

**Evaluation of the synergistic effects of *Lavandula angustifolia* extract in combination with antibiotics against multidrug-resistant *Enterococcus faecalis* and its in-vitro antioxidant activity**

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**ABSTRACT**

*Lavandula angustifolia* (lavender) is a perennial shrub known for its antibacterial and antioxidant properties, largely due to its essential oils and extracts. This study evaluated these properties using plant material collected in Lahore, Pakistan and processed at Minhaj University Lahore. The dried plant was extracted with 80% ethanol and chloroform and the crude extracts were tested against multidrug-resistant *Enterococcus faecalis*. Antibacterial activity was assessed via MIC and disk diffusion methods, while antioxidant activity was measured through hydrogen peroxide assay. The results showed significant antibacterial effects, particularly when combined with antibiotics, indicating synergistic interactions. The ethanolic extract achieved 73% inhibition while the chloroform extract showed 77.6% inhibition at a concentration of 5 mg/ml, with both also exhibiting strong antioxidant activity. These findings highlight *Lavandula angustifolia* as a promising source of bioactive compounds for the treatment of drug-resistant bacteria and the development of natural antioxidants.

**Key Words:** Medicinal plant, *Lavandula angustifolia* extract, synergism, additive, MDRs.

## INTRODUCTION

Traditional or herbal medicines are plant-based remedies widely used for the prevention and treatment of diseases. Due to their long history of use, they are generally regarded as safe at appropriate dosages. Medicinal plants are rich in phytochemicals such as flavonoids and polyphenols, which are responsible for biological activities including antimicrobial, antioxidant, and anti-inflammatory effects. A significant proportion of the global population, particularly in developing countries, relies on these natural products for primary healthcare. Consequently, medicinal plants remain an important source for the discovery of novel therapeutic agents. (Ugboko, *et al.*, 2020). (Liu, *et al.*, 2017). (Azwanida, 2015).

Lavender (*Lavandula angustifolia*) is a well-known medicinal and aromatic plant belonging to the Lamiaceae family. It has been used for centuries in traditional medicine, cosmetics, and food industries due to its pleasant fragrance and therapeutic properties. The genus *Lavandula* consists of approximately 39 species and is native to the Mediterranean region. *L. angustifolia* is the most widely used species, traditionally cultivated in countries such as France, Italy, and Spain. (Despinasse, *et al.*, 2020).

Lavender is economically valuable due to its applications in pharmaceuticals, cosmetics, and aromatherapy. It is a perennial shrub with woody stems, narrow leaves, and purple flowers arranged in spikes, adapted to dry and well-drained environments (Malakar, 2024). Lavender contains biologically active compounds, mainly linalool and linalyl acetate, present in its essential oil. These compounds contribute to its antimicrobial, antifungal, antioxidant, and anti-inflammatory properties. Lavender extracts also contain polyphenols and flavonoids, enhancing their therapeutic potential. The chemical composition may vary depending on environmental and extraction conditions. (Carrasco, *et al.*, 2016). (Radu, *et al.*, 2019).

Lavender exhibits diverse pharmacological activities, including antioxidant, antimicrobial, antifungal, and anti-inflammatory effects. It has also shown potential in wound healing, cancer inhibition, and mental health management, particularly in reducing anxiety, depression, and sleep disorders. Lavender essential oil is widely used in aromatherapy to promote relaxation and relieve stress. Additionally, it has demonstrated effectiveness in pain management, including labor pain, and shows emerging antiviral properties.

Antimicrobial resistance (AMR) is a major global health concern resulting from the overuse and misuse of antibiotics. It leads to reduced treatment effectiveness and increased mortality. Multidrug-resistant (MDR) bacteria, such as *Enterococcus faecalis*, pose serious challenges in clinical settings. (Kim *et al.*, 2024).

Oxidative stress is a key factor in the development of chronic and degenerative diseases, including cancer, cardiovascular disorders, autoimmune diseases, and neurodegenerative conditions. *Lavandula* species contain secondary metabolites such as flavonoids and phenolic acids that exhibit strong antioxidant activity. Lavender essential oil also contributes to antioxidant effects due to the presence of compounds like linalool, thymol, limonene, camphor, and 1,8-cineole. Studies have shown that these antioxidant properties are associated with additional biological effects, including lipoxygenase and hyaluronidase inhibition, as well as antibacterial activity (Sasaki *et al.*, 2015). Drug resistance may be intrinsic or acquired and includes categories such as MDR, XDR, and PDR. Bacteria develop resistance through several mechanisms, including limiting drug uptake, modifying drug targets, inactivating drugs, and active efflux of antibiotics (Mahon and Lehman, 2022).

## **MATERIAL AND METHODS**

### **Collection of Bacterial Isolates**

Multidrug-resistant bacterial isolates were obtained from blood and urine samples of hospitalized patients at Social Security Teaching Hospital. Samples were collected aseptically using sterile swabs. Species identification and confirmation of multidrug resistance were carried out according to the CLSI guidelines (Lin *et al.*, 2026).

### **Isolation of Pure Colonies**

Pure colonies of *Enterococcus faecalis* were isolated on blood agar and chocolate agar plates using the four-quadrant streaking technique. Colonies exhibiting typical morphology, characterized by small greyish-white non-hemolytic growth, were selected and further subcultured for confirmation.

### **Preparation of Culture Broth**

Culture broth was prepared for the preservation and maintenance of bacterial isolates following standard microbiological protocols. The medium was formulated using the recommended ingredients, and a total volume of 500 mL broth was prepared using the following composition. Fresh 24-hour bacterial cultures were used for inoculum preparation. Approximately 8–10 well-isolated colonies were aseptically picked from the culture plates using a sterile wire loop. The selected colonies were inoculated into 10 mL of laboratory-prepared nutrient broth and incubated at 37°C for 24 hours in the presence of 5% CO<sub>2</sub>. For sterility control, 4 mL of uninoculated broth was incubated under the same conditions to monitor possible environmental contamination.

### **Evaluation of bacterial broth**

After 24 hours of incubation, bacterial growth was evaluated by spectrophotometric analysis. The spectrophotometer was calibrated using a sterile nutrient broth blank to set the baseline and eliminate background absorbance. A 1–2 mL aliquot of the bacterial culture was then transferred into a clean cuvette, ensuring that the optical surfaces were free from any contamination such as fingerprints or smudges. Absorbance was measured at 600 nm, which is routinely used for estimating bacterial cell density. The recorded optical density (OD) values were used to assess bacterial growth and confirm culture purity.

### **Gram staining**

The selected bacterial colonies were performed to confirm their Gram-positive nature. Colonies were showed Gram-positive morphology and subjected to biochemical tests, including oxidase catalase and API 20E tests, to confirm their identity as *Enterococcus faecalis*.

### **API (Analytical Profile Index) 20E Test**

API 20E test strips were inoculated with a standardized bacterial suspension, with each strip containing 20 microtubules embedded with different biochemical substrates. Following inoculation, the strips were incubated at 35-37°C for 18–24 hours under appropriate conditions. After incubation, biochemical reactions were assessed based on visible color changes in each well, reflecting specific enzymatic or metabolic activities. The obtained reaction profile was interpreted using API web.

## Antibiotic susceptibility testing

### Disk Diffusion (Kirby-Bauer Method)

Antibiotic susceptibility testing was performed using the disk diffusion method. Antibiotic-impregnated paper disks were placed on agar plates inoculated with *Enterococcus faecalis*. After incubation, zones of inhibition around the disks were measured, and their diameters were used to assess antimicrobial activity. Results were interpreted as susceptible(S), intermediate(I), or resistant(R) according to standardized clinical and Laboratory standards Institute (CLSI) guidelines.

### Stock Solution

Bacterial isolates obtained during the early stage of the study were maintained as glycerol stocks for subsequent experimental applications. A sterile 50% (v/v) glycerol solution was prepared by combining equal volumes of sterile glycerol and sterile distilled water. The 700 µL culture was transferred into sterilized Eppendorf and 300µL sterilized 50% (v/v) glycerol for further preservation.

### Plant Preparation

Plant material of *Lavandula angustifolia* was collected from Pakistan in April 2024 and authenticated by following standard taxonomic identification procedures. The plant material was washed with deionized water, air-dried, and then oven-dried at 60°C for 6–8 hours. The dried material was ground into a fine powder and subjected to extraction using 80% ethanol and chloroform (1:16 w/v) at 25°C for 7–8 days with repeated extraction cycles. The resulting extracts were filtered and concentrated using a water bath followed by drying in a hot air oven at 60°C to remove residual solvent, yielding a semisolid crude extract, which was subsequently collected and pulverized into a fine powder for further analysis.

### Yield of Extraction

The extract of the yield calculated by using the formula (Akcaý *et al.*, 2023).

$$\% \text{Yield} = \text{Weight of extract} / \text{Weight of plant sample (10 g)} \times 10$$

### **MIC of resistant antibiotic**

Both microdilution assay was performed to determine the minimum inhibitory concentration (MIC) of antibiotics and plant extract against *Enterococcus faecalis*. A standardized bacterial suspension (0.5 McFarland) was prepared and exposed to serial two-fold dilutions in a 96-well microliter plate. Vancomycin and ampicillin were used as positive and negative controls, respectively, with PBS as a sterility control. Plates were incubated at 37°C for 24 hours in 5% CO<sub>2</sub>, and MIC values were determined using an ELISA plate reader based on inhibition of bacterial growth (Kowalska-Krochmal and Dudek-Wicher, 2021).

### **Checkerboard Board Assay**

All procedures were carried out under aseptic conditions in a biosafety cabinet, with equipment disinfected using 70% ethanol. A sterile microtiter plate was labelled for organism, antibiotic/plant extract, and respective concentrations. A standardized bacterial suspension of *Enterococcus faecalis* was prepared in Mueller–Hinton broth and adjusted to 0.5 McFarland using PBS. Serial two-fold dilutions of antibiotics (6–0.25 µg/mL) and serial dilutions of plant extracts in ethanol and chloroform were prepared, along with an ethanolic stock solution (300 mg/3 mL PBS). The prepared dilutions were dispensed into designated wells for subsequent MIC and checkerboard analysis (Bellio *et al.*, 2021).

### **Preparation of plant extract dilution with chloroform**

A chloroform plant extract stock solution was prepared at a concentration of 200 mg in 2 mL PBS and further diluted using the equation  $C_1V_1 = C_2V_2$ . A standardized inoculum of *Enterococcus faecalis* ( $5 \times 10^5$  CFU/mL) was added (100 µL) to each well of a sterile microtiter plate. Serially diluted antibiotics and plant extracts (ethanolic and chloroform) were dispensed into designated wells to create a checkerboard layout for combination testing. Vancomycin served as the positive control, ampicillin as the negative control, with additional wells for bacterial growth and PBS sterility control. Plates were incubated at 37°C for 24 hours in 5% CO<sub>2</sub>, and MIC values were determined using a BioTek ELISA microplate reader based on inhibition of bacterial growth.

### **Fractional Inhibitory Concentration (FIC)**

The Fractional Inhibitory Concentration (FIC) index used to evaluate the interaction between two antimicrobial agents, one is resistant antibiotic ampicillin and our plant extract to determine if their combined effect is synergistic, antagonistic, or indifferent by using the following formula (Fatsis-Kavalopoulos *et al.*, 2024):

$$\text{FICI} = (\text{MIC}_{\text{AB}}/\text{MIC}_{\text{A}}) + (\text{MIC}_{\text{BA}}/\text{MIC}_{\text{B}})$$

where  $\text{MIC}_{\text{AB}}$  is the minimum inhibitory concentration (MIC) of drug A tested in combination,  $\text{MIC}_{\text{A}}$  is the MIC of drug A tested alone,  $\text{MIC}_{\text{BA}}$  is the MIC of drug B tested in combination and  $\text{MIC}_{\text{B}}$  is the MIC of drug B tested alone. Synergy was defined as a FICI  $\leq 0.5$ , indifference as a FICI between  $>0.5$  and 4 and antagonism as a FICI  $> 4$ . The secondary outcomes were bactericidal activity, defined as  $>3 \log_{10}$  CFU/mL reduction in the colony count relative to the initial inoculum, and the effect of combination therapy on resistance development.

### **Combination of antibiotics and extract by disc diffusion assay**

Ethanollic and chloroform plant extracts were prepared at concentrations of 90 mg/2 mL and 70 mg/1.5 mL, respectively, and combined with ampicillin (10  $\mu\text{g}/\text{mL}$ ) for antimicrobial testing. Mueller–Hinton agar plates were inoculated with a lawn culture of *Enterococcus faecalis* using a sterile swab. Sterile filter paper discs were impregnated with extract–antibiotic combinations, ampicillin (negative control), vancomycin (positive control), and PBS (blank control), then placed onto the inoculated agar surface. Plates were incubated at 37°C for 18– 24 hours, after which zones of inhibition were measured. Antimicrobial activity and possible synergistic or antagonistic interactions were assessed based on inhibition zone diameters (Betlej *et al.*, 2024).

### **Antioxidant Activity**

Antioxidant activity was evaluated using a hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) scavenging assay. Stock solutions of ethanolic and chloroform extracts were prepared by dissolving 10 mg of each extract in 1 mL of the respective solvent. Serial two-fold dilutions were prepared in six tubes, yielding decreasing concentrations (5 to 0.16 mg). For analysis, each tube received  $\text{H}_2\text{O}_2$  and PBS, followed by the corresponding diluted extract, while a control tube contained  $\text{H}_2\text{O}_2$  and PBS without extract. All tubes were incubated at 37°C for 10 minutes, and absorbance was measured at 230 nm using

a spectrophotometer. The same procedure was applied for both extracts, and antioxidant activity was determined based on the reduction in absorbance relative to the control (Dobros *et al.*, 2022).

## RESULTS

Antimicrobial susceptibility testing of *Enterococcus faecalis* infection isolates revealed a multidrug-resistant antibiogram pattern. The isolates showed resistance to ciprofloxacin, levofloxacin, doxycycline, fosfomycin, nitrofurantoin, ampicillin, and penicillin, while remaining sensitive to vancomycin, teicoplanin, and linezolid. These findings indicated that the isolates possessed resistance against several commonly used antibiotics, whereas glycopeptide and oxazolidinone antibiotics retained effective antimicrobial activity against the tested strains.

**Table 1:** Antibiogram of *Enterococcus faecalis*

Antimicrobial susceptibility testing										
Antibiotics	Cipro	Levo	Doxy	Fose	Nitro	Amp	Peni	Vanco	Tenc	Line
(sensitive (S), resistant (R) or intermediate (I))	R	R	R	R	R	R	R	S	S	S

### *Enterococcus faecalis* on Gram stain

Morphological and microscopic examination was confirmed the bacterial isolate as *Enterococcus faecalis*. The isolate appeared as Gram-positive cocci with slightly spherical to ovoid-shaped cells. The cells were catalase-negative and measured approximately 0.5–2.0 µm in diameter, while some elongated cells measuring about 0.6–2.5 µm were also observed, contributing to the characteristic ovoid morphology of the organism.

### API 20E

The biochemical profile of *Enterococcus faecalis* was determined using standard enzymatic and carbohydrate utilization tests. The organism showed a positive reaction for arginine dihydrolase (ADH), Voges–Proskauer (VP), glucose fermentation (GLU), mannitol fermentation (MAN), and sucrose fermentation (SAC). In contrast, it was negative for ONPG, lysine decarboxylase (LDC), ornithine decarboxylase (ODC), citrate utilization (CIT), hydrogen sulfide (H<sub>2</sub>S) production, urease (URE), tryptophan deaminase (TDA), indole (IND), gelatin hydrolysis (GEL), inositol

(INO), sorbitol (SOR), rhamnase (RHA), melibiose (MEL), amygdalin (AMY), and arabinose (ARA). This biochemical pattern is consistent with the typical identification profile of *Enterococcus faecalis*.

### Minimum Inhibitory Concentration

The MIC analysis showed that the *Lavandula angustifolia* ethanol and chloroform extracts inhibited the growth of *Enterococcus faecalis* at concentrations of 150 mg/mL and 100 mg/mL, respectively. The MIC of ampicillin alone against the resistant isolate was 6 µg/mL. However, combination of ampicillin with 80% ethanol extract reduced the MIC to 2 µg/mL, while combination with 80% chloroform extract further reduced the MIC to 1 µg/mL, indicating enhanced antibacterial activity of the combined treatments.

**Table 2:** Minimum inhibitory concentration (MIC) of extracts through broth dilution method.

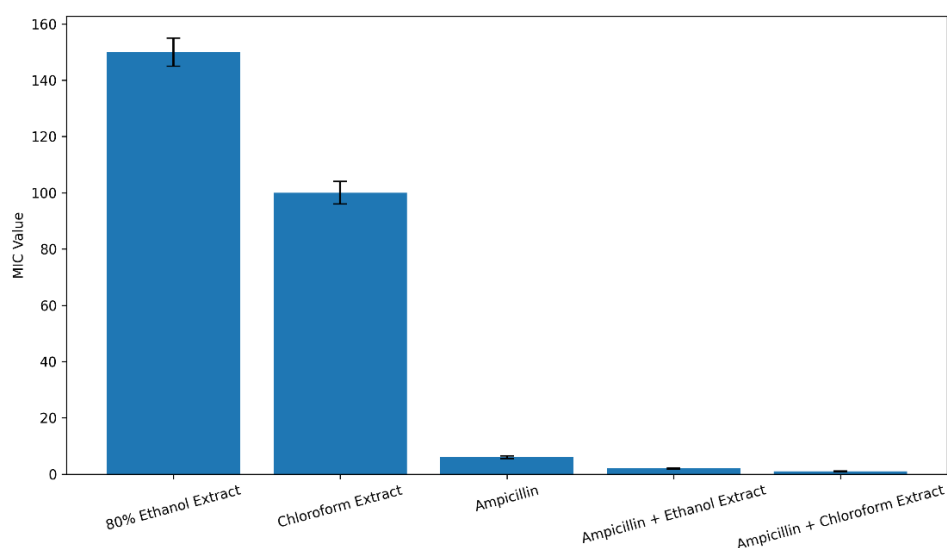
	80% Ethanol Extract (mg/ml)	Chloroform Extract (mg/ml)
<b>MIC</b>	150 mg/ml	100 mg/ml

**Table 3:** MIC of resistant antibiotic

		Resistant antibiotic
Strain	Activity	Ampicillin
<i>Enterococcus faecalis</i>	MIC	6ug

**Table 4:** Combination effects of *Lavandula angustifolia* extracts and resistant antibiotics

Strain	Ampicillin	Ampicillin + 80% Ethanol extract	Ampicillin + 80% Chloroform extract
<i>Enterococcus faecalis</i>	6ug	2ug	1ug

**Figure 1:** MIC and combination effect against *Enterococcus faecalis***Disc diffusion assay**

The combination of *Lavandula angustifolia* extracts with ampicillin showed antibacterial activity against *Enterococcus faecalis*. The ethanol extract combined with ampicillin to produced zone of inhibition, while the chloroform extract combined with ampicillin showed the highest activity with a inhibition zone. Vancomycin (positive control) showed also inhibition zone, whereas ampicillin alone and PBS showed no antibacterial activity in the following figure 3.2.



**Figure 2:** Results of combination of antibiotic and extract by disc diffusion assay

**Table 5:** Combination of resistant antibiotic with Ethanol and chloroform extracts

Zone of inhibition (mm)	
Ethanol extract + ampicillin	20
Chloroform + ampicillin	22
PC (vancomycin)	21
NC (ampicillin)	Negative
NC (PBS)	Negative

### Fractional Inhibitory Concentration Index

- **FIC Index  $\leq 0.5$ : Synergistic effect** (the combined effect of the drugs was greater than the sum of their individual effects).
- **FIC Index  $> 0.5 - 1.0$ : Additive effect** (the combined effect of the drugs was equal to the sum of their individual effects).
- **FIC Index  $> 1.0 - 2.0$ : Indifferent effect** (the combined effect of the drugs was less than the sum of their individual effects, but not significantly).
- **FIC Index  $> 2.0$ : Antagonistic effect** (the combined effect of the drugs was less effective than one or both drugs alone).

**Table 6:** Fractional Inhibitory Concentration Index Outcome

Strain	Antibiotic + extract	Results	
<i>Enterococcus faecalis</i>	Ampicillin + 80% Ethanol extract	0.76	Additive
	Ampicillin + 80% Chloroform extract	0.46	Synergistic

**Antioxidant activity**

*Lavandula angustifolia* (lavender) has shown notable antioxidant activity, particularly when exposed to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), a common oxidative stress agent. The results demonstrated that lavender extracts (Ethanol and Chloroform) are capable of scavenging free radicals and reducing oxidative stress markers in vitro, including hydrogen peroxide-induced oxidative damage. The results suggest that lavender's bioactive compounds play a protective role by neutralizing reactive oxygen species (ROS), including H<sub>2</sub>O<sub>2</sub> (Tang *et al.*, 2017).

**Table 7:** Antioxidant activity of plant extract with ethanol

Concentration (mg/ml)	Absorbance (230nm)	Inhibition (%)
5	0.5	73
2.5	0.6	68
1.25	0.81	56
0.62	1.08	42.5
0.31	1.09	42
0.15	1.1	41.4

**Table 8:** Antioxidant activity of plant extract with chloroform

Concentration (mg/ml)	Absorbance (230nm)	Inhibition (%)
5	0.42	77.6
2.5	0.52	72.1
1.25	0.65	65.1
0.62	0.67	64.3
0.31	0.68	63.4
0.15	0.69	62.9

## DISCUSSION

Medicinal plants are rich sources of bioactive compounds such as alkaloids, flavonoids, phenolic and essential oils, which exhibit significant antimicrobial and antioxidant activities. These properties have gained increasing importance due to the rise of antibiotic-resistant microorganisms, highlighting the need for alternative and complementary therapeutic strategies. Plant-based antimicrobials not only inhibit microbial growth but also enhance the efficacy of conventional antibiotics.

*L. angustifolia* is widely recognized for its therapeutic potential. Its essential oil contains active compounds such as linalool, linalyl acetate, and phenolic constituents, which contribute to its antibacterial and antioxidant effects (Nwozo *et al.*, 2023). Previous studies have demonstrated its ability to scavenge reactive oxygen species, including hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), thereby reducing oxidative stress. These antioxidant properties are largely attributed to phenolic acids and flavonoids, which act as effective free radical neutralizers (Ozougwu, 2016). In addition to its antioxidant activity, *L. angustifolia* has shown notable antimicrobial effects. Earlier research reported synergistic and additive interactions when lavender essential oil was combined with antibiotics such as chloramphenicol, ciprofloxacin, and fusidic acid against pathogens like *Pseudomonas aeruginosa*. Similar synergistic effects have also been observed with other plant essential oils in combination with antibiotics, supporting the potential of plant–drug combinations in antimicrobial therapy. In the present study, ethanolic and chloroform extracts of *L. angustifolia* exhibited significant antibacterial activity against multidrug-resistant (MDR)

*Enterococcus faecalis*, with the ethanolic extract showing slightly higher efficacy. Conversely, the chloroform extract demonstrated comparatively stronger antioxidant activity, indicating that solvent type influences the extraction of specific bioactive compounds.

Importantly, a synergistic effect was observed when *L. angustifolia* extracts were combined with ampicillin, resulting in enhanced antibacterial activity against MDR *E. faecalis*. This may be due to mechanisms such as increased membrane permeability or inhibition of resistance pathways. Notably, this is among the first studies to report such interaction against this pathogen. In conclusion, *L. angustifolia* demonstrates dual antimicrobial and antioxidant potential, along with the ability to potentiate conventional antibiotics. These findings suggest that plant-antibiotic combinations may serve as an effective strategy to combat multidrug-resistant infections while reducing drug dosage and limiting the development of resistance.

The findings of this study have several important implications. First, they underscore the potential of *Lavandula angustifolia* as a source of new antimicrobial agents. The use of plant-derived compounds in conjunction with resistant antibiotic could be a viable strategy to manage MDR infections. Moreover, this study highlights the need for further research to isolate and characterize the specific bioactive compounds responsible for the observed antimicrobial activity.

Future studies should focus on exploring the mechanisms underlying the synergistic effects observed between plant extracts and antibiotics. Additionally, in vivo studies are necessary to assess the safety, efficacy, and pharmacokinetics of these plant extracts when used alone or in combination with antibiotics. The development of standardized protocols for the extraction and use of plant-based antimicrobials is also essential to ensure consistency and reproducibility in research and clinical applications.

## CONCLUSION

The findings of this study highlight the significant antioxidant potential of *Lavandula angustifolia* (lavender) extracts, particularly their ability to scavenge free radicals and mitigate oxidative stress induced by hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Both ethanolic and chloroform extracts demonstrated notable antioxidant activity, with the chloroform extract showing slightly higher efficacy in reducing oxidative damage. At a concentration of 5 mg/ml, the chloroform extract exhibited 77.6% inhibition, while the ethanolic extract showed 73% inhibition, indicating that lavender contains

bioactive compounds capable of neutralizing reactive oxygen species (ROS). These results suggest that lavender's antioxidant properties may be attributed to its phenolic acids, such as rosmarinic acid, and flavonoids, which have been previously reported to play a crucial role in ROS scavenging. Additionally, the difference in inhibition percentages between the two extracts suggests that the solvent type influences the extraction efficiency of the bioactive compounds. Chloroform, being a non-polar solvent, may have extracted compounds more effectively than ethanol, contributing to its higher antioxidant activity. This study supports previous research on the antioxidant properties of *Lavandula angustifolia*, reinforcing its potential as a natural source for therapeutic applications aimed at combating oxidative stress. Further research is needed to isolate and characterize the specific compounds responsible for the observed antioxidant effects, which could lead to the development of novel antioxidant therapies derived from lavender.

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