Kastamonu Uni., Orman Fakültesi Dergisi, 2025, 25(3): 409-421 Research Article
Kastamonu Univ., Journal of Forestry Faculty
Doi:10.17475/kastorman.1845436

Antimicrobial Activity and Mode of Action of *Phellinus hartigii* Extract Investigated on Multidrug-Resistant *Staphylococcus aureus* Using Fourier-Transform Infrared Spectroscopy

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Received Date: 15.01.2025 Accepted Date: 23.06.2025

Abstract

Aim of study: Macrofungi have garnered attention as promising reservoirs for novel antibiotics. *Phellinus hartigii* is known to have antimicrobial properties against various microorganisms. This study aims to investigate the *in vitro* mode of action of the ethanol extract of *P. hartigii* (PH-EtOH) against multidrug-resistant (MDR) *Staphylococcus aureus* using Fourier-transform infrared (FTIR) spectroscopy.

Material and method: The composition of the extract was determined by GC-MS analysis, and its antimicrobial activity was evaluated through MIC and MBC tests. The effect of the extract was further analysed using FTIR spectroscopy.

Main results: The PH-EtOH extract demonstrated antibacterial activity with a MIC of 53 µg/mL and an MBC of 420 µg/mL. The major constituents of PH-EtOH were ethyl oleate, linoleic acid ethyl ester, and hexadecanoic acid ethyl ester. FTIR spectra showed changes mainly in proteins and nucleic acids.

Research highlights: The results of this study provide a starting point for further research into the use of *P. hartigii* ethanol extract as an antimicrobial agent against MDR *S. aureus*.

Keywords: *Phellinus hartigii*, *Staphylococcus aureus*, Antimicrobial Activity, Mode of Action, FTIR

Çoklu İlaca Dirençli *Staphylococcus aureus* Üzerinde *Phellinus hartigii* Ekstraktının Antimikrobiyal Aktivitesi ve Etki Mekanizmasının Fourier Dönüşüm Infrared Spektroskopisi Kullanılarak İncelenmesi

Öz

Çalışmanın amacı: Makrofunguslar, yeni antibiyotikler için umut vadeden rezervuarlar olarak dikkat çekmektedir. *Phellinus hartigii*'nin çeşitli mikroorganizmalara karşı güçlü antimikrobiyal özellikler taşıdığı gösterilmiştir. Bu çalışma, *P. hartigii*'nin etanol ekstraktının (PH-EtOH) çoklu ilaca dirençli (MDR) *Staphylococcus aureus*'a karşı *in vitro* etki şekline ilişkin FTIR tabanlı kanıt sağlamayı amaçlamaktadır.

Materyal ve yöntem: PH-EtOH'nin bileşimi GC-MS analizi kullanılarak belirlenmiş ve *S. aureus*'a karşı antimikrobiyal aktivitesi MİK ve MBK testleri ile değerlendirilmiştir. Ekstraktın etkisi FTIR spektroskopisi kullanılarak analiz edilmiştir.

Temel sonuçlar: PH-EtOH ekstraktı, *S. aureus*'a üzerinde 53 µg/mL'lik bir MİK ve 420 µg/mL'lik bir MBK ile antibakteriyel aktivite göstermiştir. PH-EtOH'nin ana bileşenleri etil oleat, linoleik asit etil ester ve heksadekanoik asit etil ester olarak bulunmuştur. FTIR spektrumları, başlıca protein ve nükleik asitlerde değişiklikler göstermiştir.

Araştırma vurguları: Bu çalışmanın sonuçları, *P. hartigii* etanol özütünün MDR *S. aureus*'a karşı antimikrobiyal ajan olarak kullanımına yönelik daha ileri araştırmalar için bir başlangıç noktası sağlamaktadır.

Anahtar Kelimeler: *Phellinus hartigii*, *Staphylococcus aureus*, Antimikrobiyal Aktivite, Etki Mekanizması, FTIR

Citation (Atıf): Akcay, K., Teksen, K., Senturan, U. M., Altinöz, E., Ozerkan, D., Akata, I., & Altuner, E. M. (2025). Antimicrobial Activity and Mode of Action of *Phellinus hartigii* Extract Investigated on Multidrug-Resistant *Staphylococcus aureus* Using Fourier-Transform Infrared Spectroscopy. *Kastamonu University Journal of Forestry Faculty*, 25 (3), 409-421.

Introduction

Drug resistance in bacteria is a growing concern in modern medicine, as it reduces the efficacy of antibiotics and can lead to the spread of infections that are difficult to treat (Friedman et al., 2016). Drug resistance occurs when bacteria evolve mechanisms to protect themselves from the effects of antimicrobial drugs, allowing them to survive and multiply despite the presence of the drugs (Tenover, 2006). This phenomenon can occur naturally or as a result of overuse and inappropriate use of antibiotics (Cantón et al., 2013). Addressing drug resistance in bacteria is essential, as it can cause more extended hospital stays, higher healthcare costs, and increased mortality rates (Slama, 2008; Hay et al., 2018; Li & Webster, 2018). It can also result in the spread of drug-resistant strains of bacteria, leading to the emergence of new, more dangerous infections (Alanis, 2005). Implementing measures such as using antibiotics only when necessary, promoting the development of new antibiotics, and improving infection prevention and control practices to avoid the spread of drug resistance (Canli et al., 2016a; MacGowan & Macnaughton, 2017).

Research into new antibiotics derived from natural sources has gained increased attention in recent years due to the growing threat of antibiotic-resistant bacteria (Subramani et al., 2017). Scientists are exploring various sources of natural compounds, including soil microorganisms, plants, and marine life, searching for new compounds to combat drug-resistant bacteria (Harvey, 2000; Christina et al., 2013; Cragg & Newman, 2013; Nweze et al., 2020). These compounds often have unique structures and modes of action compared to conventional antibiotics, making them more effective against bacteria resistant to existing drugs (Fjell et al., 2012). The discovery of new antibiotics from natural sources is crucial for addressing the growing threat of antibiotic resistance and ensuring effective treatments for bacterial infections (Spellberg et al., 2008; Högberg et al., 2010; Altuner & Çetin, 2018).

Macrofungi are one of these natural sources, emerging as promising sources for discovering new antibiotics (Bala et al., 2012; De Silva et al., 2013; Nelson et al., 2021). They produce a wide range of secondary metabolites with potent antimicrobial activity and offer a rich source of novel compounds for drug development (Zhong & Xiao, 2009).

Fungi can be found in diverse environments and produce various antibiotics targeting various bacteria, including drug-resistant strains (Morath et al., 2012). Furthermore, many fungi have been shown to produce antibiotics in response to competition from other microorganisms, making them ideal sources for discovering new antibiotics (Shen et al., 2017). Using fungi to extract new antibiotics is a promising approach in searching for new treatments for bacterial infections and the fight against antibiotic resistance (De Silva et al., 2013; Canli et al., 2016b).

Phellinus hartigii is a fungus that harms fir trees, leading to trunk cankers and wood rot. It infects trees through wounds or areas weakened by disease. The fungus forms persistent fruiting structures that are effused-reflexed and sessile, with a pale yellow-brown upper surface and pores ranging from grey to purplish-brown. As well as colonising living trees, it completes its lifecycle by breaking down dead wood, contributing to decomposition throughout the year (Breitenbach & Kränzlin, 1986).

Phellinus hartigii is a fungus that has been shown to have intense antimicrobial activity against various pathogens, including bacteria and yeast (Altuner & Akata, 2010; Akpi et al., 2017; Azeem et al., 2018). This activity is due to bioactive compounds in the fungus, such as polyphenols and terpenoids, which have been shown to have potent antimicrobial effects (Appiah et al., 2017). The ability of *P. hartigii* to inhibit the growth of harmful microorganisms makes it a promising candidate for use in developing new antimicrobial agents. These findings suggest that *P. hartigii* has excellent potential as a source of novel antimicrobial agents that can help combat the growing threat of drug-resistant infections (Altuner & Akata, 2010).

Staphylococcus aureus is a gram-positive bacterium that can cause a wide range of infections, from mild skin issues to severe conditions like pneumonia and sepsis. It is hazardous due to its ability to develop multidrug resistance. This multidrug

resistance not only complicates treatment options but also leads to increased morbidity and mortality rates. Therefore, combating *S. aureus*, particularly its resistant strains, is critical to safeguarding public health and ensuring effective treatment options remain available (Abebe & Birhanu, 2023).

FTIR, or Fourier Transform Infrared spectroscopy, is a powerful analytical tool for identifying substances' molecular composition (Baker et al., 2014). In microbiology, FTIR has been used to study the effects of antibiotics on bacteria by analysing the changes in the molecular structure of bacterial cells after exposure to antibiotics (Ribeiro da Cunha et al., 2020).

The method works by measuring the absorption of infrared light by the bacterial cells, which can reveal changes in the chemical bonds of the cells caused by the antibiotics (Faghihzadeh et al., 2016). This information can be used to determine the mode of action of the antibiotics and how they affect the bacterial cells. FTIR is a sensitive, cheap, and reliable method for analysing the effects of antibiotics on bacteria. It can also provide valuable information for developing new antibiotics (Ribeiro da Cunha et al., 2020).

Furthermore, FTIR can monitor bacterial population changes over time and provide information on the emergence of antibiotic-resistant strains. FTIR in the study of antibiotics and bacteria is a promising approach that can provide new insights into the mechanisms of antibiotic action and the development of new antibiotics (Alvarez-Ordóñez et al., 2011).

This study aims to present some FTIR-based evidence about the *in vitro* mode of action of the ethanol extract of *P. hartigii* (Allesch. & Schnabl) Pat. against *S. aureus*.

Material and Methods

Collection of P. hartigii Samples

P. hartigii (Allesch. & Schnabl) Pat. samples were collected from Ilgaz Mountain National Park (Kastamonu, Türkiye).

Extraction Process

Dried macrofungi (20 gr) were ground, and ethanol (EtOH) (Sigma-Aldrich, Germany) was used to extract the active compounds by shaking the fungal material at room temperature for two days. The mixture was filtered through filter paper, and EtOH was evaporated at 45°C under a vacuum using a rotary evaporator (Heidolph). The remnant was weighed, and an extract stock of 64.23 mg/mL was prepared using 1% DMSO (Merck, Germany) as solvent (Canli et al., 2020).

Gas Chromatography-Mass Spectrometry Analysis

The method described in previous studies was used to determine the composition of *P. hartigii* ethanol extract (PH - EtOH) by GC-MS analysis (Canli et al., 2014). The chemical compounds in the extract greater than 1% were considered major components.

Inoculum Preparation Process

In this study, a clinically isolated *S. aureus* with benzylpenicillin, ciprofloxacin, levofloxacin, and tetracycline-resistant was used. Bacterial culture in the exponential phase was first obtained, and a loopful of bacteria was transferred to 0.9% sterile NaCl solution. The turbidity of the suspension was adjusted to a 0.5 McFarland standard (Altuner & Canlı, 2012).

Minimum Inhibition Concentration Test

The Minimum Inhibition Concentration (MIC) test was performed using a 96-well plate. A series of two-fold dilutions of the *P. hartigii* extracts were prepared in sterile Mueller Hinton Broth, and 100 µL of the inoculum was added to each well of the 96-well plate. The plate was then incubated at 37°C for 24 hours, and the minimum concentration of the antimicrobial agent that inhibited visible bacterial growth was determined. The MIC value was recorded as the lowest concentration of the *P. hartigii* extract, which showed no visible bacterial growth after incubation (Altuner & Çetin, 2009).

Minimum Bactericidal Concentration Test

The Minimum Bactericidal Concentration (MBC) test was performed to determine the lowest concentration of the *P. hartigii* extract that killed bacteria. After conducting the MIC test, a sub-sample of the wells showing

inhibition of bacterial growth was plated onto agar plates and incubated overnight to determine the presence of viable bacteria. The MBC was defined as the lowest concentration of the *P. hartigii* extract, resulting in no detectable viable bacteria after overnight incubation at 37°C (Altuner & Çetin, 2009).

Fourier-Transform Infrared Analysis

The bacteria were grown in TSB (Merck, Germany) in glass tubes to examine the effect of the observed concentrations, and after incubation, they were precipitated through centrifugation at 5000 rpm for 10 minutes. The resulting pellets were then prepared for FTIR analysis by spreading them on a glass slide and allowing them to dry for two days. The dried pellets of three different replicates were scraped and inserted into the ATR-FTIR (Bruker Alpha, Germany) device for analysis separately (Zengin Köksal et al., 2021).

The FTIR spectra were acquired with the following parameters: 64 background scans, 32 analysis scans, a resolution of 2 cm^{-1}, and a spectral range spanning from 400 to 4000 cm^{-1}.

Data Processing

The FTIR and second derivative spectra were analysed in the 400 to 4000 cm^{-1} range. Data were processed using R Studio version 4.3.2, as mentioned in the statistics section (URL-1, 2023). Vector normalisation was used to adjust the intensity values of the spectral data. Zero-center scaling was applied by subtracting the mean value from each data point in the spectrum. Baseline correction was performed to enhance the spectral features and reduce the impact of baseline variations. Savitzky-Golay smoothing was used to reduce noise and enhance the clarity of spectral data, making it easier to analyse and interpret the underlying features. The spectra were visualised using Orange Data Mining Software version 3.36.

Statistics

The experiments were conducted in triplicate for each sample to ensure the accuracy and reproducibility of the results. The results were analysed using a t-test with a significance level of 0.05. All analyses were done using R Studio, version 4.3.2 (URL-1, 2023). In addition, a volcano plot was used, plotting $\log_2$(fold change) vs. $-\log_{10}$(p-value), to identify significant bands, showing an increase or decrease and non-significant bands in *S. aureus* following PH-EtOH treatment.

Results and Discussion

In this study, the antibacterial effect of PH - EtOH against the *S. aureus* strain was studied, and the extract's *in vitro* mode of action was evaluated by FTIR.

Antibacterial Activity of P. hartigii Ethanol Extract

As a result of the study, the minimum inhibitory concentration (MIC) for PH-EtOH was determined as 53 µg/mL, and the minimum bactericidal concentration (MBC) was found to be 420 µg/mL. These results suggest that the PH - EtOH has the potential to be an antibacterial agent, which has a bacteriostatic effect against *S. aureus*.

Altuner and Akata (2010) previously tested the antimicrobial activity of *P. hartigii* extract against *Candida albicans*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Shigella flexneri*. Their study found antibacterial activity only against *S. flexneri*.

The data showing the resistance profile of *S. aureus* in Table 1 indicate that the MIC values for all antibiotics are lower than the MIC values of PH-EtOH extract, but the MIC value for PH-EtOH is still low and shows potential as an antibacterial agent.

Major Chemical Compounds of P. hartigii Ethanol Extract

Table 2 provides information on the major chemical compounds of PH-EtOH and their relative quantities. In addition, the chromatogram of the analysis is given in Figure 1.

The results given in Table 2 show that the highest percentage composition of PH-EtOH is ethyl oleate (19.19%), followed by linoleic acid ethyl ester (16.53%) and hexadecanoic acid ethyl ester (11.14%).

Table 1. Antibiotic susceptibility profile and MIC interpretation for *S. aureus*.

Antibiotic	MIC (µg/mL)	Susceptibility Interpretation
Benzylpenicillin	>0.25	R
Gentamicin	<=0.5	S
Ciprofloxacin	2	R
Levofloxacin	0.5	I
Erythromycin	<=0.25	S
Clindamycin	<=0.12	S
Linezolid	1	S
Daptomycin	0.25	S
Teicoplanin	<=0.5	S
Vancomycin	<=0.5	S
Tetracycline	>8	R
Tigecycline	<=0.12	S
Fosfomycin	<=8	S
Nitrofurantoin	<=16	S
Fusidic acid	<=0.5	S
Mupirocin	<=1	S
Trimethoprim/ Sulfamethoxazole	<=10	S

Note: S (sensitive), I (intermediate), and R (resistant)

Table 2. The major chemical compounds and their corresponding percentages in the *P. hartigii* ethanol extract.

Major Compounds	Corresponding quantities (%)
Ethyl oleate	19.19
Linoleic acid ethyl ester	16.53
Hexadecanoic Acid, Ethyl Ester	11.14
Octadecanoic acid, ethyl ester	6.66
9-Octadecenamide	6.51
D - Allose	5.74
Palmitic acid	3.60
Anethole	3.57
Heptadecanoic acid, Ethyl Ester	2.47
Hexadecane	2.09
Oleic acid	2.04
Benzoic acid	1.67
Nonadecanamide	1.38
3-Hydroxy-4-methoxy benzoic acid	1.36
Pentadecanoic acid, Ethyl Ester	1.22
Dimethyl{bis[(2Z)-pent-2-en-1-yloxy]}silane	1.15
5,8-Octadecadienoic acid, methyl ester	1.11
Ethyl 9-hexadecenoate	1.09

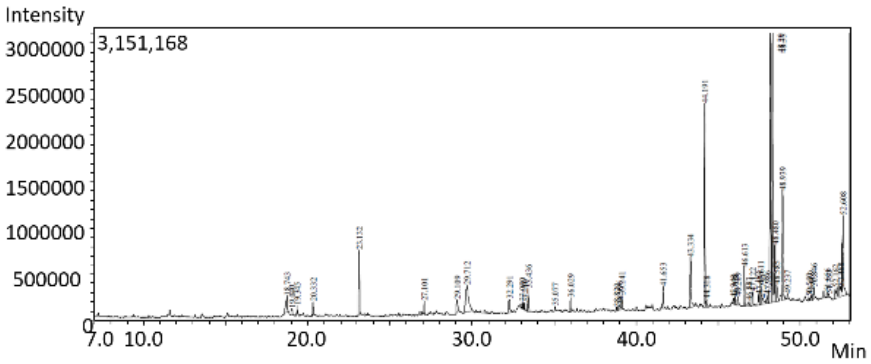

Figure 1. Chromatogram of the *P. hartigii* ethanol extract gas chromatography-mass spectrometry analysis

Fourier-Transform Infrared Spectra Analysis

The pre-processed FTIR spectra of treated and untreated *S. aureus* strains were used to assess the similarities and differences (Figure 2). Upon careful examination of Figure 2, alterations in peak intensities and positions can be seen, signifying potential chemical transformations induced by the treatment.

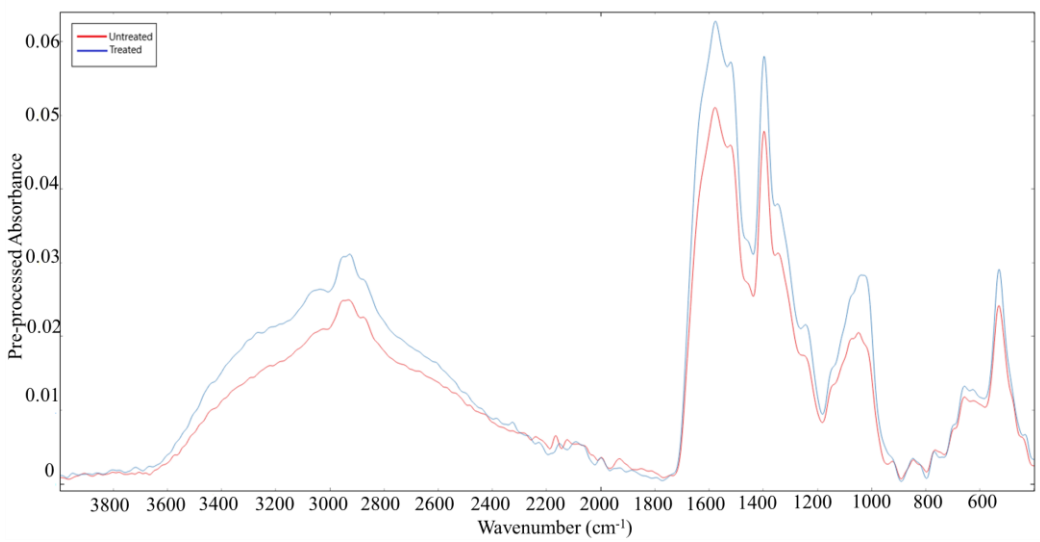

Figure 2. Fourier-transform infrared spectra of treated and untreated *S. aureus* cells (peaks are given as the average of three replicates)

In order to enhance the fine structural details and subtle changes occurring within the samples, a second derivative calculation was applied, and the second derivative spectra given in Figure 3 were prepared.

In the analysis of the second derivative spectra given in Figure 3, specific spectral ranges associated with lipids, proteins, and nucleic acids were focused on to quantify changes related to these biomolecules. Specifically, the 3000-2800 cm^{-1} range for lipids, the 1800-1200 cm^{-1} range for proteins, the 1200-900 cm^{-1} range for nucleic acids, and below 900 cm^{-1} as the fingerprint region were examined, as previously described by Zengin Köksal et al. (2021).

Concentrating on these defined regions aims to precisely evaluate the changes within these biomolecular components, enabling a comprehensive understanding of the impact of the treatment on lipids, proteins, and nucleic acids.

Figure 3 clearly shows that the second derivative calculation amplified the spectral features, making it easier to detect and analyse minor shifts or overlapping peaks that may be obscured in the original spectra given in Figure 2.

Notably, in Figure 3, several peaks exhibit noticeable shifts or variations in relative heights, suggesting modifications in molecular functional groups or structural rearrangements. These observed spectral differences between the PH - EtOH treated and untreated samples provide compelling evidence of the treatment's impact on the composition and molecular properties.

The second derivative spectra were analysed to match the untreated and treated spectra bands. Changes in the relative intensities and band shifts due to PH-EtOH treatment in *S. aureus* samples were determined. The zero-order spectrum is used to determine whether an increase or decrease is present in these corresponding bands and whether this change is statistically significant. Increasing and decreasing bands determined by statistical analysis are presented in the volcano plot given in Figure 4.

The list of the bands that were found to have significant changes and were previously assigned for cells and biological materials (Talari et al., 2017) is given in Table 3.

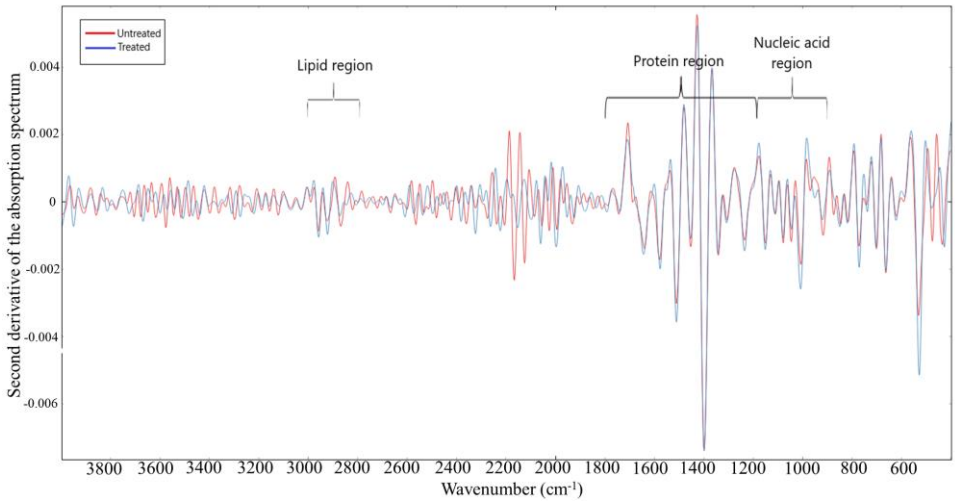

Figure 3. Second derivative spectra of the *P. hartigii* ethanol extract treated and untreated *S. aureus* cells

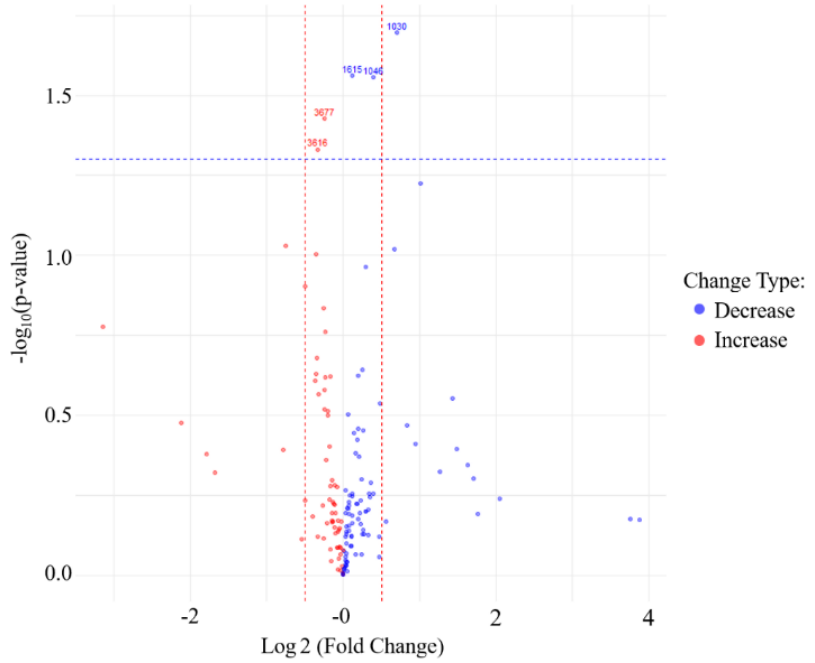

Figure 4. Volcano plot identifying significant and non-significant bands presenting increase or decrease upon treatment by PH-EtOH in *S. aureus*

Table 3. Fourier-transform infrared band assignments for cells and biological materials

Band position (cm^{-1})	Band assignments
1030	Phosphoric ester group (P–O–C) (Filip et al., 2004; Maity et al., 2013)
1046	Stretching vibrations of C–O bonds in deoxyribose (Muntean et al., 2013; Muntean et al., 2021), inner sphere complexation of the EPS phosphate groups (Adamou et al., 2016)
1615	Combination of amide and C-N stretching vibrations from R-NH_3^+ and R-NH_2 (Liauw et al., 2023), NH_3^+ asymmetric deformation (Sebben & Pendleton, 2014)
3616	Symmetric stretching vibration of water molecules (Soleimani et al., 2023) and structural OH groups (Madejová, 2003)
3677	O-H stretching vibrations associated with alcohols or hydroxyl groups (Gupta & Rana, 2021; Tsilo et al., 2024)

The nucleic acid region of the second derivative of the absorption spectrum demonstrates details about DNA or RNA, such as base pairing and secondary structure variations (Wien et al., 2021). Statistical analysis of zero-order spectra showed that the treatment caused significant changes at 1030 cm^{-1} and 1046 cm^{-1} bands.

Filip et al. (2004) and Maity et al. (2013) mentioned that the band at 1030 cm^{-1} can be assigned to the phosphoric ester group (P–O–C). The band at 1030 cm^{-1}, assigned to the phosphoric ester group (P–O–C), is directly related to the backbone of DNA and RNA. This connection arises because the phosphodiester bonds form the structural framework of DNA and RNA molecules. A decrease in the intensity of this band could indicate degradation or damage to the DNA or RNA backbone as a result of PH-EtOH treatment in *S. aureus*.

Muntean et al. (2013; 2021) attributed the peaks at 1046 cm^{-1} to the stretching vibrations of C–O bonds in deoxyribose. Adamou et al. (2016) assigned this band at 1046 cm^{-1} to the inner sphere complexation involving the phosphate groups of extracellular polymeric substances (EPS) originating from phosphodiesters of nucleic acids and proteins. A decrease in the intensity of the band at 1046 cm^{1} may indicate possible structural changes in deoxyribose and phosphate groups within DNA and RNA for both cellular and extracellular, suggesting alterations in stability and interactions that could impact their biological functions (Zhizhina & Oleinik, 1972). This reduction may reflect processes such as DNA damage, repair mechanisms, or changes in RNA structure during cellular activities, as indicated by various studies linking this band to nucleic acid characteristics and interactions.

As a result, it can be inferred that significant alterations in the bands falling into the nucleic acid region (1200 - 900 cm^{-1}) may indicate changes in various aspects of nucleic acids.

Statistical analysis of zero-order spectra showed that the treatment caused significant change at 1615 cm^{-1}, which is in the protein region of the second derivative of the absorption spectrum.

Liauw et al. (2023) assigned the 1650-1615 cm^{-1} region to the stretching vibrations of amide groups and C–N stretching vibrations from various amines, including R-NH_3^+ (protonated amines) and R-NH_2 (primary amines). Alterations in the 1615 cm^{-1} band could either change the concentration of amide-containing compounds (i.e., peptides, proteins) or changes in their conformation that enhance N-H interactions.

Sebben & Pendleton (2014) associated the band observed at 1615 cm^{-1} with the NH_3^+ asymmetric deformation vibration of the N-H bonds. A decrease in the intensity of the band at 1615 cm^{-1} may indicate a reduction in the concentration of amide-containing compounds, such as peptides and proteins, or alterations in their conformation that diminish N-H interactions (Lorenz-Fonfria, 2020). This change could reflect possible structural modifications that affect the stability and functionality of these biomolecules, potentially impacting their biological roles and interactions within cellular environments.

The region above 3000 cm^{-1} of the second derivative of the absorption spectrum demonstrates details about stretching vibrations of O–H and N–H bonds. Statistical analysis of zero-order spectra showed that the treatment caused significant changes at 3616 cm^{-1} and 3677 cm^{-1} bands.

Soleimani et al. (2023) proposed that the band observed at 3616 cm^{-1} in the FTIR spectra of bacteria can be attributed to the symmetric stretching vibration of water molecules. A change in this band could mean a change in the molecular interactions, hydration dynamics and adsorption mechanisms. Madejova (2003) associated the band observed at 3616 cm^{-1} with OH stretching vibrations of the structural OH groups.

Gupta and Rana (2021) proposed that the band observed at 3677 cm^{-1} in the FTIR spectra can be attributed to the O-H stretching. Tsilo et al. (2024) assigned this band to the stretching in −OH. A change in the peak at 3677 cm^{-1} can indeed indicate the presence of O-H stretching vibrations, particularly associated with alcohols or hydroxyl groups.

In addition to this information, extracting the absorbance intensity values from characteristic bands in FTIR spectroscopy is

necessary to analyse the structure and composition of biological molecules, including proteins, peptides, RNA, and DNA.

The amide I band, which corresponds to the stretching vibrations of the C=O bond in the peptide backbone, is typically found in the wavenumber range of 1600-1700 cm^{-1}. The amide II band, associated with a combination of N-H bending and C-N stretching vibrations, is commonly observed in the 1500-1600 cm^{-1} range. For DNA analysis, the band of interest is in the wavenumber range of 1220-1240 cm^{-1}. This region corresponds to specific vibrational modes related to the DNA backbone. Similarly, the RNA band is located in the 900-1100 cm^{-1} wavenumber range, reflecting characteristic vibrations specific to RNA molecules.

Various ratios are calculated to gain insights into the structure and composition of these biological molecules. These include the Amide I/Amide II ratio, which provides information about the relative intensities of these two bands and their contribution to the protein secondary structure. Additionally, the RNA/DNA ratio and Amide I/DNA ratio are calculated to assess the relative amounts and proportions of these biomolecules in a given sample (Table 4).

Table 4. Biochemical changes induced by treatment: absorbance intensity ratios in untreated and treated samples.

Biological significance	The ratio of absorbance intensity	Untreated	Treated
Secondary structure of proteins and DNA content	Amide I/Amide II	0.853	0.862
Transcription level	RNA/DNA	0.572	0.799
Chromatin condensation	Amide I/DNA	4.851	4.218

When considering the characteristic band intervals of Amide I (around 1600-1700 cm^{-1}), Amide II (1500-1600 cm^{-1}), DNA (1220-1240 cm^{-1}), and RNA (900-1100 cm^{-1}), as suggested by various authors, Dousseau & Pezolet (1990), Surewicz et al. (1993), Barth (2007), Movasaghi et al. (2008), several ratios have been calculated to estimate the secondary structures of proteins, such as α-helix and β-sheets.

The Amide I/Amide II ratio estimates these secondary structures, while the RNA/DNA ratio indicates gene expression and cell proliferation, reflecting the relative amounts of RNA and DNA. Amide I/DNA ratio provide information about the relative quantities of proteins and nucleic acids in the sample. These ratios examine changes in the secondary structures of proteins and nucleic acids.

Determining secondary structures and relative quantities of proteins and nucleic acids using these ratios provides valuable insights into biological molecules' composition and possible structural changes.

The lower Amide I/Amide II ratio in the untreated group suggests a higher presence of α-helix structures compared to β-sheet structures, whereas this ratio is reversed in the treated group. Based on this information, it can be inferred that the treatment caused a slight shift in the secondary structures of proteins. This protein conformation change may indicate alterations in protein function or stability.

The increased RNA/DNA absorbance ratio in the treated group demonstrates an increment in nucleic acid content, specifically RNA, and a potential increase in transcription (Ishida & Griffiths, 1993). An increase in this ratio indicates that the treatment may have affected gene expression dynamics and cellular processes associated with RNA synthesis.

The decrease in the Amide I/DNA ratio in the treated group typically indicates a decrease in protein content relative to nucleic acids. A decrease in this ratio could imply changes in the overall composition of the bacterial cells, possibly indicating alterations in the synthesis or degradation of proteins.

The observed changes in the ratios reflect alterations in the secondary structures and relative quantities of proteins and nucleic acids caused by the treatment. These changes can be associated with modifications in cellular processes, such as the expression of

specific genes and alterations in protein-nucleic acid interactions.

Conclusion

The results showed that the ethanol extract of *P. hartigii* has the potential as an antibacterial agent against *S. aureus*. In conclusion, this study contributes significantly to the growing research addressing drug resistance in bacteria. The emergence of antibiotic-resistant strains and the subsequent decline in the efficacy of conventional antibiotics have fuelled the search for novel therapeutic options. Investigating the potential of natural sources, specifically macrofungi like *P. hartigii*, is particularly noteworthy. By elucidating the *in vitro* mode of action of the ethanol extract of *P. hartigii* against multidrug-resistant *S. aureus*, this research sheds light on a promising alternative treatment strategy. The findings underscore the substantial antibacterial activity of the *P. hartigii* ethanol extract. Overall, this study highlights both the therapeutic potential of *P. hartigii* ethanol extract and the potential of FTIR spectroscopy in determining the mode of action of nature-derived extracts, contributing to the broader landscape of antimicrobial research and strategies to combat drug-resistant bacterial infections.

Considering the implications of these results and the study's limitations is essential. While the findings demonstrate the potential of the ethanol extract of *P. hartigii* as an antimicrobial agent, further research is needed to fully evaluate its efficacy and safety. Additionally, the study was performed under laboratory conditions, and it is crucial to determine the practicality and effectiveness of the extract *in vivo*. Despite these limitations, the results of this study provide a starting point for further research into the use of *P. hartigii* ethanol extract as an antimicrobial agent against MDR *S. aureus*. However, further research is needed regarding the toxicity profile of *P. hartigii* extract.

Acknowledgements

The authors thank Prof. Dr. Turhan KÖPRÜBAŞI for his valuable guidance in interpreting the zero-order and second-derivative spectra.

Ethics Committee Approval

N/A

Peer-review

Externally peer-reviewed.

Author Contributions

Conceptualisation, E.M.A.; data curation, K.T., D.O. and E.M.A.; methodology, K.A., U.M.S, E.A, and E.M.A.; supply and identification of the macrofungi, I.A.; writing, K.T., D.O. and E.M.A.; supervision, E.M.A.; project administration, E.M.A.; funding acquisition, E.M.A. All authors have read and agreed to the manuscript.

Conflict of Interest

The authors have no conflicts of interest to declare.

Funding

This work was supported by the Kastamonu University Scientific Research Coordination Unit, with project number KÜ-HIZDES/2023-51.

Declaration

During the preparation of this work, the authors used Artificial Intelligence to improve readability and language. After using this service, the authors reviewed and edited the content as needed and took full responsibility for the content of the publication.

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