

The Therapeutic Properties of *Juniperus communis* L.: Antioxidant Capacity, Bacterial growth Inhibition, Anticancer Activity and Toxicity

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ABSTRACT

Introduction: *J. communis* berry is a high antioxidant fruit which is used in several traditional medicinal systems to treat a variety of diseases including rheumatism, arthritis and gout. This study was undertaken to examine the inhibitory activity of *J. communis* berry extracts on the growth of several bacteria associated with autoimmune inflammatory disease, and to test their ability to block CaCo₂ and HeLa cancer cell proliferation. **Methods:** *J. Communis* solvent extracts were prepared using solvents of varying polarity. The extracts were investigated by disc diffusion assay for the ability to inhibit the growth of a panel of pathogenic bacteria associated with autoimmune inflammatory diseases. Their MIC values were determined to quantify and compare their efficacies. Inhibitory activity against CaCo₂ and HeLa human carcinoma cell lines was evaluated using an MTS colorimetric cell proliferation assay. Toxicity was determined using the *Artemia franciscana* nauplii bioassay. **Results:** The methanol, water and ethyl acetate *J. communis* berry extracts displayed moderate to potent growth inhibitory activity against bacterial triggers of rheumatoid arthritis, ankylosing spondylitis and multiple sclerosis. The methanol and water extracts displayed the broadest specificity, inhibiting the growth of all bacteria tested. The ethyl acetate extract also displayed antibacterial activity, inhibiting the growth of 9 of the 13 bacterial strains (69%). The ethyl acetate extract displayed the greatest potency, with MIC values substantially below 2000 µg/mL for all bacteria which it inhibited. It was most effective at inhibiting the growth of *P. mirabilis*, *P. vulgaris* and *S. aureus*, each with MIC's ≤ 500 µg/mL. The

methanol and water extracts also proved effective at blocking the proliferation of the colorectal cancer cell line CaCo₂ and HeLa cervical cancer cell growth, with IC₅₀ values in the 1300-2500 µg/mL range. All extracts were non-toxic in the *Artemia* nauplii bioassay. **Conclusion:** The lack of toxicity of the *J. Communis* berry extracts and their potent growth inhibitory bioactivity against bacteria and HeLa and CaCo₂ carcinoma cells indicates their potential in the treatment and prevention of selected autoimmune inflammatory diseases and some cancers.

Key words: Anti-bacterial activity, Traditional medicine, Juniper berry, Auto-immune inflammatory disease, CaCo₂, HeLa, *Artemia*, Antioxidant.

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INTRODUCTION

Plants have been used for thousands of years as medicines for treating a variety of different diseases and medical complaints by most, if not all civilisations. As well as being useful therapeutic agents in their own right, an understanding of these traditional medicines has provided new plant derived drug leads for allopathic medicine. Phytochemicals have provided modern medicine with new analgesics, anti-inflammatory, anti-cancer, cardiovascular and immune-modulatory drugs.¹ Furthermore, with the development of increasing numbers of antibiotic resistant bacterial strains, much recent attention has also focussed on screening traditional medicines for bacterial growth inhibitory activity and the isolation of bioactive components from promising species.¹ Traditional plant medicines have also provided modern medicine with laxatives, anti-depressants and with new leads for the treatment of diabetes mellitus.²⁻³

The genus *Juniperus* (Family Cupressaceae) consists of approximately 60 species, although taxonomists disagree on the exact number.⁴⁻⁵ Most species are distributed throughout the Northern Hemisphere, with some species extending as far south as tropical Africa. The best known and perhaps most useful species is *Juniperus communis* L. (common juniper). This species has the largest natural range of any woody plant, extending from the Arctic regions of Asia, Europe and North America south to approximately 30°N latitude, although some studies have reported that natural populations also occur in the Southern Hemisphere.⁵

J. communis produces astringent blue-black seed cones, commonly known as juniper berries. These are used as culinary components by a variety of cultures in the regions in which they occur. The berries are used to flavour meats and sauces and as the flavouring agent for the alcoholic beverage gin. They have also been used in several traditional medicine systems. *J. Communis* berries are used in traditional Turkish medicine as a diuretic, as well as for gastrointestinal problems and as a general antiseptic.⁶ They are also used for the treatment of rheumatism, arthritis and gout and are believed to have both anti-inflammatory and analgesic effects.⁷ Native Americans used *J.communis* berries as an appetite suppressant and in the treatment of diabetes.⁸ Indeed, hypoglycemic/anti-diabetic effects have been demonstrated in several laboratory studies.²⁻³

Recent studies have reported strong antioxidant activity for *J.communis* berries.⁹ Further studies have examined the inhibitory activity of *J. communis* berry essential oils against *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes*, *Cornybacterium* spp. 754 and *Staphylococcus aureus*¹⁰ and against an extended panel of pathogenic bacteria.¹¹⁻¹² However, despite the traditional usage of *J.communis* berries for the treatment of autoimmune inflammatory diseases (e.g. rheumatoid arthritis), growth inhibition of the bacterial triggers of these diseases remains relatively unexplored. This study examines the growth inhibitory

activity of *J. Communis* berry extracts on the growth of bacteria associated with autoimmune diseases with the aim of determining new leads for the prevention and treatment of these diseases. The extracts were also tested for anti-proliferative activity against HeLa and CaCo₂ carcinoma cell lines, and for toxicity.

MATERIALS AND METHODS

Plant source and extraction

Dried *Juniper communis* berries were supplied and verified by the Melbourne Food Ingredient Depot, Australia. Voucher samples have been stored in the School of Natural Sciences, Griffith University. The plant materials were stored at -30°C. Prior to use, the plant materials were thawed and freshly ground to a coarse powder. Individual 1 g quantities of the ground plant material were weighed into separate tubes and 50 mL of methanol, water, ethyl acetate, chloroform or hexane were added. All solvents were obtained from Ajax and were AR grade. The ground plant materials were individually extracted in each solvent for 24 h 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 1% DMSO).

Qualitative phytochemical studies

Phytochemical analysis of the extracts for the presence of saponins, phenolic compounds, flavonoids, polysteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by previously described assays.¹³⁻¹⁴

Antioxidant capacity

The antioxidant capacity of each sample was assessed using the DPPH free radical scavenging method¹⁵⁻¹⁶ with modifications. Briefly, a DPPH solution was prepared fresh as a 400 µM solution by dissolving DPPH (Sigma) in AR grade methanol (Ajax, Australia). The initial absorbance of the DPPH solution was measured at 515 nm using a Molecular Devices, Spectra Max M3 plate reader and did not change significantly throughout the assay period. A 2 mL aliquot of each extract was dried by evaporation and the residue resuspended in 2 mL of methanol. Each extract was added to a 96-well plate in amounts of 5, 10, 25, 50, 75 µL in triplicate. Methanol was added to each well to give a volume of 225 µL. A volume of 75 µL of the fresh DPPH solution was added to each well for a total reaction volume of 300 µL. A blank of each extract concentration, methanol solvent, and DPPH was also performed in triplicate. Ascorbic acid was prepared fresh and examined across the range 0-25 µg per well as a reference and absorbance were recorded at 515 nm. All tests were performed in triplicate and triplicate controls were included on each plate. The antioxidant capacity based on DPPH free radical scavenging ability was determined for each extract and expressed as µg ascorbic acid equivalents per gram of original plant material extracted.

Antibacterial screening

Test microorganisms

All media was supplied by Oxoid Ltd. Reference strains of *Acinetobacter baylyi* (ATCC33304), *Escherichia coli* (ATCC0157), *Klebsiella pneumoniae* (ATCC31488), *Proteus mirabilis* (ATCC21721), *Proteus vulgaris* (ATCC21719), *Pseudomonas aeruginosa* (ATCC39324) and *Staphylococcus aureus* (ATCC25923) were purchased from American Tissue Culture Collection, USA. All other microbial strains were obtained from Michelle Mendel, Griffith University. Stock cultures of

all bacterial stocks 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.¹⁷⁻¹⁹ Briefly, 100 µL of each test bacteria were grown in 10 mL of fresh nutrient broth media until they reached a count of approximately 10⁸ 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 h before incubation at 30°C for 24 h. The diameters of the inhibition zones were measured in millimetres. All measurements were to the closest whole millimetre. Each assay was performed in at least triplicate. Mean values (± SEM) are reported in this study. Standard discs of ampicillin (2 µg) and chloramphenicol (10 µg) were obtained from Oxoid Australia Ltd. 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 was determined as previously described.¹⁷⁻¹⁹ 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.

Screen for anti-cancer bioactivity Cancer cell lines

The CaCo₂ and HeLa carcinoma cell lines used in this study were obtained from American Type Culture Collection (Rockville, USA). The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies), supplemented with 20 mM HEPES, 10 mM sodium bicarbonate, 50 µg/mL streptomycin, 50 IU/mL penicillin, 2 mM glutamine and 10% foetal calf serum (Life Technologies). The cells were maintained as monolayers in 75 mL flasks at 37°C, 5% CO₂ in a humidified atmosphere until approximately 80% confluent.

Evaluation of cancer cell anti-proliferative activity

The anti-proliferation screening study was conducted using standard methods.²⁰⁻²¹ Briefly, 1 mL of trypsin (Sigma) was added to the culture flasks and incubated at 37°C, 5% CO₂ for 15 min to dislodge the cancer cells. The cell suspensions were then transferred to a 10 mL centrifuge tube and sedimented by centrifugation. The supernatant was discarded and the cells were resuspended in 9 mL of fresh media. Aliquots of the resuspended cells (70 µL, containing approximately 5000 cells) were added to the wells of a 96 well plate. A volume of 30 µL of the test extracts or cell media (for the negative control) was added to individual wells and the plates were incubated at 37°C, 5% CO₂ for 12 h in a humidified atmosphere. A volume of 20 µL of Cell Titre 96 Aqueous One solution (Promega) was subsequently added to each well and the plates were incubated for a further 3 h. Absorbances were recorded at 490 nm using a Molecular Devices, Spectra Max M3 plate reader. All tests were performed in at least triplicate and triplicate controls were included on each plate. The anti-proliferative activity of each test was calculated as a percentage of the negative control using the following formula:

$$\text{Proliferation (\% untreated control)} = \left(\frac{A_{ct}}{A_{cc}} \right) \times 100$$

A_{ct} is the corrected absorbance for the test extract (calculated by subtracting the absorbance of the test extract in media without cells from

the extract cell test combination) and A_{cc} is the corrected untreated control (calculated by subtracting the absorbance of the untreated control in media without cells from the untreated cell media combination).

Toxicity screening

Reference toxin for toxicity screening

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared as a 4 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.²²⁻²⁴ Briefly, 400 μ L of seawater containing approximately 47 (mean 46.8, n=90, SEM12.7) *A. franciscana* nauplii were added to wells of a 48 well plate and immediately used for the bioassay. A volume of 400 μ L of diluted plant extracts or the reference toxin were transferred to the wells and incubated at $25 \pm 1^\circ\text{C}$ under artificial light (1000 Lux). A negative control (400 μ L 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.

Statistical analysis

Data are expressed as the mean \pm SEM of at least three independent experiments. One way ANOVA was used to calculate statistical significance between control and treated groups with a P value < 0.01 considered to be statistically significant.

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extraction of 1 g of dried *J. Communis* berry with various solvents yielded dried plant extracts ranging from 32.1 mg (hexane extract) to 487.8 mg (methanolic extract) (Table 1). Methanol and water gave the highest yields of dried extracted material (487.8 and 378.4 mg respectively). Ethyl acetate, chloroform and hexane extracted lower masses (146.3, 218.8, and 32.1 mg respectively). The dried extracts were resuspended in 10 mL of deionised water resulting in the extract concentrations shown in Table 1. Phytochemical studies (Table 1) showed that methanol and water extracted the widest range and largest quantity of phytochemicals in this study. Both had high levels of phenolics (both water soluble and insoluble), alkaloids and tannins, as well as moderate levels of flavonoids. Lower levels of cardiac glycosides, saponins and triterpenoids were also evident. Ethyl acetate extracted similar classes of phytochemicals, although generally at lower levels. Phenolic compounds and flavonoids were present in high to moderate levels in the chloroform and hexane extracts.

Antioxidant content

Antioxidant capacity (expressed as ascorbic acid equivalents) for the *J. communis* berry (Table 1) ranged from below the level of detection to a high of 196 mg ascorbic acid equivalents per gram of dried berry extracted (methanolic extract). The aqueous extract also had a relatively high antioxidant capacity (30.3 mg ascorbic acid equivalents), whilst the ethyl acetate and hexane extract contained relatively low antioxidant capacities (1.9 and 1.7 mg ascorbic acid equivalents respectively). The antioxidant capacity of the chloroform extract was below the threshold of detection.

Anti-microbial activity

To determine the ability of the crude plant extracts to inhibit the growth of the panel of pathogenic bacteria, aliquots (10 μ L) of each extract were screened using a disc diffusion assay (Figure 1). The bacterial growth of every bacterium tested was inhibited by at least 1 of the *J. communis* berry extracts. *K. pneumoniae* was the most susceptible bacterial species, being inhibited by all of the extracts. The *Proteus* spp. were also highly susceptible to the *J. communis* berry extracts, with all extracts except the hexane extract inhibiting the growth of both *P. mirabilis* and *P. vulgaris*. The methanolic, aqueous and ethyl acetate extracts inhibited the growth of *A. baylyi* and *S. aureus* (both reference and clinical strains), whilst *E. coli* and *P. aeruginosa* (both reference and clinical strains) were inhibited only by the methanolic and aqueous *J. communis* berry extracts. All extracts appeared to be moderate bacterial growth inhibitors (as judged by zone of inhibition), with inhibition zones substantially < 10 mm for all extracts against all bacterial species. The level of inhibition was similar to that of the ampicillin control (2 μ g), which had zones of inhibition of ≤ 10 mm for most bacterial species. Chloramphenicol (10 μ g) was a substantially more potent growth inhibitor, with zones of inhibition 10-17 mm against most bacteria.

The antimicrobial efficacy was further quantified by determining the MIC values (Table 2). The ethyl acetate *J. communis* extract was a particularly effective bacterial growth inhibitor, with MIC values substantially < 1000 μ g/mL against several bacterial species. Indeed, the MIC for the ethyl acetate extract against the reference and clinical *S. aureus* strains was 370 and 425 μ g/mL respectively (this equates to approximately 4 μ g impregnated in the disc). The ethyl acetate extract was also potent against the *Proteus* spp. with MIC's of 500 μ g/mL (5 μ g impregnated in the disc) for reference strains of *P. mirabilis* and *P. vulgaris*, as well as the clinical *P. mirabilis* strain. The ethyl acetate extract also displayed good antibacterial activity against *A. baylyi* and *K. pneumoniae* (reference and clinical strains) with MIC's < 2000 μ g/mL (20 μ g impregnated in the disc). Whilst less potent bacterial growth inhibitors, the methanolic and aqueous extracts also displayed good growth inhibition with MIC's 1000-3000 μ g/mL (10-30 μ g impregnated in the disc) against most bacteria. The chloroform and hexane extracts were either inactive or showed only low inhibitory activity (MIC values > 3000 μ g/mL).

Inhibition of cancer cell proliferation

The *J. communis* berry extracts were tested against 2 cancer cell lines (CaCo₂ colorectal carcinoma cells, Figure 2; HeLa cervical cancer cells, Figure 3) to determine their ability to inhibit cancer cell growth. The methanol and water extracts displayed potent inhibitory activity against CaCo₂ cells, with proliferation inhibited to as low as 6.3 ± 2.6 % of the untreated control cell growth for the methanolic extract (Figure 2). Indeed, both of these extracts were substantially more potent inhibitors of cellular proliferation than cisplatin (10 mg/mL) which blocked approximately 50% of the cellular proliferation. The ethyl acetate, chloroform and hexane extracts also significantly inhibiting CaCo₂ proliferation, albeit to a much lesser extent. Inhibition of proliferation by the *J. communis* berry extracts was dose dependent, with the level of inhibitory activity decreasing at lower concentrations (Table 2).

The *J. communis* berry extracts were also shown to affect the proliferation of HeLa cells, although only the methanolic and aqueous extracts significantly inhibited HeLa proliferation (Figure 3). Furthermore, the inhibition was of lower efficacy than was evident with the CaCo₂ cells. The methanol extract inhibited HeLa cell proliferation to approximately 23% of the untreated control cell growth. Similarly, the aqueous extract inhibited HeLa proliferation to approximately 18% of the untreated control proliferation. In contrast to the CaCo₂ study, the proliferation rates for the HeLa cells treated with the ethyl acetate, chloroform and hexane

Table 1: The mass of dried extracted material, the concentration after resuspension in deionised water, qualitative phytochemical screenings of the plant extracts

Extract	Mass of Dried Extract (mg)	Concentration of Resuspended Extract (mg/ml)	Antioxidant capacity (μg ascorbic acid equivalents/ml)	Total Phenolics	Water Soluble Phenolics	Water Insoluble Phenolics	Cardiac Glycosides	Saponins	Triterpenes	Polysteroids	Alkaloids (Mayer Test)	Alkaloids (Wagner Test)	Flavonoids	Tannins	Free Anthraquinones	Combined Anthraquinones
M	487.8	48.8	196	+++	++	+++	++	+	+	+	++	+++	++	+++	-	+
W	378.4	37.8	30.3	+++	+++	+++	+	+	+++	-	++	+++	++	+++	-	++
E	146.3	14.6	1.9	+++	+	+++	-	+	-	-	-	++	++	+++	-	-
C	218.8	21.9	BDT	+++	++	+++	-	+	-	-	-	+	++	-	-	-
H	32.1	3.2	1.7	+++	++	+++	-	+	-	-	-	+	++	-	-	-

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay; BDT=below the detection threshold.

Table 2: Minimum inhibitory concentrations ($\mu\text{g}/\text{mL}$), HeLa and CaCo_2 antiproliferative IC_{50} values ($\mu\text{g}/\text{mL}$), and LC_{50} values ($\mu\text{g}/\text{ml}$) for *Artemia franciscana* nauplii exposed to *J. communis* berry extracts and control solutions

	JBM	JBW	JBE	JBC	JBH	PC	
MIC ($\mu\text{g}/\text{mL}$)	<i>A. baylii</i> (ATCC33304)	1923	1750	1436	-	ND	
	<i>A. baylii</i> (clinical isolate)	2238	1750	1787	-	ND	
	<i>E. coli</i> (ATCC0157)	1287	1844	-	-	ND	
	<i>E. coli</i> (clinical isolate)	945	1634	-	-	ND	
	<i>K. pneumoniae</i> (ATCC31488)	1560	1827	1523	>10,000	3200	ND
	<i>K. pneumoniae</i> (clinical isolate)	2149	1827	1687	>10,000	3200	ND
	<i>P. aeruginosa</i> ATCC39324)	2845	3266	-	-	-	ND
	<i>P. aeruginosa</i> (clinical isolate)	1243	1633	-	-	-	ND
	<i>P. mirabilis</i> (ATCC21721)	2975	3850	500	>10,000	-	ND
	<i>P. mirabilis</i> (clinical isolate)	2975	3850	500	>10,000	-	ND
	<i>P. vulgaris</i> (ATCC21719)	1435	1128	500	>10,000	-	ND
	<i>S. aureus</i> (ATCC25923)	1052	1720	370	-	-	ND
<i>S. aureus</i> (clinical isolate)	837	1577	425	-	-	ND	
Anti-proliferative IC_{50}	HeLa	2592	2157	-	-	ND	
	CaCo_2	1383	1516	-	-	ND	
Toxicity	LC_{50}	1525	1638	-	-	165	

Numbers indicate the mean MIC, IC_{50} or LC_{50} values of at least triplicate determinations. - indicates no significant bacterial growth inhibition was evident, or that inhibition of carcinoma proliferation/brine shrimp mortality did not reach 50% at any dose. ND indicates the test was not performed.

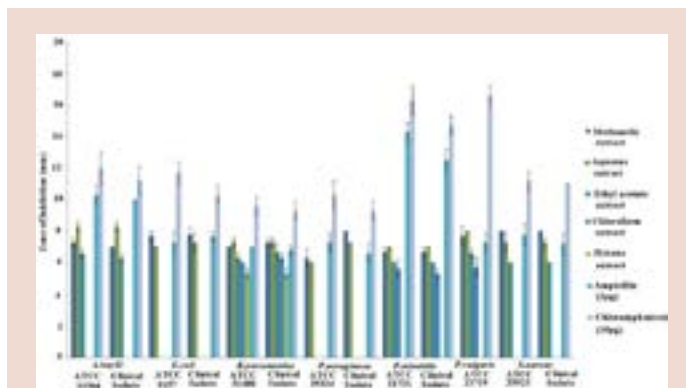


Figure 1: Growth inhibitory activity of *J. communis* berry extracts against the selected pathogenic bacterial species measured as zones of inhibition (mm). Results are expressed as mean zones of inhibition \pm SEM.

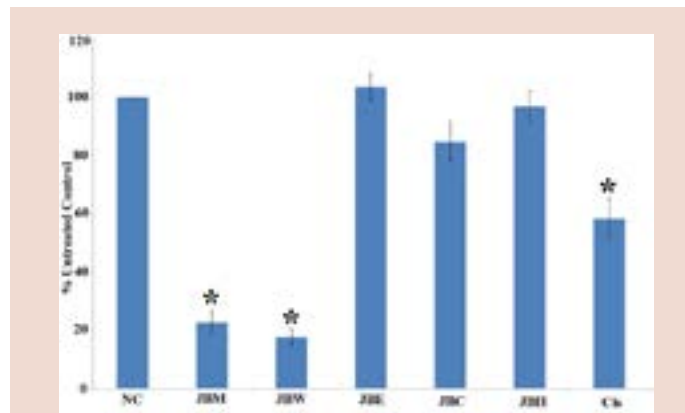


Figure 2: Anti-proliferative activity of *J. communis* berry extracts, cisplatin and untreated controls against CaCo₂ cancer cell lines measured as percentages of the untreated control cells. NC=untreated control; JBM=juniper berry methanolic extract; JBW=juniper berry aqueous extract; JBE=juniper berry ethyl acetate extract; JBC=juniper berry chloroform extract; JBH=juniper berry hexane extract; Cis=cisplatin (10 mg/mL). Results are expressed as mean percentages \pm SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p < 0.01$).

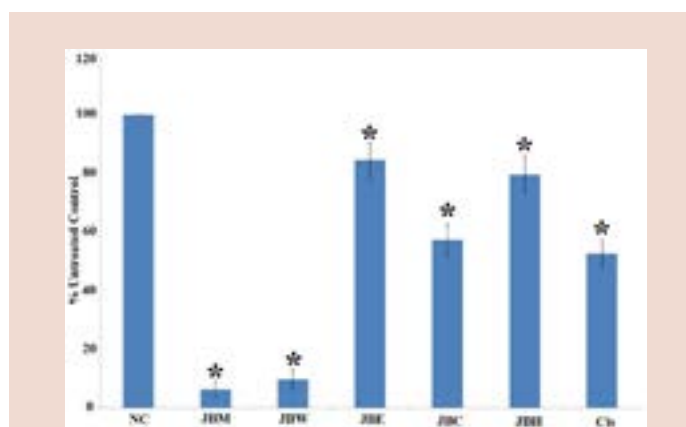


Figure 3: Anti-proliferative activity of *J. communis* berry extracts, cisplatin and untreated controls against HeLa cancer cell lines measured as percentages of the untreated control cells. NC=untreated control; JBM=juniper berry methanolic extract; JBW=juniper berry aqueous extract; JBE=juniper berry ethyl acetate extract; JBC=juniper berry chloroform extract; JBH=juniper berry hexane extract; Cis=cisplatin (10mg/mL). Results are expressed as mean percentages \pm SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p < 0.01$).

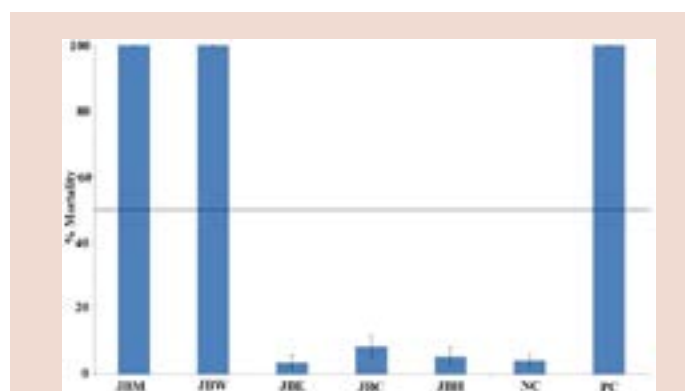


Figure 4: The lethality of the *J. communis* berry extracts and the potassium dichromate control towards *Artemia franciscana* nauplii after 24 h exposure. JBM=juniper berry methanolic extract; JBW=juniper berry aqueous extract; JBE=juniper berry ethyl acetate extract; JBC=juniper berry chloroform extract; JBH=juniper berry hexane extract; NC=untreated control; PC potassium dichromate control (1000 μ g/mL). Results are expressed as mean % mortality \pm SEM.

extracts was not significantly different to the untreated cell proliferation. Inhibition of proliferation by the methanolic and aqueous extracts was dose dependent, with the level of inhibitory activity decreasing at lower concentrations (Table 2).

Quantification of toxicity

All extracts were initially screened at 2000 μ g/mL in the assay (Figure 4). For comparison, the reference toxin potassium dichromate (1000 μ g/mL) was also tested in the bioassay. 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). Only the *J. communis* methanolic and aqueous extracts displayed $>50\%$ mortality rates at 24h. As $\geq 50\%$ mortality is indicative of toxicity,²² these extracts were further evaluated at various doses to determine their LC₅₀ values. All other extracts were deemed to be non-toxic.

Table 2 shows the LC₅₀ values of the extracts towards *A. franciscana*. No LC₅₀ values are reported for the ethyl acetate, chloroform and hexane

extracts as $<50\%$ mortality was seen for all concentrations tested. Whilst the initial toxicity screening study (Figure 4) indicated that the methanolic and aqueous extracts may be toxic due to the induction of *Artemia* nauplii mortality, both of these extracts had LC₅₀ values substantially above 1000 μ g/mL. Extracts with an LC₅₀ values greater than 1000 μ g/mL towards *Artemia* nauplii have previously been defined as being non-toxic.²² Therefore, all *J. communis* berry extracts were determined to be nontoxic.

DISCUSSION

J. communis berry has been used in a wide variety of traditional medicinal systems as an appetite suppressant, diuretic, anti-inflammatory agent and analgesic, as well for the treatment of wounds, kidney and stomach disorders.⁶⁻⁸ Several previous studies have reported growth inhibitory activity for *J. communis* berries towards multiple bacterial species.¹⁰⁻¹²

Of the bacterial species tested, the growth of *E. coli* and *S. aureus* was consistently inhibited. However, it is noteworthy that all of these studies examined the growth inhibition of *J. communis* essential oils. We were unable to find reports of similar inhibitory studies using *J. communis* berry extracts, although antibacterial activity has been reported for extracts prepared from *J. communis* leaves.²⁵ The latter study reported good growth inhibitory activity for the leaf extracts against a panel of bacteria including *E. coli*, although this study did not determine MIC values, making it difficult to compare efficacies between studies. In agreement with the earlier antibacterial studies,¹⁰⁻¹² we also detected potent growth inhibitory activity for the polar *J. communis* berry extracts against *E. coli* and *S. aureus*, with some MIC values < 1000 µg/mL (10 µg impregnated in the disc).

As well as its traditional use as a general antiseptic, *J. communis* berries are also used in the treatment of various inflammatory diseases including rheumatism, arthritis and gout.⁷ The majority of the bacteria tested in our study were selected as they are triggers of various autoimmune inflammatory diseases. Recent serotyping studies have identified bacterial triggers of several of these conditions. The major microbial trigger of rheumatoid arthritis has been identified as *Proteus mirabilis*,²⁶ a normal part of the human gastrointestinal flora. It has been postulated that *Proteus vulgaris* may also trigger this disease,²³ although this has not been verified. Several recent studies have documented the growth inhibitory activity of several Australian²⁷⁻³² and South African plants towards *P. mirabilis*,²¹ thus indicating their ability to prevent the induction of rheumatoid arthritis. *Klebsiella pneumoniae* has been identified as a trigger of ankylosing spondylitis.³³ Several studies have reported growth inhibitory activity of Australian^{27,29-30} and South African plants towards *K. pneumoniae*,²⁴ indicating their ability to prevent ankylosing spondylitis. Similarly, *Acinetobacter baylyi* and *Pseudomonas aeruginosa* have been linked with the onset of multiple sclerosis³⁴ and recent studies have reported growth inhibitory activity of native Australian plants²⁷⁻³⁰ towards *A. baylyi* and *P. aeruginosa*, indicating their ability to prevent multiple sclerosis.

Of the *J. communis* berry extracts tested in our study, the ethyl acetate had the most potent inhibitory activity against the majority of the bacterial triggers of the investigated autoimmune inflammatory diseases. It was a particularly potent inhibitor of *Proteus* spp. with MIC's of 500 µg/mL against all strains. As *Proteus* spp. (particularly *P. mirabilis*) are causative agents for rheumatoid arthritis,²⁶ *J. communis* berry ethyl acetate extracts may be useful for the prevention of this disease in genetically susceptible individuals. The ethyl acetate extract was also a good inhibitor of the growth of a bacterial trigger of ankylosing spondylitis (*K. pneumoniae*)³³ and thus also has potential in the prevention and treatment of this disease. In contrast, the therapeutic potential of this extract for multiple sclerosis was less definitive. Whilst it was a moderate inhibitor of the growth of *A. baylyi* (which can trigger multiple sclerosis),³⁴ it did not inhibit *P. aeruginosa* which has also been implicated in the induction of multiple sclerosis in genetically susceptible individuals.³⁴ Therefore, it may prove to be useful in lessening the impact of multiple sclerosis, although it is unlikely to completely block the disease. Indeed, *J. communis* berry methanol and aqueous extracts may prove to be a better therapeutic option for preventing/lessening the impact of multiple sclerosis as these extracts are effective growth inhibitors of both *A. baylyi* and *P. aeruginosa*. These extracts were also good inhibitors of *Proteus* spp. and *K. pneumoniae* growth, albeit with higher MICs. They may therefore also be useful for the treatment and prevention of rheumatoid arthritis and ankylosing spondylitis, although *in vivo* experiments are required to confirm this.

Previous phytochemical profiling of *J. communis* berry essential oils has focused on the terpenoid contents.¹⁰ A number of terpenes including α -pinene, sabinene, myrcene, p-cymene, D-limonene, γ -terpinene,

terpinolene, 1-terpinen-4-ol, β -caryophyllene and bicyclogermacrene were determined to be present in significant levels in the essential oil.¹⁰ This study also identified a number of other minor terpenoid components such as α -cubebene, α -copaene, α -humulene, germacrene-D, γ -cadinene and δ -cadinene, which were present in lower levels. Many of these terpenoids have potent broad spectrum antibacterial activity³⁵⁻³⁶ and therefore may contribute to the bacterial growth inhibitory activity in our study. Interestingly, several of these monoterpenoids including limonene³⁷⁻³⁸ and α -pinene³⁹ have also been reported to suppress NF- κ B signaling (the major regulator of inflammatory diseases). α -Pinene affects inflammation by inhibiting p65 translocation into the nucleus in LPS-induced NF- κ B signalling.³⁹ Furthermore, many other sesquiterpenes and sesquiterpene lactones also have well established anti-inflammatory activities.⁴⁰ Whilst much work is still needed to characterize the mechanisms of action of these compounds, it appears that NF- κ B inhibitory activities may be responsible. Thus, the terpene components may have a pleuripotent mechanism in blocking autoimmune inflammatory diseases and relieving their symptoms by acting on both the initiator and downstream inflammatory stages of the disease.

Whilst a detailed investigation of the phytochemistry of the *J. communis* berry extracts was beyond the scope of our study, a qualitative screening study was undertaken and is reported herein. A common feature of the methanolic, aqueous and ethyl acetate extracts (which had either potent or moderate bacterial growth inhibitory activities) was the high levels of phenolic compounds, alkaloids, flavonoids and tannins. Many studies have reported potent antibacterial activities for a wide variety of flavonoids.⁴¹ Similarly, a number of tannin compounds have bacterial growth inhibitory activity. Gallotannins have been reported to inhibit the growth of a broad spectrum of bacterial species⁴² through a variety of mechanisms including binding cell surface molecules including lipoteichoic acid and proline-rich cell surface proteins,⁴³⁻⁴⁴ and by inhibiting glucosyltransferase enzymes.⁴⁵ Ellagitannins are also highly potent inhibitors of bacterial growth, with MIC values as low as 62.5 µg/mL.^{42,44} Ellagitannins have also been reported to function via several antibiotic mechanisms including interaction with cytoplasmic oxidoreductases and by disrupting bacterial cell walls.^{42, 44}

It is likely that other phytochemical classes may also contribute to the bacterial growth inhibitory properties of these extracts. Alkaloids, anthraquinones, flavonoids, polyphenolics, phytosterols and saponins have also been linked with anti-bacterial activity in different plant species and thus may be responsible (at least in part) for the bacterial growth inhibitory activities reported here. Thus, it is likely that multiple compounds within the *J. communis* berry extracts are contributing to their bacterial growth inhibition.

Anti-proliferative activity against CaCo₂ and HeLa carcinoma cell lines was noted for the *J. communis* berry extracts (especially for the methanol and water extracts), with IC₅₀ values for CaCo₂ and HeLa cell approximately 1 500 µg/mL and 2000-2500 µg/mL respectively. These findings support and extend previous studies examining the anticancer effects of *J. communis* berry extracts against other cell lines. Aqueous *J. communis* berry extracts significantly inhibit the growth of MCF-7/AZ mammary carcinoma cells.⁴⁶ Whilst that study did show significant inhibition of MCF-7/AZ cells following 4 days exposure to the extract, an IC₅₀ was not reported as the inhibition was not greater than 50% at any dose tested. Interestingly, the highest dose tested in that study (180 µg/mL) inhibited cellular protein expression by approximately 90%. The authors postulated that the anti-proliferative effect in the MCF-7/AZ cells may be due to an inhibition of cellular receptor tyrosine kinases, insulin like growth factor receptor (IGF-1R) and c-erbB2/HER2/neu receptors. If this is indeed the case, the anti-proliferative effect reported for the aqueous *J. communis* berry extract may be due to inhibition of

cell growth rather than induction of apoptosis. However, as the test sample was a crude extract, it is possible that more than 1 anti-proliferative compound was present. Thus, the extract may function via pluripotent mechanisms. Indeed, the authors of that study also reported inhibition of 12-lipoxygenase activity. As lipoxygenases have crucial roles in apoptosis and survival of cancer cells, it is also likely that cellular survival may also be affected, although regulation of caspase activation pathways was ruled out as caspase levels remained constant.

The findings reported here demonstrate that the *J. communis* berry extracts tested in our study all had LC₅₀ values towards *Artemia franciscana* nauplii well in excess of 1000 µg/mL and were therefore deemed to be nontoxic. Extracts with LC₅₀ values <1000 µg/ml towards *Artemia* nauplii are defined as being toxic.²² However, before these extracts can be used medicinally, studies using human cell lines are required to further evaluate their safety.

CONCLUSION

The results of this study demonstrate the potential of *J. communis* berry extracts to block the growth of the bacterial triggers of rheumatoid arthritis, ankylosing spondylitis and multiple sclerosis. Furthermore, our studies also indicate that the extracts may also be useful in the treatment of some cancers. Further studies aimed at the purification of the 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.

ABBREVIATION USED

Cis: Cisplatin; **DMSO:** Dimethyl sulfoxide; **DPPH:** 2, 2-diphenyl-1-picrylhydrazyl, **IC₅₀:** The concentration required to achieve 50% of the maximal effect, **JBM:** *J. communis* berry methanolic extract, **JBW:** *J. communis* berry aqueous extract, **JBE:** *J. communis* berry ethyl acetate extract; **JBC:** *J. communis* berry chloroform extract; **JBH:** *J. communis* berry hexane extract; **LC₅₀:** The concentration required to achieve 50% mortality; **MIC:** Minimum inhibitory concentration.

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PICTORIAL ABSTRACT



SUMMARY

- *J. communis* L. berry extracts were good growth inhibitors of bacterial triggers of rheumatoid arthritis (*Proteus* spp.), ankylosing spondylitis (*K. pneumoniae*) and multiple sclerosis (*A. baylyi* and *P. aeruginosa*).
- In agreement with previous studies, the *J. communis* extracts were also potent inhibitors of *E. coli* and *S. aureus* growth.
- The ethyl acetate extract was a particularly potent growth inhibitor with MIC's \leq 500 μ g/mL against several bacteria.
- *J. communis* berry methanolic and aqueous extracts inhibited the proliferation of CaCo₂ and HeLa human carcinoma cell lines (IC₅₀ approximately 1500 μ g/mL for both extracts against each cell line).
- All *J. communis* berry extracts were non-toxic in the *Artemia nauplii* assay, with LC₅₀ values substantially > 1000 μ g/mL.

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 publications in a variety of peer reviewed journals.