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Original Article

Effects of *Hamamelis virginiana* L. Extracts on *Pseudomonas aeruginosa* Growth and Antagonism of Ciprofloxacin

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ABSTRACT

Introduction: *Pseudomonas aeruginosa* is a Muti-Drug Resistant (MDR) pathogen that causes a myriad of infectious diseases. Limited evidence exists for antibacterial properties of extract preparations from the Virginian witch hazel (WH; *Hamamelis virginiana* L.; family: Hamamelidaceae) and their interactions with conventional antibiotics, especially against this significant pathogen. **Materials and Methods**: Five solvents of varying polarity were used to prepare WH extracts that were dried and resuspended in aqueous solution (1% DMSO) for testing in agar disc diffusion and liquid microdilution MIC assays. **Results:** The water extract showed mild (but statistically insignificant) *P. aeruginosa* growth inhibition, whilst the methanolic extract produced significant inhibition on agar and an MIC value in broth assays of 587 µg/mL. Extracts prepared with ethyl acetate, hexane and chloroform were inactive. Combinations of the active extracts with ciprofloxacin (the only antibiotic used in this study that was active against *P. aeruginosa*) produced an antagonistic effect on growth inhibition. **Conclusion**: WH extracted with polar solvents inhibit *P. aeruginosa* growth but counteract the activity of the antibiotic ciprofloxacin. Mechanisms of WH extract activity towards this pathogen, and their interaction(s) with ciprofloxacin, are discussed.

Keywords: Witch hazel, *Pseudomonas aeruginosa*, Extracts, Antibacterial, Antagonism, Traditional medicine.

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INTRODUCTION

Pseudomonas aeruginosa is an intrinsically multi-drug resistance (MDR) opportunistic pathogen that causes acute and chronic infections in both immunocompromised and immunocompetent individuals.[1] It is a biofilm-forming organism that secretes a variety of virulence factors, rendering it capable of surviving in hypoxic and otherwise inhospitable environments.[2-4] Biofilm formation is an adaptive resistance mechanism of the organism and is observed in cystic fibrosis patients, leading to persistent infections and poor prognosis.[5,6] *Pseudomonas aeruginosa* is also a potential trigger for other diseases such as multiple sclerosis, bloodstream infections, and urinary tract infections.[7-9] The organism is also a prominent nosocomial pathogen that flourishes on medical devices.[10] In cases of MDR-*P. aeruginosa* infections in the respiratory system, higher mortality rates and longer periods of hospitalisation occur[11] and its economic burden in the US alone was almost US$800 million in 2017.[12] It possesses a high level of intrinsic resistance due to its restricted outer membrane permeability, as well as the presence of antibiotic efflux pumps and antibiotic-inactivating enzymes.[13,14] The various resistance mechanisms and phenotypes have rendered current pharmacotherapies unreliable, and their effective clinical therapies cannot be assured.[15]

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Due to these factors and, given the emergence of both the MDR and XDR (extensively drug-resistant) pathogens, the World Health Organisation has listed carbapenem-resistant *P. aeruginosa* as a priority 1 pathogen that urgently requires new therapies to counteract the global threat to public health.[16] Novel drugs and other therapeutic approaches are desperately required, but the standard approaches of antibiotic discovery and development are no longer cost-effective, which has dramatically slowed the production of new antibiotics.[17] There has been an increasing level of interest in the use of traditional plants as sources of new antimicrobial therapies.[18] Recently discovered plant-based antimicrobials that are either used alone or in combination with conventional antibiotics show great promise as effective antimicrobial pharmacotherapies.[19]

An example of a plant possessing promising antibacterial properties is Virginian witch-hazel (WH; *Hammamelis virginiana*

L.; family Hamamelidaceae). It has been used traditionally by Native Americans for thousands of years as treatments for dermatological wounds and inflammation.[20,21] We have previously demonstrated that extracts prepared from the plant inhibit the growth of several *Staphylococcus* and *Streptococcus* bacterial species,[22] yielding minimum inhibitory concentration (MIC) values as low as 210 µg/mL in microdilution broth assays. However, the growth inhibitory effects of the WH extracts against the intrinsically resistant *P. aeruginosa* reference strain have not been rigorously examined. The only studies that have been conducted in this regard have involved the commercial preparation whISOBAX™ (StaphOff Biotech Inc.) against *P. aeruginosa* in combination with iodine present in teat dips.[23] However, this preparation is a concentrated formula solubilised in 50% ethanol and thus not representative of an aqueous WH plant preparation. Indeed, the antibacterial activity reported in that study may be due primarily to the high ethanol concentration of the product. Furthermore, a commercial tincture elicited poor growth inhibitory activity against *P. aeruginosa* in microdilution broth assays in another study, producing mean MIC values of almost 3000 µg/mL.[24]

In the present study, WH was extracted with solvents of varying polarity and resuspended in an aqueous solution (1% DMSO) for testing against the *P. aeruginosa* reference strain to negate the antibacterial effects of any of the solvents used to generate the final preparations. Inhibition was measured on agar and in broth to approximate infections on both solid surfaces and in solutions. A selection of five antibiotics from different drug classes were also used in assays to test the *P. aeruginosa* resistance pattern, and any extracts showing antibacterial activity were combined with effective antibiotics. This work was conducted to firstly determine the effect of WH extracts against this organism, and then to ascertain whether any interactions occurred with antibiotics.

MATERIALS AND METHODS

Plant source and extractions

WH leaf material was purchased from Noodles Emporium (Australia) but was originally sourced from the US. Small (approx. 5 mm) leaf fragments were stored as voucher specimens (GU2018WHa) at the School of Environment and Science, Griffith University, Australia. Leaf material (1 g) was added to individual 50 mL tubes and then 50 mL of either sterile deionised water, methanol, ethyl acetate, hexane or chloroform were added, and plant material extracted in each solvent for 24hr at room temperature with constant agitation. All organic solvents were obtained from Ajax Fine Chemicals (Wollongong, Australia) and were AR grade. The extracts were filtered through Whatman No. 54 filter paper to remove particulate matter. Organic solvents were evaporated by air drying at 45ºC for 48 hr in a chemical fume hood. Aqueous extracts were freeze-dried by lyophilization at -80ºC in a VirTis Sentry 2.0 Bench Top Lyophilizer (SP Scientific, USA) for up to 72 hr. The dried extracts were then weighed, and were resuspended in 10 mL of sterile deionised water (containing 1% DMSO) and subjected to mild sonication (three 20 sec pulses at 1 kHz, with 30 sec rest between pulses). Extracts were then sterilised by filtration through 0.22 µm Millex-GS mixed cellulose ester membrane syringe filter units (Merck Pvt. Ltd., Baywater, Australia) and stored at 4ºC in tightly capped polypropylene tubes until required.

Bacterial cultures and assays

Pseudomonas aeruginosa (ATCC 27853) was purchased from the American Type Culture Collection (ATCC, USA) and used in this study. Antibacterial testing conditions conformed to CLSI standardised methods.[25] Powdered dehydrated media was purchased from Oxoid Ltd., (Scoresby, Australia) and the bacterial cultures were maintained at 37ºC in aerobic conditions on Mueller-Hinton (MH) agar for Disc Diffusion (DD) assays, and in MH broth for liquid cultures. 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 DD and microplate broth microdilution assays.

The antibacterial assays were conducted as previously described.[26] Briefly, the DD assays assessed bacterial susceptibility or resistance to inhibition on agar by the plant extracts or antibiotics. For microplate dilution broth assays, a standard liquid dilution MIC assay using 96-well microtitre plates was used to obtain quantitative measures of bacterial growth inhibition of the extracts and conventional antibiotics, or extract/antibiotic combinations.[26,27] Once the initial MIC values were obtained from serial two-fold dilutions of the originally prepared plant extracts, active extracts were further analysed by testing up to ten different dilutions of the original extract preparation in order to acquire more accurate MIC values for these extracts. Extract MIC values >5000 µg/mL were considered inactive; MIC values between 2000 and 5000 µg/mL were considered as low activity; 1000–2000 µg/mL were considered as moderate activity; 400–1000 µg/mL were considered as noteworthy activity; 100–400 µg/mL were considered as good activity; and <100 µg/mL were considered to be high activity.

Extracts and antibiotics were assessed in triplicate on agar and the zone of inhibition (ZOI) around each disc measured to the nearest millimetre. One-way analysis of variance (ANOVA) was used to calculate statistical significance between control and treatment groups, or between treatment groups. Although statistical analysis could not be performed with the broth microdilution assays, the reliability of MIC values was ensured by repeating the broth microdilution assays twice on separate days, with two replicates per assay (n = 4), to confirm that the results

were reproducible for all extracts, antibiotics and combinations tested.

Fractional inhibitory (FIC) and ΣFIC determinations

Only extracts and antibiotics showing activity were included in FIC determinations. A ratio of 50:50 of extract:antibiotic was tested, and interactions between the two components were examined by measuring the sum of fractional inhibitory concentrations (ΣFIC) for each combination. FIC values for each component (*A* and *E*) were calculated using the following equations, where *A* and *E* represent the antibiotic and extract components, respectively:

FIC (a) = MIC (*A* in combination with b) / MIC (*A* independently)

FIC (b) = MIC (*E* in combination with a) / MIC (*E* independently)

ΣFIC was then calculated using the formula $\Sigma FIC = FIC(A) + FIC(E)$, and the resultant values classified as synergistic ($\Sigma FIC \leq 0.5$), additive ($\Sigma FIC > 0.5– \leq 1.0$), indifferent ($\Sigma FIC > 1.0-\leq 4.0$) or antagonistic ($\Sigma FIC > 4.0$).[28]

RESULTS

Antibacterial activities on agar

The crude WH extracts (10 µL volumes) and antibiotics (penicillin, erythromycin, tetracycline, chloramphenicol, ciprofloxacin; 1 µg) were infused into 6 mm filter discs and subjected to DD assays in order to provide a semi-quantitative assessment of *P. aeruginosa* growth inhibition by these samples (Figure 1). A small ZOI was observed for the aqueous WH extract, but this was not found to be significantly different to the negative control. In contrast, a significantly larger mean ZOI value ($p < 0.001$) was obtained for the methanolic extract as compared to the aqueous extract or negative control. There was no growth inhibition on agar by the ethyl acetate, hexane and chloroform extracts. Of the antibiotics tested, only ciprofloxacin inhibited *P. aeruginosa* growth, producing large ZOI values, while penicillin, erythromycin, tetracycline and chloramphenicol were inactive against this bacterium.

MIC quantifications

The WH extracts were analysed further by initially using the undiluted crude extracts to obtain MIC values, and then with diluted extracts so that more precise MIC values could be obtained against *P. aeruginosa*. The reference antibiotics were also included in the microdilution broth assays at the highest concentration of 2.5 µg/mL. A summary of the MIC values obtained from this assay is shown in Table 1.

WH extracted with ethyl acetate, hexane and chloroform did not inhibit the growth of *P. aeruginosa* at the highest extract concentrations tested and were thus considered inactive. Similar findings were observed for four of the five antibiotics with MIC values >2.5 µg/mL. In contrast, ciprofloxacin showed activity towards *P. aeruginosa* at 0.156 µg/mL. While the aqueous WH extract showed moderate activity (1724 µg/mL) towards this pathogen, the activity of the methanolic extract was at least three-fold higher with an MIC value of 597 µg/mL, indicating a noteworthy inhibition of *P. aeruginosa* growth. Overall, the MIC values are generally concordant with activities observed on agar, where the active antibiotic and WH extracts produce ZOIs on agar and lower MIC values in the microdilution broth assays.

FIC determinations

Combination experiments could not be conducted on extracts or antibiotics that do not inhibit bacterial growth. Therefore, only one antibiotic (ciprofloxacin) and two of the WH extracts (aqueous and methanolic) were tested in combinations in FIC assays. FIC values were obtained by using 1:1 ratios of the antibiotic to the aqueous or methanolic extracts (Table 2) Notably, ΣFIC values >4.0 were obtained when extracts were combined with ciprofloxacin, indicating that the interaction(s) between them is antagonistic. Thus, the combination of these extracts with ciprofloxacin reduces the antimicrobial potency of each component used alone, and thus would be potentially detrimental to the effectiveness of the anti-pseudomonal therapy if both agents are used concomitantly.

DISCUSSION

The potential for WH extracts as a source of antimicrobial agents against *P. aeruginosa* has received little attention to date. Studies have been performed with a highly concentrated commercial WH tincture, but the findings are based on dilution of the preparation that contains 50% ethanol.[23,24] In one study, the MIC for this preparation was determined to arise from 1:20 and 1:30 dilutions of the tincture, which means that 1.7-2.5% ethanol was present at inhibition of *P. aeruginosa* growth at the MIC.[23] The presence of ethanol at these high concentrations is likely to contribute to growth inhibition but may also potentiate inhibitory activity, which would confound the findings. We opted to prepare WH extracts using different solvents of differing polarity in a way that approximated how they may have been prepared traditionally with water. To mitigate any potential antibacterial effects of the solvents, all solvents were removed by evaporation and resuspended in an aqueous solution in order to study their effects on *P. aeruginosa* growth.

The ethyl acetate, hexane and chloroform extracts were devoid of anti-pseudomonal activity on agar and in broth assays. WH extracted with water produced mild inhibition on agar and a moderate level of activity in broth, yielding an MIC value of 1724 µg/mL. The methanolic extract was far more potent, producing good inhibition on agar and a much lower MIC value of 587 µg/mL. Generally, the activities of the extracts may be reflected by the quantities and types of phytochemicals present in the samples. We have previously shown that water and methanol extract large

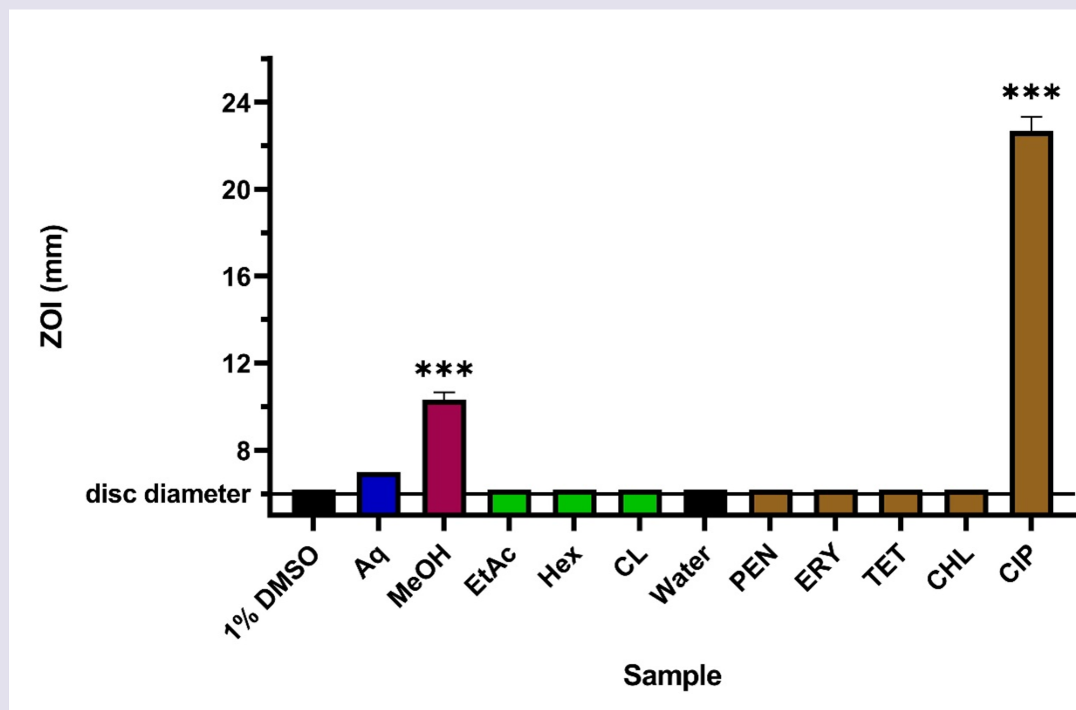

Figure 1: Agar disc diffusion assays for crude WH extracts and reference antibiotics against *P. aeruginosa*. The ZOI values were measured in mm and the disc sizes were 6 mm, as indicated on the y-axis. Negative control discs contained 1% DMSO (for crude extracts) or deionised water (for antibiotics). Aq = aqueous; MeOH = methanol; EtAc = ethyl acetate; Hex = hexane; CL = chloroform; PEN = penicillin, ERY = erythromycin, TET = tetracycline, CHL = chloramphenicol and CIP = ciprofloxacin. Values are expressed as mean ± SEM of triplicate assays. Results are shown as highly significantly different to the negative control if $p < 0.001$ (***). A highly significant difference ($p < 0.001$) between the value for the aqueous (Aq) and methanol (MeOH) WH extracts was also found (not indicated).

Table 1: MIC values (µg/mL) for WH extracts and the reference antibiotics against *P. aeruginosa*.

Extract type or antibiotic	MIC (µg/mL)
Water	1724
Methanol	587
Ethyl Acetate	>5000
Hexane	>5000
Chloroform	>5000
PEN	>2.5
ERY	>2.5
TET	>2.5
CHL	>2.5
CIP	0.156

PEN = penicillin, ERY = erythromycin, TET = tetracycline, CHL = chloramphenicol and CIP = ciprofloxacin. The range of concentrations used in the assays was 0.01-10 mg/mL for the plant extracts and 0.01-2.5 µg/mL for the reference antibiotics. Values >2.5 µg/mL for the antibiotics indicate lack of growth inhibition at the highest concentration of antibiotic examined.

amounts of flavonoids, tannins, phenolics and both water-soluble and water-insoluble phenols,[22] which may be responsible for the activities observed in these extracts. However, WH extracted with water contains lower levels of cardiac glycosides, and does not contain saponins and terpenoids, which can be found in WH extracted with methanol. It is possible that the presence and higher abundance of phytochemicals in the methanolic extract enhances inhibition of *P. aeruginosa* growth to produce stronger inhibition on agar and lower MIC values in the liquid dilution assay. Furthermore, the substantially lower abundance of phytochemicals in the ethyl acetate, hexane and chloroform extracts[22] is likely to explain their inability to inhibit bacterial growth in this study.

Plant extract preparations, or compounds isolated from plants, have received increasing levels of interest in recent years. This has occurred since evidence is accumulating that they contain either single antimicrobial compounds or that inhibition of pathogens require a combination of antimicrobial compounds and potentiator molecules.[19,29,30] Additionally, they may also synergistically enhance the effects of conventional antibiotics.[31-33] WH contains gallic acid, gallocatechin and epigallocatechin, which are known to interact with bacterial cell lipid bilayers,[34-36] which may be one mechanism by which the active WH extracts overcome the intrinsic resistance associated with *P. aeruginosa*. Interestingly, synthetic fusion of the macromolecular nucleophile

Table 2: FIC and ΣFIC values for the combinations of active aqueous and methanolic WH extracts with ciprofloxacin against the *P. aeruginosa* strain used in this study.

Extract solvent	FIC or ΣFIC values	
Aqueous	FIC_{EXT}	0.50
	FIC_{CIP}	4.01
	ΣFIC	4.51
Methanol	FIC_{EXT}	1.00
	FIC_{CIP}	8.01
	ΣFIC	9.01

chitosan with flavan-3-ols extracted from WH yielded compounds that exerted an inhibitory effect on *P. aeruginosa* growth, producing MIC values as low as 125 µg/mL,[37] and this inhibition was postulated to occur via membrane permeability alterations. WH also contains Hamamelitannin (HAMA), and while this compound appears not to have any effect against Staphylococcal species,[38] it may play a role in the inhibition of *P. aeruginosa* growth. Indeed, tannins (in addition to saponins, alkaloids and flavonoids) and have been shown to inhibit *P. aeruginosa* adhesion, swarming motility and the formation of biofilms.[39,40]

One of the antibiotics tested in this study, ciprofloxacin, inhibited *P. aeruginosa* growth, further evidence of the highly resistant nature of this microorganism. Therefore, ciprofloxacin was the only antibiotic that could be subjected to combination experiments with the two active WH extract types. In both cases, the interaction between ciprofloxacin and the WH extracts was antagonistic. This is an important finding. Ciprofloxacin operates by a mechanism that involves inhibition of the bacterial DNA gyrase enzyme.[41] It is possible that the phytochemicals present in the active WH extracts are competing with the antibiotic for the gyrase enzyme, thereby decreasing the effectiveness of the antibiotic. A previous study showed that a fraction isolated from the methanolic extract of the plant *Phyllanthus muellerianus*, when combined with ciprofloxacin, antagonised the growth of *P. aeruginosa*.[42] We have previously shown that WH water and methanolic extracts combined with ciprofloxacin yields either indifferent or additive interactions on *S. aureus* bacterial growth.[22] However, differences in the antibiotic targets and cell wall and lipid bilayer between *S. aureus* and *P. aeruginosa* may account for this difference. Isolation and further study of unique phytochemicals found in methanolic WH extracts would need to be conducted to confirm this postulated interaction.

Other mechanisms may also contribute to the antagonism of ciprofloxacin by the WH extracts, including the possible effects of WH phytochemicals on *P. aeruginosa* membrane fluidity. Ciprofloxacin affects anionic phospholipids to alter fluidity,[43] as do multiple phytochemicals including flavonoids, terpenoids and alkaloids.[44] Interactions between the antibiotic and the active extracts in the present study may be preventing ciprofloxacin entry through the *P. aeruginosa* cell membrane. Additionally, phytochemicals from many plant sources have been found to act as efflux pump inhibitors, and thus potentiate the efficacies of antibiotics.[45] Specific combinations of ciprofloxacin and WH phytochemicals on *P. aeruginosa* may manifest themselves such that efflux of the antibiotic is enhanced, although this suggested mechanism, and the possible effects of the combination on membrane fluidity, require further investigation.

Our findings suggest that the combination of WH phytochemicals from the extracts should be avoided as potential combinatorial therapies with ciprofloxacin to treat *P. aeruginosa* infection, as the effectiveness of one or both components are significantly decreased.

CONCLUSION

Whilst water WH extracts have moderate activity against *P. aeruginosa* growth on agar and broth, the methanol extracts yielded considerably higher activities against this pathogen. In contrast, extracts prepared with ethyl acetate, hexane and chloroform were inactive. Interestingly, antibacterial activities were antagonised when combined with ciprofloxacin, suggesting that this combination ameliorates the anti-pseudomonal potency.

The identity of the compounds responsible for the inhibition of *P. aeruginosa* is the aim of a future study in our laboratory, using mass spectrometry and other methodologies, to ascertain the compounds responsible for activity and for the mechanism(s) causing antagonism when combined with ciprofloxacin.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ABBREVIATIONS

ANOVA: Analysis of Variance; **DD**: Disc Diffusion; **DMSO**; Dimethyl sulfoxide; **FIC:** Fractional Inhibitory Concentration; **HAMA:** Hamamelitannin; **HPLC:** High-performance liquid chromatography; **MDR:** Multi-Drug Resistance; **MH:** Mueller Hinton; **MIC:** Minimum Inhibitory Concentration; **WH:** Witch Hazel; **XDR:** Extensively Drug Resistant; **ZOI:** Zone of Inhibition.

SUMMARY

- Aqueous and methanolic witch hazel extracts inhibited the growth of *Pseudomonas aeruginosa*.
- Inhibition of growth is mild (statistically insignificant) with the aqueous extract.
- The methanolic witch hazel extract showed good activity on both agar and in liquid broth.
- Combinations of the active extracts with ciprofloxacin antagonised the inhibition of *P. aeruginosa* growth.

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