Pharmacognosy Communications, 2024; 14(4):144-154.
https://www.phcogcommn.org

Original Article

Interactive Antibacterial Activity and Toxicity Profiles of Selected Pure Plant Compounds and Conventional Antibiotics Against Bacterial Triggers of Some Autoimmune Diseases

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ABSTRACT

Background: Curcumin, lupeol and piperine are plant compounds that have been reported to be effective against numerous medical conditions, including inflammation and against pathogenic infections. However, these compounds are yet to be tested for the ability to inhibit the growth of bacterial triggers of rheumatoid arthritis, ankylosing spondylitis and multiple sclerosis. **Materials and Methods:** Antimicrobial activity was assessed using disc diffusion and liquid dilution minimum inhibitory concentration (MIC) assays against a panel of bacterial triggers of autoimmune diseases. Interactions between the pure plant compounds and conventional antibiotics were studied and classified using the sum of the fractional inhibitory concentration (∑FIC). The toxicity of the individual samples and the combinations was assessed using the *Artemia* lethality assay (ALA) assay. **Results:** Curcumin, lupeol and piperine (in 3% DMSO) displayed clinically relevant antibacterial activity against *P. mirabilis* and *K. pneumoniae* when tested alone, but were ineffective against *A. baylyi* and *P. aeruginosa*. However, the inhibitory effects were potentiated against *A. baylyi* and *P. aeruginosa* by several plant compound-antibiotic combinations. None of the individual components (nor the combinations) were toxic in the ALA assay. **Conclusion:** The antibacterial activity of curcumin, lupeol and piperine against some triggers of autoimmune diseases and their lack of toxicity indicates that these compounds may provide leads in the development of new therapies to prevent and treat the autoimmune diseases rheumatoid arthritis and ankylosing spondylitis.

Keywords: Rheumatoid arthritis, Ankylosing spondylitis, Multiple sclerosis, Conventional antimicrobials, Synergy, Drug interaction Toxicity.

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Received: 07-09-2024;
Revised: 14-09-2024;
Accepted: 23-09-2024.

INTRODUCTION

Autoimmune inflammatory disorders are a group of debilitating conditions including rheumatoid arthritis, ankylosing spondylitis and multiple sclerosis that afflict genetically susceptible individuals.[1,2] There are no cures for these disorders. Instead, current treatment strategies aim to alleviate the symptoms (particularly pain, swelling and inflammation) with analgesics and anti-inflammatory agents and/or to modify the disease process through the use of disease modifying drugs. None of these treatments is ideal as prolonged usage of these drugs is often accompanied by unwanted side effects and toxicity.[1,2] There is a need to develop safer, more effective treatments for these conditions which will not only alleviate the symptoms, but may also cure or prevent the disease. These autoimmune disorders may be triggered in susceptible individuals by specific microbial infections. Serotyping studies have identified several of the bacterial triggers of these conditions and the bacterial antigens responsible for the induction of an immune response. The major microbial trigger of rheumatoid arthritis has been identified as *Proteus* spp. (especially *Proteus mirabilis*), which are a normal part of the human gastrointestinal flora. Similarly, *Klebsiella pneumoniae* has been shown to initiate ankylosing spondylitis and *Acinetobacter baylyi* and *Pseudomonas aeruginosa* have been linked with the onset of multiple sclerosis.[1,2] The development of antibiotic agents targeting 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.

DOI: 10.5530/pc.2024.4.24

Whilst antibiotics are available to treat infections of these bacteria, the development of multiple antibiotic resistant bacterial strains has rendered multiple clinical antibiotics of decreased efficacy, or in some cases, has rendered the antibiotics completely effective.[3] The development of alternative treatment methods is crucial and is considered by the World Health Organisation (WHO) to be one of the biggest challenge facing medical science.[4] For a number of reasons reviewed elsewhere,[3] it is unlikely that the current methods of antibiotic discovery/development will be as successful in the future. Instead, examination of natural compounds with therapeutic properties may generate new drug leads for the development of new antibiotics. Despite this, relatively few plant derived antibiotic compounds are in common use clinically. This may be because synergistic interactions are often required to potentiate the antibacterial activity and purified plant phytochemicals often have much lower activity than the crude extract that they are derived from.[5] A combinational approach that allows synergistic interaction between plant extracts (or pure plant compounds) and conventional antibiotics may be more effective in combatting bacterial pathogens, especially in antibiotic resistant strains.[6-8] Combinational therapy is already preferred over mono-therapy in multiple life-threatening infectious diseases such as malaria, tuberculosis and HIV/AIDS due to its ability to target multiple facets of a disease and to curb resistance.[3,4] A combination of plant extracts/isolated compounds with conventional antibiotics may also prove to have an economic advantage.[5] Developing a new drug requires years of extensive and costly testing. However, combinational therapy can potentially restore an existing drug to a state of significantly reduced resistance, thereby bypassing the lengthy and expensive process of discovering new antibiotic agents. Further advantages of synergistic interactions include increased efficiency, reduced side effects, increased stability and bioavailability and the requirement for lower doses in comparison to synthetic alternatives.[5]

Curcumin (Figure 1a) is a bioactive secondary metabolite that is isolated from *Curcuma longa* L. roots (commonly known as turmeric). It has a long history of use in Chinese, Indian and Middle Eastern medicine (as reviewed in).[9] Curcumin is particularly well known fot its anti-inflammatory activity and has been reported to be a potent inhibitor of various inflammation activation pathways, including TNF-dependent NF-κB activation.[9] Additionally, curcumin down regulates COX-2 expression and inhibits 5-LOX activity, as well as downregulating the production of multiple pro-inflammatory cytokines, including TNF, IL-1, IL-6, IL-8 and IFN-γ.[10,11] Additionally, curcumin has been reported to inhibit the growth of several panels of bacterial pathogens.[12-19] Lupeol also has substantial anti-inflammatory activities, including reducing prostaglandin E2 production in A23187-stimulated macrophages, inhibiting 15-LOX and decreasing the production of pro-inflammatory cytokines in LPS-stimulated macrophages.[20] Lupeol has also been reported to have antibacterial activity against a limited panel of bacterial pathogens.[21] However, that study only tested the activity of a single concentration of lupeol and MIC values were not reported, making it difficult to compare the potency between studies. Piperine also has anti-inflammatory activity via downregulation of IL-1β, IL-6, TNF-α, NF-κB and MAPKs, as well as upregulation of IL-10.[22,23] Piperine also has growth inhibitory activity against *Mycobacterium tuberculosis* and *Staphylococcus aureus* (including methicillin-resistant strains).[23] However, to the best of our knowledge, curcumin, lupeol and piperine are yet to be tested against the bacterial triggers of rheumatoid arthritis (*Proteus mirabilis*), ankylosing spondylitis (*Klebsiella pneumoniae*) and multiple sclerosis (*Acinetobacter baylyi, Pseudomonas aeruginosa*).[1,2] This study investigates the antimicrobial effects of these plant compounds and their ability to potentiate the growth-inhibitory properties of conventional antibiotics against the bacterial triggers of some autoimmune inflammatory diseases.

MATERIALS AND METHODS

Pure plant compounds

Curcumin (>80% curcumin; >94% curcuminoid content by HPLC), lupeol (>94% by HPLC) and piperine (>95%) were purchased from Sigma, Australia. The compounds were individually resuspended in DMSO (Sigma, Australia; ACS grade) and diluted in deionined water to prepare 10 mg/mL stock solutions containing either 1 or 3% DMSO.

Antibacterial analysis

Conventional antibiotics

Penicillin-G (potency of 1440-1680 µg/mg), chloramphenicol (nic% purity by HPLC), erythromycin (potency ≥pur µg/mg), ciprofloxacin (µg/% purity by HPLC) and tetracycline (pur% purity by HPLC) were purchased from Sigma-Aldrich, Australia and used for the microplate liquid dilution assay. All antibiotics were prepared in sterile deionised water at stock concentrations of 0.0 1 mg/mL and stored at 4ºC until use. For the disc diffusion studies, ampicillin (10 µg), tetracycline (10 µg) and chloramphenicol (10 µg) standard discs were obtained from Oxoid Ltd., Australia and used as positive controls.

Bacterial cultures

All bacterial strains were selected based on their ability to trigger autoimmune inflammatory diseases in genetically susceptible individuals.[24-26] Reference strains of *Proteus mirabilis* (ATCC21721), *Proteus vulgaris* (ATCC21719), *Klebsiella pneumoniae* (ATCC31488), *Acinetobacter baylyi* (ATCC33304) and *Pseudomonas aeruginosa* (ATCC39324) were purchased from American Type Culture Collection, USA. All bacteria were cultured in nutrient broth (Oxoid Ltd., Australia). Streak nutrient agar (Oxoid Ltd., Australia) plates were tested in parallel to ensure the purity of all bacterial cultures and for sub-culturing.

All bacterial cultures were incubated at 37°C for 24 hr and were subcultured and maintained in nutrient broth at 4°C until use.

Evaluation of bacterial susceptibility to growth inhibition

The susceptibility of the bacteria to the pure plant compounds and the conventional antibiotics was initially assessed using a modified disc diffusion assay.[27,28] Ampicillin (10 µg), tetracycline (10 µg) and chloramphenicol discs (10 µg) were obtained from Oxoid Ltd., Australia and used as positive controls to compare antibacterial activity. Filter discs infused with 10 µL of distilled water were used as a negative control.

Minimum Inhibitory Concentration (MIC) determination

The minimum inhibitory concentration for each extract was determined using two methods. A liquid dilution MIC assay was employed as it is generally considered the most sensitive bacterial growth inhibitory assay.[29] Furthermore, as microplate liquid dilution MIC assays are perhaps the most commonly used method of quantifying bacterial growth inhibition efficacy, use of this method allows for comparisons with other studies. A solid phase agar disc diffusion assay was also used in this study for comparison.

Microplate liquid dilution MIC assay

A standard liquid dilution MIC assay[30-32] was used to evaluate the antimicrobial activity of the plant compounds and conventional antimicrobials independently and in combinations. Briefly, 100 µL of sterilized distilled water was dispensed into each well of 96 well micro-titre plate. The plant compounds and conventional antibiotics (100 µL) were then added into separate wells of the first row of the plate. A negative control (nutrient broth), a sterile control (without bacteria) and a sample-free culture control (to ensure the media was capable of supporting microbial growth) were included on all plates. After addition of the test samples to the plate, each was serially diluted by doubling serial dilution. The relevant bacterial culture inoculum (100 µL) was then added to all wells of the plate except the sterile control wells. Each inoculum contained approximately $1x10^6$ colony forming units (CFU)/mL. All plates were subsequently incubated at 37°C. p-Iodonitrotetrazolium violet (INT) was obtained from Sigma-Aldrich, Australia and dissolved in sterile deionised water to prepare a 0.2 mg/mL INT solution. A 40 µL volume of this solution was added into all wells and the plates were incubated for a further 6 hr at 30°C. Following incubation, the MIC was visually determined as the lowest dose at which colour development was inhibited.

Disc diffusion MIC assay

The minimum inhibitory concentrations (MIC) of the extracts was also evaluated by disc diffusion assay as previously described.[33,34] Graphs of the zone of inhibition (ZOI) versus Ln of the concentration were plotted and MIC values were calculated using linear regression.

Fractional inhibitory concentration (FIC) assessment

The interactions between the pure plant compounds and conventional antimicrobials in combinations were further classified using the sum of the fractional inhibitory concentration (∑FIC). The FIC was calculated using the following equation, where (a) represents the pure plant compounds and (b) the conventional antimicrobial sample:[33-36]

$$FIC(\mathrm{i}) = \frac{MIC\ (a) in\ combination\ with\ (b)}{MIC\ (a) independently}$$

$$FIC(\mathrm{ii}) = \frac{MIC\ (b) in\ combination\ with\ (a)}{MIC\ (b) independently}$$

The ∑FIC was then calculated using the equation: $\sum FIC = FIC^{(i)} + FIC^{(ii)}$. The interactions were classified as being

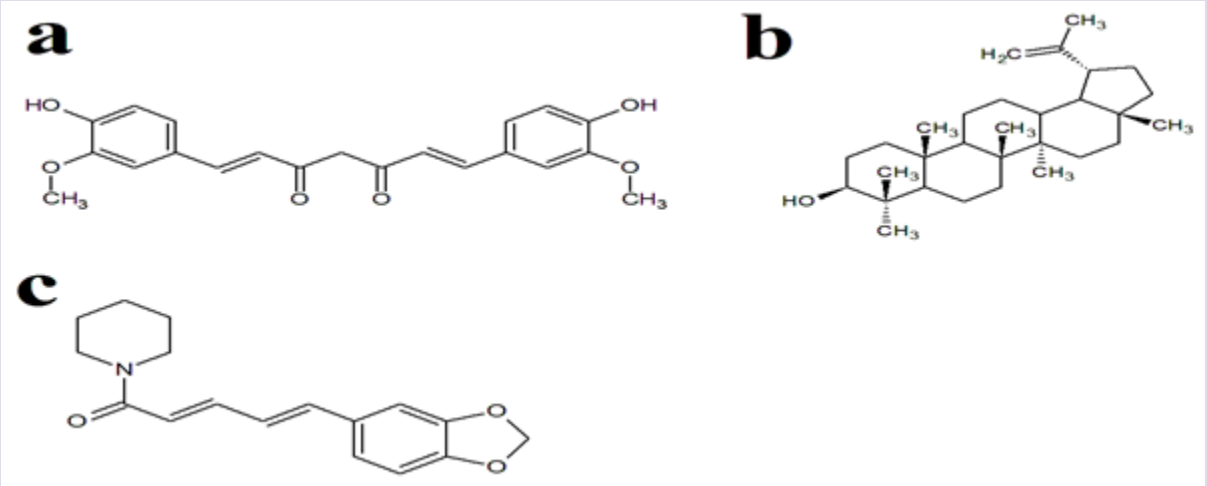

Figure 1: Structures of (a) curcumin (keto form), (b) lupeol and (c) piperine.

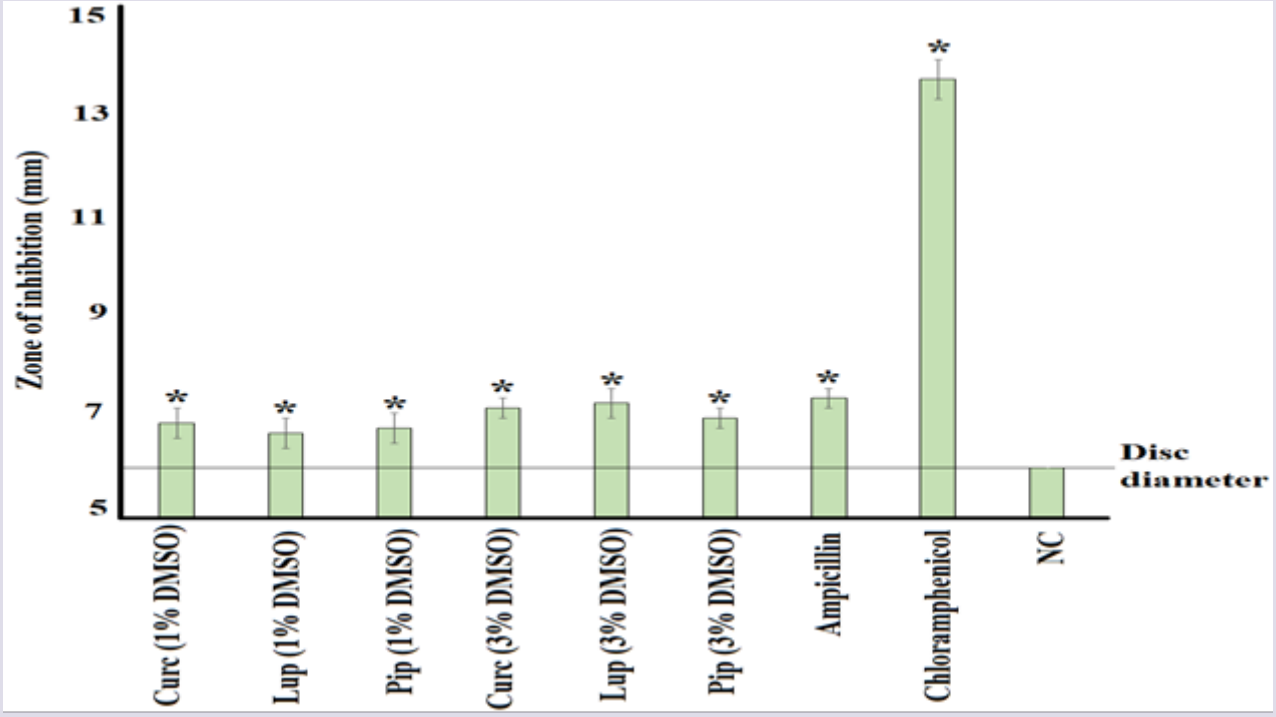

Figure 2: Antibacterial activity of selected extracts against *P. mirabilis* (ATCC21721) measured as zones of inhibition (mm). Curc=curcumin extract; Lup=lupeol; Pip=piperine; NC=negative Control; Amp=ampicillin (10 µg); Chl=chloramphenicol (10 µg); NC=negative Control (nutrient broth). Results are expressed as mean zones of inhibition of at least six replicates±SEM * indicates results that are significantly different to the negative control (p<0.01).

synergistic for $^{\Sigma}$FIC values of ≤0.5, additive (>0.5-1.0), indifferent (>1.0-≤4.0) or antagonistic (>4.0).[33-36]

Artemia franciscana lethality assay (ALA)

Toxicity of the pure plant compounds, reference toxin and conventional antibiotics was assessed using a modified *Artemia franciscana* nauplii lethality assay.[37,38] potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared in deionised water (4 mg/mL) and serially diluted in artificial seawater as a reference toxin. The LC_{50} with 95% confidence limits for each treatment was calculated using probit analysis.

Statistical analysis

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

RESULTS

Bacterial growth inhibition screening

Proteus mirabilis growth was susceptible to curcumin, lupeol and piperine, albeit with relatively small ZOIs measured (Figure 2). All of these compounds inhibited bacterial growth to approximately the same extent. Notably, the 3% DMSO solutions had larger ZOIs compared to the 1% DMSO solutions, although these differences were not statistically significant. It is possible the higher DMSO content may assist these compounds to cross the lipid membrane barrier into the cell, thereby enhancing their intracellular concentration. The bacterial growth inhibition by these compounds compared well to that of the ampicillin control (ZOI=7.4 mm), although the chloramphenicol control was a substantially stronger inhibitor of *P. mirabilis* growth (13.8 mm). As *P. mirabilis* can induce rheumatoid arthritis in genetically susceptible people,[2] these compounds may be useful in the prevention and treatment of this disease. A similar susceptibility profile was evident for the compounds when screened against *K. pneumoniae* (Figure 3). The compounds in 3% DMSO produced slightly larger ZOIs against this bacterium. As *K. pneumoniae* can trigger ankylosing spondylitis in genetically susceptible people,[2] these compounds may also be useful in preventing and treating that disease.

The pure compounds also inhibited the growth of some bacterial triggers of multiple sclerosis,[1] although the ZOIs indicate that the compounds were substantially less potent against these bacteria. Notably, only the compounds suspended in 3% DMSO inhibited the growth of *A. baylyi* tested in our study, whilst the compounds in 1% DMSO were completely ineffective at the concentration tested (Figure 4). As noted above, the higher DMSO in those samples may assist in the transport of the compound into the cell, thereby increasing the intracellular concentration. However, the ZOIs for the 3% DMSO samples were also small (≤6.6 mm) and

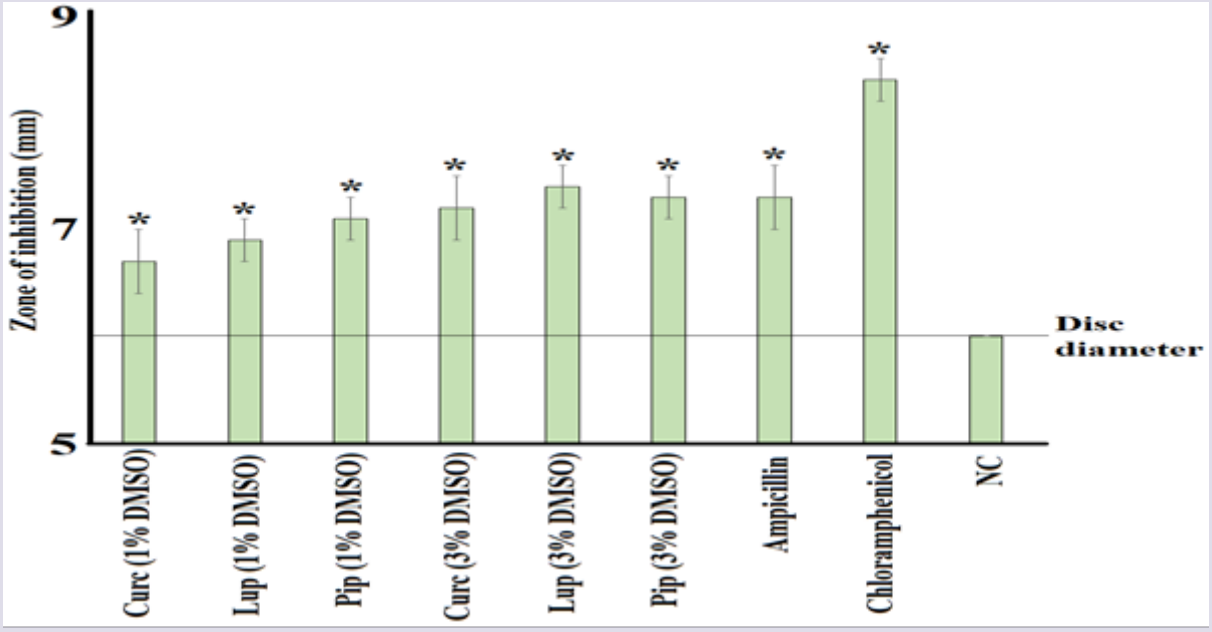

Figure 3: Antibacterial activity of selected extracts against *K. pneumoniae* (ATCC31488) measured as zones of inhibition (mm). Curc=curcumin extract; Lup=Lupeol; pip=Piperine; NC=negative Control; Amp=ampicillin (10 µg); Chl=chloramphenicol (10 µg); NC=negative Control (nutrient broth). Results are expressed as mean zones of inhibition of at least six replicates±SEM * indicates results that are significantly different to the negative control (p<0.01).

therefore indicate that these tests are also low potency against *A. baylyi*. With the exception of the curcumin test in 1% DMSO, all the test samples also inhibited the growth of *P. aeruginosa* (Figure 5). However, the small ZOIs (<7 mm for all pure compounds) indicates that these extracts have only weak activity against that bacterium. However, the *P. aeruginosa* strain tested in our study was relatively resistant to antibiotic exposure. Indeed, the ampicillin control was completely inactive against this bacterium. Similarly, previous studies in our group have reported that this bacterial strain is resistant to several other antibiotics, as well as to multiple plant extracts with reported antibacterial activity.[32-34] Therefore these compounds may still be useful against this bacterium, although testing against further bacterial strains is required to confirm this.

Quantification of minimum inhibitory concentration (MIC)

The relative level of antimicrobial activity was further evaluated by determining the MIC values using two methods: the liquid dilution MIC assay and the disc diffusion MIC assay (Table 1). Consistent with the antibacterial disc screening assays, all bacterial strains tested were susceptible to the pure compounds, although the compounds in 1% DMSO generally produced only weak inhibitory activity against all of the bacteria tested. However, these bacterial strains were also resistant to several of the antibiotic controls. Indeed, as MIC values >1 µg/mL indicate resistance in this assay,[31,33] most of the bacterial strains tested herein were resistant to all of the conventional antibiotics except ciprofloxacin. The exception was *P. aeruginosa*, which was resistant to all of the antibiotics tested including ciprofloxacin.

Proteus mirabilis was particularly susceptible to the curcumin, lupeol and piperine compounds in 3% DMSO, with an MIC of 625 µg/mL recorded for each compound. In contrast, the same compounds suspended in 1% DMSO were relatively ineffective against this bacterium. These compounds (in 3% DMSO) were even better inhibitors of *K. pneumoniae* (MIC=313 µg/mL). As these bacteria can induce rheumatoid arthritis and ankylosing spondylitis respectively in genetically susceptible people,[2] these compounds may be particularly useful in preventing and treating these diseases. In contrast, the high MIC values of the pure compounds (even in 3% DMSO) against *A. baylyi* and *P. aeruginiosa*, indicate that these compounds would be less useful against multiple sclerosis, which may be triggered in genetically susceptible people by these bacteria.[1]

Fractional inhibitory concentration (FIC) assessment

None of the combinations of the pure plant compounds and the conventional antibiotic combinations produced synergistic interactions when tested together against any of the bacteria tested (Table 2). However, several combinations (against *A. baylyi* and *P. aeruginosa*) had additive effects in the assay. Notably, with the exception of the combination of curcumin (1% DMSO) and chloramphenicol against *P. aeruginosa*, all of the potentiating

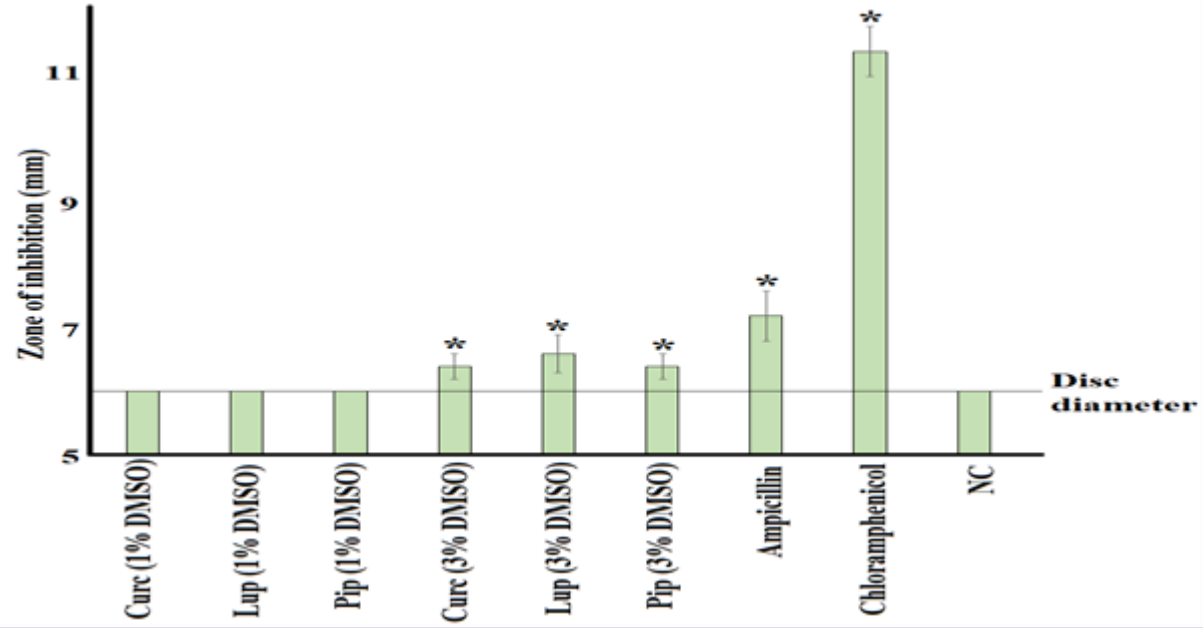

Figure 4: Antibacterial activity of selected extracts against *A. baylyi* (ATCC33304) measured as zones of inhibition (mm). Curc=curcumin extract; Lup=lupeol; Pip=piperine; NC=negative Control; Amp=ampicillin (10 µg); Chl=chloramphenicol (10 µg); NC=negative Control (nutrient broth). Results are expressed as mean zones of inhibition of at least six replicates±SEM * indicates results that are significantly different to the negative control (p<0.01).

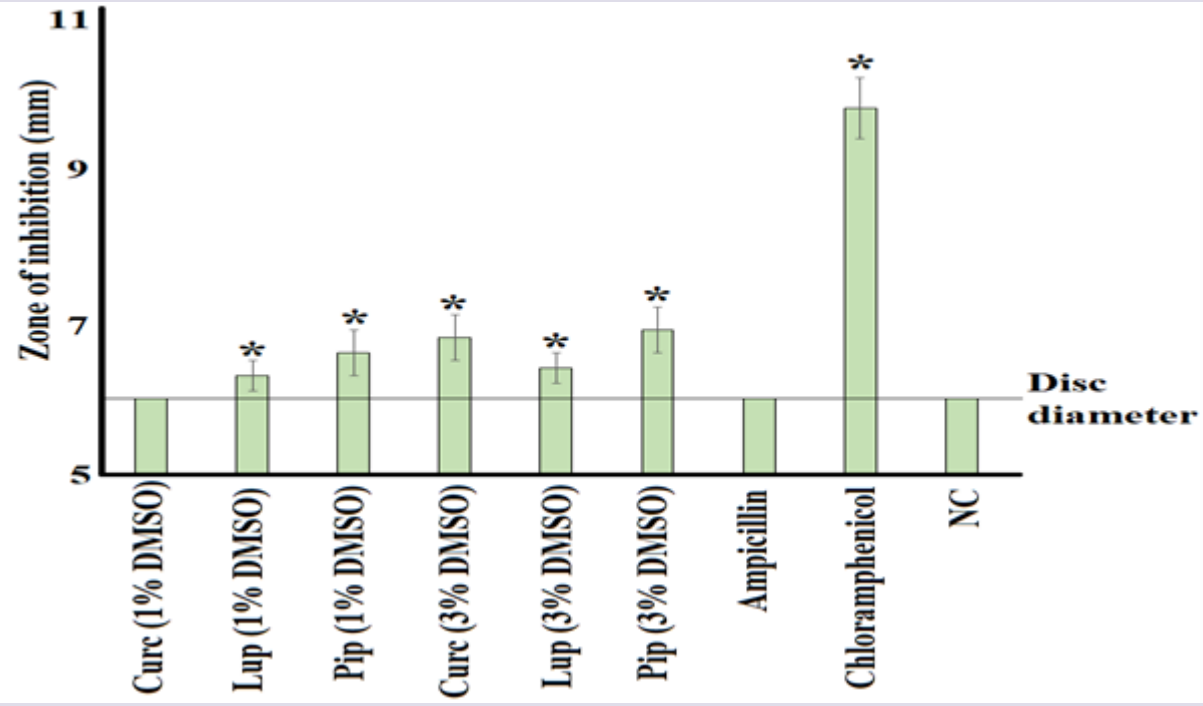

Figure 5: Antibacterial activity of selected extracts against *P. aeruginosa* (ATCC39324) measured as zones of inhibition (mm). Curc=curcumin extract; Lup=lupeol; Pip=piperine; NC=negative Control; Amp=ampicillin (10 µg); Chl=chloramphenicol (10 µg); NC=negative control (nutrient broth). Results are expressed as mean zones of inhibition of at least six replicates±SEM * indicates results that are significantly different to the negative control (p<0.01).

Table 1: Disc diffusion (DD) and liquid dilution (LD) MIC values (µg/mL) for the pure plant compounds against microbial triggers of some autoimmune inflammatory diseases.

Test Compound	*P. mirabilis*		*K. pneumoniae*		*A. baylyi*		*P. aeruginosa*	
	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC
Curcumin (1% DMSO)	>1000	>1000	>1000	>1000	-	-	-	-
Lupeol (1% DMSO)	>1000	>1000	>1000	>1000	-	-	>1000	>1000
Piperine (1% DMSO)	>1000	>1000	>1000	>1000	-	-	>1000	>1000
Curcumin (3% DMSO)	>1000	625	>1000	313	>1000	>1000	>1000	>1000
Lupeol (3% DMSO)	>1000	625	>1000	313	>1000	>1000	>1000	>1000
Piperine (3% DMSO)	>1000	625	>1000	313	>1000	>1000	>1000	>1000
Antibiotic Controls								
Ciprofloxacin	ND	0.62	ND	0.62	ND	0.62	ND	1.36
Erythromycin	ND	-	ND	-	ND	2.5	ND	-
Tetracycline	ND	-	ND	1.25	ND	1.25	ND	2.5
Chloramphenicol	ND	1.25	ND	2.5	ND	2.5	ND	-

DD=disc diffusion; LD=liquid dilution; - indicates no inhibition at any dose tested.

Table 2: ΣFIC values for the pure plant compound and conventional antibiotic combinations against susceptible bacteria.

Bacteria	Pure Compound	Ciprofloxacin	Chloramphenicol	Erythromycin	Tetracycline
P. mirabilis	Curcumin (1% DMSO)	1.02	1.02	1.02	1.02
		(IND)	(IND)	(IND)	(IND)
	Lupeol (1% DMSO)	1.02	1.02	1.02	1.02
		(IND)	(IND)	(IND)	(IND)
	Piperine (1% DMSO)	1.02	1.02	1.02	1.02
		(IND)	(IND)	(IND)	(IND)
	Curcumin (3% DMSO)	1.02	1.02	1.02	1.02
		(IND)	(IND)	(IND)	(IND)
	Lupeol (3% DMSO)	1.02	1.02	1.02	1.02
		(IND)	(IND)	(IND)	(IND)
	Piperine (3% DMSO)	1.41	1.02	1.02	1.02
		(IND)	(IND)	(IND)	(IND)
K. pneumoniae	Curcumin (1% DMSO)	2	2.5	1.02	2.03
		(IND)	(IND)	(IND)	(IND)
	Lupeol (1% DMSO)	1.02	2.5	1.02	2.03
		(IND)	(IND)	(IND)	(IND)
	Piperine (1% DMSO)	1.02	2.5	1.02	4.06
		(IND)	(IND)	(IND)	(ANT)
	Curcumin (3% DMSO)	1.02	2.5	1.02	2.03
		(IND)	(IND)	(IND)	(IND)
	Lupeol (3% DMSO)	2	2.5	1.02	2.03
		(IND)	(IND)	(IND)	(IND)
	Piperine (3% DMSO)	1.41	2.5	1.02	2.03
		(IND)	(IND)	(IND)	(IND)

A. baylyi	Curcumin (1% DMSO)	2.83	2.5	1.02	2.03
		(IND)	(IND)	(IND)	(IND)
	Lupeol (1% DMSO)	2.83	2.5	1.02	2.03
		(IND)	(IND)	(IND)	(IND)
	Piperine (1% DMSO)	2.83	2.5	1.02	2.03
		(IND)	(IND)	(IND)	(IND)
	Curcumin (3% DMSO)	0.71	0.88	1.02	2.03
		(ADD)	(ADD)	(IND)	(IND)
	Lupeol (3% DMSO)	0.71	2.5	1.25	2.03
		(ADD)	(IND)	(IND)	(IND)
	Piperine (3% DMSO)	0.71	2.5	1.02	2.03
		(ADD)	(IND)	(IND)	(IND)
P. aeruginosa	Curcumin (1% DMSO)	1.41	0.63	1.02	1.02
		(IND)	(ADD)	(IND)	(IND)
	Lupeol (1% DMSO)	2.02	1.02	1.02	1.02
		(IND)	(IND)	(IND)	(IND)
	Piperine (1% DMSO)	1.02	1.02	1.02	1.02
		(IND)	(IND)	(IND)	(IND)
	Curcumin (3% DMSO)	1.41	0.63	1.02	1.02
		(IND)	(ADD)	(IND)	(IND)
	Lupeol (3% DMSO)	1.41	0.63	1.25	1.02
		(IND)	(ADD)	(IND)	(IND)
	Piperine (3% DMSO)	1.41	2.5	1.02	1.02
		(IND)	(IND)	(IND)	(IND)

ADD=additive interaction; IND=indifferent interaction: ANT=antagonism.

combinations contained compounds suspended in 3% DMSO. It is also noteworthy that all of the additive combinations contained either ciprofloxacin or chloramphenicol as the antibiotic component of the combination. Whilst these combinations would not be as effective as synergistic combinations, they are still an improvement on using either the antibiotic or the extract alone. It may therefore be beneficial to use these combinations in the prevention and treatment of multiple sclerosis. Notably, one antagonistic combination (piperine in 1% DMSO and tetracycline) displayed antagonistic effects in the assay when tested against *K. pneumoniae*. That combination should therefore be avoided when treating ankylosing spondylitis, or other infections of that bacterium. All of the other inhibitory combinations were non-interactive. Whilst these combinations provide no added benefit over that of the individual components alone, they did not antagonise each other's effects and are therefore safe to use concurrently without risk of lessening the efficacy of either component.

Toxicity evaluation

All pure plant compounds and antibiotics were individually screened at 1000 µg/mL in the ALA assay (Table 3). The extracts were only considered to be toxic if they induced percentage mortalities greater than 50% (LC_{50}) following 24 hr of exposure to the *Artemia* nauplii.[37,38] When tested individually, the antimicrobials demonstrated no toxicity in the ALA. Similarly, none of the pure plant compounds produced mortality above 50% following 24 h exposure. Additionally, when the extract-antibiotic combinations were tested in the ALA, none of them produced mortality >50% mortality. Therefore, all combinations and individual components were deemed nontoxic. In contrast, the positive control potassium dichromate induced 100% mortality in the ALA.

DISCUSSION

This study investigated the ability of selected plant compounds to inhibit the growth of some bacterial triggers of auto-immune inflammatory diseases, both alone and in combination with conventional antibiotics. These compounds were selected for this study as they have been reported to be effective in treating inflammation, as well as against some pathogenic diseases.[9-23] However, to the best of our knowledge, none of these previous studies has tested these compounds for the ability to inhibit the growth of the bacterial triggers of autoimmune inflammatory

Table 3: Mortality (%) assessment for the plant compounds and conventional antibiotics tested individually and as combinations in the *Artemia* lethality assay.

	Test sample or combination	Mortality±SEM (%)
Conventional Antibiotics	Cip	1.8±1.4
	Ery	2.7±1.3
	Tet	1.2±0.6
	Chl	2.4±1.5
Plant Compounds	Curc (1% DMSO)	17.3±3.5
	Lup (1% DMSO)	9.6±2.9
	Pip (1% DMSO)	12.5±2.5
	Curc (3% DMSO)	21.6±2.9
	Lup (3% DMSO)	9.3±3.3
	Pip (3% DMSO)	10.9±4.8
Combinations	Curc (1% DMSO)+Cip	28.6±3.7
	Lup (1% DMSO)+Cip	18.3±2.9
	Pip (1% DMSO)+Cip	22.6±3.8
	Curc (3% DMSO)+Cip	24.9±3.4
	Lup (3% DMSO)+Cip	20.5±3.6
	Pip (3% DMSO)+Cip	19.8±3.7
	Curc (1% DMSO)+Ery	30.8±4.6
	Lup (1% DMSO)+Ery	21.4±2.8
	Pip (1% DMSO)+Ery	19.7±3.6
	Curc (3% DMSO)+Ery	23.2±3.4
	Lup (3% DMSO)+Ery	17.8±3.8
	Pip (3% DMSO)+Ery	14.5±3.3
	Curc (1% DMSO)+Tet	17.4±4.6
	Lup (1% DMSO)+Tet	19.5±3.3
	Pip (1% DMSO)+Tet	18.7±3.7
	Curc (3% DMSO)+Tet	15.7±3.7
	Lup (3% DMSO)+Tet	11.4±4.6
	Pip (3% DMSO)+Tet	9.6±3.7
	Curc (1% DMSO)+Chl	16.3±3.9
	Lup (1% DMSO)+Chl	16.6±3.5
	Pip (1% DMSO)+Chl	17.5±3.0
	Curc (3% DMSO)+Chl	14.8±3.6
	Lup (3% DMSO)+Chl	10.6±4.2
	Pip (3% DMSO)+Chl	7.3±3.8
Controls	Deionised water	2.7±1.7
	Potassium dichromate	100.00±0.00

All compounds and the potassium dichromate were tested at 1000 µg/mL; Curc=curcumin; Lup=lupeol; Pip=piperine; Cip=ciprofloxacin; Ery=erythromycin; Tet=tetracycline; Chl=chloramphenicol; SEM=standard error of mean. Results represent means±SEM of 3 independent experiments, each preformed in triplicate (n=9).

diseases. All of the compounds were effective inhibitors of *P. mirabilis* and *K. pneumoniae* growth, with clinically relevant potency. However, these compounds were generally ineffective against *A. baylyi* and *P. aeruginosa* growth. Curcumin, lupeol and piperine (in 3% DMSO) had the strongest inhibitory activity against *P. mirabilis* and *K. pneumoniae*, indicating that it may be particularly useful in preventing and treating rheumatoid arthritis and ankylosing spondylitis (as well as other infections caused by these bacteria) when used by alone.

The combinational studies combining the pure plant compounds with conventional antibiotics also yielded interesting results. Several combinations displayed enhanced potential as therapeutic agents against *A. baylyi* and *P. aeruginosa* than either the plant compounds or antibiotics alone. Indeed, seven additive combinations were noted, with four of these containing chloramphenicol and three containing ciprofloxacin. The implications of this potentiation include enhanced efficacy, the requirement for lower dose administration and a reduction in side effects, as well as possibly reduced antimicrobial resistance.[3] Importantly, only one combination produced antagonistic effects (piperine in 1% DMSO and tetracycline against *K. pneumonia*). This is an important finding as it indicates that all other combinations are safe to use without decreasing the efficacy of either component.

None of the plant compounds or conventional antibiotics demonstrated toxicity in the ALA assay when tested independently. Similarly, all combinations were nontoxic, indicating their potential for therapeutic use. The lack of toxicity of the combinations in our study also confirms their potential for therapeutic usage. However, further *in vitro* studies using human cell lines are required to verify their safety. Furthermore, *in vivo* testing is also required to confirm that the plant compounds and combinations retain efficacy and remain nontoxic in complex biological systems.

CONCLUSION

Whilst the findings reported herein support the therapeutic properties of curcumin, lupeol and piperine as preventative and therapeutic options against rheumatoid arthritis and ankylosing spondylitis, further *in vivo* investigations are required to support these *in vitro* findings. In particular, because of their low solubility in aqueous solutions, further studies are required to examine the bioavailability of the compounds *in vivo*. Furthermore, studies to determine the possible mechanism of action resulting in these effects are warranted.

ACKNOWLEDGEMENT

The financial assistance was provided by the Centre for Planetary Health and Food Security and the School of Environment and Science, Griffith University.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ALA: Brine-shrimp lethality assay; **DMSO:** Dimethyl sulfoxide; **FIC:** Fractional inhibitory concentration; **INT:** ρ-iodonitrotetrazolium chloride; **LC_{50}:** Dose of sample necessary to have a lethal effect on 50% of test organisms or cells; **MIC:** Minimum inhibitory concentration; **ΣFIC:** The sum of the fractional inhibitory concentration; **ZOI:** Zone of inhibition.

SUMMARY

- Pure curcumin, lupeol and piperine were screened for the ability to inhibit the growth of some bacterial triggers of selected autoimmune inflammatory diseases.
- The antibacterial potency was quantified by liquid dilution and disc diffusion assays.
- The plant compounds were also tested in combination with conventional antibiotics to determine whether they could potentiate the antibiotic activity.
- The toxicity of curcumin, lupeol and piperine was evaluated using the *Artemia* nauplii toxicity bioassays.

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Cite this article: Puisais A, Cock IE. Interactive Antibacterial Activity and Toxicity Profiles of Selected Pure Plant Compounds and Conventional Antibiotics against Bacterial Triggers of Some Autoimmune Diseases. Pharmacognosy Communications. 2024;14(4):144-54.