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# Essential Oil of *Chrysopogon Zizanioides* Increases Membrane Permeability, Disturbs Cell Membrane Integrity, and Suppresses the Growth of Methicillin-Resistant *Staphylococcus Aureus* (MRSA)

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## ABSTRACT

**Background & Objectives:** Given the extensive use of antibiotics and the ever-increasing drug resistance of prominent pathogen, Methicillin-resistant *Staphylococcus aureus* (MRSA), essential Oils have been in high demand to rectify inadequacies by providing a broad spectrum of activity with minimal side effects, consequently playing an important role in drug discovery.

**Material & Method:** The present research study explores the therapeutic potential of the Essential Oil extracted from *Chrysopogon zizanioides* commonly known as "*Vetiver*", against MRSA. The essential oil was extracted by hydro-distilling Vetiver grass, and its composition was assessed by GC-MS technique. The antibacterial attribute was evaluated using a growth curve, Time Kill curve, MIC, and the determining the leakage of DNA, RNA, and proteins through the bacterial cell membrane.

**Result:** GC-MS analysis detected major components such as 1,3,5-Cycloheptatriene-1-carboxamide,7-methyl- (25.27%), Diethyl Phthalate (13.78%) and 1,8-Cyclopentadecadiyne (7.42%). Low MIC values hint at the potential antimicrobial nature of the oil. The bactericidal effect of EO was determined by a decline in the number of colonies at varied time intervals. The bacterial cell constituents were expelled into the medium when the essential oil of *Chrysopogon zizanioides* was administered to the bacterial culture. The leakage of cell components was higher with increasing doses of the essential oil. The analysis revealed that *Chrysopogon zizanioides* Essential Oil has potent therapeutic properties that should be explored further.

**Keywords:** MRSA, Vetiver, *Chrysopogon zizanioides* Essential oil, Time kill curve, Leakage, MIC, GC-MS

**Abbreviations:** CzEO: *Chrysopogon zizanioides* Essential Oil; EO: Essential Oil; PCR: Polymerase Chain Reaction; MIC: Minimum Inhibitory Concentration; DMSO: Dimethyl Sulfoxide; GC-MS: Gas Chromatography-Mass Spectrometry; MSD: Mass Selective Detector; NIST: National Institute of Standard and Technology; EI: Electron ionization

## INTRODUCTION

Antibiotics discovery is acknowledged as one of medicine's biggest successes, saving millions; nonetheless, in the last decade, the emergence of antimicrobial resistance in bacteria has surged, defeating the purpose of many clinically relevant medicines [1]. It is reported that more than two million individuals are afflicted by antibiotic-resistant diseases each year, with at least 23,000 deaths as a result of the infection [2]. Gram-positive bacteria, Methicillin-resistant *Staphylococcus aureus* (MRSA) cause nosocomial infections

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and are associated with increased rates of illness and mortality due to their broad range of antibiotic resistance [3]. The rise of antibiotic resistance impairs illness treatment, mandating the development of novel medications and approaches to address this menace. Advancements and research in this sector are not catching pace with the demands for novel and effective medicinal treatments. Consequently, the scientific community is becoming deeply focused on the diversification of the plant world and its metabolites, as they represent key sources of potential compounds with a broad range of action.

Essential Oils (EOs) have been the focus of many studies and have exhibited a broad spectrum of antibacterial activity against human pathogens [4,5]. Notably, *Chrysopogon zizanioides* Essential Oil has been recognized as antibacterial, antifungal, anti-parasitic, anti-convulsant, anti-depressant, antioxidant, anti-inflammation, analgesic, and cytotoxic against cancer due to the presence of components like Cinnamaldehyde, Aromandendrene, Azulene, and Caryophyllene oxide [6]. The main mechanism of action of these compounds is to impede cell proliferation, suppress cell wall-producing enzymes, and disrupt the cell membrane [7]. New studies have also addressed the synergistic effect of EOs with other antimicrobial drugs and how these interactions might be leveraged to modify the antibacterial efficacy of specific antibiotics against antibiotic-resistant bacteria because of which the medications' minimum effective dose is minimized [8]. In a nutshell, EOs offers an effective weapon against illnesses caused by antibiotic-resistant microbes, either alone or in combination.

The current study aims at assessing the antibacterial activity of *Chrysopogon zizanioides* essential oil *in vitro* against strain of significant antibiotic-resistant bacteria, Methicillin-resistant *Staphylococcus aureus* (MRSA). The drug-resistant pathogen was isolated from soil samples in the vicinity of the hospital. MRSA was recognized utilizing PCR to detect the mecA gene. CzEO was examined to see whether it may influence the drug resistance phenomena in antibiotic-resistant strain. Finally, the activity and composition of the essential oil was investigated using MIC (Minimum Inhibitory Concentration), GC-MS (Gas Chromatography-Mass Spectrometry) analysis, Growth Curve, Time Kill curve, and expulsion of Nucleic acids and Proteins.

## MATERIAL AND METHODS

### Bacterial Characterization

### Isolation of MRSA

Soil samples were procured from a nearby area of a medical center in Mumbai, India. After performing suitable dilutions of samples in saline, they were plated on Luria Bertani agar plates for isolation. The colony morphology and Gram nature of the well-isolated colonies were determined. The bacterial culture was further confirmed by biochemical tests and molecular characterization.

### Biochemical tests

Methicillin resistance of the *Staphylococcus aureus* strains was detected using the oxacillin agar test. As a highly sensitive and efficient test, the samples were isolated on a Mannitol salt agar (MSA) consisting of 6 µg/mL of oxacillin [9]. The samples were isolated on these plates and incubated at 37°C for 24 h [10].

### 16S rRNA sequencing and Identification of gene

The CTAB method was used for the isolation of genomic DNA [11]. The 16S rRNA gene was amplified from extracted DNA using universal primers 8F and 907R **(Table 1)**. The reaction mixture volume was 25µL consisting of 18µL nuclease-free water, 2.5µL Taq Buffer,1.25µL each primer, 1µL of Template DNA, 0.5 µL dNTPs, 0.5 µL Taq DNA polymerase. Cycling conditions included 30 amplification cycles, Pre denaturation for 5 min at 95°C, 1 min of Denaturation at 95°C, 1 min of annealing at 55°C, extension for 1 min at 72°C lastly termination at 72°C for 5 min [12]. Agarose Gel Electrophoresis was used to observe the amplified products. Followed by identification of gene using PCR. The methicillin-resistant mecA gene is highly conserved in *Staphylococcal* species; therefore, they are used for the detection of MRSA. PCR amplification using the primers for these genes is a standard for identifying MRSA [13]. The PCR amplification for MRSA was performed according to the method described by Geha et al with slight modification. The primers that were used for mecA gene amplification are given in **Table 2**. 200 µM dNTPs, 2.5 mM (each) primers, 1.25 U of Taq DNA polymerase, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 µM $MgCl_2$, and 0.5% Tween were added and reaction mixture of 25 µL was prepared. The amplification steps included 33 cycles, Pre-Denaturation of 5 min at 94°C, 45 seconds of Denaturation at 94°C, 45 seconds of Annealing at 50°C, 1 min of Primer extension at 72°C and 5 min of final extension at 72°C. The gene sequences were submitted to GenBank. The isolates' accession numbers are listed in **Table 3.**

**Table 1.** Primer and its sequence for the 16S rRNA sequencing.

| | Primer sequence 16S rRNA [14] |
|---|---|
| **8F Forward primer** | 5′-GGATCCAGACTTTGATYMTGGCTCAG-3' |
| **907R Reverse primer** | 5′-CCGTCAATTCMTTTGAGTTT-3' |

**Table 2.** Primer and its sequence for the *mecA* gene.

| | Primer sequence for mecA gene [15] | Position |
|---|---|---|
| **Forward primer** | 5′-GTAGAAATGACTGAACGTCCGATAA-3′ | 318 to 342 |
| **Reverse primer** | 5′-CCAATTCCACATTGTTTCGGTCTAA-3′ | 603 to 627 |

**Table 3.** Isolate characterization and Sequence analysis.

| S No. | Isolate | Organism Name | Accession-ID |
|---|---|---|---|
| **1** | MRSA01 | *Staphylococcus aureus* | ON754237 |

**Sample collection and Extraction of Essential Oil**

*Chrysopogon zizanioides* was acquired at a regional market in Mumbai, Maharashtra. After that, it was brought to the research facility. The roots were cleaned with distilled water to remove any accumulated dirt before oil extraction. Roots were air dried for one week in the shade. The essential oil was extracted by hydrodistillation in a modified Clevenger apparatus for 2 h from 200 g of sample in 2 L of distilled water and stored at 4°C [16]. The extracted oil was further analyzed for its composition and antimicrobial properties against isolated test pathogen.

**Chemical Characterization using GC-MS**

The GC-MS technique was used to investigate the volatile components of the essential oil (Shimadzu GCMS-QP2010) [17]. The evaluation was carried out on the Rtx-5MS column (5 percent diphenyl and 95% dimethylpolysiloxane chemistry, 30 m length, 0.25 m diameter, and 0.25 mm film thickness) [18]. Helium was employed as a carrier gas with a flow rate of 14 mL/min to assess 1 µL of the essential oil in a split ratio of 1:10. To examine the CzEO components, they were subjected to regulated experimental conditions, Oven temperature, which was programmed to differ in a gradient fashion from 70°C with the isothermal environment for 1 min, with a 6 °C/min increase to 260 °C with a 10.00 min isothermal condition. The essential oil components were identified by matching their mass spectra to those in the Wiley and NIST libraries [19].

**Analysis of antibacterial activity by Minimum Inhibitory Concentration (MIC)**

Using the resazurin microplate assay, the minimum inhibitory concentration (MIC) of *Chrysopogon zizanioides* essential oil against the test culture (MRSA) was measured. The method was performed as outlined by Jha [4]. The bacterial suspension was calibrated with a saline solution (0.8%) to $10^5$ CFU/mL. To determine the minimal inhibitory concentration (MIC), several essential oil concentrations were formulated in 2 percent DMSO [20]. The microbial growth was assessed after incubation. After adding 5µL of 2 mg/mL resazurin to each well and incubating at 37°C for 30 min, the MIC was determined. The change in color of resazurin in the microplate wells was used to detect microbial growth [21]. In this assay, the antibiotic Kanamycin was utilized as a positive control.

**Kill Time Analysis**

Time-kill curves that monitor bacterial growth and fatality across a wide range of antimicrobial levels have been widely used to determine antimicrobial efficacy over time [22]. The time-kill curve for CzEO against MRSA was assessed. Bacterial cells were incubated in fresh Nutrient broth at 37°C for 24 h. Following the measurement of the O.D. of the 24 h grown cultures, dilutions of the culture were prepared with an inoculum of approximately $10^6$ CFU/mL before the addition of the *Chrysopogon zizanioides* essential oil [23]. A growth control was maintained by adding essential oil to the nutrient broth. Dilutions were agitated in an incubator set to 37°C. At 5, 10, 15, 20, 25, 30, 45, and 60-min intervals, 100 µL of the sample was retained and rinsed with 900 µL of sterile phosphate buffer (pH 7.0) [24]. After centrifugation at 1000 rpm for 10 min, the samples were re-suspended in a sterile phosphate buffer. Aliquots of each culture were recovered and plated on the Nutrient agar by the spread plate method. After 24 h at 37°C, the number of colonies on the plates was recorded [25]. A bactericidal effect was defined as a decline in the number of colonies from the initial amount over a specific period.

**Antibacterial Kinetics Assay**

Essential oils have been found to have strong antiseptic, antibacterial, antiviral, antioxidant, anti-parasitic, anti-fungal, and insecticidal properties [26]. The bactericidal effects of the essential oil were analyzed using the growth curve assay [27]. The essential oil was incubated with 24-h-old culture of Methicillin-resistant *Staphylococcus aureus* at a dilution of $10^6$ CFU/mL at 1X MIC and 2X MIC in Luria

Bertani broth. Additionally, control was maintained without the essential oil. Samples of the cultures were taken at appropriate intervals (0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, and 36 h). The numbers of colonies were calculated by plating 100µL of the sample on Luria Bertani agar plates and incubating them at 37ºC for 24 h.

**Cell membrane integrity:**

**Experiment involving leakage of DNA and RNA through the bacterial membrane**

Essential oils (EOs) and their components play a vital role in antimicrobial activity. Due to their hydrophobic nature, EOs migrate rapidly across the lipids of bacteria's cell membranes, disrupting cell wall structures and making them more permeable, resulting in ion and other cellular material leakage and, eventually, cell death [28]. The experiment was carried out as follows; MRSA was incubated for 24 h in a Luria Bertani medium at 37°C. Logarithmic growth phase bacterial cells were treated with *Chrysopogon zizanioides* essential oil at 1 × MIC and 2 × MIC levels. The culture was incubated at 37°C and hourly samples were taken. Subsequently, cells were separated by centrifugation (10,000 g) at 4°C for 15 min. The supernatant's optical density at 260 nm was evaluated using Nanodrop 2000 UV–vis spectrophotometer by measuring the amounts of Nucleic Acid (ng/µL) released from the cytoplasm.

**Experiment involving leakage of proteins through the bacterial membrane**

The characterization of proteins that release into the supernatant is one of the crucial indicators for the assessing permeability of the cell membrane [29]. The Bradford method was used to determine the protein concentrations in supernatants [30]. The test pathogen was treated with the essential oil at 1× MIC and 2× MIC concentrations, and hourly samples were taken. Cells were separated by centrifugation (10,000 g) at 4°C for 15 min. To determine the concentrations of proteins released from the cytoplasm, the supernatant was used to measure the optical density at 595 nm using Nanodrop 2000 UV-vis spectrophotometer.

## RESULT AND DISCUSSION

**Bacterial Strain Characterization**

Soil samples taken from the vicinity of the medical center were diluted in saline, plated on the LB plates, and incubated for 24 h at 37°C. Methicillin resistance of *Staphylococcus aureus* was tested using the oxacillin agar test. After incubation, the colonies that grew on the respective plates were labeled as MRSA. For the molecular identification of MRSA, genomic DNA isolation was carried out. Sequencing of the 16S rRNA was performed. The 16S rRNA genes of all isolated bacteria were amplified. The amplicons were then purified and sequenced using a column. To compare the obtained nucleotide sequences to known sequences in the National Center for Biotechnology Information (NCBI) database, the Basic Local Alignment Search Tool (BLAST) was utilized. Later, PCR amplification was carried out. PCR was used for identification and confirmation since it has many advantages over other methods like picking the true positives from the positive results obtained from the other methods [31]. PCR detection was carried out using primers for the target gene, *mecA* for MRSA. The amplified product was found to be 310 bp in the case of MRSA. As a result, it was established that the strain identified from the soil sample was MRSA. Post extraction of essential oil it was analyzed for its chemical composition and antibacterial activity.

**GC-MS Analysis of the Composition of the Essential Oil**

Analysis by Gas Chromatography-Mass Spectrometry system revealed a total of 82 components of the *Chrysopogon zizanioides* essential oil. The most common classes present in the essential oil are sesquiterpene (31.73%), which includes cyclic and oxygenated sesquiterpenes, the organic compound (29.27%), fatty acid ester (19.7 %), terpenes (3.59%), which includes cyclic and diterpenoids, and monoterpene (2.14%), which includes cyclic and oxygenated monoterpenes. The principal components of the essential oil identified were 1,3,5-Cycloheptatriene-1-carboxamide,7-methyl-(25.27%); Diethyl Phthalate (13.78%); 1,8-Cyclopentadecadiyne (7.42%) and Cyclohexanemethanol, 4-ethenyl-.alpha.,.alpha.,4-trimethyl-3-(1-methylethenyl)-(4.74%) having retention time of 17.492, 17.588, 20.305 and 18.513 respectively. The rest of the oil corresponds to other identified compounds such as Copaene, Aromandendrene, and others. Due to the numerous components present, the essential oil offers a variety of therapeutic and industrial purposes. 1,3,5-cycloheptatriene-1-carboxamide,7-methyl-, the most abundant compound in the essential oil belongs to the family of carboxamides with known antibacterial activity against Methicillin-resistant *Staphylococcus aureus* [32]. Cinnamaldehyde (0.50%) is an organic compound well known for its anti-tyrosinase activity [33]. Cinnamaldehyde is widely used as an antimicrobial against oral bacteria and effectively eliminates Gram-positive and Gram-negative bacterial biofilms by inhibiting cell wall synthesis [34,35]. Diethyl phthalate present in the essential oil is used as a potent antimicrobial agent against *Aspergillus* species and it has several industrial uses [36]. Aromandendrene being a sesquiterpene bears a reactive exocyclic methylene group, and a cyclopropane ring grants it the ability to alkylate proteins and disrupt the conformation of proteins making it effective against Methicillin-resistant *Staphylococcus aureus* [37]. Thus, this essential oil has the potential to be employed in the pharmaceutical industry owing to its many capabilities, can also be used as a cosmetical product, and has the potential to be used as a substitute for several commercially available antibiotics **(Table 4)**.

**Table 4.** Chemical composition of *Chrysopogon zizanioides* essential oil. (Run time: 42 min, Mass spectra: 50-650 m/z, Injector temperature: 250°C, Ion source temperature: 220°C).

| S No | Component Name | Molecular formula | Molecular weight | Classification | % Composition | Retention time |
|---|---|---|---|---|---|---|
| 1 | Cinnamaldehyde, (E)- | C9H8O | 132.16 | Phenylpropanoid | 0.5 | 10.206 |
| 2 | Copaene | C15H24 | 204.35 | Tricyclic sesquiterpenes | 0.1 | 11.925 |
| 3 | .alpha.-ylangene | C15H24 | 204.35 | Sesquiterpenoids | 0.04 | 12.45 |
| 4 | Copaene | C15H24 | 204.35 | Tricyclic sesquiterpenes | 0.12 | 12.552 |
| 5 | Tricyclo[7.1.0.0[1,3]]decane-2-carbaldehyde | C11H16O | 164.24 | Organic oxide | 0.11 | 12.722 |
| 6 | Bicyclo[4.1.0]-3-heptene,2-isopropenyl-5-isopropyl-7,7-dimethyl- | C15H24 | 204.35 | Organic compound | 0.15 | 12.92 |
| 7 | 8-Hydroxymethyl-trans-bicyclo[4.3.0]non-3-ene | C10H16O | 152.23 | Esters of keto-carboxylic acids or aldehydo-carboxylic acids | 0.03 | 13.05 |
| 8 | Tricyclo[4.3.1.1(2,5)]undec-3-en-10-ol, stereoisomer | C11H16O | 164.24 | Organic compound | 0.02 | 13.201 |
| 9 | Cycloheptene, 5-ethylidene-1-methyl- | C10H16 | 136.23 | Branched unsaturated hydrocarbons | 0.06 | 13.322 |
| 10 | Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,[1R-(1R*,4Z,9S*)]- | C15H24 | 204.35 | Bicyclic sesquiterpene | 1.89 | 13.566 |
| 11 | 1H-Cyclopenta[1,3]cyclopropa[1,2]benzene, octahydro-7-methyl-3-methylene-4-(1-methylethyl)-, | C15H24 | 204.35 | Tricyclic sesquiterpenes | 0.07 | 13.791 |
| 12 | trans-anti-trans-Tricyclo[7.3.0.0(2,6)]-7-dodecene | C12H18 | 162.27 | Polycyclic hydrocarbon | 0.04 | 13.877 |
| 13 | Cyclopropane, 1-(2-methylene-3-butenyl)-1-(1-methylenepropyl)- | C12H18 | 162.27 | Organic compounds | 0.31 | 14.156 |
| 14 | cis-.alpha.-Bisabolene | C15H24 | 204.35 | Sesquiterpenes | 0.89 | 14.273 |
| 15 | 2(5H)-Furanone, 4-methyl-3,5-bis(2-methyl-2-propenyl)- | C13H18O2 | 206.28 | Organic compound | 0.1 | 14.342 |
| 16 | Aromandendrene | C15H24 | 204.35 | Sesquiterpenoid | 0.04 | 14.435 |
| 17 | Doconexent | C22H32O2 | 328.5 | Omega 3 fatty acid | 0.24 | 14.559 |
| 18 | 1H-3a,7-Methanoazulene, octahydro-1,4,9,9-tetramethyl- | C15H26 | 206.37 | Polycyclic hydrocarbons | 0.04 | 14.742 |
| 19 | .alpha.-ylangene | C15H24 | 204.35 | Sesquiterpenoids | 0.94 | 14.84 |
| 20 | exo-2,7,7-trimethylbicyclo[2.2.1]heptan-2-ol | C10H18O | 154.25 | Monoterpenoid | 0.17 | 14.908 |
| 21 | 1,8-Nonadien-3-yne, 2,8-dimethyl-7-methylene- | C12H16 | 160.25 | Branched unsaturated hydrocarbons | 0.64 | 14.975 |
| 22 | Bicylo[4.1.0]heptane, 7-bicyclo[4.1.0]hept-7-ylidene- | C14H20 | 188.31 | Organic compound | 1.2 | 15.089 |
| 23 | .beta.-ylangene | C15H24 | 204.35 | Sesquiterpenoids | 0.22 | 15.145 |
| 24 | Bicyclo[4.1.0]-3-heptene, 2-isopropenyl-5-isopropyl-7,7-dimethyl- | C15H24 | 204.35 | Organic compound | 0.07 | 15.39 |
| 25 | Pentacyclo[7.5.0.0(2,8).0(5,14).0(7,11)]tetradecane | C14H20 | 188.31 | Organic compound | 0.01 | 15.449 |
| 26 | Oxacyclotetradeca-4,11-diyne | C13H18O | 190.28 | Ethyl ester | 0.31 | 15.567 |
| 27 | Azulene, 1,2,3,3a,4,5,6,7-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, | C15H24 | 204.35 | Sesquiterpene | 0.02 | 15.64 |
| 28 | 1H-Cycloprop[e]azulene, | C15H24 | 204.35 | Sesquiterpenoids | 0.43 | 15.721 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 1a,2,3,4,4a,5,6,7b-octahydro-1,1,4,7-tetramethyl-, | | | | | |
| 29 | .beta.-Vatirenene | C15H22 | 202.33 | Sesquiterpenoids | 0.37 | 15.872 |
| 30 | Norbornane, 2-chloro-1,5,5-trimethyl-, exo- | C10H17Cl | 172.69 | Bicyclic hydrocarbon | 0.24 | 16.147 |
| 31 | 7-Octen-2-ol, 2-methyl-6-methylene- | C10H18O | 154.25 | Monoterpenoid | 0.04 | 16.296 |
| 32 | 2-Octanol, 2-methyl-6-methylene- | C10H20O | 156.26 | Terpenoid | 0.13 | 16.410 |
| 33 | 2-Naphthalenemethanol, decahydro-.alpha.,.alpha.,4a-trimethyl-8-methylene-, | C15H26O | 222.37 | Sesquiterpenoids | 0.51 | 16.582 |
| 34 | 1b,5,5,6a-Tetramethyl-octahydro-1-oxa-cyclopropa[a]inden-6-one | C13H20O2 | 208.3 | Oxanes | 0.12 | 16.772 |
| 35 | Caryophyllene oxide | C15H24O | 220.35 | Sesquiterpenes | 0.88 | 17.046 |
| 36 | 1,3,5-Cycloheptatriene-1-carboxamide, 7-methyl- | C9H11NO | 149.19 | Organic compound | 25.27 | 17.492 |
| 37 | Diethyl Phthalate | C12H14O4 | 222.24 | Phthalate esters | 13.78 | 17.588 |
| 38 | Cubenol | C15H26O | 222.37 | Sesquiterpenoid | 2.36 | 17.859 |
| 39 | 6,9,12,15-Docosatetraenoic acid, methyl ester | C23H38O2 | 346.5 | Fatty acid methyl esters | 0.77 | 18.018 |
| 40 | 2-Naphthalenemethanol, 1,2,3,4,4a,5,6,8a-octahydro-.alpha.,.alpha.,4a,8-tetramethyl-, | C15H26O | 222.37 | Sesquiterpenoid | 0.56 | 18.108 |
| 41 | 8-Isopropenyl-1,3,3,7-tetramethyl-bicyclo[5.1.0]oct-5-en-2-one | C15H22O | 218.33 | Oxygenated sesquiterpenes | 1.32 | 18.358 |
| 42 | Cyclohexanemethanol, 4-ethenyl-.alpha.,.alpha.,4-trimethyl-3-(1-methylethenyl)-, | C15H26O | 222.37 | Sesquiterpenoid | 4.74 | 18.513 |
| 43 | 11-Methylene-tricyclo[4.3.1.1(2,5)]undecane | C12H18 | 162.27 | Branched unsaturated hydrocarbons | 0.89 | 18.643 |
| 44 | trans-Z-.alpha.-Bisabolene epoxide | C15H24O | 220.35 | Sesquiterpenes | 2.35 | 18.784 |
| 45 | 10-12-Pentacosadiynoic acid | C25H42O2 | 374.6 | Unsaturated acyclic monocarboxylic acids | 2.28 | 18.952 |
| 46 | 2-Pentadecyn-1-ol | C15H28O | 224.38 | Saturated long-chain fatty alcohol | 1.20 | 19.093 |
| 47 | Tetracyclo[6.1.0.0(2,4).0(5,7)]nonane, 3,3,6,6,9,9-hexamethyl-, | C15H24 | 204.35 | Bicyclic monoterpenoids | 0.42 | 19.223 |
| 48 | Caryophyllene oxide | C15H24O | 220.35 | Sesquiterpene | 1.09 | 19.346 |
| 49 | 6-Isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydro-naphthalen-2-ol | C15H24O | 220.35 | Cycloeudesmane sesquiterpenoids | 0.26 | 19.467 |
| 50 | 3H-3a,7-Methanoazulene, 2,4,5,6,7,8-hexahydro-1,4,9,9-tetramethyl-, | C15H24 | 204.35 | Sesquiterpenoid | 0.81 | 19.647 |
| 51 | Aromandendrene | C15H24 | 204.35 | Sesquiterpenoid. | 2.18 | 19.853 |
| 52 | Andrographolide | C20H30O5 | 350.4 | Terpenoid | 0.31 | 20.033 |
| 53 | 1,8-Cyclopentadecadiyne | C15H22 | 202.33 | Cyclic alkadiynes | 7.42 | 20.305 |
| 54 | Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-, | C15H24 | 204.35 | Sesquiterpenoids | 0.40 | 20.407 |
| 55 | 1H-Cycloprop[e]azulen-4-ol, decahydro-1,1,4,7-tetramethyl-, | C15H26O | 222.37 | Sesquiterpenoid | 0.69 | 20.596 |
| 56 | Preg-4-en-3-one, 17.alpha.-hydroxy-17.beta.-cyano- | C20H27NO2 | 313.4 | Lipids | 0.31 | 20.667 |
| 57 | Cycloprop[e]indene-1a,2(1H)-dimethanol, 3a,4,5,6,6a,6b-hexahydro-5,5,6b-trimethyl-, | C15H24O2 | 236.35 | Lactarane sesquiterpenoids | 0.56 | 20.749 |
| 58 | 7-Oxabicyclo[4.1.0]heptane, 1-methyl-4-(2-methyloxiranyl)- | C10H16O2 | 168.23 | Cyclic monoterpene | 0.72 | 20.938 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 59 | Lanceol, cis | C15H24O | 220.35 | Sesquiterpenes | 3.00 | 21.090 |
| 60 | 1,3-Bis-(2-cyclopropyl,2-methylcyclopropyl)-but-2-en-1-one | C18H26O | 258.399 | Ketone | 0.70 | 21.175 |
| 61 | .beta.-ylangene | C15H24 | 204.35 | Sesquiterpenoids | 1.36 | 21.358 |
| 62 | Aromandendrene | C15H24 | 204.35 | Sesquiterpenoid. | 0.71 | 21.510 |
| 63 | Cyclopropa[d]naphthalen-2(4aH)-one, 1,1a,5,6,7,8-hexahydro-4a,8,8-trimethyl-, | C14H20O | 204.31 | Sesquiterpenes | 2.79 | 21.618 |
| 64 | Tetracyclo[6.1.0.0(2,4).0(5,7)]nonane, 3,3,6,6,9,9-hexamethyl-, | C15H24 | 204.35 | Bicyclic monoterpenoids | 0.79 | 21.800 |
| 65 | Kauran-18-al, 17-(acetyloxy)-, (4.beta.)- | C22H34O3 | 346.5 | Oxygenated diterpenes | 0.89 | 21.865 |
| 66 | Bicyclo[10.1.0]trideca-4,8-diene-13-carboxylic acid (3-nitro-phenyl)-amide | C20H24N2O3 | 340.4 | Organic compound | 1.94 | 22.084 |
| 67 | Ethanol, 2-[4-(1,1-dimethylpropyl)phenoxy]- | C13H20O2 | 208.3 | Alcohol | 0.52 | 22.201 |
| 68 | 4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-Z)- | C49H74NO8P | 836.1 | Fatty acid methyl ester | 0.04 | 22.834 |
| 69 | Ledene oxide-(II) | C15H24O | 220.35 | Sesquiterpenoids | 0.12 | 22.968 |
| 70 | Adamantane-2-carbonitrile, 4-oxo- | C11H13NO | 175.23 | Adamantanones | 0.09 | 23.599 |
| 71 | Ergostane-3,5,6,12,25-pentol, 25-acetate, (3.beta.,5.alpha.,6.beta.,12.beta.)- | C30H52O6 | 508.7 | Sterols | 0.05 | 24.350 |
| 72 | Chloromethyl 5-chlorododecanoate | C13H24Cl2O2 | 283.2 | Fatty acid esters | 0.03 | 27.140 |
| 73 | 1-Hydroxy-6-(3-isopropenyl-cycloprop-1-enyl)-6-methyl-heptan-2-one | C14H22O2 | 222.32 | Ketones | 0.54 | 28.718 |
| 74 | Kaur-16-en-18-oic acid, methyl ester, (4.beta.)- | C21H32O2 | 316.5 | Diterpenoids | 0.83 | 29.345 |
| 75 | Methyl palustrate | C21H32O2 | 316.5 | Diterpenoids | 0.41 | 29.453 |
| 76 | Methyl dehydroabietate | C21H30O2 | 314.5 | Terpenes | 0.74 | 29.957 |
| 77 | Methyl abietate | C21H32O2 | 316.5 | Terpenes | 0.92 | 30.611 |
| 78 | 1-Naphthalenepentanoic acid, decahydro-5-(methoxycarbonyl)-.beta.,5,8a-trimethyl-2-methylene-, | C22H36O4 | 364.5 | Monohydroxybenzoic acid | 0.52 | 31.600 |
| 79 | Methyl abietate isomer | C21H32O2 | 316.5 | Terpenes | 0.06 | 32.021 |
| 80 | 9,12-Octadecadienoyl chloride, (Z,Z)- | C18H31ClO | 298.9 | Fatty acyl glyceric acids | 0.64 | 34.778 |
| 81 | L-Arginine, N2-[(phenylmethoxy)carbonyl]- | C14H20N4O4 | 308.33 | Amino acid | 0.06 | 35.653 |
| 82 | Propanoic acid, 2-bromo-, ethyl ester | C5H9BrO2 | 181.03 | Fatty acid | 0.07 | 36.083 |

**Antibacterial activity by MIC**

MICs are predominantly used to assess the *in vitro* efficacy of novel metabolites [38]. CzEO showed strong activity against Gram-positive MRSA the MIC values of the test organism were 15.625µg/mL. These antibacterial attributes of the essential oil can be attributed to the presence of the principal bioactive constituents, especially Cinnamaldehyde and Diethyl phthalate [39]. The primary mechanism of action of these components includes gene transcription and protein production restriction. Cinnamaldehyde has been shown to significantly inhibit antibiotic-resistant biofilms [40]. The investigated essential oil and its main active components could be potential candidates to be used as natural alternatives for further applications.

**Kill Time Analysis**

Essential oils are effective in suppressing the growth of drug-resistant bacterial strains [41]. Time-kill assays were used to investigate the cell viability (kill-time) of *Chrysopogon zizanioides* essential oil, and the results were expressed as a logarithm of colony-forming units. 24-h-old cultures of *Methicillin-resistant Staphylococcus aureus* which were diluted to $10^6$ CFU/mL were used. As reported in the **Figure 1** below the survival rate of treated MRSA after 5-10 min decreased from 8.2 log CFU/mL to 7.5 log CFU/mL over the first hour after treatment with *Chrysopogon zizanioides* essential oil. According to existing studies, *Chrysopogon zizanioides* essential oil has a quick decapitating effect on MRSA's growth, with a bactericidal effect after 1 h of incubation [42]. As previously evidenced

by GC-MS analysis, compounds such as Cinnamaldehyde [43], 1,3,5-Cycloheptatriene-1-carboxamide, 7-methyl, Diethyl Phthalate,1,8-Cyclopentadecadiyne [44-46] are commonly associated with antimicrobial properties. According to a previous report, *Chrysopogon zizanioides* essential oil contains components that have antimicrobial activity against a wide range of microorganisms, most likely due to their ability to complex with extracellular and soluble proteins as well as the bacterial cell wall; more lipophilic active compounds may disrupt microbial membranes [47]. Thus, combining various components can have synergistic or additive effects; despite being present in low concentrations, these minor components may enhance the effect of oil or have other targets in microbial species [48].

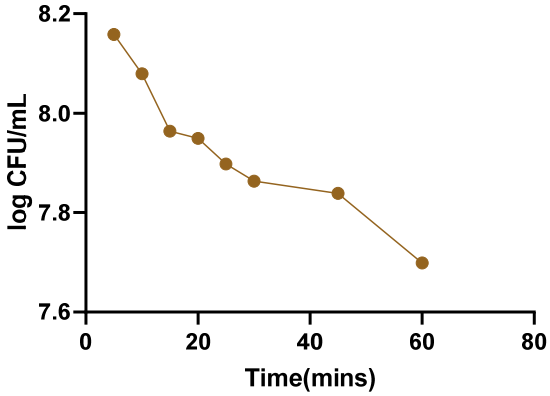


**Figure 1.** Effect of *Chrysopogon zizanioides* essential oil on viability of MRSA.

**Antibacterial Kinetics Assay**

The bactericidal effects of *Chrysopogon zizanioides* essential oil were analyzed using the growth curve assay [27]. The growth curves of MRSA affected by the essential oil are presented in **Figure 2** below. It can be observed in the graphs that the essential oil showed significant inhibition of the growth of the test isolate at 1X MIC and 2X MIC concentrations. The inhibition of the test cultures at 2X MIC was greater than 1X MIC. A steady death phase was observed for both the concentrations of the essential oils. This assay provides evidence that the essential oil of *Chrysopogon zizanioides* inhibits MRSA at all phases of bacterial growth. CzEO serves as a promising antibacterial agent.

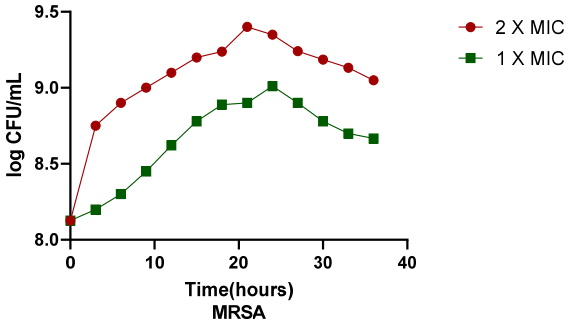


**Figure 2.** Antibacterial Kinetic assay of *Chrysopogon zizanioides* essential oil against MRSA.

**Cell membrane integrity**

**Experiment involving leakage of DNA and RNA through the bacterial membrane:**

Leakage of DNA and RNA content was evaluated to confirm the impact of *Chrysopogon zizanioides* essential oil on the permeability of bacterial cell membranes [49]. **Figure 3** illustrates the effect of Methicillin-resistant *Staphylococcus aureus* treatment with *Chrysopogon zizanioides* essential oil at increasing time intervals. When the Essential oil was added to the bacterial isolate, DNA and RNA were released throughout the six hours of incubation. At 2X MIC, the concentration of DNA and RNA released in the supernatant was higher than the concentration of both nucleic acids released at 1X MIC. According to previous research, the various components of CzEO have highly hydrophobic properties that allow them to be distributed in the lipids of cell membranes and mitochondrion. Cinnamaldehyde-(E) further affects the structural integrity and function of cell membranes, thus causing easier penetration in membranes and resulting in cell content leakage [50].

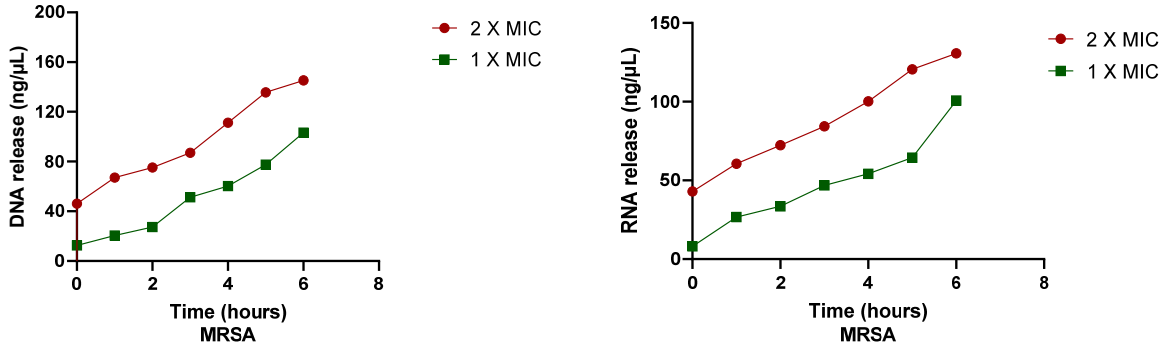


**Figure 3.** Release of genetic material (DNA & RNA) from MRSA when treated with essential oil of *Chrysopogon zizanioides.*

**Experiment involving leakage of proteins through the bacterial membrane:**

The hydrophobicity of essential oils allows them to partition into the lipids of bacteria's cellular membranes, interrupting the structure and making it more porous [51]. Proteins are essential components of microbial species. The impact of *Chrysopogon zizanioides* essential oil on protein leakage from MRSA was investigated. The results shown in the graph above indicate that the essential oil of *Chrysopogon zizanioides* induces protein leakage from the cells into the supernatant of pathogen by damaging the bacterial cell membrane. The protein release concentration was measured using Bradford's method beginning at one hour and expanding for six hours treatment period. The concentration of protein released in the supernatant at 2X MIC was higher than the concentration of protein released at 1X MIC for the isolate (**Figure 4**). Results demonstrated that the compounds Caryophyllene oxide [52]; Aromandendrene when present in high concentrations, caused cellular protein leakage by disrupting cellular integrity. Although a certain amount of bacterial cell leakage can be tolerated without exacerbating cell death, increased loss of cell contents or critical output of molecules and ions can induce cell death.

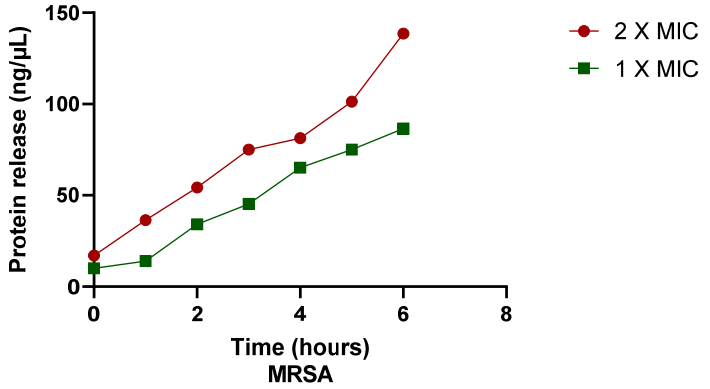


**Figure 4.** Leakage of protein from MRSA when treated with *Chrysopogon zizanioides* essential oil.

## CONCLUSION

Treatment of Methicillin-resistant *Staphylococcus aureus* (MRSA) is becoming increasingly tedious. MRSA is potentially deadly bacteria with complex pathogenic mechanisms and are associated with high rates of mortality. On investigation of the distinct characteristics of the Essential oil, the findings were successful in demonstrating the therapeutic potential. GC-MS analysis reveals various components such as 1,3,5-Cycloheptatriene-1-carboxamide,7-methyl-, Caryophyllene oxide, Aromandendrene, Diethyl Phthalate, 1,8-Cyclopentadecadiyne and Cinnamaldehyde which have a promising antimicrobial attribute. In the growth curve, essential oil showed appreciable results by inhibiting growth. The leakage of Nucleic acids and Proteins after the introduction of the essential oil further exhibits the effect of essential oil on membrane permeability. This confirmation provides a budding demand for consequent exploration of Essential oils and their mechanism of action. In addition to reducing the lowest effective dose of the medications, and reducing their potential adverse effects and treatment costs, EOs can be utilized as an alternative therapeutic application.

## 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.

**Funding:** This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

**Ethical statement:** No animals were harmed during this study.

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