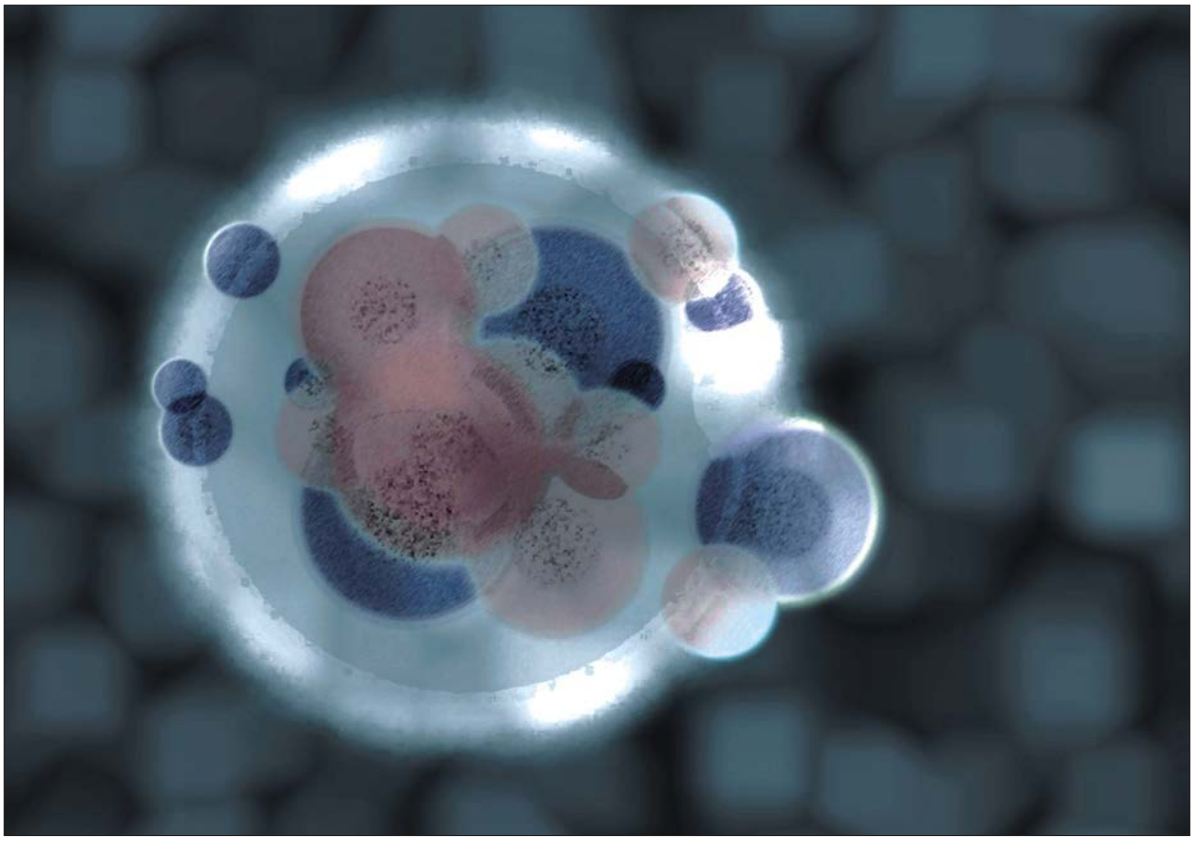

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Identification of Potentially Allelopathic Isobutylamides in *Echinacea angustifolia* D.C. Roots

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Abstract: Multiple regression models of the HPLC chromatogram peak areas and the results from lettuce seed bioassays were used to determine which combination of isobutylamides from *E. angustifolia* root extracts accounted for the allelopathic effects in the bioassay. Two fractions initially identified as peaks 3 and 6 and subsequently determined to be undeca-2E-ene-8, 10-diynoic acid isobutylamide and dodeca-2E, 4E, 8Z, 10E-tetraenoic acid isobutylamide, respectively, were identified as the compounds responsible for the allelopathic activity. The two compounds each accounted for slightly more than 6% of the total bioassay variability, but together accounted for about 73% of the variability based on the simplest non-linear model. Direct application of the purified compounds in the bioassay demonstrated that use of the genetic variability in the *Echinacea* samples allowed identification of synergistic effects without the traditional approach of fractionation and testing of all compounds and their possible combinations.

Key words: *Echinacea angustifolia*, isobutylamides, allelopathy, HPLC, lettuce seed bioassay, natural herbicide

INTRODUCTION

Plants are sessile organisms that are unable to escape environmental pressures and as a consequence have evolved an array of chemical defense mechanisms that are genetically and environmentally regulated (Weckwerth, 2003). Allelopathic compounds produced by many plant species may have a significant effect on production agriculture, both by affecting crop seed germination and weed germination (Ghafar *et al*., 2000; Hoque *et al*., 2003a, b; Kadioglu and Yanar, 2004; Nasr and Shariati, 2005; Shaukat and Siddiqui, 2002; Uremis *et al*., 2005). Many of these biologically active compounds have potential for the development of natural herbicides, pesticides and other marketable products (Duke *et al*., 2003; Macias *et al*., 2004).

Echinacea angustifolia D.C. (Asteraceae), a native to the Great Plains of North America, has been studied extensively for biologically active secondary plant compounds (Barrett, 2003; Miller, 2004; Mitscher and Cooper, 2004). The presence of biologically active compounds in this species was first suggested by Native American Plains Indians, who used this herb to alleviate ailments such as toothaches, serious infections and snakebites (Hobbs, 1995; Kindscher, 1992). Viles and Reese (1996) demonstrated that the volatile compounds and those present in aqueous extracts of *E. angustifolia* also had allelopathic potential against several native species and in a standard lettuce seed bioassay. Their study also showed that the chemical activity of specific extracts was not uniform, but dependent upon genotypic variation of the plants grown in a common garden.

The traditional approach to identification of biologically active compounds is to extract, separate and individually test compounds that have shown activity in a bioassay of a crude extract (Wagner, 1999). This approach has proven successful for many compounds but is limited if there is a large number of compounds in the crude extract or if the efficacy is the result of additive or synergistic effects. In natural product chemistry, explaining the efficacy that results from the interactions of several compounds in crude extracts has been problematic. Identifying the biologically active compounds and mechanisms involved in the activity of the extracts has proven especially difficult in evaluating the medical potential of extracts from *Echinacea* (Barrett, 2003; Mitscher and Cooper, 2004).

The newest approach to identifying potentially useful biologically active compounds from plants involves metabolic profiling of the whole metabolome or of specific fractions of primary and secondary products (Murch *et al*., 2004; Kite *et al*., 2004). The metabolites are chemically identified and the variation in their concentrations measured. Those compounds playing key

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roles in observed physiological variation or in specific bioassays are then identified using statistical analyses (e.g., principal component analysis). Such metabolomic approaches avoid problems with additive or synergistic effects by recognizing all of the components that may be involved in the assay and then relying on the statistical analysis of the data to determine which compounds control the observed phenomenon. This approach is both powerful and promising, but it requires considerable input of time and resources, as identification and characterization of an entire metabolome requires sophisticated and often costly analyses.

This study was designed to take advantage of the metabolomic approach's use of statistical analyses, but without the initial identification of all of the chemical constituents. *Echinacea* was chosen to test this statistical approach to natural products chemistry because of the extensive work that has been conducted on the secondary compounds and their variability within the species (Binns *et al*., 2002; Miller, 2004) and because previous work in our lab has demonstrated that extracts of *E. angustifolia* contained allelopathic compounds having the potential to induce inhibition of seed germination and root elongation in several plant species (Viles and Reese, 1996) and may prove valuable as natural herbicides (Duke *et al*., 2003; Macias *et al*., 2004). Using statistical models to compare biological activity and HPLC profiles of root extracts, selection and identification of the biologically active components were made and the activity of the selected compounds was compared to the activity of crude *Echinacea* root extracts.

MATERIALS AND METHODS

Plant sources: *Echinacea angustifolia* (Asteraceae) seeds were collected from locations in North Dakota, South Dakota, Kansas, Wyoming and Nebraska and planted in a common garden on the South Dakota State University Farm in Brookings, South Dakota (Feghahati and Reese, 1994). The *E. angustifolia* were planted in half-sib families and allowed to grow for three years. Plants were harvested, separated into roots, stems and leaves and flower heads. The plant parts were then placed in labeled brown paper bags and air-dried.

Extract preparation: Dried root material was ground in a Wiley No. 2 grinder fitted with a one-millimeter mesh screen. Ground root material (0.5 g aliquots) from 30 plants (6 plants from each of the 5 populations) was placed into 50 mL 95% ethanol. The extracts were placed on a shaking table and agitated for 4 h in the dark at room temperature. The extracts were centrifuged for 20 min at 1,200 x g and the supernatants saved.

Lettuce seed bioassay: Allelopathic activity of crude *Echinacea* root extracts and purified compounds from the extracts was evaluated using a lettuce (*Lactuca sativa*) seed bioassay (Rice, 1983). For the initial screening, 9 cm disposable petri plates were lined with 2 sheets of filter paper. Four milliliters of ethanolic extracts were pipetted onto the filter paper and the ethanol allowed to dry in a sterile laminar-flow hood for 4-6 h at 23°C. Four milliliters of sterile, de-ionized water were then added to the filter paper. Twenty-five lettuce seeds were placed into the dish. (For experiments using purified compounds from the *Echinacea* extracts, 6 cm disposable petri dishes were used and liquid volumes were reduced to 2 mL). In all experiments, the Petri dishes were sealed with parafilm immediately after water and seeds were placed on the filter paper. Seeds were cultured in the petri dish for four days under 16 h of light and 8 h of dark at 25°C. Percent germination was determined and root lengths were recorded. The experiments were replicated three times.

Analytical high performance liquid chromatography: Ten milliliter aliquots of each of the 30 crude ethanolic extracts were concentrated to 1 mL in glass test tubes under vacuum at room temperature, using a Savant SWJ 120 speed-vac system. The concentrate was centrifuged for 10 min at 20,000 x g and the supernatant saved. Twenty microliter samples were separated on a 150×4.6 mm i.d., 5 μm, C-18, Ultrasphere ODS column (Beckman Instrument Co., Fullerton, CA) and eluted using a 30-100% ethanol gradient over 30 min. Chemical constituents were monitored by UV at 220 nm using a Beckman 168 Diode array detector. Real-time purity of the peaks was monitored using the Beckman System Gold software. This program uses evolving factor analysis to determine differences in successive spectra (220-400 nm) during elution of a chromatographic peak and allows identification of up to 3 co-eluting compounds.

Preparative high performance liquid chromatography: Individual compounds were isolated from ethanolic extracts of a single *Echinacea* plant, selected from the South Dakota population because of its strong allelopathic activity, as shown in the initial bioassay. The individual compounds, identified as separate peaks, were isolated using a semi-preparatory 150×10 mm i.d., 5 μm C-18, Adsorbosphere column (Alltech Associates, Inc.,

Deerfield, IL). A 1 mL sample was injected and eluted using a 30 min, 30-100% ethanol gradient at a flow rate of 5 mL per min. Materials eluting as peaks 3 and 6 were collected using a fraction collector connected to the HPLC and controlled by the Beckman System Gold Software. Materials under the individual peaks from multiple injections were pooled, stored at -80°C and then lyophilized. Relative concentrations of the individual components were determined based upon the area under the peaks for the isolated compounds in comparison with the areas for the components found in the crude preparation. Lettuce seed bioassays were conducted, as above, using the individually isolated components from the roots applied at concentrations equivalent to those found in the original ethanolic extracts or fractions of those concentrations.

Preliminary chemical determination: Preliminary identification of the chemical constituents from the ethanolic root extracts as isobutylamides was made using the above described analytical HPLC method and comparing HPLC retention times and UV spectra with published characteristics of *E. angustifolia* secondary products (Bauer *et al*., 1988, 1989; Bauer and Remiger, 1989). Further confirmation was made using the HPLC protocol of Bauer and Remiger (1989) to separate the root extracts. Retention times and UV spectra (200 to 300 nm) generated with the diode array detector and confirmed using a DU 650 spectrophotometer (Beckman Instruments Co., Fullerton, CA) were compared to those published by Bauer and Remiger (1989).

Mass spectrometry: Confirmation of the chemical structures of peaks 3 and 6 was also made using high resolution mass spectrometry, with a 5890 Series II gas chromatograph and 5971 MSD (Hewlett Packard Co., Palo Alto, CA) using electron impact ionization. Separation was conducted with a 30×0.25 mm i.d. × 0.25 micron film thickness DB-5 capillary column (J and W Scientific, Folsom, CA). The mass spectrometer was operated in scanning mode, from mass 40 to 650 Da, with splitless injection and oven temperature beginning at 80°C, holding for 3 min and increasing at 10 degrees/min up to 300°C. The injection port temperature was 230°C and the GC/MS interface temperature 280°C.

Nuclear magnetic resonance: For further confirmation of the chemical structure of isolated alkamides, samples were sent to Dr. Paul Molitor at the University of Illinois, Urbana. ^{1}H and ^{13}C NMR spectra of the isolated compounds were made using a Varian 500 MHZ NMR, with samples dissolved in D_3COD.

Statistical modeling and identification of biologically active components: Statistical analyses were conducted using programs provided by SAS. Analyses of variance of the effects of the *Echinacea* extracts on germination and root elongation were calculated using factorial designs with the GLM procedures. Multiple regressions comparing peak area to seed germination and root elongation were conducted with the PROC REG procedure.

Initial evaluation of the relationship between the bioassay results and the areas under the peaks of the analytical HPLC chromatograms utilized multiple linear regression protocols. However, the residual plot for peak 6 showed a clear quadratic pattern in the plot of the residuals, indicating a violation of linearity and that use of a quadratic expression was appropriate. All possible models for combinations of the peak areas vs. germination or root elongation were generated. The models having the highest correlation coefficient (R^2 value) were then used to determine what combinations of compounds were to be tested.

RESULTS AND DISCUSSION

Lettuce seed germination and root elongation: Results of the bioassays showed that exposure to the ethanolic root extracts of *Echinacea* from the five populations reduced mean germination of lettuce seeds by more than 30% and reduced root elongation by more than 70%. Genetic variation in allelopathic potential was demonstrated by the variability in lettuce seed response to extracts from the five populations even though all of the plants were grown in a common garden in Brookings, SD.

Reduction in germination in response to individual extracts varied more between *Echinacea* plants from within the same population than between populations (Table 1). In all 5 populations, the least active root extract caused no reduction in germination, while at least one extract generated a maximum reduction in lettuce seed germination of 100, 100, 84, 84 and 88% (ND, WY, SD, NE and KS, respectively). Although root extracts from all populations significantly reduced mean germination below that of controls ($p = 0.05$), there was no significant difference between the reduction of seed germination by the *Echinacea* root extracts between populations (Table 1). Extracts from plants of all five populations also significantly reduced root elongation by about 75%. As with germination, variation in the allelopathic potential of the *Echinacea* extracts from plants within a given population was so great that differences between populations were not detectable.

Table 1: Effects of *E. angustifolia* root extracts on lettuce seed germination and seedling root elongation

	Germination (%)				Root length (mm)			
Population	Max	Min	Mean	STD	Max	Min	Mean	STD
ND	100.0	0.0	63.9	31.8	13	1	4.5	3.4
WY	100.0	0.0	68.7	33.3	12	1	5.7	4.2
SD	100.0	16.0	68.2	28.5	36	1	6.2	7.4
NE	100.0	16.0	65.8	32.5	9	1	3.7	3.3
KS	100.0	12.0	59.8	33.8	13	1	4.3	4.1
Control	100.0	100.0	100.0	00.0	25	20	21.4	1.7

Root elongation

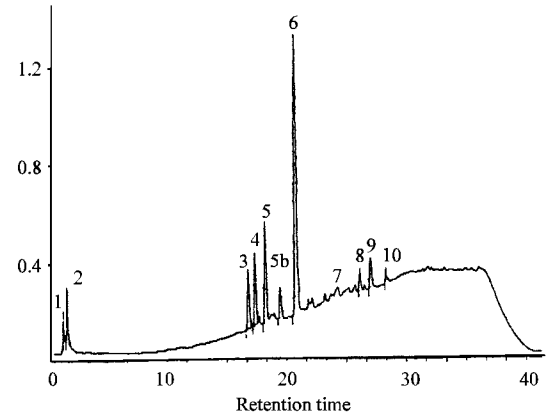

Fig. 1: Typical chromatogram of ethanolic extract of *E. angustifolia*. Twenty microliter samples were separated on a Beckman, C-18, ultrasphere ODS (5 μm), 4.6 mm × 15 cm and eluted using a 30-100% ethyl alcohol gradient

Determination of biological activity: HPLC separations of *Echinacea* extracts (Fig. 1) were made for all of the plants from the five populations that were used to examine genetic variability of allelopathic potential. The chromatograms revealed 11 peaks that were common to extracts from plants of all five populations, although all peaks were not necessarily found in all plants in any given population. Regression analyses of peak area of HPLC profiles of the ethanolic root extracts against the bioassay activity of the extracts showed that no one peak accounted for more than about 6% of the variation of the allelopathic potential (Table 2). When residuals for the regressed values were plotted for each individual peak, the plot for peak six showed that the relationship was not linear, with the residual plot being curvilinear. This curvilinear relationship required the introduction of a quadratic function. The quadratic function alone (peak 6^2) accounted for approximately 27% of the variation in the bioassay data and the three-variable regression model utilizing peaks three, six and six squared accounted for 73% of the variation ($R^2 = 0.7294$). This was the simplest model explaining the majority of the biological activity in the *Echinacea* extracts and, therefore, identification of these two peaks was pursued.

Table 2: Selected models from multiple regression analyses for comparison of HPLC chromatogram areas of *echinacea* root extracts with results of the lettuce seed bioassays

Model No.	Peak (s)	R^2
1	3	0.064
1	6	0.061
1	5	0.041
2	6&6^2	0.269
2	1&6	0.100
2	3&6	0.097
3	3&6&6^2	0.729
3	1&6&6^2	0.303
3	2&6&6^2	0.291
4	3&4&6&6^2	0.329
6	1&2&3&4&6&6^2	0.350
8	1&2&3&4&6&7&8&6^2	0.358
10	1&2&3&4&5b&6&7&8&9&6^2	0.989

Allelopathic potential of isolated compounds from root extracts of *Echinacea angustifolia*: To test the hypothesis that most of the inhibition of germination and root elongation occurred in response to the compounds associated with peaks 3 and 6 of the HPLC elutions, the lettuce seed bioassay was performed using fractionated extracts from a single *Echinacea* plant selected from the South Dakota population. The crude extract reduced germination by 85% and root elongation by 92%, as compared to controls. Purified preparations of peaks three and six were applied at rates equal to one-quarter, one-half and an amount equivalent to that of the crude extract, based upon peak areas of known volumes injected onto the HPLC. Figure 2A and B show the lettuce seed bioassay results conducted using HPLC-purified fractions from peaks 3 and 6 applied individually and in combination.

Peak 6 reduced germination by 12, 45 and 77%, at one-quarter, one-half and full strength, respectively, when applied independently. Peak 3 reduced germination by 16, 25 and 56%, at one-quarter, one-half and full strength, respectively. The combination of peaks 3 and 6 at one-quarter strength reduced germination by 79%, at one-half strength by 87% and at full strength by 90%. Peaks 3 and 6 combined were as effective as that of the crude extract, but neither compound individually reduced germination to the levels of the crude extract.

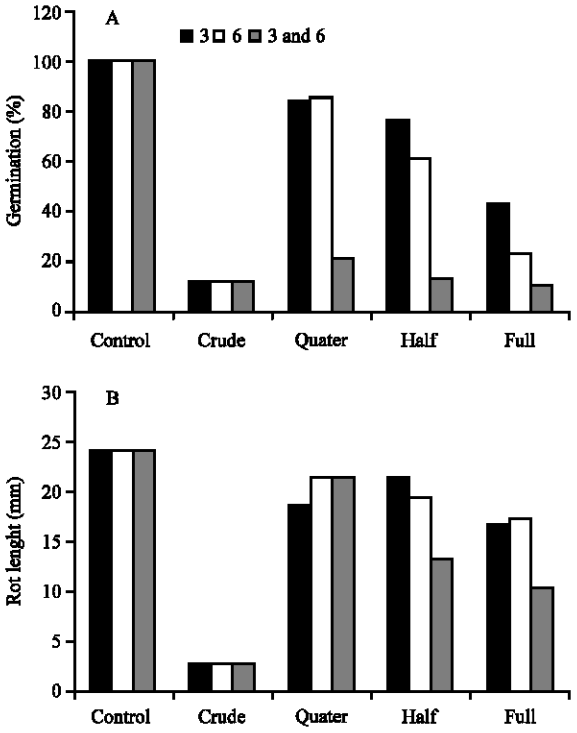

Fig. 2: Allelopathic potential, as determined by lettuce seed bioassay, of isolated compounds found in the ethanolic root extracts from a single South Dakota *Echinacea angustifolia* plant. (A) Percent germination for lettuce seeds treated with compounds isolated by HPLC and identified as peaks three and six, applied individually or combined at rates equivalent to that found in the crude extracts of fraction there of LDS = 1.8023. (B) Root length for lettuce seeds treated with compounds isolated by HPLC and identified as peaks 3 and 6, applied individually or combined at rates equivalent to that found in the crude extracts of fraction there of LSD = 13.5. Control = ethanol control, crude = crude *Echinacea* extract of the SD plant, 3 = undeca-2E-ene-8, 10-diynoic acid isobutylamide, 6 = dodeca-2E, 4E, 8Z, 10E-tetraenoic acid isobutylamide

The effects of the compounds from peaks 3 and 6 on root elongation (Fig. 2B) in the lettuce seed bioassay show a similar pattern. Peaks 3 and 6 individually at quarter and half strength reduced root length by about 21% compared to controls. Peak 3 at full strength reduced root length by 30% as compared to the controls. Peak 6 at full strength reduced root length to 28% as compared to the controls. The reduction in root length was not significantly different from the controls for lettuce seed treated with individual peak 3 or peak 6 isolates. The

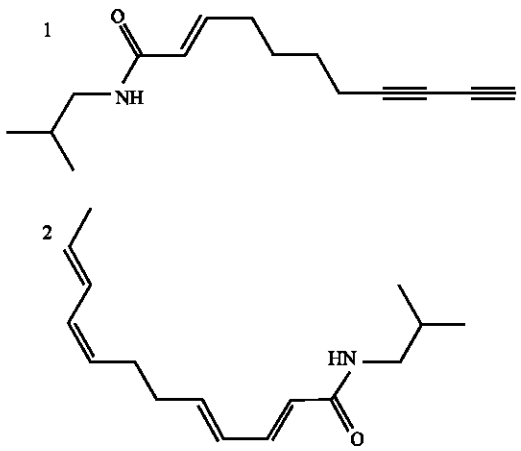

Fig. 3: (1) Structure of the compound isolated as peak three, undeca-2E-ene-8, 10-diynoic acid isobutylamide. (2) Structure of the compound isolated as peak six, dodeca-2E, 4E, 8Z, 10E-tetraenoic acid isobutylamide

combination of peaks three and six at full strength and the crude extract were the only root elongation treatments significantly different from controls.

Identification of peaks 3 and 6: Previously published characteristics of *Echinacea* extracts suggested that the allelopathic compounds in the *E. angustifolia* root extracts were alkamides (Bauer *et al*., 1988, 1989; Bauer and Remiger, 1989). UV spectra of peaks 3 and 6 showed that the compounds contain unsaturated, carbon-carbon double bonds. The spectra were almost identical to compounds identified by Bauer and Remiger (1989) as undeca-2E-ene-8, 10-diynoic acid isobutylamide (peak 3) and dodeca-2E, 4E, 8Z, 10E-tetraenoic acid isobutylamide (peak 6). Elution times were also consistent with those published for these compounds, when run under the same HPLC protcols (Bauer and Remiger, 1989)

Nuclear magnetic resonance and mass spectrometry were used to confirm the identification of peaks three and six. The data were consistent with the structures of compounds 1 and 2 (Fig. 3).

The lettuce seed bioassay was used to examine the allelopathic potential of root extracts of *Echinacea angustifolia*. Previous studies have shown that aqueous extracts and volatile components from the roots of *E. angustifolia* can inhibit germination and root elongation in grass and lettuce seed bioassays (Rice, 1983). The root extracts examined here significantly affected both lettuce seed germination and root elongation, with variation in potential between individuals from the same population appearing to be as large as that between individual plants from the various populations.

Identification of the compound or compounds that elicited the reductions in germination and growth was attempted by multiple regression of peak area with the results of the bioassays. This approach was selected to allow the identification of the active compounds even if the activity was the result of interactions of more than one constituent. Utilizing the natural variability of the plant extracts allowed a rapid and relatively simple screening of the constituents, without the need to isolate and purify the individual compounds. This protocol reduced the number of combinations of constituents that needed to be tested, which increases geometrically as the number of interacting constituents increases.

The simplest model generated suggested that the compounds associated with peaks labeled 3 and 6 (Fig. 3, molecules 1 and 2, respectively) from the HPLC separated *E. angustifolia* crude extracts were responsible for the majority of the variation in allopathic potential of the extracts. Therefore, we conducted bioassays using the isobutylamides associated with the elution of these peaks.

The first step was to demonstrate that 1 and 2, alone or in combination, affected root elongation and germination of the lettuce seed to the same extent as the crude extract. The assay showed that these compounds had an additive effect in root elongation. Individually they had little effect, but together the reduction in root elongation was equal to that of the sum of the effects produced by the two compounds. It is clear that 1 and 2 are not responsible for all of the reduction in root elongation attributed to the crude extract, as together they account for only about half of that reduction.

In contrast to root elongation, 1 and 2 appear to have a synergistic effect in the germination bioassay. Even at quarter strength, both reduced germination as almost as much as the crude extract. The difference in activity between the two assays highlights the complex nature of allelopathic interactions and indicates that the inhibition of germination and root elongation are differentially affected by the various components of the *Echinacea* root extracts.

The two identified isobutylamides, 1 and 2 may have potential value as natural herbicides. The mechanisms of toxicity, including the role of isomerization, metabolism of the compounds by microorganisms and exposure to soil, are yet to be examined (Coja *et al*., 2006; Macias *et al*., 2006). Determination of the commercial potential of the two isobutylamides will require further study. Analyses of their selectivity and stability as well as LD_{50} values are anticipated after synthesis of sufficient quantities have been completed.

CONCLUSIONS

The results of this study strongly support the hypothesis that genetically or environmentally induced variation in the secondary product composition of plants can be used to identify biologically active components by modeling of the response of their impact in a bioassay. Utilizing the natural variation within the species provided a relatively simple method for *a priori* identification of compounds that were biologically active in a complex mixture. The method is particularly useful when the biological activity is the result of synergistic interactions between two or more compounds. This approach to identification of biological activity in a complex extract has the potential to significantly reduce the need to identify all of the components in the mixture in an effort to discover those that can be used as a source of new commercial products.

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REFERENCES

Barrett, B., 2003. Medical properties of *Echinacea*: A critical review. Phytomedicine, 59: 708-717.

Bauer, R., A.I. Kahn and H. Wagner, 1988. TLC and HPLC analysis of *Echinacea pallida* and *E. angustifolia* roots. Planta Medica, 54: 426-430.

Bauer, R. and P. Remiger, 1989. TLC and HPLC analysis of alkamides in *Echinacea* drugs. Planta Medica, 55: 367-371.

Bauer, R., P. Remiger and H. Wagner, 1989. Alkamides from the roots of *Echinacea angustifolia*. Phytochemistry, 28: 505-508.

Binns, S.E., J.F. Livesey, J.T. Arnason and B.R. Baum, 2002. Phytochemical variation in *Echinacea* from roots and flowerheads of wild and cultivated populations. J. Agic. Food Chem., 50: 3673-3687.

Coja, T., J. Idinger and S. Blumel, 2006. Influence of the soil composition on the effects of benzoxazinoid allelochemicals on two soil nontarget organisms. J. Agic. Food Chem., 54: 1093-1098.

Duke, S.O., S.R. Baerson, F.E. Dayan, A.M. Rimando and B.E. Scheffler *et al*., 2003. United States Department of Agriculture-Agriculture research service research on natural products for pest management. Pest Manag. Sci., 59: 708-717.

Feghahati, S.M.J. and R.N. Reese, 1994. Ethylene, light and prechill enhanced germination of *Echinacea angustifolia* D.C. seeds. J. Am. Hortic. Sci., 119: 853-858.

Ghafar, A., B. Saleem and M.J. Qureshi, 2000. Allelopathic effects of sunflower (*Helianthus anuus* L.) on germination and seedling growth of wheat (*Triticum aestivus* L.). Pak. J. Biol. Sci., 3: 1301-1302.

Hobbs, C., 1995. *Echinacea*: The Immune Herb Handbook. 2nd Edn. Botanica Press, Santa Cruz (CA).

Hoque, A.T.M., R. Ahmed, M.B. Uddin and M.K. Hossain, 2003a. Allelopathic effect of different concentration of water extracts of *Acacia auriculiformis* leaf on some initial growth parameters of five common agricultural crops. Pak. J. Agron., 2: 92-100.

Hoque, A.T.M., R. Ahmed, M.B. Uddin and M.K. Hossain, 2003b. Allelopathic effects of different concentration of water extracts of *Eupatoriom odoratum* leaf on germination and growth behavior of six agricultural crops. OnLine J. Biol. Sci., 3: 741-750.

Kadioglu, I. and Y. Yanar, 2004. Allelopathic effects of plant extracts against seed germination of some weeds. Asian J. Plant Sci., 3: 472-475.

Kindscher, K., 1992. Medicinal wild plants of the prairie. Univ. Press of Kansas, Lawrence (KS) pp: 84-94.

Kite, G.C., M-J.R. Howes and M.S.J. Simmonds, 2004. Metabalomic analysis of saponins in crude extracts of *Quillaja saponaria* by liquid chromatograph/mass spectrometry for product authentication. Rapid Comm. Mass Spectr., 18: 2859-2870.

Macias, F.A., J.C.G. Galindo, J.M.G. Molinillo and H.G. Cutler, 2004. Allelopathy: Chemistry and mode of action of allelochemicals. CRC, New York.

Macias, F.A., D. Marin, A. Oliveros-Bastidas, D. Castellano, A.M. Simonet and J.M.G. Molinillo, 2006. Structure-Activity Relationship (SAR) studies of benzoxazinones, their degradation products and analogues. Phytotoxicity on problematic weeds *Avena fatua* L. and *Lolium rigidum* Gaud. J. Agic. Food Chem., 54: 1040-1048.

Miller, 2004. *Echinacea*: The genus *Echinacea*. Assistant H. Yu. (Ed.). CRC, Boca Raton (FL).

Mitscher, L.A. and A. Cooper, 2004. *Echinacea* and Immunostimulation. In: Herbal and Traditional Medicine: Packer, L., C.N. Ong and B. Halliwell (Eds.). Molecular Aspects of Health. CRC, New York, pp: 721-756.

Murch, S.J., H.P.V. Rupasinghe, D. Goodenowe and P.K. Saxena, 2004. A metabalomic analysis of medicinal diversity in Huang-qin (*Scutellaria baicalensis* Georgi) genotypes: Discovery of novel compounds. Plant Cell Rep., 23: 419-425.

Nasr, M. and M. Shariati, 2005. The use of allelochemicals to delay germination of *Astragalus cycluphyllus* seeds. J. Agron., 4: 147-150.

Rice, E., 1983. Allelopathy. Academic Press, Orlando.

Shaukat, S.S. and I.A. Siddiqui 2002. Allelopathic and antifungal potential of *Lantana camara* in root leachates in soil. Pak. J. Biol. Sci., 5: 51-53.

Uremis, I., M. Arslan and A. Uludag, 2005. Allelopathic effects of some *Brassica* species on germination and growth of cutleaf ground-cherry (*Physlis angulata* L.). J. Biol. Sci., 5: 661-665.

Viles, A. and R.N. Reese, 1996. Allelopathic potential of *Echinacea angustifolia*. Environ. Exper. Bot., 36: 39-43.

Wagner, H., 1999. Phytomedical research in Germany. Environ. Health Perspec., 107: 779-781.

Weckwerth, W., 2003. Metabalomics in systems biology. Ann. Rev. Plant Biol., 54: 669-689.