



ANTIBIOTIC ACTIVITY OF 2, 3-DIHYDROXYBENZOIC ACID ISOLATED FROM *FLACOURTIA INERMIS* FRUIT AGAINST MULTIDRUG RESISTANT BACTERIA

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ABSTRACT

In recent years, great numbers of research activities around the world are going on for isolating effective, non-resistant antibiotic for developing new antibacterial drug. In the present study, an antibacterial phenolic compound 2, 3-dihydroxybenzoic acid was isolated from the fruit extract of *Flacourtia inermis* Roxb by chromatographic techniques and characterized by spectroscopic analysis. Its antibacterial activity was tested against multidrug resistant MTCC bacterial strains by disc diffusion method and the activity was compared with standard antibiotics. Results showed that 2, 3-dihydroxybenzoic acid is an effective antibacterial agent against the tested strains. Among the tested strains, increased susceptibility was given by *Serratia marcescens* followed by *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Klebsiella pneumoniae*. Study showed that the isolated 2, 3-dihydroxybenzoic acid of the *Flacourtia inermis* fruit is an effective antibiotic against multi-drug resistant bacterial strains.

Key words: *Flacourtia inermis*, Loika, Lavalolika, 2, 3-dihydroxybenzoic acid, 2, 3-DHB, antibacterial, multidrug resistant strains, antibiotics.

INTRODUCTION

Since the last 50 years, science community has paid great interest for isolating natural compounds from plants as a remedy for antimicrobial treatment in place of synthetic drugs ¹. The main advantages of plant derived natural drugs are a) they are non-toxic to man because the mechanism and cellular organization of plants and animals are similar in many aspects, b) less expensive when comparing synthetic drugs and d) most of them possess manifold health protecting properties ^{2, 3}. For example, 'resveratrol', a phenolic acid found in grapes and other berries possesses anti-inflammatory, antioxidant, cardio protective and anticancer properties ^{4, 5, 6}.

In pharmacology, the natural products may serve as an immediate source of drug, or an improvising agent for preparing *de novo* drugs or may serve as an initial molecule for developing drugs after some chemical modifications ⁷. Therefore, natural products have been getting significant attention all around the world. Bacteria, fungi and protozoa are the major infectious agents account for major cause of mortality in developing countries ^{8, 9, 10}.

Although the scientific knowledge of pathogens, their interaction with host and prophylactic measures are greatly advanced, the phenomenon of drug resistance by pathogens still make a serious challenge in medicine ^{11, 12}. For developing non-resistant drug, emphasis is giving for the isolation of natural product as a source of therapeutic agent

Flacourtia inermis Roxb is a tree of Flacourtiaceae family. It is very common in the village areas of Kerala State-India where its vernacular name is Loika or Lavalolika. Its fruits are edible and have only secondary importance in the State. Due to the lack of scientific knowledge, none of its part – fruit, leaf, bark, or root is used in medicine. To substantiate its medicinal importance especially in antibacterial therapy, present study has mainly focused on isolation of antibacterial compound from fruit extract of *Flacourtia inermis* against multidrug resistant bacterial strains.

MATERIALS AND METHODS

Isolation and purification of antibacterial compound from the fruit of *Flacourtia inermis* were done by Soxhlet extraction followed by chromatography. For this, fresh fruits were collected from village areas of Kottayam District, Kerala State-India. They were washed with water, saline water followed by distilled water. Then they were wiped dry and cut into small pieces, dried in hot air oven at 60 °C and were powdered in a mixer grinder. 250 gram of the dried powder was serially extracted with 500 ml each of petroleum ether,

chloroform and acetone using a Soxhlet extractor. When these crude extracts were tested for antibacterial activity by Disc Diffusion Method, only the acetone extract was found to be active, and therefore, this fraction was used for further studies. The acetone fraction was concentrated on a vacuum rotary evaporator.

Thin Layer Chromatography (TLC)

For TLC, activated silica gel was layered over the surface of a clean glass plate of 10 cm X 5 cm size. A mixture of chloroform-methanol in the ratio 85:15 was used as the mobile phase. Five obvious spots were identified on the plate. Based on TLC study, a mixture of chloroform-methanol in the ratio of 85:15 was identified as the mobile phase for column chromatography.

Separation of active compound by column chromatography

The size of the column was 45 cm x 3 cm. The activated silica gel of 60-120 mesh size was made into slurry in chloroform and this was used as the solid phase. The acetonic extract loaded on the top of the column was then eluted out with chloroform-methanol in the ratio 85:15. The component fractions moved down were collected separately, concentrated by evaporation and the antibacterial activities of different fractions were tested using standard strains. Since impurities were observed on TLC analysis, the active fraction was again subjected to stepwise gradient elution using chloroform-methanol mixture.

The fraction eluted with chloroform-methanol in the ratio of 90:10 was found to be pure on TLC and was active during antibacterial assay. The yield of biologically active purified compound from *Flacourtia inermis* is 1.22% of the dried fruit powder. This data shows that a significant amount of antibacterial compound is present in its fruits.

Spectroscopic analysis of the antibacterial compound of *Flacourtia inermis* fruit

Gas Chromatography and Mass Spectrometry (GCMS)

GCMS was recorded by JEOL GCMATE II, using acetone as the solvent. A base peak at M/e 137.1 and a molecular ion peak at 154.1 were obtained for the compound.

CHN analysis

CHN analysis was carried out by Elementar Vario EL III. Analysis showed that the purified compound contains 54% C, 41.5 % O and 3.8 % H.

Ultraviolet-Visible (UV-VIS) Spectral Analysis

The UV-visible spectrum was recorded on a Shimadzu-160 UV- Vis spectrophotometer operating in the range 190-1100 nm. Two peaks at 294 nm and 269 nm correspond to the carbonyl group and conjugated pi skeleton in the compound.

Infrared (IR) Spectral Analysis

The IR spectrum was recorded on a Shimadzu IR 470 spectrophotometer by the KBr pellet method in the operating frequency range 4000-400 cm^{-1} . In the IR absorption peak at 3204 cm^{-1} , 1675 cm^{-1} , 1598 cm^{-1} , 1296 cm^{-1} and 1126 cm^{-1} represent -OH group, C=O group, and bonds such as C=C, C-C and C-O respectively.

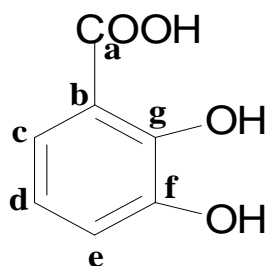
Nuclear Magnetic Resonance(NMR) spectroscopy

The ^1H NMR spectrum was recorded on a Bruker DRX500 FT-NMR spectrometer at 500 MHz. Deuterated acetone was used as solvent and tetramethylsilane (TMS) served as an internal standard. The peaks at δ 9.518 and δ 3.45 indicate -CHO or -COOH proton and -OH proton respectively.

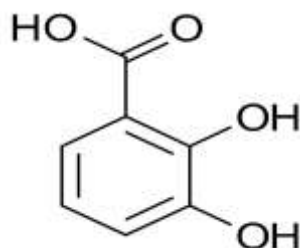
The integral value (peak area) shows the presence of two -OH groups in the compound. The two doublets at δ 6.880 and δ 7.519 and a double doublet at δ 7.449 and δ 7.469 describe the presence of aromatic or benzene ring with two adjacent -OH groups.

The molecular formula obtained from elemental analysis and mass spectral studies is $\text{C}_7\text{H}_6\text{O}_4$. So, the presence of -COOH is evident as the third substituent in the benzene ring. Therefore, based on these data, it can conclude that the unknown compound has a -COOH group with two -OH groups at the 2, 3 positions in the aromatic ring.

The ^{13}C -NMR spectrum shows a signal at 167.07 ppm and it corresponds to a C=O carbon (Ca) of the -COOH group. Signals are also obtained at 149.97, 144.73, 122.70, 122.17, 116.56 and 114.77 ppm indicate the presence of other six carbon atoms (Cb, Cc, Cd, Ce, C_f, and Cg) of the benzene ring.



Based on the data obtained from elemental analysis, GC-MS analysis, and FT IR, UV-VIS, ^1H NMR and ^{13}C NMR, the antibacterial compound purified from *Flacourtia inermis* fruit is 2, 3-dihydroxybenzoic acid, a phenolic compound with a molecular formula $\text{C}_7\text{H}_6\text{O}_4$



2, 3-dihydroxybenzoic acid

The purified 2,3-DHB of *Flacourtia inermis* was tested for antibacterial activity against multidrug resistant bacteria and the activity was compared with that of standard antibiotics.

Preparation of disc

For a quantitative analysis four discs with known quantity of 2,3-DHB were prepared. The concentration of each disc was 1mg, 2.5mg, 5mg and 10mg respectively.

Bacterial strains

MTCC strains purchased from Institute of Microbial Technology, Chandigarh (IMTECH), India were used for the study. They were.

Staphylococcus aureus, sub species *aureus* (MTCC 96)

Escherichia coli (MTCC 443)

Pseudomonas aeruginosa (MTCC 741)

Serratia marcescens (MTCC 97)

Klebsiella pneumoniae, sub species *pneumoniae* (MTCC 109)

Antibiotics used for comparison

Following eight general antibiotics purchased from Hi- media Laboratories Ltd. Mumbai, were selected for the sensitivity study. They are;

1. Amikacin (30 mcg)

2. Ampicillin (10mcg)

3. Cloxacillin (1 mcg)

4. Erythromycin (15 mcg)

5. Chloramphenicol (30 mcg)

6. Penicillin-G (10 u)

7. Streptomycin (10 mcg)

8. Gentamicin (10 mcg)

Culture media

The dehydrated Muller Hinton Agar (MHA) medium purchased from Himedia Laboratories Pvt.Ltd. Mumbai, India was used. The medium was rehydrated, sterilized in an autoclave and was poured into sterilized petri dishes and allowed to set. The plates were stored at 4 - 10 °C in refrigerator. Before inoculation, the surface of the petriplates was dried in an incubator.

Antibacterial assay by Disc Diffusion Method

The antibacterial activity and antibiotic sensitivity were tested by Disc Diffusion Method [13]. The dried plates were inoculated by test strains uniformly over the surface using a sterile cotton swab.

A sterile 6 mm Whatmann No.1 filter paper loaded with appropriate concentration was placed on the surface of the inoculum and gently pressed by a sterile forceps. The plates were incubated at 37 °C for 16 to 20 hrs. The zone of inhibition of bacterial growth around the disc was measured in millimeters. The experiment was repeated for three times and the average values were recorded.

For antibiotic comparison, sample concentration of 5mg/disc was selected because other concentrations were found to be less effective against the tested strains. The disc along with antibiotics were placed on the lawn culture, incubated for 16-18hrs, and zone of inhibition was measured if sensitive or represented as 'R' if resistant.

RESULTS

Table 1 shows the effects of different concentrations of 2,3-DHB against standard strains. Results indicate that the 2, 3-DHB at a concentration of 1mg/disc was active, but the activity was not significant. Disc concentration of 2.5mg showed moderate activity, where as 5mg/disc and 10mg/disc showed powerful activity against the tested strains. Study reveals that the zone of inhibition increases when the amount of 2, 3- DHB increases.

Among the strains, *Serratia marcescens* showed highest susceptibility followed by *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Klebsiella pneumoniae*.

The activity of 2,3-DHB is compared with that of standard antibiotics and the results are given in Table 2. Analysis of the results showed that the 2, 3-DHB is powerful against the tested strains. All the

Table -1: Shows The Antibacterial Activity of 2, 3-Dhb Against Multidrug Resistant Strains

S. No.	Bacterial Strains	Zone of inhibition(in mm) by different concentrations of 2, 3-DHB			
		1 mg	2.5 mg	5 mg	10 mg
1	<i>Serratia marcescens</i>	7	18	25	27
2	<i>Escherichia coli</i>	7	11	18	20
3	<i>Pseudomonas aeruginosa</i>	0	11	17	18
4	<i>Klebsiella pneumoniae</i>	7	10	14	15
5	<i>Staphylococcus aureus</i>	7	10	11	16

Table -2: Shows The antibiotic Comparison of 2, 3-Dhb Against Multidrug Resistant Strains

Strains	Average diameter of zone of inhibition by 2, 3-DHB (in mm)	Diameter of zone of inhibition by antibiotics (in mm)								No. of antibiotics resisted
		5mg/disc	1*	2*	3*	4*	5*	6*	7*	
<i>Escherichia coli</i>	16	22	9	R†	R	20	R	12	21	3
<i>Staphylococcus aureus</i>	22	22	R	30	R	33	R	23	25	3
<i>Klebsiella pneumoniae</i>	18	17	R	R	R	28	R	19	R	5
<i>Pseudomonas aeruginosa</i>	17	16	R	R	R	15	R	17	18	4

1* Amikacin; 2* Ampicillin; 3* Cloxacillin; 4* Erythromycin; 5* Chloramphenicol; 6* Penicillin-G; 7* Streptomycin; 8* Gentamicin; R†:-resistant.

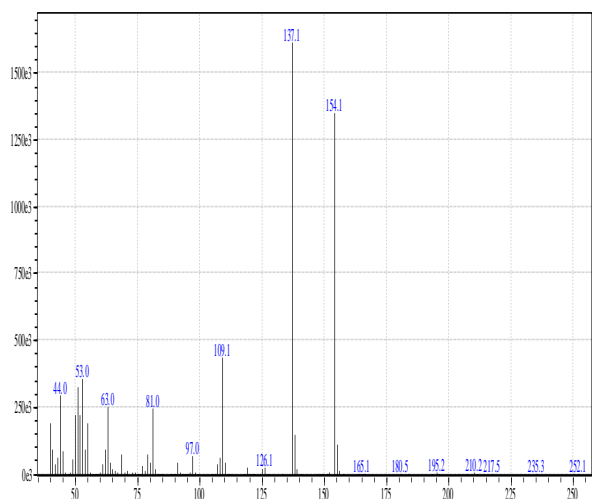


Figure -1: Mass spectrum of the purified compound

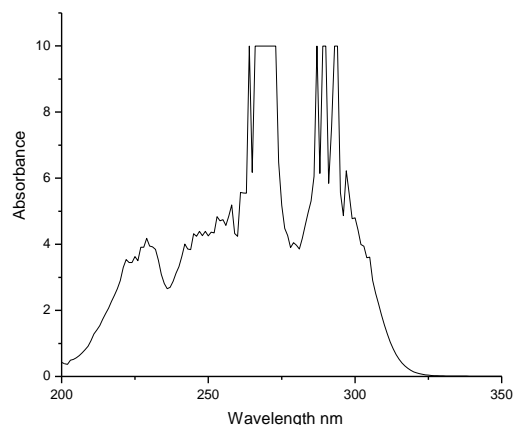


Figure -2: UV visible spectrum of the purified compound

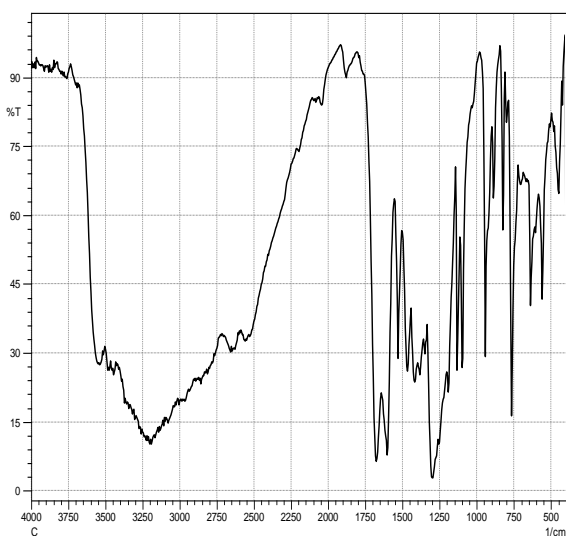


Figure -3: IR spectrum of the purified compound

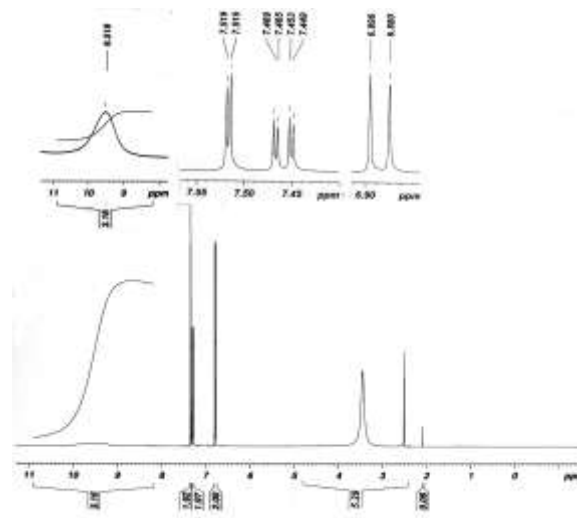


Figure -4: ¹H NMR of the purified compound

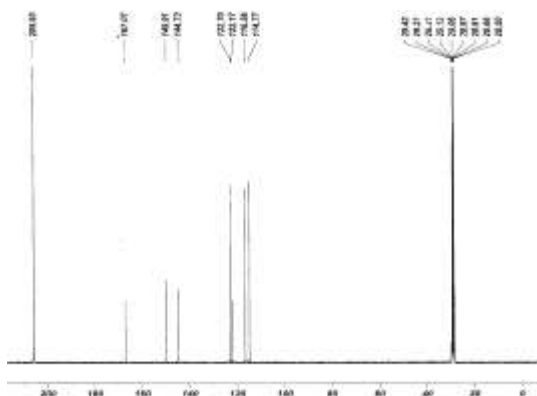


Figure -5: ¹³C NMR of the purified compound

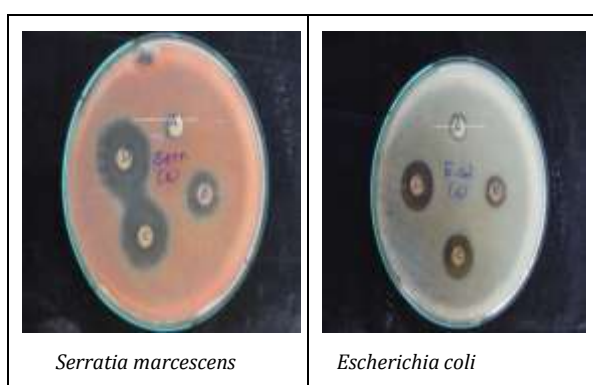


Figure -6: Antibacterial activity of different concentrations of 2,3-DHB isolated from *Flacourtia inermis* against standard multidrug resistant strains

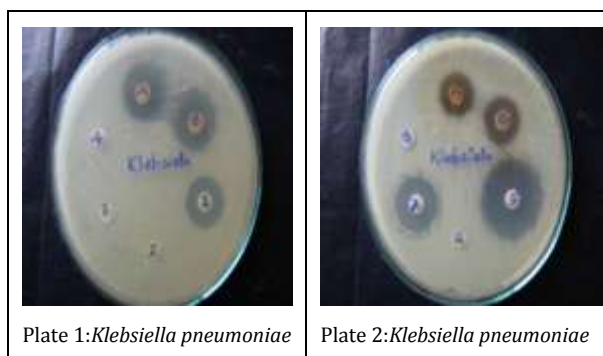


Figure -7: Antibiotic sensitivity and its comparison with 2,3-DHB of *Flacourtia inermis* against standard multidrug resistant strains
A: - Disc concentration of 5mg; B: - Disc concentration of 10mg; 1. Amikacin; 2. Ampicillin; 3. Cloxacillin; 4. Erythromycin; 5. Chloramphenicol; 6. Penicillin-G; 7. Streptomycin; 8. Gentamicin.

RESULTS

Table 1 shows the effects of different concentrations of 2,3-DHB against standard strains. Results indicate that the 2, 3-DHB at a concentration of 1mg/disc was active, but the activity was not significant. Disc concentration of 2.5mg showed moderate activity, where as 5mg/disc and 10mg/disc showed powerful activity against the tested strains. Study reveals that the zone of inhibition increases when the amount of 2, 3- DHB increases.

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The activity of 2,3-DHB is compared with that of standard antibiotics and the results are given in Table 2. Analysis of the results showed that the 2, 3-DHB is powerful against the tested strains. All the tested strains showed resistance to three or more antibiotics, which implies that the tested strains were multidrug resistant strains.

Among the resistant strains, *Klebsiella pneumoniae* showed resistance to five out of the eight antibiotics tested. *Klebsiella pneumoniae* is a dangerous pathogen and is responsible for pneumoniae and other kind of hospital acquired infectious diseases. Even though it is resistant to common antibiotics, it showed complete susceptibility to the tested compound. It is noticed that the activities of Amikacin and Streptomycin are equal to that of 5mg of 2, 3-DHB. Therefore, the 2,3-DHB is as effective as Amikacin or Streptomycin for treating *Klebsiella pneumoniae*.

Escherichia coli showed resistance to three antibiotics, but it was completely susceptible to the 2,3 -DHB. Even the sensitive antibiotics, Ampicillin and Streptomycin were less active than the tested samples. A sample concentration of 5mg/disc of 2,3-DHB can cause much higher activity than Ampicillin and Streptomycin against the multidrug resistant *E.coli*.

Staphylococcus aureus showed resistance to three antibiotics tested. However, 2,3-DHB is effective even at a concentration of 5 mg/disc against it.

Pseudomonas aeruginosa is another multidrug resistant bacterium that showed resistance to four antibiotics. Its sensitivity towards Amikacin, Chloramphenicol, Streptomycin and Gentamicin were equal to that of 5mg/disc concentration of the tested sample. Hence, in antibacterial treatment for *Pseudomonas aeruginosa*, the 2,3-DHB can be used as an alternative for Amikacin, Chloramphenicol, Streptomycin and Gentamicin.

DISCUSSION

Since the first use of antibiotics, microorganisms have developed a natural phenomenon called drug resistance and it makes a serious challenge in antimicrobial therapy. The effective antibiotics of the early days become resistant by most of the infectious organisms [14]. Hence, discovery of new, non-resistant antibiotics are necessary [15]. In this study, 2, 3-DHB isolated from fruit extract of *Flacourtia inermis* showed effective antibacterial activity against multidrug resistant strains. Previous reports are not available on its antibacterial activity. However, its antioxidant property was extensively studied [16]. Reports reveal that 2,3-DHB is very effective for reducing the oxidative tissue damaging effects of other antibiotics [17, 18, 19].

2, 3-DHB is a simple phenolic compound. Its presence is appreciably high in the fruit of *Flacourtia inermis* and it can be easily isolated and purified from the fruit without much expense. Structurally simple compounds are easy to metabolize in the human cell. Therefore, 2,3-DHB isolated from *Flacourtia inermis* has greater significance for antibacterial therapy.

CONCLUSION

From the acetonic extract of the *Flacourtia inermis* fruit, a phenolic compound named 2,3-dihydroxybenzoic acid was isolated by chromatographic techniques. It was found to be an excellent antibacterial agent against multidrug resistant strains. Previous reports show that this compound is an effective antioxidant. Based on the present study, it can be considered as an effective 'antibiotic' against pathogenic bacteria.

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