Current Clinical and Medical Education

Received 02 Mar 2024 | Revised 06 Mar 2024 | Accepted 25 Mar 2024 | Published Online 15 Apl 2024

Published By: Vision Publisher

CCME 02 (4), 21-28

ISSN 2942-0792

Original Article

Characterization of Bioactive Volatile Metabolites Released from Streptococcus pneumoniae and Evaluation of Antibacterial activity Using Four Medicinal Plants

Mays Hassan Obais Alkhalidi[1], Rabab J.H. Al Hasseny[2], Abbas K. Al-Mansoori[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:- The gram-positive bacterium *Streptococcus pneumoniae* is responsible for a number of serious diseases such as pneumonia, septicemia and meningitis. When it comes to pneumococcal pathophysiology, host nutrients including purines, pyrimidines, amino acids and carbon sources are absolutely crucial. If we want to know how *S. pneumoniae* adapts to the host environment during infection and find new therapeutic targets, we need to investigate its metabolism. The purpose of this study was to investigate the antibacterial effects of the medicinal herbs Equisetum arvense, *Althaea rosea*, Nigella sativa and *Foeniculum vulgare* by analyzing the bioactive volatile compounds generated by Streptococcus pneumoniae.

GC-MS analysis of *Streptococcus pneumoniae* found: ethyl 2-methoxycarbonyloxytetradecanoate, ethyl-N-ethoxycarbonylcabamate, prop-2-enylnonanoate, butane, 1,1-dibutoxy-3-methyl, ethanol, 2,2'-oxybis -, diacetate , hexadecane, butanedioic acid, diethyl ester, 4-methylthio-4-methyl-2-pentnone, 1-phenylethyl hydroperoxide, p-dioxne, methylene. Bioactivity of the ethanol extract of the bacterial product Streptococcus pneumoniae against four microorganisms *Bacillus cereus* (18.01±0.07, 11.90±0.03, and 15.88 ±0.04), *Klebsiella pneumoniae* (15.00±0.04, 10.05±0.02, and 12.07±0.04), *Staphylococcus epidermidis* (17.43±0.06, 14.20±0.04, and 13.34±0.04), *Enterobacter aerogenes* (16.20±0.07, 11.03±0.02, and 14.52±0.03). In Figure 1, 2, 3, and 4, the metabolites of *Streptococcus pneumoniae* demonstrated a noteworthy level of action against Bacillus cereus, (18.01±0.07).

Keywords: *Streptococcus pneumoniae*, Secondary metabolites, Antibacterial, GC/MS.

Corresponding Author: Mays Hassan Obais Alkhalidi†, Department of Genetic Engineering, College of Biotechnology, Al-Qasim Green University, Iraq

Supplementary information The online version of this article (https://doi.org/xx.xxx/xxx.xx) contains supplementary material,which is available to autho-rized users.

Introduction:

Streptococcus pneumoniae is a gram-positive, catalase-negative, facultatively anaerobic organism that grows as a single coccus, as diplococci are often recognized by their lanceolate shape and in chains of varying length. It is also capable of autonomous growth in the absence of oxygen. Anaerobic environments or conditions with 5% carbon dioxide favor increased growth. Colonies are initially grown on blood agar, but eventually become flattened and have a depression in the center [1]. It is possible that autolysis accounts for the inability of the organism to grow in subculture, although a positive Gramme stain reaction was observed in the turbid brown broth culture. α-hemolytic colonies are characterized by darkening of the medium, which is either green or brown in color. This coloration is the result of partial destruction of red blood cells. An increasing number of *Streptococcus pneumoniae* strains show resistance to the most commonly used drugs in the clinical setting, including β-lactam antibiotics and macrolides [2]. To ensure that therapies are effective in the future, there is an urgent need for new antimicrobial drugs that are effective against *S. pneumoniae* and resistant strains of the bacteria. The imminent replacement of vaccines with disease-causing serotypes that are not vaccinated presents a substantial threat due to inadequate vaccine coverage. Currently available against invasive infections caused by vaccine-type strains is a 13-valent conjugate vaccine that is relatively efficacious [3]. In order to attain the intended outcomes, pneumococcal vaccination is designed to generate efficacious antibodies, mucosal immunity, and immunological memory. Consequently, the identification of novel vaccine candidates that can offer protection against a broader spectrum of pneumococcal strains and facilitate the development of a serotype-independent vaccine has emerged as one of the current obstacles. The environment exerts a substantial influence on S. pneumoniae, impacting both the bacterial metabolism and pathogenicity [4,5]. Consequently, bacterial adaptation to the particular host compartments they confront is essential. Virulence determinants, including toxins and capsular polysaccharides, have been observed to be negatively regulated in nutrient-rich environments with regard to *Staphylococcus aureus* [6].

On the other hand, adhesins reveal a beneficial regulatory role in these settings. Research into compounds with bactericidal or bacteriostatic properties against diseases in animals and humans is also crucial for the advancement of new antibiotic treatments and disinfectants. A lot of focus in recent years has been on finding naturally occurring molecules from various sources that may have antibacterial properties, in addition to developing new chemical compounds. Bacterial antagonism, or interaction, is usually the starting point for the search for effective antibiotics for human diseases. This hostility is materialised in it, and it generates and releases compounds that hinder or stop the development of other species. When competing for resources in the wild, a creature with a microbe-secreted chemical that stunts its own growth has a natural ecological advantage. The majority of antibiotics used in medicine today are either produced by or derived from microbes. By utilising distinct metabolic adaption mechanisms in reaction to demanding conditions within host cells, S. pneumoniae is able to thrive in adverse host settings and outcompete other bacteria. Another challenge that pneumococci have to overcome is mineral restriction resistance [7]. In addition, nutrient limitations make matters worse. In spite of this, *S. pneumoniae's* ability to change its metabolic activities in an environment with low ion concentration and hostile hosts is crucial for the organism's survival and dissemination.

Another reason medicinal plant extracts are so efficient at inhibiting bacterial growth is the synergistic impact that happens between the active compounds in these extracts [8]. The synergistic effect is due to several effects. Among these effects are the following: the development of multi-target mechanisms; the presence of compounds with the ability to suppress bacterial resistance mechanisms; pharmacokinetic or physicochemical effects that enhance bioavailability, solubility, and resorption rate; and the neutralisation or reduction of toxicity and side effects. Examining the antibacterial activity of medicinal plants such *Equisetum arvense*, *Althaea rosea*, *Nigella sativa*, and *Foeniculum vulgare*, as well as the bioactive volatile chemicals emitted by *Streptococcus pneumoniae*, was the goal of this study.

Materials and Methods

Optimal environmental conditions for growth and identification of metabolites

Streptococcus pneumoniae is a gram-positive anaerobic bacterium that is cultured at temperatures between 35 and 37

degrees Celsius with 5% carbon dioxide. In the presence of a source of catalase, such as red blood cells, Streptococcus pneumoniae is able to generate hydrogen peroxide (H2O2) via a flavoenzyme system. This allows the bacteria to multiply more efficiently. In addition to being alpha-hemolytic, the bacterium produces a green zone of hemolysis when cultured on blood agar, giving the colonies a tiny gray appearance [8, 9]. When colonies are cultured for more than twenty-four hours, the colony nuclei deteriorate over time. Evaporation of metabolites was carried out using a rotary evaporator at a temperature of 45 degrees Celsius. Metabolites were extracted from the liquid culture.

Performing spectral analysis of naturally occurring bioactive chemical components of *Streptococcus pneumoniae* using gas chromatography and mass spectrometry (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 previous experiment, 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.

This study evaluated the efficacy of secondary metabolite compounds as antibacterial agents against four different types of pathogenic bacteria.

A sterile cork borer was used to cut five millimeter diameter holes in the agar. After well preparation was complete, 25 microliters of sample solutions containing metabolites generated by *Streptococcus pneumoniae* were added to the wells. The standard antibiotics GN-Gentamicin and VC-Vancomycin along with the tested pathogens Bacillus cereus, *Klebsiella pneumoniae*, *Staphylococcus epidermidis* and *Enterobacter aerogenes* were collected using swabs. Furthermore, Muller Hinton agar plates were inoculated with these pathogens [10].

Statistical analysis

A number of statistical procedures were employed during the data analysis process that was carried out on the information that had been extracted from an SPSS (Version 11.6) database. The steps involved in these processes included calculating the average and doing an ANOVA.

Results and Discussion

The identified chemicals were represented by 10 peaks in the GC-MS chromatogram. Said compounds are methyl-2-methoxycarbonyloxytetradecanoate, ethyl-N-ethoxycarbonylcabamate, prop-2-enylnonanoate, butane, 1,1-dibutoxy-3-methyl, ethanol, 2,2'-oxybis-, diacetate, hexadecane, butanedioic acid, diethyl ester, 4-methylthio-4-methyl-2-pentnone, 1-phenylethyl hydroperoxide, p-dioxne, methylene. Bioactivity of the ethanolic extract of the bacterial product Streptococcus pneumoniae against four microorganisms Bacillus cereus, Klebsiella pneumoniae, Staphylococcus epidermidis and Enterobacter aerogenes and the standard antibiotics GN-Gentamicin and VC-Vancomycin: *Bacillus cereus* (18.01±0.07, 11.90±0.03, and 15.88 ±0.04), *Klebsiella pneumoniae* (15.00±0.04, 10.05±0.02, and 12.07±0.04), *Staphylococcus epidermidis* (17.43±0.06, 14.20±0.04, and 13.34±0.04), *Enterobacter aerogenes* (16.20±0.07, 11.03±0.02, and 14.52±0.03). In Figure 1, 2, 3, and 4, the metabolites of *Streptococcus pneumoniae* demonstrated a noteworthy level of action against Bacillus cereus, (18.01±0.07).

In vitro antimicrobial activity of plant extracts on *Streptococcus pneumoniae*: The inhibition zone (mm) of bioactive components of Equisetum arvense, Althaea rosea, Nigella sativa, and Foeniculum vulgare methanolic, ethyl acetate, and ethanolic extract against Streptococcus pneumoniae was recorded as follows: 13.08±0.21, 09.83±0.17 and 09.21±0.17 mm respectively for *Equisetum arvense.* While recorded 17.79±0.31, 14.46±0.28 and 15.47±0.28 mm respectively for *Althaea rosea.* In vitro antimicrobial activity of plant extracts on Streptococcus pneumoniae recorded 13.05±0.21, 15.60±0.28 and 18.52±0.31 mm respectively for *Nigella sativa* and 17.52±0.31, 15.55±0.28 and 13.27±0.21 mm respectively for *Foeniculum vulgare* (Figure 5, 6, 7 and 8). *Nigella sativa* (Crude) 18.52±0.31 mm

was very highly active against *Streptococcus pneumoniae*.

Over the past ten years, a number of substantial clinical trials have been conducted to demonstrate the effectiveness of breath analysis in identifying the presence of bacteria in the respiratory tract or bloodstream of critically ill patients. Mass spectrometry methods are often used for this purpose because of their ability to identify an unknown substance. This approach is based on the assumption that the qualitative and quantitative determination of bacterial volatile metabolites in exhaled breath gases of patients [11] with suspected infection is necessary.

Extracts derived from medicinal plants have been documented to possess a range of biological properties, such as antioxidant, antibacterial, and anti-inflammatory effects [12]. Antimicrobial compounds derived from botanical sources possess the capacity to impede the proliferation of bacteria, fungi, viruses, and protozoa via mechanisms distinct from those of presently employed antimicrobials. Furthermore, significant clinical applications for these compounds may include the treatment of resistant microbial strains.

Furthermore, some of these compounds, despite not being effective as antibiotics by themselves, may be useful in overcoming antibiotic resistance in bacteria when paired with antibiotics. Substances that are chemically complicated have significant therapeutic potential because compared to manufactured drugs, they have fewer side effects and are also less likely to acquire resistance [13]. Bacteria can acquire resistance to treatment provided by medicinal plants if only one active ingredient is included and that chemical targets a specific target. This is a condition that is comparable to that of antibiotics.

Since there is a dearth of literature on the specific microorganisms responsible for plant resistance, additional research into the mechanisms behind this phenomenon is urgently required [14]. Bacterial extract research is intricate and multi-faceted, touching on many societal domains like as medicine, agriculture, commerce, and academia. As part of the process, we are looking for and investigating chemicals and biological processes that could provide solutions to numerous issues and offer up new opportunities [15, 16]. Screening bacterial extracts for novel antibiotic-compounds and (AMPs) with therapeutic is a frequent activity due to the number of chemicals generated by bacteria.

The discovery and isolation of potential modifiers produced by medicinal plants has dominated studies investigating the topic of plant extracts and antibiotic interactions. On the other hand, it is quite possible that such pairings may lead to adversarial interactions that have been considered irrelevant by a large number of studies and subsequently missed. On the other hand, it is of utmost importance to investigate the synergy and antagonism that exists between plant extracts and antimicrobial treatment [17]. Some common examples are as follows: In studies investigating the synergistic effects of terpenes and penicillin against MRSA and Escherichia coli, it was found that carvone and penicillin had a synergistic effect, while thymol and penicillin had an antagonistic effect [18, 19]. Several essential oils have been found to have synergistic interactions with ampicillin, cephalothin, and tetracycline, while gentamicin has predominantly antagonistic interactions [20, 21], [23]. The outcomes exhibited either synergism or antagonism when the four essential oils were combined with ciprofloxacin in the case of Staph. aureus and K. pneumoniae, or with amphotericin B in the case of *Candida albicans* strains, contingent upon the specific type of essential oil [24, 25].

Table 1. Bioactive volatile metabolites released from *Streptococcus pneumoniae*

Compounds	Formula	Molecular weight
Methyl 2-methoxycarbonyloxytetradecanoate	$C_{17}H_{32}O_5$	316.4 g/mol
Ethyl N-ethoxycarbonylcarbamate	$C_6H_{11}NO_4$	161.16 g/mol
Prop-2-enyl nonanoate	$C_{12}H_{22}O_2$	198.30 g/mol
Butane, 1,1-dibutoxy-3-methyl	$C_{13}H_{28}O_2$	216.36 g/mol
Ethanol,2,2′-oxybis-, diacetate	$C_8H_{14}O_5$	190.19 g/mol
Hexadecane	$C_{16}H_{34}O_2$	258.44 g/mol
Butanedioic acid, diethyl ester	$C_8H_{14}O_4$	174.19 g/mol
4-Methylthio-4-methyl-2-pentanone	$C_7H_{14}OS$	146.25 g/mol
1-Phenylethyl hydroperoxide	$C_8H_{10}O_2$	138.16 g/mol
p-Dioxane, methylene	$C_5H_8O_2$	100.12 g/mol

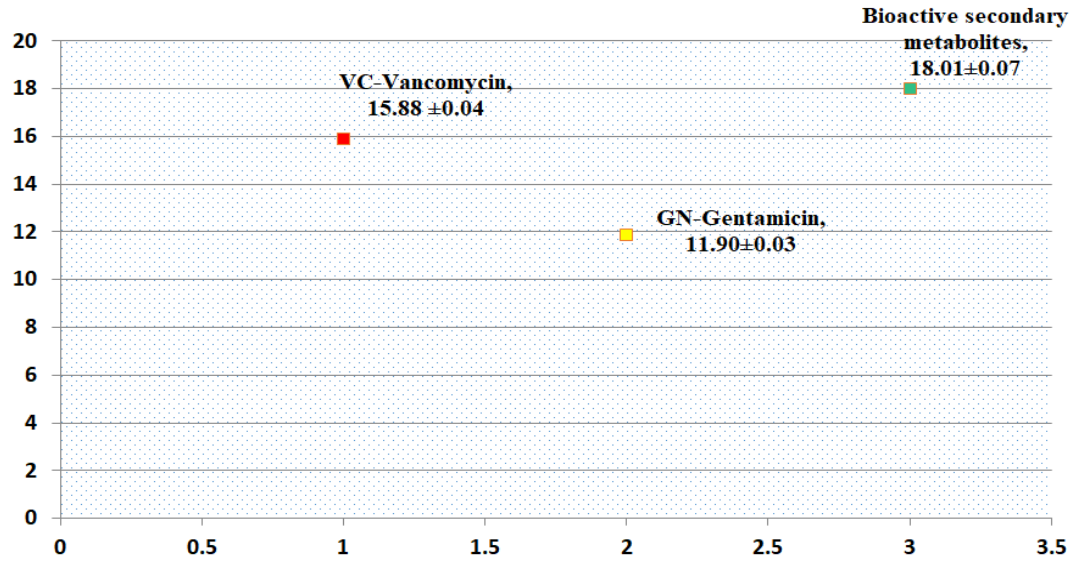

Figure 1. Bioactivity of the ethanolic extract of bioactive secondary metabolites of *Streptococcus pneumoniae* and standard antibiotics GN-Gentamicin and VC-Vancomycin against *Bacillus cereus*

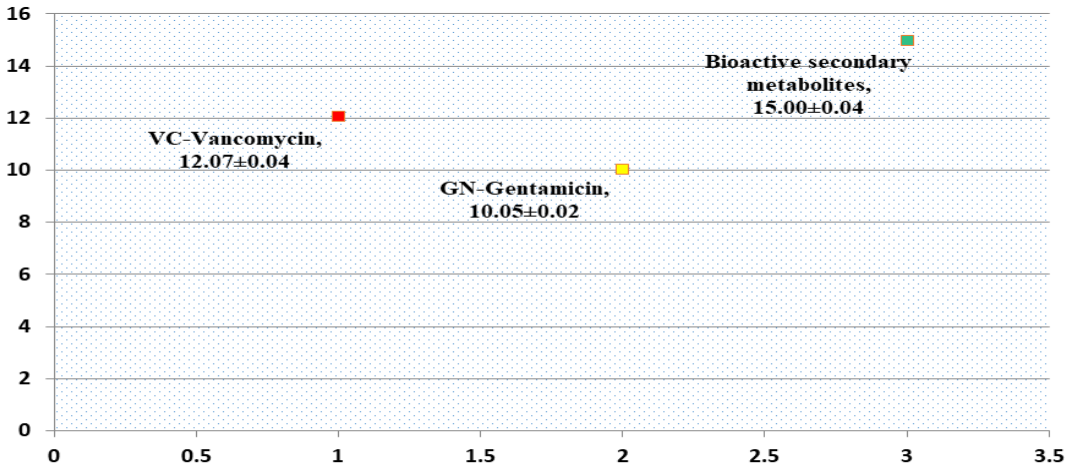

Figure 2. Bioactivity of the ethanolic extract of bioactive secondary metabolites of *Streptococcus pneumoniae* and standard antibiotics GN-Gentamicin and VC-Vancomycin against *Klebsiella pneumoniae*

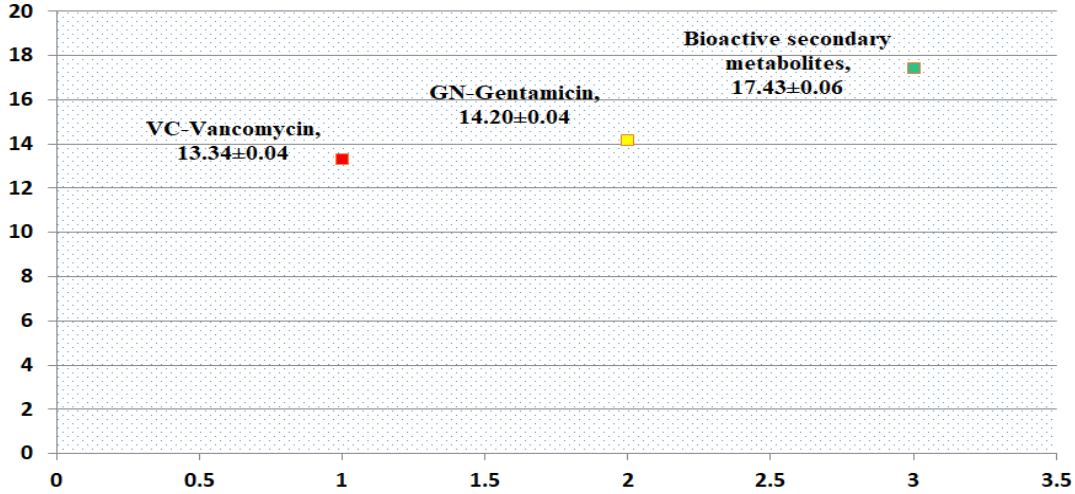

Figure 3. Bioactivity of the ethanolic extract of bioactive secondary metabolites of *Streptococcus pneumoniae* and standard antibiotics GN-Gentamicin and VC-Vancomycin against *Staphylococcus epidermidis*

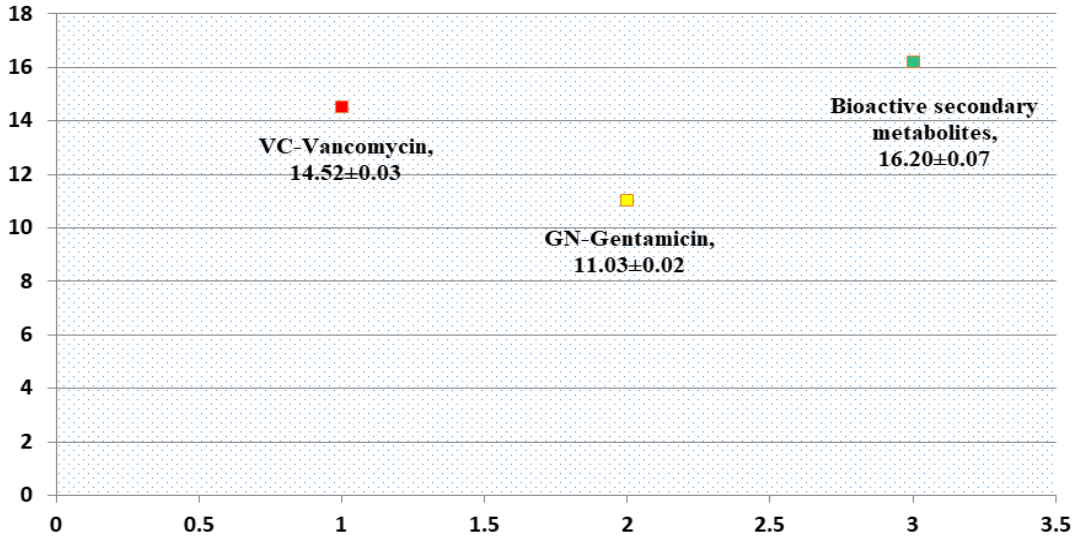

Figure 4. Bioactivity of the ethanolic extract of bioactive secondary metabolites of *Streptococcus pneumoniae* and standard antibiotics GN-Gentamicin and VC-Vancomycin against *Enterobacter aerogenes*

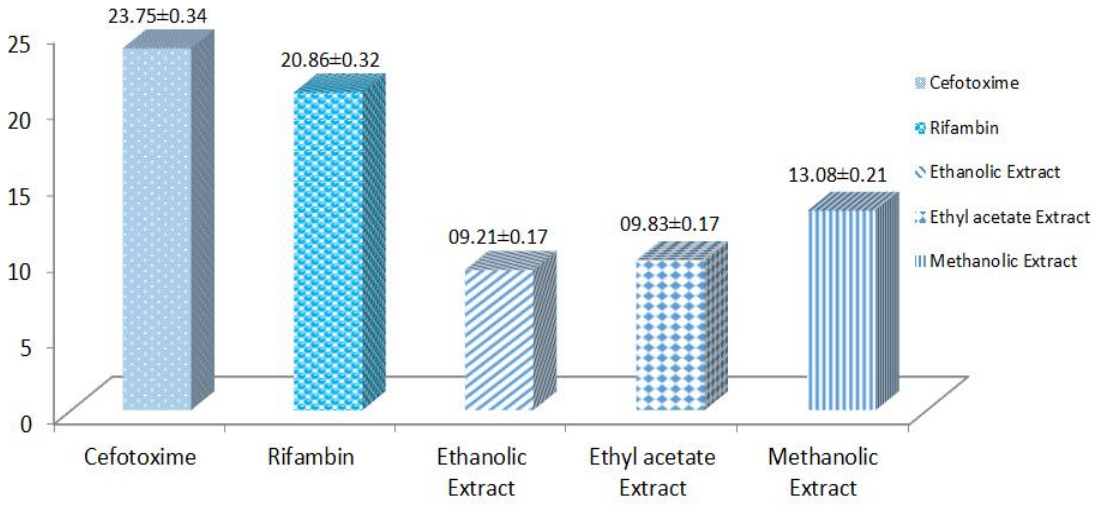

Figure 5. Inhibition Zone (mm) of bioactive compounds of *Equisetum arvense* and conventional antibiotics Rifambin and Cefotoxime against *Streptococcus pneumoniae*.

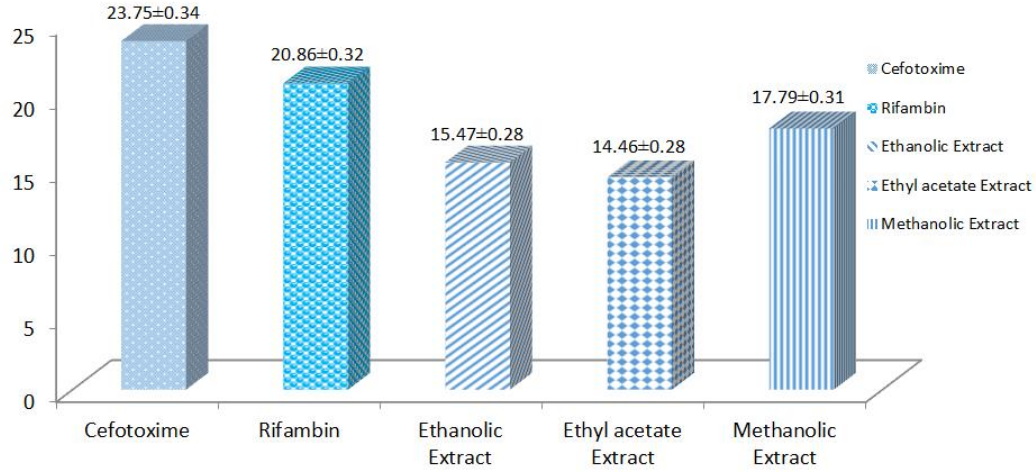

Figure 6. Inhibition Zone (mm) of bioactive compounds of *Althaea rosea* and conventional antibiotics Rifambin and Cefotoxime against *Streptococcus pneumoniae*.

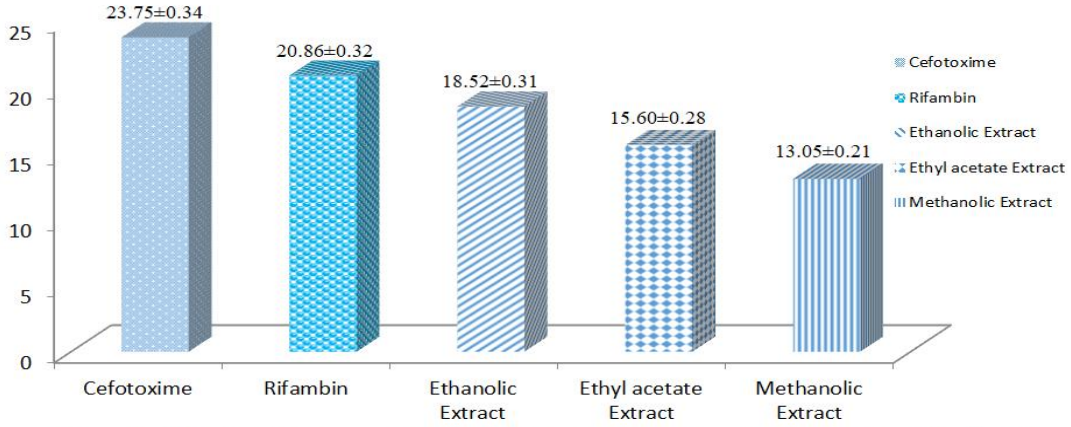

Figure 7. Inhibition Zone (mm) of bioactive compounds of *Nigella sativa* and conventional antibiotics Rifambin and Cefotoxime against *Streptococcus pneumoniae*.

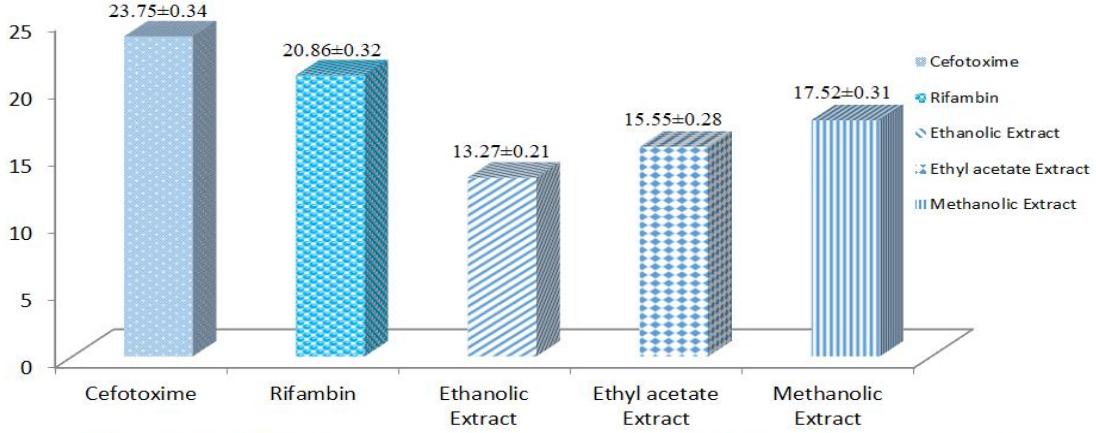

Figure 8. Inhibition Zone (mm) of bioactive compounds of *Foeniculum vulgare* and conventional antibiotics Rifambin and Cefotoxime against *Streptococcus pneumoniae*.

Conclusion

Using GC-MS, the chemical components contained in the bacterial species were identified and quantified. Several volatile inhibitory chemicals, including phenolics, esters, and ethers, are believed to be involved in the antimicrobial action process as demonstrated in this study. Metabolites of Streptococcus pneumoniae showed significant activity against *Bacillus cereus* in research with a specific activity level of 18.01±0.07. *Nigella sativa* (Crude) showed an impressive rate of efficacy against *Streptococcus pneumoniae* with a diameter of 18.52±0.31 mm. The chemical composition of the volatile molecules varies between the tested samples, raising the possibility that these differences affect antibiotic efficacy and antibacterial activity. Some strains of *Streptococcus pneumoniae* are able to produce a wide variety of secondary metabolites that have antibacterial effects. Our research also shows that these strains can make these other compounds.

References

1. Leonard, A et al. Metabolic inventory of Streptococcus pneumoniae growing in a chemical defined environment. Int J Med Microbiol. 2018.
2. Härtel T, et al. Impact of glutamine transporters on pneumococcal fitness under infection-related conditions. Infect Immun. 2011;79:44–58.
3. Hoskins J, et al. Genome of the bacterium Streptococcus pneumoniae strain R6. J Bacteriol. 2001;183:5709–17.
4. Lynch JP, Zhanel GG. Streptococcus pneumoniae. Epidemiology, risk factors, and strategies for prevention. Semin Respir Crit Care Med. 2009;30:189–209.

5. Waterer GW, Somes GW, Wunderink RG. Monotherapy may be suboptimal for severe bacteremic pneumococcal pneumonia. Arch Intern Med. 2001;161:1837–42.
6. Weiss K, et al. Clinical characteristics at initial presentation and impact of dual therapy on the outcome of bacteremic Streptococcus pneumoniae pneumonia in adults. Can Respir J. 2004;11:589–93.
7. Martínez JA, et al. Addition of a macrolide to a beta-lactam-based empirical antibiotic regimen is associated with lower in-hospital mortality for patients with bacteremic pneumococcal pneumonia. Clin Infect Dis. 2003;36:389–95.
8. Gonçalves VM, Zangirolami TC, Giordano RLC, Raw I, Tanizak MM, Giordano RC. Optimization of medium and cultivation conditions for capsular polysaccharide production by Streptococcus pneumoniae serotype 23F. Appl Microbiol Biotechnol. 2002;59:713–717.
9. Balachandran P, Hollingshead SK, Paton JC, Briles DE. The autolytic enzyme LytA of Streptococcus pneumoniae is not responsible for releasing pneumolysin. J Bacteriol. 2001;183:3108–3116.
10. Yang, S.C.; Lin, C.H.; Sung, C.T.; Fang, J.Y. Antibacterial Activities of Bacteriocins: Application in Foods and Pharmaceuticals. Front. Microbiol. 2014, 5, 241.
11. Magashi, A.M.; Bukar, A.; Omola, E.M.; Halima, B.A.; Hadiza, M.S. Bacteriocin and its application—A review. Int. J. Adv. Acad. Res. Sci. Technol. Eng. 2019, 5, 242–256.
12. Wiegers, C.; van de Burgwal, L.H.M.; Larsen, O.F.A. Probiotics for the Management of Infectious Diseases: Reviewing the State of the Art. Front. Microbiol. 2022, 13, 877142.
13. Ramirez-Olea, H.; Reyes-Ballesteros, B.; Chavez-Santoscoy, R.A. Potential Application of the Probiotic Bacillus licheniformis as an Adjuvant in the Treatment of Diseases in Humans and Animals: A Systematic Review. Front. Microbiol. 2022, 13, 993451.
14. Chrolavicius, S. Effects of pretreatment with clopidogrel and aspirin followed by long-term therapy in patients undergoing percutaneous coronary intervention: The PCI-CURE study. Lancet 2001, 358, 527–533.
15. Shankar, S, Rangarajan R, Sarada D. Evaluation of Antibacterial Activity and Phytochemical Screening of Wrightia Tinctoria L. Pharmacogn. J. 2010, 2, 19–22.
16. Lewis, K.; Ausubel, F.M. Prospects for Plant-Derived Antibacterials. Nat. Biotechnol. 2006, 24, 1504–1507. [CrossRef]
17. Ody, P. The Complete Medicinal Herbal: A Practical Guide to the Healing Properties of Herbs; Skyhorse Publishing Inc.: New York, NY, USA, 2017; pp. 1–271.
18. Ruddaraju, L, Pammi, S, Guntuku, G. A Review on Anti-Bacterials to Combat Resistance: From Ancient Era of Plants and Metals to Present and Future Perspectives of Green Nano Technological Combinations. Asian J. Pharm. Sci. 2020, 15, 42–59.
19. Reker, D, Perna, A, Rodrigues, T. Revealing the Macromolecular Targets of Complex Natural Products. Nat. Chem. 2014, 6, 1072–1078.
20. Almabruk, K, Dinh, L, Philmus B. Self-Resistance of Natural Product Producers: Past, Present, and Future Focusing on Self-Resistant Protein Variants. ACS Chem. Biol. 2018, 13, 1426–1437.
21. Arima, H.; Ashida, H.; Danno, G. Rutin-Enhanced Antibacterial Activities of Flavonoids against Bacillus cereus and Salmonella enteritidis. Biosci. Biotechnol. Biochem. 2002, 66, 1009–1014..
22. Assob, J.C.N.; Kamga, H. Antimicrobial and Toxicological Activities of Five Medicinal Plant Species from Cameroon Traditional Medicine. BMC Complement. Altern. Med. 2011, 11, 70.
23. Radulovi´c, N.S.; Blagojevi´c, P.D.; Stojanovi´c-Radi´c, Z.Z.; Stojanovi´c, N.M. Antimicrobial Plant Metabolites: Structural Diversity and Mechanism of Action. Curr. Med. Chem. 2013, 20, 932–952.
24. Saleem, M, Nazir, M, Ali, M. Antimicrobial Natural Products: An Update on Future Antibiotic Drug Candidates. Nat. Prod. Rep. 2010, 27, 238–254.
25. Vadhana, P.; Singh, B.; Bharadwaj, M.; Singh, S. Emergence of Herbal Antimicrobial Drug Resistance in Clinical Bacterial Isolates. Pharm. Anal. Acta 2015, 6, 1–7.