Antibacterial and Antioxidant Activities of 3-O-methyl Ellagic Acid 4'-rhamnoside from Stem Bark of *Polyalthia longifolia* Thw.

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Abstract

The plant *Polyalthia longifolia* (Annonaceae) is an ornamental tree that finds its reference in Indian medicinal literature owing to its popular Hindi name Ashoka i.e, *Saraca indica*. However, *P. longifolia* is equated with the name Asoka and often used as an adulterant or substitute of the genuine Asoka bark. The present investigation was carried out with an object to separate and isolate active phytomolecule(s) from stem bark of *P. longifolia* and to screen their antibacterial and antioxidant potential. Column chromatography of the butanol fraction of the hydroalcoholic extract (methanol:water, 1:1) has led to the isolation of a phenolic compound. Structural elucidation was done by IR, ^{1}H NMR, ^{13}C NMR, DEPT, COSY, HSQC, HMBC and mass spectroscopy techniques, and purity was checked by HPTLC and HPLC. Butanol fraction and the isolated compound were screened for antibacterial activity (against facultative aerobic and fastidious aerobic bacterial strains) and antioxidant potential (DPPH method). The compound was revealed to be 3-O-methyl ellagic acid 4'-rhamnoside (**1**), and the purity of the compound was 99.2%. The isolated compound comprises promising antibacterial and antioxidant activities.

Keywords

- Annonaceae
- antibacterial activity
- antioxidant activity
- phenolic compounds
- *Polyalthia longifolia*

Introduction

The plant *Polyalthia longifolia* (Annonaceae) is an ornamental tree, that finds its reference in Indian medicinal literature owing to its popular Hindi name Ashoka. Ashoka, a Sanskrit name in Ayurveda stands for the plant *Saraca indica*. However, *Polyalthia longifolia* is equated with the name Asoka and due to its easy availability, often used as an adulterant or substitute of the genuine Asoka bark [1]. As such, no medicinal attributes are accorded to *P. longifolia* [2]. *P. longifolia* is indigenous to the southernmost part of the India and to Ceylon; it has been cultivated in Bombay and other parts of India. It is useful in fever, skin diseases, ulcer, diabetes, hypertension, helminthiasis and vitiated conditions of vata and pitta [3-6]. It is also used in the treatment of burning sensation, thirst, worm infestations, wound, diarrhoea, scrofulous gland tumors and uterine disorders. The plant contains diterpenoids, alkaloids, tannins and mucilage. The chief components among others are aporphine and azafluorene alkaloids, clerodane and ent-halimane diterpenoids and sesquiterpenes [7-12]. The present study was designed to isolate phytoconstituent(s) from the hydroalcoholic extract of the bark of the plant and to study their antibacterial and antioxidant activities.

Results and Discussion

Characterization of compound 1

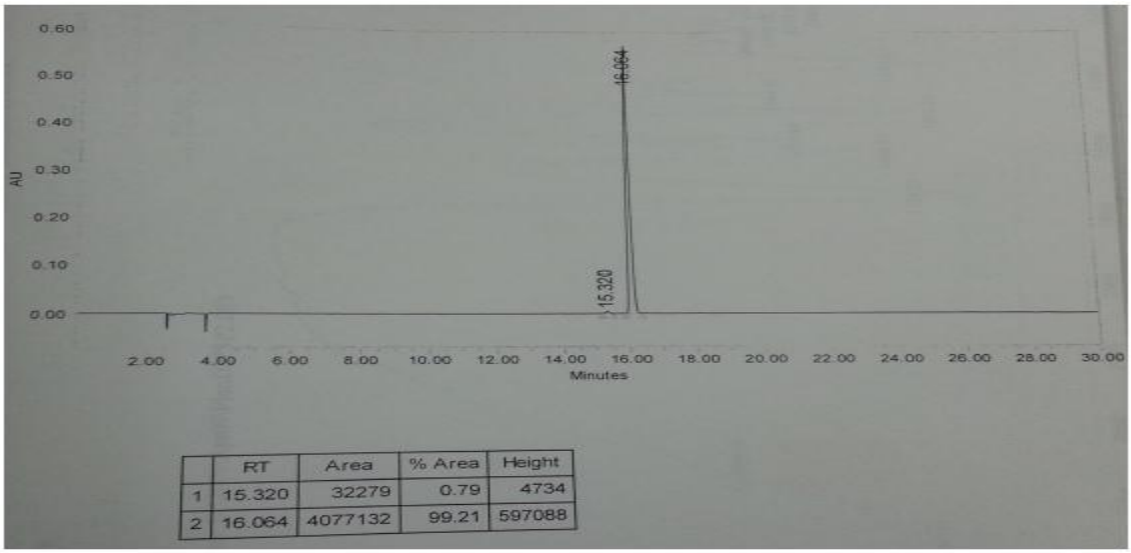
The compound isolated by chromatographic techniques was subjected to spectroscopic technique like IR, ^{1}H NMR, ^{13}C NMR, DEPT, COSY, HSQC, HMBC and Mass spectroscopy. Structure elucidation was done on the basis of spectroscopic data as follows:

Compound **1** was obtained as off white colored compound (135 mg, 0.21428 % in butanol fraction), isolated from chloroform: methanol eluents (78:22), The mass spectra displayed a molecular ion peak M+ at m/z 462 and M+-H at m/z 461 corresponding to formula $C_{21}H_{18}O_{12}$. The purity of compound **1** assigned by HPLC was 99.2% (Figure 1). The IR spectrum displayed characteristic absorptions for hydroxyl groups (3383 cm^{-1}), α,β unsaturated lactone functions (1738cm^{-1}). The ^{1}H NMR showed characteristic signals for nine proton including two aromatic methine singlets, H-5 and H-5’ (δ 7.5 and δ 7.7); a methoxyl singlet (δ 4.04), and five oxygenated sugar methine protons wherein H-1”(δ 5.4, d) is an anomeric proton, H-2”(δ 4.73, dd), H-3”(δ 3.5, dd), H-4”(δ 3.85, m), H-5”(δ 4.9,) signals along with a doublet for methyl at H-6”(1.14, J=6.1 Hz) indicates the presence of rhamnose sugar (table 1). ^{13}C NMR spectra (table 1) showed signals for twenty one carbon with characteristic signals for the two α, β-unsaturated lactone carbonyl carbons C-7 and C-7’ (δ 158.7), one methoxy carbon at (δ 60.9), two aromatic methine carbon signal C-5 and C-5’ (δ 111.4), ten quaternary carbons C-1 (δ 111.4), C-2 (δ 140), C-3 (δ 141.1), C-4 (δ 141.7), C-6 (δ 114.1), C-1’(δ 111.2), C-2’(δ 136), C-3’(δ 146.3), C-4’(δ 152.6); C-6’(δ 112.9) and six carbon of rhamnose unit C-1”(δ 100.7), C-2”(δ 70.02), C-3”(δ 69.9), C-4”(δ 69.9), C-5”(δ 71.02), C-6”(δ 17,83). The HSQC spectral data ^{1}H - ^{13}C exhibit attachment of carbon C-5 (δ111.2) with H-5 (δ7.4); C-5’ (δ 110.2) with H-5’ (δ7.4); anomeric proton of rhamnose sugar H-1” (δ 5.4) with the carbon C-1’’ (δ 100.07). The HMBC spectral data ^{1}H–^{13}C shows multiple bond correlations of methoxyl protons (δ 4.04) with carbon C-3 (δ139.6), aromatic methine proton H-5 with C-7 (δ 158.5), C-1 (δ 111.4), C-3 (δ 139.6), and H-5’ (δ7.4, δ7.4)

with the carbon C-7' (δ 158.7), C-1' (δ 111.2) C-3' (δ 140), and methyl protons of rhamnose sugar H-6" (δ1.14) with carbon at C-5" (δ71.02). In rhamnose sugar, anomeric proton H-1" (δ 5.4) shows long range correlation with the carbon C-2" (δ 70.02) and C-4' (δ 152.6). All assignments are in agreement with DEPT, COSY, HSQC and HMBC spectral data. Thus on the basis of spectral data, compound-1 is 3-O-methyl ellagic acid 4'-rhamnoside, having molecular formula $C_{21}H_{18}O_{12}$ (Figure 2). HPTLC profile of butanol fraction and the isolated compound is depicted in figure 3.

Tab. 1. NMR spectroscopic data for compound-1 (^{1}H: 400MHz, ^{13}C:100MHz, DMSO-d_6)

Position (C/H)	δ_H	δ_C	HMBC
1	-	111.4	-
2	-	140	-
3	-	141.1	-
4	-	141.7	-
5	7.5, s, 1H	111.4	C-1(111.4) C-3(141.1) C-7(158.6)
6	-	114.1	-
7	-	158.6	-
OCH3	4.04, s, 3H	60.9	C-3(141.1)
1'	-	111.2	-
2'	-	136	-
3'	-	146.3.	-
4'	-	152.6	-
5'	7.7, s, 1H	111.4	C-1'(111.2) C-3'(146.3) C-7'(158.5)
6'	-	112.9	-
7'	-	158.5	-
1''	5.4, d, 1H	100.07	C-4'(152.6), C-2''(70.02)
2''	4.73, dd, 1H	70.02	-
3''	3.5, dd, 1H	69.9	-
4''	3.85, t, 1H	69.9	-
5''	4.9, dq, 1H	71.02	-
6''	1.14(3H, d, 6.1)	17.83	C-5''(71.02)



	RT	Area	% Area	Height
1	15.320	32279	0.79	4734
2	16.064	4077132	99.21	597088

Fig. 1. HPLC chromatogram of compound 1

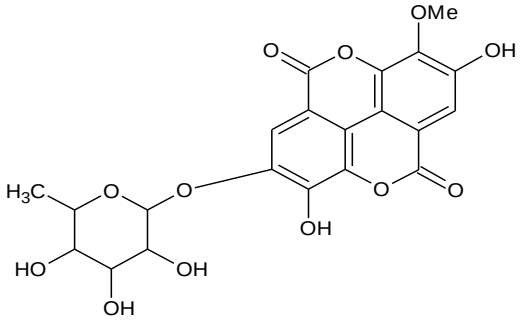


Fig. 2. 3-O-methyl ellagic acid 4’-rhamnoside

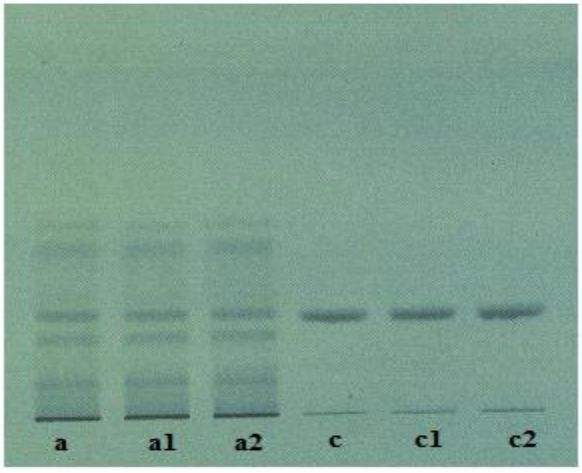


Fig. 3. HPTLC profile: a, a1, a2: butanol fraction; and c, c1, c2: compound 1

Determination of minimum inhibitory concentration

The minimum inhibitory concentration of compound **1** and butanol fraction was found to be in the range of 80-160 µg/ml and 160-320 µg/ml respectively. Compound **1** exhibited higher antibacterial potential against all most all tested bacterial strains than butanol fraction, but the potency is less as compared to standard drugs (Table 2 and 3).

Tab. 2. MIC of standard drug against microorganisms

Bacterial strain	Standard drug	MIC (µg/ml)
S. pnemoniae		0.065
S. pyogens	Erythromycin	0.125
S. viridens		0.065
S. aureus	Vancomycin	0.25
MRSA	Oxacillin	8.0
P. aeruginosa		0.25
E. coli	Ciprofloxacin	0.015
A. baumannii		0.015

Tab. 3. MIC of butanol fraction and isolated compounds

Bacterial strain	Butanol fraction	Compound-1
S. pnemoniae	160	320
S. pyogens	160	160
S. viridens	160	160
S. aureus	320	320
MRSA	320	320
P. aeruginosa	160	160
E. coli	320	320
A. baumannii	160	160

Antioxidant activity

The highest antioxidant activity of the compound was 57.95% at 40 µg/ml and butanol fraction was 66.05% at 40 µg/ml. Isolated compound exhibited better antioxidant property than the standard drug, vitamin C (Table 4).

Tab. 4. Free radical scavenging activity in DPPH method

Concentration (µg/ml)			**% free radical scavenging**		
Isolated Compounds	Butanol fraction	Vitamin C	Compound **1**	Butanol fraction	Vitamin C
2.5	25	25	08.24±0.72	12.13±2.26	23.42±0.24
5	50	50	16.32±0.86	22.60±0.78	47.26±0.72
10	100	100	26.56±0.68	29.94±1.19	58.83±0.54
20	200	200	40.48±1.06	44.14±1.48	68.38±0.36
40	400	400	57.95±2.56	66.05±2.87	81.64±0.48

Values are mean±SEM (n=3)

Literature surveys indicated that plant phenolics constitute one of the major groups of compounds found in both edible and inedible plants and reported to have multiple biological effects, including antioxidant activity [13-17]. Hence, the antioxidant and antimicrobial activities of *P. longifolia* may be due to presence of the isolated compound.

Materials and Methods

Plant material

The stem bark of *Polyalthia longifolia* (Sonn) Thw. was obtained from Banasthali University campus, Rajasthan, India and identified by Dr. Vinod Kumar Sharma, Department of Botany, Rajasthan University, Jaipur, India (Voucher No.: RUBL 211351). A voucher specimen was preserved in department of pharmacy, Banasthali University, Rajasthan for future references.

Preparation of extract

The stem bark of *P. longifolia* was first air dried for few days and then dried under controlled temperature (45^0 C). It was then crushed to smaller pieces followed by coarsely powdered in a grinding mill and stored in an air tight labeled container in a cool place till further use. Air dried and coarse powdered stem bark (2kg) was extracted by cold maceration technique with hydroalcohol (methanol:water, 1:1) at room temperature for twenty four hours, three times successively. Filtered the extracts and pooled, concentrated in rota vapor (Buchi, Switzerland) under reduced pressure, a dark brown viscous mass (178g) was obtained.

Isolation of compounds

The above hydroalcoholic extract was suspended in water and partitioned with n-butanol. 61 gm of butanol fraction was adsorbed over 90 gm of silica and column chromatographed on a silica gel column (mesh 100-200; Swambe Chemical, India) and eluted with solvent mixtures of increasing polarity and fractions (500ml) were collected and monitored on TLC: chloroform (fraction 1-4), chloroform : methanol [(98:2, fraction 5-9), (96:4, fraction 10-13), (94:6, fraction 14-16), (92:8, fraction 17-18), (90:10, fraction 19-24), (88:12, fraction 25-35), (86:14, fraction 36-43), (84:16, fraction 44-49), (82:18, fraction 50-76), (80:20, fraction 77-

89), (78:22, fraction 90-112), (76:24, fraction 113-125), (71:29, fraction 126-130), (66:34, fraction 131-134), (61:39, fraction 135-137), (56:44, fraction 138-140), (51:49, fraction 141-142), (46:54, fraction 143-145), (41:59, fraction 146-147), (36:64, fraction 148-149), (26:74, fraction 150-151), (16:84, fraction 152-153)] and methanol (fraction 154). Fractions (90 to 106) produced an off white coloured compound **1** (yield: 135 mg).

Characterization of compound 1

FTIR studies were conducted on the IR ARD/1402 (FTIR Spectrophotometer, Perkin Elmer, USA); ^{1}H and ^{13}C NMR was recorded on AVA-NCE (Bruker, Switzerland) at 400 and 100 MHz respectively. The two-dimensional experiments (HSQC, HMBC, COSY) were also performed. Samples were dissolved in DMSO based on the solubility of the sample. Mass spectra were recorded on Direct MS (Waters, USA). Purity of the isolated compound was done by HPTLC [Sample applicator: Linomat 5, stationary phase: precoated silica gel $G_{60}F_{254}$, mobile phase: chloroform:methanol:water (7:3:0.5), detection: under UV at 254 nm] and waters HPLC system equipped with waters 2996 PDA detector in combination with Empower software [column: C18(ODS), 250*4.6 mm, 5 micron, Kromasil; sample cabinet temperature: $20^{0}C$; sample prepared in methanol; injection volume of 20µl; mobile phase: acetonitrile:buffer; flow rate: 1ml/min; run time: 30min; detection: 254 nm; and purity determination by area normalization].

Antibacterial activity of butanol fraction and isolated compounds

Antibacterial activity was studied in facultative aerobic bacterial strains like *Staphylococcus aureus* 29213, methicillin resistant *Staphylococcus aureus* 562 (MRSA), *Pseudomonas aeruginosa* 27853, *Escherichia coli* 29212 and *Acinetobacter baumannii* 56231; fastidious aerobic bacterial strains like *Streptococcus pnemoniae* ATCC 49619, *Streptococcus pyogens* ATCC 19615, *Streptococcus viridens* 661. Tryptic Soya Agar Media (Becton Dickinson Microbiology Systems Sparks, USA) was used for sub culturing for inoculum preparation of facultative aerobic bacterial strains, Columbia Blood Agar Media (Becton Dickinson Microbiology Systems Sparks, USA) for sub culturing of inoculums preparation of fastidious aerobic bacterial strains and Muller Hinton broth media (Becton Dickinson Microbiology Systems Sparks, USA) for determination of minimum inhibitory concentration (MIC) value. Erythromycin (for *S. pnemoniae*, *S. pyogens* and *S. viridens*), vancomycin for *S. aureus*, oxacillin for MRSA and ciprofloxacin (for *P. aeruginosa*, *E. coli* and *A. aumannii*) were used as standard drugs.

Bacterial inoculum preparation

Picked up 3-4 isolated bacterial colonies from the respective medium plates individually with the help of inoculation needle one day prior to incubation of the plate. Picked up colonies were added in 2ml of pre sterilized saline solution (0.85% NaCl), mixed it properly with the help of vortex mixer (Remi, New Delhi) to make a homogeneous suspension. Bacterial density was adjusted to 1.0-1.1 Mac Farland for fastidious bacterial strains and 0.5-0.8 Mac Farland for facultative aerobic bacterial strains using densitometer (Biomerieux, France). Both adjusted Mac Farland absorbance were represented as 0.5-1.5 x 10^{8} cfu/ml.

Preparation of drug samples

The stock solution of 2 mg/ml of butanol extract and isolated compound was prepared in DMSO. From the stock solution different concentrations as 320, 160, 80, 40, 20, 10, 5, 2.5, 1.25, 0.625 µg/ml were prepared. The different concentrations of standard drugs used were 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.062, 0.031, 0.015 µg/ml.

Assay method

Micro titter 96 well plate was used to determine MIC value of each drug. For the good growth of fastidious organisms one of the growth supplement i.e., sheep blood (5%) was added in Mullere Hinton broth. On the other side, Muller Hinton broth was used for facultative aerobic bacteria. The prepared inoculum 0.5-1.5 $x10^8$ cfu/ml was serially diluted to make a final concentration of 1 x 10^5 cfu/ml. The final concentrations of drugs and culture inoculum (1 x 10^5 cfu/ml) were added in micro titer well as per protocol. The final volume was 200µl per micro titter well. Simultaneously DMSO control and positive control as media with inoculum were prepared. Micro titter plates were incubated at 35-37^{0}C for 24 hrs and then minimum inhibitory concentration was determined [18, 19].

Evaluation of antioxidant activity of butanol fraction and isolated compound

Antioxidant activity was measured on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical [20, 21] and compared with Vitamin C (CAS No.: 50-81-7; purity: 99%; HIMEDIA). Various concentrations of the compound and standard drug were added to 0.004% methanolic solution of DPPH. After 30 min the absorbance at 517 nm was determined, and the percentage inhibition activity was calculated using the following formula.

% inhibition = $[(Ac - At) / Ac] \times 100$

Where, Ac = absorbance of control sample and At = the absorbance of test sample.

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