

# A LC/MS-MS Guided Isolation of Laccaic Acid-A: A Potent Antimicrobial Agent

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## ABSTRACT

**Background:** Lac is traditionally used as a dye because of its coloring properties and it is obtained from *Laccifer lacca* Kerr. Now a days, it is widely used in food and coloring industry. **Purpose:** To evaluate the antioxidant, antimicrobial activity of different extracts along with identification of active constituents present in it. **Methods:** Antioxidant activity was performed by DPPH, reducing power and hydrogen peroxide scavenging activity, whereas antimicrobial activity was performed by well diffusion method to evaluate the antimicrobial potential of chloroform, ethyl acetate, methanol and water extracts. Based on the most potent activity of the extracts were run in LC-MS/MS to identify the active constituent from it. **Results:** Ethyl acetate and methanol extracts were found to have most potent antioxidant activity with more than 50% inhibition at a concentration of 16  $\mu\text{g/ml}$  where all the three methods when compared with ascorbic acid as standard. Antimicrobial activity was also found to be potent for ethyl acetate and methanol extract having a zone of inhibition of 15 mm and 18 mm for *E. coli* and *S. aureus* respectively. Finally, ethyl acetate and methanol extracts were processed for an effective LC-MS/MS run to identify the active constituent as laccaic acid-A in both the extract. **Conclusion:** Laccaic acid A is identified as the main constituent present in ethyl acetate and methanol fractions responsible for the antioxidant and antimicrobial activity.

**Key words:** *Laccifer lacca* Kerr., DPPH, Reducing power, Hydrogen peroxide, Antimicrobial assay, LC-MS/MS, Laccaic acid-A.

## INTRODUCTION

Lac is a natural polymer (resinous exudation) from the female scale insect. *Laccifer lacca* Kerr. Lac is generally obtained by crushing, sieving and washing of stick-lac. Resin is the major constituent of lac. Lac resin is a complex straight chain hydroxyl fatty acids such as butolic acid, aleuritic acid, jalaric acid, shellolic acid, laccishellolic acid etc having C-14 to C-18 carbon chain. Other than resin there are other components of lac. The percentage wise composition of lac (data adopted from Indian Lac Research Institute, ILRI) is lac resin 68%, lac wax 6%, lac dye 1-2% and others 25%.<sup>1</sup> Lac dye is a combination of anthraquinone derivatives consists of laccaic acids. Lac has multipurpose application in different fields such as military, electric appliance, printing ink, leather, plastic, metallurgy, machinery, woodworking,

food, medicine, etc.<sup>2</sup> Though lac has multipurpose use in the various industries recently it has proven its anticancer activity in MCF-7 breast cancer cell lines. It inhibits direct DNA competitive inhibitor of DNA methyl transferase I by altering the expression of the methylated gene in MCF-7.<sup>3</sup> The result of DPPH assay indicates the higher  $\text{EC}_{50}$  value of lac dye than the  $\text{EC}_{50}$  value of aluminum lake.<sup>4</sup> Lac is widely used for food coloring and also acts as a protease inhibitor in terms of both auto activations of PHBP (Plasma Hyaluronan-Binding Protein) proenzyme ( $\text{IC}_{50} = 0.35\text{--}0.55\text{g/ml}$ ) and the catalytic activity of the active enzyme ( $\text{IC}_{50} = 1.1\text{g/ml}$ ).<sup>5</sup> Lac extract has been analyzed by a high-speed counter-current chromatography for the isolation of laccaic acids A, B, C and E the major chromophores

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present in lac-dye.<sup>6</sup> For the identification of laccaic acids in jellies and stick lac samples an ESI LC-MS/MS method, has been reported using a volatile mobile phase acetylacetone.<sup>7</sup> Laccaic acids A, B and E were detected in all lac color products and were analyzed using HPLC comprising of reverse phase C-18 column with a PDA detector set at 280 nm and 490 nm.<sup>8</sup> An analytical method was developed for the toxicity studies of lac color in diet and feces of rat.<sup>9</sup> Laccaic acid A was isolated using cellulose column, preparative paper chromatography and characterized by using IR and UV-Vis.<sup>10</sup> The pKa of laccaic acid was reported to be 4.4 and 6.5.<sup>11</sup> Isolation of laccaic acid C was performed using cellulose column as a major fraction and laccaic acid E as a minor fraction, both were reported to possess similar properties like laccaic acid A and B but differs in  $\alpha$ -amino acid as a side chain.<sup>12</sup> Using silica gel column and acetone as an eluent isolation of laccaic acid D was performed from rangini stick lac.<sup>13</sup> Laccaic acid B was isolated from the polyamide column as a minor fraction and characterized using UV and IR. They also reported the presence of chelated quinine group in laccaic acid B.<sup>14</sup> From recent literature study, it was found that a HPLC-DAD-MS analysis of colorant and resinous components of lac-dye was performed. The authors reported that a comparative study was performed for the identification of laccaic acid A, laccaic acid B, laccaic acid C and laccaic acid E present in *Kerria* and *Paratachardina genera*.<sup>15</sup> However, there was no information available regarding antibacterial activity of lac dye from *Laccifer lacca*. Thus, our present work focuses on the antibacterial activity of different extracts of *Laccifer lacca* and evaluation of antioxidant activities of these extracts by different methods. Further study is also undertaken to identify the active component present in the bio-active fractions by LC-MS/MS. The antimicrobial activity is performed employing well diffusion assay by determining the zone of inhibition.

## METHODS AND MATERIALS

### Chemicals and reagents

All the chemicals and reagents used were of analytical grade. Water was purified by the Milli Q water purification system (Millipore, Milford, MA, USA). Methanol, petroleum ether 60-80°C, chloroform, ethyl acetate and formic acid used were of AR Grade purchased from Ranchem private limited. Syringe filters having the pore size of 0.22 $\mu$ m were purchased from Merck, Mumbai. Lac-dye was provided by Indian Institute of Natural Resins and Gums, IINRG, Ranchi, Jharkhand, India. DPPH (2, 2-diphenyl-1-picrylhydrazyl), hydrogen peroxide, potassium ferricyanide ( $K_3[Fe(CN)_6]$ ), phosphate

buffer ( $KH_2PO_4$ ), trichloroacetic acid, ferric chloride ( $FeCl_3$ ) and ascorbic acid used were purchased from Sigma, U.S.A. Acetonitrile, water and methanol used were of LC-MS grade purchased from Fisher Scientific (Loughborough, Leicestershire, UK). The Instrument used for the present study was UV-VIS spectrophotometer Model No 2450 and the data acquisition was carried out by UV probe 2.3 version. All the bacterial strains were procured from the Microbial Type Culture Collection and Gene Bank, an International Depository Authority, Chandigarh, India, maintained in nutrient broth at -20°C.

### Preparation of Technical grade and sample preparation of lac dye

#### Technical grade

Stick lac was washed with water then acidified with HCl (0.1% on the vol. of water) and the supernatant liquor was collected by filtration. Calcium carbonate or quicklime was used to treat the mixed filtrate until the liquor becomes colorless. By the help of filtration calcium salt of lac dye that has separated out was gathered and washed repeatedly with water. The calcium salt of lac dye (the cold suspension) in water was kept for a week after acidification at room temperature until all the dye was crystallized. Later it was washed, filtered and dried out (approx. yield was found to be 0.5% weight of stick lac). Net dye content was approximately 80-90% but it depends on the quality and age of stick lac.<sup>16</sup>

#### Sample Preparation

Technical grade lac was obtained as a powder. Successive fractionation was done, starting from a non-polar solvent to the polar solvents. Five grams of lac was added to 50 ml volumetric flask containing 50 ml of petroleum ether (60°-80°C) and allowed for ultrasonication for 10 min at a of temperature 40°C. Petroleum ether solution containing lac was transferred to a 15 ml centrifuge tube, equally distributed in 5 centrifuge tubes, centrifuged for about 10 min at 5000 rpm. The clear supernatant liquid was collected in petri-dishes and allowed to evaporate. The lac dye which has settled down after centrifugation in the centrifuge tubes were collected and dissolved in 50 ml volumetric flask containing chloroform. Further, the solution was subjected to ultrasonication for 10 min at a temperature of 40°C. Similarly, this procedure was repeated for ethyl acetate, methanol and water for the preparation of ethyl acetate, methanol and a water fraction. Total three sets of fractions were prepared, chloroform (C), ethyl acetate (EA), methanol (M) and water (W) respectively. Fractionation with petroleum ether was mainly done for defatting pur-

pose to remove the unwanted oils and unsaturated fatty acids from the samples.

### Assessment of extracts of lac dye for antioxidant activity

#### DPPH scavenging activity

The DPPH radical scavenging activities of the different extracts were estimated by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method as illustrated by Miliauskas *et al.*<sup>17</sup> The Methanolic solution of 0.1 mM DPPH was prepared. Samples with different concentrations (4, 8, 12, 16 and 20 µg/ml) were made in same solvent. Ascorbic acid was taken as a quality standard of different concentrations (4, 8, 12, 16 and 20 µg/ml) for all methods of antioxidant assay. The reaction was commenced by adding 1ml of DPPH solution to the 3 ml of standard or different extracts (aqueous, methanol, ethyl acetate and chloroform) in methanol of various concentrations. The reaction mixture was vigorously agitated and kept it for half an hour in a dark place at room temperature till the completion of the reaction. Absorbance was measured at a wavelength of 517 nm using an ultraviolet spectrophotometer.

$$\% \text{ of DPPH scavenging activity is} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

( $A_{\text{control}}$  = absorbance of control reaction,  $A_{\text{sample}}$  = absorbance in the presence of standard or sample).

#### Reducing power assay

The reducing power assay for the different extracts of lac was evaluated according to the method of Jayanthi and Lalitha with little alteration.<sup>18</sup> Samples with different concentrations (4, 8, 12, 16 and 20 µg/ml) were made in mili-Q water. One ml of sample solution assorted with  $\text{KH}_2\text{PO}_4$  buffer 2.5 ml (0.2 M, pH 6.6) and  $\text{K}_3[\text{Fe}(\text{CN})_6]$  2.5 ml. The reaction blend was retained for 20 min at a temperature of 50°C. The reaction mixture was allowed to cool, then 2.5 ml of 10% trichloroacetic acid was added and centrifuged at 2000 rpm for 10 min. The upper layer of 2.5 ml was taken to it 2.5ml of mili-Q water and 0.5 ml of  $\text{FeCl}_3$  solution (0.1%) freshly prepared was added. Absorbance was recorded at 700 nm using an ultraviolet spectrophotometer. In a similar way, control was prepared without the samples. Ascorbic acid was taken as the standard of various concentrations as mentioned above. Samples were analyzed and the data were presented as percentage inhibition. Increase in the reducing power of extracts at different concentrations indicates enhance in absorbance of the reaction blend.

$$\% \text{ of reducing power} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

( $A_{\text{control}}$  = absorbance of control reaction,  $A_{\text{sample}}$  = absorbance in the presence of standard or sample).

#### Hydrogen peroxide assay

The hydrogen peroxide scavenging assay has been carried out as stated by Ruch *et al.* with little modification.<sup>19</sup> Two molar (2 M) of hydrogen peroxide solution was freshly prepared in phosphate buffer ( $\text{KH}_2\text{PO}_4$ ) with a pH of 7.40 (0.2 M). Different extracts of various concentrations (4, 8, 12, 16 and 20 µg/ml) were made readily in mili-Q water. An amount of 2.5 ml hydrogen peroxide in phosphate buffer was mixed with 1.5 ml of extract and standard. The blend was allowed to mix and vortexed then incubated for 10 min in a dark place. Absorbance was measured at 230 nm, after 10 min of incubation. Ascorbic acid was taken as the standard of various concentrations as mentioned above. Phosphate buffer without  $\text{H}_2\text{O}_2$  served as a blank. The percentage of hydrogen peroxide scavenging activity was measured from the below-mentioned formula

$$\% \text{ of hydrogen peroxide} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

(Where  $A_{\text{control}}$  = absorbance of control reaction,  $A_{\text{sample}}$  = absorbance in the presence of standard or sample)

### Assessment of extracts of lac dye for antimicrobial activity

#### Antimicrobial activity

In the present study, the test microbe used for the antimicrobial studies were Gram-negative bacteria *Escherichia coli* (MTCC-739) and Gram-positive bacteria *Staphylococcus aureus* (MTCC-3160). Three hundred milliliter of each stock-culture was added to 3 ml of nutrient broth.<sup>20</sup> Overnight cultures were kept for 24 h at 37°C. After 24 h of incubation, bacterial suspension (inoculum) was diluted with a sterile physiological solution, to make a final cell concentration of  $10^5$  CFU/mL.

#### Bacterial media

Sterilization of Muller-Hinton Agar (MHA) media mixed with distilled water was performed in an autoclave at 15 lb pressure for 15 min. In the petri dishes, sterile media was poured and allowed it to solidify. The bacterial inoculum ( $10^5$  cells/ml) was equally distributed by a sterile cotton swab on a sterile petridish MHA. The sterilized well cork borer was used to bore the solidified plates with a diameter of 6 mm. The antibacterial studies were performed with well plates.<sup>21</sup>

### Antibacterial activity of the lac extracts

The antibacterial activity of different extracts of lac was performed taking 100 µl from the stock solution (1 mg/ml), were evaluated against *E. coli* (MTCC-739) and *S. aureus* (MTCC-3160) by well diffusion method<sup>22</sup> taking amoxicillin as standard at a concentration of 200 µg/ml.

### Well diffusion method

The method used to evaluate the antibacterial activity of different lac extracts was well diffusion. The streak plate method was used for inoculating different stains to the prepared culture plates. Wells of 6 mm in diameter were made by using sterilized cork well borer. One hundred milliliters from the stock solution (1mg/ml) of each extract were seeded in the wells by a sterile syringe. For the optimal bacterial activity plates were incubated for 24 h at 37°C. The zone of inhibition around the well was observed. Inhibition was assessed by determining the diameter of the inhibited zone throughout the well (mm) as well as the well diameter.<sup>23</sup> Readings were taken in three replicates along in three different fixed directions finally, the average values were compiled.

### Liquid Chromatography with Mass Spectroscopy

The instrument used for LC-MS/MS was of Waters ACQUITY FTN Auto-Sampler, QSM series with a quaternary pump system having PDA detector (type UPLC LG 500 nm) and ESI detector for MS set at positive ionization mode. The column employed was of reverse-phase C18, Accucore RPMS (100mm×3mm×2.6µm) and 3D Channel range 200-450 nm and resolution of 1.2 nm whereas compensation reference 2D parameter ranging from 310-410 nm. The ethyl acetate and methanol fraction were subjected to LCMS/MS using mobile phase, mixture of acetonitrile: water (5:95) in solvent reservoir A, acetonitrile in solvent reservoir B, methanol in solvent reservoir C and solvent reservoir D consisted of water + formic acid followed through an effective gradient run (Table 1) at a flow rate of 0.25 ml/min with a run time of 20 min.

## RESULTS

### Assessment of antioxidant activity

#### DPPH scavenging activity

In the radical scavenging assay, the antioxidant when coming in contact with DPPH there is an occurrence of the color change, the purple color of DPPH changed to yellow. The intense yellow color of DPPH indicates the more prominent antioxidant action of the extract been examined. This is due to the formation of a stable

**Table 1: Gradient run for ethyl acetate and methanol fraction in LC-MS/MS.**

Time	Flow rate	%A	%B	%C	%D
Initial	0.250	0.0	10.0	0.0	90.0
1.00	0.250	0.0	10.0	0.0	60.0
6.00	0.250	0.0	40.0	0.0	40.0
8.00	0.250	0.0	60.0	0.0	40.0
12.00	0.250	0.0	80.0	0.0	20.0
14.00	0.250	0.0	80.0	0.0	20.0
16.00	0.250	0.0	10.0	0.0	90.0
20.00	0.250	0.0	10.0	0.0	90.0

complex resulting from the reaction between the DPPH and the antioxidant molecule. The standard, ascorbic acid expressed 57.275% inhibition at a concentration 12 µg/ml, while ethyl acetate and methanol showed 54.200% and 52.350% inhibition respectively at a concentration 16 µg/ml which is quite promising to that of ascorbic acid. Further extracts (chloroform and aqueous) expressed relatively lower percentage inhibition when compared to standard and test fractions (ethyl acetate and methanol). The percentage inhibition for all extracts along with standard data compiled in Table 2. The results obtained from the DPPH assay for ascorbic acid, ethyl acetate and methanol extracts are quite similar.

#### Reducing power assay

The antioxidant compound when tested by reducing power assay, conversion of iron (Fe<sup>+3</sup>) in ferric chloride to ferrous (Fe<sup>+2</sup>) the oxidation form of iron depending upon formation of metal ion complex with iron. This is the basic principle involved in reducing power assay for any antioxidant compounds. In the present study ascorbic acid was taken as standard or positive control. Ascorbic acid showed 54.347% inhibition at a concentration 12 µg/ml, whereas ethyl acetate and methanol showed 52.173% and 54.347% inhibition respectively at a concentration 16 µg/ml that is quite promising to that of ascorbic acid. Other extracts (chloroform and aqueous) showed relatively lower percentage inhibition when compared to standard as well as ethyl acetate and methanol extracts. The percentage inhibition of all the extracts and standard data are summarized in Table 3. Ascorbic acid, the reference standard, resembles the activity with that of ethyl acetate and methanol extracts.

#### Hydrogen peroxide assay

Hydrogen peroxide is a weak oxidizing agent and highly sensitive to light and at the same time, it can inactivate few of the enzymes by the oxidation of thiol groups. The



**Table 2: DPPH radical scavenging assay by different extracts of *Laccifer lacca*.**

Percentage DPPH inhibition					
Concentration (µg/ml)	Ascorbic Acid	Chloroform Extract	Ethyl acetate Extract	Methanol Extract I	Aqueous Extract
4	31.475	10.900	28.075	25.525	9.475
8	44.975	18.475	34.700	34.248	16.975
12	57.275	27.225	42.575	42.475	24.950
16	63.900	36.425	54.200	52.350	33.050
20	74.700	47.150	65.050	64.825	43.975

DPPH: 1, 1-diphenyl-2-picrylhydrazyl

**Table 3: Reducing power assay by different extracts of *Laccifer lacca*.**

Percentage reducing power inhibition					
Concentration (µg/ml)	Ascorbic Acid	Chloroform Extract	Ethyl acetate Extract	Methanol Extract	Aqueous Extract
4	32.609	8.696	23.913	26.087	2.174
8	45.652	10.870	32.609	34.783	8.696
12	54.348	17.391	47.826	45.652	15.217
16	60.870	23.913	52.174	54.348	21.739
20	76.087	32.609	60.870	58.696	26.087

**Table 4: Hydrogen peroxide scavenging assay by different extracts of *Laccifer lacca*.**

Percentage hydrogen peroxide inhibition					
Concentration (µg/ml)	Ascorbic Acid	Chloroform Extract	Ethyl acetate Extract	Methanol Extract I	Aqueous Extract
4	29.987	10.928	18.933	18.297	8.767
8	41.169	17.789	33.418	33.672	12.706
12	54.244	27.065	47.776	49.047	23.634
16	61.753	35.070	53.494	52.859	31.258
20	73.189	43.964	62.008	60.483	36.213

present study reveals ethyl acetate and methanol extract exhibits 53.494% and 52.859% inhibition respectively at a concentration of 16 µg/ml. Ascorbic acid (standard) showed 54.244% inhibition at a concentration 12 µg/ml which is quite comparable to that of ethyl acetate and methanol extract. While on other hand chloroform and aqueous extract exhibited lower percentage inhibition when compared to standard, ethyl acetate and methanol extracts. The percentage inhibition of all the extracts and standard data are summarized in Table 4. These results proved ethyl acetate and methanol extracts had overall comparable to hydrogen peroxide radical scavenging activity to that of ascorbic acid.

### Assessment of antimicrobial activity

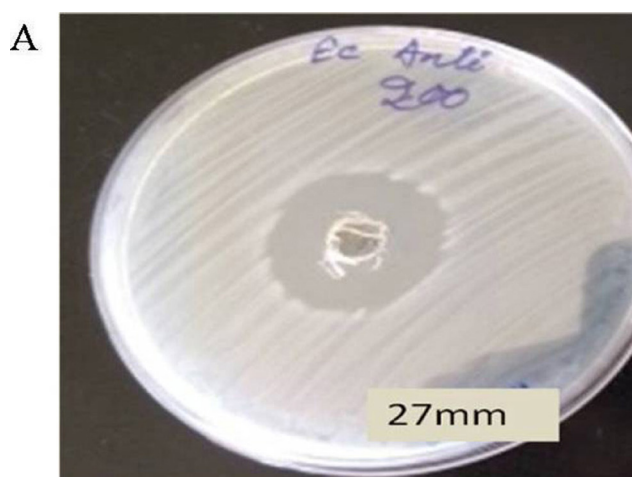
#### Antimicrobial activity

The antimicrobial activity of different extracts of lac was evaluated antibiotic susceptible microorganisms.

The antimicrobial activities of different extracts of lac were studied at a concentration of (1 mg/ml) taking 100 µl solution. Amoxicillin (200 µg/ml) was taken as standard. The antimicrobial activity was assessed in terms of zone of inhibition of bacterial growth for the different extracts of lac. The results and the figures (Figure 1A, 1B, 2A and 2B) are summarized in Table 5. The methanolic and ethyl acetate extract showed 18mm zone of inhibition against *Escherichia coli* (MTCC-739) and 15mm against *Staphylococcus aureus* (MTCC-3160). The present study focused on dose-dependent obtained by a zone of inhibition. The ethyl acetate and methanol extracts of lac showed activity against gram-positive bacteria *Staphylococcus aureus* and gram-negative bacteria *Escherichia coli*, at a high concentration of 1000 µg/ml. On the other hand, no favorable activity was noted for chloroform and water extract. From the present investigation, it may be suggested that the lac may contain



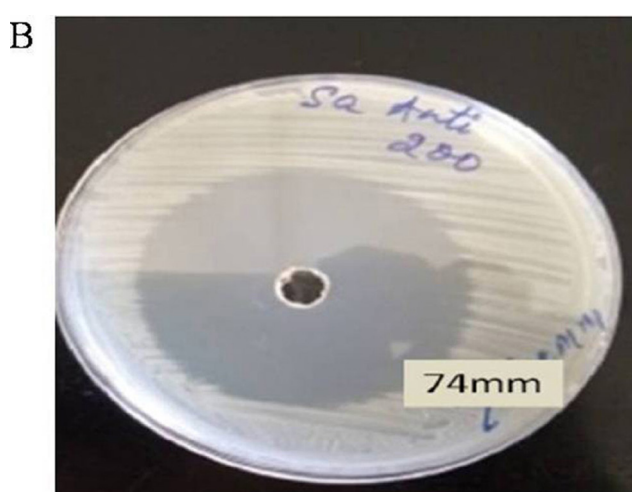
**Figure 1A:** Zone of inhibition of different extracts (9-Water, 10- Ethyl Acetate, 11- Chloroform and 12- Methanol) of lac dye on *E. coli*.



**Figure 2A:** Zone of inhibition of Amoxicillin (standard) on *E. coli*.



**Figure 1B:** Zone of inhibition of different extracts (9-Water, 10- Ethyl Acetate, 11- Chloroform and 12- Methanol) of lac dye on *S. aureus*.



**Figure 2B:** Zone of inhibition of Amoxicillin (standard) on *S. aureus*.

some components or their derivatives such as saponin, triterpenoids, steroids, glycosides, anthraquinones, flavonoids, proteins, amino acids and a phenolic compound may be responsible for possessing antimicrobial activity for ethyl acetate and methanol extracts of lac.

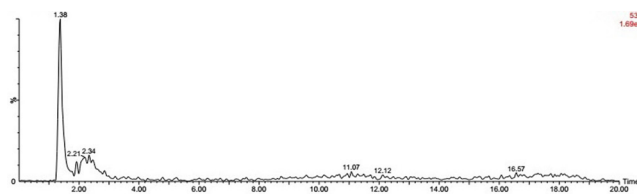
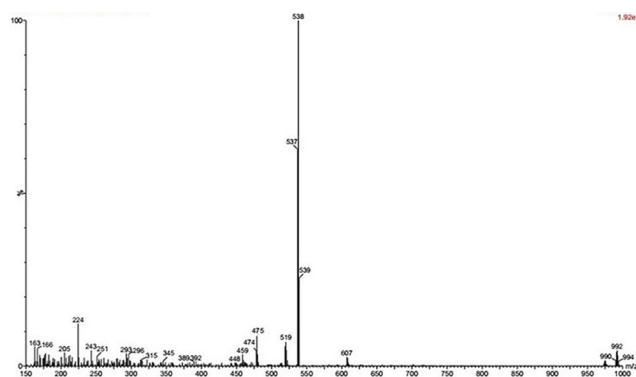
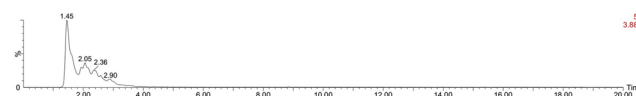
#### **Chromatographic separation of ethyl acetate and methanol fraction using LCMS/MS**

The ethyl acetate and methanol fraction were subjected to LC-MS/MS with an effective gradient run for the identification of active compounds present in these two bio-active fractions with better resolution. Compound having retention time of 1.358 min (Figure 3) shows an  $M+H^+$  prominent peak at 538  $[M+H]^+$ , other prominent daughter ion peaks of this compound were observed at 519  $[M+H-18]^+$  for the removal of  $H_2O$

and 475  $[M+H-18-44]^+$  due to the removal of  $CO_2$  probably indicating the compound may be laccaic acid A given in Figure 4 and Table 6. The same gradient run was employed for methanol fraction. Retention time of 1.423 min (Figure 5) having ES<sup>+</sup> peak at 538  $[M+H]^+$  which is as similar to that of the peak present in ethyl acetate fraction along with other daughter ion peaks at 519  $[M+H-H_2O]^+$  and 475  $[M+H-H_2O-CO_2]^+$  identified as same component present in ethyl acetate fraction i.e. laccaic acid A as identified given in Figure 6 and Table 6. The LC-MS/MS results showed a molecular ion peak of  $m/z$  538  $[M+H]^+$  for both ethyl acetate and methanol extract which is same molecular mass of laccaic acid A (537amu) with same fragmentation pattern as reported earlier.<sup>6,7</sup> The structure of laccaic acid-A is given in Figure 7.

**Table 5: Antimicrobial activity of different extracts of *Laccifer lacca*.**

	Pathogen (zone size in mm)	
	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>
Stick lac extract (1 mg/mL)		
Chloroform	-	-
Ethyl acetate	18	15
Methanol	18	15
Aqueous	-	-
Amoxicillin (Standard)	74	27

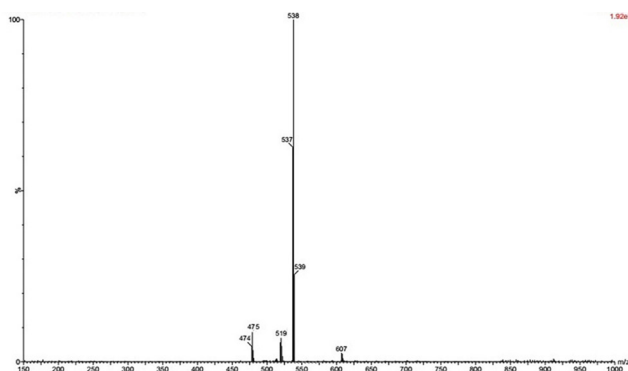
**Figure 3: Showing retention time of ethyl acetate extract of lac dye at 1.3 min on mass chromatograms.****Figure 4: Mass spectrum of ethyl acetate extract with Rt 1.3 and 538 m/z.****Figure 5: Showing retention time of methanolic extract of lac dye at 1.4 min on mass chromatograms.****Table 6: Precursor and product ions in ethyl acetate and methanolic extract of lac dye.**

Ethyl Acetate fraction of lac dye						
Sl. No.	Retention Time (min)	Precursor Ion	Product Ion			Compound Identified
		[M+H] <sup>+</sup>	[M+H-H] <sup>+</sup>	[M+H-H-H <sub>2</sub> O] <sup>+</sup>	[M+H-H-H <sub>2</sub> O-CO <sub>2</sub> ] <sup>+</sup>	
1	1.3	538	537	519	475	Laccaic Acid-A
2	16.9	391				Unknown
Methanolic fraction of lac dye						
Sl. No.	Retention Time (min)	Precursor Ion	Product Ion			Compound Identified
		[M+H] <sup>+</sup>	[M+H-H] <sup>+</sup>	[M+H-H-H <sub>2</sub> O] <sup>+</sup>	[M+H-H-H <sub>2</sub> O-CO <sub>2</sub> ] <sup>+</sup>	
1	1.4	538	537	519	475	Laccaic Acid-A
2	8.5	515				Unknown
3	11.8	269				Unknown
4	15.6	452				Unknown

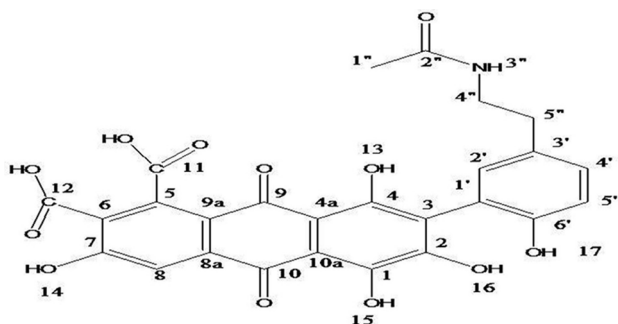
## DISCUSSION

The main focus of the present work is determination of the antimicrobial activity of two different extracts of lac dye, the ethyl acetate and methanolic extract against gram-positive bacteria *Staphylococcus aureus* and gram-negative bacteria *Escherichia coli* for the first time, also to identify the active constituent present in the bio-active fraction by LC-MS/MS. The antimicrobial activity of two extracts of lac dye was found to be active at a concentration of 1000 µg/ml having a zone of inhibition of 18 mm and 15 mm for *Staphylococcus aureus* and *Escherichia coli* respectively. The results obtained showed the antimicrobial activity of both the

lac extracts at a high concentration of 1000 µg/ml while the standard drug Amoxicillin at a concentration 200 µg/ml having a zone of inhibition of 74 mm and 27 mm for *S. aureus* and *E. coli* respectively. Other than antimicrobial activity, the ethyl acetate and methanol extract are also having potent antioxidant activity. The results obtained for ethyl acetate and methanol extract were quite comparable with the standard. Literature suggests that the free radical as well as the reactive oxygen species (ROS) generation can be prevented by the antioxidants available from different natural sources. Antioxidants act by interfering in the oxidation process of the particular substrate. The antioxidant activity or the scavenging



**Figure 6: Mass Spectrum of methanolic extract with Rt 1.4 min and 538 m/z.**



**Figure 7: Chemical structure of laccaic acid A.**

activity may be the result of the five hydroxyl groups attached to the moiety which may release the  $H^+$  ion to trap the ROS in the reaction. Both the bioactive extracts were subjected to chromatographic isolation to detect the bioactive moiety responsible for the activity. The ethyl acetate and methanolic extract were subjected to LC-MS/MS which revealed that both the extracts contained prominent  $M+H^+$  mass peak at 538  $[M+H]^+$ . Other prominent peaks were observed at 519  $[M+H-H_2O]^+$  and 475  $[M+H-H_2O-CO_2]^+$  at retention times 1.358 and 1.423 min for ethyl acetate and methanol indicating the compound to be laccaic acid A. The molecular weight of laccaic acid A (537amu) reported earlier<sup>6,7</sup> which is same as the molecular mass of the identified compound from ethyl acetate and methanol extract having similar fragmentation pattern. So, laccaic acid A may be the responsible compound for antimicrobial and antioxidant activity.

## CONCLUSION

The above study showed the antimicrobial activity of different extracts of lac dye (ethyl acetate and methanol) for the first time in *Staphylococcus aureus* and *Escherichia coli* species with a comparison to a standard. Other than antimicrobial activity both the extracts of lac dye were having a very sound antioxidant activity that to at a very low concentration. Moreover, the two bio-active frac-

tions were subjected to LC-MS/MS to identify laccaic acid A as the main component of the lac dye mainly responsible for the activity.

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## CONFLICT OF INTEREST

There is no conflict of interest among all authors.

## ABBREVIATIONS

**DPPH:** 2, 2-diphenyl-1-picrylhydrazyl; **LC-MS/MS:** Liquid Chromatography-Mass Spectroscopy/ Mass Spectroscopy; ***E. coli:*** *Escherichia coli*; ***S. aureus:*** *Staphylococcus aureus*; **ILRI:** Indian Lac Research Institute; **EC<sub>50</sub>:** Half maximal effective concentration; **PHBP:** Plasma Hyaluronan-Binding Protein; **IC<sub>50</sub>:** Half maximal inhibitory concentration; **ESI:** Electrospray Ionization; **HPLC:** High-Performance Liquid Chromatography; **PDA:** Photodiode Array; **IR:** Infrared; **UV-Vis:** Ultraviolet-Visible; **DAD:** Diode Array Detector; **IINRG:** Indian Institute of Natural Resins and Gums; **K<sub>3</sub>[Fe(CN)<sub>6</sub>]:** Potassium Ferricyanide; **KH<sub>2</sub>PO<sub>4</sub>:** Potassium Dihydrogen Phosphate Buffer or Phosphate Buffer; **FeCl<sub>3</sub>:** Ferric Chloride; **rpm:** Revolution per minute; **C:** Chloroform; **EA:** Ethyl acetate; **M:** Methanol; **W:** Water; **EtOAc:** Ethyl acetate; **h:** Hour; **H<sub>2</sub>O<sub>2</sub>:** Hydrogen Peroxide; **CFU:** Colony Forming Units; **MHA:** Muller-Hinton Agar; **mm:** Millimeter; **nm:** Nanometer; **H<sub>2</sub>O:** Water; **CO<sub>2</sub>:** Carbon dioxide; **min:** Minutes; **amu:** Atomic Mass Unit; **ROS:** Reactive Oxygen Species.

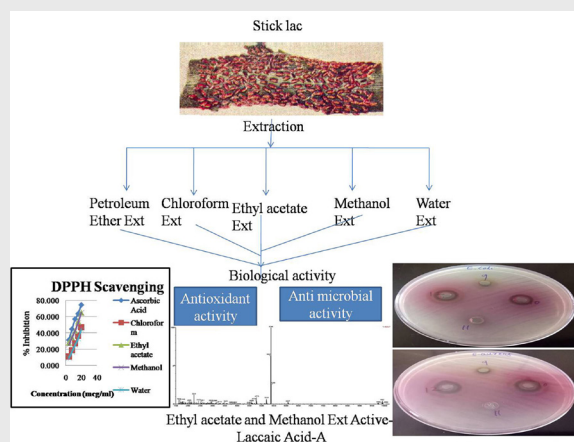
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## PICTORIAL ABSTRACT



## About Authors



**Dr. Manik Ghosh**, Assistant Professor, Department of Pharmaceutical Sciences & Technology, Birla Institute of Technology, Mesra, Ranchi. He has diverse areas of research in the field of Pharmaceutical Chemistry. His work is related to identification of plant extracts having potential antiviral or anticancer activity along with isolation and characterization of phytochemicals. He is also working in the field of analytical & bioanalytical chemistry. He has published many research articles in journals of repute.



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## Summary

Antioxidant activity of extracts of lac dye was evaluated by three different methods (DPPH, Reducing power and Hydrogen peroxide scavenging) and the results were quit comparable with the standard ascorbic acid. For the first time antimicrobial activity was carried out for different extracts of lac dye against gram-positive bacteria *Staphylococcus aureus* and gram-negative bacteria *Escherichia coli* and compared against the standard drug *amoxicillin* using well diffusion method. The bioactive fraction (ethyl acetate and methanol) was further processed for an effective LC-MS/MS run for the identification of active component (laccaic acid-A) responsible for the activity.

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