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Original Article

Characterization of Vancomycin Resistant *Enterococci* and Drug Ligand Interaction between *vanA* of *E. faecalis* with the Bio-Compounds from *Aegles marmelos*

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Objectives: *Enterococcus faecalis* is a gram positive diplococci, highly versatile and a normal commensal of the gut microbiome. Resistance to vancomycin is a serious issue in various health-care setting exhibited by vancomycin resistant Enterococci (VRE) due to the alteration in the peptidoglycan synthesis pathway. This study is thus aimed to detect the VRE from the patients with root caries from the clinical isolates of *E. faecalis* and to evaluate the in-silico interactions between vanA and the *Aegles marmelos* bio-compounds.
Methods: *E. faecalis* was phenotypically characterized from 20 root caries samples and the frequency of *vanA* and *vanB* genes was detected by polymerase chain reaction (PCR). Further crude methanolic extracts from the dried leaves of *A. marmelos* was assessed for its antimicrobial activity. This is followed by the selection of five *A. marmelos* bio-compounds for the computational approach towards the drug ligand interactions.
Results: 12 strains (60%) of *E. faecalis* was identified from the root caries samples and *vanA* was detected from two strains (16%). Both the stains showed the presence of *vanA* and none of the strains possessed *vanB*. Crude extract of *A. marmelos* showed promising antibacterial activity against the VRE strains. *In-silico* analysis of the *A. marmelos* bio-compounds revealed Imperatonin as the best compound with high docking energy (–8.11) and hydrogen bonds with < 140 TPSA (Topological polar surface area) and zero violations.
Conclusion: The present study records the VRE strains among the root caries with imperatorin from *A. marmelos* as a promising drug candidate. However the study requires further experimentation and validation.

Keywords: *E. faecali*, *vanA*, *vanB*, *A. marmelos*, health, environment

INTRODUCTION

Enterococci, which belong to the Group D *Streptococci*, are Gram-positive facultative aerobic bacteria that occur as oval pairs or short chains. *Enterococci* are generally regarded as normal flora of the gut, the oral microbiome, and the vagina. However, they are associated with a variety of recalcitrant nosocomial infections, especially urinary tract infections [1]. *Enterococcus faecalis* (*E. faecalis*) is also reported to be an important dental pathogen that causes root caries and is associated with dental procedures that cause endocarditis. Enterococci are the second most common infectious health challenge in the U.S., and in India, they are the most frequently isolated species (63.8%) [2]. Enterococci have recently attracted renewed attention because of their propensity to develop multidrug resistance against routinely used antibiotics, including vancomycin. In addition to intrinsic resistance, acquired resistance through chromosomes, plasmids, or transposons is also common. The exceptional ability of these strains to transmit genetic information between themselves and to other genera has conferred them with a high level of vancomycin resistance [3].

Multidrug-resistant enterococci have emerged as serious

infections worldwide, especially vancomycin-resistant *Enterococci* (VRE), which have rapidly emerged in healthcare settings in recent decades. VRE were identified as hospital-associated pathogens in Europe in the mid-1980s and have since spread worldwide [4]. The drug resistance mechanism of VRE is mainly encoded by *vanA* and *vanB* gene clusters, which are most commonly found in *Enterococcus faecium* and are becoming widespread worldwide [5]. The resistance mechanism of VRE involves alterations in peptidoglycan synthesis, which is mediated by a cluster of *van* genetic determinants. The *vanA* gene cluster induces resistance to vancomycin and teicoplanin (the *vanA* phenotype) and is mostly associated with various types of Tn1546 transposon variants. Alterations may be caused by point mutations, deletions, or activation of insertion sequences [6]. In *E. faecalis*, the host Inc18 and pheromone-responsive families of plasmids, which are only found in this species, have been associated with *vanA* genetic determinants [7]. VRE dynamics are influenced by mobile genetic elements that carry *vanA* Tn1546 insertion point heterogeneity, and also by clonal proliferation of various stains that acquire *vanA* [8].

1. Literature review

Targeting the *vanA* gene could be an effective strategy for combating drug-resistant strains of *E. faecalis*. Multidrug-resistant strains, such as VRE, might pose serious risks for hospitalized patients. A systematic review and meta-analysis associated VRE with high infection rates [9]. Thus, alternative treatment strategies involving the use of various plants and herbs, and their bioactive compounds are becoming popular in various developing countries, including India. Of the numerous natural herbs from India, *Aegle marmelos* (*A. marmelos*), commonly known as the 'wood apple plant', which belongs to the family *Rutaceae*, is known to possess various bioactive properties [10]. Phytochemical analyses of the various parts of *A. marmelos* have identified tannins, phenylpropanoids, flavonoids, carotenoids, and other minor compounds as possessing promising bioactive properties [11]. Various studies have shown that biocompounds extracted from *A. marmelos* using various methods might possess multiple therapeutic effects [12]. Indeed, the antibacterial effects of the various solvent extracts have exhibited promising properties against various Gram-positive and Gram-negative bacteria [13].

However, there are no recent reports on the antimicrobial effects of *A. marmelos* against drug-resistant *E. faecalis* strains. Here, we aimed to determine the antimicrobial effects of a methanolic crude extract from *A. marmelos* against VRE isolated from root caries. Because bioinformatic analysis of biocompounds to identify their targets can accelerate drug development [14], we used computational tools to evaluate potential drug–ligand interactions between the compounds, coumarin, xanthotoxol, imperatorin, aegeline, and marmeline, which were isolated from *A. marmelos*, against *vanA*.

MATERIALS AND METHODS

1. Sampling and isolation of *E. faecalis*

This prospective observational study was conducted from April 2022 to June 2022 at the Department of Microbiology at Saveetha Dental College and Hospitals. Carious scrapings excavated from twenty individuals with typical root caries were collected in sterile trypticase soy broth and immediately transferred to the microbiology lab. The reconstituted samples were then streaked onto sterile Brain Heart Infusion Agar (HiMedia Laboratories, Mumbai, India) and then incubated at 37℃ for 24 hours. Typical colonies were then identified and subjected to preliminary gram staining and routine biochemical tests to identify *E. faecalis* strains. Ethical approval for the study was granted by the institutional ethics review board (approval numbers: SRB/SDC/UG-2073/21/MICRO/061 and IHEC/SDC/UG-2073/21/MICRO/602).

2. Antibiotic susceptibility test

Antibiogram profiling of the clinical strains of *E. faecalis* was done using the standard agar, Kirby–Bauer disc diffusion method [15]. Next, lawn cultures of the fresh broth suspension of *E. faecalis* were established on sterile Mueller–Hinton agar (HiMedia Laboratories, Mumbai, India). The following antibiotics were selected based on the 2021 CLSI guidelines and introduced on the lawn surface at indicated concentrations, viz., Amoxyclav (30 μg), ceftriazone (30 μg), cefoperazone–sulbactam (75/30 μg), clindamycin (2 μg), cefixime (5 μg), levofloxacin (5 μg), linezolid (30 μg) vancomycin (30 μg), azithromycin (15 μg), amikacin, tetracyclin (30 μg), ciprofloxacin (5 μg), cefoperazone (2 μg), and gentamycin (10 μg). Vancomycin E-strips were used to identify MIC breakpoints for VRE selection. All plates were incubated at 37℃ for 24 hours, followed by zone of inhibition measurements.

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3. PCR detection of *vanA* and *vanB* in *E. faecalis*

Fresh vancomycin-resistant clinical isolates were recovered from sterile trypticase soy agar cultures after 24 hours of incubation at 37℃, followed by genomic DNA extraction using a Qiagen kit (Germany) following the manufacturer's protocol. Specific primers (Table 1) were added to the master mix followed by PCR on a thermocycler (Biorad Laboratories) to detect the presence of the genetic determinants of interest. The PCR program involved 35 cycles at an annealing temperature of 58℃. The PCR product alongside a 1.5 kb DNA ladder, was then subjected to 1% agarose gel electrophoresis, followed by ethidium bromide staining and visualization using a gel documentation system.

4. Preparation of the *A. marmelos* extract

The crude *A. marmelos* extract was prepared as described previously [16], with slight modifications. Fresh *A. marmelos* (L.) *Correa* was obtained from local regions, washed thrice with sterile distilled water, and then shade-dried before grinding the leaves into fine powder. Next, 100 g of the *A. marmelos* dried leaf powder were mixed with 100 mL of methanol. The resulting suspension was then allowed to react for a week at room temperature, on an orbital shaker (Remi Lab World). The extract was then filtered into sterile Petri dishes using a Whatman No. 1 filter paper and then evaporated. The extracts were then stored at 4℃ until use (Fig. 1).

5. Analysis of the antimicrobial effect of the *A. marmelos* extract

The crude extract recovered from the methanol was weighed to determine the final yield. Next, the extracts were weighed and dissolved in dimethyl sulphoxide at final concentrations of 100, 50, 25, 12.5, and 6.25 mg/mL. Fresh clinical strains of VRE were prepared as broth suspension and was made as a lawn onto sterile brain heart infusion agar and the wells were cut using agar puncture [17]. Next, 50 μL of the diluted extract were added into appropriate wells, followed by incubation at 37℃ for 24 hours. After the incubation, the zone of clearance was measured and recorded. The assay was repeated thrice and the results were recorded as mean values.

Table 1. The primers used for the study to detect *vanA* and *vanB* genetic determinants from the clinical isolates of VRE

Gene of target	Primers used for the study	Annealing temperature	Amplicon size
vanA	F: 5'-TCTGCAATAGAGATAGCCGC-3' R: 5'-GGAGTAGCTATCCCAGCATT-3'	58℃	400 bp
vanB	F: 5'-ATGGGAAGCCGATAGTC-3' R: 5'-GATTTCGTTCCTCGACC-3'	58℃	635 bp

6. Modeling and validation of the VanA protein structure

Because the crystal structure of VanA was unavailable in the protein data bank, SWISS-MODEL was used to predict its structure using the bacterial RQC complex 7AQC-Q chain from *Bacillus subtilis* as a template. The quality of the predicted model was then validated by assessing its residues in the favored regions using Ramachandran plots and was selected for the docking analysis.

7. Ligand preparation and optimization

The structural configurations of the bioactive derivatives were visualized using the ChemSketch software. The following *A. marmelos* biocompounds were selected for optimization and further interaction analysis: coumarin, xanthotoxol, imperatorin, aegeline, marmeline, and erythromycin. Next, selected ligands were saved as MOL files using the Open-Babel molecular

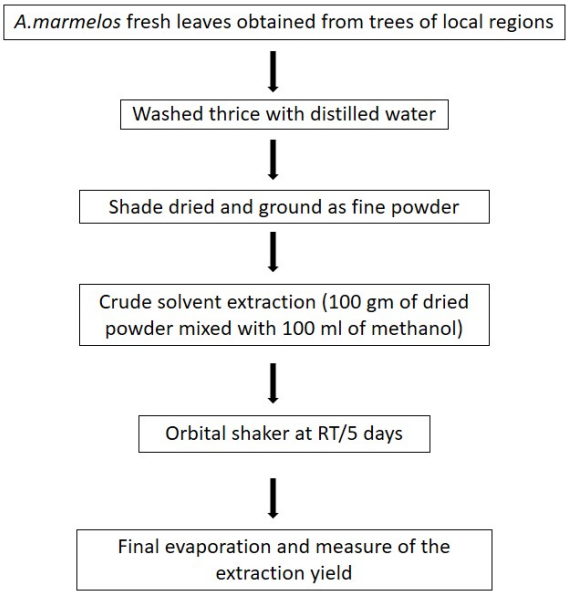

Figure 1. The schematic representation of the *A. marmelos* crude extraction procedure.

converter and then saved in PDB format.

8. Assessment of the drug properties of the selected biocompounds

The Molinspiration program was used to analyze the log P molecular descriptors for partition coefficient, compound molecular weight, and the hydrogen bond acceptor and donor counts associated with membrane permeability and bioavailability. Next, the absorption, distribution, metabolism, and elimination characteristics of the selected biocompounds were evaluated using "Lipinski's rule of five".

9. Analysis of docking interactions

Docking analysis of the affinity between each compound (coumarin, xanthotoxol, imperatorin, aegeline, marmeline, and erythromycin) and *A. marmelos*' *vanA* gene was done using the AutoDock tool. Grid maps were used to embed the *vanA* protein using an auxiliary Autogrid program, one for each type of atom present in the complex being docked. To model the hydrogen bonds and van der Waals forces, Lennard–Jones parameters of 12-10 and 12-6 were used, respectively. The force fields were evaluated in two phases and the intramolecular energetics from unbound states and bound conformations were assessed using the following equation: $\Delta G = \Delta Gvdw + \Delta Ghbond + \Delta Gelec + \Delta Gtor + \Delta Gdesolv$. Discovery Studio Visualizer was used to visualize hydrogen bonds between coumarin, xanthotoxol, imperatorin, aegeline, marmeline, or erythromycin and the *E. faecalis vanA* gene.

RESULTS

1. VRE phenotypic characterization and detection of the *vanA* gene

E. faecalis was identified and isolated from 12 strains (60%) based on its typical pinpoint colonies on trypticase soy agar plates. Gram staining revealed typical Gram-positive diplococci, which were bile esculin test positive and catalase test negative (Fig. 2). Seven multi-drug resistant isolates (58.3%) were identified using the disc diffusion method, whereas two strains

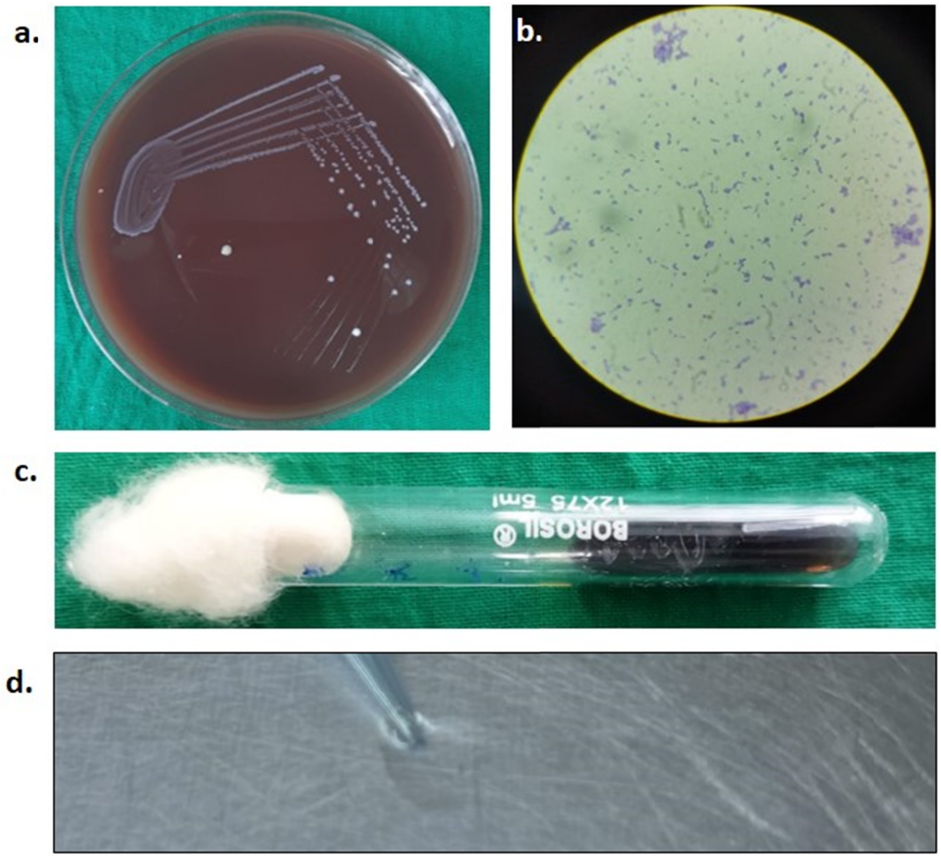

Figure 2. Phenotypic characterization of *E. faecalis* from the clinical samples (a) *E. faecalis* growth on blood agar (b) Gram staining showing gram positive diplococcic (c) positive bile esculin test (d) catalase tested negative.

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(16%) were vancomycin-resistant (VRE strains). PCR analysis revealed that both VRE strains carried the *vanA* gene (amplicon size: 400 bp, Fig. 3), but not the *vanB* gene.

2. Antimicrobial effect of the *A. marmelos* extract against VRE

The methanol extraction method yielded 23 mg of extract from 100 g of *A. marmelos* dry leaf powder. The extract exhibited promising effects against both the multidrug-resistant strains (n = 7) and the VRE strains possessing the *vanA* gene (n = 2), with a zone size of 12 mm at a concentration of 100 mg/mL, although lower concentrations (50, 25, 12.5, and 6.25 mg/mL) had no effect (Fig. 4).

3. Validation of VanA protein structure

The *E. faecalis* VanA protein entry was retrieved from Uniprot (ID: Q0WYK7). Because the VanA protein structure was not available on PDB, it was modeled using the 7AQC-Q chain template. The quality of the predicted model was regarded as being good because 90.6% of the residues were in the most favored regions, whereas only 0.3% of the residues occurred in disallowed regions (Fig. 5).

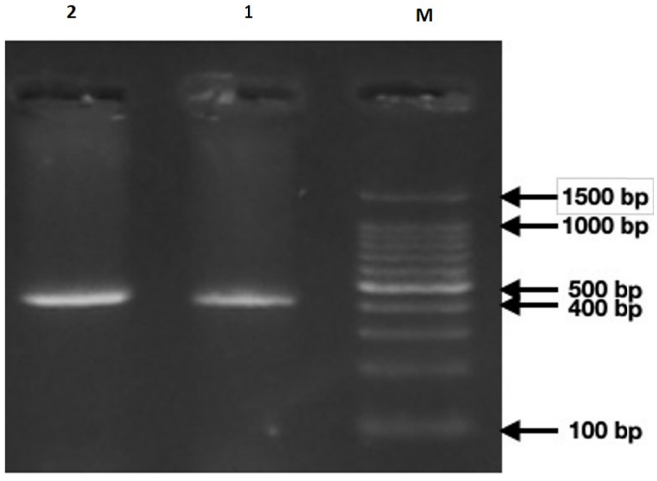

Figure 3. Electropherogram of *vanA* gene product of size 400 bp in lanes 1 and 2 with 1.5 k bp marker lane (M).

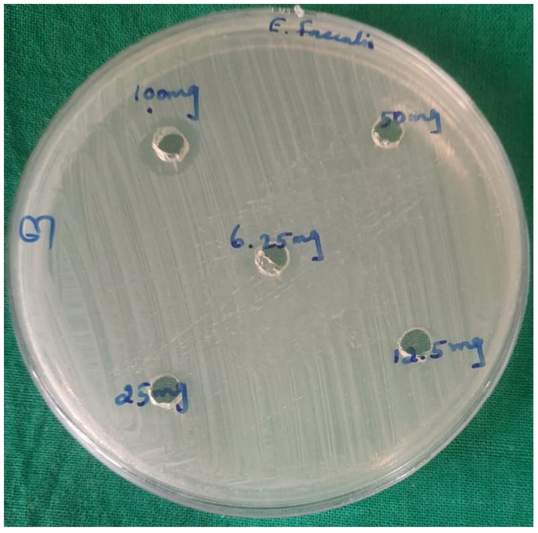

Figure 4. Antimicrobial effect of the crude methanolic extracts at varying concentrations (100 mg, 50 mg, 25 mg, 12.5 mg and 6.25 mg) of *A. marmelos* against the VRE strains of *E. faecalis*.

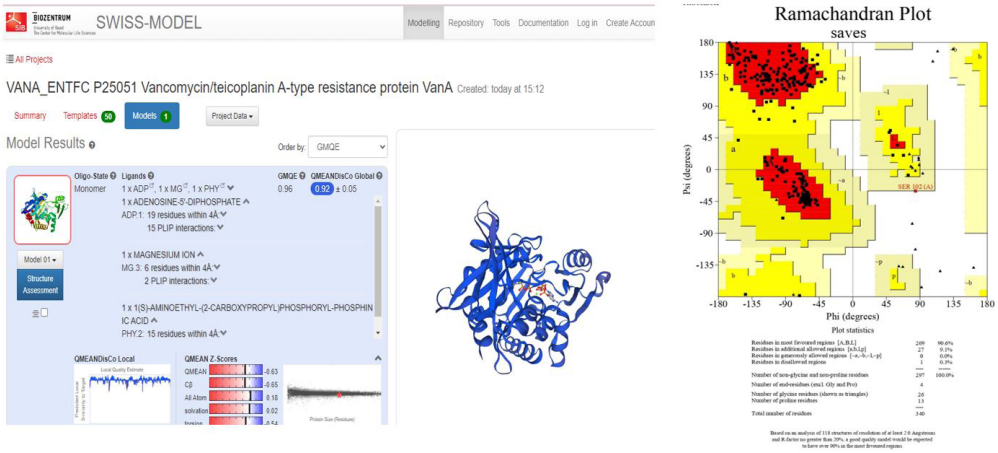

Figure 5. Prediction of vanA structure and homology modelling in Swissmodel Server and validation of the predicted structure using Ramachandran plot.

4. Assessments of the structures and drug properties of *A. marmelos* bioactive compounds

Successful optimization of the selected ligands was attained using the ChemSketch software. The three-dimensional structures of coumarin, xanthotoxol, imperatorin, aegeline, marmeline, and erythromycin were obtained and their PubChem IDs and molecular weights are presented in Fig. 6.

5. Evaluation of drug-likeness parameters

The predictions of the bioactivity of coumarin, xanthotoxol, imperatorin, aegeline, marmeline, or erythromycin against *E. faecalis vanA* protein were determined using default parameter settings, and the predicted scores are shown in Tables 1 and 2.

6. Analysis of the docking between the compounds from *A. marmelos* and the *E. faecalis* VanA protein

After the docking analysis, suitable conformers were selected using the Lamarckian Genetic Algorithm. The ball and stick models of hydrogen bond interactions between coumarin, xanthotoxol, imperatorin, aegeline, marmeline, or erythromycin and the *E. faecalis* VanA protein were visualized using Accelrys Discovery Studio (Fig. 7). The number of hydrogen bonds formed in concert with the torsional energy and the scores after

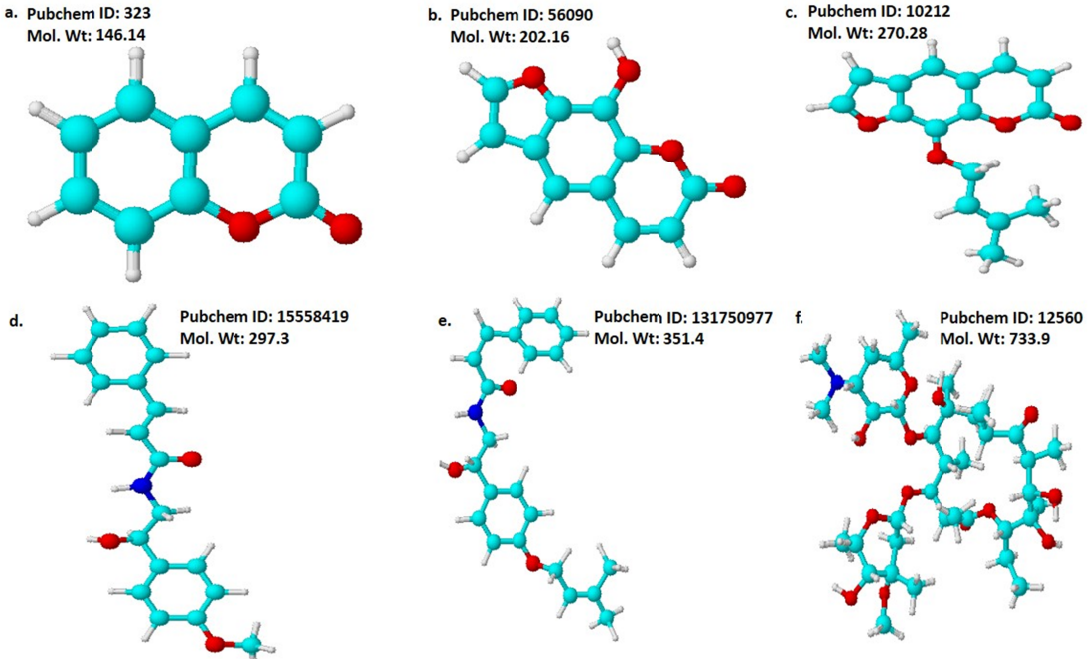

Figure 6. The 3D structures of the selected bio-compounds (a) Coumarin, (b) Xanthotoxol, (c) Imperatorin, (d) Aegeline, (e) Marmeline from *A. marmelos* and (f) Erythromycin (control) with the Pubchem ID and molecular weight.

Table 2. The drug properties of the selected bio-compounds from *A. marmelos*

Compound name	nViolations	TPSA (Å)	Rotatable bonds	Hydrogen bond donor	Hydrogen bond acceptor	miLogP	Volume	N atoms
Coumarin	0	30.21	0	0	2	2.01	128.59	11
Xanthotoxol	0	63.58	0	1	4	2.00	162.16	15
Imperatorin	0	52.59	3	0	4	3.95	240.47	20
Aegeline	0	58.56	6	2	4	2.64	281.45	22
Marmeline	0	58.56	8	2	4	4.32	342.23	26
Erythromycin	2	193.92	7	5	14	2.28	709.28	51

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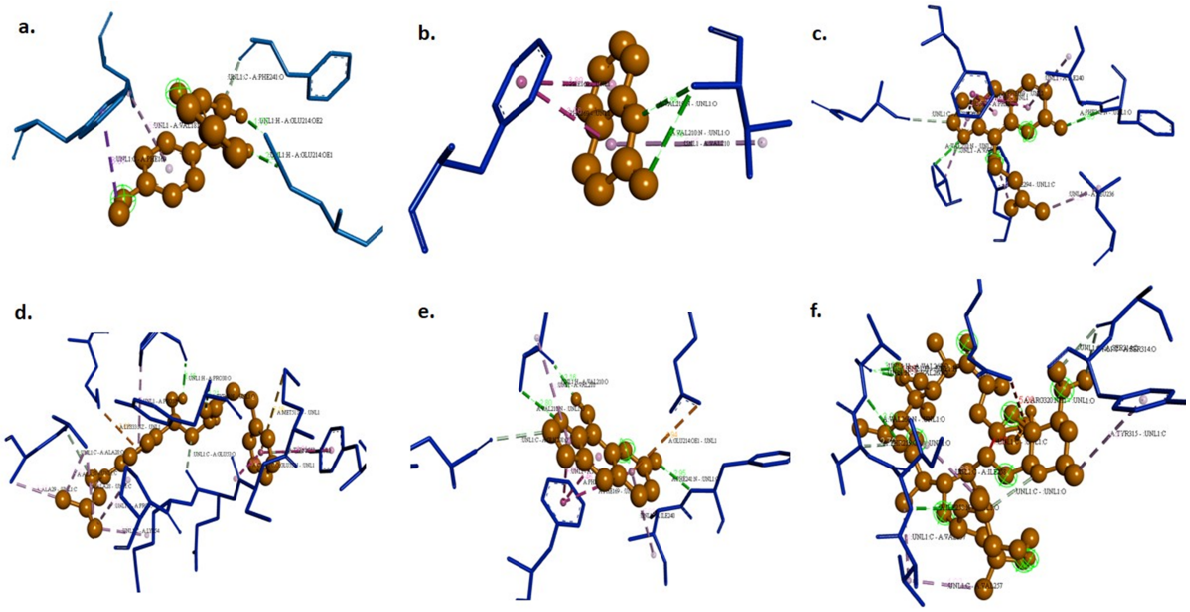

Figure 7. Visualizing hydrogen interactions between vanA with (a) coumarin (b) xanthotoxol (c) imperatorin (d) aegeline (e) marmeline (f) erythromycin.

Table 3. The docking scores of the bio-compounds from *A. marmelos* against *vanA* protein of *E. faecalis*

EfbA docking with compounds	Number of hydrogen bonds	Binding energy	Inhibition constant	Ligand efficiency	Intermolecular energy	vdW + Hbond + desolv energy	Electrostatic energy	Torsional energy	Total internal unbound
Coumarin	1	−5.99	41.01	−0.546	−5.99	−5.96	−0.03	0.0	0.0
Xanthotoxol	3	−7.14	31.4	−0.41	−6.44	−6.33	−0.11	0.3	−0.44
Imperatorin	3	−8.11	5.85	−0.48	−7.44	−7.4	−0.03	0.03	−0.03
Aegeline	3	−7.69	2.31	−0.35	−9.78	−9.26	−0.52	2.09	−1.21
Marmeline	0	−6.56	15.46	−0.25	−9.25	−9.18	−0.07	2.68	−1.78
Erythromycin	5	−7.87	1.7	−0.15	−10.26	−10.01	−0.25	2.39	−4.23

the docking between the drug and ligands (Table 3).

DISCUSSION

E. faecalis is an important oro-dental pathogen and its virulence factors are vital for the establishment of infections [18]. Our analyses indicated that the frequency of *E. faecalis* in patients with root caries is 60%, and detected the presence of the *vanA* gene in VRE strains. This frequency seems to be higher when compared with earlier studies that detected *E. faecalis* in 19% and 38%, with statistical significance [19]. This discrepancy may be caused by differences in sample size since our study had a sample size of 20. *E. faecalis* is frequently found in the apical part of the root canal, implying that its invasion occurs during endodontic therapy [20]. A link has also been reported between the presence of *E. faecalis* and the number of clinic visits, which are caused by coronal microleakage via the temporary filling used between endodontic treatment sessions [21]. These findings suggest that *E. faecalis* is more prevalent in patients with dental infections or disorders.

Resistant *E. faecalis* strains are common in healthcare settings. However, in dental settings, periodic surveillance of multidrug resistance or VRE strains remains limited. This study indicates that the presence of the *vanA* gene in VRE is up to 16% (n = 2) and did not detect the *vanB* gene. A previous global report on VRE prevalence in eight countries found the prevalence of VRE to be highest in the UK (2.9%), followed by Israel (2%) [22]. However, VRE rates in the remaining European countries

Table 4. The bioactivity scores of the selected compounds based on the score > 0.3

Compounds	Kinase inhibitor	Nuclear receptor ligand	GPCR ligand	Ion channel modulator	Enzyme inhibitor	Protease inhibitor
Coumarin	−1.57	−1.42	−1.44	−0.86	−0.58	−1.43
Xanthotoxol	−0.82	−0.75	−0.70	−0.16	−0.14	−0.94
Imperatorin	−0.56	−0.18	−0.37	−0.02	0.10	−0.60
Aegeline	−0.23	−0.23	0.19	−0.22	0.05	−0.05
Marmeline	−0.28	0.15	0.16	−0.14	0.13	−0.05
Erythromycin	−1.25	−1.12	−0.50	−1.31	−0.60	−0.18

were ≤ 1%. VRE strains with the *vanB* gene have been isolated in Slovenia, Finland, Sweden, and the UK, and they were found to be most common in Slovenia (2%) [23].

Plant bioactive compounds have the potential to control the complications of various microbial pathogens [24, 25]. In this study, we evaluated the antibacterial activity of a methanol extract from the leaves of *A. marmelos*. Using disc diffusion assays, similar studies have reported the antimicrobial activities of extracts obtained using chloroform, methanol, and water against *Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Escherichia coli*, *Salmonella paratyphi A*, and *Salmonella paratyphi B* [26]. Here, we report the promising effects of a methanol extract from *A. marmelos*, which showed significant moderate activity against clinical VRE isolates.

In this study, analysis of docking interactions between the biocompounds and *vanA*, as well as the docking scores and energies, were evaluated. Because the VanA protein structure was not available, we used SWISS-MODEL to predict its structure using the 7AQC-Q chain template. Using Ramachandran validation, which can evaluate predicted protein structures by showing similar residues in the favored region, we found that in the predicted model, 90.6% of the residues were in the favored region, whereas only 0.3% occurred in disallowed regions. Drug parameter analysis using Molinspiration revealed all the compounds to be the best, with zero violations based on Lipinsky's rule of five, and that erythromycin (control) had two violations. However, TPSA analysis revealed that the oral bioavailability scores did not exceed 140 Å, except for erythromycin. However, the nuclear receptor and enzyme inhibitor bioactivity scores, with values of > 0.3, were promising.

Analysis of bioactive properties (Table 4) with scores set at > 0.3, revealed promising scores for all *A. marmelos* ligands that were selected. Studies of the biocompounds obtained from *A. marmelos* indicate that in lung disorders (edema and fibrosis), imperatorin has anti-inflammatory effects on alveolar macrophages. Coumarin, which has systemic effects, has been reported to eliminate the symptoms of persistent brucellosis. *A. marmelos* has anti-inflammatory effects against various cancer cell lines and it has been reported to contain the anti-carcinogenic substances, beta caryophyllene and caryophyllene oxide [27]. In this study, the inhibitory property of imperatorin is promising against the *E. faecalis* VanA protein. A limitation of this study is that we did not purify the crude extract for analyses of the active biocompounds. Future studies should seek to identify novel *A. marmelos* biocompounds, determine their cytotoxicity, and perform preclinical trials to determine their suitability as alternative treatments for *E. faecalis* infections. However, this study is the first to suggest the compound imperatorin, from *A. marmelos*, as a potential drug for treating *E. faecalis* infections.

CONCLUSION

This study shows that the *vanA* gene is frequent in multi-drug-resistant *E. faecalis* strains in a dental health care setting. These findings highlight the need for periodic microbiological surveillance in healthcare settings. Our findings show that the leaves of *A. marmelos* are effective against *E. faecalis* VRE strains. Among the five biocompounds selected for analysis, imperatorin exhibits good binding energy, but further studies are required to establish its suitability as an alternative to existing antibiotics.

CONFLICTS OF INTEREST

None to declare.

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