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Phytochemical profiling of *Artocarpus lakoocha* Roxb. leaf methanol extract and its antioxidant, antimicrobial and antioxidative activities

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ABSTRACT

Objective: To explore the phytochemical profile of *Artocarpus lakoocha* Roxb. leaves both qualitatively and quantitatively, and validate its role as a potent antioxidant and antimicrobial agent.

Methods: Extraction and isolation of different compounds were done from the leaves of *Artocarpus lakoocha* based on solvent fractionation method. Subsequently, quantitative and qualitative phytochemical profiling along with antioxidant, antimicrobial and antioxidative activities were tested following standard protocols.

Results: Among the five fractions, methanol fraction of *Artocarpus lakoocha* exhibited higher content of phytochemical compounds [phenols = (3 175.21±290.43) mg GAE/g dry extract, flavonoids = (1 173.15±47.52) mg QE/g dry extract and tannins = (923.53±95.21) mg TAE/g dry extract] as compared to other fractions. The methanol fraction showed the highest antioxidant activity in DPPH and ABTS radical scavenging assays with IC_{50} of (111.98±34.20) μg/mL and (138.26±0.66) μg/mL, respectively, and the best reduction potential with a value of (316.81±2.96) mg QE/g dry extract in reducing power assay. There was significant correlation between the amount of phytochemicals and antioxidant activities. Moreover, the extract successfully protected Lambda phage DNA from damage at 5 and 6 mg/mL concentration and exhibited substantial bactericidal as well as fungicidal activity. The GC-MS analysis of methanol fraction of *Artocarpus lakoocha* revealed diethyl phthalate as the main phytochemical compound, along with 3,4-dihydroxymandelic acid, 9-octyl eicosane and 7,8-didehydro-3-methoxy-17-methyl-6-methylene morphinan.

Conclusions: The methanol fraction of *Artocarpus lakoocha* could be used as a potent antioxidant and antimicrobial agent for sustainable agriculture and pharmaceutical purposes.

1. Introduction

Plants are rich sources of secondary metabolites, such as phenols, tannins, and flavonoids, which have been found to have *in vitro* antimicrobial and antioxidant properties. Control of plant pathogens and combating plant's stress by biological means is of great significance for sustainable agriculture and restoring healthy ecosystems. At present, major research emphasis has been given to discover biologically active natural products from various plants that

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are more potent than synthetic agrochemicals and are much safer, from a health and environmental point of view. Diverse researches in the field of development of natural products such as bactericides, fungicides, and their role in plant disease control, are indicative for reduction in environmental and health hazards[1].

Artocarpus lakoocha (*A. lakoocha*), Roxb. (Moraceae) is a tropical tree distributed all through the Indian Subcontinent and Southeast Asia. It is known by different vernacular names such as monkey jack or lakuchi in India; tampang in Malaya and as lokhat in Thailand. The tree is valued for its wood and its edible fruits, which are believed to have medicinal value[2]. It is a tall (6-9 m) deciduous tree with large and leathery leaves, downy on the underside. The leaves of this tree are abundant sources of bioactive compounds. It is also highly recognized as a fodder tree in the lower foothills of the Himalayas in Nepal. The leaves contain about 16% (w/w) crude protein and a single tree produces about 60 to 200 kg fresh fodder in a year. It is fed to lactating animals and is considered one of the most important milk-producing forages[3]. Significant amount of squalene with 99.9% purity was isolated and identified from the hexane fraction of *Artocarpus* leaves[4].

The lakoocha fruits are generally eaten fresh when ripe and are believed to act as a medication for the liver problems[5,6]. Male and female flowers are borne on the same tree. The raw fruits and male flower spikes (acidic and astringent) are traditionally used in pickles and chutney. Aqueous extract of *A. lakoocha* that is prepared by boiling the wood chips is dried to form a brown powder called Puag-Haad in Thailand. This preparation has been used as a traditional antihelminthic drug for the treatment of tapeworm infection in Thailand[7]. This Puag-Haad contains oxyresveratrol, as a major active compound which is responsible for neuroprotective effect[8]. Extraction and separation of oxyresveratrol molecule were done from the extracted solution of *A. lakoocha* through membrane application[9]. Petroleum ether extracts of fruit and leaves containing triterpenoids and phytosterols have significant larvicidal activity[10]. The heartwood extract of this plant is used as a novel cosmetic ingredient which exhibited *in vitro* anti-tyrosinase and *in vivo* melanin-reducing efficacy in human volunteers[11,12]. Fruits of *A. lakoocha* are rich in flavonoids, phenols, tannins, lignins, saponins and steroids[13]. Lakoocha seeds are purgative in nature. Seeds contain *A. lakoocha* agglutinins (ALA Ⅰ and ALA Ⅱ) which exhibit high haemagglutination activity[14]. Lakoocha is reported to be widely used in the ethno-medicinal formulations by the tribal people of Jharkhand[15].

The tree of *A. lakoocha* sheds all of its large leaves in the month of December to January. So a very thick layer of leaves covers the ground. Shed leaves contain a good amount of valuable bioactive compounds which are important sources for pharmaceuticals and natural agrochemical production. Therefore, in this study, in-depth characterization of the leaf phyto-constituents as well as its antioxidant activities were performed. In addition, DNA protective activity against oxidative radical damage and antimicrobial activity were studied for the most potent bioactive extract. The GC-MS analysis was also conducted for detecting the chemical constituents of the bioactive fraction.

2. Materials and methods

2.1. Collection of plant samples

Shed *Artocarpus* leaves were collected from the campus of Indian Statistical Institute, (22.6482°N, 88.3768°E), Kolkata, India in the month of December to January 2018 and also nearby areas. The collected plant specimen was identified and further checked for verification from the Botanical Survey of India, Kolkata with specimen voucher number PB (Plant Barcode) 30. The collected leaves were dried in air for 7 d before the extraction of bioactive compounds.

2.2. Extraction, isolation, and purification of different fractions from leaves of A. lakoocha

The collected and dried *A. lakoocha* leaves were ground into a fine powder using Sample Miller Machine (Cyclotec 1093. Sample Mill, TECATOR) and 500 g of powder was soaked in 1 000 mL of methanol. The entire mixture was vortexed at a speed of 3 000 rpm using Mechanical Stirrer (Model No. DC Stirrer NZ-1000s AC220V, EYELA) for 2 h and filtered through sintered disc funnel. The brown coloured extract containing both polar and nonpolar compounds was collected and concentrated in a rotary vacuum evaporator (EYELA, Model No. N1-NW) and considered as a crude extract. This crude extract was then fractionated with solvents, namely, pentane, hexane, ethyl acetate, acetone, and methanol, respectively based on their polarity. These fractions were then purified by column chromatography and thin-layer chromatography. Five fractions were recovered from the crude extract of *Artocarpus* leaves. These extracts were weighed and stored in air-tight bottles at 4 ℃ for further study.

2.3. Quantitative phytochemical screening

Quantitative phytochemical screenings of all the five fractions of *A. lakoocha* were performed as per standard protocols to detect the amount of total flavonoids, total phenols, and tannins in each fraction.

2.3.1. Detection of total phenolic content

The total phenolic content was determined following the Folin-Ciocalteu method[16]. At first, 100 μL of the sample (plant extracts at a concentration of 2 mg/mL for each fraction) were mixed with 2 mL of 10% Folin-Ciocalteu reagent and 1.6 mL of 7.5% sodium carbonate (Na_2CO_3) followed by incubation for 30 min at room temperature. The colour generated was measured in

a spectrophotometer with absorbance reading at 765 nm. The calibration curve was prepared using a concentration range of 0.03-0.3 mg/mL of gallic acid as standard. The mean of three readings was used and the total phenolic content was expressed as mg gallic acid equivalents (GAE)/g dry extract. Standard curve of gallic acid was obtained by the following equation: y = 0.234x + (-0.005), R^2= 0.990.

2.3.2. Detection of total flavonoid content

Total flavonoid content was determined according to the method of Jia *et al*[17] with minor modifications. The preferred concentration of different fractions of leaves was 2 mg/mL. About 0.4 mL of 5% sodium nitrite ($NaNO_2$) was added to 1 mL of the sample with uniform mixing and incubated for 5 min at room temperature. Thereafter, 0.6 mL of 10% $AlCl_3$ solution was added, followed by further incubation of 5 min at room temperature. The reaction was terminated by addition of 2 mL of 1 M sodium hydroxide (NaOH) solution. The absorbance was measured at 510 nm. The calibration curve was prepared using a standard solution of quercetin in the concentration range of 0.03-0.3 mg/mL. Total flavonoid content was calculated as mg quercetin equivalent (QE)/g dry extract using the calibration curve. Quercetin standard curve was obtained by the following equation: y = 0.054x + 0.019, R^2 =0.960.

2.3.3. Detection of tannin content

The total tannin content was determined following the International Standard method[18] with slight alteration using tannic acid as standard. Sample concentrations were taken at 2 mg/mL for each of the fractions. Initially, 200 μL of the sample was mixed with freshly prepared 200 μL of 0.35% ferric ammonium citrate followed by 200 μL of 0.8% ammonia solution. Finally, volume was made up to 4 mL by adding 3.4 mL of water. The absorbance of the mixture was measured at 525 nm. The results were expressed as mg tannic acid equivalent (TAE)/g dry extracts. Standard curve of tannic acid was based on the following equation: y=0.085x + (-0.013), R^2= 0.960.

2.4. Antioxidant activities

Several mechanisms are often employed to explain the antioxidant potential of a compound or a complex substance; the main mechanisms involve the free radical scavenging, reduction capacity, and metal chelation. In this study, we used 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and reducing power assays to evaluate the antioxidant potential of different fractions of *A. lakoocha* leaves.

2.4.1. DPPH radical scavenging assay

The free radical scavenging activity of all the fractions was measured *in vitro* using DPPH radical[19]. At first, the DPPH solution (0.025 mg/mL) in methanol was prepared and then 3.9 mL of DPPH solution was mixed with 0.1 mL of sample. Plant extracts at a concentration of 2 mg/mL were used for each fraction. The mixture was shaken vigorously and left to stand for 30 min, and the absorbance was measured at 517 nm. Butylated hydroxy toluene (BHT) in the concentration range of 0.03-0.3 mg/mL was used as a standard (positive control). The capability of the samples to scavenge the DPPH radical was calculated using the following equation:

DPPH radical scavenging activity (%) =$[(A_c-A_t)/A_c]\times100$

Where A_c is the absorbance of the blank reaction and A_t is the absorbance in the presence of the sample of the extracts.

2.4.2. ABTS scavenging capacity assay

Free radical scavenging activity of all the fractions was determined by ABTS radical cation decolorization assay[20]. $ABTS^{\bullet+}$ cation radical was produced by the reaction between 7 mM ABTS in water and 2.45 mM potassium persulphate (1:1). The mixture was incubated at room temperature for 12-16 h in darkness before use. After the addition of 100 μL of the sample (plant extract at a concentration 2 mg/mL for each fraction) to 3.9 mL of diluted $ABTS^{\bullet+}$ solution, the absorbance was measured at 734 nm after 30 min incubation. The solvent was used as blank in each assay and quercetin was used as a standard substance (positive control). All the measurements were carried out in triplicates. Inhibition percentage of the samples was calculated using the formula:

ABTS scavenging effect (%) =$[(A_B-A_A)/A_B]\times100$

Where A_B is absorbance of blank reaction; A_A is absorbance in the presence of sample extract.

2.4.3. Reducing power assay

The ability of the sample fractions to reduce ferric ions was measured according to the modified method described by Oyaizu[21]. A mixture containing 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of $K_3Fe(CN)_6$ (1% w/v) was added to 1.0 mL of sample (plant extract at same concentration used previously). Quercetin at 0.03-0.3 mg/mL as positive control was prepared in distilled water. The resulting mixture was incubated for 20 min at 50 ℃, followed by the addition of 2.5 mL of trichloroacetic acid (10% w/v), which was then centrifuged at 3 000 rpm for 10 min. About 2.5 mL of the supernatant was mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_2$ (0.1% w/v). The absorbance was then measured at 700 nm against a blank sample containing phosphate buffer. Higher absorbance of the reaction mixture indicates higher reducing power. The results were expressed as mg QE/g dry extract[22].

2.5. DNA protection assay against peroxide radical-induced damage

DNA protective potential of the most potent bioactive fraction, methanol fraction of *A. lakoocha*, at different concentrations was evaluated using Lambda phage genomic DNA (Promega). Oxidative damage to DNA was induced using a hydroxyl free-radical generating system (H_2O_2/UV) in the presence of the methanol fraction of *A. lakoocha*, along with positive and negative control[23]. Gallic acid (2 mg/mL) and deionized water were used as positive and negative controls, respectively. Briefly, 10 μL aliquot of Lambda DNA (0.263 μg/mL) was added to microfuge tubes containing 10

μL each of tris buffer (50 mM, pH 7.4) and H_2O_2 (30% v/v). To this reaction mixture, 10 μL of various concentrations of the methanol fraction (1-6 mg/mL) was added. The tubes were UV irradiated using UV transilluminator (Fischer Scientific) for 45 min at room temperature. After irradiation, 5 μL of 6× Loading dye (bromophenol blue) was added to each tube. All samples were analyzed by gel electrophoresis on 1% agarose gel (containing ethidium bromide) in TAE buffer (pH 8). UV-irradiated DNA treated with gallic acid (positive control) and extracts of different concentrations of the methanol fraction were run along with untreated UV-irradiated DNA (negative control).

2.6. Fungicidal activity of methanol fraction of A. lakoocha

The effects of methanol fraction of *A. lakoocha* on fungi were assayed by the agar well diffusion method. Four fungal species including *Aspergillus niger*, *Aspergillus tamarii*, *Aspergillus fumigatus* and *Penicillium chrysogenum* were used for detecting fungicidal activity. Few fungal spores of the tested species were transferred to potato dextrose agar slants and were incubated for one week. After proliferous fungal growth was observed, a loop full of fungal spore of each species was added to the 0.01% sterile saline water (30 mL) and mixed well. Fungal spore suspension (1 mL) in water was then poured in a sterile Petri dish containing molten potato dextrose agar and allowed to solidify. A well of 78.5 mm^2 size was cut at the centre of the Petri dish and 0.8-1.0 mL of the methanol fraction was added at a concentration range from 0.5 mg/mL to 2 mg/mL. Fungicide nystatin (0.5 mg/mL) was used as a positive control and 0.8 mL of dimethyl sulfoxide (DMSO) (1%) was used as a negative control. The plates were incubated at a temperature of 28 ℃ for 24-48 h. The antifungal activity was assayed by measuring the inhibition zone diameter formed around the well. Area of inhibition zone was calculated as:

Area of inhibition at x concentration = $\pi [(TR_x)^2 - (r)^2]$

Where x = concentration used; r = radius, TR_x = Total radius of the inhibition zone at specific concentration of the sample[24].

2.7. Bactericidal activity of methanol fraction of A. lakoocha

The effects of purified methanol fraction of *A. lakoocha* on bacteria were assayed by the agar well diffusion method. Five bacterial species including *Staphylococcus aureus*, *Streptococcus aureus*, *Sarcina lutea*, *Micrococcus aureus*, and *Micrococcus roseus* were used for detecting bactericidal activity. The same procedures were followed as described in the fungicidal activity. About 0.8 mL of ampicillin (0.5 mg/mL) and 0.8 mL of DMSO (1%) was added as positive and negative controls, respectively. The plates were incubated at (37±1) ℃ for 24 h. After incubation, plates were taken out and the inhibition zone was measured. The experiments were done in triplicate. The area of inhibition zone was calculated using the formula described previously.

2.8. GC-MS analysis of methanol fraction of A. lakoocha

Purified methanol fraction of *A. lakoocha* was subjected to GC-MS Analysis (Model No. Agilent Technologies, GC-6860N Network GC System with 5973 inert Mass Selective Detector) for detecting bioactive compounds. The GC-MS analysis was done at the National Test House, Salt Lake, Sector V, Kolkata 700091, INDIA. HP-1MS column (25 m × 0.33 mm, i.d. 0.25 μm) was used. 0.1 μL of methanol fraction of *A. lakoocha* (dissolved in chloroform) was injected into GC in the split mode for analysis at an injector temperature of 280 ℃. A constant flow of helium as the carrier gas was maintained at a rate of 1 mL/min. The oven temperature was programmed as follows: 50 ℃ (1 min hold), 50 ℃ to 200 ℃ at 7 ℃/min, 200 ℃ to 300 ℃ at 6 ℃/min, 200 ℃ (2 min). The mass spectrometer employed the electron ionization mode with an ionization energy of 70 eV. A full scan mode was used with an ion source temperature of 280 ℃ and an acquisition rate of 0.2 s. The mass range was adjusted to 50-350 Da.

The identification of compounds was done by comparing the mass spectra with the spectral data of the NBS75K library provided by the GC/MS control and data processing software.

2.9. Statistical analysis

All the values were analyzed with independent sample *t*-test, and mean values were compared using the least significant difference test. The critical level of significance was set at $P < 0.05$. Correlation analyses were performed using Pearson's Correlation Coefficient. All statistical analyses were carried out using IBM SPSS Statistics 21 software.

3. Results

3.1. Quantitative phytochemical screening

The amounts of total phenolic content, total flavonoid, and tannins in the different fractions of *A. lakoocha* leaves are presented in Table 1. The amounts of total phenolic content, flavonoids and tannins of the methanol fraction significantly differed (P <0.05) from other fractions, except for tannin content in ethyl acetate, acetone and methanol fractions which had a total tannin content of (811.76±44.50), (905.88±130.28) and (923.53±95.21) mg TAE/g dry extract, respectively. Methanol fraction contained significantly higher amount of phytochemical compounds [phenols = (3 175.21±290.43) mg GAE/g dry extract and flavonoids = (1 173.15±47.52) mg QE/g dry extract]. In addition, hexane fraction contained the lowest amount of tannins (205.88±25.64) mg TAE/g dry extract as well as the lowest phenolics (83.33±10.56) mg GAE/g dry extract, whereas ethyl acetate fraction had the lowest amount of flavonoids (212.96±49.53) mg QE/g dry extract.

Table 1. Phytochemical composition in different fractions of *Artocarpus lakoocha* Roxb. leaves.

Samples	Phenols (mg GAE/g dry extract)	Flavonoids (mg QE/g dry extract)	Tannins (mg TAE/g dry extract)
Artcl-PF	269.23±95.33[a]	364.82±39.59[a]	241.18±60.06[a]
Artcl-HF	83.33±10.56[a]	341.67±44.51[a]	205.88±25.64[a]
Artcl-EF	771.36±105.87[b]	212.96±49.53[a]	811.76±44.50[b]
Artcl-AF	1 115.38±245.45[b]	412.04±60.02[a]	905.88±130.28[b]
Artcl-MF	3 175.21±290.43[c]	1 173.15±47.52[b]	923.53±95.21[b]

Values in each column with different superscripts (a,b,c) are significantly different (P<0.05). Artcl-PF: pentane fraction of *Artocarpus lakoocha*; Artcl-HF: hexane fraction; Artcl-EF: ethyl acetate fraction; Artcl-AF: acetone fraction; Artcl-MF: methanol fraction.

3.2. Antioxidant activities

3.2.1. DPPH radical scavenging activity

Table 2 shows the scavenging activities of different fractions of *A. lakoocha* leaves. The methanol fraction showed the best scavenging activity with the lowest IC_{50} value of (111.98±34.20) μg/mL, whereas pentane fraction exhibited the least antioxidant activity with the highest IC_{50} value of (423.53±40.12) μg/mL.

3.2.2. ABTS scavenging capacity

ABTS scavenging activities of different fractions of *A. lakoocha* leaves are shown in Table 2. The methanol fraction showed the highest $ABTS^{\bullet+}$ scavenging activity with the lowest IC_{50} of (138.26±0.66) μg/mL followed by ethyl acetate, acetone, hexane, and pentane fractions.

3.2.3. Reducing power ability

The highest ferric reducing power was revealed in the methanol fraction of *A. lakoocha* with (316.81±2.96) mg QE/g dry extract (Table 2). Acetone and ethyl acetate fractions showed moderate activity with (270.95±32.98) and (261.67±16.92) mg QE/g dry extract, respectively. Pentane fraction exhibited the least ferric reducing power of (9.96±2.36) mg QE/g dry extract.

A significant correlation was observed between antioxidant activity and concentrations of total phenolics, flavonoids, and tannins of all the fractions (Table 3).

Table 2. Antioxidant activities of different fractions of *Artocarpus lakoocha* Roxb. leaves.

Samples	DPPH	ABTS	Reducing power
	IC_{50} (μg/mL)		(mg QE/g dry extract)
Artcl-PF	423.53±40.12[a]	1 153.75±43.78[a]	9.96±2.36[a]
Artcl-HF	344.50±52.25[a]	558.64±44.62[b]	103.05±17.98[b]
Artcl-EF	406.78±67.17[a]	239.14±11.50[b]	261.67±16.92[b]
Artcl-AF	247.42±40.23[a]	403.08±4.81[b]	270.95±32.98[b]
Artcl-MF	111.98±34.20[b]	138.26±0.66[c]	316.81±2.96[b]

The values are expressed as mean ± SD of three independent experiments. Artcl-PF: pentane fraction of *Artocarpus lakoocha*; Artcl-HF: hexane fraction; Artcl-EF: ethyl acetate fraction; Artcl-AF: acetone fraction; Artcl-MF: methanol fraction. Values in each column with different superscripts (a,b,c) are significantly different (P<0.05).

Table 3. Correlation coefficient between antioxidant activities and phytochemical compounds of *Artocarpus lakoocha* Roxb. leaves.

Antioxidant tests	Total flavonoids		Total phenolics		Total tannins	
	r	P	r	P	r	P
DPPH	-0.893*	0.041	-0.897*	0.039	-0.624	0.260
ABTS	-0.471	0.423	-0.732	0.160	-0.962**	0.009
Reducing power	0.469	0.426	0.729	0.163	0.941*	0.017

** indicates P<0.01 and * P<0.05.

3.3. Protective activity of the methanol fraction against DNA damage

Figure 1 shows the electrophoretic pattern of DNA after the UV-photolysis of H_2O_2 in the absence and presence of different concentrations of methanol extract of *A. lakoocha*. In the presence of H_2O_2, UV irradiation led to significant oxidative DNA damage caused by OH^- radical generated from UV photolysis of H_2O_2. Untreated DNA when exposed to H_2O_2 UV photolysis degraded completely giving no clear bands. Upon addition of the methanol fraction of *A. lakoocha* to the reaction mixture at concentrations of 5 mg/mL and 6 mg/mL, the fraction successfully protected the DNA against H_2O_2 induced damage as it was evident with the clear Lambda DNA band visible in Figure 1. This activity of the methanol fraction was observed owing to its high antioxidative potential which could successfully quench the radicals generated, thus protecting the DNA.

3.4. Fungicidal activity of the methanol fraction of A. lakoocha

The methanol fraction of *A. lakoocha* showed fungicidal activity against all four fungal strains. Based on the inhibition zone diameter, the highest inhibition was observed at 1 and 2 mg/mL concentration of methanol extract of *A. lakoocha* against *Aspergillus niger* (Figure 2). In *Aspergillus tamarii*, concentrations of 0.5 and 1 mg/mL showed higher inhibition while *Penicillium chrysogenum* was inhibited more at higher concentrations of 1.5 and 2 mg/mL. In case of *Aspergillus fumigatus*, all the tested concentration exhibited an inhibition zone diameter ranging within 17.0-21.4 mm.

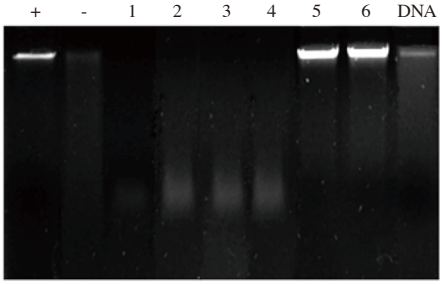

Figure 1. Protective activity of the methanol fraction of *Artocarpus lakoocha* Roxb. at different concentrations against peroxide radical induced DNA damage. Lanes marked 1 to 6 show the effects of methanol extract of *Artocarpus lakoocha* at concentrations ranging from 1 mg/mL to 6 mg/mL on UV-induced DNA damage. Lane marked '+' shows the effect of gallic acid (2 mg/mL) as the positive control. Negative control containing untreated DNA exposed to UV-photolysis is loaded in the lane marked '-'. Last row contains untreated non-UV exposed DNA.

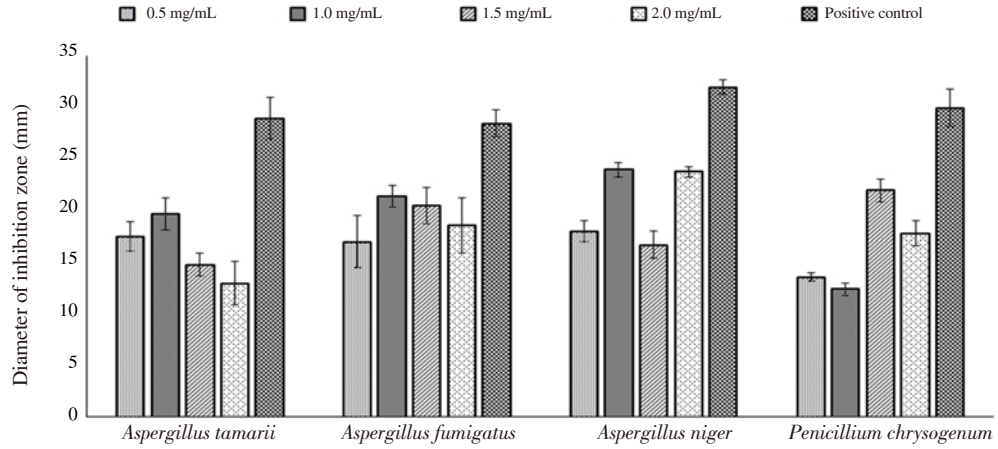

Figure 2. Fungicidal activity of methanol fraction of *Artocarpus lakoocha* Roxb. at different concentrations against *Aspergillus tamarii*, *Aspergillus fumigatus*, *Aspergillus niger* and *Penicillium chrysogenum*.

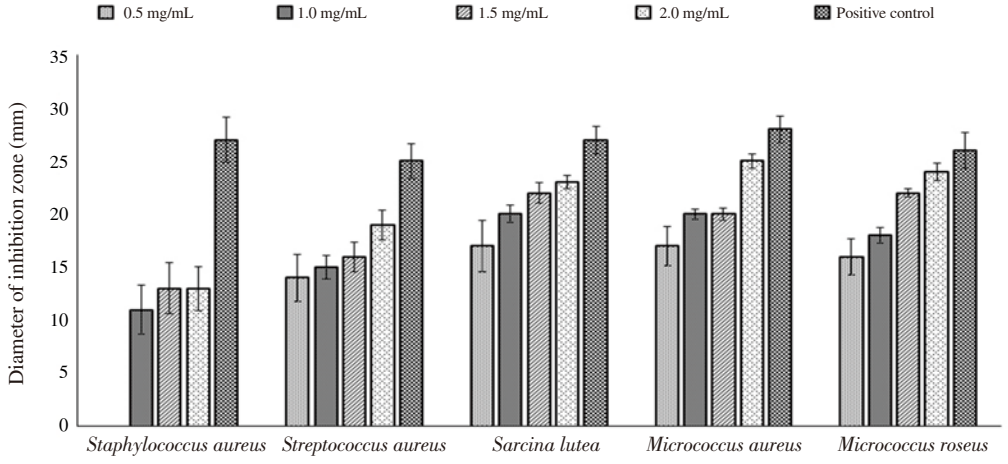

Figure 3. Bactericidal activity of methanol fraction of *Artocarpus lakoocha* Roxb. at different concentrations against *Staphylococcus aureus*, *Streptococcus aureus*, *Sarcina lutea*, *Micrococcus aureus* and *Micrococcus roseus*.

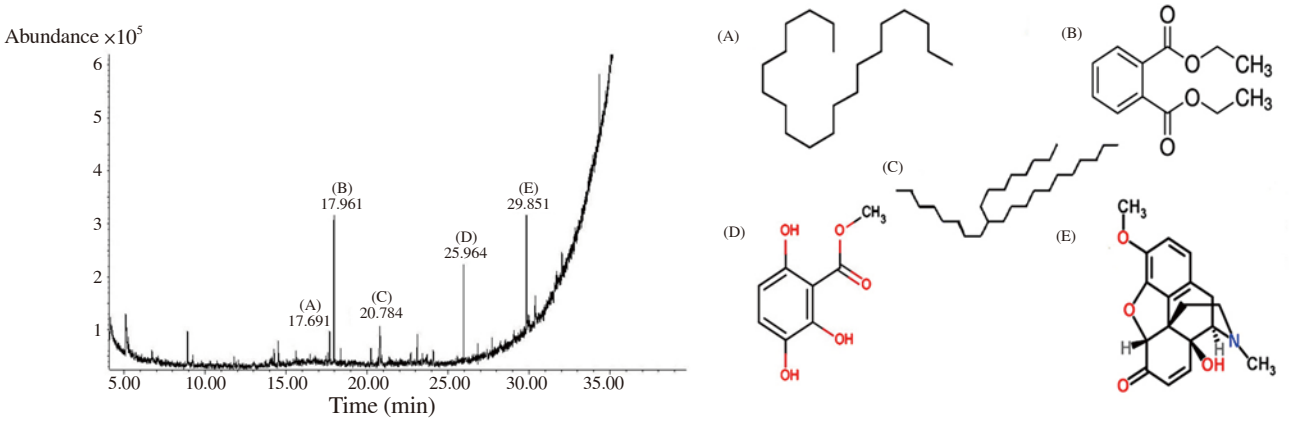

Figure 4. GC-MS spectra and phytochemical constituents of purified methanol fraction of *Artocarpus lakoocha* Roxb leaves. A) Eicosane, B) Diethyl phthalate, C) 9-Octyl eicosane, D) 3,4-Dihydroxymandelic acid, E) 7,8-Didehydro-3-methoxy-17-methyl-6-methylene, morphinan.

3.5. Bactericidal activity of the methanol fraction of A. lakoocha

The methanol fraction exhibited dose-dependent bactericidal activities against all the five bacterial species including *Sarcina lutea*, *Micrococcus aureus*, *Micrococcus roseus*, *Streptococcus aureus*, and *Staphylococcus aureus*. *Staphylococcus aureus* did not show any activity at 0.5 mg/mL concentration of the methanol fraction (Figure 3).

3.6. GC-MS analysis of the purified methanol fraction of A. lakoocha

GC-MS spectra of purified methanol fraction of *A. lakoocha* showed five distinct peaks. The peak at 17.961 was the main peak of the phytochemical constituent corresponding to the chemical compound diethyl phthalate. The other peaks located at 17.691, 20.784, 25.964 and 29.851 corresponded to the compound eicosane, 9-octyl eicosane, 3,4-dihydroxymandelic acid, and 7,8-didehydro-3-methoxy-17-methyl-6-methylene morphinan, respectively (Figure 4).

4. Discussion

Phytochemical screening of the major fractions revealed that the methanol fraction of *A. lakoocha* contained the highest amount of phenols, flavonoids, and tannins as compared to other fractions. The fraction also showed the highest antioxidant activity against DPPH and ABTS radicals as well as in reducing power assay.

The correlation coefficient between antioxidant activities and phytochemical compounds of *A. lakoocha* leaves revealed that the IC_{50} values of DPPH assay were significantly and negatively correlated with total phenolics (r=−0.897, P<0.05) and flavonoids (r=−0.893, P<0.05). However, in the case of ABTS, a highly negative correlation coefficient was observed between the IC_{50} values and total tannin content (r=−0.962, P<0.01). A high negative correlation indicates that higher the amount of phytochemicals, lower the IC_{50} value, thus a better antioxidant potential, suggesting that different classes of compounds play key roles in the inhibition and trapping of free radicals in different systems.

A strong positive correlation was detected between reducing power ability and total tannin content (r=0.941, P<0.05), indicating that tannins significantly contribute to the reduction power of all the extracts. Tannins are a class of plant polyphenolic secondary metabolites which have a molecular weight larger than 500 kDa and give typical blue coloration with $FeCl_3$ which is known as phenol reaction[25]. Tannins may be classified into hydrolyzable tannins which are esters of gallic acid (Gallo- and ellagitannins) and condensed tannins that are polyhydroxyflavan-3-ols or proanthocyanidins[26]. Fractions of *A. lakoocha* may have shown better results in ABTS and reducing power assays due to the presence of tannin class of secondary metabolites.

As the methanol fraction of *A. lakoocha* had the highest amount of phytochemicals as well as best antioxidant or radical scavenging activity compared to others, we proceeded with the methanol fraction to check whether it can protect the DNA against oxidative radical damage. Hydroxyl free radicals are one of the main reasons that damage cellular DNA in human beings, and even partial damage to DNA can lead to cell cancer. UV-photolysis of H_2O_2 generates OH^- radicals, which cause oxidative damage. OH^- binds to DNA leading to base modification, deoxysugar fragmentation and finally strand breakage[27]. The band intensity of the DNA in the presence of different concentrations of the methanol fraction is indicative of its protective effect against hydrogen peroxide-induced damage. It was found from the gel electrophoresis that 5 mg/mL and 6 mg/mL of the methanol fractions from *A. lakoocha* successfully protected the DNA from damage induced by oxidative radicals whereas the fraction at concentrations from 1 to 4 mg/mL failed to completely protect the DNA as evidenced by their faint bands. The result is in agreement with other reports indicating the potential of plants to protect against free radical-mediated DNA damage[28,29]. Methanol fraction of *A. lakoocha* was found to be a highly potent fraction among all other fractions. So, we were interested to identify compounds present in the methanol fraction which are responsible for its bioactivity.

The GC-MS analysis of the methanol fraction revealed diethyl phthalate, as the main phytochemical, along with 3,4-dihydroxymandelic acid, 9-octyl eicosane and 7,8-didehydro-3-methoxy-17-methyl-6-methylene morphinan. Di-2-ethylhexylphthalate is an ester of phthalic acid which has been used as a plasticizer in many materials[30,31]. Owing to its prolonged use, it has been extensively found in varied environments and has been classified as a pollutant with minor health-related risks. However, the recent finding of its production by plants or microorganisms as its natural source opened a serious debate about the nature of this compound and whether it is a real pollutant or a natural metabolite with few biological activities that could be put into use for human welfare[32].

Apart from such controversies, the remarkable antitumor activity of di-(2-ethylhexyl) phthalate isolated from the ethyl acetate extract of *Calotropis gigantea* flower was found against Ehrlich ascites carcinoma in mice[33]. This is the only report of diethyl phthalate found as a bioactive compound in plants. There have been numerous reports stating diethyl phthalate as a natural compound secreted by a number of microorganisms. *Streptomyces* sp. synthesized di-2-ethylhexyl phthalate showed antibacterial activities against Gram-positive bacteria and fungi[34,35]. In accordance to these reports, in our present study, the methanol fraction exhibited strong

antimicrobial activity against a number of fungal and bacterial species which may be due to presence of diethyl phthalate along with the other phytochemical compounds, unlike the antioxidant activities that are dependent mainly on the presence of polyphenols[36]. Phthalate was isolated from *Aspergillus awamori* which showed antifungal activity against *Candida albicans* and antibacterial activity against Gram-positive bacteria *Sarcina lutea*[37]. The largest microbial source secreting this compound is the fungi. Recently, it has been reported that *Penicillium janthinellum* produced di-(2-ethylhexyl) phthalate which showed promising antitumor activity against Ehrlich cells[38].

The present investigation provides a possible way to develop natural agrochemicals and natural pharmaceuticals from the shed leaves of *A. lakoocha*, thereby maintaining ecological balance. This study also re-emphasizes the fact that diethyl phthalate is a potential bioactive natural compound instead of a pollutant. However, further investigations must be conducted to confirm the mode of action, to find out effective concentration and also its effects on non-target organisms.

In conclusion, the present study reveals that methanol fraction of *A. lakoocha* leaves contain the highest amount of phytochemical compounds than other fractions that can competently provide protection against oxidative stress caused by free radicals. Besides, this fraction also showed antimicrobial activity. In GC-MS analysis, diethyl phthalate was recognized as one of the main bioactive compounds in the methanol fraction along with 3,4-dihydroxymandelic acid, 9-octyl eicosane and 7,8-didehydro-3-methoxy-17-methyl-6-methylene morphinan. Therefore, the isolation of natural bioactive compounds from the leaves of *A. lakoocha* Roxb can be a novel approach for developing new agrochemicals and natural pharmaceuticals.

Conflict of interest statement

Authors declare that they have no conflict of interests among them regarding the publication of this paper.

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Authors' contributions

SMB and EB designed the study and made the critical revision of the article. Collecting test data, drafting the article and getting a final approval of the version to be published were done by SMB, EB, RD and SC, as well as data analysis and interpretation was done by SMB, EB and SC. In addition, SMB was responsible for supervision, project administration and funding acquisition.

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