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*Original Article*

# The Effects of Storage Time on the Antibacterial Activity of *Terminalia sericea* Burch. ex DC. Extracts

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## ABSTRACT

**Introduction:** The recent development of extensively antibiotic resistant bacteria has necessitated the search for novel antibacterial therapies. An examination of aromatic plants and traditional medicines is an attractive option for drug discovery. *Terminalia sericea* Burch. ex DC. is a southern African native species, which has been reported to have antibacterial activity, although the effects of storage on the activity is yet to be determined. **Materials and Methods:** The ability of *T. sericea* leaf extracts to inhibit the growth of a panel of bacterial triggers of autoimmune diseases was quantified by disc diffusion and liquid dilution MIC assays. The potency of extracts prepared using fresh and stored leaves was compared. Toxicity was examined using the *Artemia franciscana* nauplii bioassay. **Results:** The *T. sericea* leaf methanolic and aqueous extracts displayed noteworthy growth inhibitory activity towards all bacterial pathogens tested. However, a substantial decrease in activity was seen when extracts were prepared using leaves that had been stored for two years, indicating that the leaves become less useful therapeutically over time. All extracts were non-toxic in the *Artemia* nauplii bioassay following 24hr exposure. **Conclusion:** The *T. sericea* leaf extracts had noteworthy antibacterial activity, although the potency decreased substantially following storage of the leaf material.

**Key words:** Combretaceae, Synergy, Rheumatoid arthritis, Ankylosing spondylitis, Multiple sclerosis, Antibacterial activity, Antibiotic resistant bacteria.

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## INTRODUCTION

Traditional plant-based medicines have been used for thousands of years to treat pathogenic diseases and are still widely used as a primary health modality in the majority of the world's population. Even in developed countries where allopathic medicine predominates, there has been a trend in recent years to incorporate herbal therapies as complementary and/or alternative therapies into the treatment of multiple illnesses. This is particularly evident for the treatment of bacterial diseases. The recent establishment of bacterial pathogens that are either extremely (XDR) or totally resistant (TDR) to common clinically used antibiotics[1] has resulted in the need to develop new and effective antibiotic chemotherapies. There are now limited therapeutic options for many diseases caused by bacterial pathogens and the situation is expected to worsen in the future as bacteria exchange resistance genes. Indeed, the development of alternative antibacterial treatment modalities has become crucial and is considered by the World Health Organisation (WHO) to be one of the most serious challenges facing medical science.[2] For varoius reasons, it is unlikely that the previous methods of antibiotic discovery/development will be as successful in the future and new treatment modalities are urgently required. Traditional medicines and herbal remedies have great potential for antimicrobial drug development and there has recently been a substantial increase in interest in this field.[3-17]

The genus *Terminalia* contains some of the most widely used medicinal plants worldwide. Indeed, several species are recorded in ethnobotanical literature for their analgesic,[18-19] antibacterial,[6,8,10,20-23] anticancer,[24-26] antidiarrhoeal, antifungal,[27-28] anti-inflammatory,[18-19] antioxidant,[24] antimalarial,[29] antiprotozoal[30-32] and antiviral uses.[33] Some species have also be used for wound healing and to treat cardiovascular conditions.[18] Whilst the therapeutic properties of the South Asian *Terminalia* spp. have been most extensively reported (particularly in Indian Ayuverda),[10,11,18] recent studies have also reported the therapeutic properties of African[18,21] and Australian spp.[3,8,15,16,20] Of the southern African *Terminalia* spp., *Terminalia sericea* Burch. ex DC. is one of the most useful species and is used in several traditional medicine systems to treat bacterial[18,21] and viral respiratory diseases,[21] diarrhoea,[18] fungal diseases,[28] diabetes,[34] inflammation,[19] malaria,[29] cancer[26] and other parasite infestations.[30]

The antibacterial activity of *Terminalia* spp. (including *T. sericea*) has been correlated with several phytochemical classes, including but not limited to tannins, flavonoids[3,8,16,31-32] and terpenpoids.[15] Notably, the phytochemical profiles of plant extracts can vary substantially between individual samplings and may be influenced by numerous factors including geolocation of the harvested plant, the genotype of the sampled plants, harvest season and time, the cultivation techniques (or if it is wild harvest), the climatic conditions that the plant is exposed to in the period pre-harvest and the post-harvest storage and processing.[35] Additionally, plant materials are often stored for prolonged periods at traditional markets (such as the muthi markets in which *T. sericea* is sold in South Africa) before sale, and this may have profound effects on the efficacy of the traditional medicines. This can result in highly variable results reported between different studies. In a recent study, we reported on the growth inhibitory properties of *T. sericea* leaf extracts against some bacterial triggers of selected autoimmune anti-inflammatory diseases.[7] In this study, we have used the same plant material that has been stored at 4°C for 2 years to determine if the antibacterial properties of the leaves decreases substantially over time.

## MATERIALS AND METHODS

### Collection of Plant Material and Extraction

*Terminalia sericea* Burch. ex. DC. leaves were sourced and identified by Andrew Hankey, chief botanist at the Walter Sisulu Botanical Gardens in Johannesburg, South Africa (gps coordinates 26° 05' 13.2"S, 27° 50' 24" E). Voucher specimens are deposited in the School of Natural Sciences, Griffith University, Australia (voucher number GUTS-SA-2017). The *T. sericea* leaves were air-dried until a consistent mass was recorded and

the dried leaves were ground into a coarse powder. For initial testing, individual 1g quantities of the ground plant material were weighed into separate tubes and 50 mL of methanol (Ajax Fine Chemicals, Australia, AR grade) or sterile deionised water were added. The ground plant materials were extracted in each solvent for 24 hr at 4°C with gentle shaking in an orbital shaker (30 rpm). The extracts were subsequently filtered through Whatman No. 54 filter paper under vacuum. The solvent extracts were air dried at room temperature in the shade. The aqueous extracts were lyophilised by freeze drying at -50°C. The resultant dried extracts were weighed and dissolved in 10mL deionised water containing 1 % DMSO (Ajax Fine Chemicals, Australia). To determine the effects of storage time on the antibacterial activity of the dried leaves, they were stored in a brown paper bag at 4°C for 2 years. Following the storage period, the same extraction method was repeated as outlined above to yield the "stored" extracts.

## Qualitative Phytochemical Studies

Phytochemical analyses of the *T. sericea* leaf extracts for the presence of saponins, phenolic compounds, flavonoids, phytosteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids were conducted by standard assays.[4-5]

## Antibacterial Analysis

### *Conventional antibiotics*

The standard antibiotics chloramphenicol (≥98 % purity by HPLC), ciprofloxacin (≥98 % purity), erythromycin (potency ≥850 µg/mg), gentamicin (potency of 600 µg/mg), penicillin-G (potency of 1440-1680 µg/mg) and tetracycline (≥95% purity by HPLC) used in this study were obtained from Sigma-Aldrich, Australia and used as antibiotic controls in the microplate liquid dilution assay. All antibiotics were prepared in sterile deionised water at stock concentrations of 0.01 mg/mL and stored at 4°C until use. Ampicillin (10 µg) and chloramphenicol (10 µg) standard discs were obtained from Oxoid Ltd., Australia and were used as positive controls for the disc diffusion screening studies.

## Test Micro-organisms

All media was purchased from Oxoid Ltd., Australia. The reference strains of *Acinetobacter baylyi* (ATCC 33304), *Klebsiella pneumoniae* (ATCC31488), *Proteus mirabilis* (ATCC21721), *Proteus vulgaris* (ATCC 20719), and *Pseudomonas aeruginosa* (ATCC 39324) were purchased from American Tissue Culture Collection (ATCC), USA. All bacteria were cultured individually in nutrient broth (Oxoid Ltd., Australia) until the cell count reached approximately $10^8$ cells/mL. All bacterial species were cultured at 37°C for 24 hr and were passaged and maintained in nutrient broth at 4°C until use. Streak nutrient agar (Oxoid Ltd., Australia) plates were tested in parallel to ensure the purity of all bacterial cultures.

## Evaluation of Antimicrobial Activity

Bacterial susceptibility to the *T. sericea* extracts was initially assessed using a modified disc diffusion assay.[3] Standard discs containing ampicillin (10 µg) and chloramphenicol (10 µg) were included on each agar plate as antibiotic positive controls, whilst filter paper discs infused with 10 µL of distilled water (containing 1% DMSO) were included as a negative control. A volume of 100 µL of individual bacterial cultures (in log growth phase) were spread onto the plates and the infused filter paper discs were places at regular intervals on the agar surface. Following an overnight incubation at 37°C, the zones of inhibition (ZOI) were measured to the nearest whole mm. All tests were performed three times, each with three technical replicates ($n$=9). One-way ANOVA analysis was used to calculate statistical significance between the control and treatment groups.

## Quantification of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration of each extract was quantified using both liquid and solid phase assays to approximate of the antibacterial activity of the extracts in different physiological conditions. A liquid dilution MIC assay was employed as it is generally considered the most sensitive bacterial growth inhibitory assay.[20] The microplate liquid dilution MIC assay is also one of the most commonly used method of quantifying bacterial growth inhibition efficacy, allowing for comparisons across studies.

## Microplate Liquid Dilution MIC Assay

Bacterial growth inhibitory activity of the extracts and conventional antibiotics evaluated by standard methods.[13,20] Briefly, bacterial cultures in log phase growth (determined by optical density) were diluted in the nutrient broth to produce 0.5 McFarland inoculation cultures. Sterilized nutrient broth (100 µL) was dispensed into all wells of a 96 well micro-titre plate and 100 µL of the plant extracts or conventional antibiotics were individually dispensed into wells in the top row of the plate. Each plate also included a negative control (broth), sterile control (broth without bacteria) and a sample-free culture control (to ensure the media was capable of supporting microbial growth). All tests and controls were serially diluted down each column on the plate. A 100 µL volume of the culture inoculum (~$1x10^6$ colony forming units/mL) was then added to all wells of the plate (except the sterile control) and incubated overnight at 37°C. The following day, 40 µL of *p*-iodonitrotetrazolium violet (INT; Sigma-Aldrich) was dispensed into all wells and the plates were incubated for a further 6 hr at 37°C. The MIC was visually determined as the lowest dose at which colour development was inhibited. The reliability of MIC values was ensured by repeating the 96-well microtitre plate liquid dilution assays twice on separate days, with two replicates per assay ($n$=4), to confirm that the results were reproducible for all extracts and antibiotics tested.

## Disc Diffusion MIC Assay

The MIC values of the *T. sericea* leaf extracts were also quantified by disc diffusion assays across a range of extract dilutions. Graphs of the zone of inhibition versus Ln of the concentration of extract applied to the discs were plotted. Linear regression was used to determine the MIC values.

## *Artemia franciscana* Nauplii Toxicity Screening

Toxicity was tested using an adapted *Artemia franciscana* nauplii lethality assay (ALA).[36-37] Briefly, *A. franciscana* nauplii were incubated in the presence of the extracts, reference toxin (1mg/mL potassium dichromate) or artificial seawater (negative control) at 25±1°C under artificial light. All treatments were performed three times in triplicate ($n$=9). The number of dead were counted in each well at 24hr. At the completion of the 24hr exposure period, the remaining live nauplii were sacrificed and the total number of nauplii in each well were counted and used to calculate the % mortality per well. $LC_{50}$ values were calculated for each treatment using probit analysis.

## Statistical analysis

Data are expressed as the mean ± SEM of three independent experiments with internal triplicates ($n$=9). One-way ANOVA was used to calculate statistical significance between control and treated groups, with a $p$ value <0.01 considered to be statistically significant.

# RESULTS

## Liquid Extraction Yields and Qualitative Phytochemical Screening

Extractions of the dry powdered *T. sericea* leaves (1g) with solvents of varying polarity yielded dried plant extracts ranging from 124 mg (aged *T. sericea* leaf aqueous extract) to 180 mg (fresh *T. sericea* leaf methanolic extract) (Table 1). Qualitative phytochemical screening (Table 1) showed that the fresh leaves and the leaves that had been stored for 2 years contained similar levels and diversities of each phytochemical class.

## Antimicrobial Growth Inhibition Screening

*Proteus mirabilis* growth was susceptible to all of the *T. sericea* leaf extracts produced from the fresh and stored plant material (Figure 1). However, large differences were seen between the extracts produced against the fresh and stored leaves. Both the methanolic and aqueous extracts produced from fresh leaves (13.5 mm and 12.3 mm respectively) produced significantly larger zones of inhibition (ZOIs) compared to the extracts produced using stored leaves (7.6 mm and 6.8 mm respectively). The ampicillin (13.3 mm) and chloramphenicol controls (17.7 mm) were also strong inhibitors of *P. mirabilis* growth, whilst no inhibition was noted for the negative control, indicating that the assay was function correctly.

Similar susceptibilities were recorded when *P. vulgaris* was exposed to the *T. sericea* leaf extracts. Similar to the trend noted for *P. mirabilis*, the methanolic and aqueous extracts produced from freshly processed leaves were particularly effective inhibitors of the growth of this bacterium, with ZOI values of 14.7mm and 17.3 mm respectively. This is particularly noteworthy and is comparable to the inhibition by chloramphenicol (16.8 mm) and substantially better than the inhibition by ampicillin (12.2mm). Whilst the extracts made from stored plant material also inhibited *P. vulgaris* growth, substantially smaller ZOIs were recorded (8 mm and 7.3 mm respectively).

The methanolic and aqueous *T. sericea* extracts produced from the freshly processed leaves also inhibited the growth of *K. pneumoniae* (Figure 2). Furthermore, the ZOIs (13.0±0.4 mm and 11.8±0.4 mm for the methanolic and aqueous extracts respectively) compared favourably to the inhibition by the antibiotic controls. Indeed, this bacterial strain was completely resistant to ampicillin. Notably, previous studies have also reported that this *K. pneumoniae* strain is resistant to multiple β-lactam antibiotics.[17] In contrast, chloramphenicol was a good *K. pneumoniae* growth inhibitor, with ZOIs of 12.6mm. As noted for the *Proteus* spp., the extracts prepared using the stored leaves were substantially weaker *K. pneumoniae* growth inhibitors (7.8 mm and 7.2 mm respectively).

The *T. sericea* aqueous and methanolic extracts prepared from freshly processed leaves also inhibited *A. baylyi* growth, albeit with substantially smaller ZOIs (Figure 3). The methanolic extract was the most potent *A. baylyi* growth inhibitor (9.7mm). Thus, the methanolic extract has potential in the prevention and treatment of multiple sclerosis by blocking this trigger of the disease's etiology. The aqueous extract had substantially lower efficacy (7.2mm), indicative of moderate to low inhibitory activity. In contrast, the extracts prepared using stored leaf material were completely devoid of *A. baylyi* growth inhibitory activity. Ampicillin and chloramphenicol controls were potent *A. baylyi* growth inhibitors, with ZOIs of 12 0.4 mm and 17.4 mm respectively, indicating that the assay was functioning correctly.

Interestingly, *P. aeruginosa* was resistant to most of the treatments tested (Figure 3). Indeed, only the methanolic *T. sericea* extract produced using freshly processed leaves inhibited *P. aeruginosa* growth, with a ZOI indicative of low potency (7.2mm). The freshly prepared aqueous extract, and both extracts produced using the leaves that had been stored for 2 years, were completely devoid of *P. aeruginosa* inhibitory activity. However, it is noteworthy that the *P. aeruginosa* strain tested in this study was a particularly resistant strain, with only small ZOIs recorded against ampicillin (7.5mm). Previous studies have also reported resistance of this bacterial strain towards multiple conventional antibiotics.[17] In contrast,

**Table 1: The mass of dried extracted material, the concentration after resuspension in deionised water, qualitative phytochemical screenings and antioxidant capacities of the fresh and aged *T. sericea* extracts.**

| | Mass of Dried Extract (mg) | Resuspended Extract (mg/mL) | Phenols | | | Cardiac Glycosides | Saponins | | Triterpenes | Phytosteroids | Alkaloids | | | Flavanoids | | | Tannins | | Anthraquinones | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Total Phenolics | Water Soluble | Water Insoluble | Keller-Kiliani Test | Froth Persistence | Emulsion test | Salkowski Test | Acetic Anhydride Test | Meyers Test | Wagners Test | Draggendoffs Test | Shinoda Test | Kumar test | Ferric Chloride Test | Lead Acetate Test | Free | Combined |
| M (Fresh) | 180 | 18 | +++ | ++ | ++ | - | + | + | ++ | - | - | - | - | +++ | +++ | ++ | ++ | - | - |
| M (Aged) | 137 | 13.7 | +++ | ++ | ++ | - | + | + | ++ | - | - | - | - | +++ | +++ | ++ | ++ | - | - |
| W (Fresh) | 155 | 15.5 | +++ | ++ | +++ | - | + | + | ++ | - | - | - | - | +++ | +++ | ++ | ++ | - | - |
| W (Aged) | 124 | 12.4 | +++ | ++ | +++ | - | + | + | ++ | - | - | - | - | +++ | +++ | ++ | ++ | - | - |

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay; M = methanolic *T. sericea* extract; W = aqueous *T. sericea* extract. Fresh referes to extracts that were prepared from freshly obtained. Aged refers to extracts prepared from leaves stored for 2 years.

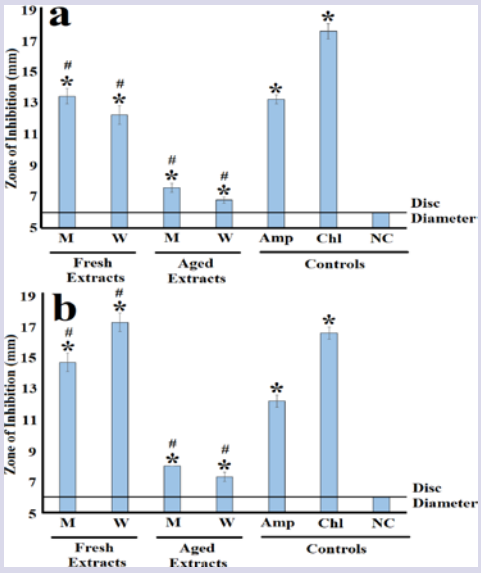


**Figure 1:** Growth inhibitory activity of *T. sericea* leaf extracts and reference antibiotics against (a) *P. mirabilis* and (b) *P. vulgaris* measured as ZOIs (mm) ± SEM. M = methanolic extract; W = aqueous extract; Amp = ampicillin (10µg); Chl = chloramphenicol (10µg); NC = negative control. All assays were completed three times, each with internal triplicates (*n*=9) and the results are expressed as mean zones of inhibition (mm) ± SEM. * = results significantly different to the negative control ($p<0.05$); # = significant differences between the equivalent extracts at different storage times ($p<0.05$).

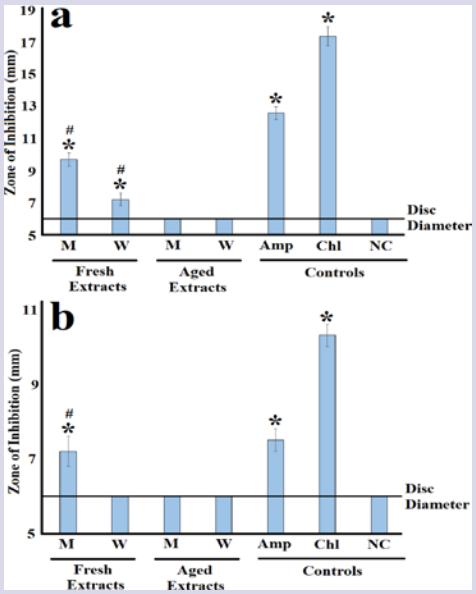


**Figure 3:** Growth inhibitory activity of *T. sericea* leaf extracts and reference antibiotics against (a) *A. baylyi* and (b) *P. aeruginosa* measured as ZOIs (mm) ± SEM. M = methanolic extract; W = aqueous extract; Amp = ampicillin (10µg); Chl = chloramphenicol (10µg); NC = negative control. All assays were completed three times, each with internal triplicates (*n*=9) and the results are expressed as mean zones of inhibition (mm) ± SEM. * = results significantly different to the negative control ($p<0.05$); # = significant differences between the equivalent extracts at different storage times ($p<0.05$).

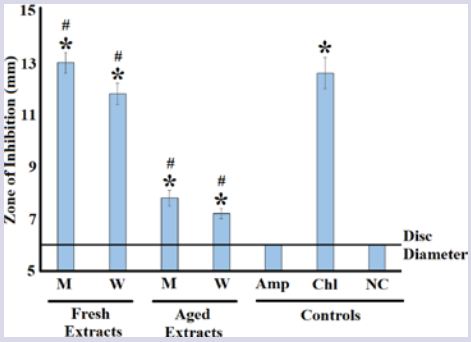


**Figure 2:** Growth inhibitory activity of *T. sericea* leaf extracts and reference antibiotics against *K. pneumoniae* measured as ZOIs (mm) ± SEM. M = methanolic extract; W = aqueous extract; Amp = ampicillin (10µg); Chl = chloramphenicol (10µg); NC = negative control. All assays were completed three times, each with internal triplicates (*n*=9) and the results are expressed as mean zones of inhibition (mm) ± SEM. * = results significantly different to the negative control ($p<0.05$); # = significant differences between the equivalent extracts at different storage times ($p<0.05$).

this *P. aeruginosa* strain was susceptible to chloramphenicol (10.3mm), confirming that the assay was functioning properly.

## Quantification of Minimum Inhibitory Concentration (MIC)

The relative antimicrobial potency was further evaluated by determining the MIC values using two methods: the liquid dilution and disc diffusion MIC assays (Table 2). The MIC values determined for the *T. sericea* leaf extracts compare relatively well between the disc diffusion and liquid dilution assays. Consistent with the antibacterial screening assays, the freshly prepared extracts had substantially lower MIC values, indicating that the *T. sericea* leaves lost a significant amount of antibacterial activity following two years storage. Indeed, the MIC values of the methanolic extracts against *P. mirabilis, P. vulgaris, K. pneumonia, A. baylyi* and *P. aeruginosa* increased from 350 to 2820 µg/mL, 325 to >5000 µg/mL, 750 to >5000 µg/mL, 580 to 2820 µg/mL, and 1650 to 3820 µg/mL respectively. Similar trends (although more pronounced) were evident for the aqueous extracts, with large increases in MIC recorded in the extracts produced from the stored leaves.

In this study, MIC values were only determined for conventional antibiotic controls in the liquid dilution assay, as commercial susceptibility discs (loaded with a single set amount of antibiotic) were used for the disc diffusion assay. Thus, the zones of only single doses was recorded for the disc diffusion MIC assay. In the liquid dilution assay, MIC values >1 µg/mL indicate antibiotic resistance. Notably, all of the bacterial strains tested were multi-antibiotic resistant strains. Indeed, the *P. mirabilis* and *A. baylyi* strains tested in our study were completely resistant to penicillin, erythromycin and tetracycline. Whilst these strains were inhibited by the other antibiotics, MIC values >1 µg/mL against chloramphenicol, ciprofloxacin (for both bacteria) and gentamicin (for *A. baylyi*) indicate that these strains were also resistant to those antibiotics. Similarly (with the exception of gentamicin against *K. pneumonmiae*), all of the bacterial pathogens screened in this study were resistant to all of the antibiotics tested.

## Quantification of Toxicity

The *T. sericea* leaf extracts prepared using freshly processed and stored leaves were individually screened at 1 mg/mL in the *Artemia* assay to evaluate their toxicity. The extracts were only considered toxic if they

**Table 2: Disc diffusion (DD) and liquid dilution (LD) MIC values for *T. sericea* leaf extracts against the bacterial triggers of some autoimmune inflammatory diseases (µg/mL).**

| Extract | *P. mirabilis* (ATCC: 33304) | | *P. vulgaris* (ATCC: 20719) | | *K. pneumoniae* (ATCC: 31488) | | *A. baylyi* (ATCC: 21721) | | *P. aeruginosa* (ATCC: 39324) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | MM MIC | LD MIC | MM MIC | LD MIC | MM MIC | LD MIC | MM MIC | LD MIC | MM MIC | LD MIC |
| M (Fresh) | 465 | 350 | 387 | 325 | 864 | 750 | 945 | 580 | 2388 | 1650 |
| M (Aged) | >5000 | 2820 | >5000 | >5000 | >5000 | >5000 | - | 2820 | - | 3820 |
| W (Fresh) | 214 | 265 | 265 | 180 | 420 | 500 | 1438 | 954 | - | - |
| W (Aged) | >5000 | 1482 | >5000 | >5000 | >5000 | 1741 | - | 2965 | - | - |
| **Controls** | | | | | | | | | | |
| Penicillin | ND | - | ND | - | ND | - | ND | - | ND | 2.5 |
| Chloramphenicol | ND | 1.25 | ND | 1.25 | ND | 1.9 | ND | 2.5 | ND | 1.25 |
| Ciproflaxacin | ND | 1.25 | ND | 1.25 | ND | 1.9 | ND | 1.25 | ND | 1.25 |
| Gentamicin | ND | 0.63 | ND | 1.25 | ND | 0.3 | ND | 1.25 | ND | 1.25 |
| Erythromicin | ND | - | ND | - | ND | 1.9 | ND | - | ND | - |
| Tetracycline | ND | - | ND | 1.25 | ND | 1.9 | ND | - | ND | 1.25 |
| Negative control | ND | - | ND | - | ND | - | ND | - | ND | - |

DD = disc diffusion; LD = liquid dilution, - = no inhibition. Numbers indicate the mean DD MIC and LD MIC values expressed in µg/mL. ND = MIC could not be determined in the disc diffusion study as a single fixed dose was tested. Bacteria are classified as resistant to an antibiotic if an MIC ≥ 1 µg/mL was recorded.

induced percentage mortalities greater than 50% ($LD_{50}$) following 24 hr of exposure to the *Artemia* nauplii (as defined by).[40] Notably, none of the extracts induced mortality greater than 50% at 1 mg/mL (results not shown) and therefore all extracts were deemed non-toxic. In contrast, the positive control (potassium dichromate) induced 100% mortality in the ALA, indicating that the assay was functioning correctly. Whilst toxicity was assessed in this study with the test organism *A. franciscana*, toxicity towards *A. franciscana* has previously been shown to correlate well with toxicity towards human cells for many toxins.[36,37] However, further studies are required to determine whether this is also true for the *T. sericea* leaf extracts examined in these studies.

## DISCUSSION

Traditional plant derived medicines have been used in most parts of the world for a variety of therapeutic purposes, including fighting microbial disease. Indeed, the ability of plant extracts to block the growth of pathogenic bacteria has become a focus of substantial recent study.[39-42] Much of the research into traditional medicinal plant use has focused on Asian,[10,11,18] African,[18,21] Middle Eastern[43-44] and South American[45] plants. However, despite the potential of plants to provide us with useful pharmaceutical agents, the field is still relatively poorly studied. Only an estimated 5-10 % of the approximately 300,000-500,000 plant species worldwide have been screened for one or more bioactivities.[46]

The development of new antibiotic therapies is particularly urgent. The recent establishment of bacterial pathogens that are either extremely (XDR) or totally resistant (TDR) to common clinically used antibiotics[1] has resulted in the need to develop new and effective antibiotic chemotherapies. There are now limited therapeutic options for many diseases caused by bacterial pathogens and the situation is expected to worsen in the future as bacteria exchange resistance genes. Indeed, the development of alternative antibacterial treatment modalities has become crucial and is considered by the World Health Organisation (WHO) to be one of the most serious challenges facing medical science.[2] For reasons reviewed elsewhere,[1] it is unlikely that the previous methods of antibiotic discovery/development will be as successful in the future and new treatment modalities are urgently required. Traditional medicines and herbal remedies have great potential for antimicrobial drug development and there has recently been a substantial increase in interest in this field.[18-28]

Notably, the *T. sericea* leaf extract prepared using freshly dried leaves displayed potent growth inhibitory activity against the bacterial triggers of rheumatoid arthritis (*Proteus* spp.), ankylosing spondylitis (*K. pneumoniae*) and multiple sclerosis (*A. baylyi* and *P. aeruginosa*). This indicates that these extracts would be useful in preventing and treating these autoimmune inflammatory diseases. In contrast, the extracts prepared using leaves that had been stored two years were substantially less potent bacterial growth inhibitors, indicating that the efficacy of *T. sericea* leaf extracts is dependent on the amount of time that the leaves have been stored. Interestingly, a similar trend has been reported by other researchers, including against *T. sericea* leaf extracts, against other bacterial species. Indeed, one study reported that the inhibitory potency of *T. sericea* leaves against *Escherichia coli* and *P. aeruginosa* decreased by 60% and 87% respectively following six weeks storage at 4°C (based of increased MIC values).[38] In contrast, the growth inhibitory activity against *Staphylococcus aureus* and *Entercoccus faecalis* were not significantly affected by six weeks of storage in that study.

The loss of growth inhibitory activity in our study (as well as the earlier study) may be due to the loss of inhibitory compounds by either physical or chemical mechanisms. Volatile components may be lost through evaporation, whilst larger compounds may conjugate and precipitate out of solution. Alternatively, the phytochemicals may be modified into an inactive form, thereby resulting in decreased activity. It is noteworthy that *Terminalia* spp. are characterised by their levels of reduced compounds with high antioxidant capacities.[18] It is likely that these compounds will oxidise over time. If these compounds contribute to the antibacterial activity of *T. sericea* leaves, this may account for the large decreases in activity noted in both studies. Interestingly, the effects of storage were even more pronounced in our study than in earlier studies,[38] which may be due to several factors:

(1) The *T. sericea* material tested in our study was stored for 2 years, whereas the *T. sericea* extracts were stored for six weeks in the earlier study before retesting. The longer storage period allows greater time for loss of bioactive components, or for their inactivation by chemical conversion.

(2) The *T. sericea* leaves were prepared and stored as extracts in the previous study, whereas our study stored the whole dried leaves and prepared fresh extracts before testing. This may have profound effects on the chemical composition as tannins, which are a major phytochemical class in *T. sericea* leaves.[18] Tannins can precipitate overtime in aqueous solutions. As tannins have noteworthy antibacterial activity,[6,8,18] this may significantly reduce the potency of the stored extracts compared to the freshly prepared extracts. Additionally, the physical state of the stored material may affect evaporation of volatile antibacterial components (e.g. monoterpenoids), resulting in different rates of loss.

(3) The *T. sericea* leaves were stored at room temperature (~23°C) in our study, whereas the *T. sericea* extracts tested in the earlier study were stored at 4°C. The storage temperature may have profound effects on the chemical composition of the tested material. Levels of volatile components (including antibacterial monoterpenoids) would decrease substantially faster at room temperature than at 4°C, thereby causing a more profound increase in MIC over time. Additionally, the higher temperature may accelerate phytochemical transformations, thereby also decreasing the concentrations of bioactive components over time.

Whatever the mechanism of inactivation, it is evident that storage time and conditions significantly influences the antibacterial activity of *T. sericea* leaves. It is therefore important that plant material be used within a relatively short time after collection. This may not always be possible, particularly when the plant material is obtained from commercial sources (e.g. a traditional muthi market) as it is difficult to ascertain the length of time that the plant material has been in stock. Ideally, bioactivity and/or phytochemical studies should use plant material collected by the researcher and the origin and collection time and conditions should be noted to allow the reader to take these effects into account. Where this is not possible and commercial material is used, any available information regarding the provenance of the material, as well as the storage conditions, should be reported.

## CONCLUSION

Methanolic and aqueous *T. sericea* leaf extracts had noteworthy growth inhibitory activity against some bacterial triggers of rheumatoid arthritis, ankylosing spondylitis and multiple sclerosis and may therefore be useful in preventing and treating these diseases in genetically susceptible people. However, the leaf material loses substantial activity over time, thereby decreasing the usefulness of stored *T. sericea* leaves (or extracts).

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## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

## ABBREVIATIONS

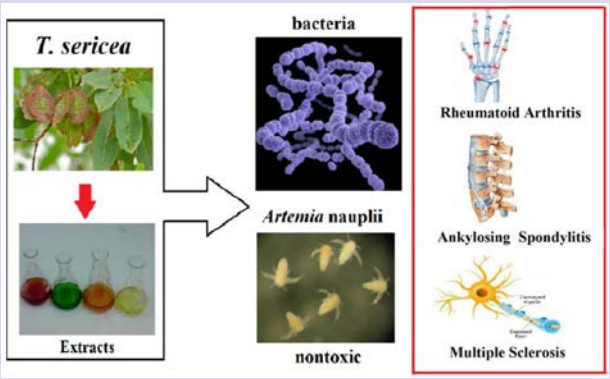
**ALA:** *Artemia* lethality assay; **DMSO:** dimethyl sulfoxide; **$LC_{50}$:** the concentration required to achieve 50 % mortality; **MIC:** minimum inhibitory concentration; **ZOI:** zone of inhibition.

## REFERENCES

1. Cheesman MJ, Ilanko A, Blonk B, *et al.* Developing new antimicrobial therapies: Are synergistic combinations of plant extracts/compounds with conventional antibiotics the solution? Pharmacogn Rev 2017;11(22):57-72. doi: 10.4103/phrev.phrev_21_17, PMID 28989242.
2. WHO. Antimicrobial resistance. World Health Organization; 2016 [cited May 10 2019]. Available from: http://www.who.int/mediacentre/factsheets/fs194/en/.
3. Sirdaarta J, Matthews B, Cock IE. Kakadu plum fruit extracts inhibit growth of the bacterial triggers of rheumatoid arthritis: Identification of stilbene and tannin components. J Funct Foods 2015;17:610-20. doi: 10.1016/j.jff.2015.06.019.
4. Ilanko A, Cock IE. The interactive antimicrobial activity of conventional antibiotics and *Petalostigma* spp. extracts against bacterial triggers of some autoimmune inflammatory diseases. Pharmacogn J 2019;11(2):292-309. doi: 10.5530/pj.2019.11.45.
5. Winnett V, Sirdaarta J, White A, *et al.* Inhibition of *Klebsiella pneumoniae* growth by selected Australian plants: Natural approaches for the prevention and management of ankylosing spondylitis. Inflammopharmacol. 2017;25(2):223-35. doi: 10.1007/s10787-017-0328-1, PMID 28239782.
6. Cheesman MJ, White A, Matthews B, *et al. Terminalia ferdinandiana* fruit and leaf extracts inhibit methicillin-resistant *Staphylococcus aureus* growth. Planta Med 2019;85(16):1253-62. doi: 10.1055/a-1013-0434, PMID 31597166.
7. Nel AL, Murhekar S, Matthews B, *et al.* The interactive antimicrobial activity of *Terminalia sericea* Burch. ex DC. leaf extracts and conventional antibiotics against bacterial triggers of selected autoimmune inflammatory diseases. S Afr J Bot 2020;133:17-29.
8. Courtney R, Sirdaarta J, Matthews B, *et al.* Tannin components and inhibitory activity of *Kakadu plum* leaf extracts against microbial triggers of autoimmune inflammatory diseases. Pharmacogn J 2015;7(1):18-31. doi: 10.5530/pj.2015.7.2.
9. Wright MH, Sirdaarta J, Matthews B, *et al.* Growth inhibitory activity of Kakadu plum extracts against the opportunistic pathogen *Clostridium perfringens*: new leads in the prevention and treatment of clostridial myonecrosis. Pharmacogn J 2016;8(2):144-53. doi: 10.5530/pj.2016.2.8.
10. Tiwana G, Cock IE, White A, *et al.* Use of specific combinations of the triphala plant component extracts to potentiate the inhibition of gastrointestinal bacterial growth. J Ethnopharmacol 2020;260:112937. doi: 10.1016/j.jep.2020.112937, PMID 32464314.
11. Mandeville A, Cock IE. *Terminalia chebula* Retz. fruit extracts inhibit bacterial triggers of some autoimmune diseases and potentiate the activity of tetracycline. Indian J Microbiol 2018;58(4):496-506. doi: 10.1007/s12088-018-0754-9, PMID 30262960.
12. Arkhipov A, Sirdaarta J, Rayan P, *et al.* An examination of the antibacterial, antifungal, anti-Giardial and anticancer properties of *Kigelia africana* fruit extracts. Pharmacogn Commun 2014;4(3):62-76. doi: 10.5530/pc.2014.3.7.
13. Ilanko P, McDonnell PA, Van Vuuren SF, *et al.* Interactive antibacterial profile of *Moringa oleifera* Lam. extracts and conventional antibiotics against bacterial triggers of some autoimmune inflammatory diseases. S Afr J Bot 2019;124:420-35. doi: 10.1016/j.sajb.2019.04.008.
14. Lee CJ, Wright MH, Arnold MSJ, *et al.* Inhibition of *Streptococcus pyogenes* growth by native Australian plants: New approaches towards the management of impetigo, pharyngitis and rheumatic heart disease. Pharmacogn Commun 2016;6(3):164-73. doi: 10.5530/pc.2016.3.6.
15. Wright MH, Sirdaarta J, White A, *et al.* GC-MS headspace analysis of *Terminalia ferdinandiana* fruit and leaf extracts which inhibit *Bacillus anthracis* growth. Pharmacogn J 2016;9(1):73-82. doi: 10.5530/pj.2017.1.14.
16. McManus K, Wood A, Wright MH, *et al. Terminalia ferdinandiana* Exell. extracts inhibit the growth of body odour-forming bacteria. Int J Cosmet Sci 2017;39(5):500-10. doi: 10.1111/ics.12403, PMID 28488331.
17. Hutchings A, Cock IE. An interactive antimicrobial activity of *Embelica officinalis* Gaertn. fruit extracts and conventional antibiotics against some bacterial triggers of autoimmune inflammatory diseases. Pharmacogn J 2018;10(4):654-62. doi: 10.5530/pj.2018.4.108.
18. Cock IE. The medicinal properties and phytochemistry of plants of the genus *Terminalia* (Combretaceae). Inflammopharmacol 2015;23(5):203-29. doi: 10.1007/s10787-015-0246-z, PMID 26226895.
19. Khumalo GP, Van Wyk BE, Feng Y, *et al.* A review of the traditional use of southern African medicinal plants for the treatment of inflammation and inflammatory pain. J Ethnopharmacol 2022;283. doi: 10.1016/j.jep.2021.114436.
20. Hübsch Z, Van Zyl RL, Cock IE, *et al.* Interactive antimicrobial and toxicity profiles of conventional antimicrobials with southern African medicinal plants. S Afr J Bot 2014;93:185-97. doi: 10.1016/j.sajb.2014.04.005.
21. Cock IE, Van Vuuren SF. The traditional use of southern African medicinal plants for the treatment of bacterial respiratory diseases: A review of the ethnobotany and scientific evaluations. J Ethnopharmacol 2020;263:Article number 113204. doi: 10.1016/j.jep.2020.113204, PMID 32730881.
22. Cock IE, Van Vuuren SF. South African food and medicinal plant extracts as potential antimicrobial food agents. J Food Sci Technol 2015;52(11):6879-99. doi: 10.1007/s13197-015-1806-3.
23. Wright MH, Shalom J, Matthews B, *et al. Terminalia ferdinandiana* Exell: Extracts inhibit *Shewanella* spp. growth and prevent fish spoilage. Food Microbiol 2019;78:114-22. doi: 10.1016/j.fm.2018.10.006, PMID 30497593.
24. Shalom J, Cock IE. *Terminalia ferdinandiana* Exell. Fruit and leaf extracts inhibit proliferation and induce apoptosis in selected human cancer cell lines. Nutr Cancer 2018;70(4):579-93. doi: 10.1080/01635581.2018.1460680, PMID 29641917.

25. Courtney R, Sirdaarta J, White A, *et al*. Inhibition of Caco-2 and HeLa proliferation by *Terminalia carpentariae* C. T. White and *Terminalia grandiflora* Benth. extracts: identification of triterpenoid components. Pharmacogn J 2017;9(4):441-51. doi: 10.5530/pj.2017.4.74.
26. Gu B, Shalom J, E. Cock IE. Anti-proliferative properties of *Terminalia sericea* Burch. ex Dc leaf extracts against $Caco_2$ and HeLa cancer cell lines. Pharmacogn J 2018;10(3):408-15. doi: 10.5530/pj.2018.3.67 ex DC.
27. Noé W, Murhekar S, White A, *et al*. Inhibition of the growth of human dermatophytic pathogens by selected Australian and Asian plants traditionally used to treat fungal infections. J Mycol Méd 2019;29(4):331-44. doi: 10.1016/j.mycmed.2019.05.003, PMID 31248775.
28. Cock IE, Van Vuuren SF. A review of the traditional use of southern African medicinal plants for the treatment of fungal skin infections. J Ethnopharmacol 2020;251:112539. doi: 10.1016/j.jep.2019.112539. PMID 31899200.
29. Cock IE, Selesho MI, Van Vuuren SF. A review of the traditional use of southern African medicinal plants for the treatment of malaria. J Ethnopharmacol 2019;245:Article number 112176. doi: 10.1016/j.jep.2019.112176, PMID 31446074.
30. Cock IE, Selesho MI, Van Vuuren SF. A review of the traditional use of southern African medicinal plants for the treatment of selected parasite infections affecting humans. J Ethnopharmacol 2018;220:250-64. doi: 10.1016/j.jep.2018.04.001, PMID 29621583.
31. Rayan P, Matthews B, McDonnell PA, *et al*. *Terminalia ferdinandiana* extracts as inhibitors of *Giardia duodenalis* proliferation: a new treatment for giardiasis. Parasitol Res 2015;114(7):2611-20. doi: 10.1007/s00436-015-4465-4, PMID 25876047.
32. Cock IE, Rayan P. Ascorbic acid potentiates the *Giardia duodenalis* growth inhibitory activity of pure *Terminalia ferdinandiana* Exell compounds. Parasitol Res 2020;119(3):1125-37. doi: 10.1007/s00436-019-06579-1, PMID 31907666.
33. Cock IE, Van Vuuren SF. The traditional use of southern African medicinal plants in the treatment of viral respiratory diseases: A review of the ethnobotany and scientific evaluations. J Ethnopharmacol 2020;262:Article number 113194. doi: 10.1016/j.jep.2020.113194, PMID 32730880.
34. Cock IE, Ndlovu N, Van Vuuren SF. The use of South African botanical species for the control of blood sugar. J Ethnopharmacol 2021;264:Article number 113234. doi: 10.1016/j.jep.2020.113234, PMID 32768640.
35. Gao CY, Lu YH, Tian CR, *et al*. Main nutrients, phenolics, antioxidant activity, DNA damage protective effect and microstructure of *Sphallerocarpus gracilis* root at different harvest time. Food Chem 2011;127(2):615-22. doi: 10.1016/j.foodchem.2011.01.053, PMID 23140708.
36. Cock IE, Ruebhart DR. Comparison of the brine shrimp nauplii bioassay and the ToxScreen-II test for the detection of toxicity associated with *Aloe vera* (*Aloe barbadensis* Miller) leaf extract. Pharmacogn Res 2009;1(2):98-101.
37. Ruebhart DR, Wickramasinghe W, Cock IE. Protective efficacy of the antioxidants Vitamin E and trolox against *Microcystis aeruginosa* and microcystin-LR in *Artemia franciscana* nauplii. J Toxicol Environ Health A 2009;72(24):1567-75. doi: 10.1080/15287390903232459, PMID 20077231.
38. Eloff JN. The antibacterial activity of 27 southern African members of the Combretaceae. S Afr J Sci 1999;95(3):148-52.
39. Kamboj VP. Herbal medicine. Curr Sci 2000;78:35-9.
40. Hostettmann K, Hamburger M. Search for new lead compounds of natural origin. In: Testa B, Kyburz E, Fuhrer W, Giger R, editors, Verlag Helvitica Acta. Perspectives in medical chemistry. Basel; 1993.
41. Mpala LN, Chikowe GR, Cock IE. Growth inhibitory properties of extracts prepared from selected Leptospermum and Melaleuca species against a panel of pathogenic bacteria. Pharmacogn Commun 2016;6(4):215-24. doi: 10.5530/pc.2016.4.4.
42. Omer E, Elshamy AI, Nassar M, *et al*. *Plantago squarrosa* Murray extracts inhibit the growth of some bacterial triggers of autoimmune diseases: GC-MS analysis of an inhibitory extract. Inflammopharmacol 2019;27(2):373-85. doi: 10.1007/s10787-018-0547-0, PMID 30446926.
43. Omer E, Elshamy A, El Gendy AN, *et al*. *Cakile maritima* Scop. extracts inhibit the growth of some bacterial triggers of autoimmune diseases: GC-MS analysis of an inhibitory extract. Pharmacogn J 2016;8(4):361-74. doi: 10.5530/pj.2016.4.9.
44. Biggs I, Sirdaarta J, White A, *et al*. GC-MS analysis of *Commiphora molmol* oleo-resin extracts which inhibit the growth of bacterial triggers of selected autoimmune diseases. Pharmacogn J 2016;8(3):191-202. doi: 10.5530/pj.2016.3.4.
45. Fernandez A, Cock IE. *Tabebuia impetiginosa* (Mart. Ex DC. Mattos) bark extracts inhibit the growth gastrointestinal bacterial pathogens and potentiate the activity of some conventional antibiotics. Pharmacog Commun 2020;10(2):75-82. doi: 10.5530/pc.2020.2.15
46. Gilani AH, Rahman AU. Trends in ethnopharmocology. J Ethnopharmacol 2005;100(1-2):43-9. doi: 10.1016/j.jep.2005.06.001, PMID 16127805.


## PICTORIAL ABSTRACT




## SUMMARY


- *Terminalia sericea* leaf extracts were screened for the ability to block the growth selected bacterial triggers of autoimmune diseases.
- Methanolic and aqueous leaf extracts had noteworthy inhibitory activity against all bacterial species tested.
- Substantial decreases in growth inhibitory activity were evident following storage of the leaves for two years.
- Toxicity of the *T. sericea* leaf extracts was determined using the *Artemia* nauplii toxicity bioassay.
- The methanolic and aqueous extracts were non-toxic.


## About Authors

**Dr Ian Cock** leads a research team in the Environmental Futures Research Institute and the School of Natural Sciences at Griffith University, Australia. His research involves bioactivity and phytochemical studies into a variety of plant species of both Australian and international origin, including Aloe vera, South Asian and South American tropical fruits, as well as Australia plants including *Scaevola spinescens, Pittosporum phylliraeoides, Terminalia ferdinandiana (Kakadu plum), Australian acacias, Syzygiums, Petalostigmas* and *Xanthorrhoea johnsonii* (grass trees). This range of projects has resulted in approximately 250 publications in a variety of peer reviewed journals.

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