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Full Length Research Paper

Discovery and partial purification of an antibiotic from lodgepole pine dwarf mistletoe (*Arceuthobium americanum*) active against Gram-positive organisms including Methicillin-resistant *Staphylococcus aureus* (MRSA)

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The dioecious parasitic flowering plant, *Arceuthobium americanum* (lodgepole pine dwarf mistletoe), was investigated for antimicrobial activity. In a step beyond typical "plant bioprospecting" studies, seasonal variation and plant gender were considered. Methanolic extracts from male and female *A. americanum* shoots collected at two times in the growing season, May and August (2006), were used to challenge a panel of microbes via a disk diffusion assay. Crude extracts were active against Gram-positive organisms including *Staphylococcus aureus* and Methicillin-resistant *S. aureus* (MRSA), with "Males-August" extracts exhibiting the most antimicrobial activity as determined by measuring inhibition zones with a digital caliper ($p<0.05$). Partial purification of the active components was accomplished using fractionation by solvent partitioning (methanol: water [9:1] vs. n-hexane followed methanol: water (6:4) vs. chloroform) coupled to a biological assay employing *S. aureus*. This process effectively concentrated the antimicrobial compound(s) in the more polar fractions, evidenced by a significant increase in inhibition zone diameter ($p<0.05$) at each purification step. Tests with crude extract introduction and removal by dilution indicated that the antimicrobial activity may be bactericidal, at least with respect to *S. aureus*. Based on these findings, *A. americanum* warrants further study as a source of potential new antibiotics, especially in light of its activity against medically-relevant MRSA.

Key words: Antibiotic, antimicrobial, *Arceuthobium americanum*, dwarf mistletoe, gender, Gram-positive, methanolic extract, Methicillin-resistant *Staphylococcus aureus* (MRSA).

INTRODUCTION

Overuse of antibiotics has provided a selection mechanism by which susceptible organisms are rapidly eliminated while those that are resistant are left to proliferate (Shibl, 2005). This abuse has led to the emergence of drug-resistant pathogens and the inefficacy of older, traditional antibiotics. Currently, the spectrum of resistance among pathogens is widening with regard to both the variety of resistant species as well as the types of antibiotics that are no longer effective.

The declining efficacy of existing antibiotics has fuelled demand for new antibiotic compounds. Plants produce such compounds to protect themselves from colonization by parasites such as bacteria and other microbes

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(McDowell and Dangl, 2000). Indeed, plants may be an important source for novel active compounds because such compounds have a "natural" origin and may be less toxic to eukaryotes than synthetically-derived agents (Roselli et al., 2007).

European or Christmas mistletoe (*Viscum album* L., family Viscaceae) is known to be a "natural" medicinal plant, having roles in cancer therapy and diabetes management (Jurin et al., 1993; Blumenthal et al., 1998; Gray and Flatt, 1999). In addition, recent literature indicates that *Viscum album* displays antibacterial properties (Oguntoye et al., 2008), suggesting that plants related to *Viscum* may also possess antimicrobial properties. Such plants might include the dwarf mistletoes which belong to the genus *Arceuthobium* and, like *Viscum*, are parasitic flowering plants in the family Viscaceae (Hawksworth and Wiens, 1996).

There are approximately forty species of *Arceuthobium* growing primarily in the Northern Hemisphere throughout Afro-Eurasia and the Americas (Hawksworth and Wiens, 1996). Each species grows on either one or several specific host Pinaceae (pines) and Cupressaceae (cedars) and are dioecious, having male and female reproductive parts on separate plants. Preliminary evidence suggests that *Arceuthobium oxycedri* (DC.) M. Bieb., which parasitizes *Juniperus* spp. and other Cupressaceae (Hawksworth and Wiens, 1996), has some activity against both Gram-positive and Gram-negative bacteria as well as the fungus (yeast) *Candida albicans* (Zaidi et al., 2006, 2008). It would be worthwhile to explore the genus *Arceuthobium* as a new source of antimicrobial agents.

A. americanum Nutt. *ex* Engelm. grows in western North America, and is found as a parasite on lodgepole pine (*Pinus contorta* subsp. *latifolia* [Engelm. *ex* Wats.] Critchfield) and jack pine (*Pinus banksiana* Lamb.) trees (Hawksworth and Wiens, 1996). Like most members of the genus, *A. americanum* is evergreen, remaining on its host throughout the year instead of falling off during the winter. Furthermore, while *A. americanum* seeds are dispersed in the fall, they do not germinate until the following spring, and thus lay exposed throughout the winter and early spring. As both the plant and seeds are exposed to many potential antagonists throughout the year, *A. americanum* has a high probability of producing a chemical means of defense.

When searching for novel antibiotics, aspects of the candidate organism's biology and ecology must be considered, as its secondary metabolite production can be influenced by many variables. The lack of such biological information has been a weakness in similar "plant bioprospecting" studies. Therefore, the purpose of this study is to obtain methanolic extracts from both male and female *A. americanum* shoots collected at two different times in the growing season and to challenge a panel of microbes with the crude extracts. If an extract possessed antibiotic activity, a bioassay-guided fractionation was investigated using the susceptible organism(s).

MATERIALS AND METHODS

Sample collection

Male and female *A. americanum* shoots were collected from *Pinus contorta* subsp. *latifolia* at two different times in the growing season (May 2006 and August 2006) resulting in four collections: Males-May, Females-May, Males-August, and Females-August. All samples were taken from a site near Stake Lake, British Columbia (BC), Canada, 50°31' latitude and 120°28' longitude (approximately 26 km southwest of Kamloops, BC). The samples, separated by both gender and date in freezer bags (about 40 g per collection type), were stored in a -80°C New Brunswick Scientific Ultra Low Temperature Freezer until further use.

Ultrasonic extraction

Frozen shoots from the separate collections were crushed to approximately 0.5 to 2 cm in length, placed in a 1 L Erlenmeyer flask containing 500 ml of methanol, and ultrasonicated at room temperature (19 to 22°C) for 3 h. The methanol was removed and the extraction repeated two additional times with fresh solvent each time for each of the four separate collections. A final extraction in fresh methanol over 17 h ended with 1 h of ultrasonication. Care was taken throughout the extraction procedure to protect the extracts from UV light by working in rooms with covered windows and storing samples in the dark. The combined methanol extracts from each collection were filtered through cheesecloth (to remove unwanted particulates) into a round-bottom flask. The solvent was removed under reduced pressure using a Buchi Rotavapor RE-114 and a 30 to 50°C water bath. Any remaining solvent was removed under high vacuum until completely dry. The solid extract from each collection was weighed on an analytical balance and stored at -80°C. At the end of each subsequent purification step, the fractions were dried and stored in the same manner outlined above.

Susceptibility testing

The organisms tested for susceptibility to the crude extracts from the four collections are described in Table 1. All organisms were grown in Mueller-Hinton liquid nutrient broth (Atlas, 2004) to a 0.5 MacFarland Standard, which was used to inoculate liquid Mueller-Hinton agar (Atlas, 2004) to 1% (v/v) via the standard pour-plate method (Parisi et al., 1973). Following the well-known Kirby-Bauer procedure for disk diffusion assays (Bauer et al., 1966), dried extracts from each treatment were weighed and suspended in methanol to yield samples with concentrations of 0.1 mg/µl. Sample aliquots of 50 µl (corresponding to 5.00 ± 0.05 mg of dried extract) were applied to 6 mm diameter paper disks and allowed to dry. Negative control disks were prepared by using only an identical volume of methanol, while positive control disks varied depending on the organism (Table 1). These controls were used only to assess growth conditions, and were not included in statistical analyses. Plates containing only the organisms were incubated as well to assess growth. Dried disks were placed on the surface of the agar with the treatment-side contacting the agar, and plates were incubated for 18 to 24 h at 37°C. Following incubation, any zones of inhibition surrounding the disks were measured with a dissecting microscope and digital calipers (Mastercraft) accurate to 0.01 mm. All four collection extracts and the positive controls were tested in triplicate.

Table 1. Microorganisms, their characteristics, and the positive controls used in testing *Arceuthobium americanum* extracts for antimicrobial activity.

Test organism	Relevant characteristics and ATCC® number	Positive control
Bacillus sp.	Gram-positive bacterium (rod), ATCC 12456	Clindamycin
Candida albicans	Yeast, ATCC 90028	None Available
Clostridium sp.	Gram-positive bacterium (rod), ATCC 53464	Ampicillin
Escherichia coli	Gram-negative bacterium (rod), ATCC 29425	Ampicillin
Methicillin-resistant *Staphylococcus aureus* (MRSA)	Gram-positive bacterium (coccus), ATCC 33591	Tetracycline
Propionibacterium acnes	Gram-positive bacterium (rod), ATCC 6919	Clindamycin
Salmonella sp.	Gram-negative bacterium (rod), ATCC 35664	Gentamicin
Staphylococcus aureus	Gram-positive bacterium (coccus), ATCC 25923	Ampicillin
Streptococcus pyogenes	Gram-positive bacterium (coccus), ATCC 19615	Erythromycin
Vancomycin-resistant *Enterococcus* (VRE)	Gram-positive bacterium (coccus), ATCC 51299	Clindamycin

The diameters of inhibition zones for the four collection extracts against each test organism were subjected to a one-way analysis of variance (ANOVA) to determine if date and gender considered together had a significant effect ($p<0.05$). The inhibition values for the positive controls were not included, as the results obtained from the pure substance would skew the analysis. Post-hoc Turkey tests were used to determine which samples had a significant inhibitory effect on a given microorganism. Where the inhibition zone sizes between different organisms challenged with the same sample extract appeared substantially different, a post-hoc Student's t-test was performed to determine if the difference was significant ($p<0.05$).

Bioassay and purification

Initial susceptibility results indicated that all the crude methanolic extracts were highly active against *S. aureus* and MRSA, with the Males-August collection extract being the most potent. Therefore, this crude extract was selected for purification using *S. aureus* as the bioassay organism. At each stage in the purification, the growth and challenge of *S. aureus* followed the same pour-plate and Kirby-Bauer protocol as was previously described for the initial crude extract susceptibility testing, using disks exposed to the corresponding solvents as additional controls. Drying and weighing of extracts from each successive stage of purification was as per the crude.

The first step in the fractionation involved suspending approximately 12 g of the Males-August dry crude extract in a 1 L separatory funnel containing 450 ml of a methanol: water (9:1) mixture. A 400 ml aliquot of n-hexane was then added to the funnel, agitated, and then allowed to separate. The bottom aqueous methanol layer was removed and set aside to be extracted three additional times with n-hexane, while each n-hexane fraction was decanted through the top of the funnel and ultimately combined.

A second solvent partitioning fractionation using the 9:1 extract (about 7 g dry) followed the same process as the initial fractionation. However, the polarity of the aqueous mixture was increased to methanol: water (6:4) and successive additions of chloroform were used to partition the compounds present in the extract.

At each stage of purification, the bioactivity of each fraction was tested in triplicate and the resulting zones of inhibition were measured with digital calipers to determine if there was an increasing amount of active compound(s) present in the fractions. In addition, the inhibition values obtained were normalized by dividing the average zone diameter by the mass of sample extract at that stage. One-tailed pair-wise Student's t-tests were performed with respect to the diameter of the zone of inhibition as well as the normalized data to determine if extracts at the various stages of purification (crude, initial fractionation, second fractionation) were significantly more active ($p<0.05$). One-tailed tests were chosen because a more conservative estimate was desired to determine if the inhibition zone or normalized value at a purification stage was significantly larger, not merely different, than the previous. Also, t-tests rather than ANOVAs were used here due to the inherent pair-wise nature of the comparisons.

Antimicrobial mode of action

A preliminary examination was performed to determine if the antimicrobial action was bactericidal or bacteriostatic. Here, *S. aureus* was grown to the logarithmic phase in Mueller-Hinton liquid broth (37°C), and sub-cultured into one of two conditions. The first served as the control and consisted of 5 ml of fresh Mueller-Hinton broth, while the second (the treatment) contained 5 ml broth plus 300 mg of the crude Males-August extract. Mueller-Hinton plates were surface-inoculated with 100 µl of the control or treatment broths at dilutions of 1/10, 1/100 and 1/1000, and then incubated an additional 24 h at 37°C. Counts of colony forming units (CFUs) were performed on each plate to then calculate the number of cells present in 1 ml of the original, undiluted solution. Recovery of a significant number of CFUs suggests a bacteriostatic mode of action, whereas a reduced number or no recovery suggests bactericidal action.

RESULTS

Susceptibility testing

Susceptibility testing results for various microorganisms subjected to four types of crude methanolic *A. americanum* extracts (and their relevant positive controls) are shown in Table 2. The antibiotic compound(s) exhibited a narrow spectrum of activity limited only to Gram-positive bacteria, and did not affect all Gram-positive organisms tested. The largest zones of inhibition were observed in MRSA and *S. aureus* challenges, with

Table 2. Diameter measurements of zones of inhibition following susceptibility testing on a panel of microorganisms. Different superscript letters [a], [b], and [c] represent a significant difference in the zone of inhibition diameter ($p<0.05$) for a given microorganism (n=3). The positive control was not included in statistical analyses. Water and solvent controls showed no zones of inhibition (data not shown).

Test organism	Zones of inhibition (mm) (X ± SEM)				
	Males-May extract	Females-May extract	Males-August extract	Females-August extract	Positive control
Bacillus sp.	8.10 ± 0.15[a]	7.55 ± 0.21[a]	8.43 ± 0.25[b]	7.33 ± 0.41[a]	25.50 ± 0.55
Candida albicans	0	0	0	0	Not available
Clostridium sp.	0	0	0	0	10.00 ± 0.60
Escherichia coli	0	0	0	0	15.00 ± 2.10
Methicillin-resistant *Staphylococcus aureus* (MRSA)	9.21 ± 0.22[a]	9.11 ± 0.34[a]	12.45 ± 0.75[b]	10.11 ± 0.16[c]	22.00 ± 0.90
Propionibacterium acnes	0	0	0	0	11.00 ± 1.10
Salmonella sp.	0	0	0	0	25.00 ± 1.05
Staphylococcus aureus	10.31 ± 0.22[a]	10.01 ± 0.33[a]	11.45 ± 0.15[b]	10.01 ± 0.33[a]	22.00 ± 1.00
Streptococcus pyogenes	8.12 ± 0.15[a]	8.02 ± 0.15[a]	8.52 ± 0.04[b]	8.12 ± 0.21[a]	15.15 ± 1.03
Vancomycin-resistant *Enterococcus* (VRE)	0	0	0	0	10.40 ± 0.32

the Males-August extracts most biologically active (12.45 ± 0.75 mm and 11.45 ± 0.15 mm, respectively). A post-hoc *t*-test suggested that the zone of inhibition values observed in MRSA were significantly larger than those for *S. aureus* ($p=0.00230$). On any given organism where activity was noted, the zone of inhibition by the Males-August extracts was significantly larger than zones from the other three collections ($p<0.05$). While MRSA was the most susceptible organism, *S. aureus* was chosen as the bioassay test organism for purification, as it was easier to obtain and safer to work with.

Bioassay and purification

Bioassays of sample extracts against *S. aureus* demonstrated that the active compound(s) repeatedly partitioned into the more polar fractions.

The initial fractionation yielded an active methanol: water (9:1) phase extract, whereas the n-hexane-derived extract demonstrated no activity (Table 3).

The second fractionation involved the more polar methanol: water (6:4) and chloroform phases. Again, activity was observed in the aqueous methanol extract, while the less polar chloroform fraction exhibited no antimicrobial activity.

With respect to zone of inhibition diameters, the p values were 0.00240 for the difference between the crude extract and the methanol: water fraction (9:1), 0.00300 for the difference between the methanol: water (9:1), and methanol: water (6:4), and 0.00066 for the difference between the crude extract and the methanol: water (6:4) fraction. Therefore, the inhibition diameter differences observed at each stage of purification were significant, indicating that the process was successful in concentrating the active compound(s).

Active fraction yields from 33.13 g of Males-August plant material are shown in Table 3. Table 3 also lists the normalized data for each stage of extraction from the initial 33.13 g of plant material, and the statistical trends were similarly significant to those observed for the zone diameters ($p=0.00300$, 0.00110, and 0.000001 for crude vs. 9:1, 9:1 vs. 6:4, and crude vs. 6:4, respectively). These significant differences again indicate the effectiveness of the purification protocol.

Antimicrobial mode of action

Plate counts showed that the *S. aureus* bacterial

Table 3. Average zone of inhibition diameter measurements from *S. aureus* bioassays using the Males-August extract (n=3). The active fraction yields from 33.13 g of Males-August plant material and normalized values for each active fraction derived from these yields are also presented. Methanol controls had no effect.

	Fraction	Zone of inhibition (mm)	Yield (active fraction)(g)	Normalized values (mm/g)
Initial fractionation	crude	8.76 ± 0.15	12.35	0.71 ± 0.01
	methanol: water (9:1)	9.45 ± 0.27	7.44	1.27 ± 0.04
	n-hexane	no activity	not applicable	not applicable
Second fractionation	methanol: water (6:4)	11.02 ± 0.63	5.64	1.95 ± 0.11
	chloroform	no activity	not applicable	not applicable

population was approximately 1000-fold lower when initially subjected to the extract compared to the control (9.07×10^6 vs. 4.00×10^9 CFU/ml). The substantially lower CFU recovery upon removal from the extract is suggestive of bactericidal properties.

DISCUSSION

Susceptibility testing

Results indicate that crude methanolic extracts from *A. americanum* had noteworthy antibiotic activity against the Gram-positive bacteria *Bacillus* sp., MRSA, *S. aureus*, and *Streptococcus pyogenes*, particularly when prepared from male shoots collected in August. However, no activity was evident against the Gram-negative bacteria species tested nor the yeast, *C. albicans*.

These results contrast those obtained by Zaidi et al. (2006, 2008), who reported that methanolic extracts of *Arceuthobium oxycedri* growing on *Juniper excelsa* M. Bieb. in Pakistan were not active against *S. aureus*, but were active against the Gram-negative *Escherichia coli* and the fungus, *C. albicans.* Their literature reports are in agreement with this study in showing *Arceuthobium* extracts are active against Gram-positive *Bacillus*, while exhibiting no activity on Gram-negative *Salmonella*. Zaidi et al. (2006, 2008) did not report results against MRSA.

The variations in biological activity between the current study and those reported by Zaidi et al. (2006, 2008) are not unexpected. Some differences could be a function of the amount of crude extract tested. Tests in this study employed a dose of 5 mg (as a 50 µl aliquot on disk), whereas doses used by Zaidi et al. (2006, 2008) were lower by about a factor of 10 (0.2 to 0.4 mg added; up to 20 µl aliquots on disk). In addition, the season of collection might play a role in dictating antibiotic activity, as could the fact that Zaidi et al. (2006, 2008) did not differentiate between male and female shoots. Perhaps more importantly, different species of dwarf mistletoe might have distinct antimicrobial properties, especially considering these two *Arceuthobium* species grow in different environments and on different hosts. Therefore, environmental conditions, species, and, in the case of parasitic plants, hosts, must be taken into consideration when searching for natural products derived from plants.

Based on the experimental inefficacy against the Gram-positive organisms VRE and *Propionibacterium acnes*, the spectrum of antibiotic activity of the *A. americanum* extract is relatively narrow. Narrow-spectrum antibiotics are believed to be better at targeting pathogens without concomitantly interacting with the body's normal microbes, and do not induce resistance to the same degree as broad-spectrum compounds (Yang et al., 2007). The finding of a relatively narrow spectrum of activity warrants further investigation of *A. americanum* as a source of antimicrobials, especially considering its effectiveness against MRSA. MRSA is a frequently nosocomially-acquired pathogen that is notoriously hard to treat in immunocompromised individuals, and is now emerging in community-acquired infections (Raygada and Levine, 2009).

Bioassay and purification

The purification procedure used a biological assay to determine which fractions contained biological activity and effectively separated compounds based on polarity. In some cases, however, purification procedures can be detrimental due to the separation of components that work in synergy or provide an additive effect (Hagelin et al., 2004).

This work shows that the *A. americanum*-derived methanolic extract can be partially purified while maintaining activity; in fact, the active compound(s) became concentrated and more effective during purification (from 0.71 mm/g to 1.95 mm/g). Further purification, isolation, characterization, structural elucidation, and pharmaceutical testing is required.

Antimicrobial mode of action

A bactericidal mode of action is suspected with respect to

S. aureus, as CFUs could not be recovered from even the lowest dose of the crude Males-August extract. Work on a purified compound at lower concentrations against a range of susceptible organisms is required. In terms of mode of action, bactericidal drugs are reported to be superior to bacteriostatic compounds for the treatment of endocarditis, meningitis, bacteremia in a neutropenic host, and possibly osteomyelitis (Chambers, 2003).

Conclusion

The present study has shown that a crude methanolic extract from *A. americanum* has antibiotic, narrow-spectrum, bactericidal properties, and was effective against several Gram-positive bacteria, including medically-relevant MRSA. The gender and time of year plants were collected had significant effects, with male plants collected in August generating the most potent extracts. The biological reason behind the difference in efficacy between males- vs. female- and August vs. May-derived extracts has not yet been ascertained, although hormonal or developmental fluctuations occurring during each gender's reproductive cycle might be an underlying influence. More work is required to illuminate the biology behind our findings. Nonetheless, biological and ecological aspects should always be considered in studies of plant products. Many current antibiotic classes are microbe-derived, increasing the likelihood that pathogens have already been exposed to them. Plants, on the other hand, provide an alternative source for the discovery of compounds not yet encountered by pathogens, and may potentially be less likely to develop resistance. *A. americanum* has revealed itself to be a good candidate as a source of promising antibiotic activity. Research should now focus on structural elucidation of the bioactive constituents, which may serve as novel lead compounds in the search for new drugs.

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