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Original Article

GC-MS Analysis of *Commiphora molmol* Oleo-Resin Extracts which Inhibit the growth of Bacterial Triggers of Selected Autoimmune Diseases

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ABSTRACT

Introduction: Myrrh has been used traditionally for the inhibition of microbial growth and for the treatment of rheumatic diseases. Despite this, myrrh extracts are yet to be tested for the ability to inhibit the growth of the bacterial triggers of autoimmune inflammatory diseases. **Methods:** Solvent extracts prepared from commercially obtained myrrh resin were analysed for the ability to inhibit the growth of bacterial species associated with initiating rheumatoid arthritis (*P. mirabilis*), ankylosing spondylitis (*K. pneumoniae*) and multiple sclerosis (*A. baylyi*, *P. aeruginosa*) by disc diffusion assay, and quantified by MIC determination. Toxicity was determined by *Artemia franciscana* bioassay. The most potent inhibitory extract was investigated using non-targeted GC-MS head space analysis (with screening against a compound database) for the identification and characterisation of individual components in the crude plant extracts. **Results:** Methanolic myrrh extract inhibited the growth of all bacterial species tested. The growth inhibition of this extract was particularly notable against *P. mirabilis* and *K. pneumoniae*, with MIC values substantially < 1000 µg/mL for both reference and clinical bacterial strains. Indeed, the MIC values of the methanolic extract against *P. mirabilis* reference and clinical strains were 572 and 463 µg/mL respectively. The methanolic extract also inhibited the growth of *A. baylyi* (MIC approximately 3000 µg/mL) and *P. aeruginosa* (MIC approximately 1800 µg/mL). However, the MICs against these bacteria was indicative of only moderate inhibitory activity. The aqueous, ethyl acetate, chloroform and hexane extracts also inhibited the growth of all bacterial species, albeit with moderate (MIC values 1000-5000 µg/mL) to low efficacy (MIC values >5000 µg/mL) against all bacterial species. All myrrh extracts were non-toxicin the *Artemia franciscana* bioassay, with LC_{50} values substantially above 1000 µg/mL. Non-biased GC-MS headspace analysis of the methanolic extracti dentified a high diversity of monoterpenoids and sesquiterpenoid. **Conclusion:** The lack of toxicity and the inhibitory activity of the methanolic myrrh extract against microbial triggers of rheumatoid arthritis, ankylosing spondylitis and multiple sclerosis indicates its potential in the treatment and prevention of these diseases.

Key words: *Commiphora molmol*, Myrrh, Terpenoid, Monoterpenoid, Sesquiterpenoid, Rheumatoid arthritis, Ankylosing spondylitis, Multiple sclerosis.

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INTRODUCTION

Myrrh is the common name for the aromatic resin produced by several small thorny trees of the genus *Commiphora* (family Burseraceae). The main myrrh producing species is *Commiphora molmol* (synonym *Commiphora myrrha*; Yemen, Somalia, Ethiopia), although the resins of *Commiphora gileadensis* (Eastern Mediterranean, Arabian Peninsula), *Commiphora wightii* (northern India) and several other species are often also referred to as myrrh.[1,2] The myrrh resin is harvested by making incisions in the tree to tap the resinous exudate. The gum subsequently hardens to produce a pale yellow resin called myrrh.

Myrrh has been traded on the Arabian Peninsula and in Northern Africa since ancient times. It has long been valued for its health promoting properties and therapeutic effects. There are frequent references to the use of myrrh in religious texts, accounting for its extensive burning as incense in Christian, Jewish and several Orthodox religious ceremonies.[3] Indeed, myrrh is perhaps best known in Western cultures through the biblical story of the 'three wise men' presenting gifts of gold, frankincense and myrrh to the newborn baby Jesus.[4] Later in the Christian bible, Jesus was also offered myrrh in wine as an analgesic prior to the crucifixion. Nowadays, the primary use of myrrh is in perfumery and for aromatherapy, although it is also used as a traditional medicine.

A wide variety of therapeutic uses have also been attributed to myrrh, including its use in the treatment of several inflammatory and skin diseases, as an antiseptic, an antipyretic, a mouth wash, to aid wound healing and as a stimulant.[5,6] Many of these conditions are related to bacterial infections and several studies have reported the growth inhibitory properties of myrrh against panels of pathogens. One study screened *Commiphora molmol* extracts for the ability to inhibit the growth of a panel of bacterial and fungal pathogens.[7] That study tested myrrh for growth inhibitory activity against 7 bacterial (*Staphylococcus aureus*, *Vibrio tubiashii*, *Sterptococcus* spp., *Cellulosimicrobium cellulans*, *Micrococcus luteus*, *Legionella pneumophila*, *Bacillus cereus*) and 2 fungal species (*Fusarium oxysporum*, *Aspergillus flavus*). The *C. molmol* extract inhibited the growth of all microbes screened, although it was particularly potent against *A. flavus, V. tubiashii*, *Sterptococcus* spp., *L. pneumophila* and *B. cereus.* However, the value of that study was limited as MIC's were not determined, making it difficult to compare this activity to that of other antibacterial chemotherapies. Another study examined the growth inhibitory potential of myrrh essential oil against *S. aureus* and reported that the oil not only inhibited bacterial growth, but also inhibited biofilm formation.[8] Furthermore, several myrrh compounds have been isolated and shown to have good antibacterial efficacies. Furanodiene-6-one and methoxyfuranoguaia-9-ene-8-one have been highlighted as being particularly potent growth inhibitors, with MIC values against three bacterial and one fungal species ranging from 0.2–2.8 µg/mL.[9] Simi-

larly, several terpenoids (including the sesquiterpenoids β-elemene and T-cadinol) isolated from *C. molmol* oleo-resin inhibited the growth of a panel of pathogenic bacteria, with MIC ranging from 4-256 µg/mL.[10]

A number of studies have also verified the anti-inflammatory, anti-pyretic, anti-histamine[11] and anti-arthritic pharmacological claims.[12] A recent study reported the myrrh inhibited the production of nitric oxide, prostaglandin E2, tumour necrosis factor-α (TNF-α), but did not affect interleukin (IL)-1β and IL-6.[13] The same study reported that myrrh also inhibited c-jun NH_2-terminal kinase (JNK), but did not affect extracellular signal related kinase (ERK), p_{38} and nuclear factor-κB (NF-κB). Furthermore, myrrh administration decreased leukocyte infiltration, providing evidence for a pleuripotent anti-inflammatory mechanism.

Despite the traditional uses of myrrh in the treatment of inflammatory disease, as well as the well-established growth inhibitory activity of myrrh against other bacterial pathogens, myrrh has not been rigorously evaluated for the ability to block the microbial triggers of autoimmune inflammatory diseases. This may result from a poor understanding of many of these disorders. However, recent serotyping studies have identified bacterial triggers of some of these conditions and the bacterial antigens responsible for the induction of an immune response, allowing for studies to examine the ability to inhibit the trigger mechanisms of these diseases. The major microbial trigger of rheumatoid arthritis has been identified as *Proteus mirabilis*,[14-16] a normal part of the human gastrointestinal flora. Similarly, *Klebsiella pneumoniae* has been shown to initiate ankylosing spondylitis[16-18] and *Acinetobacter baylyi* and *Pseudomonas aeruginosa* have been linked with the onset of multiple sclerosis.[20-22] The development of antibiotic agents targeted at the specific bacterial triggers of autoimmune inflammatory disorders would enable afflicted individuals to target these microbes and thus prevent the onset of the disease and reduce the severity of the symptoms once the disease has progressed. The current study examined the ability for myrrh extracts to block the initiating events of several autoimmune inflammatory diseases by blocking their microbial triggers.

MATERIALS AND METHODS

Myrrh source and extraction

Myrrh was originally sourced from verified *Commiphora molmol* Engler trees in Somalia by Noodles Emporium, Australia and supplied as a dry resin. Voucher samples have been stored in the School of Natural Sciences, Griffith University. Prior to use, the resin was freshly ground to a coarse powder and 1 g quantities were weighed into separate tubes. Individual 50 mL volumes of methanol, water, ethyl acetate, chloroform or hexane were added to the ground resin and extracted by maceration for 24 h at 4ºC with gentle shaking. The extracts were subsequently filtered through filter paper (Whatman No. 54) under vacuum, followed by drying by rotary evaporation in an Eppendorf concentrator 5301. The resultant dry extract was weighed and redissolved in 10 mL deionised water (containing 0.5% DMSO). All solvents were obtained from Ajax Australia and were AR grade.

Qualitative phytochemical studies

Phytochemical analysis of the myrrh extracts for the presence of saponins, phenolic compounds, flavonoids, phytosteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by previously described assays.[23-25]

Antibacterial screening
Test microorganisms

All media was supplied by Oxoid Ltd. Australia. Reference strains of *Klebsiella pneumoniae* (ATCC31488), *Proteus mirabilis* (ATCC21721), *Acinitobacter baylyi* (ATCC33304) and *Pseudomonas aeruginosa* (ATCC39324) were purchased from American Tissue Culture Collection, USA. All other clinical microbial strains were obtained from the School of Natural Sciences teaching laboratory, Griffith University. All stock cultures were subcultured and maintained in nutrient broth at 4ºC.

Evaluation of antimicrobial activity

Antimicrobial activity of all plant extracts was determined using a modified disc diffusion assay.[26-28] Briefly, 100 µL of the test bacteria were grown in 10 mL of fresh nutrient broth media until they reached a count of approximately 10^8 cells/mL. An amount of 100 µL of bacterial suspension was spread onto nutrient agar plates. The extracts were tested for antibacterial activity using 5 mm sterilised filter paper discs. Discs were impregnated with 10 µL of the test sample, allowed to dry and placed onto inoculated plates. The plates were allowed to stand at 4ºC for 2 h before incubation with the test microbial agents. Inoculated plates were incubated at 30ºC for 24 h, then the diameters of the inhibition zones were measured to the closest whole millimetre. Each antimicrobial assay was performed in at least triplicate. Mean values (± SEM) are reported in this study. Standard discs of ampicillin (10 µg) were obtained from Oxoid Ltd., Australia and served as positive controls for antibacterial activity. Filter discs impregnated with 10 µL of distilled water were used as a negative control.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) of the extracts were determined as previously described.[29,30] Briefly, the extracts were diluted in deionised water and tested across a range of concentrations. Discs were impregnated with 10 µL of the test dilutions, allowed to dry and placed onto inoculated plates. The assay was performed as outlined above and graphs of the zone of inhibition versus concentration were plotted for each extract. Linear regression was used to calculate the MIC values.

Toxicity screening
Reference toxin for toxicity screening

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared as a 1.6 mg/mL solution in distilled water and was serially diluted in artificial seawater for use in the *Artemia franciscana* nauplii bioassay.

Artemia franciscana nauplii toxicity screening

Toxicity was tested using a modified *Artemia franciscana* nauplii lethality assay.[31-33] Briefly, 400 µL of seawater containing approximately 46 (mean 46.3, n=120, SD 11.3) *A. franciscana* nauplii were added to wells of a 48 well plate and immediately used for bioassay. A volume of 400 µL of diluted plant extracts or the reference toxin were transferred to the wells and incubated at 25 ± 1ºC under artificial light (1000 Lux). A negative control (400 µL seawater) was run in triplicate for each plate. All treatments were performed in at least triplicate. The wells were checked at regular intervals and the number of dead counted. The nauplii were considered dead if no movement of the appendages was observed within 10 sec. After 24 h, all nauplii were sacrificed and counted to determine the total % mortality per well. The LC_{50} with 95% confidence limits for each treatment was calculated using probit analysis.

Non-targeted GC-MS head space analysis

Separation and quantification were performed using a Shimadzu GC-2010 plus (USA) linked to a Shimadzu MS TQ8040 (USA) mass selective detector system as previously described.[22] 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 Supelco (USA) divinyl benzene/carbowax/polydimethylsiloxane (DVB/CAR/PDMS). Chromatographic separation was accomplished using a 5% phenyl, 95% dimethylpolysiloxane (30 m×0.25 mm id×0.25 um) 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 m, then increased to 270°C over a period of 3 mins 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 70 eV. The analytes were then recorded in total ion count (TIC) mode. The TIC was acquired after a min and for duration of 45 mins utilising a mass range of 45-450 m/z.

Statistical analysis

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

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extraction of 1 g of myrrh with selected solvents yielded dried extracts ranging from 162 mg (hexane extract) to 728 mg (aqueous extract) (Table 1). Ethyl acetate extracted a relatively high yield (642 mg) similar to that of the aqueous extract. In contrast, methanol extracted a similarly low yield (181 mg) to the hexane extract. Chloroform extracted an intermediate amount (307 mg). The dried extracts were resuspended in 10 mL of deionised water (containing 1% DMSO), resulting in the extract concentrations shown in Table 1.

Qualitative phytochemical studies showed that all solvents extracted similar classes of phytochemicals, with polyphenolic compounds (particularly water insoluble polyphenolic compounds), flavonoids, saponins and triterpenoids generally present in the highest levels. All extracts were generally devoid of all other classes of phytochemicals. Interestingly, despite having much lower extraction yields, the methanolic extract contained a similar phytochemical profile and similar relative levels compared to the aqueous extract.

Antimicrobial activity

To determine the growth inhibitory activity of the myrrh extracts against the bacterial triggers of the autoimmune inflammatory diseases, aliquots (10 µL) of each extract were tested in the disc diffusion assay. Reference and clinical strains of *P. mirabilis* were inhibited by all myrrh extracts (Figure 1). The methanolic extract was the most potent *P. mirabilis* growth inhibitor (as assessed by the sizes of the zones of inhibition), with 9.5 ± 0.5 and 9.0 ± 0 mm for the reference and clinical strains respectively. The ethyl acetate and chloroform extracts were also moderate inhibitors of *P. mirabilis* growth, with zones of inhibition >8 mm for each extract against both strains. Whilst the aqueous and hexane extracts also inhibited *P. mirabilis* growth, their inhibition was weaker, with inhibition zones generally ≤7.5 mm in diameter (compared to >10.5 mm for the ampicillin control).

The growth of *K. pneumoniae* (Figure 2) was generally more susceptible to inhibition by the myrrh extracts than was *P. mirabilis* (as judged by the zones of inhibition). Both polar and low polarity extracts were generally good *K. pneumoniae* growth inhibitors, however the higher polarity aqueous and methanolic extracts were generally more potent. Indeed, both the methanolic and aqueous extracts showed zones of inhibition ≥8.3 mm against both the reference and clinical strains of the bacterium. This compares well with the growth inhibition of the positive control (ampicillin, 10 µg), which had inhibition zones of 7-7.3 mm against both strains. The lower polarity chloroform extract was also a good inhibitor of *K. pneumoniae* growth (zones of inhibition 7.3 mm). Whilst also inhibiting *K. pneumoniae* growth, the ethyl acetate and hexane extracts were substantially less potent, with smaller zones of inhibition (6.8-7.5 mm).

Both reference and clinical strains of *A. baylyi* were also inhibited by all of the myrrh extracts (Figure 3). Both strains appeared to be approximately equally susceptible to each extract. The methanolic extract was the most potent growth inhibitor, with zones of inhibition of 7.7 ± 0.3 and 8.0 ± 0.5 mm for the reference and clinical bacterial strains respectively. The ethyl acetate extract was also a good growth inhibitor (zones of inhibition of 7.5 ± 0.4 mm and 7.3 ± 0.3 mm). In addition, the aqueous, chloroform and hexane extracts all inhibited *A. baylyi* growth, with zones of inhibition of 6.6-7.0 mm. However, it is noteworthy that these

Table 1: The mass of dried extracted material, the concentration after resuspension in deionised water, qualitative phytochemical screenings and antioxidant capacities of the myrrh 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	181	18.1	+++	+	+++	-	+++	++	-	-	-	+++	-	-	-
W	728	72.8	+++	+	+++	-	+++	+++	-	-	-	+++	-	-	-
E	642	64.2	++	-	++	-	+	++	-	-	-	++	-	-	-
C	307	30.7	++	-	++	-	-	+++	-	-	-	-	-	-	-
H	162	16.2	+	-	++	-	-	-	-	-	-	-	-	-	-

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay. M=methanolic myrrh extract; W=aqueous myrrh extract; E=ethyl acetate myrrh extract; C=chloroform myrrh extract; H=hexane myrrh extract.

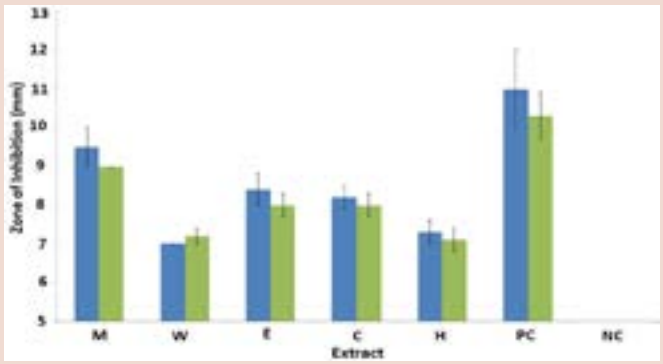

Figure 1: Antibacterial activity of the myrrh extracts against *P. mirabilis* measured as zones of inhibition (mm). The blue bars represent the inhibitory activity against the reference strain (ATCC:21721) and the green bars represent the zones of inhibition against the clinical strain. M = methanolic extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; NC = 0.5% DMSO; PC = ampicillin (10 µg) control. Results are expressed as mean zones of inhibition ± SEM.

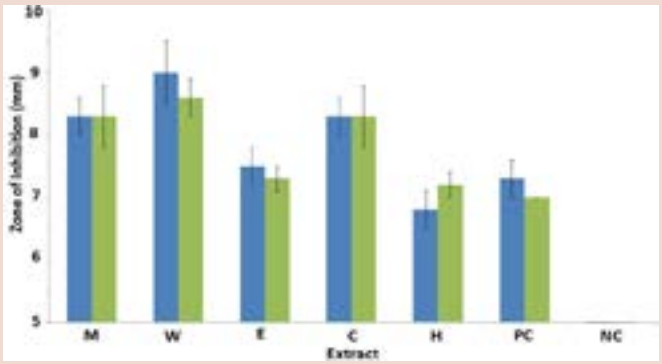

Figure 2: Antibacterial activity of the myrrh extracts against *K. pneumoniae* measured as zones of inhibition (mm). The blue bars represent the inhibitory activity against the reference strain (ATCC:213488) and the green bars represent the zones of inhibition against the clinical strain. M = methanolic extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; NC = 0.5% DMSO; PC = ampicillin (10 µg) control. Results are expressed as mean zones of inhibition ± SEM.

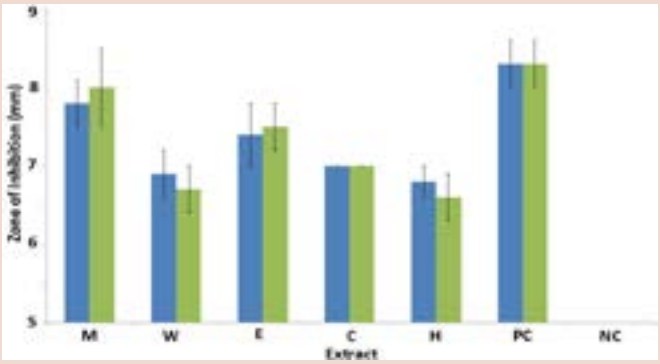

Figure 3: Antibacterial activity of the myrrh extracts against *A. baylyi* measured as zones of inhibition (mm). The blue bars represent the inhibitory activity against the reference strain (ATCC:33304) and the green bars represent the zones of inhibition against the clinical strain. M = methanolic extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; NC = 0.5% DMSO; PC = ampicillin (10 µg) control. Results are expressed as mean zones of inhibition ± SEM.

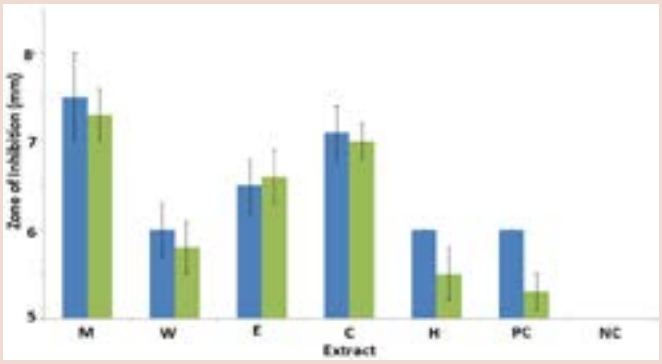

Figure 4: Antibacterial activity of the myrrh extracts against *P. aeruginosa* measured as zones of inhibition (mm). The blue bars represent the inhibitory activity against the reference strain (ATCC:39324) and the green bars represent the zones of inhibition against the clinical strain. M = methanolic extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; NC = 0.5% DMSO; PC = ampicillin (10 µg) control. Results are expressed as mean zones of inhibition ± SEM.

zones of inhibition are indicative of only weak growth inhibition, and are substantially less than the inhibition by the ampicillin control (8.3 mm for both strains).

Both reference and clinical strains of *P. aeruginosa* were also inhibited by all of the myrrh extracts, albeit with relatively small zones of inhibition (Figure 4). The methanolic extract was the best *P. aeruginosa* growth inhibitor with zones of inhibition of 7.5 ± 0.5 mm and 7.3 ± 0.3 mm for the reference and clinical strains respectively. The chloroform extract was also a moderate *P. aeruginosa* growth inhibitor (zones of inhibition of 7.0 ± 0.3 mm and 6.8 ± 0.2 mm for the reference and clinical strains respectively). All other myrrh extracts gave substantially lower zones of inhibition. The hexane extract was a particularly weak growth inhibitor, with zones of inhibition ≤6 mm against both strains. However, it is noteworthy that both *P. aeruginosa* strains were also resistant to the antibiotic control (10 µg ampicillin), with only small zones of inhibition also noted (6.0 ± 0 and 5.3 ± 0.3 mm for the reference and clinical strains respectively).

The antimicrobial efficacy was further quantified by determining the MIC values for each extract against each microbial species. The methanolic extract was generally most effective at inhibiting bacterial growth, particularly growth of *P. mirabilis* and *K. pneumoniae* (Table 2), with MIC values ≤1000 µg/mL (≤10 µg impregnated in the disc), indicating the potential of these extracts in controlling rheumatoid arthritis and ankylosing spondylitis. The methanolic extract was particularly potent against *P. mirabilis* with MIC values of 572 and 463 µg/mL (approximately 6 and 5 µg impregnated in the disc) against the reference and clinical *P. mirabilis* strains respectively. As *P. mirabilis* has been identified as a trigger of rheumatoid arthritis, methanolic myrrh extracts have potential for the prevention of this disease in genetically susceptible individuals. The methanolic extract also was a good inhibitor of *K. pneumoniae* growth with MIC values of 850-950 µg/mL (approximately 9 µg impregnated in the disc). Notably, the methanolic extract also showed moderate inhibitory activity against the bacterial triggers of multiple sclerosis (*A. baylii,* MIC approximately 3000 µg/mL; *P. aeruginosa*, approximately 1500 µg/mL). The aqueous extract was a moderate inhibitor of *K. pneumoniae* growth (MIC values of 3721 and 4866 µg/mL for the reference and clinical strains respectively). Similarly, the ethyl acetate extract was a

Table 2: Minimum bacterial growth inhibitory concentration (µg/mL) of the myrrh extracts and LC_{50} values (µg/mL) in the Artemia nauplii bioassay

	Bacterial Species	Strain	M	W	E	C	H
MIC (µg/mL)	*P. mirabilis*	ATCC:21721	572	>10,000	1774	3974	4785
		Clinical isolate	463	>10,000	2578	4528	5893
	K. pneumoniae	ATCC:213488	947	3721	>10,000	4648	6697
		Clinical isolate	847	4866	>10,000	6188	7323
	A. baylyi	ATCC:33304	2652	>10,000	>10,000	>10,000	>10,000
		Clinical isolate	3012	>10,000	>10,000	>10,000	>10,000
	P. aeruginosa	ATCC:39324	1439	>10,000	>10,000	>10,000	>10,000
		Clinical isolate	1870	>10,000	>10,000	>10,000	>10,000
LC_{50} (µg/mL)	*A. franciscana*	nauplii	2538	>10,000	>10,000	>10,000	-

Numbers indicate the mean MIC and LC_{50} values of triplicate determinations.-indicates no inhibition.

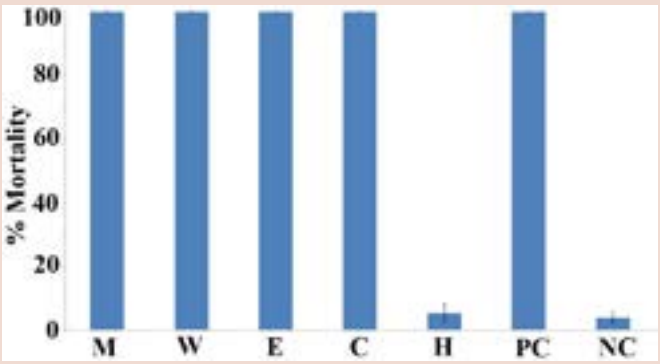

Figure 5: The lethality of the myrrh extracts and the potassium dichromate control (1000 µg/mL) towards *Artemia* nauplii. M = methanolic extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; PC = positive control (1000 µg/ml potassium dichromate); NC = negative (seawater) control. All tests were performed in at least triplicate and the results are expressed as mean ± SEM.

moderate inhibitor of *P. mirabilis* (MIC 17750-2500 µg/mL); chloroform was a moderate inhibitor of *P. mirabilis* and *K. pneumoniae* growth (MIC 1750-5000 µg/mL). Low growth inhibition was noted for all other extract/bacterium combinations, with MIC values generally>5000 µg/mL.

Quantification of toxicity

All extracts were initially screened undiluted in the *Artemia* nauplii bioassay (Figure 5). For comparison, the reference toxin potassium dichromate (1000 µg/mL) was also tested. The potassium dichromate reference toxin was rapid in its onset of mortality, inducing nauplii death within the first 3 h of exposure and 100% mortality was evident following 4-5 h (results not shown). With the exception of the hexane extract, all myrrh extracts induced 100% mortality within 24 h.

To further quantify the effect of toxin concentration on the induction of mortality, the extracts were serially diluted in artificial seawater to test across a range of concentrations in the *Artemia* nauplii bioassay. Table 2 shows the LC_{50} values of the myrrh extracts towards *A. franciscana*. All myrrh extracts were determined to be nontoxic, with LC_{50} values substantially greater than 1000 µg/mL following 24 h exposure. Extracts with an LC_{50} of greater than 1000 µg/mL towards *Artemia* nauplii have previously been defined as being nontoxic.[34]

Non-targeted GC-MS headspace analysis of the myrrh extracts

As the methanolic myrrh extract had the greatest bacterial growth inhibitory efficacy (as determined by MIC; Table 2), it was deemed the most promising extract for further phytochemical analysis. Optimised GC-MS parameters were developed and used to examine the phytochemical composition of this extract. The resultant gas chromatogram is presented in Figure 6. Major peaks were presentin the methanolic extract chromatogram at approximately 11.1, 12.9, 14.4, 17.0, 19.5, and 34.1 min. Numerous smaller peaks were also evident throughout all stages of the chromatograms. In total, 138 unique mass signals were noted for the myrrh methanolic extract (Table 3). Putative empirical formulas and identifications were achieved 36 of these compounds (26%).

DISCUSSION

The current therapeutic regimens for the treatment of autoimmune inflammatory diseases utilise disease modifying anti-rheumatic drugs (DMARDs) to alleviate the symptoms of these diseases and/or alter the disease progression. Unfortunately, none of these therapies are entirely effective and many have been associated with numerous adverse effects.[35] Furthermore, many of the current treatments are aimed at treating the symptoms without addressing the underlying causes and pathogenic mechanisms. Therefore, whilst these treatments may alleviate pain, redness, swelling etc., they do not address the tissue damage that occurs as a consequence of the disease etiology. Furthermore, all of these drugs are used as treatments and there are currently no preventative therapy options. A better understanding of the mechanisms for initiation and progression of the autoimmune inflammatory diseases is important for developing new drugs to target specific processes and thus more effectively treat autoimmune inflammatory diseases.

The studies reported here examined the ability of *Commiphora molmol* oleo-resin (myrrh) extracts to block microbial triggers of 3 autoimmune inflammatory disorders (*Proteus mirabilis*: rheumatoid arthritis; *K. pneumonia*: ankylosing spondylitis; *Acinitobacter baylyi* and *Pseudomonas aeruginosa*: multiple sclerosis). The methanolic extract was identified as being a particularly potent inhibitor of *P. mirabilis* (MIC values of 572 and 463 µg/mL for the reference and clinical strains respectively). Thus, this extract has potential for the development of rheumatoid arthritis inhibitory therapies. Interestingly, as previous studies have demonstrated that *C. molmol* oleo-resin extracts and essential oils also regulate cytokine production,[13] the methanolic myrrh extract, may well have similar prop-

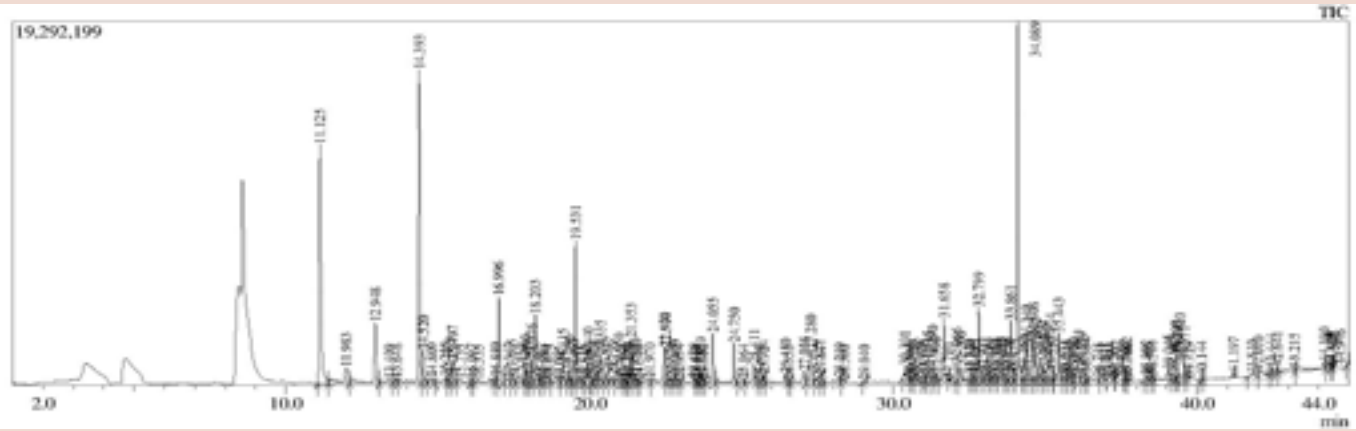

Figure 6: Head space gas chromatograms of 0.5 µL injections of methanolic myrrh extract. The extract was dried and resuspended in methanol for analysis

Table 3: GC-MS analysis of the myrrh methanolic extract, elucidation of empirical formulas and putative identification (where possible) of each compound

Molecular Mass	Molecular Formula	R. Time	Area%	Height%	Putative Identification
		11.125	8.69	8.11	
		11.983	1.47	0.54	
151	$C_8 H_9 NO_2$	12.948	2.93	2.07	Methyl N-hydroxybenzenecarboximidoate
		13.409	0.4	0.14	
		13.648	0.18	0.17	
		14.52	1.2	1.13	
144	$C_8 H_{16} O_2$	14.809	0.31	0.2	Propanoic acid, 2,2-dimethyl-, propyl ester
		15.255	0.51	0.22	
139	C8 H18 O	15.389	0.36	0.34	2-Ethyl-1-hexanol
		15.497	0.85	0.78	
		15.735	0.34	0.14	
		16.097	0.14	0.15	
154	$C_{10} H_{18} O$	16.335	0.54	0.12	Cineole
		16.889	0.24	0.22	
		17.347	0.48	0.18	
		17.445	0.4	0.31	
		17.75	0.23	0.14	
142	$C_9 H_{18} O$	17.89	0.39	0.45	Nonanal
		17.974	0.28	0.29	
122	$C_8 H_{10} O$	18.076	0.61	0.82	2,6-Dimethylphenol
		18.203	1.73	2.32	
		18.362	0.15	0.17	
110	$C_8 H_{14}$	18.5	0.1	0.08	1,2-Dimethyl-1-cyclohexene
		18.673	0.15	0.14	
		19.005	0.25	0.06	
		19.115	0.6	0.67	
152	$C_{10} H_{16} O$	19.46	0.25	0.31	(+)-Sabinol
		19.725	0.15	0.11	
		19.815	0.07	0.1	
122	$C_8 H_{10} O$	19.94	0.97	0.7	o-Xylenol

150	$C_9H_{10}O_2$	20.074	0.19	0.21	Ethyl benzoate
150	$C_{10}H_{14}O$	20.178	0.3	0.3	3-Thujen-2-one
154	$C_{10}H_{18}O$	20.335	0.79	0.95	Terpinen-4-ol
134	$C_9H_{10}O$	20.545	0.38	0.37	p-Ethylbenzaldehyde
154	$C_{10}H_{18}O$	20.739	0.3	0.25	α.-Terpineol
152	$C_{10}H_{16}O$	20.95	0.52	0.51	(-)-Myrtenol
		21.115	0.15	0.16	
254	$C_{17}H_{34}O$	21.206	0.11	0.14	(8Z)-14-Methyl-8-hexadecen-1-ol
		21.27	0.14	0.18	
150	$C_{10}H_{14}O$	21.353	1.27	1.53	Verbenone
		21.5	0.29	0.32	
		21.596	0.1	0.11	
		21.97	0.39	0.16	
		22.47	1.61	1.21	
		22.504	0.75	1.19	
134	$C_9H_{10}O$	22.67	0.23	0.18	3,4-Dimethylbenzaldehyde
		22.809	0.26	0.31	
		22.954	0.14	0.12	
		23.429	0.38	0.16	
		23.51	0.1	0.12	
		23.595	0.09	0.13	
196	$C_{12}H_{20}O_2$	23.659	0.31	0.37	L-α.-bornyl acetate
		23.76	0.12	0.13	
134	$C_{10}H_{14}$	24.055	1.57	1.71	3a,6-Methano-3aH-indene, 2,3,4,5,6,7-hexahydro-
		24.75	1.3	1.37	
		25.064	0.16	0.13	
164	$C_{11}H_{16}O$	25.411	0.69	0.64	Tricyclo[7.1.0.0[1,3]]decane-2-carbaldehyde
		25.62	0.08	0.09	
		25.724	0.25	0.26	
216	$C_{12}H_{24}O_3$	26.45	0.51	0.32	Isobutyl 3-hydroxy-2,2,4-trimethylpentanoate
		26.555	0.09	0.1	
		27.056	0.83	0.4	
		27.28	1.44	1.13	
204	$C_{15}H_{24}$	27.617	0.29	0.29	1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-cyclohexane,
		28.219	0.41	0.18	
		28.38	0.16	0.1	
		29.04	0.07	0.08	
204	$C_{15}H_{24}$	30.391	0.52	0.47	γ-Elemene
		30.545	0.08	0.09	
		30.62	0.14	0.14	
		30.715	0.23	0.26	
204	$C_{15}H_{24}$	30.945	0.17	0.15	4a,8-Dimethyl-2-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,7-octahydronaphthalene
		31.005	0.2	0.24	
204	$C_{15}H_{24}$	31.135	0.58	0.51	β-Longipinene
204	$C_{15}H_{24}$	31.39	0.27	0.28	1H-Cyclopropa[a]naphthalene, decahydro-1,1,3a
206	$C_{14}H_{22}O$	31.658	2.01	2.14	2,4-Di-tert-butylphenol
		31.79	0.01	0.02	

		32.066	0.54	0.62	
		32.2	0.57	0.5	
194	$C_{11}H_{14}O_3$	32.52	0.15	0.2	Benzoic acid, 4-ethoxy-, ethyl ester
		32.597	0.21	0.22	
		32.685	0.2	0.2	
		32.799	2.15	2.47	
		33.114	0.23	0.25	
222	$C_{15}H_{26}O$	33.195	0.1	0.11	α-o-Menth-8-ene-4-methanol
		33.311	0.24	0.23	
		33.389	0.18	0.23	
		33.555	0.32	0.32	
220	$C_{15}H_{24}O$	33.756	0.2	0.27	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1a.alpha.,4a.alpha.,7.beta.,7a.beta.,7b.alpha.)]
		33.861	2.14	2.03	
		33.975	0.16	0.21	
		34.089	9.37	12.16	
230	$C_{15}H_{18}O_2$	34.26	0.28	0.32	Epicurzerenone
		34.349	1.17	1.37	
		34.485	0.67	0.47	
220	$C_{15}H_{24}O$	34.608	1.23	1.48	Isospathulenol
222	$C_{15}H_{26}O$	34.692	0.8	0.91	γ.-Eudesmol
		34.85	0.61	0.83	
		34.952	0.31	0.37	
264	$C_{17}H_{28}O_2$	35.089	0.81	0.73	T-Cadinol acetate
		35.174	0.99	1.08	
		35.257	0.28	0.31	
		35.443	1.4	1.62	4-beta,5-α-Eremophila-1(10),11-diene
		35.715	0.21	0.22	
		35.84	0.2	0.11	
		35.92	0.22	0.25	
		36.083	0.69	0.53	
		36.229	0.55	0.44	
		36.334	0.2	0.27	
		36.718	0.28	0.11	
		36.921	0.15	0.1	
		37.035	0.06	0.05	
204	$C_{15}H_{24}$	37.265	0.01	0.02	
		37.322	0.05	0.1	
		37.615	0.07	0.12	
		37.7	0.06	0.11	
		38.302	0.15	0.18	
		38.405	0.09	0.1	
		38.516	0.15	0.16	
		39.045	0.53	0.34	
		39.185	0.29	0.25	
		39.29	0.82	0.8	
		39.345	0.47	0.86	
		39.42	2.14	1.05	

204	$C_{15}H_{24}$	39.571	0.52	0.61	4-beta,5-α-Eremophila-1(10),11-diene
		39.729	0.06	0.07	
		40.144	0.11	0.14	
		41.197	0.05	0.18	
		41.81	0.32	0.06	
		42.01	0.43	0.16	
		42.444	0.2	0.19	
		42.641	0.22	0.25	
		43.215	0.77	0.12	
		44.19	0.93	0.23	
		44.4	0.07	0.05	
		44.465	0.02	0.03	
		44.505	0.01	0.04	
		44.946	0.03	0.05	

The relative abundance expressed in this table is a measure of the area under the peak expressed as a % of the total area under, or % of the total height of all chromatographic peaks.

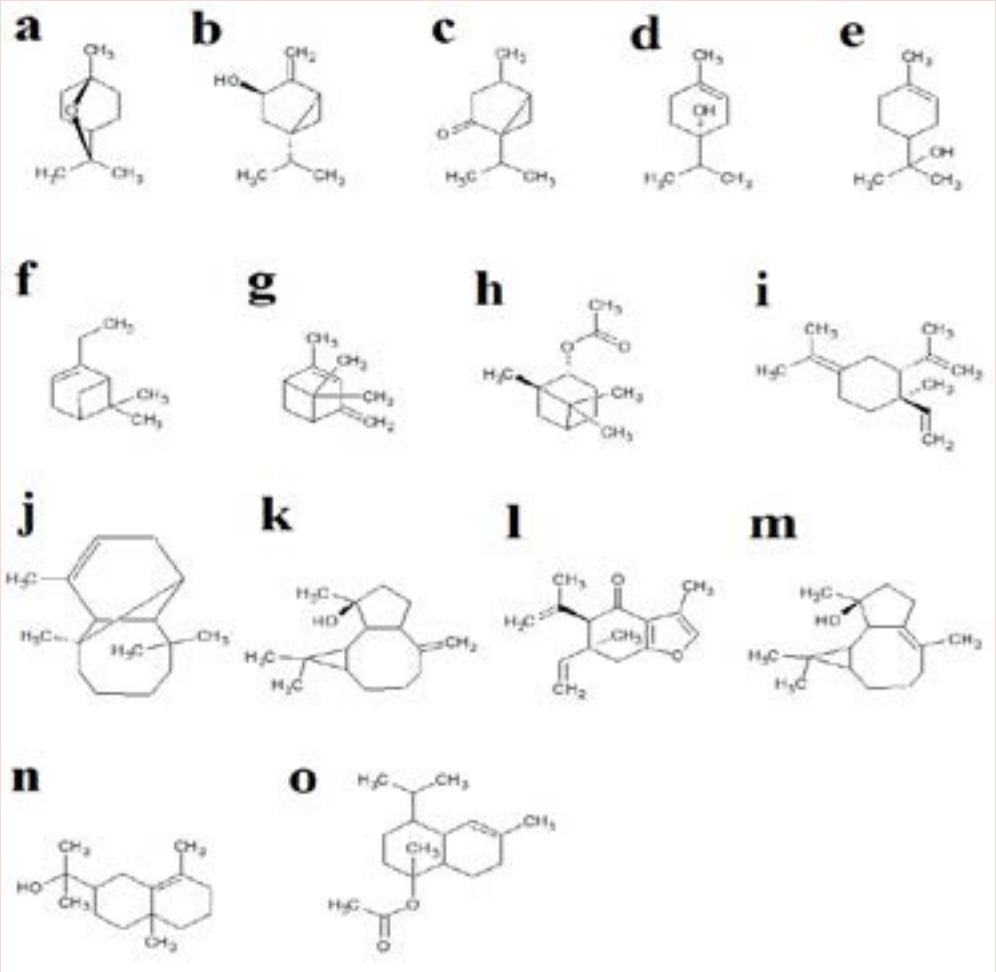

Figure 7: Monoterpenoid and sesquiterpenoid components detected in the methanolic myrrh extract: (a) cineole, (b) sabinol, (c) 3-thujen-2-one, (d)terpinene-4-ol, (e) α-terpineol, (f) myrtenol, (g) verbenone, (h)L-α-bornyl acetate, (i) γ-elemene, (j)β-longipinene, (k) 1H-cyclopropa[a]naphthalene, decahydro-1,1,3a, (l) epicurzerenone, (m) isospathulenol, (n) γ-eudesmol, (o) α-cadinol acetate

erties and further studies are required to test this. If these extracts are subsequently found to modulate cytokine production, they may prove to be particularly useful for individuals suffering from rheumatoid arthritis, as they would provide both preventative and treatment mechanisms.

The methanolic myrrh extract also displayed good *K. pneumonia* growth inhibitory properties (MIC values of 850-950 µg/mL for both the reference and clinical strains), indicating that it may also be useful in the prevention of ankylosing spondylitis. The myrrh extract may also have further effects on other phases of ankylosing spondylitis disease etiology. Indeed, it is possible that the extract may also modulate cytokine production and therefore also block later inflammatory disease events,[13] although this has yet to be tested for our extracts.

Similarly, the methanolic myrrh extract also inhibited *A. baylyi* and *P. aeruginosa* growth, albeit with higher MIC values indicative of only moderate inhibition. However, the therapeutic properties of myrrh in the treatment of autoimmune diseases may be of greater efficacy as synergistic actions may exist between various therapeutic mechanisms (antibacterial, anti-inflammatory, antioxidant, immune-stimulatory etc.), providing combined affects on these complex diseases. Of further note, the antibacterial and MIC assays performed in our study utilised agar based methods. Whilst these are effective in many cases, they often under estimate the efficacy of very low polarity mixtures such as essential oils as the low polarity compounds do not diffuse well within the agar gels. Furthermore, volatile compounds in essential oils are often lost due to evaporation, resulting in falsely low efficacies. Whilst our study tested extracts rather than oils, GC-MS analysis of the methanolic extract detected a number of low polarity, volatile terpenoids. Thus, perhaps testing by liquid dilution MIC techniques may have yielded lower MIC values, indicative of greater efficacy and further studies are required to test this.

GC-MS headspace analysis of the methanolic myrrh extract detected a number of interesting compounds, including a wide diversity of terpenoids. Monoterpenoids were particularly prevalent, with 8 monoterpenoids putatively identified. The monoterpenoids cineole (Figure 7a), sabinol (Figure 7b), 3-thujen-2-one (Figure 7c), terpinene-4-ol (Figure 7d), α-terpineol (Figure 7e), myrtenol (Figure 7f), verbenone (Figure 7g), and L-α-bornyl acetate(Figure 7h) were identified. Monoterpenes have been reported to exert a wide variety of biological effects including antibacterial, antifungal, anti-inflammatory and antitumour activities[36] and therefore may contribute to the growth inhibitory activity against the bacterial triggers of the autoimmune diseases reported here. Indeed, many of the monoterpenoids putatively identified in our study have been previously reported to have potent broad spectrum antibacterial activity.[36] A wide variety of monoterpenoids including camphor, carvone, cineole, borneol, menthone, pinene, terpinene, as well as their derivatives, inhibit the growth of an extensive panel of pathogenic and food spoilage bacteria.[37] Interestingly, several of these monoterpenoids have also been reported to suppress NF-κ B signaling (the major regulator of inflammatory diseases).[38-41] Thus, the terpene components may have a pleuripotent mechanism in blocking the autoimmune inflammatory diseases and relieving its symptoms by acting on both the initiator and downstream inflammatory stages of the disease. Further phytochemical evaluation studies and bioactivity driven isolation of active components are required to further evaluate the mechanism(s) of bacterial growth inhibition.

Several sesquiterpenoids including γ-elemene (Figure 7i), β-longipinene (Figure 7j), 1H-cyclopropa[a]naphthalene, decahydro-1,1,3a (Figure 7k), epicurzerenone (Figure 7l), isospathulenol (Figure 7m), γ-eudesmol (Figure 7n) and α-cadinol acetate (Figure 7o) were also detected in the methanolic myrrh extract. Previous studies have reported bacterial growth inhibitory activities for many sesquiterpenoids including cadinol and its derivatives,[42] caryophyllene,[43] copaene, epicubenol and cubenene.[44] Indeed, a previous study reported that several sesquiterpenoids detected in our study (including the sesquiterpenoids elemene and T-cadinol) inhibited the growth of a panel of pathogenic bacteria, with MIC ranging from 4-256 µg/mL.[10] Thus, these compounds are likely to contribute to the growth inhibitory activity determined in our study.

Notably, despite triterpenoids being detected as a major component for the methanolic myrrh extract in the qualitative screenings, no triterpenoids (nor diterpenoids or sesterterpenoids) were detected by GC-MS headspace analysis. It is perhaps not surprising that triterpenoids were not detected as a mass range cut off of 450 m/z was used in these studies. Therefore, many triterpenoids would have molecular masses that would be near this cut off and may not be detected. However, given the diversity of other terpenoids detected, it is perhaps surprising that no di-and sesterterpenoids were detected in the extract. Due to the low polarity of these compounds, perhaps an analysis of the lower polarity extracts (chloroform, hexane) may have detected these classes of phytochemical.

This highlights an important point: An important consideration of any metabolomic technique is that it will not detect all compounds in a complex mixture, but instead will only detect a portion of them. This is not necessarily a problem when a directed/biased study is undertaken to detect a particular compound or class of compounds and the separation and detection conditions can be optimised for the study. However, when the aim of the study is metabolomic profiling rather than metabolomic fingerprinting, the technique conditions must be chosen and optimised to separate and detect the largest amount of compounds, with the broadest possible physical and chemical characteristics. GC-MS analysis is limited to the detection of the lower polar compounds. Therefore, it is likely that mid and high polarity compounds are present in these extracts and these compounds may also contribute to the growth inhibitory activity reported here. HPLC-MS is a good choice for the detection of mid-highly polar compounds. Thus, further studies are required, focussing on these techniques, to build a more comprehensive understanding of the complete metabolomic profile of the myrrh resin. Furthermore, mass spectral techniques are generally not capable on their own of differentiating between structural isomers. Further studies using a wider variety of techniques are required to confirm the identity of the compounds putatively identified here.

Whilst these studies have demonstrated the potential of the myrrh extracts to treat autoimmune disease, more work is required. This study has only tested these extracts against some microbial triggers of 3 autoimmune diseases (rheumatoid arthritis, ankylosing spondylitis and multiple sclerosis). The microbial triggers for several other autoimmune inflammatory disorders are also known. *Borrelia burgdorferi* is linked with Lyme disease.[45] Similarly, members of the Enterobacteriaceae family are associated with Graves' disease and Kawasaki syndrome. *Mycoplasma pneumoniae* is associated with several demyelinating diseases.[46] Whilst microbial triggers have also been postulated for lupus, the specific causative agents are yet to be identified. It would be interesting to extend our studies to also screen for the ability of the extracts to block these microbial triggers of autoimmune diseases.

CONCLUSION

The results of this study demonstrate the ability of myrrh extracts to block the growth of bacterial species associated with the onset of rheumatoid arthritis, ankylosing spondylitis and multiple sclerosis, and thus their therapeutic potential in genetically susceptible individuals. Further studies aimed at the purification and identification of bioactive components are needed to examine the mechanisms of action of these agents.

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CONFLICTS OF INTEREST

The authors report no conflicts of interest.

ABBREVIATIONS

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

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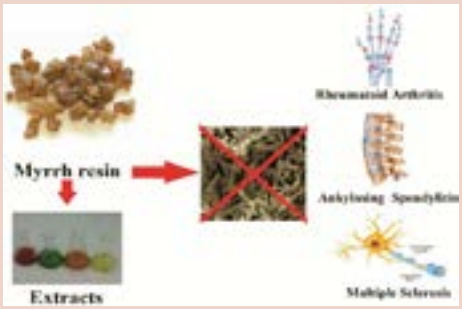
PICTORIAL ABSTRACT



SUMMARY

- Rheumatoid arthritis may be triggered by *P. mirabilis*; ankylosing spondylitisby *K. pneumoniae*; and multiple sclerosis by *A. baylyi* and *P. aeruginosa*.
- Myrrh solvent extracts inhibited these microbial triggers of autoimmune disease *in vitro*.
- The methanolic myrrh extract was most potent against *P. mirabilis* growth, (MIC approximately 500 µg/mL).
- The methanolic myrrh extract was also a good inhibitor of *K. pneumoniae*, and a moderate inhibitor of *A. baylyi* and *P. aeruginosa* growth.
- The other extracts also inhibited bacterial growth, albeit with lower efficacy.
- Phytochemical profiling highlighted several nonpolar compounds as potentially contributing to the growth inhibitory activity of the methanolic myrrh extract.

ABOUT AUTHOR

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 nearly 200 scientific publications in a variety of peer reviewed journals.