Pharmacognosy Communications, 2024; 14(4):175-184.
https://www.phcogcommn.org

Original Article

Hamamelis virginiana L. Leaf Extracts Inhibit Some Bacterial Triggers of Selected Autoimmune Inflammatory Diseases

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ABSTRACT

Background: *Hamamelis virginiana* L. leaves were used traditionally to treat bacterial infections and inflammation. Despite the traditional uses, relatively few studies have examined the ability of *H. virginiana* leaf extracts to inhibit the growth of the bacterial triggers of autoimmune inflammatory diseases. **Materials and Methods:** *Hamamelis virginiana* extracts were screened for growth inhibitory activity against bacterial triggers of multiple sclerosis (MS) and rheumatic fever (RF) by disc diffusion assays and the potency was quantified using liquid dilution MIC assays. Toxicity was evaluated using the *Artemia* nauplii cytotoxicity assay (ALA) and the therapeuitic index was calculated as a measure of therapeutic safety. **Results:** *Hamamelis virginiana* extracts strongly inhibited the growth of some bacterial triggers of MS and RF. The methanolic extract was a particularly good inhibitor of an antibiotic-resistant strain of *P. aeruginosa* (MIC=113 µg/mL), although it also had noteworthy inhibitory activity against *A. baylyi* and *S. pyogenes* (245 and 368 µg/mL respectively). The aqueous *H. virginiana* leaf extract also displayed noteworthy activity against the same bacteria, albeit with higher MIC values (560-1120 µg/mL). Both extracts have potential for preventing the onset of MS and RF, and for treating these diseases and decreasing the symptoms once they have been initiated. Additionally, the methanolic and aqueous extracts potentiated the activity of several conventional antibiotics in bacterial strains otherwise resistant to those antibiotics. Notably, both the methanolic and aqueous extracts synergised the activity of tetracycline against all bacteria tested, indicating that combinations containing tetracycline may be particularly useful in preventing MS and RF. None of the *H. virginiana* leaf extracts were toxic in the ALA toxicity assay. **Conclusion:** *Hamamelis virginiana* leaf extracts inhibit the growth of some bacterial triggers of MS and RF, as well as potentiating the activity of conventional antibiotics. Further *in vivo* studies to determine the anti-inflammatory and antibacterial mechanisms are warranted.

Keywords: Witch-hazel, North American plants, Antibacterial activity, Skin therapy, Inflammation, Multiple sclerosis, Rheumatic fever, Tannins.

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Received: 05-08-2024;
Revised: 21-08-2024;
Accepted: 04-09-2024.

INTRODUCTION

Exposure to specific antigens in genetically susceptible people may stimulate the immune system to produce self-reactive antibodies, which may result in autoimmune inflammatory diseases.[1-3] Whilst environmental and dietary stimuli (e.g. the gluten stimulus in Celiac's disease) have been identified as triggers for so autoimmune diseases, others may be triggered by specific bacterial pathogens[1] and inhibiting these bacterial triggers may block the onset of these disease in genetically susceptible people. Inhibiting the disease etiology would also prevent the later-stage inflammatory cascades and the symptoms associated with these diseases. Notably, several antigenic triggers of autoimmune inflammatory diseases have already been identified through genotyping and serotyping1 providing novel therapeutic targets to block the etiological events of these diseases. The bacterium *Proteus mirabilis* can trigger rheumatoid arthritis (RA) in genetically susceptible people and recent studies have targeted this pathogen as a preventative treatment for that disease.[4-6] Similarly, *Klebsiella pneumoniae* can initiate ankylosing spondylitis (AS) in some people and inhibition of this bacterium has also attracted recent interest for this reason.[7-9] Additionally, multiple sclerosis (MS) may be initiated by *Acinetobacter baylyi* and/or *Pseudomonas aeruginosa* infections in some people,[10] whilst rheumatic fever (RF) may be induced by *Streptococcus pyogenes* infections in people with specific genetic markers.[11]

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DOI: 10.5530/pc.2024.4.27

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Traditional medicines used to treat inflammation and/or bacterial infections may provide promising leads for the development of novel therapies for the prevention and treatment of autoimmune inflammatory diseases. The usage of plants medicinally is well documented for some cultures,[1,12] particularly for Asian and African traditional medicine systems. In contrast, there are relatively few rigorous ethnobotanical studies for many other cultures, which may hinder relevant research into traditional medicines of those ethnic groups. *Hamamelis virginiana* L. (family Hamamelidaseae; commonly known as witch-hazel, common witch-hazel and American witch-hazel) is a flowering plant (Figure 1a) that is native to eastern regions of North America (from Novia Scotia to Minnesota and south to Florida). It is a deciduous tree or shrub that grows to 6 m in height, with oval leaves up to 17 cm long and 13 cm wide (Figure 1b). The pale-yellow flowers (Figure 1c) grow in clusters from mid to late autumn/fall (Figure 1b). *Hamamelis virginiana* produces characteristic tannin compounds called hamamelitannins (Figure 1d), that may hydrolyse to release gallic acid moieties.[13] Hamamelitannins (and their gallic acid moieties) have been linked with several of the traditional uses of this plant.

Hamamelis virginiana has numerous traditional therapeutic applications, although the most frequent uses are for the treatment of inflammation and to alleviate haemorrhoids and superficial skin wounds. Traditionally, *H. virginiana* was prepared as decoctions by boiling the whole shrub (leaves and/or bark) and then applying it topically it to the skin for most therapies.[13,14] However, when used to treat to treat colds, fevers and other pathogenic diseases, the decoction was ingested orally.[13] Its uses as an astringent and for the treatment of acne and irritable scalp conditions have also been extensively documented.[13] Notably, several of these conditions are caused (or exacerbated) by bacteria. Despite this, there is a relative lack of scientific evidence for the antibacterial activity of this species. Furthermore, most studies screening the antibacterial properties of *H. virginiana* leaf extracts have focussed on skin pathogens,[13] whilst other bacteria have generally been neglected. Multiple studies have screened *H. virginiana* leaf extracts for therapeutic bioactivities and have reported substantial anti-inflammatory, antioxidant and anti-proliferative properties for *H. virginiana* leaf extracts.[15-20] Despite the traditional uses of *H. virginiana* leaves and several previous antibacterial studies,[13] their growth inhibitory properties have not been extensively tested against the bacterial triggers of MS or RF. This study aimed to address this gap in the literature by quantifying the antibacterial activity of the *H. virginiana* leaf extracts against *Acinitobacter baylyi, Pseudomonas aeruginosa* and *Streptococcus pyogenes*. Additionally, the interactions of the extracts with selected conventional antibiotics was tested to highlight combinations with potentiated activity. The extract components of any potentiating combinations may highlight future therapeutic options with substantially improved antibacterial activity, particularly against antibiotic-resistant bacteria.

MATERIALS AND METHODS

Sourcing and preparation of plant samples

Dried and ground *Hamamelis virginiana* L. leaves were purchased from Noodles Emporium, Australia. Voucher specimens are deposited in the School of Natural Sciences, Griffith University, Australia (voucher number GU2018WHa). Individual quantities (1 g) of the dried leaves were weighed into separate tubes and 50 mL of methanol, deionised water, ethyl acetate, chloroform or hexane was added. All solvents were obtained from Ajax Fine Chemicals, Australia and were AR grade. The ground leaf materials was extracted in each solvent for 24 hr at 4ºC with gentle shaking and were then filtered through Whatman No. 54 filter paper under vacuum. The solvent extracts were subsequently air dried at room temperature, whilst the aqueous extract was lyophilised by freeze drying at -50ºC. The resultant dried extracts were weighed and dissolved in 10 mL of deionised water (containing 1% DMSO) and stored as aliquots at -30ºC until use.

Qualitative phytochemical studies

Qualitative phytochemical analysis of the *H. virginiana* leaf extracts to detect the presence of saponins, phenolic compounds, flavonoids, phytosteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids (and evaluate their relative abundances) was conducted using standard assays.[21-23]

Antibacterial analysis

Conventional antibiotics

Penicillin-G (potency of 1440-1680 µg/mg), chloramphenicol (≥98 % purity by HPLC), erythromycin (potency ≥850 µg/mg), ciprofloxacin (≥98 % purity by HPLC), gentamicin (potency of 600 µg/mg), and tetracycline (≥95% 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 0.01 mg/mL and stored at 4ºC until use. Standard discs of ampicillin (10 µg) and chloramphenicol (10 µg) were obtained from Oxoid Ltd., Australia and used as positive controls in the disc diffusion susceptibility assays.

Bacterial cultures

The bacterial pathogens screened in this study were selected for study as they are can trigger autoimmune inflammatory diseases in genetically susceptible individuals.[1,2] Reference strains of *Acinetobacter baylyi* (ATCC33304), *Pseudomonas aeruginosa* (ATCC39324) and *Streptococcus pyogenes* (ATCC12384) were purchased from American Type Culture Collection, USA. All bacteria were cultured at 37ºC in nutrient broth (Oxoid Ltd., Australia) for 24 h and maintained in nutrient broth at 4ºC until

use. Streaked nutrient agar (Oxoid Ltd., Australia) plates were prepared to the manufactures specifications and were tested in parallel to ensure the purity of all bacterial cultures.

Evaluation of bacterial susceptibility to growth inhibition

Bacterial susceptibility to the *H. virginiana* extracts and the conventional antibiotics was assessed using standard disc diffusion assays.[24] Ampicillin (10 µg) and chloramphenicol discs (10 µg) were obtained from Oxoid Ltd., Australia and included in the assays as positive controls. Filter discs infused with 10 µL of distilled water were included as a negative control.

Minimum inhibitory concentration (MIC) determination

The antibacterial activity of the individual *H. virginiana* extracts and conventional antibiotics was quantified using standard liquid dilution MIC assays.[25-27] Briefly, 100 µL of sterilized distilled water was first dispensed into each well of 96 well micro-titre plate. The *H. virginiana* extracts or conventional antibiotics (100 µL) were then individually dispensed into the first row of the plate. Nutrient broth (negative control) and a sterile control (media without bacteria) were included on all plates to verify that the assay was functioning correctly. Each test well was then serially diluted down each column by doubling serial dilution. Individual bacterial cultures (100 µL containing approximately $1x10^6$ Colony Forming Units (CFU)/mL) were then added to all wells of the plate (excluding the sterile control wells) and incubated at 37°C for 24 hr. The colourimetric indicator p-iodonitrotetrazolium violet (INT) (Sigma-Aldrich, Australia) was prepared in sterile deionised water as a 0.2 mg/mL INT stock solution. Following the incubation, 40 µL of the INT stock solution was dispensed into all wells and the plates were incubated for 6 hr at 30 °C to allow full colour development. The lowest dose at which colour development was completely inhibited was classified as the MIC of the test.

Fractional inhibitory concentration (FIC) assessment

Interactions between the combinations of plant samples and conventional antimicrobials were,

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

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

The $^{\Sigma}$FIC was then calculated using the equation: $^{\Sigma}FIC=FIC^{(i)}+FIC^{(ii)}$. The interactions were classified as being synergistic for $^{\Sigma}$FIC values of ≤0.5, additive (>0.5-1.0), indifferent (> 1.0-≤ 4.0) or antagonistic (> 4.0).[24-27]

Toxicity screening

Toxicity evaluations of the *H. virginiana* extracts, conventional antibiotics and the reference toxin were assessed using adapted

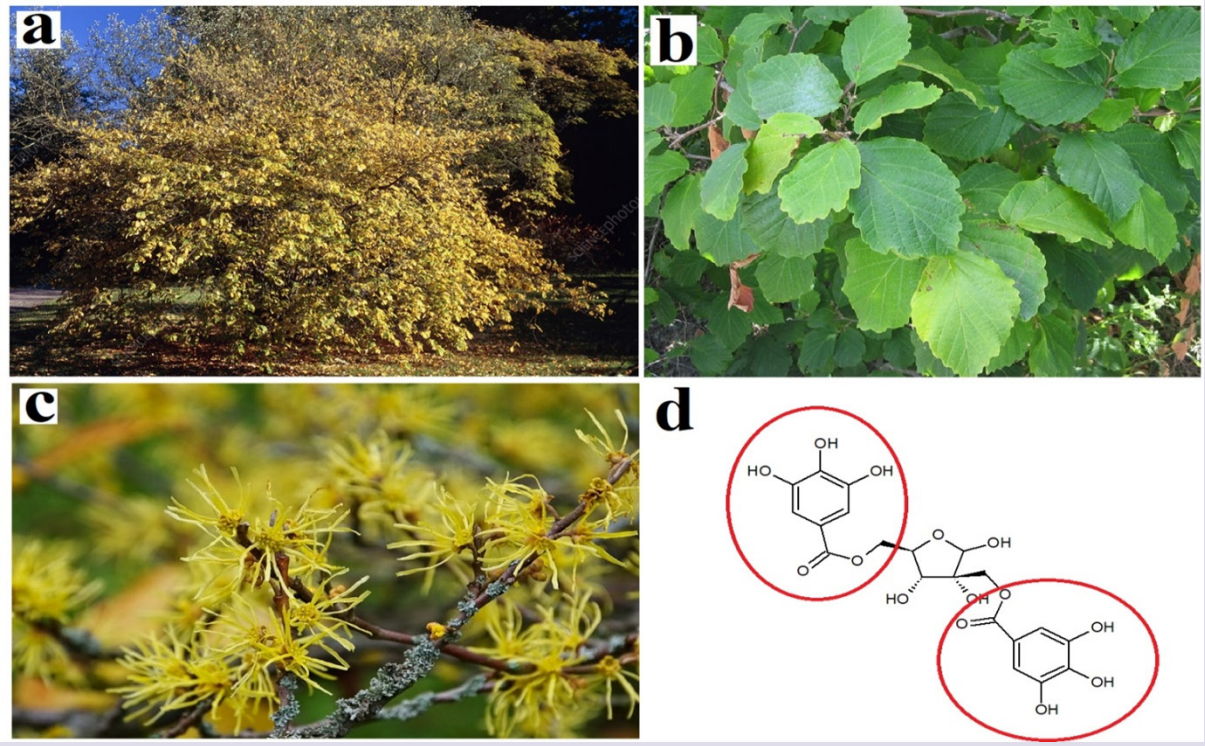

Figure 1: *Hamamelis virginiana* L. (a) whole plant, (b) leaves and (c) flowers, as well as (d) hamamelitannin (with hydrolysable gallic acid moieties highlighted).

Artemia franciscana nauplii Lethality Assays (ALA).[28] 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 mortality induction of all tests and controls was assessed following 24 hr exposure and is expressed as a % of the untreated control. The LC_{50} for each treatment was calculated using Probit analysis.

Statistical analysis

All data is expressed as the mean±SEM of 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

Liquid extraction yields and qualitative phytochemical screening

Extractions of the dried *H. virginiana* leaf material (1 g) with solvents of varying polarity yielded dried plant extracts ranging from 2 mg (*H. virginiana* ethyl acetate extract) to 332 mg (*H. virginiana* methanolic extract) (Table 1). Qualitative phytochemical screening showed that the higher polarity solvents (methanol and water) extracted the greatest amount and widest diversity of phytochemical classes.

Inhibition of bacterial triggers of multiple sclerosis (*A. baylyi* and *P. aeruginosa*)

The growth inhibitory activity of the *H. virginiana* leaf extracts was screened against two bacterial triggers of MS (*A. baylyi, P. aeruginosa*). The methanolic and aqueous *H. virginiana* leaf extracts were effective inhibitors of *A. baylyi* growth, with ZOIs of 15.2 and 10.3 mm respectively (Figure 2). These results compare well to the inhibition of these bacteria by the control antibiotics. Indeed, the aqueous extract produced similar ZOIs as the ampicillin control (10.3 and 10.7 mm respectively), whilst substantially bigger ZOIs were noted for the methanolic extract and the chloramphenicol control (ZOIs of 15.2 and 16.8 mm respectively). These results are noteworthy because the control antibiotics were tested as relatively high doses of pure compounds, whereas the extracts are complex mixtures of compounds, of which the antibacterial compound(s) would be expected to account for a minor %. Thus, the noteworthy activity of the methanolic and aqueous *H. virginiana* leaf extracts are particularly promising for inhibiting *A. baylyi* infections, and therefore for inhibiting MS. In contrast, the ethyl acetate, chloroform and hexane extracts were completely ineffective at inhibitors of *A. baylyi* growth.

The methanolic and aqueous *H. virginiana* leaf extracts were also effective at inhibiting *P. aeruginosa* growth (another trigger of MS). The methanolic extract was particularly promising, with a ZOI of 11.2 mm measured (Figure 3). Notably, this strain of *P. aeruginosa* has previously been reported to be resistant to multiple antibiotics.[24,27,29] Indeed, ampicillin was a completely ineffective inhibitor of *P. aeruginosa* growth in the disc diffusion

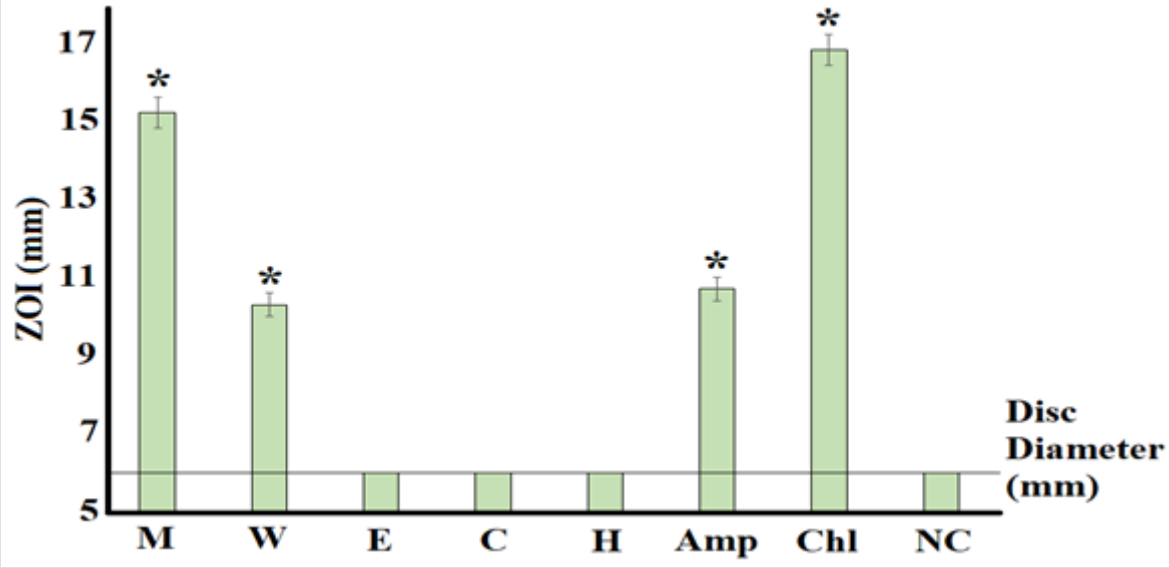

Figure 2: Antibacterial activity of the *H. virginiana* extracts against *A. baylyi* (ATCC33304), measured as zones of inhibition (mm). M=methanolic extract; W=aqueous extract; E=ethyl acetate extract; C=chloroform extract; H=hexane extract; Amp=ampicillin (10 µg); Chl=chloramphenicol (10 µg); NC=negative Control (1% DMSO). Results are expressed as mean zones of inhibition of three replicates, each with internal triplicated (n=9)±SEM * indicates results that are significantly different to the negative control (p<0.01).

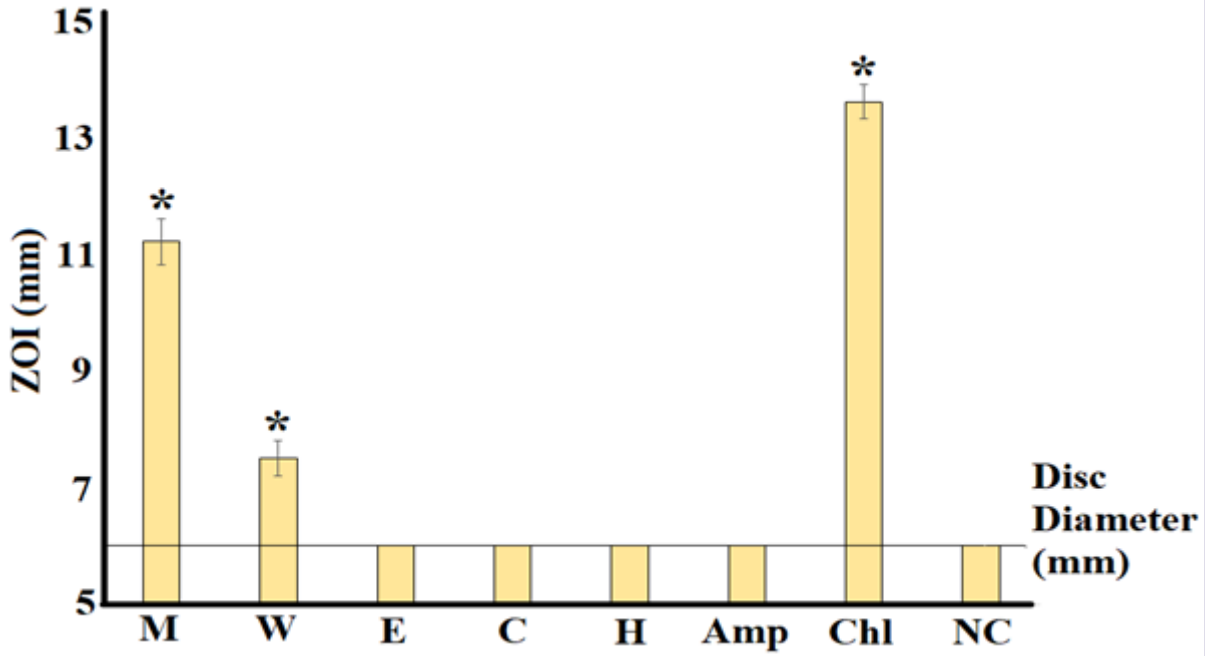

Figure 3: Antibacterial activity of the *H. virginiana* extracts against *P. aeruginosa* (ATCC39324) measured as zones of inhibition (mm). M=methanolic extract; W=aqueous extract; E=ethyl acetate extract; C=chloroform extract; H=hexane extract; Amp=ampicillin (10 µg); Chl=chloramphenicol (10 µg); NC=negative Control (1% DMSO). Results are expressed as mean zones of inhibition of three replicates, each with internal triplicated (n=9)±SEM * indicates results that are significantly different to the negative control (p<0.01).

assay. Therefore, this extract may be particularly useful for treating β-lactam resistant strains of *P. aeruginosa*. In contrast, this bacterial strain was highly susceptible to chloramphenicol, confirming that the assay was functioning correctly. Whilst the aqueous *H. virginiana* leaf extract also inhibited *P. aeruginosa* growth, the measured ZOI was substantially smaller (7.5 mm), indicating lower potential for that extract for preventing and treating MS (and other diseases caused by *P. aeruginosa* infections). As noted for *A. baylyi*, the ethyl acetate, chloroform and hexane extracts were ineffective at inhibiting the growth of *P. aeruginosa*.

Inhibition of the bacterial trigger of rheumatic fever (*S. pyogenes*)

The methanolic and aqueous *H. virginiana* leaf extracts displayed noteworthy inhibition of *S. pyogenes* growth (Figure 4), with ZOIs of 12.3 and 9 mm respectively. Notably, this *S. pyogenes* strain was completely resistant to the ampicillin control in this assay, indicating that these extracts may be particularly useful in preventing and treating RF in genetically susceptible people, as well as treating other diseases caused by this bacterium. In contrast, the chloramphenicol positive control was a strong inhibitor of *S. pyogenes* growth (ZOI of ~13 mm). The ethyl acetate, chloroform and hexane extracts were completely ineffective against the *S. pyogenes* strain screened in our study.

Quantification of minimum inhibitory concentration (MIC)

The antimicrobial activity of the *H. virginiana* extracts and conventional antibiotics was further evaluated by determining the MIC values by liquid dilution MIC assays (Table 2). MIC values >1 µg/mL for the pure conventional antibiotic standards have previously been defined as indicative of antibiotic resistance.[25-27] Notably, all of the bacterial strains tested were resistant to penicillin-G, chloramphenicol, erythromycin and tetracycline. In contrast, all bacterial strains were susceptible to ciprofloxacin and gentamycin (MIC values 0.32-0.64 µg/mL). The methanolic and aqueous *H. virginiana* extracts also displayed noteworthy growth inhibitory activity against A. *baylyi, P. aeruginosa* and *S. pyogenes* (Table 2). The methanolic *H. virginiana* leaf extract was the most potent inhibitor of the growth of all bacteria, with MIC values of 245, 113 and 368 µg/mL against *A. baylyi, P. aeruginosa* and *S. pyogenes* respectively. Substantially higher MICs were determined for the aqueous extract (560, 560 and 1120 µg/mL), although these values also indicate noteworthy growth inhibition. Therefore, the methanolic and aqueous *H. virginiana* extracts may be useful for preventing MS and RF, and for treating those conditions (as well as other infections caused by infections of those bacteria) once infections are established.

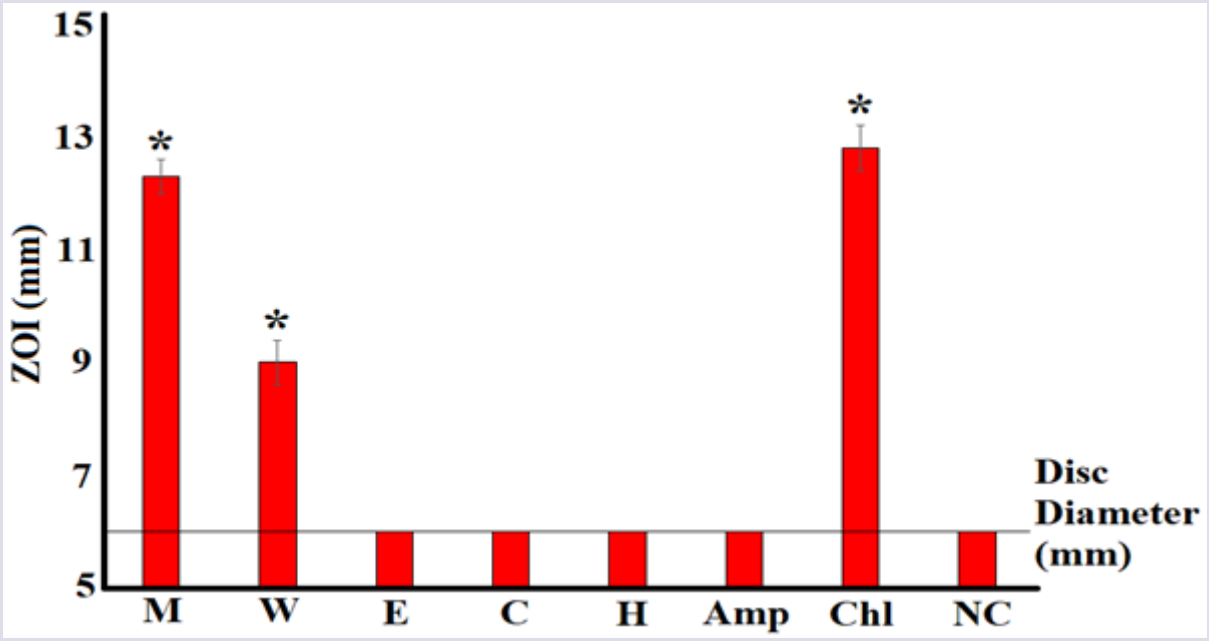

Figure 4: Antibacterial activity of the *H. virginiana* extracts against *S. pyogenes* (ATCC12384) measured as zones of inhibition (mm). M=methanolic extract; W=aqueous extract; E=ethyl acetate extract; C=chloroform extract; H=hexane extract; Amp=ampicillin (10 µg); Chl=chloramphenicol (10 µg); NC=negative Control (1% DMSO). Results are expressed as mean zones of inhibition of three replicates, each with internal triplicated (n=9)±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 *T. bellericia* extracts against microbial triggers of some autoimmune inflammatory diseases.

Extract	Mass of Dried Extracted Material (mg)	Conventration of ectract (mg/mL)	Phenols			Cardiac	Saponins	Triterpenes	Phytosterols	Alkaloids		Flavanoids		Tannins	Anthraquinones	
			Total Phenolics	Water Soluble	Water Insoluble	Keller-Kiliani Test	Froth Persistence	Salkowski Test	Acetic Anhydride Test	Meyers Test	Wagners Test	Shinoda Test	Kumar test	Ferric Chloride Test	Free	Combined
Methanol	332	33	+++	+++	+++	-	++	+	-	-	-	+++	+++	+++	-	-
Water	179	18	+++	+++	+++	-	-	-	-	-	-	+++	+++	+++	-	-
Ethyl Acetate	2	0.2	+	+	+	-	-	-	-	-	-	+	+	+	-	-
Chloroform	19	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hexane	6	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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

Table 2: MIC values of the *H. virginiana* extracts and conventional antibiotics (µg/mL) against some bacterial triggers of selected autoimmune anti-inflammatory diseases.

Extract or Antibiotic		*A. baylyi* (ATCC33304)	*P. aeruginosa* (ATCC39324)	*S. pyogenes* (ATCC12384)
Extracts	Methanol	245	113	368
	Aqueous	560	560	1120
	Ethyl Acetate	-	-	-
	Chloroform	-	-	-
	Hexane	-	-	-
Conventional Antibiotics	Penicillin-G	2.5	1.25	2.5
	Chloramphenicol	2.5	1.25	1.25
	Erythromycin	1.25	2.5	2.5
	Tetracycline	1.25	1.25	1.25
	Ciprofloxacin	0.32	0.64	0.32
	Gentamicin	0.32	0.64	0.32

- indicates no inhibition at any dose tested.

Table 3: ΣFIC values for the *H. virginiana* extracts in combination with conventional antibiotics against some bacterial triggers of autoimmune diseases.

	A. baylyi		*P. aeruginosa*		*S. pyogenes*	
	M	W	M	W	M	W
Penicillin-G	1.08	1.26	1.1	1.22	1.66	2.27
	(IND)	(IND)	(IND)	(IND)	(IND)	(IND)
Chloramphenicol	1.14	1.4	1.37	1.18	1.92	1.83
	(IND)	(IND)	(IND)	(IND)	(IND)	(IND)
Erythromycin	0.41	2.5	1	1	0.95	1.26
	(SYN)	(IND)	(ADD)	(ADD)	(ADD)	(IND)
Tetracycline	0.13	0.14	1.13	0.38	0.38	0.38
	(SYN)	(SYN)	(IND)	(SYN)	(SYN)	(SYN)
Ciprofloxacin	0.13	2.03	1	1	2.38	4.25
	(SYN)	(IND)	(ADD)	(ADD)	(IND)	(ANT)
Gentamicin	2	1.25	4.13	1	1	1
	(IND)	(IND)	(ANT)	(ADD)	(ADD)	(ADD)

M=Methanolic extract; W=Aqueous extract; SYN=Synergistic interaction; ADD=Additive interaction; IND=Indifferent interaction; ANT=Antagonistic interaction.

ΣFIC Determination

Six of the combinations of the *H. virginiana* extract and conventional antibiotics produced synergistic effects when tested together against the bacteria screened in this study (Table 3). The majority of the synergistic interactions contained tetracycline as the antibiotic component in bacteria otherwise resistant to the effects of that antibiotic. With few exceptions, bacteria generally develop tetracycline resistance through the expression of tetracycline-specific efflux pumps.[30,31] Thus, the *H. virginiana* extract components may function as efflux pump inhibitors, thereby effectively increasing the intracellular concentration of tetracycline and increasing the effectiveness of the therapy. However, in some cases, tetracycline resistance may be due to bacterial ribosome modification.[30,31] Further studies are required to determine whether the methanolic and aqueous extract are functioning via efflux pump inhibition.

Several other combinations containing erythromycin, ciprofloxacin or gentamicin produced additive interactions, particularly against *P. aeruginosa* and *S. pyogenes*. Whilst the potentiation noted for these combinations was not as great as for the synergistic combinations, they still substantially increase the effectiveness of the different components when used separately. Therefore, those combinations are still beneficial for use against those bacteria. The majority of the other combinations had

non-interactive effects. Whilst these combinations have no added benefit over using the extract or antibiotic components separately, they also don't decrease each other's activities and therefore would be safe to use in combination without decreasing the efficacy. This is important information as many users of traditional and herbal medicine use those therapies in combination with allopathic therapies, often without informing their medical practitioner. Notably, two antagonistic interactions were also noted, indicating that those combinations should be avoided when treating *P. aeruginosa* and *S. pyogenes* infections.

Toxicity studies

All of the *H. virginiana* extracts (1000 µg/mL) and the conventional antibiotics (10 µg/mL) were tested individually in the *Artemia* lethality assay (ALA) (Table 3). The compounds were only considered toxic if they induced percentage mortalities greater than 50% following 24 hr of exposure to the *Artemia* nauplii.[28] All of the conventional antibiotics and *L. tridenta* extracts induced substantially <50 % mortality. Therefore, all extracts and antibiotics were deemed to be nontoxic. In contrast, the positive control potassium dichromate induced 100 % mortality in the ALA, indicating that the assay was functioning correctly.

DISCUSSION

This study evaluated and quantified the growth inhibitory properties of *H. virginiana* leaf extracts against bacterial triggers of multiple sclerosis and rheumatic fever.[1,2] There are currently no widely available effective cures for these diseases. Instead, most current therapies target the disease symptoms (particularly inflammation and pain) using anti-inflammatory drugs (particularly non-steroidal anti-inflammatory drugs (NSAIDs)) and analgesics. These treatments alleviate the patient's discomfort and suffering, although they do not alter the disease progression or decrease the auto-immune damage to self-tissue. Furthermore, prolonged usage of allopathic anti-inflammatory therapies is often toxic and induces numerous unwanted side-effects.[32] For example, the use of cyclooxygenase-2 (COX-2) inhibitors over an extended period increases the risk of myocardial infarction.[33] Chemotherapies that target the etiology of the autoimmune diseases yet also reduce inflammation may be particularly effective for preventing these diseases, as well as treating them once they have been initiated. This approach may inhibit neuronal myelin and cardiac tissue degradation during MS and RF in genetically susceptible people, as well as downregulating the inflammatory symptoms caused by these diseases.

Surprisingly, studies analysing the potential of *H. virginiana* leaf extracts as MS and RF inhibitors by blocking the growth of the trigger pathogens have been largely neglected. Our study focussed on the trigger mechanisms of these diseases by inhibiting the growth of their bacterial triggers. *Acinitobacter baylyi* and *P. aeruginosa* can induce MS, whilst *S. pyogenes* is a trigger of RF in genetically susceptible people.[1,2] Whilst several previous studies have screened *H. virginiana* leaf extracts for antibacterial activity, most studies have concentrated on other bacterial species. One study reported weak growth inhibitory activity for a *H. virginiana* distillate against *Staphylococcus aureus* and *Staphylococcus epidermidis*.[34] However, the distillate tested in that study contained a high level of urea (5%). As urea itself has substantial antibacterial activity, this may account for the reported activity and it is uncertain of the distillate's contribution to this activity.[35] In contrast, another study reported strong anti-*Staphylococcus* activity towards these two bacterial strains for a commercial product marketed as whISOBAX (available from StaphOff Biotech Inc., Hopkinton, USA) that contains *H. virginiana* as the bioactive component.[36] However, that product is supplied as a tincture, and contains high concentrations of ethanol, which are likely to contribute substantially to the reported strong antibacterial activity of that product. Similar studies tested Dickinson's® Witch Hazel (T.N. Dickinson Co. USA), a commercial *H. virginiana* preparation, and reported that the preparation inhibited *Staphylococcus mutans* growth and decreased tooth biofilm formation.[37,38] However, Dickinson's® Witch Hazel contains 14% ethanol, which may have provided a falsely high evaluation of bacterial growth inhibitory activity of that preparation. More recently, a study from our group screened *H. virginiana* extracts that were devoid of non-extract adulterants that may affect antibacterial activity and reported noteworthy growth inhibitory activity against *Streptococcus oralis, Streptococcus pyogenes, S. aureus* and *S. epidermidis* (MIC values 200-500 µg/mL).[13]

The current study determined that *H. virginiana* leaf methanolic and aqueous extracts were also strong inhibitors of the growth of some bacterial triggers of MS and RF. The methanolic leaf extract displayed particularly noteworthy inhibitory activity against antibiotic-resistant strains of *P. aeruginosa* (MIC=113 µg/mL), *A. baylyi* (MIC=245 µg/mL) and *S. pyogenes* (368 µg/mL). Similarly, the aqueous leaf extract was also a good inhibitor of the same bacterial strains (560-1120 µg/mL). Therefore, both the methanolic and aqueous extracts may be useful for preventing the onset of MS and RF, and for treating these diseases once they are established. However, this remains to be verified in *in vivo* assay models. Interestingly, the methanolic and aqueous *H. virginiana* leaf extracts also potentiated the activity of several conventional antibiotics against the antibiotic-resistant strains tested herein. The potentiation of the methanolic and aqueous *H. virginiana* leaf extracts were particularly noteworthy in combination with tetracycline. Indeed, synergistic interactions for combinations containing tetracycline were noted against all of the bacteria tested, indicating that combinations containing tetracycline and *H. virginiana* leaf methanolic and aqueous extract combinations may be particularly useful for preventing and treating MS and RF.

Combinational studies such as this presented herein are important to not only identify potentiating combinations that may be useful in the treatment of bacterial infections, but also to provide valuable information about therapeutic interactions, which may inform future clinical therapeutic usage. Many users of traditional and complementary medicines use these therapies concurrently with allopathic medicines, often without informing their medical practitioner. Mixing therapies may profoundly impact the efficacy of one or both components of the combination and my compromise the effectiveness of the treatment and may jeopardise the patient's health and safety.

Determination of the antibiotic-potentiating mechanism(s) of the *H. virginiana* leaf extracts was beyond the scope of this study. However, as the majority of the synergistic combinations reported herein contained tetracycline, it is reasonable to assume that the potentiating extract component(s) may function by blocking bacterial tetracycline resistance mechanisms. As tetracycline-resistance is generally achieved in most bacteria by the expression of tetracycline-specific efflux pumps,[30,31] it is possible that the potentiation reported in our study was due to inhibition of tetracycline efflux pumps. However, these remains to be verified and future studies to examine the mechanism of potentiation are planned. Similarly, the bioactive components of the extracts were not identified in our study, although the quantitative phytochemical studies reported herein highlighted the relatively high abundance of tannins in the methanolic and aqueous extracts. Previous studies have also reported that *H. virginiana* leaves contain high tannin contents and are particularly rich in hamamelitannin (HAMA). However, previous studies have reported that HAMA may not be a significant contributor to the antibacterial activity of *H. virginiana* extracts. Despite inhibiting the growth of *Staphylococcus* spp. infections *in vitro*,[39] the potency of purified HAMA was determined to be too low to account for the antibacterial activity of the extracts.[40,41] However, those studies did not test whether HAMA can inhibit tetracycline efflux pumps and future studies are required to test this possibility. Notably, multiple other tannins have been reported to bind to cell membrane proteins,[42-44] so it is possible that *H. virginiana* tannins (including HAMA) may bind to bacterial tetracycline-efflux pumps, thereby blocking them and effectively increasing the intracellular tetracycline concentration, although this remains to be tested.

Our study tested the potential of the *H. virginiana* leaf extracts to inhibit the etiology of MS and RF by blocking the bacterial triggers. More direct inflammatory modulation effects of the extracts were not tested herein and future studies directed at examining the immunomodulatory and anti-inflammatory effects of the extracts are warranted. If such effects are ultimately detected, the *H. virginiana* leaf extracts may be particularly useful in the prevention and treatment of MS and RF as they would block both the trigger events of these diseases, as well as the later phase inflammatory effects. Notably, one of the *H. virginiana* leaf extracts tested in this study were toxic in the ALA toxicity assay. However, further *in vitro* toxicity studies using other human cell lines are required to verify the safety of these extracts prior to clinical usage. Future studies should also use *in vivo* toxicity assays to confirm the safety of these compounds and combinations in complex biological systems.

CONCLUSION

Whilst the findings reported herein indicate the potential of *H. virginiana* leaf extracts to inhibit the etiological events of MS and RF, further *in vivo* investigations are required to support these *in vitro* findings. Furthermore, studies to determine the therapeutic mechanisms of the extracts are warranted. Additionally, further studies are required to determine whether *H. virginiana* leaf extracts can also modulate other immunological and inflammatory events in MS and RF disease progression.

ACKNOWLEDGEMENT

The financial assistance of the Centre for Planetary Health and Food Security, and the School of Environment and Science, Griffith University is hereby acknowledged.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ALA: *Artemia nauplii* lethality assay; **DMSO:** Dimethyl sulfoxide; **INT:** ρ-iodonitrotetrazolium chloride; **LC_{50}:** Concentration of sample necessary to have a lethal effect on 50% of test organisms or cells; **MIC:** Minimum inhibitory concentration; **MS:** Multiple sclerosis; **RF:** Rheumatic fever; **ZOI:** Zone of inhibition.

SUMMARY

- *Hamamelis virginiana* leaf extracts were screened for growth-inhibitory activity in the disc diffusion assay against bacterial triggers of multiple sclerosis and rheumatic fever.
- The ability of the extracts to inhibit the growth of the bacteria was quantified by liquid dilution MIC assays.
- Combinational effects between the extracts and selected conventional antibiotics were examined by ΣFIC determination.
- Toxicity of the individual compounds and combinations was evaluated using the *Artemia* nauplii bioassay.

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Cite this article: Nguyen C, Cock IE. *Hamamelis virginiana* L. Leaf Extracts Inhibit Some Bacterial Triggers of Selected Autoimmune Inflammatory Diseases. Pharmacognosy Communications. 2024;14(4):175-84.