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INVESTIGATION OF *Plantago lanceolata* L.: A MULTIDIMENSIONAL STUDY ON ITS BIOCHEMICAL PROFILING, ANTIOXIDANT CAPACITY, AND BIOLOGICAL ACTIVITIES

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Abstract

Plantago lanceolata L. is a medicinal and aromatic plant recognized for its antimicrobial and antioxidant effects. This research focused on evaluating its biological activity, antioxidant capacity, and volatile compound composition through Gas Chromatography-Mass Spectrometry (GC-MS) analysis. The extract's effects on biofilms formed by pathogenic bacteria were evaluated, showing significant biofilm inhibition and disruption. The antimicrobial activity was assessed based on minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values. The extract exhibited strong effects against Staphylococcus aureus MRSA, Streptococcus mutans, and S. aureus MRSA+MDR strains. The antioxidant potential was assessed through the 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical assay, demonstrating a notable ability to scavenge free radicals. These findings suggest that P. lanceolata could serve as a protective or supportive agent against diseases associated with oxidative stress. GC-MS analysis identified the volatile components, with high concentrations of fatty acid derivatives such as linolenic acid and hexadecenoic acid. The study demonstrated that P. lanceolata possesses notable antibiofilm, antimicrobial, and antioxidant properties, making it a valuable natural resource. Owing to its bioactive compounds, this species exhibits significant potential for applications in the pharmaceutical, food, and cosmetic industries. It may also function as an important phytochemical in the drug developers' search for resources. Further research is required to expand its potential applications and prove its clinical efficacy.

1. INTRODUCTION

Microorganisms constitute a significant portion of global biodiversity, encompassing the domains *Archaea* and *Bacteria*, which represent two of the three fundamental domains of life [1]. The majority of microorganisms consist

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of bacteria [2] and this group of organisms is critically important for human life [3]. Indeed, while many bacterial species contribute positively to vital processes, opportunistic pathogens can cause severe infections, particularly in cases of a weakened immune system [4]. Bacteria can employ both phenotypic and genetic strategies to develop resistance against environmental stressors, such as antibiotics, that threaten their survival [5]. These strategic adaptations enhance the ability of microorganisms to adapt and acquire resistance [6].

As reported by the World Health Organization [7], the major challenge of the modern era is the increasing antimicrobial resistance. To prevent this rising resistance, the discovery of new antimicrobial compounds is essential [8]. The discovery of antibiotics is regarded as one of the greatest achievements in the field of medicine in the modern era. The clinical use of antibiotics has provided an effective treatment for bacterial infections, significantly reducing morbidity and, consequently, mortality rates [9]. Since the 1990s, the discovery of new antibiotics has declined, and newly introduced antibiotics are merely optimized and modified versions of existing ones, without changes in their antibacterial mechanisms [6]. According to data from the Centers for Disease Control and Prevention (CDC), antibiotic-resistant bacteria cause infections in approximately 2 million people annually in the United States alone [10]. Inappropriate treatment practices and the misuse of antibiotics have contributed to the emergence of antibiotic-resistant bacteria, presenting serious challenges for future public health [11]. Resistant bacteria complicate treatment processes and gradually diminish the effectiveness of existing drugs [12]. This situation encourages scientists to develop new drugs and treatment methods; however, the number and effectiveness of current research efforts remain insufficient. The rapid ability of bacteria to develop resistance mechanisms makes the exploration of alternative treatments even more urgent and crucial [13]. Alongside advancements in synthetic technologies, plant-derived compounds play a crucial role in the development of antimicrobial agents [14]. In traditional medical practices, plant-derived substances have been widely used throughout history and have gained prominence due to their pharmacological effects [15].

Throughout history, many civilizations have used various plant extracts for wound healing, infection treatment, and even the management of numerous chronic diseases [16] Medicinally important plants continue to be key sources of bioactive compounds in pharmaceutical research and development [17]. In the study of antibiotic resistance, a major issue of our time, scientists worldwide and in Turkey are increasingly focusing on plants, which are the foundation of traditional medicine [18,19]. Plants synthesize a wide variety of secondary metabolites for environmental adaptation, competitive advantage, and defense [20]. Recent studies reveal that some of these metabolites possess antimicrobial potential and can be considered in alternative treatment strategies, particularly against global health issues such as antibiotic resistance [18, 21].

The genus *Plantago* belongs to the *Plantaginaceae* family and consists of seed-bearing plants [22]. This family, which includes approximately 275 species, is represented in Turkey by 22 species, two of which are endemic [23, 24]. It is a perennial herbaceous plant that can be found in grasslands and along roadsides [25]. It is used worldwide as a functional food and in certain diets for the treatment of various diseases [26]. It has extensive pharmaceutical applications, with *P. lanceolata* L. standing out particularly due to its phytochemical effects [27]. This plant has traditionally been utilized for treating various health conditions, including wound healing and inflammation [28]. This plant has traditionally been utilized for treating various health conditions, including wound healing and inflammation [29]. The high concentrations of flavonoids and phenolic compounds in its leaves constitute the primary biochemical basis for its pharmacological activities [30]. Recent studies have demonstrated that extracts of *Plantago lanceolata* exhibit antibacterial and biofilm-inhibitory activities against pathogenic bacteria such as *Borrelia burgdorferi* [31] The extract of the plant has also been reported to exhibit antifungal activity against certain dermatophytic fungi, suggesting the potential of *Plantago lanceolata* for dermatological applications [30].

This study was carried out primarily on P. lanceolata L., which is widespread in the Anatolian region. The biological activity of *P. lanceolata*, which has ethnobotanical importance and grows in our geography, was evaluated with 48 microorganisms. The bioactive compounds it contains were examined by GC-MS and its antioxidant capacity was evaluated accordingly. The data obtained contribute significantly to the accumulation of knowledge in the literature on the biological properties of the species in question.

2. MATERIALS AND METHODS

2.1 Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH, Sigma-Aldrich): Used as a stable free radical in the antioxidant capacity assay. Ethanol (Sigma-Aldrich): Absolute ethanol was used during the extraction process. Dimethyl Sulfoxide (DMSO, Sigma-Aldrich): Utilized at a 2% concentration in combination with distilled water for the preparation of extracts in antimicrobial tests. LB Broth (Merck): Employed as a liquid growth medium in antimicrobial assays. Mueller-Hinton Agar (MHA, Oxoid): Used as a solid culture medium for antimicrobial testing. Saline Solution (0.9% NaCl): Used for bacterial suspension preparation according to the McFarland standard. Ascorbic Acid (Carlo Erba): Served as the positive control in the antioxidant assay.

2.2 Preparation of *Plantago lanceolata* extract

P. lanceolata samples were collected from local markets. To extract its secondary metabolites, the plant material was finely pulverized using an IKA

grinder. A precisely measured 15-gram portion of the powdered sample was placed into an Erlenmeyer flask, and 200 milliliters of 99.8% ethanol was added. The mixture was then subjected to continuous agitation at 160 rpm for 72 hours using an orbital shaker to enhance the extraction process [32]. Following the shaking process, the mixture was filtered using Whatman No.1 filter paper to remove solid residues. The extract was then concentrated using a rotary evaporator (Buchi Labortechnik AG) at 35°C [33]. The remaining substance in the flask was weighed, yielding 0.686 grams. Finally, it was dissolved in ethanol to obtain a 15 mL extract. The extract was made into DMSO-water (2% DMSO) extract for the antimicrobial activity tests performed in the study (for MIC, MBC and Anti-biofilm tests). For bioactive content analysis, the extract was prepared with 99.8% absolute ethanol.

The ethanol extracted *P. lanceolata* solution was adjusted to a concentration of 1 mg/mL for antioxidant activity assay. Similarly, ascorbic acid solution used as a positive control was prepared at the same concentration of 1 mg/mL. Ascorbic acid (vitamin C) was used as a positive control due to its well-established antioxidant activity. It reacts with DPPH radicals, leading to a measurable color change, and thus serves as a reliable reference for comparing the antioxidant potential of samples. In the negative control group, only the DPPH solution was used to establish the baseline absorbance of the system.

2.3 Inoculum preparation

The inoculum of microorganisms used in the experiment were selected from exponentially growing colonies on nutrient media that exhibited similar morphological characteristics. The isolated colonies were transferred into tubes containing a 0.9% sterile sodium chloride (NaCl) solution, and the turbidity of the cell suspensions was adjusted to match the 0.5 McFarland standard. This standard corresponds to approximately 1.5×10^8 CFU/mL for bacteria and 1.5×10^7 CFU/mL for yeasts. Maintaining this standard is essential for ensuring a consistent cell concentration under experimental conditions, thereby enabling reproducible and reliable results. During the inoculum preparation, the optical density of the suspensions was assessed using a densitometer [33].

2.3.1 Microorganisms

The microbial strains used in this study were obtained from the Microbiology Laboratory of the Faculty of Science, Dokuz Eylül University.

The tested microorganisms consisted of a total of 48 strains, including 45 bacterial strains and 3 yeast strains. Standard isolation microorganisms, *Bacillus subtilis DSMZ 1971, Enterobacter aerogenes ATCC 13048, Enterococcus faecalis ATCC 29212, Escherichia coli ATCC 25922, Listeria monocytogenes ATCC 7644, Pseudomonas aeruginosa DSMZ 50071, Pseudomonas fluorescens P1, Salmonella enteritidis ATCC 13076, Salmonella typhimurium SL1344,*

Staphylococcus aureus ATCC 25923, Staphylococcus epidermidis DSMZ 20044, Staphylococcus hominis ATCC 27844, Staphylococcus warneri ATCC 27836, Bacillus cereus RSKK 863, Shigella flexneri RSKK 184, Acinetobacter baumannii CECT 9111, Food isolated microorganisms, Enterococcus durans, Enterococcus faecium, Klebsiella pneumoniae, Listeria innocua, Salmonella infantis, Salmonella kentucky, Escherichia coli, Clinical isolated microorganisms, Staphylococcus aureus, Streptococcus mutans, Staphylococcus hominis, Staphylococcus haemolyticus, Staphylococcus lugdunensis, Shigella boydi, Acinetobacter baumannii, Shigella flexneri, Staphylococcus aureus, Enterococcus faecalis, Klebsiella pneumoniae, multi-drug resistance microorganisms, Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii, Enterobacter aerogenes, Serratia odorifera, Proteus vulgaris, Streptococcus pneumonia, Staphylococcus aureus MRSA, Staphylococcus aureus MRSA+MDR, Providencia rustigianii, Achromobacter sp. The yeasts are Candida albicans DSMZ 1386, Candida tropicalis, Candida glabrata.

2.4 Antimicrobial activity test

2.4.1 Disc diffusion test

In the disk diffusion assay used to assess antimicrobial activity [34], blank antibiotic susceptibility disks (Oxoid, 6 mm diameter) were impregnated with the extract at three different volumes (50, 100, and 150 µL). The corresponding extract concentrations on the disks were calculated as follows: 50 µL – 2.28 mg, 100 µL – 4.57 mg, and 150 µL – 6.86 mg. The extract-loaded disks were then allowed to dry at 30°C to facilitate ethanol evaporation. Following this, 40 mL of Mueller-Hinton Agar (BD Difco) was dispensed into sterilized 90 mm Petri dishes and left to solidify [35]. Inside a biosafety cabinet, pre-inoculated microorganism suspensions were evenly spread over the agar surface. After complete evaporation of ethanol, disks containing only the active compound were appropriately placed in each Petri dish. The plates were subsequently incubated at 37°C for 24 hours for bacterial cultures, while yeast cultures were incubated at 27°C for 48 hours. Gentamicin and Tobramycin antibiotics were used as positive controls in the study. At the end of the experiment, the sizes of the inhibition zones were determined in millimeters with a ruler and noted.

2.4.2 Minimum Inhibitory Concentration (MIC) test

The Minimum Inhibitory Concentration (MIC) assay is a crucial antimicrobial evaluation method that identifies the minimal extract concentration required to prevent bacterial proliferation. In this study, the extract obtained from *P. lanceolata* was prepared as a 2% Dimethyl Sulfoxide (DMSO)-water solution. To maintain sterility, the extract was passed through a 0.45 micrometer membrane filter. The broth microdilution method was performed in 96-well microplates to evaluate antimicrobial activity [36]. The bacterial strains used in the experiments were adjusted to the 0.5 McFarland standard. Subsequently, an

appropriate amount of culture medium was added to each well of a 96-well microplate, and the extract was serially diluted to achieve decreasing concentrations. A predetermined amount of inoculum was then added to each well. For the positive control, only the culture medium and microbial suspension were used, whereas for the negative control, wells containing only the extract and culture medium, without the microbial inoculum, were prepared. To ensure the repeatability and reliability of the experiments, all procedures were performed in three independent replicates. Following the incubation period, the wells were inspected, and the minimum extract concentration that showed no detectable bacterial proliferation was identified. This value was documented as the Minimum Inhibitory Concentration (MIC). Bacteria and culture medium without plant extract were used as positive control. As negative control, only culture medium and plant extract were selected.

2.4.3 Minimum Bactericidal Concentration (MBC) test / Minimum Fungicidal Concentration (MFC) test

The Minimum Bactericidal Concentration (MBC) is defined as the smallest amount of an antimicrobial agent necessary to effectively eliminate the bacterial population. In this study, after determining the Minimum Inhibitory Concentration (MIC) value of *P. lanceolata*, 10 µL samples were taken from all wells where bacterial growth was either inhibited or absent. These samples were then transferred onto MHA plates. The inoculated plates were incubated at 37°C for 24 hours. The MBC value was determined by observing bacterial growth before and after incubation. Based on these findings, the minimum antimicrobial concentration that completely inhibited bacterial presence on the plates was determined as the MBC value [37]. Positive and negative controls included in the MIC test were used.

The Minimum Fungicidal Concentration (MFC) is the minimum concentration of an antimicrobial agent needed to completely eradicate the yeast population. In this study, following the determination of the MIC of *P. lanceolata*, 10 µL samples were collected from all wells where yeast growth was either inhibited or absent. These samples were subsequently inoculated onto MHA plates. The plates were under controlled condition 27°C for 48 hours, after which yeast growth was assessed. The MFC value was determined as the lowest antimicrobial concentration at which no yeast colonies were observed. Positive and negative controls included in the MIC test were used.

2.4.4 Anti-biofilm test

To determine the anti-biofilm potential of *P. lanceolata* ethanol extract in different microbial strains, concentrations below the MIC values were first identified. Bacterial cell suspensions were standardized to the 0.5 McFarland turbidity level and dispensed into each well of a 96-well microplate. The prepared plates were incubated at 37°C for 48 and 72 hours, respectively. The

exposure durations were established using a previously performed biofilm detection assay [38]. After the incubation period, the plates were rinsed with distilled water to remove free-floating cells and then treated with 0.1% crystal violet for 15 minutes. Following this process, any excess dye was removed by an additional wash with distilled water. Subsequently, a 7:3 mixture (70% ethanol: 30% acetone) was added to dissolve the strain. In the final step, the optical density of the liquid in each well was assessed at 550 nanometers. The experiment was performed in triplicate [39].

2.4.5 Determination of antioxidant activity

The ability of the *P. lanceolata* ethanol extract to neutralize free radicals was assessed by monitoring the decolorization of the persistent DPPH radical. This approach is based on the capacity of antioxidant compounds to neutralize DPPH radicals, leading to a reduction in the intense purple coloration observed at a wavelength of 515 nanometers.

For this purpose, 0.0039 g of DPPH preapared in 50 mL ethanol. Prepared DPPH was added to the specified columns with the serial dilution method [40]. The mixture was kept at ambient temperature in the absence of light for 30 minutes. After this period, the optical measurement was recorded at 515 nanometers using a microplate reader (Biotek Microplate Spectrophotometer). Ascorbic acid, a commercially available antioxidant, was used as the positive control. All tests were repeated three times.

The percentage of DPPH radical neutralization was determined using the following formula:

$$\text{DPPH Radical Scavenging (\%)} = \left(\frac{A_1 - A_0}{A_0}\right) \times 100$$

In the equation, A_0 refers to the initial optical density of the DPPH mixture (control), while A_1 represents the optical density of the extract-treated samples. This calculation quantifies the percentage of DPPH radicals scavenged by the *P. lanceolata* ethanol extract, providing an assessment of its antioxidant potential [21].

2.6 Biochemical composition analysis

To analyze the biochemical profile of the plant extract, GC-MS analyses were conducted using an Agilent 8890 GC system coupled with an Agilent 5977B mass spectrometer (Agilent Technologies Inc.) [41]. An HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) was used for the separation of volatile compounds. Helium (99.999% purity) was employed as the carrier gas at a constant flow rate of 1.0 mL/min. The injection volume was 1 μL with a split ratio of 1:10, and the injector temperature was set at 250 °C. The oven temperature was initially set at 50 °C (held for 2 minutes), then increased by 10 °C/min to 280 °C and held for 10 minutes, resulting in a total run time of

approximately 35 minutes. The MS detector operated in electron ionization (EI) mode at 70 eV, scanning a mass range of m/z 50–550. The ion source temperature was maintained at 230 °C. The obtained mass spectra and retention times were compared with entries in the Wiley-NIST mass spectral library for compound identification. The data were analyzed multiple times to verify the presence and quantity of the identified compounds. Depending on the solvent system, certain analytical parameters were adjusted as needed. In this study, compounds constituting at least 0.5% of the total composition were classified and reported as predominant components. The results obtained through this approach provide a comprehensive profile of the extract's fundamental chemical composition [42].

2.7 Statistical analysis

Data from three independent experiments for each antioxidant activity were reported as the mean ± standard deviation (SD). EC_{50} values were subsequently estimated through the Four-Parameter Logistic Regression method with a 95% confidence interval [43]. Statistical analyses were performed in R Studio **(**version 2024.09.0**)** using One-Way Analysis of Variance (ANOVA) and the Pearson correlation test. The level of statistical significance was set at $p \leq 0.05$.

3. RESULTS

3.1 Antimicrobial activity test

The ethanol extract of *P. lanceolata* was tested for antimicrobial activity against various bacterial and yeast strains using the disk diffusion method. Gentamicin and Tobramycin were employed as positive controls, and the inhibition zone measurements for extract-containing disks are provided in Tables 1–4. These antibiotics were included in the assay to facilitate comparative evaluation.

To evaluate the differences among independent trials, an ANOVA test was applied, resulting in a p-value of 0.00111. Since this value is below 0.05, it demonstrates a notable variation among the dose groups. However, Pearson correlation analysis revealed a low correlation coefficient ($r = 0.0365$, $p = 0.4491$) between extract concentration (50 microliters, 100 microliters, and 150 microliters) and inhibition zone diameter. This result suggests that the increase in extract concentration did not produce a clear dose-dependent effect on the inhibition zone.

TABLE 2. The disk diffusion method was employed to assess the antimicrobial response of food-isolated strains, with results recorded in millimeters. A value of (0.00±0.00) indicates no inhibition. Data are presented as mean ± standard deviation (n = 3), derived from three independent trials that yielded consistent results

Strains	50µL	100µL	150µL	Gentamicin	Tobramycin
Bacillus subtilis DSMZ 1971	7.33± 0.58	7.00± 0.00	7.33± 0.58	30.00± 0.00	26.00± 0.00
Candida albicans DSMZ 1386	7.33± 0.58	0.00± 0.00	8.00± 0.00	12.00± 0.00	13.00± 0.00
Enterobacter aerogenes ATCC 13048	0.00± 0.00	0.00± 0.00	0.00± 0.00	24.00± 0.00	18.00± 0.00
Enterococcus faecalis ATCC 29212	0.00± 0.00	0.00± 0.00	0.00± 0.00	12.00± 0.00	8.00± 0.00
Escherichia coli ATCC 25922	0.00± 0.00	0.00± 0.00	0.00± 0.00	22.00± 0.00	20.00± 0.00
Listeria monocytogenes ATCC 7644	0.00± 0.00	0.00± 0.00	0.00± 0.00	28.00± 0.00	24.00± 0.00
Pseudomonas aeruginosa DSMZ 50071	0.00± 0.00	0.00± 0.00	0.00± 0.00	15.00± 0.00	22.00± 0.00
Pseudomonas fluorescens P1	0.00± 0.00	0.00± 0.00	0.00± 0.00	13.00± 0.00	12.00± 0.00
Salmonella enteritidis ATCC 13076	7.00± 0.00	7.00± 0.00	7.33± 0.58	21.00± 0.00	20.00± 0.00
Salmonella typhimurium SL1344	0.00± 0.00	0.00± 0.00	0.00± 0.00	24.00± 0.00	15.00± 0.00
Staphylococcus aureus ATCC 25923	0.00± 0.00	0.00± 0.00	0.00± 0.00	21.00± 0.00	14.00± 0.00
Staphylococcus epidermidis DSMZ 20044	7.67± 0.58	9.00± 1.00	10.00± 1.00	22.00± 0.00	20.00± 0.00
Staphylococcus hominis ATCC 27844	8.33± 0.58	8.00± 1.00	9.00± 1.00	18.00± 0.00	16.00± 0.00
Staphylococcus warneri ATCC 27836	0.00± 0.00	0.00± 0.00	0.00± 0.00	23.00± 0.00	18.00± 0.00
Bacillus cereus RSKK 863	0.00± 0.00	0.00± 0.00	0.00± 0.00	24.00± 0.00	18.00± 0.00
Shigella flexneri RSKK 184	0.00± 0.00	0.00± 0.00	0.00± 0.00	18.00± 0.00	17.00± 0.00
Acinetobacter baumannii CECT 9111	0.00± 0.00	0.00± 0.00	0.00± 0.00	13.00± 0.00	22.00± 0.00

TABLE 2. The disk diffusion method was employed to assess the antimicrobial response of food-isolated strains, with results recorded in millimeters. A value of (0.00±0.00) indicates no inhibition. Data are presented as mean ± standard deviation (n = 3), derived from three independent trials that yielded consistent results

Strains	50µL	100µL	150µL	Gentamicin	Tobramycin
Enterococcus durans	0.00±0.00	0.00±0.00	0.00±0.00	11.00± 0.00	13.00± 0.00
Enterococcus faecium	0.00±0.00	0.00±0.00	0.00±0.00	28.00± 0.00	15.00± 0.00
Klebsiella pneumoniae	0.00±0.00	0.00±0.00	0.00±0.00	19.00± 0.00	23.00± 0.00
Listeria innocua	0.00±0.00	0.00±0.00	0.00±0.00	13.00± 0.00	15.00± 0.00
Salmonella infantis	0.00±0.00	0.00±0.00	0.00±0.00	17.00± 0.00	14.00± 0.00
Salmonella kentucky	0.00±0.00	0.00±0.00	0.00±0.00	12.00± 0.00	16.00± 0.00
Escherichia coli	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00± 0.00

TABLE 3. The disk diffusion method was used to evaluate the antimicrobial response of clinically isolated strains, with results expressed in millimeters. A value of (0.00±0.00) signifies no inhibition. Data are presented as mean ± standard deviation (n = 3), based on three independent trials that demonstrated consistent results

Strains	50µL	100µL	150µL	Gentamicin	Tobramycin
Staphylococcus aureus	7.67±0.58	9.00±1	10.00±0.00	22.00± 0.00	18.00± 0.00
Streptococcus mutans	7.00±0.00	8.00±0.00	9.00±5.20	22.00± 0.00	24.00± 0.00
Staphylococcus hominis	0.00±0.00	0.00±0.00	0.00±0.00	9.00± 0.00	11.00± 0.00
Staphylococcus haemolyticus	0.00±0.00	7.00±0.00	8.00±0.00	10.00± 0.00	10.00± 0.00
Staphylococcus lugdunensis	0.00±0.00	0.00±0.00	0.00±0.00	17.00± 0.00	18.00± 0.00
Shigella boydi	0.00±0.00	0.00±0.00	0.00±0.00	20.00± 0.00	18.00± 0.00
Acinetobacter baumannii	0.00±0.00	0.00±0.00	0.00±0.00	18.00± 0.00	16.00± 0.00
Shigella flexneri	0.00±0.00	0.00±0.00	0.00±0.00	16.00± 0.00	14.00± 0.00
Staphylococcus aureus	0.00±0.00	0.00±0.00	0.00±0.00	22.00± 0.00	16.00± 0.00
Enterococcus faecalis	8.33±0.58	9.00±1	9.67±0.58	12.00± 0.00	10.00± 0.00
Klebsiella pneumoniae	0.00±0.00	0.00±0.00	0.00±0.00	18.00± 0.00	18.00± 0.00
Candida tropicalis	0.00±0.00	0.00±0.00	0.00±0.00	0.00± 0.00	0.00± 0.00
Candida glabrata	0.00±0.00	0.00±0.00	0.00±0.00	7.00± 0.00	8.00± 0.00

TABLE 4. The disk diffusion method was employed to evaluate the antimicrobial activity against multi-drug-resistant strains, with results expressed in millimeters. A value of (0.00±0.00) denotes the absence of inhibition. Data are reported as mean ± standard deviation (n = 3), based on three separate trials that yielded consistent results

Strains	50µL	100µL	150µL	Gentamicin	Tobramycin
Escherichia coli	0.00± 0.00	0.00± 0.00	0.00± 0.00	8.00± 0.00	9.00± 0.00
Klebsiella pneumoniae	8.33± 0.58	8.00± 0.00	8.00± 1.00	15.00± 0.00	20.00± 0.00
Acinetobacter baumannii	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00
Enterobacter aerogenes	0.00± 0.00	0.00± 0.00	0.00± 0.00	16.00± 0.00	18.00± 0.00
Serratia odorifera	0.00± 0.00	0.00± 0.00	0.00± 0.00	7.00± 0.00	9.00± 0.00
Proteus vulgaris	0.00± 0.00	0.00± 0.00	0.00± 0.00	11.00± 0.00	11.00± 0.00
Streptococcus pneumonia	0.00± 0,00	0.00± 0.00	0.00± 0.00	10.00± 0.00	8.00± 0.00
Staphylococcus aureus MRSA	9.33± 0.58	10.00± 1.00	11.00± 0.00	0.00± 0.00	7.00± 0.00
Staphylococcus aureus MRSA+MDR	10.00± 1.00	11.00± 0.00	11.67± 0.58	22.00± 0.00	21.00± 0.00
Providencia rustigianii	0.00± 0.00	0.00± 0.00	0.00± 0.00	16.00± 0.00	19.00± 0.00
Achromobacter sp.	8.00± 0.00	9.00± 0.00	9.67± 0.58	9.00± 0.00	0.00± 0.00

These results indicate that *P. lanceolata* ethanol extract exhibited inhibition effects against 13 out of 48 tested strains, demonstrating significant antimicrobial activity against specific microorganisms. Among standard isolated bacteria (Table 1), *S. epidermidis* DSMZ 20044 showed a 10 mm inhibition zone, while *S. hominis* ATCC 27844 exhibited a 9 mm inhibition zone. No antimicrobial activity was observed among food isolated strains (Table 2). In clinical isolates (Table 3), the highest inhibition zone was recorded for *S. aureus*, measuring 10 mm. Among multidrug-resistant (MDR) strains (Table 4), *S. aureus* MRSA, *S. aureus* MRSA+MDR, and *Achromobacter* sp. showed the greatest sensitivity to the applied extract concentrations. However, the extract's efficacy varied among different microorganisms, emphasizing the importance of strain-specific susceptibility.

TABLE 5. Evaluation of MIC, MBC, and MFC values of *P. lanceolata*: Unveiling its antimicrobial and antifungal efficacy

Strains	**MIC (mg/ml)**	**MBC/MFC (mg/ml)**
Bacillus subtilis DSMZ 1971	> 22,867	> 22,867
Candida albicans DSMZ 1386	> 22,867	> 22,867
Salmonella enteritidis ATCC 13076	> 22,867	> 22,867
Staphylococcus epidermidis DSMZ 20044	> 22,867	> 22,867
Staphylococcus hominis ATCC 27844	> 22,867	> 22,867
Klebsiella pneumoniae	> 22,867	> 22,867
Staphylococcus aureus MRSA	0,175	0,179
Staphylococcus aureus MRSA +MDR	5,717	5,717
Achromobacter sp.	> 22,867	> 22,867
Staphylococcus aureus	0,175	0,715
Streptococcus mutans	0,175	0,179
Staphylococcus haemolyticus	0,175	11,433
Enterococcus faecalis	> 22,867	> 22,867

MIC and MBC values were also determined (Table 5). MIC values varied between, 0.175 to 22.867 milligrams per milliliter. The microorganisms with the lowest MIC values were *S. aureus* MRSA, *S. aureus*, *S. mutans*, and *S. haemolyticus*, each with an MIC of 0.175 mg/mL. MBC values ranged between 0.179 and 11.433 mg/mL. Notably, *S. aureus* MRSA and *S. mutans* exhibited the lowest MBC values (0.179 mg/mL), indicating high susceptibility to bactericidal effects. For other strains, such as *B. subtilis* DSMZ 1971, *S. epidermidis* DSMZ 20044, and *C. albicans* DSMZ 1386, MIC and MBC values were recorded as >22.867 mg/mL, suggesting that the required extract concentration for antimicrobial activity exceeded the tested range.

These findings demonstrate that *P. lanceolata* ethanol extract is effective against *S. aureus* MRSA, *S. mutans*, and *S. haemolyticus* even at low concentrations. The notable antimicrobial effect detected at minimal concentrations indicates that *P. lanceolata* could be a strong natural antimicrobial agent.

3.2 Anti-biofilm activity

The antibiofilm activity of *P. lanceolata* ethanol extract against different bacterial strains was evaluated. Biofilm activity was determined through OD550 absorbance measurements, and percentage changes were calculated relative to the control group. This research evaluated the antibiofilm activity of the tested plant solution against various bacterial species.

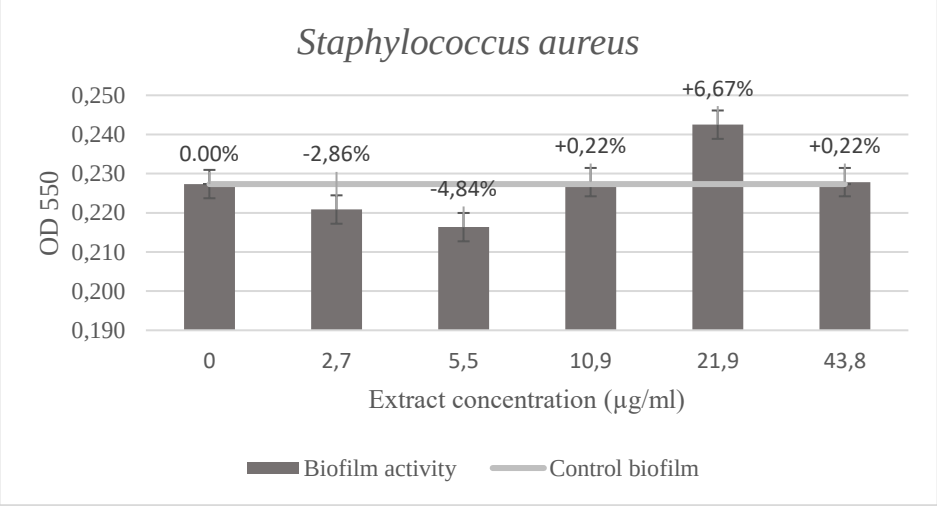

FIGURE 1. Effects of *Plantago lanceolata* on inhibition of biofilm formation. The line is normal amount of biofilm observed-positive controls. *S. aureus* from clinically isolated strain

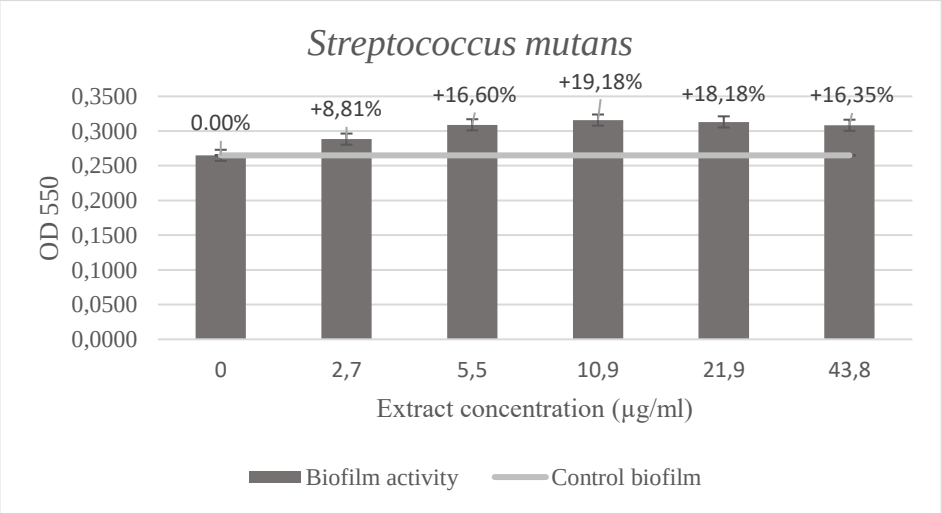

FIGURE 2. Impact of *Plantago lanceolata* on Biofilm Development Inhibition. The line is normal amount of biofilm observed-positive controls. *S. mutans* from clinically isolated strain

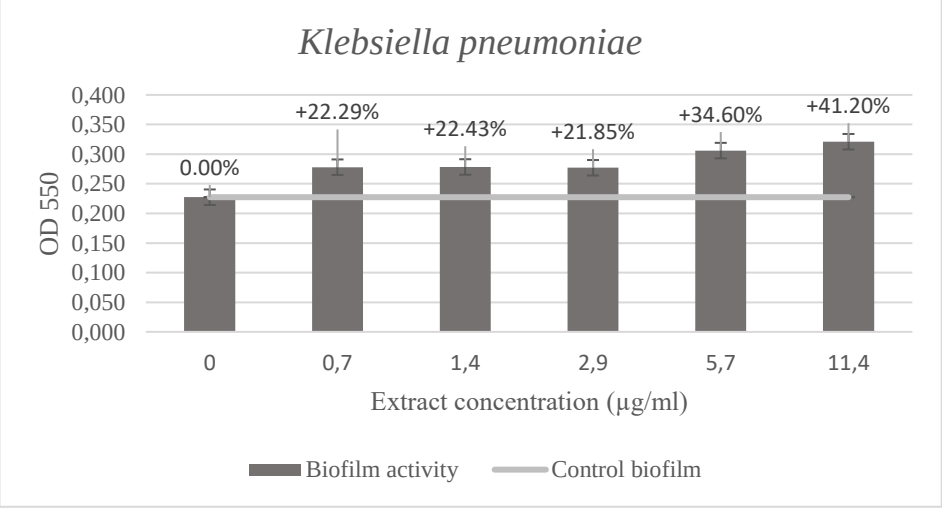

FIGURE 3. Impact of *Plantago lanceolata* on Biofilm Development Inhibition. The line is normal amount of biofilm observed-positive controls. *K. pneumoniae* from strains with multi-drug resistance

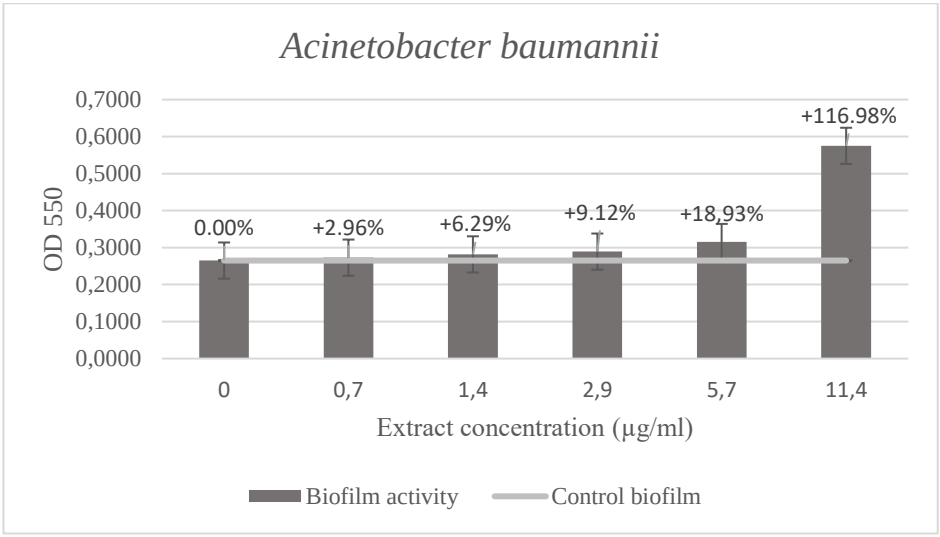

FIGURE 4. Impact of *Plantago lanceolata* on Biofilm Development Inhibition. The line is normal amount of biofilm observed-positive controls. *A. baumannii* from strains with multi-drug resistance

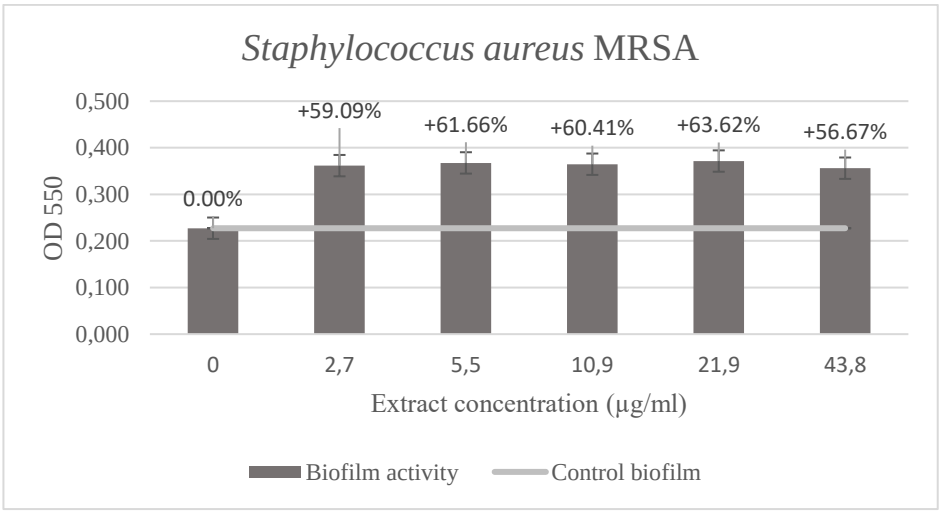

FIGURE 5. Impact of *Plantago lanceolata* on Biofilm Development Inhibition. The line is normal amount of biofilm observed-positive controls. *S. aureus* MRSA from strains with multi-drug resistance

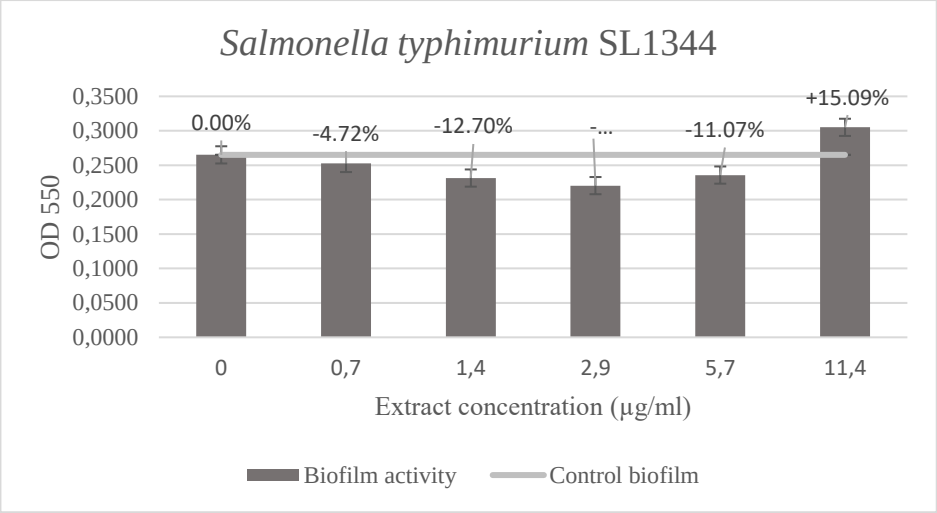

FIGURE 6. Effects of *Plantago lanceolata* on inhibition of biofilm formation. The line is normal amount of biofilm observed-positive controls. *S. typhimurium* SL1344 from standart isolates

In *S. aureus* (Figure 1) and *S. typhimurium* SL1344 (Figure 6) strains, biofilm inhibition was observed at lower extract concentrations, whereas an increase in biofilm formation was recorded at higher concentrations. Specifically, in *S. aureus*, biofilm production decreased by 4.84% at 5.5 µg/mL, while an increase of 6.67% was observed at 21.9 µg/mL. Similarly, *S. typhimurium* was the only strain where biofilm formation was suppressed at lower concentrations (16.86% reduction), yet at the highest concentration, biofilm production increased by 15.09%. These findings indicate that the solution may act in a dose-dependent manner, demonstrating antibiofilm properties at low concentrations while potentially promoting biofilm formation once specific threshold concentrations are exceeded.

In contrast, *S. mutans* (Figure 2), *K. pneumoniae* (Figure 3), *A. baumannii* (Figure 4), and methicillin-resistant *S. aureus* (MRSA) (Figure 5) exhibited a significant increase in biofilm formation upon treatment with the extract. The highest increase in *S. mutans* was observed at 10.9 µg/mL (19.18%), while in *K. pneumoniae*, biofilm production increased by 41.20% at 11.4 µg/mL. In the Gram-negative pathogen *A. baumannii*, biofilm formation dramatically increased by 116.98% at 11.4 µg/mL. Similarly, in *S. aureus* MRSA, biofilm activity significantly increased across all tested concentrations, with a 61.66% increase at 5.5 µg/mL and reaching 63.62% at 21.9 µg/mL.

These findings suggest that *P. lanceolata* extract may induce different biofilm responses depending on the bacterial species and applied concentration.

3.3 Antioxidant activity

The antioxidant activity of *P. lanceolata* ethanol extract was measured using the DPPH free radical neutralization test. Ascorbic acid was employed as the positive control, and ethanol without the extract was used as the negative control.

TABLE 6. DPPH free radical neutralization activity of *Plantago lanceolata* ethanol extract and ascorbic acid (%) expressed as mean ± SD

Concentration (µg/mL)	**DPPH Radical Neutralization Activity of *P. lanceolata* (%)**	**DPPH Radical Neutralization Activity of Ascorbic Acid (%)**
1000	62.70 ± 0.21	94.70 ± 0.00
500	60.80 ± 0.56	94.30 ± 0.05
250	63.20 ± 1.01	92.40 ± 0.01
125	41.00 ± 1.87	91.00 ± 0.01
62.5	23.40 ± 6.91	73.00 ± 0.04
31.25	18.90 ± 2.65	40.20 ± 0.07
15.625	10.60 ± 1.71	23.20 ± 0.27
7.81	0.51 ± 1.14	11.80 ± 0.04

Upon examining the antioxidant activity of the extract, the highest inhibition rate was determined to be 62.7% ± 0.21. Furthermore, the EC_{50} value was determined to be 0.0858 milligrams per milliliter. In contrast, the EC_{50} value of ascorbic acid was determined to be 0.04 milligrams per milliliter, indicating that the antioxidant capacity of the extract is lower than that of ascorbic acid. To assess the statistical significance of differences between concentrations, an ANOVA test was conducted, yielding a *p*-value of $p < 2 \times 10^{-16}$. This outcome confirms a statistically significant difference between ascorbic acid and the extract. Furthermore, Pearson correlation analysis was conducted to assess the correlation between extract concentration and DPPH scavenging activity, yielding an r value of 0.753 ($p = 2.335 \times 10^{-14}$). This strong positive correlation indicates that as the extract concentration increases, the ability to neutralize DPPH radicals increases proportionally. In conclusion, while *P. lanceolata* ethanol extract exhibits notable antioxidant activity, its efficacy is not as strong as that of ascorbic acid. However, a significant increase in antioxidant capacity was observed with increasing extract concentrations.

3.4 Biochemical composition analysis (GC-MS)

The biochemical profile of the *P. lanceolata* ethanol extract was examined through GC-MS analysis. Compounds present in concentrations above 0.5% were categorized as primary components. Table 7 outlines the identified chemical constituents, including their chemical structure, molecular formula, and molecular weight.

TABLE 7. GC-MS analysis of *Plantago lanceolata* ethanol extract profile

Retention Time	Area %	Compound Name	Formula	Molecular Weight (g/mol)
35.005	0.31	Myristic acid	$C_{14}H_{28}O_2$	228.37
36.532	4.10	Neophytadiene	$C_{20}H_{38}$	278.50
39.794	17.95	Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42
44.189	5.14	Linoleic Acid	$C_{18}H_{32}O_2$	280.40
44.357	26.46	Linolenic Acid	$C_{18}H_{30}O_2$	278.40
44.484	7.95	Linolenyl alcohol	$C_{18}H_{32}O$	264.40
44.621	26.37	Linolenic Acid	$C_{18}H_{30}O_2$	278.40
44.881	4.64	cis,cis,cis-7,10,13-Hexadecatrienal	$C_{16}H_{26}O$	234.38
45.208	2.26	Octadecanoic acid	$C_{18}H_{36}O_2$	284.5
47.906	0.19	Sorbitol	$C_6H_{14}O_6$	182.17
49.331	0.26	Eicosanoic acid	$C_{20}H_{40}O_2$	312.5
53.985	0.34	Docosanoic acid	$C_{22}H_{44}O_2$	340.6
56.723	0.22	Eicosane	$C_{20}H_{42}$	282.5
65.710	0.32	1-Nonadecene	$C_{19}H_{38}$	266.5
70.871	0.23	1-Docosene	$C_{22}H_{44}$	308.6
72.875	0.61	Phytyl stearate	$C_{38}H_{74}O_2$	563.0

The major components identified in the GC-MS analysis were Neophytadiene ($C_{20}H_{38}$) – 4.10%, Hexadecanoic acid ($C_{16}H_{32}O_2$, Palmitic Acid) – 17.95%, and Linolenic acid ($C_{18}H_{30}O_2$) – 52.83%, as detailed in Table 7.

This analysis indicates that *P. lanceolata* ethanol extract possesses a composition rich in lipid derivatives and fatty acids. Notably, hexadecanoic acid is a well-known compound with reported antimicrobial and antioxidant properties [44].

Among the compounds identified in the GC-MS analysis, sorbitol ($C_6H_{14}O_6$) was detected at 0.19%. Sorbitol metabolism plays a crucial role in plants as a sugar

alcohol with various biological functions [45]. It is involved in the plant's defense mechanisms against environmental stress factors. To sum up, the major components identified in *P. lanceolata* ethanol extract provide significant insights into the plant's potential pharmacological and biological activities.

4. DISCUSSION

Bacteria pose significant risks in the modern world and have become one of the greatest threats of our time. The resistance mechanisms that bacteria have developed against antibiotics and antimicrobial agents have driven scientists to focus on discovering novel antimicrobial compounds. In this search for alternative bioactive compounds, plants play a crucial role. Plants contain important biochemical compounds that contribute to various physiological and ecological processes, including environmental adaptation. These biochemical compounds, classified as secondary metabolites, exhibit diverse biological properties, including antimicrobial, antioxidant activities [46].

Our comprehensive study on *P. lanceolata* offers an in-depth evaluation of its antimicrobial, antibiofilm, and antioxidant properties. The results of the chemical composition analysis offer valuable insights into the plant's potential antimicrobial and antioxidant effects. The findings align with previous studies in existing literature, further supporting the pharmacological capability of *P. lanceolata*. This study was conducted using a broad-spectrum microorganism collection, ensuring a comprehensive evaluation of its bioactive properties.

The antimicrobial activity of *P. lanceolata* ethanol extract was initially evaluated using the disk diffusion test. To ensure the consistency of the results across replicates, ANOVA statistical analysis was performed, yielding a *p*-value of 0.00111. This result indicates a statistically significant similarity among repeated replicates. The correlation coefficient between the extract doses (50 microliters, 100 microliters, and 150 microliters) and inhibition zone diameters was found to be low, suggesting that increasing extract concentration did not have a clear dose-dependent effect on inhibition zones. The extract was tested against 48 microorganism strains, with the most pronounced inhibitory effects observed among multidrug-resistant and clinically relevant strains. *P. lanceolata* extract exhibited the highest antimicrobial effectiveness against multidrug-resistant *S. aureus* MRSA+MDR, forming a 12 mm inhibition zone, while among clinical isolates, it demonstrated a 10 mm inhibition zone against *E. faecalis***.** The Gram-positive bacterium *S. aureus* has shown significant susceptibility to *P. lanceolata* in previous studies [47]. A study in existing literature investigated the differences in the antimicrobial effectiveness of *P. lanceolata* extracts prepared using different solvents. The results demonstrated that the same bacterial strains exhibited varying susceptibilities depending on the solvent used [48]. Antimicrobial activity studies with different solvents are conducted to explore how each solvent's unique chemical properties influence the extraction and bioactivity of plant compounds.

In antimicrobial activity assays, bacterial growth in response to varying concentrations of the plant extract was assessed using the MIC test. The obtained results showed that the extract displayed strong antimicrobial activity against specific microorganisms. Among the tested strains, the most susceptible bacteria were multidrug-resistant *S. aureus* MRSA, clinically isolated *S. aureus*, and *S. haemolyticus*, with an MIC of 0.175 milligrams per milliliter. This finding indicates that *P. lanceolata* extract is effective against these strains even at low concentrations. Evaluation of the MBC test revealed that the MBC value for *S. aureus* MRSA and *S. mutans* was 0.179 mg/mL, suggesting strong bactericidal activity. In contrast, the MIC value for *S. haemolyticus* was recorded as 11.433 mg/mL, indicating a higher concentration requirement for antimicrobial efficacy against this strain. The observation of low MIC and MBC values against antibiotic-resistant strains such as *S. aureus* MRSA suggests that the plant extract has potential for further investigation as an antimicrobial agent. Among the standard strains, microorganisms such as *B. subtilis* DSMZ 1971 and *S. enteritidis* ATCC 13076 exhibited resistance beyond the tested extract concentrations.

The results obtained from the MIC and MBC experiments indicate that the antimicrobial activity of *P. lanceolata* extract aligns with findings from previous studies in the literature [48]. Similarly, studies available have explored the antimicrobial properties of other members of the *Plantaginaceae* family, such as *P. lanceolata* and *P. major*. The findings suggest that the presence of antioxidant activity within this plant family may contribute to its potential applications in various fields [49].

The antibiofilm activity of *P. lanceolata* ethanol extract was evaluated against different microorganism strains, revealing that biofilm inhibition varied with the type of bacterial species and extract concentration. The analyses indicated that the extract exhibited a selective effect on biofilm inhibition. While biofilm suppression was observed at low concentrations in certain strains, such as clinically isolated *S. aureus* and standard strain *S. typhimurium* SL1344, an increase in biofilm formation was detected in pathogenic strains including *S. mutans*, *K. pneumoniae*, *A. baumannii*, and *S. aureus* MRSA.

The presence of saturated fatty acids detected in the GC-MS analysis was investigated because of their potential influence on biofilm formation, which may be either positively or negatively affected. This effect appears to be dependent on environmental conditions [50]. The findings suggest that *P. lanceolata* extract exhibits a selective effect on biofilm inhibition, acting as a suppressor in some bacterial species while promoting biofilm formation in others. Research has examined the antimicrobial properties of species within the *Plantago* genus. Studies have examined how extracts obtained from different plant organs using various solvents can influence biofilm formation in bacteria, highlighting the impact of solvent selection on antimicrobial activity [51]. In the literature, the antibiofilm activity of *P. lanceolata* extract has been investigated

against *K. pneumoniae* and *E. coli* bacteria [52]. In our antibiofilm study, the analysis of multidrug-resistant strains suggested the presence of certain factors that may promote biofilm formation. This finding provides a valuable contribution to the literature by offering a deeper understanding of the biofilm-modulating effects of *P. lanceolata* extract.

The antioxidant property of *P. lanceolata* ethanol extract was assessed using the DPPH assay. The free radical neutralization capacity of DPPH was compared with that of vitamin C (ascorbic acid). The results showed that the extract exhibited antioxidant activity, although its effectiveness was less than ascorbic acid. To quantify the antioxidant activity, the EC_{50} value was used. The EC_{50} represents the concentration of the plant extract needed to neutralize half of the DPPH free radicals. The EC_{50} value of *P. lanceolata* ethanol extract was determined as 0.0858 mg/mL, whereas for ascorbic acid, this value was 0.0400 mg/mL. The higher EC_{50} value of the plant extract indicates that a higher amount is needed to produce the same antioxidant effect as ascorbic acid.

To determine whether the antioxidant activity of *P. lanceolata* extract is concentration-dependent, Pearson correlation analysis was performed, yielding an r value of 0.753 ($p < 0.0001$). This strong positive correlation indicates that as the concentration of the extract rises, the DPPH scavenging capacity also increases proportionally. Additionally, ANOVA analysis confirmed statistically significant differences among the different extract concentrations.

While ascorbic acid is identified as a pure compound, the plant extract exhibits a complex composition. Antioxidant effects can occur in synergistic or antagonistic interactions among its constituents. This suggests that the bioactivity of *P. lanceolata* extract could be enhanced through the isolation of its compounds into purer fractions. *P. lanceolata* extract presents potential as a natural antioxidant source.

Several studies have assessed the antioxidant capacity of *P. lanceolata* obtained from different sources using the DPPH assay [53]. The obtained results indicated that *P. lanceolata* extracts from different locations exhibited varying antioxidant activities. In the literature, the total antioxidant capacity of *P. lanceolata* extracts has been measured using both the DPPH and ABTS assays [54]. These studies suggest that the antioxidant activity of *P. lanceolata* has potential applications in various fields.

The biochemical profiling of *P. lanceolata* ethanol extract was analyzed using GC-MS. The results indicated that the extract primarily consists of lipid derivatives, fatty acids, aliphatic hydrocarbons, and terpenoids. A study in the literature [55] reported the presence of fatty acids in *P. lanceolata* extract based on GC-MS analysis. Among the dominant components, fatty acids such as linolenic acid ($C_{18}H_{30}O_2$) (26.46% and 26.37%) and hexadecanoic acid ($C_{16}H_{32}O_2$, palmitic acid) (17.95%) were identified. Linolenic acid has previously been shown to exhibit strong antioxidant, cardiovascular protective

effects [56]. Hexadecanoic acid, detected in the GC-MS analysis, is a fatty acid known for its antibacterial properties [57]. The compound has been reported to exhibit antibacterial effects against pathogens such as *S. aureus* and *P. aeruginosa*. In our study, the low MIC values observed against *S. aureus* MRSA and *S. mutans* may be associated with the presence of this compound. Additionally, Neophytadiene ($C_{20}H_{38}$) (4.10%) has been documented in the literature as a compound capable of inhibiting biofilm formation in certain bacterial species [58]. This compound is considered a significant secondary metabolite with the potential to contribute to the inhibitory effects observed in plant extracts against microorganisms. The results suggest that the components identified in the GC-MS analysis of *P. lanceolata* ethanol extract may be associated with its antioxidant and antimicrobial activities.

According to the literature, P. lanceolata is a plant with high drought tolerance, fast growth and deep rooting [59]. Plantago lanceolata's tolerance to drought conditions and deep rooting ability make it an important candidate for forage production and environmental sustainability. The inhibitory effect of aucubin compound on nitrification in the soil indicates the potential of this species to reduce nitrogen losses and N_2O emissions. The genotype and seasonal variability of aucubin content in leaves and roots reveals the importance of genotype selection for this purpose [60].

5. CONCLUSIONS

As a poster with the title “Antimicrobial Activity of *Plantago lanceolata* L. (Ribwort Plantain) with Ethnobotanical Significance” a part of this study was presented in International Congress of New Searches in Multidisciplinary Studies. This study extends the findings summarized in our presentation 'Antimicrobial Activity of *Plantago lanceolata* L. (Ribwort Plantain) with Ethnobotanical Significance' by analyzing them in more detail and compares them with existing information in the literature.

This study extends the findings summarized in our presentation 'Antimicrobial Activity of *Plantago lanceolata* L. (Ribwort Plantain) with Ethnobotanical Significance' by analyzing them in more detail and compares them with existing information in the literature.

This study demonstrates that *Plantago lanceolata* L. possesses antimicrobial, antibiofilm, and antioxidant properties. The plant extract exhibited significant antimicrobial activity, particularly against *S. aureus* MRSA, *S. mutans*, and *S. aureus* MRSA+MDR strains. The antioxidant capacity, determined by the DPPH assay, indicated a strong free radical scavenging potential.

GC-MS analysis revealed a volatile compound profile rich in fatty acid derivatives, particularly linolenic acid and hexadecenoic acid. The study employed a broad-spectrum microorganism collection, contributing valuable

data to the literature. The findings suggest that *P. lanceolata* represents a promising natural resource for pharmaceutical, food, and cosmetic applications.

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Declaration of Competing Interests The authors declare that there is no conflict of interest related to the content of this study.

Ethical Statement This research did not involve human participants or animals. Therefore, no ethical approval was required.

Use of Artificial Intelligence No artificial intelligence-based tools or applications were used in the preparation of this study. The entire content of the study was produced by the author(s) in accordance with scientific research methods and academic ethical principles.

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