



RESEARCH ARTICLE

In vitro antibacterial and antifungal activities of methanolic leaf extracts of *Eucalyptus lanceolatus* and *Lawsonia inermis*, individually and in combination

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ABSTRACT

The growing problem of antimicrobial resistance has boosted interest in plant-derived compounds as potential sources of novel antimicrobial drugs. The present study was conducted to investigate the in vitro antibacterial and antifungal activities of crude methanolic leaf extracts of *Eucalyptus lanceolatus* and *Lawsonia inermis* alone and in combination at equal ratio. Dried leaves were powdered and extracted with methanol. The antibacterial activity against *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* was tested by agar-well diffusion method. Individual extracts were tested at 400 and 1000 µg/mL and the combination at a nominal concentration of 1000 µg/mL. Antifungal activity was tested against *Aspergillus fumigatus* and *Aspergillus flavus* on Sabouraud dextrose agar at 1000 µg/mL, terbinafine was used as positive control and dimethyl sulfoxide as negative control. *L. inermis* and *E. lanceolatus* showed 75% and 50% relative inhibition against *K. pneumoniae* and 35% and 30% against *P. aeruginosa* at 1000 µg/mL, respectively. The combination showed 78.2% inhibition against *S. aureus* as against the individual extracts which showed 65.2% and 39.1%. In these settings, the inhibition of fungi was modest. *L. inermis* showed 22% against *A. flavus*, while *E. lanceolatus* showed 15% against *A. fumigatus*. These preliminary results require more standardized susceptibility testing, formal interaction assays, phytochemical characterization, and cytotoxicity evaluation to prove any therapeutic significance.

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1. Introduction

Antimicrobial resistance (AMR) poses a major threat to global public health and affects the efficacy of existing antibacterial and antifungal treatments and the clinical and economic burden of infectious disease. Resistant Gram-negative and Gram-positive infections remain high on the World Health Organization's list of surveillance and research priorities [1, 2]. The GBD 2021 analysis estimated a large global mortality burden from bacterial AMR. These results highlight the need for standardizing the in vitro screening approach and for further exploring the naturally

occurring compounds with chemical diversity as promising beginning points for constructing antibacterial medicines. Medicinal plants are a source of various secondary metabolites like Phenolics, Quinones, Terpenoids, Tannins, flavonoids, and other compounds that can affect cellular targets such as microbial membranes, enzymes, redox balance, etc. Hence, it is of great importance to research plant-derived antimicrobials at the discovery stage. Activity depends not only on potency but also on solubility, molecular diffusion, inoculum density, and composition of

the medium, diffusion-based assays should be carefully examined [3,4].

Species of *Eucalyptus* (Myrtaceae) have been thoroughly explored for their antibacterial, antioxidant, and anti-inflammatory activities. The major ingredients of essential oils of certain *Eucalyptus* species are known to be 1,8-cineole (eucalyptol), α -pinene, and other terpenoids. The phytochemical content is highly dependent on species, plant part, location, and the extraction process [3, 4]. Bhagat [5], investigated the antibacterial activity of essential oils produced from different sections of *Eucalyptus lanceolatus*. They identified eucalyptol, α -pinene, β -pinene, and α -eudesmol as the main components of the oils. Note that the chemistry of an essential oil is not comparable to that of a methanolic crude extract, and so mechanistic attribution necessitates direct profiling of the extract in question.

L. (henna; family Lythraceous) is one of the oldest cosmetic and therapeutic plants and is widely used in South Asia, the Middle East and North Africa. It includes naphthoquinones (including lawsone (2-hydroxy-1,4-naphthoquinone)), polyphenols, and other helpful substances in its leaves. Recently, research has also underlined the significance of chemical standardization and additional mechanistic validation of *L. inermis* for its antibacterial, antioxidant, anti-inflammatory, and wound-associated pharmacological action [6-8]. Recent experimental studies have shown the important bactericidal action of methanolic extracts of henna [9].

Extensive investigation has been made on both plants, although the antibacterial activity of mixed crude methanolic extracts of both the plants has been researched to a limited extent. The present investigation was carried out to evaluate the antibacterial and antifungal activities of methanolic leaf extract of *E. lanceolatus* and *L. inermis* singly and in combination against selected bacterial and fungal isolates. The data reported herein were interpreted without synergy because the original experiment was not performed with a combination analysis with MIC. Mixed effect of extract not synergistic (more pronounced reaction) [10].

2. Materials and Methods

2.1 Study design

In the present laboratory work, crude methanolic leaf extracts of *E. lanceolatus* and *L. inermis* were tested for activity against three bacterial test species and two filamentous fungus species. Individual extracts of the antibiotic were assessed at 400 and 1000 $\mu\text{g/mL}$. The combined extract was evaluated at the reported nominal concentration of 1000 $\mu\text{g/mL}$. Antifungal susceptibility testing was performed at 1000 $\mu\text{g/mL}$. The experimental values reproduced in this publication are those published in the original study record.

2.2 Plant material and authentication

Leaves of *E. lanceolatus* were taken from Shahbaz Azmat Khel, Bannu, Pakistan, and shade-dried. *L. inermis* leaves were purchased from the local market in Bannu. Taxonomical identification of plant material was done by the Department of Botany, Government Degree College No. 1, Bannu. For future journal publication, we encourage deposition of voucher specimens in a recognized herbarium and inclusion of voucher numbers, as botanical traceability is a critical prerequisite for reproducible medicinal plant research.

2.3 Preparation of methanolic extracts

The dried leaves were pulverized into a fine powder. The plants were ground, and 20g of leaf powder was transferred separately into a lidded conical flask containing methanol and shaken for around 12 hours. The suspension was filtered with Whatman filter paper. The filtrate was allowed to evaporate at room temperature for about three days, and the dried crude extract was collected in microcentrifuge tubes and stored for antimicrobial testing. The extraction yield and phytochemical composition can be affected by grinding, solvent selection, extraction duration, and agitation; thus, these parameters must be reported consistently, and preferably, extraction yield should be quantified [7,8].

2.4 Test microorganisms

The antibacterial activity was evaluated against two Gram-negative bacteria, *K. pneumoniae* and *P. aeruginosa*, and one Gram-positive pathogen, *S. aureus*. Its antifungal activity was evaluated against *A. fumigatus* and *A. flavus*. The source paper does not disclose the strain accession numbers and exact isolate provenance; these should be supplied in any confirmatory analysis.

2.5 Antimicrobial test

The antibacterial activity was assessed by the agar-well diffusion method, which is a common screening approach for plant extracts [11]. Bacterial cultures were grown on nutrient broth at 37 °C for 24 hours. Nutrient agar was prepared by dissolving 6.5 g in 100 mL of deionized water and autoclaved at 121 °C for 15 min. The medium was poured into each Petri dish (ca. 30 mL) and allowed to solidify. The suspension of bacteria was spread on the surface of the agar with a sterile brush. Wells were made with a sterile cork borer (3-6 mm).

The negative control was dimethyl sulfoxide (DMSO; 10 μL). The positive control was done using a regular antibiotic. The antimicrobial comparator was not identified, and its concentration was not stated in the corresponding study record and should be disclosed in future studies. Ten microliters of each distinct extract at 400 and 1000 $\mu\text{g/mL}$ were added into the respective wells. The mixture of *E. lanceolatus* + *L. inermis* combination preparation was tested at the previously reported nominal value of 1000 $\mu\text{g/mL}$, in equal proportions. Plates were incubated for 24

h, and inhibitory zones were found. The original data expressed activity as relative inhibition (%), with the positive-control response set to 100%. Due to the absence of raw zone dimensions, the percentages presented in the present paper are given without reconstructing unreported observations.

2.6 Antifungal assay

Antifungal activity was assessed by a solid medium inhibition of growth experiment. Sabouraud dextrose agar (7.9 g) was dissolved in 120 mL deionized water and sterilized by autoclaving at 121 °C for 15 min. Five milliliters of medium were added to each tube. A 67 µL aliquot of *E. lanceolatus* extract, *L. inermis* extract, or the equal-parts combination extract was added at the reported concentration of 1000 µg/mL. DMSO was utilized as the negative control and terbinafine as the positive control. The tubes were kept in a slant position until the agar solidified, infected with the relevant fungal species, plugged with cotton, and incubated at 30 °C. Growth inhibition was measured after seven days.

This approach is to be viewed as a preliminary growth-inhibition screen and not as a standardized MIC determination. For confirmatory antifungal susceptibility studies with filamentous fungi, broth microdilution according to the CLSI M38 reference standard is chosen since it specifies inoculum preparation, medium, incubation, and endpoint conditions [12].

2.7 Interpretation of activities of mixed extracts

The equal-part mixture was descriptively compared with the activities of individual extracts. The increased percentage inhibition for the combo was viewed only as an enhanced combined response. Formal interaction methods such as the checkerboard MIC assay with calculation of the fractional inhibitory concentration index (FICI) and/or time-kill approach are required to confirm antimicrobial synergy; simple comparison of diffusion-zone percentages is not sufficient to determine synergy [10,11].

2.8 Analysis of data

The available trial record presented single percentage-inhibition results for each treatment condition but not replicate-level measures, standard deviations, confidence intervals, or inferential statistics. Therefore, results are presented descriptively, and p-values or assertions of statistical significance are not given. This method does not require the precision not available in the underlying record.

3. Results

3.1 Antibacterial activity

Both plant extracts demonstrated detectable antibacterial activity and showed greater inhibition, usually at 1000 µg/mL than at 400 µg/mL. The susceptibility pattern was different in different bacterial species (Table 1). *L. inermis*

was the most active individual extract against *K. pneumoniae* with 75% relative inhibition at 1000 µg/mL and 58.3% at 400 µg/mL. *E. lanceolatus* produced 50% and 25% inhibition at the given doses. The combined extract exhibited 50% inhibition at 1000 µg/mL and did not show higher activity than *L. inermis* alone.

Activity against *P. aeruginosa* was often lower. *E. lanceolatus* showed 35% inhibition at 1000 µg/mL and 20% at 400 µg/mL. *L. inermis* showed 30% and 20%, respectively. The combined extract showed 25% inhibition at 1000 g/mL. The highest combined extract activity was reported against *S. aureus*, where the mixture showed 78.2% inhibition at 1000 µg/mL compared to 65.2% for *L. inermis* and 39.1% for *E. lanceolatus*. The separate extracts showed 30.4 and 21.7% inhibition at 400 µg/mL, respectively.

4. Discussion

The present study demonstrated that the crude methanolic leaf extracts of *E. lanceolatus* and *L. inermis* had considerable in vitro antibacterial and antifungal activity, although the degree and pattern of inhibition were organism specific. The maximum antibacterial activity was seen for *L. inermis* against *K. pneumonia* [11]. The combined extract showed the highest overall activity against *S. aureus*. The antifungal effects were somewhat moderate, and no additional effect of the combination above the best-performing individual extract was seen.

This strong activity of *L. inermis* is in keeping with its phytochemical profile. The antibacterial activity of henna leaves is due to the presence of Lawson and other phenolic and polyphenolic chemicals [6, 7, 9, 13]. The antibacterial activity of *L. inermis* alcoholic and methanolic extracts against major clinical pathogens, such as *S. aureus* and Gram-negative bacteria [4, 14]. The 75% inhibition of *K. pneumoniae* obtained in these experiments is in keeping with the wider evidence of substantial antibacterial activity of methanolic henna extracts. But the results of the research cannot be directly compared, because the extraction techniques, standardization of the inoculum, assay formats, and endpoint criteria are different.

E. lanceolatus was also found to possess antibacterial and antifungal properties, which have been previously reported for this species. The main volatile compounds of *E. lanceolatus* essential oils were eucalyptol, α -pinene, β -pinene, and α -eudesmol [11]. They displayed antibacterial and antifungal action. Many species of *Eucalyptus* have been generally reported to have antibacterial properties. The efficiency is strongly reliant on the chemical composition [3,12,13,14]. The active components of essential oils often operate on the microbial membranes and affect the permeability of membranes, ion balance, and cellular homeostasis [15]. As the present experiment used methanolic leaf extracts instead of essential oils, these mechanisms should be considered as hypotheses rather than confirmed explanations. The methanolic extract should be

analyzed by LC-MS, HPLC or GC-MS to correlate the activity with specific constituents.

Fig. 1. Methodology Schematic diagram (Source: Bio Render)

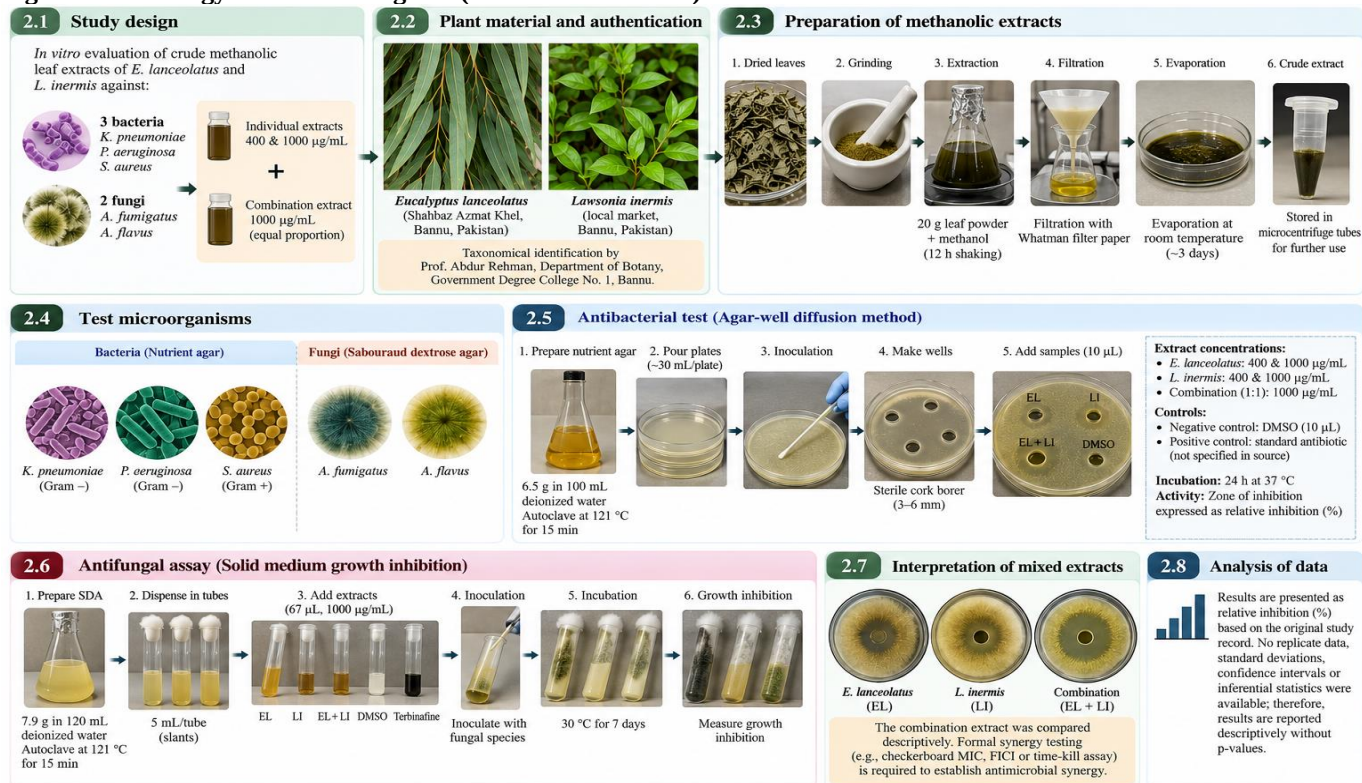


Table 1. Relative antibacterial inhibition (%) of methanolic leaf extracts

Bacterial species	<i>E. lanceolatus</i> 1000 µg/mL	<i>E. lanceolatus</i> 400 µg/mL	<i>L. inermis</i> 1000 µg/mL	<i>L. inermis</i> 400 µg/mL	Combined extract 1000 µg/mL
<i>Klebsiella pneumoniae</i>	50	25	75	58.3	50
<i>Pseudomonas aeruginosa</i>	35	20	30	20	25
<i>Staphylococcus aureus</i>	39.1	21.7	65.2	30.4	78.2

Note: The positive-control antibiotic response was normalized to 100% in the original record. Raw inhibition-zone diameters and replicate statistics were not available.

The least vulnerable bacterial species to the tested compounds was *P. aeruginosa*, with inhibition values of 25–35% at 1000 µg/mL. Interestingly, this pattern is observed although *P. aeruginosa* has been proven to be less resistant than Gram-positive bacteria to plant-derived antimicrobials and essential oils [16,17], Elaissi evaluated 20 *Eucalyptus* essential oils and showed that the most susceptible bacteria were *S. aureus* and the most resistant was *P. aeruginosa*. The observed activity is quite modest, which may reflect the known permeability and intrinsic

defense systems of this organism. In the present experiment, it is not possible to demonstrate a mechanism. *S. aureus* requires special attention [19]. The combined extract at 1000 µg/mL gave 78.2 % relative inhibition, while *L. inermis* and *E. lanceolatus* showed 65.2 % and 39.1 % inhibition, respectively. The above tendency reflects the increased activity of the chemical in the observed conditions. However, the discovery is technically improper to be defined as pharmacological synergy, as no MIC values, checkerboard concentration matrix, or time-kill data were produced. Synergy is a concept of interaction, which

requires the quantitative assessment of the effects of medications alone and in combination, usually by checkerboard analysis based on FICI or kinetic killing assays [Odds 2003]. Hence, this result should be viewed as a positive sign for the recommendations for cautious interaction testing. The pooled extract showed no efficacy against *K. pneumoniae* or *P. aeruginosa*. *K. pneumoniae*, the mixture was equivalent to *E. lanceolatus* (50%) but inferior to *L. inermis* alone (75%). The mixture (25%) was less active against *P. aeruginosa* at 1000 µg/mL than *E. lanceolatus* (35%) and *L. inermis* (30%). However, the species-specific aspects suggest that the overall effects are not generalizable across the microbial species. Interaction between crude extracts may be additive, neutral, or antagonistic depending on the relative amount of active and inert chemicals, physicochemical compatibility, and the biology of the microbial target. Antifungal research also revealed species-specific trends. *L. inermis* had higher activity against *A. flavus* (22%) than *A. fumigatus* (7%), while *E. lanceolatus* had higher activity against *A. fumigatus* (15%) than *A. flavus* (11%). The blend generated moderate yields (20 and 10%, respectively) and thus showed no evidence of increased activity. The literature reviews of *L. inermis* and *Eucalyptus* spp. suggest the antifungal potential of both plants; however, the low inhibition obtained here indicates that crude methanolic preparations at the concentration tested would need further optimization and MIC/MFC confirmation before any meaningful antifungal claims can be made [3, 5, 6].

Some methodological constraints reduce the strength of inference. First, raw inhibition-zone diameters, number of replicates, and estimates of variation were not provided, making statistical testing and assessment of repeatability problematic. Second, the identity of the strain and the antibacterial drug used as a positive control were not mentioned. Thirdly, standardization of the doses up to the extraction yield, volume of methanol used, and the chemical fingerprints proved difficult to report. Fourthly, diffusion assays can underestimate weakly diffusing medicines and may not produce MIC or bactericidal/fungicidal endpoints. Finally, for the equal-part combination, just one nominal concentration was evaluated, which is not enough to mimic the interaction.

Future experiments in pursuit of a high-impact journal standard should be performed using authentic voucher specimens, standardized extract yield, phytochemical fingerprinting by LC-MS/HPLC and GC-MS as appropriate, reference microbial strains with well-characterized clinical isolates, biological replicates with mean $\pm$ standard deviation, MIC and minimum bactericidal/fungicidal concentration measurements, and formal checkerboard or time-kill analysis for combination effects. If confirmation is needed, susceptibility testing of filamentous fungi should be performed following the CLSI M38 methods [12]. Selectivity index test and cytotoxicity test should be done before suggesting for biomedical usage [20].

Figure 2. Antibacterial inhibition of single and mixed leaf extracts in methanol. Values are taken from the original study record. Error bars cannot be provided as replicate-level data were not reported.

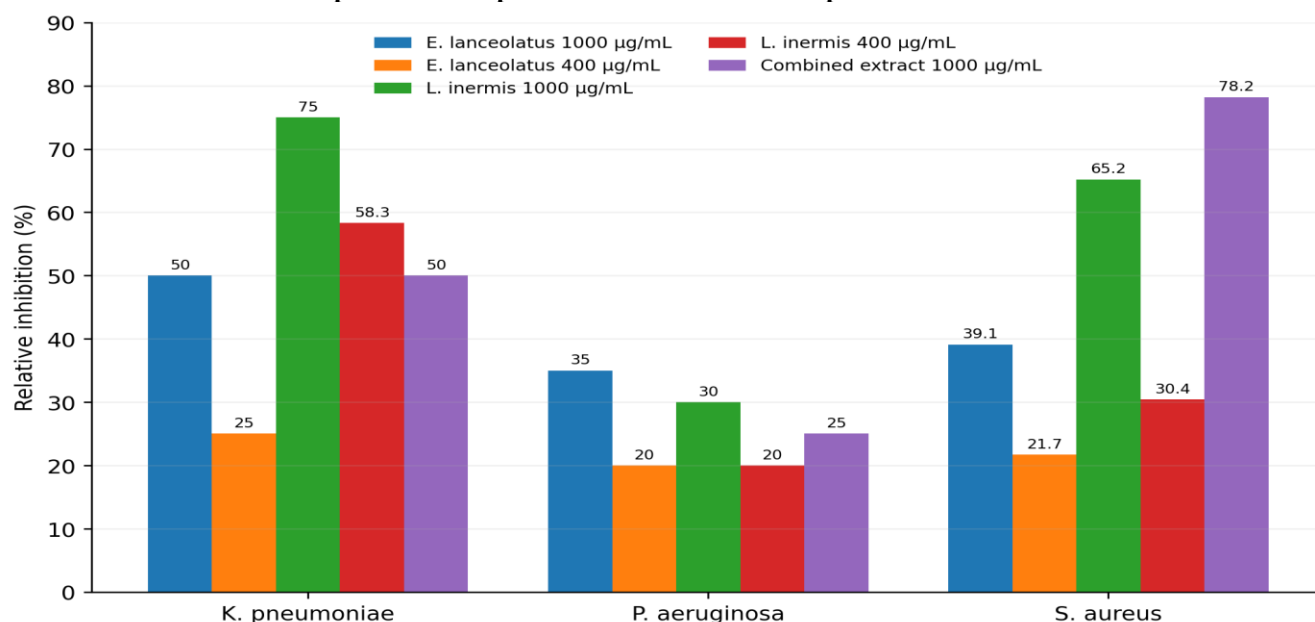
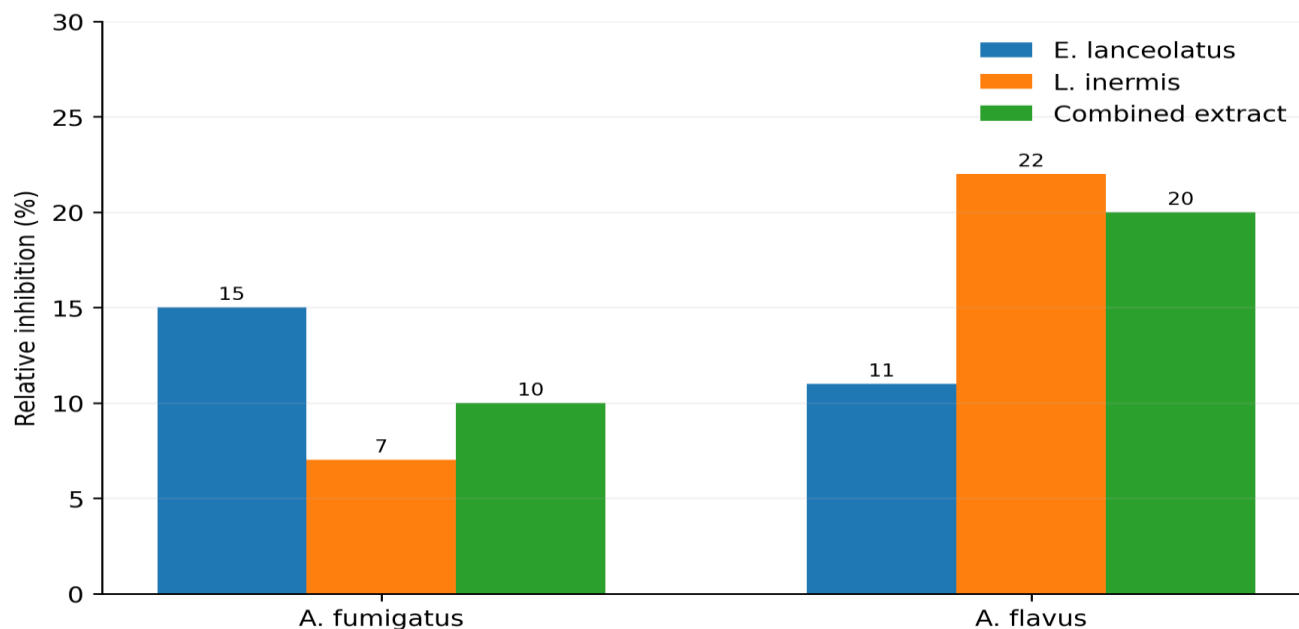


Table 2. Relative antifungal inhibition (%) at 1000 µg/mL

Fungal species	<i>E. lanceolatus</i>	<i>L. inermis</i>	Combined extract
<i>Aspergillus fumigatus</i>	15	7	10
<i>Aspergillus flavus</i>	11	22	20

Figure 3. Antifungal inhibition of methanolic leaf extracts relative to the control at 1000 µg mL⁻¹. Terbinafine was used as a positive control (100% original record).



5. Conclusion

Methanolic extracts of leaves of *E. lanceolatus* and *L. inermis* showed preliminary in vitro antibacterial and antifungal activity against the tested microorganisms. *L. inermis* had significant activity against *K. pneumoniae*, and the same combination exhibited the maximum antibacterial activity against *S. aureus*. Comparative testing showed considerable efficiency against *P. aeruginosa* and against both *Aspergillus* spp. Combined extract vs *S. aureus* is fantastic but should be taken as improved activity and not proven synergy. These data merit further investigation using standardized MIC-based approaches, quantitative interaction testing, statistical analysis at the replicate level, and phytochemical characterization.

6. Study limitations and submittal details

Information not provided with the supplied study record (which should not be fabricated): extract yield; volume of methanol used for extraction; voucher-specimen numbers; microbial-strain accession numbers; identity/concentration of the antibacterial positive-control drug; raw inhibition-zone diameters; replicate-level observations; standard deviations; p-values; MIC/MBC/MFC values; and a formal synergy metric. These exclusions do not contradict the early screening

results, but they significantly constrain the claims about potency, repeatability, and synergy. This data will have to be written in the lab notebook before submission if it is to be included.

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