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Antimicrobial activity of selected Native American seeds

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Antibacterial activity profiles of 22 seed extracts against five pathogens were evaluated using disc diffusion assay. *Helenium flexuosum, Helenium autumnale, Epilobium angustifolium, Epilobium coloratum* and *Epilobium glandulosum* showed activity against *Staphylococcus aureus, Enterobacter aerogenes*, *Shigella flexneri* and *Pseudomonas aeruginosa*. The two *Helenium* species exhibited moderate activity. Bioassay guided chemical examination of *H. autumnale* and afforded the flavonoids apigenin and luteolin of which luteolin showed weak activity against *S. aureus*.

Key words: Antibacterial, *Helenium autumnale*, flavonoid, apigenin, luteolin.

INTRODUCTION

The growing disparity between the need for new anti-infective drugs to treat pathogens resistant to multiple antibiotics and the lean developmental pipeline was highlighted by the infectious Diseases Society of America (Boucher et al., 2009). Most newly introduced anti-infective agents are newer generations of established skeletal classes such as fluoroquinolones and carbapenems (Fischbach and Walsh, 2009). However, the persistent threat that microbes may rapidly develop resistance to the new generation anti-infective drugs limits their use and necessitates the discovery of new structural classes of anti-infective agents.

Natural products based drug discovery played a crucial role in the development of modern medicine (Li and Vederas, 2009). About 70% of all known anti-bacterial agents owe their development to a natural product (Newman and Cragg, 2007). These statistics continue to drive the systematic screening of plants for biologically active constituents (McCutcheon et al., 1992; Locher et al., 1995; Kumar et al., 2006; Cock, 2008). Some of these studies rely on exploiting the traditional wisdom of the local populations. For example, Native American medical traditions employed various parts such as stem and leaves of hundreds of different plants to treat a number of diseases (Moerman, 1998). Of the plants screened in this study, *Baptisia alba, Baptisia australis, Baptisia bracteata, Baptisia tinctoria*, *Epilobium angustifolium, Helenium autumnale, Lysimachia quadriflora* and *Lysimachia thyrsiflora* were included in the Native American Ethnobotany (Moerman, 1998), although the use of seeds is not cited in the book. A systematic study of seeds of Mississippi river basin was reported recently (Borchardt et al., 2008) and the authors aptly point out to the dearth of chemical investigations into seed extracts.

As part of our investigations on antimicrobial compounds from natural sources we have embarked on the systematic screening of local flora. In this paper, we report the antibacterial activity profiles of 22 seed extracts (Table 1) and the results of bioassay guided chemical examination of *H. autumnale,* the most active extract of

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Table 1. Antimicrobial activity of seed extracts.

Plant	Common name	Zone of inhibition (mm)				
		SA	EC	PA	SF	EA
Baptisia alba	White Wild Indigo	na	na	na	na	na
Baptisia australis	Blue Wild Indigo	na	na	na	na	na
Baptisia bracteata	Cream Wild Indigo	7	na	na	na	na
Baptisia minor	Dwarf Blue Indigo	na	na	na	na	na
Baptisia sphaerocarpa	Lg. Yellow Wild Indigo	na	na	na	na	na
Baptisia tinctoria	Sm. Yellow Wild Indigo	8	na	na	na	na
Cassia fasciculate	Partridge Pea	na	na	na	na	na
Cassia hebecarpa	Wild Senna	na	na	na	na	na
Cassia marilandica	Maryland Senna	na	na	na	na	na
Dodecatheon amethystinum	Amethyst Shooting Star	na	na	na	na	na
Dodecatheon maedia	Midland Shooting Star	na	na	na	na	na
Epilobium angustifolium	Fireweed	13	na	12	13	13
Epilobium coloratum	Cinnamon Willow Herb	16	na	17	16	15
Epilobium glandulosum	Northern Willow Herb	16	na	17	14	16
Gaura biennis	Biennial Gaura	na	na	na	na	na
Gaura longiflora	Large-Flowered Gaura	na	na	na	na	na
Helenium autumnale	Sneezeweed	20	na	na	na	na
Helenium flexuosum	Purple-headed Sneezeweed	24	na	na	na	na
Lysimachia ciliate	Fringed Loosestrife	na	na	na	na	na
Lysimachia quadriflora	Prairie Loosestrife	7	na	na	na	na
Lysimachia terrestris	Swamp Candles	8	na	na	na	na
Lysimachia thyrsiflora	Tufted Loosestrife	na	na	na	na	na
Chloramphenicol (Positive control)		17	23	na	40	26

Na, No activity; **SA,** *S. aureus*; **EC,** *E. coli*, **PA,** *P. aeruginosa*; **SF,** *S. flexneri*; **EA,** *E. aerogenes*. None of the extracts inhibited *E. coli*.

the ones tested.

MATERIALS AND METHODS

Twenty two (22) seeds of native and naturalized species were evaluated in the antimicrobial assay. The seeds were obtained from Prairie Moon Nursery, Winona, MN. Extracts from these seeds were tested against a mini panel of microbes, namely *Staphylococcus aureus* (ATCC 27661), *Pseudomonas aeruginosa* (ATCC 27853), *Shigella flexneri, Enterobacter aerogenes,* and *Escherichia coli* (ATCC 14948) using the disc diffusion assay. All bacterial specimens were bought from Carolina Biological Sciences.

Preparation of extracts

Seeds (7.08 g) were ground in a kitchen grinder except for *Cassia fasciculata, Gaura biennis* and *Gaura longiflora* (28.35 g each) and *L. quadriflora, L. terrestris* and *L. thyrsiflora* (3.54 g each). The ground powder was transferred to 15 ml conical tubes and extracted overnight in methanol (10 ml). The solutions were filtered under vacuum and the seeds were soaked in fresh methanol again and vacuum filtered the next day. The combined methanol extracts were evaporated to dryness using a rotavapor. The extract residues were transferred into vials for storage at 4°C. The weights of the residues ranged from 160 mg to 1.18 g. All extracts were tested for antimicrobial activity using the disk diffusion assay (Bauer et al., 1966).

Disc diffusion assay

The test organisms were grown overnight in Luria Broth (LB) at 37°C and then diluted to approximate optical density of about 0.5 using a spectrophotometer. Mueller Hinton Agar (MHA) plates were inoculated with freshly diluted cultures (100 µl per plate) using a sterile L shaped sterile glass rod. After 30 min, 6 mm sterile paper discs impregnated with the test extracts were placed on the inoculated MHA plates and incubated (18 to 24 h) at 37°C. Zones of inhibition were read on three axes and averaged. Each extract was tested in triplicate and then averaged and reported in Table 1. Chloramphenicol discs (sensi disc BD-BBL, 30 µg) were used as positive control and dimethylsulfoxide (DMSO) (15 µl/disc) was used as negative control. Extracts with inhibition zones of >8 mm are considered to be significant (McCutcheon et al., 1992).

Preparation of impregnated discs

Sterile 6 mm paper discs were from Fisher Scientific. Each plant extract (30 mg) was dissolved in DMSO (250 µl) and stored at 4°C. Before impregnation, the vials were thawed and 15 µl of each extract was pipetted onto the 6 mm paper discs. DMSO (15 µl) was used as negative control. The paper discs thus impregnated were used in the disc diffusion assay.

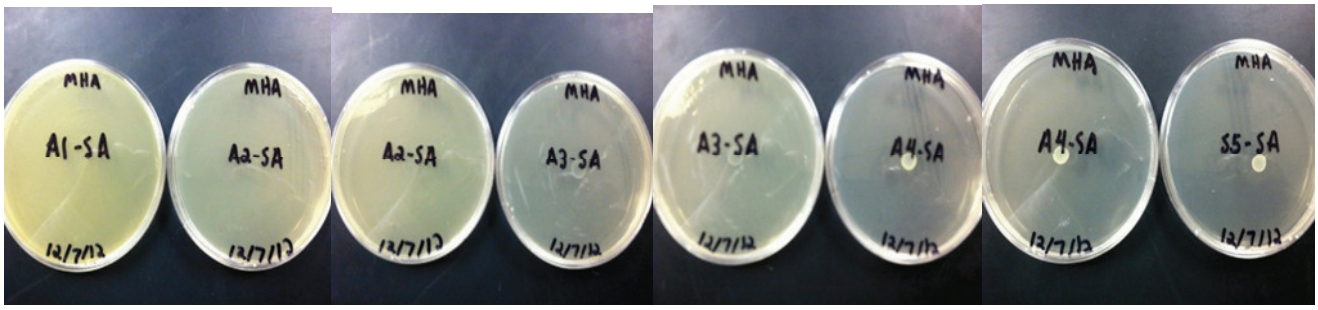

Figure 1. MHA plates with various concentrations of *H. autumnale* extract tested on *S. aureus* (10 µl). Growth after 18 h. A1 (1800 µg/ml); A2 (900 µg/ml); A3 (450 µg/ml); A4 (225 µg/ml); A5 (112.5 µg/ml). The minimum inhibitory concentration was between 900 and 450 µg/ml.

Minimum inhibitory concentration

H. autumnale extract (216 mg) was dissolved in DMSO (6 ml) and the solution was transferred into three vials (2 ml each) labeled A1. Four 2-fold serial dilutions were made from A1 and were labeled A2, A3, A4 and A5.

MHA (19 ml) was transferred to fifteen 50 ml Erlenmeyer flasks prior to sterilizing. The sterilized MHA was cooled to 50°C in a water bath and 1 ml of each concentration was added to obtain concentrations of 1800, 900, 450, 225 and 112.5 µg/ml. The agar was thoroughly mixed and immediately poured into 10 cm sterile Petri dishes. Each of the 15 plates (3 replicates at each concentration) was inoculated with 10 µl of freshly diluted culture of *S. aureus*. The plates were incubated for 18 h at 37°C and the growth of *S. aureus* was recorded (Figure 1).

Bioassay guided examination of *Helenium autumnale*

H. autumnale seeds (454 g) were obtained from Prairie Moon Nursery and were ground to powder. The powder was extracted in methanol (1.3 L × 5) for 2 days, and filtered. The combined methanolic extract was rotavapped below 35°C down to a dry residue (33.6 g). The residue was reconstituted in methanol water mixture (9:1, 200 ml) and it was partitioned with hexane (200 ml × 3) to obtain hexane extract (F003, 9.4 g). Remaining aqueous methanol was diluted to methanol water (3:2) and partitioned with methylene chloride (300 ml × 3) to obtain the methylene chloride extract (F004, 4.67g). The methylene chloride extract exhibited moderate antibacterial activity against *S. aureus* (14 mm, 180 µg/disc) and was subjected to chemical examination.

F004 was dissolved in methanol and loaded on a Sephadex LH-20 set in methanol. The column was eluted with methanol as the mobile phase and the resulting fractions were pooled into eight fractions (F007-F014) by comparing their thin layer chromatography profiles. Each of these fractions was subjected to disc diffusion assay (180 µg/disc) and the activity was spread across the fractions. Fractions F013 (inhibition zone 12 mm) and fraction F014 (inhibition zone 11 mm) were subjected to further chemical examination.

Fraction F013 (71 mg) was adsorbed on silica gel and separated on a Biotage SNAP cartridge (KP-Sil 10 g) using a Biotage Isolera IV system using a gradient of hexane - ethylacetate as the mobile phase. The separation was monitored using ultraviolet (UV) detection at 254 nm. Four fractions were isolated of which F016 (5 mg) and F017 (11.7 mg) were identified as the flavonoids apigenin and luteolin, respectively. Separation of fraction F014 (20 mg) under identical conditions resulted in the isolation of fraction F020 (3 mg) which is again luteolin.

RESULTS AND DISCUSSION

Natural products from various parts of plants were studied in great detail but very few studies were done on the seeds (Borchardt et al., 2008). In this study, we report the antibacterial activity of 22 plant seeds and the bioassay guided chemical examination of *H. autumnale.*

Nine (9) of the 22 extracts inhibited the growth of *S. aureus* with the two *Helenium* species exhibiting modest zones of inhibition (Table 1). The average inhibition zone of the chloramphenicol (30 µg) disc on *S. aureus* and *E. coli* were 17 and 23 mm, respectively, whereas DMSO serving as negative control had virtually no inhibition zone.

Extracts of *H. autumnale, Helenium flexuosum, E. angustifolium, Epilobium coloratum* and *Epilobium glandulosum* showed moderate zones of inhibition on *S. aureus*. The inhibition zone varied from 13 mm for *E. angustifolium* to 24 mm for *H. flexuosum* (Figure 2). The two species of *Helenium* were significantly more effective at inhibiting *S. aureus* compared to the three *Epilobium* species ($p < 0.05$). The three *Epilobium* species were not significantly different from each other in their activity against *S. aureus* ($p = 0.11$). *H. flexuosum* is significantly more active than *H. autumnale* ($p = 0.03$). Null hypothesis (0.05 level) results show that the order of inhibitions of *S. aureus* is as follows: *H. flexuosum* > *H. autumnale* > *E. coloratum* ≥ *E. glandulosum* > *E. angustifolium*.

The active extracts (*H. autumnale, H. flexuosum, E. angustifolium, E. coloratum* and *E. glandulosum*) were also tested at 180 µg/disc. At this concentration, only the 2 *Helenium* species exhibited moderate activity. The inhibition zones were 13 mm for *H. autumnale* and 15 mm for *H. flexuosum*.

Various parts of *H. autumnale* (Family Asteraceae) were used by the Cherokee, Comanche, Mahuna, Menominee and Meskwaki tribes of North America for a host of ailments (Moerman, 1998). A literature search revealed that a study was conducted on the flavonoid distribution in 22 taxa of *Helenium* (Bierner, 1973) and reported 6-O-methylluteolin and methylated and glycosylated derivatives of apigenin from *H. autumnale*. A

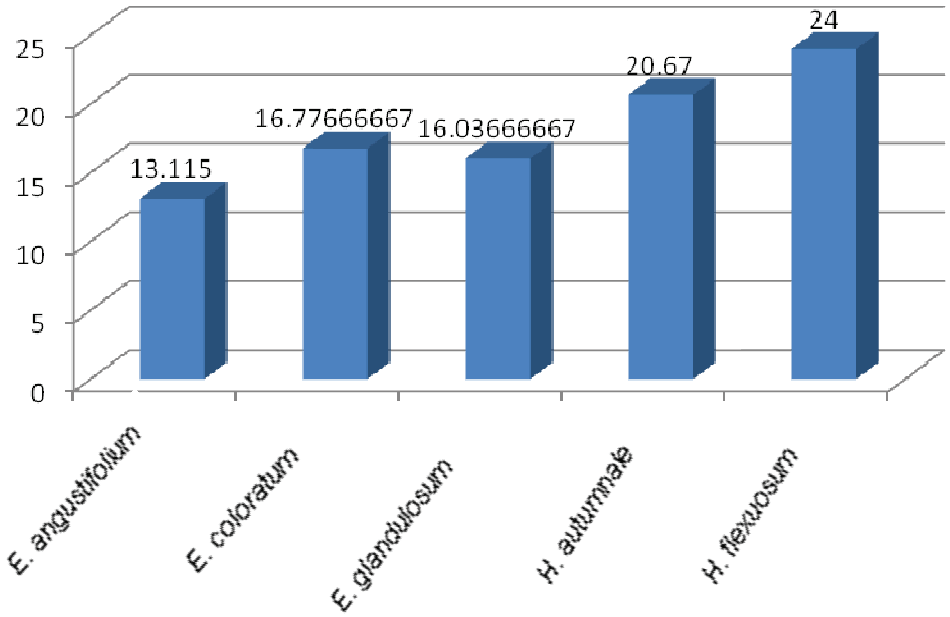

Figure 2. Graphical representation of the zones of inhibition (mm) of active extracts versus *S. aureus*.

Scifinder search revealed 43 studies were reported on *H. autumnale*.

Bio-assay guided chemical examination of the methylene chloride extract (F004) of *H. autumnale* resulted in the isolation of two compounds F016 and F017 which were identified as apigenin (Wawer and Zielinska, 2001) and luteolin (Wawer and Zielinska, 2001; Burns et al., 2007; Lopez-Lazaro, 2009), respectively on the basis of nuclear magnetic resonance (NMR) and mass spectrometric data. However, to the best of our knowledge, this is the first report of the occurrence of these flavonoids in *H. autumnale*. There is slight discrepancy in the ^{13}C values of some carbons (Δδ ranging from -0.5 to +0.9) of luteolin between our data and that reported in literature (Wawer and Zielinska, 2001) but our data was in perfect agreement with another literature report (Burns et al., 2007). Luteolin was found to inhibit the growth of *S. aureus* (ATCC 27661) at 180 µg/disc (inhibition zone 13 mm) and its occurrence in the seeds may serve to protect the seeds from microbial invasion.

Although bioassay guided separation of *H. autumnale* was successful in isolating luteolin with activity against *S. aureus*, it belongs to a well studied class of compounds. Therefore, the search for an effective antibacterial compound that belongs to a new structural class continues to drive our efforts.

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