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Original Article

# Antibacterial Activity and an Upscaled Extraction Protocol for *Terminalia ferdinandiana* Exell. Leaves

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## ABSTRACT

**Background:** *Terminalia ferdinandiana* Exell. is an endemic Australian plant with a high anti-oxidant capacity. Liquid solvent extractions of *T. ferdinandiana* leaves are strong inhibitors of the growth of numerous bacterial pathogens. Despite these promising therapeutic properties, methods for the rapid extraction of large quantities of *T. ferdinandiana* leaves are lacking. This study aimed to develop a rapid supercritical extraction method to produce extracts which retain therapeutic properties and phytochemistry characteristics. **Materials and Methods:** *Terminalia ferdinandiana* leaves were extracted by both solvent maceration extraction and Supercritical fluid extraction (SCE). The extracts were tested for the ability to inhibit bacterial growth using the disc diffusion assay. The antibacterial potency was further quantified using liquid dilution MIC assays. Toxicity was evaluated using an *Artemia franciscana* nauplii bioassay and GC-MS headspace analysis was used to evaluate phytochemical similarity between the extracts. **Results:** *Terminalia ferdinandiana* leaf SCE displayed strong bacterial growth inhibitory activity against bacterial triggers of autoimmune inflammatory diseases, with efficacies similar to the smaller laboratory scale methanolic liquid extractions. The growth inhibition of the ethyl acetate and methanolic solvent extracts and the SCE were particularly noteworthy against *P. mirabilis* (MICs = 128, 227 and 208µg/mL respectively) and *A. baylyi* (MICs = 452, 780 and 104 µg/mL respectively). GC-MS analysis of the berry SCE revealed similar terpenoid components and similar abundances to those in methanolic solvent leaf extraction. Furthermore, all liquid extracts and the SCE were non-toxic in the *Artemia franciscana* toxicity assay. **Conclusion:** The *T. ferdinandiana* SCE retained the tested therapeutic properties, was non-toxic and had similar phytochemical profiles as the smaller scale liquid solvent extractions. Thus, SCE is a viable method of rapidly extracting large masses of *T. ferdinandiana* leaves to produce quality extracts that retain therapeutic properties.

**Keywords:** Kakadu plum, Combretaceae, Supercritical fluid extraction, Antibacterial activity, Autoimmune inflammatory disease, Extraction optimisation, Toxicity.

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## INTRODUCTION

*Terminalia ferdinandiana* Exell. (family Combretaceae), commonly known as Kakadu plum or gubinge, is an endemic Australia plant which has received substantial recent interest due to its high antioxidant content.[1] Indeed, the fruit of this plant has the highest ascorbic acid levels of any plant in the world, with levels reported as high as 6% of the recorded wet weight.[2] This is approximately 900 times higher (g/g) than the ascorbic acid content in blueberries (which were used as a standard). As a further comparison, oranges and grapefruit (which are considered good sources of ascorbic acid) only contain approximately 0.007% wet weight (0.5% dry weight).[3] Additionally, *T. ferdinandiana* fruit and leaves contain many other noteworthy compounds, including benzoic acids, flavanols and flavanones.[4] *Terminalia ferdinandiana* fruit is also a good source of gallic and ellagic acids,[3,4] which have strong antioxidant activity *in vitro*. Lipophilic *T. ferdinandiana* fruit extracts are also rich in lutein (a carotenoid antioxidant compound associated with eye health), and in vitamin E and vitamin E analogs.[3,4] Hesperitin, as well as the glycosides kaempferol, luteolin, and quercetin are some of the other antioxidants present in *T. ferdinandiana* fruit.[4]

Based on its high antioxidant capacity, the medicinal potential of *T. ferdinandiana* has received much recent attention. *T. ferdinandiana* fruit and leaf extracts have been reported to have a wide variety of bioactivities which would confer therapeutic potential. The bacterial growth inhibitory activity of *T. ferdinandiana* has been particularly well reported. *T. ferdinandiana* fruit and leaf extracts inhibit the growth of a broad panel of bacteria that cause food spoilage and/or cause gastrointestinal distress, diarrhoea and dysentery.[5,6] Those studies reported potent growth inhibitory

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activity, with MIC values generally substantially <1000 µg/mL. Leaf extracts were generally more potent bacterial growth inhibitors than the fruit, and the growth of both Gram positive and Gram negative bacteria were inhibited. Furthermore, both mesophilic and psychrotropic bacteria were affected by the *T. ferdinandiana* extracts, as were spore forming bacteria. The extracts were similarly potent inhibitors of the growth of bacteria associated with skin diseases (*Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes*).[6] *Terminalia ferdinandiana* extracts have even been reported to be effective inhibitors of the growth of the bacteria which cause anthrax (*Bacillus anthricis*)[7] and gas gangrene (*Clostridium perfringens*).[8] Both of these bacteria are endospore formers and are generally considered to be difficult to treat and decontaminate.

The potential of *T. ferdinandiana* leaf extract in treating inflammation has also been reported.[9] Other recent studies have also highlighted the potential of *T. ferdinandiana* extracts in inhibiting the initiating events of selected autoimmune inflammatory diseases and thus have potential in the prevention and treatment of rheumatoid arthritis, ankylosing spondylitis and multiple sclerosis.[10-13] It is likely that *T. ferdinandiana* extracts have pluripotent effects against inflammation and inflammatory diseases and they may be particularly useful therapeutic agents against these diseases. The inhibitory properties of *T. ferdinandiana* extracts against the growth of several eukaryotic cells have also been reported. Berry and leaf *T. ferdinandiana* extractions are potent inhibitors of *Giardia duodenalis* growth.[14,15] Furthermore, *T. ferdinandiana* extracts have anti-cancer activity against a panel of carcinoma cell lines.[16-18] Those studies examined the effect of *T. ferdinandiana* leaf extract on Caco2 colorectal carcinoma cells, HeLa cervical carcinoma cells, MC3T3-E1 and MG63 osteosarcoma cells, as well as Jeg-3 and JAR choriocarcinoma cells. The extracts blocked proliferation in all cell lines. A significant increase in apoptosis was also reported in the Caco2 cells, which corresponded to elevated caspase 3 activity. Several recent reports have examined the phytochemical composition of *T. ferdinandiana* extracts.[7,10,12,14,16,19-23] These phytochemical evaluations have utilised LC-MS techniques to examine polar and mid-polarity compounds in fruit and extracts. Similarly, lower polarity compounds have been detected and identified using GC-MS techniques.[12,17,24] Of the volatile terpenoid components, camphor, cineole, linalool and terpineol are consistently reported in relative abundance in *T. ferdinandiana* extracts[12,17,24] and therefore are suitable marker compounds for *T. ferdinandiana* extracts.

Whilst conventional laboratory extraction has been useful in initial studies for screening *T. ferdinandiana* fruit and leaves for therapeutic properties, upscaling the extraction to produce commercially relevant levels would use large amounts of expensive solvent, which would be time consuming for drying. Furthermore, there are substantial environmental pollution concerns associated with large scale usage of organic solvents. Up-scaled extraction protocols that provide a high yield of *T. ferdinandiana* phytochemicals are therefore of considerable interest. However, larger scale extraction procedures would need to produce extracts with similar phytochemical profiles and therapeutic properties as those reported in the laboratory scale pilot studies. Supercritical fluid extraction (SCE) offers a number of advantages compared to conventional liquid extraction for larger scale extract preparation. Significantly less solvent is required, extraction is significantly more rapid than for extraction with an organic solvent and the properties of the supercritical fluid can be readily altered by varying the temperature and pressure, allowing for greater selectivity in extraction. Thus, SCE is ideally suited to the preparation of larger scale extract preparations.

In this study, we describe a preparative scale extraction of *T. ferdinandiana* leaf material using a SCE method which results in considerably higher extract yields at a reasonable cost. Furthermore, the therapeutic potential of these upscaled extractions was compared to the properties of the laboratory scale extractions by comparing their ability to inhibit the growth of selected bacteria associated with selected autoimmune inflammatory diseases. Furthermore, the phytochemistries of the most promising solvent and supercritical extracts were compared by GC-MS headspace analysis, targeting several marker compounds.

## MATERIALS AND METHODS

### Laboratory scale extraction

*Terminalia ferdinandiana* Exell. leaves were obtained from David Bohme of Northern Territory Wild Harvest, Australia. The leaves were dried in a Sunbeam food dehydrator and subsequently ground into a coarse powder. The powdered leaf material was extracted by standardised solvent extraction methods.[25] Briefly, an amount of 1g of powdered plant material was weighed into each of five tubes and five different extracts were prepared by adding 50 mL of methanol, water, ethyl acetate, chloroform, or hexane, respectively. Extraction in each solvent was undertaken for 24 hr at 4°C with gentle shaking. All solvents were obtained from Ajax, Australia and were AR grade. The extracts were filtered through filter paper (Whatman No. 54) under vacuum, followed by drying by centrifugal evaporation in an Eppendorf concentrator 5301. The resultant dry extract was weighed and redissolved in 10 mL deionised water (containing 1% DMSO).

### Sub-critical extraction of *Terminalia ferdinandiana* leaves with dimethyl ether

Ground *T. ferdinandiana* leaves were packed into the biomass chamber of the extraction system as previously described.[26] The system was sealed and evacuated before the plant material in the biomass chamber was covered with compressed solvent (dimethyl ether). The compressed gas was cycled repeatedly across the plant

material for 20 min. Sub-critical DME extraction was carried out at room temperature and at a pressure of 500MPa. The solvent was recycled and stored in a solvent reservoir. After the compressed gas (the dimethyl ether) had been removed, the material that had been extracted from the plant biomass was collected in a separate vessel.

## Qualitative phytochemical studies

Phytochemical analysis of the *T. ferdinandiana* leaf extracts for the presence of saponins, phenolic compounds, flavonoids, polysteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by previously described assays.[27-29]

## Antibacterial screening

### *Test microorganisms*

All media was supplied by Oxoid Ltd., Australia. Reference strains of *Acinitobacter baylyi* (ATCC33304), *Klebsiella pneumoniae* (ATCC31488), *Proteus mirabilis* (ATCC21721), and *Pseudomonas aeruginosa* (ATCC39324) were purchased from American Tissue Culture Collection, USA. All stock cultures were subcultured and maintained in nutrient broth at 4°C.

## Evaluation of antimicrobial activity

Antimicrobial activity of all plant extracts was determined using a modified disc diffusion method.[30,31] Briefly, 100 µL of the test bacteria were grown in 10 mL of fresh nutrient broth until they reached a count of approximately $10^8$ cells/mL as determined by direct microscopic determination. One hundred microliters of microbial suspension was subsequently spread onto the agar plates. The extracts were applied onto 5 mm sterilised filter paper discs. Discs were infused with 10 µL of the test sample, allowed to dry and placed onto inoculated plates. The plates were allowed to stand at 4°C for 2 hr before incubation with the test microbial agents. The plates were then incubated at 30°C for 24 hr and the diameters of the inhibition zones were measured in millimetres. All measurements were to the closest whole millimetre. Each antimicrobial assay was performed three times, each with internal triplicates and mean values were determined. Standard discs of ampicillin (10 µg) and chloramphenicol (10 µg/mL) were obtained from Oxoid Ltd., Australia and served as positive controls. Filter paper discs impregnated with 10 µL of distilled water were used as negative controls.

## Minimum Inhibitory Concentration (MIC) determination

The minimum inhibitory concentration for each extract was determined using two methods. A liquid dilution MIC assay was employed as it is generally considered the most sensitive bacterial growth inhibitory assay.[32] Furthermore, as microplate liquid dilution 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 assay was also used in this study for comparison.

## Microplate liquid dilution MIC assay

A standard liquid dilution MIC assay[32] was used to evaluate the antimicrobial activity of the plant samples and conventional antimicrobials independently and in combinations. Briefly, 100 µL of sterilized distilled water was dispensed into each well of 96 well micro-titre plate. The plant samples and conventional antibiotics (100 µL) were then added into separate wells of the first row of the plate. The *T. ferdinandiana* extracts were introduced at a starting concentration of 32 mg/mL whilst the conventional antibiotics were introduced at a starting concentration of 0.01 mg/mL. A negative control (nutrient broth), a sterile control (without bacteria) and a sample-free culture control (to ensure the media was capable of supporting microbial growth) were included on all plates. After addition of the test samples to the plate, each was serially diluted by doubling dilutions. The relevant bacterial culture inoculum (100 µL) was then added to all wells of the plate except the sterile control wells. Each inoculum contained approximately $1x10^6$ Colony forming units (CFU)/mL. All plates were subsequently incubated at 37°C. p-Iodonitrotetrazolium violet (INT) was obtained from Sigma-Aldrich, 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.

## Disc diffusion MIC assay

The Minimum inhibitory concentrations (MIC) of the extracts was also evaluated by disc diffusion assay as previously described.[30,31] Graphs of the Zone of inhibition (ZOI) versus Ln of the concentration were plotted and MIC values were calculated using linear regression.

## Toxicity screening

### *Reference toxins for biological screening*

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared as a 2 mg/mL solution in distilled water and was serially diluted in synthetic seawater for use in the *A. franciscana* nauplii bioassay.

## *Artemia franciscana* Lethality Assays (ALA)

Toxicity was tested using a modified *Artemia franciscana* nauplii lethality assay.[33-35] Briefly, *A. franciscana* cysts were obtained from North American Brine Shrimp, LLC, USA (harvested from the Great Salt Lake, Utah). Synthetic seawater was prepared using Reef Salt, AZOO Co., USA. Seawater solutions at 34 g/L

distilled water were prepared prior to use. An amount of 1 g of *A. franciscana* cysts were incubated in 500 mL synthetic seawater under artificial light at 25°C, 2000 Lux with continuous aeration. Hatching commenced within 16-18 hr of incubation. Newly hatched *A. franciscana* (nauplii) were used within 10h of hatching. Nauplii were separated from the shells and remaining cysts and were concentrated to a suitable density by placing an artificial light at one end of their incubation vessel and the nauplii-rich water closest to the light was removed for biological assays. The extracts and positive control were also serially diluted in artificial seawater for $LC_{50}$ determination. A volume of 400 µL of seawater containing approximately 44 (mean 44.3, $n$ = 125, SD 10.6) nauplii were added to wells of a 48-well plate and immediately used for bioassay. The plant extracts were diluted to 4 mg/mL in seawater for toxicity testing, resulting in a 2 mg/mL concentration in the bioassay. A volume of 400 µL of diluted plant extract 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 moribund if no movement of the appendages was observed within 10 sec. After 48 hr, all nauplii were sacrificed and counted to determine the total number per well. The $LC_{50}$ with 95% confidence limits for each treatment was calculated using probit analysis.

## Non-targeted GC-MS head space analysis

Separation and quantification of phytochemical components were performed using a Shimadzu GC-2010 plus (USA) linked to a Shimadzu MS TQ8040 (USA) mass selective detector system as previously described.[24] Briefly, the system was equipped with a Shimadzu auto-sampler AOC-5000 plus (USA) fitted with a Solid Phase Micro-Extraction fibre (SPME) handling system utilising a Supelco (USA) divinyl benzene/carbowax/polydimethylsiloxane (DVB/CAR/PDMS). Chromatographic separation was accomplished using a 5% phenyl, 95% dimethylpolysiloxane (30 m x 0.25 mm id x 0.25 µm) capillary column (Restek USA). Helium (99.999%) was employed as a carrier gas at a flow rate of 0.79 mL/min. The injector temperature was set at 230°C. Sampling utilised a SPME cycle which consisted of an agitation phase at 500 rpm for a period of 5 sec. The fibre was exposed to the sample for 10 min to allow for absorption and then desorbed in the injection port for 1 min at 250°C. The initial column temperature was held at 30°C for 2 min, increased to 140°C for 5 min, then increased to 270°C over a period of 3 min and held at that temperature for the duration of the analysis. The GC-MS interface was maintained at 200°C with no signal acquired for a min after injection in split-less mode. The mass spectrometer was operated in the electron ionisation mode at 70eV. The analytes were then recorded in Total Ion Count (TIC) mode. The TIC was acquired after a min and for duration of 45 min utilising a mass range of 45-450 m/z.

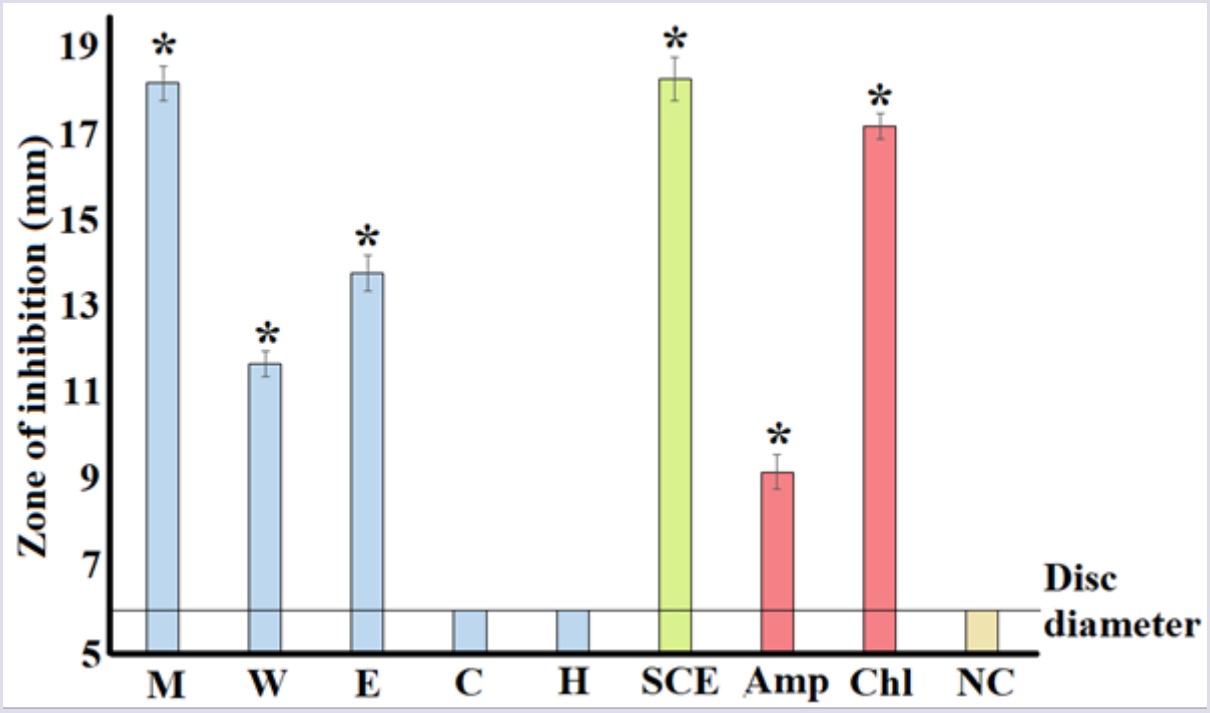


**Figure 1:** Antibacterial activity of *T. ferdinandiana* extracts measured as zones of inhibition (mm) against *Proteus mirabilis*. Results are expressed as mean ± SEM of three repeats, each with internal triplicates ($n$ = 9). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; SCE = supercritical extract; Amp = ampicillin control (10 µg); Chl = chloramphenicol control; NC = negative control; * = results that are significantly different to the untreated control.

## Statistical analysis

Data are expressed as the mean ± SEM of at least three independent experiments. 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

## Extraction yields and qualitative phytochemical screening

Extraction of 1 g of dried *T. ferdinandiana* leaves with various solvents yielded dried plant extracts ranging from approximately 59 mg (*T. ferdinandiana* ethyl acetate extract) to 352 mg (*T. ferdinandiana* methanolic extract) (Table 1). Water and methanol gave the highest yields of dried extracted material for all *T. ferdinandiana* plant materials tested, whilst ethyl acetate, chloroform and hexane extracted substantially lower masses. The dried extracts were resuspended in 10 mL of deionised water (containing 1% DMSO) resulting in the extract concentrations shown in Table 1. The supercritical extraction of *T. ferdinandiana* leaves also yielded high amounts of extract, which were prepared to a comparable concentration as the methanolic small scale extract as a direct comparison of potency (Table 1).

Phytochemical studies (Table 1) show that methanol and water extracted the widest range and largest amount of phytochemicals of the laboratory scale extractions. The aqueous and methanolic extracts both showed moderate to high levels of total phenolics (water soluble and insoluble phenolics), flavonoids and tannins. The methanolic and aqueous extract also contained moderate levels of saponins. Similar classes of phytochemicals were detected in the ethyl acetate extract, albeit generally at lower levels than in the aqueous and methanolic extracts. Most of the phytochemical classes were not evident in the chloroform or hexane extracts. The Supercritical extractions (SCE) displayed similar phytochemical profiles as the laboratory scale aqueous and methanolic extractions, with moderate to high levels of polyphenolics, flavonoids and tannins. Lower levels of saponins were also generally detected in the SCE *T. ferdinandiana* leaf extract. Thus, qualitative phytochemical analysis indicates that the larger SCE extractions contain similar classes of phytochemical as evident in the laboratory scale extractions which were prepared in the same way as extractions with previously published therapeutic properties.[26]

The mass of dried extracted material, the concentration after resuspension in deionised water (mg/mL) and qualitative phytochemical screenings of the *T. ferdinandiana* extracts.

## Antibacterial activity

Aliquots (10 µL) of each extract were tested in the disc diffusion assay against bacterial species associated with the induction of rheumatoid arthritis (*Proteus mirabilis*, Figure 1), ankylosing

**Table 1: The mass of dried extracted material, the concentration after resuspension in deionised water (mg/mL) and qualitative phytochemical screenings of the *T. ferdinandiana* extracts.**

| Extract | Mass of Dried Extract (mg) | Concentration of Resuspended Extract (mg/mL) | Total Phenolics | Water Soluble Phenolics | Water Insoluble Phenolics | Cardiac Glycosides | Saponins | Triterpenes | Phytosteroids | Alkaloids (Mayer Test) | Alkaloids (Wagner Test) | Flavonoids | Tannins | Free Anthraquinones | Combined Anthraquinones |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| M | 352 | 35.2 | +++ | +++ | +++ | - | ++ | + | - | + | + | +++ | +++ | - | - |
| W | 456 | 45.6 | +++ | +++ | +++ | - | ++ | - | - | - | - | +++ | +++ | - | - |
| E | 59 | 5.9 | ++ | ++ | ++ | - | - | - | - | - | - | ++ | ++ | - | - |
| C | 78 | 7.8 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| H | 64 | 6.4 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| SCE | ND | 30 | +++ | +++ | +++ | - | + | + | - | - | - | +++ | ++ | - | - |

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; SCE = supercritical extract; ND = not determined.

spondylitis (*Klebsiella pneumoniae*, Figure 2), and multiple sclerosis (*Acinitobacter baylyi*, Figure 3; *Pseudomonas aeruginosa*, Figure 4). Consistent with previous studies,[10-12] the methanolic and ethyl acetate leaf extracts were potent inhibitors of reference and clinical strains of *P. mirabilis* (Figure 1), with zones of inhibition approximately 13.8-18.2 mm. Whilst the aqueous leaf extract also inhibited *P. mirabilis* growth (ZOI = 11.7 mm), it had lower efficacy (as judged by the zone of inhibition) than was evident for the corresponding methanolic and ethyl acetate extracts, whilst the chloroform and hexane extracts were completely devoid of inhibitory activity. The SCE leaf extract was also a good inhibitor of *P. mirabilis* growth, with a 18.3 mm zone of inhibition. Thus, the SCE leaf extract had similar efficacy to the small-scale laboratory extraction (as judged by zone of inhibition). As *P. mirabilis* can trigger rheumatoid arthritis in genetically susceptible people,[10,36] these extracts may be useful in the prevention and treatment of this disease.

The methanolic and ethyl acetate *T. ferdinandiana* leaf extracts also inhibited *K. pneumoniae* growth, albeit with smaller zones of inhibition (Figure 2). The methanolic leaf extract was the most potent growth inhibitor, with zones of inhibition of 10.6 mm. The ethyl acetate extract was also a noteworthy inhibitor of *K. pneumoniae* growth (ZOI = 8.6 mm). In contrast, the aqueous, chloroform and hexane extracts were completely devoid of *K. pneumoniae* growth inhibitory activity. Notably, this strain of *K. pneumoniae* was resistant to the ampicillin control, but highly susceptible to the chloramphenicol control (ZOI = 17.2 mm). The SCE leaf extract (ZOI = 8.8 mm) had similar potency to the small scale methanolic and ethyl acetate extractions. As *K. pneumoniae* can trigger ankylosing spondylitis in genetically susceptible individuals,[11,36] these extracts have potential in the prevention and treatment of this disease, as well as other infections of that bacterium.

The *T. ferdinandiana* extracts were also screened for growth inhibitory activity against bacterial triggers of multiple sclerosis (*Acinitobacter baylyi*, Figure 3; *Pseudomonas aeruginosa*, Figure 4).[13,36] The methanolic and ethyl acetate leaf extracts were the most potent *A. baylyi* growth inhibitors, with zones of inhibition ranging from 14.4-18.6 mm. smaller inhibition zones (11.8 mm) were measured for the aqueous extract. Similarly, the SCE leaf extract (ZOI = 19.2 mm) was also a good inhibitor of *A. baylyi* growth. In comparison, this bacterial strain was completely resistant to ampicillin, but was highly sensitive to the chloramphenicol control (ZOI = 16.8 mm).

The methanolic, aqueous and ethyl acetate laef extracts also inhibited *P. aeruginosa* growth (Figure 4), albeit with relatively small zones of inhibition. The strongest inhibition (ZOI = 7.8 mm) was recorded for the methanolic leaf extract. The SCE leaf extract also inhibited *P. aeruginosa* growth, although the zones of inhibition indicate only weak inhibitory activity, with an inhibition zones of 6.3 mm. Whilst this inhibition is relatively poor in comparison the other bacterial species screened, it is

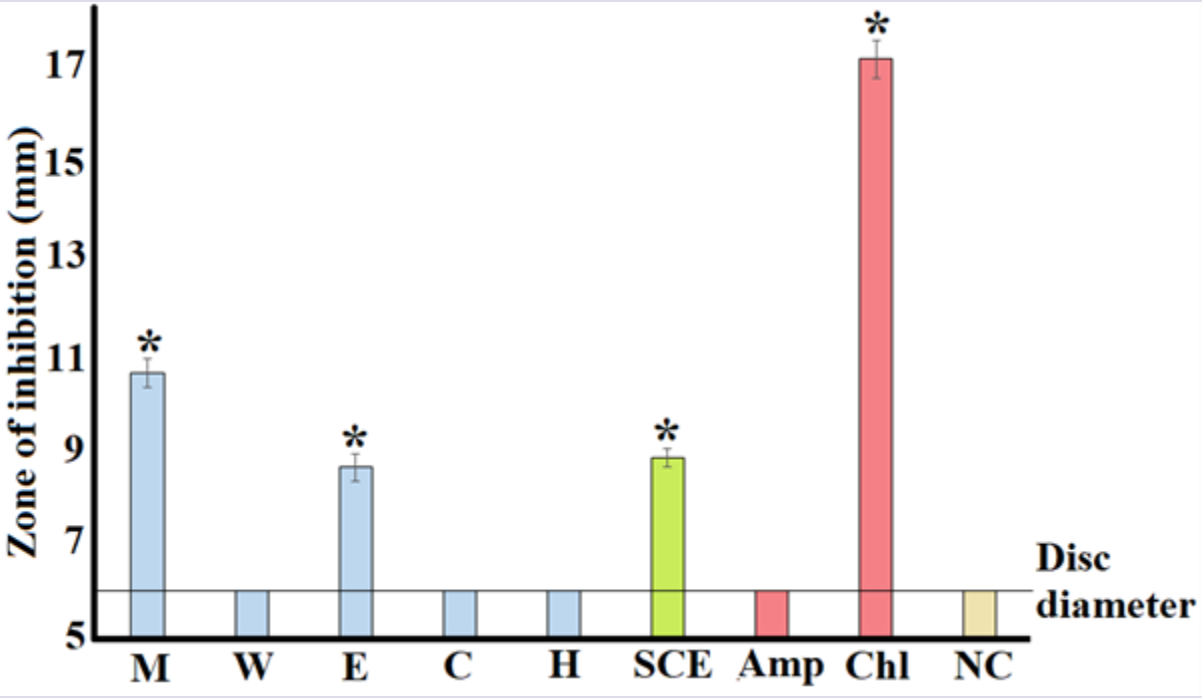


**Figure 2:** Antibacterial activity of *T. ferdinandiana* extracts measured as zones of inhibition (mm) against *Klebsiella pneumoniae*. Results are expressed as mean ± SEM of three repeats, each with internal triplicates ($n$ = 9). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; SCE = supercritical extract; Amp = ampicillin control (10 µg); Chl = chloramphenicol control; NC = negative control; * = results that are significantly different to the untreated control.

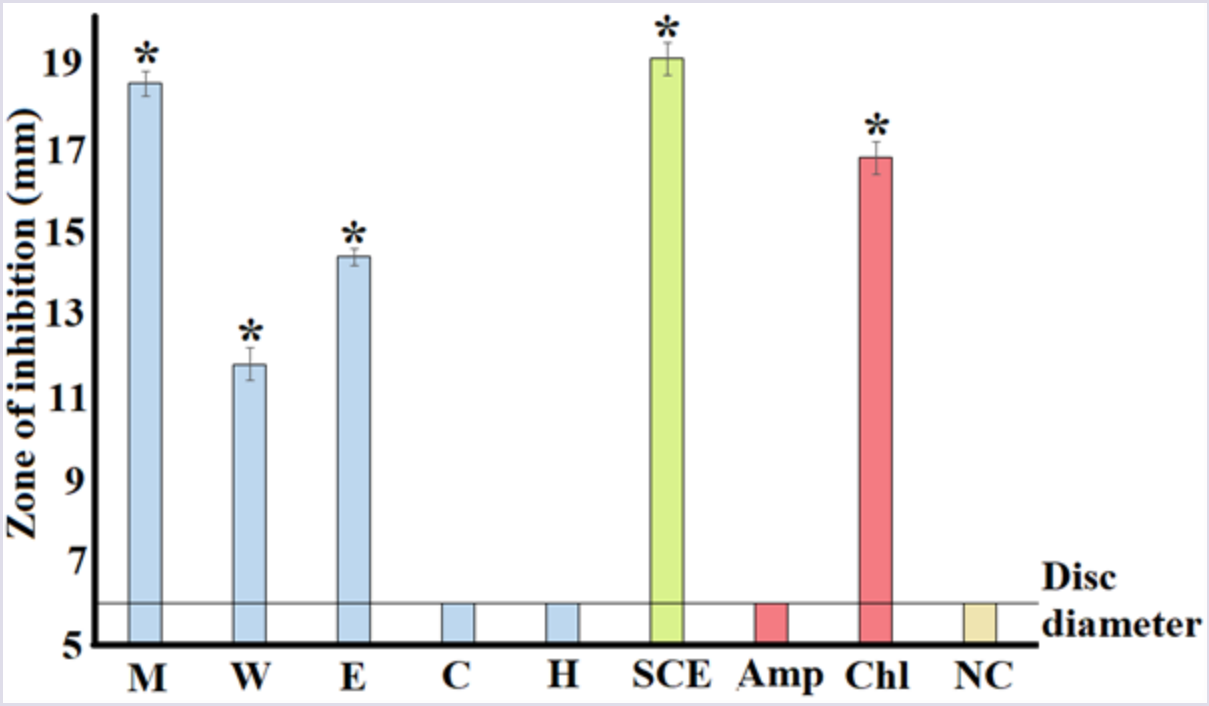


**Figure 3:** Antibacterial activity of *T. ferdinandiana* extracts measured as zones of inhibition (mm) against *Acintobacter baylyi*. Results are expressed as mean ± SEM of three repeats, each with internal triplicates ($n$ = 9). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; SCE = supercritical extract; Amp = ampicillin control (10 µg); Chl = chloramphenicol control; NC = negative control; * = results that are significantly different to the untreated control.

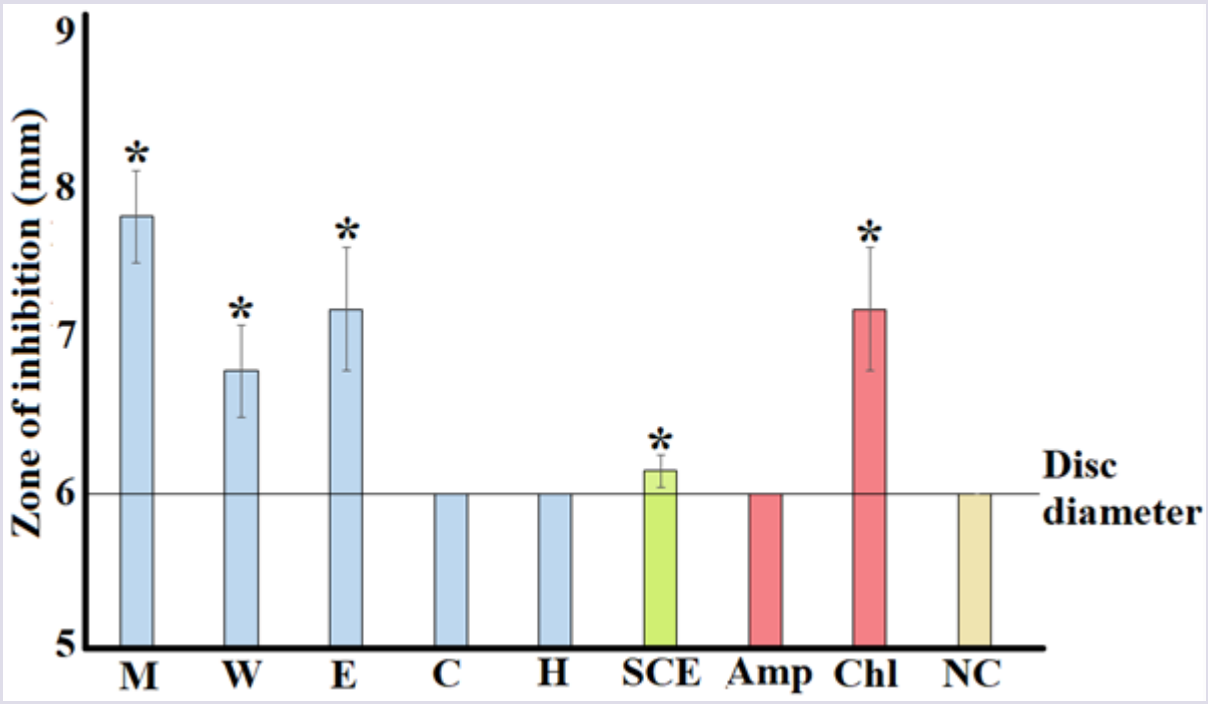


**Figure 4:** Antibacterial activity of *T. ferdinandiana* extracts measured as zones of inhibition (mm) against *Pseudomonas aeruginosa*. Results are expressed as mean ± SEM of three repeats, each with internal triplicates ($n$ = 9). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; SCE = supercritical extract; Amp = ampicillin control (10 µg); Chl = chloramphenicol control; NC = negative control; * = results that are significantly different to the untreated control.

**Table 2: Disc Diffusion (DD) and Liquid Dilution (LD) MIC values (µg/mL) for *T. ferdinandiana* leaf extracts against microbial triggers of some autoimmune inflammatory diseases.**

| | *P. mirabilis* | | *K. pneumoniae* | | *A. baylyi* | | *P. aeruginosa* | | ALA toxicity |
|---|---|---|---|---|---|---|---|---|---|
| | DD MIC | LD MIC | DD MIC | LD MIC | DD MIC | LD MIC | DD MIC | LD MIC | $LC_{50}$ |
| M | 356 | 227 | 1920 | 2033 | 925 | 780 | 1239 | 1220 | 1183 |
| W | 925 | 683 | - | - | 1063 | 1188 | 2728 | 2656 | 1420 |
| E | 119 | 128 | 1092 | 1550 | 625 | 452 | 1382 | 1568 | 1254 |
| C | 1571 | 1263 | - | - | - | - | - | - | CND |
| H | 1571 | 1450 | - | - | - | - | - | - | CND |
| SCE | 405 | 208 | 2856 | 3333 | 328 | 104 | 2685 | 2240 | 1438 |
| Pen | ND | 2.5 | ND | - | ND | - | ND | - | ND |
| Amp | ND | 2.5 | ND | - | ND | - | ND | - | ND |
| Chl | ND | 1.25 | ND | 2.5 | ND | 2.5 | ND | - | ND |
| Ery | ND | - | ND | - | ND | 2.5 | ND | - | ND |
| Tet | ND | - | ND | 1.25 | ND | 1.25 | ND | 2.5 | ND |
| ALA PC | ND | | ND | | ND | | ND | | 63 |

M = methanol extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane; SCE = supercritical extract; ALA PC = $LC_{50}$ of potassium dichromate in the ALA assay; DD = disc diffusion; LD = liquid dilution; - indicates no inhibition at any dose tested; ND = not tested; CND = could not determine as % mortality was <50% at all concentrations tested.

noteworthy that both the reference and clinical *P. aeruginosa* strains are antibiotic resistant strains. Indeed, it was completely resistant to 10 µg ampicillin control used in our studies, and only relatively small (7.2 mm) zones of inhibition were recorded for the chloramphenicol control. This finding is supported by previous studies which have also reported these strains to be antibiotic resistant.[12,19] Thus, despite the relatively small inhibition zones, the *T. ferdinandiana* extracts may still be useful for the inhibition of *P. aeruginosa* growth. As both *A. baylyi* and *P. aeruginosa* can trigger multiple sclerosis in genetically susceptible individuals,[13,36] these extracts have potential in the prevention and treatment of that disease.

The relative level of antimicrobial activity was further evaluated by determining the MIC values (Table 2) for each extract against the bacterial species that were shown to be susceptible by disc diffusion assays. Most of the extracts were effective at inhibiting microbial growth at low concentrations, with MIC values against the bacterial species that they inhibited often substantially <1000 µg/mL, indicating the potent antimicrobial activity of these extracts. The ethyl acetate leaf extracts was a particularly potent bacterial growth inhibitor, with MIC values as low as 128 µg/mL against *P. mirabilis*. This extract was similarly potent against *A. baylyi* (MIC values = 452 µg/mL) and was a moderate inhibitor of *K. pneumoniae* and *P. aeruginosa* growth (MIC values ~1500 µg/mL). The methanolic extract also displayed noteworthy inhibition of each of the bacteria, albeit with MIC values slightly higher than recorded for the ethyl acetate extract. These MIC values compare favourably with the inhibition of the pure ampicillin standard which was tested using 10 µg per disc. Indeed, all bacteria were completely resistant to this antibiotic except *P. mirabilis*, which had an MIC of 2.5 µg/mL against ampicillin. As MIC values >1 µg/mL are considered to indicate resistance for pure antibiotics in this assay,[37,38] this bacterium was also deemed to be resistant to ampicillin.

The SCE leaf extracts were also effective at inhibiting bacterial growth at low concentrations, with MIC values against the bacterial species that they inhibited as low as <104 µg/mL. The SCE leaf extracts were particularly potent against *P. mirabilis* and *A. baylyi* (MICs of 208 and 104 µg/mL respectively). In contrast, the SCE leaf extract was a less potent inhibitor of *K. pneumoniae* and *P. aeruginosa*, with MIC values substantially >2000 µg/mL. Whilst the SCE leaf extract was not as potent at inhibiting the growth of *K. pneumoniae* and *P. aeruginosa* as the corresponding methanolic and ethyl acetate extracts, these MIC values are still indicative of moderate bacterial growth inhibition. Thus, the SCE extract also has potential in the prevention and treatment of diseases associated with these bacteria.

## Quantification of toxicity

The *T. ferdinandiana* leaf extracts were diluted to 4000 µg/mL (to give a bioassay concentration of 2000 µg/mL) in artificial seawater for toxicity testing in the *Artemia* nauplii lethality bioassay. For comparison, the reference toxin potassium dichromate was also tested in the bioassay. Potassium dichromate was rapid in its induction of mortality, with mortality evident within 4 hr of exposure (unpublished results). All of the *T. ferdinandiana* leaf extracts were slower at inducing mortality, with ≥ 12 hr needed for mortality induction. Despite the slower onset of mortality, several of the small-scale solvent extractions induced mortality significantly above that of the artificial seawater control (Figure

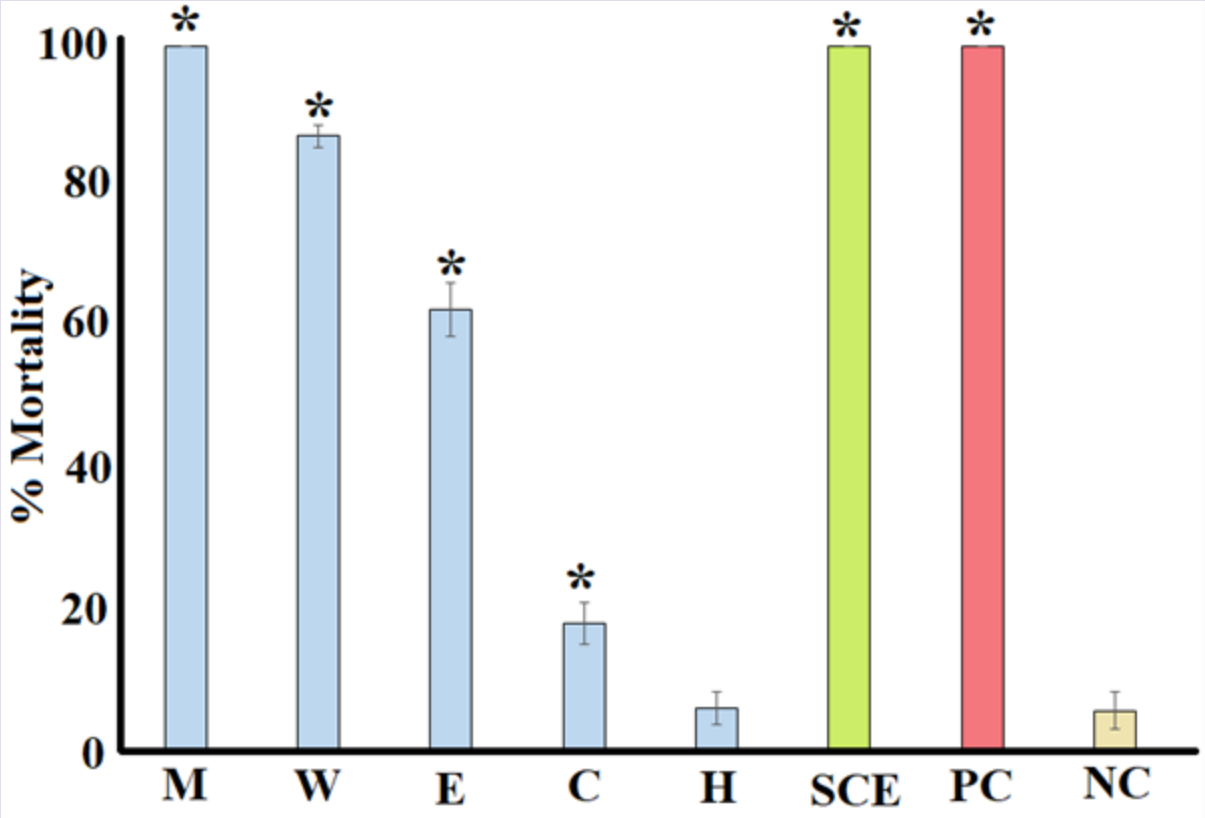


**Figure 5:** The % lethality of the *T. ferdinandiana* extracts, potassium dichromate control (PC; 1000 µg/mL) and seawater (NC; negative control). Results are expressed as mean ± SEM of three repeats, each with internal triplicates ($n$ = 9). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; SCE = supercritical extract; PC = potassium dichromate control (1000 µg/mL); NC = negative control; * = results that are significantly different to the untreated control.

5). Similarly, the supercritical fluid extractions induced similar mortality levels at 24 hr. To further evaluate the toxicity, each extract was tested across a range of concentrations in the *Artemia* nauplii bioassay. Table 2 shows the extract and control toxin concentrations required to achieve 50% mortality ($LC_{50}$) following 24 hr exposure. As toxicity of crude plant extracts has previously been defined as 24 hr $LC_{50}$ values <1000 µg/mL,[35] the measured $LC_{50}$ values indicate that none of the small-scale *T. ferdinandiana* solvent extractions were toxic. Similarly, the SCE leaf extraction also had an $LC_{50}$ value substantially >1000 µg/mL and was thus also deemed to be non-toxic.

## Non-targeted GC-MS headspace analysis of the *T. ferdinandiana* extracts

As the methanolic *T. ferdinandiana* leaf extract and the SCE extract generally had the most potent bacterial growth inhibitory efficacy (as determined by MIC; Table 2), they were deemed the most promising extracts for further phytochemical analysis. Optimised GC-MS parameters were developed and used to examine the phytochemical composition of these extracts. The resultant gas chromatograms are presented in Figures 6a and Figure 6b, respectively. Several marker compounds (camphor, cineole, limonene, terpineol) were selected as a comparison between the extracts as these have previously been reported to be present in relative abundance in *T. ferdinandiana* leaf extracts.[12,17,24] All of these compounds were detected in both the methanolic solvent extract and in the SCE extract. A major peak in both extracts eluted at approximately 14.1 min. This compound was identified as linalool by database spectral comparison and by co-elution with an authentic standard. This peak was relatively abundant in both extracts, although there was a higher relative abundance noted in the SCE extract. Another major peak (identified as cineole) was present in approximately equal relative abundances in both the methanolic and SCE leaf extracts at 15.5 min. Whilst camphor was a major component in both extracts, it was present in higher relative abundance in the SCE extract compared to the smaller scale methanol solvent extract. Conversely, terpineol was present in slightly higher relative abundance in the methanolic extract compared to the SCE extract. Thus, the overall composition between the 2 extracts appears to be similar although there are some variations in the relative abundances of the selected marker compounds.

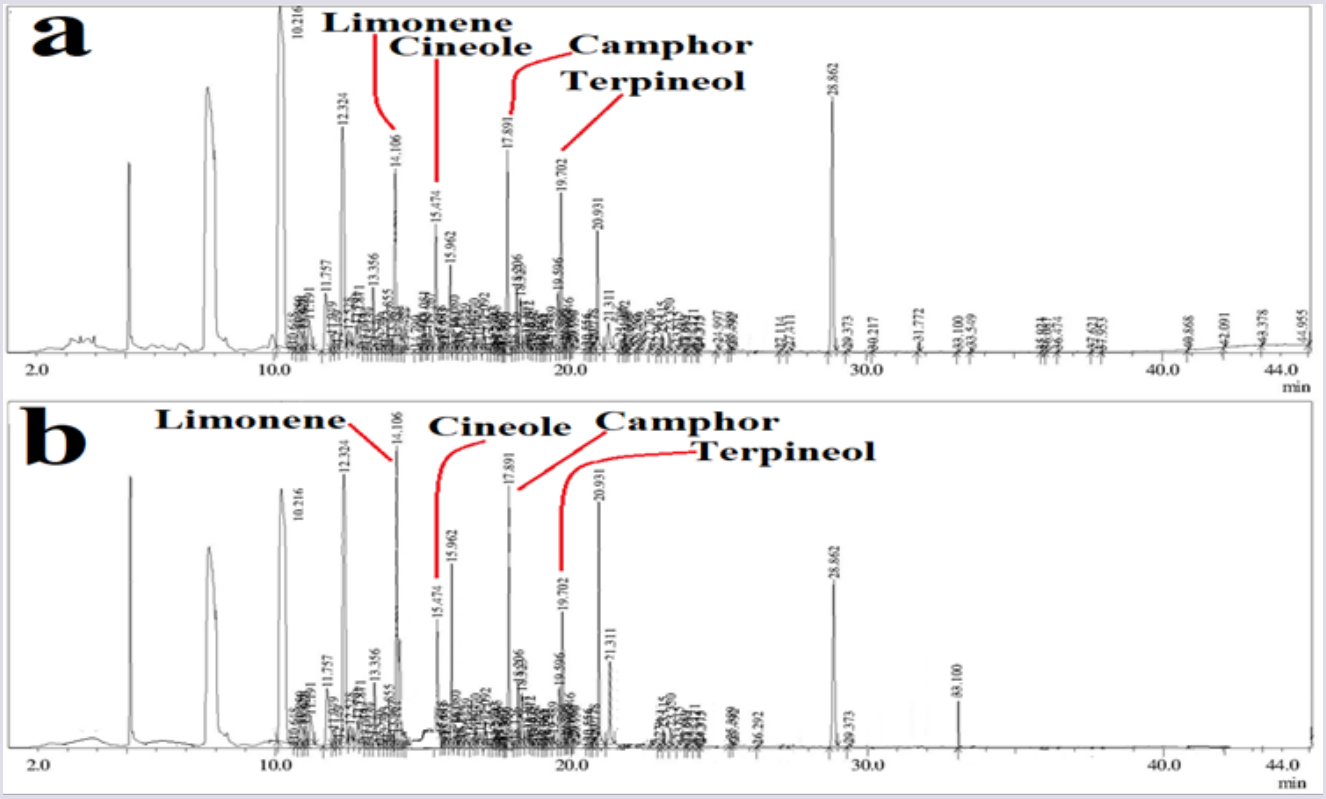


**Figure 6:** Head space gas chromatogram of 0.5 µL injections of *T. ferdinandiana* leaf (a) methanolic extract and (b) SCE leaf extract. The extracts were dried and resuspended in methanol. Major phytochemical components are identified as a comparison between the extraction methods.

## DISCUSSION

*Terminalia ferdinandiana* fruit and leaf extracts have been highlighted for their medicinal properties. Recent studies have reported that fruit extracts have especially high antioxidant capacities,[1,2] as well as strong antibacterial activity,[6-8,10-12,19-21] anti-inflammatory activity[9] and anti-Giardial activity.[14,15] Far less extensive studies have reported on the therapeutic properties of the leaves, although they have also been reported to have high antioxidant contents,[16,19] as well as potent antibacterial activities[19-21] Notably, *T. ferdinandiana* leaf extracts have been reported to have substantially stronger antibacterial activity than fruit extracts against many bacterial species,[19-21] including the bacterial triggers of several autoimmune inflammatory diseases.[19] *Terminalia ferdinandiana* leaf extracts have also been reported to inhibit cellular proliferation in several carcinoma cell models, as well as inducing apoptosis in Caco2 cells.[16] However, it is noteworthy that all of these studies utilised small scale laboratory extraction methodologies to prepare extracts for biological screening.

The aim of this study was to develop a quick and simple extraction method which would allow the extraction of substantially increased masses of plant material without significantly affecting the quality of the extract produced. Supercritical Fluid Extraction (SFE) was selected for the development of an extraction protocol as it has several advantages over other methods:

- Supercritical fluids (SF) have similar solvating powers to liquid organic solvents but higher diffusivities, lower viscosities and lower surface tension.
- Alteration of the temperature and pressure conditions can readily be adjusted, allowing for rapid separation of analytes.
- The polarity of the SF can readily be modified to provide more selective separation.
- The extracts produced by SCE do not have residual solvent present (as may be the case in solvent extraction protocols). This is extremely favourable for extracts with pharmaceutical or food applications.
- Diffusivities are much faster in SFs than in liquid solvents. Therefore, SCE is substantially more rapid than liquid solvent extraction. Less time is required to produce large amounts of extract using SFs.
- Substantially less costly solvent is used than for liquid solvent extraction. As well as decreasing processing costs, this results in significant reductions in drying time and substantially less environmental risk due to solvent pollution.

With consideration of these potential benefits, an ethanol dimethyl ether-based SCE protocol was developed and trialled. As previous studies have reported the therapeutic potential of the leaves, these plant materials were both extracted using our SCE protocol and tested in parallel with extracts produced by liquid solvent extraction. All extracts were screened for the ability to inhibit the growth of bacterial species that are triggers for autoimmune inflammatory diseases as these would provide insights into the ability to block bacterial growth, as well as indicating therapeutic options for the treatment of some inflammatory diseases.

Screening the SCE leaf extract for antibacterial activities demonstrated that it had potent antibacterial properties, with similar efficacies to the smaller scale liquid solvent extractions. The MIC values of the SCE leaf extract against several bacterial species were <1000 µg/mL, indicating the potential of this extract in the prevention and treatment of some autoimmune inflammatory diseases. The MIC values of the SCE against *P. mirabilis* (208 µg/mL) and *A. baylyi* (104 µg/mL) indicate that it may be especially useful for the prevention of rheumatoid arthritis and multiple sclerosis. Thus, whilst the supercritical extraction method used in these studies resulted in a far greater extraction throughput as well as reduced extraction time and costs, the resultant SCE retained therapeutic activities at or near the potency of the smaller scale liquid solvent extracts. Furthermore, toxicity screening in the *Artemia* nauplii bioassay indicate that the SCE leaf extract is nontoxic, with $LC_{50}$ values >1000 µg/mL. Thus, SCE can produce substantially greater masses of extracted material with similar therapeutic potential as the smaller liquid solvent extraction techniques.

A comparison of the phytochemical composition of the liquid solvent extracts and the supercritical fluid extracts was used as a further quality control measure. Several important terpenoids were selected for this study on the basis of their known bioactivities relevant to the therapeutic properties tested in this study, and reports of their relevant abundance in *T. ferdinandiana* extracts.[12,17,24] The selected terpenoids have been previously reported to have potent broad-spectrum antibacterial activity[16,39] and therefore are likely to contribute to the inhibitory activity reported in this study. Furthermore, these terpenoids have also been reported to suppress NF-κB signalling (the major regulator of inflammatory diseases) and are thus likely to contribute to the anticancer and anti-inflammatory properties of *T. ferdinandiana.*[18,39] Minor changes in phytochemical abundance were evident between the extracts produced by the different methods. Both extracts contained similar abundances of cineole. The small-scale methanolic extracts contained higher relative levels of terpineol than the SCE extract, whereas the SCE extract contained higher levels of camphor and linalool.

Whilst this study provides insight into the phytochemical compositions of the *T. ferdinandiana* extracts, it is noteworthy that no single technique will detect and identify all compounds responsible for any therapeutic property in an extract. Our study utilised a GC-MS headspace technique to examine the extracts. This technique was chosen as it is simple and rapid, and several biomarker compounds are known to be detected using these techniques.[12,17,24] As detection of volatile, relatively nonpolar compounds is suited to GC-MS headspace analysis, this was an appropriate analytical tool for detecting colatile terpenoids components. However, these extracts are likely to contain many more polar compounds that were not detected in this study. Indeed, several previous studies utilising LC-MS analysis have highlighted the phytochemical complexity of *T. ferdinmandiana* extracts.[10,16,19-22] Thus, whilst extraction by SCE techniques does not appear to have resulted in dramatic changes in phytochemical composition (at least for the volatile lower polarity compounds), further studies using LC-MS analysis are required to ensure that more dramatic differences between the extracts are not apparent with respect to their polar components.

## CONCLUSION

The results of this study demonstrate that supercritical fluid extraction is a viable method of extraction of large masses of *T. ferdinandiana* leaves rapidly and with high yields. Similarly, the supercritical extracts generally retain their therapeutic properties, are relatively non-toxic and have similar profiles and abundances of several therapeutically important compounds as the smaller scale liquid solvent extractions.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## ABBREVIATIONS

**ALA:** *Artemia* lethality assay; **DMSO:** Dimethyl sulfoxide; **$LC_{50}$:** The concentration required to achieve 50% mortality; **MIC:** Minimum inhibitory concentration; **SCE:** Supercritical fluid extraction; **SF:** Supercritical fluid; **ZOI:** Zone of inhibition.

## SUMMARY

- *T. ferdinandiana* leaf solvent and SCE extracts were screened for bacterial growth inhibitory activity against bacterial triggers of autoimmune inflammatory diseases.
- Antibacterial activities were quantified and compared using solid phase and liquid dilution MIC assays.
- Toxicities of the extracts were evaluated by *Artemia* nauplii assays.

• GC-MS analysis was used to compare the low molecular weight volatile components between the SCE and the solvent extractions.

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