

# Activity of plant essential oils against antibiotic resistant *Enterococcus faecalis* isolated from diarrheic children

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**Abstract:** Activity of plant essential oils and their fractions was evaluated against characterized isolates of antibiotic resistant *Enterococcus faecalis* recovered from diarrheic children. The isolates were confirmed by polymerase chain reaction (PCR) targeting 16S rRNA gene amplification followed by nucleotide sequencing and accession numbers retrieved were MW349990.1, MW349859.1, MW332122.1, MW356805.1, MW349975.1, MW349988.1, MW356790.1, MW356244.1, MW341593.1 and MW332549.1. These isolates were screened for antibiotic susceptibility to a wide range of antibiotic groups and mean zone of inhibition (ZOI) of all antibiotics were recorded. Antibacterial activity of plant essential oils (n=05) was checked against three antibiotic resistant isolates of *E. faecalis*. Three plant essential oils having higher ZOI including *Cinnamomum verum*, *Syzygium aromaticum* and *Nigella sativa* were used against resistant *E. faecalis* isolates to determine minimum inhibitory concentration (MIC). The lowest MIC observed was of *S. aromaticum* (11.39±3.94 mg mL<sup>-1</sup>). The *S. aromaticum* n-hexane plus chloroform fraction displayed higher mean ZOI (16.67±2.51 mm), while the lowest MIC was of n-hexane oil fraction. Based upon gas chromatography-mass spectrometry (GC/MS) analysis, the most effective fatty acid was eugenol which is present in higher proportion in both fractions. These fractions of essential oils proved safe for the treatment of antibiotic resistant diarrheic cases of children caused by *E. faecalis*.

**Keywords:** *Enterococcus faecalis*, antibiotic resistance, *Syzygium aromaticum*, Eugenol, GC/MS.

## INTRODUCTION

*Enterococcus faecalis* is a lactic acid fermenter bacteria which is normal inhabitant of gastrointestinal tract of humans as well as animals and causes nosocomial infections (He *et al.*, 2018). Among enterococci, *E. faecalis* is most frequent in human feces (De Almeida *et al.*, 2019) and can be transmitted via person-to-person through contaminated food or environment and causes soft tissue infections, bacteremia, endocarditis and urinary tract infections (Golob *et al.*, 2019). The *E. faecalis* and *E. faecium* are significant cause of a bacteremia especially in immunocompromised and healthcare-associated individuals with high mortality rate. The problem of enterococci infections in the last decade was directly related to the emergence of antimicrobial resistance. The alternative therapeutic options for enterococci are vaccines, nutraceuticals, immunomodulation agents, probiotics, bacteriophages, etc. (Loubet *et al.*, 2020). Most of the enterococci strains are vancomycin resistant and can transfer the resistance carrying genes (Stepień-Pyśniak *et al.*, 2018). Phytochemicals can be used as source of drug due to antibiotic resistance ability of bacteria. The plant origin compounds include essential oils, volatile compound mixture and aromatic compounds which have antibacterial activity. Essential oils contain

phenolic compounds (secondary metabolites) which are important for antibacterial activity (Liu *et al.*, 2020).

Cinnamon oil extracted from bark and leaves by steam distillation is effective against cold or flu infections, arthritis, skin infections and menstrual cramps (Wajs-Bonikowska *et al.*, 2021). *Nigella sativa* (black seed) and main chemicals including thymoquinone, linoleic acid,  $\alpha$ -pinene and thymohydroquinone has most therapeutic properties (Badri *et al.*, 2018). *Syzygium aromaticum* (Hamad *et al.* 2017) having eugenol, eugenol acetate and gallic acid as active components have broad spectrum antibacterial activity (Zhang *et al.*, 2017). Clove oil can be used as antifungal, insecticidal, antioxidant, antiseptic and antimicrobial (Qureshi *et al.*, 2019). Essential oils in combination with other antimicrobials can broaden the antimicrobial effectiveness against the infectious diseases. Components of EOs are usually derived from terpenes and their oxygenated derivatives and terpenoids. The components evaluated such as cinnamaldehyde (the major component of cinnamon oil) is effective against gram-positive infections. The GC/MS is the technique used for the fractionation of chemical components of essential oils (Behbahani *et al.*, 2019).

Present study was conducted to determine the antibacterial activity of plant essential oils as alternate therapeutics against antibiotic resistant *E. faecalis* isolates

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as well chemical component analysis of effective plant essential oil by GC/MS analysis.

## MATERIALS AND METHODS

### *Characterization of Enterococcus faecalis*

*E. faecalis* (n=10) isolates from children suffering with diarrhea were procured from the Institute of Microbiology, University of Veterinary and Animal Sciences (UVAS), Lahore. These procured isolates were previously characterized by colonial morphology, microscopic appearance and biochemical profile. Bead stocks were cultured on nutrient agar and incubated aerobically at 37°C for 24 hours. Bacterial microscopic appearance and microscopic characteristics were confirmed by Gram's staining. Deoxyribose nucleic acid (DNA) was extracted by manufacturer recommendations of DNA extraction kit. The extracted DNA was visually confirmed by agarose gel electrophoresis using 0.8% agarose gel. The isolates were confirmed by PCR using 16S rRNA gene specific primers following the method of Asghar *et al.* (2016) and the primer sequences used were Forward: 8FLP: 5'-AGTTTGATCCTGGCTCAG-3', Reverse: XB4: 5'-GTGTGTACAAGGCCCGGGAAC-3'. Reaction mixture was prepared by mixing 2µL of DNA template with 12.5µL of 2X PCR master mix and 1µL of each forward and reverse primer (10 pmol). To make the final volume of reaction mixture up to 25 µL DNase and RNase free water (8.5µL) was added. The amplification was carried out at 94°C for 10 minutes, followed by 35 cycles at 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes and final extension at 72°C for 10 minutes.

Amplification product was confirmed by agarose gel electrophoresis using 1.5% gel concentration having ethidium bromide 0.5µg mL<sup>-1</sup>. Gel results were visualized and recorded as mentioned above. The amplicons were subjected to sequencing and FASTA files were retrieved. These sequences were cleaned by using JUSTbio software and on the basis of these sequences phylogenetic analysis was performed using Molecular Evolutionary Genetics Analysis across Computing Platforms (MEGA X) software. These sequences were submitted to National Center for Biotechnology Information (NCBI) through Gen Bank and accession numbers were received.

### *Antibiotic resistance pattern*

The isolates of *E. faecalis* (n=10) which confirmed on the basis of 16S rRNA amplification were subjected to antibiotic susceptibility. For this purpose, disk diffusion method (Kirby Bauer Method) was performed following the guidelines provided by clinical and laboratory standards institute (CLSI-2020). Inoculum 0.5 McFarland was prepared by adjustment of suspension optical density to 0.1 at 630 nm. The inoculum was cultured on nutrient agar plates by spread plate method. Commercially available antibiotic disks of six antibiotics classes viz.,

penicillin, cephalosporin, carbapenems, macrolides, tetracycline, vancomycin and aminoglycosides were placed on agar plates by disk dispenser and pressed for firm contact with agar plates. After 24 h of incubation at 37°C, plates were observed for a confluent lawn of growth and clear ZOI. Diameter of ZOI was measured in millimeter by the help of a ruler and compared with CLSI-2020 guidelines to mark them as sensitive or resistant (Wayne 2020).

### *Activity of essential oils against antibiotics resistant E. faecalis*

Isolates of *E. faecalis* (n=03) which were resistant to multiple antibiotics were subjected to antibacterial activity of commercially available plant essential oils (n=05) including *Curcuma longa*, *S. aromaticum*, *C. verum*, *N. sativa* and *Prunus dulcis* by well diffusion method. For this purpose nutrient agar plates were seeded with 24hours inoculum of 0.5 McFarland and wells of 6mm in diameter were cut using sterile borer. Oils and dimethyl sulfoxide (DMSO) in 1:1 ratio were poured in the well and DMSO was used as negative control followed by incubation at 37°C for 18-24 hrs. The antibacterial activity was determined by measuring the ZOI in mm around the wells.

### *MIC of essential oils*

MIC was performed to evaluate the inhibition of visible growth with minimum amount of essential oils following the method of Jones and his colleagues with minor modifications (Jones *et al.*, 2021). A 96 well flat bottom micro titration plate was taken and 100µL of nutrient broth was pipetted in from 1<sup>st</sup> to 12<sup>th</sup> well then 2 fold serial dilution of 100µL of essential oils was made from 1<sup>st</sup> to 10<sup>th</sup> well. Bacterial suspension 100µL was poured in from 1<sup>st</sup> to 11<sup>th</sup> well, in 12<sup>th</sup> well nutrient broth was added and optical density (O.D.) was noted at 630nm at 0 time and 24 hour after the incubation at 37°C.

### *Fractionation of essential oil*

Oil fractions of *S. aromaticum* were isolated using different solvents. Two columns were selected of length 55cm and 25mm diameter. Columns were washed carefully with detergent and distilled water. Finally rinsed with methanol and left for drying. Silica gel (70-230) activated at 120°C in oven for one hour. Slurry was prepared by using 50g silica gel in a beaker using n-Hexane (other solvents for particular fractionation). Slurry was poured with help of glass stirrer in columns containing glass wool at narrow edge to stop leakage. These columns were left until silica gel slurry got settle. Essential oil 15g was measured in glass vial and loaded upon silica slurry added columns. The n-hexane 250mL poured upon the column and allowed to flow through the column bed (sample added) in drop wise manner. Fractions were collected in conical flasks. After running 250mL solvent through column, fractions were air dried

on rotary evaporator and collected in air tight glass vials. Oil fractions were prepared in n-hexane, n-hexane plus chloroform, chloroform, chloroform plus ethyl acetate, ethyl acetate, ethyle acetate plus methanol, methanol, methanol plus acetonitrile and acetonitrile.

Antimicrobial activity through agar well diffusion assay and MIC of oil fractions was performed along with solvents as mentioned above for plant oils (mentioned above).

#### **Cytotoxicity analysis of fractions of plant essential oils**

For cytotoxicity of fractions of essential oils Baby hamster kidney 21 (BHK-21) cell line was used. BHK21 cell line was grown in sterile Glasgow minimum essential medium (GMEM) medium containing 8-10% Fetal Calf Serum (FCS) in 96 flat well micro titration plate. Each well of micro titration plate was inoculated with  $1 \times 10^5$  cells per 300mL. After growth and 80-90% confluency, cell monolayer was washed twice with sterile PBS (100 mL in each well). Optical density was taken at 570nm. Two fold serial dilutions of fractions of essential oils were prepared in sterile GMEM (2-5% FCS). Each dilution, 100 $\mu$ L was added on micro titration plate containing cell growth from well 1<sup>st</sup>-10<sup>th</sup>. In 11<sup>th</sup> well GMEM was added as negative control and 12 well was kept as live cell untreated control (Dos Santos *et al.*, 2021). After 24 hours of incubation under 5% CO<sub>2</sub> at 37°C, cells were washed with PBS sterile solution. Cells were stained with 1% crystal violet solution mixed in equal volume of 3% formalin. Staining solution 80 $\mu$ L added in each well and incubated for 30 to 60 minutes at room temperature. After washing with sterile PBS stained cell lines were air dried overnight. DMSO 50 $\mu$ L was added in each well of micro titration plate and optical density was taken at 570nm by ELISA plate reader. Cell survival percentage (CSP) was calculated as:

$$\text{CSP} = \frac{\text{O.D of test} - \text{O.D of negative control}}{\text{O.D of live cell control} - \text{O.D of negative control}} \times 100$$

#### **GC/MS analysis**

*S. aromaticum* n hexane and n-hexane plus chloroform fractions were subjected to GC-MS for essential active components analysis. GC-MS analysis was performed following the method of Kaur *et al.* (2019). For GC-MS, CARBOWAX capillary column along with helium as carrier gas was used. Injector heated at 260°C and sample (essential oil n-hexane and n-hexane + chloroform solvent fractions) injected at 1 $\mu$ L/minute rate. Active compounds in test samples were detected by comparison of retention time with standard compound.

#### **STATISTICAL ANALYSIS**

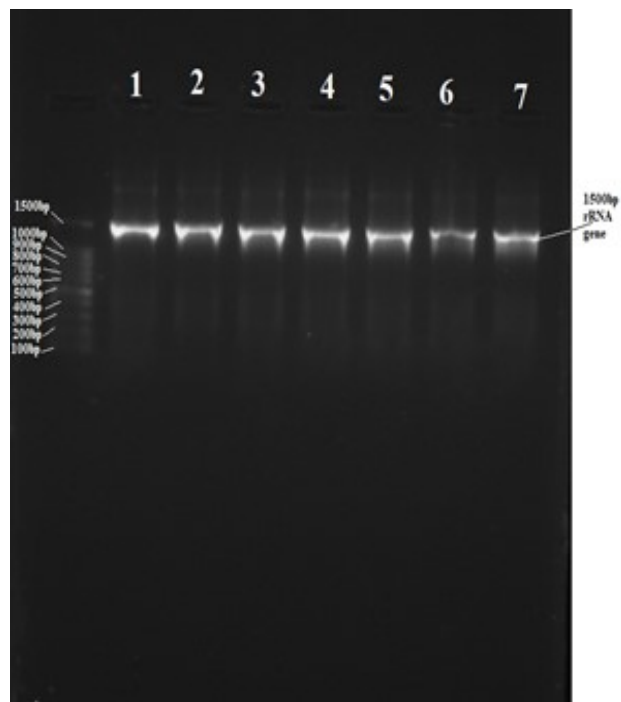
Data based on zones of inhibition and MIC was analyzed through one way analysis of variance (ANOVA) followed by post hoc Duncan's multiple range test through

Statistical package for social sciences (SPSS) version 20.0 and for statistical analysis, level of significance (p-value) of 0.05 was selected.

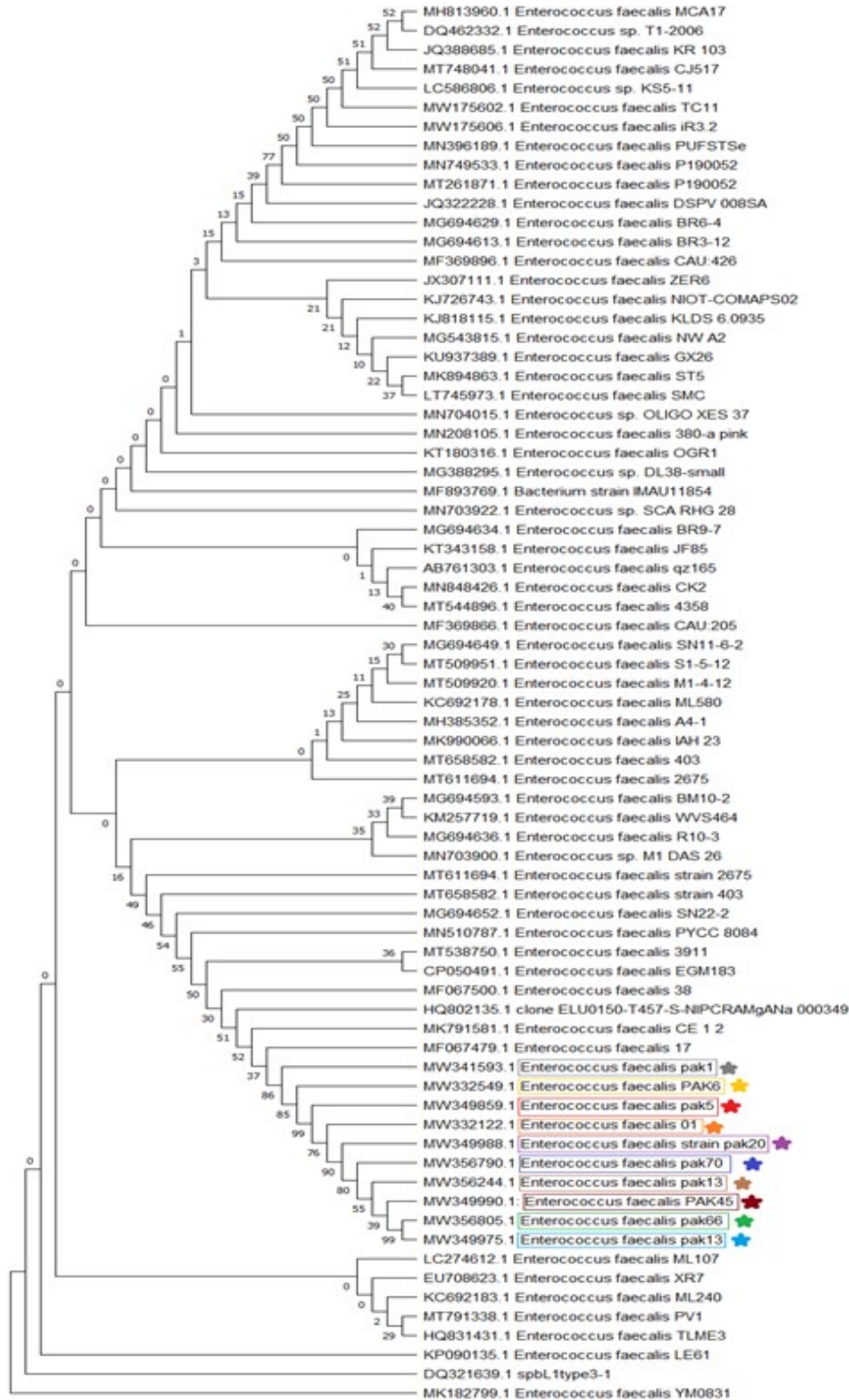
## **RESULTS**

#### **Characterization of *Enterococcus faecalis***

*Enterococcus faecalis* (n=10) recovered from children suffering with diarrhea, identified based upon culture and biochemical profile were procured from IOM, UVAS, Lahore. Isolates were confirmed by PCR targeting 16S rRNA gene followed by nucleotide sequencing. Purity of DNA extracted from fresh broth culture of isolates was estimated using Nano drop and visualized by agarose gel electrophoresis using gel documentation system at 260nm wavelength. Highest concentration of extracted DNA ranged from 444.5 to 57.2ng  $\mu$ L<sup>-1</sup>. Amplification of 16S rRNA gene by PCR using optimized conditions revealed 1500 base pair bands upon electrophoresis and visualized by gel documentation system (Fig 1). Amplified DNA products sequenced by Sanger di-deoxy sequencing method and original FASTA files were received. Each of the FASTA sequence file was checked for sequence alignment by n-Blast post cleaning by Just Bio tool in few cases. Sequences were submitted to NCBI and accession numbers received were MW349990.1, MW349859.1, MW332122.1, MW356805.1, MW349975.1, MW349988.1, MW356790.1, MW356244.1, MW341593.1 and MW332549.1.



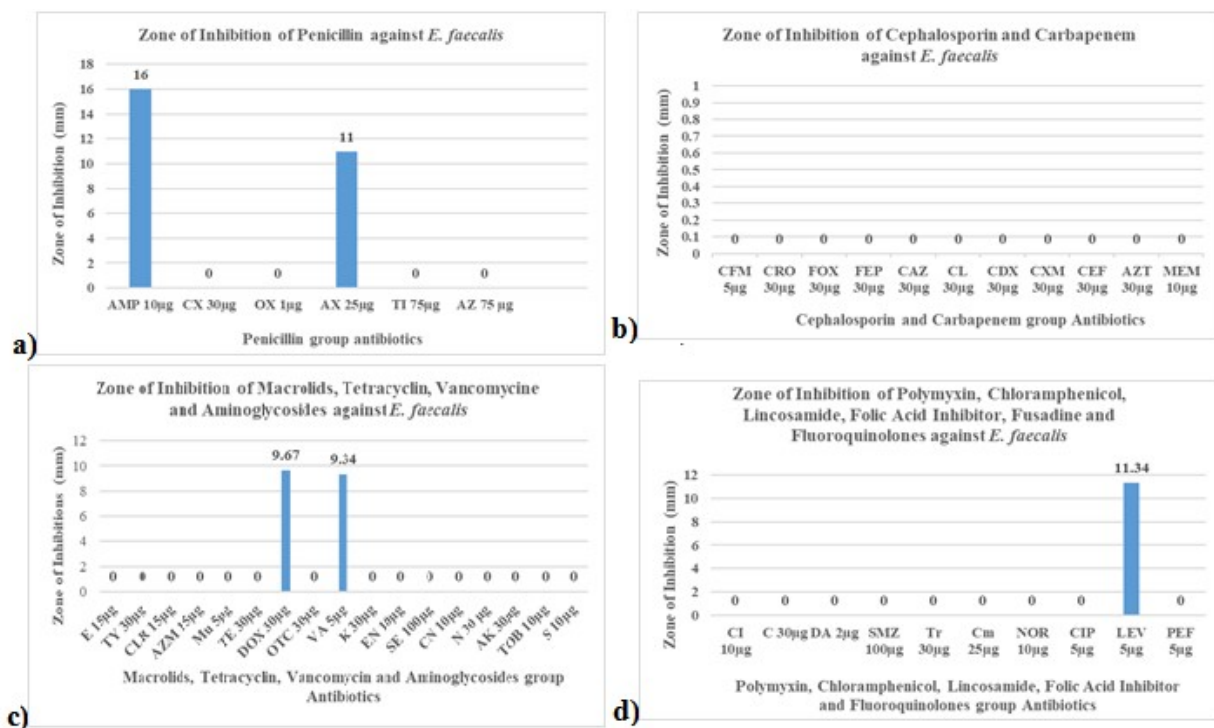
**Fig. 1:** 16S rRNA amplified gene (1500bps amplicon band) electrophoresis pattern in agarose gel visualized on gel documentation system.



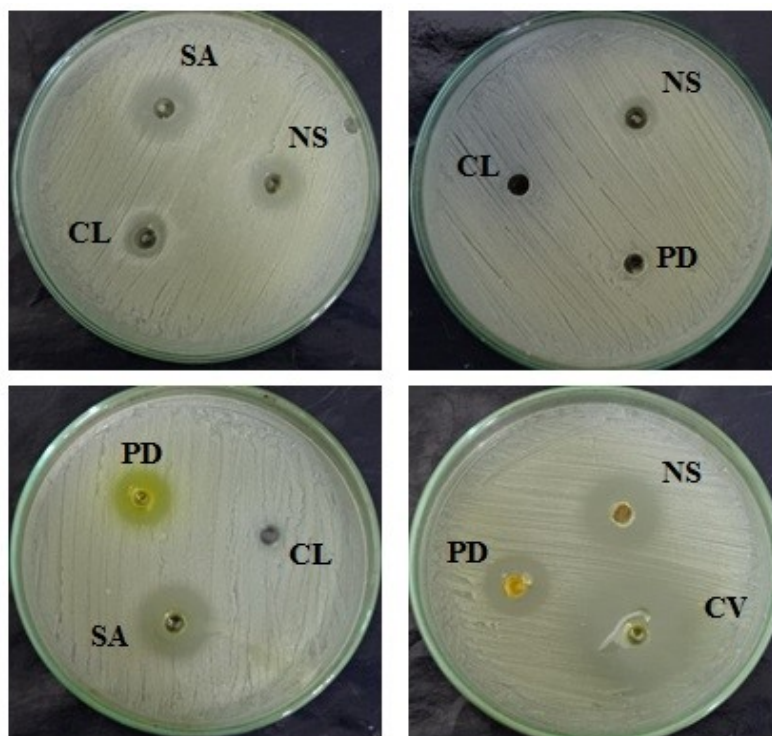
**Fig. 2:** Phylogenetic analysis of *Enterococcus faecalis* isolates using MegaX tool

Nucleotide sequences were used to construct phylogenetic tree for genetic homology with online available isolates data using clustal-w and MegaX softwares (fig. 2). Phylogenetic tree for *E. faecalis* was made using 16SrRNA sequences, neighbor joining algorithm,

bootstrap as phylogeny method and 2000 bootstrap replication. In phylogenetic tree Pakistan *E. faecalis* sequences with accession number represented as colored star. *E. faecalis* (star) present in one cluster. These bacteria were not related to ML107.



**Fig. 3:** Resistance pattern of *E. faecalis* isolates against different groups of antibiotics. a) ZOI of penicillin group antibiotics i.e. AMP ( $16.00 \pm 2.65$ mm) and AX ( $11.00 \pm 1.73$ mm), b) ZOI of cephalosporins and carbapenem group's antibiotics showed non-significant results c) ZOI of macrolids, tetracycline, vancomycin and aminoglycosides group antibiotics i.e. DOX ( $9.67 \pm 3.21$ mm) and VA ( $9.34 \pm 3.06$  mm), d) ZOI of polymyxin, chloramphenicol, lincosamide, folic acid inhibitor, fusidine, fluoroquinolones group i.e. LEV ( $11.34 \pm 4.72$ mm) exhibited significant standard deviation. All other antibiotics mentioned exhibited non-significant results.



**Fig. 4:** Activity of essential oils against antibiotic resistant *E. faecalis* (bacterial lawn on media plates) CV (*C. verum*); SA (*S. aromaticum*); NS (*N. sativa*); PD (*P. dulcis*); CL (*C. longa*)

**Table 1:** Fatty acid profile of selected *Syzygium aromaticum* fractions by GCMS analysis

S. No.	Type of fraction	Fatty acids	Retention Time (RT)	Percentage
01	n-hexane	Stearic acid	19.6	2.80
		Palmitic acid	20.5	13.6
		Eugenic acid	21.3	53.7
		Linoleic acid	25.6	4.03
		Oleic acid	26.5	18.5
02	n-hexane plus chloroform	Eugenic acid	21.36	66.32
		Linoleic acid	23.67	2.17
		Octadec-11-enoic acid	24.5	19.15
		Stearic acid	28.21	3.33
03	Ethyl acetate	Butyric acid	7.3	3.7
		3-bis(1,1-dimethylethyl	19.5	1.5

MW349975.1 and MW356805.1, 99% evolutionary related to each other. MW341593.1 and MW332549.1 *E. faecalis* 86% related to each other. MW349859.1 was 99% evolutionary related to MW332122.1.

#### **Antibiotic resistance pattern**

*Enterococcus faecalis* (n=10) were screened for antibiotic resistance to a panel of antibiotic classes. ZOI's were measured in millimeter and compared with standard inhibition zones according to CLSI. Complete resistance was exhibited by tested isolates to multiple antibiotics with zero ZOI including cloxacillin, oxacillin, ticarcillin, azlocillin, cefixim, ceftriaxone, cefoxitin, cefipime, ceftazidime, cephalixin, cefadroxil, cefuroxime sodium, cefamandole, aztreonam, meropenem, erythromycin, tylosin, clarithromycin, azithromycin, mupirocin, tetracycline, oxytetracycline, kanamycin, enoxacin, spectinomycin, gentamicin, neomycin, amikacin, tobramycin, streptomycin, colistin, chloramphenicol, clindamycin, sulfamethoxazole, trimethoprim, cotrimazine, norfloxacin, ciprofloxacin and pefloxacin. Highest mean ZOI against tested isolates was observed in case of Ampicillin (16.00±2.65mm) followed by Levofloxacin (11.34±4.72mm), Amoxicillin (11.00±1.73mm), Doxycycline (9.67±3.21mm), Vancomycin (9.34±3.06 mm), respectively (fig. 3). The ZOI of all the antibiotics were declared as resistant after comparison with CLSI-2020 data.

#### **Activity of essential oils against antibiotic resistant *E. faecalis***

Antibacterial activity of plant essential oils (n=05) was checked against three selected *E. faecalis* isolates including MW349990.1, MW356805.1 and MW356790.1 which were resistant to multiple antibiotics. All of the essential oils exhibited antibacterial activity. The highest mean ZOI was observed in case of *C. verum* (15.34±0.57mm) followed by *S. aromaticum* (10.00±1.00mm), *N. sativa* (9.34±2.30mm), *P. dulcis* (5.67±4.16mm) and lowest of *C. longa* (3.34±4.04 mm), respectively. Activity of *C. verum* differed significantly with activity of other four tested plant essential oils (fig.

4). Three plant essential oils having higher ZOI's against resistant *E. faecalis* isolates including *C. verum*, *S. aromaticum* and *N. sativa* were used to determine MIC by dilution method. Lowest effective MIC observed was of *S. aromaticum* (11.39±3.94 mg mL<sup>-1</sup>) followed by *C. verum* (32.96±21.58mg mL<sup>-1</sup>) and *N. sativa* (74.44±32.24mg mL<sup>-1</sup>), respectively. Statistically, *S. aromaticum* differed significantly with MIC values of other two tested plant essential oils.

#### **Activity of *S. aromaticum* oil fractions**

Mean ZOI of *S. aromaticum* fractions against resistant *E. faecalis* isolates (n=03) recorded by well diffusion test was the highest for n-hexane and chloroform combination (16.67±2.51mm) followed by n-hexane (15.34±1.1mm), ethyl acetate (12.34±5.8mm), ethyl acetate + methanol (11.34±2.51mm), methanol + acetonitrile (8.67±7.09mm), methanol (8.34±7.02mm), chloroform + ethyl acetate (4.33±5.78mm), chloroform (4.00±5.19 mm) and least in case of acetonitrile (0.00±0.00). Moreover, all the solvents used for fractionation showed no antibacterial activity for *E. faecalis*. The fractions n-hexane and n-hexane plus chloroform differed non-significantly with each other and significantly with activity of other fractions. Based upon inhibition zones three fractions were used to determine MIC values against *E. faecalis* and the lowest mean MIC value (mg ml<sup>-1</sup>) recorded was of n-hexane fraction (1.97±1.29) followed by n-hexane plus chloroform (18.09±7.84) and ethyl acetate (74.55±32.25) fractions, respectively. Statistically mean MIC of n-hexane differed significantly with others.

#### **Cytotoxicity and GC/MS analysis of fractions**

Cytotoxicity analysis was performed using MTT assay to evaluate the safety of mean MIC values of selected plant essential oil solvent fractions and effective concentration 50 calculated in each fraction was higher than the MIC value. Percentage cell survival recorded was 51.42, 51.86 and 54.60 at concentrations of 13.56, 14.20 and 55.93 mg ml<sup>-1</sup> for *S. aromaticum* selected solvent fractions n-hexanes, n-hexane plus chloroform and ethyl acetate, respectively.

The selected fractions were processed by GC/MS analysis for qualitative and quantitative fatty acid analysis. The GCMS chromatograms and detailed fatty acid analysis presented (table 1). Highest percentage in n-hexane fraction of *S. aromaticum* was of Eugenol acid (53.7%) followed by Oleic acid (18.5%) and least of Stearic acid (2.80%). The major fatty acid in n-hexane plus chloroform fraction was of eugenol acid (66.32%) least was of Linoleic acid (2.17%). The major component in ethyl acetate fraction was Butyric acid (3.7%). Based upon GCMS analysis the most effective fatty acid against antibiotic resistant *E. faecalis* isolates was Eugenol acid which was common and in higher proportion in solvent fractions of *S. aromaticum*.

## DISCUSSION

*Enterococcus faecalis* bacteria have become important pathogen in human and veterinary medicine due to emergence of antimicrobial resistance (Shil and Chichger, 2021). *Enterococcus faecalis* can be isolated from waste water, coastal marine environment, pristine waters, aquacultures, sea foods, foals feces, root canals of infected teeth and from stool samples (Shettima and Iregbu, 2019). In present study, *E. faecalis* isolate (n=10) were characterized by PCR targeting 16S rRNA gene followed by nucleotide sequencing. Genotypic identification based upon 16S rRNA gene sequence has emerged as accurate, objective, reliable method for bacterial identification with defining taxonomic relationships among bacteria (Braun et al., 2021). The phylogenetic analysis of the 16S rRNA gene of the present 10 isolates of *E. faecalis* with the selected 63 published sequences of *E. faecalis* discovered random grouping of sequences into different clusters irrespective of species of origin. Sequences analysis revealed identity range from 85-100%. Ribosomal RNA 16S sequencing analysis resolved the problem of false negative results for both biochemical and PCR identification (Braun et al., 2021). In agreement to present study, *E. faecalis* were identified on the basis of 16S rRNA sequence analysis from fecal and water samples (Xiao et al., 2020).

Antibiotic resistance patterns can be determined by performing disc diffusion method following the methods and standards provided by CLSI-2020. In present research *E. faecalis* isolates displayed resistant to cephalosporins, penicillin group, vancomycin, aminoglycosides, folic acid inhibitors, quinolones, meropenems, macrolides, tetracycline and chloramphenicol. Enterococci acquired resistance gene, act as the reservoir for resistance genes and transmit these genes to human, animals and environmental bacterial populations (Dejoies et al., 2021). Globally antibiotic-resistant infections cause more morbidity and mortality. Antimicrobial resistance pattern in enterococci depends upon three reasons including intrinsic resistance to antimicrobial agents like beta-

lactams and aminoglycosides, acquired resistance through mobile elements like transposon and plasmids against glycopeptides, quinolones, tetracycline lines, macrolides and streptogramin and by horizontal transfer of resistance genes. *E. faecalis* and *E. faecium* are naturally resistant to cephalosporins, aminoglycosides (low-level resistance), macrolides, sulphonamides, clindamycin and dalfopristin. Enterococci usually show a potential resistance pattern and some strains are multidrug-resistant (MDR). Ampicillin, gentamicin and vancomycin are the significant antimicrobials for the treatment of MDR enterococcal infections (Golob et al., 2019). Vancomycin-resistant enterococci are a major concern in healthcare practices because of major therapeutic use against the MDR enterococci (Raza et al., 2018).

Antimicrobial activity of EO's against *E. faecalis* was quite significant either in planktonic or biofilm state (Willett et al., 2021). In present research antibacterial activity of essential oils was determined through well diffusion assay and MIC assay against *E. faecalis*. Highest mean ZOI was observed in case of *C. verum* (15.34±0.57mm) followed by *S. aromaticum* (10.00±1.00 mm), *N. sativa* (9.34±2.30 mm), *P. dulcis* (5.67±4.16 mm) and lowest of *C. longa* (3.34±4.04 mm). Some essential oils are effective against the vancomycin-resistant enterococci (VRE) such as *Eucalyptus globulus*, *Kadsura longipedunculata*, *Sideritis erythrantha*, *Citrus limon*, *Citrus sinensis* and *Citrus bergamia* (Solórzano-Santos and Miranda-Novales, 2012). In present study, lowest effective MIC observed was of *S. aromaticum* (11.39±3.94 mg ml<sup>-1</sup>) followed by *C. verum* (32.96±21.58 mg ml<sup>-1</sup>) and *N. sativa* (74.44±32.24 mg ml<sup>-1</sup>), respectively. Antibacterial activity of *N. sativa* was determined to be higher against clinical bacterial isolates from human (Bereksi et al., 2018). Similarly, *N. sativa* showed activity against multidrug resistant bacteria including species of Enterococcus, Staphylococcus, Pseudomonas, Klebsiella and Acinetobacter (Saleh et al., 2018). Correspondingly, *in-vitro* activity of *N. sativa* oil and methanol extracts was determined against microorganism of buccal cavity (Kiari et al., 2018). In agreement to present study, activity of *S. aromaticum* have been recorded on pathogenic microorganisms of oral cavity (Oluwasina et al., 2019). *S. aromaticum* was also effective alone and in combination with antibiotics against multidrug resistant *S. aureus* (Abou et al., 2018). Based upon the results of present study, essential oils especially *C. verum*, *S. aromaticum*, *N. sativa* are better alternate to treat the infections caused by antibiotic resistant *E. faecalis* in human and animals.

Essential oils are used against various microorganisms to treat different infections and before *in-vivo* studies, *in-vitro* evaluation of essential oils is very necessary. For this purpose, cytotoxicity assays are performed on eukaryotic cell lines. It was reported that cytotoxic effect

of essential oils is dependent upon the concentration. Higher the concentration of essential oils, higher will be the cytotoxicity. In case of *S. aromaticum* oil, cytotoxicity was performed on HT29 cell line and by increasing the concentration of the oil, increase in cytotoxicity was observed (Behbahani *et al.*, 2019). Similar results were observed in this research when cytotoxicity was evaluated on BHK21 cell line. Highest viability (93-100%) was estimated at 1.6-1.77 mg ml<sup>-1</sup> concentration of oil solvent fractions. The inhibitory concentration 50% (IC<sub>50</sub>) of *C. verum* essential oil was observed as 20µg ml<sup>-1</sup> against vero cells (Azeredo *et al.*, 2014). At 0.125% *C. verum* essential oil concentration viability of oral keratinocytes reduced to 48% and at ≤0.0625% concentration no loss of viability was observed (LeBel *et al.*, 2017). In the recent study, *C. verum* essential oils solvent fractions cytotoxicity was evaluated on BHK-21 cell lines depicted effective concentration (EC<sub>50</sub>) at 14.10-28.28 mg ml<sup>-1</sup>.

The *S. aromaticum* essential oil was studied for active components compositions through GC-MS. The eugenol (75.11%) was found in abundant and present as major compound. Caryophyllene (14.05%) was second highest active compound (Behbahani *et al.*, 2019). Chemical composition of clove oils obtained from Java and Manado revealed eugenol as largest bioactive compound in oils with 55.606% and 74.647% abundance, respectively (Amelia *et al.*, 2017). Hamad and coworkers revealed that the largest active component of *S. aromaticum* leaves was p-Eugenol (75.19%) (Hamad *et al.*, 2017). In agreement to all these findings, in present study *S. aromaticum* GCMS analysis revealed eugenol as most abundant compound in oil fraction of n-hexane and n-hexane + chloroform. Octadec-11-enoic acid was found 2<sup>nd</sup> active compound in n-hexane + chloroform fraction of oil. *C. verum* and *S. aromaticum* essential oils are better alternate for treatment of antibiotic resistant cases of *E. faecalis*. Due to least cytotoxicity, these oils can be given to children orally for treatment of diarrhea caused by *E. faecalis*.

## CONCLUSION

*Syzygium aromaticum* plant essential oil and its n-hexane and n-hexane plus chloroform fractions can be the better alternate for the treatment of antibiotic resistant *E. faecalis* infection especially diarrheic cases of children as well these oil fractions exhibited less cytotoxicity and are better therapeutic option via oral route of administration.

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