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Original article

Stephania suberosa Forman extract synergistically inhibits ampicillin- and vancomycin-resistant Enterococcus faecium

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ABSTRACT

Increasing antibiotic resistance in enterococci is among the most serious public health problems worldwide. The new naturally occurring antibacterial agents were explored. This study, therefore, investigated the antibacterial potential of *Stephania suberosa* extract (SSE) and its synergism with ampicillin (AMP) or vancomycin (VAN) against AMP- and VAN-resistant *Enterococcus faecium*. Disc diffusion assay revealed that SSE inhibited *E. faecium* DMST 12829, 12852, 12970, and a reference strain of *Enterococcus faecalis* ATCC 29,212 in a dose-dependent manner. The minimum inhibitory concentration (MIC) of SSE against all *E. faecium* isolates was 0.5 mg/mL. *E. faecium* DMST 12,829 and 12,852 were highly resistant to AMP, as indicated by high MIC values, and *E. faecium* DMST 12,829 and 12,970 were resistant to VAN. *Enterococcus* spp. were killed by SSE at the minimum bactericidal concentrations (MBCs) ranging from 0.5 to 4 mg/mL. Checkerboard determination showed that SSE plus AMP and SSE plus VAN combinations exhibited synergistic interaction against *E. faecium* isolates. The killing curve assay of *E. faecium* isolates confirmed the antibacterial and synergistic activities of combined agents by dramatically reducing the viable counts compared to a single agent. Scanning electron microscope elucidated the cell damage and abnormal cell division. Enterococcal proteases were also inhibited by SSE. These findings support that SSE could reverse the activity of AMP and VAN. Moreover, it can synergistically inhibit AMP- and VAN-resistant *E. faecium*. Our combined agents could be attractive candidates for developing new combinatorial agents to resurrect the efficacy of antibiotics for treating AMP- and VAN-resistant *E. faecium* infections.

1. Introduction

Enterococci are ubiquitous Gram-positive bacteria belonging to the family Enterococcaceae. *Enterococcus* spp., particularly *Enterococcus faecium*, is a member of the ESKAPE that can cause life-threatening infections in humans (Santajit and Indrawattana 2016). They are classified as a high priority on the World Health Organization (WHO) priority list of microorganisms required for developing new antimicrobial agents (Dhingra et al., 2020). Enterococci are notable opportunistic pathogens for several human ailments by infecting different parts of humans, including the urinary system, intra-abdominal, soft-tissue, and cardiopulmonary system (Semedo et al., 2003, Guzman Prieto et al., 2016). They are more frequently resistant to penicillin, amoxicillin, cephalosporins, and vancomycin than Streptococci (Williamson et al., 1985, Cetinkaya et al., 2000).

Enterococci have become clinically important pathogens to humans since the resistance to vancomycin, and other antibiotics have been increasingly documented worldwide, corresponding to

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the increasing use of antibiotics (Bell et al., 1998, Miller et al., 2014). These bacteria can intrinsically and extrinsically develop resistance by target modification, drug inactivation, overexpression of efflux pumps, and a cell envelope alteration (Shepard and Gilmore 2002, Hollenbeck and Rice 2012). Ampicillin (AMP) resistance in Enterococci is mediated by producing low-affinity penicillin-binding protein 5 (PBP5). While, resistance to glycopeptide, especially vancomycin (VAN), is mediated by modification of peptidoglycan precursor called UDP-N-acetylmuramyl-pentadepsi peptide (Wade 1995). *E. faecium* exhibits higher resistance to antimicrobial agents than *E. faecalis*, with a higher fatality rate in immunocompromised patients (Top et al., 2008). Boccella et al. (2021) reported that *E. faecium* clinical samples at the University Hospital "San Giovanni di Dio e Ruggi d'Aragona" in Salerno, Italy, showed a high prevalence of antibiotic resistance to AMP (84.5%), AMP and sulbactam combination (82.7%), and imipenem (86.7%), but *E. faecalis* had much lower resistance rate to these antibiotics. Besides, *E. faecium* exhibited a high resistance rate to gentamicin (59 %) and streptomycin (46%) (Boccella et al., 2021). An epidemiological analysis of VAN-resistant enterococci in Europe demonstrated an increasing resistance rate from 8.1% in 2012 to 19% in 2018 across Europe (Ayobami et al., 2020).

The consequence of antibiotic resistance results in difficulty in antibiotic administration due to ineffective antibiotics increasing. Some are relatively toxic, expensive, and have undesirable adverse reactions. The development of new antibacterial drugs and strategies is of far-reaching importance. Humans have used medicinal plants to treat several diseases, including infectious diseases (Pejin et al., 2017). Due to plant-derived antimicrobials having diverse mechanisms of action in the inhibition of microbial pathogens, they are exciting sources of novel antimicrobial agents (Savoia 2012, Pejin et al., 2014). Many plants possess attractive biological activities with potential therapeutic applications. *Stephania suberosa* Forman is a native plant widely cultivated in Africa, India, Southeast Asia, and northern and eastern Australia. This herbal plant has traditionally been employed to treat various diseases, including asthma, tuberculosis, diarrhea, hyperglycemia, malignancy, pyrexia, abdominal pain, insomnia, and inflammation (Patra et al., 1987, Blanchfield et al., 2003, Semwal et al., 2010). Regarding the antibacterial spectrum, *S. suberosa* extract (SSE) exhibited bactericidal activity and synergistic interaction with AMP against AMP-resistant *S. aureus* (Teethaisong et al., 2014). More recently, SSE synergistically revived the activity of colistin against colistin resistance in *Enterobacter cloacae* (Suknasang et al., 2019).

Combinations of antibiotic/antibiotic, phytochemical/phytochemical, or phytochemical/antibiotic have been proven to helpfully treat antimicrobial-resistant bacteria and delay the development of antibiotic resistance (Teethaisong et al., 2018, Farha and Brown 2019). The drug combination approach is a promising and attractive strategy for treating complex diseases and recalcitrant infections, including cancer, tuberculosis, and antibiotic-resistant microbes (Güvenç Paltun et al., 2021). Here, the antibacterial activity of SSE and its synergistic interaction with AMP or VAN against AMP- and VAN-resistance *E. faecium* were investigated in the present study.

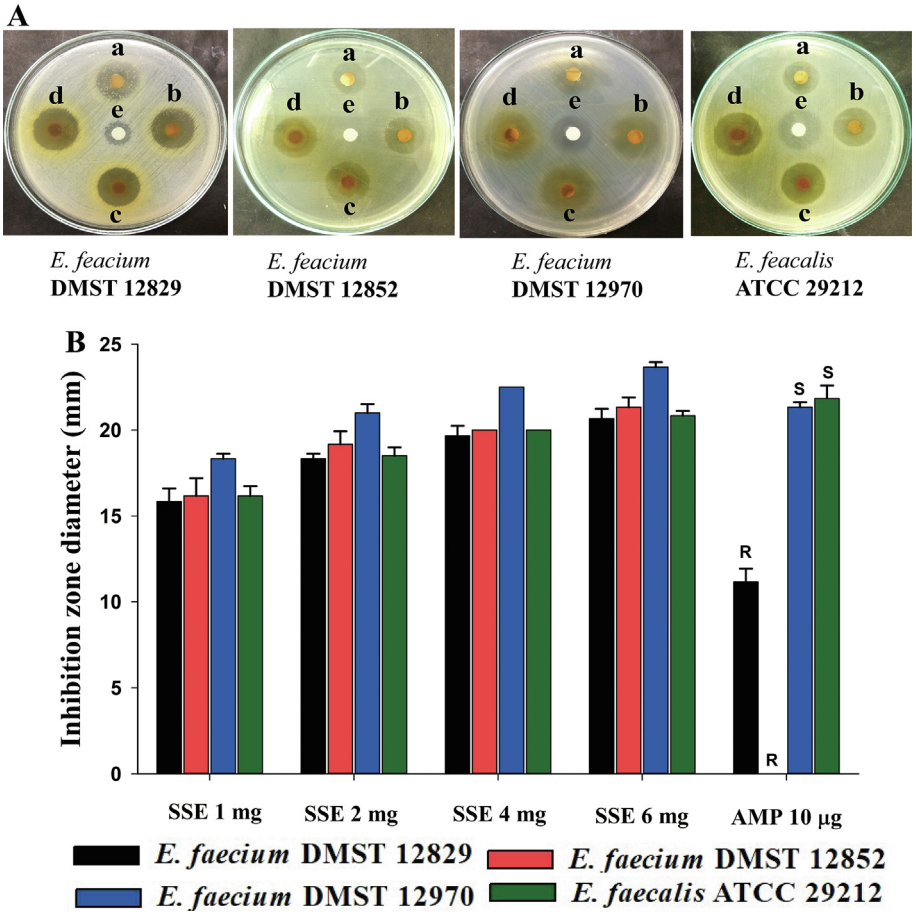

Fig. 1. Disc diffusion assay (A) and inhibition zone diameter (B) of *S. suberosa* extract (SSE) and ampicillin (AMP). **a** = SSE at 1 mg/disc; **b** = SSE extract at 2 mg/disc; **c** = SSE at 4 mg/disc; **d** = SSE at 6 mg/disc; **e** = Amp at 10 µg/disc; R = resistant to ampicillin; S = susceptible.

2. Materials and methods

2.1. Bacterial strains, Antibiotics, and plant specimen

The bacterial strains employed in this study included *E. faecium* DMST 12829, *E. faecium* DMST 12852, *and E. faecium* DMST 12,970 obtained from the Department of Medical Science of Thailand (DMST), Thailand. *E. faecalis* ATCC 29,212 was used as a control strain for the antibacterial susceptibility testing. AMP and VAN antibiotics were purchased from Sigma-Aldrich, Singapore. The dried powder of the root of *S. suberosa* was obtained from Lamtakhong Research Station, Nakhon Ratchasima, Thailand.

2.2. Ethanol extraction of S. Suberosa

The dried powder of *S. suberosa* was extracted using a Soxhlet apparatus with 95% ethanol at 75 ℃ for 8 h. Subsequently, the extracts were filtered through Whatman filter paper No 1 prior to being concentrated using a rotatory evaporator and dried by a freeze dryer (Teethaisong et al., 2014). The dried extract was stored at −20 ℃ before use.

2.3. Bacterial suspension standard curve

This assay was determined to know the exact number of bacterial suspensions. Briefly, an 18-h culture was collected and washed twice before adjusting the bacterial suspension to achieve different optical densities (0.05, 0.1, 0.15, 0.2, and 0.25) at a wavelength of 600 nm. Each optical density was diluted with normal saline to enumerate bacterial colonies as described by the Miles and Misra method (Teethaisong et al., 2018).

2.4. Disc diffusion assay

Disc diffusion or the Kirby-Bauer was used to screen the antibacterial activity of SSE. The SSE discs were prepared to obtain 1, 2, 4, and 6 mg/disc concentrations. AMP discs (10 μg/disc) were used as a reference antibiotic. Disc diffusion assay was performed in accordance with Clinical Laboratory Standard Institute (CLSI). Briefly, a sterile swab was soaked with bacterial suspension of 1×10^8 cfu/mL followed by spreading thoroughly on cation-adjusted Mueller Hinton Agar (CAMHA). SSE and AMP discs were placed equidistantly on the agar surface. Then, incubated at 37 °C for 24 h, the inhibition zone diameters were measured (Clinical Laboratory Standards Institute 2010).

2.5. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

A resazurin microtiter plate coupled with a standard guideline described by CLSI was used to evaluate MIC (Clinical Laboratory Standards Institute 2012). Resazurin has been widely used for determining the viability of cells and antimicrobial susceptibility of natural products (Sarker et al., 2007, Teethaisong et al., 2018). To determine MIC, SSE was dissolved with sterile distilled water to prepare a stock of 160 mg/mL. Antibiotics were prepared following the manufacturer's instructions to achieve a stock solution of 10,240 μg/mL. The resazurin was dissolved with sterile water and was sterilized through a 0.2 μm-pore filter. An 18-h culture was collected, washed, and adjusted (5×10^6 cfu/mL) according to the bacterial suspension standard curve prior to adding 20 μL to the well containing 180 μL of CAMHB medium with serial concentrations of SSE (0.015–32 mg/mL) or antibiotics (0.5–1024 μg/

Table 1
Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of S. suberosa extract (SSE), ampicillin (AMP), and vancomycin (VAN).

Bacterial Isolates	MIC			MBC		
	SSE (mg/mL)	AMP (μg/mL)	VAN (μg/mL)	SSE (mg/mL)	AMP (μg/mL)	VAN (μg/mL)
E. faecium DMST 12,829	0.5^{ND}	128^{R}	64^{R}	0.5	256	256
E. faecium DMST 12,852	0.5^{ND}	1024^{R}	8^{I}	4	>1024	64
E. faecium DMST 12,970	0.5^{ND}	4^{S}	32^{R}	1	256	256
E. faecalis ATCC 29212*	0.25^{ND}	4^{S}	2^{S}	1	16	8

* Reference bacterial strains for antimicrobial susceptibility testing of *Enterococcus* spp. ^{S} = susceptible; ^{I} = intermediate; ^{R} = Resistant; ND = No data in CLSI breakpoint.

Table 2
Drug interaction of S. suberosa extract (SSE) in combination with ampicillin (AMP) or vancomycin (VAN) against Enterococcus spp.

Bacterial Isolates	Checkerboard			
	Combination of agents	MIC (in combination)	FIC index	Type of interaction
E. faecium DMST 12,829	SSE	0.125	0.5	Synergism
	AMP	32		
	SSE	0.125	0.27	Synergism
	VAN	1		
E. faecium DMST 12,852	SSE	0.125	0.28	Synergism
	AMP	32		
	SSE	0.125	0.31	Synergism
	VAN	0.5		
E. faecium DMST 12,970	SSE	0.125	0.75	Partial synergism
	AMP	2		
	SSE	0.125	0.28	Synergism
	VAN	1		
E. faecalis ATCC 29212*	SSE	0.0625	0.75	Partial synergism
	AMP	2		
	SSE	0.125	0.75	Partial synergism
	VAN	0.5		

Fraction inhibitory concentration index (FICI) ≤ 0.5 indicates synergistic interaction; FICI > 0.5 to < 1.0 indicates partial synergism; FICI = 1 indicates addition; FICI > 1 to ≤ 4.0 indicates indifference; FICI > 4.0 indicates antagonism.

mL), and resazurin (20 μg/mL). Control wells were antibacterial agents and bacteria-free. After incubation at 37 °C for 18 h, the MIC results were visually observed. MIC was noted as the lowest concentration that shows no color change from blue to pink.

The MBC is defined as the lowest concentration of the agents that produced no visible growing colonies on the agar medium. To evaluate MBC, 100 μL from the wells that MIC was taken and subcultured onto the agar medium and spread evenly on the agar surface. The culture plates were subsequently incubated at 37 °C overnight. The experiment was carried out in three independent replications (Rukayadi et al., 2009).

2.6. Determination of drug interaction by Checkerboard

The interaction between two combined drugs was evaluated by a checkerboard assay as indicated by the fractional inhibitory concentration (FIC) index. The experiment procedure was performed following previous studies (Bonapace et al., 2002, Teethaisong et al., 2018). This experiment combined SSE with AMP or VAN to investigate the synergistic activity against three *E. faecium* strains and one *E. faecalis*. This experiment was performed similarly to the MIC assay. Differently, 120 μL CAMHB were mixed with 20 μL of serial concentrations of antibiotics (0.25–56 μg/mL), 20 μL of different concentrations of SSE (0.03–2 mg/mL), 20 μL of resazurin, and 20 μL of 5×10^6 cfu/mL tested bacteria. The microplates were subsequently incubated at 37 °C overnight. The FIC index was calculated by the equation below (Timurkaynak et al., 2006, Teethaisong et al., 2018).

$$\text{FIC index} = \text{FIC}_A + \text{FIC}_B = \frac{\text{Conc.of A in MICs of A} + \text{B}}{\text{MIC of A alone}} + \frac{\text{Conc.of B in MICs of A} + \text{B}}{\text{MIC of B alone}}$$

Where FIC index $\leq$ 0.5 indicates synergism, FIC index > 0.5 to < 1.0 shows partial synergism, FICI = 1.0 indicates addition, FIC index > 1.0 to $\leq$ 4.0 denotes indifference, and FIC index > 4.0 denotes antagonism.

2.7. Killing curve determination

This assay was performed to verify the antibacterial and synergistic activities of the combination of SSE with VAN or AMP against *E. faecium* DMST 12,829 and DMST 12852, respectively. Bacteria were treated with SSE alone, antibiotic alone, the combination of SSE plus antibiotic, and without an agent, then incubated for 0, 1, 2, 4, 6, 8, 20, and 24 h. After incubation, 100 μL was transferred to 900 μL normal saline in a microcentrifuge tube. Then, 10 μL of 10-fold diluted solution series was dropped on CAMHA medium that was performed in 9 replications. The growing colonies were counted after incubation at 37 °C overnight. The cfu/mL was computed as follows; mean counted colonies × dilution factor × 100. Decreases of $\geq$ 2 log10 cfu/mL between the combination compared to the most active single agent at 24 h denoted synergistic interactions, while reductions of < 2 log10 cfu/mL and increases of $\geq$ 2 log10 cfu/mL compared to mono-treatment denoted additive or indifferent interactions and antagonism, respectively. Furthermore, $\geq$ 3log10 decreases or < 3log10 reductions in cfu/mL suggested bactericidal activity and bacteriostatic activity, respectively, compared to the initial inoculum (Lee and Burgess 2013).

2.8. Protease activity

Protease is among the virulence factors playing a significant role in the pathogenesis of bacteria. The inhibitory effect of SSE on bacterial protease activity was elucidated by a skim-milk agar plate method following a previous report, with some modifications (Fu et al., 2018). Two percent skim milk was sterilized by boiling for 15 min before adding a sterile Mueller–Hinton agar medium. After the skim-milk and medium cooled down, SSE at 0.25, 0.125, 0.0625, and 0 mg/mL was mixed with skim-milk medium. Then, 25 mL were poured into sterile petri dishes. Bacterial isolates were spectrophotometrically adjusted to achieve OD 0.5 at a wavelength of 600 nm before dropping 5 μL onto skim-milk agar in the presence or absence of SSE. After incubation at 37 °C overnight, the clear zone around bacterial spots was visually assessed.

2.9. Scanning electron microscope (SEM)

Morphological change of *E. faecium* DMST 12,852 after exposure to SSE or AMP was visualized by SEM. The samples were prepared and proceeded following the modified method described by a previous study (Richards et al., 1993). A log-phase grown bacteria were treated with AMP or SSE alone, AMP plus SSE combination,

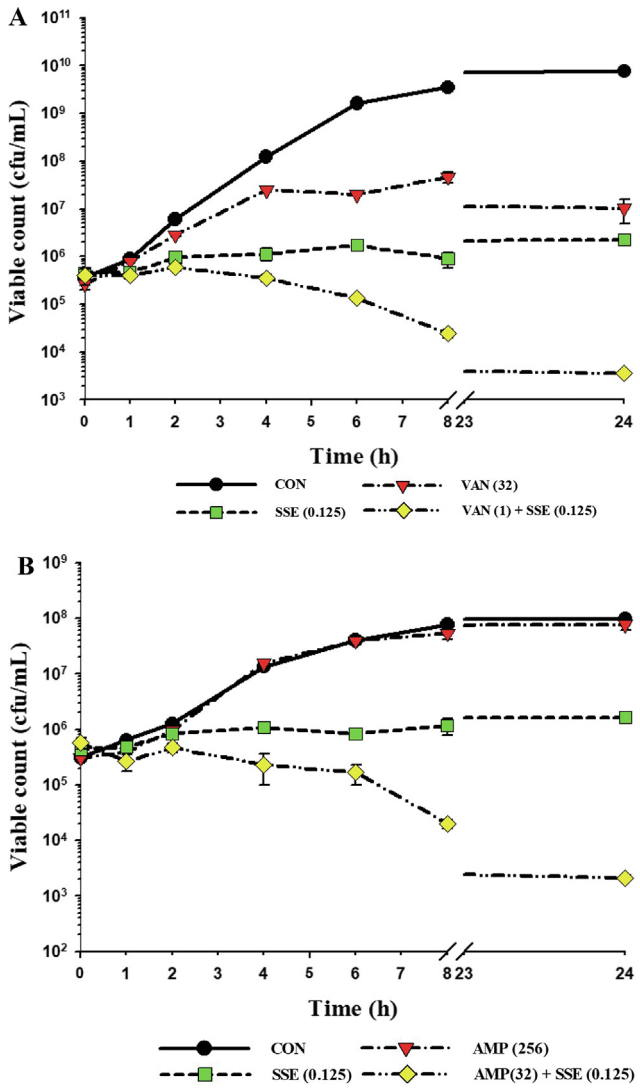

Fig. 2. Killing curves of *E. faecium* DMST 12,829 (A) and DMST 12,852 (B) after exposure to *S. suberosa* extract, vancomycin, ampicillin alone, and in combination. CON = untreated control; SSE (0.125) = *S. suberosa* extract at a concentration of 0.125 mg/mL; VAN (32) = vancomycin at a concentration of 32 μg/mL; VAN (1) + SSE (0.125) = vancomycin at a concentration of 1 μg/mL plus *S. suberosa* extract at a concentration of 0.125 mg/mL; AMP (32) + SSE (0.125) = ampicillin at a concentration of 32 μg/mL plus *S. suberosa* extract at a concentration of 0.125 mg/mL. Data were expressed as mean ± standard error of the mean.

and untreated control. Samples were centrifuged at 6,000 rpm for 15 min and were then fixed with 2.5% (w/v) glutaraldehyde overnight at 4 °C. Samples were washed 3 times with phosphate-buffered saline (PBS), followed by staining with 1% osmium tetroxide for 2 h, washed 3 times with distilled water, and centrifuged at 8,000 rpm for 5 min. Cell dehydration was carried out using 20%, 40%, 60%, 80%, 100%, and 100% graded acetone. The cells were transferred to glass slides and then air-dried. The samples were attached on stubs using carbon tape and were subsequently coated with gold. Cell morphology was finally captured by a scanning electron microscope.

3. Results

3.1. Disc diffusion assay

The results demonstrated that *Enterococcus* spp. isolates were inhibited by SSE in a concentration-dependent manner, as indicated by increasing inhibition zone diameters at higher concentrations. No inhibition zone diameter was seen in the AMP disc against *E. faecium* DMST 12852, indicating that this strain is highly resistant to AMP. According to CLSI, the susceptibility breakpoints for AMP are $\geq$ 17 mm (Clinical Laboratory Standards Institute 2022). Hence, *E. faecium* DMST 12,829 and 12,852 were resistant to AMP (Fig. 1).

3.2. MIC and MBC determinations

The MIC values of SSE, AMP, and VAN were evaluated against *E. faecium* DMST 12829, *E. faecium* DMST 12852, *E. faecium* DMST 12970, and *E. faecalis* ATCC 29212, as shown in Table 1. SSE had a MIC of 0.5 mg/mL against all 3 *E. faecium* isolates and 0.25 mg/mL against an *E. faecalis* strain. MICs of AMP against *E. faecium* DMST 12,829 and 12,852 were resistant at 128 and 1024 μg/mL, respectively. MIC of AMP was in the susceptible range against *E. faecium* DMST 12970. *E. faecalis* ATCC 29212, a reference strain, was sensitive to AMP and VAN. In addition, the MICs of VAN were 64, 8, and 32 μg/mL against *E. faecium* DMST 12829, 12852, and 12970, respectively (Clinical Laboratory Standards Institute 2022).

MBC values of SSE in killing all enterococci ranged from 0.5 to 4 mg/mL. MBCs of both AMP and VAN against *E. faecium* DMST 12,829 and 12,970 were all at 256 μg/mL, while *E. faecium* DMST 12,852 had MBCs at > 1024 and 64 μg/mL, respectively. In contrast, the MBC values of SSE, AMP, and VAN against the reference strain were 1, 16, and 128 μg/mL, respectively (Table 1).

3.3. Checkerboard assay

The interactions of SSE in conjunction with AMP or VAN for treating *Enterococcus* spp. are shown in Table 2. SSE plus AMP or VAN combinations exhibited synergistic interactions against *E. faecium* DMST 12,829 and 12,852 (FIC indices ranging between 0.27 and 0.5). The lowest FIC index was found in the combination of SSE and VAN against *E. faecium* DMST 12829. Furthermore, *E. faecium* DMST 12,970 was also synergically inhibited by SSE plus VAN mix, with an FIC index of 0.28. The combinations of SSE plus AMP and SSE plus VAN against the control strain were partially synergistic.

3.4. Killing curve assay

Kinetic inhibition monitoring by a killing curve assay elucidated that the untreated control group grew continuously from starting

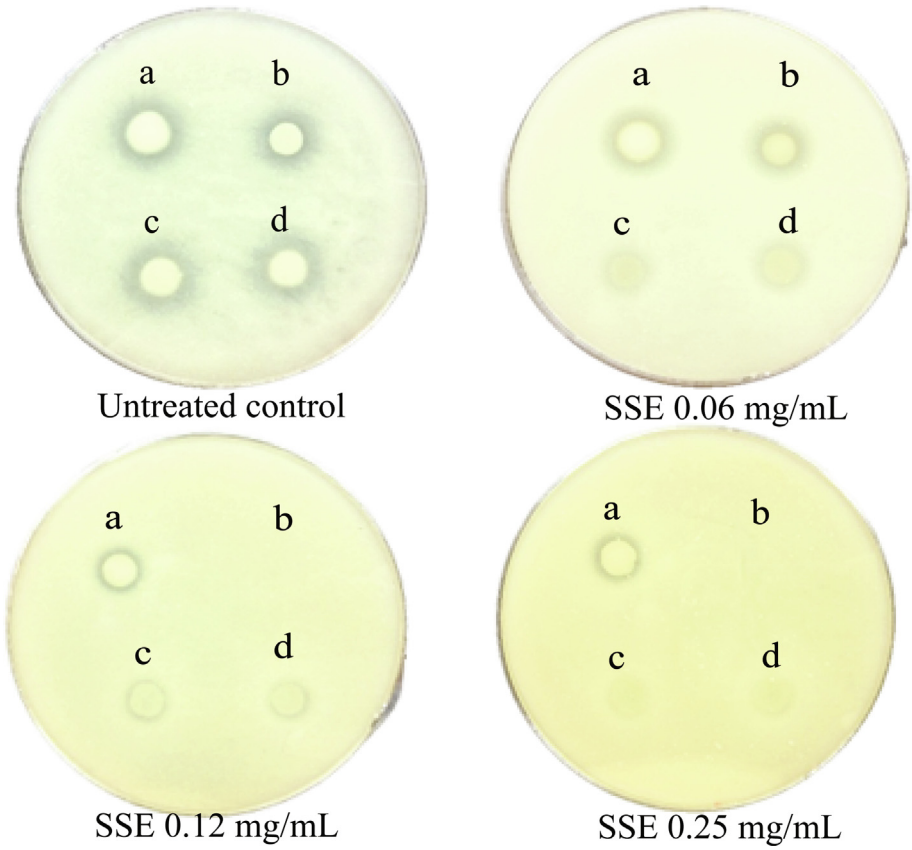

Fig. 3. Anti-protease activity of *S. suberosa* extract (SSE) at sub-inhibitory concentrations of 0.25, 0.12, and 0.06 mg/mL. **a** = *E. faecium* DMST 12852; **b** = *E. faecium* DMST 12829; **c** = *E. faecium* DMST 12970; **d** = *E. faecalis* ATCC 29212.

to 24 h of the experiment. In SSE (0.125 mg/mL)-treated group, the cells were statistically inhibited by SSE in both *E. faecium* DMST 12,829 (Fig. 2A), and DMST 12,852 (Fig. 2B) compared to untreated cells. The cells exposed to VAN alone at a concentration of 32 μg/mL grew gradually over 24 h of exposure (Fig. 2A), while *E. faecium* DMST 12,852 treated with 256 μg/mL AMP exhibited no growth inhibition compared to untreated cells, indicating that this particular strain is highly resistant to AMP. Both combinations of SSE plus VAN and SSE plus AMP clearly inhibited the growth of *E. faecium* DMST 12,829 (Fig. 2A) and DMST 12,852 (Fig. 2B). At 24 h of treatment, the viable cells in both combined treatment of SSE plus VAN and SSE plus AMP showed reduction > $2\log_{10}$ cfu/mL compared to SSE alone, confirming the synergistic interactions in those combinations (Lee and Burgess 2013).

3.5. Protease activity

Sub-inhibitory concentrations of SSE, including 0.25, 0.125, and 0.0625 mg/mL were chosen for qualitative analysis of protease activity produced by enterococci. The results exhibited that all tested enterococci produced protease. At 24 h of incubation, all concentrations of SSE inhibited the protease activity of all tested enterococci. Decreased clear zone diameter was observed in SSE 0.25 and 0.125 mg/mL against all enterococci strains, particularly protease of *E. faecium* DMST 12829, which was completely inhibited by SSE (Fig. 3).

3.6. Scanning electron microscope

A change in cell morphology was observed by SEM. Untreated control displayed intact morphology and no damage on the entire surface of cell envelopes (Fig. 4A). *E. faecium* DMST 12,852 treated with SSE at a 0.25 mg/mL concentration showed slight damage on the cell surface. Abnormal morphology, rough and protruding cell surface and cell debris were also seen in SSE-treated cells (Fig. 4B). In cells treated with SSE (0.125 mg/mL) combined with AMP (16 μg/mL) exhibited remarkable damage to the cell surface, with dimpled, protruded, and abnormal morphology with the long septum (Fig. 4C). SEM micrograph of cells treated with 512 μg/mL AMP showed no substantial damage to bacterial cells compared to other groups, suggesting *E. faecium* DMST 12,852 is highly resistant to AMP (Fig. 4D).

4. Discussion

Resistance to antibiotics in *E. faecalis* and *E. faecium* has become one serious concern in public health, with increasing incidence worldwide. Enterococci confer intrinsic resistance to several β-lactam antibiotics by expressing low-affinity PBP5 (Kristich et al., 2014). The number of newly approved antibiotics for clinical use is inverse to the antibiotic resistance rate, resulting in lower choices for treating enterococcal infections. A drug combination is a promising avenue to conquer antibiotic-resistant enterococci and delay

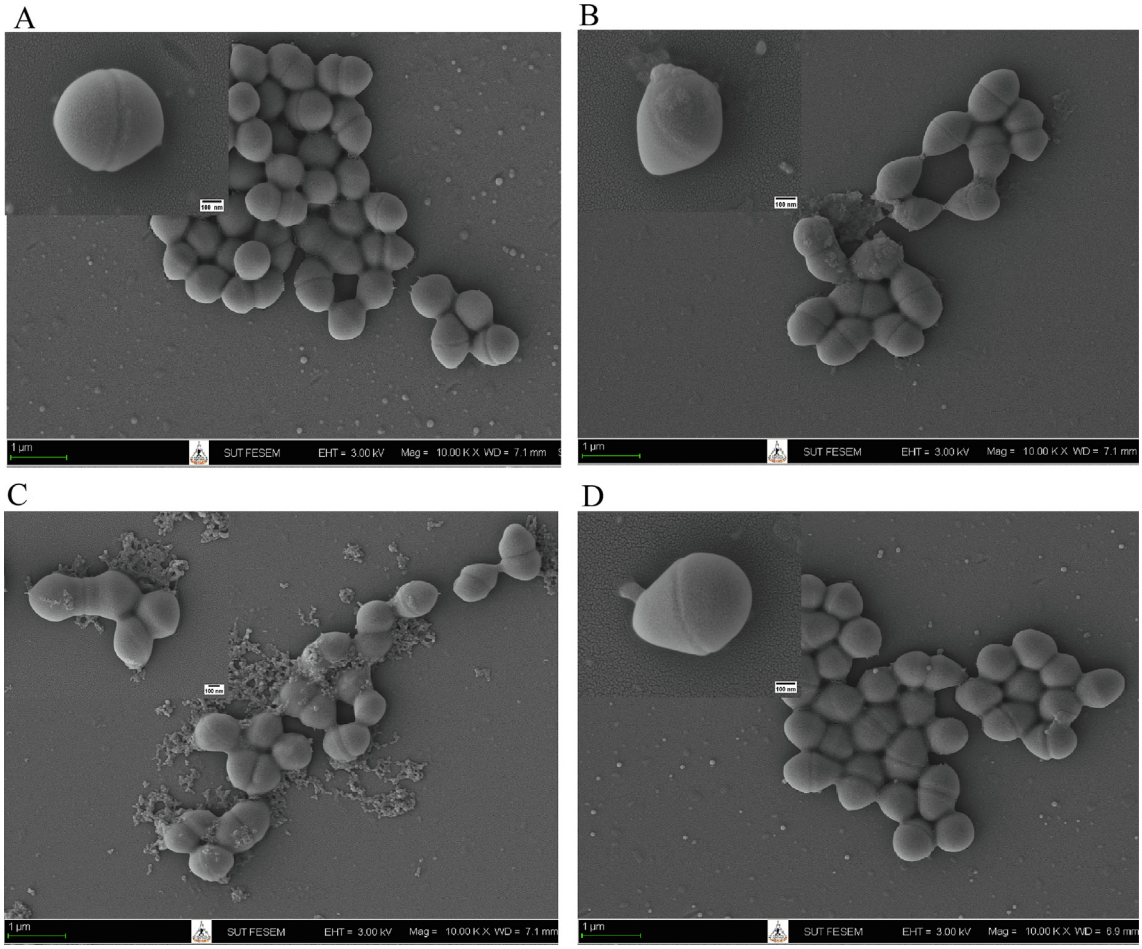

Fig. 4. Scanning electron micrographs of untreated *E. faecium* DMST 12,829 (**A**), *E. faecium* DMST 12,829 treated with *S. suberosa* extract at 0.25 mg/mL (**B**), *E. faecium* DMST 12,829 treated with the combination of 0.125 mg/mL *S. suberosa* extract plus 16 μg/mL ampicillin (**C**), and *E. faecium* DMST 12,829 treated with 512 μg/mL ampicillin (**D**). Bar = 1 μm and magnification 10,000x; Inset, Bar = 100 nm and magnification = 50,000x.

current antibiotic resistance development (Mitchison 2012). Using phytochemical compounds to resurrect the efficacy of antibiotics that have lost their potential to kill antibiotic-resistant bacteria is among the methods of choice for conquering the antibiotic resistance crisis (Siriwong et al., 2016, Cheypratub et al., 2018).

In vitro antimicrobial susceptibility testing indicated SSE showed good activity by having MICs between 0.25 and 0.5 mg/mL and MBCs ranging from 0.5 to 4 mg/mL against Enterococcal isolates. *E. faecium* DMST 12,829 was found to be resistant to both AMP and VAN. Resistance to both AMP and VAN in the same strain is uncommon in enterococci (Gagetti et al., 2019), but the present study found this phenomenon. MICs of VAN and AMP against the reference strain of *E. faecalis* ATCC 29,912 are in the MIC quality control range, following the CLSI guidelines. Interestingly, SEE can revive and potentiate the antibacterial efficacy of AMP and VAN against AMP- and VAN-resistant *E. faecium,* which is consistent with previous studies reporting that SSE revives the antibacterial activity of AMP against AMP-resistant *S. aureus*, as well as colistin against colistin-resistant *E. cloacae* (Teethaisong et al., 2014, Suknasang et al., 2019). This evidence supports that SSE may be a broad-spectrum compound against both Gram-positive and Gram-negative bacteria. Based on the SEM study, we assume that SSE inhibits *E. faecium* by interfering with cell division and damaging bacterial cell envelop, resulting in abnormal cell shape. Significant cell damage was seen in SSE combined with AMP. It is noteworthy that the synergistic interaction of two compounds could be achieved by different antibacterial targets (Teethaisong et al., 2018). It is well-established that, like other β-lactam antibiotics, AMP inhibits enterococci by targeting PBPs (Peechakara et al., 2022). Regarding the bioactive compound constituents in SSE, our previous study found that tetrahydropalmatine, palmitic acid, and roemerine were among 25 compounds identified by a GC–MS analysis that possessed antibacterial activity (Suknasang et al., 2019).

5. Conclusions

SSE possesses antibacterial activity against AMP- and VAN-resistant *E. faecium*. This herbal plant also showed synergistic interaction when combined with AMP and VAN against these refractory bacteria. The best synergistic activity was found in SSE plus VAN against *E. faecium* DMST 12829. Also, SSE inhibited protease activity, an important bacterial virulent factor for pathogenesis. SEM result suggests that AMP plus SSE combination induces significant cell damage and possibly interferes with cell division. The efficacy and toxicity of combinations showing synergistic activity should be further validated in animal models or human subjects.

6. Availability of data and material

All data generated during this study are presented in an analyzed format in this manuscript. Raw datasets used for analysis in this study are available from the corresponding author upon reasonable request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Ayobami, O., Willrich, N., Reuss, A., Eckmanns, T., Markwart, R., 2020. The ongoing challenge of vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis* in Europe: an epidemiological analysis of bloodstream infections. Emerg. Microbes. Infect. 9, 1180–1193. https://doi.org/10.1080/22221751.2020.1769500.

Bell, J.M., Paton, J.C., Turnidge, J., 1998. Emergence of vancomycin-resistant enterococci in australia: Phenotypic and genotypic characteristics of isolates. J. Clin. Microbiol. 36, 2187–2190. https://doi.org/10.1128/jcm.36.8.2187-2190.1998.

Blanchfield, J.T., Sands, D.P.A., Kennard, C.H.L., Byriel, K.A., Kitching, W., 2003. Characterisation of alkaloids from some australian *Stephania* (menispermaceae) species. Phytochemistry. 63, 711–720. https://doi.org/10.1016/S0031-9422(03)00240-1.

Boccella, M., Santella, B., Pagliano, P., De Filippis, A., Casolaro, V., Galdiero, M., Borrelli, A., Capunzo, M., Boccia, G., Franci, G., 2021. Prevalence and antimicrobial resistance of *Enterococcus* species: a retrospective cohort study in Italy. Antibiotics (Basel). 10 (12), 1552. https://doi.org/10.3390/antibiotics10121552.

Bonapace, C.R., Bosso, J.A., Friedrich, L.V., White, R.L., 2002. Comparison of methods of interpretation of checkerboard synergy testing. Diagn. Microbiol. Infect. Dis. 44, 363–366. https://doi.org/10.1016/s0732-8893(02)00473-x.

Cetinkaya, Y., Falk, P., Mayhall, C.G., 2000. Vancomycin-resistant enterococci. Clin. Microbiol. Rev. 13, 686–707. https://doi.org/10.1128/CMR.13.4.686.

Cheypratub, P., Leeanansaksiri, W., Eumkeb, G., 2018. The synergy and mode of action of *Cyperus rotundus* extract plus ampicillin against ampicillin-resistant *Staphylococcus aureus*. Evid. Based. Complement. Alternat. Med. 2018, 1–11. https://doi.org/10.1155/2018/3438453.

Dhingra, S., Rahman, N.A.A., Peile, E., Rahman, M., Sartelli, M., Hassali, M.A., Islam, T., Islam, S., Haque, M., 2020. Microbial resistance movements: an overview of global public health threats posed by antimicrobial resistance, and how best to counter. Front. Public Health. 8, 1–22. https://doi.org/10.3389/fpubh.2020.535668.

Farha, M.A., Brown, E.D., 2019. Drug repurposing for antimicrobial discovery. Nat. Microbiol. 4, 565–577. https://doi.org/10.1038/s41564-019-0357-1.

Fu, Y., Yu, Z., Liu, S., Chen, B., Zhu, L., Li, Z., Chou, S.H., He, J., 2018. C-di-gmp regulates various phenotypes and insecticidal activity of gram-positive *Bacillus thuringiensis*. Front. Microbiol. 9, 45. https://doi.org/10.3389/fmicb.2018.00045.

Gagetti, P., Bonofiglio, L., García Gabarrot, G., Kaufman, S., Mollerach, M., Vigliarolo, L., von Specht, M., Toresani, I., Lopardo, H.A., 2019. Resistance to β-lactams in enterococci. Rev. Argent. Microbiol. 51, 179–183. https://doi.org/10.1016/j.ram.2018.01.007.

Güvenç Paltun, B., Kaski, S., Mamitsuka, H., 2021. Machine learning approaches for drug combination therapies. Brief. Bioinform. 22, 1–16. https://doi.org/10.1093/bib/bbab293.

Guzman Prieto, A.M., van Schaik, W., Rogers, M.R., Coque, T.M., Baquero, F., Corander, J., Willems, R.J., 2016. Global emergence and dissemination of enterococci as nosocomial pathogens: attack of the clones? Front. Microbiol. 7, 1–15. https://doi.org/10.3389/fmicb.2016.00788.

Hollenbeck, B.L., Rice, L.B., 2012. Intrinsic and acquired resistance mechanisms in enterococcus. Virulence. 3, 421–433. https://doi.org/10.4161/viru.21282.

Institute, C.L.S., 2010. Performance standards for antimicrobial disk susceptibility tests; approved standard Clinical and Laboratory Standards Institute. Wayne, Pennsylvania.

Clinical Laboratory Standards Institute, 2012. Method for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. CLSI document M7-A7, ninth ed. In: A. W. Matthew, Franklin, R.C., William, A.C., Micheal, N.D., George, M.E., David W.H. Janet, F.H., Mary, J.F., Jana, M.S., Donal, E.L., Danie, J.S., Fred, C. T., John, D.T., Melvin, P.W., & Barbara, L.Z (Eds), Clinical and Laboratory Standards Institute Wayne, Pennsylvania.

Clinical Laboratory Standards Institute, 2022. Performance standards for antimicrobial susceptibility testing. CLSI document M100, Thirty-second ed., Wayne, Pennsylvania.

Kristich, C.J., Rice, L.B., Arias, C.A., 2014. Enterococcal infection—treatment and antibiotic resistance. In: Gilmore MS, Clewell DB, Ike Y, et al (Eds), Enterococci: From Commensals to Leading Causes of Drug Resistant Infection. Boston, Massachusetts Eye and Ear Infirmary.

Lee, G.C., Burgess, D.S., 2013. Polymyxins and doripenem combination against KPC-producing *Klebsiella pneumoniae*. J. Clin. Med. Res. 5, 97–100. https://doi.org/10.4021/jocmr1220w.

Miller, W.R., Munita, J.M., Arias, C.A., 2014. Mechanisms of antibiotic resistance in enterococci. Expert Rev. Anti. Infect. Ther. 12, 1221–1236. https://doi.org/10.1586/14787210.2014.956092.

Mitchison, D.A., 2012. Prevention of drug resistance by combined drug treatment of tuberculosis. Handb. Exp. Pharmacol. 87–98. https://doi.org/10.1007/978-3-642-28951-4_6.

Patra, A., Montgomery, C.T., Freyer, A.J., Guinaudeau, H., Shamma, M., Tantisewie, B., Pharadai, K., 1987. The protoberberine alkaloids of *Stephania suberosa*. Phytochemistry. 26, 547–549.

Peechakara, B.V., Basit, H., Gupta, M., 2022. Ampicillin. Statpearls. Treasure Island (FL), StatPearls Publishing Copyright © 2022. StatPearls Publishing LLC.

Pejin, B., Savic, A., Sokovic, M., Glamoclija, J., Ciric, A., Nikolic, M., Radotic, K., Mojovic, M., 2014. Further in vitro evaluation of antiradical and antimicrobial activities of phytol. Nat. Prod. Res. 28, 372–376. https://doi.org/10.1080/14786419.2013.869692.

Pejin, B., Ciric, A., Dimitric Markovic, J., Glamoclija, J., Nikolic, M., Sokovic, M., 2017. An insight into anti-biofilm and anti-quorum sensing activities of the selected anthocyanidins: The case study of *Pseudomonas aeruginosa* PAO1. Nat. Prod. Res. 31, 1177–1180. https://doi.org/10.1080/14786419.2016.1222386.

Richards, R.M., Xing, J.Z., Gregory, D.W., Marshall, D., 1993. An electronmicroscope study of the effect of sulphadiazine and trimethoprim on *Enterobacter cloacae*. J. Med. Microbiol. 38, 64–68. https://doi.org/10.1099/00222615-38-1-64.

Rukayadi, Y., Lee, K., Han, S., Yong, D., Hwang, J.-K., 2009. In vitro activities of panduratin a against clinical staphylococcus strains. Antimicrob. Agents Chemother. 53, 4529–4532. https://doi.org/10.1128/AAC.00624-09.

Santajit, S., Indrawattana, N., 2016. Mechanisms of antimicrobial resistance in eskape pathogens. Biomed. Res. Int. 2016, 1–8. https://doi.org/10.1155/2016/2475067.

Sarker, S.D., Nahar, L., Kumarasamy, Y., 2007. Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. Methods. 42, 321–324. https://doi.org/10.1016/j.ymeth.2007.01.006.

Savoia, D., 2012. Plant-derived antimicrobial compounds: alternatives to antibiotics. Future Microbiol. 7, 979–990. https://doi.org/10.2217/fmb.12.68.

Semedo, T., Santos, M.A., Lopes, M.F., Figueiredo Marques, J.J., Barreto Crespo, M.T., Tenreiro, R., 2003. Virulence factors in food, clinical and reference enterococci: a common trait in the genus? Syst. Appl. Microbiol. 26, 13–22. https://doi.org/10.1078/072320203322337263.

Semwal, D.K., Badoni, R., Semwal, R., Kothiyal, S.K., Singh, G.J., Rawat, U., 2010. The genus *Stephania* (menispermaceae): Chemical and pharmacological perspectives. J. Ethnopharmacol. 132, 369–383. https://doi.org/10.1016/j.jep.2010.08.047.

Shepard, B.D., Gilmore, M.S., 2002. Antibiotic-resistant enterococci: the mechanisms and dynamics of drug introduction and resistance. Microbes Infect. 4, 215–224. https://doi.org/10.1016/S1286-4579(01)01530-1.

Siriwong, S., Teethaisong, Y., Thumanu, K., Dunkhunthod, B., Eumkeb, G., 2016. The synergy and mode of action of quercetin plus amoxicillin against amoxicillin-resistant *Staphylococcus epidermidis*. BMC Pharmacol. Toxicol. 17, 1–14. https://doi.org/10.1186/s40360-016-0083-8.

Suknasang, S., Teethaisong, Y., Kabkhunthod, S., Mingsiritom, N., Chueakwon, P., Eumkeb, G., 2019. Antibacterial activity of colistin is resurrected by *Stephania suberosa* forman extract against colistin-resistant *Enterobacter cloacae*. Lett. Appl. Microbiol. 69, 128–135. https://doi.org/10.1111/lam.13187.

Teethaisong, Y., Autarkool, N., Sirichaiwetchakoon, K., Krubphachaya, P., Kupittayanant, S., Eumkeb, G., 2014. Synergistic activity and mechanism of action of *Stephania suberosa* forman extract and ampicillin combination against ampicillin-resistant *Staphylococcus aureus*. J. Biomed. Sci. 21, 1–11. https://doi.org/10.1186/s12929-014-0090-2.

Teethaisong, Y., Pimchan, T., Srisawat, R., Hobbs, G., Eumkeb, G., 2018. *Boesenbergia rotunda* (l.) mansf. extract potentiates the antibacterial activity of some β-lactams against β-lactam-resistant staphylococci. J. Glob. Antimicrob. Resist. 12, 207–213. https://doi.org/10.1016/jjgar.2017.10.019.

Timurkaynak, F., Can, F., Azap, Ö.K., Demirbilek, M., Arslan, H., Karaman, S.Ö., 2006. In vitro activities of non-traditional antimicrobials alone or in combination against multidrug-resistant strains of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolated from intensive care units. Int. J. Antimicrob. Agents. 27, 224–228. https://doi.org/10.1016/j.ijantimicag.2005.10.012.

Top, J., Willems, R., Bonten, M., 2008. Emergence of cc17 *Enterococcus faecium*: From commensal to hospital-adapted pathogen. FEMS Immunol. Med. Microbiol. 52, 297–308. https://doi.org/10.1111/j.1574-695X.2008.00383.x.

Wade, J.J., 1995. The emergence of *Enterococcus faecium* resistant to glycopeptides and other standard agents-a preliminary report. J. Hosp. Infect. 30 (Suppl), 483–493. https://doi.org/10.1016/0195-6701(95)90052-7.

Williamson, R., le Bouguénec, C., Gutmann, L., Horaud, T., 1985. One or two low affinity penicillin-binding proteins may be responsible for the range of susceptibility of *Enterococcus faecium* to benzylpenicillin. J. Gen. Microbiol. 131, 1933–1940. https://doi.org/10.1099/00221287-131-8-1933.