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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 7 (10): 1667-1670, 2004
ISSN 1028-8880

Purification and Identification of Antibacterial Compound of Atung (*Parinarium glaberrimum* Hassk) Seed

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Abstract: The present study was carried out to isolate and identify antibacterial compound of atung (*Parinarium glaberrimum* Hassk) seed. Based on the MS and NMR spectroscopic data, the chemical structure of compound was determined as nonanedioic acid (azelaic acid). This compound exhibited considerably strong antibacterial activities against six tested strains of food spoilage and pathogenic bacteria with 20-30 μg mL^{-1} of minimum inhibitory concentration.

Key words: *Parinarium glaberrimum* Hassk, antibacterial compound, minimum inhibitory concentration, nonanedioic acid, azelaic acid

INTRODUCTION

The spoilage and poisoning of food by microbes remain problems, despite the range of techniques available for preserving food. There has recently been a tendency to avoid the use of synthetic preservatives due to consumer health concerns. Therefore, a new effective and safe natural preservative is actively demanded. The use of antibacterial substances contained in spices for the preservation of food is one good sample of the utilization of biological protective of plants.

Parinarium glaberrimum Hassk, locally known as "atung" is classified into (Rosaceae) Family, (*Parinarium*) Genus and (*P. glaberrimum* Hassk) Species[1]. It is indigenous of Indonesia and the Philippines to Fiji and to the Caroline islands in Micronesia[2]. In Ambon, Indonesia, atung seed is used in the traditional preservation of fish, flavoring in pineapple salads and raw fish, treatment of diarrhea and bleeding in pregnant women. On the other hand, crushed seed of atung used to scent coconut oil in Fiji, Tonga, Samoa and Uvea.

The raw powder of atung seed has shown to increase the shelf-life of tiger shrimp (*Penaeus monodon* Fab) from 6 to 17 h[1]. Previous study shows that the ethyl acetate extracts of atung seed had the most effective antimicrobial activity against all tested bacteria, fungi and yeast[3]. Therefore this study was conducted to purify and isolate the antibacterial compound that may be contained in atung seed.

MATERIALS AND METHODS

Atung fruits (*Parinarium glaberrimum* Hassk) were collected in June, 2000 from Ambon, the province of Maluku, Indonesia (0°-10°S and 120°-130°E). The plant was identified by Dr. T. Moniharapon from Pattimura University Ambon, Indonesia. A voucher specimen was deposited at the Faculty of Agriculture, Kagoshima University, Japan. The fruits were cut into two pieces vertically then separated into seeds and peels.

Extraction: Air-dried powder of atung seeds (854 g) were extracted subsequently with 500 mL of hexane, ethyl acetate and methanol (3 times). After three days, the extracts were filtered and concentrated under reduced pressure.

Fractionation: The ethyl acetate extract of atung seed (61.5 g) was fractionated into basic, acidic, phenolic and neutral fraction[4].

Purification using silica gel column chromatography: The acidic fraction (3 g) was purified using silica gel column chromatography. About 45 g of silica gel (C-300, Wako Pure Chemical, Ltd, Osaka) were packed in

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glass column (2.5x40 cm). Then, it was eluted with 50 mL of various ratio of mixture hexane and ethyl acetate at a flow rate of 2.6 mL min^{-1}.

Purification using ODS column chromatography: Fifty milligram of fraction 8 was dissolved in a minimum volume of 80% methanol and applied to reverse phase ODS-Q3 packed column (20x500 mm) pre-equilibrated with 80% MeOH. This then was eluted with 80% MeOH and MeOH at a flow rate of 0.25 mL min^{-1}. Three milliliters of fraction were collected and eluant was monitored at 211 nm. Each peak was pooled, concentrated and tested antibacterial activity by paper disc method.

Antibacterial activity assay: The following microorganisms were used to assess the antibacterial activity: *Bacillus cereus*, *Bacillus subtilis* IFO-13719, *Micrococcus luteus* IFO-12708, *Staphylococcus aureus* IFO-14462, *Escherichia coli* IFO-3301 and *Salmonella enteritidis* IFO-3313. The culture media for bacteria consisted of peptone (1%), meat extract (1%) and NaCl (0.5%) at pH 6.5-6.6. The antibacterial activity was measured by the disc assay procedure[5]. The minimum inhibitory concentration (MIC) of isolated compound was measured by using serial dilution method[6].

Spectral analysis: UV was measured by UV-150A (Shimadzu). IR spectrum was measured by Perkin Elmer Spectrum One. ^{1}H-NMR and ^{13}C-NMR were recorded at 400 and 100 MHZ, respectively in DMSO, d_6 with TMS as an internal standard by a JNM-GSX 400 spectrometer (JEOL). HRMS was recorded by JMS-102A mass spectrometer (Nihon Denshi) in the direct inlet EI mode. Low resolution MS was measured by Polaris Q (Thermo Electron) under the following condition: ionization mode, EI (70 eV); ion trap temperature 200°C; scan range, m/z 30~400 and scan rate, 0.44 s/scan. Melting point was observed by melting point apparatus Mitamura, Japan.

RESULTS AND DISCUSSION

Extraction and isolation antibacterial compound of atung seed: As the active extract ethyl acetate extract (61.5 g) was further fractionated into basic (0.54 g), acidic (14.0 g), phenolic (0.85 g) and neutral fraction (42.5 g). Antibacterial test showed that only acidic fraction had antibacterial activity (Table 1). On the basis of the results, acidic fraction was selected for subsequent isolation of antibacterial compound. The acidic fraction was purified using silica gel column chromatography and the antibacterial activity of 11 fractions were determined.

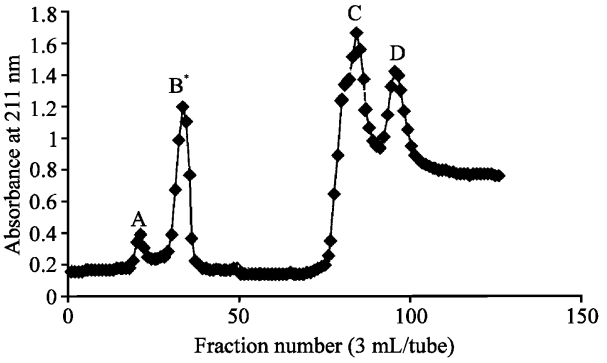

Fig. 1: Elution profile ODS column chromatographic of fraction 8. *Shows antibacterial activity

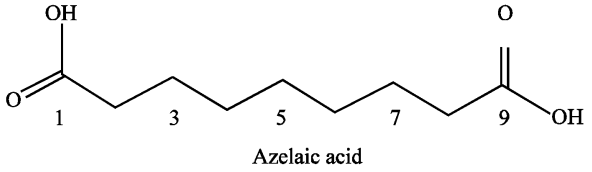

Fig. 2: Chemical structure of isolated antibacterial compound of atung seed

Antibacterial test showed that fraction 7 (eluted with hexane:ethylaceate = 40:60), fraction 8 (eluted with hexane:ethylaceate = 30:70) and fraction 9 (eluted with hexane:ethylaceate = 20:80) had strong antibacterial activity (Table 2). The 8 fraction (50.0 mg) which showed the strongest antibacterial activity was continuously purified using ODS column chromatography. The resulting fractions were pooled into four main fractions, A, B, C and D on the basis of UV monitoring at 211 nm (Fig. 1). Fraction B (25.0 mg) only showed antibacterial effect, while the rests were none. Then, it was evaporated naturally and the colorless crystals were afforded (12.0 mg).

Identification of antibacterial compound of atung seed
Nonanedioic acid (azelaic acid): Colorless needles from methanol; mp 104-105°C; UV λ_{max} (MeOH) nm (ϵ): 211(<100); IR ν_{max} (u-ATR crystal) cm^{-1}: 2931, 1689 (s), 1405, 1250, 1193 and 923; HRMS m/z $[M+H]^+$: Calcd. for $C_9H_{16}O_4$: 188.1044. Found: 188.1032. EIMS m/z (rel.int.): 55 (100), 83 (78), 124 (57), 41 (39), 152 (35), 137 (14), 111 (10), 153 (4), 188 [$(M+H)^+$, 1.1] ; NMR δ_H (DMSO): 1.25 (6H, br s, H-4, 5, 6), 1.48 (4H, m, H-3, 7), 2.18 (4H, t, J = 7.2, H-2, 8); NMR δ_C (DMSO): 174 s (C-1, 9), 33.6 t (C-2, 8), 28.3 t (C-3, 7), 24.6 t (C-4, 5, 6). The chemical structure of isolated antibacterial compound of atung seed as shown in Fig. 2.

Table 1: Antibacterial activity of ethyl acetate extract fractions of atung seed

	Antibacterial activity[a,b]					
Fractions	*E.c*	*S.e*	*B.c*	*B.s*	*M.l*	*S.a*
Basic fraction	-	-	-	-	-	-
Acidic fraction	+	++	++	++	+++	++
Phenolic fraction	-	-	-	-	-	-
Neutral fraction	-	-	-	-	-	-

Table 2: Antibacterial activity of different fraction of ethyl acetate acidic fraction

	Gradient elution			Antibacterial activity [a,b]					
Fractions	Hexane	:	Ethyl acetate	*E.c*	*S.e*	*B.c*	*B.s*	*M.l*	*S.a*
1	100	:	0	-	-	-	-	-	-
2	90	:	10	-	-	-	-	-	-
3	80	:	20	-	-	-	-	-	-
4	70	:	30	-	-	-	-	-	-
5	60	:	40	-	+	+	+	+	+
6	50	:	50	-	+	+	+	+	+
7	40	:	60	+	++	++	+	++	++
8	30	:	70	++	+++	+++	++	+++	+++
9	20	:	80	+	++	++	+	++	++
10	10	:	90	+	+	+	+	+	+
11	0	:	100	+	+	+	+	+	+

[a] Diameter inhibitory zones: - (negative) = 0 mm; + (positive) = 1-8 mm; ++ (moderately positive) = 9-15 mm and ; +++ (strongly positive) ≥16 mm;
[b] Fraction concentration: 40 µg/disc; *E.c*: *Escherichia coli*, *S.e*: *Salmonella enteritidis*, *B.c*: *Bacillus cereus*, *B.s*: *Bacillus subtilis*, *M.l*: *Micrococcus luteus*, *S.a*: *Staphylococcus aureus*

Table 3: Minimum inhibitory concentration of isolated antibacterial compound of atung seed

Test organisms	Minimum inhibitory concentration (µg mL^{-1})
Escherichia coli	30
Salmonella enteritidis	20
Bacillus cereus	20
Bacillus subtilis	20
Micrococcus luteus	25
Staphylococcus aureus	20

As ethyl acetate extract had the strongest antibacterial activity, it was further fractionated into basic, acidic, phenolic and neutral fraction. The antibacterial activity was only observed in the acidic fraction (Fig. 1). This finding is in agreement with the previous study[4]. It was found acidic and phenolic fractions have strong antibacterial effect in Kumazasa (*Sasa albo-marginata*) extracts[7]. The acidic fraction of *Eucalyptus perriniana* showed a potent antibacterial activity[8]. In the previous study, however, it was found that neutral fraction has shown the strongest antibacterial activity in bamboo stem extract[4].

The HR mass spectrum of isolated compound gave a molecular weight of 188.1032, which agrees with the molecular formula $C_9H_{16}O_4$ (calcd. 188.1044). The UV spectrum of isolated compound indicated the presence of saturated carboxylic acid. The IR absorption bands at 2931 and 1689 cm^{-1} are consistent with the presence of hydroxyl and carbonyl groups, respectively. The ^{1}H and ^{13}C-NMR data showed that the molecule contained seven methylenes and two carbonyls. The molecular formula indicated that two OH groups were attached to C-1 and C-9. All these observations enabled the structure to be established as nonanedioic acid (azelaic acid). This was supported by preliminary data[9].

Minimum inhibitory concentration (MIC) is the lowest concentration of an antibiotic, which may inhibit the growth of a particular microbe. Based on the MIC result, antibacterial isolated azelaic acid effective against food pathogenic and spoilage bacteria (Table 3). This result agreement with Cho *et al.*[9], they found that azelaic acid as antibacterial substances from buckwheat and effective against food pathogenic bacteria. Therefore, recently, in pharmacy azelaic acid has already been successfully applied to the treatment of acne, rosacea and its anti-pigmentary activity to the treatment of melasma[10-14]. To establish the safety of using azelaic acid in food system, some scholars did study in the toxicity of azelaic acid[15-17]. They found that azelaic acid is non toxic and it can be administered orally without general ill effects and harmful side product. Therefore, this study may give the possibility of azelaic acid become a potential candidates for natural antibacterial compound to be used as food preservatives in the future.

ACKNOWLEDGMENTS

The authors would like to thank Ms. KeikoYamaguchi for high resolution mass spectra analysis. Thanks are also addressed to Dr. Terumasa Honmura and Dr. D.W. Widjajanto for their valuable suggestions and assistance.

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