ORIGINAL PAPER

Isolation and characterization of p-Coumaric acid from *Diospyros melanoxylon* medicinal plant endemic to Western Ghats, India

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ABSTRACT

Diospyros melanoxylon has been traditionally for the treatment of fistula, relieving arthritis, and skin care. The bark extracts of the plant have been investigated in terms of phytochemical and pharmacological potential, while the leaf extract has been untapped. The present study aimed to evaluate the methanol extract of *D. melanoxylon* (DMM) in terms of the antibacterial ($P<0.05$), antioxidant (1.2-1.6-fold) and anti-inflammatory potential (IC_{50}: 80 µg/mL). DMM exhibited effective antibacterial, antioxidant, and anti-inflammatory activities at significantly higher levels than the standards. In addition, the HR-LCMS analysis of MBI revealed the presence of a few active compounds, which belonged to the class of phenolic acids and flavonoids at greater concentrations than other phytochemicals ($n>20$). The activity-guided repeated fractionation of the methanol extract using silica gel column chromatography yielded a single compound, which exhibited remarkable antioxidant activity. The physicochemical and spectroscopic analyses (UV, IR, 1 H NMR, 13 C NMR, and MS) indicated that the bioactive isolated compound was p-coumaric acid, the effect of which was on par with the standard antioxidant, antibacterial, and anti-inflammatory drugs. Conversely, the effects of the extract on these pharmacological attributes enhanced, confirming that the better activity observed in the study was mainly due to the synergistic effects exerted by various compounds in the extract. *In-silico* studies have also confirmed the potential of the compound in these effective antibacterial properties. Therefore, the *D. melanoxylon* extract is a strong therapeutic agent with pharmacological potential.

Keywords: Phenolic acids, p-Coumaric acid, Methanol extract, Free radicals, Anti-inflammatory

Introduction

Diospyros melanoxylon is an endemic species of the Western Ghats region in India and Sri Lanka, which belongs to the Ebenaceae family. This plant was first described by William Roxburgh in his book Flora Indica.[1] Most of the species in this family are a valuable source of wood and ornamental flowers. Most species of the *Diospyros* genus are mainly confined to the tropics, with approximately 300 species found in Asia.[2] India alone houses 66 species belonging to this genus, which are known for their therapeutic potential.[3] The World Conservation Monitoring Centre has recorded 12 major biodiversity hotspots, among which India contains two, including Western Ghats and Eastern Ghats, containing 15,000-18,000 flowering plants.

Among various regions of the Western Ghats, the Shimoga district of the Malnad ranges is less exploited, which is located in the heart of the Western Ghats region in the Karnataka state; it is one of the biodiversity hotspots in India. In our previous study, a thorough screening of various plants in this region was performed, providing evidence on the least exploited species. In the current research, we aimed to evaluate the therapeutic potential of *D. melanoxylon*, which is extensively found in the Western Ghats region

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of the Southern India. Apart from its use as timber and a material to wrap beedi, this endemic species is known for its numerous pharmacological properties (antioxidant, antidiabetic, and nephroprotective properties), which are mainly attributed to the presence of polyphenols, flavonoids, alkaloids, and anthocyanins.[4] Traditionally, *D. melanoxylon* has been used for the treatment of arthritis, relieving fistula and abdominal pain, and skin care.

Large amounts of phenolics, alkaloids, steroids, ascorbic acid, tannins, and saponins have been observed in the bark extract of *D. melanoxylon*.[5] Formulations prepared from the leaves of the plant have also been examined in terms of the effects on the improvement of intestinal flatulence and digestion issues. In the present study, the optimum activity among various bioactive compounds in the extracts of *D. melanoxylon* was observed in the extract containing p-coumaric acid. This secondary metabolite is a phenolic acid belonging to the hydroxycinnamic acid family, and p-coumaric acid is essential to secondary metabolism since it could be converted into phenolic acid, flavonoid, and lignin derivatives.[6, 7]

The chemoprotectant and the antioxidant potential of p-coumaric acid have attracted the attention of researchers to identify the plants that could be used as potential sources for the extraction of p-coumaric acid.[8] Furthermore, p-coumaric acid is known for its potent antibacterial activity against a broad spectrum of pathogens in synergy with other compounds.[9]

Based on the previous reports and a preliminary phytochemical screening in our study, the present study aimed to describe the isolation and characterization of p-coumaric acid from the bark of *D. melanoxylon* and assess its potential as an antimicrobial, antioxidant, and anti-inflammatory agent.

Materials and Methods

Plant materials and extraction

Fresh, healthy leaves of *D. melanoxylon* were collected from the Western Ghats in Shimoga district, located in Karnataka, India during May 2017. The leaves were washed, shade dried, and milled using a 50 mesh.

Isolation and identification of the bioactive compound

At this stage, 500 g of the dried leaves were used to extract the bioactive compound sequentially using the solvents of increasing polarity *viz.,* petroleum ether, chloroform, ethyl acetate, acetone, methanol, and water. Afterwards, the diverse extracts were filtered, and the solvent was excluded using a rotary evaporator (model: Rotavapor R-200, Buchi, Switzerland). The concentrated extracts were assayed in terms of the pharmacological activities *viz.* and antibacterial, anti-inflammatory, and radical-scavenging properties (DPPH, ABTS, and superoxide). Since the yield (145 g/kg) and radical scavenging activities of the methanol extract were higher, the extract was selected for the isolation of the bioactive compound. The phytochemical screening for the methanol extract was also performed in accordance with the standard protocols as described by Harborne.[10] In order to isolate the bioactive compound, 50 grams of *D. melanoxylon* methanol extract (DMM) was dispersed in water and successively extracted twice, each time using petroleum ether, ethyl acetate, and n-butanol to obtain the yield of petroleum ether (6.3 g), ethyl acetate (7.9 g), n-butanol (3.7 g), and H_2O-soluble (15 g) fractions. The subsequent fractions were subjected to pharmacological activities, while the activities of ethyl acetate soluble fraction (ESF) were applied for the isolation of the bioactive compound.

At the next stage, the ESF was subjected to silica gel column chromatography (100-200 mesh, 1.5 kg; length: 80 cm, diameter: 7 cm; elution rate: 2 mL/min, flow total elution: 500 mL) and eluted using a gradient of dichloromethane (methanol 100:1 [5 l], 90:10 [10 l], 80:20 [5 l], 70:30 [10 l], 60:40 [5 l], 50:50 [5 l], 25:75 [5 l], 0:100 [3 l]) in order to acquire fractions ESF 1 (2.2 g), ESF 2 (4.0 g), ESF 4 (6.6 g), ESF 4 (3.5 g), ESF 5 (6.9 g), ESF 6 (5.3 g), ESF 7 (4.4 g), and ESF 8 (5.7 g). Fractions ESF 1, ESF 2, and ESF 5 exhibited potent

biological activities with the following order: ESF 5>ESF 1>ESF 2. As a result, fraction ESF 5 was subjected to the silica gel column chromatography (length: 50 cm, diameter: 3 cm; elution rate: 1 mL/min; flow total elution: 100 mL) and eluted with the linear gradients of petroleum ether (acetone [95:5, 90:10, 85:15, 80:20, 70:30, and 60:40 v/v]) to obtain six major sub-fractions. Sub-fraction three was further separated using the silica gel CC and petroleum ether acetone (85:15), followed by re-chromatography on a Sephadex LH-20 column with acetone as the eluting solvent to obtain p-coumaric acid as the single compound (24.5 mg).

Thin-layer chromatography

The collected fractions were spotted onto silica gel F254 plates (25×25 cm; Merck, Germany). The solvent system contained chloroform, methanol, and formic acid (85:10:5 v/v/v), revealing optimum separation. Following that, the developed plates were air dried, and the spots were visualized by spraying a ferric chloride solution (0.5% w/v). The retention factor (R_f) of the isolated compound (R_f=0.79) and standard value (R_f=0.79) were observed to be similar when calculated and compared.

Identification of the bioactive compound using analytical methods

The melting points were determined on an electrically heated VMP-III melting point apparatus in an uncorrected manner. The infrared (IR) spectra were recorded on the NICOLET 380 FT IR spectrometer (Thermo Fisher Scientific, France) using potassium bromide (KBr) pellets. In addition, the nuclear magnetic resonance (NMR) spectra was recorded on a Bruker DRX-400 spectrometer (Bruker Biospin Co., Karlsruhe, Germany) with 1H NMR at 400 MHz and 13C NMR at 100 MHz. Following that, the isolated compound was prepared using deuterated methanol (99.8 atom% of deuterium) and tetramethylsilane (TMS) as an internal standard in five-millimeter NMR tubes.

Data were measured in CDCl3 with chemical shifts based on the TMS signal and expressed in parts per million (δ). The ultraviolet spectrum of p-coumaric acid in methanol was also recorded using a Shimadzu UV-1800 spectrophotometer. The elemental analysis of the compound was performed using the Perkin-Elmer 2400 elemental analyzer, and the mass spectrum was recorded using the Q-TOF Waters Ultima instrument (QTOF GAA 082, Waters, Manchester, UK) with an electron spray ionization source. The positive ion mode with the spray voltage of 3.5 kV at the source temperature of 80 °C was set to obtain the spectra, and the mass spectra were recorded under electron impact ionization at the energy of 70 eV. The sample was prepared within the concentration range of 0.25-0.50 mg/mL and injected using the flow analysis at the flow rate of 10 µL/min; the recorded mass was within the range of 100-500 m/z.

Determination of total phenol

The total phenolic content (TPC) of the methanol extract and fractions was determined using the Folin-Ciocalteu method[11] and expressed as gallic acid equivalents (GAE) in mgs per gram of the sample.

Determination of total flavonoids

The total flavonoid content (TFC) of the methanol extract and fractions was determined using the aluminum chloride technique[12] and expressed as quercetin equivalents (QE) in mgs per gram of the sample.

Determination of total proanthocyanidin

The total proanthocyanidin (TCC) content of the methanol extract and fractions was determined using the method described in[13] and expressed as GAE in mgs per gram of the sample.

High-resolution liquid chromatography and mass spectrometry (HR-LCMS) analysis

The fresh, healthy leaves of *D. melanoxylon* were extracted using methanol and subjected to high-resolution liquid chromatography and mass spectrometry (HR-LCMS) analysis, which was carried out at the

Sophisticated Analytical Instrument Facility (SAIF) at IIT Bombay in Mumbai, India. The chemical finger prints of the selected medicinal plant extracts were prepared using the Agilent HR-LCMS (model: G6550A) with 0.01% mass resolution.

The components in the extract were identified and interpreted on the mass spectrum, and HR-LCMS was performed using the database of the SAIF with more than 62,000 patterns. In addition, the spectrum of the unknown component was compared with the known components that were stored in the SAIF library. The name, molecular weight, and structure of the components of the test materials were determined as well.

Antioxidant assays

In the present study, three standard methods *viz.* of DPPH free radical, ABTS cation radical, and superoxide anion radical scavenging activities were determined and measured.[14] Radical scavenging activities were expressed as EC_{50} values, which signified 50% of the free, cation, and anion radicals scavenged by the tested samples. Moreover, the antioxidant butylated hydroxyl anisole (BHA) was used as the positive control.

Antibacterial activity

Three gram-positive bacteria (*Bacillus cereus, Micrococcus luteus,* and *Staphylococcus aureus*) and six gram-negative bacteria (*Klebsiella pneumonia, Enterobacter aerogenes, Escherichia coli, Pseudomonas fluorescens, Pseudomonas aeruginosa*, and *Salmonella enteritidis*) were selected for the study. The bacterial strains were obtained from Institute of Microbial Technology in Chandigarh (MTCC). The bacterial stock cultures were incubated at the temperature of 37 °C for 24 h on nutrient agar and stored in a refrigerator at the temperature of 4 °C.

In order to evaluate the antibacterial activity of DMM, fractions, and p-coumaric acid, the antibacterial agar well-diffusion assay was employed following the methods described previously[15] with slight modifications. The nutrient agar medium was inoculated with nine foodborne pathogenic bacterial strains. Subsequently, the sterile discs (diameter: 6 mm) containing the samples (1 mg/mL) were placed on the inoculated nutrient agar media.

The impregnated discs with diverse samples (prepared with methanol) were dried, placed on the inoculated plates, and incubated at the temperature of 37 °C for 24-48 h. The diameter of the inhibition zone around the disc was measured in millimetres, and the lowest concentration of the nano-compounds required to inhibit the growth of the organisms was estimated based on the minimum inhibitory concentration (MIC). In this process, amoxicillin (1 mg/mL) and methanol were used as the positive and negative control, respectively.

Anti-inflammatory activity

Inhibition of the protein denaturation method

The inhibition of protein denaturation was determined using the described method with minor modifications.[16] To this end, a reaction mixture was prepared with diverse concentrations of the test samples and 1% BSA (aqueous solution), and the pH was set using 1 N HCl. Following that, the reaction mixture was incubated at the temperature of 37 and 58 °C for 25 and 20 min, respectively. The obtained mixture was cooled, and the absorbance was measured at 660 nanometers for turbidity. The protein denaturation inhibitory activity was expressed in the inhibition rate using the Eq. 1:

$$\text{Inhibition (\%)} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \text{X}100 \quad (1)$$

where *A* shows the absorbance. IC_{50} values were determined using the curve of the inhibition rate of the individual sample to the concentration of the sample.

Membrane stabilization test

Membrane stabilization was performed using red blood cells (RBCs) using the described method.[17] To do so, fresh human blood was collected from healthy volunteers in heparinized centrifuge tubes and subjected to centrifugation for 15 min at 2,500 rpm. Afterwards, they were washed thrice with an

equal volume of normal saline solution (0.9%), and a 10% (v/v) suspension was prepared using saline. Furthermore, 2 mL of the reaction mixture was prepared, which contained the test sample (1 mL) and 10% RBC suspension (1 mL). The resulting mixture was incubated at the temperature of 58 °C for 30 min and subjected to centrifugation for 5 min at 3,000 rpm. Following that, the supernatant was separated, and the absorbance was measured at 560 nanometers in order to assess the rate of the membrane stabilization activity. Aspirin was used as the positive control in all the anti-inflammatory assays, and the ability of the sample to stabilize the membrane was calculated using the same equation as the one used for the protein denaturation activity.

Proteinase inhibitory assay

The inhibitory assay for proteinase was performed using the described method[18] with slight modifications. To this end, the reaction mixture was prepared with 0.06 mg of trypsin dissolved in 1 mL of Tris-HCl buffer (20 mM; pH: 7.4) and 1 mL of the test samples. Following that, the resulting mixture was added to 1 mL of 0.80% casein after incubation at the temperature of 37 °C for 10 min. The reaction was terminated by adding 2 mL of 70% perchloric acid after incubation at the temperature of 37 °C for 20 min. Finally, the solution was centrifuged for 20 min at 2,500 rpm, and the absorbance of the supernatant was measured at 210 nanometers against blank (Tris-HCl buffer). Based on the optical density, the inhibition rate was calculated as described for protein denaturation activity.

Molecular modeling study

The binding site of the procured protein structure was analyzed using the ligand explorer of the RCSB PDB server. In this process, the 2D structure of p-coumaric acid was generated using the ChemSketch tool, and the generated 2D structure was saved as a .mol file and used to generate the 3D structure, where hydrogen was added. In addition, 3D coordinates were generated using the Open Babel tool and saved in the PDB format. Additionally, the procured β-lactamase structure from RCSB PDB was further refined by removing the water residues, followed by the addition of gasteiger (-marsili) charges and merging the non-polar hydrogen using AutoDock V.4.0. Upon the refinement of the protein, its structure was selected for rigid molecule, and the 3D structure of the ligand was selected for the map type.

Based on the binding site residues, the grid box was set, where all the binding site residues fit inside the grid box. As a result, the grid box was set with the dimensions of X:48, Y:48, and Z:50. Upon saving the grid, the grid parameter file (gpf) was generated, and the saved grid parameter file was run using Autogrid 4. Upon the successful completion of Autogrid, the molecular docking of the ligand was carried out using the genetic algorithm in AutoDock 4, followed by the generation of the docking parameter file (dpf). Finally, the saved docking parameter file was used to run Autodock 4.

Statistical analysis

The experiments were performed in triplicate, and the results were expressed as mean and standard error (SE). Comparison of the treatment and control groups was performed using one-way analysis of variance (ANOVA) and Duncan's multiple range test in SPSS version 21.0 (Chicago, USA) at the significance level of $P \leq 0.05$. Moreover, Pearson's correlation-coefficient was performed to determine the correlations between the total phenolic, flavonoid, and proanthocyanidin contents and radical scavenging activity of the test samples. GraphPad Prism software version 4.03 was also employed for the calculation of the IC_{50} values.

Results and Discussion

A prospective compound with various biological activities was yielded through the successive solvent extraction, followed by the recurrent silica gel column chromatography of the *D. melanoxylon* leaf powder; such examples were the antioxidant, antimicrobial, and anti-inflammatory activities of the methanol extract, which were selected for further purification using the activity-guided repeated column

chromatography (Table 1). Fig. 1 shows elution with solvents such as methanol and dichloromethane, yielding a bioactive compound in the petroleum ether in the form of acetone fraction (85:15 v/v). The eluted fractions were analyzed using TLC and HPLC, showing 85% purity of the bioactive compound. Moreover, the resilient biological activities of the fraction led to structural elucidation by various spectroscopic methods (UV, IR,[1] HNMR,[13] CNMR, and MS). The elucidation of the structures was as follows:

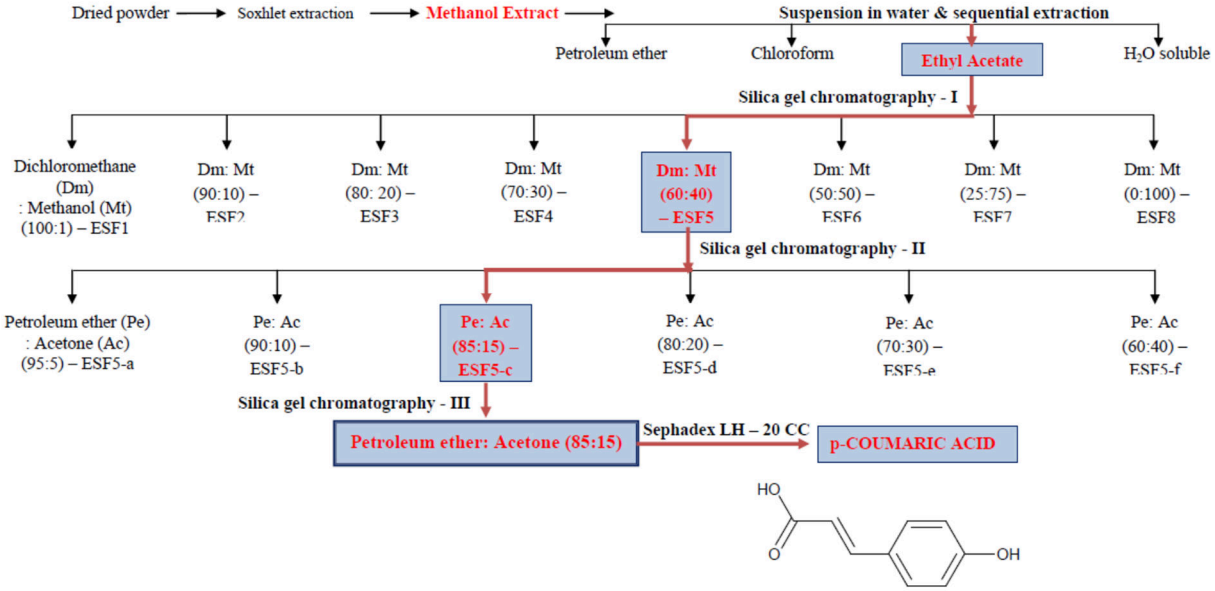


Fig. 1. Separation scheme of p-Coumaric acid from methanol extract of *Diospyros melanoxylon* and its structure

Table 1. Antibacterial activity by disc diffusion for methanol extract of *Diospyros melanoxylon* leaves (DMM), diverseethyl acetate soluble fractions (ESF) and isolated compound p-Coumaric acid (PC)

	Zone of inhibition* (in "mm")					
	Std.	DMM	ESF	ESF5	ESF5-c	PC
Gram positive						
Bacillus cereus	11.02 ± 0.09	16.05 ± 1.33	11.34 ± 1.04	09.64 ± 1.11	07.35 ± 0.13	05.67 ± 2.23
Micrococcus luteus	14.25 ± 0.06	10.13 ± 1.05	07.86 ± 0.36	07.19 ±0.31	05.04 ± 0.03	03.19 ±3.16
Staphylococcus aureus	18.67 ± 0.09	20.13 ± 0.86	18.43 ± 1.07	18.00 ±2.22	14.61 ± 0.49	11.22 ± 0.33
Gram negative						
Klebsiella pneumoniae	11.08 ± 0.15	10.27 ± 1.17	10.01 ± 1.14	05.88 ± 0.61	5.67 ± 0.15	4.98 ± 0.15
Enterobacter aerogenes	15.25 ± 0.88	10.21 ± 0.91	08.84 ± 0.18	07.54 ± 0.28	06.56 ± 0.90	03.33 ± 0.25
Escherichia coli	32.04 ± 0.53	34.31 ± 1.78	33.16 ± 1.25	33.03 ± 0.18	28.83 ± 0.42	25.15 ± 1.17
Pseudomonas fluorescens	18.09 ± 0.31	08.51 ± 2.69	08.04 ± 2.03	07.66 ± 0.09	05.50 ± 3.10	03.17 ± 1.12
Pseudomonas aeruginosa	25.06 ± 0.80	10.06 ± 3.46	08.55 ± 0.10	05.65 ±0.16	05.58 ± 0.25	03.95 ± 0.10
Salmonella enteritidis	18.40 ± 0.32	20.91 ± 1.55	20.81 ± 1.57	17.01 ± 044	16.24 ± 0.24	16.01 ± 0.17

* Values are expressed as mean ± SE. (Std.): Amoxicillin; (–): inactive

p-coumaric acid: (E)-3-(4-hydroxyphenyl) acrylic acid

Buff crystalline solid, melting point: 208-210 °C; IR (KBr): 1680 (C=C), 1,750 (COOH), 3,650 cm^{-1} (phenolic-OH); ^{1}H NMR (CDCl3): δ 4.5 (bs, 1H, Ar-OH), 6.5 (d, J=7.5 Hz, 1H, COCH), 6.8-7.2 (m, 4H, Ar-H), 7.4 (d, J=7 Hz, 1H, Ar-H);[13] C NMR (DMSO-d6): δ 115.4, 115.8 (2), 127.9 (3), 148.1, 157.6, 170.5; LC-MS: m/z 165 (M+1); analytical calculated data for $C_9H_8O_3$(164): C, 65.85; H, 4.91; found: C, 65.89; H, 4.96%.

The identity of the compound was deciphered based on the mentioned results and after comparison with the NMR and MS data as reported in the literature.[19-21]

Antibacterial activity

Tables 1 and 2 show the antibacterial activity (disc-diffusion) and MIC values exhibited by the DMM, diverse ESF, and p-coumaric acid isolated compound against nine food pathogens. Methanol was considered as the negative control and could not inhibit the growth of the tested bacterial strains. The tested samples revealed dissimilar antibacterial activity, and the potency of the antibacterial activity varied depending on the species of the bacterial strains, diverse ethyl acetate soluble fractions, methanol extract, and isolated compound. In general, DMM exhibited potent inhibitory activity compared to the diverse soluble fractions and isolated compounds. Moreover, the antibacterial activity of MBI (1 mg/mL) was on par with the standard antibiotic activity of amoxicillin, which was used as the positive control.

The results obtained from the agar well-diffusion method were used to determine the MIC values (Table 2; Fig. 2.) In terms of the MIC values (mg/mL), DMM evidently possessed potent inhibition capacity in case of the gram-positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*) and gram-negative bacteria (*Escherichia coli* and *Salmonella enteritidis*), and the effect was more significant compared to amoxicillin ($P<0.05$). In general, the MIC values of DMM, isolated p-coumaric acid, and all the tested fractions were within the range of 0.80-7.99 mg/mL.

Table 2. The minimum inhibitory concentration (MIC) for methanol extract of *Diospyros melanoxylon* leaves (DMM), diverse ethyl acetate soluble fractions (ESF) and isolated compound p-Coumaric acid (PC)

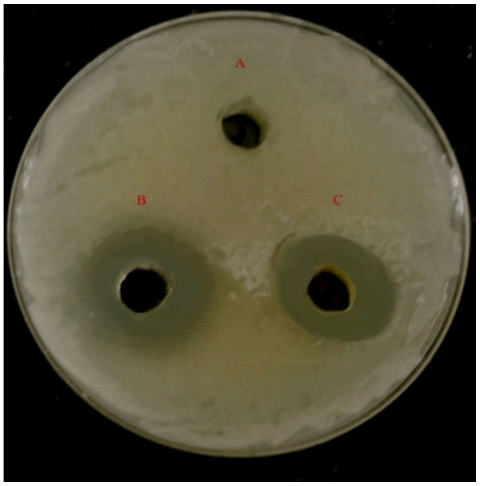
	MIC* (in "mg/mL")					
	Std.	DMM	ESF	ESF5	ESF5-c	PC
Gram positive						
Bacillus cereus	1.34 ± 0.61	1.05 ± 0.25	1.30 ± 1.10	1.77 ± 0.13	3.78 ± 0.65	3.85 ± 0.55
Micrococcus luteus	2.50 ± 0.26	2.67 ± 0.21	3.05 ± 1.08	4.50 ± 0.84	4.61 ± 3.44	6.28± 1.63
Staphylococcus aureus	3.10 ± 0.12	2.77 ± 2.47	2.98 ± 0.41	3.33 ± 2.19	3.25 ± 1.54	3.90 ± 1.88
Gram negative						
Klebsiella pneumoniae	4.05 ± 0.38	4.58 ± 1.89	4.98 ±0.02	6.06 ± 1.14	7.02 ± 0.82	7.76 ± 1.40
Enterobacter aerogenes	1.75 ± 0.25	1.85 ± 0.43	2.01 ± 0.48	2.69 ± 0.20	4.44 ± 2.31	5.15 ±5.65
Escherichia coli	1.00 ± 0.20	0.80 ± 0.35	0.94 ± 0.58	1.34 ± 2.27	1.47 ± 0.15	1.77 ± 0.38
Pseudomonas fluorescens	3.08 ± 0.95	4.74 ± 1.06	5.31 ± 2.32	6.59 ± 0.32	7.86 ± 1.24	7.99 ± 1.05
Pseudomonas aeruginosa	2.50 ± 0.04	3.61 ± 0.24	4.83 ± 0.40	5.55 ± 0.58	5.97 ± 0.46	6.56 ± 0.71
Salmonella enteritidis	1.95 ± 0.45	1.34 ± 0.79	1.55 ± 1.22	2.54 ± 1.11	2.64 ± 1.24	3.00 ± 2.50

* Values are expressed as mean ± SE. (Std.): Amoxicillin

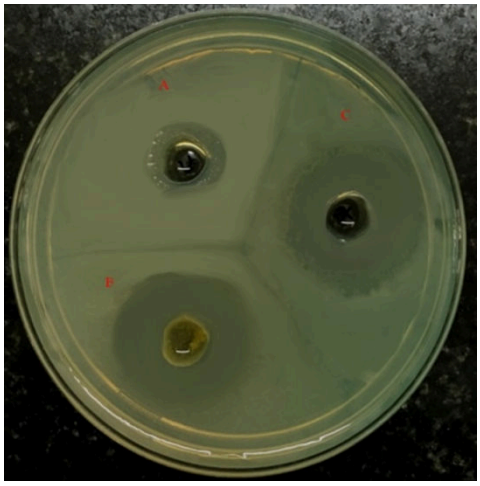
Previous studies have clearly demonstrated that the antibacterial effects of p-coumaric acid are exerted through the dual damage mechanism, which involves the increased permeability of the membrane, along with binding to the phosphate group of DNA.[22] In addition, some studies have reported that the derivatives of p-coumaric acid have higher antibacterial activity compared to the compound alone.[23-25] According to the results of the present study, the activity of the extract was more prominent compared to the compound alone, which suggested that the collective effects of various phytochemicals in the extract are responsible for the its potent antibacterial activity.

In the current research, the antibacterial activity of *D. melanoxylon* was recorded against *Klebsiella*, *Pseudomonas*, *E. coli*, and *S. aureus*, and the plant extract that was dissolved in bloodless and warm water showed variable antibacterial effects against in these bacterial pathogens. The leaf extract of *D. melanoxylon* changed *in-vitro* against *Klebsiella*, *Pseudomonas*, *E. coli*, and *S. aureus*. In addition, the exhibited inhibition zones (mm) were listed in the inhibition sector using the methanol herbal extract, becoming subtracted from the inhibition zone. The leaf extract of *D. melanoxylon* that

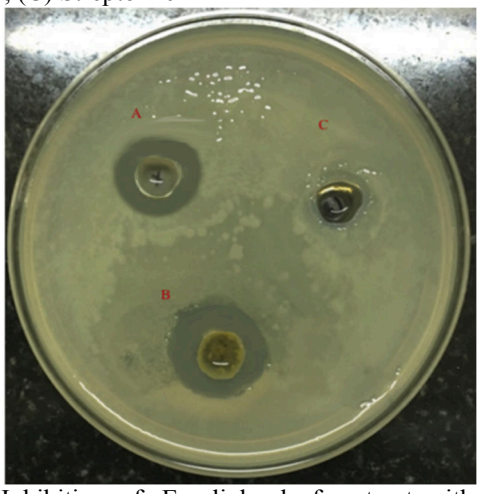
was dissolved in bloodless water showed the maximum inhibition zone in case of *Klebsiella* (17 mm), *Pseudomona s* (16 mm), *E. coli* (15 mm), streptomicin (19 mm), and *S. aureus* (16 mm).



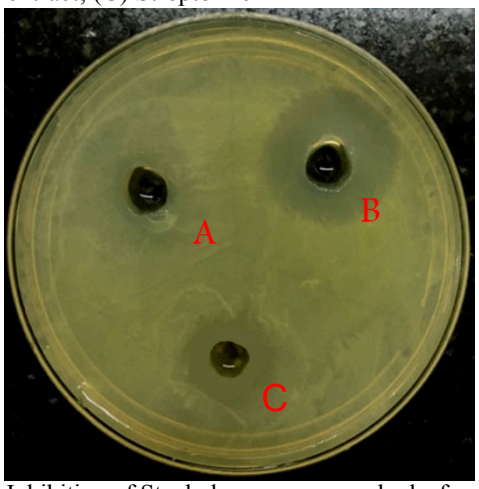
Inhibition of klebsiella by leaf extract with solvents (A) methanol; (B) methanolic extract ; (C) Streptomicin



Inhibition of Pseudomonas by leaf extract with solvents (A) methanol; (B) methanolic extract; (C) Streptomicin



Inhibition of E-coli by leaf extract with solvents (A) methanol; (B) Methanolic extract; (C) Streptomicin



Inhibition of Staphylococcus aureus by leaf extract with solvents (A) methanolic extract; (B) Methanol; (C) Streptomicin

Fig. 2. Antibacterial activities for methanol extract of *Diospyros melanoxylon* leaves

Antioxidant activity

Table 3 shows the comparative radical scavenger activity of DMM, diverse ESF, and isolated p-coumaric acid in terms of the EC_{50} values. Accordingly, the methanol extract of *D. melanoxylon* showed maximum radical scavenging activity when tested with DPPH, ABTS+, and superoxide radical scavenging assays. Conversely, the diverse soluble fractions and isolated p-coumaric acid exhibited low activity (1.2-1.6 times lower than the methanol extract). Moreover, DMM was more effective than BHA (positive control) in the scavenging of free (DPPH), cation (ABTS), and anion (superoxide) radicals. In general, the tested samples showed more significant inhibitory effects in the following order: DMM>BHA>ESF>ESF5>ESF5-c> p-coumaric acid.

Interestingly, the antioxidant activity of the DMM fraction was more significant, which also provided maximum phenolic compounds during

the preliminary phytochemical screening. This is consistent with the previous studies in this regard, suggesting the higher antioxidant potential of phenolic acids. Although high phenolic content does not necessarily indicate greater antioxidant potential, assessment of the individual components of herbal extracts could provide evidence on their antioxidant capacity.[26] Nevertheless, the observed effects in the present study could be attributed to the redox potential of the phenolic acids found in the extract, rendering it a reducing agent and hydrogen donor.

Table 3. Total phenolic (TPC), flavonoid (TFC) proanthocyanidin (TCC) contents and antioxidant activity of methanol extract of *Diospyros melanoxylon* leaves (DMM), diverse ethyl acetate soluble fractions (ESF) and isolated compound p-Coumaric acid (PC)

Sample	TPC (mg GAE/g)	TFC (mg QE/g)	TCC (mg GAE/g)	EC_{50} [x, y] (µg/mL) Radical scavenging activities		
				DPPH	ABTS	Superoxide
DMM	234.15±0.05[d]	96.12±0.14[d]	157.15±0.80[d]	30.90±0.21[a]	25.15±0.89[a]	64.02±0.17[a]
ESF	165.23±1.00[c]	42.23±0.55[c]	86.53±0.25[c]	37.16±2.14[c]	30.53±0.48[b]	79.09±0.05[c]
ESF5	98.54±0.82[b]	30.64±2.02[b]	55.95±0.49[b]	41.03±0.23[d]	37.77±0.50[c]	85.46±1.11[d]
ESF5-c	66.45±0.20[a]	19.19±0.75[a]	30.04±0.02[a]	49.30±0.22[e]	45.00±0.19[d]	92.70±2.02[e]
PC	-	-	-	55.55±0.12[f]	53.01±0.86[e]	98.89±2.22[f]
BHA	-	-	-	35.55±0.01[b]	30.16±1.28[b]	66.57±0.34[b]

[x] Values are expressed as mean ± SE. Means in the same column with distinct superscripts are significantly different ($p \leq 0.05$) as separated by Duncan multiple range test

[y] The EC_{50} value is defined as the effective concentration of the test samples to show 50% of antioxidant activity under assay conditions

Correlation between antioxidant activity and TPC, TFC, and TCC

We performed the correlation analysis of TPC, TFC, and proanthocyanidin (TCC) contents with the EC_{50} values of the radical scavenging ability of the methanol extract of *D. melanoxylon*, isolated compound, and diverse soluble fractions. According to the findings, TPC, TFC, and TCC were significantly correlated (R^2=0.505-0.956) with the DPPH, ABTS, and superoxide radical scavenging (Table 4). Several studies have confirmed that the presence of alkaloids and phenolic acids could enhance the antioxidant ability of herbal extracts.[27-29] Previous findings on *D. melanoxylon* have also confirmed the potent antioxidant activity of its bark extract.[30, 31] However, this was the first report regarding the methanol leaf extract of this plant, which exhibited promising antioxidant activity in line with the previous findings regarding the potential of phenols and flavonoids.

Table 4. Correlation between EC_{50} of radical scavenging activities and total phenolic, flavonoid, proanthocyanidin content of methanol extract of *Diospyros melanoxylon* leaves (DMM), diverse ethyl acetate soluble fractions (ESF) and isolated compound p-Coumaric acid (PC)

	Correlation (R)*		
	Phenolic	Flavonoid	Proanthocyanidin
DPPH			
DMM	0.956	0.912	0.856
ESF	0.912	0.867	0.825
ESF5	0.825	0.756	0.741
ESF5-c	0.793	0.700	0.666
PC	0.702	0.654	0.614
ABTS			
DMM	0.889	0.875	0.875
ESF	0.826	0.854	0.814
ESF5	0.745	0.702	0.733
ESF5-c	0.667	0.643	0.694
PC	0.606	0.608	0.662
Superoxide			
DMM	0.812	0.713	0.785
ESF	0.756	0.695	0.745
ESF5	0.701	0.630	0.674
ESF5-c	0.645	0.542	0.620
PC	0.599	0.505	0.544

* Values are expressed as mean ± SE

Anti-inflammatory activity

As part of the study on the mechanism of anti-inflammation activity, the ability of methanol extract of *D. melanoxylon*, isolated compound, and diverse soluble fractions to inhibit protein denaturation was assessed in the present study. According to the findings, the extract could effectively inhibit albumin denaturation with the IC_{50} of 69 µg/mL (Table 5). On the other hand, aspirin was considered as the standard anti-inflammatory drug (positive control) in our research, which could inhibit albumin denaturation with the IC_{50} of 80 µg/mL. Additionally, p-coumaric acid (IC_{50}=119 µg/mL) isolated from DMM was observed to be a potent inhibitor, while inhibition was slightly lower compared to DMM, diverse ESF, and aspirin. Albumin denaturation is an indicator of inflammation, which was assessed in the present study in order to determine the anti-inflammatory potential of the herbal extract.

In the current research, the RBC membrane stabilization method was applied for DMM, diverse ESF, and the isolated compound in order to investigate the *in-vitro* anti-inflammatory activity as the stabilization of the lysosomal membrane prevents inflammation and further damage to the surrounding tissues by preventing the release of its enzymes. In the present study, DMM could effectively inhibit the heat-induced hemolysis with the IC_{50} of 80 µg/mL (Table 5).

Table 5. Albumin denaturation, membrane protection/stabilization and proteinase inhibition potential of methanol extract of *Diospyros melanoxylon* leaves (DMM), diverse ethyl acetate soluble fractions (ESF) and isolated compound p-Coumaric acid (PC)

Anti-inflammatory	IC_{50}[x, y] (µg/mL)					
	DMM	ESF	ESF5	ESF5-c	PC	Aspirin[#]
Albumin denaturation	69.12±0.24[a]	81.06±0.93[b]	97.85±1.13[c]	106.66±1.05[d]	119.20±3.75[e]	80.11±0.11[b]
Membrane protection	80.21±2.52[a]	89.95±1.01[b]	96.65±0.24[c]	133.32±0.71[d]	144.41±0.27[e]	90.80±0.40[b]
Proteinase inhibition	91.19±1.36[a]	101.00±0.05[c]	137.52±1.28[d]	143.56±0.27[e]	149.95±0.28[f]	100.42±0.17[b]

[x] Values are expressed as mean ± SE. Means in the same row with distinct superscripts are significantly different ($p \leq 0.05$) as separated by Duncan multiple range test

[y] The IC_{50} value is defined as the inhibitor concentration to inhibit 50% under assay conditions

[#]Aspirin was used as positive control

In the current research, DMM and ESF (IC_{50}=89 µg/mL) exhibited slightly higher inhibition potential than the standard drug (IC_{50}=90 µg/mL), while p-coumaric acid (IC_{50}=144 µg/mL) had slightly lower inhibitory activity. In addition, proteinase was observed to be instrumental in the repair of tissue damage during inflammation. DMM (IC_{50}=91 µg/mL) exhibited substantial anti-proteinase activity compared to the standard drug (IC_{50}=100 µg/mL), while the inhibitory effects of proteinase based on the IC_{50} values of ESF (IC_{50}=137 µg/mL), ESF 5 (IC_{50}=143 µg/mL), and ESF5-c (IC_{50}=149 µg/mL) were comparatively less significant ($P<0.05$) compared to the therapeutic drug aspirin (IC_{50}=100 µg/mL). Therefore, it could be concluded that the anti-inflammatory potential of the leaf extract of *D. melanoxylon* should be further evaluated in terms of the mechanism of action. These findings are consistent with the previous studies in this regard, suggesting that anti-inflammatory activity of the extract was directly correlated with its phytochemical constituents.[32, 33]

HR-LCMS profile study of the active methanol extract

The potent antibacterial, antioxidant, and anti-inflammatory effects of the methanol

extract were investigated using the HR-LCMS analysis, which revealed approximately 20 compounds (Table 6), five of which were identified as phenolic acids (p-coumaric acid, homoveratric acid, chlorogenic acid, eudesmic acid, and tuberonic acid) and one flavonoid (rutin). Furthermore, the HR-LCMS analysis of the methanol extract of *D. melanoxylon* leaves demonstrated nine and six principal peaks, respectively, which confirmed the presence of diverse phytochemical materials.

Table 6. Chemical profile of the methanol extract of *Diospyros melanoxylon* leaves by HR – LCMS in +ESI mode

Compound detected	Molecular formula	DB Diff (ppm)
+ESI mode		
Chlorogenic acid	$C_{16}H_{18}O_9$	9.21
7-[2-Trifluoromethyl-4- (2-Hydroxyphenyl)-1,3- Dioxan-cis-5-YL]-HEPT-5Zenoic acid	$C_{18}H_{21}F_3O_5$	25.02
Eudesmic acid	$C_{10}H_{12}O_5$	7.72
Homoveratric acid	$C_{10}H_{12}O_4$	8.08
Deacetyl-Nmonodemethyldiltiazem	$C_{19}H_{22}N_2O_3S$	14.37
12R-acetoxy-punaglandin 3	$C_{27}H_{35}ClO_9$	21.01
p-Coumaric acid	$C_9H_8O_3$	8.10
Tuberonic acid	$C_{12}H_{18}O_4$	9.23
Rutin	$C_{27}H_{30}O_{16}$	9.89
5-Phenylvaleric acid	$C_{11}H_{14}O_2$	8.41
-ESI mode		
Piceid	$C_{21}H_{24}O_8$	15.80
3,9,15-Docosatriynoic acid	$C_{22}H_{32}O_2$	15.64
4,7,10,13,16- docosapentaenoic acid	$C_{22}H_{34}O_2$	15.44
7-[2-Trifluoromethyl-4- (2-Hydroxyphenyl)-1,3- Dioxan-cis-5-YL]-HEPT-5Zenoic acid	$C_{18}H_{21}F_3O_5$	6.67

In the assessment of the excessive resolution liquid chromatography and mass spectra of the constituents with the primary library, many of these compounds were characterized and identified, including dihydromyricetin, dihydrorobinetin, rutin, cosmosiin, barbituric acid, 5-ethyl-five-(2-hydroxyethyl), 2,2,nine,nine- tetramethyl-undecan-1,10-diol,sinomenine, dihydrodeoxy-streptomycin, hexadecanediocacid, thosu-ximide M5, hydroxy anastrozole, 7-desmethylpapaverine, lyxosylamine, isovaleric acid, taurine, minoxidil, 4-trimethyl amminobutanal, 6-beta naltrexol-3-glucuronide, and glucosylgalactosyl hydroxylysine.

Among the compounds detected in the extract were tannins, alkaloids, saponins, glycosides, flavonoids, and steroids using the simple phytochemical technique. The antibacterial activity was also terminated using poison techniques, while notable results have been observed in case of *Colletotrichum capsici*, while excellent results have been yielded toward *Fusarium oxysporum* compared to fungicides. In the current research, the plant extract play a pivotal role in controlling plant diseases, while the phytochemical properties are also effective in the treatment of various diseases. Therefore, new herbal medicines could be proper therapeutic alternatives for chronic diseases.

Molecular modeling

As is depicted in Figs. 3-5, p-coumaric acid could bind to β-lactamase at the MEG binding site, interacting with the binding site residues ASN109, PHE91, PRO90, TYR89, TRP94, and LYS87. Furthermore, p-coumaric acid had good affinity toward the MEG binding site with the binding energy of -4.6 Kcal. It is also notable that p-coumaric acid acted as a competitive inhibitor of β-lactamase by binding to the MEG binding site, while preventing MEG as a natural substrate of β-lactamase, thereby inhibiting the action of the β-lactamase enzyme. The inhibition of β-lactamase limits the ability of the organism to degrade the penicillin class of

antibiotics. Therefore, the antibiotics that are fortified with p-coumaric acid could contribute to the treatment of drug-resistant microorganisms through the dual damage effect exerted by the compound on the microbial membrane.

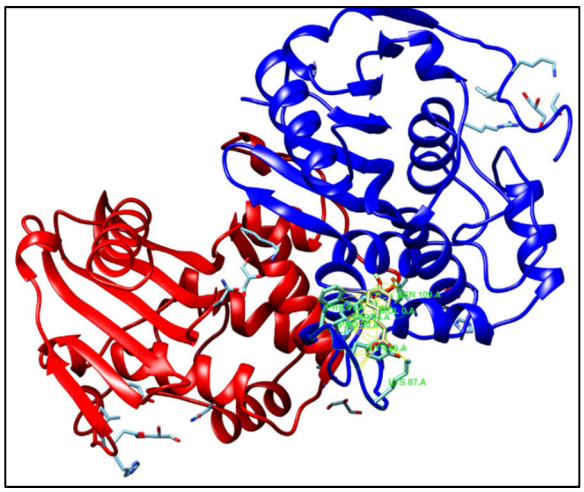


Fig. 3. Image showing p-Coumaric acid bound to β-Lactamase at MEG binding site

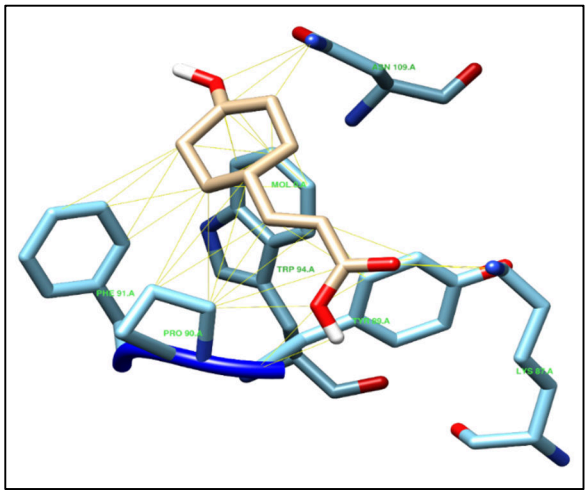


Fig. 4. Image showing interaction of p-Coumaric acid with the binding site residues ASN109, PHE91, PRO90, TYR89, TRP94 and LYS87

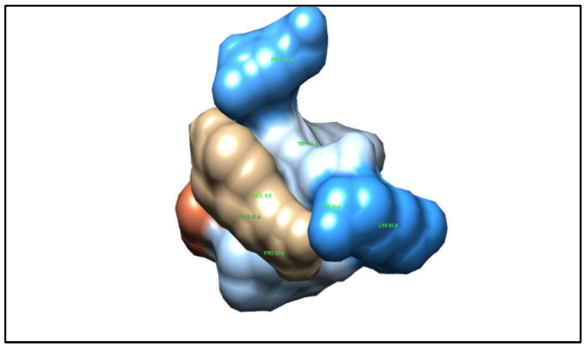


Fig. 5. Image showing hydrophobic interaction of p-Coumaric acid with MEG binding site residue of β-Lactamase

Conclusion

According to the results, the analytical activity guided the characterization of the leaf extract of *D. melanoxylon*, confirming the presence of various compounds, such as p-coumaric acid, homoveratric acid, chlorogenic acid, eudesmic acid, and tuberonic acid. Moreover, the antibacterial, antioxidant, and anti-inflammatory activity of the extract was more prominent compared to the isolated compound, which suggested the synergistic effects of the components of the extract to have higher potential as a therapeutic agent compared to the p-coumaric acid extract from the fraction. The results uphold the positive effects of the extract, highlighting the higher *in-vivo* potential of the extract to reconfirm its potential health benefits.

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