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Research with title:

Determination of the antibacterial activity of ethanolic extract of edible mushroom on drug resistant bacteria

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عملا بقرار السيد/ رئيس الجامعة رقم (59) لسنة2026م, الصادر في 2026/01/21م , القاضي بتشكيل لجنة لمناقشة رسالة علمية للحصول على درجة الإجازة العالية (الماجستير) في تخصص: الأحياء الدقيقة المقدمة من الطالبة: بثينةعبدالرزاق الشريف كلية:العلوم،وعنوانها:(**تحديد التأثير التثبيطي للمستخلص الايثانولي للفطريات القابلة للأكل علي البكتيريا المقاومة للمضادات الحيوية**). وتتكون اللجنة من الاساتذة:

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

﴿سَنُرِيهِمْ آيَاتِنَا فِي الْآفَاقِ وَفِي أَنفُسِهِمْ حَتَّىٰ يَتَبَيَّنَ لَهُمْ أَنَّهُ الْحَقُّ ۗ أَوَلَمْ يَكْفِ بِرَبِّكَ أَنَّهُ عَلَىٰ كُلِّ شَيْءٍ شَهِيدٌ﴾

صدق الله الْعَظِيمُ

الاهداء

إلى ذلك الذي تعلمت منه الصفح قبل العتاب،

وتعلمت منه أن النقاء قوة لا ضعف،

إلى من لا يُحسن التعبير بالكلام، لكن أفعاله دومًا تقول الكثير،

وإلى ذلك الذي لم يسألني يومًا شيئًا، ولم يُنقصني شيئًا،

الى ابي.

-

وإلى من سهَّلت صعاب الطريق،

وبسَّطت مخاوفي،

إلى أمي، إليكِ أهدي ثمرة هذا الجهد.

-

والى كل من كان سببا في بدايتي واستمراري؛ هذا الانجاز لكم بقدر ماهو لي.

الشكر والتقدير

فالشكر أولًا وآخرًا لربٍّ يسّر الطريق، وأمدّني بنور الفهم، وثبّتني بالصبر، ومنحني من عزمه ما يكفي لإتمام هذه الرسالة.

يسعدني أن أعبر عن بالغ الشكر والامتنان إلى كل من...

- البروفيسور الدكتور ابراهيم دغمان والدكتور عبدالله ابوقميزة، اللذان كان لهما دور كبير في توجيهي، وتقديم المشورة والخبرة القيمة طوال فترة إعداد هذا البحث.
- مختبر الفا زليتن للتحاليل الطبية، لما قدمه من بيئة بحثية مثالية ودعم تقني أسهم في إتمام هذا العمل بنجاح.
- واخيراً؛ الى زوجي الذي ألفتُ فيه لُطف الشعور ورُشدُ الفكر، على مساندته ودعمه لي.

Abstract

The study aimed to evaluate the antibacterial activity of the ethanolic extract of *Agaricus bisporus*, an edible mushroom, at concentrations of 25%, 50%, 75%, and 100% using the Agar well diffusion method, against drug-resistant bacteria: Methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Escherichia coli*. The antibacterial effects were compared with Tigecycline, an antibiotic used as a positive control. Furthermore, the antibacterial activity was assessed against four non-resistant bacterial species: *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella oxytoca*.

Results demonstrated that the antimicrobial effect of the extract was concentration-dependent, with statistically significant activity observed only at higher concentrations 75% and 100%, with a p-value < 0.001. Non-resistant bacteria, *Pseudomonas aeruginosa* showed the highest sensitivity, with a mean zone of inhibition of 15.33 mm at 100%, comparable to the effect of the positive control (Tigecycline) without a statistically significant difference. *Klebsiella oxytoca*, *Escherichia coli*, and *Staphylococcus aureus* exhibited measurable sensitivity at 100% with mean zone of inhibition of 11.667mm, 10.667mm, 8.00mm, respectively, both with statistically significant difference and p-value < 0.001 although to a lesser extent. In contrast, at 25% and 50%, most strains showed negligible or no inhibition.

Against antibiotic-resistant strains, a similar concentration-dependent pattern was observed. No inhibitory effect was observed at 25% and 50% concentrations across all tested strains. However, significant zones of inhibition were recorded at 75% and 100%

concentrations ($p < 0.001$). At 100%, *Pseudomonas aeruginosa* and MRSA exhibited the highest inhibition (10.00 mm), while *Klebsiella pneumoniae* and *Escherichia coli* showed lower sensitivity (9.00 mm and 4.67 mm, respectively), all with statistically significant difference and p-value < 0.001. Despite these statistically significant differences, the inhibition zones remained notably smaller than those produced by Tigecycline for all strains.

تحديد الفعالية المضادة للبكتيريا للمستخلص الإثانولي للفطر الصالح للأكل ضد البكتيريا المقاومة للمضادات الحيوية

الملخص

هدفت هذه الدراسة إلى تقييم الفعالية المضادة للبكتيريا للمستخلص الإيثانولي لفطر *Agaricus bisporus*، وهو فطر صالح للأكل، وذلك باستخدام طريقة الانتشار في الآجار (Agar Well Diffusion Method) وبتركيزات مختلفة (25%، 50%، 75%، و100%) ضد سلالات بكتيرية مقاومة للمضادات الحيوية، شملت: *Methicillin-resistant Staphylococcus aureus (*MRSA*)*، و*Pseudomonas aeruginosa*، و*Klebsiella pneumoniae*، و *Escherichia coli*. وقد تمت مقارنة التأثيرات المضادة للبكتيريا للمستخلص مع تأثير مضاد حيوي (Tigecycline) كمجموعة ضابطة إيجابية. كما تم تقييم النشاط المضاد للبكتيريا ضد أربع سلالات غير مقاومة، وهي: *Escherichia coli*، و*Staphylococcus aureus*، و*Pseudomonas aeruginosa*، و*Klebsiella oxytoca*. أظهرت النتائج أن الفعالية المضادة للبكتيريا كانت تعتمد على التركيز، حيث لوحظ نشاط معنوي إحصائي عند التركيزين 75% و100% فقط ($p < 0.001$).

بالنسبة للسلالات غير المقاومة، أظهرت *Pseudomonas aeruginosa* أعلى حساسية عند تركيز 100%، بمتوسط قطر منطقة التثبيط بلغ 15.33 ملم، وهو تأثير مماثل

للضابط الإيجابي بدون فروق معنوية. كما أظهرت كل من *Klebsiella oxytoca* و*Escherichia* coli و*Staphylococcus aureus* حساسية عند تركيز 100%، بمتوسط أقطار مناطق التثبيط بلغت 11.667 ملم، و10.667 ملم، و8.00 ملم على التوالي، مع فروق معنوية إحصائية ($p < 0.001$).

أما بالنسبة للبكتيريا المقاومة للمضادات، فقد تم تسجيل نمط مشابه من حيث الاعتماد على التركيز، حيث لم يُلاحظ أي تأثير مثبط عند تركيزي 25% و50%، بينما لوحظت مناطق تثبيط معنوية إحصائياً عند التركيزين 75% و100% ($p < 0.001$). عند تركيز 100%، أظهرت كل من *P. aeruginosa* وMRSA أعلى تأثير مثبط بمتوسط قطر 10.00 ملم، في حين كانت الاستجابة أقل في كل من *K. pneumoniae* (9.00 ملم) وE. coli (4.67 ملم)، مع فروق ذات دلالة معنوية ($p < 0.001$). ومع ذلك، كانت مناطق التثبيط الناتجة عن المستخلص أقل بوضوح مقارنة بتلك الناتجة عن Tigecycline لجميع السلالات.

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List of Abbreviations

Abbreviation	Full Term
WHO	World health organization
CDC	Central for disease control
DNA	DeoxyriboNucleic Acid
ARB	Antibiotics-Resistant Bacteria
ARG	Antibiotics-Resistant Genes
MRSA	Methicillin-resistant *Staphylococcus aureus*
VRSA	Vancomycin-resistant *Staphylococcus aureus*
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
MDR	Multi Drug Resistant
EtOAc	Ethyl acetate
ZOI	Zone Of Inhibition
MAE	Microwave-Assisted Extract
UAE	Ultrasound-Assisted Extract
TLC	Thin-Layer Chromatography
PC	Positive Control
DMSO	Dimethyl Sulfoxide
CFU	Colony Forming Unite
MANOVA	Multivariate Analysis Of Variance

Introduction

1. Introduction

Antibiotics are considered one of the most important weapons used to protect humanity from bacterial infection, since 1928, since the scientist Fleming discovered the first one by chance, they have become one of the most important discoveries of mankind (Hutchings *et al.*, 2019). Since then, the continuous development of this weapon began, as the annual production of antibiotics was estimated at approximately 100-200 thousand tons worldwide (Serwecińska, 2020). Hence, with the increase of the number of antibiotics, the wrong and excessive use also increased, and thus it speeds up the bacteria to acquire resistance against them. Previously, the acquisition of antibiotic-resistant did not pose a great threat, as new classes of antibiotics were developed that were considered magic drugs that bacteria could not overcome, such as vancomycin and methicillin (Mancuso *et al.*, 2021).

Unfortunately, with its overuse, new resistant strains such as MRSA and VRSA have emerged, and in 2017 the World health organization (WHO) has published a list of 12 bacterial families that pose a major threat to human health, this drug resistance microbes can causes untreatable, and fatal diseases such as pneumonia, tuberculosis, gonorrhoea and bloodstream infections, examples include *Enterococcus faecium* and *Staphylococcus aureus*, which are resistant to several important antibiotics such as vancomycin and fluoroquinolones (Mancuso *et al.*, 2021). Central for disease control (CDC) reported in 2019 antibiotic-resistant bacteria and fungi rendered more than 2.8 million and 35,000 infections and deaths, respectively, in the United States alone and 1.27 million people worldwide (García *et al.*, 2020), and about $4.6 billion annually counted as the national cost of treating infections from 6 multidrug-resistant germs (Nelson *et al.*, 2021).

As the acquisition of antibiotic resistance continues to increase, a high rate of uncontrolled hospital infections increases, research should be intensified to find alternative and practical solutions against these resistant microbes with fewer side effects and materials that are not dangerous to the consumer's health with less cost.

Nowadays, there is growing interest in natural antimicrobial agents derived from bacteria, fungi, and plants (Hossain *et al.*, 2024). These sources have become increasingly significant, with many new drug molecules now being primarily derived from them.

Generally, mushrooms are a macro fungus, visible to the naked eye, picked by hand, and is characterized by a unique, special fruiting structure, mushrooms are a very rich source of dietary fiber, protein, minerals, and vitamins, and are low in calories and fats (Assemie & Abaya, 2022; Navarro-Simarro *et al.*, 2024). Mushrooms long time have been used as a source of essential nutrients in our diet, in addition to being a good source for pharmaceutical properties. Mushroom extract provide a good source of antitumor, antioxidant, antiobesity, antiviral, and antimicrobial drugs (Binsaleh *et al.*, 2025; Maha *et al.*, 2021). Currently, mushrooms consider one of the greatest sources of antibacterial agents and have a large pharmacological activity, and the fruiting bodies and mycelium of mushrooms exhibit health-promoting worths (Al Qutaibi & Kagne, 2024; Shakeri, 2016).

Mushrooms inhibitory effect contributed by several bioactive compounds as secondary metabolites or as a part of their cellular compounds, such as terpenoids, flavonoids, tannins, alkaloids, polysaccharides, and Stearic acid (Huguet *et al.*, 2022; K. Kumar *et al.*, 2021; Tee *et al.*, 2024) by breaking the DNA protein and devastating the permeability of the cell membrane of bacteria, and to name just a few, this occurred by *A. bisporus* (Dash *et al.*, 2024; Erdoğan Eliuz, 2022; Singh *et al.*, 2025).

A. bisporus is a white button mushroom a member of the Agaricaceae family, and the most common cultivated mushroom in the world (Dong *et al.*, 2024), where it is extensively cultivated up to approximately 70% of total edible mushrooms, considered a suitable origin of K, Fe, Zn, Cu, Na, Se, Co, and Mn (Valchev, 2020), and so many amino acids (1 g of *A. bisporus* contained 48.8–64.2 mg of free amino acids). Nevertheless, it has a 1-3 days shelf life which make it effortless to cultivate fairly compared to other mushrooms. (Iqbal *et al.*, 2024; Tsai *et al.*, 2007)

The chemical composition of *A. bisporus* is rich in carbohydrates, proteins, and many vitamins like niacin, riboflavin, vitamin B1, vitamin B3, L-ascorbic acid, and α-tocopherol, and fat in significant amount (Atila *et al.*, 2017; Valchev, 2020; Zhang *et al.*, 2024), recent research tested the influence of *A. bisporus* in many therapeutic investigations including life-threatening diseases, and many experiments approved its ability to inhibit the bacterial growth. (Fadhil & Mous, 2020; Krishnamoorthi *et al.*, 2022; Lysakova *et al.*, 2024) *A. bisporus* have demonstrated significant inhibitory effects against a range of pathogenic microorganisms, including both Gram-positive and Gram-negative bacteria, as well as certain fungi (Jegathchandran *et al.*, 2024). These effects are believed to be due to the presence of bioactive compounds such as phenols, flavonoids, and other secondary metabolites. Research has shown that aqueous and ethanol extracts of *A. bisporus* can inhibit the growth of *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Candida albicans* (Jankov *et al.*, 2024a).

Furthermore*, A. bisporus* was approved to have an antiviral activity, where it showed effectiveness against two significant viruses: Human Respiratory Syncytial Virus (HRSV) and Herpes Simplex Virus Type 1 (HSV-1) (Doğan *et al.*, 2025). The potential for using

these extracts as natural antimicrobial agents opens new opportunities for food preservation, pharmaceuticals, and alternative medicine.

Problem statement

2. Problem statement

Antibiotics have long been considered one of the most significant medical achievements of the 20th century (Pham *et al.*, 2024), playing a crucial role in the treatment and prevention of bacterial infections. However, the overuse of the antibiotics has demonstrated increasing resistance to multiple classes of antibiotics, rendering conventional treatments less effective and contributing to higher rates of morbidity and mortality. In response, there is a growing scientific interest in identifying alternative antimicrobial agents, particularly from natural sources, to combat resistant and non-resistant bacterial strains.

Many studies have tended for a long time to use natural extracts from plant leaves or extracts of well-known medicinal plants themselves. Plants and mushrooms provide acceptable natural compounds with different mechanisms of action, notably lower side effects, and favorable effects when given with conventional antibiotics as a synergistic route (Moussa *et al.*, 2022). Also, many studies showed the effectiveness of edible mushroom extract in stopping the growth of specific bacterial species such *Escherichia coli, Salmonella typhi, Klebsiella pneumonia*, and other bacterial species (Devi *et al.*, 2024; Stojanova *et al.*, 2024).

Therefore, this study aims to evaluate the antibacterial efficacy of the ethanolic extract of the edible mushroom *Agaricus bisporus* against a selection of clinically relevant bacterial strains. Specifically, it will assess its inhibitory effect on four antibiotic-resistant species: MRSA, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Escherichia coli*,

will be compared with their non-resistant counterparts: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella oxytoca*. The objective is to determine whether the ethanolic extract demonstrates significant antimicrobial activity and to explore its potential as a natural alternative in combating the growing challenge of antibiotic resistance.

Objectives of the study

3. Objectives of the study

1. To analyze the *in vitro* effectiveness of *A. bisporus* ethanolic extract against resistant bacteria, in comparison with Tigecycline (15 µg) as a positive control.
2. To evaluate the effect of *A. bisporus* ethanolic extract on resistant versus non-resistant bacterial strains.

Literature reviews

3. Literature reviews

Antibacterial activity of mushrooms extracts against different bacterial types

In 2019. Gebreyohannes *et al*., investigated the antimicrobial activity of indigenous wild mushrooms (Thirty-five indigenous wild mushrooms were used) against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus,* and some other clinical organisms, and the results showed that 3 mushroom types, namely, *Trametes* spp, *Trametes* spp, and *Microporus* spp from 35 types that used in the experiment have a bright antimicrobial activities against six bacterial pathogens, including Methicillin-Resistant *Staphylococcus aureus* (MRSA).

Hassan *et al.* (2019), assayed the antibacterial activities of 75 mushroom types against bacterial strains using a methanol and water extracts, the results exhibited that the water extract of *Polyporus squamosus, Ganoderma applanatum, Lentinellus subaustralis, Laetiporus sulphureus*, *G. lucidum,* and *Trametes versicolor* have a substantial antibacterial activity against all bacterial types tested.

Another study conducted by Salihović *et al.* (2020), were they examine the antimicrobial activity of mushrooms used in the diet, on Nine microbial strains, four gram positive, four gram negative. They found that wild mushrooms exhibit notable antibacterial activity against *S. aureus, MRSA* and *B. subtilis*, registered inhibition zones of the extract were 18.11± 0.20mm against *S. aureus*, 18.10 ± 0.17mm against *B. subtilis*, and 20.03 ± 0.08mm against *MRSA*.

In addition, Ogidi *et al.* (2021), estimated the antimicrobial properties of methanol and aqueous extracts of wild edible mushrooms using agar well diffusion method, the results showed that both solvents expose the antibacterial sensitivity of Gram-positive bacteria, demonstrating the inhibition zone diameters as 10±1.0mm and 9.3 ± 0.3mm for *Staphylococcus sp*. For other Gram-negative bacteria, the inhibition zones are 9.0 ± 1.0mm and 9.0 ± 1.0mm for *E. coli*, 9.2 ± 0.8mm and 19 ± 1.0mm for *Shigella sp*, 9.6 ± 0.2mm and 7.3 ± 0.3mm for *Vibrio sp*, for aqueous and methanol extracts, respectively.

In Romania, Fogarasi *et al.* (2020), investigated the potential effect of five mushroom species (*Agaricus bisporus, Pleurotus ostreatus, Cantharellus cibarius, Boletus edulis, and Lactarius piperatus*) against **5** bacterial strains, the result showed different zones of inhibition ranging from 9.5 to 13.24mm (*E.coli*), followed by *B. cereus* (10.09 to 12.04mm), *P. aeruginosa* (8.75 to 11.98mm), *S. aureus* (9.25 to 11.65mm), and *S. Typhimurium* (10 to 11.18mm).

On the other hand, Barros *et al.* (2007), investigated the antimicrobial activities of Portuguese wild edible mushrooms (*Lactarius deliciosus, Sarcodon imbricatus and Tricholoma portentosum*), extracted with the phenol, and evaluated the MIC with cap and stipe of the mushrooms, and the result indicated that the extracts had an inhibitory effect on Gram-positive bacteria, while the Gram-negative bacteria were not affected. Also, Ren *et al.* (2014), assessed the inhibitory effect of eight edible mushroom species (Polysaccharides extracts) against five bacterial strains using disc diffusion method and microdilution method, and the result showed that all the mushroom extracts at both concentrations of 3 and 15 mg/mL did not show antibacterial activity using the disc diffusion method, but in the single microdilution method, antibacterial activity was

observed with *C. sinensis* against *B. subtilis* and *S. epidermidis*, and with *P. australis* against *S. epidermidis*. In comparison, antibiotics proved more effective at creating inhibition zones around the paper discs.

In 2012, Alves *et al.*, assessed the antibacterial effect of 13 mushroom species against resistance and non-resistance bacteria using microdilution method, and the result pointed clearly that the majority of extracts did not show any inhibitory effect against gram negative bacteria and resistant bacteria.

In addition, Nwobodo *et al.* (2021), examined the antibacterial effect of *Pleurotus ostreatus* and *Agaricus bisporus* against pathogenic bacteria, and the results revealed that the pathogenic bacteria were resistant to 75% of mushroom extracts used in the experiment, with a mean zone of inhibition 8.00 ± 1.40mm and 6.0±0.11mm against the Gram-positive.

Erdoğan Eliuz (2022), conducted a comparison of antimicrobial activity between *Lentinula edodes* and *Agaricus bisporus* ethanolic extract, and the result exhibited the largest inhibition zone observed was 5.1mm from *L. edodes* against *E. faecalis*, while the smallest was 2.4mm from *A. bisporus* against *S. aureus*. And this effect mainly is due to protein and DNA breakage and interruption of cell membrane permeability by the cellular components of mushrooms.

In contrast, Krishnamoorthi *et al.* (2022), evaluated the radical scavenging, antibacterial, and antifungal effects and the medicinal importance of *A. bisporus* in treating human pathogens, and they observed that *A. bisporus* extract has a remarkable antimicrobial activity against *Staphylococcus aureus* (14.3 ± 0.24mm) and *Candida albicans* (14.2 ± 0.12mm).

Huguet *et al.* (2022), assessed the inhibitory effect of France mushroom against wild-type bacteria and multidrug-resistant (MDR) bacteria, they found that 95% of extracts possess antibacterial compounds, particularly the EtOAc extracts and clearly showed that the all extracts were more effective against gram positive than gram negative bacteria, moreover, they ensured that the most promising extract against wild-type bacteria were globally less effect against MDR, this experiment demonstrated the potential of macromycetes as a source of antibacterial compounds. However, additional assays are required to evaluate fungal compounds as viable candidates for combating antibiotic resistance.

Another study by Jankov *et al.* (2024a), evaluated the potential antibacterial effect of *A. bisporus* against different bacterial species, including antibiotic-resistant bacteria, and the results indicated that four extracts possessed antibacterial properties, but all bacterial strains were able to grow within the inhibitory zones.

In 2018, Hussein *et al*., examined the antibacterial effect of aqueous and alcoholic extracts of *A. bisporus* against two bacterial species, *Salmonella typhi* and *Staphylococcus aureus*. The result showed that the aqueous extract did not show any effect against both species; meanwhile, the alcoholic extract represented a ZOI of 15mm and 9mm against *Staphylococcus aureus* and *Salmonella typhi*, respectively.

Kumar *et al.* (2016), tested *A. biporus* methanol and alcoholic extracts antibacterial activity, and the results indicated that the crude extract of *A. bisporus* exhibited limited activity, with an inhibition zone against *B. subtilis* 6.5mm and 9mm and *S. aureus* of 9mm and 10mm, respectively, while against *E. coli*, they were 7.5mm and 8.5mm for the aqueous

and methanolic extracts, respectively. In general, the antibacterial effects of *A. bisporus* were less potent than those of commercial antibiotics.

Zatout and Kacem Chaouche (2023), assessed the bioactive value of dried fruiting bodies of *Agaricus litoralis* and found that these fruiting bodies contain diverse metabolites, including alkaloids, flavonoids, tannins, sterols, triterpenoids, carbohydrates, and others, which are believed to contribute to its antibacterial effect. They noted that in the preliminary screening, the aqueous and organic extracts exhibited high antimicrobial efficacy.

Another study by Gargano *et al.* (2025), evaluated the nutritional contents and antimicrobial activity of a dry sample of *Leccinum scabrum* using microwave-assisted and ultrasound-assisted extraction techniques (UAE and MAE). The result displayed inhibitory activity against all tested bacteria. Yazdi *et al.* (2023), also supports the use of mushrooms as a source of medically bioactive compounds. In their study, they tested the antibacterial effect of *Lentinus tigrinus* fruiting bodies against Gram-positive spore-forming bacterium *Bacillus cereus*, and they found that the *B. cereus* growth was inhibited by acetone extract at 31.25 µg/ML concentration.

In 2022, S. Kumar, evaluated the antibacterial effects of *Agaricus bisporus, Calocybe indica, Flammulina velutipes, Pleurotus florida,* and *Volvariella volvacea* using acetone, aqueous, and methanol extracts. The effectiveness of these extracts was tested against six bacterial strains: *Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes, Shigella dysenteriae, Salmonella typhi, and Escherichia coli*, using the agar well diffusion method. The results confirmed that all the extracts exhibited significant antibacterial effects against all tested bacterial strains.

Moreover, Dutta *et al.* (2024), analyzed the exopolysaccharide and mycelia antibacterial efficacy of two Lentinus species against four pathogenic bacteria. and they discovered that both of them have an inhibitory effect on Gram-positive and gram-negative bacteria. Sitara *et al.* (2023) tested the antifungal and antibacterial activity of three Pleurotus species against five pathogenic fungi and five food pathogenic bacteria, the results showed that *Pleurotus ostreatus* exhibits significant antifungal and antibacterial activity against all tested pathogens.

Same as the study of Haq *et al.* (2022), were they investigated the antibacterial effect of *Morchella conica* and *M. esculenta* (Different extracts of Ascomata) against typhoidal and nontyphoidal Salmonella species using agar diffusion assay. The result declared that all extracts inhibited the growth of typhoidal and nontyphoidal Salmonella species, with MIC: 3.33 ± 0.6 to 16.0 ± 0 mg/mL for bacteriostatic effects and MBC: 8-16 mg/mL for bactericidal effects, which significantly sustain the uses of natural mushroom extract in antibacterial pharmaceuticals.

In Turkey, Karakas *et al.* (2023), conducted an study evaluating the antibacterial and antioxidant potentials of 13 wild-growing edible mushrooms sold in a public market in Bolu. They found that all tested species of *Lactarius* exhibited robust antibacterial activity, particularly against *Staphylococcus aureus* and *Staphylococcus epidermidis*. Furthermore, mushrooms such as *Marasmius oreades*, *Agaricus campestris*, *Tricholoma terreum*, *Hydnum repandum*, and *Ramaria* sp. also demonstrated strong antibacterial effects against a broad spectrum of human pathogens, which suggests the potential of these mushrooms as natural antimicrobial agents, also documented the remarkable growth inhibition of *Marasmius oreades* against *S. aureus*, with a zone of inhibition measuring 29.33 ± 1.11mm,

suggesting that these species can be integrated into functional foods or used in therapeutic contexts.

In 2022, Nameni *et al*., evaluated *A. bisporus* methanolic extract chemical composition and tested its antibacterial activity against three bacterial species. The result showed the crude protein content was found to be 46.62 ± 0.19 g/100g, crude fat was 10.59 ± 0.13 g/100g, fiber content measured 17.76 ± 0.32 g/100g, and carbohydrates amounted to 1.56 ± 0.27 g/100g. The total energy value was 288.3 ± 2.61 Kcal, and the greatest inhibitory activity was observed against *Escherichia coli*, exhibiting a zone of inhibition measuring 22 ± 0.2mm.

Additionally, Sutthisa and Chaiyacham (2022), tested the antibacterial effect of *Lentinus squarrosulus* against human pathogenic bacteria. They prepared the ethanol extracts from both the mycelium and basidiocarp of the mushroom using a 50% ethanol solvent. The mycelium extract showed significant antibacterial activity, particularly against *Bacillus cereus*, with higher concentrations proving most effective. It also demonstrated some activity against *Staphylococcus aureus*, though at a lower level. Suggesting that the mycelium extract was found to be an excellent source of antimicrobial compounds, highlighting its potential for use in natural antimicrobial applications.

Jankov *et al.* (2024b), Investigated the antibacterial effect of ethanol and acetone extracts of *Agaricus bisporus* on four bacterial strains (*Bacillus subtilis*, *Staphylococcus aureus*, *methicillin-resistant Staphylococcus aureus (MRSA)*, *and Escherichia coli*) using the agar well diffusion method, the result revealed that all extracts presented inhibition zones on all tested bacteria. supporting the uses of edible mushrooms in treating pathogenic bacteria as a natural alternative for antibiotics.

Additionally, Shafiq and Sahib (2022), assessed the antagonistic activity of *Agaricus Campestris* extracts on some human pathogenic bacteria and the result indicated that all extracts exhibited efficacy against all tested bacteria, with gram-positive being more affected than gram-negative.

Udeh *et al.* (2021), examined the antibacterial and antioxidant activity of wild medicinal (*agaricomycetous*) mushroom (hot water, cold water, and ethanol extracts) against *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella pneumonia* (multidrug antibiotic-resistant types) with the agar disc diffusion method. Results demonstrated that the agaricomycetous mushrooms exhibited both antibacterial properties against bacterial pathogens and antioxidant activity, which can be attributed to their high phenolic content.

Also, Risan *et al.* (2017), investigated the antibacterial activity of *A. bisporus* and *p. ostreatus* using different extracts (hot water, cold water, ethanol, chloroform and acetone extract), against *Escherichia coli* and *Staphylococcus aureus*, with different concentrations (25%, 50%, 75%, and 100%). The result indicated that the cold and hot water extracts exhibited the highest inhibition at a 75% concentration (ZOI 33.3mm). Additionally, ethanol, chloroform, and acetone extract of these mushrooms, at their maximum concentration (75%), resulted in the greatest reduction in growth of bacteria (ZOI 36.6mm).

Furthermore, Waqas *et al.* (2019), investigated the antibacterial activity of *A. bisporus* against four plant pathogenic bacteria, the result indicated that the greatest ZOI diameter showed by ethyl acetate extract (30, 20, 19, and 28.67mm) at 100 mg/ml concentration. Similarly, Imtiaj *et al.* (2007), evaluated the antibacterial and antifungal activity of different extracts of *Stereum ostrea* against 5 bacteria species and 3 plant pathogenic fungi, they

confirmed that the extracts obtained from *S. ostrea* exhibit promising bioactive compounds that can inhibit the growth of both bacterial and fungal pathogens.

Quereshi *et al.* (2010), estimated the antibacterial activity of different extracts of *Ganoderma lucidum* against six bacterial species. The results showed the potential effectiveness of these extracts against bacteria, indicating that *Klebsiella pneumonia* was the most affected bacteria with ZOI 31.60mm. Erjavec *et al.* (2016) Investigated in vitro microtiter plate assays of the antibacterial activity of 15 extracts of wild mushrooms against plant pathogenic bacteria (*Ralstonia solanacearum*), and the result indicated that out of the tested samples, 11 completely inhibited bacterial growth, while 4 exhibited partial inhibitory effects. Of the 15 extracts, five were selected for further evaluation, and three demonstrated the ability to slow disease progression and reduce disease severity in artificially inoculated tomato and potato plants.

Yamaç and Bilgili (2006) evaluated the antibacterial activity of fruiting bodies and mycelial cultures from selected mushroom isolates was investigated against eight bacterial species and one yeast species. Results showed antimicrobial efficacy against both yeast and bacteria. The most noteworthy point was that the observed antimicrobial activity was predominantly bacteriostatic, and the bioactive compounds responsible were found to be heat-stable.

Also, Praveen *et al.* (2012), investigated the antibacterial activity of the methanolic extract and crude culture filtrate of *Stereum ostrea* against two Gram-negative bacteria and three Gram-positive using disc diffusion method, The crude culture filtrate exhibited the highest inhibition zone against *Bacillus subtilis* (15.9mm) and the lowest against *Klebsiella*

pneumoniae (9.1mm). They suggest that the crude filtrate possesses potential broad-spectrum antimicrobial efficacy.

Chomcheon *et al.* (2013), assessed the antibacterial activity of crude culture extract of *Pleurotus citrinopileatus* and *Tricholoma crissum* against *E. coli* and *S. aureus* using disc diffusion method. Result confirmed that the crude extract of *P. citrinopileatus* culturing on PDB (Potato dextrose broth) displayed ZOI 37.47mm against *E. coli* and 33.73mm against *S. aureus* with minimum inhibitory concentration 5.0 mg/ml and 2.5 mg/ml, respectively.

Another study conducted by Sharma *et al.* (2015), demonstrated the antibacterial potential of *Agaricus bisporus* through the use of methanolic and acetone extracts tested against the pathogenic bacteria *Escherichia coli* and *Staphylococcus aureus*. Using the agar well diffusion method across varying concentrations (25%, 50%, 75%, and 100%), both extracts exhibited notable antibacterial effects. Among them, the methanolic extract displayed the highest inhibitory activity against both bacterial strains. These findings highlight the potential of *A. bisporus* as a source of bioactive compounds.

Also, Huguet *et al.* (2022), confirmed the promising potential of macromycetes as a reservoir of bioactive antibacterial agents, they conducted a study used TLC-direct bioautography to evaluate mushroom extracts for antibacterial activity and they found that most extracts demonstrated the presence of antibacterial compounds, Extracts showed stronger activity against Gram-positive bacteria compared to Gram-negative strains. While activity was generally lower against multidrug-resistant bacteria, some mushroom species still exhibited inhibitory effects. Stearic acid was identified as a common compound potentially involved in the antibacterial properties of mushrooms.

Chaiharn *et al.* (2018), investigated the antibacterial effect of edible mushroom against food-born pathogenic bacteria. Result showed that the extracts of *Flammulina velutipes*, *Ganoderma lucidum*, *Pleurotus ostreatus*, and *Pleurotus pulmonarius* demonstrated inhibitory effects against both Gram-positive and Gram-negative bacterial strains. Notably, the aqueous extract of *Pleurotus pulmonarius* exhibited significant antibacterial activity, particularly against Gram-positive species such as *Bacillus cereus* and *Micrococcus luteus*. In contrast, Gram-negative bacteria, including *Escherichia coli* and *Salmonella typhimurium*, showed resistance to most of the tested extracts.

Hirasawa *et al.* (1999), extracted a kind of antibacterial substances from an edible mushroom, *Lentinus edodes* (Shiitake mushrooms), using chloroform, ethylacetate, and water, and results confirmed that the extracts possess a significant antibacterial activity against *Streptococcus* spp*., Actinomyces* spp*., Lactobacillus* spp*., Prevotella* spp*., and Porphyromonas* spp, and did not inhibit the growth of some other. Kandasamy *et al.* (2020) assessed the antibacterial capability of the wild edible mushroom *Pleurotus sajor-caju* methanolic extract against the multidrug-resistant *Salmonella typhi*, the results demonstrated that the growth of *S. typhi* was reduced with a zone of inhibition of 15mm with an MIC of 100 µg/mL. indicating that the *P. sajor-caju* extract could be a potential pharmacological source against MDR bacteria.

Furthermore, Solak *et al.* (2006), investigated the antibacterial effect of *Clitocybe alexandri* and *Rhizopogon roseolus* (wild edible mushrooms) against 13 microorganisms using the disk diffusion method. The results indicated that the two mushroom extracts demonstrated considerable activity against *E. coli, Bacillus subtilis,* and *Enterobacter aerogenes*. Olawuyi *et al.* (2010), examined the antibacterial ability of water, ethanol, and

chloroform extracts of *Fomes lignosus* against *Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Bacillus cereus, and Staphylococcus aureus*, using the well diffusion method and the disk diffusion method. the results showed that the extract inhibited the growth of the tested bacteria with considerable microbial specificity, and with *Staphylococcus aureus* being most affected, with a ZOI of 5mm - 11mm.

Another study by Tamrakar *et al.* (2017) proved that wild mushrooms from Nepal possess significant antibacterial properties, indicating their potential as novel candidates for incorporation into the functional ingredients industry. Gaur and Rao (2017) tested the antibacterial activity of two strains of *Macrocybe gigantea* (Agaricomycetes) and assessed their phenolic profile and bioactive compounds using gas chromatography–mass spectrometr. The result showed that both strains possess bioactive compounds, and most compounds have antibacterial activity.

Nwachukwu and Uzoeto (2010), investigated the antibacterial activity of ethanol, hot and cold extracts of mushrooms species including: *Russula vesca, Auricularia auricular, Pleurotus squarrosulus, and Volvariaella vulvae* against gram-positive and gram-negative bacteria using agar disk diffusion method. The results showed that the ethanolic and aqueous extracts of the mushroom species, particularly *P. squarrosolus*, demonstrated inhibitory effects against the majority of the tested microbial isolates.

Materials and Methods

5 Materials and Methods

5.1 Materials and Reagents used

Petri dishes, micropipettes, test tube, cork borer (6mm), filter paper, sterile cotton, sterile loop, cotton swap, forceps, Erlenmeyer flask, syringe (5ml,10ml), sterile plastic container, water bath, medical scalpel, Distilled water, ethanol alcohol (75%), Dimethyl sulfoxide (DMSO; 99.9%), McFarland standards, Tigecycline (15 µg).

5.2 Instruments and Equipment used

Many devices were used in the study, as shown in Table 1:

Table (1): Instruments and Equipment Used

Instruments/ Equipment	Manufacturer company
Sensitive electronic weigher	Sartorius/ Germany
Vortex	MD Group/ China
Electric grinder	Nima/ Japan
Autoclave	Witeg/ China
Incubator	Medicana M.B. L
Rotary vacuum evaporator	Heidolph/ Germany

5.3 Bacterial culture media

Three types of bacterial culture media were used in the study (Table 2):

Table (2): Bacterial culture media

Culture media	Manufacturer company
Nutrient agar	Ismailia free zone/ Eygpt
Blood agar	Biolife/ Italy
MacConoky agar	Biolife/ Italy

5.4 Mushroom sample

Fresh fruit bodies of *A. bisporus* (white button mushroom), (harvested one day prior to extraction) were employed in the preparation of the ethanolic extract. The fruit bodies were obtained from Benghazi mushroom farm, Libya. As shown in Figure (1).

Figure (1): *Agaricus bisporus* fruit bodies

5.5 Selected bacterial isolates

Eight bacterial isolates were used in the study, separated into two groups: **antibiotic-resistant bacteria**; three were gram-negative (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae*), and one was gram-positive, Methicillin-resistant *Staphylococcus aureus* (MRSA); **non-resistant bacteria** were also three gram-negative (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella oxytoca)*, and one gram-positive (*Staphylococcus aureus*), each bacterial strain was cultivated on a growth medium appropriate for its specific requirements (MacConoky agar and Blood agar). All bacterial isolates were obtained from Zliten Central Hospital and identified with the Phoenix automated microbiology system

5.6 Preparation of fruit bodies for extraction

The preparation technique was conducted as cited in Risan *et al.* (2017) study, fresh mushroom fruiting bodies were initially rinsed thoroughly with distilled water to remove any surface contaminants. Subsequently, they were carefully cut into small pieces using a sterile medical scalpel (Figure 2). The sliced tissues were then placed on filter paper and left to air dry in the shade at room temperature (20–25 °C) for six days. After complete dehydration, the dried fruiting bodies were ground into a fine powder using an electric grinder. The resulting powder was stored in a sealed plastic container and kept at 4 °C in a refrigerator until further use for extraction.

Figure (2): Sliced mushroom fruit bodies

5.7*A. bisporus* ethanolic extract preparation

The ethanolic extract was prepared following the method outlined by Risan *et al.* (2017). Fifty grams (50g) of powdered fruit bodies were immersed in 200 ml of ethanol (75%), in a conical flask (Figure 3), and covered with aluminum foil. The flask was kept in the dark for 7 days at room temperature and shaken twice daily. Subsequently, the extract was filtered through multiple layers of Whatman No. 1 filter paper; the extract was then evaporated and concentrated using a rotary evaporator (Figure 4) at a temperature of 35–45 °C and stored in a sterile container in the refrigerator at 4 °C until use. The process was repeated multiple times to obtain a sufficient quantity of the extract.

Figure (3): Mushroom ethanolic extract

s

Figure (4): Vacuum rotary evaporator

5.8 Preparation of different concentrations of *A. bisporus* ethanolic extract

The preparation of various concentrations was conducted as described in Risan *et al.* (2017) study. 0.1 gram (100mg) of the concentrated extract was dissolved in 10 ml of 5% dimethyl sulfoxide (DMSO) to a final concentration of 10 mg/mL as a stock solution, the extract was sterilized using a Whatman membrane filter with a pore size of 0.45 micrometers. From the stock solution, additional concentrations of 25%, 50%, 75% and 100% were prepared.

5.9 Bacterial suspension preparation

Eight bacterial suspensions have been prepared, each with a 0.5 McFarland turbidity standard (1.5×10^8 CFU/mL), using the Phoenix Spec system at Zliten Central Hospital, (Figure 5), following the method described by Mahesh *et al.* (2025).

Figure (5): Bacterial Turbidity Meter, Phoenix Spec

5.10 Determination of antimicrobial activities of *A. bisporus* ethanolic extract

The antibacterial potential of the ethanolic extract of *A. bisporus* was assessed employing the agar well diffusion assay, in accordance with the protocol described by Risan *et al.* (2017). A 100 µL aliquot of bacterial suspension, adjusted to a 0.5 McFarland turbidity standard, was aseptically inoculated onto the surface of nutrient agar plates using a sterile cotton swab to ensure uniform distribution. Subsequently, five wells (6 mm in diameter) were aseptically bored into each Petri plate using a sterile cork borer. Each well was loaded with 200 µL of *A. bisporus* extract at concentrations of 25%, 50%, 75%, and 100%. One well was designated as the negative control (DMSO), while Tigecycline (15 µg) served as the positive control. All assays were conducted in triplicate for each bacterial strain. The inoculated plates were incubated at 37 °C for 24 h under aerobic conditions.

Antibacterial efficacy was quantified by measuring the diameter of the zone of inhibition (ZOI), including the well diameter, using a precision digital caliper. All experimental procedures were performed under aseptic conditions in the Microbiology Unit, Alpha Zliten Laboratory.

6 Statical analysis

All quantitative data were analyzed utilizing Multivariate Analysis of Variance (MANOVA) to examine the effects of stock solution concentration, bacterial type, and their interaction on the dependent variables and inferential statistics, post hoc tests, specifically the Dunnett t-test, with a significance level set at $p \leq 0.01$(Appendix 1).

Results and Discussion

7 Results and Discussion

7.1 Verification of *A. bisporus* identity based on morphological characteristics

The *A. bisporus* fruiting bodies used in the study were purchased from Benghazi mushroom farm, Libya. The identity of the mushroom was verified solely based on its macroscopic morphological characteristics (Figure 6), and its external appearance was in full agreement with the standard descriptions reported in the literature (Kumar, S. 2022a) .

Figure 6: Fruiting bodies of *A. bisporus* showing typical cap shape, gill arrangement, and stipe structure (Kumar, S. 2022a)

Figure 7: Fruiting bodies of *A. bisporus* used in the present study

7.2 Activation of bacterial isolates on appropriate culture media, both resistant and non-resistant types

Bacterial isolates obtained from Zliten Medical Center were reactivated by culturing each species on the appropriate medium. The bacteria included four antibiotic-resistant species: three gram-negative, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*, and one gram-positive, MRSA, as shown in Figure (8). Each isolate was classified as strongly or moderately resistant, showing resistance to at least four antibiotics. The non-resistant bacteria included three gram-negative species, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella oxytoca*, and one gram-positive, *Staphylococcus aureus,* as shown in Figure (9). All Bacterial strains were identified by Phoenix automated microbiology system

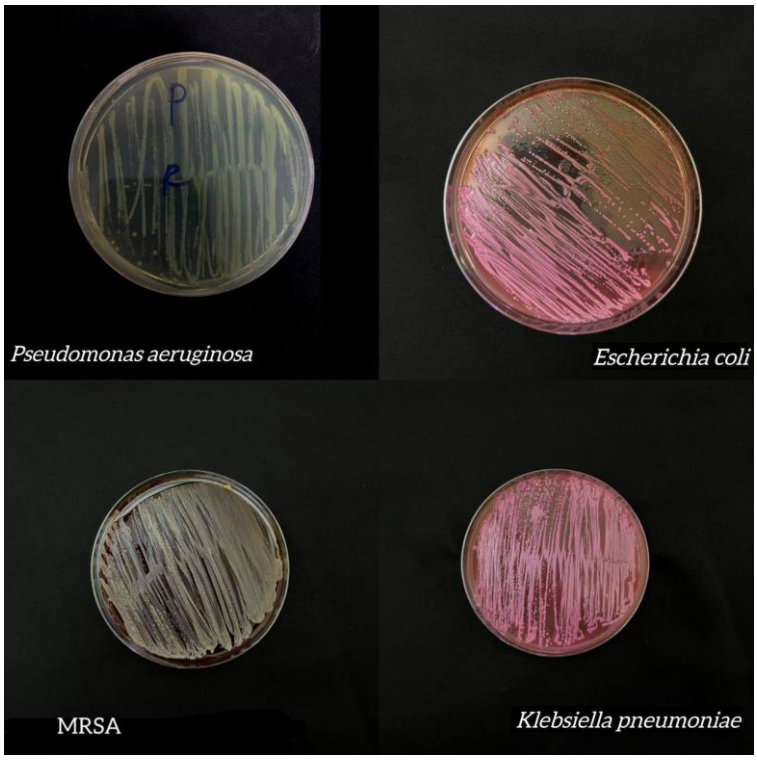

Figure 8: Antibiotics-resistant bacterial isolates

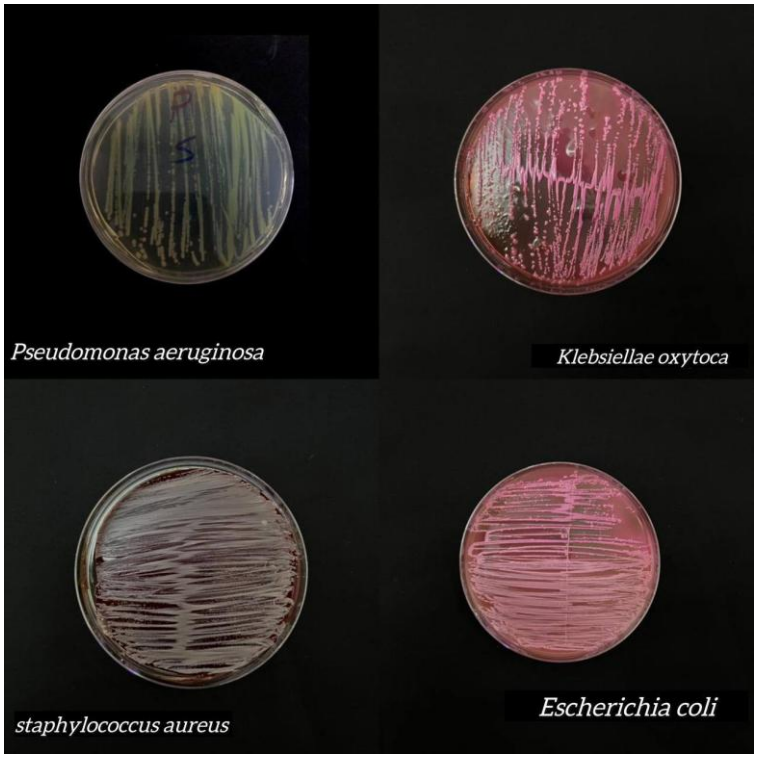

Figure 9: Non-resistant bacterial isolates

7.3 The effect of *A. bisporus* ethanolic extract against non-resistant bacteria

The following table (Table 3) presents the ZOI observed for the *A. bisporus* ethanolic extract categorized by extract concentration and bacterial strain. Statistical analysis confirmed that dilution of the stock solution significantly decreased its antibacterial activity, with higher concentrations demonstrating greater efficacy. No significant inhibition zones (ZOI ≈ 0 mm) were observed at lower concentrations (25% and 50%) for most of the tested bacteria, indicating minimal or no effect at these levels.

In contrast, significant inhibition was observed at higher concentrations (75% and 100%), with *P. aeruginosa* showing a mean ZOI of 11.00mm at 50% ($p < 0.001$) and

13.667mm at 75% ($p < 0.001$) and the highest sensitivity at 100%, with a mean ZOI of 15.333mm, matching with the Tigecycline (15.333mm), without a statistically significant difference with p-value = 1.000. *K. oxytoca* exhibited mean ZOI of 10.000mm and 11.667mm at 75% and 100% concentrations, respectively, with a significant statistical difference and p-value < 0.001. While *E. coli* responded only at higher concentrations, with mean ZOI of 10.667mm, ($p < 0.001$). *S. aureus* was the most sensitive to the Tigecycline (25.333mm), but required 100% extract concentration to achieve measurable inhibition of 8.000mm, $p < 0.001$ (Figure 10). Overall, the given results indicated that dilution of the stock solution reduces its antibacterial effect, with **higher concentrations being more effective**. the extract demonstrated statistically significant antibacterial effects only at higher concentrations, suggesting potential activity, though not equivalent to the positive control (Appendix 2,3).

Table (3): Estimated marginal means of non-resistant bacteria concentrations

Bacterial type	ZOI of *Agaricus bisporus* ethanolic extract concentrations (mm)				TIG (15 µg)
	25%	50%	75%	100%	
P. aeruginosa	-	11.000*	13.667*	15.333	15.333
K. oxytoca	-	-	10.000*	11.667*	23.667
E. coli	-	-	-	10.667*	23.667
s.aureus	-	-	-	8.000*	25.333

* The mean difference is significant at the 0.001 level

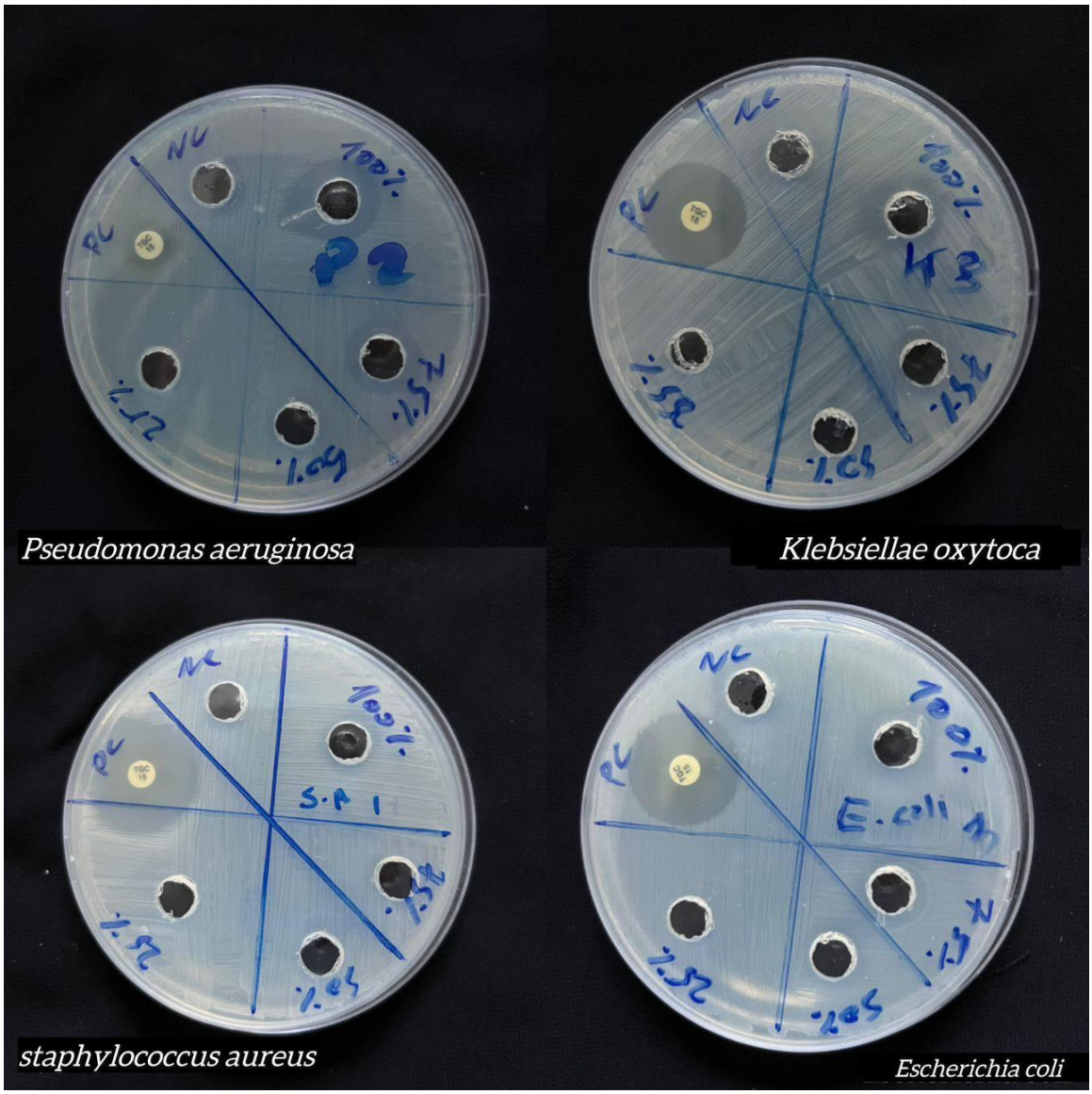

Figure (10): ZOI of non-resistant bacteria

The next Figure (Figure 11) shows that the interaction effect between stock solution concentration and bacteria type was significant for non-resistant bacteria with $p < .001$. This indicates that the effect of the extract concentration on antibacterial activity depends on the type of bacteria being tested, and vice versa.

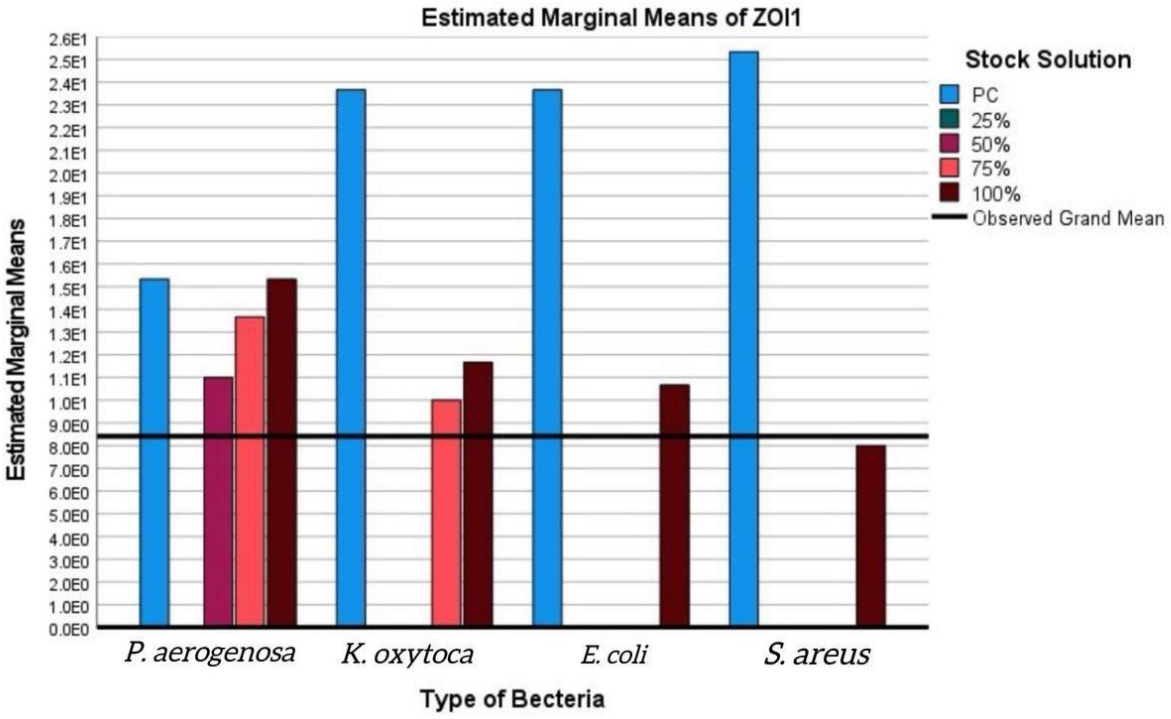

Figure (11): Estimated marginal means of non-resistant bacteria (stock solution concentrations × bacterial type)

7.4 The effect of *A. bisporus* ethanolic extract against resistant bacteria

According to the result represented in Table (4), the antimicrobial activity of the extract against antibiotic-resistant bacteria was concentration-dependent and statistically significant only at higher concentrations. At concentrations of 25% and 50%, no inhibition zones were observed (ZOI ≈ 0mm) for all tested strains. In contrast, at 75% and 100%, measurable inhibition zones were recorded with p-values less than 0.001 for all bacteria tested. *P. aeruginosa*, showed a zone of 8.333mm and 10.000mm at 75% and 100% concentrations, respectively ($p < 0.001$). Similarly, *K. pneumoniae* detected significant effects at 75% (6.000mm) and 100% (9.000mm), with $p < 0.001$. *E. coli* demonstrated the highest level of resistance, with small inhibition zone at 100% concentration (4.667mm, p

< 0.001). In contrast, MRSA demonstrated significant inhibition at 75% (7.333mm) and 100% (10.000mm), both with $p < 0.001$ (Figure 12). Overall, the statistical analysis revealed that stock concentration had a significant main effect ($p < 0.001$), while bacterial type alone was not significant ($p = 0.112$) (Appendix 4,5).

Table (4): Estimated marginal means of resistant bacteria concentrations

Bacterial type	ZOI of *Agaricus bisporus* ethanolic extract concentrations				TIG (15 µg)
	25%	50%	75%	100%	
P. aeruginosa	-	-	8.333*	10.000*	14.667
K. pneumoniae	-	-	6.000*	9.000*	16.333
E. col	-	-	-	4.667*	22.667
MRSA	-	-	7.333*	10.000*	17.000

* The mean difference is significant at the 0.001 level

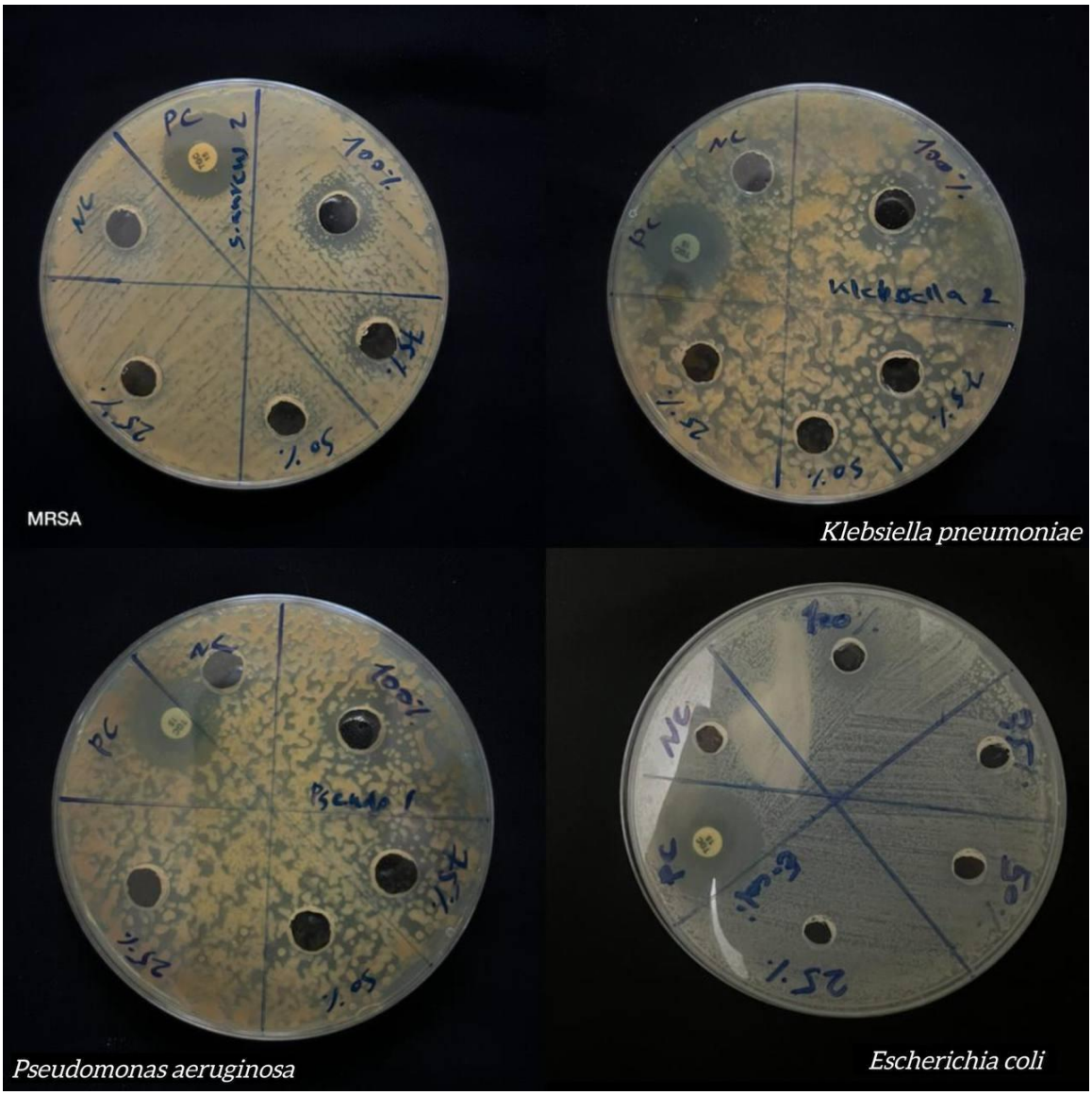

Figure (12): ZOI of resistant bacteria

However, the interaction between bacterial type and stock concentration was highly significant ($p < 0.001$), suggesting that the response to the extract varied depending on both the bacterial strain and the concentration used (Figure 13). These findings confirm that the extract has measurable antibacterial activity against resistant strains, but only at higher concentrations and with varying levels of effectiveness across different bacterial types, yet still less than the positive control.

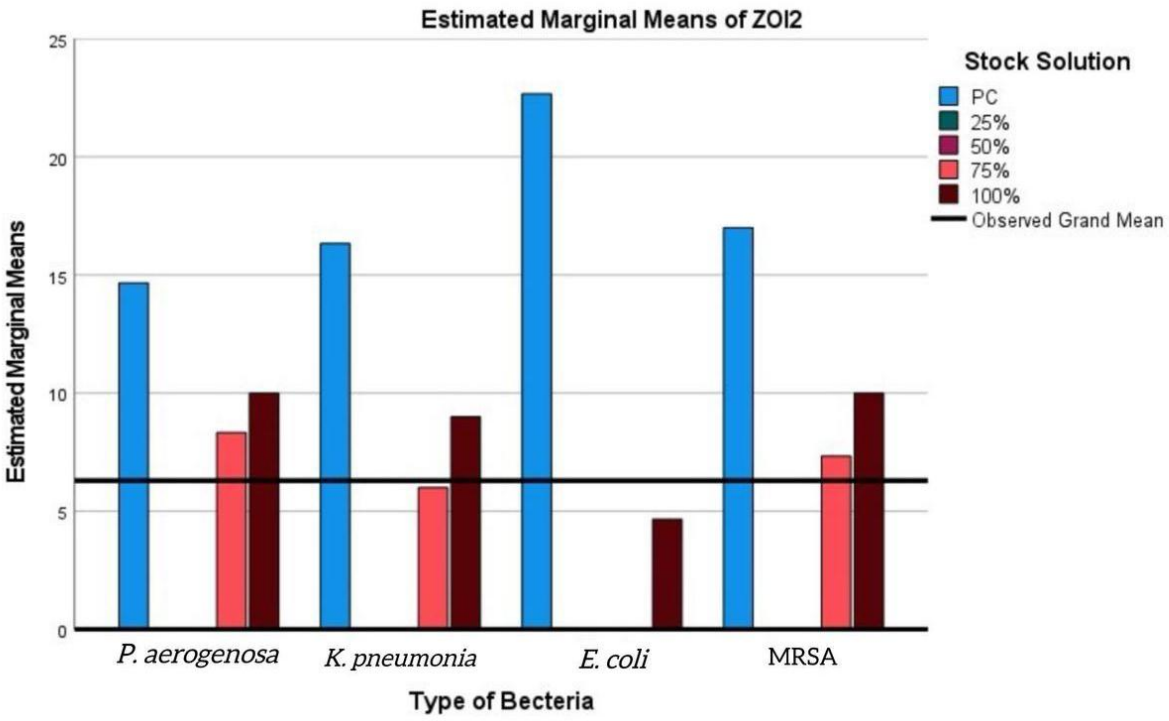

Figure (13): Estimated marginal means of resistant bacteria (stock solution concentrations × bacterial type)

The results indicate that both bacterial type and the concentration of the stock solution have significant effects on the dependent variables, non-resistant bacteria and resistant bacteria. However, the effect of bacterial type is more pronounced on non-resistant bacteria than on resistant bacteria.

The significant interaction effect suggests that the relationship between bacteria type and stock solution concentration is complex, and the effect of one factor depends on the level of the other factor. This implies that the effectiveness of the stock solution in influencing non-resistant bacteria and resistant bacteria varies depending on the type of bacteria being tested, which agrees with the study of (Huguet *et al.*, 2022) which highlights

the selective antibacterial properties of compounds found in mushrooms. And they explained that certain compounds can inhibit specific bacterial species while having no effect on others. For instance, some compounds target *Enterococcus faecalis* without affecting *Staphylococcus* species, while others act against *E. faecalis* and *S. aureus* but not *S. epidermidis*. Additionally, some polar compounds are active against *Staphylococcus* species yet do not influence *E. faecalis*. These observations emphasize the chemical diversity and targeted action of antibacterial agents in mushrooms.

For the resistant bacteria, the bacterial colonies were observed within the inhibition aura, which consistent with the findings of Jankov *et al.* (2024a) study, which confirmed that these natural extracts were less effective against MDR bacteria.

The post hoc tests confirm that all concentrations of the stock solution significantly differ from the control group (TIG) except for *P. aeruginosa*, with higher concentrations generally leading to greater differences in non-resistant bacteria and Resistant bacteria values. Which is consistent with Kumar *et al.* (2016) and Sutthisa and Chaiyacham (2022) and Kandasamy *et al.* (2020) study. Also, our findings indicated that the extract inhibited the resistant type of *S. aureus* (MRSA) with a ZOI of 10.000mm and showed a smaller ZOI against the non-resistant type (8.000mm), which consistent with the study of Huguet *et al.* (2022).

Moreover, our findings demonstrated that gram-negative bacteria exhibited greater susceptibility to the ethanolic extract compared to gram-positive bacteria. This observation is consistent with the results reported by (Sharma *et al.*, 2015) and (Olawuyi *et al.*, 2010) study, further supporting the differential sensitivity between bacterial types. Although the antibiotic was more effective on both resistant and non-resistant bacteria, the extract had a

bactericidal effect against non-resistant bacteria and showed a maximum ZOI against *P. aeruginosa* of 15.333mm, with a bacteriostatic effect against antibiotic-resistant bacteria, this aligns with the research conducted by (Kumar *et al.*, 2016).

However, it is important to acknowledge the limitations of this study. The extract was crude and not chemically characterized; therefore, the specific compounds responsible for the observed effects remain unidentified. Additionally, the study relied on the agar well diffusion method, which, while useful, does not capture the full spectrum of antimicrobial mechanisms, particularly those involving biofilm disruption or intracellular inhibition. Another potential limitation lies in the choice of solvent; the use of ethanol as the extraction medium may have influenced the observed activity, and future work should consider water-based extraction to better mimic physiological conditions. Moreover, the type of mushroom used in this study was an edible strain of *Agaricus bisporus*; previous research has suggested that wild, non-edible strains may exhibit higher antimicrobial potential due to their richer secondary metabolite profiles (Hassan *et al.*, 2019).

These findings highlight the potential use of the extract as a natural candidate antibacterial agent. Further research is needed using different extraction techniques to characterize the bioactive compounds and their underlying mechanisms of action as well as in vivo assessments of efficacy and toxicity. Furthermore, assessing their activity against additional pathogenic and multidrug-resistant (MDR) bacterial strains will provide valuable insights into their selectivity and therapeutic potential, paving the way for their development as future antimicrobial drug candidates.

Conclusion

8 Conclusion

This study aimed to evaluate the antibacterial activity of the ethanolic extract of *A. bisporus* against antibiotic-resistant bacteria (s*taphylococcus aureus (MRSA), Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Escherichia coli*), compared with non-resistant bacterial strains (*Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli,* and *Klebsiella oxytoca*), using the agar well diffusion method, with different concentrations (25%, 50%, 75%, and 100%). The results demonstrated a bactericidal effect against non-resistant bacteria, with the largest ZOI against *Pseudomonas aeruginosa* of 15.333mm, equal to the positive control (TIG), and a bacteriostatic effect against resistant bacteria, with the largest ZOI against *Pseudomonas aeruginosa* and *MRSA* with 10.000mm, yet still less than the positive control (TIG).

The statical analysis confirmed that the effectiveness of the extract varied depending on the bacterial species, with some strains showing greater sensitivity than others. These findings suggest that *A. bisporus* may serve as a potential candidate natural source of antimicrobial compounds particularly if advanced extraction techniques are applied and the bioactive constituents are accurately characterized, and combined with antibiotics in a synergistic manner, especially in light of the growing challenge of antibiotic resistance. Further studies are recommended to isolate and identify the active compounds and to explore their mechanisms of action in more detail.

Recommendations

9 Recommendations

1. Further studies are required to evaluate the efficacy of the extract against a wider range of clinically relevant bacterial species, in order to confirm its broad-spectrum antimicrobial potential.
2. The use of mushroom-derived extracts, such as those obtained from *A. bisporus*, should be encouraged as alternative antimicrobial agents, given their demonstrated effectiveness, low toxicity, affordability, low concentrations required to give the effect, and ease of cultivation.
3. Advanced extraction and purification techniques should be employed to isolate and chemically characterize the active bioactive compounds and secondary metabolites responsible for the observed antibacterial activity.
4. Additional investigations are needed to elucidate the biological and bactericidal mechanisms through which natural antimicrobial compounds exert their effects, contributing to a deeper understanding of their mode of action.
5. Research should be directed toward evaluating possible synergistic interactions between mushroom-derived compounds and conventional antibiotics, with the aim of enhancing antibacterial efficacy and supporting the development of improved therapeutic strategies.
6. It is recommended to encourage the promotion and wider adoption of mushrooms in daily dietary practices due to their rich nutritional profile, including essential vitamins, minerals, dietary fiber, and bioactive compounds that support overall health and disease prevention.

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Appendices

Appendices

Appendix (1): detailed statistical analysis procedures applied in this study:

The present study aimed to evaluate the inhibitory effect of the ethanolic extract of *Agaricus bisporus* on antibiotic-resistant bacteria: three gram-negative (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae*), and one gram-positive (MRSA), comparing its efficacy against non-resistant bacteria: three gram-negative (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella oxytoca*), and one gram-positive (*Staphylococcus aureus*). The experiment was designed to assess the antibacterial activity of the extract at varying concentrations (25%, 50%, 75%, and 100%) using the **Agar well diffusion method**, with zones of inhibition for resistant bacteria and zone of inhibition for non-resistant bacteria serving as the primary measures of antibacterial efficacy. To analyze the data, **SPSS version 27** was employed, utilizing **Multivariate Analysis of Variance (MANOVA)** to examine the effects of stock solution concentration, bacterial type, and their interaction on the dependent variables (resistant and non-resistant bacterial). Post hoc tests, specifically the **Dunnett t-test**, were conducted to compare the extract's effectiveness across different concentrations relative to the control group (PC).

Independent Variables (Factors):

1. **Stock Solution Concentration:**

The stock solution of the ethanolic extract was prepared at concentrations of **25%, 50%, 75%, and 100%** (10 mg/ml). These concentrations were used to determine the dose-dependent effect of the extract on bacterial growth inhibition.

2. **Type of Bacteria:**

The study included both **antibiotic-resistant** and **non-resistant** strains of bacteria:

- **Resistant strains:** *E. coli*, *MRSA*, *P. aerogenosa*, *K. pneumoniae*.
- **Non-resistant strains:** *E. coli*, *S. areus*, *P. aerogenosa*, *K. oxytoca*.

3. **Method of Application:**

The **Agar well diffusion method** was used to apply the ethanolic extract to the bacterial cultures. This method allows for the measurement of the zones of inhibition (resistant and non-resistant bacteria), which indicate the antibacterial activity of the extract.

10.1 Hypotheses:

1. **Null Hypothesis (H_0):** The ethanolic extract of *Agaricus bisporus* has no significant inhibitory effect on antibiotic-resistant bacteria.
    - **Alternative Hypothesis (H_1):** The ethanolic extract of *Agaricus bisporus* has a significant inhibitory effect on antibiotic-resistant bacteria.
2. **Null Hypothesis (H_0):** There is no significant difference in the inhibitory effect of the extract on antibiotic-resistant vs. non-resistant bacteria.

- **Alternative Hypothesis (H_1):** There is a significant difference in the inhibitory effect of the extract on antibiotic-resistant vs. non-resistant bacteria.

3. **Null Hypothesis (H_0):** The ethanolic extract of *Agaricus bisporus* is not significantly more effective than traditional antibiotics against antibiotic-resistant bacteria.

    - **Alternative Hypothesis (H_1):** The ethanolic extract of *Agaricus bisporus* is significantly more effective than traditional antibiotics against antibiotic-resistant bacteria.

- MANOVA (Multivariate Analysis of Variance) is an appropriate choice for this research because:

1. Multiple dependent variables (Non-resistant bacteria and Resistant bacteria, representing zones of inhibition for different bacteria strains).
2. Examining the effects of multiple independent variables (stock solution concentration, type of bacteria, and their interaction).
3. MANOVA allows assess the overall effect of these factors on the dependent variables while controlling for Type I error.

1. **Main Effect of Bacteria Type:**

The main effect of bacterial type was significant for Non-resistant bacteria with p-value < .001, but not for Resistant bacteria with p-value = .112. This suggests that the type of bacteria significantly influences the antibacterial activity of the extract for Non-resistant bacteria, but not for Resistant bacteria (Table A1).

2. **Main Effect of Stock Solution Concentration:**

The MANOVA results showed a significant main effect of stock solution concentration on both Non-resistant bacteria and Resistant bacteria ($p < .001$). This indicates that the concentration of the ethanolic extract significantly affects its antibacterial activity, with higher concentrations generally leading to larger zones of inhibition.

3. **Interaction Effect (Stock Solution × Bacteria Type):**

The interaction effect between stock solution concentration and bacteria type was significant for both Non-resistant bacteria and Resistant bacteria ($p < .001$). This indicates that the effect of the extract concentration on antibacterial activity depends on the type of bacteria being tested, and vice versa.

4. **Post Hoc Tests (Dunnett t-test):**

Post hoc tests revealed that all concentrations of the stock solution (25%, 50%, 75%, 100%) were significantly different from the control group (PC) for both Non-resistant bacteria and Resistant bacteria ($p < .001$). This confirms that the ethanolic extract has a dose-dependent effect on bacterial growth inhibition.

Table (A1): Test between- subjects effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Non-resistant bacteria	4611.250[a]	19	242.697	728.092	.000
	Resistant bacteria	2858.600[b]	19	150.453	57.866	.000
Intercept	Non-resistant bacteria	4250.417	1	4250.417	12751.250	.000
	Resistant bacteria	2381.400	1	2381.400	915.923	.000
Bectiria	Non-resistant bacteria	193.650	3	64.550	193.650	.000
	Resistant bacteria	16.600	3	5.533	2.128	.112
Stock	Non-resistant bacteria	3632.500	4	908.125	2724.375	.000
	Resistant bacteria	2566.100	4	641.525	246.740	.000
Bectiria * Stock	Non-resistant bacteria	785.100	12	65.425	196.275	.000
	Resistant bacteria	275.900	12	22.992	8.843	.000
Error	Non-resistant bacteria	13.333	40	.333		
	Resistant bacteria	104.000	40	2.600		
Total	Non-resistant bacteria	8875.000	60			
	Resistant bacteria	5344.000	60			
Corrected Total	Non-resistant bacteria	4624.583	59			
	Resistant bacteria	2962.600	59			

a. R Squared = .997 (Adjusted R Squared = .996)

b. R Squared = .965 (Adjusted R Squared = .948)

Appendix (2): SPSS result for non-resistant bacteria

Dependent Variable: Non-resistant bacteria					
				95% Confidence Interval	
Type of Becteria	Stock Solution	Mean	Std. Error	Lower Bound	Upper Bound
P.aerogenosa	PC	15.333	.333	14.660	16.007
	25%	-1.776E-15	.333	-.674-	.674
	50%	11.000	.333	10.326	11.674
	75%	13.667	.333	12.993	14.340
	100%	15.333	.333	14.660	16.007
K.oxytoca	PC	23.667	.333	22.993	24.340
	25%	8.882E-16	.333	-.674-	.674
	50%	1.776E-15	.333	-.674-	.674
	75%	10.000	.333	9.326	10.674
	100%	11.667	.333	10.993	12.340
E.col	PC	23.667	.333	22.993	24.340
	25%	8.882E-16	.333	-.674-	.674
	50%	8.882E-16	.333	-.674-	.674
	75%	.000	.333	-.674-	.674
	100%	10.667	.333	9.993	11.340
S.areus	PC	25.333	.333	24.660	26.007
	25%	5.329E-15	.333	-.674-	.674
	50%	1.776E-15	.333	-.674-	.674
	75%	8.882E-16	.333	-.674-	.674
	100%	8.000	.333	7.326	8.674

Appendix (3): Tukey post-hoc comparisons of ethanolic mushroom extract concentrations against non-resistant bacteria

Dependent Variable: Non-resistant bacteria							
						95% Confidence Interval for Difference[b]	
Type of Becteria	(I) Stock Solution	(J) Stock Solution	Mean Difference (I-J)	Std. Error	Sig.[b]	Lower Bound	Upper Bound
P.aerogenosa	PC	25%	15.333*	.471	.000	14.381	16.286
		50%	4.333*	.471	.000	3.381	5.286
		75%	1.667*	.471	.001	.714	2.619

		100%	-1.421E-14	.471	1.000	-.953-	.953
	25%	PC	-15.333-*	.471	.000	-16.286-	-14.381-
		50%	-11.000-*	.471	.000	-11.953-	-10.047-
		75%	-13.667-*	.471	.000	-14.619-	-12.714-
		100%	-15.333-*	.471	.000	-16.286-	-14.381-
	50%	PC	-4.333-*	.471	.000	-5.286-	-3.381-
		25%	11.000*	.471	.000	10.047	11.953
		75%	-2.667-*	.471	.000	-3.619-	-1.714-
		100%	-4.333-*	.471	.000	-5.286-	-3.381-
	75%	PC	-1.667-*	.471	.001	-2.619-	-.714-
		25%	13.667*	.471	.000	12.714	14.619
		50%	2.667*	.471	.000	1.714	3.619
		100%	-1.667-*	.471	.001	-2.619-	-.714-
	100%	PC	1.421E-14	.471	1.000	-.953-	.953
		25%	15.333*	.471	.000	14.381	16.286
		50%	4.333*	.471	.000	3.381	5.286
		75%	1.667*	.471	.001	.714	2.619
K.oxytoca	PC	25%	23.667*	.471	.000	22.714	24.619
		50%	23.667*	.471	.000	22.714	24.619
		75%	13.667*	.471	.000	12.714	14.619
		100%	12.000*	.471	.000	11.047	12.953
	25%	PC	-23.667-*	.471	.000	-24.619-	-22.714-
		50%	-1.776E-15	.471	1.000	-.953-	.953
		75%	-10.000-*	.471	.000	-10.953-	-9.047-
		100%	-11.667-*	.471	.000	-12.619-	-10.714-
	50%	PC	-23.667-*	.471	.000	-24.619-	-22.714-
		25%	1.776E-15	.471	1.000	-.953-	.953
		75%	-10.000-*	.471	.000	-10.953-	-9.047-
		100%	-11.667-*	.471	.000	-12.619-	-10.714-
	75%	PC	-13.667-*	.471	.000	-14.619-	-12.714-
		25%	10.000*	.471	.000	9.047	10.953
		50%	10.000*	.471	.000	9.047	10.953
		100%	-1.667-*	.471	.001	-2.619-	-.714-
	100%	PC	-12.000-*	.471	.000	-12.953-	-11.047-
		25%	11.667*	.471	.000	10.714	12.619
		50%	11.667*	.471	.000	10.714	12.619
		75%	1.667*	.471	.001	.714	2.619
E.col	PC	25%	23.667*	.471	.000	22.714	24.619
		50%	23.667*	.471	.000	22.714	24.619
		75%	23.667*	.471	.000	22.714	24.619
		100%	13.000*	.471	.000	12.047	13.953
	25%	PC	-23.667-*	.471	.000	-24.619-	-22.714-
		50%	.000	.471	1.000	-.953-	.953

		75%	8.882E-16	.471	1.000	-.953-	.953
		100%	-10.667-*	.471	.000	-11.619-	-9.714-
	50%	PC	-23.667-*	.471	.000	-24.619-	-22.714-
		25%	.000	.471	1.000	-.953-	.953
		75%	8.882E-16	.471	1.000	-.953-	.953
		100%	-10.667-*	.471	.000	-11.619-	-9.714-
	75%	PC	-23.667-*	.471	.000	-24.619-	-22.714-
		25%	-8.882E-16	.471	1.000	-.953-	.953
		50%	-8.882E-16	.471	1.000	-.953-	.953
		100%	-10.667-*	.471	.000	-11.619-	-9.714-
	100%	PC	-13.000-*	.471	.000	-13.953-	-12.047-
		25%	10.667*	.471	.000	9.714	11.619
		50%	10.667*	.471	.000	9.714	11.619
		75%	10.667*	.471	.000	9.714	11.619
S.areus	PC	25%	25.333*	.471	.000	24.381	26.286
		50%	25.333*	.471	.000	24.381	26.286
		75%	25.333*	.471	.000	24.381	26.286
		100%	17.333*	.471	.000	16.381	18.286
	25%	PC	-25.333-*	.471	.000	-26.286-	-24.381-
		50%	3.553E-15	.471	1.000	-.953-	.953
		75%	4.441E-15	.471	1.000	-.953-	.953
		100%	-8.000-*	.471	.000	-8.953-	-7.047-
	50%	PC	-25.333-*	.471	.000	-26.286-	-24.381-
		25%	-3.553E-15	.471	1.000	-.953-	.953
		75%	8.882E-16	.471	1.000	-.953-	.953
		100%	-8.000-*	.471	.000	-8.953-	-7.047-
	75%	PC	-25.333-*	.471	.000	-26.286-	-24.381-
		25%	-4.441E-15	.471	1.000	-.953-	.953
		50%	-8.882E-16	.471	1.000	-.953-	.953
		100%	-8.000-*	.471	.000	-8.953-	-7.047-
	100%	PC	-17.333-*	.471	.000	-18.286-	-16.381-
		25%	8.000*	.471	.000	7.047	8.953
		50%	8.000*	.471	.000	7.047	8.953
		75%	8.000*	.471	.000	7.047	8.953

Appendix (4): SPSS result for resistant bacteria

Estimates					
Dependent Variable: Resistant bacteria					
Type of Becteria	Stock Solution	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
P.aerogenosa	PC	14.667	.931	12.785	16.548
	25%	1.776E-15	.931	-1.882-	1.882
	50%	3.553E-15	.931	-1.882-	1.882
	75%	8.333	.931	6.452	10.215
	100%	10.000	.931	8.118	11.882
K. pneumoniae	PC	16.333	.931	14.452	18.215
	25%	.000	.931	-1.882-	1.882
	50%	1.776E-15	.931	-1.882-	1.882
	75%	6.000	.931	4.118	7.882
	100%	9.000	.931	7.118	10.882
E.col	PC	22.667	.931	20.785	24.548
	25%	1.776E-15	.931	-1.882-	1.882
	50%	3.553E-15	.931	-1.882-	1.882
	75%	1.776E-15	.931	-1.882-	1.882
	100%	4.667	.931	2.785	6.548
S.areus	PC	17.000	.931	15.118	18.882
	25%	.000	.931	-1.882-	1.882
	50%	.000	.931	-1.882-	1.882
	75%	7.333	.931	5.452	9.215
	100%	10.000	.931	8.118	11.882

Appendix (5): Tukey post-hoc comparisons of ethanolic mushroom extract concentrations against resistant bacteria

Type of Becteria	(I) Stock Solution	(J) Stock Solution	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
P.aerogenosa	PC	25%	14.667*	1.317	.000	12.006	17.328
		50%	14.667*	1.317	.000	12.006	17.328
		75%	6.333*	1.317	.000	3.672	8.994
		100%	4.667*	1.317	.001	2.006	7.328
	25%	PC	-14.667-*	1.317	.000	-17.328-	-12.006-
		50%	-1.776E-15	1.317	1.000	-2.661-	2.661
		75%	-8.333-*	1.317	.000	-10.994-	-5.672-
		100%	-10.000-*	1.317	.000	-12.661-	-7.339-
	50%	PC	-14.667-*	1.317	.000	-17.328-	-12.006-
		25%	1.776E-15	1.317	1.000	-2.661-	2.661
		75%	-8.333-*	1.317	.000	-10.994-	-5.672-
		100%	-10.000-*	1.317	.000	-12.661-	-7.339-
	75%	PC	-6.333-*	1.317	.000	-8.994-	-3.672-
		25%	8.333*	1.317	.000	5.672	10.994
		50%	8.333*	1.317	.000	5.672	10.994
		100%	-1.667-	1.317	.213	-4.328-	.994
	100%	PC	-4.667-*	1.317	.001	-7.328-	-2.006-
		25%	10.000*	1.317	.000	7.339	12.661
		50%	10.000*	1.317	.000	7.339	12.661
		75%	1.667	1.317	.213	-.994-	4.328
K. pneumoniae	PC	25%	16.333*	1.317	.000	13.672	18.994
		50%	16.333*	1.317	.000	13.672	18.994
		75%	10.333*	1.317	.000	7.672	12.994
		100%	7.333*	1.317	.000	4.672	9.994
	25%	PC	-16.333-*	1.317	.000	-18.994-	-13.672-
		50%	-1.776E-15	1.317	1.000	-2.661-	2.661
		75%	-6.000-*	1.317	.000	-8.661-	-3.339-
		100%	-9.000-*	1.317	.000	-11.661-	-6.339-
	50%	PC	-16.333-*	1.317	.000	-18.994-	-13.672-
		25%	1.776E-15	1.317	1.000	-2.661-	2.661
		75%	-6.000-*	1.317	.000	-8.661-	-3.339-
		100%	-9.000-*	1.317	.000	-11.661-	-6.339-
	75%	PC	-10.333-*	1.317	.000	-12.994-	-7.672-
		25%	6.000*	1.317	.000	3.339	8.661
		50%	6.000*	1.317	.000	3.339	8.661
		100%	-3.000-*	1.317	.028	-5.661-	-.339-

	100%	PC	-7.333-*	1.317	.000	-9.994-	-4.672-
		25%	9.000*	1.317	.000	6.339	11.661
		50%	9.000*	1.317	.000	6.339	11.661
		75%	3.000*	1.317	.028	.339	5.661
E.col	PC	25%	22.667*	1.317	.000	20.006	25.328
		50%	22.667*	1.317	.000	20.006	25.328
		75%	22.667*	1.317	.000	20.006	25.328
		100%	18.000*	1.317	.000	15.339	20.661
	25%	PC	-22.667-*	1.317	.000	-25.328-	-20.006-
		50%	.000	1.317	1.000	-2.661-	2.661
		75%	.000	1.317	1.000	-2.661-	2.661
		100%	-4.667-*	1.317	.001	-7.328-	-2.006-
	50%	PC	-22.667-*	1.317	.000	-25.328-	-20.006-
		25%	.000	1.317	1.000	-2.661-	2.661
		75%	1.776E-15	1.317	1.000	-2.661-	2.661
		100%	-4.667-*	1.317	.001	-7.328-	-2.006-
	75%	PC	-22.667-*	1.317	.000	-25.328-	-20.006-
		25%	.000	1.317	1.000	-2.661-	2.661
		50%	-1.776E-15	1.317	1.000	-2.661-	2.661
		100%	-4.667-*	1.317	.001	-7.328-	-2.006-
	100%	PC	-18.000-*	1.317	.000	-20.661-	-15.339-
		25%	4.667*	1.317	.001	2.006	7.328
		50%	4.667*	1.317	.001	2.006	7.328
		75%	4.667*	1.317	.001	2.006	7.328
S.areus	PC	25%	17.000*	1.317	.000	14.339	19.661
		50%	17.000*	1.317	.000	14.339	19.661
		75%	9.667*	1.317	.000	7.006	12.328
		100%	7.000*	1.317	.000	4.339	9.661
	25%	PC	-17.000-*	1.317	.000	-19.661-	-14.339-
		50%	.000	1.317	1.000	-2.661-	2.661
		75%	-7.333-*	1.317	.000	-9.994-	-4.672-
		100%	-10.000-*	1.317	.000	-12.661-	-7.339-
	50%	PC	-17.000-*	1.317	.000	-19.661-	-14.339-
		25%	.000	1.317	1.000	-2.661-	2.661
		75%	-7.333-*	1.317	.000	-9.994-	-4.672-
		100%	-10.000-*	1.317	.000	-12.661-	-7.339-
	75%	PC	-9.667-*	1.317	.000	-12.328-	-7.006-
		25%	7.333*	1.317	.000	4.672	9.994
		50%	7.333*	1.317	.000	4.672	9.994
		100%	-2.667-*	1.317	.050	-5.328-	-.006-
	100%	PC	-7.000-*	1.317	.000	-9.661-	-4.339-
		25%	10.000*	1.317	.000	7.339	12.661
		50%	10.000*	1.317	.000	7.339	12.661
		75%	2.667*	1.317	.050	.006	5.328