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Contributed Paper

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

Pankaj Kumar Jain [a], Arjun Patra*+ [b], Swaha Satpathy [b], Sonika Jain [c] and Saleemulla Khan [d]

[a] Department of Pharmacy, Banasthali University, Rajasthan, India.

[b] Institute of Pharmacy, Guru Ghasidas Vishwavidyalaya, Bilaspur (C.G.), India.

[c] Department of Chemistry, Banasthali University, Rajasthan, India.

[d] Manipal College of Pharmaceutical Sciences, Manipal University, Manipal, Karnataka, India.

* Author for correspondence; e-mail: drarjunpatra22@gmail.com

+ **Present Address:** College of Pharmacy, Department of Pharmaceutical & Biomedical Sciences, California Health Sciences University, Clovis-93612, California, USA.

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ABSTRACT

Polyalthia longifolia (Annonaceae) is an ornamental plant that is used in Indian traditional medicine and mistakenly known in Hindi as Ashoka. It is often used as an adulterant or substitute of the genuine Asoka (*Saraca indica*) bark. *P. longifolia* is used traditionally for treatment of a number of complications. The present investigation was carried out with an objective to separate and isolate active phytomolecules 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 (determined by DPPH and ABTS methods). The compound was identified as 3-O-methyl ellagic acid (compound **1**) and the purity of compound **1** was 99.6%. The isolated compound comprised of promising antibacterial and antioxidant activities. MIC of compound **1** and butanol fraction against tested bacterial strains were in the range of 80-160 and 160-320 µg/ml. The IC_{50} value in DPPH method was 24.28 µg/ml for compound **1** and 266.59 µg/ml for butanol fraction. Further, the total antioxidant capacity (ABTS method) of compound **1** and butanol fraction was 2486.94±10.20 and 207.93±9.91 µmol TE/ g dry weight of sample. In the present study, we have reported a phenolic compound, 3-O-methyl ellagic acid from stem bark of *P. longifolia* with antibacterial and antioxidant activities.

Keywords: Annonaceae, antibacterial activity, antioxidant activity, phenolic compounds, *Polyalthia longifolia*

1. INTRODUCTION

The plant *Polyalthia longifolia* (Annonaceae) is an ornamental plant that is used in Indian traditional medicine and mistakenly known in Hindi as 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]. *P. longifolia* is indigenous to the southernmost part of 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 [2-3]. It has been used in the treatment of burning sensation, thirst, worm infestations, wound, diarrhea, scrofulous gland tumors and uterine disorders. The plant contains diterpenoids, alkaloids, tannins and mucilage. The chief compounds of the plant are aporphine and azafluorene alkaloids, clerodane and ent-halimane diterpenoids, and sesquiterpenes [4-7]. The plant has been studied for antimalarial [8], antinociceptive [9], antifungal [10], antioxidant, antityrosinase [11], anticancer [12], hepatoprotective [13], anti-inflammatory [14] and antiulcer [15] activities. Among the seven clerodane diterpenoids isolated, (-)-16α-hydroxycleroda-3,13(14)*Z*-dien-15,16-olide was most potent against *Staphylococcus aureus* and *Sporothrix schenckii* [16]. The objective of present study was to isolate phytoconstituents from the stem bark of the plant and to study the antibacterial and antioxidant activities of the compounds.

2. MATERIALS AND METHODS

2.1 Plant Material

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

2.2 Preparation of Extract

The stem bark of *P. longifolia* was first air dried for seven days at room temperature and then dried under controlled temperature (45 °C). It was then broken down into smaller pieces, ground to coarse powder in a grinding mill and stored in a labelled, air-tight 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. The extracts were filtered, concentrated in rotary evaporator (Buchi, Switzerland) under reduced pressure, resulting in a dark brown viscous mass (178g).

2.3 Isolation of Compounds

The above hydroalcoholic extract was suspended in water and partitioned with n-butanol. Sixty-one (61) g of butanol fraction was adsorbed over 90 g of silica and column chromatographed on a silica gel column (mesh 100-200; Swambe Chemical, India) and eluted with solvent mixtures of increasing polarity. Several fractions (500ml) were collected and monitored on TLC: chloroform (fractions 1-4), chloroform : methanol [(98:2, fractions 5-9), (96:4, fractions 10-13), (94:6, fractions 14-16), (92:8, fractions 17-18), (90:10, fractions 19-24), (88:12, fractions 25-35), (86:14, fractions 36-43), (84:16, fractions 44-49), (82:18, fractions 50-76), (80:20, fractions 77-89), (78:22, fractions 90-112), (76:24, fractions 113-125), (71:29, fractions 126-130), (66:34, fractions

131-134), (61:39, fractions 135-137), (56:44, fractions 138-140), (51:49, fractions 141- 142), (46:54, fractions 143-145), (41:59, fractions 146-147), (36:64, fractions 148-149), (26:74, fractions 150-151), (16:84, fractions 152-153)] and methanol (fraction 154). The yellow colored mother liquor from fraction 54 to 65 produced a yellow colored crystal. The crystals were dissolved in minimum amount of methanol by warming and set aside for recrystallization. Yellow colored crystals of compound **1** were obtained (yield: 43 mg).

2.4 Characterization of Compound 1

FTIR studies were conducted on the IR ARD/1402 (FTIR Spectrophotometer, Perkin Elmer, USA); ^{1}H and ^{13}C NMR were recorded on AVA-NCE (Bruker, Switzerland) at 400 and 100 MHz. The two-dimensional experiments (HSQC, HMBC, COSY) were also performed. Samples were dissolved in DMSO for NMR studies. 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: toluene: ethyl acetate: formic acid: methanol, detection: under UV at 254 nm] and Waters HPLC system equipped with waters 2996 PDA detector in combination with Empower software. The column used was Kromasil: C18(ODS), 250*4.6 mm, 5 micron. The HPLC conditions used were as follows: cabinet temperature: 20 °C; sample prepared in methanol with injection volume of 20 µl; mobile phase: acetonitrile:buffer (potassium dihydrogen orthophosphate); flow rate: 1ml/min; run time: 30min; detection: 254 nm; and purity determination by area normalization.

2.5 Antibacterial Activity of Butanol Fraction and Isolated Compound

Antibacterial activity was studied in facultative aerobic bacterial strains such as *Staphylococcus aureus* 29213, methicillin resistant *Staphylococcus aureus* 562 (MRSA), *Pseudomonas aeruginosa* 27853, *Escherichia coli* 29212 and *Acinetobacter baumannii* 56231; and fastidious aerobic bacterial strains such as *Streptococcus pneumoniae* ATCC 49619, *Streptococcus pyogenes* ATCC 19615, and *Streptococcus viridans* 661. Tryptic Soya Agar Media and Columbia Blood Agar Media (Becton Dickinson Microbiology Systems Sparks, USA) were used for sub-culturing for inoculum preparation of facultative and fastidious aerobic bacterial strains. Muller Hinton broth media (Becton Dickinson Microbiology Systems Sparks, USA) was used for determining minimum inhibitory concentration (MIC). Erythromycin, vancomycin, oxacillin and ciprofloxacin were used as standard drug.

2.5.1 Bacterial inoculum preparation

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

2.5.2 Preparation of drug samples

The stock solution of 2 mg/ml of butanol fraction 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.

2.5.3 Assay method

96 well microtiter plate was used to determine MIC value of each drug. For growth of fastidious organisms, sheep blood (5%) was added as growth supplement in Muller Hinton broth. Muller Hinton broth without the growth supplement was used for facultative aerobic bacteria. The prepared inoculum 0.5-1.5 × 10^8 cfu/ml was serially diluted to make a final concentration of 1 × 10^5 cfu/ml. The final concentrations of drugs and culture inoculum (1 × 10^5 cfu/ml) were added into micro titer well as per protocol. The final volume was 200 µl per microtiter well. Simultaneously DMSO control and positive control as media with inoculum were prepared. Microtiter plates were incubated at 35-37 °C for 24 hrs and then minimum inhibitory concentration was determined [17, 18].

2.6 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 [19] and compared with vitamin C standard (CAS No.: 50-81-7; purity: 99%; HIMEDIA). Various concentrations of the compounds, gallic acid and vitamin C were added to 0.004% methanolic solution of DPPH. After 30 min the absorbance at 517 nm was determined, and the percentage inhibition was calculated using the following formula.

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

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

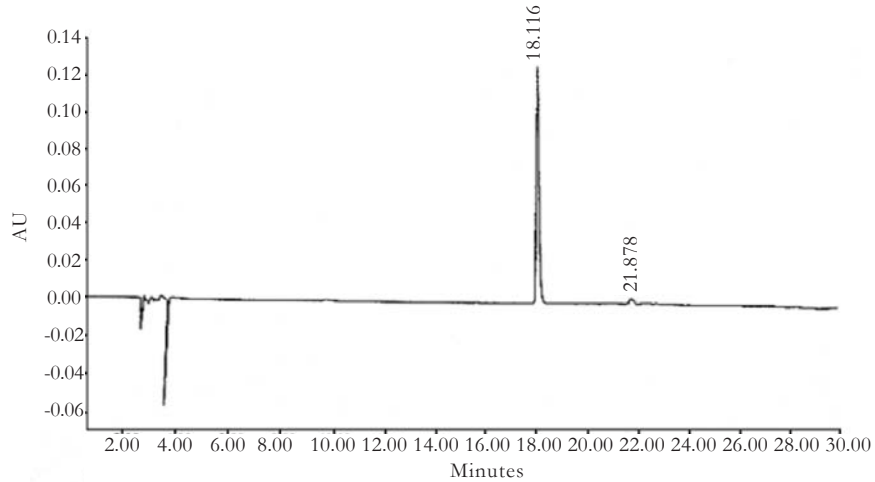
Further, total antioxidant activity of butanol fraction and isolated compound was also determined by ABTS method. This assay was carried out spectrophotometrically to assess the ability of different test samples to scavenge $ABTS^{\bullet+}$ radical cation compared to trolox standard according to reported method. The $ABTS^{\bullet+}$ radical cation was generated by mixing 7 mM ABTS and 2.45 mM potassium persulfate, and incubating for 16 h in dark at room temperature (23 °C). The $ABTS^{\bullet+}$ solution was equilibrated to an absorbance of 0.700 ± 0.020 by diluting with 80% HPLC grade ethanol. Test samples (butanol fraction and compound 1) were suitably diluted and 30 µl was mixed properly with 3.0 ml of diluted $ABTS^{\bullet+}$ solution. The reaction mixture was allowed to stand at room temperature for 6 min and the absorbance was immediately measured at 734 nm. Trolox standard solutions of different concentrations (0, 0.25, 0.5, 1.0, 1.5, 2.0 and 2.5 mM) were assayed under similar conditions. All experiments were repeated three times and appropriate solvent blanks were used in each assay. The percentage inhibition of the absorbance was measured and plotted as a function of concentration of trolox (standard) and test sample to determine the trolox equivalent antioxidant capacity (TEAC) in terms of µmol trolox equivalent per g dry weight of sample [20, 21].

3. RESULTS AND DISCUSSION

3.1 Characterization of Compound 1

The compound isolated by chromatographic techniques was subjected to spectroscopic techniques such as 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 yellow colored crystals

(43 mg, 0.06825% in butanol fraction), isolated from chloroform : methanol eluents (82:18). The purity of the compound by HPLC was found to be 99.6% (Figure 1). The mass spectra displayed a molecular ion peak M+ at m/z 316 and M+-H at m/z 315 corresponding to molecular formula $C_{15}H_8O_8$. The IR spectrum displayed characteristic peaks for hydroxyl groups (3279 cm^{-1}), α,β-unsaturated lactone group (1718 cm^{-1}). The ^1H NMR (Table 1) showed characteristic signals for two aromatic methine singlets H-5 and H-5' (δ 7.4 and δ 7.4) and a methoxy signal (δ 4.02). ^{13}C NMR spectra (Table 1) showed signals for fifteen 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 signals at C-5 (δ 111.2) and C-5' (δ 110.2), ten quaternary carbons at C-2 (δ 148.1), C-3 (δ 139.6), C-4 (δ 144.1), C-2' (δ 136), C-3' (δ 140), C-4' (δ 152); C-1 (δ 107.2), C-6 (δ 112.3), C-1' (δ 107.2), C-6' (δ 112). The HSQC spectral data ^1H-^{13}C exhibited attachment of carbon C-5 (δ 111.2) with proton H-5 (δ 7.4) and C-5' (δ 110.2) with H-5' (δ 7.4). The HMBC spectral data ^1H-^{13}C showed multiple bond correlations of methoxyl protons (δ 4.04) with carbon C-3 (δ 139.6), H-5 (δ 7.4) with the carbonyl carbon at C-7 (158.5), C-3 (δ 139.6), C-4 (δ 144.1), C-6 (δ 112.3); H-5' (δ7.4) with the carbonyl carbon C-7' (δ 158.7), C-3' (δ 140), C-4' (δ 152), C-6' (δ 112.3). All assignments were 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 (Figure 2), having molecular formula $C_{15}H_8O_8$. HPTLC profile of butanol fraction and the isolated compound are shown in Figure 3 and 4 (A, B).



Sl. No.	RT	Area	% Area	Height
1	18.116	1026970	99.60	130745
2	21.878	4156	0.40	881

Figure 1. HPLC chromatogram of compound 1.

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

Position (C/H)	δH	δC	HMBC
1	-	107.2	-
2	-	148.1	-
3	-	139.6	-
4	-	144.1	-
5	7.4, s, 1H	111.2	C-3 (139.6), C-4 (144.1), C-6 (112.3), C-7 (158.7)
6	-	112.3	-
7	-	158.7	-
1'	-	107.2	-
2'	-	136	-
3'	-	140	-
4'	-	152	-
5'	7.4, s, 1H	110.2	C-3'(140), C-4'(152), C-6'(112), C-7'(158.7)
6'	-	112	-
7'	-	158.7	-
OCH_3	4.02, s, 3H	60.8	C-3 (139.6)

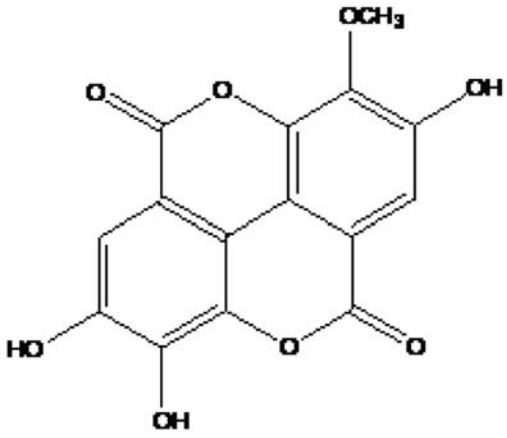


Figure 2. Chemical structure of 3-O-methyl ellagic acid.

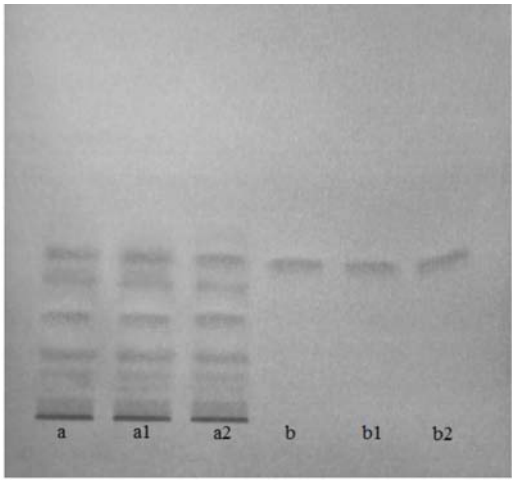


Figure 3. HPTLC profile of butanol fraction (a, a1, a2) and compound 1 (b, b1, b2).

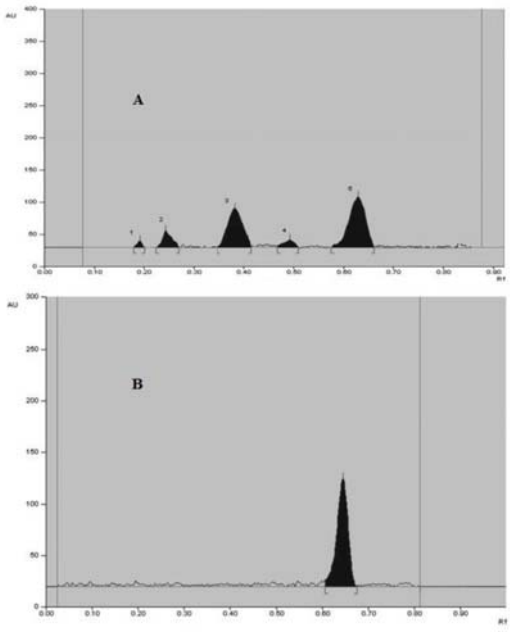


Figure 4. HPTLC chromatogram: A, butanol fraction; B, compound 1.

3.2 Determination of Minimum Inhibitory Concentration

MIC is the lowest concentration of the test sample that inhibits the growth of a given strain of bacteria. The MIC of compound 1 and butanol fraction was found to be in the range of 80-160 mg/ml and 160-320 mg/ml respectively. Compound **1** exhibited higher antibacterial potential against all tested bacterial strains than butanol fraction, but the potency was less compared to standard drugs (Table 2). Antimicrobial activity of different parts of *P. longifolia* against various microorganisms has been studied [22-23] and clerodane diterpenoids as antibacterial leads have been isolated from the leaves and berries, but present study demonstrated the antibacterial activity of compound 1 against both facultative and fastidious aerobic bacterial strains for the first time. Compound 1, a derivative of ellagic acid produced antibacterial activity as extracts with ellagic acid showed similar activity in previous study [24]. The antibacterial activity might be due to the ability of the compound to form complex with extracellular and soluble proteins, and bacterial cell wall or by disruption of microbial membrane.

Table 2. MIC of standard drug, butanol fraction and isolated compound against different microorganisms.

Bacterial strain	MIC in µg/ml		
	Standard drug	Butanol fraction	Compound 1
S. pneumoniae	0.065[@]	160	160
S. pyogenes	0.125[@]	160	80
S. viridans	0.065[@]	160	80
S. aureus	0.25[#]	320	80
MRSA	8.0[€]	320	160
P. aeruginosa	0.25[∂]	160	80
E. coli	0.015[∂]	320	80
A. baumannii	0.015[∂]	160	80

[@], Erythromycin; [#], Vancomycin; [€], Oxacillin, [∂], Ciprofloxacin

3.3 Antioxidant Activity

In DPPH method the antioxidant activity of compound 1 and butanol fraction was 69.81% at 40 µg/ml and 66.05% at 400 µg/ml respectively. The IC_{50} value of compound 1 and butanol fraction was 24.28 and 266.59 µg/ml respectively. Isolated compound exhibited significant antioxidant property compared to the standard drug, vitamin C and butanol fraction (Table 3), but the activity was less compared to gallic acid. DPPH method has been accepted widely for determining free radical scavenging activity of plant extracts. DPPH molecule is characterized as a stable free radical and it accepts an electron or hydrogen radical to become a stable diamagnetic molecule [25, 26]. In the present study the reduction of DPPH radical was determined by measuring the decrease in absorbance at 517 nm by the samples and it was revealed that the tested samples have the ability of scavenging free radicals.

Table 3. Free radical scavenging property of compound 1, gallic acid, butanol fraction and Vitamin C in DPPH method.

Concentration (µg/ml)				% free radical scavenging			
Compound 1	Gallic acid	Butanol fraction	Vitamin C	Compound 1	Gallic acid	Butanol fraction	Vitamin C
2.5	2.5	25	25	10.36±0.69	35.46±1.10	12.13±2.26	23.42±0.24
5	5	50	50	28.17±1.03	45.80±1.80	22.60±0.78	47.26±0.72
10	10	100	100	34.83±0.78	80.46±1.85	29.94±1.19	58.83±0.54
20	20	200	200	45.80±2.48	99.13±0.72	44.14±1.48	68.38±0.36
40	40	400	400	69.81±4.42	99.8±0.20	66.05±2.87	81.64±0.48

Values are mean ± SEM (n=3).

Antioxidant potential was also carried out to evaluate the ability of the test samples to scavenge free radicals by the improved $ABTS^{\bullet+}$ method. The percentage inhibition of absorbance of $ABTS^{\bullet+}$ as a function of standard trolox solutions of different concentration is furnished in Figure 5. The total antioxidant capacity of compound **1** and butanol fraction in terms of µmol trolox equivalent per g dry weight of sample was 2486.94±10.20 and 207.93±9.91 respectively. $ABTS^{\bullet+}$ [(2, 2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)], chemically produced for the assay procedure is a stable radical and not found in human body. Antioxidants reduce $ABTS^{\bullet+}$ to ABTS and decolorize it. The loss of color by addition of test samples was measured spectrophotometrically and compared with trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) as the standard [25, 26]. Both butanol fraction and compound **1** showed radical scavenging activity in ABTS method.

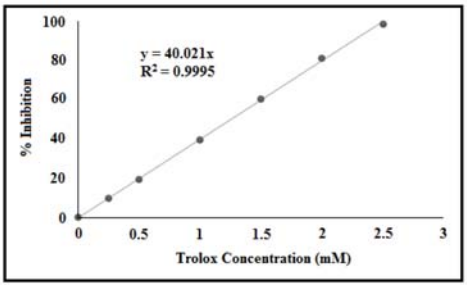


Figure 5. Percentage inhibition of absorbance of $ABTS^{\bullet+}$ for different concentrations of standard trolox solution at 734 nm.

Free radicals contain one or more unpaired electrons and are highly unstable and damaging to other molecules by taking electrons from them for stability. Over production of free radicals or weak antioxidant defense mechanism or damage to cell in the body causes increased risk of many life threatening diseases. Enzymatic and non-enzymatic antioxidants act by various mechanisms as free radical scavenging, reducing ability, quenching of singlet oxygen etc. Exploring the natural antioxidants is paramount and important as these can reduce/prevent the disease progression [25, 26]. A large number of *in vivo* and *in vitro* methods have been developed for antioxidant screening but a few are simple, reliable and cost effective. DPPH method is the most commonly used screening technique because it is easy, simple and inexpensive [26]. More than one method needs to be performed for antioxidant capacity measurement to determine different modes of action of the test samples. Hence, in the present study the free radical scavenging ability of the test samples have been performed using DPPH and ABTS methods.

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 [27, 28]. We have already reported the antibacterial and antioxidant activities of 3-O-methyl ellagic acid 4′-rhamnoside from *P. longifolia* [29]. Further, ellagic acid and extracts with ellagic acid have been studied earlier for antibacterial and antioxidant activities [30]. Hence the antibacterial and antioxidant properties of *P. longifolia* may be due to 3-O-methyl ellagic acid 4′-rhamnoside and compound **1** (derivatives of ellagic acid) along with the other reported bioactives.

4. CONCLUSION

The present study has shown that the butanol fraction and compound 1 from *P. longifolia* possess strong antibacterial and antioxidant activities. However, *in vivo* studies are also needed to ascertain the mechanisms of action as antioxidant and antimicrobial agents.

DECLARATION OF INTEREST

The authors have no declarations of interest.

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