Journal of Medicinal Plants Research Vol. 6(6), pp. 938-949, 16 February, 2012
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR10.710
ISSN 1996-0875 ©2012 Academic Journals

Full Length Research Paper

Optimization of screening of native and naturalized plants from Minnesota for antimicrobial activity

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Accepted 7 March, 2011

The White Earth Tribe of Ojibwe and the University of Minnesota have partnered to identify antimicrobial properties in native plant species in the Upper Mississippi River and Red River Basins. Optimization of harvest time, tissue preparation, and extracting solvent methods was completed using extracts from two species with known antimicrobial activity, *Betula papyrifera* and *Rhus typhina*. Tissue was collected at three different times (July, August, and September) corresponding to different developmental states (juvenile, reproductive, post-reproductive) and extracts were prepared from fresh, frozen, or dried tissue using one of three solvents, acetone, ethanol, or methanol. Using optimized methods plant extracts from 265 above ground plant components (flower, leaves, stems, berries) of 130 species were tested against four microorganisms (*Escherichia coli*, *Pseudomonas aeruginosa, Candida albicans*, *Staphylococcus aureus*). Eighty extracts were found to inhibit at least one microorganism. Sixteen extracts inhibited two microorganisms, six extracts inhibited three microorganisms, and three extracts inhibited all four microorganisms. Extracts from *R. typhina* and *B. papyrifera* leaves were also tested against soil borne pathogens, *Fusarium solani, Phytophthora sojae, Rhizoctonia solani, and Pythium* spp, in order to assess potential uses as seed protectants. *Rhus* spp extracts inhibited *F. solani and Pythium spp* as well as a commercial fungicide seed treatment applied as a control.

Key words: Antimicrobial, native plants, antifungal seed treatment.

INTRODUCTION

Plants produce a multitude of organic compounds and some of which have antimicrobial activity. These natural products are found in stem, berry, bark, leaf, flower, and root tissue (Borchardt, 2008b) as well as essential oils (Afolayan et al., 2009; Hassan et al., 2009) and include such compounds as alkaloids, anthocyanins, anthraquinoes, flavonoids, glycosides, phenols, saponins, tannins, and terpenoids (Afolayan and Ashafa, 2009; Banso, 2009; Cowan, 1999; Ekpo and Etim, 2009; Hassan et al., 2009; Hemaiswarya et al., 2008; Ladan et al., 2009).

These compounds which are soluble in a variety of solvents including ethanol and water, and are safe for human exposure, have been shown to be highly effective in the preparation of biologically active extracts (Afolayan and Ashafa, 2009; Banso, 2009; Ekpo and Etim, 2009; Hassan et al., 2009; Rasool et al., 2008). The synthesis of many of these products of secondary metabolism is induced by stress in response to pathogen and herbivore

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attack or other abiotic signals whereas others are constitutively produced. They have demonstrated antibacterial and/or antifungal activities and are bactericidal and bacteriostatic influencing lag time, growth rate and maximum growth of microorganisms (Borchardt, 2008b; Cutter, 2000).

Native and naturalized plants in Minnesota have the potential to provide antimicrobial properties for use against drug-resistant microorganisms, to be marketed as natural preservatives, and to produce other value-added agricultural goods. New antimicrobial agents are needed to treat human, plant and animal illness because of the emergence of drug-resistant pathogens whose effects are felt from hospitals to corn fields (Cowan, 1999; Hemaiswarya et al., 2008). Studies have shown that water and ethanol extracts of a variety of plants have antimicrobial activity against nosocomial infections (Abubakar, 2009; Jyh-Ferng et al., 2009), aquaculture fish diseases (Bhuvaneswari and Balasundaram, 2009), dental caries (Babpour et al., 2009), human skin infections (Ekpo and Etim, 2009), and seed/post-harvest crop diseases (Akinjogunla et al., 2009; Al-Bakri and Afifi, 2007; Broders et al., 2007; Chapin et al., 2006; Chen and Huang, 2009; Mari and Guizzardi, 1998). Additionally, studies have shown that natural compounds are effective for improving food quality and safety. Natural products and essential oils from plants such as garlic and rosemary have been used to increase shelf life in fresh pasta (Del et al., 2009), fresh sprouts (Gamage et al., 2009) and to kill foodborne pathogens and spoilage bacteria in lettuce, meat, and milk (Gutierrez et al., 2009). Water and ethanol extractions of raisins have shown anti-fungal activity in bread products (Wei et al., 2009). Furthermore, the use of plant derived compounds addresses the increasing consumer demand for natural products to be used in place of synthetic compounds in food, medicinal, and agricultural goods (Cowan, 1999; Mari and Guizzardi, 1998).

An additional use of these natural products is for the control of plant pathogens in agricultural systems. Certified organic crop producers are limited to the use of cultural practices or natural products for crop disease management. Only a few compounds are available and their efficacy is limited so that the risk of losses to plant disease in organic crop production remains high (Munzer et al., 2000). Conventional farmers use highly effective synthetic seed treatment chemicals such as mefenoxam even though public opinion is shifting against the use of many chemical pesticides (Broders, 2007). These conventional methods can be effective but are expensive and development of fungicide resistance in diverse populations of pathogenic fungi can make targeted treatments less successful. Fungi cause pre-and-post emergence damping-off of emerging seedlings resulting in poor stands and reduced vigor and yield, with significant losses to farmers as a consequence (Broders et al., 2007). Moreover, several studies have reported fungicide resistance in *Fusarium* spp (Chen and Huang, 2009; Munzer et al., 2000), downy mildew (Falloon et al., 2000), and ascoshyta blight (Gan et al., 2006) to several commercially available synthetic products resulting in decreased yield in many important crops such as tomato, pea, potato, wheat, and chickpea (Chapin et al., 2006; Chen and Huang, 2009; Errampalli et al., 2006; Falloon et al., 2000; Gan et al., 2006). There is a need to identify and develop compounds as seed treatments and post-harvest disease management tools that can target a broad spectrum of plant pathogens and are certifiable for use in organic crop production systems. Native plant extracts provide an important source of biologically active compounds that have the potential to be developed into cost effective solutions accessible to producers of organic crops worldwide.

In addition to the commercial benefits that would result from the use of plant derived natural products, there exists a real opportunity to engage indigenous cultures in knowledge preservation and environmental conservation efforts. North American Indian knowledge of plants in the Upper Mississippi River basin is well documented (Densmore, 1974; Moerman, 2004) and indicates that this group of people have identified many native and naturalized plants that have a broad spectrum of activity against differing classes of microorganisms (Borchardt et al., 2008a, 2008b). There is a possibility to utilize indigenous knowledge of herbal medicine to identify promising plant species and their component parts. Indigenous knowledge of herbal medicines is extensive and includes which plant species, method of plant preparation, and the specific plant part to be used. Moreover, there is possibility to work with these communities to transition land out of commercial crop production to a traditional land use while providing an economic benefit.

On the White Earth reservation, there is substantial area (6-10 thousand acres) dedicated to commercial agriculture (corn and soybean rotation). However, this is not a traditional landscape usage. By developing mixtures of plant materials with appropriate uses, it may be possible to transit the agricultural land into a native and naturalized plant polyculture, which will have numerous benefits for the environment and White Earth traditional knowledge preservation. This would increase the number of perennial species and biodiversity on the landscape enhancing ecosystem services such as increased ground cover, carbon sequestration, and erosion control. It would also increase biomass yields available for use in biofuels (Tscharntke et al., 2005).

The objectives of this research include optimization of the screening process by determining key harvest times that maximize the accumulation of antimicrobial phytochemicals, the best methods of preparing plant tissue for extraction, and identification of the most effective extracting agent to maximize the activity of plant material. We used existing knowledge from the White

Earth Tribe of Ojibwe to identify as yet uncollected native or naturalized plants with potential for antimicrobial activity, collected, and tested them. Finally, we tested the most promising plant extracts against common soil borne pathogens and explored their impact on soybean seed germination as a step towards developing seed disease management tools.

MATERIALS AND METHODS

Plant collection and screening optimization

In 2008, aerial parts (leaves, stems, and flowers) were collected and screened using methods extending from those previously described by Borchardt et al. (2008a). In the field, plant material was immediately put into air-tight bags and placed in a cooler to be processed in the lab within 48 h. To streamline the process and assess the best way to maximize antimicrobial activity, three different sets of treatments, using the bioassay methods described below, were conducted with *Betula papyrifera* (BP)*, Rhus typhina* leaves (RT), and *R. typhina* flowers (RTF). The aim of these experiments is to determine the most appropriate harvest time, plant tissue preparation method, and solvent to extract the antimicrobial agents, which could then be applied to all of the plant species collected. To analyze harvest time, material was collected at three dates, July 1, August 15 and September 30, which roughly correspond to three developmental life stages, vegetative, reproductive, and post-reproductive.

The different developmental stages for each species were determined by using the USDA NRCS database. After collection, plant material was immediately frozen (-20ºC), dried in an oven (65ºC), or taken fresh to be tested for antimicrobial activity. Fresh material was processed within 48 h. Bulk plant material was then extracted using one of three different solvents, acetone, ethanol or methanol. All possible combinations of all three sets of treatments (time of harvest, tissue preparation, and extraction solvent) were used in the antimicrobial assay to determine the combination of plant material treatments yielding the most antibacterial activity. These results were then applied to the collection of native and naturalized plant species found within the White Earth Tribal boundaries and throughout greater Minnesota. White Earth Reservation is situated at the joining of three biomes; the Laurentian Mixed Forest province, Eastern Broadleaf forest province, and the Prairie parkland province, towards Northwest Minnesota.

Extract preparation and antimicrobial testing

The extract preparation and antimicrobial disk diffusion screening procedures used in this paper were previously described by Borchardt et al. (2008) and Bauer et al. (1966), respectively. Antimicrobial activity was tested against four representative microorganisms that cause diseases and spoilage including: gram-positive *Staphyloccocus aureus* (ATCC 12600), Gram-negative *Escherichia coli* (ATCC 8677) and *Pseudomonas aeruginosa* (ATCC 9721), and the yeast *Candida albicans* (ATCC 10231). To prepare the extract, ten grams of plant materials were combined with 80:20 aqueous solvent (ethanol, methanol, or acetone) solutions and kneaded in a stomacher for 2 min. This pulp was allowed to soak 24 h after which a 6 mm sterile paper disk was impregnated with 50 µl of the supernatant. Following a 60 min solvent evaporation, the disks were placed onto Mueller-Hinton plates onto which a lawn had been swabbed with one of each of the microorganisms. Discs with evaporated solvent were used as a negative control and an antibiotic disc (ticarcillin, 75 mcg or chloramphenicol 30 mcg) was used as a positive control. The plates were incubated at 37°C for 18 h and zones of inhibition were measured with a ruler across three axis with the mean diameter (mm) reported. Previous studies measuring antimicrobial activity of plant extracts have defined a significant inhibition zone at 8 to 10 mm when using 6 mm discs (Borchart et al., 2008a; McCutcheon et al., 1992; Omar et al., 2000; Tepe et al., 2005). In this paper, we used more stringent antimicrobial designations of: >15 mm = high activity; 10 to 15 mm = medium activity; <10 mm = low activity.

Tests of antifungal properties

Promising plant extracts were tested for their effectiveness against plant pathogens. Because of the pronounced antimicrobial activity demonstrated in prior experiments (Borchardt et al., 2008), extracts of *B. papyrifera* and *R. typhina* were evaluated for their effects on growth of the filamentous fungi, *Fusarium solani* and *Rhizoctonia solani* and on the oomycetes, *Phytophthora sojae, and Pythium* spp. Leaf tissue was collected from *B. papyrifera (*Paper birch) and *R. typhina (*Staghorn sumac) in September 2009 and stored in a freezer (-20ºC). Extract preparation was previously described by Borchardt et al. (2008) and Bauer et al. (1966). Tissue was thawed and then dried at 95ºC for 48 h. Fifty grams of the dried plant material were combined with 200 ml of 80:20 aqueous ethanol solvent in a Polytron PT10/35 homogenizer (Capitol Scientific, Inc. Austin, TX) and processed for 2 min. The mixture of tissue and solvent was then allowed to stand for 18 to 24 h. The supernatant was then filtered to remove leaf tissue and used as the plant extract. Leaf tissue remaining after filtering was centrifuged and the resulting supernatant was filtered and added to extract. The extracts were then stored at 0ºC until they were to be used.

Each of the pathogens, *F. solani, Phytophthora sojae, R. solani, and Pythium* spp. was tested in a completely random design with eight treatments. The treatments consisted of extracts of either *B. papyrifera* or extracts of *R. typhina* applied at three rates, one fungicide, and an untreated control. The experiment was replicated three times. A media-only plate provided a negative control and untreated comparison for the experiment and either of two commercial fungicidal treatments, metalaxyl or fludioxonil provided a positive control. Metalaxyl (Apron®, Subdue®), considered effective in limiting growth of oomycetes, was incorporated into V-8 media at rate of 5 ug/mL and inoculated with either *Pythium* spp. or *P. sojae*. Similarly, Fludioxonil (Maxim®, Medallion®), an inhibitor of growth by filamentous fungi, was incorporated into PDA media at rate of 5 ug/ml and inoculated with either *F. solani* and *R. solani* (Nelson, 2006). The treatments were 0.1, 0.05, or 0.01 ml of extracts of either *B. papyrifera* or *R. typhina*, added to 20.0 ml of media. In each treatment, 1.0 ml of ethanol was allowed to vaporize prior to addition of media to each plate. Cultures of actively growing fungi, consisting of plugs of inoculated media, were added to each treatment plate. Dimensions of the *Pythium* spp. *and R. solani* colonies and of the *F. solani* and *P.sojae* were measured at three days and five days respectively *(*Broders, 2007; Munzer, 2000). Colony growth rate was then expressed as mm of growth/day. Growth by the plant extracts and commercial standards were calculated as a percentage of the growth rate that occurred in the media-only control. Inhibition of plant pathogen growth was considered to have occurred if the percentage was less than 100%.

Seed treatment assay

Plant extracts were also tested for their ability to affect germination of soybean seeds. Whatman paper was treated by soaking with either 0.1 ml of *R. typhina* extract/1.0 ml of distilled water, 0.1 ml of *B. papyrifera* extract/1.0 ml of distilled water, 0.1 ml of

ethanol/1.0 ml of distilled water, or distilled water only. Ten soybean seeds were then placed on each treated germination paper in a petri dish and stored in the dark. Five days later germination was assessed by counting the number of seeds showing any root and cotyledon emergence.

Statistical analysis

All experiments were conducted in a completely randomized design with three replications. Analysis of variance (ANOVA) was performed on the growth rate data using SAS Proc GLM (SAS Institute, Cary, NC). Means were compared using a Fisher protected LSD at the 95% confidence level.

RESULTS AND DISCUSSION

Plant activity

Eighty ethanolic plant extracts (30%) of the 265 extracts tested, representing 68 species, inhibited at least one of the four microorganisms (Table 1). Of the 130 species tested, six species (4.6%) inhibited three microorganisms, while 11 (8.4%) inhibited at least two microorganisms.

Extracts of 3 species (2%) inhibited growth of all four microorganisms. It is important to note that different plant parts exhibited varying rates of antimicrobial activity. For example, *Dianthus armeria* (Deptford pink) flowers, *Fragaria virginana* (Virginia wild strawberry) leaves and *Helianthus pauciflorus* (stiff sunflower) stems inhibited all four microorganisms. Furthermore, the antimicrobial activity of the plant organs, which was tested and varied by plant part with 24, 27, 35 and 28% of all the stems, leaves, flowers, and seeds tested, respectively, were found to exhibit antimicrobial properties (Table 1). Extracts from 100 species showed no antimicrobial activity (Table 2).

Plant extracts inhibiting four microorganisms

Plant extracts from three species showed inhibitory activity against all four microorganisms (Table 1). Extracts of *D. armeria* (Deptford pink) flowers were most effective against *S. aureus* and *E. coli* and slightly inhibited the other two microorganisms. Previously in a study of Micmac and Malecite, medicinal plants *D. armeria L.* was found to contain sterols (campesterol, stigmasterol, and β-sitosterol) and triterpenes (α-amyrin and β-amyrin), which have been reported to have antibacterial activity (Hooper and Chandler, 1984). *F. virginana* (Virginia wild strawberry) leaf extracts had slight inhibition against *E. coli* and *P. aeruginosa* and were very inhibitory against *S. aureus* and the fungus *C. albicans*. In a study by Webster et al. (2008), testing plant extracts against human pathogenic fungal isolates *F. virginana* leaf aqueous extracts showed strong antifungal activity against such fungi as *C. albicans, Aspergillus* spp., and *Fusarium* spp. *H. pauciflorus* (stiff sunflower) stem extracts showed very strong inhibition against all microorganisms suggesting superior broad spectrum antimicrobial activity. This is the first study to report antimicrobial activity of extracts made from *H. pauciflorus* stems.

Plant extracts inhibiting three microorganisms

Plant extracts from six species displayed activity against three microorganisms (Table 1). Extracts made from *Comandra umbellata* leaves, *Geum triflorum* leaves, and *Tilia americana* flowers showed very strong inhibition against one microbe, *S. aureus, S. aureus*, and *C. albicans*, respectively, and inhibition to a lesser degree against two microbes. Several studies have been reported on tradition Mexican ethnomedicinal uses of *T. americana* as an antinociceptive (Martinez et al., 2009), sedative, and anxiolytic effector (Agiurre-Hernandez et al., 2007; Aguirre-Hernandez et al, 2007; Maribel et al., 2008; Perez-Ortega et al., 2008).

The remaining three species showed strong-medium inhibition against two microbes and limited activity against one microbe. *Heuchera americana* flower extract was inhibitory against the gram positive *S. aureus* and gram negative *P. aeruginosa* even though a previous study found expelled juice from *H. Americana* to have no activity against the gram positive *Bacillus substilus* or gram negative *E. coli* (Sanders et al., 1945). Extracts made from *Nuphar lutea* flowers and *Populus tremuloides* leaves inhibited *E. coli* and *C. albicans*; and *S. aureus* and *C. albicans*, respectively. A study by Silici and Katluca (2005) examining antibacterial activity of bee propolis derived partly from *P. tremuloides* bud exudates found that the inhibitory activity of the propolis partly mirrored the activity found here with strong action against *S. aureus* and weak action against *E. coli* and *P. aeruginosa*.

General impact on inhibition

There was an effect of plant type indicating that different genera had different levels of microbial inhibition. Focusing on two strong broad spectrum inhibitors, the effect of genus on microbial inhibition is quite clear; *R. typhina* shows more activity than *B. papyrifera* (Figures 1, 2 and 3). In terms of plant part extracts made from *R. typhina* flowers and leaves changed rank depending on which microorganism being tested against.

Time of harvest impact on inhibition

Because phytochemicals accumulate in different plant species and parts at varying rates, it is important to identify key time periods of compound accumulation for

Table 1. Antimicrobial activity of 80% aqueous ethanol plant extracts from aerial parts of plants collected in Minnesota in 2008.

Botanical name	Common name	Plant part tested	Microorganism[1]			
Inhibition against four microorganisms			**Inhibition zones in mm**			
			Ec	**Sa**	**Pa**	**Ca**
Dianthus armeria	Deptford pink	Flowers	6	8	0.1	0.1
Fragaria virginiana	Virginia strawberry	Leaves	0.1	12	0.1	13
Helianthus pauciflorus	Stiff sunflower	Stems	16	20	10.0	20
Inhibition against three microorganisms			**Ec**	**Sa**	**Pa**	**Ca**
Comandra umbellata	Bastard toadflax	Leaves	0.1	13	0.1	
Geum triflorum	Prairie smoke	Leaves	0.1	12	0.1	
Heuchera americana	Alumroot	Flowers	0.1	14	7.0	
Nuphar lutea	Yellow pond lily	Flowers	10		0.1	10
Populus tremuloides	Quaking aspen	Leaves		11	0.1	10
Tilia americana	Basswood	Flowers	0.1		0.1	18
Inhibition against two microorganisms			**Ec**	**Sa**	**Pa**	**Ca**
Acer saccharum	Sugar maple	Leaves		15		20
Acer spicatum	Mountain maple	Stems		20		20
Acer spicatum	Mountain maple	Leaves		15		0.2
Alliaria petiolata	Wild sasparilla	Flowers		9	12.0	
Cornus racemosa	Grey dogwood	Flowers		10		20
Cornus racemosa	Grey dogwood	Leaves		10		20
Geum triflorum	Prarie smoke	Flowers		7	0.2	
Lespedeza capita	Roundhead lespedeza	Stems		0.2	0.0	12
Rosa arkansas	Arkansas rose	Leaves		12	0.2	
Rosa arkansas	Arkansas rose	Flowers	0.2		0.1	
Rosa suffalata	Prairie rose	Leaves		8		20
Rosa suffalata	Prairie rose	Flowers		0.2		18
Rubus alleghanesis	Alleghany blackberry	Flowers		8	0.2	
Rubus alleghanesis	Alleghany blackberry	Leaves		8	0.2	
Artemisia caudata	Wild wormwood	Leaves		9		15
Inhibition against a single microorganism			**Ec**	**Sa**	**Pa**	**Ca**
Allium canadense	Meadow garlic, wild onion	Flowers		6		
Amelanchier spp	Downy serviceberry, juneberry, shadbush, service tree, sarvis-tree	Stems		0.2		
Apios americana	Groundnut	Stems		10		
Apocynum androsaemifolium	Spreading dogbane	Leaves		10		
Apocynum androsaemifolium	Spreading dogbane	Flowers		8		
Arisaema triphyllum	Jack in the pulpit	Leaves				14
Baptista australis	Blue Indigo	Flowers	0.2			
Circuta maculata	Water hemlock	Flowers		0.2		
Comandra umbellata	Bastard toadflax	Flowers			8.0	
Comandra umbellata	Bastard toadflax	Stems			0.2	
Cornus alternifolia	Alternate leaf dogwood	Flowers				10
Crateagus spathulata	Little-hip hawthorn	Leaves		8		
Dicentra cucullaria	Dutchman's breeches	Leaves			0.2	

Table 1. Contd.

			Ec	Sa	Pa	Ca
Echinacea purpurea	Eastern purple coneflower	Flowers		10		
Eupatorium perfoliatum	Common boneset	Flowers		15		
Galium boreale	Northern bedstraw	Flowers		7		
Geum triflorum	Purple avens	Stems		7		
Helianthus pauciflorus	Stiff sunflower	Leaves		15		
Heracleum lanatum	Cow Parsnip	Flowers				10
Heracleum lanatum	Cow Parsnip	Stems	0.2			
Heuchera americana	American alumroot	Stems		51		
Krigia coiflora	Two flower dwarf dandelion	Flowers		10		
Lespedeza capitata	Roundhead lespedeza	Leaves		8		
Lespedeza capitata	Roundhead lespedeza	Petals		7		
Linum sulentum	Grooved flax	Seeds		0.2		
Lychnis alba	White campion	Stems		10		
Polygala spp. *(Milkwort)*	*Milkwort*	Seeds			8.0	
Patibola columitera		Leaves		10		
Patibola columitera		Flowers		0.2		
Penstamom grandiforus	Nutt. large beardtongue	Seeds				14
Penstemom palliclus	Small pale beardtongue	Flowers/stems		8		
Phlox divorcica	Wild blue phlox	Leaves		15		
Phlox divorcica	Wild blue phlox	Flowers		10		
Polygonum amphibium	Water knotweed	Leaves		10		
Polygonum amphibium	Water knotweed	Flowers			0.2	
Potentilla recta	Sulphur cinquefoil	Leaves		0.2		
Prunus pensylvanica	Pin cherry	Leaves/stems		0.2		
Prunus serotina	Black cherry	Stems/w		8		
Prunus serotina	Black cherry	Leaves		0.2		
Psoralea argophylla	Silver leaf Indian breadroot	Leaves		20		
Psoralea argophylla	Silver leaf Indian breadroot	Stems		0.2		
Pycnanthemum fenvofolium	Narrow leaf mountain mint	Leaves/stems		8		
Rosa arkansas	Arkansas rose	Stems		7		
Rubus alleghanesis	Alleghany blackberry	Stems	0.2			
Sambucus racemosa	Red elderberry	Leaves/stems		0.2		
Scrophularia lanceolata	Lance leaf figwort	Leaves				0.2
Streptopus lanceolatus	Twisted stalk	Leaves		0.2		
Tanacetum vulgare	Common tansy	Flowers		0.2		
Thalictrum polygamum	Tall meadow rue	Leaves		12		
Unknown ornamental gentia		Leaves/stems		8		
Picea glauca	White spruce	Leaves		10		
Desmodium illinoense A.	Illinois tre tickfoil	Leaves		10		15
Asclepia verticallus	Whorled milkweed	Stems		12		
Artemisia caudata	Wild wormwood	Flowers		15		
Liatris aspera	Tall blazing star	Flowers				

[1]Ec, *Escherichia coli*; Sa, *Staphylococcus aureus*; Pa, *Pseudomonas aeruginosa*; Ca, *Candida albicans*

wild crafting and harvest. This will better enable these plants to be incorporated into current or newly designed agricultural systems. Both harvest date in general and according to species type showed an effect on the bioactivity of extracts.

In general, harvesting later in the growing season before the post- reproductive life cycle was best indicating that developmentally mature plants have increased stores of bioactive phytochemicals (Figure 1). Although, it is important to remember that novel compounds may be present at any time during the plant lifecycle and in different plant parts; therefore, it will be a

Table 2. Alphabetical list of species that did not show antimicrobial activity.

Actaea rubra	*Leonurus cardiaca*
Allium canadense	*Lilium philadelphium*
Amelanchier spp	*Linum sulentum*
Amorpha fruiticosa	*Lithospermum canescens*
Amphicarpaea bracteata	*Lonicera japonica*
Anemone canadensis	*Lupinus perennis*
Anemonella thalictroides	*Lychnis alba*
Antemara platyginofia/neglecta	*Maianthemum canadense*
Apios americana	*Morus* spp.
Apocynum androsaemifolium	*Osmorhiza claytoni*
Apocynum cannabinum	*Ostrya virginiana*
Aquilegia canadensis	*Panic grass seed head*
Aralia nuclicaulis	*Panicum* spp.
Arisaema triphyllum	*Parthenocissus quinquefolia*
Articum minus	*Patibola columitera*
Asclepia verticallus	*Penstemom palliclus*
Asparagus spp	*Phalaris arundinacea*
Aster ericoides	*Phleum pratense*
Aster macrophyllus	*Phlox divorcica*
Aster oblongiflora	*Physalis heterophylla*
Aster sericeus	*Platanthera psycodes*
Astragalus canadensis	*Poa prantense*
Athyrium filix-femina	*Polygala vulgaris*
Baptista australis	*Polygonum amphibium*
Brassica nigra	*Populus tremuloides*
Campanula rotundifolia	*Potentilla recta*
Castilleja linariaefolia	*Robinia pseudoacacia*
Ceanthus cuneatus	*Rosa suffalata*
Cicuta maculata	*Sambucus canadensis*
Circium vulgare	*Sambucus racemosa*
Circuta macullata	*Saxifraya pensylvanica*
Comandra umbellata	*Senecio aureus*
Convolvulus arvensis	*Silene latifolia*
Coronilla varia	*Silphium laciatum*
Cypripedium calceolus	*Sisyrinchium aujustifolium*
Dalea purpuruem	*Sium suave*
Delphinium virescens	*Solanum dulcamara*
Desmodium canadense	*Sorbus americana*
Dianthus armeria	*Stachys palustris*
Eupatorium perfoliatum	*Streptopus lanceolatus*
Fragaria virginiana	*Thalictrum polygamum*
Fraxinus pennsylvanica	*Tilia americana*
Galium boreale	*Tribolium arvense*
Gentianella quinquefolia	*Trifolium pratense*
Heliopsis helianthoides	*Unknown ornamental gentia*
Hepatica nobilis	*Viburnum dentatum*
Heterostipa spartea	*Viburnum dentatum*
Heterotheca villosa	*Vicia villosa*
Hypoxis hirsota	*Zanthoxylem americanum*
Koeleria macrantha	*Zigadenus venenosus*

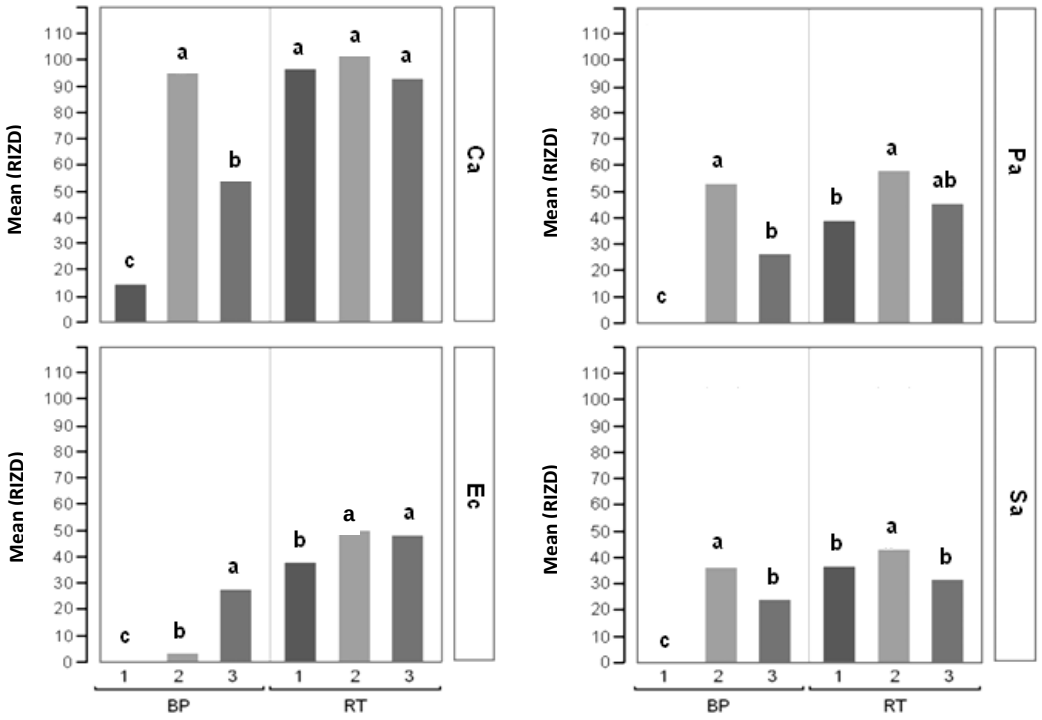

Figure 1. Effect of plant maturity (1, vegetative; 2, reproductive; 3, post-reproductive) on mean inhibition zone diameter (RIZD), measured in mm, (as % of commercial control) by species (BP, *Betula papyrifera;* RT, *Rhus typhina* leaves) and microorganism (Ca, *Candida albicans;* Ec, *Escherichia coli*, Pa, *Pseudomonas aerugunosa*; Sa, *Staphyloccus aureus).* Different superscripts indicate statistical significant differences among the treatments at p-value = 0.05.

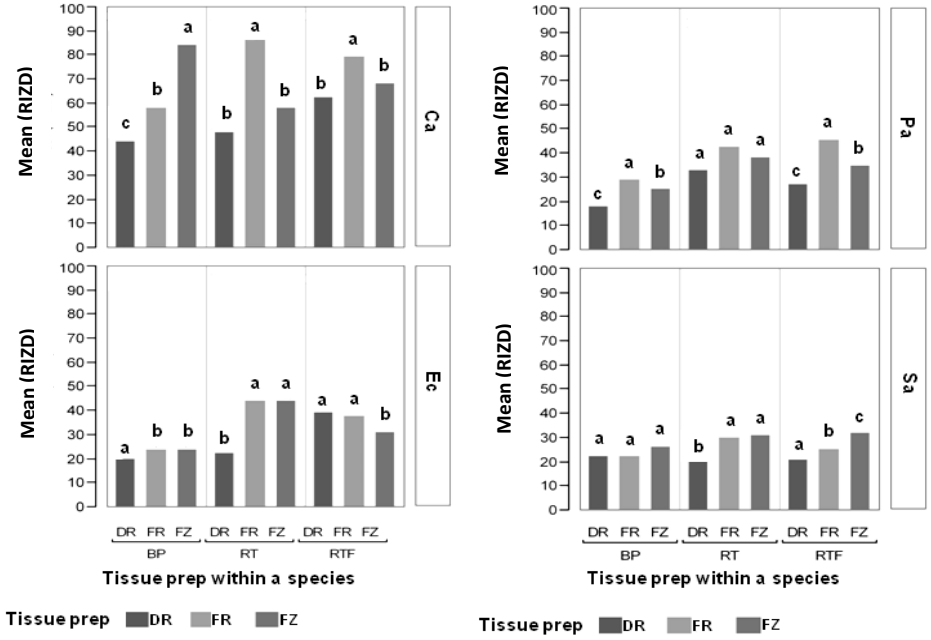

Figure 2. Effect of tissue state (DR, dried; FR, fresh; FZ, frozen) on mean inhibition zone diameter (RIZD), measured in mm, (as % of commercial control) by species (BP, *Betula papyrifera;* RT, *Rhus typhina* leaves, RTF-*Rhus typhina* flowers) and microorganism (ca, *Candida albicans*; ec, *Escherichia coli*, pa, *Pseudomonas aerugunosa;* sa, *Staphyloccus aureus.* Different superscripts indicate statistical significant differences among the treatments at a p-value = 0.05.

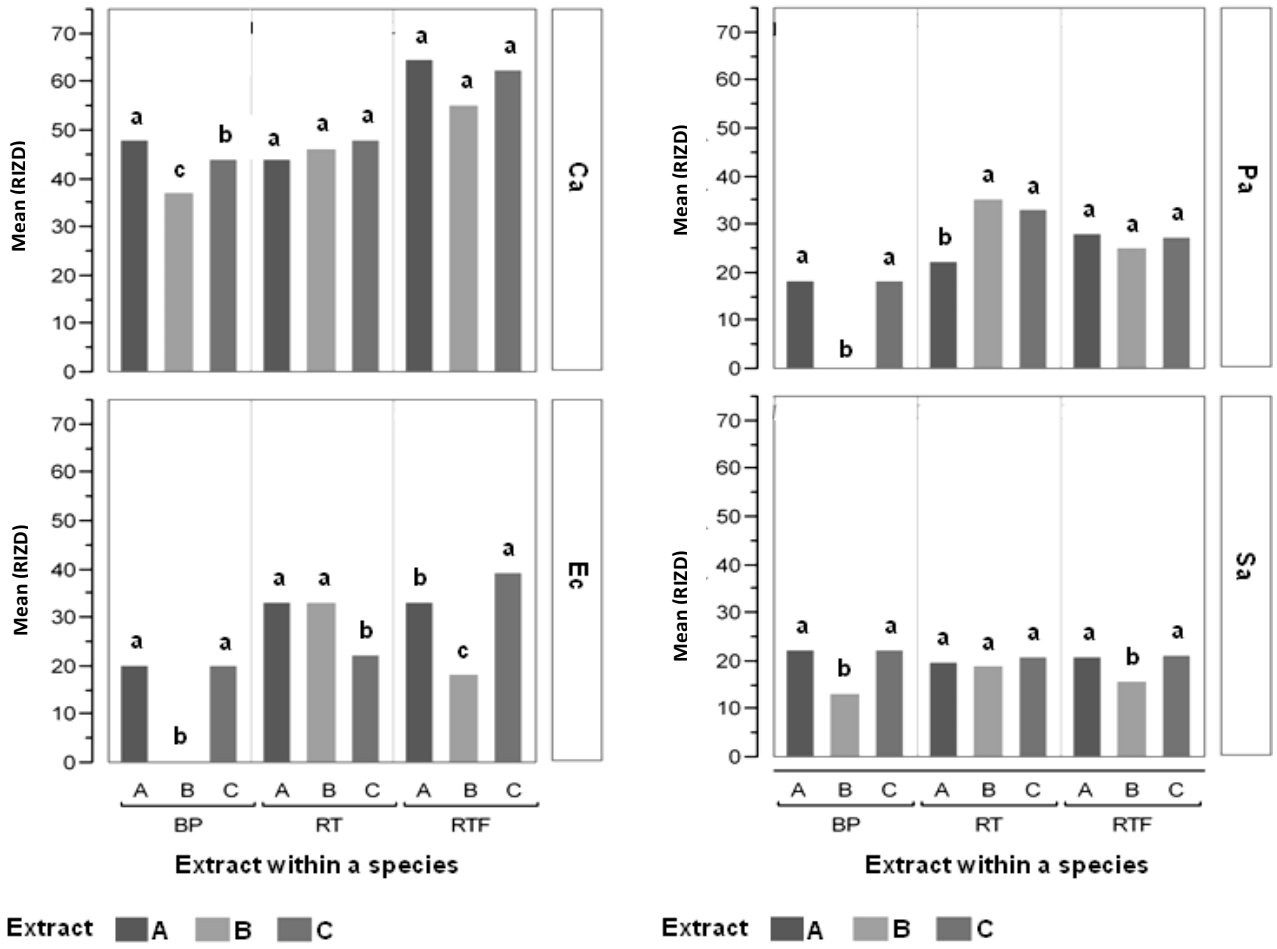

Figure 3. Effect of extracting solvent (A, acetone; B, methanol; C, ethanol) on mean inhibition zone diameter (RIZD), measured in mm, (as % of commercial control) by species (BP, *Betula papyrifera;* RT, *Rhus typhina* leaves; RTF, *Rhus typhina* flowers) and microorganism (ca, *Candida albicans*; ec, *Escherichia coli*; pa, *Pseudomonas aerugunosa*; sa, *Staphyloccus aureus.* Different superscripts indicate statistical significant differences among the treatments at a p-value = 0.05.

key to develop plant specific harvesting guidelines in order to obtain the most potent extract.

Tissue preparation impact on inhibition

Plant tissue condition post harvest affects the chemical stability of its bioactive compounds and different states can lead to variable relative concentrations of the chemical constituents present. In this experiment, three plant tissue preparations (fresh, frozen, and dried) were compared. There was an effect of tissue preparation on antimicrobial activity between species; all extracts made from dried tissue had decreased bioactivity compared to those prepared from frozen or fresh tissue, which were similar (Figure 2).

While there were differences in the amount of inhibition among tissue preparation states, it is important to note that all three resulted in some inhibition of microorganisms. Therefore, extracts prepared from tissue in any of the three states will show bioactivity – just to a lesser degree in dried tissue preparations.

Solvent impact on phytochemical extraction

This experiment was designed to assess the extracting abilities of different organic solvents in order to identify the cheapest and most environmentally friendly solvent that would perform the best for higher throughput testing. The results of these tests were clouded because of species and plant part interactions so that while there was an effect of solvent, there was also an interaction between species and the solvent used to make the extract. However in general, acetone and ethanol were roughly equivalent in extracting active compounds based on the plant species and species of microbes being tested against, and they were both superior to methanol, which was a poor extracting solvent overall (Figure 3). These findings are supported by several studies and

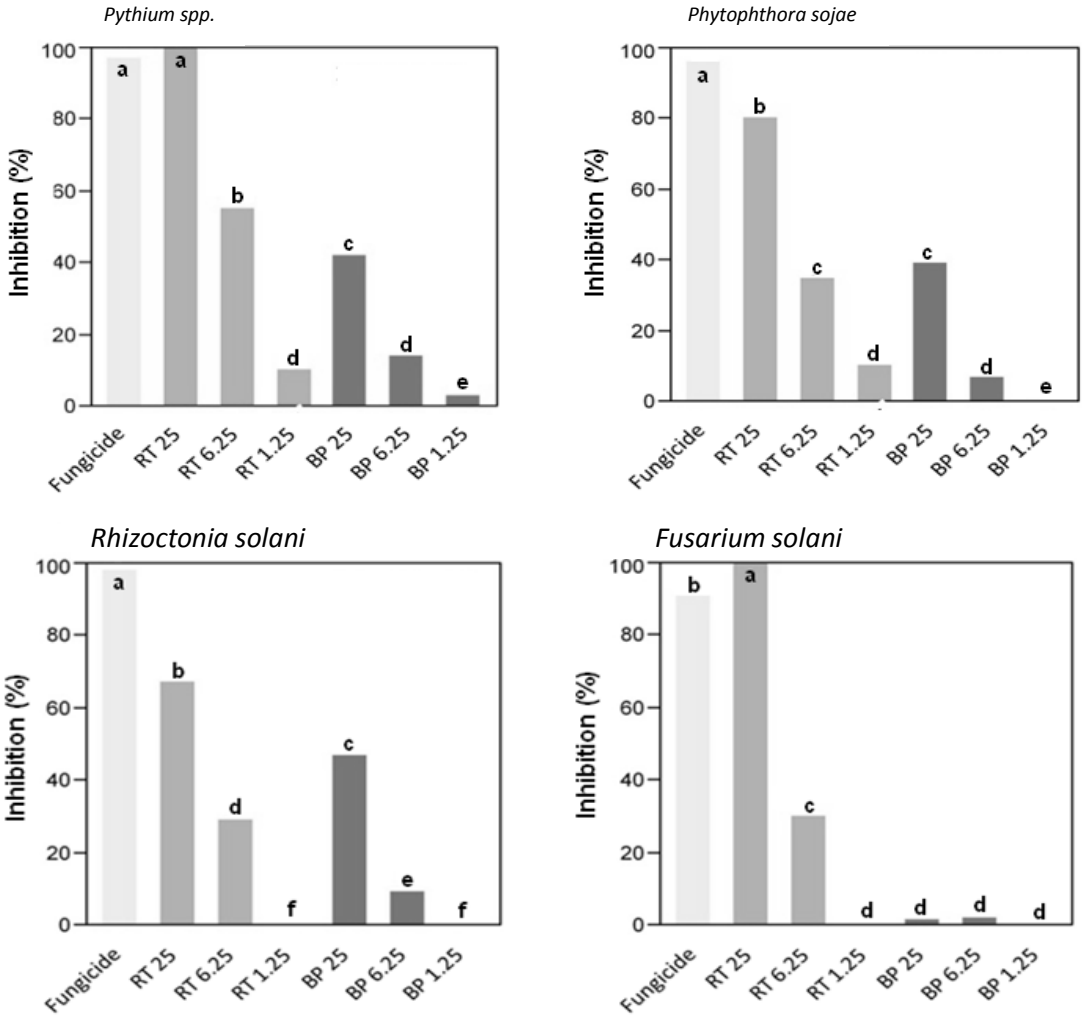

Figure 4. Inhibition of growth of the plant pathogens: *Pythium spp*., *Phytopthora sojae, Rhizoctonia solani*, and *Fusarium solani* by either a commercial fungicide or three concentrations of extracts from either *Rhus typhina* (RT) or *Betula papyrifera* (BP). Inhibition is expressed as a percentage of growth of the pathogen observed on unamended media. Different superscripts indicate statistical significant differences among the treatments at a p-value = 0.05, n = 3. Fungicide applied in media inoculated with *P. sojae* and *Pythium spp* is metalaxyl. Fungicide applied in media inoculated with *F. solani* and *R. solani* is fludioxonil.

found out that ethanolic extractions of plant material have the highest antimicrobial activity, superior to acetone extractions (Durga et al., 2009) and even the commercial antibiotic gentamicin (Hassan and Amjid, 2009). Moreover, ethanol extractions subject to cytotoxicity assays have proven to be not lethal to mice (Rasool et al., 2008) providing support for the environmentally friendly benefits of this solvent. Thus, ethanol was used to extract the greater collection of plants beyond those used for the screening optimization experiments.

Antifungal properties

Both plant species and extract concentration influenced the inhibitory activity of the plant extracts on colony growth of the four plant pathogens. Addition of metalaxyl inhbited oomycete growth while addition of fludioxonil inhibited growth of filamentous fungi by greater than 90%. Inhibition of growth increased with increasing concentrations of extracts except for *B. papyrifera* extracts added to tested against *F. solani* which was unaffected by the extract amendment. The highest concentration of the *R. typhina* extract reduced the growth of *F. solani* and *Pythium spp* as effectively as either of the commercial fungicides *(*Figure 4). The antifungal activity of extract from *R. typhina* was superior to the activity of extract from *B. papyrifera* at every concentration. Only the highest concentration of *B. papyrifera* extract (25 µg/ml) inhibited pathogen colony growth as much as the intermediate concentration (6.25 µg/ml) of *R. typhina* extract which reduced the growth of

R. solani by 30%, *F. solani*, *Pythium spp. and P. sojae* by greater than 30% (Figure 4). Extract from *B. papyrifera* did not affect the growth of *F. solani* at any of the rates tested. Neither *R. typhina* nor the *B. papyrifera* extracts impacted germination of soybean seeds indicating that natural extracts have potential as seed treatments to control soilborne disease.

Conclusion

This study has identified 80 plant extracts from 68 species, several which have never been previously studied, with antimicrobial activity, thereby increasing the knowledge of biologically active Minnesota plants. Optimization of collection and extraction methods were determined including harvesting developmentally mature plants, using a fresh or frozen plant preparation, and extracting in ethanol or acetone, solvents relatively safe for the environment and human uses. These results have important implications for the development of plant specific agronomic practices, storage of harvested material, and safety both for the environment and humans. Specific practices resulting in accumulation of the highest concentrations of active compounds in specific plant species will need to be determined for growth in commercial production. For storage purposes, drying material is ideal, so although fresh and frozen material showed stronger inhibition, the fact that dried material is still active will be important for large-scale production.

This study shows promise in using plant extracts as fungicide seed treatments. Some extracts do not inhibit seed germination and control soil borne pathogens as well as commercial standards. More work is needed to test active extracts in the soil with additional seed stocks. Finally, it is necessary to explore agricultural systems to cultivate these plants in an economical way; whether that is wild crafting or in a traditional agricultural production system still needs to be determined. The possibilities for using plant extracts as antimicrobial agents are far-reaching making this an exciting area for future research efforts.

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