JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Human Gene Expression as a Tool To Determine Horticultural Maturity in a Bioactive Plant (*Echinacea purpurea* L. Moench)

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A phenological study was conducted to determine the impact of harvest maturity on the immune-modulating properties of *Echinacea purpurea*. The aerial parts of this plant were collected during seven stages of development and were assayed for a common botanical marker for this species, cichoric acid. Plants of selected development stages were also assayed for total polysaccharides and compared for their immune-modulating effects on the THP-1 monocyte/macrophage cell line by means of a gene expression study. Although the concentration of cichoric acid did not change significantly during the course of the study, stage 1 (advanced vegetative) had the highest concentration of total polysaccharides and exhibited the most potent induction activity on immune-modulating cytokines such as interferon-γ and tumor necrosis factor-α. These findings suggest that the use of gene expression may be an effective tool not only to standardize botanical extracts but also to optimize harvest time.

KEYWORDS: Harvest maturity; *Echinacea*; botanical marker; cichoric acid; polysaccharides; immune-modulating effects; gene expression

INTRODUCTION

By definition, "horticultural maturity is the stage of development when a plant or plant part possesses the prerequisites for utilization by the consumer *for a particular* purpose." It is typically at this stage when plants are harvested (*1*). Depending on the plant, this purpose ranges from fresh consumption, to processing, to medicinal use, and therefore maturity or harvesting indices should be set according to the final use of the commodity. For fresh-market commodities, maturity indices focus on sensory characteristics, such as texture, color, or aroma (*2*). For bioactive or medicinal plants, harvest maturity has centered around the level of specific phytochemicals (*3*−*5*), but it has failed to address medicinal or functional considerations.

The functional properties of *Echinacea* (also known as the American coneflower) have been broadly recognized given this plant's property to modulate the immune system. Three species within the genus *Echinacea* have been assigned medicinal value: *E. purpurea*, *E. angustifolia*, and *E. pallida* (*6*). Recent clinical work has substantiated the functional activity of this plant (*7*−*9*), and although no report has conclusively identified the active fraction, cichoric acid, alkylamides, and polysaccharides have all been ascribed activity (*10*).

Cichoric acid is one of the major constituents of *E. purpurea*'s aerial parts and is often used as a botanical marker (*11*). A few studies have reported changes in the concentration of cichoric acid as the plant develops. Wang et al. (*12*) observed that the maximum concentration of cichoric acid in the plant occurred at the blooming stage. According to Letchamo et al. (*13*), the concentration of cichoric acid in plants at the flower bud stage is more than double that in the developed flower stage. Stuart and Wills (*14*), on the other hand, reported steady levels in the concentration of this acid from preflower

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10.1021/jf0505372 CCC: $30.25
Published on Web 09/16/2005

Table 1. Stages of Development of *E. purpurea*

stage	description	characteristics
1	advanced vegetative	no flower buds are present
2	hidden bud formation	leaves surrounding the apical meristem are unfolded, confirming the presence of brachts and denoting the initial stages of the inflorescence
3	diminutive bud formation	flower buds are ~18 mm in diameter
4	bud enlargement	flower buds are ~21 mm in diameter
5	cone formation	composite of densely arranged florets is formed in the shape of a cone
6	erected green or white ligules	ligules are visible along the perimeter of the cone
7	full bloom of ligules with color	ligules completed elongation, are downward drooping and pink in color

to mature stage and a significant decrease when senescence sets in.

It is difficult to find a functional impact for all of these compositional changes when no active fraction has been elucidated to date. Rather than relying on compositional aspects as a function of plant development, a different approach consists of relating a plant's developmental stages to mechanistic observations in in vitro human cell systems. One such example is the use of the THP-1 bioassay to better understand *Echinacea*'s function. This bioassay is based on the human THP-1 cell line, which is derived from peripheral blood and is commonly used as a model for circulating blood monocytes and macrophages (*15*). *E. purpurea* activates immune cells such as macrophages and monocytes so they increase their secretions of proinflammatory substances, such as cytokines (*16*, *17*). An inflammatory response is necessary to initiate tissue swelling and leukocyte infiltration that beget antibody mobilization to fight the infecting organisms. Produced de novo in response to an immune stimulus, cytokines are small proteins that generally act at very low concentrations (*18*). Cytokines produced by activated monocytes and macrophages include tumor necrosis factor-α (TNF-α), IL-1, IL-6, and IL-8 (*19*). TNF-α is a pleiotropic cytokine that is key for immune response initiation, whereas IL-1 and IL-6 stimulate proliferation and differentiation of T cells and B cells, respectively (*20*−*22*). Among the cytokines with chemoattracting or chemokine functions are IL-8, with selectivity for neutrophil activation, and macrophage-inflammatory protein (MIP)-1α, which attracts leukocytes to infection sites (*23*, *24*). Another group of cytokines are the interferons (IFN) such as IFN-γ, which activates macrophages (*25*). Some of these functions are shared as most cytokines have redundant functions, except IL-10, which in an autocrine manner inhibits proinflammatory cytokine production in monocytes and macrophages (*26*, *27*). Measuring the expression of the genes coding for these cytokines is thus a promising mechanistic approach to evaluate the potency of different *Echinacea* preparations.

MATERIALS AND METHODS

Plant Material. *E. purpurea* was harvested from a 6-year old field during the end of June to the beginning of July 2003, in the temperate climate of Trout Lake, WA, situated at an elevation of 1750 ft above sea level. Plants were pruned at ~5 cm above the ground at seven stages of development. Four composite replications, consisting of four entire plants each, were collected for each stage of development. Each stage is described in **Table 1**.

Soluble Solids Determination. The fresh aerial parts of the plant were used for determining soluble solids by blending an average of 50 g in 300 mL of distilled water. After removal of particles by filtering through cheesecloth, a drop of solution was tested by means of a hand-held refractometer (Kernco Instruments Co.mpany Inc., El Paso, TX), and results were reported as percentage of soluble solids.

Drying and Preparation of Samples. Each sample was manually chopped into pieces of ~1−2 in. and dried at 54 °C until constant weight. All samples were then ground using a coffee grinder and frozen. A fraction of sample was tested for endotoxin contamination using the LAL/endotoxin reaction assay (Associates of Cape Cod, East Falmouth, MA).

Cichoric Acid Assay by HPLC with Diode Array Detection. For this internally developed and validated method, a methanolic extract was obtained by suspending 0.5 g of dried plant material in 20 mL of 80% MeOH at 60 °C for 1 h. During the hour, each suspension was shaken manually for 10 s every 10 min. Samples were then removed from the water bath and placed on a shaker for 1 h before filtering and diluting as necessary for analysis. Each replication was analyzed by HPLC using a Waters Alliance liquid chromatograph (Milford, MA) with a photodiode array detector set at 330 nm. Separation was achieved in a 250 mm × 4.6 mm (i.d.), 5 μm Symmetry C18 column (WATO54215, Waters). Samples and standard were injected (10 μL) and subjected to a gradient elution program consisting of 0.2% phosphoric acid in water (solution A), 100% methanol (solution B), and 100% acetonitrile (solution C). The elution program condition started with 70% A/20% B/10% C with a linear gradient leading to 54% A/36% B/10% C for 25 min, a linear gradient to 25% A/40% B/35% C for 5 min, and a linear gradient to 70% A/20% B/10% C, for 10 min, followed by a 10 min equilibration prior to the next injection. The flow rate was 0.6 mL/min, and the column temperature was 25 °C. Cichoric acid was expressed as milligrams per 100 mg of dry plant material.

Crude Polysaccharide Gravimetric Assay. A crude extract was prepared by adding 40 mL of water to 5 g of plant material and incubating this suspension at 90 °C for 1 h in a water bath. Each sample was cooled to room temperature and then centrifuged at 4000 rpm for 15 min. The supernatant was filtered and the volume adjusted to 20 mL with water. Polysaccharide precipitation was induced by the addition of 30 mL of 99% ethanol followed by vortexing and refrigeration for 15 min. The sample was then centrifuged for 15 min at 4000 rpm, and the supernatant was discarded. The pellet was resuspended in 8 mL of water. After the addition of 32 mL of 99% ethanol, the sample was vortexed and refrigerated for 15 min. The supernatant was discarded, and the pellet was dried at 65 °C (~15 h). The dry mass was then recorded as the crude polysaccharide fraction and expressed in percentage. This gravimetric assay was based on reported properties of water soluble polysaccharides (*28*).

Polysaccharide Derivatization. The dried pellet was resuspended in 2 mL of 6 N HCl and heated in a water bath at 90 °C for 1 h to hydrolyze the polysaccharides into their constituent monosaccharides. The solution was then cooled to room temperature and the pH adjusted within the range of 5.6−6.1 with concentrated ammonium hydroxide. The solution was then centrifuged at 4000 rpm for 10 min, the supernatant was collected and the volume adjusted to 3 mL with water. The production of alditol acetates from the released monosaccharides was achieved following the method of Blakeney et al. (*29*).

Gas chromatographic analysis was accomplished using an HP5890 GC equipped with a 6890 autoinjector, an FID detector, and a DB-225 15 m × 0.32 mm column with a 6 m × 0.53 mm guard. The injection port temperature was set at 230 °C and the detector at 240 °C. Helium was used as the carrier with the head pressure set at 30 psi and the column oven at 150 °C. A 2 μL injection was made with the column at 150 °C. This temperature was held for 2 min and then increased at 5 °C/min to reach 220 °C (14 min). The oven was held at 220 °C for 2 min before returning to 150 °C to re-equilibrate for the next injection.

Table 2. Compositional Changes of *E. purpurea* As Affected by the Plant's Stage of Development[a]

stage of development	soluble solids (%)	cichoric acid (mg/100 mg of dry sample)	total crude polysaccharides (%, w/w)
1	2.0 ± 0.18a	3.58 ± 0.18a	4.20 ± 0.62a
2	1.9 ± 0.07a	3.44 ± 0.31a	
3	1.7 ± 0.07a	3.14 ± 0.20a	2.49 ± 0.18b
4	1.8 ± 0.03a	3.51 ± 0.22a	
5	2.2 ± 0.17a	3.44 ± 0.25a	2.30 ± 0.36b
6	2.0 ± 0.05a	3.39 ± 0.29a	
7	2.2 ± 0.05a	2.69 ± 0.20a	1.91 ± 0.39b

[a] All data represent the mean ± SEM. Means within the column followed by the same letter are not significantly different at the $P < 0.05$ level by LSD multiple-range test.

The alditol acetates of rhamnose, arabinose, xylose, mannose, galactose, and glucose eluted at 4.5, 5.6, 6.7, 9.1, 9.6, and 10.0 min, respectively, with the internal standard, inositol, eluting at 10.5 min. Quantification of the peaks was accomplished by comparison to a calibration curve of alditol acetates of commercial standards of the individual monosaccharides.

Gene Induction Assay. The TPH-1 cell line (ATCC, Manassas, VA) was maintained in RPMI-1640 medium containing 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate along with supplementation with 0.05 mM 2-mercaptoethanol and 10% fetal bovine serum. Twenty-four hours prior to experimentation, cells (10^6 cells/mL) were seeded in 12-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) and incubated in a 5% CO_2 atmosphere at 37 °C. Each experiment consisted of a 6 h incubation of THP-1 cells with *E. purpurea* extracts at a dosage concentration of 100 μg/mL in growth media, where extracts were prepared as 50 mg/mL stock solutions by adding the solvent 50% dimethyl sulfoxide (DMSO)/30% ethanol/20% water to each dried plant material followed by vortexing and then sonicating in a room temperature water bath for 10 min. Lipopolysaccharide (LPS) at a dosage concentration of 500 ng/mL was used as a positive control. The cells were then harvested by centrifugation, and their RNA was extracted using conventional trizol/guanidine isothiocyanate-based lysis. The isolated RNA was digested with RNase-free DNase I to remove any DNA contamination and then reverse transcribed to cDNA using random hexamer as well as oligo(dT) primers according to the manufacturer's instructions (Stratagene, La Jolla, CA). Quantitative real-time PCR was performed using validated Taqman FAM-labeled specific primers for IL-1 β (PN 4327035F), IL-1 α (PN 4327032F), IL-6 (PN 4327040F), IL-8 (PN 4327042F), IL-10 (PN 4327043F), IFN-γ (PN 4327052F), TNF-α (PN 4327055F), MIP-1 α (PN 4324585F), and GAPDH (PN 4333764F) obtained from Applied Biosystems (Foster City, CA). All reactions were carried out in triplicate, and the relative amount of mRNAs in treated versus untreated samples was calculated using the comparative C_T method established by Applied Biosystems (*30*). Replicate values in all cases were within a 3% range of the mean.

Data Analysis. The statistical analysis of data was performed using the StatGraphics software package, version 5.1. The treatment means were compared using the LSD multiple-range test, and the differences were considered to be significant if the associated P value was <0.05.

RESULTS

***Echinacea* Plant Composition at Different Developmental Stages.** The concentrations of cichoric acid and soluble solids over the course of the study are shown in **Table 2**. Results indicate that neither the levels of soluble solids nor the levels of cichoric acid were significantly different among the stages of development studied. Total crude polysaccharides, on the other hand, were significantly higher in stage 1 than in stages 3, 5, and 7 (**Table 2**).

The authenticity of the isolated fractions to contain polysaccharides based on gravimetry was qualitatively confirmed by analysis of the constituent monosaccharides that were liberated after acid hydrolysis of the polysaccharide fractions (*31*). The constituent monosaccharides liberated as a result of acid hydrolysis mainly consisted of arabinose, galactose, glucose, mannose, and xylose, with the first two being the major constituents in all of the samples.

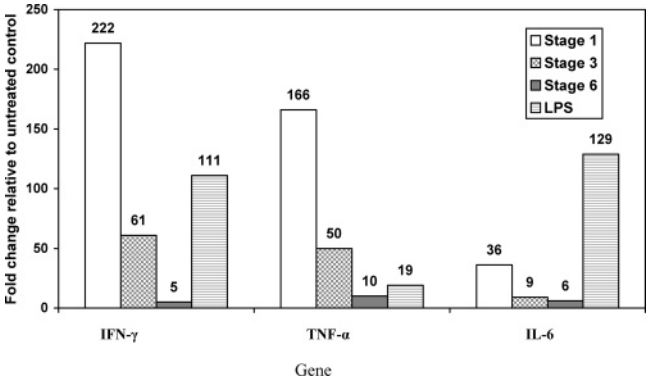

Figure 1. Genes showing a strong response following THP-1 cell treatment with *Echinacea* extracts from three harvest stages. THP-1 cells were treated with 100 mg/mL select *Echinacea* extracts, and a standard LPS solution was used as positive control. The levels of mRNA for IL-6, TNF-α, and IFN-γ were measured and normalized to GAPDH. The average gene expression response is presented relative to the untreated control. Replicate variation in gene expression in all cases was <3%.

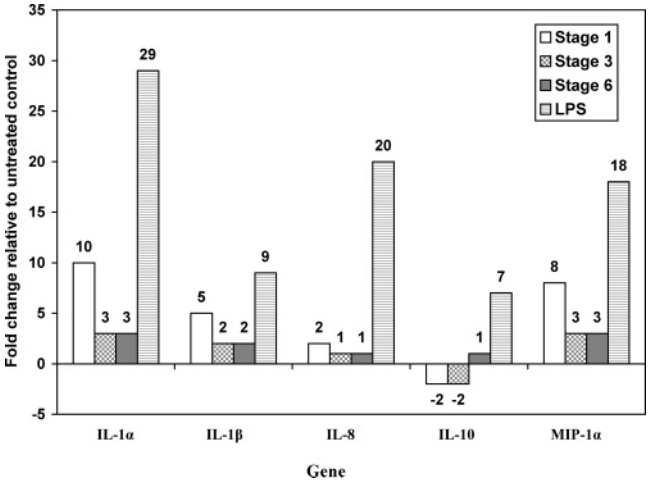

Figure 2. Genes showing a moderate or small response following THP-1 cell treatment with *Echinacea* extracts from three harvest stages. THP-1 cells were treated with 100 μg/mL select *Echinacea* extracts, and a standard LPS solution was used as positive control. The levels of mRNA for IL-1α, IL-1β, IL-8, IL-10, and MIP-1α were measured and normalized to GAPDH. The average gene expression response is presented relative to the untreated control. Replicate variation in gene expression in all cases was <3%.

Gene Expression Analysis. Expression levels of TNF-α, IFN-γ, IL-1α, IL-1β, IL-6, IL-8, IL-10, and MIP-1α were measured at the transcription level and normalized to the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). After a 6-h treatment, the most pronounced induction of mRNA expression was noted with plant material from stage 1. IFN-γ, TNF-α, and IL-6 were interestingly the genes that exhibited the largest fold induction (**Figure 1**). Conversely, IL-1α, IL-1β, IL-8, and MIP-1α were modestly induced, and IL-10 was down-regulated, consistent with the presence of a signal to maximize cytokine production (**Figure 2**).

Given that LPS is known to activate monocytes and macrophages with an ensuing increase in the gene expression of

multiple cytokines, we used this archetypal bacterial product as a positive control in the THP-1 bioassay. IL-1α, IL-6, IL-8, IL-10, and MIP-1α were major responders to LPS treatment as they showed at least a 2-fold larger expression than that caused by the most effective *Echinacea* extract (**Figures 1** and **2**). Interestingly, TNF-α induction by LPS amounted to only a small fraction of the response to *E. purpurea* stage 1 extract (**Figure 1**). It is also noteworthy that LPS was not as effective as vegetative *Echinacea* extract to induce IFN-γ gene expression.

Except for IL-10 gene regulation, it was apparent that the vegetative *Echinacea* plant extract and LPS treatments caused similar responses in human monocytes/macrophages. Therefore, it was essential to check *Echinacea* preparations for endotoxin (or LPS) contamination. Gene expression responses were concluded not to be due to endotoxin contamination as *Echinacea* preparations tested negative in the Limulus assay.

The results of this study reveal that *E. purpurea* in advanced vegetative stage has bioactive compounds with strong effects on the production of immunostimulatory cytokines. Whereas conventionally *Echinacea* is harvested during bloom, it appears that anticipating the harvest does not impair the capacity to regulate immunostimulatory genes.

DISCUSSION

Similarly to Stuart and Wills (*14*), no significant changes in the concentration of cichoric acid were observed from stages 1 through 7. Our study was discontinued before the flowers reached senescence; thus, no comparisons can be made with these author's findings during flower senescence. It is interesting to note the nonsignificant drop that occurred between stages 6 and 7. Blooming within the *Echinacea* plant is irregular, and it is possible that the variability of blooming stages within this plant precludes the declaration of this drop in concentration as significant.

It has been reported that of the three classical phytochemical moieties in *Echinacea*, the flowers and root have the highest concentrations of polyphenols and alkylamides, whereas the leaves and stems are rich in polysaccharides (*32*). This phytochemical distribution in the plant can explain the higher concentration of crude polysaccharides in the advanced vegetative plant. To date, there are reports of several known polysaccharides from aerial parts of *E. purpurea*. These polysaccharides are heteroxylan (molecular mass = 35 000 Da), an acidic arabinogalactan (molecular mass = 450 000 Da), and 4-*O*-methylglucuronoarabinoxylan (unknown molecular mass) (*33*). As in our study, the major sugars constituting these polysaccharides are arabinose, galactose, and xylose (*34*). Research interest has also focused on the arabinogalactan protein (AGP) portion of the cell wall polysaccharides as highly biologically active. Recently, the AGP was characterized in cell wall polysaccharides derived from a pressed juice product (*35*).

The findings presented in this study on the capacity of *Echinacea* to modulate the transcription of cytokines are consistent with previous results (*36*, *37*) as well as with studies on cytokine protein levels reported by Burger et al. (*38*, *39*). We have extended the research by comparing mRNA levels in THP-1 cells stimulated with extracts from *Echinacea* at different developmental stages, and the data demonstrate that they all induced proinflammatory cytokine gene expression, particularly IFN-γ and TNF-α. Interestingly, extracts from nonflowering plants elicited the strongest induction of such transcription. Extracts from less developed *Echinacea* plants also suppressed IL-10 mRNA expression, which is significant because it is associated with increased and prolonged IFN-γ-potentiated expression of TNF-α (*40*). These observations are particularly interesting in light of the work by Gertsch et al. (*36*) showing the action of *Echinacea* to be the reduction of adverse effects from excessive TNF-α produced during times of stress and bacterial or viral invasion. Furthermore, the production of cytokines such as IFN-γ and TNF-α has been related to the activation of natural killer (NK) cells that are pivotal to the cleaning of viral infections (*41*). The observations of our study are thus in agreement with the notion that *E. purpurea* serves as an immunomodulator for innate immune responses by stimulating macrophages and NK cells (*17*, *42*, *43*).

When the immunomodulatory effects of different preparations of *Echinacea* were studied in vivo by Goel et al. (*44*), the effects were of dose specificity to the concentrations of cichoric acid, polysaccharides, and alkylamides. These authors found an enhanced release of cytokines (such as TNF-α and IFN-γ) in response to *Echinacea* components, supporting its prophylactic and therapeutic effects against the common cold. Although these authors found cichoric acid as a key driver for *Echinacea*'s activity, we found no apparent association between cichoric acid and induction of the gene panel under investigation.

Arabinogalactan was found to activate macrophages and was associated with an increased production of TNF-α, IL-β, and INF-β_2 or IFN-γ. This polysaccharide interacts predominantly with cells of the macrophage lineage, stimulating them to various functions both in vitro and in vivo (*43*). Arabinogalactan induced TNF-α in a dose-dependent manner and was highly effective in inducing the production of IFN-β_2 to a level comparable to that induced by LPS stimulation. However, most of the studies on *Echinacea* polysaccharides have been on those derived from tissue cultures of *E. purpurea*, where the structure of polysaccharides differs from that of the aerial parts of the naturally grown plant.

Although the exact mode of action of *Echinacea* is yet to be clearly understood, it is likely that its stimulatory activities are comparable to those of immunological adjuvants widely used to stimulate animal immune systems. Immunological adjuvant substances normally consist of complex foreign lipids, carbohydrates, and/or proteins that are able to stimulate the immune system to improve antibody production or vaccine response (*45*). This may offer an explanation by which *Echinacea* decreases the severity and reduces the duration of colds and upper respiratory infections, especially when taken early in the course of an infection (*46*). Furthermore, because isolated cichoric acid, polysaccharides, and glycoproteins have shown immunostimulatory activities (*43*, *47*, *48*), the functional assessment of the plant matrix or an extract thereof, rather than the quantification of any one of this plant's constituents, may provide a more accurate evaluation of performance.

This study has opened the possibility of expanding the harvest window beyond traditional recommendations. Although crude polysaccharides, rather than cichoric acid, seemed to be more closely related to the induction of immune modulation, no firm conclusions can be drawn on isolated chemistries. Various phytochemicals have been ascribed activity in *Echinacea*, and in the evaluation of a whole plant extract, it is critical to consider balances and interactions, especially when there are diverse compositional changes during plant ontogeny. In this context, it is apparent that linking bioactivity, as given by human gene expression, to stages of maturity can render valuable information to define an appropriate harvest window. This approach can guide harvesting decisions to optimize the consistency and efficacy of a botanical extract.

ACKNOWLEDGMENT

We thank Luis Cisneros-Zevallos, Andrew Burt, John Thor Arnason, and Sylina M. Espinosa for their valuable contributions during the execution of the experiments and the preparation of the manuscript.

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Received for review March 9, 2005. Revised manuscript received August 3, 2005. Accepted August 5, 2005.

JF0505372