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GC-MS Analysis of Volatile Constituents, Antibacterial Activity, and Mechanism of Action of the Essential Oil from *Cinnamomum Cassia* Bark Against Vancomycin-Resistant *Enterococci*

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ABSTRACT

Background & Objectives: An alarming rise in multi-drug resistant bacterial pathogen like Vancomycin-resistant *Enterococci* (VRE) has culminated in a global health threat in today's healthcare environment and has caused significant morbidity and mortality in patients. The field of medicinal herbs has developed at an exponential rate in subsequent decades. Acknowledging the antimicrobial mechanisms of essential oils and their components is vital for developing them as a potential new source of antibiotics that can work as an alternative treatment for multi-drug resistant bacterial infections.

Methods: The current study investigates the physiochemical properties and a variety of analytical and biological activities to determine the activity of *Cinnamomum cassia* essential Oil against drug-resistant microorganisms by utilizing a Time kill curve and evaluating the Minimum Inhibitory Concentration (MIC) of oil. The antimicrobial properties were assessed using a growth curve and the cell membrane's integrity. The essential oil was retrieved by hydro-distilling Cinnamon bark, and the volatile components of the essential oil were determined by Gas Chromatography-Mass Spectrometry (GC-MS).

Result: GC-MS analysis detected major components such as Cinnamaldehyde-(E) (37.40%); 2-Propenal, 3-phenyl (11.70%) and 1,6-Octadien-3-ol,3,7-dimethyl- (5.97%). The minimum inhibitory concentration (MIC) and growth curves against VRE were also evaluated. The liberation of cellular contents, as well as changes in cell membrane permeability, have been studied. The MIC value of the essential oil against Vancomycin-resistant *Enterococci* was 15.625 µg/mL speculating that the essential oil seemed to be effective against the drug-resistant culture. When the essential oil of *Cinnamomum cassia* was administered to the bacterial culture, the constituents of the bacterial cell were expelled into the medium.

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Keywords: Multi-Drug Resistance, Vancomycin-Resistant *Enterococci*, VRE, *Cinnamomum cassia* Essential Oil, Time Kill Curve, Leakage, MIC, GC-MS

Abbreviations: *Cinnamomum cassia* Essential Oil (CcEO), Vancomycin-resistant *Enterococci* (VRE), Polymerase Chain Reaction (PCR), Dimethyl sulfoxide (DMSO), Minimum Inhibitory Concentration (MIC), Gas Chromatography-Mass Spectrometry (GC-MS), Mass Selective Detector (MSD), National Institute of Standard and Technology (NIST), Electron ionization (EI)

Introduction

The prevalence of microorganisms that are adaptable to extreme pharmacological treatments has substantially grown in recent years. There is presently no working equation that can correctly evaluate the risks posed by antibiotic misuse and the accompanying rapid emergence of antimicrobial resistance to antibiotics. The potential risk of ingesting antibiotics is the main source of concern over the health risk posed by antibiotic residues in the environment. The rise of antibiotic-resistant bacteria has become a serious concern globally, with significant clinical and economic implications. According to reports, more than two million people suffer from antibiotic-resistant illnesses annually, and at least 23,000 of them lose their lives because of the infection. Gram-positive bacteria, such as Vancomycin-resistant *Enterococci* (VRE), which is also a notable example of multi-drug resistance (MDR), cause nosocomial infections and are associated with increased rates of morbidity and mortality due to their broad range of antibiotic resistance [1]. Alternative treatments for these bacteria are becoming increasingly inadequate, and illness outcomes are substantially impacted. It is possible to take several steps to reduce the harm posed by recently

resistant species [2]. The development of novel antimicrobials has been the first line of defence for many years. The availability of new drugs that can contain resistant microbes, however, is not anticipated for several years [3]. Newer agents, even when they are accessible, are almost always more expensive and frequently more hazardous than the ones that organisms have developed resistance to. Hence, to tackle these limitations, additional tactics are required. Newer alternatives must be studied to keep up with evolving nature of bacterial strains concerning their drug-resistant nature [4].

Most of the plant's natural flora greatly enhances the therapeutic effects [5]. Natural products with a wide range of uses and minimal side effects are in demand to overcome the drawbacks of synthetic drug use. Essential oils are complex, volatile, naturally occurring substances with low molecular weight and potent aromas [6]. They are primarily found in the vapour state because their vapour pressure is sufficient at air pressure and room temperature. Many researchers have focused on essential oils (EOs), which have demonstrated a broad spectrum of antibacterial action against human infections. The chemical classifications of the essential oils derived from aromatic and therapeutic plants include alcohols, aldehydes, ketones, ethers or oxides, esters, amides, amines, phenols, heterocyclic compounds, and primarily terpenes [7]. Terpene alcohol-carrying essential oils had the second-highest antibacterial activity, followed by those containing aldehydes or phenols, such as cinnamaldehyde, citral carvacrol, eugenol, or thymol [8]. The impacts of other essential oils that contain ketone or esters, like myrcene, thujone, or geranyl acetate, are much weaker. It has been revealed that the terpenoids in EOs impede the enzymatic processes of energy metabolism [9].

Notably, *Cinnamomum cassia* has been identified as plants possessing antibacterial and antifungal properties. CcEO contains compounds that may promote heart health and aid in the treatment of diabetes. Its antioxidant properties may aid in the prevention of cancer and the treatment of skin inflammation [5]. The oil can be used in aromatherapy to overcome stress and increase alertness. Essential oil illustrated anticancer activity against prostate, lung, and breast cancers [10]. The primary mechanisms of action of the chemical cinnamaldehyde are to impede the enzymes that produce cell walls, rupture cell membranes, and restrict cell growth [11]. New research has also looked at the synergistic impact of EOs with other antimicrobial agents, as well as how these interactions may be used to change the antibacterial activity of certain antibiotics against antibiotic-resistant bacteria [12]. In view, *Cinnamomum cassia*, either alone or in combination, may be a significant factor against diseases brought on by antibiotic-resistant microorganisms [10]. The objective of the current study is to evaluate the antibacterial effectiveness of CcEO against a significant antibiotic-resistant bacteria strain Vancomycin-resistant *Enterococci* (VRE). Finally, MIC (Minimum Inhibitory Concentration), GC-MS (Gas Chromatography Mass Spectrometry) analysis, and Time Kill curve were used to study the activity and composition of EO.

Material and Methods

Bacterial Characterization

Isolation of VRE

VRE was isolated from soil samples collected from the vicinity of a medical centre in the region of Mumbai, India. The soil samples were diluted in sterile saline and plated on Luria Bertani agar. Prominent colonies were further identified based on Biochemical testing and Molecular characterization.

Biochemical Tests

Vancomycin-resistant *Enterococci* can be selectively isolated on Bile esculin azide (BEA) agar containing Vancomycin [13]. The isolates were streaked on Bile esculin azide (BEA) agar containing 6µg/mL of Vancomycin for isolation of VRE. The plates were incubated at 37^0C for 24 hours and growth was observed after 24 hours.

16S rRNA sequencing and PCR amplification

Genomic DNA was extracted from the isolate by the standard CTAB method [14]. The quality of DNA was evaluated using agarose gel electrophoresis (AGE).

Using universal primers 8F and 907R, the 16S rRNA gene was amplified from isolated DNA (Table 1). The reaction mixture volume was 25µL, which included nuclease-free water, 1X Taq Buffer, 0.5µM of each primer, 100ng of Template DNA, 0.2mM of dNTPs, and 2.5 U of Taq DNA polymerase. 35 amplification cycles were included in the cycling conditions. Pre-denaturation at 95°C for 5 minutes, denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute, and termination at 72°C for 5 minutes [15]. Amplicons were purified and sequenced at Eurofins India Pvt Ltd. The sequence data was submitted to the GenBank for BLAST analysis. Phylogenetic analysis was performed using MEGA X. The gene sequence was submitted to GenBank having accession number ON754239.

The Polymerase Chain Reaction (PCR) was carried out for the detection of the vanA gene in Vancomycin-resistant *Enterococci*. *Van* (*vanA, B, D, E, G*) genes are widely conserved among VRE out of which vanA is the most prevalent [16]. The *vanA* gene was amplified using the primers (Table1) described by He et al.. The sequence of the primer *vanA1* and *vanA2* is mentioned in the table given below. The 25µL reaction system consists of 0.5µM forward primer, 0.5µM reverse primer, 1X Taq Buffer, 100ng of Template DNA, 0.2mM of dNTPs. 35 amplification cycles were included in the cycling conditions. Pre-denaturation at 95°C for 3 minutes, denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute, extension at 72°C for 1 minute, and termination at 72°C for 5 minutes. Amplicons were purified and sequenced at Eurofins India Pvt Ltd. The sequence data was analysed using BLAST tool of NCBI.

Table 1: Primers and its Sequences Used in The Study

Gene	Primers	Sequences
16S rRNA gene [19]	Forward primer 8F	5′-GGATCCAGACTTTGATYMTGGCTCAG-3’
	Reverse primer 907R	5′-CCGTCAATTCMTTTGAGTTT-3’
vanA gene [18]	Forward primer vanA1	5’- GCAAGTCAGGTGAAGATGGA - 3’
	Reverse primer vanA2	5’- GCTAATACGATCAAGCGGTC - 3’

Sample collection and Extraction of Essential Oil

Cinnamomum cassia (Cinnamon) bark was procured from the local market in the region of Mumbai, India. The essential oil was extracted from the bark of the plant. The bark was crushed and then subjected to hydro-distillation with distilled water for 24 hours using a Clevenger apparatus [19]. The essential oil was separated from the hydrosol water using a separatory funnel,

and the hydrosol water was recycled into the flask containing the boiling plant material. The essential oil of cinnamon bark was collected and stored at 4°C for further analysis [20].

Essential oil composition by GC-MS Analysis

GC-MS analysis (Shimadzu GCMS-QP2010) was used to evaluate the volatile components of the essential oil. The Rtx-5MS column with 5% diphenyl and 95% dimethylpolysiloxane chemistry having 30m length, 0.25m diameter, and 0.25m film thickness was used for the assessment [21]. Helium was used as a carrier gas at a flow rate of 54mL/min to evaluate 1µL of essential oil in a split ratio of 1:10. The CcEO components were subjected to regulated experimental conditions oven temperature, which was programmed to vary in a gradient fashion from 40°C with the isothermal environment for 3 min, then ramping up to 120°C for 3 minutes at a rate of 5°C/min, then kept at 180°C for 3 minutes at a rate of 2°C/min, and finally to 230°C for 3 minutes at a rate of 5°C/min. The components of the EO were identified by comparing their mass spectra to those in the Wiley and NIST libraries [22].

Antimicrobial activity by Minimum Inhibitory Concentration (MIC)

Cinnamomum cassia essential oil against VRE was tested for the determination of MIC. MIC results were established by using the 96-well microtiter plate method. Essential oil of varying concentrations was formulated in DMSO [23]. 24-hour-old culture of VRE was adjusted to 10^5 CFU/mL using saline solution [24,25]. Essential oil with no culture was used as a control. Kanamycin was employed as a positive control. 10µL of cultures was seeded into each well. The plate was incubated for 24 hours followed by the addition of resazurin dye for evaluation of results.

Kill Time Analysis

Time-kill curves, which evaluate the growth of bacteria and mortality, were used with essential oils to investigate antibacterial efficacy. The bactericidal activity of *Cinnamomum cassia* essential oil against the test organisms was evaluated by analysing the decrease in CFU/mL during a certain period. Inoculating bacterial cultures in nutrient broth, they were then incubated at 37°C for 24 hours. Overnight cultures of the test pathogen were adjusted to 10^6 CFU/mL [26]. To the cultures was introduced 200µL of the essential oil. Growth control of the essential oil in nutrient broth was maintained. After intervals of 5, 10, 15, 20, 25, 30, 45, and 60 minutes, 100µL of the sample was collected. It was then promptly washed with 900µL of sterile phosphate buffer (pH 7) [27]. Later it was centrifuged for 10 minutes at 1000 rpm before being resuspended in Phosphate buffer. Using the spread plate method, the samples were plated on nutrient agar. Following incubation, microbial colonies were counted, with the findings being recorded.

Antibacterial Kinetics Assay

The growth curve assay was employed to evaluate the essential oil's bactericidal effect. By performing suitable dilutions on the 24-hour-old culture of the test pathogen they were treated with the essential oil at 1 X MIC, 2 X MIC concentrations, and the control containing only DMSO (1%) [28]. The bacteria were then grown at 37 °C for 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, and 36 hours, respectively, while being shaken at 180 rpm. 100µL of the culture was plated on Luria Bertani agar plates at specified intervals and incubated at 37°C for 24 hours to calculate the effect of essential oil on the test pathogen.

Experiment involving leakage of DNA and RNA through the bacterial membrane

CcEO is renowned for its biological antibacterial and antioxidant capabilities [29]. Damaging the cytoplasmic membrane, cytoplasmic coagulation, membrane protein damage, cell wall degradation and increased permeability leading to cell content leakage are a few of the mechanisms essential oils utilize to inhibit bacterial growth [30]. Vancomycin-resistant *Enterococci* was incubated for 24 hours at 37°C in Luria Bertani broth. Bacterial isolates in the log phase were treated with the essential oil at 1 X and 2 X MIC concentrations. Control of only bacterial cells was also maintained. The control and test samples were incubated at 37°C for 24 hours. After 24 hours the bacterial cells were separated from the media by centrifugation at 10,000g at 4°C for 5 minutes. The optical density of the supernatant was evaluated using Nanodrop 2000 UV–vis spectrophotometer by measuring the amounts of Nucleic Acid (ng/µL) released from the cytoplasm at 260nm.

Experiment involving leakage of proteins through the bacterial membrane

Due to their hydrophobic/lipophilic nature, essential oils can effectively pass through the lipid bilayer of bacterial cell membranes, which interfere with the ion transport, cause the leakage of cellular materials, alternate the proton motive force-mediated electron flow, and eventually lead to apoptosis of the bacterial cell [31]. Test pathogens were incubated for 24 hours at 37° C in Luria Bertani broth. Bacterial isolates in the exponential phase were treated with the essential oil at 1X and 2X MIC concentrations. Control without essential oil was maintained. The control and test samples were incubated at 37°C for 24 hours. After 24 hours the bacterial cells were separated from the media by centrifugation at 10,000g at 4°C for 5 minutes. The concentration of protein in the supernatant was determined by the Bradford method [32]. The optical density was measured at 595nm using Nanodrop 2000 UV–vis spectrophotometer to determine the concentration of proteins released.

Result and Discussion

Bacterial Strain Identification

Soil samples collected from the vicinity of the medical center were diluted in 0.8% saline, plated on Luria Bertani agar, and, incubated for 24 hours at 37°C. Bile esculin azide agar was used to assess the Vancomycin resistance of *Enterococci*. Post incubation, the colonies which were observed on the respective plates were referred to as VRE. For absolute confirmation of the strains, molecular identification using genomic DNA was carried out. Upon verifying the quality of DNA, PCR amplification was carried out using primers for the target gene, 16S rRNA, and *vanA* gene for VRE. The amplified product was found to be 721bp for VRE. Based on molecular characterization, it was confirmed that the bacterial isolate was Vancomycin-resistant *Enterococci* (VRE).

Chemical Composition by GC-MS analysis

Components in essential oils can be identified by comparing their relative retention times or indices to their mass spectra (MS) [33]. A total of 35 components of *Cinnamomum cassia* essential oil extracted from the bark of the Cinnamon plant were identified using a Gas Chromatography-Mass Spectrometry method. The data obtained reported Cinnamaldehyde-(E) (37.40%); 2-Propenal, 3-phenyl (11.70%); 1,6-Octadien-3-ol,3,7-dimethyl- (5.97%); Acetic acid-cinnamyl ester (4.42%); cis-. beta. -Farnesene (3.41%); L-. alpha. -Terpineol (2.86%); Phenol, 2-methoxy-4-(2-propenyl)-, acetate (2.84%); 6,6-dimethyl-2-(3-oxobutyl) bicyclo (3,1,1) heptan-3-one (2.68%); 3-Cyclohexene-1-methanol, alpha-alpha-4-trimethyl- (1.46%) and a few other minor components were revealed. The retention times for the compounds were 19.402, 19.452, 13.909, 18.721, 24.878, 16.749, 22.299, 11.558,

and 21.855 minutes, respectively. *Cinnamomum cassia* essential oil has a wide range of therapeutic and commercial applications owing to the inherent compounds observed. Moreover, edible antimicrobial films made from foods like fruits and vegetables have Cinnamaldehyde incorporated into them [34]. It has been evidenced to have potent antifungal [35] and antitumor [36] characteristics. β-Farnesene is widely speculated to have anti-cancer, antibacterial, antifungal, and DPPH free radical scavenging properties [37]. 3-Cyclohexene-1-methanol has been reported to have anticancer activity [38]. Because of its pleasant lilac-like odour, α-Terpineol is widely used in the manufacturing of cosmetics, soaps, perfumes, and antiseptic agents, and is considered one of the most frequently used fragrance compounds [39]. α-Terpineol demonstrated anti-proliferative (antioxidant) activity, which could be used in cancer prevention or treatment. It also demonstrated a potential antioxidant capacity effect against different human cancer cell lines [40]. Although the antimicrobial properties of *Cinnamomum cassia* essential oil and its components have been reviewed previously, the mechanism of action has not been thoroughly studied [41]. Additional in vivo studies and clinical trials would be needed to validate and then further assess these compounds potential as antimicrobial agents [42].

Table 2: Chemical composition of *Cinnamomum cassia* essential oil by GC-MS (Run time: 67 minutes, Mas spectra: 40-1000 m/z, Ion source temperature=220°C)

Sr no.	Component name	Molecular formula	Molecular weight	Classification	Composition %	Retention time
1	Spiro {6,6-dimethyl-2,3-diazobicyclo [3.1.0] hex-2-ene-4,1'-cyclopropane	$C_8H_{12}N_2$	136.19	Organic compound	0.37	8.309
2	1,2-Benzenedicarboxaldehyde	$C_8H_6O_2$	134.13	Organic compound	0.28	9.257
3	beta. -Myrcene	$C_{10}H_{16}$	136.23	monoterpene	0.44	9.709
4	o-Cymene	$C_{10}H_{14}$	134.22	Organic compound	1.09	11.341
5	Limonene	$C_{10}H_{16}$	136.23	monoterpene	0.43	11.474
6	6,6-Dimethyl-2-(3-oxobutyl) bicyclo [3.1.1] heptan-3-one	$C_{13}H_{20}0_2$	208.30	Organic compound	2.68	11.558
7	2,4,6-trimethyl-1,3,6-heptatriene	$C_{10}H_{16}$	136.23	monoterpenoid	0.09	12.476
8	Furan, 2-(2-nitroethenyl)-5-(2-pyrimidylthio)-	$C_{15}H_{12}N_4O_2$	280.28	Adenosine receptor	0.07	13.174
9	Ethanone, 1-(3-ethylcyclobutyl)-	$C_8H_{14}O$	126.20	Organic compound	0.44	13.487
10	1,6-Octadien-3-ol, 3,7-dimethyl-	$C_{10}H_{18}O$	154.25	acyclic monoterpenoids	5.97	13.909
11	Phenylethyl Alcohol	$C_6H_5CH_2CH_2OH$	122.16	Alcohol	0.45	14.342
12	2-(2-Nitrovinyl) furan	$C_6H_5NO_3$	139.11	Organic compound	0.07	14.943
13	Terpinen-4-ol	$C_{10}H_{18}0$	154.25	menthane monoterpenoids	0.51	16.293
14	Ethanone, 1-[5-(1-hydroxyethylidene)-1,3-cyclopentadien-1-yl]-	$C_9H_{10}O_2$	150.17	Organic compound	0.13	16.585
15	L-. alpha. -Terpineol	$C_{10}H_{18}O$	154.25	terpineol	2.86	16.749
16	Cyclohexanol, 1-methyl-4-(1-methylethylidene)-	$C_{10}H_{20}O$	156.26	Organic compound	0.29	16.942
17	1,5-Dimethyl-1-vinyl-4-hexenyl butyrate	$C_{14}H_{24}O_2$	224.34	acyclic monoterpenoids	0.22	18.658
18	Acetic acid, 2-phenylethyl ester	$C_{10}H_{12}O_2$	164.20	acetate ester	1.37	18.721
19	Cinnamaldehyde, (E)-	C_9H_8O	132.16	Organic compound	37.40	19.402
20	2-Propenal, 3-phenyl-	C_9H_8O	132.16	cis-cinnamic aldehyde	11.70	19.452
21	Cinnamaldehyde, (E)-	C_9H_8O	132.16	Organic compound	15.47	19.507
22	1-Butyn-3-one, 1-(6,6-dimethyl-1,2-epoxycyclohexyl)-	$C_{12}H_{16}O_2$	192.25	Organic compound	2.04	19.706
23	2-Propen-1-ol, 3-phenyl-	$C_9H_{10}O$	134.17	cinnamyl alcohol	0.28	20.435
24	3-Cyclohexene-1-methanol.,.alpha.,.alpha.,4-trimethyl-,	$C_{12}H_{20}O_2$	196.29	Organic compound	1.46	21.855
25	Phenol, 2-methoxy-4-(2-propenyl)-, acetate	$C_{12}H_{14}O_3$	206.24	phenylpropanoid	2.84	22.299
26	1-Octyne	C_8H_{14}	110.20	Organic compound	0.26	23.287
27	cis-. beta. -Farnesene	$C_{15}H_{24}$	204.35	beta-farnesene	3.41	24.878
28	1,3,6,10-Dodecatetraene, 3,7,11-trimethyl-, (Z,E)-	$C_{15}H_{24}$	204.3511	Organic compound	0.58	25.591

29	Acetic acid, cinnamyl ester	$C_{11}H_{12}O_2$	176.21	acetate ester	4.42	26.162
30	3-Octen-5-yne, 2,7-dimethyl-, (Z)-	$C_{10}H_{16}$	136.23	Organic compound	0.15	26.400
31	Propanoic acid, pentafluoro-, 1-phenylethyl ester	$C_{11}H_{14}O_2$	178.23	benzyloxycarbonyls	0.07	26.823
32	1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-, [S-(Z)]-	$C_{15}H_{26}O$	222.37	Organic compounds	0.68	32.769
33	3,3-Dimethyl-1-(2-carboxyphenyl) triazene	$C_9H_{11}N_3O_2$	193.20	Organic compound	0.07	33.555
34	10-12-Pentacosadiynoic acid	$C_{25}H_{42}O_2$	374.6	diacetylene	0.12	37.354
35	Benzyl Benzoate	$C_{14}H_{12}O_2$	212.24	benzoate ester	1.32	42.134

Minimum Inhibitory Concentration

The aim of the broth dilution technique is to identify the minimum inhibitory concentration (MIC), or lowest concentration of the tested antimicrobial agent, that, when used in specific test circumstances, prevents the observable development of the under-investigation bacterium [4]. MIC values are used to assess the efficacy of new antimicrobial medications as well as the drug susceptibilities of bacteria [8]. Treatment for Vancomycin-resistant *Enterococci* (VRE) is becoming more and more challenging. *Cinnamomum cassia* essential oil inhibited the test organism, with MIC of 15.625µg/mL for VRE. Researchers discovered that *Cinnamomum cassia* essential oil is mostly constituted of cinnamaldehyde, accounting for around 85% of the total, with almost 9% o-methoxy cinnamaldehyde [43]. *Cinnamomum cassia* essential oil's antibacterial activities can be influenced by the presence of the primary bioactive ingredients, namely Cinnamaldehyde (37.47%) [44]. The bacteriostatic activity of an EO is determined by the presence of specific dominating chemical groups, but synergistic action between the numerous biological components is also crucial [3]. Gene transcription and protein synthesis were both severely hampered [6]. *Cinnamomum cassia* essential oil can be used as an additive in pharmaceutical formulations as well as a substitute for outdated drugs. Another advantage of using essential oils as microbial inhibitory factors is that they are plant-derived and pose zero to no threat to the environment [43]. They are also fit to be vegan products, which are great characteristics towards making their products friendlier to the environment and social standards. To find new antibiotics, it would be useful to conduct more research on the trans-cinnamaldehyde, o-methoxy-cinnamaldehyde, cinnamyl cinnamate, and benzyl cinnamate of *Cinnamomum zeylanicum* and *Cinnamomum cassia*.

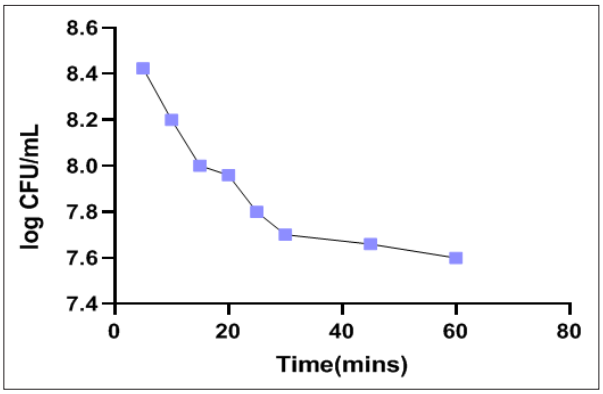

Figure 1: Kill Time curve of *Cinnamomum cassia* essential oil against VRE

Antimicrobial drug resistance has prompted researchers to develop novel antimicrobial compounds to treat a variety of human pathogens [45,46]. In this regard, utilizing essential oils to regulate epidemic multidrug-resistant pathogenic microorganisms can help combat a variety of infectious diseases [46]. Time-kill assays were used to investigate the cell viability (kill-time) of *Cinnamomum cassia* essential oil, and the results were expressed as a logarithm of colony-forming units. 24-hour old Vancomycin-resistant *Enterococci* culture at a dilution of 10^6 log CFU/mL was used. The essential oil showed activity against *Enterococci* resistant to vancomycin. Following 10 minutes of treatment, the bacteria's growth rate significantly dropped from 8.4 log CFU/mL to 7.5 log CFU/mL within an hour, indicating a higher killing rate against pathogenic VRE. Existing research indicates that *Cinnamomum cassia* essential oil has a rapid decapitating effect on VRE growth, with a bactericidal effect after 1 hour of incubation [47]. As previously confirmed by GC-MS analysis, the antimicrobial activity reported was almost certainly due to the presence of antimicrobial compounds in the EO. Compounds such as Cinnamaldehyde- (E) [34]; 3-Cyclohexene-1-methanol [38]; L- alpha-Terpineol [40] are previously known components in essential oils contributing to their antimicrobial properties. The primary element of CcEO extracted from the bark was cinnamaldehyde, which has inhibitory activity against some microorganisms by inhibiting cell enzyme synthesis, disrupting the cell wall structure, resulting in cytoplasm shortage, cytoplasmic granulation, cytoplasm acidity, and depletion of intracellular ATP collect [48]. Resultantly, integrating various components can have additive and synergistic effects; although if present in decreased concentrations, these minor components may strengthen the effect of oil or have other targets in the bacteria population [49].

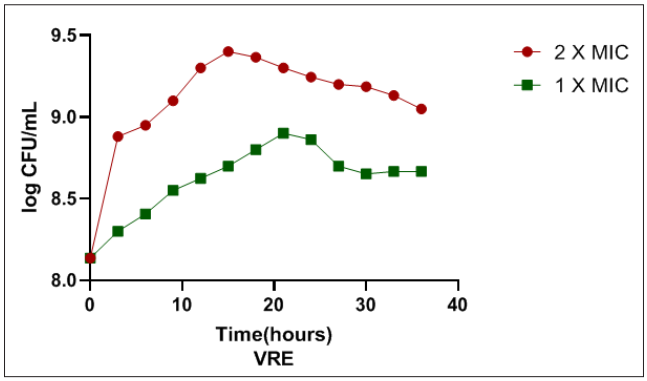

Figure 2: Antibacterial Kinetics Assay of VRE affected by the *Cinnamomum cassia* essential oil

To pass as a good alternative therapy against MDR strains, the selected essential oil must show an adverse influence on the normal growth rate of the bacterial culture. For this, the growth rate should be altered to limit or slow down the proliferation of bacteria. If the essential oil meets this criterion, it can be further used in drug formulations. The Growth Curve Assay was used to analyze the bactericidal effects of *C. cassia* essential oil. Discerning the graph it can be seen that the growth of the test strain was considerably

influenced by the 2X concentration of the MIC value of the oil giving desirable results. There was no clear line between the growth phases in broth concentrations, the lag phase of VRE lasted for approximately 6 hours, the exponential phase from the 9th hour to the 27th hour, and the death phase from the 28th to the 36th hour.

Experiment involving leakage of DNA and RNA through the bacterial membrane

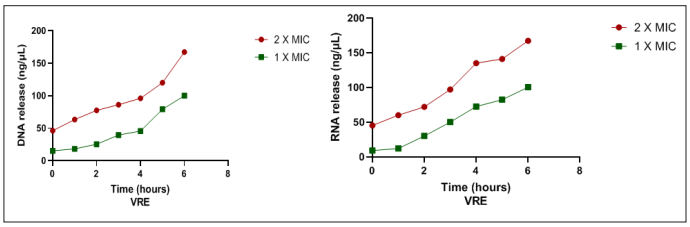

Figure 3: Leakage of DNA and RNA from VRE after treatment with essential oil

The hydrophobicity of the major antibacterial compounds of essential oils enables them to partition into the lipids of the bacterial cell membrane, they disturb the cell structure and increase the membrane permeability which leads to the leakage of nucleic acids and ultimately cell death [50]. The bactericidal effects of the essential oil were tested against Vancomycin-resistant *Enterococci*. The results in the above graphs express that essential oil can cause DNA and RNA leakage by increasing membrane permeability. The concentration of Nucleic acids released in the supernatant increased with the incubation time for VRE. The concentration of DNA and RNA released in the supernatant at 2 X MIC was greater than the concentration of both nucleic acids released at 1 X MIC. This was observed for the bacterial isolate which confirms that the essential oil damages the cell membrane causing the leakage of macromolecules including DNA and RNA. There are several components present in *Cinnamomum cassia* essential oil which contribute to its antibacterial activity. It was revealed by GC-MS results that Cinnamaldehyde is the most abundant compound present in the essential oil. Due to its broad-spectrum bactericidal efficacy, safety, and low toxicity, limonene offers a wide range of potential applications in antibacterial and food preservation [51]. p-Cymene, also known as 1-methyl-4-(1-methylethyl)-benzene, is present in more than 100 plant species and is utilized in both food and medicine. It exhibits a wide spectrum of biological activity, including anti-inflammatory, anxiolytic, anti-nociceptive, antioxidant, and antibacterial properties [52].

Experiment involving leakage of proteins through the bacterial membrane

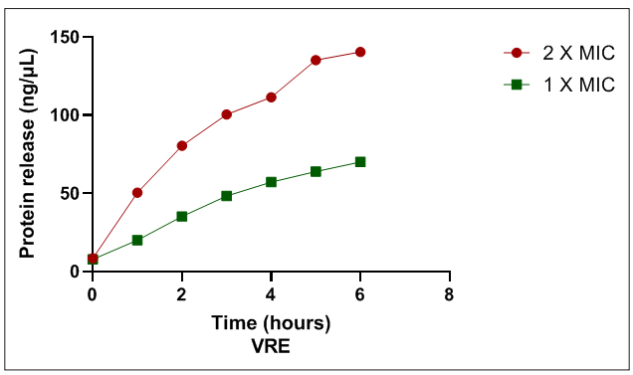

Figure 4: Expulsion of protein from VRE after treatment with essential oil

Essential oils can damage the cellular membrane, enabling cellular materials to leak out, which finally causes the bacterial cell to undergo apoptosis [31]. The graphs reveal that the essential oil can cause protein leakage, proving its bactericidal capabilities against Vancomycin-resistant *Enterococci*. The concentration of proteins released in the supernatant increased with the incubation time for VRE which was measured using Bradford's method. The concentration of proteins released in the supernatant at 2X MIC was greater than the concentration of proteins released at 1X MIC. This was observed for the bacterial isolate. Studies reveal that Cinnamaldehyde present in the essential oil is capable of causing protein leakage by damaging cell permeability, bacterial cell morphology, and membrane integrity [53-55]. It was revealed through SEM data that limonene might alter the cellular morphology of several bacteria. Cell death could result from the breakdown of the cell wall and cell membrane as well as from the extravasation of protoplasm and other intracellular molecules [51]. *Cinnamomum cassia* essential oil is capable of protein leakage which further proves the antibacterial potential of the essential oil.

Conclusion

Treatment of Vancomycin-resistant *Enterococci* (VRE) is getting increasingly difficult. VRE is a fatal bacterium with complicated pathogenic pathways and a high fatality rate. The outcomes of an examination of the specific properties of the *Cinnamomum cassia* essential oil were effective in establishing its therapeutic properties. *Cinnamomum cassia* essential oil has been displayed to inhibit bacteria by altering the lipid profile, membrane porins, motility, and biofilm formation. GC-MS analysis indicates many components with promising antibacterial properties, including Cinnamaldehyde-(E); 2-Propenal, 3-phenyl; 1,6-Octadien-3-ol,3,7-dimethyl-; Acetic acid-cinnamyl ester; cis-. beta. -Farnesene; L-. alpha. -Terpineol and Phenol, 2-methoxy-4-(2-propenyl)-, acetate. Essential oil inhibited development in the growth curves. The leaking of nucleic acids and proteins following the addition of essential oil demonstrates the essential oil's influence on membrane permeability. This confirmation paves the way for further investigation of essential oils and their mechanisms of action. EOs can reduce the lowest effective dose of drugs as well as their possible side effects and treatment costs.

Ethics declarations

Ethics approval and consent to participate: Not applicable.

Data Availability Statement: All data generated or analyzed during this study are included in this published article.

Author Contributions: All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Ethical statement: No animals were harmed during this study.

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