

# The Interactive Antimicrobial Activity of *Withania somnifera* (L.) Dunal Root Extracts and Conventional Antibiotics Against some Bacterial Triggers of Autoimmune Inflammatory Diseases

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## ABSTRACT

**Background:** *Withania somnifera* (L.) Dunal roots are known for their anti-inflammatory and rejuvenative properties. This study focuses on the growth inhibitory activity of *W. somnifera* root extracts against some bacterial triggers of autoimmune inflammatory diseases, as well as the extracts in combination with conventional antibiotics. **Methods:** *W. somnifera* root powder was extracted with solvents of varying polarity and screened for inhibition of bacterial growth. Inhibition on agar was assessed by disc diffusion techniques, whilst the minimum inhibitory concentrations (MICs) were quantified by liquid dilution assays. To screen for combinatorial effects, the *W. somnifera* root extracts were combined with a range of conventional antibiotics and tested against each bacterial strain using liquid dilution assays. Extract toxicities was examined using *Artemia* nauplii bioassays, while their phytochemical profiles were determined using standard methodologies. **Results:** *W. somnifera* root extracts were unable to inhibit the growth of *P. mirabilis*, *K. pneumoniae*, *A. baylyi* and *Pseudomonas aeruginosa*. However, the methanolic and aqueous extracts were good inhibitors of *Y. enterocolitica*, with MICs of 696 and 358 µg/mL respectively. These extracts also inhibited *S. pyogenes* growth, albeit with substantially higher MIC values indicative of only mild inhibition. Only small zones of inhibition were observed on agar for these extracts against both strains. Combinations of the *W. somnifera* extracts and conventional antibiotics generally

produced additive or indifferent interactions, indicating that they are safe to use concomitantly without compromising the efficacy of either component. A single antagonistic combination (aqueous extract and tetracycline) against *Y. enterocolitica* was observed. All extracts were determined to be non-toxic as assessed in *Artemia* nauplii bioassays. **Conclusions:** Methanolic and aqueous *W. somnifera* extracts were strong inhibitors of *Y. enterocolitica* growth and mild inhibitors of *S. pyogenes*. These extracts were also non-toxic in the *Artemia* nauplii bioassay, indicating their potential for the prevention and treatment of Hashimoto's disease.

**Key words:** Ashwagandha, Multi-drug resistant bacteria, Combinational therapies, Rheumatoid Arthritis, Hashimoto's disease.

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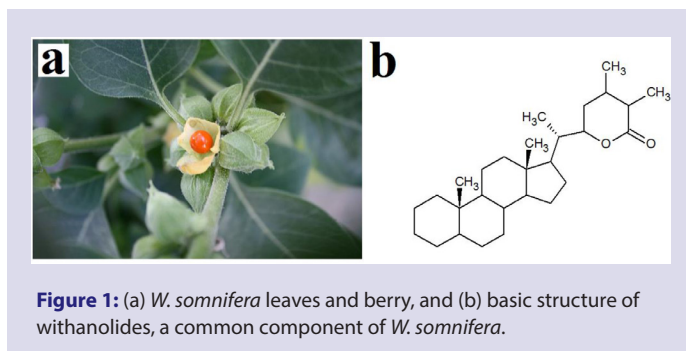
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## INTRODUCTION

In recent years, there has been an increase in bacterial resistance to many conventional antibiotics and several strains of medicinally important bacterial pathogens are now multi (MDR), extensively (XDR) or totally drug resistant (TDR).<sup>1</sup> There are now limited therapeutic options for the diseases caused by these pathogens. This problem is expected to be exacerbated in the future as bacteria exchange resistance genes and more strains develop MDR. The development of alternative antibacterial treatment modalities has become crucial and is considered by the World Health Organisation (WHO) to be one of the most serious challenges facing medical science.<sup>2</sup> For a number of reasons (reviewed elsewhere),<sup>1</sup> it is unlikely that the previous methods of antibiotic discovery/development will be as successful in the future and new treatment modalities are urgently required. Traditional medicines and herbal remedies have great potential for antimicrobial drug development and there has recently been a substantial increase in interest in this field.<sup>3-5</sup>

The traditional Indian medicinal system Ayurveda uses a variety of natural plant and fruit products to treat numerous ailments.<sup>3,6,7</sup> *Withania somnifera* (L.) Dunal (Figure 1 a; commonly known as ashwagandha) root has been used for thousands of years in Indian Ayurveda to treat a wide variety of conditions.<sup>8</sup> *W. somnifera* root is best known as an effective treatment for the promotion of physical and mental health, and a pharmacological basis exists for its use in the treatment of a variety of central nervous system disorders.<sup>9</sup> While it also appears to possess anti-

oxidant and anticancer properties,<sup>10-12</sup> evidence is lacking for the ability of *W. somnifera* extracts to inhibit the growth of specific bacteria known to trigger autoimmune inflammatory diseases. Whilst much of the phytochemistry is yet to be elucidated, *W. somnifera* is rich in a variety of withanolides (Figure 1b).<sup>13</sup> Previous studies that identified the bacterial triggers of some autoimmune inflammatory diseases in genetically susceptible humans have facilitated drug therapies targeting the initiating events of these diseases, thereby providing prophylactic chemotherapeutic options. *Acinetobacter baylyi* and *Pseudomonas aeruginosa* were identified as bacterial triggers for multiple sclerosis, *Klebsiella pneumoniae* has been linked to ankylosing spondylitis and *Proteus mirabilis* was found to be a bacterial trigger of rheumatoid arthritis.<sup>14,15</sup> *Streptococcus pyogenes* is associated with rheumatic fever, pharyngitis and impetigo<sup>16</sup> while *Yersinia enterocolitica* induces the autoimmune disease Hashimoto's thyroiditis.<sup>17</sup> The present study aimed to investigate the growth inhibitory activity of *W. somnifera* root extracts against *P. mirabilis*, *K. pneumoniae*, *A. baylyi*, *Y. enterocolitica*, *S. pyogenes* and *P. aeruginosa*. The extracts were also tested in conjunction with conventional antibiotics to evaluate any interactive effects.



**Figure 1:** (a) *W. somnifera* leaves and berry, and (b) basic structure of withanolides, a common component of *W. somnifera*.

## MATERIALS AND METHODS

### Plant material and extraction

The *Withania somnifera* (L.) Dunal root powder used in this study was sourced from verified plants in India by Noodles Emporium (Australia) and supplied as a dried, ground powder. A voucher sample (NSC2017wsic) has been stored at the School of Environment and Science, Griffith University, Australia. Individual 1.5 g quantities of the material were weighed into separate tubes and 50 mL of methanol, deionised water, chloroform, hexane or ethyl acetate were added. All solvents were obtained from Ajax, Australia and were AR grade. The ground plant materials were individually extracted in each solvent for 24 hours at 40°C with gentle shaking. The extracts were subsequently filtered through filter paper (Whatman No. 54) under vacuum, and dried at 40°C, with the resultant extracts weighed and resuspended in 10 mL deionised water (containing 1 % DMSO). The suspensions were briefly sonicated (3 x 20 s pulse cycles, at 2 kHz) and then sterilised by filtration through a 0.2 µm membranes and stored at 4°C until required for further analysis.

### Qualitative phytochemical studies

Phytochemical analyses of the *W. somnifera* root extracts for the presence of saponins, phenolic compounds, flavonoids, phytosterols, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids were conducted by previously described assays.<sup>18</sup>

### Antibiotics

Penicillin-G (potency of 1440-1680 µg/mg), chloramphenicol (≥98 % purity by HPLC), erythromycin (potency ≥850 µg/mg), ciprofloxacin (≥98 % purity by HPLC), and tetracycline (≥95% purity by HPLC) were purchased from Sigma-Aldrich, Australia and were used as controls for the microplate liquid dilution assay. All antibiotics were prepared in sterile deionised water at stock concentrations of 0.01 mg/mL and stored at 4°C until use.

### Bacterial cultures

Reference strains of *Proteus mirabilis* (ATCC 21721), *Klebsiella pneumoniae* (ATCC 31488), *Acinetobacter baylyi* (ATCC 33304), *Streptococcus pyogenes* (ATCC 12384) and *Pseudomonas aeruginosa* (ATCC 39324) were purchased from American Type Culture Collection, USA. Clinical isolate strains were obtained from the School of Environment and Science teaching laboratory. All bacteria were cultured in nutrient broth (Oxoid Ltd., Australia). Streaked 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 h and were subcultured and maintained in nutrient broth at 4°C until use.

### Evaluation of antibacterial activity on agar

Antibacterial activity screening of the *W. somnifera* root extracts on solid agar was assessed using a modified disc diffusion assay method.<sup>19-21</sup> Extracts (10 µL) were infused onto Whatman #1 filter discs (6 mm in diameter), with negative control discs containing 10 µL of extract solvent (1% DMSO). Penicillin, erythromycin, ciprofloxacin, tetracycline and chloramphenicol discs (1 µg) were prepared on discs and used as positive controls to compare antibacterial activity, while filter discs infused with 10 µL of distilled water were used as a negative control for the antibiotics.

### Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration for each extract was determined using a microplate liquid dilution MIC method,<sup>5,22</sup> as it is generally considered the most sensitive bacterial growth inhibitory assay. 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. All microplates were incubated at 37°C for 24 h following addition of samples. p-Iodonitro-tetrazolium violet (INT) was obtained from Sigma-Aldrich (Australia) and dissolved in sterile deionised water to produce 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 hours at 24-30°C. Following incubation, the MIC was visually determined as the lowest dose at which colour development was inhibited.

### Fractional inhibitory concentration (FIC) assessment

Interactions between the *W. somnifera* root extracts and the conventional antibiotics were examined by determination of the sum of fractional inhibitory concentrations (ΣFIC) for each combination.<sup>5</sup> The FIC values for each component (a and b) were calculated using the following equations where a represents the plant extract sample and b represents the conventional antibiotic:

$$FIC(a) = \left( \frac{MIC[a \text{ in combination with } b]}{MIC[a \text{ independently}]} \right)$$

$$FIC(b) = \left( \frac{MIC[b \text{ in combination with } a]}{MIC[b \text{ independently}]} \right)$$

The ΣFIC was then calculated using the formula ΣFIC = FIC(a) + FIC(b). The interactions were classified as synergistic (ΣFIC ≤0.5), additive (ΣFIC >0.5-1.0), indifferent (ΣFIC >1.0-4.0) or antagonistic (ΣFIC >4.0).<sup>22</sup>

### *Artemia* nauplii toxicity screening

Potassium dichromate (K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>) (AR grade, Chem-Supply, Australia) was prepared in deionised water (4 mg/mL) and serially diluted in artificial seawater for use as a reference toxin. Toxicity of the *W. somnifera* root extracts, the reference toxin and the conventional antibiotics was assessed using a modified *Artemia franciscana* nauplii lethality assay.<sup>23,24</sup> The LC<sub>50</sub> with 95% confidence limits for each treatment was calculated using probit analysis.

### Statistical analysis

Data are expressed as the mean ± SEM of at least three independent experiments. One-way ANOVA was used to calculate differences between the control and treated groups, with a *P* value < 0.01 considered to be significant.

**Table 1: Mass of dried extracted material, concentration after resuspension in deionised water, and qualitative phytochemical screenings of the *W. somnifera* root extracts.**

Extract	Mass of Dried Extract (mg)	Resuspended Extract (mg/mL)	Phenols			Cardiac Glycosides	Saponins		Triterpenes	Phytosteroids	Alkaloids			Flavonoids		Tannins		Anthraquinones	
			Total Phenolics	Water Soluble	Water Insoluble		Keller-Kiliani Test	Froth Persistence			Emulsion test	Salkowski Test	Acetic Anhydride Test	Meyers Test	Wagners Test	Draggendorff's Test	Shinoda Test	Kumar test	Ferric Chloride Test
Methanol	200	20	+++	+++	+++	-	++	++	+	-	+	-	-	+++	+++	+	+	-	-
Water	320	32	+++	+++	++	-	+++	+	-	-	+	-	-	+++	+++	+	+	-	-
Ethyl Acetate	12	1.2	+	+	+	-	+	++	+	-	+	-	-	++	++	-	-	-	-
Chloroform	77	7.7	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Hexane	60	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay.

## RESULTS

### Liquid extraction yields and qualitative phytochemical screening

*W. somnifera* root extractions (1.5 g material) using various solvents yielded dried plant extracts ranging from 12 mg to 320 mg (Table 1). Aqueous and methanolic extracts provided significantly greater yields of extracted material relative to the ethyl acetate, chloroform and hexane counterparts, which gave low to moderate yields. The dried extracts were resuspended in 10 mL of deionised water (containing 1 % DMSO), resulting in the concentrations presented in Table 1.

Qualitative phytochemical studies showed that methanol and water extracted the greatest amount, and widest range of, phytochemicals (Table 1). These solvents extracted high levels of phenolic compounds and flavonoids, as well as moderate levels of saponins and low levels of tannins and triterpenoids. The ethyl acetate extracts generally extracted similar but lower phytochemical profiles as compared to the methanolic and aqueous extracts. In contrast, the chloroform and hexane extracts were generally devoid of detectable levels of all classes of the screened phytochemicals.

### Antibacterial activity

To determine the ability of the crude plant extracts to inhibit the growth of the bacterial triggers of selected autoimmune inflammatory diseases, aliquots (10  $\mu$ L) of each extract were screened using a disc diffusion assay. None of the *W. somnifera* extracts tested caused inhibition of the growth of the bacterial triggers of rheumatoid arthritis (*P. mirabilis*), ankylosing spondylitis (*K. pneumoniae*) and multiple sclerosis (*A. baylyi* and *P. aeruginosa*) (results not shown). In contrast, the methanolic and aqueous *W. somnifera* extracts were good inhibitors of bacterial triggers of rheumatic fever (*S. pyogenes*; Figure 2a) and Hashimoto's disease

(*Y. enterocolitica*; Figure 2b) on solid agar, indicating that they may be useful in the prevention and treatment of these diseases.

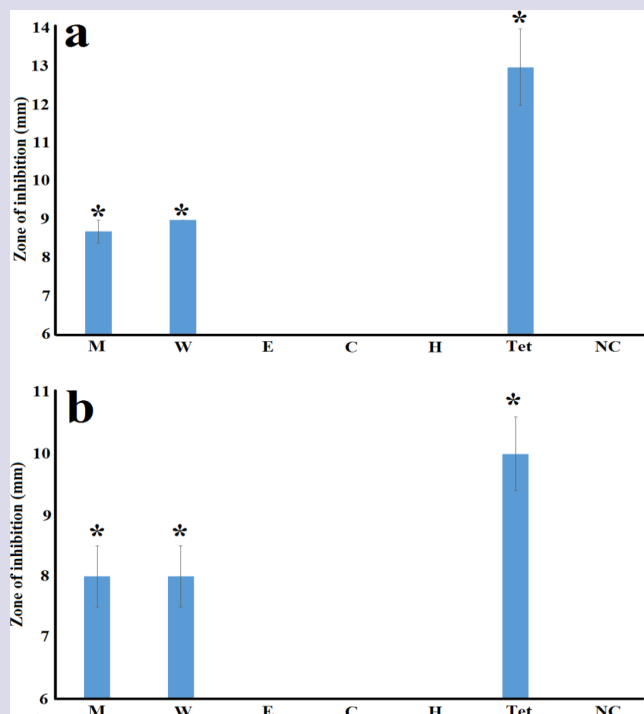
### Quantification of minimum inhibitory concentration (MIC)

The antimicrobial efficacies of the *W. somnifera* extracts were further quantified by determining the MIC values (Table 2). The methanolic and aqueous extracts were particularly effective at inhibiting growth of *Y. enterocolitica*, with MIC values of 696 and 358  $\mu$ g/mL, respectively. *S. pyogenes* growth was inhibited at MIC values of 6260 and 9690  $\mu$ g/mL, respectively, for the methanolic and aqueous extracts, indicating a much weaker antimicrobial activity towards this strain as compared to *Y. enterocolitica* was found in liquid cultures.

### Determination of combinational effects: Fractional inhibitory concentration (FIC) assessment

Fractional inhibitory concentration (FIC) determination was performed using a 1:1 ratio of each *W. somnifera* extract and conventional antibiotic and sums of FIC ( $\Sigma$ FIC) were calculated (Table 3). As all bacterial species were completely resistant to the ethyl acetate, chloroform and hexane extracts, no  $\Sigma$ FIC values could be determined for combinations containing these extracts. Similarly, as *P. mirabilis*, *K. pneumoniae*, *A. baylyi* and *P. aeruginosa* were resistant to all extracts, no  $\Sigma$ FIC values could be determined for these bacteria.  $\Sigma$ FIC values were calculated for the remaining combinations and the class of interaction was determined for each.

Additive interactions between the aqueous extract and penicillin or ciprofloxacin were observed against *S. pyogenes*, with an additive interaction also observed between the methanolic extract and ciprofloxacin for the same strain. A single combination produced an additive interac-



**Figure 2:** Antibacterial activity of *W. somnifera* root extracts against (a) *S. pyogenes* and (b) *Y. enterocolitica* measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. Positive control = Tet (tetracycline; 1 µg). Negative control (NC) = 1% DMSO. Results are expressed as mean zones of inhibition of at least three replicates ± SEM. \* indicates results that are significantly different to the negative control ( $P < 0.01$ ).

tion against *Y. enterocolitica* (methanolic extract with tetracycline) while three combinations were found to have an additive effect against *S. pyogenes* (methanolic extract with ciprofloxacin, and aqueous extracts with penicillin or ciprofloxacin). These are promising findings since the antibacterial activity of these combinations is substantially enhanced against these strains, albeit not to the extent expected for a synergistic interaction. However, the additive growth inhibitory effects of such combinations show that they would be preferable as a growth inhibitor of these bacteria compared to treatment with the individual antibiotic or extract components. Of the remaining combinations, seven were determined to be non-interactive. Whilst co-administering these extracts and antibiotics would be of no therapeutic benefit, they would not counteract/lessen the activity of the individual components and therefore may be deemed safe to use concomitantly.  $\Sigma$ FIC values could not be determined for the remaining interactive combinations (Table 3) except for an antagonistic interaction ( $\Sigma$ FIC > 4.0) between the aqueous *W. somnifera* extract and tetracycline. This suggests that this combination of extract and antibiotic significantly reduces the antimicrobial effect on *Y. enterocolitica*, indicating that this specific *W. somnifera*: antibiotic combination may need to be avoided to maintain antibacterial activity. Finally, no synergistic interactions ( $\Sigma$ FIC  $\leq 0.5$ ) were evident for any combination against any bacterial species.

### Quantification of toxicity

All extracts were initially screened in the *Artemia* nauplii assay at 2000 µg/mL (Figure 3). Additionally, potassium dichromate was also tested in the bioassay as a reference toxin. The reference toxin was rapid in its onset of mortality, promoting nauplii death within the first 3 h of exposure, with 100% mortality evident within 5 h (data not shown). The methanolic and aqueous extracts also induced 100% mortality following 24 h exposure. All other extracts induced <50% mortality and were therefore deemed to be non-toxic. To further quantify the effects of toxin concentration on the initiation of mortality, the methanolic and aqueous extracts were serially diluted in artificial seawater to test across

**Table 2:** MIC values for *W. somnifera* root extracts against *P. mirabilis*, *K. pneumoniae*, *A. baylyi*, *P. aeruginosa*, *S. pyogenes* and *Y. enterocolitica* growth, and  $LC_{50}$  values against *Artemia* nauplii (µg/mL).

	Antibacterial MIC (µg/mL)						Toxicity ALA (µg/mL)
	<i>P. mirabilis</i>	<i>K. pneumoniae</i>	<i>A. baylyi</i>	<i>P. aeruginosa</i>	<i>S. pyogenes</i>	<i>Y. enterocolitica</i>	
<i>W. somnifera</i> extracts							
M	-	-	-	-	6260	696	1585
W	-	-	-	-	9670	358	2286
E	-	-	-	-	-	-	-
C	-	-	-	-	-	-	-
H	-	-	-	-	-	-	-
Antibiotic controls							
Pen	-	-	-	-	0.002	-	ND
Ery	-	-	-	-	0.005	-	ND
Chl	-	1.11	3.33	-	3.3	1.11	ND
Tet	-	0.37	0.041	0	0.123	0.041	ND
Cip	0.002	0.002	0.002	0	0.37	0.002	ND

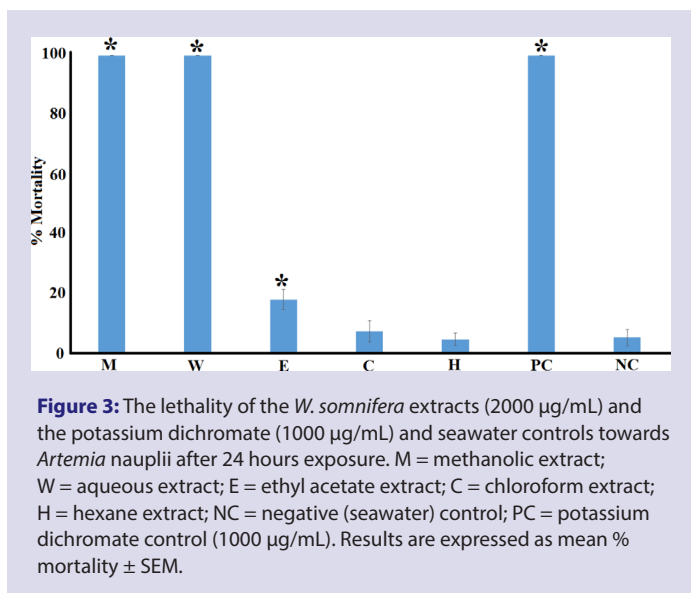
ALA = *Artemia* lethality assay; M = methanol extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Pen = penicillin; Ery = erythromycin; Chl = chloramphenicol; Tet = tetracycline; Cip = ciprofloxacin; - = an MIC or  $LC_{50}$  was not determined as there was no inhibition at highest concentration tested. ND =  $LC_{50}$  was not determined.



**Table 3:**  $\Sigma$ FIC values of *W. somnifera* extracts in combination with conventional antibiotics against *S. pyogenes* and *Y. enterocolitica*.

Extract	Penicillin	Erythromycin	Chloramphenicol	Tetracycline	Ciprofloxacin
<i>S. pyogenes</i>					
M	1.53	1.40	CND	1.60	0.57
W	0.53	1.40	CND	1.61	0.68
E	ND	ND	ND	ND	ND
C	ND	ND	ND	ND	ND
H	ND	ND	ND	ND	ND
<i>Y. enterocolitica</i>					
M	ND	ND	ND	0.68	1.01
W	ND	ND	ND	18.01	1.01
E	ND	ND	ND	ND	ND
C	ND	ND	ND	ND	ND
H	ND	ND	ND	ND	ND

M = Methanol extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; CND = indicates that a FIC could not be determined as the combination was not inhibitory; ND =  $\Sigma$ FIC could not be determined as at least one component of the combination was not inhibitory at any concentration tested. Additive (>0.5 to  $\leq$ 1.0); Indifferent (>1.0 to  $\leq$ 4.0); Antagonistic (>4.0).



**Figure 3:** The lethality of the *W. somnifera* extracts (2000  $\mu$ g/mL) and the potassium dichromate (1000  $\mu$ g/mL) and seawater controls towards *Artemia nauplii* after 24 hours exposure. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; NC = negative (seawater) control; PC = potassium dichromate control (1000  $\mu$ g/mL). Results are expressed as mean % mortality  $\pm$  SEM.

a range of concentrations in the *Artemia nauplii* bioassay. The 24 h  $LC_{50}$  values of the *W. somnifera* root extracts towards *A. nauplii* are displayed in Table 2. No  $LC_{50}$  are reported for the ethyl acetate, chloroform or hexane extracts as < 50 % mortality was seen in all tested concentrations.  $LC_{50}$  values substantially >1000  $\mu$ g/mL were determined for the methanolic and aqueous extracts. As extracts with  $LC_{50}$  values >1000  $\mu$ g/mL towards *Artemia nauplii* have been defined as being non-toxic in this assay,<sup>23</sup> each of the *W. somnifera* root extracts were deemed to be non-toxic.

## DISCUSSION

*W. somnifera* is used in Ayurveda for a wide variety of therapeutic purposes. It is perhaps best known as a potent booster of the immune system, as a general tonic for well-being, and as a rejuvenative agent.<sup>8</sup> The root extracts also have documented uses for inhibiting bacterial growth

and suppress the production of pro-inflammatory cytokines, thereby blocking inflammatory conditions, including arthritis, as reviewed elsewhere.<sup>25</sup> Therefore, this study aimed to confirm the antibacterial activity of a panel of root extracts against bacterial species known to trigger autoimmune diseases in genetically susceptible individuals.<sup>14-17</sup> Whilst we did detect some antibacterial activity in this study, most bacterial species examined were refractory to inhibition by the extracts. Only *S. pyogenes* and *Y. enterocolitica* were susceptible to inhibition by the methanolic and aqueous extracts, and the MIC values against *S. pyogenes* were indicative of only mild inhibitory activity. In contrast, the MIC values (358 and 696  $\mu$ g/mL for the aqueous and methanolic extracts respectively) against *Y. enterocolitica* indicated much greater potencies. The relatively low antibacterial strength determined in our study contrasts with several studies that have reported strong antibacterial activity for a variety of *W. somnifera* extracts. One publication reported potent inhibition of *Salmonella typhimurium* and *Escherichia coli* by *W. somnifera* leaf methanolic and hexane extracts.<sup>26</sup> However, the definition of potency in that study was based purely on the size of the zone of inhibition in disc diffusion assays. The authors did not evaluate the MIC values for the extracts, rendering a comparison of the potencies impossible. Furthermore, that study screened a single high concentration of each extract (100mg/mL). Inhibition at this concentration is not necessarily indicative of high potency, and dose response studies are required. It is also noteworthy that this earlier study involved different bacterial species to those examined in the present study, which may also contribute to the differences.

A more recent report by Alam *et al.*<sup>27</sup> examined a different panel of bacteria, including some of the species included in our study. That study reported large zones of inhibition against *P. aeruginosa* and *K. pneumonia* (approximately 20-26 mm for the ethanolic extract). Whilst we did not prepare ethanolic extracts in our study, we did include methanolic extracts. These two solvents have similar polarity and can generally extract similar chemical constituents. The previous study<sup>27</sup> also determined MIC values and described the antibacterial activity as high based on these results. However, the MIC values reported for the *W. somnifera* root ethanolic extract were 50 mg/mL. These values would generally be regarded as inactive in most studies rather than the strong antibacterial activity

which had been described in that report. Indeed, in our study, we class  $>10\text{mg/mL}$  as being inactive,  $5\text{--}10\text{mg/mL}$  as low activity,  $1\text{--}5\text{ mg/mL}$  as moderate activity and  $<1\text{mg/mL}$  as strong activity. If those criteria were used, the previously reported MIC values in the earlier study would instead be classed as inactive.

The combinational studies involving extracts potentiating the activity of conventional antibiotics are perhaps of more interest. Several additive combinatorial interactions were noted. In particular, the water extract potentiated the activity of penicillin and ciprofloxacin against *S. pyogenes*. The methanolic extract potentiated ciprofloxacin against *S. pyogenes* and tetracycline against *Y. enterocolitica*. As such, these combinations would be beneficial against rheumatic fever and Hashimoto's disease, respectively, as they have greater efficacy than either component alone. Of the remaining combinations, the majority were either non-interactive, or were unable to be determined as at least one of the components in the combination showed no inhibition of bacterial growth when tested alone. Therefore, whilst using these combinations would have no additional therapeutic benefit, the components would not impede the activity of the other component. Notably, a single combination (aqueous extract in combination tetracycline) produced an antagonistic effect against *Y. enterocolitica*. This suggests that this combination should be avoided in the treatment of Hashimoto's disease (and any other diseases caused by this bacterium).

Whilst a thorough examination of the phytochemical composition of the *W. somnifera* extracts was beyond the scope of this study, we detected high levels of flavonoids in the extracts that were found to exert anti-bacterial effects. Flavonoids have previously been reported to have good growth inhibitory activity against a broad panel of pathogenic bacteria<sup>28-30</sup> and it is likely that they may contribute to the potent bacterial growth inhibitory activity observed in our study. Other phytochemical classes may also contribute to this activity. Saponins and triterpenes were also detected in the *W. somnifera* methanolic and aqueous extracts. This is consistent with previous studies which reported withanolides (Figure 1b) as abundant in similar extracts.<sup>13</sup> Some terpenoids have potent broad spectrum antibacterial activity<sup>31</sup> and it is therefore likely that they also contribute to the inhibition of the bacteria examined in our study. Further evaluation of the phytochemical composition and isolation of the active components is required to more rigorously assess the mechanism of bacterial growth inhibition. Of further note, all extracts displayed low toxicity in the *Artemia nauplii* bioassay. Indeed,  $LC_{50}$  values  $\geq 1000\text{ }\mu\text{g/mL}$  are generally deemed to be non-toxic for crude plant extracts. Only the methanolic and aqueous extracts induced any mortality and in both cases were substantially  $>1000\text{ }\mu\text{g/mL}$ . These findings are also consistent with studies which have demonstrated the *W. somnifera* root extracts to be non-toxic in rodents.<sup>32,33</sup> Therefore, it is likely that the *W. somnifera* extracts are safe for both prophylactic and therapeutic use. However, further toxicity studies using mammalian cell lines are required to confirm the safety of these extracts.

## CONCLUSION

The strong growth inhibitory activity of *W. somnifera* against *Y. enterocolitica* and its lack of toxicity render it a promising option for the prevention and treatment of Hashimoto's disease and any other infections of this bacterium. Further studies are required to isolate the compound(s) responsible for this activity and to elucidate the antibacterial mechanisms involved.

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## CONFLICTS OF INTEREST

The authors report no conflicts of interest.

## ABBREVIATIONS

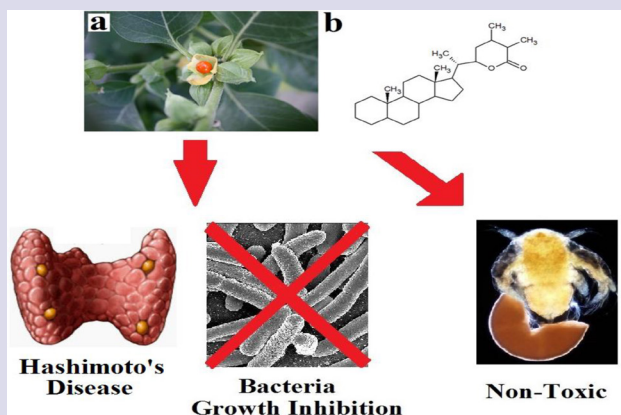
DMSO: Dimethyl sulfoxide;  $LC_{50}$ : The concentration required to achieve 50 % mortality; MIC: minimum inhibitory concentration.

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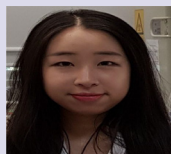
### PICTORIAL ABSTRACT



### HIGHLIGHTS OF PAPER

- Methanolic and aqueous *W. somnifera* root extracts were good inhibitors of *Y. enterocolitica*, (MICs of 696 and 358 µg/mL respectively).
- The methanolic and aqueous extracts also inhibited *S. pyogenes* growth, albeit with substantially higher MIC values indicative of only mild inhibition.
- *W. somnifera* root extracts were unable to inhibit the growth of *P. mirabilis*, *K. pneumoniae*, *A. baylyi* and *P. aeruginosa*.
- Combinations of the *W. somnifera* extracts and conventional antibiotics generally produced additive or indifferent interactions.
- All extracts were determined to be non-toxic as assessed in *Artemia* nauplii bioassays.

### ABOUT AUTHORS



**Wenjing Lai** is a visiting scholar from Guangdong Pharmaceutical University in China. Her interests lie in the identification of novel therapeutic compounds from traditional medicinal plants, and the application of Traditional Chinese Medicine.



**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 Australian 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.



**Dr. Matthew Cheesman** is a lecturer from Griffith University and is a member of the Quality Use of Medicines (QUM) Network. His laboratory group studies the antimicrobial properties of medicinal plants and the mechanisms underlying microbial resistance