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Original Research

GC-MS profiling of Volatile Metabolites produced by *Enterococcus faecalis* and Evaluation of Its Antibacterial and Antifungal Activity

Mays Hassan Obais Alkhalidi [1], Rabab J.H. Al Hasseny [2], Sabreen M. Abes [3]

[1]Department of Genetic Engineering, College of Biotechnology, Al-Qasim Green University, Iraq.
[2]Medical Microbiology, Department of Health Food and Nutrition, College of Food Science, Al-Qasim Green University, Iraq.
[3]Department of Genetic Engineering, College of Biotechnology, Al-Qasim Green University, Iraq.

Abstract:

Aims and Objectives:

The purpose of this laboratory study was to study the truly biologically active chemical compounds produced by *Enterococcus faecalis* and to evaluate the antibacterial and antifungal bioactivity of these products.

Method:

In this research, (GC-MS) techniques were used to investigate the biochemical components that are commonly referred to as bioactive substances. At the same time, these bioactive substances are referred to as bioactive secondary metabolites. Furthermore, the ethanolic extract of *Enterococcus faecalis* was tested in an experimental laboratory to determine whether it actually has effective antibacterial and antifungal properties.

Results:

Using GC-MS analysis on *Enterococcus faecalis*, the presence of the following bioactive components was experimentaly identified: 2-methyl-1-propenol, 2-pentene, 4-methyl-2,4-diphenyl, 2,4-methyl Dioxo-4-phenylbutanoate, 2 -pentnone, -phenylbutan-2-yl acetate, methyl 2-(butn-2-ylsulfanyl)acetate, trithylene glycol bis(2-ethyl butyrate), methyl 2-(butan-2-ylsulfanyl)) Acetate, triethylene glycol PS (2-ethyl butyrate), 3-mercapto-2-pentenone, ethyl 2-ethylhexanoate, 3-methylbutyl acetate. Metabolites of *Enterococcus faecalis* showed significant activity against Proteus mirabilis (20.18 ± 0.04). Metabolites of *Enterococcus faecalis* were highly active against *Fusarium oxyporum* (13.76 ± 0.17).

Keywords: *Enterococcus faecalis*, Secondary metabolites, Antibacterial, GC/MS.

Introduction:

These microscopic Gram-positive anaerobes are facultatively known as enterococci and can be observed and identified laboratory under a light microscope as single, double, or very short chains of cocci [1]. Although enterococci are a major part of the microflora of the gastrointestinal tract, they are common opportunistic pathogens. Here we must mention the presence of a number of known strains of enterococci that are capable of actually causing cases of infection acquired from the same community or acquired from the hospital, for example, especially in infected people and participants who actually suffer from known weak immunity. Hence, endocarditis as well as urinary tract infection (UTI) are examples of diseases that can be caused by enterococci [3]. *E. faecalis* and E. faecium are two of the most important and common species capable of causing infections and raise significant concerns about resistance to known antibiotics, with *E. faecalis* accounting for the majority of infections [4]. Of the most important of these different species that have actually been found, E. faecalis is the only one actually responsible for most of the recorded and known cases of infection. There are 38 different known species belonging to the pathogenic Enterococcus genus. The two most important species are *Enterococcus faecalis*, both of which colonize the human intestine. Hospitals in the United States and around the world frequently isolate these bacteria because they are the source of a variety of diseases, including bacteremia, urinary tract infections (UTIs), endocarditis, and intra-abdominal and pelvic infections, as well as nosocomial and iatrogenic infections [5]

Because enterococci are so common, it is critical that hospitals have the technology to distinguish them from other types of bacteria when testing hospitalised patients. It is also critical for labs to be able to differentiate between strains of the same species of Enterococcus and between species of Enterococcus themselves [6]. The developed world needs better surveillance and speciation procedures are either insufficient or cumbersome; developing countries lack trained hospital staff and money for adequate identification of enterococci, but sadly, enterococci are becoming more and more common as serious pathogens worldwide. level of the genus or species [7]. The objective of this research is to identify volatile metabolites generated by *Enterococcus faecalis* and analyse their antimicrobial and antifungal properties using gas chromatography-mass spectrometry.

Materials and Methods:

Optimal environmental conditions for growth and identification of metabolites

An isolated strain of Enterococcus faecalis was grown on nutrient agar for forty-eight hours at a temperature of 22 degrees Celsius. Subcultures were obtained. For ten minutes, the solution was heated to a temperature of four degrees Celsius and then subjected to stirring at a speed of one hundred and thirty revolutions per minute. This process was repeated for ten minutes. A rotary evaporator was used to evaporate metabolites at a temperature of 45 degrees Celsius [8, 9]. Metabolites were then extracted from the liquid culture and separated from the culture.

Conducting a spectral study of the bioactive natural chemical components of Enterococcus faecalis using (GC-MS).

An Agilent 789 A instrument was used to perform the examination, which was performed using a GC–MS approach. The gas chromatography column used was a DB-5MS column purchased from J&W Scientific in Folsom, California. The following measurements were made for this column: The film thickness is 0.25 µm and the diameter is 30 m with an internal diameter of 0.25 mm. Compared to the earlier experiment [10], the temperature in the furnace was kept at the same level throughout the process. The carrier gas used was helium and the flow rate was set at one milliliter per minute each time. Effluent from the gas chromatography (GC) column was directly injected into the mass spectrometer (MS) source via a transfer line that was heated to 250 degrees Celsius. 230 degrees Celsius (°C) was the temperature that was maintained at the ion source while the ionization process took place at a

voltage of 70 electron volts (eV). A total of 41 atomic mass units (amu) were included in the measuring range, which reached up to 450.

Evaluation of the antibacterial efficacy of secondary metabolite chemicals against three pathogenic bacteria.

A sterile cork borer was used to create wells in the agar with a diameter of five millimeters. Then, 25 microliters of sample solutions containing metabolites generated by Enterococcus faecalis were introduced into the wells. Swabs were used to collect test pathogens, which included E. coli, Proteus mirabilis and Staph. epidermidis. These pathogens were then plated on Muller Hinton agar plates. The solvent used for the control was ethanol.

Determination of antifungal activity of Enterococcus faecalis secondary metabolite compounds

The microbes that were tested were plated on Muller-Hinton agar plates. *Enterococcus faecalis* extract was placed in drilled wells in a volume of 70 microliters. Antifungal activity was determined by determining the zone of inhibition against the microorganisms that were tested. Ethanol was used for solvent control purposes. These two antifungal agents, ketoconazole and amphotericin B, have been used as usual treatment. Experiments were performed three times to ensure accuracy. Antifungal activity was evaluated by determining the diameter of the inhibition zone, which was observed after an incubation period of 48 hours.

Statistical Analysis

If the p-value was less than or equal to 0.05, we used Student's t-test to find out whether the parametric data was statistically significant.

Results and Discussion:

In recent years, Enterococcus faecalis has emerged as a grave source of urinary tract infections (UTIs) that are acquired in hospitals as well as in the community. These UTIs can result in life-threatening consequences such as bacteremia [11, 12]. Despite the fact that there is no specific group of genes that has been suggested to be connected with urinary tract infections (UTIs), there has been no comprehensive analysis undertaken to demonstrate phenotype–genotype concordance for this clinically significant type of infection. We launched the current study with the intention of phenotypically and genotypically investigating and correlating virulence factors in E. faecalis clinical isolates from patients in Iraq who had community-acquired urinary tract infections (UTIs). These factors could include biofilm formation, gelatine and casein hydrolysis, as well as blood hemolysis. This study was guided by a previous study that investigated enterococci isolated from different tissues.

The GC-MS chromatogram exhibited peaks that corresponded to the compounds that were previously identified. The following chemicals are mentioned: 2-Methyl-1-Propnol, 2-Pentene, 4-mthyl-2,4-diphnyl-, methyl 2,4-dioxo-4-phenylbutanoate, 2-Pentnone, -phenylbtan-2-yl acetate, Methyl 2-(btan-2-ylsufanyl)cetate, Triethylene glycol bis(2-ethlbutyrate), Methyl 2-(butan-2-ylsulfanyl)acetate, Triethylne glycol bs(2-ethylbutyrate), 3-Mercapto-2-pentnone, Ethyl 2-ethylhxanoate, 3-Methlbutyl Acetate. It was investigated whether the secondary metabolites produced by Enterococcus faecalis have antibacterial properties against three dangerous pathogens. In the current research, the biological activity of the ethanolic extract of Enterococcus faecalis, as well as the conventional antibiotics Amikacin and Vancomycin, against three different pathogens were investigated.

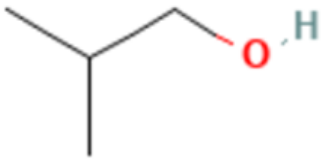
Bacillus subtilis (15.28±1.73, 22.93±2.31, and 19.00±2.04), Proteus mirabilis (20.18±0.04, 17.09±0.03, and 21.34±0.01), and *Staphylococcus saprophyticus* (16.02±1.75, 04.65±0.93, and 11.05±0.26). Enterococcus faecalis metabolites were shown to show remarkable activity against Proteus mirabilis, with a mean value of 20.18±0.04. The bioactivity of the ethanolic extract of Enterococcus faecalis and the conventional antibiotics Amphotericin B and Ketoconazole against five different types of fungi and yeasts was investigated. Secondary metabolites of *Enterococcus faecalis*. *Aspergillus flavus* (11.73 ± 0.13, 17.01 ± 0.19 and 20.71 ±

1.08) Figure 4, *Fusarium oxyporum* (13.76 ± 0.17, 19.73 ± 1.02 and 16.09 ± 0.97) Figure 5, *Cladosporium herbarum* (12.19 ± 0.14, 20.80 ± 1.01 and 23.01 ± 1.53) Figure 6, *Alternaria alternaria* (09.86 ± 0.11, 18.07 ± 1.00 and 15.16 ± 0.86) Figure 7, *Candida albicans* (10.24 ± 0.12, 20.03 ± 1.01 and 24.50 ± 1.81) Figure 8. *Enterococcus faecalis* metabolites was very highly active against *Fusarium oxyporum* (13.76 ± 0.17).

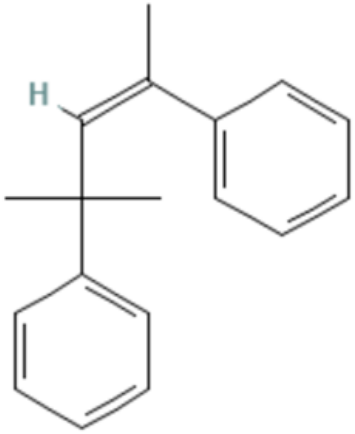
As a gold standard for identifying nosocomial disease pathogens, culture of microorganisms isolated from human material has emerged in recent years. The patient undergoes empirical treatment with broad-spectrum antibiotics for up to three days before the findings of this approach are known [13]. Invasive sampling of human specimens is required, and patients are required to undergo invasive sampling, even though this method is very specific, sensitive, and informative about antibiotic resistance. Qualified personnel are required to perform the procedure. This is why, in addition to the conventional microbiological methods, alternative analytical approaches are being pursued for the aim of bacterial disease diagnosis. Variations in the rate of microbial synthesis of volatile organic molecules are associated with either the bacterial cell load or metabolic activity. The metabolites produced by early-stage bacteria may also contribute to clinical practice since they may be signs of an infection that is only starting to take hold [14, 15]. As the infection develops or clears up (maybe as a consequence of the antibiotic treatment), it may be possible to monitor the secretion of chemicals whose levels are directly related to the overall bacterial load. With the increasing incidence of multidrug-resistant strains (for example, vancomycin-resistant enterococci [VRE]), E. faecalis strains show both intrinsic and acquired resistance to a wide range of antibiotics [16]. Since vancomycin is known as an antibiotic of last resort, VRE poses a significant risk to human health. In addition, E. faecalis plays a role in the transfer of resistance genes to other medically important bacterial species such as *Staphylococcus aureus*. Recently, a connection between oxidative stress and the bactericidal activity of antimicrobial substances has been considered [17]. This is because the prevalence of antibiotic-resistant bacteria has increased in recent years. However, the idea underlying the effect of a direct interaction between reactive oxygen species (ROS) and antibiotics has recently been challenged by a number of different groups. These groups argued that the link between ROS induction and antibiotics is not as strong as previously thought.

On the other hand, E. faecalis is substantially more likely to be associated with episodes of primary endodontic infections that are asymptomatic. In addition, the presence of E. faecalis is significantly more likely to be discovered in case of unsuccessful endodontic therapy. Through the administration of the antibacterial intracanal drug, microorganisms can be eliminated from the root canal systems of both adults and children. Because of this, the discomfort and irritation that are experienced by the pulpal and periapical tissues are diminished [18, 19]. This represents the most effective strategy for disinfecting root canals, which ensures that root canal therapy will have a high percentage of success. E. faecalis was shown to be the most abundant type of bacteria that was found in the root canal environment of a patient who was sick. The unfortunate reality is that this species is able to flourish in hostile settings that are devoid of nutrients and have an alkaline pH that may reach up to 11.5; this implies that it will

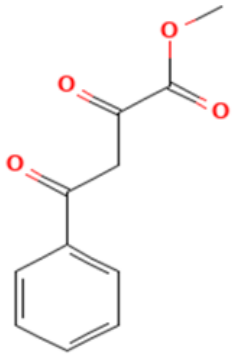
be tough to eliminate completely from the root canal system.



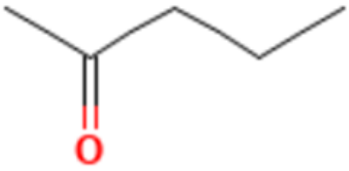
2-Methyl-1-Propanol
Molecular Formula: $C_4H_{10}O$
Molecular Weight: 74.12 g/mol



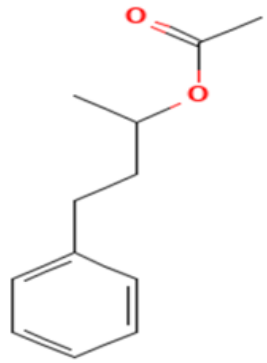
2-Pentene, 4-methyl-2,4-diphenyl-
Molecular Formula: $C_{18}H_{20}$
Molecular Weight: 236.4 g/mol



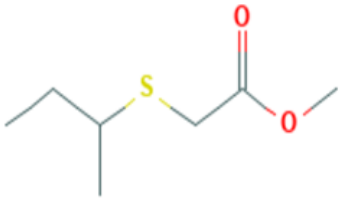
methyl 2,4-dioxo-4-phenylbutanoate
Molecular Formula: $C_{11}H_{10}O_4$
Molecular Weight: 206.19 g/mol



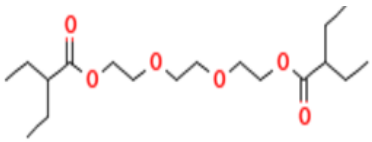
2-Pentanone
Molecular Formula: $C_5H_{10}O$
Molecular Weight: 86.13 g/mol



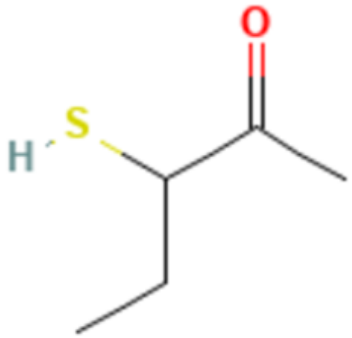
4-phenylbutan-2-yl acetate
Molecular Formula: $C_7H_{14}O_2$
Molecular Weight: 192.25 g/mol



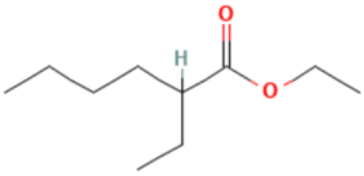
Methyl 2-(butan-2-ylsulfanyl)acetate
Molecular Formula: $C_7H_{14}O_2S$
Molecular Weight: 162.25 g/mol



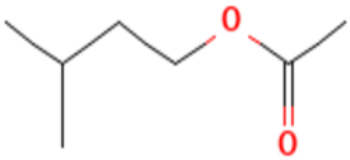
Triethylene glycol bis(2-ethylbutyrate)
Molecular Formula: $C_{18}H_{34}O_6$
Molecular Weight: 346.5 g/mol



3-Mercapto-2-pentanone
Molecular Formula: $C_5H_{10}OS$
Molecular Weight: 118.20 g/mol



Ethyl 2-ethylhexanoate
Molecular Formula: $C_{10}H_{20}O_2$
Molecular Weight: 172.26 g/mol



3-Methylbutyl Acetate
Molecular Formula: $C_7H_{14}O_2$
Molecular Weight: 130.18 g/mol

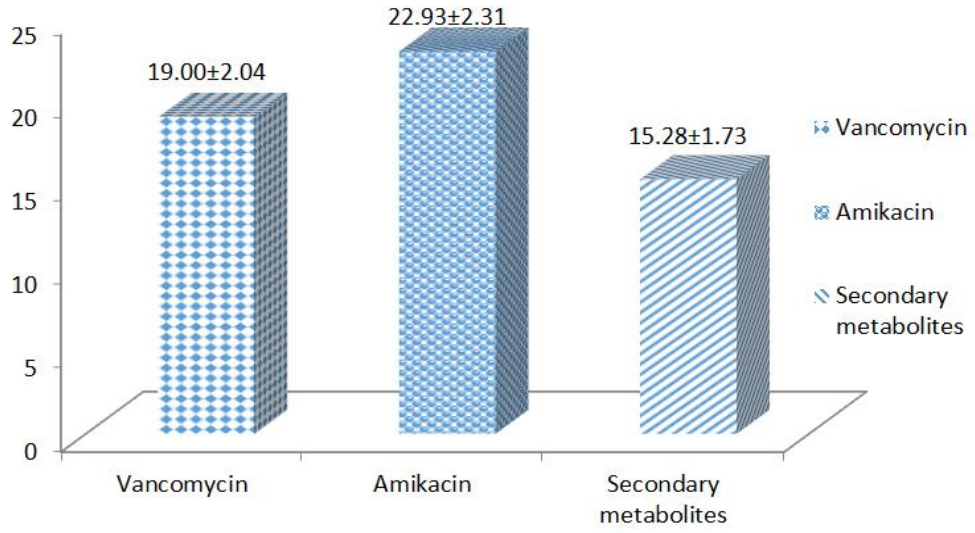


Figure 1. Metabolite products of *Enterococcus faecalis* , Amikacin and Vancomycin as anti-bacterial activity against *Bacillus subtilis.*

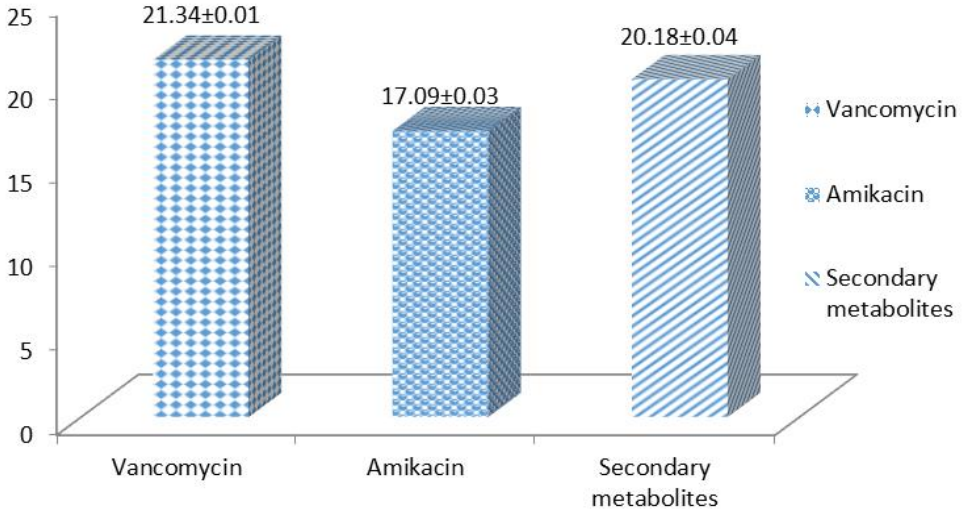


Figure 2. Metabolite products of *Enterococcus faecalis* , Amikacin and Vancomycin as anti-bacterial activity against *Proteus mirabilis.*

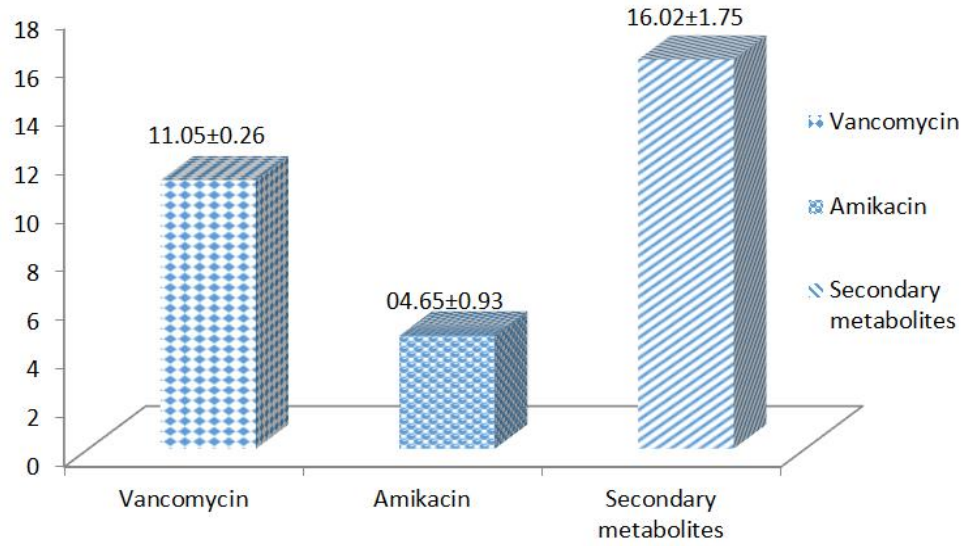


Figure 3. Metabolite products of *Enterococcus faecalis* , Amikacin and Vancomycin as anti-bacterial activity against *Staphylococcus saprophyticus.*

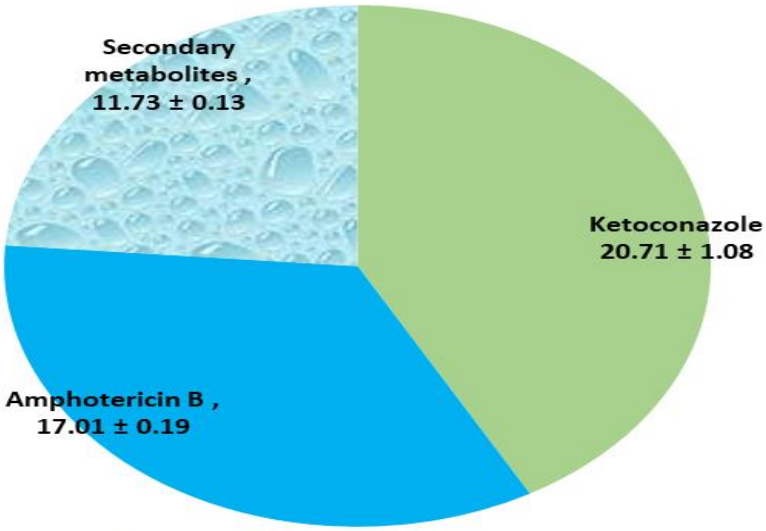


Figure 4. Metabolite products, Amphotericin B, and Fluconazol as anti-Fungal activity against *Aspergillus flavus*

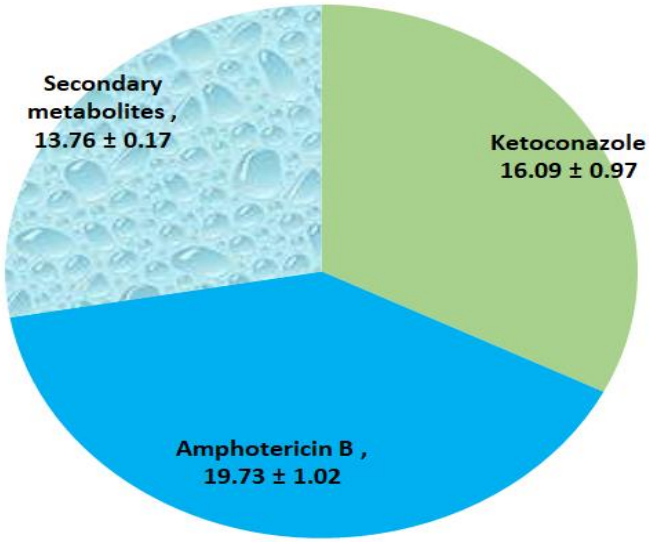


Figure 5. Metabolite products, Amphotericin B, and Ketoconazole as anti-Fungal activity against *Fusarium oxyporum*

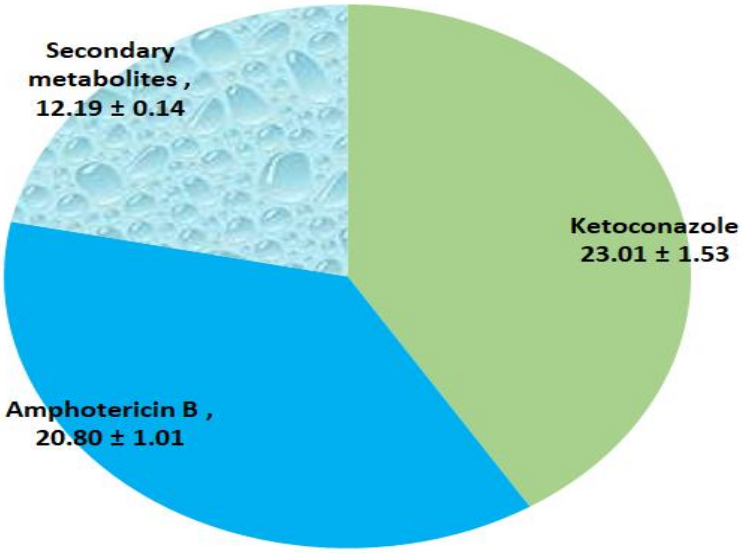


Figure 6. Metabolite products, Amphotericin B, and Ketoconazole as anti-Fungal activity against *Cladosporium herbarum*

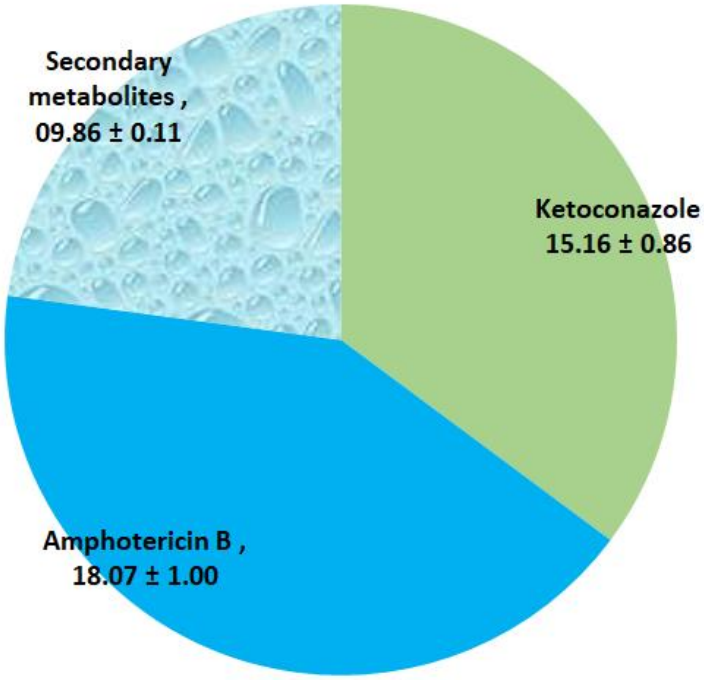


Figure 7. Metabolite products, Amphotericin B, and Ketoconazole as anti-Fungal activity against *Alternaria alternaria*

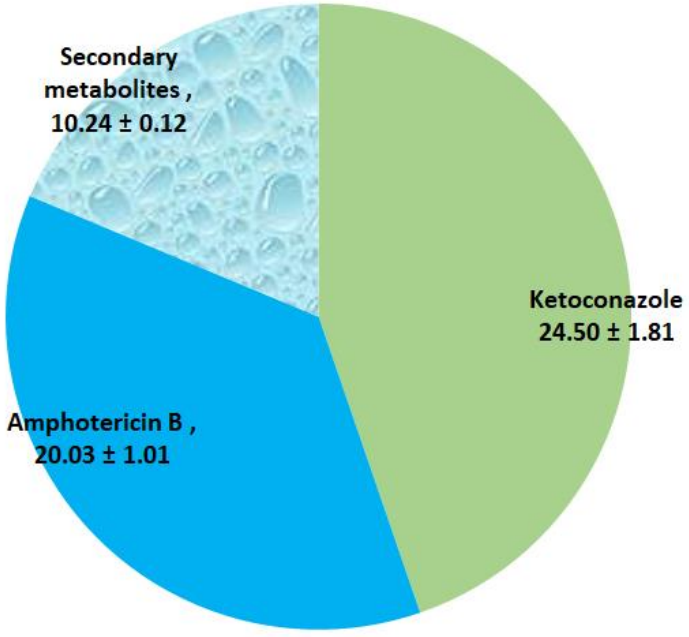


Figure 8. Metabolite products, Amphotericin B, and Ketoconazole as anti-Fungal activity against *Candida albicans*

Conclusion:

Based on the results of the antibacterial activity test, it was determined that the metabolites of *Enterococcus faecalis* demonstrated a noteworthy level of activity against *Proteus mirabilis*, with a specific activity level of 20.18±0.04. The metabolites produced by Enterococcus faecalis shown a remarkable level of activity against *Fusarium oxyporum* (9.76±0.17). According to the findings of the most recent research, there are a variety of common substances that are not only freely accessible but also have the potential to be utilised as alternatives to conventional therapy as well as supplements to conventional therapy.

References:

1. Poh CH, Oh HML, Tan AL. Epidemiology and clinical outcome of enterococcal bacteraemia in an acute care hospital. J Infect. 2006;52 (5):383–386.
2. Jett BD, Huycke MM, Gilmore MS. Virulence of enterococci. Clin Microbiol Rev. 1994;7(4):462–478.
3. Ruoff KL, De La Maza L, Murtagh MJ, Spargo JD, Ferraro MJ. Species identities of enterococci isolated from clinical specimens. J Clin Microbiol. 1990;28(3):435–437.
4. Kreft B, Marre R, Schramm U, Wirth R. Aggregation substance of Enterococcus faecalis mediates adhesion to cultured renal tubular cells. Infect Immun. 1992;60(1):25–30.
5. Budavari S M, Saunders G L, Liebowitz L D, Khoosal M, Crewe-Brown H. Emergence of vancomycin-resistant enterococci in South Africa. S Afr Med J. 1997; 87(11): 1557
6. Cetinkaya Y, Falk P, Mayhall C. Vancomycin-resistant enterococci. Clin Microbiol Rev. 2000; 13(4): 686–707
7. Chan E D, Iseman M. Multidrug-resistant and extensively drug-resistant tuberculosis: a review. Curr Opin Infect Dis. 2008; 21(6): 587–595
8. Manson JM, Keis S, Smith JMB, Cook GM. 2003. Characterization of a vancomycin-resistant Enterococcus faecalis (VREF) isolate from a dog with mastitis: further evidence of a clonal lineage of VREF in New Zealand. J. Clin. Microbiol. **41**:3331–3333. 10.1128/JCM.41.7.3331-3333.2003
9. Smart KF, Aggio RBM, Van Houtte JR, Villas-Bôas SG. 2010. Analytical platform for metabolome analysis of microbial cells using methyl chloroformate derivatization followed by gas chromatography-mass spectrometry. Nat. Protoc. **5**:1709–1729. 10.1038/nprot.2010.108
10. Aggio R, Villas-Bôas SG, Ruggiero K. Metab: an R package for high-throughput analysis of metabolomics data generated by GC-MS. Bioinformatics. 2011; 27:2316–2318.
11. Otto M. Targeted immunotherapy for staphylococcal infections. BioDrugs. 2008;22(1):27–36.
12. Patti JM, Allen,BL, McGavin MJ, Hook M. MSCRAMM-mediated adherence of microorganisms to host tissues. Annu Rev Microbiol. 1994;48(1):585–617.
13. Donlan RM. Biofilms: microbial life on surfaces. Emerg Infect Dis. 2002;8(9):881–890.
14. Mohamed JA, Huang DB. Biofilm formation by enterococci. J Med Microbiol. 2007;56:1581–1588.
15. Ike Y, Hashimoto H, Clewell DB. Hemolysin of Streptococcus faecalis subspecies zymogenes contributes to virulence in mice. Infect Immun. 1984;45(2):528–530.
16. Poh CH, Oh HML, Tan AL. 2006. Epidemiology and clinical outcome of enterococcal bacteraemia in an acute care hospital. J. Infect. 2005; 52:383–386.
17. Livermore DM. Has the era of untreatable infections arrived? J. Antimicrob. Chemother. 2009; 64(Suppl 1):i29–i36.
18. Neely AN, Maley MP. Survival of enterococci and staphylococci on hospital fabrics and plastic. J. Clin. Microbiol. 2000; 38:724–726.
19. Weigel LM, Clewell DB, Gill SR, Clark NC, McDougal LK, Flannagan SE, Kolonay JF, Shetty J, Killgore GE, Tenover FC. Genetic analysis of a high-level vancomycin-resistant isolate of Staphylococcus aureus. Science. 2003; 302:1569–1571.