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Research Letter

Antibacterial activity of selected australian syzygium species

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ABSTRACT: **Introduction:** Many species of Syzygium are known to have antiseptic activity. Australian Syzygium species had roles as traditional bush medicines for Australian Aborigines although their antiseptic potential has not been rigorously studied. **Methods:** Methanol extracts of leaves from *Syzygium forte*, *Syzygium francissi*, *Syzygium moorei*, *Syzygium puberulum* and *Syzygium wilsonii* were tested for antimicrobial activity by disc diffusion assay. Antibacterial strength was measured by MIC determination. Toxicity was determined using the *Artemia franciscana* nauplii bioassay. **Results:** *S. forte*, *S. francissi*, *S. moorei*, *S. puberulum* and *S. wilsonii* leaf methanolic extracts inhibited the growth of 5 (36%), 3 (21%), 3 (21%), 5 (36%) and 2 (14%) of the 14 bacteria tested respectively. Both Gram-positive and Gram-negative bacterial growth was inhibited by the Syzygium extracts, although Gram-positive bacteria appeared slightly more susceptible. With the exception of *S. forte*, all Syzygium leaf extracts tested also displayed low toxicity (LC_{50} > 1000 μg/ml) in the *Artemia fransiscana* bioassay. *S. forte* had a 48 h LC_{50} of 392.4 ± 33.0 μg/ml, making it slightly more toxic than Mevinphos (495.0 ± 35.1 μg/ml) and approximately 28 fold less toxic than potassium dichromate (14.0 ± 2.4 μg/ml) at 48 h. **Conclusions:** The antibacterial activity and low toxicity of the Syzygium spp. validate their medicinal usage by Australian Aborigines and indicate their potential as antibacterial medicinal agents.

KEYWORDS: *Syzygium forte*, *Syzygium francissi*, *Syzygium moorei*, *Syzygium puberulum*, *Syzygium wilsonii*, Australian plants, medicinal plants, antibacterial

INTRODUCTION

Plants have long been used as medicines for treating a variety of different diseases and complaints. In many parts of the world plant preparations and medications continue to be used in the treatment of numerous disorders including eczema, malaria, respiratory disorders and infectious diseases.[1] Plant based medicines have a long history for the treatment of microbial infections in many traditional medicinal systems. For some of these plant treatments, antimicrobial activity has been proven. However, for many plant based antiseptics the evidence is anecdotal, or at best, epidemiological. Many traditionally used antiseptic agents have yet to be subjected to rigorous scientific investigation.

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Syzygium is a large genus of evergreen flowering plants of the family Myrtaceae which consists of approximately 500 species.[2] Plants of this genus are widespread, occurring in tropical and subtropical regions of South-East Asia, Australia and Africa.[3] Many Syzygium species produce edible berries (eg. *Syzygium leuhmannii*). In the commercially most important species *Syzygium aromaticum* (clove), the unopened flower bud is used as a spice. This plant also has uses in traditional medicine due to its anaesthetic properties.[4] The antibacterial activity of *S. aromaticum* is also well known. Numerous studies have reported on the antibacterial[5] and antifungal[6] activities of oils and extracts from this plant. Other Syzygium species from South East Asia (*Syzygium jambos*)[7] and India (*Syzygium lineare* and *Syzygium cumini*)[8] have also been shown to have antimicrobial activity. Much of our knowledge about the antimicrobial activities of Australian Syzygium species is anecdotal, although Australian Aborigines are known to have used some species as medicinal agents.[9]

Recent studies within our laboratory have reported antibacterial activity in methanolic leaf extracts from the

Australian species *Syzygium australe* (Bush Cherry) and *Syzygium leuhmannii* (Riberry).[10,11] The current study was undertaken to examine the antiseptic potential of other Australian Syzygium species against a panel of bacteria and fungi and examine their toxicity to determine their suitability for use as antibiotic medicinal agents.

MATERIALS AND METHODS

Plant material

Collection of plant samples

Syzygium forte, *Syzygium francissi*, *Syzygium moorei*, *Syzygium puberulum* and *Syzygium wilsonii* leaves were obtained from and identified by Philip Cameron, senior botanic officer, Brisbane Botanical Gardens, Mt Cootha, Australia. Leaves of each species were obtained from single trees, washed in deionised water and processed within 4 hours of collection.

Preparation of crude extracts

S. forte, *S. francissi*, *S. moorei*, *S. puberulum* and *S. wilsonii* leaves were dried in a Sunbeam food dehydrator and the dried material was ground to a coarse powder. 1 g of each of the dried plant materials was extracted extensively in 50 ml methanol (Ajax, AR grade) for 24 hours at 4 °C with gentle shaking. The extract was filtered through filter paper (Whatman No. 54) under vacuum followed by drying by rotary evaporation in an Eppendorf concentrator 5301. The resultant pellet was dissolved in 10 ml of deionised water. The extract was passed through 0.22 μm filter (Sarstedt) and stored at 4 °C.

Qualitative phytochemical studies

Phytochemical analysis of Syzygium spp. extracts for the presence of saponins, phenolic compounds, flavonoids, polysteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by previously described assays.[12–14]

Antibacterial screening

Test microorganisms

All media was supplied by Oxoid Ltd. Microbial strains were obtained from Tarita Morais, Griffith University. Stock cultures of *Aeromonas hydrophilia*, *Alcaligenes feacalis*, *Bacillus cereus*, *Bacillus subtilis*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeuroginosa*, *Pseudomonas fluorescens*, *Salmonella salford*, *Serratia marcescens*, *Staphylococcus aureus* and *Yersinia enterocolitia* were subcultured and maintained in nutrient broth at 4 °C. *Aspergillus niger*, *Candida albicans*, and *Saccharomyces cerevisiae* were maintained in Sabouraud media at 4 °C.

Evaluation of antimicrobial activity

Antimicrobial activity of all plant extracts was determined using a modified disc diffusion assay.[15–18] Briefly, 100 μl of the test bacteria were grown in 10 ml of fresh media until they reached a count of approximately 10^8 cells/ml. 100 μl of microbial suspension was spread onto nutrient agar plates.

The *Syzygium* leaf extracts components were diluted to 10 mg/ml with deionised water and tested for antibacterial activity using 5 mm sterilised filter paper discs. Discs were impregnated with 10 μl (100 μg) of the test sample, allowed to dry and placed onto inoculated plates. The plates were allowed to stand at 4 °C for 2 hours before incubation with the test microbial agents. Plates inoculated with *Alcaligenes feacalis*, *Aeromonas hydrophilia*, *Bacillus cereus*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas fluorescens*, *Serratia marcescens*, were incubated at 30 °C for 24 hours, then the diameters of the inhibition zones were measured in millimetres. Plates inoculated with *Escherichia coli*, *Salmonella newport*, *Shigella sonnei*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pyogenes* were incubated at 37 °C for 24 hours, then the diameters of the inhibition zones were measured. All measurements were to the closest whole millimetre. Each antimicrobial assay was performed in at least triplicate. Mean values (± SEM) are reported in this study. Standard discs of ampicillin (2 μg) and chloramphenicol (10 μg) were obtained from Oxoid Ltd. and served as positive controls for antimicrobial activity. Filter discs impregnated with 10 μl of distilled water were used as a negative control.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) of the Syzygium methanolic extracts were determined as previously described.[19] Briefly, the plant extracts were diluted in deionised water and tested across a range of concentrations. Discs were impregnated with 10 μl of the test dilutions, allowed to dry and placed onto inoculated plates. The assay was performed as outlined above and graphs of the zone of inhibition versus concentration were plotted for each extract. Linear regression was used to calculate the MIC values.

Toxicity screening

Reference toxins for toxicity screening

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared as a 1.6 mg/ml solution in distilled water and was serially diluted in artificial seawater for use in the *Artemia franciscana* nauplii bioassay. Mevinphos (2-methoxycarbonyl-1-methylvinyl dimethyl phosphate) was obtained from Sigma-Aldrich as a mixture of cis (76.6%) and trans (23.0%) isomers and prepared as a

4 mg/ml stock in distilled water. The stock was serially diluted in artificial seawater for use in the bioassay.

Artemia franciscana nauplii toxicity screening

Toxicity was tested using a modified *Artemia franciscana* nauplii lethality assay.[20–23] Briefly, 400 µl of seawater containing approximately 45 (mean 43.8, n = 145, SD 18.6) *A. franciscana* nauplii were added to wells of a 48 well plate and immediately used for bioassay. 400 µl of diluted plant extracts and the reference toxins were transferred to the wells and incubated at 25 ± 1°C under artificial light (1000 Lux). A negative control (400 µl seawater) was run in at least triplicate for each plate. All treatments were performed in at least triplicate. The wells were checked at regular intervals and the number of dead counted. The nauplii were considered dead if no movement of the appendages was observed within 10 seconds. After 72 h all nauplii were sacrificed and counted to determine the total % mortality per well. The LC_{50} with 95% confidence limits for each treatment was calculated using probit analysis.

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extraction of 1 g of dried plant material from each species yielded dried plant extracts ranging from 108.4 mg to 435.1 mg (Table 1). The greatest amount of material (435.1 mg) was extracted from *S. puberulum* leaf. Methanolic extraction of all other Syzygium species leaves resulted in relatively consistent amounts of dried extracts (approximately 110–150 mg). The dried extracts were resuspended in 10 ml of deionised water, resulting in the extract concentrations shown in Table 1.

Phytochemical studies (Table 1) show that the methanolic extracts from all species contained high levels of phenolic phytochemicals (both water soluble and water insoluble phenolics). All extracts (except the *S. forte* extract) also showed moderate to large responses for tannins. *S. forte* and *S. moorei* also extracted high levels of saponins whilst all other species only displayed low responses in this assay. High levels of triterpenes were also evident for the *S. moorei* extract. Low to moderate responses were seen for alkaloids for all species. Low levels of flavanoids were also detected in all extracts. Also noteworthy is the large response seen for free anthraquinones in the *S. forte* extract.

Antibacterial activity

Syzygium leaf extracts were tested for antimicrobial activity at a concentration of 10 mg/ml in the disc diffusion assay against 14 bacteria (Table 2). *S. forte* and *S. puberulum* were the most effective antibacterial agents in terms of the diversity of the bacteria inhibited, each inhibiting the growth of 5 of the 14 bacteria tested (36%). *S. francisii*, *S. moorei* and *S. wilsonii* were less effective, inhibiting the growth of 3 (21%), 3 (21%) and 2 (14%) of the 14 bacteria tested respectively.

Both Gram-positive and Gram-negative bacteria were affected by the Syzygium methanolic leaf extracts although the Gram-positive bacteria appeared slightly more susceptible to the extracts of most of the Syzygium extracts tested. Of the 10 Gram-negative bacteria tested, only 2 bacteria (20%) were inhibited by *S. forte* leaf extract. In contrast, *S. forte* leaf extract inhibited the growth of 3 of the 4 Gram-positive bacteria tested (75%). *S. francisii*, *S. moorei* and *A. wilsonii* each inhibited the growth of 2 (20%) Gram-negative bacteria whilst *S. puberulum*

Table 1: The mass of dried extracted material, the concentration of extracts after resuspension in deionised water and qualitative phytochemical screenings of Syzygium spp. methanolic extractions

Extract	Mass of Dried Extract (mg)	Resuspended Extract Concentration (mg/ml)	Total Phenolics	Water Soluble Phenolics	Water Insoluble Phenolics	Cardiac Glycosides	Saponins	Triterpenes	Polysteroids	Alkaloids (Meyer test)	Alkaloids (Wagners test)	Flavanoids	Tannins	Free Anthraquinones	Combined Anthraquinones
S. forte	113.3	11.3	+++	+++	++	–	+++	–	–	–	–	+	+	+++	–
S. francissi	157.6	15.8	+++	+++	+++	–	+	–	–	+	+	+	+++	+	–
S. moorei	141.6	14.2	+++	+++	+++	–	+++	+++	–	++	+	+	++	+	–
S. puberulum	435.1	43.5	+++	+++	+++	–	+	–	–	+	+	+	+++	-	–
S. wilsonii	108.4	10.8	+++	+++	++	–	+	++	–	+	+	+	+++	+	–

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; – indicates no response in the assay.

Table 2: Antibacterial activity of Syzygium leaf extracts and antibiotic controls

	S. forte	*S. francisii*	*S. moorei*	*S. puberulum*	*S. wilsonii*	Amp	Chl
Gram negative rods							
A. faecalis	–	6.0 ± 0	–	10.0 ± 0	8.0 ± 1.0	15.2 ± 1.2	6.3 ± 0.6
A. hydrophilia	8.0 ± 0	–	7.3 ± 0.6	8.0 ± 0	–	12.0 ± 1.0	28.7 ± 1.6
C. freundi	–	–	–	–	–	8.3 ± 0.6	15.7 ± 1.2
E. coli	–	10.0 ± 0	9.6 ± 1.3	–	8.0 ± 0	14.7 ± 0.6	17.3 ± 0.6
K.pneumoniae	8.0 ± 0	–	–	7.0 ± 0.5	–	10.3 ± 0.6	21.3 ± 1.5
P. mirabilis	–	–	–	–	–	17.3 ± 0.6	8.7 ± 0.6
P. fluroscens	–	–	–	–	–	18.2 ± 0.5	21.2 ± 1.2
S. newport	–	–	–	–	–	18.7 ± 0.6	20.3 ± 0.6
S. marcenscens	–	–	–	–	–	0 ± 0	14.7 ± 0.6
S. sonnei	–	–	–	–	–	14.0 ± 0	14.3 ± 0.6
Gram positive rods							
B. cereus	7.3 ± 0.3	6.6 ± 0.3	6.0 ± 0	7.3 ± 0.6	–	26.7 ± 0.6	13.3 ± 1.2
Gram positive cocci							
S. aureus	–	–	–	–	–	11.7 ± 2.1	16.0 ± 1.0
S. epidermidis	7.0 ± 0	–	–	9.0 ± 0.3	–	26.3 ± 1.5	12.3 ± 0.6
S. pyogenes	9.6 ± 0.6	–	–	–	–	17.0 ± 1.0	24.0 ± 1.0

Numbers indicate the mean diameters of inhibition (mm) of at least triplicate experiments ± SEM. – indicates no growth inhibition. Chloramphenicol (10 µg) and ampicillin (2 µg) were used as positive controls.

inhibited the growth of 3 (30%) Gram-negative bacteria. In contrast, 1 (25%), 1 (25%), 0 (0%) and 2 (50%) of Gram-positive bacteria were inhibited by *S. francisii*, *S. moorei*, *A. wilsonii* and *S. puberulum* respectively.

The relative antibacterial activity strength of the extracts was evaluated by determining the MIC values for each extract against the bacteria which were shown to be susceptible by disc diffusion assays. MIC's were evaluated in the current studies across a range of concentrations. This has previously been determined to be a valid method of MIC determination as MIC values determined by disc diffusion correlate well with those determined by broth dilution assays.[24] The antibacterial activity was generally strongest against Gram-negative bacteria (Table 3). *S. francisii* in particular was a good inhibitor of the Gram-negative bacillus *A. faecalis* (MIC 229.1 ± 17.2 µg/ml) and *E. coli* (MIC 256.1 ± 21.5 µg/ml). In contrast, the

Table 3: Minimum inhibitory concentrations (µg/ml) of Syzygium leaf extracts against susceptible bacteria

	MIC (µg/ml)						
	A. faecalis	*A. hydrophilia*	*E. coli*	*K.pneumoniae*	*B. cereus*	*S. epidermidis*	*S. pyogenes*
S. francisii	229 ± 17	–	256 ± 21	–	890 ± 62	–	–
S. wilsonii	718 ± 46	–	1040 ± 65	–	–	–	–
S. puberulum	2525 ± 131	5269 ± 252	–	8084 ± 307	8603 ± 378	835 ± 43	–
S. moorei	–	473 ± 41	792 ± 56	–	–	–	–
S. forte	–	619 ± 45	–	854 ± 45	2391 ± 93	1632 ± 66	227 ± 26

Numbers indicate the mean MIC values of at least triplicate determinations. – indicates no growth inhibition observed.

antibacterial activity of *S. forte* was strongest against the Gram-positive cocci *S. pyogenes* (MIC 227.0 ± 26.3 µg/ml).

Qantification of toxitity

The *Syzygium* leaf extracts were diluted to 2000 µg/ml in artificial seawater for toxicity testing, resulting in a 1000 µg/ml concentration in the *Artemia franciscana* lethality bioassay. For comparison, the reference toxins potassium dichromate (800 µg/ml) (Figure 1f) and Mevinphos (2000 µg/ml) (Figure 1g) were also tested in the *Artemia franciscana* lethality bioassay. *S. francisii* (Figure 1b), *S. puberulum* (Figure 1d) and *S. wilsonii* (Figure 1e) exposure resulted in no increase in mortality above that of the seawater control. In contrast, both *S. forte* (Figure 1a) and *S. moorei* (Figure 1c) exposure resulted in increased mortality. Both reference toxins were more rapid in their induction of the onset of mortality than the *S. forte* and *S. moorei* leaf extracts at

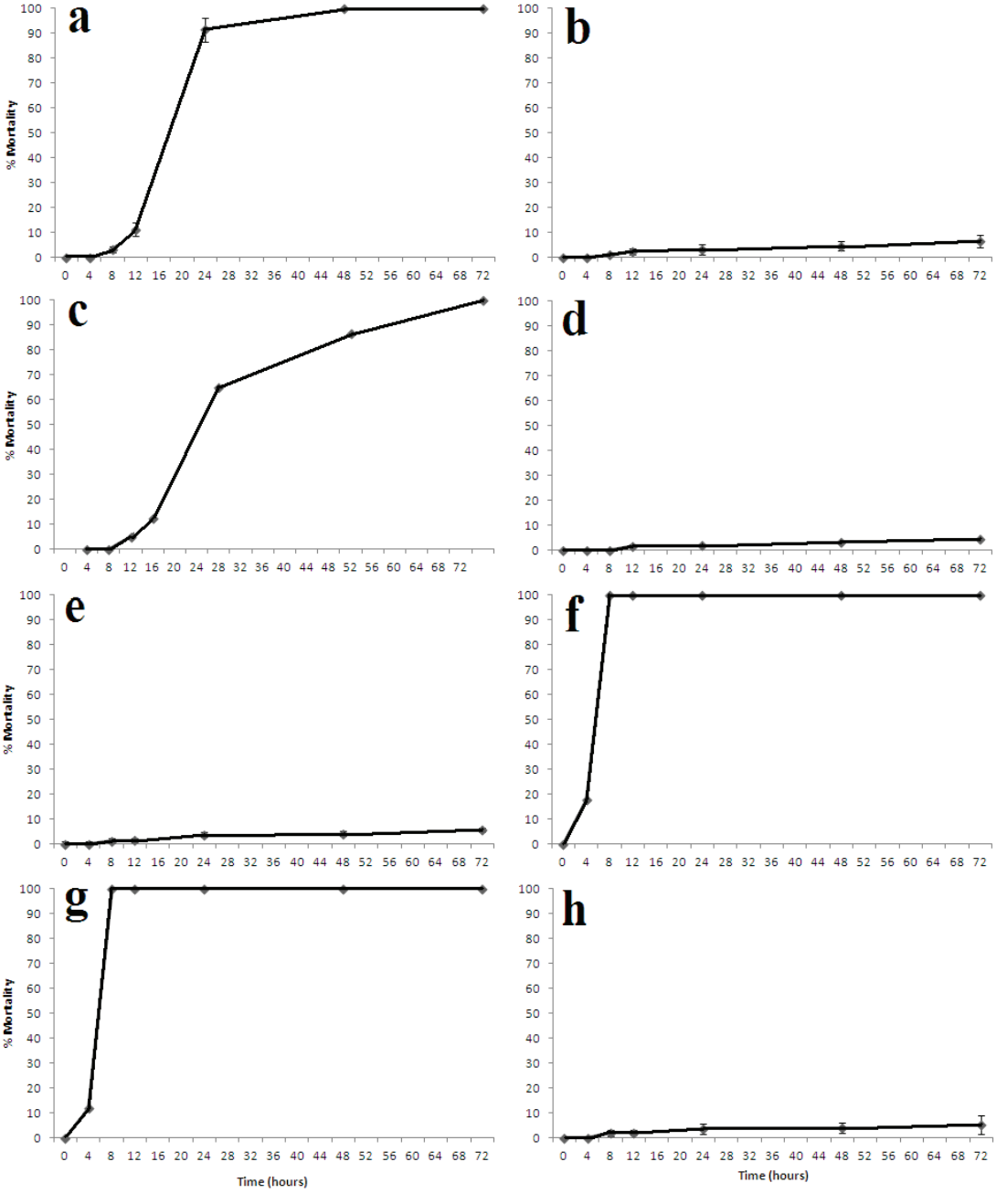

Figure 1. Brine shrimp lethality of (a) *S. forte* leaf extract (1000 µg/ml), (b) *S. francisii* (1000 µg/ml), (c) *S. moorei* leaf extract (1000 µg/ml), (d) *S. puberulum* (1000 µg/ml), (e) *S. wilsonii* leaf extract (1000 µg/ml), (f) potassium dichromate (800 µg/ml) and (g) Mevinphos (2000 µg/ml), (h) seawater control. All bioassays were performed in at least triplicate and are expressed as mean ± SEM.

Table 4: LC_{50} (95% confidence interval) for brine shrimp nauplii exposed to Syzygium extracts or the reference toxins potassium dichromate and Mevinphos

Sample	LC_{50} (µg/ml) at time (h)		
	24 h	48 h	72 h
S. francisii	–	–	–
S. wilsonii	–	–	–
S. puberulum	–	–	–
S. moorei	1312 ± 90	1108 ± 86	1108 ± 86
S. forte	392 ± 33	384 ± 39	371 ± 28
Mevinphos	1320 ± 125	495 ± 35	115 ± 12
Potassium Dichromate	78 ± 5	14 ± 2	3 ± 2

– denotes values that were not obtained as ≥ 50% mortality was not obtained at this time point.

the concentrations tested. For the reference toxins, the induction of mortality was seen within the first 4 hours of exposure. 100% mortality was evident following 8 hours of exposure. In contrast, 12 hours was required for *S. forte* and *S. moorei* extracts to induce the onset of mortality. 48 hours was required by *S. forte* to kill 100% of the brine shrimp whilst 72 hours was required by *S. moorei* to induce 100% mortality.

To determine the effect of toxin concentration on the induction of mortality, the extract was serially diluted in artificial seawater to test across the concentration range 2000 µg/ml to 15 µg/ml in the *Artemia* nauplii bioassay at 24, 48 and 72 hours. Table 4 shows the LC_{50} values of the *Syzygium* leaf extracts towards *A. franciscana*. No LC_{50} values are reported for the *S. francisii*, *S. puberulum* and *S. wilsonii* leaf extracts as less than 50% mortality was seen for all concentrations tested for the 72 h test period. *S. moorei* displayed low toxicity (>1000 µg/ml) at all times tested. The *S. forte* leaf extract was the only *Syzygium* extract to display appreciable toxicity, being substantially more toxic than Mevinphos at 24 hours (24 h LC_{50} values of 392.4 ± 33.0 µg/ml and 1320.0 ± 125.4 respectively). The *S. forte* leaf extract was also slightly more toxic than Mevinphos at 48 h (48 h LC_{50} values of 384.0 ± 39.2 µg/ml and 495.0 ± 35.1 respectively) but less toxic at 72 h (72 h LC_{50} values of 371.6 ± 28.7 µg/ml and 115.0 ± 12.8 respectively). Potassium dichromate was substantially more toxic than either the *S. forte* leaf extract or Mevinphos at all times tested (24 h LC_{50} 78.0 ± 5.6; 48 h LC_{50} 14.0 ± 2.4; 72 h LC_{50} 3.9 ± 2.0).

DISCUSSION

The current study reports on the antimicrobial activity and toxicity of leaf methanolic extracts of selected Australian Syzygium species. The ability of the leaf extracts to inhibit the growth of both Gram-positive and Gram-negative bacteria is in agreement with previous reports of the antibacterial activity of other Syzygium spp.[10,25–27] The greater susceptibility of Gram-positive bacteria seen in this study agrees with the previously reported specificities of South American,[28] African[29] and Australian[30] plant extracts. Results within this laboratory have also confirmed the greater susceptibility of Gram-positive bacteria towards other Australian plant extracts.[10,31,32] The Gram-negative bacterial cell wall outer membrane is thought to act as a barrier to many substances including antibiotics.[33] The uptake of the Syzygium extract antibiotic agents by Gram-negative bacteria is presumably affected by the cell wall outer membrane of some bacteria. Individual Syzygium leaf components responsible for the antisceptic potential of the Syzygium leaf extracts were not identified in the current study. Further phytochemical studies are needed to purify and identify the antibacterial components from these extracts.

The findings reported here also indicate that the majority of the Syzygium extracts examined in this study displayed low toxicity (>1000 µg/ml) in the Artemia nauplii bioassay. Indeed, we were unable to obtain LC_{50} values for *S. francisii*, *S. puberulum* and *S. wilsonii* as less than 50% mortality was recorded, even at longer exposure times (72 h). In contrast, *S. forte* was more toxic than was Mevinphos at 24 h and 48 h, and a third as toxic at 72 h. This toxicity would impact on the usefulness of the *S. forte* extract as a medicinal antiseptic agent. Toxicity was assessed in this study with the test organism *A. franciscana*. Whilst, toxicity towards *A. franciscana* has previously been shown to correlate well with toxicity towards human cells for many toxins,[34] further studies are required to determine whether this is also true for the Syzygium extracts examined in these studies. Toxic antibacterial extracts may still be useful as non-medicinal antibacterial agents (eg. surface disinfectants and topical antiseptics). Likewise, toxic plant extracts may also still have medicinal potential even if they are not antimicrobial. Previous studies have demonstrated that toxicity in the *A. franciscana* bioassay may indicate anti-cancer potential.[34] Toxic extracts such as the *S. forte* leaf extract should therefore also be tested against human cancer cell lines to determine their potential as anticancer drugs.

In conclusion, the results of this study indicate that the Syzygium leaf extracts examined in this report are worthy of further study due to their antibacterial activity and generally low toxicity. Further evaluation of the antibacterial properties of these extracts against a more extensive

panel of microbial agents is warranted. Likewise, purification and identification of the bioactive components is needed to examine the mechanisms of action of these agents. Whilst the extracts examined in this report have potential as antimicrobial agents, caution is needed before these compounds can be applied to medicinal purposes. In particular, further toxicity studies using human cell lines are needed to determine the suitability of these extracts for these purposes.

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