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**BIOACTIVE 4-HYDROXYCINNAMIDE AND
BIOACTIVITIES OF *POLYALTHIA CERASOIDES***

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ABSTRACT

Constituents from *Polyalthia cerasoides*, stem bark methanol extract, were previously documented. This study reports the first isolation of bioactive *N*-(4-hydroxy- β -phenethyl)-4-hydroxycinnamide (**1**) from ethyl acetate extract of the plant species including stigmasterol and a mixture of triterpenes from hexane and dichloromethane extracts. Trace essential elements were found in the hexane extract in ppm level. The plant extracts were evaluated for their antimicrobial and antioxidative activities. The dichloromethane extract displayed the highest activity against *Corynebacterium diphtheriae* NCTC 10356 with MIC of 32 μ g/mL, as well as, the highest SOD activity with an IC₅₀ of 4.51 μ g/mL.

Keywords: *Polyalthia cerasoides*, 4-hydroxycinnamide, antioxidants, antimicrobials, trace elements

INTRODUCTION

Polyalthia cerasoides (Roxb.) Benth. Ex Bedd. (Annonaceae), a medicinal plant used in Thai indigenous medicine in particular, its roots as a tonic and febrifuge (Pharmaceutical sciences, 1996) and stem barks as a pain relief and kidney dysfunction (Smitinand, 1980). Diverse groups of

bioactive compounds from the roots of *P. cerasoides* were reported, e. g. aporphine and benzyloquinoline alkaloids, triynoic acid and triterpenes (Kanok-medhakul et al., 2007). Previously, stem bark polar extracts (methanol) of the plant species were reported to contain sesquiterpene and benzopyrans (González et al., 1995, 1996) including oxoprotoberberine

(Zafra-Polo et al., 1996; González et al., 1997). So far, nonpolar and less polar plant extracts were not documented in the literature. The present study reports the isolation and bioactivities of the stem barks; hexane, dichloromethane and ethyl acetate extracts of the plant species as well as trace elements.

MATERIALS AND METHODS

General

Melting points were determined on an Electrothermal 9100 melting point apparatus and are uncorrected. ^1H - and ^{13}C -NMR spectra were recorded on a Bruker AVANCE 300 NMR spectrometer (operating at 300 MHz for ^1H and 75 MHz for ^{13}C). Infrared spectra (IR) were obtained on a Perkin Elmer System 2000 FTIR. Mass spectra were recorded on a Finnigan INCOS 50. Column chromatography was carried out using silica gel 60 (0.063–0.200 mm). Analytical thin layer chromatography (TLC) was performed on silica gel 60 PF₂₅₄ aluminium sheets (cat. No. 7747 E., Merck). Solvents were distilled prior to use. Chemicals and reagents for cell culture and assays were of analytical grade.

Plant material

Stems of *P. cerasoides* were collected from Praputtath, Saraburi Province, Thailand. It has been identified by the Forest Herbarium, Royal Forest Department, Bangkok, Thailand.

Extraction

The air dried milled stem barks of *P. cerasoides* (6 kg) was extracted with hexane (3×5 days), then filtered and evaporated *in vacuo* to give a hexane extract (16.5 g). Similarly, the extraction was carried out using dichloromethane, ethyl acetate and methanol to give the corresponding dichloromethane (12.5 g), ethyl acetate (11.2 g) and methanol (58 g) extracts, respectively.

Sample preparation

Plant extract (0.3 g) was added conc H_2SO_4 (2 mL) and 30 % H_2O_2 (5 mL), then heated at 300 °C for 5 h. After cooling, the solution was added to distilled water (100 mL), then filtered to give a digested solution.

Isolation

Hexane extract (15.5 g) was placed onto a silica gel (400 g) column, eluted with hexane: CHCl_3 (9:1 to 1:1) to give a pale yellow solid (3.7 g). Recrystallization by hexane: CHCl_3 afforded stigmasterol 0.92 g of m.p. 168–170 °C.

Dichloromethane extract (11.1 g) was separated by silica gel (300 g) column to provide stigmasterol (0.17 g) and a mixture of triterpenes after elution with hexane: CH_2Cl_2 (1:1).

Ethyl acetate extract (9.4 g) was subjected to silica gel (250 g) column. Initial elution with hexane: CH_2Cl_2 (1:1) gave β -sitosterol 6.2 mg. Further elution with CH_2Cl_2 : MeOH (95:5) provided paprazine (1) as a white amorphous (4.7 mg); mp 250–251 °C [mp 250–251 °C (Ee et al., 2009), 253–254 °C (Yamamoto et al., 1991)] together with a mixture of yellow oils (17.8 mg).

Paprazine; IR (KBr) ν_{max} (cm^{-1}) 3434 (OH), 3310 (br OH), 3175 (br NH), 1662 (C=O), 1174, 1105, 1050 (C-O); ^1H -NMR (CD_3OD): δ 2.75 (t, 2H, H-7, J = 7.4 Hz), 3.46 (t, 2H, H-8, J = 7.4 Hz), 6.37 (d, 1H, H-8', J = 15.7 Hz), 6.72 (d, 2H, H-3, H-5, J = 8.5 Hz), 6.78 (d, 2H, H-3', H-5', J = 8.6 Hz), 7.04 (d, 2H, H-2, H-6, J = 8.5 Hz), 7.39 (d, 2H, H-2', H-6', J = 8.6 Hz), 7.44 (d, 1H, H-7', J = 15.7 Hz); ^{13}C -NMR (CD_3OD): δ 35.7 (C-7), 42.4 (C-8), 116.2 (C-3, C-5), 116.6 (C-3', C-5'), 118.3 (C-8'), 127.6 (C-1'), 130.4 (C-2', C-6'), 130.6 (C-2, C-6), 131.2 (C-1), 141.7 (C-7'), 156.7 (C-4), 160.3 (C-4'), 169.1 (CO); LRMS (EI): m/z (%) = 283 (8.81) [M^+], 164 (73), 147 (100), 120 (56), 107 (30), 91 (26), 77 (17).

Elemental analysis

Trace elements of the plant digested solution was analyzed by ICP-AES (Inductively couple plasma-atomic emission spectrometry), Seiko Instruments, SPS 7000 using an argon as plasma gas, carrier gas and auxiliary gas with the flow rate of 6–7 L/min. The determination was performed in triplicate.

Biological activities

Antimicrobial assay

Antimicrobial activity of the tested compounds was performed using agar dilution method as previously described (Prachayasittikul et al., 2009a). Briefly, the tested compounds dissolved in DMSO were individually mixed with 1 mL Mueller Hinton (MH) broth. The solution was then transferred to the MH agar solution to yield the agar plates with the final concentration of compounds ranging from 4 to 256 $\mu\text{g/mL}$. Tested microorganisms, cultured in MH broth at 37 °C for 24 h, were diluted with 0.9 % normal saline solution to adjust the cell density of 1×10^8 cell/mL. The organisms were inoculated onto each plate and further incubated at 37 °C for 24–48 h. Cell growth was analyzed as compared to the control culture plates. The DMSO was tested in parallel with the compounds and showed no antimicrobial action on the tested organisms. Twenty-seven strains (18 reference strains and 9 clinical isolates) of the tested microorganisms were **gram negative bacteria**: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Serratia macescens* ATCC 8100, *Salmonella typhimurium* ATCC 13311, *Salmonella choleraesuis* ATCC 10708, *Achromobacter xylosoxidans* ATCC 2706, *Pseudomonas aeruginosa* ATCC 15442, *Pseudomonas stutzeri* ATCC 17587, *Shigella dysenteriae*, *Salmonella enteritidis*, *Morganella morganii*, *Aeromonas hydrophila*, *Citrobacter freundii*, *Plesiomonas shigelloides*; **gram positive bacteria**: *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* ATCC 25923, *Staphylococcus*

epidermidis ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecalis* ATCC 33186, *Micrococcus luteus* ATCC 10240, *Corynebacterium diphtheriae* NCTC 10356, *Bacillus subtilis* ATCC 6633, *Streptococcus pyogenes*, *Listeria monocytogenes*, *Bacillus cereus* and **diploid fungus (yeast)**: *Candida albicans* ATCC 90028, *Saccharomyces cerevisiae* ATCC 2601.

Radical scavenging: DPPH assay

Reduction of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was measured via spectrophotometry upon reaction with an antioxidant (Prachayasittikul et al., 2009b). Briefly, 0.1 mM of DPPH in methanol was freshly prepared. The scavenging activity was calculated from the absorbance (517 nm) after 30 min incubation at the absence (Abs. control) and presence of the tested compounds (Abs. sample) as the following equation:

$$\text{Radical scavenging activity (\%)} = \left[1 - \frac{\text{Abs.sample}}{\text{Abs.cont}} \right] \times 100$$

UV-1610, Shimadzu was used in the experiments and vitamin E was used as a standard.

Superoxide scavenging: SOD assay

The tested compounds were assessed for the SOD activity by photoreduction of nitro blue tetrazolium (NBT) as previously described (Prachayasittikul et al., 2009b). Purified SOD from bovine erythrocytes (Sigma-Aldrich) was used as a standard.

RESULTS AND DISCUSSIONS

Isolation

P. cerasoides extracts (hexane, dichloromethane and ethyl acetate) were isolated by silica gel column using gradient elution with increasing polarity. Structures of the isolates were confirmed by comparison of their spectral data with the literature reported. The hexane and dichloromethane extracts gave stigmasterol and a mixture of

triterpenes. Previously, sterols were isolated from leaves and stems ethanol extracts of *P. cerasoides* (Dan et al., 1985). Whereas the ethyl acetate extract provided β -sitosterol and paprazine (*N*-(4-hydroxy- β -phenethyl)-4-hydroxycinnamide, **1**, Fig. 1). In addition, the obtained mixture of yellow oils when tested with the Dragendorff's reagent (Farnworth, 1966), the strong yellow-orange color was observed. It could possibly assume that such mixture oils were alkaloids. Structure of the amide **1** was determined by comparison of its IR, ^1H -, ^{13}C -NMR and MS with the literature data (Ee et al., 2009). In addition, 2D-NMR (COSY, HMQC and HMBC) were also performed. Paprazine is a bioactive amide isolated from many plant species, e. g. the genus *Piper*; *Piper nigrum* (Ee et al., 2009) and *Piper kadsura* (Lin et al., 2006), *Erycibe hainanensis* (Song et al., 2010), *Dendrobium candidum* (Wang et al., 2010) and *Cannabis sativa* L. (Yamamoto et al., 1991).

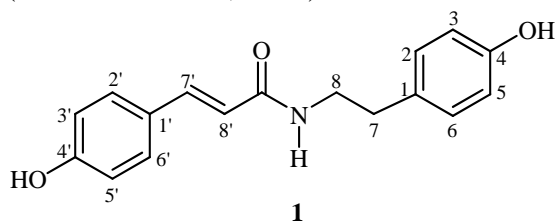


Figure 1: Chemical structure of paprazine (**1**)

Trace elements analysis

Metal ions play important roles in life and biological systems (Hegde et al., 2004; Nahar et al., 2010), e. g. enzymes containing Mn, Cu, Ni and Zn. Such components formed through coordination of endogenous ligands constituting *N*-, *O*- and *S*-atom electron donors with the metal ions. Our previous studies revealed that the endogenous ligand like nicotinic acid formed metal (Cu) complexes *via N*- and *O*-electron donors leading to the compounds with SOD and antimicrobial actions (Sukrichavalit et al., 2008, 2009). In fact, plant species are known to contain a variety of bioactive metabolites, such as alkaloids,

flavonoids and triterpenes, that are capable to form such metal complexes. These came to the interesting idea that trace elements of the plant species should be analyzed. Therefore, the content of trace elements was carried out using the ICP-AES. Results showed that the hexane extract contained (ppm) Mg (1.46), Al (1.36), Ca (1.48), Zn (0.13) and Ba (0.09). Unfortunately, the other plant extracts were not possible to prepare digested solution for the analysis. Among these, Mg, Ca and Zn are essential elements that play vital roles in biological process (Hegde et al., 2004; Nahar et al., 2010).

Antimicrobial activity

The extracts were tested using the agar dilution method (Prachayasittikul et al., 2009a) against 27 strains of microorganisms. It was found (Table 1) that only the hexane and dichloromethane extracts selectively displayed antigrowth activity against gram-positive bacteria; *C. diphtheriae* NCTC 10356, *B. subtilis* ATCC 6633 and *S. pyogenes* with minimum inhibitory concentrations (MICs) range 32-128 $\mu\text{g/mL}$. The dichloromethane extract exhibited the highest activity against *C. diphtheriae* NCTC 10356 with the MIC of 32 $\mu\text{g/mL}$, whereas the MIC for *B. subtilis* ATCC 6633 and *S. pyogenes* was 64 $\mu\text{g/mL}$. In addition, the dichloromethane extract also showed growth inhibition against *B. cereus* and *M. luteus* ATCC 10240 with the MIC of 128 and 256 $\mu\text{g/mL}$, respectively. However, the antimicrobial activity of the stem bark of the *P. cerasoides* was not found in the literature. The root extracts of *P. cerasoides* were reported to be active against *Plasmodium falciparum* (Kanokmedhakul et al., 2007). The isolated paprazine was reported to display potent antiinflammatory activity with an IC_{50} of $8.4 \pm 1.3 \mu\text{M}$ (Lin et al., 2006) and to exhibit cataleptogenic effect including to cause hypothermia and motor incoordination in mice (Yamamoto et al., 1991).

Table 1: Antimicrobial activity^a of *P. cerasoides*

Extract	Microorganism	MIC ^d (µg/mL)
Hexane	<i>C. diphtheriae</i> NCTC 10356	64 ^b
	<i>B. subtilis</i> ATCC 6633, <i>S. pyogenes</i>	128
Dichloromethane	<i>C. diphtheriae</i> NCTC 10356	32 ^c
	<i>B. subtilis</i> ATCC 6633, <i>S. pyogenes</i>	64
	<i>B. cereus</i>	128
	<i>M. luteus</i> ATCC 10240	256
Ethyl acetate	inactive	–
Methanol	inactive	–

^aAmpicillin at 10 µg/mL was used as a control of antibacterial testing system; it showed complete inhibition against *S. typhimurium* ATCC 13311, *P. stutzeri* ATCC 17587, *S. aureus* ATCC 29213, *S. aureus* ATCC 25923, *S. epidermidis* ATCC 12228, *M. luteus* ATCC 10240, *C. diphtheriae* NCTC 10356, *S. choleraesuis* ATCC 10708, *S. pyogenes*, *L. monocytogenes* and *P. shigelloides*. Partial inhibition (50 %) at ^b32 µg/mL and ^c16 µg/mL. ^dMIC: Minimum inhibitory concentration was the lowest concentration that inhibited the growth of microorganisms.

Antioxidative activity

The tested plant extracts (Table 2) all exerted SOD activity. The highest activity was observed for dichloromethane extract with an IC₅₀ of 4.51 µg/mL, followed by methanol and ethyl acetate extracts with the IC₅₀ of 12.06 and 39.08 µg/mL, respectively. However, the hexane extract showed weak NBT inhibition (35.29 %) at 300 µg/mL. In case of DPPH activity, only the dichloromethane extract was shown to be an antioxidant with the IC₅₀ of 100.76 µg/mL. It is notable that the extract (dichloromethane) with the highest antimicrobial activity displayed the strongest SOD activity. Recently, stem barks of *P. cerasoides* were reported to show DPPH (of methanol extract) and SOD (of alcoholic extract) activities with the IC₅₀ of 25 and 80 µg/mL, respectively as equivalent to tannic acid (Ravikumar et al., 2008; Krishnaiah et al., 2010). This study, the antioxidative activity of dichloromethane extract, could possibly arise from triterpenes. Stigmasterol was previously reported to be an antioxidant (Hung and Yen, 2001; Ramadan et al., 2007). Some plant extracts, e. g. *Hydnophytum formicarium* Jack. (Prachayasittikul et al., 2008),

Spilanthes acmella Murr. (Wongsawatkul et al., 2008; Prachayasittikul et al., 2009c) and *Eclipta prostrata* Linn. (Prachayasittikul et al., 2010) containing stigmasterol and triterpenes were reported to be antioxidants.

Table 2: Antioxidative activity of *P. cerasoides*

Extract	IC ₅₀ (µg/mL)	
	DPPH assay ^a	SOD assay ^b
Hexane ^c	inactive	-
Dichloromethane	100.76	4.51
Ethyl acetate	inactive	39.08
Methanol	inactive	12.06

^a α-tocopherol with IC₅₀ of 5.35 µg/mL

^b SOD from bovine erythrocytes with IC₅₀ of 0.39 µg/mL

^c Hexane extract at 300 µg/mL displayed 35.29 % inhibition (SOD assay)

CONCLUSION

Bioactive amide (**1**) is firstly isolated from the stem bark ethyl acetate extract of *P. cerasoides*. The hexane and dichloro-

methane extracts afford stigmasterol and a mixture of triterpenes. Trace essential elements were found in the hexane extract in ppm level. Bioactivity testings show that the hexane and dichloromethane extracts are active antimicrobials, the latter being the most active. The dichloromethane extract also exhibits the strongest superoxide scavenging activity. The findings imply a protective effect of the plant species that supports its medicinal applications.

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