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Original Article

# *Eucalyptus major* (Maiden) Blakely and *Melaleuca alternifolia* (Maiden and Betche) Cheel Leaf Solutions Inhibit the Growth of Antibiotic-Sensitive and β-Lactam Resistant Bacterial Pathogens

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## ABSTRACT

**Background:** The increased prevalence of antibiotic-resistant bacterial pathogens has substantially decreased the efficacy of some antibiotics and has rendered others completely ineffective. Widespread bacterial resistance to β-lactam antibiotics (including resistance to the second-generation drug methicillin) is particularly concerning and new antibiotic therapies are urgently needed. **Materials and Methods:** The antibacterial activity of commercially sourced water-soluble *Eucalyptus major* and *Melaleuca alternifolia* leaf solutions was screened against β-lactam resistant and sensitive bacterial strains of *Escherichia coli, Klebsiella pneumoniae* and *Staphylococcus aureus* using disc diffusion assays. The activity was quantified by broth microdilution minimum inhibitory concentration (MIC) assays. Toxicity was evaluated by testing *Artemia* nauplii mortality assays. **Results:** Methanolic and aqueous extracts prepared from *Eucalyptus major* and *Melaleuca alternifolia* leaves displayed noteworthy growth inhibitory activity against all of the bacteria tested, including against methicillin resistant *Staphylococcus aureus* MRSA and extended spectrum β-lactamase (ESBL) strains of *E. coli* and *K. pneumoniae*. The methanolic *E. major* leaf extract had particularly good antibacterial activity (MICs=39-625 µg/mL) against all bacterial strains. Notably, this extract had similar potency against an ESBL strain of *E. coli* as against the corresponding antibiotic-sensitive strain (MICs=78 µg/mL against each) and was significantly more potent against an ESBL *K. pneumoniae* strain (MIC=39 µg/mL) than against the corresponding antibiotic sensitive strain (MIC=78 µg/mL). All extracts were non-toxic in the *Artemia* nauplii lethality assay (ALA), indicating their safety for topical use. **Conclusion:** The potency of the *E. major* and *M. alternifolia* extracts against multi-antibiotic resistant bacteria and their lack of toxicity highlight these species as potential targets for antibiotic drug development. Further phytochemical and mechanistic studies of these species are warranted.

**Keywords:** Myrtaceae, Australian plants, Multi-drug resistant bacteria, Superbugs, β-lactamase, ESBL, MRSA.

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## INTRODUCTION

The clinical introduction of penicillin almost 100 years ago revolutionised healthcare and allowed many previously difficult to treat diseases to be safely and effectively treated, thereby saving countless lives and easing suffering globally. Indeed, penicillin was one of the first medications to be effective against *Staphylococcus* spp. (including *Staphylococcus aureus*) and *Streptococcus* spp. However, the incorrect and overuse of antibiotics provided selective pressure for bacteria to develop resistance to penicillin and other β-lactam antibiotics through the production β-lactamase enzymes that degrade the β-lactam structure, rendering those antibiotics ineffective or of low efficacy.[1] The discovery of multiple other classes of antibiotics, particularly during the "golden years of antibiotic discovery in the 1940's to 1960's, has allowed medical science to maintain a pipeline of effective antibiotics to combat developing strains with resistance to previous therapies. Additionally, synthetic chemists have sought to overcome antibiotic-resistance by modifying natural antibiotic scaffolds through the addition of specific functional groups. This has been particularly effective

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for the β-lactam structure and has resulted in the development of multiple semi-synthetic β-lactam antibiotics (e.g. methicillin, oxacillin), which evade the actions of the original β-lactamase enzymes.

Unfortunately, since the introduction of these semi-synthetic antibiotics, bacteria have rapidly evolved to produce modified β-lactamase enzymes with substantially broader specificity, allowing them to recognise and inactivate most β-lactam antibiotics.[1] Indeed, the development of methicillin resistant strains of *S. aureus* (MRSA) has rendered that antibiotic ineffective against many infections. Additionally, as many MRSA strains also developed resistance to multiple other antibiotics, there are now few effective treatment options remaining for these pathogens and new therapies are urgently required. The increase in MRSA infections has dramatically increased the incidence of *S. aureus*-associated mortality and this bacterium now being considered to a major cause of death globally.[2] Similarly, the development of extended spectrum β-lactamase (ESBL) enzymes (especially in the family Enterobacteriaceae) has rendered multiple classes of β-lactam antibiotics ineffective against many bacterial pathogens. For an increasing number of bacteria (particularly MRSA), vancomycin is now considered to be the only effective therapy. However, vancomycin-resistant MRSA strains have now also developed[3] and new antibiotic therapies that are effective against these resistant strains are urgently needed. Indeed, the World Health Organisation (WHO) considers the development of novel, effective and safe antibiotics to be one of the most urgent challenges facing medical science.[4]

Prior to the clinical introduction of penicillin, plant medicines were commonly used to treat bacterial infections and these preparations are still commonly used in multiple traditional healing systems such as Ayurveda, or traditional chinese medicine (TCM). Re-examining plant-based antibacterial therapies is a promising approach to develop new antibiotic chemotherapies. Even in Westernised medicinal systems, some plant-based extracts and oils are still used to treat bacterial infections, particularly for topical treatments of cuts and abrasions. Indeed, oils and soluble extracts prepared from medicinal plants are used globally to treat bacterial skin infections. Notably, plant-based therapies often contain multiple anti-bacterial compounds, which provide them with greater efficacy, as well as reducing the development of further

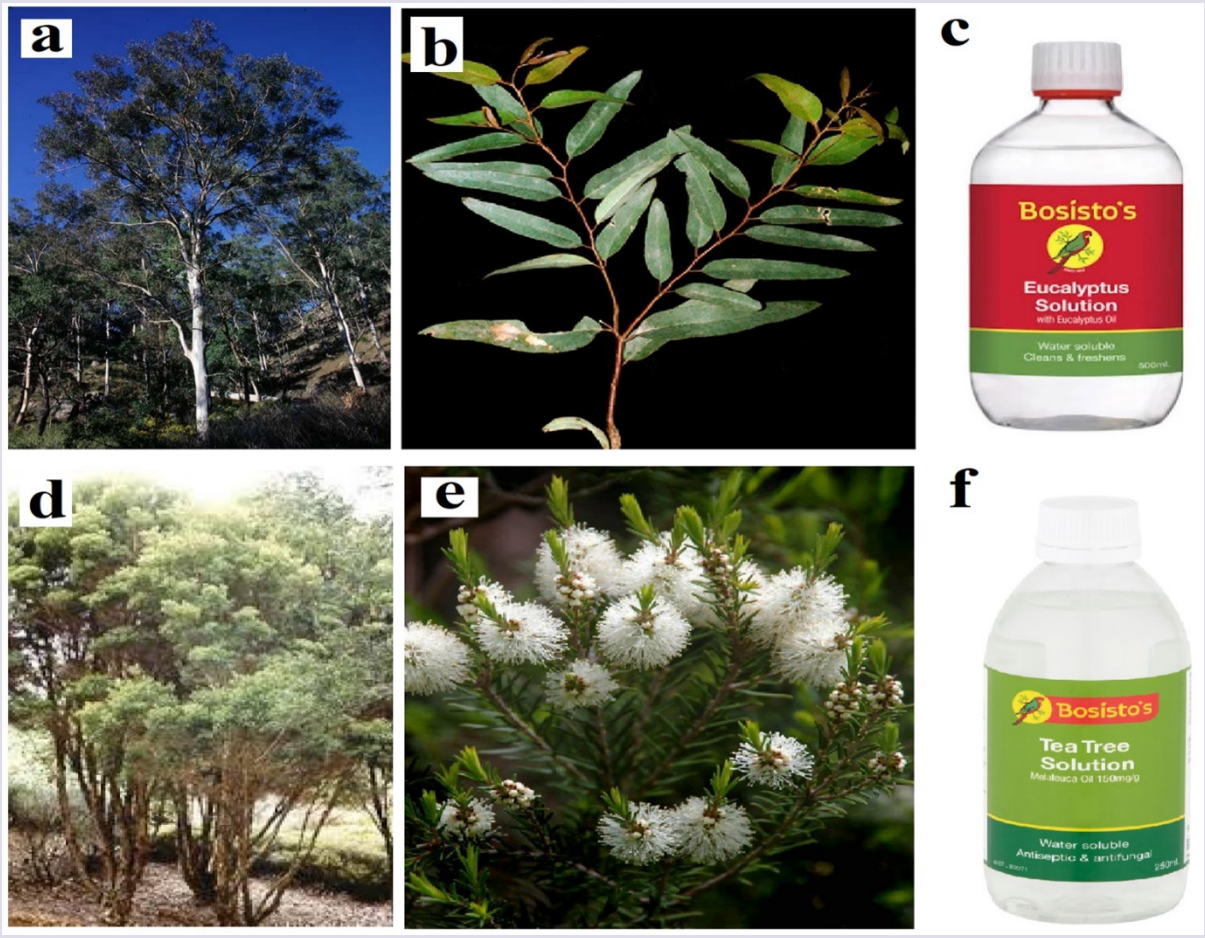


**Figure 1:** *Eucalyptus major* (a) whole plant, (b) leaves and (c) commercial *E. major* solution, as well as *M. althernifolia* (d) whole plant, (e) leaves and flowers and (f) commercial *M. alternifolia* solution.

bacterial resistance towards the extract's components. For example, epicatechin gallate (ECG), which is a relatively common component of many plant preparations,[5,6] has been reported to be effective for the treatment of MRSA infections.[7,8] Interestingly, ECG circumvents MRSA resistance mechanisms by inserting into the bacterial cytoplasmic membrane. The membrane changes induce disruptions in the penicillin-binding-protein 2a (PBP2a) resistance mechanism. Therefore, ECG not only has noteworthy antibiotic properties in its own right, but it also blocks bacterial β-lactam resistance mechanisms, allowing β-lactam antibiotics (including methicillin) to function, even in β-lactam resistant bacterial strains.

The Australian plants *Eucalyptus major* (Maiden) Blakely (Figures 1a and 1b) and *Melaleuca alternifolia* (Maiden and Betche) Cheel (Figures 1d and 1e) are particularly well known for their antibiotic properties,[9-12] and are available from pharmacies in many regions of the world as an antiseptic for skin cuts and abrasions. The inhibitory activity of *Eucalyptus* spp. extracts and oils have been relatively well documented.[13-16] Similarly, the antibacterial properties of *Melaleuca* spp. have also been reported against multiple bacteria.[17,18] However, these studies generally only tested *Eucalyptus* spp. and *Melaleuca* spp. preparations against antibiotic-sensitive strains and testing against resistant strains has been largely neglected. Indeed, we are only aware of one study that screened *Eucalyptus globulus* Labill., *Corymbia citriodora* (Hook.) K.D. Hill and L.A.S. Johnson and *Eucalyptus radiata* Sieber ex DC. against antibiotic resistant bacteria, including an MRSA strain.[19] That study examined the antibacterial activity of an essential oil and water-soluble preparations were not evaluated. Our study examines the antibacterial activity of commercially sourced water-soluble preparations produced from *E. major* and *M. alternifolia* leaves against a panel of β-lactam resistant bacteria and compare it to their activity against their antibiotic-sensitive counterparts.

## MATERIALS AND METHODS

### Plant source and extraction

The *E. major* (Maiden) Blakely and *M. alternifolia* (Maiden and Betche) Cheel leaf solutions used in this study were purchased from Bosistos, Australia as water soluble solutions (Figure 1 c and 1 f respectively). Individual 100 mL volumes of each solution was dried by lyophilisation. A 1-gram mass of each dried extract was extracted with 10 mL of either sterile deionised water or AR grade methanol (Ajax Fine Chemicals, Australia). The extracts were filtered through Whatman No. 54 filter paper, dried and the extract yield was determined. A 200 µL volume of DMSO was added to the dried masses to aid in solubilising the material and the volumes were adjusted to 20 mL with sterile deionised water. The subsequent extracts were stored at 4°C until use.

### Qualitative phytochemical studies

Phytochemical analysis of the *E. major* and *M. alternifolia* leaf extracts for the presence of cardiac glycosides, alkaloids, saponins, tannins, flavonoids, phenolic compounds, phytosterols, flavonoids and triterpenoids was achieved as previously described.[20-22]

### Antibacterial screening

#### *Test bacterial strains*

All media and components were supplied by Oxoid Ltd., Australia. Reference strains of β-lactam-sensitive *Escherichia coli* (ATCC 25922), methicillin-sensitive *Staphylococcus aureus* (MSSA; ATCC 25923), methicillin-resistant *Staphylococcus aureus* (MRSA; ATCC 43300), β-lactam-sensitive *Klebsiella pneumoniae* (ATCC 13883) and an ESBL *K. pneumoniae* (ATCC 700603) were purchased from American Type Culture Collection, USA. A clinical isolate strain of *E. coli* which has been reported to be resistant to multiple β-lactam antibiotics, including second and subsequent generation β-lactams was obtained from Gold Coast University Hospital, Australia. Bacterial stock cultures were initially streaked onto individual Mueller-Hinton agar plates to ensure that pure colonies were used and that the culture was not contaminated. Single colonies of each bacteria was inoculated into separate flasks of Mueller-Hinton broth and grown aerobically until they reached log growth phase. A 100 µL aliquot of the individual cultures were subsequently transferred into fresh media and incubated at 37°C for 24 hr prior to use. All media and agar powders were purchased from Oxoid Ltd., Australia.

### Evaluation of antibacterial activity

Antibacterial activity screening of the *E. major* and *M. alternifolia* leaf extracts was achieved using modified disc diffusion assays.[23,24] Briefly, 100 µL of each individual bacteria was grown separately in 20 mL of broth until an approximate count of $10^8$ cells/mL was reached. A volume of 100 µL of each bacterial suspension was spread onto Mueller-Hinton agar plates. Filter paper discs (6 mm diameter) were infused with 10 µL of the individual extracts, allowed to dry and placed onto the inoculated plates. The plates were left to stand at 4°C for 2 hr before incubation to allow the bacteria to settle into the agar surface. All plates were incubated aerobically at 37°C for 24 hr. The diameters of the inhibition zones (ZOIs) were measured to the closest whole millimetre. Each assay was completed three times, each with internal triplicates ($n$=9). Mean values (±SEM) are reported in this study. Ampicillin (10 µg) and vancomycin (5 µg) discs were obtained from Oxoid Ltd., Australia and used as positive controls to compare antibacterial activity. Filter discs infused with 10 µL of distilled water (containing 0.5% DMSO) were used as negative controls.

## Minimum Inhibitory Concentration (MIC) determination

The minimum inhibitory concentration for each extract was determined using a liquid dilution MIC assay as it is generally considered the most sensitive bacterial growth inhibitory assay.[25] Additionally, as microplate liquid dilution (LD) MIC assays are perhaps the most commonly used method of quantifying bacterial growth inhibition efficacy, use of this method allows for comparisons with other studies. A solid phase agar disc diffusion (DD) assay was also used in this study as this bioassay was deemed to provide a closer representation of the environment and conditions relevant to solid axillary and foot skin systems.

## Microplate liquid dilution MIC assay

The MICs of the extracts were evaluated by standard methods.[25-28] Briefly, overnight bacterial cultures were added dropwise to fresh liquid broth and the turbidity was visually adjusted to produce a McFarlands number 1 standard culture. This was subsequently diluted 1 in 50 with fresh broth, resulting in the MIC assay inoculum culture. A volume of 100 µL sterile broth was added to all wells of a 96 well plate. Test extracts or control antibiotics (100 µL) were then added to the top row of each plate and 1 in 2 serial dilutions were prepared in each column of wells by transferring 100 µL from the top well to the next well in each column, etc. A growth control (without extract) and a sterile control (without inoculum) were included on each plate. A volume of 100 µL of bacterial culture inoculum was added to all wells except the sterile control wells. The plates were then incubated aerobically at 37 °C for 24 hr. p-Iodonitrotetrazolium violet (INT) was obtained from Sigma, Australia and dissolved in sterile deionised water to prepare a 0.2 mg/mL INT solution. A 40 µL volume of this solution was added into all wells and the plates were incubated for a further 6 hr at 30°C. Following incubation, the MIC was visually determined as the lowest dose at which colour development was inhibited.

## Toxicity screening

### *Reference toxin for toxicity screening*

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared in deionised water (4 mg/mL) and serially diluted in artificial seawater for use in the *Artemia franciscana* nauplii bioassay.

## *Artemia franciscana* nauplii toxicity screening

Toxicity was assessed using a modified *Artemia franciscana* nauplii lethality assay.[29,30] Briefly, 400 µL of seawater containing ~38 (mean 37.6, n=125, SD 12.5) *A. franciscana* nauplii were

**Table 1: The mass of dried extracted material, the concentration after resuspension in deionised water (0.5% DMSO) and qualitative phytochemical screenings of the *E. major* and *M. alternifolia* leaf extracts.**

| | | | *E. major* | | *M. alternifolia* | |
|---|---|---|---|---|---|---|
| | | | Methanolic extract | Aqueous extract | Methanolic extract | Aqueous extract |
| Mass of extracted material (mg) | | | 280 | 220 | 160 | 130 |
| Concentration of resuspended extract (mg/mL) | | | 14 | 11 | 8 | 6.5 |
| Qualitative Phytochemical Tests | Phenols | Total phenols | +++ | +++ | +++ | +++ |
| | | Water soluble phenols | +++ | +++ | +++ | +++ |
| | | Insoluble phenols | + | + | +++ | +++ |
| | Saponins | Froth persistence | + | + | + | ++ |
| | | Emulsion test | + | + | + | ++ |
| | Cardiac glycosides | Keller-Kiliani Test | - | - | - | - |
| | Tri-terpenoids | Salkowski Test | - | - | - | - |
| | Phyto-sterols | Acetic Anhydride Test | - | - | - | - |
| | Alkaloids | Meyer's Test | - | - | - | - |
| | | Wagner's Test | - | - | - | - |
| | | Draggendoff's Test | - | - | - | - |
| | Flavo-noids | Kumar Test | ++ | +++ | ++ | ++ |
| | Tannins | Ferric Chloride Test | ++ | + | ++ | ++ |
| | | Lead Acetate Test | + | + | + | + |
| | Anthra-quinones | Free | - | - | - | - |
| | | Combined | - | - | - | - |

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

added to wells of a 48 well plate and immediately used in the bioassay. A volume of 400 µL of the reference toxin or the diluted plant extracts were transferred to the wells and incubated at 25±1°C under artificial light (1000 Lux). For each plate, a 400 µL seawater negative control was run in triplicate. The wells were assessed at regular intervals and the number of dead counted. The nauplii were deemed dead if no movement of the appendages was observed within 10 sec. After 24 hr, 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.

### Statistical analysis

Data is expressed as the mean±SEM of at least three independent experiments, each with internal triplicates ($n$=9). One-way analysis of variance (ANOVA) was used to calculate statistical significance between the negative control and treated groups, with $p$ values <0.05 considered to be statistically significant.

## RESULTS

### Liquid extraction yields and qualitative phytochemical screening

Dried *E. major* and *M. alternifolia* leaf solutions (1 g masses) were extracted with methanol and water resulting in yields ranging from 130 mg (*M. alternifolia* aqueous extract) to 280 mg (*E. major* methanolic extract) (Table 1). In general, higher extraction yields were noted for *E. major* leaf than the corresponding *M. alternifolia* extracts and methanol was a better extractant (based on extraction yields) than water. The dried extracts were resuspended in 10 mL of deionised water (containing 0.5% DMSO), resulting in the concentrations presented in Table 1. Qualitative phytochemical studies showed that the *E. major* and *M. alternifolia* extracts had similar phytochemical profiles. All contained high levels of phenolic compounds, as well as moderate to high levels of flavonoids and tannins and low levels of saponins.

### Inhibition of bacterial growth

To determine the ability of the *E. major* and *M. alternifolia* leaf extracts to inhibit the growth of the panel of β-lactam sensitive and β-lactam resistant bacteria, 10 µL of each extract was screened using a disc diffusion assay. *Escherichia coli* growth was inhibited by both the methanolic and aqueous extraxt of both *E. major* and *M. alternifolia* (Figure 2). The methanolic *E. major* extract produced substantially larger ZOIs than the aqueous extract, indicating greater potency. Interestingly, the potency of the *E. major* extracts was the same for against the β-lactam resistant *E. coli* strain as for the antibiotic sensitive strain, indicating that the bioactive extract components are functioning by different mechanisms to β-lactam antibiotics. Alternatively, the extract may contain components that counteract the ESBL

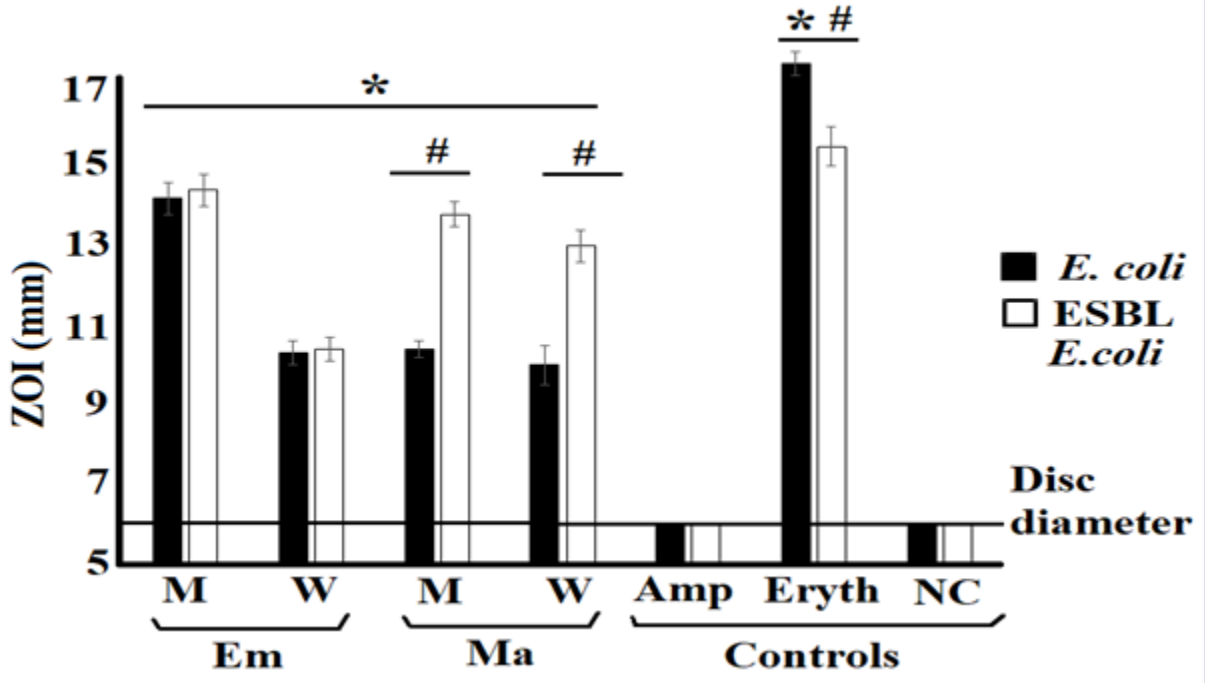


**Figure 2:** Growth inhibitory activity of the *E. major* and *M. alternifolia* leaf extracts against β-lactam sensitive and ESBL *E. coli* measured as zones of inhibition (mm). Em=*E. major*; Ma=*M. alternifolia*; M=Methanolic extract; W=aqueous extract; Amp=Ampicillin (10 µg); Eryth=Erythromycin (10 µg); NC=Negative control (0.5% DMSO). Results are expressed as mean zones of inhibition±SEM. * indicates results that are significantly different to the negative control ($p$<0.05); # indicates significant differences between the antibiotic sensitive and resistant bacterial species ($p$<0.05).

activity of the bacteria, allowing other extract components to function, even in bacteria resistant to their effects. Notably, the control antibiotic ampicillin was completely ineffective towards both *E. coli* strains, demonstrating that even the strain designated "antibiotic-sensitive" itself had inherent antibiotic resistance. In contrast, erythromycin was a potent inhibitor of both strains, with ZOIs of 17.6 and 15.5 mm recorded against the sensitive and resistant bacterial strains respectively. No inhibition was noted for the negative water control, indicating that the assay was functioning correctly.

A different trend was noted for the *M. alternifolia* leaf extracts (Figure 2). The methanolic and aqueous extracts had similar potency (as determined by the similar ZOIs). This may indicate that similar bioactive and/or potentiating extract compounds are present in both extracts. Whilst methanol extracts compounds with widely varying polarities, water exclusively extracts higher polarity compounds, indicating that polar molecules are most important for the bacterial growth inhibitory effects evident in our study. Of greater interest, the methanolic and aqueous *M. alternifolia* leaf extracts were both significantly more effective against the ESBL *E. coli* strain compared to the β-lactam sensitive strain. This trend is interesting as the *E. coli* strain screened in our study is highly resistant to most classes of antibiotic, with the exception of the amino glycoside gentamicin.[31] This strain was particularly resistant to β-lactam antibiotic monotherapies (but not to Augmentin®) and was also resistant to chloramphenicol, macrolides, tetracyclines and fluoroquinolones. It is possible that *M. alternifolia* extract components may function via a novel mechanism, although this remains to be determined.

The *E. major* and *M. alternifolia* leaf extracts were also effective inhibitors of β-lactam sensitive and β-lactam resistant *K. pneumoniae* growth (Figure 3), although different trends were noted compared to *E. coli* inhibition. The methanolic and aqueous *E. major* extracts had similar potency (as determined by ZOI). However, unlike the trends noted for *E. coli*, both *E. major* extracts were significantly more potent against the β-lactam resistant *K. pneumoniae* strain than the corresponding sensitive strain. This is a particularly interesting result and may indicate that *E. major* extract components may function via novel mechanisms. Alternatively, some extract components may not only be blocking the effects of the ESBL enzyme, but may also be potentiating the activity of the antibiotic components. However, these remains to be verified and future studies focussing on the antibacterial mechanisms of the extract components are required. Also, in contrast to the *E. coli* inhibition results, the *M. alternifolia* extracts were approximately as effective against the β-lactam sensitive *K. pneumoniae* strain as the *E. major* extracts. However, unlike the trend seen for *E. coli*, the ESBL *K. pneumoniae* strain was significantly ($p<0.05$) less effective against the ESBL strain than against the β-lactam sensitive *K. pneumoniae* strain, indicating that some of the extract components may be inactivated by the ESBL enzyme, although this remains to be verified.

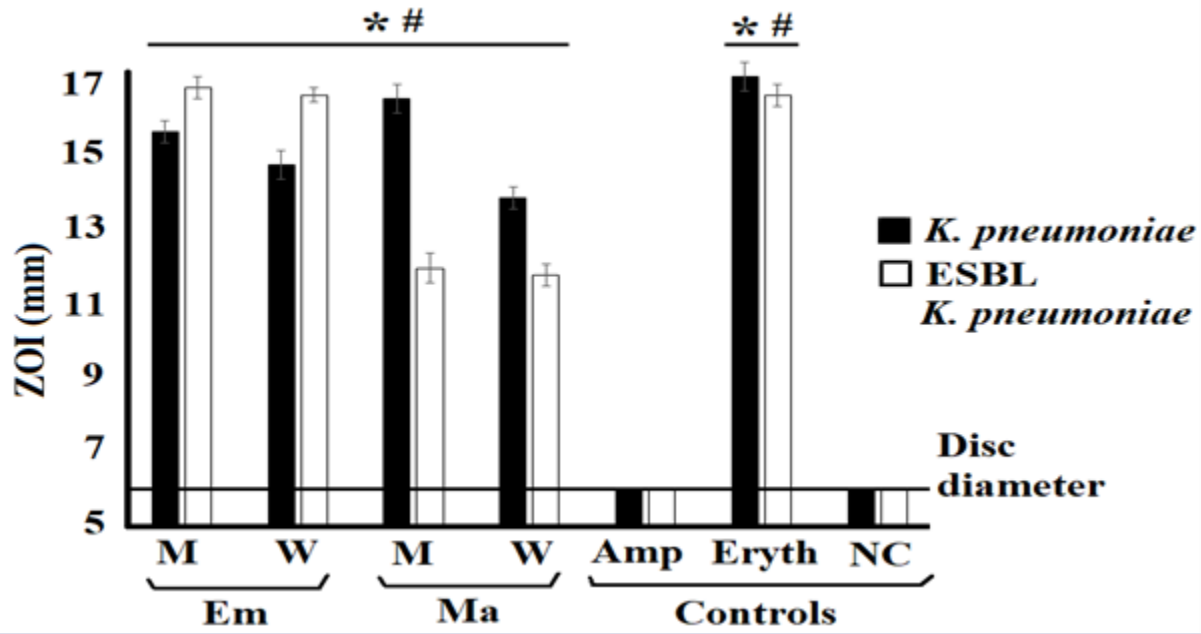


**Figure 3:** Growth inhibitory activity of the *E. major* and *M. alternifolia* leaf extracts against β-lactam sensitive and ESBL *K. pneumoniae* measured as zones of inhibition (mm). Em=*E. major*; Ma=*M. alternifolia*; M=Methanolic extract; W=aqueous extract; Amp=Ampicillin (10 µg); Eryth=Erythromycin (10 µg); NC=Negative control (0.5% DMSO). Results are expressed as mean zones of inhibition±SEM. * indicates results that are significantly different to the negative control ($p<0.05$); # indicates significant differences between the antibiotic sensitive and resistant bacterial species ($p<0.05$).

The methanolic and aqueous extracts produced from the leaves of both *E. major* and *M. alternifolia* were also effective inhibitors of the growth of both the β-lactam sensitive *S. aureus* strain and the MRSA strain, with ZOIs up to 14.6 mm (methanolic *E. major* extract against antibiotic-sensitive *S. aureus* strain) (Figure 4). Similar ZOIs were measured for both the methanolic and aqueous extracts of both species against the β-lactam sensitive *S. aureus* strain. However, substantially different trends were noted for the extracts against the antibiotic-sensitive *S. aureus* strain compared to the MRSA strain. The aqueous *E. major* extract was significantly less effective (as judged by ZOI size) than the corresponding methanolic extracts against both *S. aureus* strains. Similarly, the aqueous *M. alternifolia* extract was also significantly less effective against the MRSA strain than against the β-lactam sensitive *S. aureus* strain. In contrast, the aqueous *M. alternifolia* extract was significantly more potent against the β-lactam sensitive *S. aureus* strain than against MRSA.

## Quantification of Minimum Inhibitory Concentration (MIC)

The relative level of antimicrobial activity was further evaluated by determining the MIC values (Table 2) for each extract against the bacteria which were shown to be susceptible in the disc diffusion screening assays. Noteworthy antibacterial activity (MIC values substantially <1000 µg/mL) were calculated for all of the methanolic and aqueous *E. major* and *M. alternifolia* leaf extracts against all of the bacteria screened in this study. The methonolic *E. major* extract was a particularly effective inhibitor of the antibiotic-resistant strains, with MICs of 78, 39, 625 µg/mL against ESBL *E. coli*, ESBL *K. pneumoniae* and MRSA respectively. This extract was similarly effective against the antibiotic-sensitive strains of *E. coli* and *K. pneumoniae* as against the corresponding resistant strains and was substantially more potent against the antibiotic-sensitive *S. aureus* strain than against MRSA.

Notably, The MICs calculated for the control antibiotics indicate that all of the bacterial strains tested in our study (including the strains designated as "antibiotic-sensitive" strains) were actually antibiotic-resistant strains. In this assay, bacteria with MIC values >1 µg/mL against pure control antibiotics are defined as resistant to that antibiotic.[32] Notably, ampicillin was ineffective against all of the bacterial strains tested in our study, indicating that all strains were β-lactam resistant (at least against ampicillin). Additionally, with the exception of the antibiotic-sensitive *S. aureus* strain, MIC values >1 µg/mL were noted for chloramphenicol, ciprofloxacin, erythromycin and tetracycline against all other bacterial strains. Indeed, only gentamicin was highly effective against all of the bacterial strains screened in this study (MICs ≤0.04 µg/mL). Thus, all bacterial strains tested herein are Multidrug Resistant (MDR) strains.

## Quantification of toxicity

All extracts were initially screened in the *Artemia* nauplii assay at 2000 µg/mL (Figure 5). Additionally, potassium dichromate was also tested in the bioassay as a reference toxin. Potassium dichromate was rapid in its onset of mortality, promoting nauplii death within the first 3 hr of exposure, with 100% mortality evident within 5 hr (unpublished results). In contrast, the methanolic and aqueous *E. major* and *M. alternifolia* leaf extracts all induced substantially <50% moratlity following 24 hr exposure. As 24

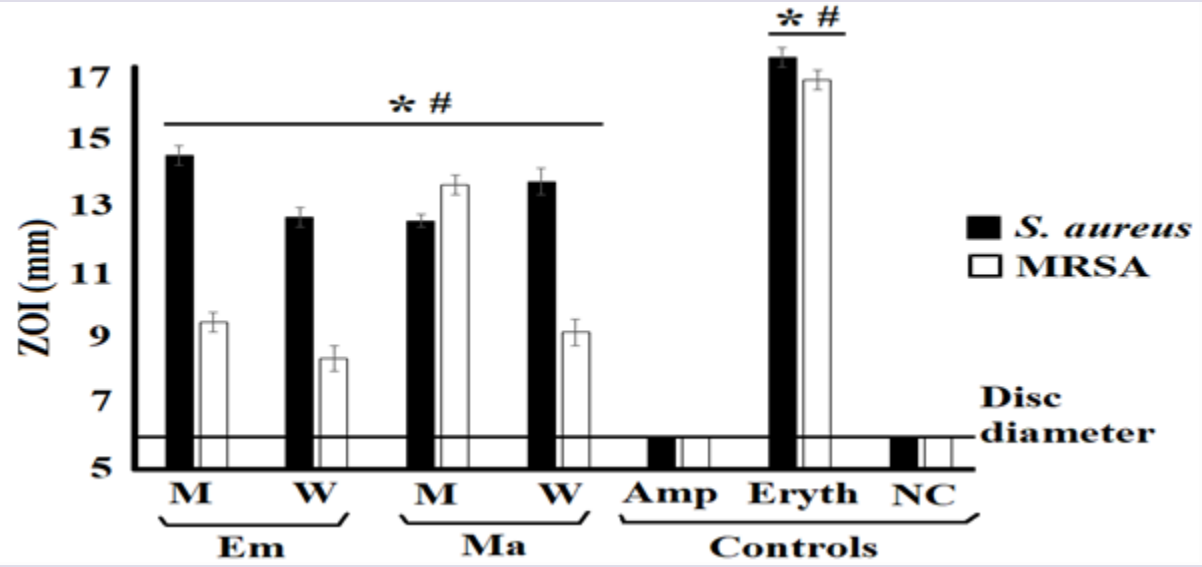


**Figure 4:** Growth inhibitory activity of the *E. major* and *M. alternifolia* leaf extracts against β-lactam sensitive and methicillin resistant *S. aureus* measured as zones of inhibition (mm). Em=*E. major*; Ma=*M. alternifolia*; M=Methanolic extract; W=aqueous extract; Amp=Ampicillin (10 µg); Eryth=Erythromycin (10 µg); NC=Negative control (0.5% DMSO). Results are expressed as mean zones of inhibition±SEM. * indicates results that are significantly different to the negative control ($p$<0.05); # indicates significant differences between the antibiotic sensitive and resistant bacterial species ($p$<0.05).

**Table 2: MIC values (µg/mL) of the *E. major* and *M. alternifolia* leaf extracts against antibiotic-sensitive and antibiotic-resistant bacterial strains.**

| | *E. coli* | ESBL *E. coli* | *K. pneumoniae* | ESBL *K. pneumoniae* | *S. aureus* | MRSA |
|---|---|---|---|---|---|---|
| Em M | 78 | 78 | 78 | 39 | 156 | 625 |
| Em W | 312 | 312 | 156 | 78 | 468 | 938 |
| Ma M | 625 | 312 | 156 | 312 | 312 | 312 |
| Ma W | 625 | 468 | 312 | 312 | 468 | 938 |
| Ampicillin | - | - | - | - | - | - |
| Chloramphenicol | - | - | 1.25 | 1.25 | 0.31 | - |
| Ciprofloxacin | 2.5 | - | 2.5 | 1.25 | 0.62 | 2.5 |
| Erythromicin | 1.25 | 1.25 | 1.25 | 1.25 | 1.25 | 1.25 |
| Gentamicin | 0.04 | 0.04 | 0.03 | 0.03 | 0.03 | 0.03 |
| Tetracycline | - | - | - | - | 1.25 | - |
| Negative control | - | - | - | - | - | - |

Em M=*E. major* methanolic extract; Em W=*E. major* aqueous extract; Ma M=*M. alternifolia* methanolic extract; Ma W=*M. alternifolia* aqueous extract; - indicates no inhibition at any concentration tested. MIC values of triplicate determinations are expressed in µg/mL.

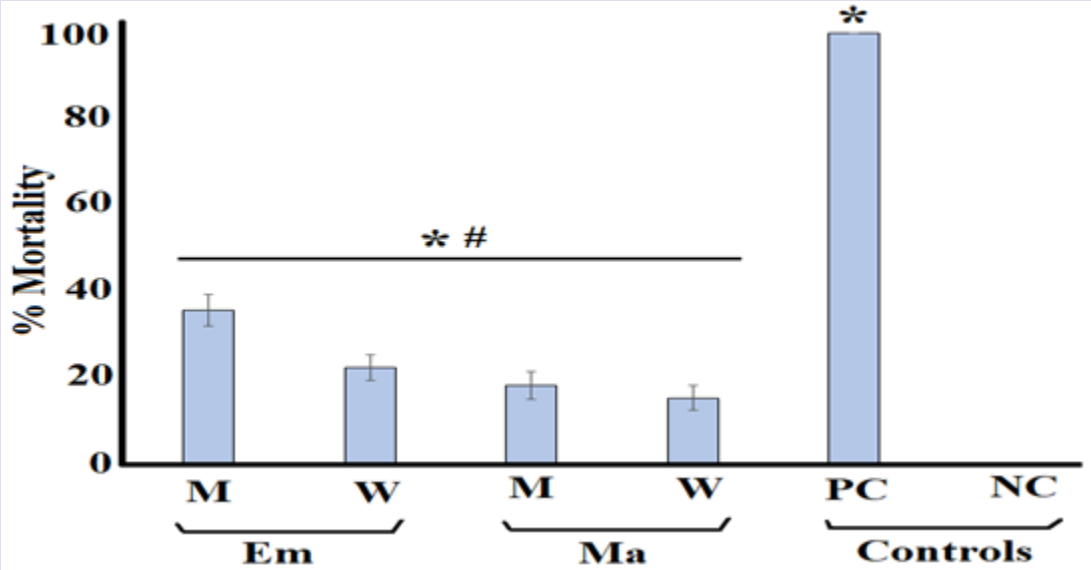


**Figure 5:** The lethality of the *E. major* and *M. alternifolia* leaf extracts (1000 µg/mL) and the potassium dichromate (1000 µg/mL) and seawater controls towards *Artemia franciscana* nauplii after 24 hr exposure. Em=*E. major*; Ma=*M. alternifolia*; M=Methanolic extract; W=aqueous extract; PC=Potassium dichromate control; NC=Negative (seawater) control. Results are expressed as mean zones of inhibition±SEM. * indicates results that are significantly different to the negative control ($p<0.05$); # indicates significantly <50% mortality ($p<0.05$).

hr $LC_{50}$ values >1000 µg/mL have previously been defined as nontoxic in this assay,[29,30] all of the extracts were deemed to be nontoxic and their $LC_{50}$ values were therefore not determined.

## DISCUSSION

This study evaluated the antibacterial properties of extracts prepared from commercially sourced *E. major* and *M. alternifolia* hydrosols against ESBL and methicillin-resistant bacterial pathogens and compared the inhibition to the activity of the extracts against β-lactam sensitive bacterial strains. The bacterial strains selected for this study were included as they were previously shown to be resistant to multiple β-lactam antibiotics.[31] Additionally, the previous study also reported that the bacteria screened in our study were also resistant to an extended panel of antibiotics of multiple classes. Indeed, the authors of that study reported that the ESBL *E. coli* and ESBL *K. pneumoniae* strains were only strongly inhibited by cefotoxin and Augmentin®. The previous study also reported that the MRSA strain also had

broad-spectrum antibiotic resistance, although it was sensitive to ciprofloxacin.

*Eucalyptus major* and *M. alternifolia* were selected for screening in our study as they are widely available commercially and have a record of ethnobotanical usage to treat bacterial infections.[33] Furthermore, the antibacterial properties of these species are well established.[13-19] However, all of those studies have evaluated the antibacterial activity of the extracts against bacterial strains that are relatively sensitive to common clinically used antibiotics, including β-lactams. It is important to establish whether these plants can also inhibit the growth of antibiotic-resistant pathogen strains as the development of multi-drug resistance in recent years has substantially reduced the therapeutic options against bacterial infections. New chemotherapies with novel antibacterial mechanisms against these antibiotic-resistant strains is urgently required. This study was undertaken to extend previous studies into the antibacterial activity of *E. major* and *M. alternifolia* preparations by evaluating their inhibitory properties against a panel of multi-antibiotic resistant bacterial strains.

Good antibacterial activity was noted for all of the *E. major* and *M. alternifolia* leaf extracts against all bacterial strains (both antibiotic-sensitive and antibiotic-resistant strains) that were tested in this study. The methanolic *E. major* leaf extract had particularly good antibacterial activity against all bacterial strains (MICs 39-625 µg/mL). Notably, this extract was more potent against the ESBL *K. pneumoniae* strain than against the antibiotic-sensitive reference strain, indicating that extract compounds may be functioning via mechanisms different to β-lactams. Alternatively (or additionally), the components in the methanolic *E. major* leaf extract may be blocking bacterial ESBL activity and potentiating the activity of other leaf components, although this remains to be verified. If future studies confirm this, the methanolic *E. major* leaf extract (and its individual components) may have substantial therapeutic applications as it may indicate that some extract components allow current β-lactam antibiotics (and perhaps other classes of antibiotics) to function with substantially greater efficacy, even against bacterial strains otherwise resistant to their effects. This would extend their useful lifespan, as well as increasing their efficacy. Additionally, as extract components may be negating bacterial resistance mechanisms, future studies should also screen the activity of these extracts in combination with conventional antibiotics. Where potentiating activity is detected, the potentiating compounds should be identified and their potentiation mechanism(s) should be evaluated.

Identification of the bioactive compounds in the *E. major* and *M. alternifolia* leaf extracts was beyond the scope of this study. However, the qualitative phytochemical evaluations included herein highlight the presence of specific phytochemical classes, which may be useful for focusing future phytochemical identification studies. Moderate to high levels of polyphenolics, flavonoids and tannins were detected in all extracts. Interestingly, a wide variety of flavonoids have been reported to inhibit bacterial growth via multiple mechanisms, including by binding to and inactivating bacterial cell wall proteins and modulating cellular redox status by inactivating intracellular oxidoreductases.[34,35] Similarly, multiple tannins have good antibacterial activity against a broad-spectrum of bacteria.[36-39] Gallotannins (including gallic acid) function by binding and and precipitating bacterial cell surface proteins, rendering them nonfunctional.[37,38] They may also bind and inhibit the activity of intracellular glucosyltransferase enzymes.[40] Ellagitannins also inhibit bacterial growth via modulating bacterial redox status and by disrupting bacterial cell walls.[38]

Notably, none of the extracts tested herein were toxic in the ALA assay. 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. Notably, the major ethnobotanical uses of both of the species screened in this study are related to topical treatment. Therefore, even if future studies using human cell lines detect toxicity, this may not greatly impact their suitability for topical usage, although it could greatly affect their suitability for oral administration.

## CONCLUSION

The findings reported herein highlight the antibacterial activity of *E. major* and *M. alternifolia* leaves against selected β-lactam resistant strains of *E. coli, K. pneumoniae* and *S. aureus*. Furthermore, all of the extracts were non-toxic in an *Artemia* nauplii toxicity assay. Further studies to evaluate their therapeutic mechanisms and to identify the antibacterial and/or potentiating components are warranted.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## ABBREVIATIONS

**DMSO:** Dimethyl sulfoxide; **$LC_{50}$:** The concentration required to achieve 50% mortality; **MIC:** Minimum inhibitory concentration; **ZOI:** Zone of inhibition.

## SUMMARY

- *Eucalyptus major* and *Melaleuca alternifolia* leaf solutions were screened for inhibitory activity against antibiotic-resistant bacteria.

• All extracts inhibited the growth of ESBL and MRSA resistant bacterial strains.

• The methanolic *E. major* leaf extract had particularly good antibacterial activity (MICs=39-625 µg/mL) against all bacterial strains.

• The *E. major* and *M. alternifolia* leaf extracts was determined to be non-toxic in the Artemia nauplii toxicity bioassay.

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