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Research Article

Carvacrol from *Plectranthus Amboinicus* as Antibiofilm and Anti-Inflammatory Compound

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ABSTRACT

Background: The present study investigated the bioactive potential of *Plectranthus amboinicus* leaf extract with a focus on the isolation, characterization, and functional evaluation of its principal phytochemical constituents. **Materials and Methods:** Leaves were subjected to Soxhlet extraction using methanol, followed by purification through Thin-Layer Chromatography (TLC) and column chromatography. Phytochemical characterization using GC–MS and FTIR analyses identified Carvacrol as the major constituent of Fraction 2. **Results:** Enhanced bioactivity was observed upon fractionation, with Fraction 2 exhibiting the most pronounced effects. Carvacrol accounted for approximately 32% of its composition. Carvacrol demonstrated significant antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, along with potent antibiofilm efficacy, exhibiting low IC_{50} values comparable to the standard antibiotic tetracycline. Mechanistic investigations revealed that Carvacrol effectively inhibited key virulence factors of *P. aeruginosa*, including elastase production, pyocyanin synthesis, and swarming motility. Additionally, Carvacrol exhibited notable immunomodulatory and anti-inflammatory properties in RAW 264.7 macrophage cell lines by modulating critical inflammatory enzymes such as Cyclooxygenase (COX), Lipoxygenase (LOX), and Myeloperoxidase (MPO). **Conclusion:** Overall, these findings highlight Carvacrol as a multifunctional phytochemical with substantial antibacterial, antibiofilm, and immunomodulatory activities, supporting its therapeutic potential for biomedical and pharmaceutical applications.

Keywords: *Plectranthus amboinicus*, Carvacrol, GC-MS, FTIR, Thin Layer Chromatography, *S. aureus*, *P. aeruginosa*.

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INTRODUCTION

The essential oil obtained from the leaves and stem explants of *Plectranthus amboinicus* is shown to contain a total of 76 volatile constituents, some of which are pharmaceutically appreciated for various culinary properties. The quality as well as quantity of chemical compounds occurring in the essential oil is directly related to its biological functions. *P. amboinicus* oil is rich in oxygenated monoterpenes, monoterpene hydrocarbons, sesquiterpene hydrocarbons and oxygenated sesquiterpenes (Arumugam *et al*., 2016). The essential oil extracted from its leaves contains compounds like carvacrol, thymol, and eugenol, which are known for their antibacterial and antifungal effects.

Studies have demonstrated its efficacy against *Staphylococcus aureus, Escherichia coli, Candida albicans*, and *Aspergillus niger*. The antimicrobial activity is attributed to the disruption of microbial cell membranes and inhibition of biofilm formation, making it a potential candidate for developing natural antimicrobial agents (Arumugam *et al*., 2016). The leaves of *P. amboinicus* have been traditionally used to treat inflammatory conditions such as arthritis and skin irritations. Research has shown that the plant's extracts can inhibit the production of pro-inflammatory cytokines like TNF-α and IL-6, thereby reducing inflammation (Lukhoba *et al*., 2006). *P. amboinicus* has been used in traditional medicine to treat gastrointestinal disorders such as indigestion, flatulence, and ulcers. Studies have shown that its extracts can protect the gastric mucosa by reducing acid secretion and enhancing mucus production. The gastroprotective effects are attributed to its antioxidant and anti-inflammatory properties, which mitigate oxidative damage and inflammation in the gastric lining (Algasoumi *et al*., 2009).

Though the antimicrobial activity of *P. amboinincus* have been proved, the individual efficacy of the compounds contained within was an insight in this study. The various compounds contained in the crude extracts and their independent pharmacological

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activities could add an added contribution and significance in terms of synergistic / antagonistic activity of the extracts.

MATERIALS AND METHODS

Preparation of crude extract

Fresh leaves of *P. amboinicus* were harvested and after drying, the samples were ground into a powder. The resulting dried and ground plant material was then subjected to Soxhlet extraction using 100% methanol. The extract was concentrated to dryness (Vishnupriya *et al*., 2025).

Purification of the Crude Extracts of *Plectranthus amboinicus*

Thin Layer Chromatography: The crude leaf extracts were subjected to analytical TLC using commercially available silica TLC plate 20 cm x 20 cm (GF 25460; Merck 250 mm thick) according to the procedure laid by Vishnupriya *et al*., (2025). The solvent system consisted of Hexane: Ethyl acetate (7:3 v/v) which gave a better separation of compounds. The Retention Factor (Rf) values of each compound were calculated.

Column Chromatography: The best solvent system exhibiting good separation of compounds in TLC was selected for the isolation of pure compounds by Column Chromatography according to the procedure by Adorisio *et al*., (2019) and Kaaniche *et al*., (2019). The crude leaf extracts of *P. amboinicus* were subjected to Column Chromatography for fractionation on silica gel (60-120 mesh) using gradient solvent system of hexane: ethyl acetate (7:3v/v) and finally 100% methanol in the order of increasing polarity. Following quantification of all the eluted compounds, all the fractions were screened by TLC on a silica plate (MERCK silica gel 60 PF254 aluminium sheets, 250 mm thick, 20 cm x 20 cm) under visible light and ultraviolet light in both short and long wavelengths.

Antibacterial activity

Agar well-diffusion method was followed to determine the antimicrobial activity of all eluted column fractions. 30 µL of extract corresponding to 20 mg/mL of each fraction was added to each well. DMSO was used as negative control. All the plates were incubated at 37°C for 24 hr. Zone of inhibition of test organism's growth around each well was measured in mm (Jahangirian *et al*., 2013).

Characterization of Fractionated Compound of Plectranthus amboinicus

Fourier Transform Infrared Spectroscopy (FTIR) Analysis: Analysis of the functional groups of the purified compounds was performed using the FT-IR spectrometer spectrum two (Perkin-Elmer, USA). The absorbance spectra were acquired over the range 600-4000 cm^{-1} at a resolution of 4 cm^{-1} with 5 scans per spectrum.

Gas Chromatography-Mass Spectroscopy (GCMS) Analysis: Analysis was performed by injecting 2 µL of the sample in a splitless. Helium gas (99.9995%) was used as the carrier gas at a flow rate of 1 mL/min. The analysis was performed in the EI (electron impact) mode with 70 eV of ionization energy. The compounds were identified after comparing the spectral configurations obtained with that of available mass spectral database (NIST -08 SPECTRAL DATA).

Biofilm Inhibition of the Bioactive Compound

Anti-biofilm activity by Crystal violet assay: Plant extract was dissolved in DMSO at a concentration of 500 ug/mL. This was further to obtain test concentrations of 250, 125, 62.5, 31.25, 15.6, 7.8 and 3.9 ug/mL. A 100 µL sample of the diluted culture of *S. aureus* and *P. aeruginosa* strains was added to a microtiter plate and incubated at 37°C for 24 hr followed by washing with PBS (pH 7.4). Subsequently, 125 µL of a freshly prepared 0.1% crystal violet solution was added to the dried pellet and incubated for 10 min. After staining and washing, 200 µL of 30% acetic acid was added to the pellet and incubated for 15 min to dissolve the stain followed by transfer of 100 µL aliquot and the optical density was recorded at 600 nm using an ELISA reader (Biorad, USA). The reduction in biofilm formation in the presence of plant extracts was calculated as percent inhibition using the formula:

$$[(\text{OD of control} - \text{OD of treated}) / \text{OD of control}] \times 100.$$

Las B Elastolytic assay: The Elastin Congo Red assay was performed according to the protocol of Akshay, (2025). Cells were cultured in LB broth at 37°C for 14 hr and then centrifuged at 10,000 rpm for 10 min; subsequently 50 µL aliquot of the resulting supernatant was mixed with 2 mL of 10 mM Na_2HPO_4 containing 30 mg of Elastin Congo Red, incubated at 37°C for 14 hr, followed by centrifugation. The absorbance of the released Congo Red was measured at 495 nm.

Pyocyanin Quantification Assay: The assay was carried out following the method of Akshay, (2025). Post culturing the cells in LB broth at 37°C for 24-48 hr, the mixture was subjected for centrifugation followed by the addition of 3 mL of chloroform to 5 mL sample of the culture supernatant. The separated lower organic phase was mixed with 2 mL of 0.2 M HCl followed by the absorbance measurement at 520 nm. The pyocyanin concentration was expressed in µg/mL of culture supernatant and calculated by multiplying the optical density by 17.072.

Swarming Motility Assay: A 5 µL sample of the overnight culture was inoculated onto 0.4% nutrient agar and incubated for 24 hr. The swarm diameter on the agar plates was then measured in millimeters.

Anti-Inflammatory Assays

RAW 264.7 cells were initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecco's

modified Eagles medium, DMEM (Sigmaaldrich, USA). The cells were grown to 60% confluency followed by activation with 1 µL lipopolysaccharide (LPS: 1 µg/mL). LPS stimulated RAW cells were exposed with different concentration (25,50,100 µg/mL) of sample solution and incubated for 24 hr. After incubation the anti-inflammatory assays were performed using the cell lysate for Cyclooxygenase, Lipoxygenase and Myeloperoxidase (Thomas, 2022).

Cyclooxygenase (COX) activity: The efficacy of lycopene in inhibiting COX enzyme was performed and COX activity was determined by reading absorbance at 632 nm and the results were expressed in terms of percentage of COX inhibition.

5-Lipoxygenase (LOX) activity: The LOX activity assay was performed and the formation of 5-hydroxyeicosatetraenoic acid reflected the LOX activity which was monitored by reading the absorbance at 234 nm.

Myeloperoxidase activity: MPO in the sample was activated by the addition of 50 mM phosphate buffer (pH 6) containing 1.67 mg/mL guaiacol and 0.0005% hydrogen peroxide. The change in absorbance at 460 nm was measured. MPO activity was presented as units per mL of cell lysate. One unit of MPO activity was defined as that degrading 1 µM of peroxide per minute at 25°C.

Statistical Analysis

All the experiments were performed a triplicate and the results are expressed as Means ± SD (n=3). The results were analysed for statistical significance using the unpaired Students T-test, One- way ANOVA, and Dunnets test (SPSS Inc. 20. 0 version). Probability values (p) ≤ 0.05 were statistically significant.

Table 1: Antibacterial screening of *P. amboinicus* column fractions.

Organisms	Column fractions of *P. amboinicus* extract (20 mg/mL)	Zone of inhibition (mm)			Mean ± SD
		Trial 1	Trial 2	Trial 3	
Pseudomonas aeruginosa	1	25	23	24	21.7±1.25
	2	22	20	23	24 ±0.82
	3	15	16	15	15.3±0.5
	4	22	23	22	22.3±0.5
	5	18	17	17	17.3±0.5
	6	18	16	15	16.3±1.25
	7	14	15	14	14.3±0.5
	8	13	15	12	13.3±1.25
	9	12	14	13	13±0.82
	10	13	14	12	13±0.82
	11	13	15	14	14±0.82
	12	12	14	13	13±0.82
	13	12	14	15	13.7±1.25
Staphylococcus aureus	1	17	16	15	16±0.82
	2	18	17	16	17±0.82
	3	16	15	14	15±0.82
	4	14	15	13	14±0.82
	5	14	13	15	14±0.82
	6	12	11	12	11.7±0.5
	7	13	14	12	13±0.81
	8	12	10	13	11.7±1.25
	9	15	13	14	14±0.85
	10	14	12	13	13±0.85
	11	11	09	10	10±0.82
	12	11	10	09	10±0.82
	13	11	08	10	9.7±1.25

Data shown are the average and standard deviation based on triplicate runs (Mean +Standard Deviation).

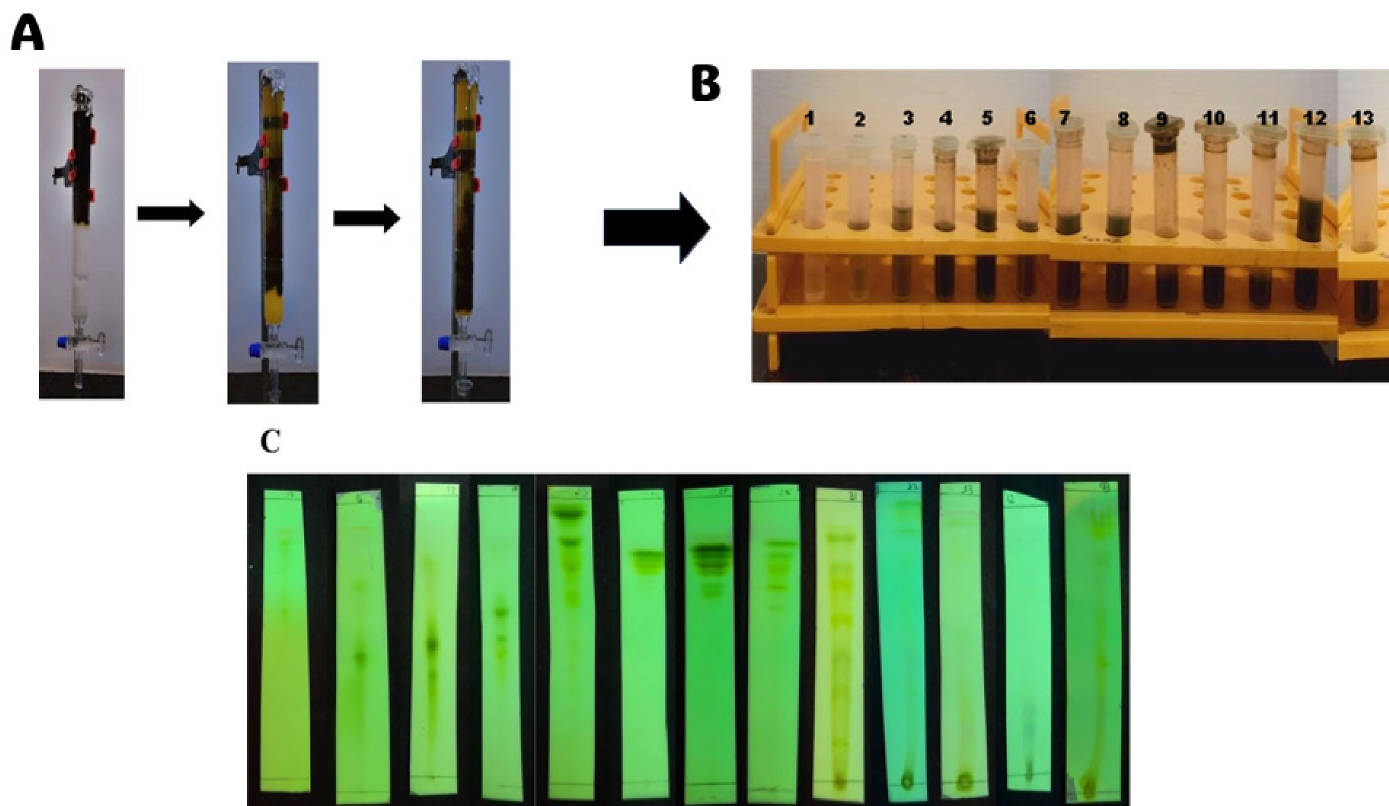

Figure 1: Pictorial representation of the Purification of crude extract of *P. amboinicus* (A) Column Chromatography (B) Collected column fractions (C) TLC of the column fractions.

RESULTS

Extraction of Plectranthus amboinicus

The total yield of leaf extracts of *Plectranthus amboinicus* from a total weight of 50 g of dried leaves were found to be 5.3 g.

Purification of Crude extract

Thin Layer Chromatography: The crude leaf extract of *P. amboinicus* was separated on a silica gel TLC plate with standardized mobile phase of Hexane: Diethyl ether (7:3 v/v). The extract separated into 11 distinct bands. The TLC plates were visualized under Visible light and UV of long and short wavelength. The corresponding R_f values were also recorded.

Column Chromatography: The crude extract (5 g) of the leaf extract of *P. amboinicus* was subjected to column chromatography for fractionation on silica gel using a tertiary gradient solvent system of hexane-di ethyl ether (7:3 v/v), and finally 100% methanol with gradually increasing the polarity to yield 13 fractions. The net yield of all the fractions was noted. TLC was performed with all the fractions (1-13) with hexane: diethyl ether (7:3 v/v) The obtained chromatograms of were visualized under visible, UV long and short wavelength (Figure 1).

Antibacterial Activity by the well-diffusion method

The antimicrobial activity of all the thirteen column fractions corresponding to 20 mg/mL of the *Plectranthus amboinicus* leaf extract was assessed against *P. aeruginosa* and *S. aureus* wherein Fraction 2 exhibited potent activity against *P. aeruginosa* and *S. aureus* with a zone of inhibition of 24 mm and 17 mm respectively (Table 1).

Characterization and Identification of Fraction 2 from *P. amboinicus* flower extract

Fourier Transform Infrared Spectroscopy (FTIR) Analysis: The functional group analysis of fraction 2 (F2) through FTIR spectroscopic analysis showed a strong broad stretching peak of alcohol (O-Hstr), weak and bending C-H peak of aromatic compound and a strong sharp = C-H out of plane bending trans-alkene peak at 3428.93, 1655.76, and 1007.81 [cm^{-1}] respectively

Gas Chromatography-Mass Spectroscopy (GCMS) Analysis: The GCMS analysis of the fractionated compound F2 was identified to be Carvacrol, a monoterpenoid phenol compound and an essential oil with the retention time of 15.94 min. The percentage of Carvacrol in Fraction 2 accounted to 32% along with other minor compounds such as glycerin (17%), phytol (11%), Octadecatrienoic acid (7.2%) and hexadecenoic acid (3.7%). The MS peak of carvacrol along with structure and the FTIR peak is depicted in Figure 2.

Assessment of Virulence factors of *Pseudomonas aeruginosa* in the presence of Carvacrol

The Carvacrol was able to reduce the biofilm, elastase production, pyocyanin production and swarming motility assay in *P. aeruginosa* (Figure 3A, 3B, 3C & 3D).

Antibiofilm Activity by Crystal Violet Assay Method: The biofilm inhibition activity of Carvacrol was performed against *S. aureus and P. aeruginosa*. The inoculum without the sample was taken as the control and Tetracycline was used as the positive control. Test concentrations of the sample were taken from 250 – 3.9 mg/mL for sample. The IC_{50} values of Carvacrol against *S. aureus and P. aeruginosa* were 66.3 & 66.45 mg/mL respectively.

Las B Elastolytic Assay: The elastase inhibition with Carvacrol at 65 ug/mL (corresponding to IC50 value) were measured at 495 nm. OD value of the control was found to be 0.397 and the positive control was 0.355 and test sample was 0.367. There was 92.4% elastase inhibition with Carvacrol when compared to the positive control of 89.4%.

Pyocyanin Reduction Assay: Carvacrol were able to inhibit the production of pyocyanin. The OD value of the control was

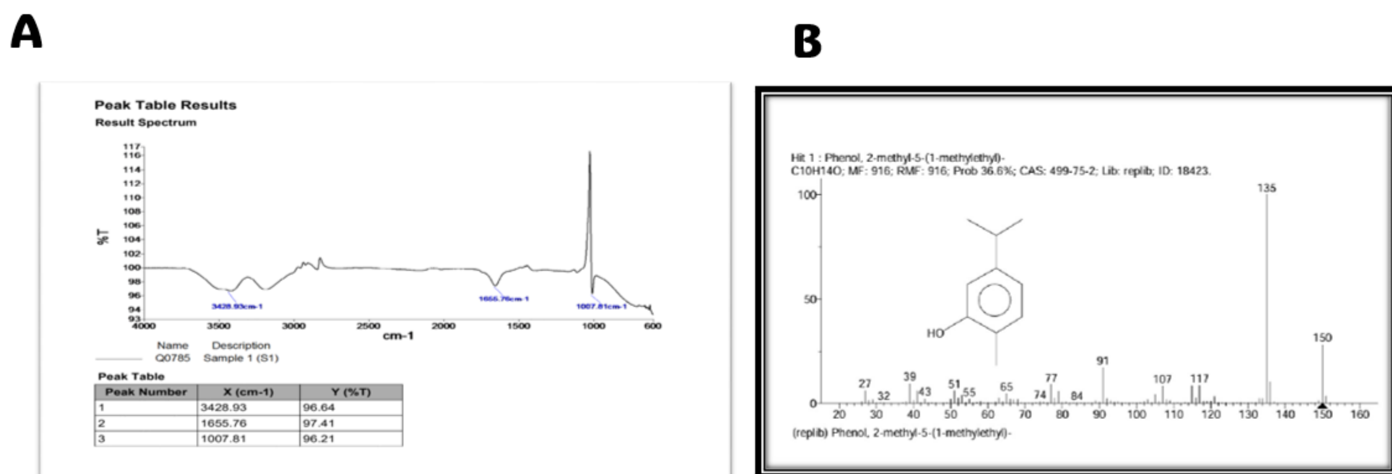

Figure 2: (A) FTIR spectrum of fraction 2; (B) GC-MS of fraction 2 identified as carvacrol.

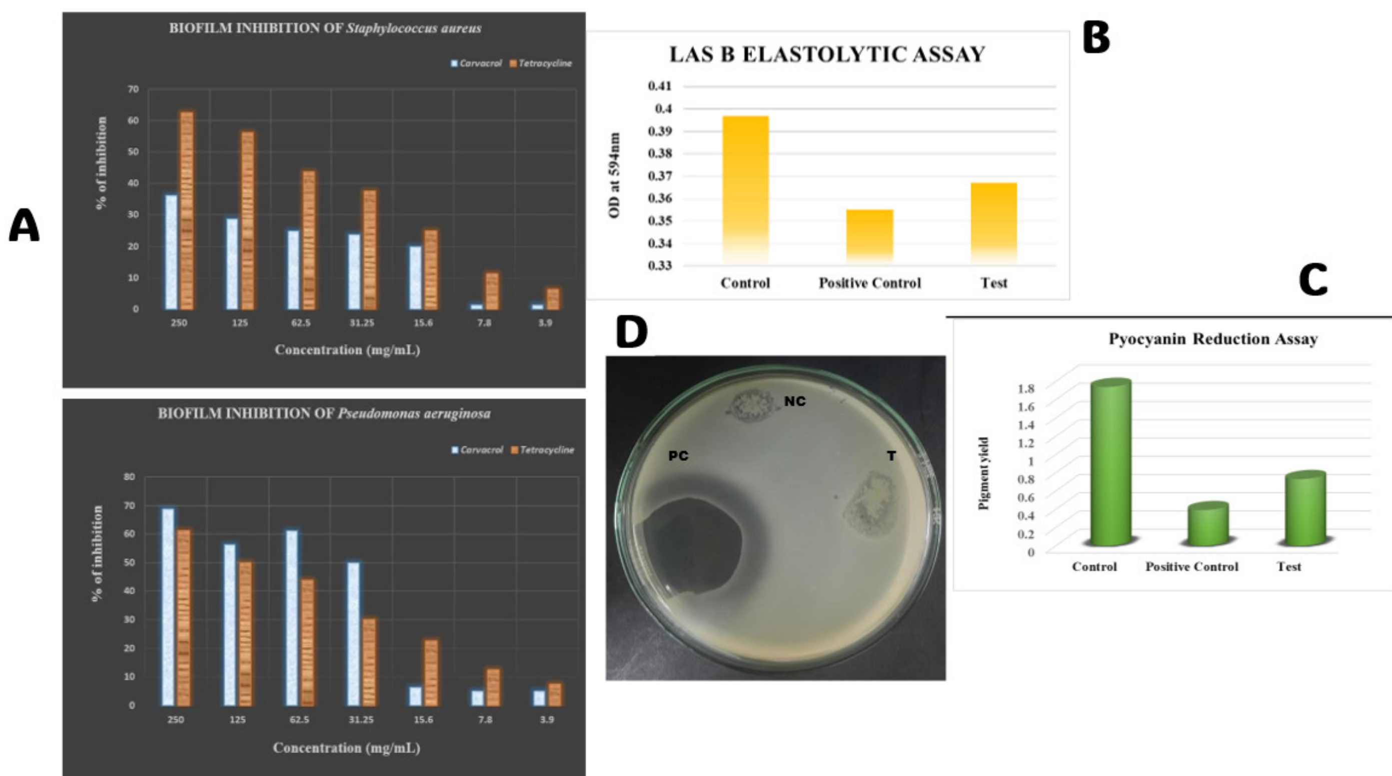

Figure 3: (A) Percentage of biofilm inhibition against S. aureus and *P. aeruginosa*; (B) Las B Elastolytic Assay of carvacrol against *P. aeruginosa*; (C) Pyocyanin Reduction Assay of carvacrol against *P. aeruginosa*; (D) Swarming motility assay against *P. aeruginosa*.

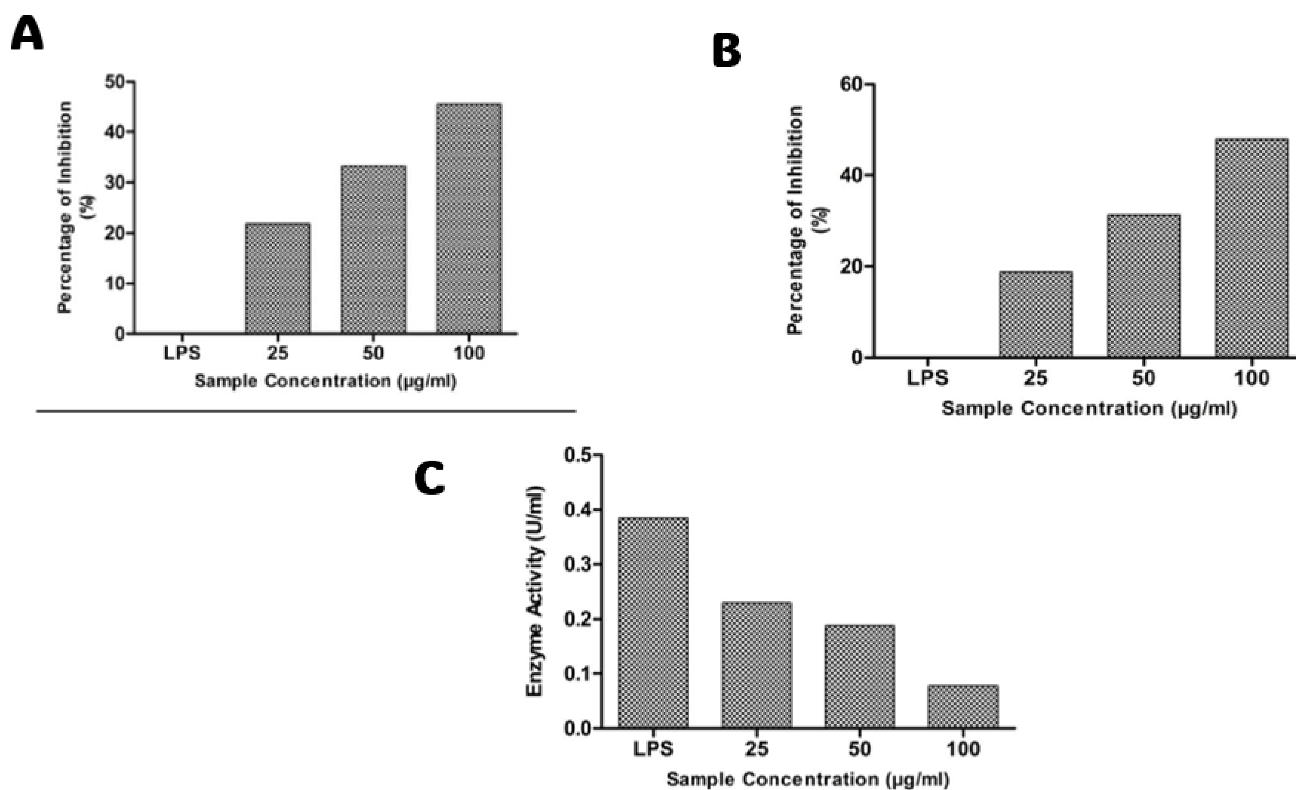

Figure 4: Graphical representation depicting the Anti-inflammatory activity (A) COX of carvacrol; (B) LOX of carvacrol; (C) Activity MPO of Carvacrol.

measured as 0.102 and the positive control was measured as 0.023 and test sample 0.043 at 520 nm. The pigment yield of the control and the samples were calculated by multiplying with 17.072. The pigment yield of the control was calculated as 1.74 μg/mL and Carvacrol was found as 0.73 μg/mL and PC was found as 0.39 μg/mL. There was 58% pigment reduction by Carvacrol and 77.6% pigment reduction by positive control.

Swarming Motility Assay: There was a significant difference in the swarming motility exhibited by the control and test and positive control. The control showed 11 mm diameter zone of inhibition whereas Carvacrol showed 21 mm diameter zone of inhibition and positive control shown 47 mm zone of inhibition.

Anti-inflammatory Activity of Carvacrol by *in vitro* assays

Cyclooxygenase Activity: The anti-inflammatory activity of carvacrol on 264.7 RAW macrophage cell line was evaluated post inducing the cell culture with LPS following which carvacrol was added in the same concentrations wherein the IC_{50} value w.r.t. to % of inhibition was recorded at 118.42 ug/mL.

Lipoxygenase (LOX) activity: The % of inhibition of the compound in LPS induced cell line for measuring the anti-inflammatory activity was recorded at 106.249 ug/mL.

Myeloperoxidase activity: Carvacrol was found to exhibit excellent immunomodulatory effect and anti-inflammatory compound wherein the degradation of peroxide was calculated every minute. The graphical representation of all the anti-inflammatory compounds and its inhibition is depicted in Figure 4.

CONCLUSION AND DISCUSSION

The study demonstrated the *Plectranthus amboinicus* leaf is extracted by Soxhlet extraction using methanol. Further bioactivity enhancement was observed upon purification through TLC and column chromatography— especially in Fraction 2. GC-MS and FTIR characterization identified Carvacrol as the major constituent in Fraction 2, accounting for 32% of the fraction's composition. Identifying Carvacrol as the principal bioactive compound. Carvacrol exhibited significant antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, alongside potent antibiofilm efficacy with low IC_{50} values, closely comparable to tetracycline.

Mechanistic assays revealed that Carvacrol significantly inhibited key virulence factors of *P. aeruginosa*, including elastase production, pyocyanin synthesis, and swarming motility. Furthermore, it showed immunomodulatory and anti-inflammatory effects in RAW 264.7 macrophage cell lines by modulating key enzymes such as COX, LOX and MPO.

These findings support the therapeutic potential of Carvacrol as a multi-functional phytochemical with promising biomedical applications. Carvacrol holds significant potential for therapeutic and pharmaceutical development. However, further studies

involving *in vivo* validation, toxicity profiling, and formulation development are essential to translate these findings into clinical or industrial applications. Expanding research into its synergistic effects, delivery systems, and mechanism of action will help optimize its efficacy and safety for real-world use.

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ABBREVIATIONS

ANOVA: Analysis of Variance; **COX:** Cyclooxygenase; **DMEM:** Dulbecco's Modified Eagles Medium; **DMSO:** Dimethyl Sulfoxide; **FTIR:** Fourier Transform Infrared Spectroscopy; **GC-MS:** Gas Chromatography-Mass Spectroscopy; **HCl:** Hydrochloric Acid; **IC_{50}:** Half Maximal Inhibitory Concentration; **LB:** Luria-Bertani; **LOX:** Lipoxygenase; **LPS:** Lipopolysaccharide; **MPO:** Myeloperoxidase; **NCCS:** National Centre for Cell Sciences; **OD:** Optical Density; **PBS:** Phosphate Buffered Saline; **SD:** Standard Deviation; **TLC:** Thin Layer Chromatography; **UV:** Ultraviolet.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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