR, MS, and ^{1}H and ^{13}C NMR, and it was identified as a known compound, gallic acid (Fig. 2): yellow-green solid; TLC: (hexane:ethyl acetate:acetic acid, 2:1:0.3 v/v/v) R_f 0.24; UV λ_{max} (ethanol) nm: 220, 271; IR (KBr) ν cm^{-1}: 3491, 3377, 1703, 1617, 1539, 1453, 1254 cm^{-1}; ^{1}H NMR (acetone-d_6): δ H 7.15 (2H, *s*, H-3 and H-7); ^{13}C NMR (acetone-d_6): δ C 167.39 (C-1), 144.94 (C-4 and C-6), 137.77 (C-5), 120.81 (C-2), 109.14 (C-3 and C-7); HRMS (ESI) m/z 169.0137 $[M-H]^-$ (calcd. for $C_7H_5O_5$, 169.0137).

The active substance in the fraction FD was identified as gallic acid by chromatographic and spectroscopic analyses. Gallic acid is a simple phenolic acid possessing a single aromatic ring. It is widely distributed in angiosperms and is also found in some green algae (Waterman & Mole, 1994). It is a well-known antioxidant, and has been previously reported to possess anti-inflammatory activity (Kroes, Van den Berg, Quarles van Ufford, Van Dijk, & Labadie, 1992), and antitumor activity (Miki et al., 2001). In addition, antibacterial and antifungal activities were also found for this substance (Akiyama, Fujii, Yamasaki, Oono, & Iwatsuki, 2001; Panizzi et al., 2002; Penna et al., 2001; Shukla, Srivastava, Kumar, & Kumar, 1999).

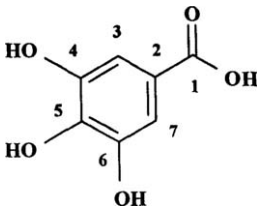

Fig. 2. Structure of gallic acid.

4. Conclusions

In conclusion, this study demonstrated the antimicrobial activities of several extracts from *C. mimosoides*. Furthermore, we successfully isolated an active antimicrobial substance, gallic acid, from its ethanolic extract. Gallic acid exhibited the activity against the bacteria *S. typhi* and *S. aureus* with the MIC values of 2500 and 1250 μg/ml, respectively.

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