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Original Article

GC-MS Analysis of Frankincense Extracts which Inhibit the Growth of Bacterial Triggers of Selected Autoimmune Diseases

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ABSTRACT

Introduction: Frankincense has been used traditionally for the inhibition of microbial growth and for the treatment of rheumatic diseases. Despite this, frankincense 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 frankincense 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 extracts were 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 and aqueous frankincense extracts inhibited the growth of all bacterial species. The growth inhibition of these extracts was particularly notable against *P. mirabilis* and *K. pneumoniae*, with MIC values generally ≤ 1000 µg/mL for both reference and clinical bacterial strains. Indeed, the MIC values of the methanolic extract against *P. mirabilis*, and for the aqueous extract against *K. pneumonia,* were as low as 59.6 and 75.2 µg/mL respectively. The methanolic and aqueous extracts also inhibited the growth of *A. baylyi* and *P. aeruginosa*. However, with the exception of the growth inhibition of *A. baylyi* by the aqueous extract (MIC=4313 µg/mL: moderate inhibitory activity), the MICs against these bacteria was indicative of only low inhibitory activity. The ethyl acetate, chloroform and hexane extracts also inhibited the growth of all bacterial species, albeit with low efficacy (MIC values generally >5000 µg/mL against all bacterial species). All frankincense extracts were non-toxic in the *Artemia franciscana* bioassay, with LC_{50} values substantially above 1000 µg/mL. Non-biased GC-MS headspace analysis of the methanolic and aqueous extracts putatively identified a high diversity of monoterpenoids and sesquiteriterpenoids. **Conclusion:** The lack of toxicity and the inhibitory activity of the methanolic and aqueous frankincense extracts against microbial triggers of rheumatoid arthritis and ankylosing spondylitis indicates their potential in the treatment and prevention of these diseases.

Key words: *Boswellia*, Terpenoid, Monoterpene, Sesquitertene, Rheumatoid Arthritis, Ankylosing spondylitis, Multiple sclerosis.

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INTRODUCTION

Frankincense resin, obtained from trees of the genus *Boswellia* (family Burseraceae), 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. Its use is depicted in an Egyptian wall mural dating to approximately 1458 BC, as well as in the religious texts of several major religions.[1] Three main *Boswellia* spp. (*B. carteri* Birdw.,*B. frereana* Birdw. and *B. serrata* Roxb.) account for nearly all of the global production of frankincense.[2]. The resin is harvested by cutting incisions through the papery bark of mature trees and allowing the resinous exudates to seep out and harden into the orange-brown resin commonly known as frankincense.[3]

There are frequent references to the use of frankincense in religious texts, accounting for its extensive burning as incense in Christian, Jewish and several Orthodox religious ceremonies.[4] Nowadays, the primary use of frankincense is in perfumery and for aromatherapy. However, a wide variety of therapeutic uses have also been attributed to frankincense, including the treatment of skin diseases, urinary tract infections, respiratory infections, to aid in wound healing, as well as for digestive and gastrointestinal disorders.[5] Many of these conditions are related to bacterial infections and several studies have reported the growth inhibitory properties of frankincense against panels of pathogens. One study screened *B. serrata* extracts for the ability to inhibit the growth of an extended panel of microbial pathogens associated with skin infections.[6] That study tested 18 aerobic and 9 anaerobic bacterial strains (including antibiotic resistant strains), as well as 3 fungal strains. The *B. serrata* extracts inhibited the growth of 10 of the 18 aerobic bacteria, and 5 of the 9 anaerobic bacterial strains tested. Furthermore, the MIC values reported in that study were as low as 1 µg/mL against some *Streptococcus* spp., *Cornybacterium* spp., *Clostridium* spp., *Propionobacterium* spp. and *Porphyromonas* spp. In contrast, none of the fungal species tested was affected by the *B. serrata* extracts. In another study, essential oils produced by steam distillation of frankincense resins were reported to inhibit the growth of reference bacterial strains of *Bacillus cereus*, *Escherichia coli* and *Staphylococcus aureus*, as well as the fungal pathogen *Candida albicans,* with MIC values indicative of moderate growth inhibitory activity.[7] A more recent study reported growth inhibitory activity of a frankincense extract against a broader panel of bacterial species,[8] although the value of that study was limited as the extract was only tested at very high concentrations (25-100 mg/mL), making it difficult to compare this activity to that of other antibacterial chemotherapies.

Frankincense has also been used traditionally in the treatment of several inflammatory diseases including rheumatoid arthritis[9] and Crohn's disease.[10] The anti-inflammatory activities and mechanisms of frankincense have been relatively well studied and apleuripotent mechanism has been proposed. One study reported that *B. carterii* extract regulates the production of cytokines in mouse cell cultures.[11] Specifically, the extract inhibited the production of IFN-γ and IL-2. In contrast, the same study reported that production of IL-4 and IL-10 was increased substan-

tially following exposure to the *B. carterii* extract (more than double at some extract concentrations). Other studies have also demonstrated that *B. serrata* extracts strongly inhibit cellular and humoral immune responses in mice.[12] Oral administration of the *B. serrata* extract strongly inhibited antibody production and cellular responses, as well as inhibiting polymorphonuclear leucocyte infiltration.

Despite the traditional uses of frankincense in the treatment of inflammatory disease, as well as the well-established growth inhibitory activity of frankincense against other bacterial pathogens, frankincense has not been studied 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 (Table 1) 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,*[13-5] a normal part of the human gastrointestinal flora. Similarly, *Klebsiella pneumoniae* has been shown to initiate ankylosing spondylitis[16-8] and *Acinetobacter baylyi* and *Pseudomonas aeruginosa* have been linked with the onset of multiple sclerosis.[19-21] 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 frankincense extracts to block the initiating events of several autoimmune inflammatory diseases by blocking their microbial triggers.

MATERIALS AND METHODS

Frankincense source and extraction

Frankincense was originally sourced from verified *Boswellia carteri*-Birdw. trees in Oman 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 hours at 4°C 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 fruit extracts for the presence of saponins, phenolic compounds, flavonoids, polysteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by previously described assays.[22-4]

Antibacterial screening

Test microorganisms

All media was supplied by Oxoid Ltd. Australia. Reference strains of *Klebsiella pneumoniae* (ATCC31488), *Proteus mirabilis* (ATCC21721), *Acinitobacterbaylyi* (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 4°C.

Evaluation of antimicrobial activity

Antimicrobial activity of all plant extracts was determined using a modified disc diffusion assay.[25-7] 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 4°C for 2 hours before incubation with the test microbial agents. Inoculated plates were incubated at 30°C for 24 hours, 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.[28-9] Briefly, the plant extracts were diluted in deionised water and tested across a range of concentrations. Discs were impregnated with 10 µL of the test dilutions, allowed to dry and placed onto inoculated plates. The assay was performed as outlined above and graphs of the zone of inhibition versus concentration were plotted for each extract. Linear regression was used to calculate the MIC values.

Toxicity screening

Reference 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.[30-2] Briefly, 400 µL of seawater containing approximately 48 (mean 47.8, n = 120, SD 10.7) *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 ± 1°C 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 seconds. After 24 h, all nauplii were sacrificed and counted to determine the total % mortality per well. The LC50 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.[21] 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×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

Table 1: The bacterial triggers of the selected autoimmune inflammatory diseases as well as the bacterial antigen and host susceptibility antigen sequences

Disease	Bacterial Trigger	Bacterial Antigen	Bacterial Sequence	Host Antigen	Host Sequence	References
Rheumatoid arthritis	*Proteus mirabilis* and possibly also other Proteus spp.	haemolysin	ESRRAL	MHC class 2 allele HLA-DR4	EQ/KRRAA	13-15
		urease	IRRET	type XI collagen	LRREI	13-15
Ankylosing spondylitis	*Klebsiella pneumoniae*	nitrogenase reductase enzyme	QTDRED	MHC class 1 allele HLA-B27	QTDRED	16-18
		pullulanase	DRDE	MHC class 1 allele HLA-B27	DRED	16-18
		pullulanase	GxP	types I, III and IV collagen	GxP	16-18
Multiple sclerosis	*Pseudomonas aeriginosa*	ϒ-CMLD	TRHAYG	Myelin-neuronal antigen MBP	SRFSYG	19-21
	Acinetobacter spp.	4-CMLD	SRFAYG	Myelin-neuronal antigen MBP	SRFSYG	19-21
		3-OACT-A	LTRAGK	Myelin-neuronal antigen MOG	LYRDGK	19-21
		Acinetobacter regulatory protein	*KKVEEI	Neurofilament-M protein	*KKVEEI	19-21

MOG = myelin oligodendrocyte glycoprotein; MBP = myelin basic protein; 4-CMLD = 4-carboxy-muconolactone decarboxylase; 3-OACT-A = 3-oxoadipate CoA-transferase; ϒ-CMLD = ϒ-carboxy-muconolactone decarboxylase. * indicates the sequence likely to be responsible for cross-reactivity, although this is yet to be confirmed.

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 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 frankincense with the solvents yielded dried extracts ranging from 124 mg (aqueous extract) to 869 mg (methanolic and chloroform extract) (Table 2). With the exception of the aqueous extract, all solvents extracted similar quantities of frankincense material (800-870 µg/mL). This is indicative of the high amounts of mid-low polarity compounds in the frankincense resin. The dried extracts were resuspended in 10 mL of deionised water (containing 1% DMSO), resulting in the extract concentrations shown in Table 2.

Qualitative phytochemical studies showed that all solvents extracted similar classes of phytochemicals. All had low to moderate levels of phenolics. Whilst all extracts also contained saponins, the levels were high in the methanolic, aqueous and hexane extracts, but low in the aqueous and chloroform extracts. The levels of flavonoids was another difference between the extracts, only being detected in the methanolic and aqueous extracts in moderate to high levels, and in low levels in the hexane extract. All other extracts lacked detectable levels of flavonoids. All extracts were generally devoid of all other classes of phytochemicals.

Antimicrobial activity

To determine the growth inhibitory activity of the frankincense 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 frankincense 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 9.3 ± 0.3 and 8.9 ± 0.6 mm for the reference and clinical strains respectively. The ethyl acetate extract was also a moderate inhibitor of *P. mirabilis* growth, with zones of inhibition of approximately 8 mm for both strains. Whilst the other extracts also inhibited *P. mirabilis* growth, their inhibition was substantially 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 frankincense 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 of > 8 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 ethyl acetate, chloroform and hexane extracts also inhibited *K. pneumoniae* growth, albeit with smaller zones of inhibition (6.9-7.5 mm) than the higher polarity extracts.

Both reference and clinical strains of *A. baylyi* were also inhibited by all of the frankincense extracts (Figure 3). Both strains appeared to be

Table 2: The mass of dried extracted material, the concentration after resuspension in deionised water, qualitative phytochemical screenings and antioxidant capacities of the frankincense 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	869	86.9	++	+	++	-	+++	+	-	-	-	+++	-	-	-
W	124	12.4	++	+	++	-	+	+	-	-	-	++	-	-	-
E	808	80.8	+	-	+	-	+++	-	-	-	-	-	-	-	-
C	869	86.9	++	-	++	-	+	-	-	-	-	-	-	-	-
H	840	84	+	-	+	-	+++	-	-	-	-	+	-	-	-

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

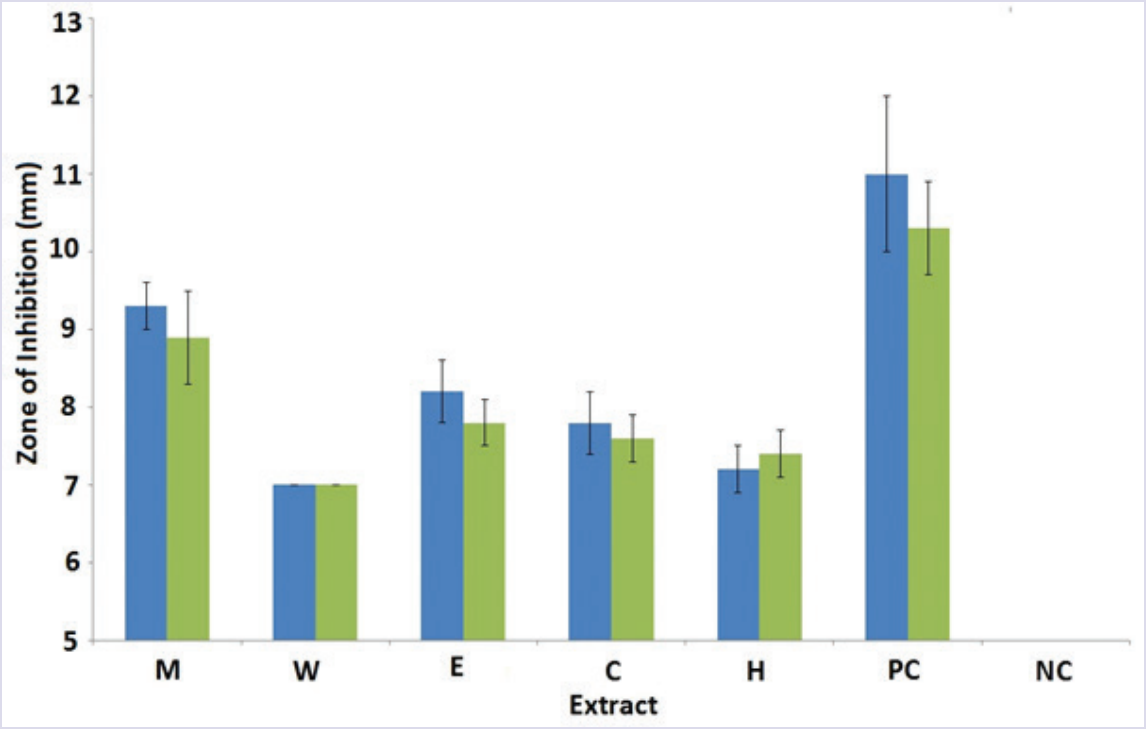

Figure 1: Antibacterial activity of the frankincense extracts against *P. mirabilis* measured as zones of inhibition (mm). The blue bars represent the inhibitory activity against the reference strain (ATCC:21721) and the green bars represent the zones of inhibition against the clinical strain. M = methanolic 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.

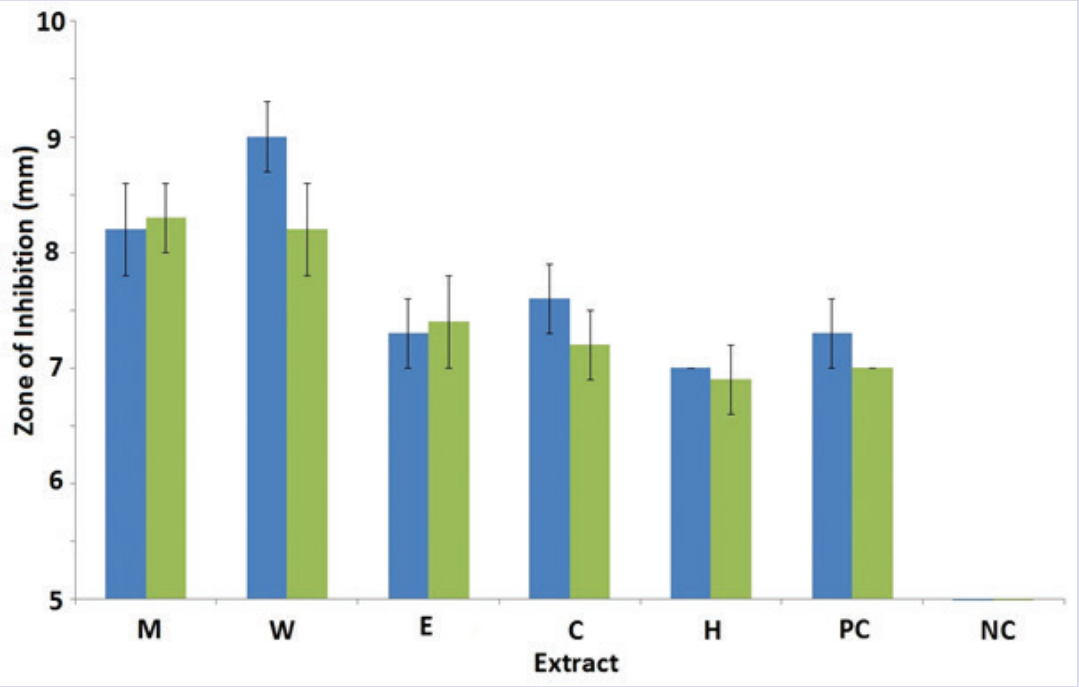

Figure 2: Antibacterial activity of the frankincense extracts against *K. pneumoniae* measured as zones of inhibition (mm). The blue bars represent the inhibitory activity against the reference strain (ATCC:213488) and the green bars represent the zones of inhibition against the clinical strain. M = 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.

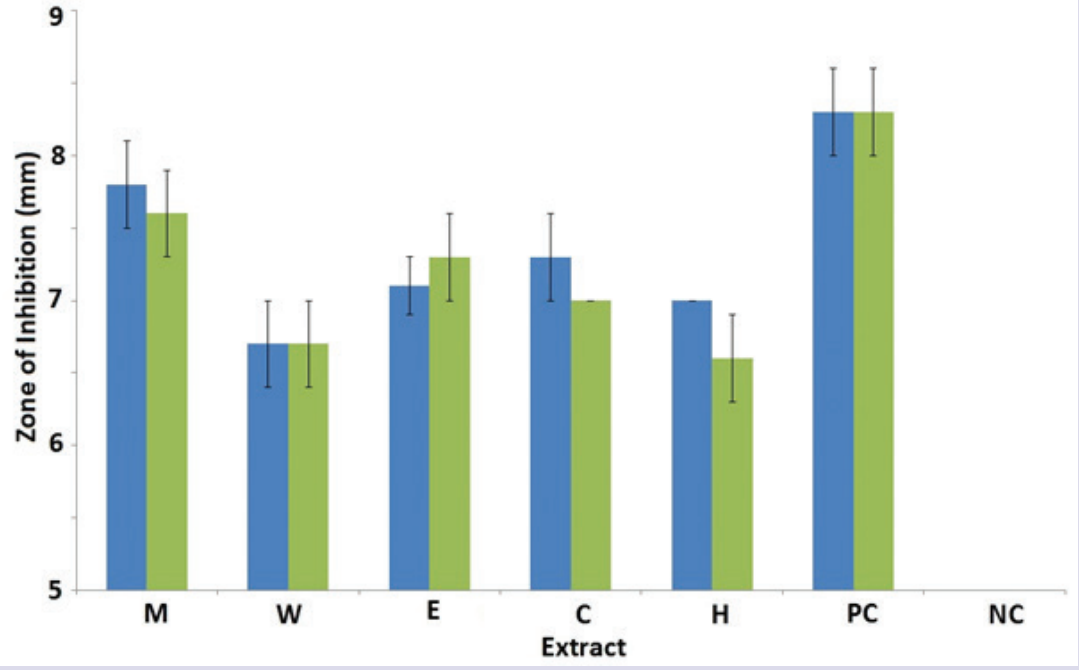

Figure 3: Antibacterial activity of the frankincense extracts against *A. baylyi* measured as zones of inhibition (mm). The blue bars represent the inhibitory activity against the reference strain (ATCC:33304) and the green bars represent the zones of inhibition against the clinical strain.M = methanolic 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.

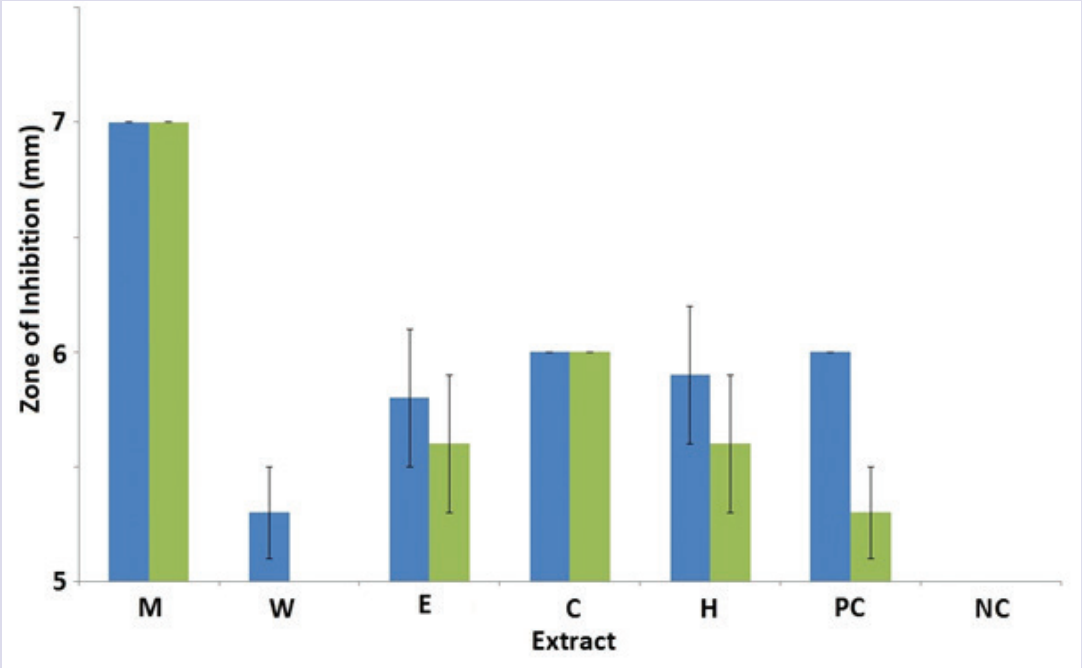

Figure 4: Antibacterial activity ofthe frankincense extracts against *P. aeruginosa* measured as zones of inhibition (mm). The blue bars represent the inhibitory activity against the reference strain (ATCC:39324) and the green bars represent the zones of inhibition against the clinical strain.M = methanolic 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.

Table 3: Minimum bacterial growth inhibitory concentration (µg/mL) of the frankincense 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	59.6	1000	8910	3391	>10,000
		Clinical isolate	123.8	1000	9235	3728	>10,000
	K. pneumoniae	ATCC:213488	1083.5	148	>10,000	7697	>10,000
		Clinical isolate	927.3	75.2	>10,000	8323	>10,000
	A. baylyi	ATCC:33304	>10,000	4313	>10,000	>10,000	>10,000
		Clinical isolate	>10,000	4313	>10,000	>10,000	>10,000
	P. aeruginosa	ATCC:39324	>10,000	>10,000	>10,000	>10,000	>10,000
		Clinical isolate	>10,000	>10,000	>10,000	>10,000	>10,000
LC_{50} (µg/mL)	*A. franciscana*	nauplii	>10,000	4406	>10,000	>10,000	>10,000

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

approximately equally susceptible to each extract. The methanolic extract was the most potent growth inhibitor, with zones of inhibition of 7.8 ± 0.3 and 7.6 ± 0.3 mm respectively.In addition, the aqueous, ethyl acetate, chloroform and hexane extracts all inhibited *A. baylyi* growth, with zones of inhibition of 6.6-7.3 mm. However, it is noteworthy that these 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 frankincense extracts, albeit with relatively small zones of inhibition (Figure 4). The methanolic extract was the best *P. aeruginosa* growth inhibitor, although it only produced zones of inhibition of 7 mm. All other frankincense extracts gave substantially lower zones of inhibition. The aqueous extract was a particularly weak growth inhibitor. Indeed, the aqueous extract only inhibited growth of the reference strain, whilst growth of the clinical *P. aeruginosa* isolate was completely uninhibited. 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 the microbial species which were determined to be susceptible.The methanolic and aqueous extracts were most effective at inhibiting the growth, particularly of *P. mirabilis* and *K. pneumoniae* (Table 3), 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, with MIC values of 59.6 and 123.8 μg/mL (approximately 0.6-1.3 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 frankincense 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 927-1084 μg/mL (approximately 10 μg impregnated in the disc). In contrast, the aqueous extract was a more potent inhibitor of *K. pneumoniae* growth (MIC values of 148 and 75.2 μg/mL for the reference and clinical strains respectively). The aqueous extract was also a good inhibitor of *P. mirabilis* growth, with MIC values of 1000 μg/mL for both strains of the bacterium. Notably, the aqueous extract also showed moderate inhibitory activity against one of the bacterial triggers of multiple sclerosis (*A. baylii,* MIC values of approximately 4300 μg/mL against both strains). The chloroform extract also gave moderate growth inhibition against *P. mirabilis* growth, with MIC values of approximately 3500 μg/mL for both strains. Low growth inhibition was noted for all other extract/bacterium combinations, with MIC values >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 hours of exposure and 100% mortality was evident following 4-5 hours (results not shown). Similarly, all frankincense 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 3 shows the LC_{50} values of the frankincense extracts towards *A. franciscana*. All frankincense 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.[33]

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

As the methanolic and aqueous frankincense extracts had the greatest bacterial growth inhibitory efficacy (as determined by MIC; Table 3), 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 for the methanolic and aqueous extracts are presented in Figures 6a and Figure 6b respectively. Major peaks were present in both the methanolic and aqueous extracts at approximately 15.3, 17.5, 19.1, 20.3, 21.0 and 21.4 min. A further major peak was also noted in the aqueous frankincense extract at 15.9 min, which corresponded to a minor peak in the methanolic extract. Numerous overlapping peaks were also evident throughout all stages of the chromatograms. In total, 74 unique mass signals were noted for the frankincense methanolic and aqueous extracts (Table 4). Putative empirical formulas and identifications were achieved for all of these compounds.

DISCUSSION

Plant remedies are becoming increasingly sought after in the treatment of a myriad of diseases and disorders due both to their perception of greater safety than synthetic drugs, and the failure of current drug regimens to effectively treat many diseases. This is especially true for the autoimmune inflammatory diseases. The current treatments utilising disease modifying anti-rheumatic drugs (DMARDs) to alleviate the symptoms of these diseases and/or alter the disease progression are not entirely effective and have been associated with numerous adverse effects.[34] Furthermore, many of the current treatments are aimed at treating the symptoms without addressing the underlying causes and pathogenic mechanisms. 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 *Boswelliacarteri* resin (frankincense) extracts to block microbial triggers of 3 autoimmune inflammatory disorders (*Proteus mirabilis*: rheumatoid arthritis; *K. pneumonia*: ankylosing spondylitis; *Acinitobacterbaylyi* and *Pseudomonas aeruginosa*: multiple sclerosis). The methanolic extract was identified as being a particularly potent inhibitor of *P. mirabilis* (MIC values of 59.6 and 123.8 μg/mL for the reference and clinical strains respecitively).Thus this extract has potential for the development of rheumatoid arthritis inhibitory therapies. Interestingly, as previous studies have also demonstrated that *B. carterii* extracts regulate cytokine production,[11] the methanolic frankincense extract (which was prepared using *B. carterii* resin), may well have similar properties and further studies are required to test this. If our 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 aqueous extract also displayed good *P. mirabilis* growth inhibitory properties (MIC values of 1000 μg/mL for both the reference and clinical strains), indicating that it may also be useful in the prevention of rheumatoid arthritis. However, the MIC values for the aqueous extract were substantially higher than the methanolic extract. In contrast, the aqueous extract appeared to have greater potential for the prevention and treatment of ankylosing spondylitis. The aqueous frankincense extract had MIC values of 148 and 75.2 μg/mL for reference and clinical *K. pnuemoniae* strains respecitively (compared to approximately 1000 μg/mL for the methanolic extract). As previously discussed for rheumatoid arthritis, it is possible that the aqueous 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, although this has yet to be tested.

GC-MS headspace analysis of the methanolic and aqueous frankincense extracts detected a number of interesting compounds, including a wide diversity of terpenoids. Monoterpenoids were particularly prevalent, with approximately 35 monoterpenoids putatively identified. The monoterpoids 2,4(10)-thujadiene (Figure 7a1), cis-p-mentha-2, 8-dien-1-ol (Figure7a2), cineole (Figure7a4), α-pinene (Figure 7a6), 1,2-dimethyl-1-cyclohexene (Figure 7b1), thujone (Figure 7b2), (1S,3S,4S,5R)-(+)-3-thujanol (Figure 7b3), (+)-sabinol (Figure 7b4), L-camphor (Figure 7b5), α-phellandren-8-ol (Figure 7b6), sabina ketone (Figure 7c1), pinocarvone (Figure 7c2), endo-borneol (Figure 7c3), verbenone (Figure 7c4), terpinene-4-ol (Figure 7c5), cis-m-menth-8-ene(Figure 7c6), α-terpineol (Figure 7d1), (-)-myrtenol (Figure 7d2), sabinyl acetate (Figure 7d3), 2-pinen-7-one (Figure 7d4), 3,4-dimethylbenzaldehyde (Figure 7d5), p-cumic aldehyde (Figure 7e2), D-carvone (Figure 7e3), p-cymen-7-ol (Figure 7e5) and thymol (Figure 7e6)

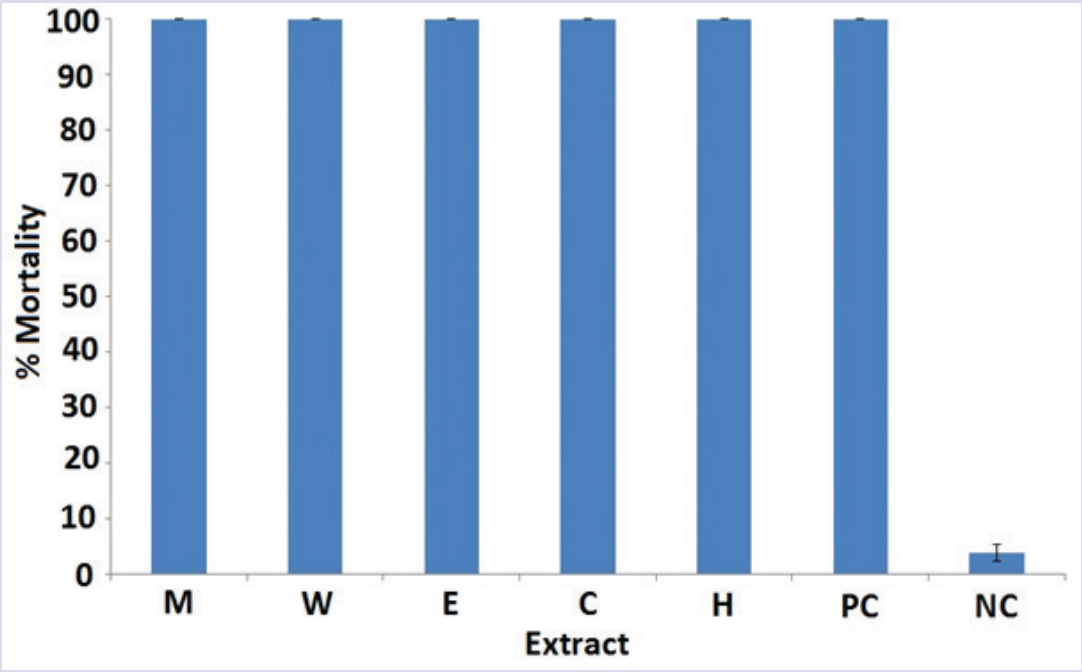

Figure 5: The lethality of the undiluted frankincense extracts and the potassium dichromate control (1000 µg/mL) towards *Artemia* nauplii. M = methanolic extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; PC = positive control (1000 µg/ml potassium dichromate);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

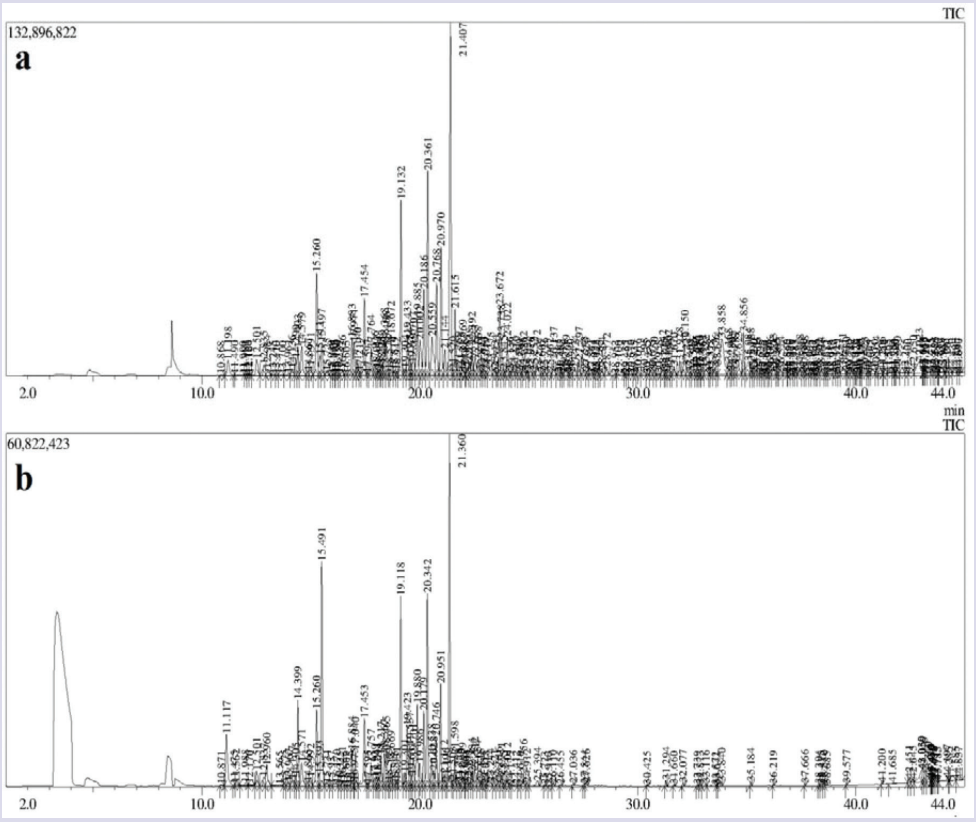

Figure 6: Head space gas chromatograms of 0.5 µL injections of (a) methanolic and (b) aqueous frankincense extracts. The extracts were dried and resuspended in methanol for analysis.

Table 4: GC-MS analysis of the frankincense methanolic and aqueous extracts, elucidation of empirical formulas and putative identification of each compound

Putative Identification	Empirical Formula	Molecular Mass (Da)	Retention Time (min)	Relative Abundance (% Area)	
				M	W
1,3,5,5-Tetramethyl-1,3-cyclohexadiene	$C_{10}H_{16}$	136	10.868	0.04	0.07
Methyl N-hydroxybenzenecarboximidoate	$C_8H_9NO_2$	151	11.198	0.88	3.53
2,4(10)-Thujadiene (isomer 1)	$C_{10}H_{14}$	134	12.501	1.21	0.88
CompName:(1S,3S,5S)-1-Isopropyl-4-methylenebicyclo [3.1.0] hexan-3-yl acetate	$C_{12}H_{18}O_2$	194	12.835	0.47	-
6-Methyl-5-heptene-2-one	$C_8H_{14}O$	126	13.961	0.08	0.2
cis-p-Mentha-2,8-dien-1-ol	$C_{10}H_{16}O$	152	14.136	0.3	0.46
2-Menthene	$C_{10}H_{18}$	138	14.299	0.55	-
Myrtenylisovalerate	$C_{15}H_{24}O_2$	236	14.579	1.2	1.54
Bicyclo [3.1.0] hexan-2-ol, 2-methyl-5-(1-methylethyl)-, (1.alpha.,2.alpha.,5.alpha.)-	$C_{10}H_{18}O$	154	14.991	0.39	0.55
p-Cymene	$C_{10}H_{14}$	134	15.26	3.53	3.41
Cineole	$C_{10}H_{18}O$	154	15.497	1.1	9.34
γ-Terpineol	$C_{10}H_{18}O$	154	15.94	0.02	-
α.-Pinene	$C_{10}H_{16}$	136	16.436	0.21	0.25
Toluene	C_7H_8	92	16.971	0.93	-
1,1-Dimethyl-decyl-mercaptan	$C_{12}H_{26}S$	202	17.219	0.12	-
p-Cymenene	$C_{10}H_{12}$	132	17.454	2.38	-
Linalool	$C_{10}H_{18}O$	154	17.764	0.82	1
1,2-Dimethyl-1-cyclohexene	C_8H_{14}	110	18.011	0.19	0.33
Thujone	$C_{10}H_{16}O$	152	18.368	1.04	1.41
Dehydrosabinene ketone	$C_9H_{12}O$	136	18.466	0.91	1.37
Camphen-6-ol	$C_{10}H_{16}O$	152	18.672	1.12	0.8
2,4(10)-Thujadiene (isomer 2)	$C_{10}H_{14}$	134	18.819	0.16	-
(1S,3S,4S,5R)-(+)-3-Thujanol	$C_{10}H_{18}O$	154	18.942	0.12	0.23
(+)-Sabinol	$C_{10}H_{16}O$	152	19.132	5.02	6.89
L-camphor	$C_{10}H_{16}O$	152	19.304	0.22	0.51
α-Phellandren-8-ol	$C_{10}H_{16}O$	152	19.433	1.19	2.35
Sabina ketone	$C_9H_{14}O$	138	19.707	0.9	1.13
Pinocarvone	$C_{10}H_{14}O$	150	19.885	1.96	3.48
endo-Borneol	$C_{10}H_{18}O$	154	20.002	1.48	0.94
Verbenone	$C_{10}H_{14}O$	154	20.186	2.63	2.61
Terpinen-4-ol	$C_{10}H_{18}O$	154	20.361	5.79	6.93
p-Cymen-8-ol	$C_{10}H_{14}O$	150	20.559	2.04	1.16
cis-m-Menth-8-ene	$C_{11}H_{20}$	138	20.63	-	0.59
α-Terpineol	$C_{10}H_{18}O$	154	20.768	2.8	1.91
(-)-Myrtenol (isomer 1)	$C_{10}H_{16}O$	152	20.97	3.9	3.57
Sabinyl acetate	$C_{12}H_{18}O2$	194	21.144	0.94	0.49
2-Pinen-7-one	$C_{10}H_{14}O$	150	21.407	11.93	12,24
3,4-Dimethylbenzaldehyde	$C_9H_{10}O$	134	21.52	0.12	0.2
Carveol	$C_{10}H_{16}O$	152	21.615	2.97	1.09
cis-p-mentha-1(7),8-dien-2-ol	$C_{10}H_{16}O$	152	21.955	-	0.46
4(10)-Thujen-3-ol, acetate	$C_{12}H_{18}O_2$	194	22.185	0.25	-
p-Cumic aldehyde	$C_{10}H_{12}O$	148	22.278	0.37	0.14
D-Carvone	$C_{10}H_{14}O$	150	22.392	0.78	0.49
(-)-Bornyl acetate	$C_{12}H_{20}O_2$	196	23.672	2.01	-
p-Cymen-7-ol	$C_{10}H_{14}O$	150	23.738	1.08	0.51
Thymol	$C_{10}H_{14}O$	150	24.022	1.07	0.38
(-)-Myrtenol (isomer 2)	$C_{10}H_{16}O$	152	24.923	0.15	0.12
Limonene oxide	$C_{10}H_{16}O$	152	25.252	0.13	-

continued...

Table: 4 Cont'd.

(1S,3S,4S,5R)-1-Isopropyl-4-methylbicyclo [3.1.0] hexan-3-ol	$C_{10}H_{18}O$	144	25.605	0.08	-
Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester	$C_{12}H_{24}O_3$	216	26.465	0.09	0.04
Copaene	$C_{15}H_{24}$	204	26.719	0.34	-
Guaia-1(10),11-diene	$C_{15}H_{24}$	204	27.297	0.53	-
1-Ethyl-5,5-dimethyl-1,3-cyclopentadiene	C_9H_{14}	122	28.364	0.17	-
Caryophyllene	C15 H24	204	28.572	0.31	-
(3S,4aR,5S,8aS)-4a,5-Dimethyl-3-(prop-1-en-2-yl)-2,3,4,4a,5,6-hexahydronaphthalen-1(8aH)-one	$C_{15}H_{22}O$	218	29.67	0.07	-
cis-muurola-3,5-diene	$C_{15}H_{24}$	204	29.825	0.02	-
Humulene	$C_{15}H_{24}$	204	30.016	0.1	-
Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1R-(1.alpha.,7. beta.,8a.alpha.)]-	$C_{15}H_{24}$	204	30.298	0.15	-
epsilon-Muurolene	$C_{15}H_{24}$	204	30.75	0.14	-
Eudesma-4(14),11-diene	$C_{15}H_{24}$	204	31.142	0.37	-
(+)-Ledene	$C_{15}H_{24}$	204	31.402	0.37	-
2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	206	31.67	0.25	0.04
γ-Muurolene	$C_{15}H_{24}$	204	31.928	0.41	-
Ethyl 4-ethoxybenzoate	$C_{11}H_{14}O_3$	194	32.15	1.08	0.49
Cubenene	$C_{15}H_{24}$	204	32.408	0.05	-
Sabinylisobutanoate	$C_{14}H_{22}O_2$	194	32.695	0.13	-
5-Cyclodecen-1-ol, 4,10-bis(methylene)-7-(1-methylethyl)-, (1R,5E,7S)-	$C_{15}H_{24}O$	220	32.962	0.26	-
Epicubenol	$C_{15}H_{26}O$	222	34.623	1.14	-
tau-Cadinol	$C_{15}H_{26}O$	222	34.856	1.11	-
2-((2R,4aR,8aS)-4a-Methyl-8-methylenedecahydronaphthalen-2-yl)prop-2-en-1-ol	$C_{15}H_{24}O$	220	35.431	0.13	-
Azunol	$C_{15}H_{18}$	198	35.505	0.1	-
α-Phellandrene	$C_{10}H_{16}$	136	37.448	0.18	-
Squalene	$C_{30}H_{50}$	410	39.411	0.42	-
1-Propylpentyl laurate	$C_{20}H_{40}O_2$	312	40.954	0.1	-

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

were detected in both the methanolic and aqueous extracts. Seven further monoterpenoids (myrtenylisovalerate (Figure 7a3), γ-terpineol (Figure 7a5), 4(10)-thujen-3-ol, acetate(Figure 7e1), (-)-bornyl acetate (Figure 7e4), limonene oxide (Figure 7f1), sabinylisobutanoate (Figure 7f2), α-phellandrene (Figure 7f3)) were detected only in the methanolic extract, whilst cis-p-mentha-1(7),8-dien-2-ol (Figure 7d6) was only detected in the aqueous extract.

Monoterpenes have been reported to exert a wide variety of biological effects including antibacterial, antifungal, anti-inflammatory and anti-tumour activities[35] 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.[35] 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.[36] Interestingly, several of these monoterpenoids have also been reported to suppress NF-κBsignaling (the major regulator of inflammatory diseases).[37-40] 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 is required to further evaluate the mechanism(s) of bacterial growth inhibition.

Several sesquiterpenoids including copaene (Figure 8a), guaia-1(10), 11-diene (Figure 8b), caryophyllene (Figure 8c), cis-muurola-3, 5-diene (Figure 8d), humulene (Figure 8e), epsilon-muurolene (Figure 8f), eudesma-4(14), 11-diene (Figure 8g), (+)-ledene (Figure 8h), γ-muurolene (Figure 8i), cubenene (Figure 8j), epicubenol (Figure 8k), tau-cadinol (Figure 8l), azunol Figure 8m) were also detected in the methanolic and aqueous frankincense extracts. Previous studies have reported bacterial growth inhibitory activities for many sesquiterpenoids including cadinol,[41] caryophyllene,[42] copaene, epicubenol and cubenene.[43]

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

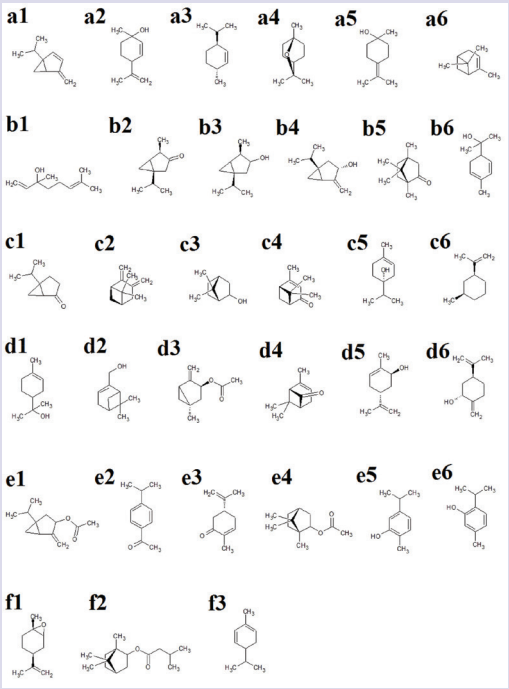

Figure 7: Monoterpenoid components detected in the aqueous and methanolic frankincense extracts: (a1) 2,4(10)-thujadiene, (a2) cis-p-mentha-2,8-dien-1-ol, (a3) myrtenylisovalerate, (a4) cineole, (a5) γ-terpineol, (a6) α-pinene, (b1) 1,2-dimethyl-1-cyclohexene, (b2) thujone, (b3) (1S,3S,4S,5R)-(+)-3-thujanol, (b4) (+)-sabinol, (b5) L-camphor, (b6) α-phellandren-8-ol, (c1) sabina ketone, (c2) pinocarvone, (c3) endo-borneol, (c4) verbenone, (c5) terpinene-4-ol, (c6) cis-m-menth-8-ene, (d1) α-terpineol, (d2) (-)-myrtenol, (d3) sabinyl acetate, (d4) 2-pinen-7-one, (d5) 3,4-dimethylbenzaldehyde, (d6) cis-p-mentha-1(7), 8-dien-2-ol, (e1) 4(10)-thujen-3-ol, acetate, (e2) p-cumic aldehyde, (e3) D-carvone, (e4) (-)-bornyl acetate, (e5) p-cymen-7-ol, (e6) thymol, (f1) limonene oxide, (f2) sabinylisobutanoate, (f3) α-phellandrene.

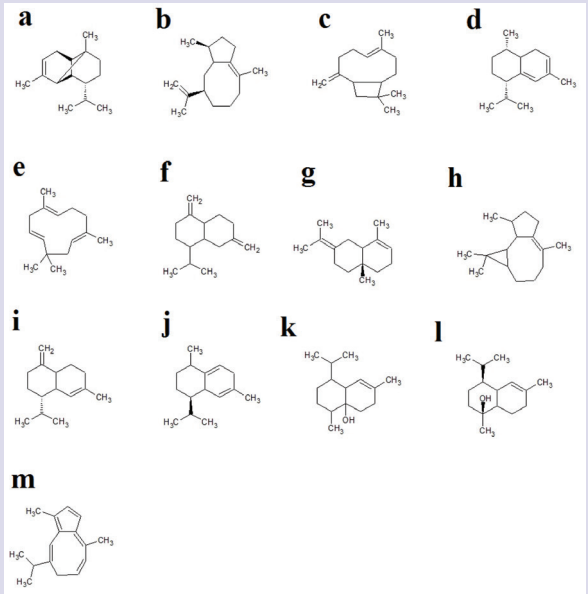

Figure 8: Sesquiterpenoid components detected in the aqueous and methanolic frankincense extracts: (a) copaene, (b) guaia-1(10), 11-diene, (c) caryophyllene, (d) cis-muurola-3,5-diene, (e) humulene, (f) epsilon-muurolene, (g) eudesma-4(14),11-diene, (h) (+)-ledene, (i) γ-muurolene, (j) cubenene, (k) epicubenol, (l) tau-cadinol, (m) azunol.

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 compounds of mid-highly polar compounds. Thus, further studies are required, focussing on these techniques, to build a more complete understanding of the complete metabolomic profile of the frankincense extracts. Furthermore, it is noteworthy that 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 frankincense 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.[44] Similarly, members of the *Enterobacteriaceae* family are associated with Graves' disease and Kawasaki syndrome. *Mycoplasma pneumoniae* is associated with several demyelinating diseases.[45] 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 frankincense extracts to block the growth of bacterial species associated with the onset of rheumatoid arthritis and ankylosing spondylitis 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.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors report no conflicts of interest.

ABBREVIATION USED

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

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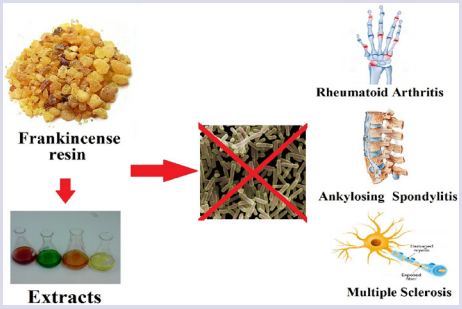
PICTORIAL ABSTRACT



SUMMARY

- Rheumatoid arthritis may be triggered by *P. mirabilis*; ankylosing spondylitisby K. pneumoniae; and multiple sclerosis by *A. baylyi* and *P. aeruginosa*.
- Aqueous and methanolic frankincense solvent extracts inhibited these microbial triggers of autoimmune disease *in vitro*.
- The methanolic frankincense extract was a particularly potent inhibitor of *P. mirabilis* (MIC 60 µg/mL).
- The aqueous extractswere alsoa potent inhibitor of *K. pneumoniae* (MIC 75 µg/mL).
- The other extracts also inhibited bacterial growth, albeit with lower efficacy.
- Phytochemical profiling highlighted several terpenoids as potentially contributing to the growth inhibitory activity of the methanolic frankincense extract.

ABOUT AUTHORS

Dr Ian Cock: Leads a research team in the Environmental Futures Research Institute and the School of Natural Sciences at Griffith University, Australia. His research involves bioactivity and phytochemical studies into a variety of plant species of both Australian and international origin including *Aloe vera*, South Asian and South American tropical fruits, as well as Australia plants including *Scaevola spinescens*, *Pittosporum phylliraeoides*, *Terminalia ferdinandiana* (Kakadu plum), Australian Acacias, Syzygiums, Petalostigmas and *Xanthorrhoea johnsonii* (grass trees). This range of projects has resulted in nearly 200 scientific publications in a variety of peer reviewed journals.