Pharmacognosy Communications, 2026; 16(2):95-108.
https://www.phcogcommn.org

Original Article

Syzygium australe (J.C.Wendl. ex Link) B.Hyland and *Syzygium luehmannii* (F.Meell.) L.A.S. Johnson Extracts Inhibit Methicillin-Resistant *Staphylococcus aureus* and ESBL *Escherichia coli* and *Klebsiella pneumoniae*

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ABSTRACT

Background: Recent increases in antibiotic-resistant bacteria infections has focussed researcher's attention on alternative therapies, including traditional plant-based medicines. The rapid development β-lactam resistant bacterial strains with resistance to later generation antibiotics including methicillin is particularly worrisome due to the reliance of medical science on β-lactam antibiotics to treat many common infections. **Materials and Methods:** The antibacterial activity of *Syzygium australe* and *Syzygium luehmannii* leaf and fruit extracts towards β-lactam resistant and sensitive bacterial strains was investigated and quantified by disc diffusion assays and broth microdilution assays. Combinational effects were evaluated in broth microdilution assays by determining ΣFIC values. Toxicity was assessed using *Artemia* nauplii cytotoxicity assays and therapeutic indices were calculated. **Results:** Most of the *S. australe* and *S. luehmannii* leaf and fruit extracts displayed noteworthy growth inhibitory activity against all of the bacteria tested, including MRSA and ESBL *E. coli* and *K. pneumonia* strains. The *S. australe* fruit ethyl acetate extract had particularly good antibacterial activity, with MIC values generally between 350 and 710 µg/mL. Notably, this extract was a better inhibitor of the ESBL *E. coli* strain than against the antibiotic-sensitive strain. The methanolic and aqueous *S. australe* fruit extracts were also generally good bacterial growth inhibitors (MIC values <1000 µg/mL), although they were less effective against both *S. aureus* strains. Several combinations of the extracts and either ciprofloxacin or gentamicin had potentiated activity (either synergistic or additive effects). All of the extracts were either nontoxic or of low toxicity in the *Artemia* nauplii lethality assay and the calculated therapeutic indexes (TIs) of the majority of the extracts indicated their safety for use as antibiotic chemotherapies. **Conclusion:** Our study demonstrated that *S. australe* (and to a lesser extent, *S. luehmannii*) leaf and fruit extracts have potential to treat antibiotic-resistant bacterial infections and potentiate the activity of ciprofloxacin and gentamicin. The low toxicity and relatively high therapeutic indexes (TIs) of many of the extracts indicated their safety for use as antibiotic chemotherapies. Further phytochemical and mechanistic studies of these extracts are warranted.

Keywords: Myrtaceae, brush cherry, riberry, antibiotic-resistant bacteria, MRSA, β-lactamase, ESBL.

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Received: 22-12-2025;
Revised: 19-01-2026;
Accepted: 09-03-2026.

INTRODUCTION

The recent development of bacterial pathogens that are resistant to multiple antibiotics has reduced clinical therapeutic options and has rendered some first-choice antibiotics ineffective against common infections.[1] Indeed, the WHO considers the development of multi-drug resistant bacteria to be perhaps the most serious threat to human health.[2] The rapid development of bacterial resistance to β-lactam antibiotics is of particular concern due to reliance of medical science on this class of antibiotics to treat a wide variety of infections. The overuse and misuse of β-lactam (especially penicillin) induced bacteria to evolve to produce β-lactamase enzymes, which break the β-lactam ring structure of these antibiotics, producing inactive metabolites. In response, chemists have developed new antibiotics by chemically modifying existing drugs. Modification of the β-lactam scaffold through the addition of bulky functional groups resulted in second

DOI: 10.5530/pc.2026.2.10

generation β-lactam antibiotics (e.g. methicillin, oxacillin) that evade bacterial β-lactamases and allow them to function, even in β-lactam resistant bacterial strains.

Unfortunately, continued misuse and overuse of these modified β-lactam antibiotics provided additional selective pressure, inducing many bacteria to evolve to produce modified enzymes with extended-spectrum β-lactamase (ESBL) activity, as well as other mexhanisms to overcome the antibiotic's actions. ESBL enzymes have modified binding sites, allowing them to bind and inactivate the majority of β-lactam antibiotics. The development of methicillin-resistance *S. aureus* (MRSA) has been particularly worrisome, and has caused substantial increases in mortality associated with *S. aureus*. Indeed, recent statistics show that MRSA infections cause substantially greater loss of life globally than human immunodeficiency virus (HIV) infections or emphysema.[3] Similarly, bacteria (particularly Enterobacteriaceae) also cause substantial mortality globally and are particularly difficult to treat. Indeed, effective treatments for many β-lactam-resistant strains is now very limited, vancomycin often being the only remaining effective antibiotic option (especially for MRSA). However, the increased reliance of medical science on vancomycin has induced several bacteria to develop of several vancomycin-resistant/methicillin-resistant and/or ESBL strains and new and effective antibiotic therapies are urgently needed.[4]

A re-examination of traditional plant-based medicines may provide leads that could be useful in the development of novel antibiotic therapies. Plants of the genus *Syzygium* may be particularly promising for the development of new antibiotics due to the widespread traditional use of multiple *Syzygium* spp. to treat a variety of pathogenic bacterial diseases. The genus *Syzygium* consists of 1200-1800 species that are dispersed widely in subtropical and tropical regions of the world, with the greatest diversity in Australia and South East Asia.[5] *Syzygium* spp. are widely used therapeutically for a variety of purposes, although their use in South Asian traditional medicine systems (particularly Ayurveda) has been most extensively documented. They are used traditionally for a wide range of therapeutic purposes, although their uses against pathogenic diseases such as colds and influenzas, diarrhoea and dysentery, pneumonia, and sexually transmitted diseases have been particularly well reported.[5]

The antibacterial activity of the South Asian species *Syzygium aromaticum* (L.) Merr. & L.M. Perry (commonly known as cloves) has been particularly well studied.[6-9] The antibacterial activity of several other Asian species including *Syzygium cumini* (L.) Skeels (Prtabhakaran *et al.*, 2011; Shafi *et al.*, 2002; Mohamed *et al.*, 2013), *Syzygium jambos* L. (Alston),[10,11] *Syzygium alternifolium* Walp. and *Syzygium samarangense* (Blume) Merr. & L.M.Perry have also been verified against a wide variety of bacteria.[12] Similarly, the African species *Syzygium cordatum* (Hochst.) extracts inhibit the growth of numerous.[13,14] In contrast, relatively few studies have examined the antibacterial properties of Australian *Syzygium* spp., despite Australia having the greatest diversity of *Syzygium* spp. Recently, the species *Syzygium australe* (H.L.Wendl. ex Link) B.Hyland and *Syzygium leuhmannii* (F.Muell.) L.A.S. Johnson have attracted substantial interest due to their extremely high antioxidant contents.[15,16] Several studies have also reported noteworthy growth inhibitory activity of *S. australe* and *S. luehmannii* extracts against a broad panel of bacteria, including *Aeromonas hydrophilia, Alicaligenes feacalis, Bacillus anthracis, Bacillus cereus, Bacillus subtilis, Citrobacter freundii, Enertobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeuroginosa, Pseudomonas fluorescens, Salmonella salford, Serratia marcescens, Staphylococcus aureus* and *Yersinia enterocolitia*.[17-19] Notably, those studies screened the extracts against antibiotic sensitive strains and their effects against resistant bacteria was not explored. Furthermore, the previous studies screened the effects of the extracts in isolation and their effects in combination with conventional antibiotics was not tested. This study aimed to address this gap in the literature by testing the growth inhibitory effects of *S. australe* and *S. luehmannii* extracts against methicillin-resistant *S. aureus* and some ESBL bacteria, both alone and in combination with conventional antibiotics.

MATERIALS AND METHODS

Sourcing of plant samples and preparation of extracts

Syzygium australe (J.C.Wendl. ex Link) B.Hyland and *Syzygium luehmannii* (F.Meell.) L.A.S.Johnson leaves and ripe fruit were collected from Mt Cootha Botanical Gardens, Brisbane and were identified by the botanical gardens botanical officer. Voucher specimens are stored in the School of Environment and Science, Griffith University, Australia. The leaves and fruit from each *Syzyium* spp. were washed in deionised water and then air-dried separately in the shade at room temperature until they were completely dry (as determined by consistent masses following further drying time). The dried plant materials were subsequently ground to produce a coarse powder. 1 g masses of each of the powdered leaves and fruits were extracted separately by maceration in 50 mL of methanol, sterile deionised water, or ethyl acetate. All solvents were AR grade and were purchased from Ajax Fine Chemicals, Australia. Following extraction for 24 hr at room temperature, the extracts were filtered through Whatman No. 54 filter paper to remove the particulate matter. The filtrates were then air dried in a vacuum oven at 40°C and the mass of the resultant dried extracts were recorded to determine the extract yield (Table 1). The dried extracts were resuspended in 10 mL deionised water (containing 1% DMSO), filtered through a 0.22 µm filter (Sarstedt) and stored at 4°C until use.

Qualitative phytochemical studies

Qualitative phytochemical evaluations of the *Syzygium* spp. leaf and fruit extracts to detect and evaluate the relative abundances

of phenolic compounds, flavonoids and tannins were conducted using standard assays.[20-22]

Antibacterial analysis

Conventional antibiotics

Ciprofloxacin (≥98%), gentamicin (600 µg/mg) and erythromycin (≥95% purity) were purchased from Sigma-Aldrich, Australia. All antibiotics were prepared as 0.01 mg/mL stocks in sterile deionised water and used as control antibiotics in the broth microdilution assay. For the disc diffusion screening studies, pre-loaded ampicillin (10 µg), chloramphenicol (10 µg) and ciprofloxacin (5 µg) susceptibility discs (Oxoid Ltd., Australia) were used to screen the bacterial strains for antibiotic susceptibility, and as positive assay controls.

Bacterial cultures

Methicillin-sensitive *Staphylococcus aureus* (ATCC 25923) and β-lactam sensitive *Escherichia coli* (ATCC 25922) and *Klebsiella pneumoniae* (ATCC 31488) were purchased from American Type Culture Collection, USA (ATCC) and used in this study. A reference strain of MRSA (ATCC 43300) was also included in this study. Extended spectrum β-lactamase (ESBL) resistant clinical isolate strains of *E. coli* and *K. pneumoniae* were kindly donated by Dr. Matthew Cheesman of the School of Pharmacy and Medicinal Sciences, Griffith University and were originally obtained from Gold Coast University Hospital, Australia. All *E. coli* and *K. pneumoniae* strains were cultured using Mueller-Hinton agar or broth (Oxoid Ltd., Australia). The antibiotic-sensitive and MRSA *S. aureus* strains were grown using the same media supplemented with 2% NaCl. All bacteria were cultured at 37°C for 24 hr, with the exception of the MRSA strain, which was maintained and tested at 35°C. Streaked agar plates were tested in parallel throughout these experiments to ensure the purity of all bacterial cultures.

Evaluation of bacterial susceptibility to growth inhibition

Bacterial susceptibility to the *Syzygium* spp. extracts and the conventional antibiotics was assessed by standard disc diffusion assays.[23] Discs preloaded with ampicillin (10 µg), chloramphenicol (10 µg) and ciprofloxacin (5 µg) were included in the assays as positive controls. Filter discs (5 mm diameter) infused with 10 µL of distilled water were included as a negative control. Following an overnight incubation at 37°C (or 35°C for MRSA) on Mueller-Hinton agar plates, the zones of inhibition (ZOIs) were measured to the nearest whole mm. All tests were performed three times, each with three technical replicates (n = 9) and the results are presented herein as the means ± SEM. One-way ANOVA analysis was used to calculate statistical significance between control and treatment groups, with $p < 0.01$ classified as significant.

Minimum inhibitory concentration (MIC) determination

The antibacterial activity of the individual *Syzygium* spp. leaf and fruit extracts, as well as the conventional antibiotics, was quantified using standard liquid dilution MIC.[24-26] Briefly, log phase bacterial cultures (determined by optical density measurements) were diluted in broth to produce a 0.5 McFarland turbidity standard. Sterilized broth (100 µL) was dispensed into all wells of 96 well micro-titre plate and 100 µL of the extracts or antibiotics were added separately to individual wells and diluted by two-fold serial dilutions down the individual columns of the plate. Mueller-Hinton broth (negative control) and a sterile control (media without bacteria) were included on all plates to verify that the assay was functioning correctly. Bacterial culture (100 µL containing approximately $1x10^6$ colony forming units (CFU)/mL) was subsequently added to all wells of the plate (excluding the sterile control wells) and the plates incubated at 37°C (or 35°C for the *S. aureus* and MRSA plates) for 24 hr. *p*-Iodonitrotetrazolium violet (INT) colourimetric indicator (Sigma-Aldrich, Australia) was prepared as a 0.2 mg/mL solution in sterile deionised water and 40 µL of the INT stock solution was dispensed into all wells. The plates were incubated for 6 hr at 30°C to allow full colour development. The MIC was classified as the lowest dose at which colour development was inhibited. To ensure the reliability of the calculated MIC values, each assay was repeated on separate days, with two technical replicates included on individual days (n = 4).

Toxicity screening: *Artemia franciscana* Kellogg nauplii lethality assay

The toxicity of the *Syzygium* spp. leaf and fruit extracts was evaluated using a modified *Artemia franciscana* nauplii lethality assay (ALA).[27] Dried *A. franciscana* cysts were purchased from Ocean Nutrition, USA and were hatched and assayed in Tropic Marine Salt (Australia) artificial seawater. Potassium dichromate (1 mg/mL; AR grade, Chem-Supply) and artificial seawater were used as a reference toxin and negative control respectively. All tests were evaluated across a range of concentrations and the LC_{50} values were calculated using Probit analysis.

Evaluation of therapeutic index

The therapeutic indexes (TI) of the *Syzygium* spp. leaf and fruit extracts were calculated using the following formula and are included as a measure of the suitability of the extracts for therapeutic usage:

$$\text{Therapeutic index} = (LC_{50})/(\text{MIC})$$

Statistical analysis

All data presented herein is expressed as the mean ± SEM of three independent experiments, each with internal triplicates (n = 9) unless otherwise stated. One-way analysis of variance analysis

(ANOVA) was used to calculate statistical significance between the negative control and treated groups, with a $P < 0.01$ considered to be statistically significant.

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Maceration of the powdered *Syzygium* spp. leaves and fruit (1 g) in the solvents yielded dried plant extracts ranging from 105 mg (*S. australe* leaf ethyl acetate extract) to 380 mg (*S. luehmannii* fruit methanol extract) (Table 1). Qualitative phytochemical screening showed that the extracts of all *Syzygium* spp. extracts contained high relative abundances of flavonoids. The leaf and fruit methanolic and aqueous extracts of both *Syzygium* spp. also contained low to moderate relative abundances of saponins, triterpenoids and tannins.

Inhibition of bacterial growth

Susceptibility of reference (ATCC 25922) and ESBL Escherichia coli strains

The *Syzygium* spp. leaf and fruit extracts were screened against a reference (ATCC 25922; Figure 1a) and ESBL *E. coli* strains (Figure 1b) to evaluate the antimicrobial potential. The antibiotic resistance was verified by screening these bacteria against a variety of clinical antibiotic therapies. Not surprisingly, a large ZOI (11.2 mm) was measured when the β-lactam sensitive *E. coli* strain was screened against ampicillin (Figure 1a). Chloramphenicol and ciprofloxacin were also effective against the reference *E. coli* strain, with ZOIs of ~14 mm measured against for both antibiotics. In contrast, a substantially smaller ZOIs were measured for ampicillin against the ESBL strain (Figure 1b) than for the β-lactam sensitive *E. coli* strain, confirming the resistance of that strain to β-lactam antibiotics. Interestingly, the ESBL *E. coli* strain was also resistant to ciprofloxacin (with a substantially smaller ZOI measured than for the reference strain), and chloramphenicol was completely ineffective. Thus, this strain was not only resistant to β-lactam antibiotics, but was also resistant to other classes of antibiotics. This is consistent with previous studies that have also reported this strain to be resistant to multiple classes of antibiotics.[28]

Several of the *Syzygium* spp. leaf and fruit extracts also significantly inhibited the growth of both β-lactam sensitive and ESBL *E. coli* strains. The methanolic and aqueous extracts were substantially stronger inhibitors of the growth of both strains of *E. coli* than the ethyl acetate extract. However, the extracts were initially tested undiluted in the assay, as an approximation of how they would be used traditionally. It is noteworthy that the ethyl acetate extracts were substantially lower concentrations than the methanolic and

Table 1: The mass of dried extracted material, the concentration after resuspension in deionised water (1% DMSO) and qualitative phytochemical screenings of the *Syzygium* spp. extracts.

Plant species	Part used	Solvent	Mass dried extract (mg)	Extract concentration (mg/ml)	Qualitative phytochemical tests						
					Cardiac glycosides	Saponins	Triterpenoids	Phytosterols	Alkaloids	Flavonoids	Tannins
S. australe	Leaf	M	360	36	-	+	++	-	-	+++	+
		W	180	18	-	+	++	-	-	++	+
		E	105	10.5	-	-	-	-	-	++	-
	Fruit	M	360	36	-	-	++	-	-	+++	+
		W	240	24	-	+	-	-	-	+++	+
		E	110	11	-	-	-	-	-	+	-
Syzygium luehmannii	Leaf	M	290	29	-	+	+	-	-	+++	+
		W	188	18.8	-	+	+	-	-	++	+
		E	132	13.2	-	-	-	-	-	++	-
	Fruit	M	380	38	-	+	+	-	-	+++	+
		W	130	13	-	+	+	-	-	+++	+
		E	120	12	-	-	-	-	-	+	-

M = methanolifc extract; W = aqueous extract; E =ethyl acetate extract; +++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay.

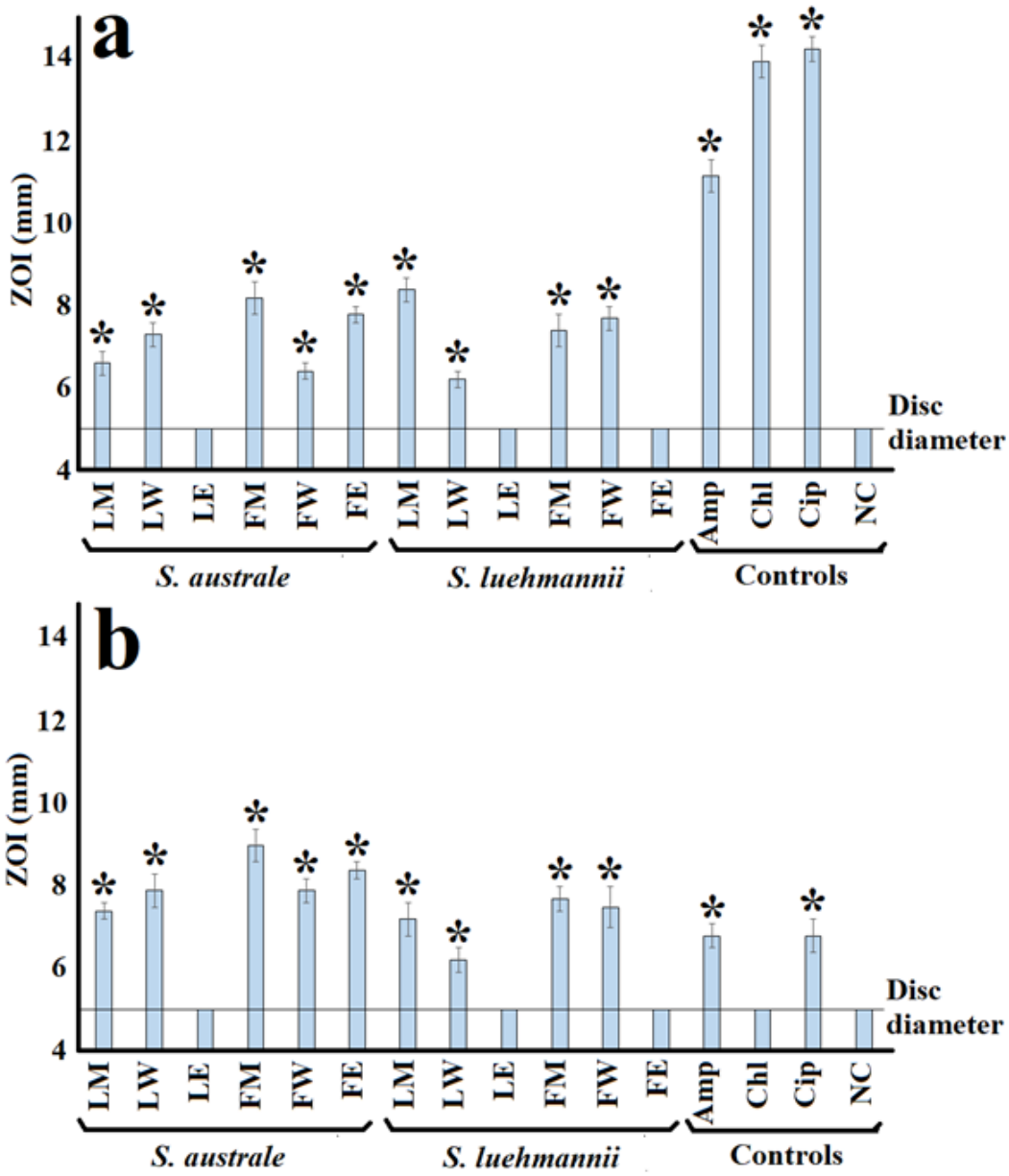

Figure 1: Antibacterial activity of the *Syzygium* spp. leaf and fruit extracts against (a) *E. coli* (ATCC 25922) and (b) ESBL *E. coli* measured as zones of inhibition (mm). LM = methanolic leaf extract; LW = aqueous leaf methanol; LE = ethyl acetate leaf extract; FM = methanolic fruit extract; FW = aqueous fruit extract; FE = fruit ethyl acetate extract; Amp = ampicillin (10 µg); Chl = chloramphenicol (10 µg); Cip = ciprofloxacin (5 µg); NC = negative control (1% DMSO). Results are expressed as mean zones of inhibition of three replicates, each with internal triplicated (n = 9) ± SEM.; * indicates results that are significantly different to the negative control ($p < 0.01$).

aqueous extracts, and therefore the lower apparent potency of the ethyl acetate extracts may be due to the lower concentrations tested. Interestingly, the susceptibility profiles of both the reference and ESBL *E. coli* strains were similar, with similar ZOIs measured against both strains. This may indicate that the antibacterial compounds in the *Syzygium* spp. extracts may exert their growth inhibitory properties by mechanisms distinct from the β-lactams, or that their structures are distinct enough from the β-lactam structure to allow them to avoid being inactivated by the ESBL enzyme.

Susceptibility of reference (ATCC 31488) and ESBL *Klebsiella pneumoniae* strains

The growth of the reference β-lactam sensitive *K. pneumoniae* reference strain (ATCC 31488) was also inhibited by ampicillin, with a ZOI of 7.3 mm (Figure 2a). This strain was also susceptible to the non-β-lactam antibiotics chloramphenicol (ZOI = 7.4 mm) and ciprofloxacin (ZOI = 7.3 mm). In contrast, the ESBL *K. pneumoniae* strain was completely resistant to ampicillin and chloramphenicol (Figure 2b), confirming the β-lactam resistant status of this strain, although it had similar susceptibility to ciprofloxacin as the reference β-lactam sensitive.

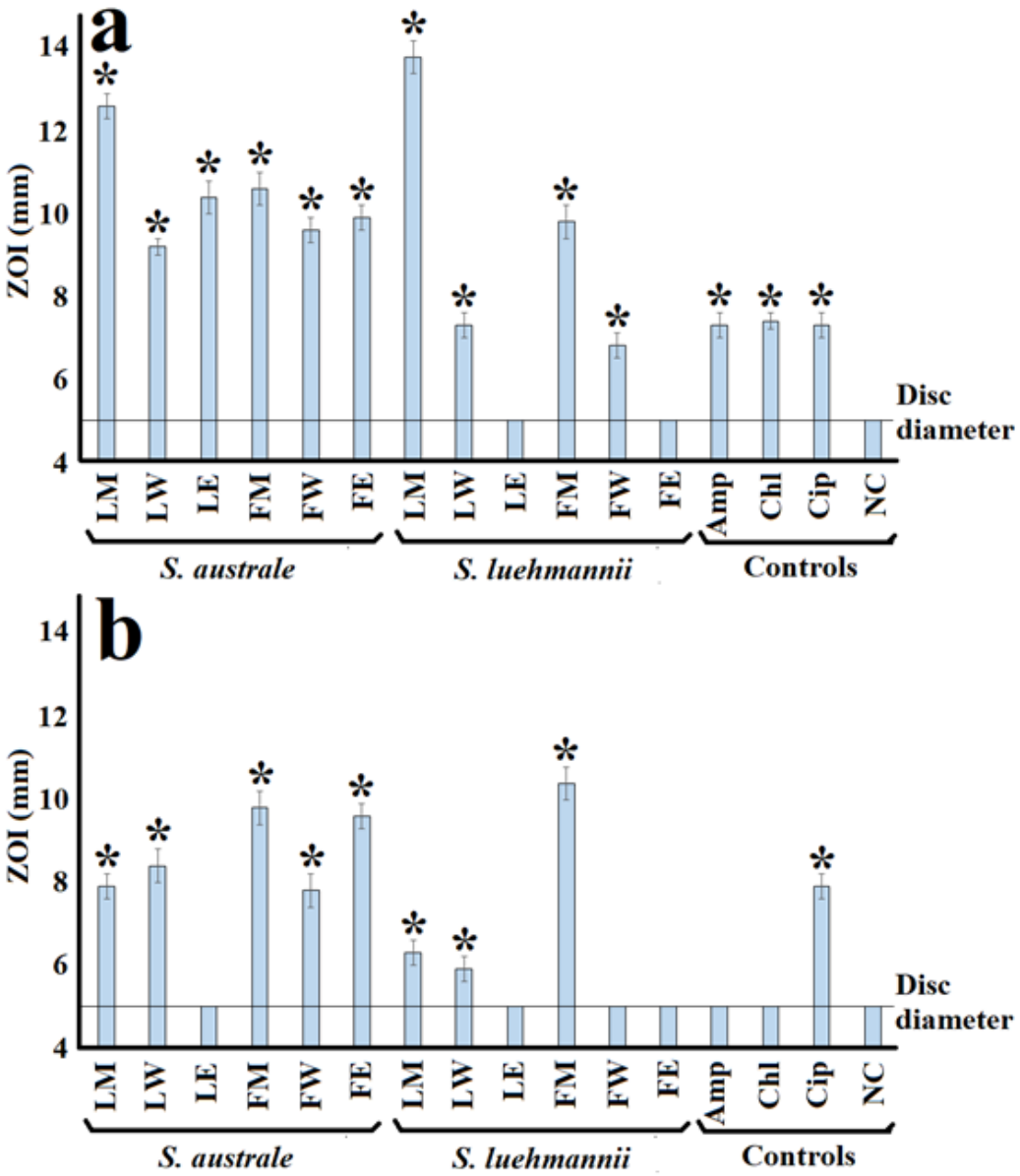

Figure 2: Antibacterial activity of the *Syzygium* spp. leaf and fruit extracts against (a) *K. pneumoniae* (ATCC 31488) and (b) ESBL *K. pneumoniae* (clinical isolate strain) measured as zones of inhibition (mm). LM = methanolic leaf extract; LW = aqueous leaf methanol; LE = ethyl acetate leaf extract; FM = methanolic fruit extract; FW = aqueous fruit extract; FE = fruit ethyl acetate extract; Amp = ampicillin (10 µg); Chl = chloramphenicol (10 µg); Cip = ciprofloxacin (5 µg); NC = negative control (1% DMSO). Results are expressed as mean zones of inhibition of three replicates, each with internal triplicated (n = 9) ± SEM.; * indicates results that are significantly different to the negative control (p < 0.01).

Whilst both *K. pneumoniae* strains were susceptible to the *Syzygium* spp. leaf and fruit extracts, the substantially smaller ZOIs measured for most of the extracts against the ESBL strain indicate that it also has some resistance to some of the extract components. The methanolic fruit extracts were the exception to this trend, with similar diameter ZOIs measured for those extracts against both *K. pneumoniae* strains. The similar potency of the methanolic fruit extract against the β-lactam sensitive and resistant *K. pneumoniae* strains indicates that it is unlikely that they function via inactivation of ESBL enzymes. Instead, compounds in those extracts may function via other mechanisms and substantially more work is required to elucidate the antibacterial mechanisms of those extracts. The leaf methanolic extracts, as well of many of the aqueous extracts, also inhibited the growth of both *K. pneumoniae* strains, although generally with lower potency against the ESBL strain. Therefore, several of the *Syzygium* spp. extracts may be particularly useful for the treatment of *K. pneumoniae* infections.

Susceptibility of reference *Staphylococcus aureus* (ATCC25923) and MRSA strains

The *Syzygium* spp. leaf and fruit extracts were screened against reference strains of antibiotic sensitive *S. aureus* (Figure 3a) and a methicillin-resistant strain (MRSA) (Figure 3b) to evaluate

the antimicrobial potential. The ATCC 25923 reference strain of *S. aureus* was highly susceptible to ampicillin, with an ZOI of approximately 15 mm. This strain has also previously been shown to be sensitive to multiple β-lactam antibiotics, including methicillin, cefoxitin, oxacillin, and the combinational therapy Augmentin® (amoxicillin and clavulanic acid) (Cheesman *et al.*, 2019) with ZOIs generally >22 mm (Figure 1a). Notably, this *S. aureus* strain was also highly susceptible to chloramphenicol (ZOI ~19 mm). It was also inhibited by ciprofloxacin, although the small ZOI (~7 mm) indicated only low susceptibility to that antibiotic. The MRSA strain was also susceptible to ampicillin and chloramphenicol, albeit with substantially smaller ZOIs than measured against the reference/methicillin-sensitive strain. Additionally, previous studies in our laboratory have confirmed that this *S. aureus* strain is resistant to methicillin.[28]

The methanolic and aqueous extracts produced from both the leaves and fruit of *S. australe* and *A. luehmannii* generally inhibited the growth of both *S. aureus* strains, with similar activity profiles. Notably, with the exception of the *S. australe* fruit ethyl acetate extract against the MRSA strain, the ethyl acetate extracts were generally devoid of growth inhibitory activity against both *S. aureus* strains. However, the ethyl acetate extracts were tested at substantially lower concentrations than the corresponding methanolic and aqueous extracts, which may account for the lack of inhibitory activity of these extracts. Interestingly, several of the extracts were notably stronger against the MRSA strain than against the reference/methicillin-sensitive *S. aureus* strain, indicating that the antibacterial components in this extract may function via different mechanism(s) than the β-lactam antibiotics, although this remains to be verified.

Quantification of minimum inhibitory concentration (MIC)

The antimicrobial activity of the methanolic *Syzygium* spp. extracts and the conventional antibiotics was further evaluated by determining the MIC values using liquid dilution MIC assays, to allow for comparisons with other studies and to benchmark the activity between extracts (Table 2). MIC values >1 µg/mL have previously been defined as demonstrating antibiotic resistance for pure antibiotics in this bioassay.[24-26] Using that definition, the ESBL *E. coli* strain was resistant to all of the conventional antibiotics, whilst the ESBL *K. pneumoniae* strain was resistant to all antibiotics except gentamicin. Similarly, the MRSA strain was resistant to all antibiotics except ciprofloxacin. In contrast the

Table 2: MIC values (µg/mL) for the *S. australe* and *S. luehmannii* extracts against antibiotic-sensitive and antibiotic-resistant bacterial pathogens.

Extract component	Part used	Solvent	Bacteria					
			E. coli	ESBL *E. coli*	*K. pneumoniae*	ESBL *K. pneumonia*	*S. aureus*	MRSA
S. australe	Leaf	M	2590	1650	830	1650	310	1240
		W	1730	1300	860	1730	650	2590
		E	-	-	6620	-	-	-
	Fruit	M	710	350	350	710	2820	710
		W	1680	840	200	1680	5040	1680
		E	450	310	310	460	-	610
Syzygium luehmannii	Leaf	M	360	3560	2670	5340	3560	560
		W	3750	3750	2810	3750	3750	5620
		E	-	-	-	-	-	-
	Fruit	M	-	3830	1620	290	290	580
		W	780	1170	1560	-	-	-
		E	-	-	-	-	-	-
Conventional antibiotics	Ampicillin		>2.5	>2.5	>2.5	>2.5	>2.5	>2.5
	Methicillin		>2.5	>2.5	>2.5	>2.5	0.32	1.56
	Oxacillin		>2.5	>2.5	>2.5	>2.5	0.16	>2.5
	Erythromicin		>2.5	>2.5	>2.5	>2.5	1.25	>2.5
	Ciprofloxacin		0.16	2.5	1.25	0.94	2.5	1.25
	Gentamicin		0.93	1.25	0.63	1.25	0.63	0.63

M = methanolic extract; W = aqueous extract; E = ethyl acetate; Extracts with noteworthy activity (MIC < 1000 µg/mL) or antibiotics (MIC <1 µg/mL) are indicated in bold.

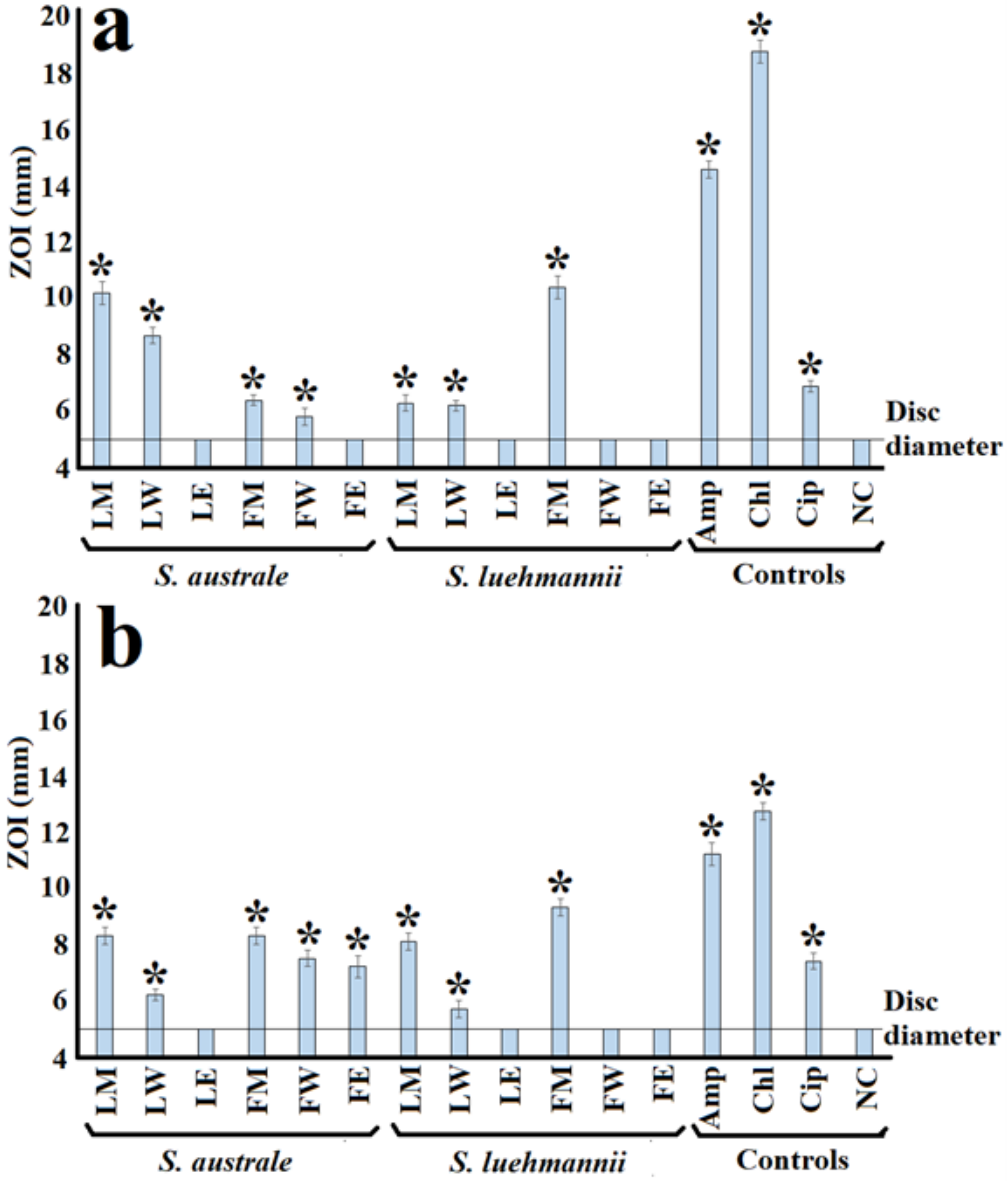

Figure 3: Antibacterial activity of the *Syzygium* spp. leaf and fruit extracts against (a) *S. aureus* (ATCC 25923) and (b) MRSA (ATCC 43300) measured as zones of inhibition (mm). LM = methanolic leaf extract; LW = aqueous leaf methanol; LE = ethyl acetate leaf extract; FM = methanolic fruit extract; FW = aqueous fruit extract; FE = fruit ethyl acetate extract; Amp = ampicillin (10 µg); Chl = chloramphenicol (10 µg); Cip = ciprofloxacin (5 µg); NC = negative control (1% DMSO). Results are expressed as mean zones of inhibition of three replicates, each with internal triplicated (n = 9) ± SEM.; * indicates results that are significantly different to the negative control (p < 0.01).

methicillin-sensitive *S. aureus* strain was sensitive to methicillin, oxacillin and gentamicin. The reference *E. coli* was only sensitive to ciprofloxacin and gentamicin, while the reference *K. pneumoniae* strain was only sensitive to gentamicin, and resistant to all of the other reference antibiotics.

Several of the *Syzygium* spp. extracts displayed noteworthy growth inhibitory activity against several of the tested bacterial strains (Table 2). With the exception of the ethyl acetate extracts, the extracts MIC quantification results are generally consistent with the disc diffusion susceptibility testing results. For the purposes of this study, MIC values <1000 µg/mL were classified as strong; MIC values between >1000 µg/mL and 2000 µg/mL were classified as noteworthy; values between >2000 to 3000 µg/mL were classified as moderate activity; MICs between >3000 µg/mL and 5000 µg/mL were classed as low activity; whilst MIC values >5000 µg/mL were deemed to lack inhibitory activity. Interestingly, the best bacterial growth inhibitory activity was generally noted for the methanolic and ethyl acetate *S. australe* fruit extracts. Both of those extracts displayed strong growth inhibitory activity against all of the bacterial strains tested, except the methicillin-sensitive strain. The aqueous *S. australe* fruit extract was also a strong inhibitor of ESBL *E. coli* and the reference *K. pneumoniae* strain, and had noteworthy inhibitory activity against most of the other bacterial strains tested. It is

expected that there would be considerable overlap between the phytochemical profiles of these extracts. The methanolic extract would contain compounds with varying polarity, whilst both the ethyl acetate extract (which would mainly contain medium polarity compounds), or with the aqueous extract (which would mainly contain higher polarity compounds) would contain many of the same compounds. This indicates that the strongest antibacterial components in *S. australe* fruit may be medium polarity compounds such as flavonoids. Therefore, focusing on the ethyl acetate extract of the species may be beneficial for future phytochemical isolation studies.

Table 3: Combinational effect of the *S. australe* and *S. luemhmannii* extracts and conventional antibiotics measured as the sum of fractional inhibition concentrations (ΣFIC).

Plant species	Bacteria	Plant part	Solvent	Antibiotic					
				Ampicillin	Methicillin	Oxacillin	Erythromycin	Ciprofloxacin	Gentamicin
Syzygium australe	*E. coli*	Leaf	W	-	-	-	-	4.5	1.17
			M	-	-	-	-	4.55	1.21
			E	-	-	-	-	-	-
		Fruit	W	-	-	-	-	2.5	1.67
			M	-	-	-	-	3	1.33
			E	-	-	-	-	8.67	2
	ESBL *E. coli*	Leaf	W	-	-	-	-	*0.92*	1.17
			M	-	-	-	-	*0.75*	*1*
			E	-	-	-	-	-	-
		Fruit	W	-	-	-	-	1.69	1.88
			M	-	-	-	-	1.59	2.25
			E	-	-	-	-	1.5	2
	K. pneumoniae	Leaf	W	-	-	-	-	1.5	2
			M	-	-	-	-	1.5	2
			E	-	-	-	-	1.5	1.25
		Fruit	W	-	-	-	-	1.25	1.5
			M	-	-	-	-	2.25	2.5
			E	-	-	-	-	1.5	3
	ESBL *K. pneumoniae*	Leaf	W	-	-	-	-	1.75	*1*
			M	-	-	-	-	1.17	*1*
			E	-	-	1.48	-	-	-
		Fruit	W	-	-	-	-	*0.83*	1.13
			M	-	-	-	-	1.33	1.88
			E	-	-	-	-	**0.5**	*0.63*
	S. aureus	Leaf	W	-	2.36	1.77	2	1.58	1.76
			M	-	*0.86*	1.34	4	1.46	2.75
			E	-	-	-	-	-	-
		Fruit	W	-	1.82	1.78	**0.42**	**0.29**	*0.67*
			M	-	*0.83*	1.28	**0.46**	**0.19**	**0.38**
			E	-	-	-	-	-	-
	MRSA	Leaf	W	-	-	-	-	*0.83*	1.33
			M	-	-	-	-	1.75	1.25
			E	-	-	-	-	-	-
		Fruit	W	-	-	-	-	*0.75*	1.5
			M	-	-	-	-	1.25	2.25
			E	-	-	-	-	-	-

Syzygium luehmanii	*E. coli*	Leaf	W	-	-	-	-	2.25	-
			M	-	-	-	-	-	-
			E	-	-	-	-		-
		Fruit	W	-	-	-	-	6.75	1.75
			M	-	-	-	-	4.5	1.17
			E	-	-	-	-	-	-
	ESBL *E. coli*	Leaf	W	-	-	-	-	**0.25**	1.67
			M	-	-	-	-	**0.25**	1.13
			E	-	-	-	-	-	-
		Fruit	W	-	-	-	-	*0.75*	*1*
			M	-	-	-	-	*0.75*	*1*
			E	-	-	-	-	-	-
	K. pneumoniae	Leaf	W	-	-	-	-	*0.75*	1.25
			M	-	-	-	-	**0.38**	1.88
			E	-	-	-	-	-	-
		Fruit	W	-	-	-	-	1.17	1.17
			M	-	-	-	-	1.17	1.17
			E	-	-	-	-	-	-
	ESBL *K. pneumoniae*	Leaf	W	-	-	-	-	-	-
			M	-	-	-	-	2.07	1.9
			E	-	-	-	-	-	-
		Fruit	W	-	-	-	-	1.17	*0.75*
			M	-	-	-	-	*1*	*0.83*
			E	-	-	-	-	-	-
	S. aureus	Leaf	W	-	1.76	2.04	-	-	-
			M	-	1.38	1.77	**0.42**	**0.29**	**0.33**
			E	-	-	-	-	-	-
		Fruit	W	-	2.83	3.24	1.83	*0.56*	1.5
			M	-	2.16	1.88	1.83	*0.75*	*0.75*
			E	-	-	-	-	-	-
	MRSA	Leaf	W	-	-	-	-	-	-
			M	-	-	-	-	-	-
			E	-	-	-	-	-	-
		Fruit	W	-	-	-	-	1.67	2.67
			M	-	-	-	-	*1*	1.53
			E	-	-	-	-	-	-

Synergistic ΣFIC values (≤0.5) are shown in **bold and green**; additive values (>0.5-⩽1.0) are shown *initalics and blue*; non-interactive values (>1.0-⩽4.0) are shown in plain text; antagonistic (>4,0) are shown underlined and in red text. M = methanolic extract; W = aqueous extract; E = ethyl acetate.

Assessment of combinational effects: ΣFIC

A range of interactions were noted for combinations of the *Syzygium* spp. leaf and fruit extract in combination with some selected conventional antibiotics (Table 3). The majority of the combinations were not tested for combinational effects as one or both components were completely inactive when tested alone in the liquid dilution MIC assay. Therefore, it is not possible to determine FIC values (and thus ΣFIC values) for those components and combinations. The majority of the combinations that ΣFIC values were determined for contained either ciprofloxacin or gentamicin as the antibiotic component because the majority of the bacterial strains tested were susceptible to one or both of those antibiotics, even if they were resistant to all other antibiotics tested. Of the ΣFIC values calculated, 78 (approximately 67%) were

non-interactive. A further 23 combinations (19.7%) had additive effects and therefore those combinations would be beneficial for treating *E. coli, K. pneumoniae* and *S. aureus* infections (of either β-lactam resistant and sensitive strains). compared to either component alone. Additionally, 11 combinations (9.4%) of the combinations were synergistic and therefore would have substantial additional value as an antibiotic therapy. Interestingly, the majority of the synergistic interactions (~67%) were against the β-lactam sensitive or MRSA *S. aureus* strains. Notably, there was also a correlation between the antibiotic component of the combination and synergy, with the majority of the synergistic combinations containing ciprofloxacin.

A further 23 combinations (19.7%) were determined to have antagonistic effects. Several trends were also evident for the antagonistic interactions. Interestingly, antagonism was only noted against the β-lactam sensitive *E. coli* strain. Additionally, all of the antagoniostic combinations contained ciprofloxacin as the antibiotic component. There was no clear correlation between the type of extract used in the combination, nor the plant type or *Syzygium* spp., with each inducing antagonism at approximately equal rates. Therefore, combinations containing ciprofloxacin and *Syzygium* spp. extracts should be avoided for the treatment of infections.

Evaluation of toxicity

All of the *Syzygium* spp. leaf and fruit extracts were serially diluted and tested across a range of concentrations in the *Artemia* nauplii lethality assay (ALA) to allow for an evaluation of toxicity by calculating the LC_{50} values (Table 3). Notably, LC_{50} values substantially >1000 µg/mL were calculated for all of the *S. australe* leaf extracts, whilst LC_{50} values close to 1000 µg/mL were noted for the *S. luehmannii* leaf extracts. As LC_{50} values >1000 µg/mL are defined as non-toxic for crude extracts in this assay,[27,29] the leaf extracts for both *Syzygium* spp. were deemed to be non-toxic or of low toxicity. In contrast, LC_{50} <1000 µg/mL were generally determined for fruit extracts of both species. However, it is noteworthy that *Syzygium* spp. fruit have high antioxidant capacities due to the presence of organic acids. Whilst the *Artemia* nauplii toxicity assay is generally considered to be robust, the nauplii are susceptible to pH changes,[30] as would occur in high antioxidant capacity extracts. Indeed, previous studies have reported high antioxidant capacities for extracts prepared from *S. australe* and *S. luehmannii* fruit by similar methods.[31] It is therefore likely that the *Artemia* nauplii toxicity assay used in this study may have greatly over-estimated the toxicity of the fruit extracts screened in this study. Further toxicity studies using human cell lines are required to more accurately evaluate the safety of these extracts.

Therapeutic index calculation

To further evaluate the suitability of the *Syzygium* spp. leaf and fruit extracts as therapeutic agents, their therapeutic indexes (TI) were calculated (Table 4). For this study, TI values ≥2.5 were considered noteworthy. The safety of the methanolic and ethyl acetate *S. australe* fruit extracts were determined to be noteworthy against the ESBL *E. coli* strains (TI values of 2.81 and 3.02 respectively), whilst methanolic *S. leuhmannii* leaf extract had the highest apparent TI value against the reference *E. coli* strain (TI = 2.68). The methanolic and ethyl acetate *S. australe* fruit extracts (as well as the aqueous fruit extract) each had TI values >2.5 against the reference *K. pneumoniae* strain, although each of these extracts had substantially lower TI values against the ESBL *K. pneumoniae* strain. Therefore, caution should be used when using these extracts to treat ESBL *K. pneumoniae* infections until the therapeutic indexes can be further evaluated using human cell line assays.

DISCUSSION

With the increase in multidrug resistant bacterial pathogen strains, the development of new safe and effective antibiotic therapies is increasingly important. This study evaluated the antibacterial activity of *S. australe* and *S. leuhmannii* extracts against some important bacterial pathogens. Whilst extracts prepared from these species have previously been reported to inhibit the growth of a broad-spectrum of bacteria,[17-19] none of the earlier screened the extracts against antibiotic-resistant bacterial strains. To the best of our knowledge, this is the first study that has screened these extracts against ESBL and MRSA bacterial strains. In this study, we screened *S. australe* and *S. leuhmannii* leaf and fruit extracts against ESBL strains of *E. coli* and *K. pneumoniae*, as well as against MRSA, and compared their inhibitory activity to their activity against β-lactam sensitive strains of the same bacteria. The resistance of these bacterial strains to a broad panel of β-lactam antibiotics (as well as against multiple classes of antibiotics) has previously been reported,[28] confirming their validity for use in this study. Notably, the ESBL *E. coli* and *K. pneumoniae* strains were resistant to a particularly broad-spectrum of antibiotics. Both strains were partially or fully resistant to nearly all of the conventional antibiotics tested in the earlier study.[28] Indeed, only cefotoxin and Augmentin® inhibited the growth of those strains strongly. The MRSA strain tested herein was also resistant to a broad-spectrum of antibiotics, but was sensitive to ciprofloxacin.

The Australian *Syzygium* spp. screened in our study were selected because the antibacterial properties of other species within this genus has been well established. The bacterial growth inhibitory properties of the Asian species *S. aromaticum*,[11,23,24,27] *S. cumini*,[32-34] *S. jambos*,[10,11] *S. alternifolium* and *S. samarangense* have been particularly well reported against a broad spectrum of bacterial pathogens.[12] Similarly, the antibacterial activity of the African species *S. cordatum* has been documented against multiple bacteria.[13,14] In contrast, the antibacterial activity of the Australian *Syzygium* spp. has been substantially less extensively reported, although noteworthy antibacterial activity has been

Table 4: Toxicity of the *Syzygium* spp. extracts (expressed as LC_{50} in µg/mL), and the therapeutic index (TI) for the extracts and positive controls.

Plant	Extract	Toxicity (LC_{50} in µg/mL)	Therapeutic index					
			E. coli (ATCC 25922)	ESBL *E. coli*	*K. pneumoniae* (ATCC 31488)	ESBL *K. pneumoniae*	*S. aureus* (ATCC 25923)	MRSA
S. australe	LM	1926	0.74	0.83	2.32	1.17	***6.21***	1.55
	LW	2753	1.59	2.12	***3.2***	1.67	***4.23***	1.06
	LE	1386	CND	CND	0.21	CND	CND	CND
	FM	985	1.39	***2.81***	***2.81***	1.39	0.35	1.39
	FW	820	0.49	0.98	***4.1***	0.49	0.16	0.49
	FE	935	2.08	***3.02***	***3.02***	2.03	CND	1.53
S. luehmannii	LM	963	***2.68***	0.27	0.36	0.18	0.27	1.72
	LW	1155	0.31	0.31	0.41	0.31	0.31	0.16
	LE	924	CND	CND	CND	CND	CND	CND
	FM	805	CND	0.21	0.5	***2.78***	***2.78***	1.39
	FW	1080	1.38	0.92	0.69	CND	CND	CND
	FE	927	CND	CND	CND	CND	CND	CND

Results represent means of 3 independent experiments, each preformed in triplicate (n = 9); MRSA = methicillin-resistant *S. aureus*; ESBL = extended spectrum β-lactamase resistant strain; LM = methanolic leaf extract, LW = aqueous leaf extract; LE = ethyl acetate leaf extract; FM = methanolic fruit extract; FW = aqueous fruit extract; FE = ethyl acetate fruit extract; CND as the extract was not toxic and/or inactive at all concentration tested; Notable TI values >2.5 are presented in ***bold red and italics***.

reported for *S. australe* and *S. luehmannii* leaf and fruit extracts against multiple bacteria.[17-19] However, those studies only evaluated the extracts against bacterial strains that were sensitive to most common antibiotics (including β-lactams). The current study aimed to extend the earlier studies by evaluating *S. australe* and *S. luehmannii* leaf and fruit extracts against extensively resistant bacterial strains, which are recalcitrant to the actions of most β-lactam antibiotics.

Noteworthy antibacterial activity was recorded for the methanolic and aqueous *S. australe* and *S. cordatum* leaf and fruit extracts against all of the bacterial strains tested, including the antibiotic-resistant strains. The *S. australe* fruit ethyl acetate extract also displayed strong inhibitory activity against all bacteria tested, except the methicillin-sensitive *S. aureus* strain. Indeed, this extract was a particularly good inhibitor of ESBL bacteria, with MICs of 310 and 460µg/mL against ESBL *E. coli* and ESBL *K. pneumoniae* respectively. Notably, this extract was substantially more potent against the ESBL *E. coli* strain than against the β-lactam sensitive strain, indicating that extract compounds may function via different mechanisms to the β-lactams. Alternatively (or additionally), extract components may function to block bacterial ESBL activity via a similar mechanism to clavulanic acid, although this remains to be verified.[1] If this ultimately proves to be the case, this extract may be particularly useful as is may "reactivate" multiple β-lactam antibiotics towards bacteria that are otherwise resistant to their effects. This would increase the efficacy of the β-lactam antibiotics, as well as extending their effective lifespan. In contrast, all other ethyl acetate extracts were generally devoid of antibacterial activity.

Whilst our study screened the *Syzygium* spp. extracts in combination with conventional antibiotics against the antibiotic sensitive and resistant bacterial panel, we did not test combinations in which one or both of the combination components were ineffective when tested alone. As all bacterial strains were resistant to ampicillin, we did not test any combinations containing this antibiotic and therefore were unable to determine if the extracts could re-sensitise the bacteria to this antibiotic. Similarly, as methicillin, oxacillin, and the macrolide antibiotic erythromycin were previously shown to be ineffective against all bacteria except the methicillin-sensitive *S. aureus* strain,[28] we were also unable to determine the ΣFICs of combinations containing these antibiotics against the other bacterial strains. However, as ciprofloxacin and gentamicin were effective against all of the tested bacterial strains, we were able to determine ΣFIC values for most combinations containing these antibiotics against all bacteria. Several synergistic combinations were noted, with nearly all of these determined to be against the methicillin-sensitive *S. aureus* strain. Additionally, two combinations containing ciprofloxacin also produced synergistic effects against ESBL *E. coli*, whilst a further ciprofloxacin containing combination was synergistic against ESBL *K. pneumoniae*. Due to their substantially enhanced

potency, these combinations would be particularly useful against infections of these bacteria.

Additionally, twenty-two combinations containing either ciprofloxacin or gentamicin had additive effects against the bacteria strains screened. No clear trend was noted for the additive combinations with regards to the extract component of the combination, or the bacteria that the effect was noted against. Whilst the potentiating effects were not as strong for these combinations as for the synergistic combinations, their use against these bacterial pathogens would still be advantageous compared to the use of either component alone. The majority of the other combinations tested were non-interactive. Whilst there is no advantage to using these combinations, this is still important information as it indicates that using the extract and antibiotic components concurrently would not decrease the activity of either individual component. As many people use traditional and allopathic medicines together (often without informing their medical practitioner), it is important to verify that the co-usage would not lessen the efficacy of the therapy. Four antagonistic combinations were also noted. All of these combinations contained ciprofloxacin as the antibiotic component and the antagonistic effects were only noted in *E. coli* strain with normal β-lactam sensitivity. Therefore, these combinations should be avoided for the treatment of *E. coli* infections unless the antibiotic susceptibility of the strain is established.

Notably, all of the *Syzygium* spp. extracts tested in this study were either nontoxic or of low toxicity in the ALA toxicity assay. Furthermore, noteworthy TI values (>2.5) were calculated for several extracts, with the TIs of the *S. australe* fruit extracts being particularly noteworthy. Indeed, TI values substantially >3 were noted for all of the *S. australe* fruit extracts against the β-lactam sensitive *K. pnuemoniae* strain, indicating that these extracts are particularly safe for therapeutic usage. In contrast, substantially lower TI values were recorded for these extracts against the corresponding ESBL strain. However, further *in vitro* toxicity studies using human cell lines are required to verify the safety of these extracts before they are adapted for clinical usage. Future studies should also use *in vivo* toxicity assays to confirm the safety of these extracts (and isolated compounds) in complex biological systems.

CONCLUSION

The findings reported herein indicate the potential of *Syzygium australe* and *Syzygium luehmannii* leaf and fruit extracts to inhibit the growth of selected β-lactam resistant strains of *E. coli, K. pneumoniae* and *S. aureus*. Both *Syzygium* spp. displayed noteworthy inhibitory activity against β-lactam sensitive and resistant strains of *E. coli, K. pneumoniae* and *S. aureus*. The *S. australe* fruit methanolic extract is particularly promising for antibiotic discovery, with MIC values substantially <1000 µg/mL against both ESBL strains, and against the MRSA. This extract had similar activity against most of the β-lactam sensitive bacterial strains. Our studies also showed that all of the extracts were either nontoxic, or of low to moderate toxicity in the *Artemia* nauplii toxicity assay. Further studies to isolate the antibacterial component(s) and to evaluate the therapeutic mechanisms of the extracts are warranted.

ACKNOWLEDGEMENT

Financial assistance for this project was provided by the Centre for Planetary Health and Food Security, and the School of Environment and Science, Griffith University.

ABBREVIATIONS

ALA: Artemia lethality assay; **ESBL:** Extended spectrum β-lactamase; **HDF:** Human dermal fibroblasts; **LC_{50}:** The concentration that induces 50% lethality; **MIC:** Minimum inhibitory concentration; **MRSA:** Methicillin-resistant *Staphylococcus aureus*; **ZOI:** Zone of inhibition.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

- *Syzygium australe* and *Syzygium luehmannii* leaf and fruit extracts were screened for growth inhibitory activity against β-lactam antibiotic sensitive and resistant bacterial strains.
- All methanolic and aqueous *S. australe* and *S. luehmannii* extracts had noteworthy bacterial growth inhibitory activity against most bacteria.
- The *S. australe* fruit ethyl acetate extract was a particularly good inhibitor of bacterial growth (MIC values generally 350-710 µg/mL)
- Several of the *Syzygium* spp. extracts also potentiated the antibacterial activity of ciprofloxacin and gentamicin.
- All of the extracts were either nontoxic or of low toxicity in the *Artemia* nauplii toxicity bioassay.

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Cite this article: Cordon ME, Cock IE. *Syzygium australe* (J.C.Wendl. ex Link) B.Hyland and *Syzygium luehmannii* (F.Meell.) L.A.S. Johnson Extracts Inhibit Methicillin-Resistant *Staphylococcus aureus* and ESBL *Escherichia coli* and *Klebsiella pneumoniae*. Pharmacognosy Communications. 2026;16(2):95-108.