**ADDIS ABABA UNIVERSITY**

**COLLEGE OF HEALTH SCIENCES**

**SCHOOL OF PHARMACY**

**DEPARTMENT OF PHARMACEUTICAL CHEMISTRY & PHARMACOGNOSY**

# Synthesis, *In vitro* Antimicrobial and *In silico* Studies of Sulfonated Derivatives of Carvacrol

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**June, 2025**

**College of Health Sciences**

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# Synthesis, *In vitro* Antimicrobial and *In silico* Studies of Sulfonated Derivatives of Carvacrol

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**A Thesis Submitted to the Department of Pharmaceutical Chemistry and Pharmacognosy in Partial Fulfilment of the Requirements for the Degree of Master of Science in Medicinal Chemistry**

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This is to certify that the thesis prepared by Ayne Teje, entitled: "**Synthesis, *In vitro* Antimicrobial and *In silico* Studies of Sulfonated Derivatives of Carvacrol**" and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Medicinal Chemistry complies with the regulations of the university and meets the accepted standards concerning originality and quality.

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Chair of the Department

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## List of acronyms and abbreviations

| | |
|---|---|
| $^{13}$C NMR | Carbon thirteen Nuclear Magnetic Resonance |
| $^{1}$H NMR | Proton Nuclear Magnetic Resonance |
| ADMET | Absorption, Distribution. Metabolism. Excretion, Toxicity |
| BBB | Blood Brain Barrier |
| CDC | Center for Disease Control and Prevention |
| CFU | Colony Forming Unit |
| CLSI | Clinical and Laboratory Standards Institute |
| DHPS | Dihydropteroate Synthase |
| DMSO | Dimethylsulfoxide |
| EIDs | Emerging Infectious Diseases |
| LRIs | Lower Respiratory Infections |
| MDR | Multi-Drug Resistance |
| MIC | Minimum Inhibitory Concentration |
| NMR | Nuclear Magnetic Resonance |
| PDP | Protein Data Bank |
| Po/w | Partition coefficient (Octanol/Water) |
| Rf | Retention Factor |
| SDF | Structure-Data Forma |
| SMILES | Simplified Molecular-Input Line-Entry System |
| TLC | Thin layer Chromatography |
| WHO | World Health Organization |
| ZOI | Zone of Inhibition |

## Abstract

**Synthesis, *In vitro* Antimicrobial and *In silico* Studies of Sulfonated Derivatives of Carvacrol**

BY: Ayne Teje

Addis Ababa University, 2025

The emergence of new pathogens and antimicrobial resistance pose significant challenges in treating infectious diseases. These ongoing challenges necessitate the discovery of new drugs. In medicinal chemistry research, carvacrol has become a focus of research as a lead compound due to its multifaceted pharmacological activities particularly its notable antimicrobial activity. Additionally, numerous studies have highlighted the substantial antibacterial potential of sulfonated compounds including sulfonic acids and sulfonate esters. Consequently, this study aimed to synthesize sulfonate derivatives of carvacrol, assess their *in vitro* antimicrobial activity against 26 bacterial and 4 fungal strains and conduct an *in silico* analysis. In this study, we synthesized two sulfonated derivatives of carvacrol, compound **8** (5-isopropyl-2-methylphenyl benzenesulfonate) and compound **9** (5-isopropyl-2-methylphenyl hydrogen sulfate). The synthesis was carried out via $SN_2$ nucleophilic substitution reactions by refluxing carvacrol with sulfonylating agents, either benzenesulfonyl chlorides or chlorosulfonic acid, in the presence of pyridine. The chemical structure of the synthesized compounds was confirmed through $^{1}H$ and $^{13}C$-NMR spectroscopy. Both compounds demonstrated a broad range of antibacterial activity against the tested bacterial strains. Specifically, compound **8** exhibited the highest activity against *Shigella sonnei* 1 and *Shigella boydii* D13629, with a minimum inhibitory concentration (MIC) of 10 µg/mL and a zone of inhibition (ZOI) measuring 16 mm.

Additionally, compound **8** showed strong activity against *Vibrio cholerae* (MIC = 25 µg/mL) and various multidrug-resistant *Escherichia coli* strains (MIC = 50-100 µg/mL). Both compounds exhibited similar antifungal activity against *Candida albicans* ATCC 10231 (MIC = 1000 µg/mL), *Penicillium notatum* ATCC 11625 (MIC = 800 µg/mL), and *Penicillium funiculosum* NCTC 287 (MIC = 800 µg/mL). However, against *Aspergillus niger* ATCC 6275, compound **9** showed enhanced activity with a lower MIC of 400 µg/mL. Given that dihydropteroate synthase (DHPS) is a key antibacterial target, particularly for sulfonamide antibiotics, molecular docking was performed against *E. coli* DHPS (PDB ID: 1AJ2). Both compounds **8** and **9** interacted with active site residues via hydrogen bonding and hydrophobic interactions. Compound **8** showed a binding energy of –7.3 kcal/mol, while compound **9** had –6.4 kcal/mol. Additionally, ADMETlab 3.0 predictions indicated that both compounds possess favorable physicochemical, ADMET, and drug-likeness profiles within recommended ranges. In general, the carvacrol derivatives (compounds **8** and **9**) synthesized in this study demonstrated promising antimicrobial activity, favorable drug-likeness and effective target binding, making them a potential lead compounds for antimicrobial drug development.

**Keywords:** Carvacrol, antimicrobial activity, antimicrobial resistance, antibacterial activity, antifungal activity, sulfonated carvacrol, synthesis, *in vitro*, *in silico* studies

# 1. Introduction

The World Health Organization (WHO) defines that infectious diseases are illnesses caused by harmful invaders known as pathogens, including viruses, bacteria, fungi, and parasites. Infectious diseases can spread in several ways such as spread from person to person, through contaminated objectives, food or water, and via bug bites.

The global burden of infectious diseases is a significant public health concern, causing a substantial number of deaths worldwide. WHO (2024) reported on the top 10 causes of death, lower respiratory infections (LRIs), including pneumonia, remain one of the leading causes of death globally. LRIs were the leading cause of death among the top ten causes of death in low-income countries, responsible for approximately 2.5 million deaths. Despite the global decline, 8 of the top 10 causes of death in 2021 in low-income countries were communicable diseases. In this report, infectious diseases remain leading causes of death in Ethiopia as well.

The emergence of new infections and antimicrobial resistance has been current challenge in treating infectious diseases. According to the 2019 Center for Disease Control and Prevention (CDC) report, more than 2.8 million antimicrobial-resistant infections occur in the U.S. each year, and more than 35,000 people die as a result. The persistent challenges in treating infectious diseases, coupled with the emergence of new pathogens and antimicrobial resistance, underscore the urgent need for ongoing drug discovery efforts (Ohashi et al., 2022).

Numerous investigations have revealed that sulfonated compounds such as sulfonic acids and sulfonate esters possess significant pharmacological effects, particularly remarkable antibacterial properties (Liu et al., 2024; Xie et al., 2022). Therefore, based on the antibacterial activity of sulfonated compounds and the well-documented antimicrobial potential of carvacrol and its derivatives, we synthesized two novel sulfonated derivatives of carvacrol in this study.

## 1.2. Statement of problem

Antimicrobial resistance (AMR) is a critical global health threat, with millions of infections and significant mortality each year. CDC (2019) reported on the antibiotic resistance threats report, bacterial AMR was responsible for 1.27 million deaths globally, with over 2.8 million resistant infections reported annually in the U.S. alone. In Ethiopia, high resistance levels have been observed in both Gram-positive and Gram-negative bacteria. For example, *S. aureus* shows 47% resistance to anti-Staphylococcal penicillin and over 50% of *K. pneumoniae* and *E. coli* are resistant to amoxicillin/clavulanic acid (Berhe et al., 2021).

Beyond existing AMR, the emergence of new infectious diseases has been a growing concern with a notable increase in the number of emerging infectious diseases (EIDs) observed over the decades due to various ecological, environmental, and social factors. The emergence of new pathogens including viruses or bacteria is a constant threat to global public health. These pathogens appeared in a population and spread rapidly (Doorn, 2017). Hence there is a call for new drug discovery to combat these emerging pathogens.

Furthermore, existing antimicrobials often have a limited spectrum of activity. This means they may only be effective against specific types of bacteria or fungi. Narrow-spectrum antibiotics, which are designed to target specific bacterial strains, may not provide adequate coverage for polymicrobial infections (Böttcher & Gersbach, 2022), In many clinical situations, patients are required to take multiple drugs to cover all these disease-causing microbes. This polypharmacy can lead to cumulative adverse effects, paradoxically worsening their health. Additionally, patients taking multiple drugs often struggle with adherence. The effectiveness of narrow-spectrum antibiotics depends on accurate diagnostics to identify the specific pathogen. Therefore, there is a need for the discovery and market introduction of broad-spectrum drugs.

To address these urgent challenges, there is a critical need to develop new and broad-spectrum antimicrobial agents. This study may propose the synthesis of sulfonated derivatives of carvacrol as potential new and broad therapeutic agents with improved efficacy against resistant and emerging pathogens.

## 1.3. Objectives

### 1.3.1. General objective

To synthesize sulfonated derivatives of carvacrol, evaluate their *in vitro* antimicrobial activity, and perform *in silico* studies to support their potential mechanism of action.

### 1.3.2. Specific objectives

- To synthesize sulfonated derivatives of carvacrol (compound **8** and **9**) via nucleophilic substitution reactions.
- To purify the synthesized carvacrol derivatives from reaction mixture using crystallization.
- To characterize the chemical structure of the synthesized sulfonated derivatives of carvacrol using $^{1}H$ and $^{13}C$-NMR spectroscopy.
- To evaluate *in vitro* antibacterial activity of sulfonated derivatives of carvacrol on 26 bacterial strains
- To evaluate *in vitro* antifungal activity of sulfonated derivatives of carvacrol on selected fungal species.
- To conduct *in silco* study of sulfonated derivatives of carvacrol

## 1.4. Significance of the study

Antimicrobial resistance (AMR) presents a serious global health challenge, necessitating the development of innovative therapeutic agents. This study directly addresses this urgent need for new antimicrobials by synthesizing two novel sulfonated derivatives of carvacrol, thereby expanding the chemical space of potential antibacterial and antifungal activities These compounds may offer broad-spectrum activity, providing effective action against both resistant and emerging pathogens for which current treatments are ineffective.

Furthermore, these compounds could significantly reduce the need for polypharmacy in treating polymicrobial infections, minimizing adverse effects and improving patient adherence. Their broad-spectrum nature may also lessen the reliance on extensive diagnostic procedures often required for narrow-spectrum antibiotics, ultimately lowering healthcare costs and improving treatment accessibility.

Our research combines synthetic chemistry with advanced computational methods, including molecular docking studies that revealed key interactions between the compounds and the DHPS enzyme, providing a lead compounds for designing more potent inhibitors. The in vitro antimicrobial activity, along with in silico analyses such as molecular docking and ADMET profiling, offer valuable insights into the efficacy, pharmacokinetics, and drug-likeness of these synthesized compounds. Their enhanced activity and predicted drug-likeness position them as promising lead compounds for preclinical development, aligning directly with global AMR mitigation strategies and World Health Organization (WHO) guidelines. Future work will focus on synthesizing a wider array of derivatives, detailing their mechanisms of action, and conducting comprehensive in vivo evaluations and pharmacokinetic/pharmacodynamic (PK/PD) profiling against diverse bacterial and fungal strains.

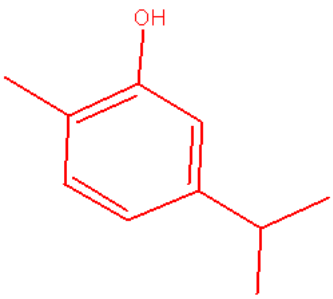
# 2. Literature review

## 2.1. Chemistry of carvacrol

Carvacrol (**1**) is a monoterpenoid phenol primarily extracted from plants such as oregano and thyme (Hao et al., 2021). Carvacrol (**1**), chemically known as 5-isopropyl-2-methylphenol is a thick, pale-yellow liquid monoterpenic phenol with a characteristic pungent, spicy odor. It has molecular formula of $C_{10}H_{14}O$, molecular weight 150.22, boiling point 238°C, and melting point 3°C. It is insoluble in water but very soluble in ethanol and diethyl ether (Gandova et al., 2023).

## 2.2. Pharmacological activities of carvacrol and Its derivatives

Carvacrol exhibits a wide range of pharmacological activities, including antimicrobial, anticancer, anti-inflammatory, antioxidant and immunomodulatory activities. Notably, it is particularly recognized for its antimicrobial and anti-inflammatory effects. The compound's capacity to modulate various biological pathways makes it a promising candidate for further research (Imran et al., 2022; Wanda, 2023).




**Carvacrol (1)**

Figure 1: The structure of carvacrol

### 2.2.1. Antimicrobial Activity of carvacrol

Numerous research findings have confirmed that carvacrol possesses significant antibacterial and antifungal activity.

It has attracted the attention of researchers due to its potential as an antimicrobial agent. Consequently, extensive studies have been conducted to evaluate its activities.

Carvacrol exhibits bactericidal activity against *Streptococcus pyogenes*, with a MIC of 125 µg/m. It has shown killing effect on this pathogen (Wijesundara et al., 2021). Another study has shown that carvacrol is effective against various bacterial strains, including *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella Typhimurium* with MIC of 400, 200, 100 µg/m respectively. It also exhibited significant inhibitory effects on *S. Typhimurium* biofilms, substantially reducing bacterial counts at higher concentrations (Trevisan et al., 2018).

Carvacrol is renowned for its broad spectrum of antibacterial activity, with numerous studies confirming its effectiveness as a promising antibacterial agent against both Gram-positive and Gram-negative bacteria. These studies showed that this compound has strong antibacterial activities against a range of pathogens, including *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Streptococcus pneumoniae*, *Proteus mirabilis*, Serratia spp., and Enterobacter species (Imran et al., 2022; Kachur & Suntres, 2019; Nostro et al., 2007). In another *in vivo* and *in vitro* study, carvacrol has shown significant antimicrobial activity against strains of *K. pneumonia* (Hellen et al., 2021). These research findings make carvacrol an interesting lead compound for further study aimed at structural modification to enhance its activity and pharmacokinetic properties.

Carvacrol has demonstrated both antifungal and antibacterial activities in various studies. It has shown significant antibacterial effects against several bacterial strains, particularly methicillin-resistant *Staphylococcus aureus* (MRSA) and other nosocomial pathogens. Additionally, it has exhibited potent antifungal effects against *Candida albicans* (Antonia & Teresa, 2012; Wanda, 2023).

Additional studies showed that carvacrol exhibits potent antifungal activity, (Lima et al., 2013) reported that carvacrol exhibits potent antifungal activity with a MIC of 24.96µg/ml against C. albicans strains. This MIC was notably lower than that of nystatin, a conventional antifungal drug. A separate study assessed carvacrol's effectiveness against 111 Candida strains and found it exhibited strong fungicidal effects across all tested strains. The results of this study indicated that carvacrol is more effective than fluconazole (Ahmad et al., 2011). In another study, carvacrol exhibited antifungal activity against strains of *Cryptococcus neoformans*. In this study the MIC was reported to be from 25 to 81 µg/mL (Nóbrega et al., 2016).

The findings from these studies underscore the potential of carvacrol as lead compound for development of antimicrobial agents.

### 2.2.2. Antimicrobial Activity of Carvacrol Derivatives

Given the promising antibacterial and antifungal activities of carvacrol, medicinal chemists have synthesized various derivatives using it as a lead compound. Current research focuses on modifying carvacrol to enhance its biological activity, improve membrane permeability, increase water solubility and improve volatility. This involves functionalizing its hydroxyl groups through ether or ester linkages and altering its benzene ring to create a more diverse chemical profile. Strategic modifications at the C-4, C-6 and hydroxyl positions identified as pharmacological hotspots, offer significant potential for synthesis of various carvacrol derivatives. As a result, several carvacrol derivatives have been identified.

Lazarevic et al., (2022) synthesized 25 carvacrol ester derivatives (compound **2-26**) and assessed their antimicrobial activity against various bacterial and fungal strains. While the esters demonstrated limited antibacterial activity, they exhibited significant antifungal activity against *Aspergillus niger* and *Candida albicans*, comparable to that of the parent compound carvacrol.

The study concluded that carvacrol ester derivatives generally possess weaker antibacterial activity than carvacrol, the researchers concluded that this is may be due to the absence of a free hydroxyl group. However, these derivatives maintained considerable antifungal activity, suggesting that lipophilicity may play a crucial role in their antifungal activity.

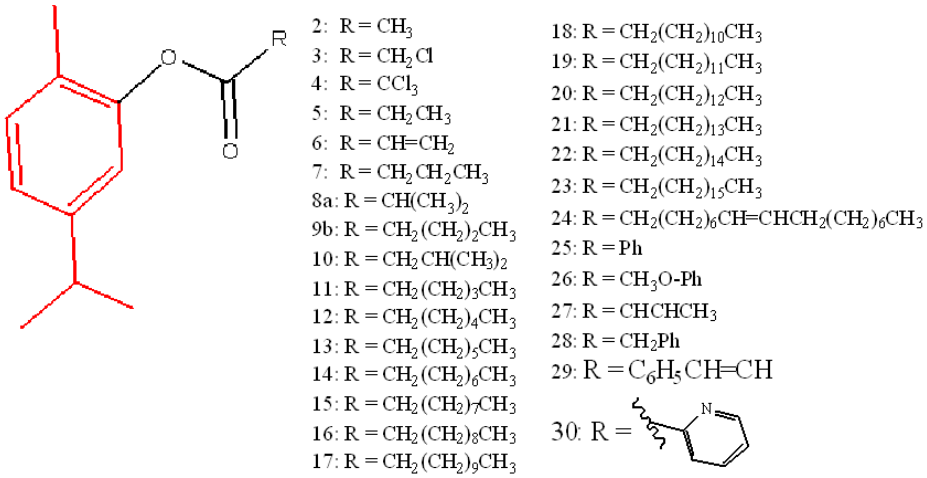
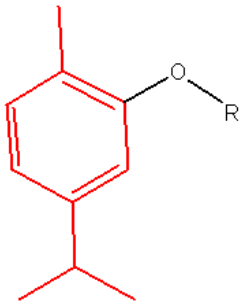



Figure 2: Carvacrol ester derivatives

In another study on the synthesis and testing antibacterial activity of ester derivatives of carvacrol, researchers synthesized 7 ester derivatives of carvacrol (compound **2, 5, 8a, 10, 25, 27, 28**) and evaluated their activities against both Gram-positive and Gram-negative bacteria. The results indicated that the ester derivatives of carvacrol exhibited weaker antibacterial activity compared to carvacrol itself. The researchers concluded that carvacrol esters are likely to demonstrate reduced antibacterial activity due to the absence of a free hydroxyl group (Myangar & Patel, 2014).

Wang et al., (2019) investigated the antifungal activity of carvacrol esters with heteroaromatic substituents. The results showed that some carvacrol esters showed good to excellent antifungal activity, and compound **30** exhibited a broad antifungal spectrum.

In another study chemists synthesized a series of ethers (compound **31–36**) and esters (compound **28, 29**) featuring both saturated and unsaturated aliphatic side chains, as well as aromatic units. These compounds were tested for their antimicrobial activity against Gram-positive and Gram-negative bacterial strains, revealing a superior antimicrobial response compared to carvacrol. Among the ethers, the carboxymethyl derivative (compound **36**) stood out for its activity, while the phenyl acetate (compound **28**) derivative in the esters produced the most active compound (Dewang et al., 2003).

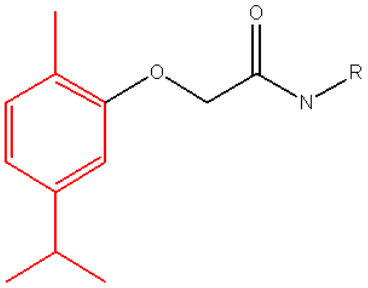



31: R=$CH_3$
32: R=$CH_2CH_2CH_3$
33: R=$CH_2CH_2CH_2CH_3$
34: R=$CH{=}CHCH_2$
35: R=$C_6H_5CH_2$
36: R=$HOOCCH_2$
37: R= 3-methylbenzyl
38: R= 4-methylsulfonylbenzyl

Figure 3: Ether derivatives of carvacrol

In a recent study by (Sisto et al., 2020) a series of carvacrol ether derivatives were synthesized and tested against multiple resistant strains of Helicobacter pylori. The results demonstrated significant antimicrobial activity, with compound **37** exhibiting the lowest MIC of 2µg/mL, and compound **38** showed an MIC of 32µg/mL.

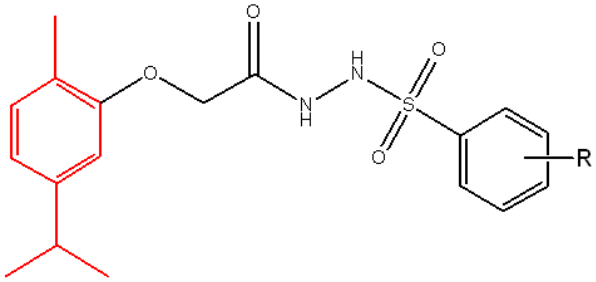
Linking carvacrol with various acetanilides via ether bonds (Patil et al., 2010) synthesized six ether derivatives (compound **39-44**) and these compounds were tested against various strains of bacteria. Among the tested ethers, compound **39** was the only compound that showed no activity against any of the selected bacteria. Compound **41** and **42** were identified as the most active compounds. Notably, the compound **43** displayed comparable antimicrobial activity to that of carvacrol.




39: R= $C_6H_5$
40: R= *p*-$CH_3C_6H_4$
41: R= *m*-$NO_2C_6H_4$
42: R= *m*-$ClC_6H_4$
43: R= *m,p*-$ClC_6H_3$
44: R= $C_{10}H_7$

Figure 4: Acetanilamide ether derivatives of carvacrol

Bagul & Rajput, (2017) synthesized seven new 5-isopropyl-2-methylphenolhydrazide-based sulfonamide derivatives (compound **44-50**) at OH position of carvacrol. The newly synthesized derivatives were screened for their antimicrobial activities against three fungal (*Aspergillus niger*, *A. flavus* and *A. fumigatus*) and three bacterial (*Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*) species, of which compounds **46** and **47** were found to have good antifungal activity and compounds **47** and **50** exhibited good antibacterial activities.




44: R= 2-F
45: R= 4-F
46: R= 2-Cl
47: R= 4-Cl
48: R= 2-Br
49: R= 4-Br
50: R= 3-trifluoromethyl

Figure 5: Hydrazide-based sulfonamide derivatives of carvacrol

In another study (Oliveira et al., 2016) synthesized nine new sulfonamide derivatives of carvacrol at 4-position and evaluated their antibacterial activity against resistant strains of *S. aureus*. Among the synthesized compounds, three compounds (compound **51, 52, 53**) showed exceptional antibacterial activity against resistant strains of *S. aureus*, with MIC values ranging from 3.9 - 62.50 ppm. The derivative from 4-methylaniline (compound **51**) exhibited the best performance across all tested strains with MIC from 3.9 - 15.62 ppm.

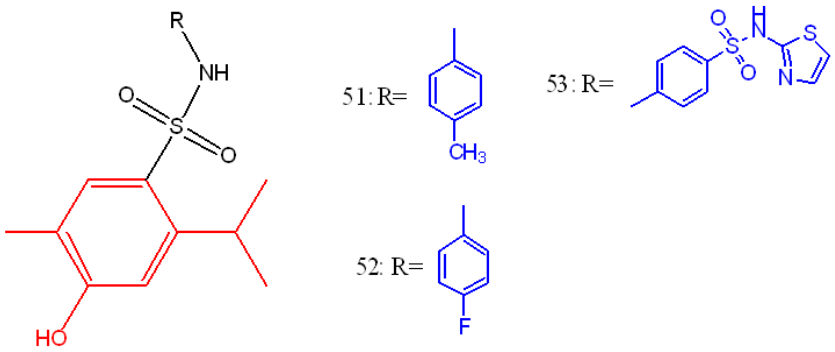



Figure 6: Sulfonamide derivatives of carvacrol at 4-position

Carvacrol's effectiveness is limited by its low water solubility, volatility, and strong aroma. To address these challenges, researchers synthesized sulfonation derivatives of carvacrol, aiming to preserve its antimicrobial activities while improving water solubility and reducing volatility. The results indicate that the sulfonation derivatives at the 4-position, specifically compounds **54** and **55** demonstrate significant antibacterial activity surpassing that of the parent molecule, along with improved water solubility (Bassanetti et al., 2017).

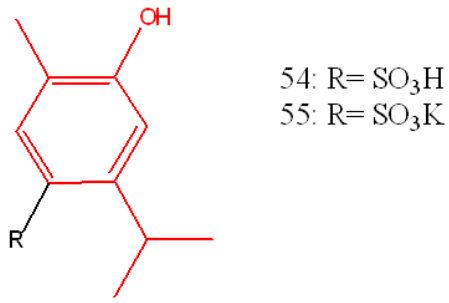



Figure 7: Sulfonation derivatives of carvacrol at 4-position

Even though medicinal chemists have successfully synthesized various carvacrol derivatives to improve carvacrol limitations, including poor water solubility, volatility, and strong aroma, and to enhance its potency, no carvacrol derivatives have currently entered clinical trials or been approved as drugs.

## 2.3. Potential mechanism of action of Carvacrol

### 2.3.1. Cell membrane disruption

Helander et al., (1998) investigated the antibacterial action of carvacrol and thymol on Gram-negative bacteria specifically E. *coli* and *S. typhimurium*. Their findings indicated that carvacrol and thymol exhibit potent antimicrobial activity by disrupting the outer membrane of bacteria, leading to the release of membrane-associated materials which in turn leads to disruption of energy balance. Carvacrol and thymol significantly decreased the intracellular ATP levels while increasing extracellular ATP, indicating a disruptive effect on the cytoplasmic membrane. They also highlight the diversity in their mechanisms of action, ranging from membrane disruption to potential interactions with intracellular targets.

Researches consistently show that carvacrol and thymol exert their antibacterial effects primarily by damaging the integrity of bacterial cell membranes. Their hydrophobic nature enables them to penetrate and integrate into the cell membrane, disrupting its structure and function. As a result, the bacterial cell membrane's normal function is compromised, becoming abnormally permeable. This increased permeability leads to the loss of ATP and other cellular components (Walsh et al., 2003; Gill and Holley, 2006a). Carvacrol is a hydrophobic compound with a log Po/w value of 3.64. Compounds with a log Po/w value higher than 3 tend to partition deeply into cell membranes and they accumulate within the fatty acid chains of the membrane. This accumulation can lead to conformational changes and structural disruption of the lipid bilayer of cell membrane. This conformational and structural changes in the membrane lipid bilayer causes increased permeability and leakage of cytoplasmic contents such as nucleic acids, enzymes, ATP, ions from bacterial cells and ultimately the death of bacterial cell (Ultee et al., 2002; Ben Arfa et al., 2006; Khan et al., 2017).

### 2.3.2. In direct inhibition of membrane bound ATPases

In addition to disrupting cell membranes, carvacrol exhibits antibacterial activity by indirectly inhibiting the ATP synthase enzyme (F-type ATPase). carvacrol acts as a proton exchanger, which disrupts the proton gradient across the cytoplasmic membrane, which leads to the collapse of the proton motive force (PMF), which is essential for ATP synthase to produce ATP.

Carvacrol has hydroxyl group and a delocalized electron system (benzene ring), characterized by double bonds. These structural features enable carvacrol to function as a proton exchanger. The presence of double bonds in carvacrol creates a delocalized electron system. This system allows carvacrol to stabilize the negative charge that results from proton exchange, making it easier for carvacrol to accept and donate protons. This proton exchange disrupts the proton gradient across the cytoplasmic membrane. This disruption collapses the proton motive force, which relies on a high concentration of protons outside the cell membrane.

The collapse of the PMF inhibits the ATP synthase enzyme, which utilizes the energy stored in the PMF to synthesize ATP from ADP and inorganic phosphate (Pi). In normal physiology, as protons flow back into the cell through ATP synthase, they drive the rotation of its subunits, catalyzing the formation of ATP. Therefore, when the PMF collapses, the ATP synthase enzyme cannot function, leading to a depletion of the ATP. Since ATP is essential for various cellular processes, ultimately this depletion of ATP leads to bacterial cell death. While there is substantial evidence supporting carvacrol's ability to disrupt cell membranes and reduce ATP synthesis by indirectly inhibiting the ATP synthase enzyme, direct evidence of its inhibition of ATP Synthase activity remains limited (Ultee et al., 2002; Gill and Holley, 2006b).

### 2.3.3. Biofilm reduction

A biofilm is defined as an assemblage of microbial cells that are irreversibly attached to a surface and enclosed in a matrix primarily composed of polysaccharides, proteins, lipids, and DNA. The Extracellular polysaccharide (EPS) acts as glue, facilitating adhesion to surfaces, the formation of enclosing matrix and intercellular communication among the microbial community. While bacterial biofilms are predominantly composed of bacteria, they can also include other microorganisms such as fungi, archaea, and protozoa, forming complex multi-species communities. Not all bacterial species form biofilms, but many have the capability to do so. Some bacteria are well-known for their strong biofilm-forming capabilities, such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, while others may not form biofilms effectively or at all. Bacteria living in a biofilm have a higher degree of resistance to host defense mechanisms and antibiotic therapy (Zhao et al., 2023).

Carvacrol has demonstrated significant potential in reducing biofilm formation across various bacterial species, showing its effectiveness as an antimicrobial agent. Carvacrol has been shown to inhibit biofilm formation by up to 86-100% for *Staphylococcus aureus* and 74-88% for *Pseudomonas aeruginosa* depending on the concentration used. This highlights its strong efficacy against common pathogens associated with biofilm-related infections. Carvacrol's ability to inhibit bacterial biofilm formation is primarily due to its effects on quorum sensing, reduction of extracellular polysaccharide (EPS) production (Peng et al., 2023; Walczak et al., 2021).

### 2.3.4. Inhibition of motility

Traditionally seen as a motility organelle, the flagellum also plays a significant role in bacterial adhesion and invasion. Study on *Escherichia coli* with sublethal concentrations (1 mM) of carvacrol showed downregulation of flagellin, a key flagellum protein. Higher concentrations of carvacrol (up to 5 mM) reduced motility in a concentration-dependent manner, with 10 mM causing immediate cessation of motility (Kachur & Suntres, 2019).

### 2.3.5. Other intracellular targets

Although the mechanisms listed above are the confirmed ways carvacrol acts, studies suggest that this compound could have other intracellular targets linked to its antimicrobial activity (Trombetta et al., 2005). Further exploration into carvacrol's interaction with intracellular components may reveal novel mechanisms of action. Various in silico studies have shown that thymol, an isomer of carvacrol with a similar mechanism of action, inhibits other intracellular targets such as DHPS (Cruz et al., 2022). Therefore, it is reasonable and logical to consider that DHPS might be an intracellular target for carvacrol and to study its binding affinity with DHPS.

Generally, carvacrol's multifaceted actions, including cell membrane disruption, indirect inhibition of membrane-bound ATP synthase, biofilm reduction, and motility inhibition, make it a promising candidate for combating bacterial infections and antibiotic-resistant bacteria.

# 3. Materials and Methods

## 3.1. Materials

### 3.1.1. Chemicals and reagents

The chemicals and reagents that were used for the synthesis process are: hexane, chloroform, ethyl acetate (99.8%), ice water, HCl (10 M), anhydrous pyridine, Benzenesulfonyl chloride (98%), carvacrol (99%) and chlorosulfonic acid (99%) (all from Sigma-Aldrich Co., MO, USA). All the solvents and reagents are of analytical grade.

### 3.1.2. Instruments

For the analysis of the compounds, silica gel TLC plates (60 F254, 0.2 mm thick, Merck KGaA, Darmstadt, Germany) and capillary tube were used. For conducting the chemical reactions, a reaction flask, magnetic stirrer with a condenser, stir bar, measuring cylinder and pipette fillers were used. To measure the synthesized compounds mass electrical weighing balance was used. To visualize the TLC chromatogram plates, UV-visible Spectrophotometer (Evolution 60S) was used. The $^{1}H$ and $^{13}C$-NMR spectra were recorded using a JEOL 400 MHz NMR (Japan), Tetramethylsilane (TMS) as internal standard, where chloroform was used as solvent for the NMR spectroscopy.

For antimicrobial test, incubator (Modell 100-800 memmert), autoclave, biosafety cabinet (biostarmodell50/60), vortex (Fisher Brand), UV-visible Spectrophotometer (Evolution 60S), water bath, inoculating loops, pipette tips, pipette filler, test tube, multichannel pipette, Microtiter Plates, Agar Plates, Ruler and Sterile Filter Paper Disks.

### 3.1.3. Media, microbial strains, and standard drugs

For the media preparation, the Nutrient Agar (NA), Mueller-Hinton Broth (MHB), Sabouraud Dextrose Agar (SDA) and Sabouraud Dextrose Broth (SDB) were used. In addition, Ciprofloxacin was used as a positive control for antibacterial test while 1% DMSO was used as a negative control. For the case of antifungal test, Griseofulvin was used as a reference standard.

In vitro antibacterial assays were performed on the following Gram-positive bacterial strains: *Staphylococcus aureus* MDR10, *Staphylococcus aureus* MDR 1*, *Staphylococcus aureus* MDR 2*, *Bacillus pumilus* 82 and *Bacillus subtilis* ATCC 6633. The Gram-negative bacterial strains used were: *Escherichia coli* K99, *Escherichia coli* K88, *Escherichia coli* 306, *Escherichia coli* LT37, *Escherichia coli* 872, *Escherichia coli* ROW 7/12, *Escherichia coli* 3:37C, *Escherichia coli* CD/99/1, *Escherichia coli* HB101*, *Escherichia coli* C600*, *Salmonella typhi* Ty2, *Salmonella enterica* TD 01, *Shigella dysentery* 8, *Shigella soneii* 1, *Shigella boydii* D13629, *Shigella flexneri* Type 6, *Vibrio cholerae* 1313, *Vibrio cholerae* 293, *Vibrio cholerae* 1315, *Vibrio cholerae* 85 and *Pseudomonas aeruginosa* MDR 1*. All the bacterial strains were procured from the Department of Technology, Jadavpur University, Central Drugs Laboratory, Kolkata and Institute of Microbial Technology, Chandigarh, India. Before sensitivity tests were performed on the test samples, the purity of the strains was checked in accordance with the standard microbiological, cultural, and biochemical tests.

On the other hand, the antifungal activity testing was performed on: *Candida albicans* ATCC 10231, *Aspergillus niger* ATCC 6275, *Penicillium notatum* ATCC 11625 and *Penicillium funiculosum* NCTC 287. All the fungal species were provided from Central Drugs Laboratory, Kolkata Institute of Microbial Technology, Chandigarh, India.

## 3.2. Methods

### 3.2.1. Synthesis of sulfonated derivatives of carvacrol (8 and 9)

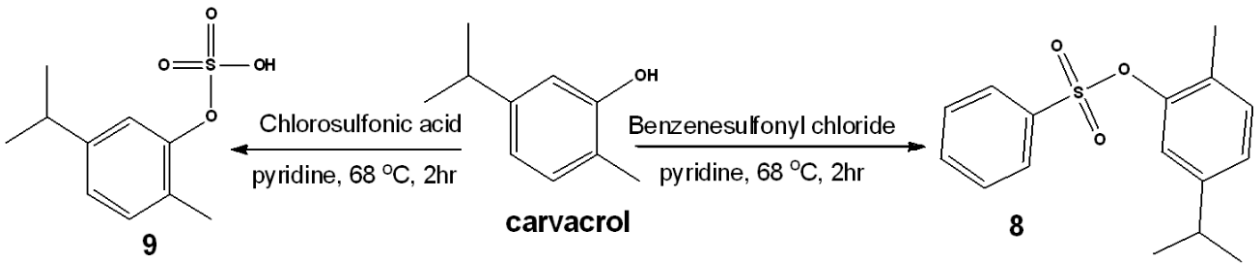
Two sulfonated derivatives of carvacrol (5-isopropyl-2-methylphenyl benzenesulfonate (**8**) and 5-isopropyl-2-methylphenyl hydrogen sulfate (**9**)) were successfully synthesized through a nucleophilic substitution reaction at sulfur by refluxing carvacrol with sulfonylating agents—either benzenesulfonyl chlorides or chlorosulfonic acid—in the presence of pyridine (Bagul et al., 2017).

#### 3.2.1.1. Synthesis of 5-isopropyl-2-methylphenyl benzenesulfonate (8)

Benzenesulfonyl chloride (1.0 mmol) was added dropwise to a solution of carvacrol (1.0 mmol) in 5 mL of anhydrous pyridine. The reaction mixture was heated under reflux for 2 h, with the reaction progress monitored by TLC. After completion, the reaction mixture was cooled to room temperature, poured into ice water, and acidified with 10M HCl. A precipitate was formed, collected by filtration.

#### 3.2.1.2. Synthesis of 5-isopropyl-2-methylphenyl hydrogen sulfate (9)

Compound **9** was synthesized following the same procedure used for compound **8**, with chlorosulfonic acid substituted for benzenesulfonyl chloride. The reaction schemes for both compounds **8** and **9** are shown in Scheme 1.




Scheme 1: Synthesis of sulfonated derivatives of carvacrol (**8** and **9**)

### 3.2.2. Structural elucidations of the synthesized compounds

The structures of compounds 8 and 9 were identified by analyzing their $^{1}H$ and $^{13}C$-NMR spectra data. Chemical shifts are denoted in ppm and signal multiplicities are indicated as follows: s = singlet; brs = broad singlet; d = doublet; t = triplet; dd = doublet of doublet and m = multiplet.

### 3.2.3. In vitro antibacterial and antifungal activity assay

#### 3.2.3.1. Disk diffusion method

The Disk Diffusion antimicrobial experiment was conducted according to the CLSI guideline (CLSI, 2015). All culture media used were prepared according to the manufacturer instruction. The media was sterilized at 121 $^{0}C$ for 15 minutes using an autoclave and allowed cool to 45 $^{0}C$-50 $^{0}C$ in water bath. Then 20 ml of media was poured into 100 mm diameter size petri-dishes under aseptic condition inside level II Bio-safety Cabinet and allowed some time to solidify.

For antibacterial experiments 4-5 bacterial colony of each tested organisms were inoculated with inoculating loop in to Petri dishes containing agar medium. Each bacterial test organisms were incubated for 18-24 h at 37 °C. Standardization was accomplished by preparing a suspension of 3-5 inoculations from a fresh, pure culture of the specimen bacterium in Mueller Hinton broth. The produced suspension's absorbance was measured using an ultraviolet-visible (UV) spectrophotometer at 625 nm having a path length of 1cm until the absorbance reading was 0.08 to 0.1, which is proportional to $1x10^{8}$ CFU/ml bacteria.

The standardized solution was then further diluted with Muller Hinton Broth at a 1:100 ratio to yield a colony suspension containing $1x10^{6}$ CFU/mL bacteria. For the broth dilutions, final suspensions of 5 $x10^{5}$ CFU/mL were employed, and they were employed to evaluate the antibacterial activity of the compounds in next broth dilution experiment (CLSI, 2015).

To investigate the synthesized compounds' antibacterial and antifungal activities, a stock solution containing 2 mg/ml was made in 1% DMSO. By diluting the stock solution in the appropriate quantities of 1% DMSO, concentrations of 200 μg/mL for antibacterial activity tests and 2000 μg/mL for antifungal activity tests were prepared.

Dip a sterile cotton swab into the standardized bacterial suspension (1x108 CFU/mL bacterial suspension) and was evenly swabbed the Petri dish (MHA plate), next the test samples were impregnated onto 6 mm filter paper discs which were then placed on the medium's surface. After that, the Petri dishes were incubated for 24 hours at a temperature of 37 °C, and the diameter of the inhibitory zone was measured in millimeters. Ciprofloxacin was used as positive control and then ZOI was compared. A negative control of 1% DMSO was used for the experiment

We used similar technique in the disk diffusion antifungal activity experiment, except saborauds dextrose media was used in preparing the culture media for the fungal strains. Hence the compounds with concentration of 2000 μg/mL were assessed against the fungal pathogens which had been inoculated on saborauds dextrose media. After three days of incubation at a room temperature, the diameter of the ZOI in the Petri dishes was determined in millimeters. Here, the Griseofulvin served as the standard for comparison. A negative control of 1% DMSO was used also for the experiment.

#### 3.2.3.2. Broth dilution method

The Broth Dilution antimicrobial experiment is conducted to determine the MIC of the synthesized compounds against a specific bacterium according to the CLSI guidelines (CLSI, 2015). For bacterial and fungal growth, the Mueller–Hinton broth and Saborauds dextrose broth were utilized respectively. Stock solutions, a concentration of 2 mg/ml, of the both compounds were prepared by dissolving in 1% DMSO.

A 100µl of Muller Hinton broth was added into each well of the 96-well microplates. From a stock solution, the serial dilutions were made ranging from 800 µg/ mL to 5 µg/mL using Mueller–Hinton broth in 96-well microplates. Hence concentrations of 5, 10, 25, 50, 100, 200, 400 and 800 µg/ mL of synthesized compounds were made for antibacterial activity test. The bacterial suspension containing approximately 5 × 105 CFU/ml was prepared from a refreshed culture. From this suspension, 100 µl was inoculated into each well and incubated for 18-24 hour at 37 °C. After incubation, the MIC values were visually determined by observing the presence and absence of turbidity. Absence of turbidity in each well marked the MIC. A negative control of 1% DMSO was used for the experiment

In MIC experiment of fungal activity, the serial dilutions of the compounds were made from a stock solution to prepare concentrations of 50, 100, 200,400, 800, 1000, 1500 and 2000 µg/mL using saborauds dextrose broth in 96-well microplates. The fungal suspension containing approximately 5 × 105 CFU/ml was prepared from a refreshed culture. From this suspension, 100 µl was inoculated into each well and incubated three days at room temperature. Absence of turbidity in the wells marked the MIC. A negative control of 1% DMSO was used for the experiment.

## 3.2.4. *In silico* studies

### 3.2.4.1. Molecular docking study

Using Autodock Vina in conjunction with the MGLTools suite (version 1.5.7), molecular docking of the synthesized compounds was performed on *E. coli* DHPS protein (PDB: 1AJ2) as potential target for antibacterial activity. The docking process involved four key steps: protein preparation, ligand preparation, grid receptor generation, and molecular docking, all utilizing default values.

**Protein preparation**

*E. coli* DHPS protein (PDB: 1AJ2) was obtained from the PDB (www.rcsb.org) on December 20, 2024, as a binary complex. Using PyMOL (version 3.1.1) both ligands (6-hydroxymethyl-7,8-dihydropterin diphosphate and sulfate ion) are removed and saved as a pdb file for further preparation by AutoDock MGL Tools. Using AutoDock MGL Tools water molecules were deleted, polar hydrogens and kollman charges were added and saved as pdbqt file format.

**Ligand preparation**

The synthesized compounds were drawn on ChemDraw Ultra (version 12.0.2) then these 2D structures of the molecule were copied to Chem3D pro 12.0 to get their 3D structure then saved as sdf files. These sdf files were converted to pdb files by PyMOL for further process by AutoDock MGL Tools. Using AutoDock MGL Tools gasteiger charges were added for both compounds and each compound's non-polar hydrogen merged, rotatable bond detected and TORSDOF was set and saved as pdbqt file format.

**Receptor grid box generation**

Using AutoDock MGL Tools, the protein structure was selected in the workspace to define the ligand-binding site. The grid box parameters were set as follows: the number of points in the x, y, and z directions were each 40; the spacing was 0.375; and the center coordinates were x = 38.901, y = 1.179 and z = 11.438. This configuration was then saved in a text file format.

**Ligand docking**

The configuration file was prepared by setting the energy range, exhaustiveness, and num_modes to 3, 8, and 9, respectively.

Using the grid parameters of the grid box, the docking of the synthesized compounds was then conducted on the prepared protein using AutoDock Vina via command lines.

The docking protocol was validated by the native ligands 6-hydroxymethyl-7,8-dihydropterin diphosphate (2PH) for antibacterial target.

**Visualization and Analysis of AutoDock Results**

The output.pdbqt file was converted to output.pdb format using PyMOL. The output.pdb file was then opened in Biovia Discovery Studio 2024 to visualize and analyze the molecular interactions between the ligands and the amino acids in the active site.

### 3.2.4.2. Pharmacokinetics and drug-likeness properties

The physicochemical, pharmacokinetic, medicinal chemistry friendliness and toxicity profiles of the synthesized compounds were evaluated using ADMETlab 3.0's online software tools, following the methodology described by (Fu et al., 2024). For this analysis, the compounds' structures were converted to SMILES format using ChemDraw Ultra 12.0 software. The generated SMILES representations for each compound were then submitted to the web server for assessment.

# 4. Results and Discussion

## 4.1. Synthesis of sulfonated derivatives of carvacrol (8 and 9)

Several studies have demonstrated that sulfonated compounds involving sulfonic acids and sulfonate esters possess strong antibacterial properties. This is due to the fact that sulfonic acid and sulfonate ester groups easily form hydrogen bonds with protein target, which makes the parent compound bind better to the protein target and also possess strong affinity for lipids, which facilitates easy penetration across cell membranes to exert their effects (Liu et al., 2024; Xie et al., 2022). Thus, in this study, we successfully synthesized two sulfonated derivatives of carvacrol, 5-isopropyl-2-methylphenyl benzenesulfonate (**8**) and 5-isopropyl-2-methylphenyl hydrogen sulfate (**9**). These compounds were prepared via nucleophilic substitution reaction at sulfur by refluxing carvacrol with sulfonylating agents—either benzenesulfonyl chlorides or chlorosulfonic acid—in the presence of pyridine. The physical properties of the synthesized compounds (8 and 9) are presented in Table 1.

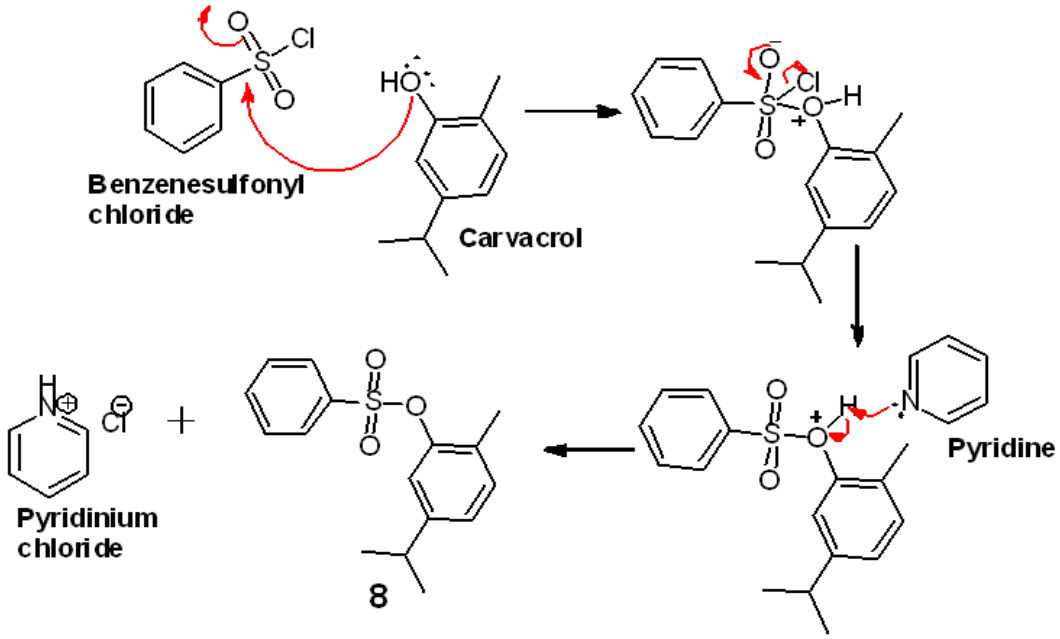
Table 1: Physical properties of the synthesized compounds 8 and 9

| compound | Molecular Formula | Predicted MW(g/mol) | Physical State | Color | % Yield | Rf value |
|---|---|---|---|---|---|---|
| **8** | $C_{16}H_{18}O_3S$ | 290.38 | Crystal | Colorless | 91% | 0.85 |
| **9** | $C_{10}H_{14}O_4S$ | 230.28 | Crystal | Colorless | 74% | 0.35 |

### 4.1.1. 5-Isopropyl-2-methylphenyl benzenesulfonate (8)

Compound **8** was obtained as white crystals with a yield of 91% (w/w). Its synthesis involves a multi-step reaction mechanism characterized by nucleophilic substitution reaction at sulfur. In this process, the hydroxyl group (-OH) of carvacrol acts as a nucleophile and directly attacks the electrophilic sulfur atom of benzenesulfonyl chloride. This nucleophilic attack temporarily disrupts the sulfur-oxygen double bond.

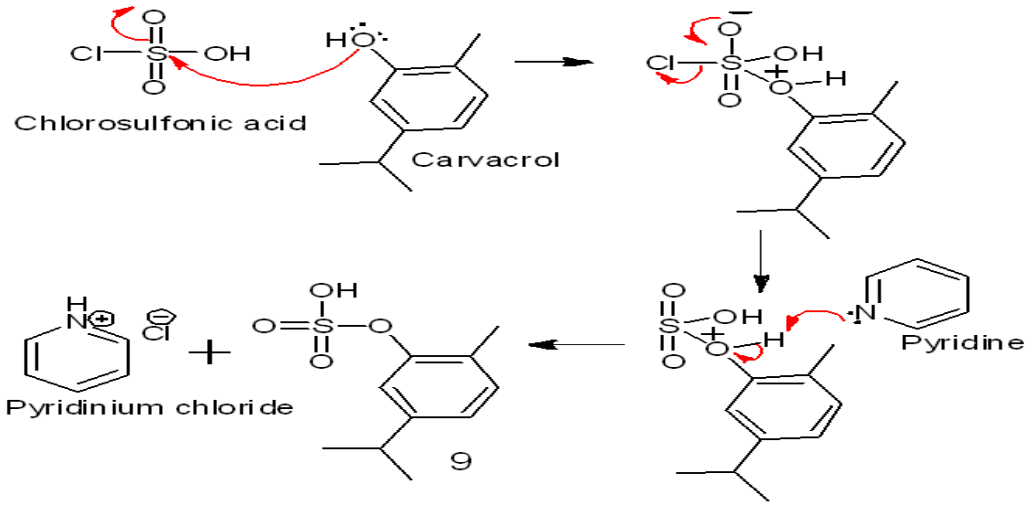
As the reaction proceeds, π-bond donation restores the S=O bond, resulting in the displacement of the chloride ion, a good leaving group. Subsequently, the proton on the positively charged oxygen is abstracted by the base catalyst, pyridine. This sequence leads to the formation of the final product, Compound **8**, along with pyridinium chloride as a side product, formed from the reaction between the pyridinium cation and the chloride anion. The reaction mechanism is depicted in Scheme 2.




Scheme 2: Mechanism of nucleophilic substitution reaction to form compound **8**

### 4.1.2. Synthesis of 5-isopropyl-2-methylphenyl hydrogen sulfate (9)

Compound **9** was synthesized via nucleophilic substitution reaction at sulfur, following the same procedure described above for compound **8**. The only difference was the use of chlorosulfonic acid in place of benzenesulfonyl chloride. In this reaction mechanism, the hydroxyl group (-OH) of carvacrol acts as the nucleophile, while chlorosulfonic acid serves as the electrophile. The reaction mechanism is identical to that of compound **8** and is illustrated in detail in Scheme 3.




Scheme 3: Mechanism of nucleophilic substitution reaction to form compound **9**

## 4.2. Structural elucidations of the synthesized compounds (8 and 9)

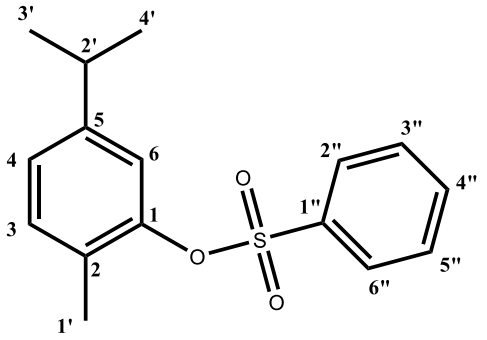
The structures of compounds **8** and **9** were confirmed through detailed analysis of their $^{1}$H and $^{13}$C-NMR spectral data. The chemical shifts, multiplicities, and integration values observed in the $^{1}$H-NMR spectrum, along with the carbon identified in the $^{13}$C-NMR spectrum, were consistent with the proposed structures. Detailed data of their spectral data are presented below.

### 4.2.1. Structural elucidation of 5-isopropyl-2-methylphenyl benzenesulfonate (8)

The $^{1}$H and $^{13}$C-NMR spectra of compound **8** suggest the presence of two substituted aromatic rings and a branched aliphatic side chain. Aromatic proton signals at δ 7.08 (1H, *dd*, *J* = 8, 4 Hz), 6.98 (1H, *dd*, *J* = 8, 4 Hz), and 6.69 (1H, *d*, *J* = 4 Hz) indicate a trisubstituted benzene ring, supported by carbon signals between δ 120.21 and 131.45 ppm. Downfield carbon signals at δ 148.34, 148.16, and 128.81 ppm suggest quaternary aromatic carbons bearing electron-donating or electron-withdrawing groups. In the aliphatic region, a singlet at δ 2.08 (3H) corresponds to a methyl group (C-1′, δC 16.02), while a multiplet at δ 2.77 (1H) and a doublet at δ 1.11 (6H) are consistent with a substituted isopropyl group, with associated carbon shifts at δ 33.43 and 23.81 ppm.

The second aromatic system shows a mono-substituted phenyl pattern with signals at δ 7.88 (2H, *dd*, *J* = 8, 4 Hz), 7.54 (2H, *t*, *J* = 8 Hz), and 7.68 (1H, *t*, *J* = 8 Hz), and corresponding carbon resonances from δ 128.61 to 136.29 ppm. Detailed 1H and 13 C-NMR assignments are presented in Table 2. Finally, based on these data, compound **8** was found to be consistent with the proposed structure of 5-isopropyl-2-methylphenyl benzenesulfonate.

Table 2: $^{1}H$ and $^{13}C$-NMR spectral data of compound **8**




Compound **8**

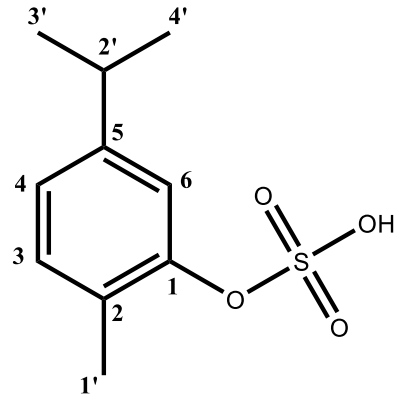
| Position | $\delta_H$ (ppm) | $\delta_C$ (ppm) |
|---|---|---|
| 1 | - | 148.34 |
| 2 | - | 128.81 |
| 3 | 7.08 (1H, *dd*, *J*= 8, 4 Hz) | 131.45 |
| 4 | 6.98 (1H, *dd*, *J*= 8, 4 Hz) | 125.35 |
| 5 | - | 148.16 |
| 6 | 6.69 (1H, *d*, *J*= 4 Hz) | 120.21 |
| 1' | 2.08 (3H, *s*) | 16.02 |
| 2' | 2.77 (1H, *m*) | 33.43 |
| 3', 4' | 1.11 (6H, *d, J=8 Hz*) | 23.81 |
| 1'' | - | 136.29 |
| 2'', 6'' | 7.88 (2H, *dd*, *J*= 8, 4 Hz) | 128.61 |
| 3'', 5'' | 7.54 (2H, *t*, *J*= 8 Hz) | 129.24 |
| 4'' | 7.68 (1H, *t*, *J*= 8 Hz) | 134.21 |

### 4.2.2. Structural elucidation of 5-isopropyl-2-methylphenyl hydrogen sulfate (9)

The $^{1}H$ and $^{13}C$ NMR spectra of compound **9** reveal a substituted aromatic ring with an aliphatic isopropyl-like side chain and hydrogen sulfate group. Three aromatic proton signals appear at δ 7.16 (1H, *dd*, *J* = 8, 4 Hz), 7.07 (1H, *dd*, *J* = 8, 4 Hz), and 7.04 (1H, *brs*), indicating a trisubstituted benzene ring.

Their corresponding carbon shifts at δ 117.13 to 130.38 ppm are consistent with protonated aromatic carbons, while downfield signals at δ 151.03, 146.68, and 129.71 ppm suggest quaternary aromatic carbons bearing electron-withdrawing or donating substituents. In the aliphatic region, a singlet at δ 2.29 (3H) and its carbon at δ 16.00 ppm confirm a methyl group. A multiplet at δ 3.03 (1H) and a doublet at δ 1.38 (6H, $J$ = 4 Hz) correspond to a methine and two methyl groups, indicating an isopropyl unit (C-2′, 3′, 4′) with associated carbon shifts at δ 33.34 and 24.05 ppm. A singlet at δ 9.21 ppm reveals the presence of sulfonic acid hydrogen (-$SO_4H$ group). The remaining $^{1}H$ and $^{13}C$-NMR chemical shifts are provided in Table 3.

Table 3: $^{1}H$ and $^{13}C$-NMR spectral data of compound **9**




Compound **9**

| Position | $\delta_H$ (ppm) | $\delta_C$ (ppm) |
|---|---|---|
| 1 | - | 151.03 |
| 2 | - | 129.71 |
| 3 | 7.16 (1H, *dd*, *J*= 8, 4 Hz) | 130.38 |
| 4 | 7.07 (1H, *dd*, *J*= 8, 4 Hz) | 120.32 |
| 5 | - | 146.68 |
| 6 | 7.04 (1H, *brs*) | 117.13 |
| 1' | 2.29 (3H, *s*, CH3) | 16.00 |
| 2' | 3.03 (1H, *m*, CH) | 33.34 |
| 3', 4' | 1.38 (6H, *d*, *J*=4 Hz, $2CH_3$) | 24.05 |
| -$SO_4H$ | 9.21 (1H, *s*, -$SO_4H$) | - |

## 4.3. Antimicrobial activities of synthesized compounds

### 4.3.1. Antibacterial activity

The antibacterial evaluation of synthesized compounds **8** and **9** against a wide range of clinically significant bacterial strains has yielded promising results, as summarized in Table 4. Both compounds exhibited strong antibacterial activity, with ZOIs ranging from 6 to 16 mm at 200 µg/mL and MICs ranging from 10 to 800 µg/mL (Table 4).

The antibacterial activity of compound **8** against *Shigella* species was particularly significant. In the present study, both *S. soneii* and *S. boydii* were highly susceptible to compound **8,** with low MICs of 10 µg/mL and ZOIs of 16 mm at 200 µg/mL. These excellent activities are highly relevant given the increasing prevalence of antibiotic-resistant enteric pathogens and the global burden of shigellosis and typhoid fever (Kesika & Balamurugan, 2012; Shad & Shad, 2021). Against *Salmonella typhi* Ty2 and *Salmonella enterica* TD01, compound **8** demonstrated inhibition zones of 14 mm and MICs of 50 µg/ml, while compound **9** showed much weaker activity (MIC >800 µg/ml).

Compound **8** demonstrated the second-highest activity against multiple strains of *Vibrio cholerae*, a major pathogen in waterborne diseases. With MICs values of 25 µg/mL and ZOIs ranging from 11 to 15 mm, compound **8** showed comparable activity as of the standard ciprofloxacin in some isolates, placing it as a potential viable candidate for treating cholera, especially in regions facing resistance to conventional antibiotics. Additionally, cholera remains a critical global public health threat, with the emergence of new *V. cholerae* strains and antibacterial resistance posing substantial concerns (Danso et al., 2020). Compound **9**, by contrast, exhibited weaker activity, with MICs uniformly at 100 µg/ml.

Compound **8** also exhibited strong antibacterial activity across most *E. coli* strains, with ZOI ranging from 12 to 14 mm and a MIC of 50 µg/ml. Interestingly, this compound showed efficacy even against two multi-drug resistant strains of *E. coli* (*E. coli* HB101 and *E. coli* C600), with MIC of 100 µg/ml. While compound **9** showed similar ZOI values but consistently weaker activity, with MICs of 100 µg/ml across nearly all *E. coli* strains tested. Ciprofloxacin has been widely used to treat infections caused by *E. coli*. However, the emergence of resistance to this antibiotic has become a major global health concern. Alarmingly high resistance rates have been reported, with one study showing that 73% of *E. coli* isolates were resistant to ciprofloxacin (Mandal et al., 2012). In the present study, the two multidrug-resistant *E. coli* strains*, E. coli* HB101 *and E. coli* C600, were found to be susceptible to compound **8**. This highlights the potential of compound **8** as a promising candidate for combating infections caused by multidrug-resistant *E. coli*.

Against *P. aeruginosa* MDR 1—a notoriously difficult-to-treat pathogen due to intrinsic resistance mechanisms—both compounds demonstrated moderate activity (ZOI = 14 mm, MIC = 200 µg/ml). While these values are not superior to ciprofloxacin, they are within a therapeutically relevant range and warrant further exploration, particularly in combination therapies or structural modifications to enhance activity. Unfortunately, both compounds were largely ineffective against *Bacillus pumilus* and *Bacillus subtilis*, with no ZOIs and high MICs (>400 µg/ml), suggesting limited utility against these spore-forming Gram-positive species.

Since both compounds exhibit broad-spectrum antibacterial activity, they may have potential for treating polymicrobial infections. Although neither compound produced larger ZOIs than ciprofloxacin, they demonstrated comparable activity.

Finally, these results suggest that the synthesized compounds hold significant potential as candidates for the development of new antimicrobial agents. Their activity against a variety of bacterial strains, including drug-resistant pathogens, is particularly promising in light of the growing global challenge of antibiotic resistance.

Table 4: Zone of inhibition and minimum inhibitory concentration of the synthesized compounds (**8** and **9**) against the tested bacterial strains

| Bacteria | ZOI in mm (at 200 µg/ml) | | | MIC (µg/ml) | |
|---|---|---|---|---|---|
| | Compound **8** | Compound **9** | Ciprofloxacin | Compound **8** | Compound **9** |
| *E. coli* K99 | 12 | 13 | 16 | 50 | 100 |
| *E. coli* K88 | 13 | 12 | 17 | 50 | 100 |
| *E. coli* 306 | 13 | 13 | 17 | 50 | 100 |
| *E. coli* LT37 | 13 | 12 | 16 | 50 | 100 |
| *E. coli* 872 | 12 | 13 | 16 | 50 | 100 |
| *E. coli* ROW 7/12 | 12 | 12 | 17 | 50 | 100 |
| *E. coli* 3:37C | 12 | 12 | 17 | 50 | 100 |
| *E. coli* CD/99/1 | 13 | 13 | 17 | 50 | 100 |
| *E. coli* HB101* | 14 | 15 | 16 | 100 | 100 |
| *E. coli* C600* | 14 | 14 | 16 | 100 | 100 |
| *Salmonella typhi* Ty2 | 14 | 12 | 16 | 50 | >800 |
| *Salmonella enterica* TD 01 | 14 | 12 | 19 | 50 | >800 |
| *Shigella dysenteriae* 8 | 14 | 12 | 20 | 50 | 100 |
| *Shigella soneii* 1 | 16 | 12 | 20 | 10 | 50 |
| *Shigella boydii* D13629 | 16 | 14 | 20 | 10 | 50 |
| *Shigella flexneri* Type 6 | 16 | 16 | 21 | 50 | 50 |
| *S. aureus* MDR10 | 15 | 12 | 18 | 400 | 400 |
| *S. aureus* MDR 1* | 14 | 14 | 18 | 100 | 100 |
| *S. aureus* MDR 2* | 14 | 14 | 18 | 100 | 100 |
| *Bacillus pumilus* 82 | 6 | 6 | 19 | 400 | >800 |
| *Bacillus subtilis* ATCC 6633 | 6 | 6 | 18 | 400 | >800 |
| *Vibrio cholerae* 1313 | 13 | 12 | 18 | 25 | 100 |
| *Vibrio cholerae* 293 | 15 | 12 | 19 | 25 | 100 |
| *Vibrio cholerae* 1315 | 15 | 11 | 19 | 25 | 100 |
| *Vibrio cholerae* 85 | 14 | 12 | 19 | 25 | 100 |
| *Pseudomonas aeruginosa* MDR 1* | 14 | 14 | 17 | 200 | 200 |

### 4.3.2. Antifungal Activity

The antifungal activity of synthesized compounds **8** and **9** was evaluated against four fungal strains, including *Candida albicans, Aspergillus niger, Penicillium notatum,* and *Penicillium funiculosum*, using griseofulvin as a reference standard. The results indicate that both compounds possess moderate antifungal properties, with ZOIs ranging from 12 to 15 mm at 2000 µg/mL and MICs between 400 and 1000 µg/mL, as presented in Table 5.

Compound **8** demonstrated slightly better activity overall compared to compound **9** in the disk diffusion mehtod. Against *C. albicans*, both compounds exhibited similar ZOIs of 13 mm for compound **8** and 12 mm for compound **9**, with MICs of 1000 µg/mL. For *A. niger*, compound **8** showed a larger ZOI (14 mm) than compound **9** (13 mm), with MICs of 800 µg/mL and 400 µg/mL, respectively—suggesting that while compound **9** had higher potency in terms of MIC, compound **8** produced a more pronounced inhibition zone. These results highlight the importance of both compounds in fighting against *A. niger* ATCC 6275, a clinically significant fungal strain associated with opportunistic infections, particularly in immunocompromised individuals. This strain is primarily linked to respiratory infections, including invasive pulmonary aspergillosis, and is often isolated from patients with weakened immune systems (e.g., HIV/AIDS, transplant recipients, or those undergoing immunosuppressive therapy) (Buyi et al., 2023).

Both compounds displayed comparable activity against *P. notatum* and *P. funiculosum*, with ZOIs of 13–15 mm and MICs of 800 µg/mL. None of these compounds have shown higher zone of inhibition than Griseofulvin yet they have shown closer activity to the standard compound in the three fungal species except in one fungal species (*C. albicans* ATCC 10231).

Finally, these findings highlight the potential of compounds **8** and **9** as lead structures for further optimization and development of new antifungal agents, especially in the context of increasing resistance to existing treatments. Their activity against both yeast and filamentous fungi suggests a broad-spectrum potential worth exploring in future studies.

Table 5: Zone of inhibition and minimum inhibitory concentration of the synthesized compounds (8 and 9) against the tested fungi strains

| Fungi | ZOI in mm (at 2000 µg/ml) | | | MIC (µg/ml) | |
|---|---|---|---|---|---|
| | Compound **8** | Compound **9** | Griseofulvin | Compound **8** | Compound **9** |
| *Candida albicans* ATCC 10231 | 13 | 12 | 16 | 1000 | 1000 |
| *Aspergillus niger* ATCC 6275 | 14 | 13 | 15 | 800 | 400 |
| *Penicillium notatum* ATCC 11625 | 15 | 14 | 15 | 800 | 800 |
| *Penicillium funiculosum* NCTC 287 | 13 | 13 | 14 | 800 | 800 |

## 4.4. In silico Studies

### 4.4.1. Molecular docking

Although carvacrol is known to exert its antimicrobial effects through mechanisms such as membrane disruption, inhibition of membrane-bound ATP synthase, biofilm reduction, and motility inhibition, some studies suggest it may also target intracellular components (Trombetta et al., 2005). Particularly, thymol, an isomer of carvacrol, has been shown to bind strongly to the active site of *E. coli* DHPS with a binding energy of –17.84 kcal/mol (Cruz et al., 2022), indicating significant DHPS-thymol interaction. Given the structural and functional similarities between thymol and carvacrol, it is reasonable to hypothesize that DHPS could also be a potential intracellular target for carvacrol and its derivatives, warranting further investigation into their binding affinities.

Thus, based on the molecular docking analysis shown in Figures 8-13, compounds **8** and **9** were assessed for their binding affinity and interaction profiles with the active site of *E. coli* dihydropteroate synthase (DHPS), using its crystal structure (PDB ID: 1AJ2). These figures illustrate both 2D and 3D representations of how each compound interacts with the enzyme, providing detailed insight into their docking behavior and binding orientations.

The docking protocol was validated using the native ligand, 6-hydroxymethyl-7,8-dihydropterin diphosphate (2PH), which is associated with the antibacterial target. When 2PH was redocked into the active site of the *E. coli* DHPS enzyme, it produced a docking score of –8.4 kcal/mol. This result indicates a strong binding affinity and provides a reliable reference point, showing a relatively stronger binding strength than both compound **8** and **9**.

Compound **8** demonstrated a strong binding affinity toward the active site of *E. coli* dihydropteroate synthase (DHPS), as detailed in Table 6. Molecular docking results revealed a favorable docking score of –7.3 kcal/mol, indicating a strong and stable interaction with the enzyme. Specifically, compound **8** engaged in multiple hydrogen bonds with key active site residues, including ARG255 and ASN22, which are likely critical for enhancing binding stability, as illustrated in Figures 8 through 10.

Beyond hydrogen bonding, compound **8** also formed several hydrophobic interactions with nonpolar amino acid residues such as PHE190, LYS221, ARG63, ILE117, and HIS257. These interactions further reinforce the compound's stable accommodation within the enzyme's binding pocket. Three-dimensional structural visualization confirmed that compound **8** is well-fitted within the active site cavity, showing its alignment with the relevant residues. This spatial compatibility underscores the potential of compound **8** as a promising DHPS inhibitor through both polar and nonpolar molecular interactions.

Table 6: Docking scores and interactions of ligands with active site amino acid residues

| Ligands | Docking score (kcal/mol) within 1AJ2 | Interaction with amino acid residues | | | |
|---|---|---|---|---|---|
| | | H-bonds | Hydrophobic | Electrostatic | miscellaneous |
| **8** | -7.3 | ARG255 and ASN22 | PHE190, LYS221, ARG63, ILE117 and HIS257 | | |
| **9** | -6.4 | GLN149 and GLY19 | PRO64 and ALA151 | | |
| Carvacrol | -5.7 | GLN149 and ASN144 | PRO64 and PRO152 | | |
| 2PH | -8.4 | ASN22, LYS221, HIS257, ASN115, ASP185, ASP96 and THR62 | | LYS221, ARG55, ARG63 and HIS257 | MET139 |

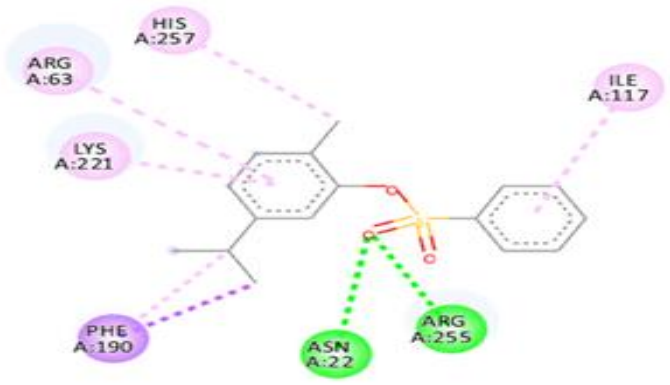



Figure 8: 2D interaction between compound **8** and *E. coli* DHPS (PDB ID: 1AJ2)


Figure 9: 3D interaction between compound **8** and *E. coli* DHPS (PDB ID: 1AJ2)

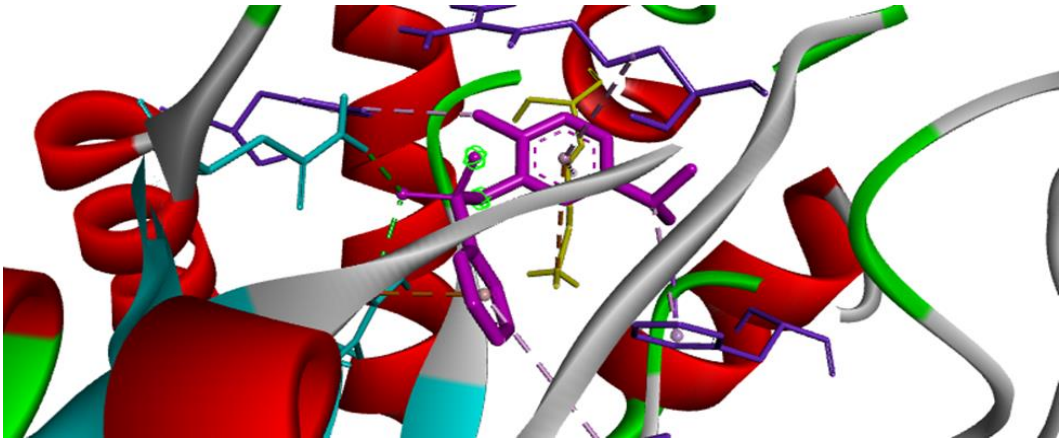


Figure 10: Compound **8** in the active site of *E. coli* DHPS (PDB ID: 1AJ2)

Compound **9** also exhibited effective binding to the active site of *E. coli* DHPS, as illustrated in Figures 11-13. With a docking score of –6.4 kcal/mol, compound **9** demonstrated a favorable binding profile, as of compound **8**. The docking analysis revealed that compound **9** establishes specific interactions with several key amino acid residues within the enzyme's active site.

Compound **9** forms two hydrogen bonds with critical residues such as GLN149 and GLY19. These hydrogen bonds likely play a significant role in enhancing both the specificity and stability of the interaction. In addition to these polar interactions, hydrophobic contacts were observed between compound **9** and nonpolar residues, including PRO64 and ALA151. These hydrophobic interactions further reinforce the compound’s binding affinity by promoting a stable fit within the enzyme's binding pocket. The three-dimensional interaction diagrams confirm that compound **9** is well-oriented within the active site cavity, aligning closely with functionally important residues. This spatial arrangement suggests that compound **9** may exert a potent inhibitory effect on the DHPS enzyme by effectively occupying and interacting with its catalytic region.

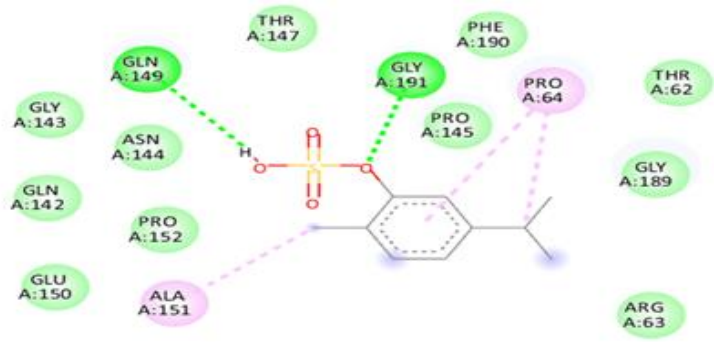



Figure 11: 2D interaction between compound **9** and *E. coli* DHPS (PDB ID: 1AJ2)

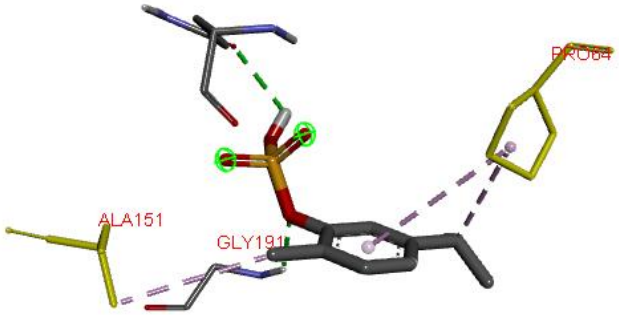



Figure 12: 3D interaction between compound **9** and *E. coli* DHPS (PDB ID: 1AJ2)

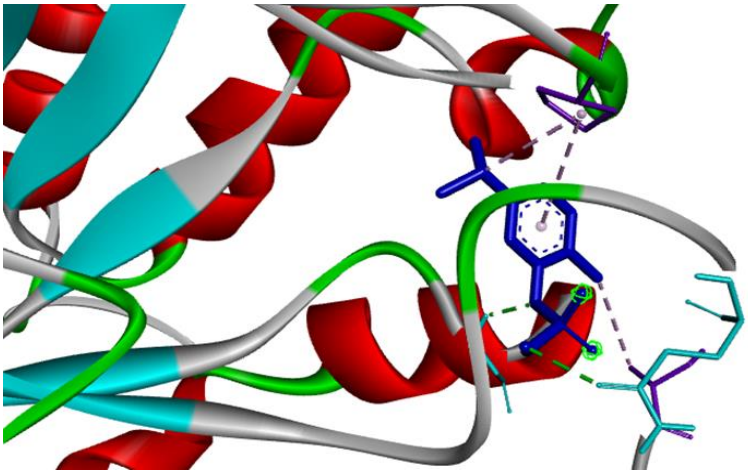


Figure 13: Compound **9** in the active site of *E. coli* DHPS (PDB ID: 1AJ2)

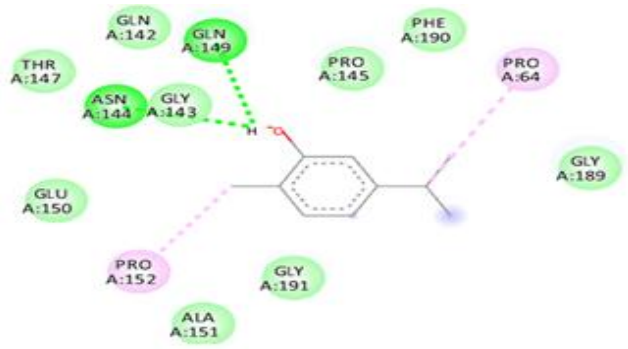



Figure 14: 2D interaction between carvacrol and *E. coli* DHPS (PDB ID: 1AJ2)

We also conducted a molecular docking study on the starting compound, carvacrol, which yielded a binding score of –5.7 kcal/mol, indicating comparatively weaker binding affinity than compound **8** and compound **9**. Carvacrol forms two hydrogen bonds: one with ASN144 and another with GLN149, both mediated through its hydroxyl group. In addition, it engages in alkyl hydrophobic interactions with PRO64 and PRO152. These interactions occur via the methylene group of its isopropyl side chain and the 2-methyl group, respectively, as illustrated in Figure 14.

In conclusion, both compounds **8** and **9** demonstrate promising molecular docking results with strong affinities and specific interactions within the DHPS active site. Their engagement through hydrogen bonding and hydrophobic contacts highlights their potential as effective inhibitors, which warrants further experimental validation for antibacterial or antimicrobial applications.

### 4.4.2. Physicochemical and ADMET Properties and Medicinal Chemistry Friendliness

In the realm of drug design and optimization, in silico models used to predict physicochemical properties, ADMET profiles and medicinal chemistry friendliness are invaluable tools for researchers.

These models assist in guiding the design and refinement of lead compounds by evaluating key Physicochemical properties, ADMET profiles and medicinal chemistry friendliness of the lead compounds.

During the early stages of drug development, they help to screen out compounds with undesirable characteristics and offer rapid insights into ADMET and medicinal chemistry friendliness profiles, thus facilitating the optimization and synthesis of lead compounds (Fu et al., 2024).

In this study, the ADMET 3.0 online server was employed to physicochemical properties, ADMET profiles and medicinal chemistry friendliness of the synthesized compounds. This tool offers a user-friendly interface for assessing ADMET properties and provides valuable insights into the physicochemical and ADMET properties, and drug-likeness of the compounds.

The physicochemical properties of compound **8** and **9** are summarized in Table 7. While most physicochemical parameters fall within the recommended range, compound **8** slightly exceeds the limits for lipophilicity (LogP = 4.253, LogD = 3.591) and water solubility (LogS = -4.394). Hence, compound **8** is predicted to be moderately water soluble and weakly basic drug (pKa = 8.906). In contrast, compound **9** meets all physicochemical parameters within the acceptable range and is acidic drug (pka=1.534).

Table 7: Prediction of physicochemical properties of compounds **8** and **9** by ADMETlab 3.

| Compound | MW | nHA | nHD | nRot | TPSA | LogS | LogP | LogD | pka |
|---|---|---|---|---|---|---|---|---|---|
| **8** | 290.1 | 3 | 0 | 4 | 43.37 | -4.394 | 4.253 | 3.591 | 8.906 |
| **9** | 230.06 | 4 | 1 | 3 | 63.6 | -2.098 | 1.605 | 2.045 | 1.534 |
| Carvacrol | 150.1 | 1 | 1 | 1 | 20.23 | -2.115 | 3.218 | 2.971 | 10.551 |
| Recommended values | | 0-12 | 0-7 | 0-11 | 0-140 | -4-0.5 | 0-3 | 1-3 | |

MW= Molecular Weight, nHA= number of hydrogen bond acceptors, nHD= number of hydrogen bond donors, nRot= number of rotatable bonds, TPSA=topological polar surface area, Log P = partition coefficient, Log S = aqueous solubility,

Log D= distribution coefficients at pH=7.4, pka= refers to the strength of a drug molecule's acidity or basicity.

Medicinal chemistry friendliness parameters of compound 8 and 9 are summarized in Table 8. All Lipinski's Rule of 5 (molecular weight ≤ 500, LogP ≤5, no more than 5 HBD, and no more than 10 HBA), which asses the drug-likeness of a compound, are satisfied in both compounds (**8** and **9**). Additionally, the quantitative estimate of drug-likeness (QED) falls within the recommended range for both compounds.

Regarding synthetic accessibility, both compounds can be efficiently synthesized based on their SA scores and GASA values. Furthermore, an assessment using the PAINS filter confirms that neither compound contains Pan Assay Interference substructures, indicating a favorable profile for further development.

Table 8: Medicinal chemistry friendliness of compounds **8** and **9** by ADMETlab 3.0

| Compound | LR-5 | QED | SA score | GASA | PAINS |
|---|---|---|---|---|---|
| 8 | Accepted | 0.802 | 1 | 0.0 | 0 alerts |
| 9 | Accepted | 0.809 | 2 | 0.0 | 0 alerts |
| Carvacrol | Accepted | 0.652 | 1 | 0.0 | 0 alerts |
| Recommended values | | > 0.67 | ≤ 6 | | |

LR-5=Lipinski rule of 5; QED= Quantitative Estimate of Drug-likeness; SA score=synthetic accessibility score; GASA (Graph Attention-based assessment of Synthetic Accessibility) = The output value represents the probability of being difficult to synthesize, ranging from 0 to 1, Values closer to 0: Indicate that the molecule is considered Easy to Synthesize;

PAINS (Pan Assay Interference Compounds) = refer to the presence and absence of Pan Assay Interference Compounds (PAINS) substructures within the molecule.

Both compounds (**8** and **9**) demonstrate favorable absorption profiles, with Caco-2 permeability, Pgp-S (P-glycoprotein substrate), and predicted Human Intestinal Absorption (HIA) falling within recommended ranges. This suggests a potential for good oral activity. Regarding distribution, parameters such as Volume of Distribution (VD) and Blood-Brain Barrier (BBB) penetration are within acceptable limits, except both compounds exhibit high plasma protein binding (PPB). The predicted low probability of BBB penetration is advantageous, as it minimizes the risk of central nervous system (CNS) side effects.

Neither compound is predicted to inhibit the major drug-metabolizing enzymes CYP3A4 or CYP2D6, suggesting a lower likelihood of interactions with the majority of currently marketed drugs. However, Compound **8** was identified as an inhibitor of CYP2C9, CYP2C19, and CYP1A2. In contrast, Compound **9** is not predicted to inhibit any of these tested CYP450 enzymes.

The predicted plasma clearance for compound **8** is 4.111 ml/min/kg, indicating low clearance rate as it falls below the 5 ml/min/kg threshold and its predicted half-life ($T_{1/2}$) is 0.529 that is within (0.7-1) range, is in short half-life range. In contrast, compound **9** exhibits slightly higher predicted clearance and its half-life is 0.816 again within (0.7-1) range, is in short half-life range. Assessment of potential toxicity via hERG and Ames tests indicate that both compounds are predicted to be safe.

Table 9: ADMET prediction of compounds **8** and **9** by ADMETlab 3.0

| Compound | Caco-2 | Pgp-S | HIA | PPB (%) | VD | BBB | CYP1A2-I | CYP2C19-I | CYP2C9-I | CYP2D6-I | CYP3A4-I | CL | T½ | hERG | Ames |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **8** | -4.5 26 | 0.002 | 0 | 98.396 | 0.744 | 0.06 | 0.86 | 0.927 | 0.875 | 0.178 | 0.119 | 4.111 | 0.529 | 0.136 | 0.167 |
| **9** | -4.6 54 | 0.079 | 0.027 | 98.296 | 0.501 | 0.038 | 0 | 0 | 0.009 | 0 | 0 | 5.324 | 0.816 | 0.028 | 0.152 |
| Carvacrol | -4.4 | 0.064 | 0.012 | 91.894 | 0.206 | 0.179 | 0.952 | 0.799 | 0.859 | 0.006 | 0.153 | 11.932 | 0.847 | 0.106 | 0.398 |
| Recommended values | > -5.15 | 0-0.3 | 0-0.3 | ≤ 90% | 0.04 - 20 | 0-0.3 | Category 0-1 | Category 0-1 | Category 0-1 | Category 0-1 | Category 0-1 | ≥ 5 | 0-0.3 excellent<br>0.3-0.7 medium<br>0.7-1 poor | 0-0.3 excellent<br>0.3-0.7 medium | 0-0.3 excellent<br>0.3-0.7 medium |

Caco-2 = human colon adenocarcinoma cell lines; Pgp-S = P-glycoprotein substrate; HIA=Human intestinal absorption; PPB=plasma protein binding; VD = Volume of distribution; BBB = blood–brain barrier; CYP450=cytochrome P450, category 0 non-inhibitor, category 1-inhibitor; CL = clearance of a drug; T½= half-life; hERG (human ether-a-go-go related gene) = hERG toxicity test; AMES = a test for mutagenicity

# 5. Conclusion

In this study, we successfully synthesized and thoroughly characterized two sulfonated derivatives of carvacrol, 5-isopropyl-2-methylphenyl benzenesulfonate (**8**) and 5-isopropyl-2-methylphenyl hydrogen sulfate (**9**). Antibacterial evaluation revealed that compound **8** exhibited superior antibacterial activity, showing particularly potent effects against *Shigella sonnei* and *Shigella boydii,* with MICs of 10 µg/mL. It also demonstrated strong activity against *Vibrio cholerae* (MIC: 25 µg/mL), the causative agent of cholera. In contrast, compound **9** was more effective in antifungal assays, indicating a differing spectrum of activity between the two derivatives. Molecular docking studies further supported the antibacterial potential of both compounds by predicting strong interactions with *E. coli* dihydropteroate synthase (DHPS, PDB: 1AJ2), a critical enzyme in the bacterial folate biosynthesis pathway. Additionally, *in silico* analyses of physicochemical properties, ADMET (absorption, distribution, metabolism, excretion, and toxicity), and medicinal chemistry parameters suggest that both compounds possess favorable drug-like characteristics, making them promising candidates for further development.

# 6. Recommendation

Based on the findings of this study, the following recommendations are proposed:

— Experimental validation is necessary to confirm the *in silico* results, including molecular docking outcomes and the predicted physicochemical and pharmacokinetic properties of the synthesized compounds.

— Further synthesis of sulfonated carvacrol derivatives is recommended to expand the compound library, assess their antimicrobial activities, and conduct structure–activity relationship (SAR) studies to identify key functional groups contributing to bioactivity.

— Given that carvacrol exhibits a wide range of pharmacological effects—including anticancer, anti-inflammatory, antioxidant, anti-obesity, antidiabetic, and immunomodulatory properties—the newly synthesized derivatives should also be evaluated for these potential therapeutic activities.

## Reference


Ahmad, A., Khan, A., Akhtar, F., Yousuf, S., & Xess, I. (2011). Fungicidal activity of thymol and carvacrol by disrupting ergosterol biosynthesis and membrane integrity against Candida. *Mycology Research*, *115*(1), 41–50.

Antonia, N., & Teresa, P. (2012). Antimicrobial activity of carvacrol: current progress and future prospectives. *Bentham science*, *7*(1), 28–35.

Bagul, S. D., & Rajput, J. D. (2017). Design, synthesis and biological activities of novel 5-isopropyl-2-methylphenolhydrazide-based sulfonamide derivatives. *Research on Chemical Intermediates*, *43*(4), 2241–2252.

Bagul, S. D., Rajput, J. D., Patil, M. M., & Bendre, R. S. (2017). Synthesis, characterization and antioxidant activity of carvacrol based sulfonates. *Medicinal Chemistry*, *7*(10), 294–298.

Bassanetti, I., Carcelli, M., Buschini, A., Montalbano, S., Leonardi, G., Pelagatti, P., Tosi, G., Massi, P., Fiorentini, L., & Rogolino, D. (2017). Investigation of antibacterial activity of new classes of essential oils derivatives. *Food Control*, *73*(2), 606–612.

Ben Arfa, A., Combes, S., Preziosi-Belloy, L., Gontard, N., & Chalier, P. (2006). Antimicrobial activity of carvacrol related to its chemical structure. *Letters in Applied Microbiology*, *43*(2), 149–154.

Berhe, D. F., Beyene, G. T., Seyoum, B., Gebre, M., Haile, K., Tsegaye, M., Boltena, M. T., Tesema, E., Kibret, T. C., Biru, M., Siraj, D. S., Shirley, D., Howe, R., & Abdissa, A. (2021). Prevalence of antimicrobial resistance and its clinical implications in Ethiopia: a systematic review. *Antimicrobial Resistance and Infection Control*, *10*(1), 1–14.

Böttcher, L., & Gersbach, H. (2022). A refunding scheme to incentivize narrow-spectrum antibiotic development. *Mathematical Biology* 84(6), 59.

Buyi, J., Mwambi, B., & Taremwa, I. M. (2023). Prevalence of pulmonary aspergillosis and drug susceptibility testing amongst adult HIV patients. *Student's Journal of Health Research Africa*, *4*(4), 9.

CDC. (2019, April 11). *Antibiotic Resistance Threats Report*. available at https://www.cdc.gov/antimicrobial-resistance/data-research/threats/index.html (Accessed: 28 October 2024).

Clinical and Laboratory Standards Institute. (2015). *Performance standards for antimicrobial susceptibility testing* (CLSI document M100-S25).

Cruz, J. N., Silva, S. G., Pereira, D. S., Souza Filho, A. P., Oliveira, M. S., Lima, R. R., & Andrade, E. H. (2022). *In silico* evaluation of the antimicrobial activity of thymol—major compounds in the essential oil of *Lippia thymoides* mart. & Schauer (Verbenaceae). *Molecules*, *27*(15), 4768.

Danso, E. K., Asare, P., Otchere, I. D., Akyeh, L. M., Asante-Poku, A., Aboagye, S. Y., Osei-Wusu, S., Opare, D., Ntoumi, F., Zumla, A., Duodu, S., & Yeboah-Manu, D. (2020). A molecular and epidemiological study of Vibrio cholerae isolates from cholera outbreaks in southern Ghana. *PLOS ONE*, *15*(7), 1–16.

Dewang, P. M., Nikumbh, V. P., Tare, V. S., & Mahulikar, P. P. (2003). Eco-friendly pest management using monoterpenoids II - antifungal efficacy of menthol derivatives. *Journal of Scientific and Industrial Research*, *62*(10), 990–995.

Doorn, H. R. (2017). Emerging infectious diseases. *Medicine*, *45*(12), 798–801.

Fu, L., Shi, S., Yi, J., Wang, N., He, Y., Wu, Z., Peng, J., Deng, Y., Wang, W., Wu, C., Lyu, A., Zeng, X., Zhao, W., Hou, T., & Cao, D. (2024). ADMETlab 3.0: an updated comprehensive online ADMET prediction platform enhanced with broader coverage, improved

performance, API functionality and decision support. *Nucleic Acids Research*, *52*(1), 422–431.

Gandova, V., Lazarov, A., Dimov, M., Stankov, S., Denev, P., Ercisli, S., Stoyanova, A., Gulen, H., Assouguem, A., Farah, A., Ullah, R., Kara, M., & Bari, A. (2023). Physicochemical and biological properties of carvacrol. *Open Chemistry*, *21*(1), 20220319.

Gill, A. O., & Holley, R. A. (2006a). Disruption of *Escherichia coli*, *Listeria monocytogenes* and *Lactobacillus sakei* cellular membranes by plant oil aromatics. *International Journal of Food Microbiology*, *108*(1), 1–9.

Gill, A. O., & Holley, R. A. (2006b). Inhibition of membrane bound ATPases of *Escherichia coli* and *Listeria monocytogenes* by plant oil aromatics. *International Journal of Food Microbiology*, *111*(2), 170–174.

Hao, Y., Li, J., & Shi, L. (2021). A Carvacrol-rich essential oil extracted from oregano (*Origanum vulgare*) exerts potent antibacterial effects against *Staphylococcus aureus*. *Frontiers in Microbiology*, *12*(November), 741861.

Hellen, G., Souza, D. A. De, Alencar, J., Vaz, S. M., Esther, K., Leite, T., Id, F., Barbosa, S., & Id, S. S. (2021). *In vitro* and *in vivo* antibacterial activity assays of carvacrol: A candidate for development of innovative treatments against KPC-producing *Klebsiella pneumoniae*. *PLOS ONE* 16(2): 1–12.

Imran, M., Aslam, M., Alsagaby, S. A., Saeed, F., Ahmad, I., Afzaal, M., Umair, M., El-, M. A. A. A. H., Khames, G. A., Ali, M., Arslan, S., Muzamal, A., & Ali, H. (2022). Therapeutic application of carvacrol. *Food science and nutrition*. *10*(11), 3544–3561.

Kachur, K., & Suntres, Z. (2019). The antibacterial properties of phenolic isomers, carvacrol and thymol. *Food Science and Nutrition*, *60*(18), 1–12.

Kesika, P., & Balamurugan, K. (2012). Studies on *Shigella boydii* infection in Caenorhabditis elegans and bioinformatics analysis of immune regulatory protein interactions. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, *1824*(12), 1449–1456.

Khan, I., Bahuguna, A., Kumar, P., Bajpai, V. K., & Kang, S. C. (2017). Antimicrobial potential of carvacrol against uropathogenic Escherichia coli via membrane disruption, depolarization, and reactive oxygen species generation. *Frontiers in Microbiology*, *8*(11), 1–9.

Lazarevic, J., Markovic, A., Smelcerovic, A., Stojanovic, G., Ciuffreda, P., & Santaniello, E. (2022). Carvacrol derivatives as antifungal agents: synthesis, antimicrobial activity and in silico Studies on carvacryl Esters. *Acta Chimica Slovenica*, *69*(3), 571–583.

Lima, I. O., Deliveira, P. F., Deliveira, W. A., Deoliveira, L. E., Menezes, E. A., Cunha, F. A., & Formiga, D. M. (2013). Antifungal activity and mode of action of carvacrol against Candida albicans strains. *Journal of Essential Oil*, *25*(2), 138–142.

Liu, Y. H., Li, C. K., Nie, M. Y., Wang, F. L., Ren, X. L., Jin, L. H., & Zhou, X. (2024). Sulfonate derivatives bearing an amide unit: design, synthesis and biological activity studies. *BMC Chemistry*, *18*(1), 1–13.

Mandal, J., Srinivas, A. N., Buddhapriya, D., & Parija, S. C. (2012). Antibiotic resistance pattern among common bacterial uropathogens with a special reference to ciprofloxacin resistant *Escherichia coli*. *Indian Journal of Medical Research*, *136*(5), 842–849.

Myangar, K. N., & Patel, N. H. (2014). Synthesis and in vitro antibacterial activity of new oxoethylthio-1,3,4-oxadiazole derivatives. *Journal of Saudi Chemical Society*, *18*(2), 101–106.

Nóbrega, R. O., Teixeira, A. P., Oliveira, W. A., & Lima, I. O. (2016). Investigation of the antifungal activity of carvacrol against strains of Cryptococcus neoformans. *Pharmaceutical Biology*, *54*(11), 2591–2596.

Nostro, A., Roccaro, A. S., Bisignano, G., Marino, A., Cannatelli, M. A., Pizzimenti, F. C., Cioni, P. L., Procopio, F., & Blanco, A. R. (2007). Effects of oregano, carvacrol and thymol on *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. *journal of medical microbiology*, 56(4), 519–523.

Ohashi, T. K., Nagashima, M. N., Kawai, N. W., & Ohmagari, N. L. (2022). Expert review of anti-infective therapy a narrative review on drug development for the management of antimicrobial- resistant infection crisis in Japan. *Expert Review of Anti-Infective Therapy*, *20*(12), 1603–1614.

Oliveira, A. S., Llanes, L. C., Maria, I., Brighente, C., Nunes, R. J., Yunes, R. A., & Jr, N. M. (2016). New Sulfonamides Derived from Carvacrol: Compounds with High Antibacterial Activity against Resistant Staphylococcus aureus Strains. *Journal of biosciences and medicines 4*(7), 105–114.

Patil, J. U., Suryawanshi, K. C., Patil, P. B., Chaudhary, S. R., & Pawar, N. S. (2010). Synthesis and antibacterial activity of carvacryl ethers. *Journal of Asian Natural Products Research*, *12*(2), 129–133.

Peng, Q., Tang, X., Dong, W., Zhi, Z., Zhong, T., Lin, S., Ye, J., Chen, F., & Yuan, W. (2023). Carvacrol inhibits bacterial polysaccharide intracellular adhesin synthesis and biofilm formation of *Staphylococcus aureus*. *Royal society of chemistry*, *13*(41), 28743–28752.

Shad, A. A., & Shad, W. A. (2021). *Shigella sonnei*: virulence and antibiotic resistance. *Archives of Microbiology*, *203*(1), 45–58.

Sisto, F., Carradori, S., Guglielmi, P., Traversi, C. B., Spano, M., Sobolev, A. P., Secci, D., Carmela, M., Marcantonio, D., Haloci, E., Grande, R., & Mincione, G. (2020). Synthesis and biological evaluation of carvacrol-based derivatives as dual inhibitors of H. pylori Strains and AGS Cell Proliferation. *pharmaceuticals*, 13(11), 405.

Trevisan, D. C., Dasilva, A. F., Negri, M., Deabreu, B. A., Machinski, J. M., Patussi, E. V., Campanerut. A. Z., & Mikcha, M. G. (2018). Antibacterial and antibiofilm activity of carvacrol against Salmonella enterica serotype typhimurium. *Brazilian Journal of Pharmaceutical Sciences*, *54*(1), 1–8.

Trombetta, D., Castelli, F., Sarpietro, M. G., Venuti, V., Cristani, M., Daniele, C., Saija, A., Mazzanti, G., & Bisignano, G. (2005). Mechanisms of antibacterial action of three monoterpenes. *Antimicrobial Agents and Chemotherapy*, *49*(6), 2474–2478.

Ultee, A., Bennik, M. H. J., & Moezelaar, R. (2002). The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen Bacillus cereus. *Applied and Environmental Microbiology*, *68*(4), 1561–1568.

Walczak, M., Michalska-Sionkowska, M., Olkiewicz, D., Tarnawska, P., & Warżyńska, O. (2021). Potential of carvacrol and thymol in reducing biofilm formation on technical surfaces. *Molecules, 26*(9), 2723

Walsh, S. E., Maillard, J. Y., Russell, A. D., Catrenich, C. E., Charbonneau, D. L., & Bartolo, R. G. (2003). Activity and mechanisms of action of selected biocidal agents on Gram-positive and -negative bacteria. *Journal of Applied Microbiology*, *94*(2), 240–247.

Wanda, M. (2023). Carvacrol — A Natural Phenolic Compound with Antimicrobial Properties. *Antibiotics*, 12(5), 824.

Wang, K., Jiang, S., Yang, Y., Fan, L., Su, F., & Ye, M. (2019). Synthesis and antifungal activity of carvacrol and thymol esters with heteroaromatic carboxylic acids. *Natural Product Research*, *33*(13), 1924–1930.

WHO. (2024). *Infectious diseases*. available at https://www.emro.who.int/health-topics/infectious-diseases/index.html (Accessed: 26 October 2024).

WHO. (2024, August 7). *The top 10 causes of death*. available at www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death (Accessed: 26 October 2024).

Wijesundara, N. M., Lee, S. F., Cheng, Z., Davidson, R., & Rupasinghe, P. V. (2021). Carvacrol exhibits rapid bactericidal activity against Streptococcus pyogenes through cell membrane damage. *Scientific Reports*, *11*(1), 1487.

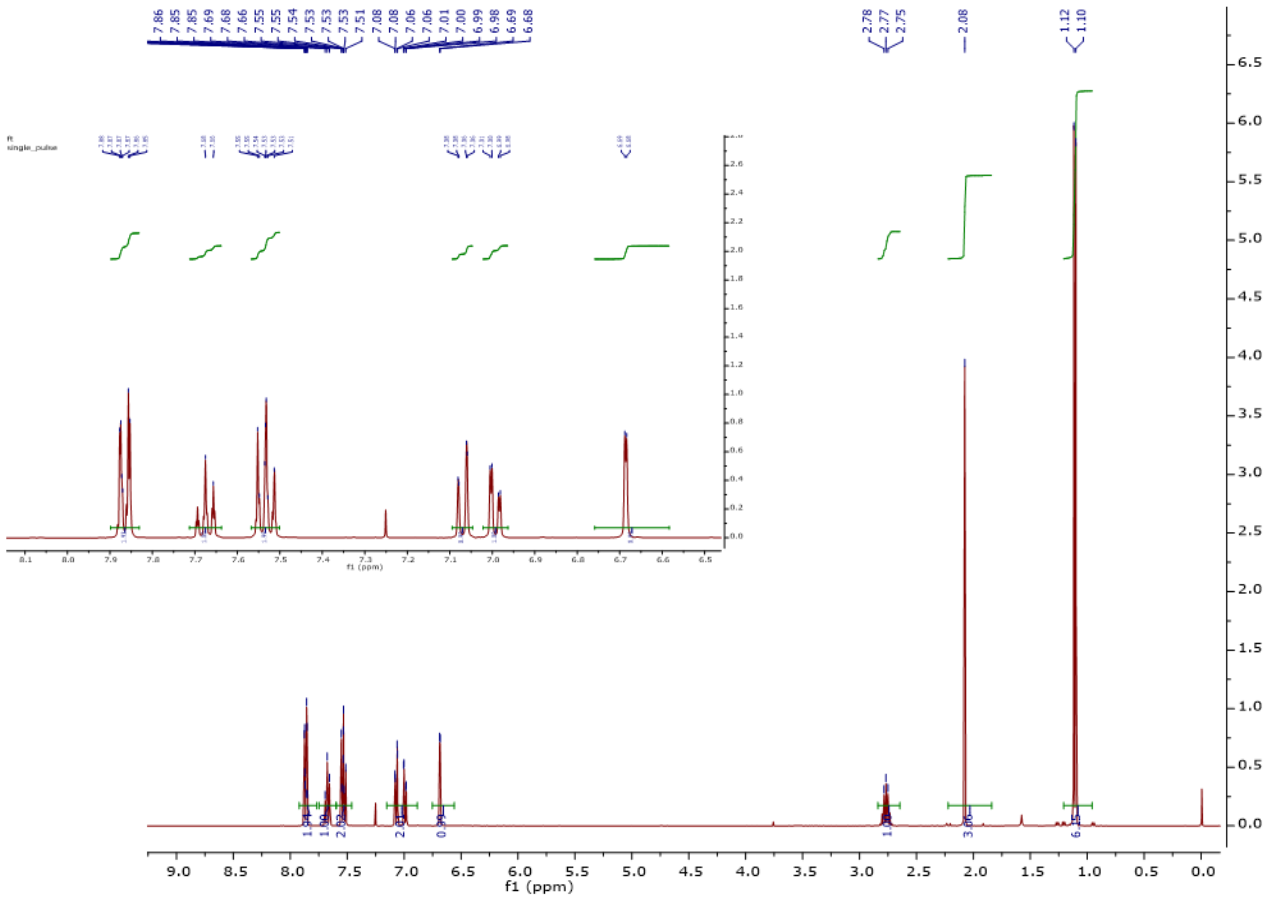
Xie, D. X., Yang, Z. W., Hu, X. R., & Wen, Y. H. (2022). Synthesis and antibacterial and activities of novel capsaicin derivatives containing a sulfonic acid esters Moiety. *Frontiers in Chemistry*, *10*(50), 1–8.

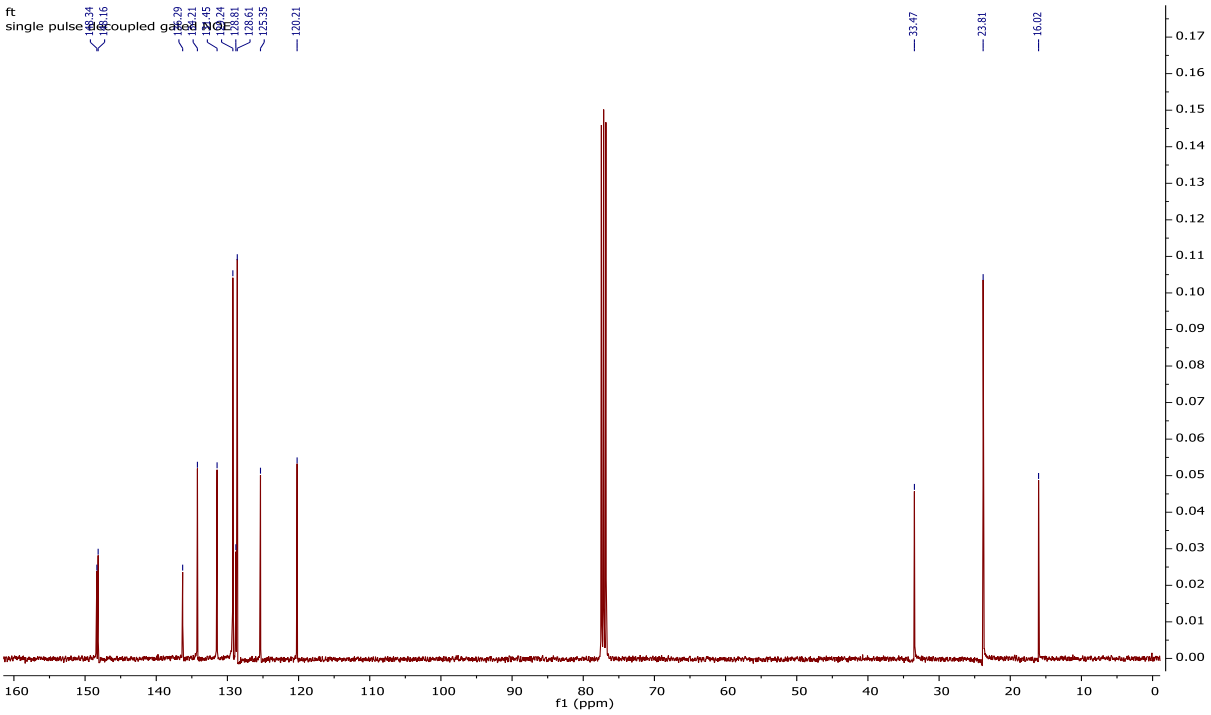
Zhao, A., Sun, J., & Liu, Y. (2023). Understanding bacterial biofilm: from definition to treatment strategies. *Frontiers in Cellular and Infection Microbiology*, *13*(5), 1–23.

# Appendices

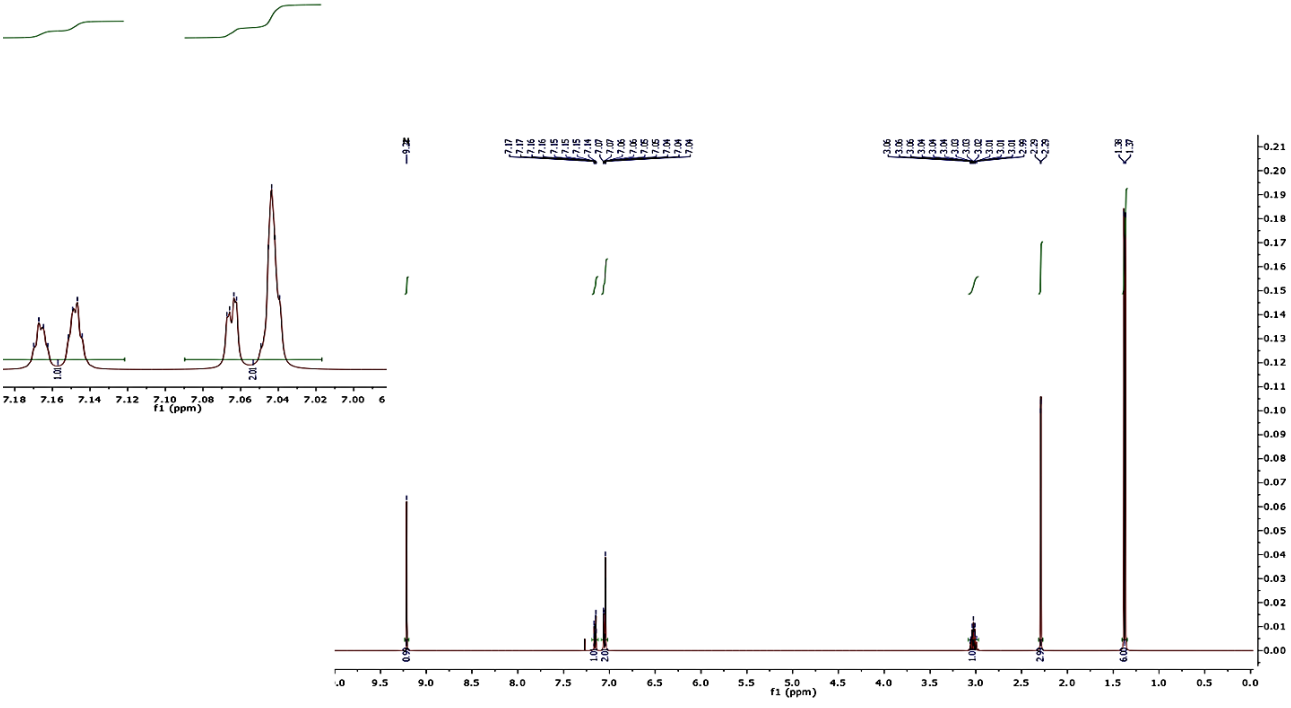
Appendix 1. The $^{1}H$ and $^{13}C$ NMR of the synthesized compounds

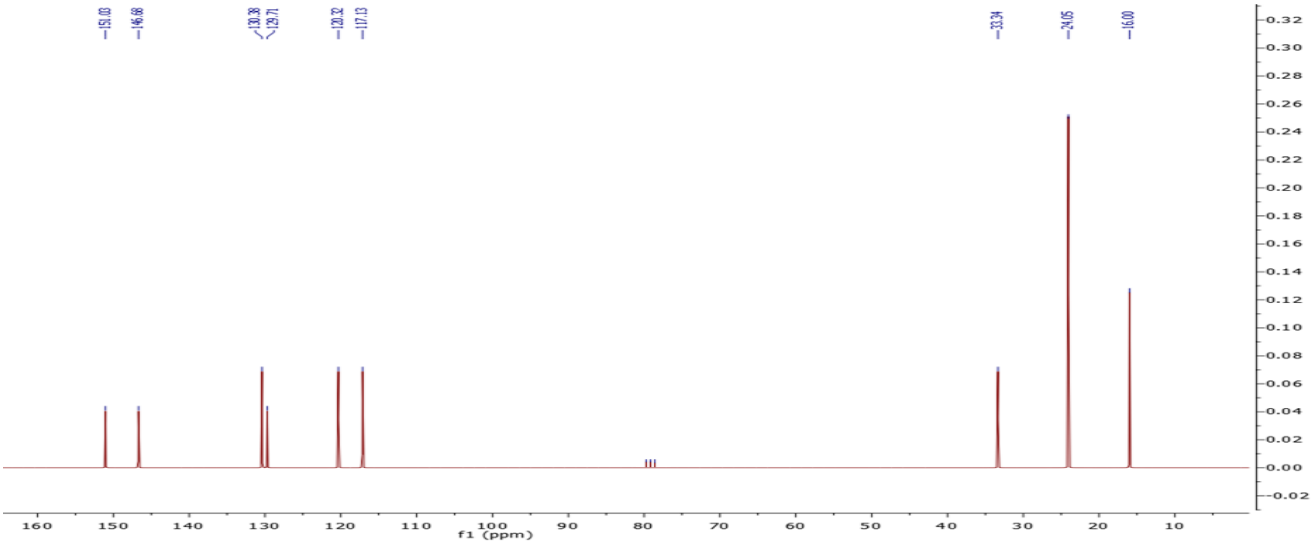
$^{1}H$ and $^{13}C$ NMR spectra of compound **8**






$^{1}H$ and $^{13}C$ NMR spectra of compound **9**






Appendix 2. TLC chromatogram of synthesized compounds

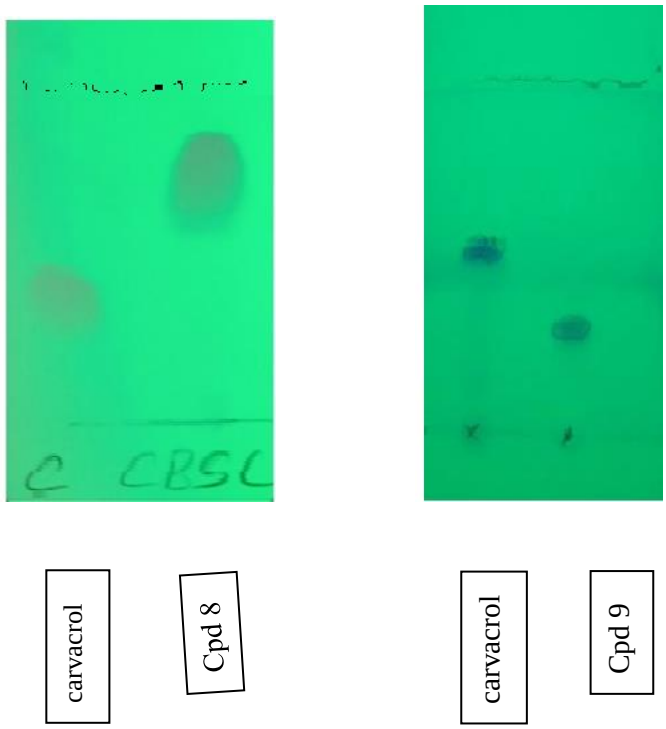



Figure I: Normal Phase TLC chromatograms of compound 8 viewed under UV 254 using solvent system Hexane: chloroform (1:1) and compound 9 viewed under UV 254 using solvent system chloroform: ethyl acetate (1:1).

Appendix 3. Binding interactions of the synthesized compounds in *E. coli* DHPS (PDB ID: 1AJ2) displayed in different diagram of the protein

**Compound 8**

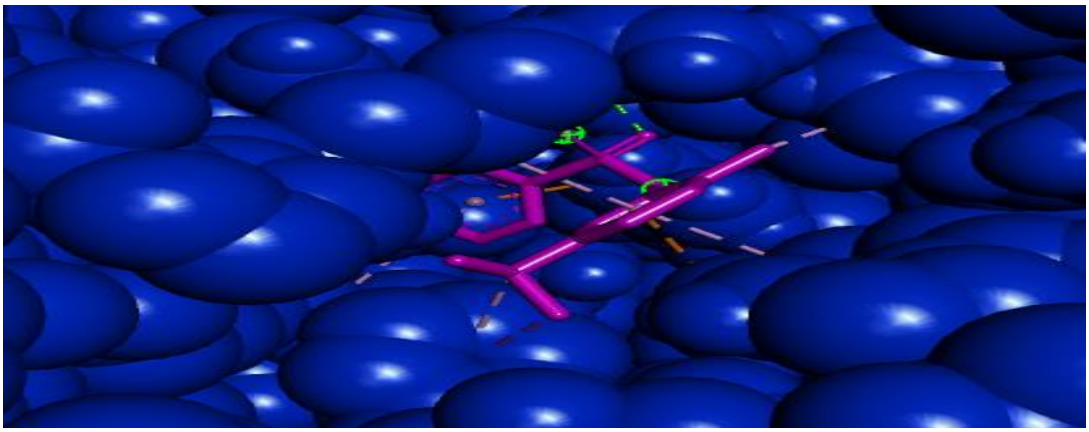


Figure II: compound 8 in binding pocket in space filling model of the protein

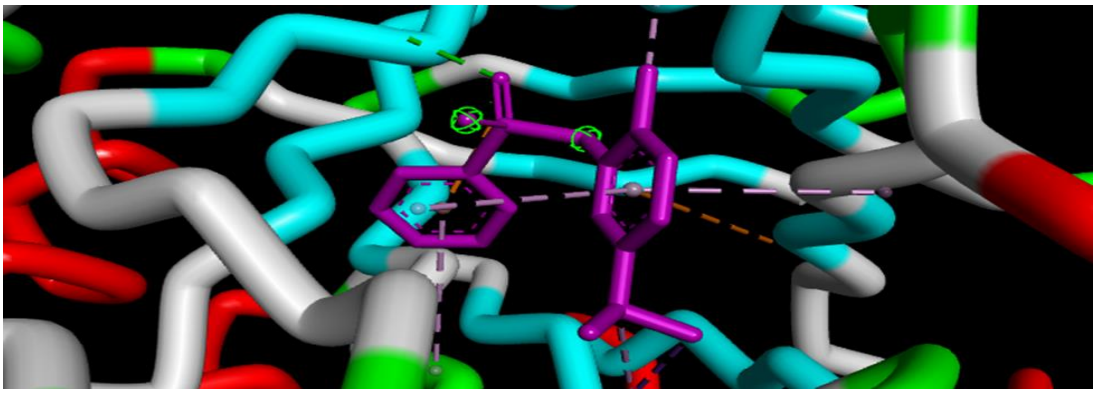


Figure III: compound 8 in binding pocket in solid ribbon diagram of the protein

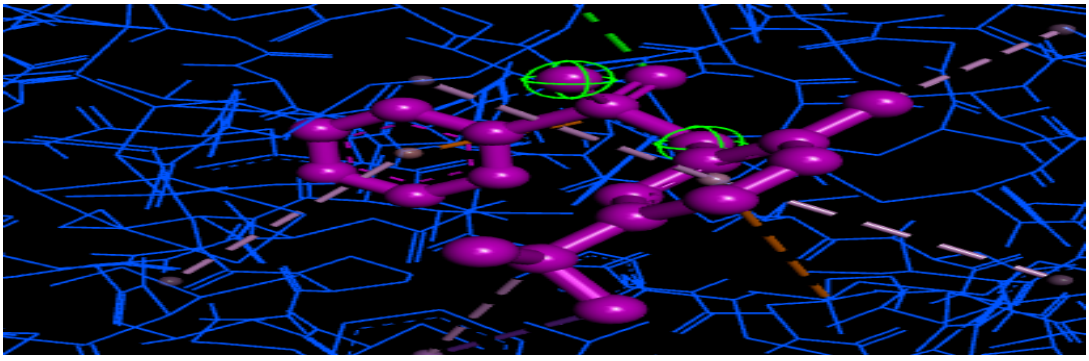


Figure IV: Compound **8** in binding pocket in line ribbon diagram of the protein

**Compound 9**

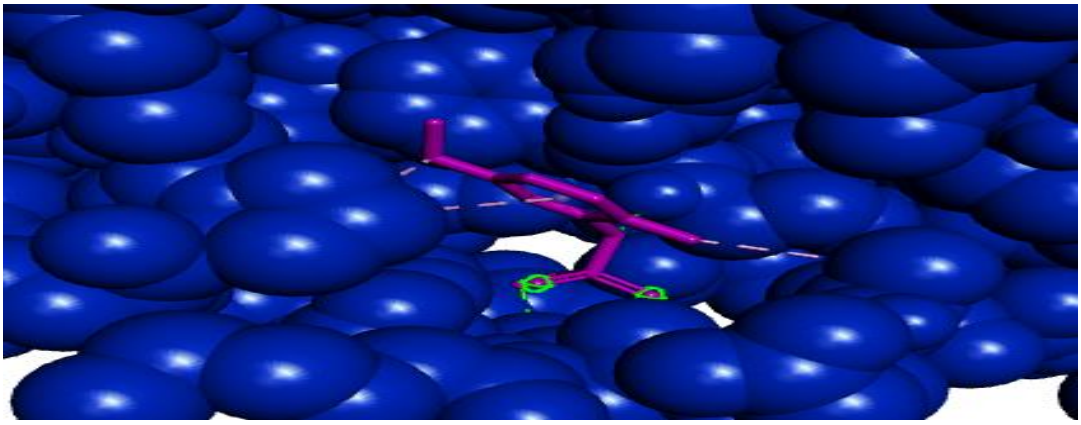


Figure V: compound **9** in binding pocket in space filling model of the protein

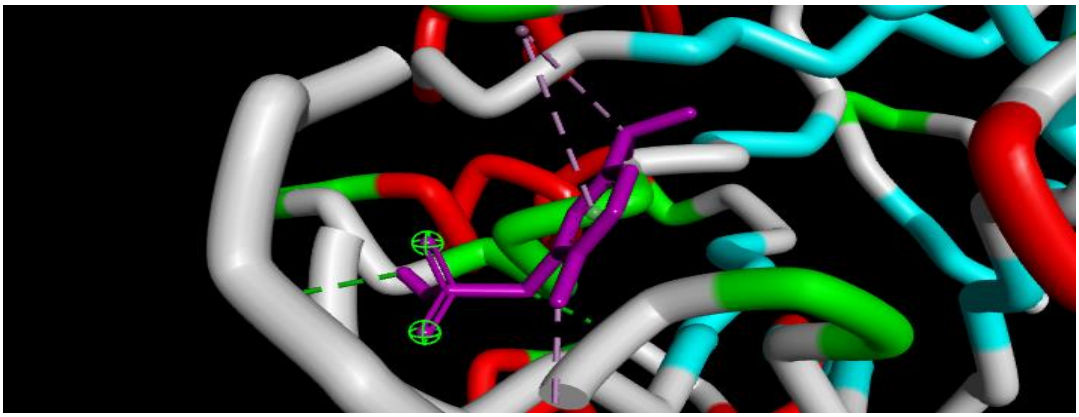


Figure VI: Compound **9** in binding pocket in solid ribbon diagram of the protein

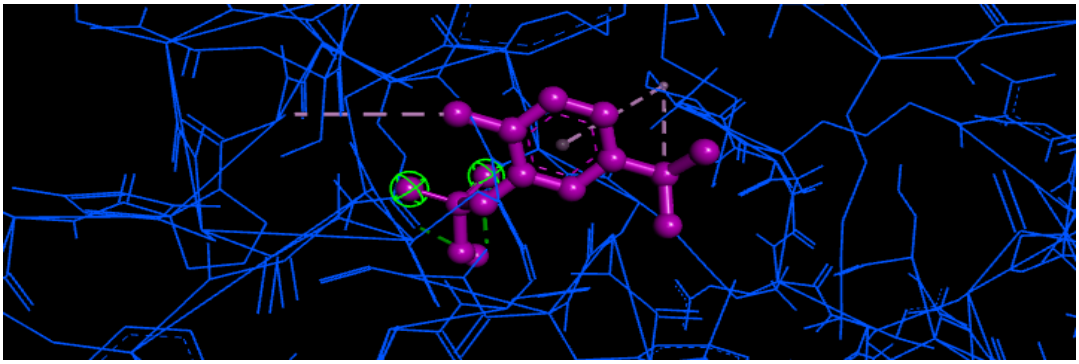


Figure VII: Compound **9** in binding pocket in line ribbon diagram of the protein

# Draft Manuscript from the thesis

# Synthesis and Antimicrobial Activity of Carvacrol Sulfonate Derivatives with *In silico* analysis

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## Abstract

The emergence of new pathogens and antimicrobial resistance pose significant challenges in treating infectious diseases. In this study, we synthesized two sulfonated derivatives of carvacrol, compound **8** (5-isopropyl-2-methylphenyl benzenesulfonate) and **9** (5-isopropyl-2-methylphenyl hydrogen sulfate). The structure of the synthesized compounds was confirmed via $^{1}H$ and $^{13}C$-NMR spectroscopy. Both compounds demonstrated a broad range of antibacterial activity against the tested bacterial strains. Specifically, compound **8** exhibited the highest activity against *Shigella sonnei* 1 and *Shigella boydii* D13629, with a minimum inhibitory concentration (MIC) of 10 µg/mL and a zone of inhibition (ZOI) measuring 16 mm. Against *Aspergillus niger* ATCC 6275, compound **9** showed the highest activity with a lower MIC of 400 µg/mL. Both compounds **8** and **9** interacted with active site residues of *E. coli* DHPS via hydrogen bonding and hydrophobic interactions. Both compounds possess favorable drug-likeness properties.

**Keywords:** Carvacrol, antimicrobial activity, antimicrobial resistance, antibacterial activity, antifungal activity, sulfonated carvacrol, synthesis, *in vitro*, *in silico* studies

## 1. Introduction

The World Health Organization (WHO) defines that infectious diseases are illnesses caused by harmful invaders known as pathogens. The global burden of infectious diseases is a significant public health concern. WHO (2024) reported on the top 10 causes of death, lower respiratory infections (LRIs), one of the leading causes of death globally and were responsible for approximately 2.5 million deaths in low-income countries. According to this report, 8 of the top 10 causes of death in 2021 were communicable diseases.

The emergence of new infections and antimicrobial resistance has been current challenge in treating infectious diseases. According to the 2019 Center for Disease Control and Prevention (CDC) report, more than 2.8 million antimicrobial-resistant infections occur in the U.S. The persistent challenges in treating infectious diseases, coupled with the emergence of new pathogens and antimicrobial resistance, underscore the urgent need for ongoing drug discovery efforts (Ohashi et al., 2022).

Numerous investigations have revealed that sulfonated compounds possess significant antibacterial properties (Liu et al., 2024; Xie et al., 2022). Therefore, based on the antibacterial activity of sulfonated compounds and the well-documented antimicrobial potential of carvacrol and its derivatives, we synthesized two novel sulfonated derivatives of carvacrol in this study.

# 2. Materials and Methods

## 2.1. Materials

### 2.1.1. Chemicals and reagents

The chemicals and reagents that were used for the synthesis process are: hexane, chloroform, ethyl acetate (99.8%), ice water, HCl (10 M), anhydrous pyridine, Benzenesulfonyl chloride (98%), carvacrol (99%) and chlorosulfonic acid (99%) (all from Sigma-Aldrich Co., MO, USA).

### 2.1.2. Instruments

For the analysis of the compounds, silica gel TLC plates (60 F254, 0.2 mm thick) and a capillary tube were used, with a UV-visible Spectrophotometer (Evolution 60S) for visualization, and an electrical weighing balance for mass measurements. Chemical reactions were conducted using a reaction flask, magnetic stirrer with a condenser, stir bar, measuring cylinder, and pipette fillers. For structural characterization, $^{1}H$ and $^{13}C$-NMR spectra were recorded on a JEOL 400 MHz NMR using TMS as an internal standard and chloroform as the solvent. Antimicrobial testing utilized an incubator, autoclave, biosafety cabinet (model 50/60), vortex, UV-visible Spectrophotometer (Evolution 60S), water bath, inoculating loops, pipette tips, pipette filler, test tubes, multichannel pipettes, microtiter plates, agar plates, a ruler, and sterile filter paper disks.

### 2.1.3. Media, microbial strains, and standard drugs

Nutrient Agar (NA), Mueller-Hinton Broth (MHB), Sabouraud Dextrose Agar (SDA) and Sabouraud Dextrose Broth (SDB) were used for media preparation. Ciprofloxacin and Griseofulvin were used as a positive control for antibacterial and antifungal test while 1% DMSO was used as a negative control. Antibacterial assays were performed on the following Gram-positive bacterial strains: *Staphylococcus aureus* MDR10, *S. aureus* MDR 1*, *S. aureus*

MDR 2*, *B. pumilus* 82 and *B. subtilis* ATCC 6633. The Gram-negative bacterial strains used were: *E. coli* K99, *E. coli* K88, *E. coli* 306, *E. coli* LT37, *E. coli* 872, *E. coli* ROW 7/12, *E. coli* 3:37C, *E. coli* CD/99/1, *E. coli* HB101*, *E. coli* C600*, *S. typhi* Ty2, *S.enterica* TD 01, *S. dysentery* 8, *S. soneii* 1, *S. boydii* D13629, *S. flexneri* Type 6, *V. cholerae* 1313, *V. cholerae* 293, *V. cholerae* 1315, *V. cholerae* 85 and *P. aeruginosa* MDR 1*. The antifungal activity testing was performed on: *C. albicans* ATCC 10231, *A. niger* ATCC 6275, *P. notatum* ATCC 11625 and *P. funiculosum* NCTC 287.

## 2.2. Methods

### 2.2.1. Synthesis of sulfonated derivatives of carvacrol (8 and 9)

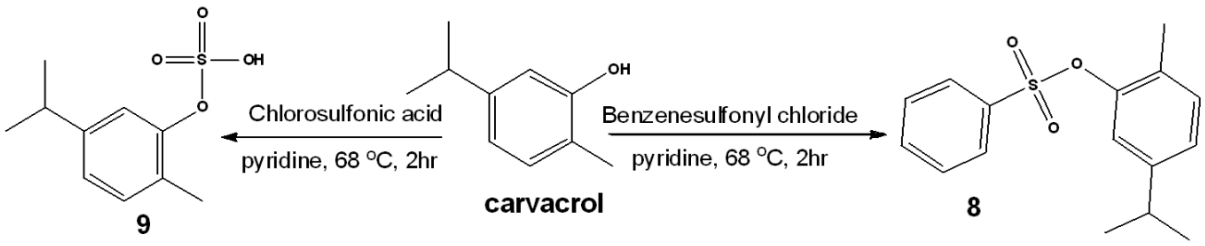
Two sulfonated carvacrol derivatives, 5-isopropyl-2-methylphenyl benzenesulfonate (**8**) and 5-isopropyl-2-methylphenyl hydrogen sulfate (**9**), were synthesized via a reaction of nucleophilic substitution at sulfur. This involved refluxing carvacrol with either benzenesulfonyl chlorides or chlorosulfonic acid in the presence of pyridine (Bagul et al., 2017).

#### 2.2.1.1. Synthesis of 5-isopropyl-2-methylphenyl benzenesulfonate (8)

Benzenesulfonyl chloride (1.0 mmol) was added dropwise to a solution of carvacrol (1.0 mmol) in 5 mL of anhydrous pyridine. The reaction mixture was heated under reflux for 2 h, with the reaction progress monitored by TLC. After completion, the reaction mixture was cooled to room temperature, poured into ice water, and acidified with 10M HCl. A precipitate was formed, collected by filtration.

#### 2.2.1.2. Synthesis of 5-isopropyl-2-methylphenyl hydrogen sulfate (9)

Compound **9** was synthesized following the same procedure used for compound **8**, with chlorosulfonic acid substituted for benzenesulfonyl chloride. The reaction schemes for both compounds **8** and **9** are shown in Scheme 1.




Scheme 1: Synthesis of sulfonated derivatives of carvacrol (8 and 9)

### 2.2.2. Structural elucidations of the synthesized compounds

The structures of compounds 8 and 9 were identified by analyzing their $^{1}H$ and $^{13}C$-NMR spectra data. Chemical shifts are denoted in ppm and Signal multiplicities are indicated as follows: s = singlet; brs = broad singlet; d = doublet; t = triplet; dd = doublet of doublet and m = multiplet

### 2.2.3. In vitro antibacterial and antifungal activity assay

#### 2.2.3.1. Disk diffusion method

The Disk Diffusion antimicrobial experiment was conducted according to the CLSI guideline (CLSI, 2015). Nutrient agar media were sterilized (121°C, 15 min), cooled to 45–50°C, and poured into Petri dishes under a biosafety cabinet. Bacterial inoculum was standardized to $1\times10^{8}$ CFU/mL (0.08–0.1 absorbance at 625 nm) and diluted to $1\times10^{6}$ CFU/mL. Test compounds (2 mg/mL in 1% DMSO) were diluted to 200 µg/mL (antibacterial) or 2000 µg/mL (antifungal). Sterile swabs coated with bacterial suspension ($1\times10^{8}$ CFU/mL) were streaked onto Mueller-Hinton agar, while antifungal tests used Sabouraud dextrose agar. Filter discs impregnated with 200 µg/mL (antibacterial) or 2000 µg/mL (antifungal) compounds were placed on agar.

Plates were incubated (37°C, 24 h for bacteria; 25°C, 72 h for fungi), and zones of inhibition (ZOI) were measured. Controls included ciprofloxacin (bacterial), griseofulvin (fungal), and 1% DMSO.

#### 2.2.3.2. Broth dilution method

Minimum inhibitory concentrations (MICs) were determined using CLSI protocols (CLSI, 2015). Serial dilutions of compounds (2 mg/mL in 1% DMSO) in Mueller-Hinton broth (5–800 µg/mL for bacteria) or Sabouraud dextrose broth (50–2000 µg/mL for fungi) were prepared in 96-well plates. Bacterial ($5\times10^5$ CFU/mL) or fungal suspensions were added to wells and incubated (37°C, 18–24 h for bacteria; 25°C, 72 h for fungi). MICs were identified as the lowest concentration showing no turbidity. Controls included 1% DMSO.

### 2.2.4. *In silico* studies

#### 2.2.4.1. Molecular docking study

Using Autodock Vina in conjunction with the MGLTools suite (version 1.5.7), molecular docking of the synthesized compounds (**8** and **9**) was performed on *E. coli* DHPS protein (PDB: 1AJ2) as potential target for antibacterial activity. The docking process involved four key steps: protein preparation, ligand preparation, grid receptor generation, and molecular docking, all utilizing default values.

#### 2.2.4.2. Pharmacokinetics and drug-likeness properties

The physicochemical, pharmacokinetic, medicinal chemistry friendliness and toxicity profiles of the synthesized compounds were evaluated using ADMETlab 3.0's online software tools, following the methodology described by (Fu et al., 2024).

# 3. Results and Discussion

## 3.1. Synthesis of sulfonated derivatives of carvacrol (8 and 9)

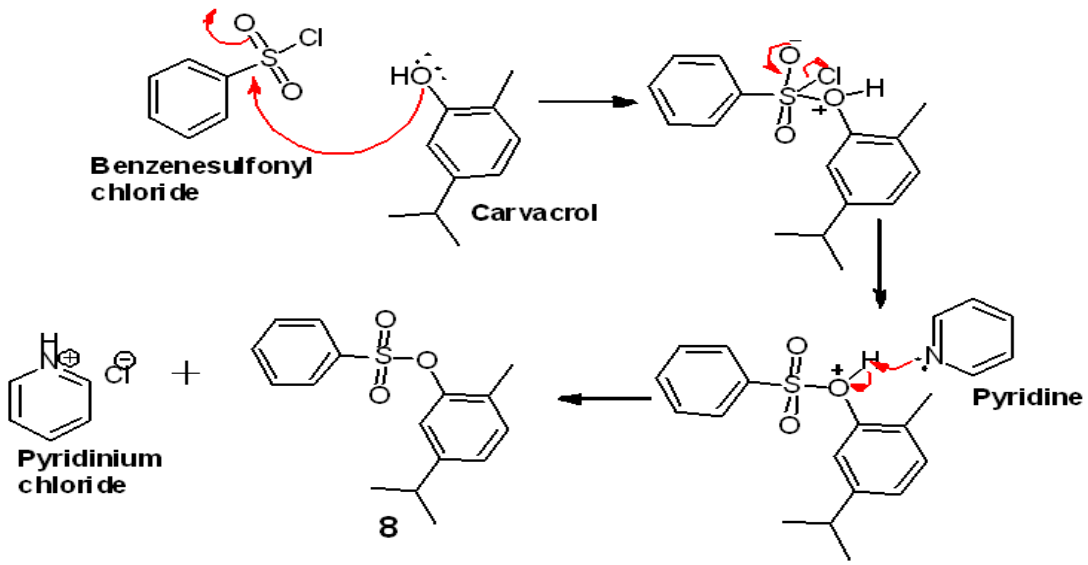
Sulfonated compounds exhibit strong antibacterial properties due to their ability to form hydrogen bonds with proteins and affinity for cell membrane lipids (Liu et al., 2024; Xie et al., 2022). Thus, in this study, we synthesized two novel sulfonated derivatives of carvacrol, 5-isopropyl-2-methylphenyl benzenesulfonate (**8**) and 5-isopropyl-2-methylphenyl hydrogen sulfate (**9**). These compounds were prepared via nucleophilic substitution reactions by refluxing carvacrol with benzenesulfonyl chlorides or chlorosulfonic acid. The physical properties of the synthesized compounds (8 and 9) are presented in Table 1.

Table 1: Physical properties of the synthesized compounds **8** and **9**

| compound | Molecular Formula | Predicted MW(g/mol) | Physical State | Color | % Yield | Rf value |
|---|---|---|---|---|---|---|
| **8** | $C_{16}H_{18}O_3S$ | 290.38 | Crystal | Colorless | 91% | 0.85 |
| **9** | $C_{10}H_{14}O_4S$ | 230.28 | Crystal | Colorless | 74% | 0.35 |

### 3.1.1. 5-Isopropyl-2-methylphenyl benzenesulfonate (8)

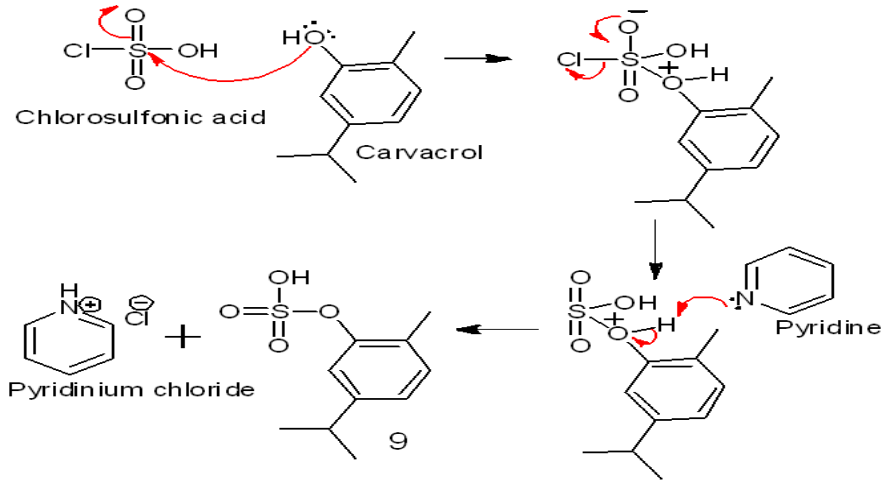
Compound **8**, a white crystalline solid, was obtained in 91% yield through of nucleophilic substitution at sulfur. In this reaction, carvacrol's hydroxyl group acted as a nucleophile, attacking the electrophilic sulfur of benzenesulfonyl chloride, displacing the chloride ion to form Compound 8 and pyridinium chloride. The mechanism is shown in Scheme 2.




Scheme 2: Mechanism of nucleophilic substitution reaction to form compound **8**

### 3.1.2. Synthesis of 5-isopropyl-2-methylphenyl hydrogen sulfate (9)

Compound **9** was synthesized via of nucleophilic substitution at sulfur identical to that of compound 8, with the sole difference being the use of chlorosulfonic acid as the electrophile instead of benzenesulfonyl chloride. In this mechanism, carvacrol's hydroxyl group acts as the nucleophile. The full reaction mechanism is detailed in Scheme 3.




Scheme 3: Mechanism of nucleophilic substitution reaction to form compound **9**

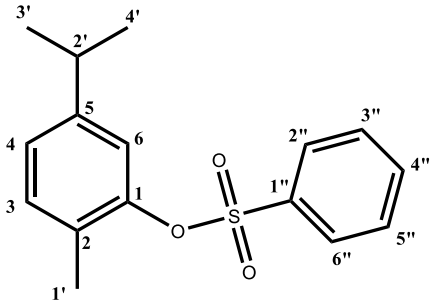
## 3.2. Structural elucidations of the synthesized compounds (8 and 9)

The structures of compounds **8** and **9** were confirmed through detailed analysis of their $^{1}$H and $^{13}$C-NMR spectral data. The chemical shifts, multiplicities, and integration values observed in the $^{1}$H-NMR spectrum, along with the carbon identified in the $^{13}$C-NMR spectrum, were consistent with the proposed structures. Detailed data of their spectral data are presented below.

### 3.2.1. Structural elucidation of 5-isopropyl-2-methylphenyl benzenesulfonate (8)

The $^{1}$H and $^{13}$C-NMR spectra of compound **8** suggest the presence of two substituted aromatic rings and a branched aliphatic side chain. Aromatic proton signals at δ 7.08 (1H, *dd*, *J* = 8, 4 Hz), 6.98 (1H, *dd*, *J* = 8, 4 Hz), and 6.69 (1H, *d*, *J* = 4 Hz) indicate a trisubstituted benzene ring, supported by carbon signals between δ 120.21 and 131.45 ppm. Downfield carbon signals at δ 148.34, 148.16, and 128.81 ppm suggest quaternary aromatic carbons bearing electron-donating or electron-withdrawing groups. In the aliphatic region, a singlet at δ 2.08 (3H) corresponds to a methyl group (C-1′, δC 16.02), while a multiplet at δ 2.77 (1H) and a doublet at δ 1.11 (6H) are consistent with a substituted isopropyl group, with associated carbon shifts at δ 33.43 and 23.81 ppm. The second aromatic system shows a 1,4-disubstituted phenyl pattern with signals at δ 7.88 (2H, *dd*, *J* = 8, 4 Hz), 7.54 (2H, *t*, *J* = 8 Hz), and 7.68 (1H, *t*, *J* = 8 Hz), and corresponding carbon resonances from δ 128.61 to 136.29 ppm. Detailed 1H and 13 C-NMR assignments are presented in Table 2. Finally, based on these data, compound **8** was found to be consistent with h the proposed structure of 5-isopropyl-2-methylphenyl benzenesulfonate.

Table 2: $^{1}H$ and $^{13}C$-NMR spectral data of compound **8**




Compound **8**

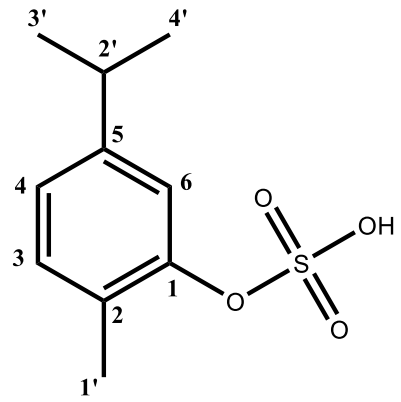
| Position | $\delta_H$ (ppm) | $\delta_C$ (ppm) |
|---|---|---|
| 1 | - | 148.34 |
| 2 | - | 128.81 |
| 3 | 7.08 (1H, *dd*, *J*= 8, 4 Hz) | 131.45 |
| 4 | 6.98 (1H, *dd*, *J*= 8, 4 Hz) | 125.35 |
| 5 | - | 148.16 |
| 6 | 6.69 (1H, *d*, *J*= 4 Hz) | 120.21 |
| 1' | 2.08 (3H, *s*) | 16.02 |
| 2' | 2.77 (1H, *m*) | 33.43 |
| 3', 4' | 1.11 (6H, *d*, *J= 8 Hz*) | 23.81 |
| 1'' | - | 136.29 |
| 2'', 6'' | 7.88 (2H, *dd*, *J*= 8, 4 Hz) | 128.61 |
| 3'', 5'' | 7.54 (2H, *t*, *J*= 8 Hz) | 129.24 |
| 4'' | 7.68 (1H, *t*, *J*= 8 Hz) | 134.21 |

### 3.2.2. Structural elucidation of 5-isopropyl-2-methylphenyl hydrogen sulfate (9)

The $^{1}H$ and $^{13}C$ NMR spectra of compound **9** reveal a substituted aromatic ring with an aliphatic isopropyl-like side chain and hydrogen sulfate group. Three aromatic proton signals appear at δ 7.16 (1H, *dd*, *J* = 8, 4 Hz), 7.07 (1H, *dd*, *J* = 8, 4 Hz), and 7.04 (1H, *brs*), indicating a trisubstituted benzene ring. Their corresponding carbon shifts at δ 117.13 to 130.38 ppm are consistent with protonated aromatic carbons, while downfield signals at δ 151.03, 146.68, and 129.71 ppm suggest quaternary aromatic carbons bearing electron-withdrawing or donating substituents.

In the aliphatic region, a singlet at δ 2.29 (3H) and its carbon at δ 16.00 ppm confirm a methyl group. A multiplet at δ 3.03 (1H) and a doublet at δ 1.38 (6H, *J* = 4 Hz) correspond to a methine and two methyl groups, indicating an isopropyl unit (C-2′, 3′, 4′) with associated carbon shifts at δ 33.34 and 24.05 ppm. A singlet at δ 9.21 ppm reveals the presence of a sulfonic acid hydrogen (-$SO_4H$ group). The remaining $^1H$ and $^{13}C$-NMR chemical shifts are provided in Table 3.

Table 3: $^1H$ and $^{13}C$-NMR spectral data of compound **9**




Compound **9**

| Position | $\delta_H$ (ppm) | $\delta_C$ (ppm) |
|---|---|---|
| 1 | - | 151.03 |
| 2 | - | 129.71 |
| 3 | 7.16 (1H, *dd*, *J*= 8, 4 Hz) | 130.38 |
| 4 | 7.07 (1H, *dd*, *J*= 8, 4 Hz) | 120.32 |
| 5 | - | 146.68 |
| 6 | 7.04 (1H, *brs*) | 117.13 |
| 1' | 2.29 (3H, *s*, CH3) | 16.00 |
| 2' | 3.03 (1H, *m*, CH) | 33.34 |
| 3', 4' | 1.38 (6H, *d*, *J*=4 Hz, $2CH_3$) | 24.05 |
| -$SO_4H$ | 9.21 (1H, *s*, -$SO_4H$) | - |

## 3.3. Antimicrobial activities of synthesized compounds

### 3.3.1. Antibacterial activity

The antibacterial evaluation of synthesized compounds **8** and **9** against a wide range of clinically significant bacterial strains has yielded promising results, as summarized in Table 4.

Both compounds exhibited strong antibacterial activity, with ZOIs ranging from 6 to 16 mm at 200 µg/mL and MICs ranging from 10 to 800 µg/mL (Table 4).

Compound **8** showed particularly significant antibacterial activity against *Shigella* species, with low MICs of 10 µg/mL and ZOIs of 16 mm (at 200 µg/mL) against both *S. soneii* and *S. boydii*. This is highly relevant given the rising antibiotic resistance in enteric pathogens (Kesika & Balamurugan, 2012; Shad & Shad, 2021). Furthermore, compound **8** demonstrated the second-highest activity against multiple *Vibrio cholerae* strains, a key waterborne pathogen. With MICs of 25 µg/mL and ZOIs from 11-15 mm, its activity was comparable to ciprofloxacin in some isolates, positioning it as a potential cholera treatment candidate, especially amidst increasing resistance (Danso et al., 2020). Compound **9**, by contrast, exhibited weaker activity, with MICs uniformly at 100 µg/ml.

Compound **8** exhibited strong antibacterial activity against most E. coli strains, showing ZOIs from 12 to 14 mm and a MIC of 50 µg/mL. While compound 9 had similar ZOI values, its activity was consistently weaker with MICs of 100 µg/mL across nearly all tested E. coli strains. This is particularly relevant given the global health concern of increasing ciprofloxacin resistance in E. coli, with reported resistance rates as high as 73% in some studies (Mandal et al., 2012).

Against *P. aeruginosa* MDR 1 both compounds demonstrated moderate activity (ZOI = 14 mm, MIC = 200 µg/ml). Unfortunately, both compounds were largely ineffective against *B. pumilus* and *B. subtilis*, with no ZOIs and high MICs (>400 µg/ml), suggesting limited utility against these spore-forming Gram-positive species.

Since both compounds exhibit broad-spectrum antibacterial activity, they may have potential for treating polymicrobial infections.

Finally, these results suggest that the synthesized compounds hold significant potential as candidates for the development of new antimicrobial agents. Their activity against a variety of bacterial strains, including drug-resistant pathogens, is particularly promising in light of the growing global challenge of antibiotic resistance.

Table 4: Zone of inhibition and minimum inhibitory concentration of the synthesized compounds (**8** and **9**) against the tested bacterial strains

| Bacteria | ZOI in mm (at 200 µg/ml) | | | MIC (µg/ml) | |
|---|---|---|---|---|---|
| | Compound **8** | Compound **9** | Ciprofloxacin | Compound **8** | Compound **9** |
| *E. coli* K99 | 12 | 13 | 16 | 50 | 100 |
| *E. coli* K88 | 13 | 12 | 17 | 50 | 100 |
| *E. coli* 306 | 13 | 13 | 17 | 50 | 100 |
| *E. coli* LT37 | 13 | 12 | 16 | 50 | 100 |
| *E. coli* 872 | 12 | 13 | 16 | 50 | 100 |
| *E. coli* ROW 7/12 | 12 | 12 | 17 | 50 | 100 |
| *E. coli* 3:37C | 12 | 12 | 17 | 50 | 100 |
| *E. coli* CD/99/1 | 13 | 13 | 17 | 50 | 100 |
| *E. coli* HB101* | 14 | 15 | 16 | 100 | 100 |
| *E. coli* C600* | 14 | 14 | 16 | 100 | 100 |
| *Salmonella typhi* Ty2 | 14 | 12 | 16 | 50 | >800 |
| *Salmonella enterica* TD 01 | 14 | 12 | 19 | 50 | >800 |
| *Shigella dysenteriae* 8 | 14 | 12 | 20 | 50 | 100 |
| *Shigella soneii* 1 | 16 | 12 | 20 | 10 | 50 |
| *Shigella boydii* D13629 | 16 | 14 | 20 | 10 | 50 |
| *Shigella flexneri* Type 6 | 16 | 16 | 21 | 50 | 50 |
| *S. aureus* MDR10 | 15 | 12 | 18 | 400 | 400 |
| *S. aureus* MDR 1* | 14 | 14 | 18 | 100 | 100 |
| *S. aureus* MDR 2* | 14 | 14 | 18 | 100 | 100 |
| *Bacillus pumilus* 82 | 6 | 6 | 19 | 400 | >800 |
| *Bacillus subtilis* ATCC 6633 | 6 | 6 | 18 | 400 | >800 |
| *Vibrio cholerae* 1313 | 13 | 12 | 18 | 25 | 100 |
| *Vibrio cholerae* 293 | 15 | 12 | 19 | 25 | 100 |
| *Vibrio cholerae* 1315 | 15 | 11 | 19 | 25 | 100 |
| *Vibrio cholerae* 85 | 14 | 12 | 19 | 25 | 100 |
| *Pseudomonas aeruginosa* MDR 1* | 14 | 14 | 17 | 200 | 200 |

### 3.3.2. Antifungal Activity

The antifungal activity of synthesized compounds **8** and **9** was evaluated against four fungal strains using griseofulvin as a reference standard. The results indicate that both compounds possess moderate antifungal properties, with ZOIs ranging from 12 to 15 mm at 2000 µg/mL and MICs between 400 and 1000 µg/mL, as presented in Table 5.

Compound **8** generally showed slightly better activity than compound 9 in disk diffusion. For *A. niger* ATCC 6275, compound 8 had a larger ZOI (14 mm) compared to compound 9 (13 mm), though compound 9 exhibited higher potency with an MIC of 400 µg/mL versus 800 µg/mL for compound 8. These findings highlight the significance of both compounds against *A. niger*, a relevant fungal strain in opportunistic infections like pulmonary aspergillosis (Buyi et al., 2023).

Finally, these findings highlight the potential of compounds **8** and **9** as lead structures for further optimization and development of new antifungal agents, especially in the context of increasing resistance to existing treatments.

Table 5: Zone of inhibition and minimum inhibitory concentration of the synthesized compounds (**8** and **9**) against the tested fungi strains

| Fungi | ZOI in mm (at 2000 µg/ml) | | | MIC (µg/ml) | |
|---|---|---|---|---|---|
| | Compound **8** | Compound **9** | Griseofulvin | Compound **8** | Compound **9** |
| *Candida albicans* ATCC 10231 | 13 | 12 | 16 | 1000 | 1000 |
| *Aspergillus niger* ATCC 6275 | 14 | 13 | 15 | 800 | 400 |
| *Penicillium notatum* ATCC 11625 | 15 | 14 | 15 | 800 | 800 |
| *Penicillium funiculosum* NCTC 287 | 13 | 13 | 14 | 800 | 800 |

## 3.4. In silico Studies

### 3.4.1. Molecular docking

Carvacrol is known for its antimicrobial effects via membrane disruption, ATP synthase inhibition, and biofilm/motility reduction, with some evidence suggesting intracellular targets (Trombetta et al., 2005). Particularly, its isomer, thymol, strongly binds to E. coli DHPS with a binding energy of –17.84 kcal/mol (Cruz et al., 2022), Due to their structural and functional similarities, it's possible that DHPS could also be an intracellular target for carvacrol.

Compound **8** demonstrated a strong binding affinity toward the active site of *E. coli* dihydropteroate synthase (DHPS), as detailed in Table 6. Molecular docking results revealed a favorable docking score of –7.3 kcal/mol, indicating a strong and stable interaction with the enzyme. Specifically, compound **8** engaged in hydrogen bonds and multiple hydrophobic interactions with active site residues, as illustrated in Figures 8 through 9.

Table 6: Docking scores and interactions of ligands with active site amino acid residues

| Ligands | Docking score (kcal/mol) within 1AJ2 | Interaction with amino acid residues | | | |
|---|---|---|---|---|---|
| | | H-bonds | Hydrophobic | Electrostatic | miscellaneous |
| **8** | -7.3 | ARG255 and ASN22 | PHE190, LYS221, ARG63, ILE117 and HIS257 | | |
| **9** | -6.4 | GLN149 and GLY19 | PRO64 and ALA151 | | |
| Carvacrol | -5.7 | GLN149 and ASN144 | PRO64 and PRO152 | | |
| 2PH | -8.4 | ASN22, LYS221, HIS257, ASN115, ASP185, ASP96 and THR62 | | LYS221, ARG55, ARG63 and HIS257 | MET139 |

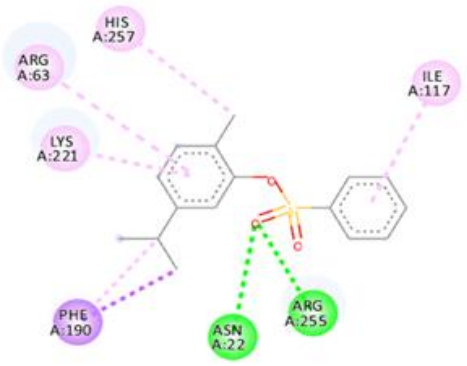



Figure 8: 2D interaction between compound **8** and *E. coli* DHPS (PDB ID: 1AJ2)

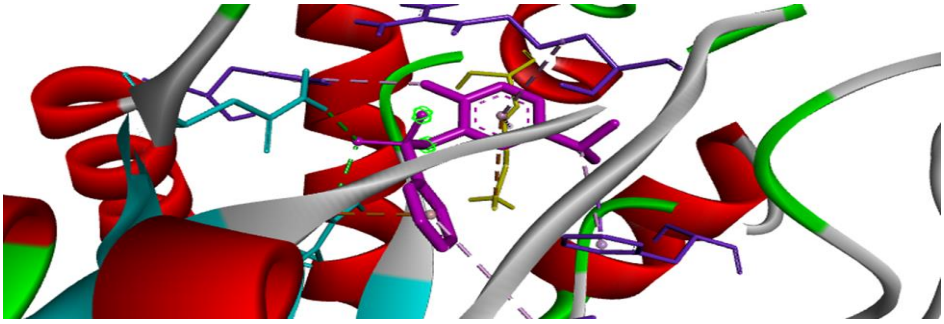


Figure 9: Compound **8** in the active site of *E. coli* DHPS (PDB ID: 1AJ2)

Compound **9** also exhibited effective binding with a docking score of –6.4 kcal/mol, compound **9** demonstrated a favorable binding profile, as of compound **8**. Compound **9** forms two hydrogen bonds with critical residues such as GLN149 and GLY19. These hydrogen bonds likely play a significant role in enhancing both the specificity and stability of the interaction. In addition to these polar interactions, hydrophobic contacts were observed between compound **9** and nonpolar residues, including PRO64 and ALA151. These interactions reveal the potential of compound **8** as a promising DHPS inhibitor.

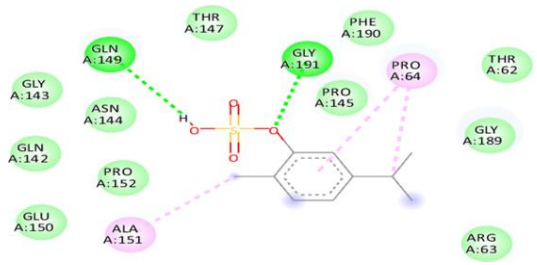



Figure 10: 2D interaction between compound **9** and *E. coli* DHPS (PDB ID: 1AJ2)

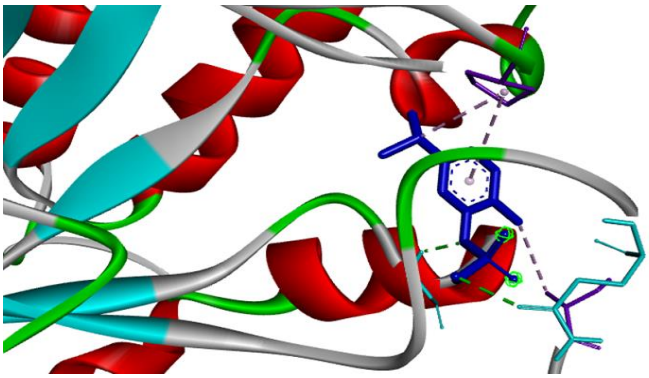


Figure 11: Compound **9** in the active site of *E. coli* DHPS (PDB ID: 1AJ2)

We also conducted a molecular docking study on the starting compound, carvacrol, which yielded a binding score of –5.7 kcal/mol, indicating comparatively weaker binding affinity than compound **8** and compound **9**.

In conclusion, both compounds **8** and **9** demonstrate promising molecular docking results with strong affinities and specific interactions within the DHPS active site. Their engagement through hydrogen bonding and hydrophobic contacts highlights their potential as effective inhibitors, which warrants further experimental validation for antibacterial or antimicrobial applications.

### 3.4.2. Physicochemical and ADMET Properties and Medicinal Chemistry Friendliness

The physicochemical properties of compound **8** and **9** are summarized in Table 7. While most physicochemical parameters fall within the recommended range, compound **8** slightly exceeds the limits for lipophilicity (LogP = 4.253, LogD = 3.591) and water solubility (LogS = -4.394).

Table 7: Prediction of physicochemical properties of compounds **8** and **9** by ADMETlab 3.

| compound | MW | nHA | nHD | nRot | TPSA | LogS | LogP | LogD | pka |
|---|---|---|---|---|---|---|---|---|---|
| **8** | 290.1 | 3 | 0 | 4 | 43.37 | -4.394 | 4.253 | 3.591 | 8.906 |
| **9** | 230.06 | 4 | 1 | 3 | 63.6 | -2.098 | 1.605 | 2.045 | 1.534 |
| Carvacrol | 150.1 | 1 | 1 | 1 | 20.23 | -2.115 | 3.218 | 2.971 | 10.551 |
| Recommended values | | 0-12 | 0-7 | 0-11 | 0-140 | -4-0.5 | 0-3 | 1-3 | |

Table 8 summarizes the medicinal chemistry friendliness parameters for compounds **8** and **9**. Both compounds satisfy all Lipinski's Rule of 5 criteria (molecular weight ≤ 500, LogP ≤ 5, ≤ 5 HBD, and ≤ 10 HBA), indicating good drug-likeness. Additionally, neither compound contains Pan Assay Interference (PAINS) substructures, confirming a favorable profile for further development.

Table 8: Medicinal chemistry friendliness of compounds **8** and **9** by ADMETlab 3.0

| compound | LR-5 | QED | SA score | GASA | PAINS |
|---|---|---|---|---|---|
| 8 | Accepted | 0.802 | 1 | 0.0 | 0 alerts |
| 9 | Accepted | 0.809 | 2 | 0.0 | 0 alerts |
| Carvacrol | Accepted | 0.652 | 1 | 0.0 | 0 alerts |
| Recommended values | | > 0.67 | ≤ 6 | | |

Both compounds (**8** and **9**) demonstrate favorable absorption profiles, with Caco-2 permeability, Pgp-S (P-glycoprotein substrate), and predicted Human Intestinal Absorption (HIA) falling within recommended ranges. This suggests a potential for good oral activity.

Neither compound is predicted to inhibit the major drug-metabolizing enzymes CYP3A4 or CYP2D6. Assessment of potential toxicity via hERG and Ames tests indicate that both compounds are predicted to be safe.

Table 9: ADMET prediction of compounds **8** and **9** by ADMETlab 3.0

| Compound | Caco-2 | Pgp-S | HIA | PPB (%) | VD | BBB | CYP1A2-I | CYP2C19-I | CYP2C9-I | CYP2D6-I | CYP3A4-I | CL | T½ | hERG | Ames |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **8** | -4.526 | 0.002 | 0 | 98.396 | 0.744 | 0.06 | 0.86 | 0.927 | 0.875 | 0.178 | 0.119 | 4.111 | 0.529 | 0.136 | 0.167 |
| **9** | -4.654 | 0.079 | 0.027 | 98.296 | 0.501 | 0.038 | 0 | 0 | 0.009 | 0 | 0 | 5.324 | 0.816 | 0.028 | 0.152 |
| Carvacrol | -4.4 | 0.064 | 0.012 | 91.894 | 0.206 | 0.179 | 0.952 | 0.799 | 0.859 | 0.006 | 0.153 | 11.932 | 0.847 | 0.106 | 0.398 |
| Recommended values | > -5.15 | 0-0.3 | 0-0.3 | ≤ 90% | 0.04 - 20 | 0-0.3 | Category 0-1 | Category 0-1 | Category 0-1 | Category 0-1 | Category 0-1 | ≥ 5 | 0-0.3 excellent<br>0.3-0.7 medium<br>0.7-1 poor | 0-0.3 excellent<br>0.3-0.7 medium | 0-0.3 excellent<br>0.3-0.7 medium |

## 4. Conclusion

In this study, we synthesized and characterized two sulfonated derivatives of carvacrol, 5-isopropyl-2-methylphenyl benzenesulfonate (**8**) and 5-isopropyl-2-methylphenyl hydrogen sulfate (**9**). Compound **8** exhibited superior antibacterial activity, showing particularly potent effects against *S. sonnei* and *S. boydii,* with MICs of 10 μg/mL and strong activity against *V. cholerae* (MIC= 25 μg/mL). In contrast, compound **9** was more effective in antifungal assays, indicating a differing spectrum of activity between the two derivatives. Molecular docking studies further supported the antibacterial potential of both compounds by predicting strong interactions with *E. coli* DHPS. Additionally, *in silico* analyses of physicochemical properties, ADMET, and medicinal chemistry parameters suggest that both compounds possess favorable drug-like characteristics, making them promising candidates for further development.

## Reference


Bagul, S. D., & Rajput, J. D. (2017). Design, synthesis and biological activities of novel 5-isopropyl-2-methylphenolhydrazide-based sulfonamide derivatives. *Research on Chemical Intermediates*, *43*(4), 2241–2252.

Bagul, S. D., Rajput, J. D., Patil, M. M., & Bendre, R. S. (2017). Synthesis, characterization and antioxidant activity of carvacrol based sulfonates. *Medicinal Chemistry*, *7*(10), 294–298.

Berhe, D. F., Beyene, G. T., Seyoum, B., Gebre, M., Haile, K., Tsegaye, M., Boltena, M. T., Tesema, E., Kibret, T. C., Biru, M., Siraj, D. S., Shirley, D., Howe, R., & Abdissa, A. (2021). Prevalence of antimicrobial resistance and its clinical implications in Ethiopia: a systematic review. *Antimicrobial Resistance and Infection Control*, *10*(1), 1–14.

Böttcher, L., & Gersbach, H. (2022). A refunding scheme to incentivize narrow-spectrum antibiotic development. *Mathematical Biology* 84(6), 59.

Buyi, J., Mwambi, B., & Taremwa, I. M. (2023). Prevalence of pulmonary aspergillosis and drug susceptibility testing amongst adult HIV patients. *Student's Journal of Health Research Africa*, *4*(4), 9.

CDC. (2019, April 11). *Antibiotic Resistance Threats Report*. available at https://www.cdc.gov/antimicrobial-resistance/data-research/threats/index.html (Accessed: 28 October 2024).

Clinical and Laboratory Standards Institute. (2015). *Performance standards for antimicrobial susceptibility testing* (CLSI document M100-S25).

Cruz, J. N., Silva, S. G., Pereira, D. S., Souza Filho, A. P., Oliveira, M. S., Lima, R. R., & Andrade, E. H. (2022). *In silico* evaluation of the antimicrobial activity of thymol—major compounds in the essential oil of *Lippia thymoides* mart. & Schauer (Verbenaceae). *Molecules*, *27*(15), 4768.

Danso, E. K., Asare, P., Otchere, I. D., Akyeh, L. M., Asante-Poku, A., Aboagye, S. Y., Osei-Wusu, S., Opare, D., Ntoumi, F., Zumla, A., Duodu, S., & Yeboah-Manu, D. (2020). A molecular and epidemiological study of Vibrio cholerae isolates from cholera outbreaks in southern Ghana. *PLOS ONE*, *15*(7), 1–16.

Doorn, H. R. (2017). Emerging infectious diseases. *Medicine*, *45*(12), 798–801.

Fu, L., Shi, S., Yi, J., Wang, N., He, Y., Wu, Z., Peng, J., Deng, Y., Wang, W., Wu, C., Lyu, A., Zeng, X., Zhao, W., Hou, T., & Cao, D. (2024). ADMETlab 3.0: an updated comprehensive

online ADMET prediction platform enhanced with broader coverage, improved performance, API functionality and decision support. *Nucleic Acids Research*, *52*(1), 422–431.

Kesika, P., & Balamurugan, K. (2012). Studies on *Shigella boydii* infection in Caenorhabditis elegans and bioinformatics analysis of immune regulatory protein interactions. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, *1824*(12), 1449–1456.

Lazarevic, J., Markovic, A., Smelcerovic, A., Stojanovic, G., Ciuffreda, P., & Santaniello, E. (2022). Carvacrol derivatives as antifungal agents: synthesis, antimicrobial activity and in silico Studies on carvacryl Esters. *Acta Chimica Slovenica*, *69*(3), 571–583.

Liu, Y. H., Li, C. K., Nie, M. Y., Wang, F. L., Ren, X. L., Jin, L. H., & Zhou, X. (2024). Sulfonate derivatives bearing an amide unit: design, synthesis and biological activity studies. *BMC Chemistry*, *18*(1), 1–13.

Mandal, J., Srinivas, A. N., Buddhapriya, D., & Parija, S. C. (2012). Antibiotic resistance pattern among common bacterial uropathogens with a special reference to ciprofloxacin resistant *Escherichia coli*. *Indian Journal of Medical Research*, *136*(5), 842–849.

Ohashi, T. K., Nagashima, M. N., Kawai, N. W., & Ohmagari, N. L. (2022). Expert review of anti-infective therapy a narrative review on drug development for the management of antimicrobial-resistant infection crisis in Japan. *Expert Review of Anti-Infective Therapy*, *20*(12), 1603–1614.

Shad, A. A., & Shad, W. A. (2021). *Shigella sonnei*: virulence and antibiotic resistance. *Archives of Microbiology*, *203*(1), 45–58.

Trombetta, D., Castelli, F., Sarpietro, M. G., Venuti, V., Cristani, M., Daniele, C., Saija, A., Mazzanti, G., & Bisignano, G. (2005). Mechanisms of antibacterial action of three monoterpenes. *Antimicrobial Agents and Chemotherapy*, *49*(6), 2474–2478.

WHO. (2024). *Infectious diseases*. available at https://www.emro.who.int/health-topics/infectious-diseases/index.html (Accessed: 26 October 2024).

WHO. (2024, August 7). *The top 10 causes of death*. available at www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death (Accessed: 26 October 2024).

Xie, D. X., Yang, Z. W., Hu, X. R., & Wen, Y. H. (2022). Synthesis, antibacterial and insecticidal activities of novel capsaicin derivatives containing a sulfonic acid esters moiety. *Frontiers in Chemistry*, *10*(50), 1–8.