ISOTOPIC LABELING OF ISOFLAVONES FROM PLANT CELL AND ORGAN CULTURE FOR METABOLIC TRACKING IN ANIMAL MODELS

BY

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THESIS

Submitted in partial fulfillment of the requirements for the degree of Master of Science in Nutritional Sciences in the Graduate College of the University of Illinois at Urbana-Champaign, 2010

Urbana, Illinois

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ABSTRACT

Kudzu and red clover isoflavones are phytoestrogens that have been investigated as alternatives to estrogen replacement for treatment of osteoporosis, cardiovascular disease, and neurological disorders. However, the pharmacokinetics of these compounds are not clearly understood. In the present studies, we utilized $^{14}$C-radiolabeled crude isoflavone extracts to track the absorption, distribution, and elimination of kudzu and red clover isoflavones in rats. A dose of approx. 0.148 MBq (4µCi) of radiolabeled extract was found to be sufficient to be metabolically tracked in rats using a scintillation counter. Analysis showed radiolabel accumulated in all major tissues, including bones, and that kudzu isoflavone extracts underwent enterohepatic circulation. We then produced a $^{14}$C-radiolabeled kudzu isoflavone-enriched fraction from a high isoflavone yielding kudzu hairy root genotype in vitro, which was used to track the absorption, distribution, and elimination of kudzu isoflavones in rats. Pharmacokinetics showed that kudzu isoflavones were rapidly absorbed, distributed into all tissues including bones, underwent enterohepatic circulation, and was largely eliminated in the urine and feces. Femur bone extracts were analyzed by HPLC-ESI-MS and found to contain puerarin, daidzein, and puerarin glucuronide, which suggests that kudzu isoflavones may be able to have a role in promoting bone health. To improve kudzu isoflavone yields in vitro for further studies, kudzu hairy root cultures were elicited with acetate and salicylic acid in varied concentrations and treatment durations. However, acetate and salicylic acid failed to significantly improve isoflavone synthesis in kudzu hairy roots.
To my family and friends
ACKNOWLEDGEMENTS

While it is highly likely that most of the individuals that I name in this section will never read these acknowledgements, or any part of my thesis for that matter, it does not make them any less worthy of mention for their tremendous contributions to my growth as a scientist and as an individual.

Without a doubt, the most significant person in my graduate career has been my advisor, Dr. Mary Ann Lila. Her focused impact on my scientific and technical writing style has been the single most critical development in my training as a scientist. Reading and reviewing every word of my thesis from beginning to end is undeniable proof of her tremendous patience and support, for which I am most grateful. I am especially thankful to Dr. Lila for not only providing me with the financial means to study at this university, but also for offering me the opportunity to learn from the many intelligent individuals on this campus and others including, Purdue University, the University of Alabama at Birmingham, and Rutgers University. Dr. Lila’s mentorship has been truly invaluable and I couldn’t thank her enough for her dedication.

I would also like to thank my thesis committee members, who generously offered their time and ideas to enhance my scientific knowledge and the quality of my research. Dr. Elizabeth Jeffery, who served as my committee chair, offered remarkable insights into the pharmacological aspects of my research and challenged me with many great, thought-provoking questions. Dr. Elvira de Mejia played an equally critical role as part of my committee in directing my attention to experimental design issues and providing guidance to possible solutions. Together, their contributions have been meaningful and genuinely appreciated.
It takes a village to raise a grad student and accordingly I owe a debt of gratitude to my fellow lab members, who have been with me every step of this journey and provided me with a wealth of knowledge, inspiration, and encouragement. I am grateful to Randy Rogers, who has worked hard to make the lab a comfortable, well-organized workplace and has always been a reliable source of helpful technical and horticultural advice. I would also like to thank Gad Yousef for his guidance with using the HPLC and the radiolabeling chamber, Lynn Wang for her help with troubleshooting HPLC problems, and Mary Grace for teaching me about phytochemical separation techniques using solvent extraction, ion-exchange chromatography, and gel filtration chromatography. I owe many thanks to my fellow graduate students in the Lila Lab: Tristan Kraft (for convincing me that Illinois would be a great place to attend graduate school and for being an awesome role model), Adam Reppert (for showing me the intricacies of initiating kudzu tissue cultures and radiolabeling), Josh Kellogg (for his chemistry and HPLC expertise, enthusiasm for science, and for making the lab a fun workplace), Diana Cheng (for always patiently answering my seemingly endless list of questions), and Nancy Engelmann (for emphasizing the importance of professional development).

I am especially indebted to my mentors at Purdue University and Rutgers University. Dr. Connie Weaver, Dr. Elsa Janle, Pam Lachcik, and Mike Grannan have taught me much of what I know about research using animal models, surgical techniques, and metabolic tracking using radioisotopes. Dr. Jim Simon and Dr. Qing Li Wu provided a great deal of assistance with the identification of kudzu isoflavones. Dr. Simon was also responsible for first exciting my interest in biologically active plants and their role in human health during my undergraduate career, which led to my fascination with human nutrition. Dr. Tom Gianfagna and Logan Logendra also
played a strongly supportive role in nurturing my growth as a scientist for four years as an undergraduate and are two individuals that I continue to look up to.

Among the many exceptional members of this university community, I wish to extend special thanks to Dr. Art Spomer for providing feedback on my practice defense and to Linda Barenthin, Linda Harvey, and Dr. Jessica Hartke for providing the departmental assistance that no graduate student could do without. I am especially grateful to Meredith Barnes for always being around just in time to lend a hand and to her brother, Phil Barnes, who let me crash on his couch for a whole summer so that I could do research at Purdue. I am also indebted to two loyal and trustworthy friends, Naiman Khan and Chris Moulton, for their endless encouragement and inspiration.

Lastly, I want to acknowledge my family for their love, sacrifice, and incredible support; I can neither thank them enough, nor can I find the space to list all that they have contributed to shape the person that I have become.
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Estrogens are hormone molecules that are involved in the development and maintenance of female physical traits, sexual reproduction\(^{(1)}\), plasma lipid levels, and bone mineral density\(^{(2)}\). Though present in both men and women, estrogens are found at significantly higher quantities in women. Estrogens are produced and secreted by the ovaries, adrenal glands, and other organs. These hormones circulate throughout the body via the bloodstream and target cells in specific areas of the body, which contain estrogen receptors. These sites include the breast, uterus, brain, bone, liver and heart\(^{(3)}\). Estrogen influences cell activity when it binds to estrogen receptors to form a complex. This estrogen-receptor complex can then bind to specific DNA regions called estrogen response elements, to activate genes that initiate mRNA transcription and protein synthesis\(^{(3)}\).

**Influence of phytoestrogens in human health maintenance**

Phytoestrogen is a term used to classify a number of plant-derived compounds that possess estrogenic activity\(^{(4, 5)}\). Among these compounds are isoflavones. Isoflavones are estrogen-like compounds that bind to estrogen receptors and are produced primarily by leguminous species\(^{(6)}\), which include soy, kudzu, and red clover. In recent years, soy isoflavones have been of much clinical interest, particularly for menopausal and post-menopausal women. Hormone replacement therapy is an effective treatment for menopausal symptoms, but concerns about risk for heart attack, stroke, and breast cancer have excited interest into alternative therapies using isoflavones\(^{(3)}\). It has been reported that Asian women
exhibit fewer menopausal symptoms than U.S. and European women and from this finding, it has been suggested that this may be attributed to the inclusion of soy foods in Asian diets (6). Other epidemiological studies showed that people who consume diets rich in soy foods have fewer breast, uterine, and prostate cancers, less coronary heart disease and osteoporosis, and fewer menopausal symptoms (7). The weak estrogenic effects of isoflavones are believed to play a role in alleviating vasomotor symptoms associated with menopause, improving plasma lipid levels, and slowing bone loss.

However, a number of recent studies have shown conflicting data on isoflavones’ effects on the human body. A recent meta-analysis by the American Heart Association has stated that soy isoflavones had no effect on HDL cholesterol, triglycerides, lipoprotein(a), or blood pressure, did not reduce vasomotor symptoms of menopause, decreased LDL cholesterol by only about 3% and showed mixed data on soy’s ability to slow post menopausal bone loss (8). In two double-blind placebo-controlled trials, red clover extracts were found to have no effect on hot flashes or other menopausal symptoms (9). Although the research evidence is conflicted, the roles of isoflavones in human health require additional investigation. For instance, the use of a whole-food as opposed to an extract may yield vastly different data. Other factors such as the source and composition of isoflavones, age of the patient at the onset of supplementation, composition of an individual’s intestinal flora (10), placebo effect (11), and starting plasma lipid levels (12) may all play a role in the effects of isoflavones on human health.

Kudzu (Pueraria sp.), one of the aforementioned leguminous plants, is becoming increasingly attractive as a rich source of isoflavones (5). Believed to have originated in eastern Asia, kudzu has long been a part of the culture and pharmacopeia of that region (13). Kudzu root has historically been used in traditional Chinese medicines since as early as 200 B.C. as an
antipyretic, antidiarrhetic, diaphoretic, and anti-emetic agent and later came into use as an amethystic (anti-alcohol intoxication) agent and an antidipsotropic (anti-alcohol abuse) agent around 600 A.D. and 1200 A.D. respectively \(^{(13)}\).

While kudzu has been used medicinally for many years, its mechanisms and bioactive constituents are only recently being studied. Kudzu roots accumulate significantly higher concentrations of isoflavones than the leaves and stems and have consequently been the target of much kudzu research \(^{(5)}\). The most common isoflavones of kudzu roots have been identified to be puerarin, daidzin, daidzein, formononetin, and daidzein diglucosides \(^{(14)}\). Puerarin (daidzein-8-C-glucoside), the most abundant bioactive isoflavone of kudzu root \(^{(15)}\), accounts for up to 80% of the total kudzu root phytoestrogens \(^{(16)}\) and has been of much interest due to its chemical structure and biological activities. The C-glycosidic bonds present in certain isoflavones such as puerarin are resistant to hydrolysis by glucosidases, resulting in rapid absorption in the intestines without metabolism and the capacity to cross the blood brain barrier \(^{(17)}\). While the O-glucosides of kudzu isoflavones such as daidzin (daidzein 7-O-glucoside) are also absorbed into the intestines, they are more susceptible to hydrolysis and their bioavailability is lower \(^{(14, 17)}\). It is believed that the enzyme lactase phlorizin hydrolase (LPH) hydrolyzes these O-glucosidic bonds and the released aglycone is either absorbed by the intestinal enterocytes or metabolized by gut microflora to products, which include equol, dihydrodaiadzein, and O-desmethylangolensin \(^{(17)}\). Equol is the most abundant circulating isoflavone in rats and mice and was also the first isoflavone to be identified in human blood and urine, which led to the discovery that soy is a rich source of isoflavones \(^{(18)}\).

Recent research into the potential health benefits of kudzu root has confirmed the antidipsotropic properties of kudzu on rat and hamster models and its effects are attributed to
daidzin and daidzein (13). Investigation into the effects of kudzu on diabetes has demonstrated a dose-dependent antihyperglycemic effect of puerarin on streptozotocin-induced diabetic rats (19). An observed increase in mRNA and protein levels of glucose transporter, GLUT 4, in soleus muscle after repeated intravenous puerarin treatments suggest that puerarin can improve glucose utilization by muscles and thereby reduce plasma glucose concentrations in diabetic rats (19).

Puerarin has also been associated with preventing osteoporosis. Recent work has shown that kudzu-derived puerarin encourages bone formation in rat osteoblasts (20) and decreases bone resorption in rat osteoclasts (21) in vitro. Another study demonstrated that surgically-created rabbit parietal bone defects grafted with puerarin and collagen mixture formed 554% more new bone than defects that were grafted collagen matrix alone (22).

While the mechanisms by which puerarin promote bone health have not been completely elucidated, current evidence suggests that puerarin may increase bone formation through activation of the PI3K/Akt pathway. Akt is a serine/threonine protein kinase that regulates the intracellular signaling of many growth factors, which serve biological functions including gene expression, survival, and cell proliferation (20). Akt does not refer to its function, but is named after a transforming leukemia virus in the AKR-strain of mice, which has a high frequency of spontaneous lymphoma (23, 24). One study showed that treatment of cultured osteoblasts with puerarin significantly increased cell viability, alkaline phosphatase (ALP) activity, and mineral nodules formation in osteoblasts, which indicated that puerarin stimulated calcium deposition, osteoblast differentiation, and bone formation (20, 25, 26). Puerarin also stimulated Akt activation dose-dependently and this effect was attenuated by inhibition of the upstream enzyme phosphatidylinositol 3-kinase (PI3K), by the PI3K-specific inhibitor, LY294002 (20).
Another rich source of natural isoflavones is red clover (*Trifolium pratense* L.) (27). Over 50 years ago, it was noted that subterranean clover (*Trifolium subterraneum*) was associated with an infertility disorder in sheep known as clover disease (28-31). Since then, isoflavones have been identified to be the compounds that were producing the estrogenic effects associated with clover disease (28) and that red clover yields the highest isoflavone content among the *Trifolium* species studied by Wu et al. (32). While intake of isoflavones from clover has been linked with infertility in sheep, cattle (28), cheetahs, and some birds, the consumption of foods containing isoflavones has not been associated with infertility in most other species, including man (29). This disparity may be attributed to the varied effects of isoflavones on the balance between estrogenic and anti-estrogenic activity, which is determined by the proportion of phytoestrogen to endogenous estrogen in different species (28).

The major isoflavones of red clover have been identified to be formononetin and biochanin A, with smaller concentrations of daidzein and genistein (32). In humans and animals, formononetin and biochanin A are metabolized to form daidzein and genistein respectively by gut microflora (32, 33). Genistein and biochanin A may then be further metabolized and broken down to non-estrogenic p-ethyl phenol and organic acids, while formononetin and daidzein are demethylated and reduced to the more estrogenic metabolite, equol. The isoflavone composition of red clover differs from kudzu, but the weakly estrogenic properties of red clover have been associated with some similar potential health benefits including prevention against breast and prostate cancer, cardiovascular disease (27), protection from oxidative damage by UV radiation exposure (34), atherosclerosis, osteoporosis, and postmenopausal symptoms (35). Although red clover has been traditionally used by Native Americans to treat whooping cough, gout, and
cancer\textsuperscript{\textordmasculine9}, and by other groups for the treatment of asthma, bronchitis, coughs, athlete’s foot, eczema, and psoriasis\textsuperscript{\textordmasculine32}, little clinical evidence substantiates these claims.

In U.S. and European markets, red clover-derived isoflavone supplements are available for women suffering from menopausal symptoms\textsuperscript{\textordmasculine32}. However, studies present conflicting data on the effectiveness of red clover extracts against hot flashes and other menopausal symptoms\textsuperscript{\textordmasculine9, 36}. Similarly, data are inconsistent with regard to the effects of red clover on plasma lipid levels\textsuperscript{\textordmasculine37, 38}. Treatment of osteoporosis with red clover isoflavones does however appear to be effective, showing increases in bone mineral density of the proximal radius and ulna by 4.1\% over 6 months in postmenopausal women with 57 mg/day of isoflavones\textsuperscript{\textordmasculine37}. In ovariectomized rat models, treatment with red clover isoflavones increased bone mineral content, tibia strength, femoral weight and femoral density, prevented the rise of serum ALP, and reduced the number of osteoclasts compared to ovariectomized control rats\textsuperscript{\textordmasculine39}. A double-blind, randomized, placebo-controlled study of the effects of red clover isoflavones on postmenopausal women, aged 49-65, further supported this data, showing that women taking the isoflavone supplement experienced a reduction in bone mineral content loss and bone mineral density loss in the lumbar region of the spine\textsuperscript{\textordmasculine40}. In this study, neither hip bone mineral content and bone mineral density nor markers of bone resorption and bone composition exhibited any statistically significant treatment effects. However, the bone formation markers, bone-specific alkaline phosphatase and N-propeptide of collagen type I were both significantly increased\textsuperscript{\textordmasculine40}. In vitro, red clover extracts promoted ALP activity in human osteoblastic osteosarcoma (HOS58) cells, which normally show reduced ALP activity with age, which suggested that red clover isoflavones were associated with osteoblastic bone formation\textsuperscript{\textordmasculine26}. Isoflavones from red clover also appeared to be effective in improving skin condition in estrogen deficient, ovariectomized rats\textsuperscript{\textordmasculine41}. Estrogen
played a significant role in maintaining skin health and was involved in the aging process. This association has been demonstrated with ovariectomy-induced, estrogen-deprived rats, which experienced skin changes comparable to those seen in postmenopausal women\(^{(41)}\). The ovariectomized control rats exhibited a reduction in epidermal thickness and keratinization, glands were fewer, vascularity was poor, and collagen bundles and elastic fibers exhibited an altered morphology and lacked uniformity. The ovariectomized treated rats that had received a daily oral dose of 20 and 40 mg red clover isoflavones, however, showed no differences in dermal or epidermal growth as compared to the non-ovariectomized control rats, thereby demonstrating the protective effects on the skin\(^{(41)}\). In another study, these protective effects were again displayed, when genistein and the red clover metabolites, equol, dehydroequol, and isoequol, were shown to be effective in protecting against inflammatory edema, contact hypersensitivity, and immunosuppression induced by UV radiation when applied topically to hairless mice\(^{(34)}\). Equol, which was the most effective of the compounds tested, revealed a dose-dependent protective effect against UV radiation-induced inflammation and immunosuppression. These photoprotective properties of equol suggested that equol has much potential as an ingredient in sun-protective cosmetics\(^{(34)}\).

**Plant tissue culture methods for secondary metabolite production**

The use of plant cell and tissue culture systems has been studied for many years for its capacity to produce useful phytochemicals, specifically secondary metabolites\(^{(42)}\). Plant cell and tissue cultures show great potential for being renewable sources of medicinal compounds, flavors, fragrances, and cosmetics, especially when the compounds of interest are structurally complex.
and cannot be chemically synthesized cost-effectively \(^{(42, 43)}\). Production of these compounds from field-grown or wild crafted plants is often influenced by a number of environmental and geopolitical instabilities, which has further contributed to exploration into the use of plant cell and tissue cultures for phytochemical production \(^{(42)}\). In a majority of cases, low product yields of secondary metabolites by plant cell and tissue cultures have limited large-scale industrial application of these cultivation techniques \(^{(42)}\). However, new methods have been developed in recent years, which exhibit strong potential for improving yields, product synthesis, and product accumulation, including hairy root induction and cell culture elicitation \(^{(42, 44, 45)}\).

Hairy roots, the result of an infection in plants by the soil-borne pathogenic bacterium, *Agrobacterium rhizogenes*, are characterized by vigorously growing, highly branched roots \(^{(42, 44)}\). In addition, infected plants produce new compounds, called opines, which are strain-specific, are not metabolized by the plant, and are excreted by the plant, to be catabolized by this *Agrobacterium* \(^{(46, 47)}\). These physiological changes in the plant are the result of genetic transformation of the infected plants’ cells. The mechanism by which transformation occurs involves the root-inducing (Ri) plasmid carried within the bacterium \(^{(48, 49)}\). Within Ri plasmids are specific DNA sequences, called transferred DNA (T-DNA) regions, which are transferred into plant cells, as well as virulence (vir) regions, which facilitate this transfer \(^{(50)}\). Wounded and metabolically-active plant cells produce compounds such as acetosyringone and \(\alpha\)-hydroxyacetosyringone, which activate expression of the vir regions, thereby initiating T-DNA transfer \(^{(50)}\). After the T-DNA has been transported into the plant cell, it is incorporated into the cell’s genome. The T-DNA transferred from *A. rhizogenes* contains *rol* (rooting locus) genes \(^{(48, 49)}\), which confer an increased sensitivity to endogenous auxin and in some strains, genes for auxin biosynthesis \(^{(51)}\). Expression of the T-DNA genes in the transformed plant induces hairy
root production (42). This genetic transformation enhances root growth and does not impair the root’s ability to produce secondary metabolites and is consequently seen as a promising technique for producing phytochemicals, which may be useful in pharmaceuticals, cosmetics, and food additives (44, 48).

To validate gene transfer by A. rhizogenes into the plant genome and gene expression opine analysis may be employed (48, 52). Opines are compounds which cannot be synthesized by non-transformed plants and hence, opine manufacture is an unambiguous indicator of transformation in hairy root cultures (42, 46, 53). Five different types of opines are produced and these can be used to categorize strains of A. rhizogenes as agropine, cucumopine, mannopine, nopaline, or octopine-type strains (44). To analyze root tissue for the presence of opines, high voltage paper electrophoresis (HVPE) can be employed effectively to separate and visualize opines (54). This process first involves preparation of an aqueous root extract by tissue maceration and removal of cellular material by filtration. The extract is then spotted on filter paper, placed into electrophoresis running buffer consisting of formic acid, acetic acid and water in a 3:6:91 ratio, and subjected to high voltage (54). Following electrophoretic separation, the electrophoretogram is dried and opines may be visualized by spraying with a reagent such as the Pauly imidazole reagent, alkaline silver nitrate, or phenanthrenequinone reagent (54, 55). Electrophoretic mobility and staining properties of the visualized compounds can then be compared with genuine standards and untransformed negative controls for confirmation (54).

The use of hairy root technology in kudzu research has significantly improved secondary metabolite production in that more vigorous growth of hairy roots results in higher yields, without inhibiting metabolite synthesis. The puerarin content in kudzu species, P. phaseoloides, hairy roots was approximately 1.190 mg/g dry weight and equated to 1.067 times the content in
untransformed roots\textsuperscript{(48)}. This showed that hairy root induction by \textit{A. rhizogenes} did not inhibit secondary metabolite biosynthesis in kudzu and that hairy roots could be exploited for their vigorous growth rates to produce larger quantities of kudzu phytochemicals.

Another means of improving phytochemical synthesis and accumulation is through the use of elicitors. Elicitors are varied stimuli, which include climatic stress, microenvironment, physical and chemical stresses that change the quantity or composition of bioactive plant secondary metabolites\textsuperscript{(45, 56)}. The use of elicitors not only improves phytochemical synthesis, but also often results in synthesis of compounds that would otherwise be undetectable in nonelicited plant tissues\textsuperscript{(45)}. The manner by which elicitors work is through stimulation of biosynthetic activity in the enzymatic pathways involved in phytochemical synthesis\textsuperscript{(45)}. The phenylpropanoid pathway for example, is one that includes an extensive collection of biologically active compounds such as isoflavones\textsuperscript{(56)}. The elicitation for isoflavones has been investigated with regard to chemical elicitors\textsuperscript{(45, 57-60)}, fungal pathogens\textsuperscript{(61)}, nutritional stress\textsuperscript{(56)}, light\textsuperscript{(61, 62)} and UV irradiation\textsuperscript{(56)} with promising results.

The use of acetate as a chemical elicitor was found to be strongly effective in inducing anticancer and antimicrobial activity in plant roots, including those of the Leguminaceae family\textsuperscript{(45)}. This enhanced potency following elicitation of roots with acetate may be attributed to an increase in isoflavones\textsuperscript{(58)} that possess antimicrobial and anticancer properties\textsuperscript{(63, 64)}. In a study of acetate, methyl jasmonate, methyl salicylate, and chitosan elicitors on a variety of hydroponically grown plants, 0.1\% acetic acid was found to be the most effective in stimulating production of bioactive secondary metabolites and improving anticancer and antimicrobial activity of plant root extracts across a wide range of species\textsuperscript{(45)}. When applied to red clover as a foliar spray, acetic acid produced an average 15\% increase in total isoflavone content in the
The highest increase in biochanin A compared to control was in the 500 mM treatment and the highest increase in total isoflavones was in the 100 mM treatment group. To eliminate treatment effects of solution pH, 1 N KOH was used to bring acetic acid treatments to a pH of 6.0.

Another chemical compound used as an elicitor of bioactive constituents in plants is salicylic acid. Shown to increase in response to infection and exposure to UV light and ozone, salicylic acid has been suggested to be part of a systemic signaling process in plants, as opposed to directly possessing antimicrobial activity. In a study with hydroponically grown yellow lupin (Lupinus luteus) roots, elicitation with salicylic acid caused significantly more secretion of genistein and other UV-absorbing compounds from the roots. At a concentration of 800 μM, salicylic acid induced 24 times more secretion of genistein in root exudates than water alone. Elicitation of salicylic acid produced a bell-shaped dose-response curve in genistein secretion by L. luteus, revealing that at high concentrations, genistein rhizosecretion may be inhibited by toxic effects. It has also been demonstrated in a wide variety of species that at 800 μM concentration, methyl salicylate was more effective than acetic acid and controls in stimulating production of biologically active secondary metabolites possessing antibacterial activity against P. aeruginosa.

In addition to chemical elicitors, environmental factors have been used to elicit bioactive secondary metabolite synthesis. One such factor is nutritional stress, which caused an increase in phenylpropanoid concentrations to increase in roots and root exudates of bean (Phaseolus vulgaris L.) plants. A nutrient stress such as low nitrogen levels stimulates release of nodulation (nod) gene-inducing flavonoids and isoflavonoids, as well as chemoattractants for nitrogen-fixing symbionts. Another environmental factor that induces secondary metabolite
synthesis is light. Exposure to UV light induces synthesis of UV-absorbing compounds, which are believed to protect DNA from dimerization and breakage\(^{(56)}\). To study the effects of light on isoflavonoid synthesis and soluble proteins in legumes, seedlings of six leguminous species including, kudzu, soybean, garbanzo bean, fava bean, mung bean, and adzuki bean were exposed to 24 hour light and dark environment treatments until the emergence of the first true leaves\(^{(61)}\). The data showed an increase in isoflavonoid and soluble protein concentrations in all six species when exposed to a constant light environment. Under the constant light conditions, kudzu (\(P. montana\)) exhibited the greatest upregulation of isoflavonoids and soluble proteins among the species tested at increases of 80% and 30% respectively\(^{(61)}\). The use of light as an environmental elicitor has also been demonstrated in kudzu (\(P. phaseoloides\)) hairy roots in vitro. While biomass of hairy roots cultured in the dark was greater than hairy roots cultured in white light, the total isoflavone content of roots cultured in white light was 1.19 times that in the dark\(^{(62)}\). Exposure to blue light however, decreased root biomass and inhibited accumulation of puerarin in hairy roots\(^{(62)}\).

Another significant asset to tissue culture systems for production of bioactive components is the potential for biolabeling in these in vitro systems. While our understanding of phytochemical constituents from specific plant sources has greatly improved in past years, we still know little about how these dietary constituents are absorbed, metabolized, and deposited in the human body\(^{(66, 67)}\). This is attributed to the difficulty in examining the absorption of phytochemicals within a complex diet and discriminating between newly absorbed phytochemicals and previously existing levels in the body\(^{(66, 68)}\). One approach to studying phytochemical bioavailability, while circumventing these problematic issues, is the use of stable isotope- or radioisotope-labeled compounds\(^{(66)}\), which offers a means of tracking an
administered dose of phytochemicals in humans or animal models. A key advantage of using radiolabeled compounds over non-radioactive compounds in such studies is that sensitive detection systems make it possible to administer diet-relevant doses and still be able to track absorption, metabolism, and accumulation.

At the moment, development of stable isotope- and radioisotope-labeled phytochemicals can only be produced through in planta synthesis. To achieve optimal labeling of phytochemical compounds of interest, chemically stable atoms such as carbon isotopes are preferred over more readily hydrolysable protons or amino groups. One method of radiolabel incorporation into phytochemicals is the use of labeled carbon dioxide. Phytochemical labeling with this approach results in the fixation of labeled carbon in all organic molecules. However, this process requires that the cells possess photosynthetic capacity for carbon fixation and restrict certain cell and tissue culture types. Labeled organic precursor molecules have also been used in phytochemical labeling, but vary in isotope enrichment efficiency, due to oxidation and loss or incorporation into other compounds.

Tissue and cell culture systems are often favored for phytochemical labeling due to their capacities to produce higher concentrations of secondary metabolites in shorter periods of time than whole plants. The preferred carbon source for most cell and tissue culture systems is sucrose. However, the use of $^{14}$C-sugar in cell and tissue culture systems results in the production of $^{14}$CO$_2$ due to $^{14}$C-sugar oxidation for metabolic energy. For this reason, precautions must be taken to safely collect and contain the gaseous radiolabel. One system for safely labeling phytochemicals with $^{14}$C-sucrose has been developed, which utilizes an enclosure for cell cultures to proliferate in, while trapping gaseous radiolabeled compounds. In a study employing this labeling chamber, $^{14}$C-sucrose was used for the in vitro introduction of carbon
isotopes into ohelo berry (Vaccinium pahalae) and grape (Vitis vinifera) phytochemical compounds. Results of this study showed that 23% and 20% of the administered $^{14}$C-sucrose was incorporated into the flavonoid-rich fractions of ohelo berry and grape cultures respectively (67). In a study on the role of 15-cis-phytoene in prostate cancer prevention, $^{14}$C-sucrose was used in tomato cell cultures to produce $^{14}$C-15-cis-phytoene. Approximately 17.5% of the administered $^{14}$C-sucrose dose was recovered in the tomato cells (70). From these cells, an average of 1.0 μg/g $^{14}$C-phytoene was produced (70).

Metabolic tracking of radiolabeled isoflavones in rats

The use of radioisotope-enriched phytochemicals has enhanced our ability to measure and understand metabolite bioavailability, metabolism, and accumulation (67). These aspects may be studied through in vitro and in vivo models. An in vivo study utilizing $^{14}$C-labeled ohelo berry phytochemicals demonstrated that it was possible to track the absorption, bioavailability, and distribution of flavonoids in an animal model (69). By drawing blood samples from a male Sprague-Dawley rat at set time points following gavage, assessment of absorption rate and bioavailability was achieved. Analysis of the brain was accomplished with accelerator mass spectrometry to track the accumulation of $^{14}$C-labeled compounds (69). In another pilot study, 4-$^{14}$C-genistein was administered to female Sprague-Dawley rats to gain a better understanding of genistein uptake and excretion. Through the collection of bile through a bile duct cannula and intestinal perfusate through isolated segments of the duodenum or proximal ileum, genistein was found to be highly bioavailable in rats and may accumulate within the gastrointestinal tract (71). Advances in the production and tracking of biolabeled flavonoids and carotenoids in plant cell
culture systems, and subsequent use in metabolic tracking has led to our very recent focus on biolabeling of isoflavones (72, 73).
HYPOTHESIS

The central hypothesis for this study is that ingested isoflavones are capable of being distributed in serum and interstitial fluid, incorporated into bone tissue, and passing the blood-brain barrier, which enables them to contribute to cardiovascular protection, bone health, and cognitive function.

OBJECTIVES

To test the hypothesis, the goals of this project are: to metabolically track $^{14}$C-radiolabeled extracts containing isoflavones from kudzu and red clover in rats; to maximize isoflavone yield from kudzu tissue cultures through hairy root induction, genotype selection, and elicitation; to radiolabel kudzu hairy roots and produce a $^{14}$C-labeled, isoflavone-rich fraction; and to evaluate distribution of the $^{14}$C-radiolabeled kudzu isoflavone-rich fraction in rat tissues, particularly in bones.
SPECIFIC AIMS

1. Evaluate the absorption, distribution, metabolism, and elimination of kudzu and red clover phytochemicals in rats, using $^{14}$C-radiolabeled crude extracts. (Chapter 2)

2. Establish hairy root cultures of four kudzu genotypes, identify and quantify the major isoflavones in each hairy root genotype, and select the genotype with the highest isoflavone yield for radiolabeling and elicitation studies. (Chapter 3)

3. Radiolabel kudzu hairy roots of the selected genotype using $^{14}$C-labeled sucrose, extract the isoflavones, and isolate a radiolabeled, isoflavone-rich fraction. (Chapter 3)

4. Track the pharmacokinetics of the radiolabeled, kudzu isoflavone-rich fraction in rats and determine whether isoflavones are capable of reaching bone tissues. (Chapter 3)

5. Elicit kudzu hairy root cultures using sodium acetate and salicylic acid to attempt to promote increased isoflavone synthesis. (Chapter 4)


22. Wong RWK, Rabie ABM. Effect of puerarin on bone formation. International Association for Dental Research (IADR) General Convention & Exhibition; June 28, 2006; Brisbane, Australia2006.


72. Reppert A, Yousef GG, Rogers RB, Lila MA. Elicitation of *Pueraria lobata* (kudzu) cell and root cultures for radiolabeling of isoflavones. The Society for In Vitro Biology Meeting; Minneapolis, MN2006.

CHAPTER 2: In Vivo Metabolic Tracking of $^{14}$C-Radiolabeled Isoflavones in Kudzu and Red Clover Extracts

ABSTRACT

Absorption, distribution, and elimination of $^{14}$C-labeled isoflavone-containing extracts from kudzu (Pueraria lobata) root culture and red clover (Trifolium pratense) cell culture, were investigated in an in vivo rat model. The predominant isoflavones in the kudzu extract were the glycosides puerarin, daidzin, and malonyl daidzin, while in the red clover extract, the major isoflavones were formononetin and its derivatives; genistein, and biochanin A, with radioactivities of 3.770 MBq/g (101.9 µCi/g) and 7.256 MBq/g (196.1 µCi/g) respectively. Male Sprague-Dawley rats, implanted with a jugular catheter and a subcutaneous ultrafiltrate probe, were gavaged with $^{14}$C-labeled isoflavone extracts from either kudzu or clover cell cultures, and serum, interstitial fluid (ISF), urine and feces were collected using a Culex Automated Blood Collection System for 24 h. Analysis of bone tissues revealed that radiolabel accumulated in the femur, tibia, and vertebrae at 0.04%, 0.03%, and 0.01% of the administered dose respectively in both kudzu and red clover treatments. The liver accumulated the greatest concentration of radiolabel among tissues tested, at 1.99% and 1.54% of the administered kudzu and red clover extracts, respectively. Serum and ISF analysis showed that both extracts were rapidly absorbed, distributed in various tissues, and largely eliminated in the urine and feces. Urine and feces contained 8.53% and 9.06% of the kudzu dose, respectively and 3.60% and 5.64% of the red

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clover dose, respectively. Serum pharmacokinetics suggest that extracts from kudzu may undergo enterohepatic circulation.

INTRODUCTION

Estrogens are hormones that are involved in the development and maintenance of female physical traits, sexual reproduction (1), plasma lipid levels, and bone mineral density (2). Isoflavones are a class of plant-derived compounds that bind to estrogen receptors to yield estrogenic activity. Kudzu (Pueraria sp.) and red clover (Trifolium pratense) are leguminous species that are rich sources of isoflavones (3,4) and have demonstrated positive effects on bone health in vitro and in vivo. Kudzu-derived puerarin promoted new bone formation in vitro in treated rat osteoblasts (5) and in vivo in repair of rabbit parietal bone defects when grafted with puerarin solution mixed with collagen matrix (6). Extracts from both P. mirifica and P. lobata have also prevented bone loss in ovariectomized rats (7) and mice (8), respectively. Red clover isoflavones at a dosage of 57 mg/day have similarly exhibited potential to improve bone mineral density of the proximal radius and ulna by 4.1% over 6 months in postmenopausal women (9). A double-blind, randomized, placebo-controlled study of the effects of red clover isoflavones on postmenopausal women, aged 49-65, further supported this data, showing that women taking the isoflavone supplement experienced a reduction in bone mineral content loss and bone mineral density loss in the lumbar region of the spine (10).

While our understanding of kudzu and red clover phytochemicals has greatly improved in recent years, little is known about how these dietary constituents are absorbed, distributed, metabolized, and excreted in the human body (11,12). This is attributed to the difficulty in
examining the phytochemical absorption within a complex diet and discriminating between newly absorbed phytochemicals and previously existing levels in the body \(^{(11, 13)}\). One approach to studying phytochemical bioavailability, while circumventing these problematic issues, is the use of stable isotope- or radioisotope-labeled compounds \(^{(11)}\), which offers a means of tracking an administered dose of phytochemicals in humans or animal models \(^{(12)}\). A key advantage of using radiolabeled compounds over non-radioactive compounds in such studies is that sensitive detection systems make it possible to administer diet-relevant doses and still be able to track pharmacokinetics and biodistribution \(^{(14)}\).

This study was conducted to investigate the absorption, distribution, and elimination of \(^{14}\)C-labeled isoflavone-containing extracts from kudzu root culture and red clover cell culture, in an *in vivo* rat model.

**MATERIALS AND METHODS**

**Chemicals and Reagents**

Standards for puerarin, daidzein, genistein, formononetin, and biochanin A were purchased from Sigma-Aldrich (St. Louis, MO) for isoflavone identification and quantification. Standards for daidzin, genistin, and malonyl-genistin were purchased from LC Laboratories (Woburn, MA). Standards for ononin (formononetin 7-O-β-D-glucoside) and sissotrin (biochanin A 7-O-β-D-glucoside) were purchased from Indofine Chemical Company, Inc. (Hillsborough, NJ). Isoflavones that could not be authenticated by standards due to commercial unavailability were instead quantified by a method of molecular weight adjustment of related standards \(^{(15)}\).
Plant Material

Kudzu (*Pueraria lobata*) seed was obtained in October 2003 from the Binkley Rd population in Johnston City (Williamson County, IL). This kudzu accession was identified by Terry Esker (Natural Heritage Biologist, Illinois Department of Natural Resources) and a voucher specimen (voucher number 227615) was deposited in the Illinois Natural History Survey herbarium. Late-flowering red clover (*Trifolium pratense* ‘Mammoth’) seed was obtained from Johnny’s Selected Seeds (Winslow, ME).

Kudzu seeds were surface scarified with a scalpel and agitated in tap water containing 2 drops of polyoxyethylene sorbitan monolaurate (Tween-20; Sigma-Aldrich, St. Louis) per liter for 14 h to overcome seed dormancy and promote germination. Seeds were surface disinfested in 0.9% sodium hypochlorite for 15 to 30 minutes. Seeds were rinsed three times with sterile distilled water and explanted to test tubes containing 15 mL of 2.2 g/L Murashige-Skoog (MS) basal media \(^{(16)}\) with 10 mL/L rose vitamins \(^{(17)}\), 0.1 g/L myoinositol (PhytoTechnology Laboratories, Shawnee Mission, KS), 30 g/L sucrose, 6 g/L agar (Sigma-Aldrich) and pH-adjusted to 5.7.

Following germination, roots were excised and transferred to 250 mL flasks containing 40 mL of root culture media, which consisted of 4.3 g/L MS basal media with 10 mL/L rose vitamins, 0.1 g/L myoinositol, 30 g/L sucrose, 1.0 mg/L 1-naphthaleneacetic acid (NAA) \(^{(18)}\), and pH-adjusted to 5.7. Cultures were maintained in the dark on a rotary shaker (150 rpm) at 25 °C and subcultured at 21 day intervals by replacing all but 5 mL of the spent medium with 35 mL of fresh root culture medium. Root cultures were divided approximately every 9 weeks, after they had accumulated about 25% new growth.
Red clover seeds were surface disinfested by wrapping in filter paper, submerging in 70% ethanol for 1 minute, and then in a 0.9% sodium hypochlorite solution with 2 drops of polyoxyethylene sorbitan monolaurate (Tween-20) per liter for 10 minutes. Seeds were rinsed five times with sterile water and explanted to MS basal medium with 10 mL/L rose vitamins, 0.1 g/L myoinositol, 6 g/L bacteriological grade agar and pH-adjusted to 5.7. Petiole segments from 21 day-old seedlings were placed onto 15 mL of solid red clover callus-inducing medium (19), consisting of 3.08 g/L Gamborg B5 salts (20), B5 vitamins, 0.1 g/L myoinositol, 20 g/L sucrose, 2.25 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 2.0 mg/L NAA, and 2.12 mg/L kinetin (Sigma-Aldrich, St. Louis, MO) and 6 g/L bacteriological grade agar in culture tubes. After callus cultures had developed, the undifferentiated cells were transferred to 125 mL flasks containing 80 mL of callus induction medium, without agar added. Cells maintained as suspension cultures in the dark were transferred to fresh callus media (3 mL settled cell volume in 7 mL spent medium) every 14 days.

Radiolabeling

Uniformly labeled, crystalline $^{14}$C-sucrose with a specific activity of 374 MBq/mmol (10 mCi/mmol) (ICN Biomedicals Inc., Irvine, CA) was utilized as the label source. A stock solution of $^{14}$C-sucrose was prepared in sterile, pH 5.7-adjusted, double distilled water and filter-sterilized. Concentrated media, at 80% and 90% of the final volume (32 and 72 mL per 250 mL flask for kudzu and red clover respectively), containing all constituents except $^{14}$C-sucrose, was prepared and autoclaved. Following transfer of established cultures to fresh, concentrated medium as described above, 8 mL of stock $^{14}$C-sucrose solution was added to kudzu root or red
clover cell suspension cultures to bring the final media volumes up to 40 mL and 80 mL, respectively. The final concentration of $^{14}$C-sucrose added was 0.248 and 0.126 MBq/mL (6.7 and 3.4 µCi/mL) medium for kudzu root cultures and red clover cell cultures, respectively.

Culture flasks were then placed into an enclosed Plexiglas labeling chamber, designed to siphon and capture respired $^{14}$CO$_2$ (12). Kudzu root cultures were grown in this chamber for 21 days, before being harvested. Red clover cultures were permitted to grow for 11 days, before being removed, and treated with 40 µL of a 50 µM CuCl$_2$ solution, as an elicitor for isoflavone synthesis (21). Red clover cultures were returned to the labeling chamber and were exposed to the CuCl$_2$ elicitor for 3 days, then harvested.

Extraction and Chemical Analysis of Isoflavone Extracts

At harvest, root and cell suspension cultures were vacuum filtered using a Buchner funnel with Whatman #4 filter paper. Cells and roots were rinsed with distilled, deionized water to wash away residual media and $^{14}$C-sucrose that was not taken up. Excess moisture was removed by suction and fresh weight was recorded. Fresh roots or cells were then placed into a blender with 80% methanol in a 1:5 w/v ratio and blended for 2 minutes. The methanolic extract was separated by filtration with Whatman #4 filter paper and stored in a flask at -20 ºC. The extraction procedure was repeated twice using the same volume of solvent, with the third extraction held overnight at 4 ºC, prior to filtration. The 3 extractions were combined, filtered with Whatman #1 filter paper, concentrated by rotoevaporation (Buchi Rotavapor, Buchi Labortechnik, Switzerland), and dried down completely by lyophilization in a freeze dryer (Labconco Freezone 4.5, Kansas City, MO).
Characterization of Isoflavones in Extracts

An LCQ Deca XP mass spectrometer (150-1000 m/z) attached to photodiode array detector (PDA; UV: 262mm) (Thermo Finnigan Corp., San Jose, CA) was utilized in high performance liquid chromatography-electro spray ionization-mass spectrophotometer (HPLC-ESI-MS) analysis on a C18-reversed-phase column (2.1 x 150 mm, VYDAC, Cat. #: 201 SPS215). The mobile phase was composed of 95% double-distilled water with 5% acetonitrile and 0.1% trifluoroacetic acid (solvent A) and 5% double-distilled water with 95% acetonitrile and 0.1% trifluoroacetic acid (solvent B). A step gradient of 0%, 70%, 100%, and 0% of solvent B was used at 0, 30, 35, and 40 minutes, respectively. Samples were prepared by dissolving approximately 20 mg dry extract in 4 mL 80% methanol (5 mg/mL), sonicating, and filtering through a 0.45 µm nylon syringe filter (Fisher Scientific, Pittsburgh, PA). 5 µL of each sample were injected for analysis at a flow rate of 200 µL/min. Commercial standards were also used in this analysis to verify UV absorption, retention time, and molecular weights of isoflavones present in extract.

Surgical Protocol

Male Sprague-Dawley rats (body weight 275-350 g), acclimatized to a 12 h photoperiod, were anesthetized with 3-5% isoflurane and delivered with oxygen at 2-4 L/min. A jugular catheter was inserted into the right jugular vein and an ultrafiltrate probe (Bioanalytical Systems, Inc., West Lafayette, IN) was implanted subcutaneously down the dorsal midline. Following surgery, the rats were administered 0.3 mL of 10% buprenex in 0.9% NaCl (w/v) and connected to a Culex® Automated Blood Sampling System (Bioanalytical Systems, Inc.) for blood, ISF,
urine and feces collection. Rats were given 48 h for recovery. Food (AIN93M, DyetsInc, PA) and water were provided *ad libitum* and the catheter was flushed with 10 units heparin/mL saline (0.9% NaCl (w/v)) every 15 minutes. All experimental procedures involving animals were approved by the Purdue Animal Care and Use Committee (PACUC), which adheres to policies set forth by the United States Department of Agriculture (USDA) and the United States Public Health Service (USPHS).

**Gavage Protocol**

Preliminary experiments demonstrated that a dose of 0.148 MBq (4 µCi) of $^{14}$C-labeled extract could be traced effectively *in vivo* using a scintillation counter and were used as a basis for choosing dose sizes for radiolabeled kudzu and red clover extracts. Rats were food-deprived for 8 h prior to gavage and 200 µL of blood was drawn as the baseline. Approximately 100 mg/kg bw of $^{14}$C-labeled kudzu extract (3.770 MBq/g; 101.9 µCi/g) or 75 mg/kg bw of red clover extract (7.256 MBq/g; 196.1 µCi/g) in 1 mL of distilled water were administered to the rats (n=6 and n=4 for kudzu and red clover treatments respectively) by gavage, followed by an immediate rinse of the gavage needle with 0.5 mL of distilled water. The Culex system automatically extracted approximately 200 uL blood at 5, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 480, 600, 720, and 1440 minutes after gavage and collected ISF at 60 minute intervals for a total of 24 h. Urine and feces were collected for 24 h. Food was returned 240 minutes after gavage.
Rat Sample Collection

Rats were anesthetized 24 h after gavage with approximately 0.3 mL of ketamine (100 mg/mL) and xylazine (100 mg/mL). Blood was drawn from the jugular catheter until no more blood could be collected. This blood was transferred to heparinized microcentrifuge tubes, centrifuged at 5000 x g for 10 minutes, and plasma was collected for analysis. An incision was made through the left jugular vein and the rat was perfused through the right jugular catheter with 240 mL chilled saline, which was sufficient to produce clear perfusate that exited the incision in the left jugular vein. The stomach, small intestines, large intestines, brain, heart, lungs, liver, kidneys, and testes were placed into scintillation vials. The contents of the stomach, small intestines and large intestines were collected by flushing the respective organs through with 10 mL saline (0.9% NaCl (w/v)). Femurs, tibias, and vertebrae were carefully removed, wrapped in saline-saturated gauze, and placed into scintillation vials. All samples were stored at -80 °C until ready for analysis.

Rat Sample Analysis

Tissue samples were prepared by macerating with a homogenizer (Bio-homogenizer model M133/1281-0, Biospec Products, Inc. Bautlesville, OK). Tissue homogenates were weighed and transferred to scintillation vials in duplicate. Bones were cleaned, dried at 50 °C, and ground to a fine powder with a mortar and pestle. Weighted samples of ground bone were placed into scintillation vials in duplicate and dissolved in concentrated nitric acid overnight to achieve uniformity. Feces were dried in an oven at 50 °C, 20 mL of 50% methanol was added to extract 14C-labeled compounds overnight, and the methanolic extract was transferred to
scintillation vials. Gastrointestinal contents were prepared by adding 10 mL methanol to each sample and the methanolic extract was transferred to scintillation vials. Serum, plasma, ISF, and urine required no additional preparation for analysis.

After all homogenized tissues, dissolved bones, bodily fluids, and methanolic extracts of feces and gastrointestinal contents were placed into scintillation vials in duplicate, 20 mL Ecolite scintillation cocktail (MP Biomedicals, Solon, OH) was added. Samples were allowed to sit overnight in the dark, prior to analysis with a Beckman LS 6500 scintillation counter (Beckman Coulter, Inc., Fullerton, CA).

ISF was collected at an average rate of 60 µL/hr. The ultrafiltrate probe tubing was 48” in length, with a total internal volume of approximately 12 µL. This internal volume presents a delay between the time ISF is collected and the time ISF reaches the sample collection vial. To account for this delay and to more accurately reflect the amount of $^{14}$C label present in the ISF at any given time, the ISF collection time points illustrated in Figures I & II were shifted 12 minutes earlier than actually collected. Total serum and ISF volumes were approximated based on the body weight of each rat (22) and were used to estimate the percent of $^{14}$C-labeled compounds remaining in each respective fluid 24 h after gavage.

**RESULTS AND DISCUSSION**

The predominant isoflavones in the kudzu and red clover extracts were unambiguously identified by HPLC-ESI-MS and HPLC-PDA using retention time, molecular weight, and comparison to known commercial isoflavone standards. Kudzu isoflavones were confirmed as the glycosides puerarin, daidzin, and malonyl daidzin (Figure 2.1 & Table 2.1) (23, 24). In the red
clover extract, the major isoflavones were formononetin and its derivatives, genistein, and biochanin A (Figure 2.2 & Table 2.2) \(^{(25)}\). Since \(^{14}\)C-sucrose was used as the radiolabel source, \(^{14}\)C was incorporated into all biosynthesized phytochemicals. The kudzu and red clover extracts had average radioactivities of 3.770 MBq/g (101.9 µCi/g) and 7.256 MBq/g (196.1 µCi/g) respectively, with standardized isoflavones composing 19.7% and 20.3% of the total extracts respectively.

Analysis of baseline serum, ISF, urine, and feces by scintillation counter established a naturally-occurring, background level of \(^{14}\)C in each animal, which was subtracted from levels measured after gavage. Serum analysis showed that kudzu and red clover isoflavone extracts were rapidly absorbed, with peak serum concentrations of \(^{14}\)C attained 15-30 minutes after gavage, at an average of 0.30% and 0.33% of the total administered \(^{14}\)C dose per mL serum in kudzu and red clover treatments, respectively (Figures 2.3 and 2.4). Labeled compounds from both extracts were also rapidly distributed, as seen in the extracellular fluid, collected by the ultrafiltrate probe. A second peak in serum concentration approximately 1 h after gavage of kudzu extract may be attributed to the varied rates of absorption and distribution of different phytochemicals \textit{in vivo}, such that one compound may already be undergoing distribution into tissues or elimination from the body, while another is still being absorbed. A plateau in serum concentration approximately 4 h (Figure 2.3) after gavage of the kudzu extract is consistent with the kudzu isoflavones, puerarin \(^{(26)}\), daidzein \(^{(27)}\), and genistein \(^{(28)}\), undergoing enterohepatic circulation, while also being eliminated from the body. Plasma collected 24 hours after gavage showed no difference in \(^{14}\)C activity compared to serum collected 24 hours after gavage. Peak ISF concentrations of 0.18% and 0.22% of the administered dose per mL were attained 1-2 h after gavage of kudzu and red clover extracts respectively (Figures 2.3 and 2.4). Analysis of
serum and ISF over the course of 24 h showed that kudzu and red clover phytochemicals were rapidly metabolized and eliminated. Excrement contained the greatest amount of the administered dose at 9.06% and 8.53% in the urine and feces respectively in the kudzu treatment (Table 2.3). While this was also true in the red clover treatment, only 4.44% and 3.59% of the administered red clover dose were accounted for in the urine and feces (Table 2.3). Total $^{14}$C in all analyzed bones, tissues, excrement, and bodily fluids did not fully account for the total administered dose and this indicated that $^{14}$C label was incorporated into other tissues such as muscle and adipose, and/or metabolism and elimination of labeled compounds in the form of exhaled $^{14}$CO$_2$.

For isoflavones to have an effect on bone health, they must reach the target tissues. $^{14}$C-labeled compounds were detected in the bones and tissues 24 h after gavage. In animals gavaged with either kudzu or red clover extracts, radiolabel accumulated in femurs, tibias, and vertebrae, at 0.04%, 0.03%, and 0.01% of the administered dose respectively (Table 2.3). Successful delivery of $^{14}$C-labeled kudzu and red clover extract to the bones supports the in vitro and in vivo studies that have demonstrated increased bone formation$^{(5, 6)}$ and reduced bone loss$^{(7, 29)}$ upon treatment with isoflavones.

The liver accumulated the greatest quantity of radiolabel among tissues analyzed, at 1.99% and 1.54% of the total dose for kudzu and red clover treatments respectively (Table 2.1). These data are consistent with the fact that compounds which are absorbed by the digestive system are first transported to the liver before reaching the rest of the body, a phenomenon also known as the first pass effect$^{(28)}$. These observations further support the notion that isoflavones are absorbed from the intestine, travel to the liver via the portal vein, are enzymatically conjugated and secreted in the bile, and may be hydrolyzed by gut microflora and reabsorbed$^{(30)}$. 
Analysis of gastrointestinal tract contents indicated that only about 0.12% of the gavaged dose remained in the stomach, 24 h after gavage. Larger quantities of extract remained in the small and large intestines at 1.33% and 2.66% of the administered kudzu dose and at 0.99% and 0.51% of the administered red clover dose respectively. While radioactivities of the intestinal contents were lower in the small intestines than the large, the small intestinal tissues contained a greater quantity of radiolabel than the large intestines in both treatments. The other major tissues examined include the lungs, kidneys, heart and testes, which accumulated 0.07%, 0.21%, 0.04%, and 0.19% of the dose respectively in the kudzu treatment and 0.07%, 0.15%, 0.04%, 0.22% respectively in the red clover treatment (Table 2.3).

A comparison of the areas under the serum concentration time curves (AUC) shows that on average, a dose of 100 mg/kg body weight of kudzu extract yielded an exposure of 6.69% of dose*hr/mL, while a dose of 75 mg/kg body weight of red clover extract yielded an exposure of 4.22% of dose*hr/mL. These values will be used to establish suitable kudzu and red clover extract doses in future pharmacokinetic studies. Future studies will also include analysis for 14C-labeled metabolites to better understand the biochemical mechanisms by which these phytochemicals exert an effect.

CONCLUSIONS

Biosynthesized 14C-labeled isoflavones were administered to rats to determine the absorption and distribution in in vivo model. These radiolabeled compounds were detected in various rat organ tissues. The results of this metabolic tracking study support the hypothesis that
isoflavones may play a role in bone health by demonstrating that radiolabeled kudzu and red
clover-derived extracts were capable of reaching and accumulating in bone tissues.

ACKNOWLEDGEMENTS

There are no conflicts of interest associated with this contribution. Authors Mun, Grannan, and Reppert are students who conducted these experiments; authors Yousef, Rogers, and Lachcik are senior research associates who aided in the analysis, and authors Janle, Weaver, and Lila are faculty members who supervised the research and took charge of the outcomes. This work was supported by the National Center for Complementary and Alternative Medicine sponsored Purdue-UAB Botanicals Center for Dietary Supplement Research (NIH, 2 P50 AT000477-06).
TABLES

Table 2.1: Isoflavones in radiolabeled kudzu root culture extracts detected by LC-MS analysis

Table 2.2: Isoflavones in radiolabeled red clover cell culture extracts detected by LC-MS analysis

Table 2.3: Analysis of $^{14}$C-labeled extract distribution in rats 24 hours after gavage by scintillation counter (percent of the orally administered dose)
Table 2.1: Isoflavones in radiolabeled kudzu root culture extracts detected by LC-MS analysis.

<table>
<thead>
<tr>
<th>t_R (min)</th>
<th>Major ion [M + H^+] (m/z)</th>
<th>Product ions m/z</th>
<th>Compound name</th>
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<tbody>
<tr>
<td>8.7</td>
<td>433</td>
<td></td>
<td>Hydroxy-puerarin</td>
</tr>
<tr>
<td>9.9</td>
<td>417</td>
<td></td>
<td>Puerarin</td>
</tr>
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<td>11.3</td>
<td>417 255</td>
<td></td>
<td>Daidzin</td>
</tr>
<tr>
<td>12.0</td>
<td>503 447, 255</td>
<td></td>
<td>Unassigned</td>
</tr>
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<td>13.3</td>
<td>433 271</td>
<td></td>
<td>Genistin</td>
</tr>
<tr>
<td>13.5</td>
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<td></td>
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</tr>
<tr>
<td>15.3</td>
<td>519 271</td>
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<td>17.2</td>
<td>255</td>
<td></td>
<td>Daidzein</td>
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<td>20.3</td>
<td>271</td>
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<td>Genistein</td>
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t_R, retention time in minutes; m/z, mass-to-charge ratio

See Figure 1 for LC-MS spectra of kudzu root culture extract.

Data in Table 1 correspond with Figure 1.
**Table 2.2:** Isoflavones in radiolabeled red clover cell culture extracts detected by LC-MS analysis

<table>
<thead>
<tr>
<th>t&lt;sub&gt;R&lt;/sub&gt; (min)</th>
<th>Major ion [M + H&lt;sup&gt;+&lt;/sup&gt;] (m/z)</th>
<th>Product ions (m/z)</th>
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* t<sub>R</sub>, retention time in minutes; m/z, mass-to-charge ratio

See Figure 2 for LC-MS spectra of red clover cell culture extract.

Data in Table 2 correspond with Figure 2.
Table 2.3: Analysis of $^{14}$C-labeled extract distribution in rats 24 hours after gavage by scintillation counter (percent of the orally administered dose)

<table>
<thead>
<tr>
<th>Tissue/Sample</th>
<th>Treatment</th>
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FIGURES

**Figure 2.1:** Representative LC-MS spectra showing total ion current (TIC, top) and LC-UV chromatogram (UV 262 nm, middle), and MS spectrum (bottom) of isoflavone extract from kudzu root cultures

**Figure 2.2:** Representative LC-MS spectra showing total ion current (TIC, top) and LC-UV chromatogram (UV 262 nm, middle), and MS spectrum (bottom) of isoflavone extract from red clover cell cultures

**Figure 2.3:** Distribution of $^{14}$C-label for kudzu isoflavone extract in serum & interstitial fluid of rats during 24 hours after administration

**Figure 2.4:** Distribution of $^{14}$C-label for red clover isoflavone extract in serum & interstitial fluid of rats during 24 hours after administration
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Figure 2.2: Representative LC-MS spectra showing total ion current (TIC, top) and LC-UV chromatogram (UV 262 nm, middle), and MS spectrum (bottom) of isoflavone extract from red clover cell cultures.
Figure 2.3: Distribution of $^{14}$C-label for kudzu isoflavone extract in serum & interstitial fluid of rats during 24 hours after administration
Figure 2.4: Distribution of $^{14}$C-label for red clover isoflavone extract in serum & interstitial fluid of rats during 24 hours after administration.
LITERATURE CITED


CHAPTER 3: Tracking Deposition of a $^{14}$C-Radiolabeled Kudzu Hairy Root-Derived Isoflavone-Rich Fraction into Bone

ABSTRACT

Hairy roots were induced in four genotypes from three kudzu species (*Pueraria montana* var. *lobata*, *P. lobata*, and *P. phaseoloides*) *in vitro* using *Agrobacterium rhizogenes* to stimulate rapid secondary metabolite synthesis. Hairy roots from *P. montana* var. *lobata* (USDA no. PI 434246) yielded the highest puerarin and total isoflavone content and the greatest new biomass per growth cycle among the genotypes evaluated. Hairy roots from this genotype were selected for radiolabeling using $^{14}$C- sucrose as a carbon source. Isoflavones from radiolabeled kudzu hairy root cultures were extracted with 80% methanol, partitioned by solvent extraction, and then subfractionated by Sephadex LH-20 gel filtration. Radiolabeled isoflavones were isolated in a highly enriched fraction, which contained predominantly puerarin, daidzin, and malonyl-daidzin and had an average radioactivity of 8.614 MBq/g (232.8 µCi/g) dry fraction. The $^{14}$C-radiolabeled, isoflavone-rich fraction was orally administered at a dose of 60 mg/kg body weight to male Sprague-Dawley rats implanted with a jugular catheter, a subcutaneous ultrafiltrate probe, and a brain microdialysate probe. Serum, interstitial fluid (ISF), brain microdialysate (BM), urine and feces were collected using a Culex® Automated Blood Collection System for 24 h. At the end of this period, rats were sacrificed and major tissues were collected. Analysis by scintillation counter confirmed that a bolus dose of $^{14}$C-radiolabeled, isoflavone-rich kudzu fraction reached bone tissues, which accumulated 0.011%, 0.09%, and 0.003% of the administered dose in femur, tibia, and vertebrae, respectively. Femurs extracted with 80% methanol were analyzed by high performance liquid chromatography with electrospray
ionization-mass spectrometry (HPLC-ESI-MS) and were found to contain trace quantities of puerarin, daidzein, and puerarin glucuronide. This study demonstrates that kudzu isoflavones and metabolites are capable of reaching bone tissues, where they may contribute to the prevention of osteoporosis and the promotion of bone health.

INTRODUCTION

Osteoporosis is a metabolic bone disease characterized by skeletal fragility and an increased susceptibility to fracture due to low bone mineral density (BMD) and weakening of internal bone structure (1). This condition affects an estimated 200 million people worldwide and is caused by insufficient bone formation by osteoblasts, increased bone resorption by osteoclasts, or a combination of both (2, 3). Osteoporosis incidence increases with age and impacts both men and women, though women have a higher risk of developing osteoporosis, in part due to differences in skeletal size and overall bone mineral content (BMC) between sexes. In addition, risk for developing osteoporosis is greatest in women with estrogen deficiency (4). Estrogen functions by binding to estrogen receptors, which regulate gene expression. 17-β-estradiol, the primary estrogen in mammals, binds to both estrogen receptor α (ER-α), which is expressed most highly in reproductive tissues, and estrogen receptor β (ER-β), which is expressed in many different tissues including the central nervous system, the cardiovascular system, and the skeletal system (5). Declining estrogen levels during menopause promote activation of osteoclasts to increase bone remodeling, which in turn increases daily calcium loss and over time decreases BMC and BMD (3, 6).
Hormone replacement therapies (HRTs) have been used to mitigate this loss of bone calcium by supplying exogenous estrogen and progesterone \(^3\). However, while HRT use has demonstrated reduction of fracture risk, its long-term use has also been shown to increase coronary heart disease risk \(^7\), breast cancer risk \(^8,9\), and stroke risk \(^10\). These negative outcomes far outweigh the benefits and have consequently led to increased investigation into identifying alternative treatments, which include selective estrogen receptor modulators (SERMs). SERMs are compounds that bear structural and functional similarities to estrogen and bind to estrogen receptors, but exert different estrogenic or anti-estrogenic activities depending on the specific tissue type \(^6\). Currently, phytoestrogens are being studied as potential SERMs that will preserve the health-promoting, estrogenic effects, while reducing or eliminating morbidity risks.

Isoflavones are a class of phytoestrogens that are structurally similar to estrogen and exhibit estrogen receptor binding activity that is characteristic of SERMs \(^11,12\). Soy, a major dietary source of isoflavones in southeast Asian diets for several thousand years and more recently in European and North American diets, has been linked to many health benefits, including the prevention of osteoporosis \(^13\). However, results from these studies have been largely inconsistent and have often been confounded by factors which include synergy or antagonism between different isoflavone combinations, hormonal status, age, and interactions between genetics and the environment \(^14\). Kudzu, another leguminous species that produces isoflavones, has also been examined as a potential source of SERMs. Historically, kudzu has been used in Traditional Chinese Medicine as an antioxidant and antidipsotropic agent \(^15,16\) and has more recently exhibited benefits to cardiovascular health \(^17\), neurological health \(^18,19\), bone health \(^20-23\), and blood glucose homeostasis \(^17,24\). Puerarin (daidzein-8-C-glucoside), the
predominant isoflavone in kudzu\(^{(25)}\), is unique in that its C-glycosides are resistant to hydrolysis in the gut, have better bioavailability than O-glycosides, and are taken up from the intestines largely unmetabolized\(^{(21)}\). \textit{In vitro}, puerarin has been shown to increase bone formation in rat osteoblasts\(^{(23)}\) and decrease bone resorption in rat osteoclasts\(^{(20)}\). Findings from these \textit{in vitro} experiments have been supported \textit{in vivo}. Surgically-created rabbit parietal bone defects grafted with a puerarin and collagen mixture formed over 500% more new bone following a two week recovery period than defects grafted with collagen alone, as quantified by computer image analysis of histological samples\(^{(22)}\). In another study, loss of BMD and BMC in trabecular bone associated with ovariectomy in rats was reported to be completely reversed by daily gavage of kudzu (\textit{Pueraria mirifica}) crude extract for 90 days in a dose dependent manner\(^{(6)}\).

While puerarin and kudzu extracts containing puerarin and other isoflavones have shown potential for supporting bone health \textit{in vitro} and \textit{in vivo}, their underlying mechanisms remain obscure. In particular, for kudzu isoflavones to have an effect on bone health, they must reach the target tissues. Previously, we showed that \(^{14}\)C-labeled phytochemicals can be isolated from kudzu (\textit{P. lobata}) roots as a crude extract, identified, quantified\(^{(26)}\), and tracked \textit{in vivo} to evaluate absorption, distribution into organs, and clearance from the body\(^{(27)}\). In this study, we have significantly optimized the radiolabeling system by 1) screening kudzu germplasm to select an elite, high-puerarin-yielding genotype, 2) inducing hairy roots by inoculation with \textit{Agrobacterium rhizogenes} to further magnify isoflavone accumulation\(^{(28)}\), and 3) purifying an isoflavone-enriched radiolabeled subfraction from the crude extract, which was then tracked \textit{in vivo} to determine bone accumulation.
MATERIALS AND METHODS

Induction and maintenance of kudzu hairy root cultures

Kudzu seeds from four genotypes, *Pueraria montana* var. *lobata* (USDA no. PI9227, Origin: Japan), *P. montana* var. *lobata* (USDA no. PI 434246, Origin: USA), *P. lobata* (Binkley Rd population, Williamson County, Illinois, USA) (26), and *P. phaseoloides* (Origin: Guatemala), were scarified, soaked overnight in tap water containing polyoxyethylene sorbitan monolaurate (0.1% v/v, Tween 20, Sigma-Aldrich, St. Louis, MO), surface disinfested in 0.9% sodium hypochlorite for 10 minutes, and rinsed with sterilized distilled water. Seeds were then explanted onto media (29), containing ½ x (2.165 g L\(^{-1}\)) Murashige and Skoog (MS) basal salts (Phytotechnology Laboratories, Shawnee Mission, K.S.) (30), Gamborg B5 vitamins (31), 0.1 g L\(^{-1}\) myoinositol (Phytotechnology Laboratories), 30 g L\(^{-1}\) sucrose, 0.1% FeEDTA, and 2 g L\(^{-1}\) gellan gum (PhytoTechnology Laboratories, Shawnee Mission, KS ). Seeds were allowed to germinate and grow for seven days under fluorescent lights. Sterile Luria-Bertani broth (LB) (Phytotechnology Laboratories, Shawnee Mission, K.S.) was prepared, inoculated with the cucumopine-producing *A. rhizogenes* strain, K599, and allowed to culture overnight. The following day, seedlings were harvested and cotyledon explants were wounded on the abaxial side with a scalpel. Cotyledon explants were placed in LB media containing *A. rhizogenes* and 250 μL acatosyringone for 5-10 minutes and then set onto sterile Petri dishes lined with sterile filter paper to co-cultivate for two days under fluorescent lights. The cotyledon explants were then transferred to Petri dishes containing a growth-regulator-free MS media, containing 4.330 g L\(^{-1}\) MS basal salts, Gamborg B5 vitamins, 0.1 g L\(^{-1}\) myoinositol, 30 g L\(^{-1}\) sucrose, 0.1% FeEDTA, 2 g L\(^{-1}\) gellan gum, and 500 mg L\(^{-1}\) carbenicillin to inhibit bacterial growth. After two weeks, explants were transferred to MS media with 250 mg L\(^{-1}\) carbenicillin. When hairy roots were
sufficiently large, they were severed from the cotyledon and transferred to dark growing conditions at 25 °C and sub-cultured every two weeks onto MS media containing 250 mg L\(^{-1}\) carbenicillin until no signs of bacterial contamination were evident, at which point roots were subcultured to carbenicillin-free media. Kudzu hairy roots on growth-regulator-free media were identified by their rapid growth rates and extensively branching phenotype. Hairy roots were further authenticated by high voltage paper electrophoresis (HVPE) of aqueous kudzu extracts for the synthesis of cucumopine, which is unique to hairy roots of this strain\(^{(32, 33)}\). An authentic cucumopine standard dissolved in distilled water and filtered, aqueous extracts from 4 kudzu hairy root genotypes and 4 non-transgenic kudzu genotypes were blotted onto Whatman #3 filter paper and allowed to dry. Filter paper was placed into a formic-acetic acid buffer of pH 1.8 and subjected to electrophoresis at 290 V for 70 min. Electrophoretograms were dried thoroughly in a stream of warm air and then visualized by spraying lightly with Pauly reagent\(^{(32)}\). Samples of kudzu hairy roots were dried on a freeze-dryer (Labconco Freezone 4.5, Kansas City, MO) and stored at -80 °C until ready for extraction and isoflavone analysis.

**Extraction of isoflavones from hairy roots**

Methanol (80%) was added to root samples at a ratio of approximately 25 mL methanol to 1 g dry roots and homogenized for 30 seconds. Methanolic extract was filtered with a Whatman #4 filter and root biomass was homogenized again with 80% methanol. This second extract was filtered with a Whatman #4 filter and 80% methanol was added to the remaining root biomass to extract overnight and then filtered. All three filtered extracts were combined, filtered through a Whatman #1 filter, and placed on a rotoevaporator (Buchi Rotavapor, Buchi
Labortechnik, Switzerland) to remove solvent. Residual water was removed with a freeze-dryer. Dried samples were weighed and stored at -80 °C. Extracts were prepared for analysis by dissolving 20 mg in 4 mL of 80% methanol and filtering through a 0.45 µm nylon syringe filter (Fisher Scientific, Pittsburgh, PA).

**Identification and quantification of isoflavones**

Isoflavones were identified by high performance liquid chromatography equipped with electrospray ionization-mass spectrometry (HPLC-ESI-MS) using a LCQ Deca XP mass spectrometer under positive ESI mode (m/z 100-1000) attached to a photodiode array (PDA) detector (Thermo Finnigan Corp., San Jose, CA) at ultraviolet (UV) wavelength 262 nm. Separations were performed on a 2.1 mm x 150 mm, 3.5 µm, Waters XBridge C18-reversed phase column (Waters Corp., Milford, MA) using a mobile phase consisting of 5% acetonitrile (ACN) in double-distilled H₂O with 0.1% formic acid (Fisher Scientific) in solvent A and 95% ACN in double-distilled H₂O with 0.1% formic acid in solvent B. The step gradient was composed of 0%, 70%, 100% and 0% of solvent B at 0, 30, 35, and 40 min, respectively. Injection volume for each sample was 5 µL and the solvent flow rate was 200 µL/min.

Isoflavones were quantified by high performance liquid chromatography with photodiode array detection (HPLC-PDA), using an Agilent 1100 HPLC system (Agilent Technologies, Inc., Santa Clara, CA) with an autosampler (G1313A), a degasser (G1322A), a quadratic pump (G1311A), and a temperature-controlled column compartment (G1316A). Separations were performed using a 250 mm x 4 mm, 5 µm Supelcosil LC-18 reversed-phase column (25 °C) (Supelco, Bellefonte, PA, USA) with a mobile phase consisting of 10% ACN in double-distilled
H₂O with 0.1% trifluoroacetic acid (TFA) (Sigma-Aldrich, St. Louis, MO) in solvent A and 90% ACN in double-distilled H₂O with 0.1% TFA in solvent B. The elution gradient was composed of 0%, 70%, 100% and 0% of solvent B at 0, 30, 35, and 40 min, respectively and UV detection was at a wavelength of 262 nm. The injection volume was 15 µL/min and the flow rate was 1.5 mL/min. Data were processed using ChemStation Software for LC 3D systems (Rev. A.10.02, Agilent Technologies, Inc.).

Authentic standards for puerarin, daidzein, and genistein from Sigma-Aldrich and daidzin, genistin, and malonyl-genistin from LC Laboratories (Woburn, MA) were used to verify UV absorption, retention time, and molecular weight of isoflavones in each extract. Isoflavones that could not be authenticated with standards due to commercial unavailability were identified by comparison to literature data and quantified by a method of molecular weight adjustment of related standards (34).

Comparison of kudzu hairy root genotypes

Hairy roots from four kudzu genotypes were cultured on petri dishes containing 20 mL solid MS media (containing 2 g L⁻¹ gellan gum) for 2 weeks. Fresh hairy root mass was recorded from 30 uniformly-growing cultures of each genotype after the 2 week culture period. Measurements were taken after four separate culture periods (n=4) for each genotype. Average fresh mass per hairy root culture was calculated for each culture interval. Isoflavones were extracted from approx. 10 g fresh kudzu hairy roots (n=4) of each genotype, identified by HPLC-ESI-MS, and quantified by HPLC-PDA. Total isoflavone content of the four genotypes were compared by one-way analysis of variance (ANOVA), followed by post hoc analysis with
Tukey’s Least Significant Difference multiple comparison test at a level of significance of 0.05 using SPSS Statistics software (Release 17.0, SPSS Inc., Chicago, IL)

**Radiolabeling of kudzu hairy roots in vivo**

$^{14}$C-sucrose (MP Biomedicals, Inc., Irvine, CA), 120 mg, with a specific activity of 370.0 MBq/mmol (10.00 mCi/mmol) was dissolved in 125 mL distilled water to create a concentrated stock solution, which was then filter sterilized (0.2 µm filter, Nalgene, Rochester, NY). Concentrated liquid MS media (without gellan gum) was prepared by dissolving all constituents in distilled water at 80% of the final concentration and then sterilized by autoclave.

Tip segments of approx. 15 cm from actively growing kudzu ($P. montana$ var. $lobata$ - USDA no. PI 434246) hairy roots were transferred from solid MS media to 40 mL concentrated liquid MS media (without gellan gum) in 250 mL flasks, on a gyrotory shaker (New Brunswick Scientific Co., Edison, NJ) at 150 rpm. 10 mL $^{14}$C-sucrose stock solution 10.79 MBq (291.7 µCi) was then added to each flask to bring the final media volume to 50 mL. Twelve cultures were treated in this manner and hairy roots were allowed to grow in the dark for a period of 3 weeks in an enclosed Plexiglas labeling chamber, designed to capture respired $^{14}$CO$_2$.$^{(27, 35)}$

Hairy roots were harvested by emptying cultures into a Buchner funnel containing a Whatman #4 filter, connected to a suction filtration flask. Fresh weight was recorded, roots were frozen at -20°C, and frozen samples were loaded onto a freeze-dryer until all roots were completely dry. Hairy roots were extracted with 80% methanol as previously described.
Fractionation of radiolabeled isoflavone-containing extracts

Radiolabeled kudzu dry crude extract was successively extracted with petroleum ether, ethyl acetate, butanol and water fractions by liquid-liquid partitioning as follows: The crude extract was mixed with distilled water in a separatory funnel, and partitioned with petroleum ether. This process was repeated 4 more times, adding fresh petroleum ether each time. Petroleum ether extracts were combined and solvent was removed using a rotary evaporator to afford the petroleum ether extract. The aqueous phase was then partitioned with ethyl acetate a total of 10 times. The ethyl acetate was removed by a rotary evaporator to afford the ethyl acetate extract. The aqueous layer was finally partitioned with butanol 5 times, and solvent evaporated to afford the butanol extract. The aqueous layer was then freeze dried. Dried ethyl acetate and butanol fractions were then combined and partitioned again by the procedure above.

The combined ethyl acetate and butanol fractions from the second partitioning procedure was then fractionated on a Sephadex LH-20 gel filtration column (Sigma-Aldrich, St. Louis, MO), using ethanol as the eluting solvent, followed by methanol to wash the column of residual extract. Sephadex fractions were then tested by thin layer chromatography (TLC) on Silica Gel 60 F254 Plates (EMD Chemicals Inc., Gibbstown, NJ), using a solvent system consisting of ethyl acetate, methanol, and distilled water (77:13:10). TLC plates were visualized using vanillin/sulfuric acid reagent and heating to 105 °C for 5 minutes. According to TLC profiles, similar fractions were combined, solvent was removed with a rotoevaporator, and residual water was removed with a freeze-dryer. Isoflavone content of each fraction was evaluated by HPLC-PDA and the isoflavone-rich fractions were combined.
Metabolic tracking of $^{14}$C-labeled, isoflavone-rich fraction in rats

Male Sprague-Dawley rats (n=7, body weight 250-300g), acclimatized to a 12 hour photoperiod and a polyphenol-free diet (AIN93G, Dyets Inc., Bethlehem, PA) for 7 days were anesthetized by intraperitoneal injection (ip) with ketamine-xylazine (0.1 mL/100 g body weight ip, 100 mg ketamine-10 mg xylazine/mL, Henry Schein Inc., Melville, NY). A locking intracerebral guide and stylet (MD-2251, Bioanalytical Systems, Inc., West Lafayette, IN) was inserted stereotaxically into the hippocampal fissure of the brain, using the three-dimensional stereotaxic coordinates from a rat brain atlas. After completion of the stereotaxic surgery, the anesthesia was changed to 3-5% isofluorane, delivered with oxygen at 2-4 L/min, and a femoral catheter (CX-2020S, Bioanalytical Systems, Inc.) was inserted into the right femoral vein and an ultrafiltrate probe (MF-7023/UF-3-12, Bioanalytical Systems, Inc.) was implanted subcutaneously along the dorsal midline. Following surgery, the rats were administered buprenex (0.1 mL/100 g body weight ip, 0.03 mg buprenex/mL, Henry Schein Inc.) in saline (0.9% NaCl w/v) for pain relief and connected to a Culex® Automated Blood Sampling System (Bioanalytical Systems, Inc.) for blood, interstitial fluid (ISF), brain microdialysate, urine, and feces collection. Rats were given 48 hours for recovery. Food (AIN93G) and water were provided ad libitum and the catheter was flushed with 10 µL of heparinized saline (10 units heparin/mL saline (0.9% NaCl w/v)) every 15 minutes. The ultrafiltrate probe was attached to a peristaltic pump to collect ISF at a rate of 1 µL/min. All procedures were approved by the Purdue Animal Care and Use Committee (PACUC), which adheres to policies set forth by the United States Department of Agriculture (USDA) and the United States Public Health Service (USPHS).
Rats were food-deprived for 8 hours prior to gavage. One hour before dosing, the brain microdialysate stylet was removed from the intracerebral guide and a brain microdialysate probe with a 4 mm membrane (MD-2204/BR-4, Bioanalytical Systems, Inc.) was inserted into the hippocampal fissure through the intracerebral guide. The probe inlet was attached to a syringe pump and perfused with artificial cerebrospinal fluid at a rate of 1 µL/min. Microdialysate was collected from the probe outlet, ISF was collected from the ultrafiltrate probe, and 200 µL blood was drawn prior to gavage as the baseline.

Approximately 60 mg/kg bw of $^{14}$C-labeled, isoflavone-rich kudzu extract 8.614 MBq/g (232.8 µCi/g) in 1 mL of distilled water was administered to the rats by gavage, followed by an immediate rinse of the gavage needle with 0.5 mL of distilled water. The Culex system automatically sampled 200 µL blood at 5, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 480, 600, 720, and 1440 minutes after gavage and collected ISF and brain microdialysate at 60 minute intervals for a total of 24 hours. Urine and feces were collected for 24 hours. Food was returned 240 minutes after gavage.

Rats were sacrificed 24 hours after gavage. They were anesthetized with ketamine-xylazine (0.1 mL/100 g body weight ip, 100 mg ketamine-10 mg xylazine/mL, Henry Schein Inc.) and exsanguinated. Blood was transferred to heparinized microcentrifuge tubes, centrifuged at 5000 x g for 10 minutes, and plasma was collected for analysis. The vascular system was perfused with cold saline to remove blood from tissues. An incision was made through the left jugular vein and the rat was perfused through the right femoral catheter with 240 mL chilled saline, which was sufficient to produce clear perfusate that exited the incision in the left jugular vein. Perfusate was also collected for analysis. The stomach, small intestines, large intestines, brain, heart, lungs, liver, kidneys, and testes were placed into scintillation vials. The contents of
the stomach, small intestines and large intestines were collected by flushing the organs through with 3, 5, and 10 mL saline (0.9% NaCl), respectively. Femurs, tibias, and vertebrae were carefully removed and placed into scintillation vials. All samples were stored at -80 °C until ready for analysis.

14C activity from serum, ISF, gut contents, feces, urine, bone, and tissue samples was measured with a Beckman LS 6500 scintillation counter (Beckman Coulter, Inc., Fullerton, CA). Bio-Safe II scintillation cocktail (Research Products International Corp., Mt. Prospect, IL), 20 mL, was added to all sample vials and allowed to sit overnight in the dark, prior to analysis in the scintillation counter. Tissue samples, gut contents, and feces were prepared by freeze-drying, grinding dried samples to a fine powder, and extracting 14C-labeled compounds overnight with 80% methanol. Bones were cleaned, freeze-dried, and ground to a fine powder. Samples of ground bone of a known mass were dissolved in concentrated nitric acid overnight to achieve uniformity for scintillation counting. Residual radiolabel in each animal carcass was analyzed by liquefying in 10% potassium hydroxide for one week, neutralizing with 12 N hydrochloric acid, and homogenizing for even consistency. Brain microdialysate was analyzed by accelerator mass spectrometer (AMS) (Purdue University, West Lafayette, IN)

ISF was collected at an average rate of 60 µL/hr. The ultrafiltrate probe tubing was 48” in length, with a total internal volume of approximately 12 µL. This internal volume presents a delay between the time ISF is collected and the time ISF reaches the sample collection vial. To account for this delay and to more accurately reflect the amount of 14C label present in the ISF at any given time, the ISF collection time points illustrated in Figure 3.5 were shifted 12 minutes earlier than actually collected. Total serum and ISF volumes were approximated based on the
body weight of each rat \(^{(36)}\) and were used to estimate the percent of \(^{14}\)C-labeled compounds remaining in each respective fluid 24 hours after gavage.

Approximately 1 g of dried, finely ground femurs was added to 50 mL of 80% methanol for extraction of metabolites. Methanolic extract was centrifuged at 5000 RPM for 10 minutes and filtered with a Whatman #4 filter, a Whatman #1 filter, and then a Millipore 0.20 \(\mu\)m nylon filter (Millipore, Billerica, MA). Filtered bone extract was dried with a rotoevaporator and a freeze-dryer. Dried extract was weighed, redissolved in 500 \(\mu\)L of 80% methanol, and filtered with a 0.45 \(\mu\)m nylon syringe filter. A 200 \(\mu\)L sample of the femur extract was spiked with commercial isoflavone standards (puerarin, daidzin, genistin, daidzein, and genistein) for comparison. The remaining 300 \(\mu\)L and the spiked extract were analyzed by HPLC-ESI-MS using the same methodology previously described.

**RESULTS AND DISCUSSION**

**Induction of hairy roots in kudzu genotypes**

Transformation of kudzu hairy roots was positively confirmed by visual observation of the typical hairy root phenotype, rapid root growth in growth-regulator-free media, and analysis for the presence of cucumopine by HVPE. The presence of cucumopine in all 4 hairy root extracts was confirmed by comparison to the authentic standard, as shown in Figure 3.1. Cucumopine was absent in all non-transgenic kudzu root extracts, which served as negative controls. Synthesis of cucumopine in hairy root samples verified the transfer of genetic material from *A. rhizogenes* into each kudzu genotype \(^{(33)}\). Endogenous histidine was present in all root
samples and was stained in the electrophoretogram when sprayed with Pauly reagent, while no histidine was present in the cucumopine standard.

The major isoflavones were identified using HPLC-ESI-MS, in the positive ion mode, in each kudzu hairy root genotype as hydroxy-puerarin, puerarin, daidzin, apiosyl/xylosyl-glucosyl-genistein, genistin, malonyl daidzin, malonyl genistin, daidzein, and genistein at retention times 7.3, 8.7, 10.2, 10.8, 11.8, 12.4, 14.2, 15.7, and 18.7 min, respectively. Quantification using HPLC-PDA showed that *P. montana* var. *lobata* (USDA no. PI 434246, Origin: USA) yielded the highest puerarin content and total isoflavone content at 53.4 and 129.9 mg/g dry extract (Table 3.1 and Figure 3.2), respectively. *P. lobata* exhibited lower total isoflavone content at only 58.9 mg/g dry extract. *P. phaseoloides* yielded no detectable quantities of hydroxy-puerarin, puerarin, apiosyl/xylosyl-glucosyl-genistein, or genistin, but instead produced the highest quantities of daidzein and genistein among the genotypes evaluated at 21.9 and 18.8 mg/g dry extract, respectively. The isoflavone profile of *P. montana* var. *lobata* from Japan was similar to that of *P. montana* var. *lobata* from the USA, however the Japanese genotype contained lower puerarin and total isoflavone contents at 50.5 and 108.2 mg/g dry extract, respectively. *P. montana* var. *lobata* from the USA yielded a significantly greater total isoflavone content than the other 3 genotypes tested (p < 0.001).

Fresh mass measurements showed that *P. montana* var. *lobata* (USDA no. PI 434246, Origin: USA) yielded the greatest amount of new fresh mass per 2 week subculture cycle among the 4 genotypes tested at approx. 3.57 g per culture (Table 3.2). This genotype also yielded the greatest amount of isoflavones per quantity of fresh roots at 58.5 mg isoflavones per 10 g fresh root mass. Based on the isoflavone yields and isoflavone profiles of the 4 genotypes tested, hairy
roots from *P. montana var. lobata* (USDA no. PI 434246, Origin: USA) were selected for subsequent radiolabeling trials.

**Extraction of radiolabeled isoflavones**

From each 15 cm root segment (fresh mass approx. 0.1 g at time of subculture) of *P. montana var. lobata* (USDA no. PI 434246, Origin USA), final root cultures averaging 13.1 g each were harvested after 21 days. This indicates that on average, 99.2% of the fresh root mass from each of the 12 harvested root cultures was comprised of new growth. New growth incorporates and utilizes both $^{14}$C-radiolabeled and unlabeled sucrose without discrimination. From this root growth data, we conclude that all new root tissue and its synthesized phytochemicals were homogeneously radiolabeled with $^{14}$C. Radiolabeled kudzu hairy roots (156.7 g fresh weight) were lyophilized to a final dry weight of 8.8 g. Dry hairy roots were extracted with 80% methanol, roteovaporated, and then freeze-dried. The crude extract (3.8 g) contained homogeneously-labeled isoflavones at a concentration of 52.4 mg/g extract. In the current study, approx. 17.0% of the total $^{14}$C dose was incorporated into the crude extract, which had an average radioactivity of 5.839 MBq/g (157.8 µCi/g) extract, which was comparable to previous research with non-transgenic kudzu (*P. lobata*) roots that reported radiolabel incorporation of 18.7% of the $^{14}$C dose$^{(26)}$. The predominant isoflavones in the crude, radiolabeled kudzu extract were puerarin, malonyl-daidzin, and daidzin at 27.2, 11.4, and 4.1 mg/g extract, respectively, as identified by HPLC-ESI-MS, quantified by HPLC-PDA, and shown in Table 3.3 and Figure 3.3.
Enrichment of radiolabeled isoflavones

Partitioning of the crude extract produced isoflavone-enriched ethyl acetate and butanol fractions, while removing undesirable, unidentified compounds in petroleum ether and water fractions. After the first solvent extraction, 65.8% of the crude extract was isolated in the water-soluble fraction and 1.8% in the petroleum ether fraction, both of which contained negligible quantities of isoflavones. The ethyl acetate fraction contained primarily puerarin, apiosyl/xylosyl-glucosyl-genistein, daidzin, and malonyl-genistin at 109.0, 24.5, 12.5, and 9.4 mg/g, respectively, while the butanol fraction contained predominantly puerarin, malonyldaidzin, malonyl-genistin, and daidzin at 61.1, 28.0, 7.5, and 6.9 mg/g, respectively (Table 3.4). Enrichment of major isoflavones increased from 5.2% in the crude extract to 17.8% in the ethyl acetate fraction and 11.9% in the butanol fraction. Ethyl acetate and butanol fractions were combined, repartitioned by the same method, dried down, and reanalyzed. Additional undesirable material was separated out through the second partitioning, in the water and petroleum ether fractions, which contained very low concentrations of isoflavones, as indicated in Table 3.5. The percentage of total isoflavones in ethyl acetate and butanol fractions improved in the second partitioning from 17.8% to 31.5% and from 11.9% to 20.7%, respectively. While liquid-liquid partitioning greatly improved the concentration of the major isoflavones in these two fractions over the crude extract, these two fractions were combined again and further fractionated to boost enrichment.

Sephadex LH-20 column fractionation of the combined ethyl acetate and butanol fractions yielded 83 new fractions, each of which were rotoevaporated, freeze-dried, and evaluated by thin layer chromatography for the presence of isoflavones. Fractions containing high concentrations of isoflavones were combined and evaluated by HPLC-PDA, as summarized
in Table 3.6. Fractions 5-8, 10-12, 24-28, and 72-82 were combined based on their estimated enrichment, dried down, and reassessed by HPLC-ESI-MS (Figure 3.4) and HPLC-PDA (Table 3.3). The resulting fraction weighed 322.7 mg, contained 588.1 mg isoflavones/g fraction (58.8% enrichment), and had an average radioactivity of 8.614 MBq/g (232.8 µCi/g) fraction. The predominant isoflavones in the isoflavone-enriched fraction were puerarin, daidzin, and malonyl-daidzin at 395.4, 65.2, and 57.0 mg per g fraction, respectively, as shown in Table 3.3. The level of enrichment of total isoflavones in the final fraction was over 10-fold higher than in the crude extract and the concentration of individual isoflavones was increased in the enriched fraction by as much as 15 times. Loss of malonyl-genistin in the isoflavone-rich fraction despite an increase in concentration of every other major isoflavone may be attributed to its strong susceptibility to degradation \(^{(37)}\). The presence of genistein in the isoflavone-rich fraction despite its absence in the crude extract was due to its rise in concentration into detectable levels.

Metabolic tracking of radiolabeled, isoflavone-rich fraction in rats

In a previous study, we demonstrated that a dose of 0.1480 MBq (4 µCi) of \(^{14}\)C-labeled crude extract containing isoflavones could be traced effectively \textit{in vivo} using a scintillation counter \(^{(27)}\) and these results were used as a basis for calculating the dose size for the current study. With an average radioactivity of 8.614 MBq/g (232.8 µCi/g) fraction, an oral dose of 60 mg of \(^{14}\)C-labeled, kudzu isoflavone-rich fraction per kg body weight was needed for each rat. Naturally occurring levels of \(^{14}\)C in each rat were established by analysis of baseline serum, ISF, urine, and feces by scintillation counter.
Serum pharmacokinetics showed rapid absorption of the isoflavone-rich fraction, with serum reaching peak $^{14}$C-label concentrations approx. 30 minutes after gavage, at an average of 0.053% of the administered $^{14}$C dose per mL serum (Figure 3.5). As seen previously in the metabolic tracking of radiolabeled kudzu crude extract $^{(27)}$, a second peak in serum concentration was observed 1 h after gavage of the isoflavone-rich fraction and may be attributed to varied rates of isoflavone absorption. Isoflavone aglycones, for instance, are absorbed faster and in higher amounts than their glucosides $^{(38)}$. A plateau in serum concentration at 4 h after gavage, which followed the trend seen in the metabolic tracking of $^{14}$C-kudzu crude extract $^{(27)}$, is consistent with the enterohepatic circulation of kudzu isoflavones $^{(39)}$. Plasma collected 24 hours after gavage showed no difference in $^{14}$C activity compared to serum collected 24 hours after gavage. Approximately 0.459% of the $^{14}$C-labeled dose remained in the perfusate 24 hours after gavage.

ISF concentrations reached peak levels of 0.029% of the administered dose 1-2 h after gavage. Analysis of serum and ISF over the course of 24 h showed that kudzu phytochemicals were rapidly metabolized and eliminated. Excrement contained the greatest amount of the administered dose at 8.384% and 26.157% in the urine and feces, respectively (Table 3.7). The amount of $^{14}$C in the urine was likely underestimated, due to losses in the urine-collection funnel.

The radiolabeled, isoflavone-rich fraction accumulated an average of 0.016% of the $^{14}$C-dose in the brain, which is consistent with puerarin (and/or its metabolites) crossing the blood-brain barrier $^{(40)}$. The liver accumulated 0.335% of the isoflavone-rich dose and accumulated the greatest quantity of radiolabel among tissues analyzed (Table 3.7), which is consistent with the distribution of $^{14}$C-labeled kudzu crude extract $^{(27)}$. Kidneys, lungs, heart, testes and spleen
accumulated 0.029%, 0.012%, 0.004%, 0.042%, and 0.004% of the administered dose, respectively.

Stomach, small intestine, and large intestinal tissues contained 0.030%, 0.335%, and 0.883% of the total dose, respectively and the gastrointestinal contents contained a significant portion of the isoflavone-rich dose, at 0.042%, 1.306%, and 11.821% in the stomach contents, small intestinal contents, and large intestinal contents respectively. In this study, a much greater percentage of the $^{14}$C-labeled dose was accounted for in the large intestinal contents and feces than was previously observed for the metabolic tracking of kudzu crude extract (27). This may be attributed to the freeze-drying of each sample and scintillation counting of a sample of dry mass rather than by scintillation counting of fecal slurry in the previous study, resulting in a more accurate measure of radiolabel in each sample.

The radiolabeled fraction also reached muscle and adipose tissues, accumulating an average of 0.011%, and 0.019% of the total $^{14}$C dose in each gastrocnemius muscle and in the total abdominal adipose of each animal, respectively. All residual $^{14}$C in the carcass of each rat was successfully analyzed by scintillation counting of chemically liquefied bones and tissues. Each rat retained an average of 5.208% of the administered dose in the tissues and bones that were not removed for individual analysis.

All tissues accumulated less $^{14}$C-label when rats were gavaged with the radiolabeled isoflavone-rich fraction in the current study, than was observed when rats were gavaged with the radiolabeled crude kudzu extract in an earlier study (27). This may be attributed to the removal of non-target phytochemicals from the crude extract, including sugars, which would also have acquired radiolabel, and would have been readily absorbed and distributed throughout all animal
tissues. Rats gavaged with the isoflavone-rich kudzu fraction accumulated 0.011%, 0.09%, and 0.003% of the administered dose in each femur, tibia, and vertebrae, respectively (Table 3.7). While these values were lower than those collected from the metabolic tracking of $^{14}$C-labeled kudzu crude extract (27), it was with greater certainty that kudzu isoflavones reached each target tissue and were available to exert an effect on bone health. This conclusion was further supported by HPLC-ESI-MS analysis of the methanolic extract from femur bones. A peak that eluted at retention time ($t_R$) 8.7 min in the UV chromatogram (262 nm) contained peaks in the MS spectrum with $m/z$ (mass-to-charge ratio) 593 and 417 (Figure 3.6b). These peaks are indicative of the presence of puerarin ($m/z$ 417) and puerarin glucuronide ($m/z$ 593, 417) as observed by Prasain et al. in organs of spontaneously hypertensive rats that were gavaged with puerarin (39). The presence of puerarin glucuronide in bone tissue is also consistent with the identification of glucuronidated metabolites of puerarin by Luo et al. in serum, liver, and intestines of rats administered with intravenous puerarin (41). Another peak at $t_R$ 15.7 in the UV chromatogram (262 nm, $m/z$ 255) (Figure 3.6c) corresponded to daidzein in the femur extract that was spiked with several isoflavone standards. Peaks that corresponded with isoflavone-metabolites in isoflavone-treated rat femur extracts (Figure 3.6) were not present in sham-treated rat femur extracts (Figure 3.7).

**CONCLUSIONS**

_in vitro_ production of kudzu isoflavones was enhanced through the induction of hairy root synthesis in 4 kudzu genotypes and the selection of one genotype with the highest isoflavone yields. Kudzu hairy root cultures from the most productive genotype were
radiolabeled using $^{14}$C-sucrose and extracted for isoflavones. Isoflavone enrichment was improved through solvent extraction and Sephadex filtration to produce a $^{14}$C-radiolabeled, isoflavone-rich fraction, which was metabolically tracked in rats. The data presented in this study demonstrated that bioactive isoflavones from kudzu when orally administered at a dose of 60 mg/kg body weight to male Sprague-Dawley rats accumulated in bone tissues at 0.011%, 0.009%, and 0.003% of the administered dose in femur, tibia, and vertebrae, respectively. This study therefore supports the hypothesis that kudzu isoflavones are capable of reaching bone tissues to exert effects that promote bone formation and prevent bone loss. Furthermore, these results suggest that puerarin, daidzein, and puerarin glucuronide may be implicated in the beneficial effects of kudzu on bone health and warrant future investigation.

**ACKNOWLEDGEMENTS**

This work was supported by the National Center for Complementary and Alternative Medicine sponsored Purdue-UAB Botanicals Center for Dietary Supplement Research (NIH, 2 P50 AT000477-06).
TABLES

Table 3.1: Average isoflavone content of kudzu (*Pueraria* spp.) hairy root genotypes

Table 3.2: Average yields of kudzu (*Pueraria* spp.) hairy root cultures

Table 3.3: Isoflavone profiles of the crude extract and the isoflavone-rich fraction from *Pueraria montana* var. *lobata*

Table 3.4: Isoflavone profiles of the partitioned crude extract from *Pueraria montana* var. *lobata*

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Table 3.6: Isoflavone profiles of Sephadex fractions from *Pueraria montana* var. *lobata*

Table 3.7: Analysis of $^{14}$C-labeled kudzu isoflavone-rich fraction distribution in rats 24 h after gavage by scintillation counter (percent of the orally administered dose)
Table 3.1: Average isoflavone content of kudzu (Pueraria spp.) hairy root genotypes

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>t_R (min)</th>
<th>P. montana var. lobata (Origin: Japan)</th>
<th>P. montana var. lobata (Origin: USA)</th>
<th>P. lobata (Origin: USA)</th>
<th>P. phaseoloides (Origin: Guatemala)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxy-puerarin</td>
<td>7.3</td>
<td>8.1 ± 0.5</td>
<td>11.4 ± 0.0</td>
<td>5.7 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Puerarin</td>
<td>8.7</td>
<td>50.5 ± 2.4</td>
<td>53.4 ± 0.8</td>
<td>15.5 ± 0.1</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Daidzin</td>
<td>10.2</td>
<td>19.1 ± 1.2</td>
<td>17.5 ± 0.2</td>
<td>12.5 ± 0.1</td>
<td>6.5 ± 0.3</td>
</tr>
<tr>
<td>Api/Xyl-G-genistein</td>
<td>10.8</td>
<td>7.1 ± 0.8</td>
<td>6.2 ± 0.0</td>
<td>1.8 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Genistin</td>
<td>11.8</td>
<td>1.4 ± 0.1</td>
<td>2.8 ± 0.0</td>
<td>2.3 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Malonyl-daidzin</td>
<td>12.4</td>
<td>15.2 ± 0.8</td>
<td>19.7 ± 0.1</td>
<td>13.3 ± 0.2</td>
<td>7.0 ± 0.1</td>
</tr>
<tr>
<td>Malonyl-genistein</td>
<td>14.2</td>
<td>1.4 ± 0.1</td>
<td>2.3 ± 0.1</td>
<td>1.8 ± 0.0</td>
<td>4.8 ± 0.0</td>
</tr>
<tr>
<td>Daidzein</td>
<td>15.7</td>
<td>4.7 ± 0.3</td>
<td>14.8 ± 1.1</td>
<td>5.3 ± 0.2</td>
<td>21.9 ± 0.0</td>
</tr>
<tr>
<td>Genistein</td>
<td>18.7</td>
<td>0.7 ± 0.1</td>
<td>1.7 ± 0.5</td>
<td>0.8 ± 0.0</td>
<td>18.8 ± 0.3</td>
</tr>
<tr>
<td>Total Isoflavones</td>
<td></td>
<td>108.2 ± 3.0</td>
<td>129.9 ± 1.5</td>
<td>58.9 ± 0.3</td>
<td>59.1 ± 0.4</td>
</tr>
</tbody>
</table>

Values represent the sample mean ± the standard error, n=4.
Table 3.2: Average yields of kudzu (*Pueraria* spp.) hairy root cultures

<table>
<thead>
<tr>
<th>Kudzu Hairy Root Genotype</th>
<th>Fresh Root Mass per Culture (g)</th>
<th>Isoflavones Recovered per 10 g Fresh Roots (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. montana</em> var. <em>lobata</em> (Origin: Japan)</td>
<td>1.46 ± 0.20</td>
<td>39.6 ± 2.1</td>
</tr>
<tr>
<td><em>P. montana</em> var. <em>lobata</em> (Origin: USA)</td>
<td>3.57 ± 0.73</td>
<td>58.5 ± 1.0</td>
</tr>
<tr>
<td><em>P. lobata</em> (Origin: USA)</td>
<td>2.89 ± 0.29</td>
<td>35.3 ± 0.2</td>
</tr>
<tr>
<td><em>P. phaseoloides</em> (Origin: Guatemala)</td>
<td>2.76 ± 0.32</td>
<td>27.6 ± 0.3</td>
</tr>
</tbody>
</table>

Values represent the sample mean ± the standard error, n=4.
Table 3.3: Isoflavone profiles of the crude extract and the isoflavone-rich fraction from *Pueraria montana* var. *lobata*

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>t&lt;sub&gt;R&lt;/sub&gt; (min)</th>
<th>[M + H'] (m/z)</th>
<th>Product Ions (m/z)</th>
<th>Isoflavone Concentration (mg isoflavone/g fraction)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Crude Extract</td>
</tr>
<tr>
<td>Hydroxy-puerarin</td>
<td>7.4</td>
<td>433</td>
<td></td>
<td>1.9</td>
</tr>
<tr>
<td>Puerarin</td>
<td>8.5</td>
<td>417</td>
<td></td>
<td>27.2</td>
</tr>
<tr>
<td>Daidzin</td>
<td>9.8</td>
<td>417</td>
<td>255</td>
<td>4.1</td>
</tr>
<tr>
<td>Api/Xyl-G-genistein</td>
<td>10.5</td>
<td>565</td>
<td>433, 271</td>
<td>2.9</td>
</tr>
<tr>
<td>Genistin</td>
<td>11.8</td>
<td>433</td>
<td>271</td>
<td>0.9</td>
</tr>
<tr>
<td>Malonyl-daidzin</td>
<td>12.4</td>
<td>503</td>
<td>255</td>
<td>11.4</td>
</tr>
<tr>
<td>Malonyl-genistin</td>
<td>14.3</td>
<td>519</td>
<td>271</td>
<td>3.6</td>
</tr>
<tr>
<td>Daidzein</td>
<td>15.5</td>
<td>255</td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>Genistein</td>
<td>18.8</td>
<td>271</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Isoflavones</td>
<td></td>
<td></td>
<td></td>
<td>52.4</td>
</tr>
</tbody>
</table>

t<sub>R</sub>, retention time; m/z, mass:charge ratio; Api, Apiosyl; Xyl, Xylosyl; G, Glucosyl.

Retention times in Table 3.4 correspond with Figure 1.
Table 3.4: Isoflavone profiles of the partitioned crude extract from *Pueraria montana* var. *lobata*

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Petroleum Ether</th>
<th>Ethyl Acetate</th>
<th>Butanol</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxy-puerarin (mg/g fraction)</td>
<td>0.4</td>
<td>5.6</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td>Puerarin (mg/g fraction)</td>
<td>6.7</td>
<td>109.0</td>
<td>61.1</td>
<td></td>
</tr>
<tr>
<td>Daidzin (mg/g fraction)</td>
<td>1.1</td>
<td>12.5</td>
<td>6.9</td>
<td>0.8</td>
</tr>
<tr>
<td>Api/Xyl-G-genistein (mg/g fraction)</td>
<td></td>
<td>24.5</td>
<td>2.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Genistin (mg/g fraction)</td>
<td>0.1</td>
<td>1.3</td>
<td>1.8</td>
<td>0.7</td>
</tr>
<tr>
<td>Malonyl-daidzin (mg/g fraction)</td>
<td>2.2</td>
<td>8.5</td>
<td>28.0</td>
<td>7.6</td>
</tr>
<tr>
<td>Malonyl-genistin (mg/g fraction)</td>
<td></td>
<td>9.4</td>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td>Daidzein (mg/g fraction)</td>
<td>0.4</td>
<td>4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genistein (mg/g fraction)</td>
<td></td>
<td>1.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Isoflavones (mg/g)</td>
<td>10.9</td>
<td>177.7</td>
<td>119.0</td>
<td>8.9</td>
</tr>
</tbody>
</table>

Mass of Total Fraction (mg) 58.2  291.7  741.4  2102.3
Table 3.5: Isoflavone profiles of *Pueraria montana* var. *lobata* from the partitioned solvent fractions

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Solvent Fraction - Second Partitioning</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Petroleum Ether</td>
</tr>
<tr>
<td>Hydroxy-puerarin (mg/g fraction)</td>
<td>0.9</td>
</tr>
<tr>
<td>Puerarin (mg/g fraction)</td>
<td>15.2</td>
</tr>
<tr>
<td>Daidzin (mg/g fraction)</td>
<td>2.9</td>
</tr>
<tr>
<td>Api/Xyl-G-genistein (mg/g fraction)</td>
<td>5.8</td>
</tr>
<tr>
<td>Genistin (mg/g fraction)</td>
<td>1.0</td>
</tr>
<tr>
<td>Malonyl-daidzin (mg/g fraction)</td>
<td>1.7</td>
</tr>
<tr>
<td>Malonyl-genistin (mg/g fraction)</td>
<td>1.0</td>
</tr>
<tr>
<td>Daidzein (mg/g fraction)</td>
<td>0.6</td>
</tr>
<tr>
<td>Genistein (mg/g fraction)</td>
<td>0.6</td>
</tr>
<tr>
<td>Total Isoflavones (mg/g)</td>
<td>29.1</td>
</tr>
<tr>
<td>Mass of Total Fraction (mg)</td>
<td>11.8</td>
</tr>
</tbody>
</table>
## Table 3.6: Isoflavone Profiles of Sephadex fractions from *Pueraria montana* var. *lobata*

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Sephadex Fraction Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxy-puerarin (mg/g fraction)</td>
<td></td>
</tr>
<tr>
<td>Puerarin (mg/g fraction)</td>
<td></td>
</tr>
<tr>
<td>Daidzin (mg/g fraction)</td>
<td></td>
</tr>
<tr>
<td>Api/Xyl-G-genistein (mg/g fraction)</td>
<td></td>
</tr>
<tr>
<td>Genistin (mg/g fraction)</td>
<td></td>
</tr>
<tr>
<td>Malonyl-daidzin (mg/g fraction)</td>
<td></td>
</tr>
<tr>
<td>Malonyl-genistin (mg/g fraction)</td>
<td></td>
</tr>
<tr>
<td>Daidzin (mg/g fraction)</td>
<td></td>
</tr>
<tr>
<td>Genistein (mg/g fraction)</td>
<td></td>
</tr>
<tr>
<td>Total Isoflavones (mg/g fraction)</td>
<td></td>
</tr>
<tr>
<td>Mass of Total Fraction (mg)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Sephadex Fraction Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxy-puerarin (mg/g fraction)</td>
<td></td>
</tr>
<tr>
<td>Puerarin (mg/g fraction)</td>
<td></td>
</tr>
<tr>
<td>Daidzin (mg/g fraction)</td>
<td></td>
</tr>
<tr>
<td>Api/Xyl-G-genistein (mg/g fraction)</td>
<td></td>
</tr>
<tr>
<td>Genistin (mg/g fraction)</td>
<td></td>
</tr>
<tr>
<td>Malonyl-daidzin (mg/g fraction)</td>
<td></td>
</tr>
<tr>
<td>Malonyl-genistin (mg/g fraction)</td>
<td></td>
</tr>
<tr>
<td>Daidzin (mg/g fraction)</td>
<td></td>
</tr>
<tr>
<td>Genistein (mg/g fraction)</td>
<td></td>
</tr>
<tr>
<td>Total Isoflavones (mg/g fraction)</td>
<td></td>
</tr>
<tr>
<td>Mass of Total Fraction (mg)</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.7: Analysis of $^{14}$C-labeled kudzu isoflavone-rich fraction distribution in rats 24 h after gavage by scintillation counter (percent of the orally administered dose)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Average Percent of Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right Femur</td>
<td>0.011 ± 0.001</td>
</tr>
<tr>
<td>Right Tibia</td>
<td>0.009 ± 0.001</td>
</tr>
<tr>
<td>Vertebrae</td>
<td>0.003 ± 0.000</td>
</tr>
<tr>
<td>Liver</td>
<td>0.335 ± 0.027</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.029 ± 0.002</td>
</tr>
<tr>
<td>Lung</td>
<td>0.012 ± 0.001</td>
</tr>
<tr>
<td>Heart</td>
<td>0.004 ± 0.000</td>
</tr>
<tr>
<td>Testes</td>
<td>0.042 ± 0.007</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.004 ± 0.000</td>
</tr>
<tr>
<td>Brain</td>
<td>0.016 ± 0.002</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>0.011 ± 0.000</td>
</tr>
<tr>
<td>Abdominal Adipose</td>
<td>0.019 ± 0.004</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.030 ± 0.002</td>
</tr>
<tr>
<td>Small Intestines</td>
<td>0.335 ± 0.037</td>
</tr>
<tr>
<td>Large Intestines</td>
<td>0.883 ± 0.132</td>
</tr>
<tr>
<td>Stomach Contents</td>
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<tr>
<td>Small Intestinal Contents</td>
<td>1.306 ± 0.272</td>
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<tr>
<td>Large Intestinal Contents</td>
<td>11.821 ± 2.680</td>
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<tr>
<td>Urine</td>
<td>8.384 ± 0.909</td>
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<tr>
<td>Feces</td>
<td>26.157 ± 3.326</td>
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<tr>
<td>Carcass</td>
<td>5.208 ± 3.005</td>
</tr>
<tr>
<td>Perfusate</td>
<td>0.459 ± 0.047</td>
</tr>
</tbody>
</table>

Values represent mean percentages ± standard error of $^{14}$C activity, n=7.
FIGURES

**Figure 3.1**: Paper electrophoretogram of cucumopine extracts from kudzu hairy roots

**Figure 3.2**: Isoflavone profiles of hairy root-induced kudzu genotypes

**Figure 3.3**: HPLC-ESI-MS spectra showing total ion current (a), HPLC-UV chromatogram (262 nm) (b), and MS spectrum (c) of ¹⁴C-labeled, crude kudzu extract.

**Figure 3.4**: HPLC-ESI-MS spectra showing total ion current (a), HPLC-UV chromatogram (262 nm) (b), and MS spectrum (c) of ¹⁴C-labeled, isoflavone-rich kudzu extract.

**Figure 3.5**: Distribution of ¹⁴C-label for isoflavone-rich kudzu fraction in serum and interstitial fluid of rats during 24 hours after administration

**Figure 3.6**: HPLC-ESI-MS spectra showing total ion current (a), HPLC-UV chromatogram (UV 262 nm) (b), and MS spectrum at t_R 8.7 and t_R 15.7 (c and d, respectively) of methanolic extract of isoflavone-treated rat femurs.

**Figure 3.7**: HPLC-ESI-MS spectra showing total ion current (a), HPLC-UV chromatogram (UV 262 nm) (b), and MS spectrum (c) of methanolic extract of sham-treated rat femurs.
Figure 3.1: Paper electrophoretogram of cucumopine extracts from kudzu hairy roots

His, Histidine; Ccm, Cucumopine; HR, Hairy Roots; NR, Non-transformed Roots
Figure 3.2: Isoflavone profiles of hairy root-induced kudzu genotypes

![Graph showing isoflavone profiles of kudzu genotypes](image)

- Api, Apiosyl; Xyl, Xylosyl; G, Glucosyl. Error bars represent the standard error, n=4.

Data in Figure 3.2 correspond to data in Table 3.1.
Figure 3.3: HPLC-ESI-MS spectra showing total ion current (a), HPLC-UV chromatogram (262 nm) (b), and scan of MS spectrum (c) of $^{14}$C-labeled, crude kudzu extract. $m/z$, mass-to-charge ratio.
Figure 3.4: HPLC-ESI-MS spectra showing total ion current (a), HPLC-UV chromatogram (262 nm) (b), and MS spectrum (c) of $^{14}$C-labeled, isoflavone-rich kudzu extract. *m/z*, mass-to-charge ratio.
**Figure 3.5**: Distribution of $^{14}$C-label for isoflavone-rich kudzu fraction in serum and interstitial fluid of rats during 24 hours after administration.
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Figure 3.7: HPLC-ESI-MS spectra showing total ion current (a), HPLC-UV chromatogram (UV 262 nm) (b), and MS spectrum (c) of methanolic extract of sham-treated rat femurs.
LITERATURE CITED


CHAPTER 4: Elicitation of kudzu (P. montana var. lobata) hairy roots to promote synthesis of isoflavones in vitro

ABSTRACT

Kudzu hairy roots were elicited in vitro with acetate and salicylic acid at varied concentrations and exposure durations in an attempt to identify conditions favoring maximal isoflavone production. Treatment of kudzu hairy roots with acetate resulted in no significant increase in isoflavone content, and in fact a significant decline in puerarin and total isoflavone content was observed as exposure time increased at 0.10% and 1.00% acetate concentrations. Puerarin and total isoflavone yield in kudzu hairy roots declined upon initial exposure to salicylic acid, then rose after exposure to salicylic acid for 24 h, followed by a decline in isoflavone levels after 72 h exposure. Puerarin levels, but not total isoflavone levels, were significantly higher in hairy roots that were treated with salicylic acid at all concentrations for 24 h than non-elicited hairy roots. None of the treatment combinations for each elicitor yielded significantly greater puerarin or total isoflavone content than at least one of the non-elicited groups, given these particular treatment conditions.

INTRODUCTION

Kudzu (Pueraria montana var. lobata), a leguminous species native to eastern Asia, is considered invasive throughout the southeastern United States \(^{(1)}\). Kudzu root has been used for
2200 years in traditional Chinese herbal medicines as an antipyretic, antidiarrhetic, diaphoretic, antiemetic, and an anti-alcohol abuse agent (1, 2). The health-promoting qualities of kudzu root have been associated with the presence of a class of plant secondary metabolites called isoflavones. The predominant kudzu isoflavone, puerarin, is unique among the isoflavones produced by other leguminous species due to its C-glycosidic linkage, which confers resistance to hydrolysis in the gastrointestinal tract during digestion, resulting in the uptake of intact puerarin into the body (3). Recent studies of kudzu root extracts and puerarin have demonstrated benefits to bone health (4-7), cardiovascular health (8), neurological health (9, 10), and blood glucose homeostasis (8, 11).

More efficient, reproducible production methods for isoflavones are needed, as they are not readily produced synthetically and require in planta synthesis (12). One approach for improving isoflavone production is to identify genotypes with consistently high isoflavone yields (13, 14). Wild-crafted plants have been used as a source of secondary metabolites; however their metabolite synthesis is highly variable and conditioned by environmental and ecological factors (15). Alternatively, plants grown under the controlled environmental conditions of in vitro culture can be regulated towards highly reproducible secondary metabolite synthesis and by virtue of the simpler structure of cultured cells or tissue, the yield can be enhanced (16). Kudzu roots yield greater concentrations of isoflavones than shoots or leaves (17). However, in vitro root cultures exhibit a slow growth rate (18, 19). To circumvent this problem, the soil-borne bacterium, Agrobacterium rhizogenes, has been used to induce synthesis of hairy roots, which are characterized by highly branching, rapid growth rate, without inhibition of secondary metabolite synthesis (18). Another promising technique for increasing efficiency of secondary metabolite synthesis is the treatment of plant tissues with an elicitor. Elicitors are varied biotic or abiotic
stimuli, which include climatic, microenvironmental, chemical, and physical stressors that influence the synthesis and accumulation of bioactive secondary metabolites (20). These stimuli often induce the enzymatic pathways involved in a defense response to pathogen attack, ultraviolet light, or wounding (21, 22). The phenylpropanoid pathway leads to the synthesis of an extensive collection of biologically active compounds, including isoflavones, and is capable of being influenced by various chemical elicitors (20, 22-25). Acetate and salicylic acid are both candidate elicitors that have been investigated for their capacity to stimulate the biosynthesis of phytochemicals with antibacterial, antifungal, and anticancer activity in a wide variety of plants for drug discovery (20).

The efficacy of acetate as an elicitor was demonstrated in species from nine different plant families, including Fabaceae (or Leguminosae) (20). In this study, acetate increased bioactivity in 13 of the 22 elicited leguminous species (20). When exposed to plants or plant parts, acetic acid increased cytotoxicity of plant root extracts against breast, central nervous system, and lung cancer cells in over 40% of the plant species screened and antibacterial or antifungal activity in over 30% of plant species screened (26). Foliar application of acetic acid to red clover (Trifolium pratense L.) demonstrated that the total isoflavone content in the foliage could be increased by an average of 15%, at both 2 and 8 days after spraying (22). Radioisotope tracer studies of 14C-labeled acetate and 14C-labeled shikimic acid have established that acetate is a flavonoid precursor that provides a six carbon ring to flavonoid biosynthesis (27, 28), though it is still not clear whether acetate also plays a part in activating specific biosynthetic pathways.

Salicylic acid has also demonstrated potential to elicit synthesis of antibacterial, anticancer, and antifungal phytochemicals across a wide range of plant species (20). In transgenic
tobacco and *Arabidopsis thaliana* that express the bacterial enzyme, salicylate hydroxylase, plants are unable to accumulate salicylic acid and are more susceptible to viral, fungal, and bacterial infection (29). Salicylic acid likely plays a role in localized and systemic disease resistance as an endogenous defense signal and has been shown to accumulate in response to viral (30) and bacterial (31) infections, as well as ultraviolet light and ozone exposure (32). The disease resistance associated with salicylic acid is in part due to synthesis of pathogenesis-related proteins, which possess antimicrobial activity (26), but is also attributed to the increased phenylalanine ammonia lyase (PAL) activity and synthesis of new PAL protein (33). PAL is a crucial enzyme involved in the formation of the class of plant secondary metabolites, phenylpropanoids, which include isoflavones. In hydroponically cultivated yellow lupine (*Lupinus luteus* L.) plants, genistein biosynthesis and secretion from root tissues was more than 24 times greater in roots that were removed from nutrient solution and placed into distilled water containing salicylic acid than those placed in only distilled water (23). This study also demonstrated relationships between time and dose response to methyl jasmonate, chitosan, and KCN elicitors in yellow lupine. Genistein rhizosecretion produced a bell-shaped dose-response curve with increasing elicitor concentration and increased for a period of one day following elicitor treatment, followed by a gradual decline (23).

In the current study, we investigated the potential of acetate and salicylic acid to increase isoflavone synthesis in highly productive kudzu hairy roots *in vitro* at varied elicitor concentrations and exposure durations.
MATERIALS AND METHODS

Plant material

Prior analyses of isoflavone profiles and yields from four hairy root-induced kudzu genotypes demonstrated that P. montana var. lobata (USDA no. PI 434246, Origin: USA) was capable of producing the greatest quantity of puerarin and total isoflavones among the genotypes evaluated. Therefore, this particular genotype was selected for elicitation trials. Hairy roots were induced using the cucumopine-producing Agrobacterium rhizogenes strain, K599, as previously described. Kudzu hairy roots were maintained on growth regulator-free Murashige and Skoog (MS) media, containing 4.330 g L\(^{-1}\) MS basal salts\(^{(34)}\), Gamborg B5 vitamins\(^{(35)}\), 0.1 g L\(^{-1}\) myoinositol (Phytotechnology Laboratories, Shawnee Mission, K.S.), 30 g L\(^{-1}\) sucrose, 0.1% FeEDTA, and 2 g L\(^{-1}\) gellan gum (Phytotechnology Laboratories, Shawnee Mission, KS). Hairy roots were grown in the dark and subcultured every 2 weeks.

Elicitation of kudzu hairy roots to promote synthesis and accumulation of isoflavones

Approximately 15 cm segments of actively growing, kudzu hairy root tips were transferred from solid MS media to 50 mL liquid MS media (without gelrite) in 250 mL flasks, on a Gyrotory Shaker (New Brunswick Scientific Co., Edison, NJ) at 150 rpm. Hairy roots were allowed to grow for 3 weeks. Culture media was then completely replaced with approximately 48 to 49 mL of fresh liquid MS media.

Distilled water was sterilized by autoclave and used as a control. Sodium acetate (Sigma-Aldrich, St. Louis, MO) stock solutions were prepared at concentrations of 0.04, 0.42, and 4.16
mM. Stock solution pH was adjusted to 5.7 with HCl prior to filter sterilization (0.2 µm nylon filter, Nalgene, Rochester, NY). 2 mL of each stock solution or distilled water were added to each designated culture flask containing 48 mL fresh media to bring final treatment volumes to 50 mL and treatment concentrations to 0, 1.67, 16.65, and 166.52 mM sodium acetate. These treatment concentrations are relatively equivalent to solutions of 0, 0.01%, 0.10%, and 1.00% acetic acid, respectively. Salicylic acid (Sigma-Aldrich) was prepared in stock solutions of 4, 40, and 400 mM, and stock solution pH was adjusted to 5.7 with NaOH prior to filter sterilization with a 0.2 µm nylon filter. 1 mL of each stock solution was added to its designated culture flask containing 49 mL media to bring final treatment volumes to 50 mL and treatment concentrations to 0, 80, 800, and 8000 µM salicylic acid. Elicitor stock solutions or distilled water were added to the fresh MS media 72 h, 24 h, and 12 h prior to harvest. All hairy roots were removed from their culture flasks at the time of harvest, patted gently with a paper towel to remove excess media, weighed, and frozen at -80 °C. Roots were lyophilized on a freeze-dryer (Labconco Freezone 4.5, Kansas City, MO) until all water had been removed from each sample and stored at -80 °C until ready for extraction. Dry root weights were recorded.

Each elicitation trial consisted of 48 hairy root cultures, each assigned to one of four treatment concentrations (five, including the control) and one of three treatment durations to create treatment combinations with n=4. Means were compared by two-way analysis of variance (ANOVA), followed by post hoc analysis with Tukey’s Least Significant Difference multiple comparison test and the Games-Howell test, at a level of significance of 0.05 using SPSS Statistics software (Release 17.0, SPSS Inc., Chicago, IL). Outliers were determined by the modified z-score method by calculating the median of absolute deviation (MAD) about the median. Modified z-scores greater than three and a half were labeled as outliers (36).
Extraction of kudzu hairy roots for isoflavones

Isoflavones were extracted by adding 80% methanol to each dried kudzu hairy root sample at a ratio of approximately 25 mL methanol to 1 g dry roots and homogenized for 30 seconds. Methanolic extract was filtered with a Whatman #4 filter and root biomass was homogenized again with 80% methanol. This second extract was filtered with a Whatman #4 filter and 80% methanol was added to the remaining root biomass to extract overnight and then filtered. All three filtered extracts were combined, filtered through a Whatman #1 filter, and placed on a rotoevaporator (Buchi Rotavapor, Buchi Labortechnik, Switzerland) to remove solvent and water. Residual water was removed with a freeze-dryer. Dried samples were weighed and stored at -80 °C. Extracts were prepared for analysis by dissolving 20 mg in 4 mL of 80% methanol and filtering through a 0.45 μm nylon syringe filter (Fisher Scientific, Pittsburgh, PA).

Identification and quantification of kudzu hairy root isoflavones

Isoflavones were identified by high performance liquid chromatography electrospray ionization-mass spectrometry (HPLC-ESI-MS) using a LCQ Deca XP mass spectrometer in positive ESI mode (m/z 100-1000) attached to a photodiode array (PDA) detector (Thermo Finnigan Corp., San Jose, CA) at ultraviolet (UV) wavelength 262 nm. Separations were performed on a 3.5 μm, 2.1 mm x 150 mm, Waters XBridge C18-reversed phase column (Waters Corp., Milford, MA) using a mobile phase consisting of 5% acetonitrile (ACN) in double-distilled H₂O with 0.1% formic acid (Fisher Scientific) in solvent A and 95% ACN in double-
distilled H$_2$O with 0.1% formic acid in solvent B. The step gradient was composed of 0%, 70%, 100% and 0% of solvent B at 0, 30, 35, and 40 min, respectively. Injection volume for each sample was 5 µL and the solvent flow rate was 200 µL/min.

Isoflavones were quantified by high performance liquid chromatography-photodiode array detection (HPLC-PDA), using an Agilent 1100 HPLC system (Agilent Technologies, Inc., New Castle, DE) with an autosampler (G1313A), a degasser (G1322A), a quadratic pump (G1311A), and a temperature-controlled column compartment (G1316A). Separations were performed using a 250 mm x 4 mm x 5 µm Supelcosil LC-18 reversed-phase column (25 °C) (Supelco, Bellefonte, PA, USA) with a mobile phase consisting of 10% acetonitrile (ACN) in double-distilled H$_2$O with 0.1% trifluoroacetic acid (TFA) (Sigma-Aldrich, St. Louis, MO) in solvent A and 90% ACN in double-distilled H$_2$O with 0.1% TFA in solvent B. The elution gradient was composed of 0%, 70%, 100% and 0% of solvent B at 0, 30, 35, and 40 min, respectively and UV detection was at a wavelength of 262 nm. The injection volume was 15 µL/min and the flow rate was 1.5 mL/min. Data were processed using ChemStation Software for LC 3D systems (Rev. A.10.02, Agilent Technologies, Inc.).

Authentic standards for puerarin, daidzein, and genistein from Sigma-Aldrich and daidzin, genistin, and malonyl-genistin from LC Laboratories (Woburn, MA) were used to verify UV absorption, retention time, and molecular weight of isoflavones in each extract. Isoflavones that could not be authenticated with standards due to commercial unavailability were identified by comparison to literature data and quantified by a method of molecular weight adjustment of related standards $^{37}$. 

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RESULTS AND DISCUSSION

Kudzu crude extracts from both elicited and non-elicited cultures consisted predominantly of puerarin and daidzin, but also contained minute quantities of malonyl-daidzin, genistin, and daidzein. Figure 4.1 shows a representative HPLC-ESI-MS spectra of crude extract from non-elicited kudzu hairy roots.

Elicitation of kudzu hairy roots with acetate

Treatment of kudzu hairy roots with acetate demonstrated increased isoflavone content at 0.01% acetate concentrations in the first elicitation trial (n=4). Consequently, treatment of kudzu hairy roots with acetate was repeated twice under the same conditions to increase sample size from n=4 to n=12 to minimize biological variance. Kudzu hairy roots showed a trend of decreasing dry mass with increasing acetate concentration in 24 h and 72 h treatment groups, but exhibited an increase in dry mass after exposure to 0.01% and 0.10% acetate for 12 h (Table 4.1). However, this increase in dry mass was not reflected by a parallel increase in either puerarin content or total isoflavone content of the crude extracts. The 24 h exposure of hairy roots to 0.01% acetate did not significantly increase the puerarin and total isoflavone content in crude extracts above quantities measured in control groups (Tables 4.3 and 4.5). The 1.00% acetate treatment group significantly decreased dry mass, puerarin content, and total isoflavone content, suggesting that the treatment concentration may have been too high. At a concentration of 0.10% acetate, a negative impact on root mass and isoflavone synthesis was evident as exposure duration increased and indicated that treatment concentrations greater than 0.10% may not
produce desirable outcomes. The mean differences in total isoflavone content between acetate concentration and exposure duration variables were significant at the 0.05 level, but interaction between the two factors was not statistically different.

Differences in media color were observed in acetate treatment concentrations from all exposure durations at time of harvest, with the most noticeable difference in the 72 h treatment group, as seen in Figure 4.2. Isoflavones may have been present in the media as a root exudate to produce the observed media pigmentation at 1.00% and 0.10% concentrations; However, isoflavone content in the media was not analyzed due to the slow rate of evaporation of water from the media and the high concentration of soluble solids, such as MS salts and sucrose, which would severely dilute the enrichment of isoflavones measured in the dried media.

Elicitation of kudzu hairy roots with salicylic acid

Unlike the results following acetate elicitation treatment, treatment of kudzu hairy roots with salicylic acid did not result in media discoloration. There was no significant difference between the salicylic acid treatment concentration and exposure duration in the average root dry mass, nor was there any significant interaction between the two independent variables at the 0.05 level. In spite of this, differences in puerarin and total isoflavone content were observed in hairy roots treated with salicylic acid. Puerarin content in hairy roots treated with salicylic acid for 12 h was significantly lower at all treatment concentrations than non-elicited roots, but increased significantly after another 12 h to levels significantly higher than the control. The level of puerarin in hairy roots treated with 80 µM salicylic acid remained elevated after 72 h, but
declined in 800 and 8000 µM salicylic acid treatment groups (Table 6). The time and dose response to salicylic acid observed in this trial was consistent with observations made by Kneer et al. in the exposure of Lupinus luteus L. roots to various chemical elicitors \textsuperscript{(23)} and by Park et al. in the exposure of Pueraria lobata cell cultures to yeast extracts \textsuperscript{(38)}. The total isoflavone content decreased in the 12 h treatment groups at all salicylic acid concentrations, increased by 24h, then declined again after 72 h. However, the level of total isoflavones after 24 h exposure was not statistically different from levels in control roots.

Overall, treatment of kudzu hairy roots with acetate and salicylic acid in these trials demonstrated that chemical stimuli were capable of altering the bioactive constituents of plants. However, puerarin and total isoflavone synthesis in elicited kudzu hairy roots was not significantly improved over non-elicited levels under the circumstances provided in these trials. The replenished nutrient availability from replacing spent media with fresh media prior to elicitation could have played a role in these outcomes. While replacement of spent media with distilled water may have perturbed root growth due to altered osmolarity, the nitrogen available in the fresh media may have impacted isoflavone synthesis, since nutritional stresses such as low nitrogen induce synthesis and accumulation of phenylpropanoids in roots and root exudates \textsuperscript{(21)}. Consequently, nutrient availability should be considered in future elicitation trials, in addition to elicitor concentration and exposure duration, for maximization of secondary metabolite synthesis.
CONCLUSIONS

Isoflavone production was improved through the induction of hairy roots in 4 kudzu genotypes and the selection of a high isoflavone-yielding kudzu hairy root genotype. Treatment of kudzu hairy roots with acetate or salicylic acid failed to significantly improve isoflavone yields under the present experimental conditions and will require further study to develop this isoflavone production method.
TABLES

Table 4.1: Dry root mass per kudzu hairy root culture following treatment with acetate

Table 4.2: Dry root mass per kudzu hairy root culture following treatment with salicylic acid

Table 4.3: Total isoflavone content per kudzu hairy root culture following treatment with acetate

Table 4.4: Total isoflavone content per kudzu hairy root culture following treatment with salicylic acid

Table 4.5: Puerarin content per kudzu hairy root culture following treatment with acetate

Table 4.6: Puerarin content per kudzu hairy root culture following treatment with salicylic acid
**Table 4.1:** Dry root mass per kudzu hairy root culture following treatment with acetate

<table>
<thead>
<tr>
<th>Treatment Concentration</th>
<th>Exposure Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>72 h</td>
</tr>
<tr>
<td>Control</td>
<td>1.12 ± 0.04</td>
</tr>
<tr>
<td>0.01%</td>
<td>1.08 ± 0.03</td>
</tr>
<tr>
<td>0.10%</td>
<td>0.93 ± 0.02 *</td>
</tr>
<tr>
<td>1.00%</td>
<td>0.91 ± 0.01 *</td>
</tr>
</tbody>
</table>

Values represent the sample mean ± the standard error; n=12

* indicates a significant difference between the treatment group from the control within a column, p<0.05

**Table 4.2:** Dry root mass per kudzu hairy root culture following treatment with salicylic acid

<table>
<thead>
<tr>
<th>Treatment Concentration</th>
<th>Exposure Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>72 h</td>
</tr>
<tr>
<td>Control</td>
<td>1.08 ± 0.02</td>
</tr>
<tr>
<td>80 µM</td>
<td>1.11 ± 0.03</td>
</tr>
<tr>
<td>800 µM</td>
<td>1.13 ± 0.05</td>
</tr>
<tr>
<td>8000 µM</td>
<td>1.14 ± 0.05</td>
</tr>
</tbody>
</table>

Values represent the sample mean ± the standard error; n=4

* indicates a significant difference between the treatment group from the control within a column, p<0.05
Table 4.3: Total isoflavone content per kudzu hairy root culture following treatment with acetate

<table>
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</tr>
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<tbody>
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<td></td>
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</tr>
<tr>
<td>Control</td>
<td>1082.0 ± 58.7</td>
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<tr>
<td>0.01%</td>
<td>1074.6 ± 101.9</td>
</tr>
<tr>
<td>0.10%</td>
<td>575.2 ± 110.3 *</td>
</tr>
<tr>
<td>1.00%</td>
<td>111.6 ± 19.7 *</td>
</tr>
</tbody>
</table>

Values represent the sample mean ± the standard error; n=12
* indicates a significant difference between the treatment group from the control within a column, p<0.05

Table 4.4: Total isoflavone content per kudzu hairy root culture following treatment with salicylic acid

<table>
<thead>
<tr>
<th>Treatment Concentration</th>
<th>Exposure Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>72 h</td>
</tr>
<tr>
<td>Control</td>
<td>1237.6 ± 102.1</td>
</tr>
<tr>
<td>80 µM</td>
<td>1268.5 ± 13.5</td>
</tr>
<tr>
<td>800 µM</td>
<td>1093.4 ± 105.4</td>
</tr>
<tr>
<td>8000 µM</td>
<td>973.7 ± 27.8 *</td>
</tr>
</tbody>
</table>

Values represent the sample mean ± the standard error; n=4
* indicates a significant difference between the treatment group from the control within a column, p<0.05
**Table 4.5:** Puerarin content per kudzu hairy root culture following treatment with acetate

<table>
<thead>
<tr>
<th>Treatment Concentration</th>
<th>Exposure Duration</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>72 h</td>
</tr>
<tr>
<td>Control</td>
<td>522.3 ± 14.3</td>
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<tr>
<td>0.01%</td>
<td>497.3 ± 33.2</td>
</tr>
<tr>
<td>0.10%</td>
<td>366.5 ± 72.3 *</td>
</tr>
<tr>
<td>1.00%</td>
<td>24.2 ± 5.0 *</td>
</tr>
</tbody>
</table>

Values represent the sample mean ± the standard error; n=12

* indicates a significant difference between the treatment group from the control within a column, p<0.05

---

**Table 4.6:** Puerarin content per kudzu hairy root culture following treatment with salicylic acid

<table>
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<th>Treatment Concentration</th>
<th>Exposure Duration</th>
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</thead>
<tbody>
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<td></td>
<td>72 h</td>
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<tr>
<td>Control</td>
<td>679.0 ± 50.0</td>
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<tr>
<td>80 µM</td>
<td>682.5 ± 14.8</td>
</tr>
<tr>
<td>800 µM</td>
<td>560.9 ± 60.1 *</td>
</tr>
<tr>
<td>8000 µM</td>
<td>494.2 ± 16.0 *</td>
</tr>
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</table>

Values represent the sample mean ± the standard error; n=4

* indicates a significant difference between the treatment group from the control within a column, p<0.05
FIGURES

**Figure 4.1:** Typical HPLC-ESI-MS spectra showing total ion current (a), HPLC-UV chromatogram (UV 262 nm) (b), and MS spectrum (c) of crude extract from non-elicited kudzu (*P. montana var lobata*) hairy roots. m/z, mass-to-charge ratio.

**Figure 4.2:** Representative photograph of media collected from hairy root cultures treated with acetate
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**Figure 4.2:** Representative photograph of media collected from hairy root cultures treated with acetate

From left to right, media from control, 0.01%, 0.10%, and 1.00% acetate treatment concentrations and 72 h exposure duration, at time of harvest
LITERATURE CITED


Isoflavones, a class of plant-derived compounds with estrogenic activity, are currently being investigated as alternatives to estrogen replacement for treatment of osteoporosis\textsuperscript{(1-4)}, cardiovascular disease\textsuperscript{(5)}, and neurological disorders\textsuperscript{(6, 7)}. These compounds are capable of binding to estrogen receptors, but exert estrogenic or anti-estrogenic activities depending on the specific tissue type\textsuperscript{(8)}. Kudzu (\textit{Pueraria sp.}) and red clover (\textit{Trifolium pratense}) are leguminous species that are rich sources of isoflavones\textsuperscript{(9, 10)} and have shown positive effects on bone health both \textit{in vitro} and \textit{in vivo}. However, the absorption, tissue distribution, metabolism, and elimination of these compounds in the human body are not well understood, in part due to the difficulty in examining phytochemical distribution within a complex diet and discriminating between newly absorbed phytochemicals and previously existing levels in the body\textsuperscript{(11, 12)}. The use of stable isotope or radioisotope-labeled compounds\textsuperscript{(12, 13)} to study isoflavone pharmacokinetics offers a promising novel research approach, which circumvents these problems.

In the first study (Chapter 2 – \textit{in Vivo Metabolic Tracking of \textsuperscript{14}C-Radiolabeled Isoflavones in Kudzu and Red Clover Extracts}), \textsuperscript{14}C-radiolabeled extracts containing isoflavones from kudzu (\textit{P. lobata}) root cultures\textsuperscript{(14)} and red clover (\textit{T. pratense}) cell cultures\textsuperscript{(15)} were tracked in an \textit{in vivo} rat model. The pharmacokinetics of radiolabeled phytochemicals in the two extracts was traced for a period of 24 h through an analysis of serum, interstitial fluid, urine, feces, and gastrointestinal contents by scintillation counter. Serum pharmacokinetics indicated that kudzu and red clover extracts were rapidly absorbed, distributed throughout the body, and largely eliminated in the urine and feces. In addition, serum pharmacokinetics were consistent with the kudzu isoflavones puerarin\textsuperscript{(16)}, daidzein\textsuperscript{(17)}, and genistein\textsuperscript{(18)} undergoing enterohepatic
circulation. Analysis of rat organ tissues that were collected 24 h after oral administration showed that a dose of approx. 0.148 MBq (4µCi) of radiolabeled kudzu and red clover-derived extracts was capable of reaching and accumulating in all major tissues, including the liver, kidneys, testes, lungs, heart, femur, tibia, and vertebrae. The liver accumulated the greatest concentration of radiolabel among the tissues tested, at 1.99 and 1.54% of the administered kudzu and red clover extracts, respectively. Analysis of bone tissues revealed that radiolabel accumulated in the femur, tibia and vertebrae at 0.04, 0.03 and 0.01% of the administered dose, respectively, in both kudzu and red clover treatments (19). While the distribution of radiolabel was capable of being tracked in vivo, radiolabeled isoflavones comprised only a portion of the total dose. This necessitated a second study to track the distribution of an isoflavone-rich fraction in vivo (Chapter 3 – Tracking Deposition of a 14C-Radiolabeled Kudzu Hairy Root-Derived Isoflavone-Rich Fraction into Bone).

In this study, in vitro kudzu isoflavone production and enrichment was significantly enhanced by inducing hairy root synthesis in the available kudzu germplasm using Agrobacterium rhizogenes, and by screening kudzu hairy root cultures for a high-isoflavone yielding genotype. The predominant isoflavone in many kudzu species is puerarin (daidzein-8-C-glucoside) (20), which is unique in that C-glycosides are resistant to hydrolysis in the gut, have better bioavailability than O-glycosides, and are taken up from the intestines largely unmetabolized (2). Puerarin has also been shown to increase new bone formation in rat osteoblasts (4) and decrease bone resorption in rat osteoclasts (1) in vitro. The puerarin content, total isoflavone content, and average culture biomass of each hairy root-induced genotype were used to evaluate genotype productivity. P. montana var. lobata, (USDA no. PI 434246) hairy
roots yielded the highest puerarin, total isoflavone content, and average fresh biomass and were used for subsequent studies.

_P. montana_ var. _lobata_ (USDA no. PI 434246) hairy roots were radiolabeled using $^{14}$C-labeled sucrose, isoflavones were extracted using 80% methanol, the crude extract was enriched through solvent extraction and Sephadex filtration, and the resulting $^{14}$C-radiolabeled, isoflavone-rich fraction was metabolically tracked in rats. Pharmacokinetics of the labeled compounds was traced using an oral dose of approx. 0.148 MBq (4µCi) kudzu isoflavone-rich fraction. Radiolabeled phytochemicals accumulated in all organs, including bone tissues. Femur bones were extracted using 80% methanol, analyzed by HPLC-ESI-MS, and found to contain puerarin, daidzein, and puerarin glucuronide. These new results not only validated and reinforced the previous study, but also provided further evidence to support a role of isoflavones in optimizing bone health.

To further improve isoflavone production in kudzu hairy roots from the selected genotype, elicitors were investigated for their potential to induce secondary metabolite synthesis (Chapter 4 – Elicitation of kudzu (_P. montana_ var. _lobata_) hairy roots to promote synthesis of isoflavones in vitro). Elicitors are biotic or abiotic stimuli, such as climatic, microenvironmental, chemical, and physical stressors, which alter the synthesis and accumulation of bioactive secondary metabolites (21). The enzymatic pathways that are induced by these stimuli are often involved as a defense response to pathogen attack, ultraviolet light, or wounding (22, 23). Acetate and salicylic acid were prioritized as candidates for elicitation trials due to their capacity to increase synthesis of phytochemicals with antibacterial, antifungal, and anticancer activity in a wide variety of plants, including leguminous species (21).
In this study, kudzu hairy root segments were grown for a period of 3 weeks, at which point culture media was replaced with fresh media to standardize the volume and concentration of each elicitor treatment. Sterilized, pH-adjusted sodium acetate or salicylic acid solutions of varying concentrations or distilled water, as a control, were used to treat each hairy root culture. Distilled water or concentrated stock solutions of each elicitor were added to each culture flask 72, 24, and 12 h before harvest to bring each hairy root culture to the final treatment concentration and volume. All kudzu hairy root cultures were then harvested, freeze-dried, weighed, and extracted for isoflavones. HPLC-PDA analysis showed that elicitation with acetate or salicylic failed to significantly improve isoflavone synthesis in kudzu hairy roots, within the parameters set in these experiments. While isoflavone production was significantly enhanced through the induction of hairy root synthesis and selection of a high isoflavone-yielding genotype, further research will be required to develop elicitation into an effective and economical secondary metabolite production method in kudzu hairy roots.

The evidence presented in these studies supports the hypothesis that ingested isoflavones are capable of being distributed in serum and interstitial fluid, incorporated into bone tissue, and passing the blood-brain barrier, facilitating their potential contribution to cardiovascular protection, bone health, and cognitive function. The radiolabeling of plant tissue cultures and metabolic tracking of $^{14}$C-radiolabeled isoflavones in an animal model has demonstrated the tremendous potential of this approach to studying the pharmacokinetics of isoflavones. This methodology need not be limited to isoflavone research and can be extended to the study of other bioactive constituents, which can be generated in vitro via cell or organ cultures and can be enhanced through hairy root-induction, germplasm screening, or elicitation. Another avenue of future research, which could be facilitated using the radiolabeling and tracking techniques, could
be the elucidation of exact metabolic targets, biochemical pathways, and phytochemical metabolites in animal tissues. By investigating the biochemical pathways involved in the metabolism of phytochemicals, we gain a stronger understanding of not only the health promoting effects associated with their use, but also their potential negative consequences.


APPENDIX

Appendix 1: Typical HPLC-ESI-MS spectra showing total ion current (a), HPLC-UV chromatogram (UV 262 nm) (b), and MS spectrum (c) of crude extract from *P. montana var lobata* (Origin: Japan) hairy roots. *m/z*, mass-to-charge ratio.

Appendix 2: Typical HPLC-ESI-MS spectra showing total ion current (a), HPLC-UV chromatogram (UV 262 nm) (b), and MS spectrum (c) of crude extract from *P. montana var lobata* (Origin: USA) hairy roots. *m/z*, mass-to-charge ratio.

Appendix 3: Typical HPLC-ESI-MS spectra showing total ion current (a), HPLC-UV chromatogram (UV 262 nm) (b), and MS spectrum (c) of crude extract from *P. lobata* (Origin: USA) hairy roots. *m/z*, mass-to-charge ratio.

Appendix 4: Typical HPLC-ESI-MS spectra showing total ion current (a), HPLC-UV chromatogram (UV 262 nm) (b), and MS spectrum (c) of crude extract from *P. phaseoloides* (Origin: Guatemala) hairy roots. *m/z*, mass-to-charge ratio.
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