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# Botanicals for age-related diseases: from field to practice<sup>1-4</sup>

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## ABSTRACT

The Purdue–University of Alabama Botanicals Research Center for Age Related Disease joins novel technologies to study the bioavailability of bioactive polyphenolic constituents and their relation to health. Many diseases that manifest with age relate to oxidative stress and tissue damage. Our goal is to follow the fate of bioactive constituents from a complex mixture to the organ affected by the disease and relate that to a protective mechanism. Equally important is to screen commercially available botanicals for their efficacy and safety. Botanicals and their relation to bone antiresorptive capacity, cognitive function, vascular effects, and cancer are principal themes in our center. *Am J Clin Nutr* 2008;87(suppl):493S–7S.

**KEY WORDS** Botanicals, isoflavones, catechins, bone resorption, cognitive function, cancer

## INTRODUCTION

A multidisciplinary team and innovative approaches are needed to identify and evaluate bioactive ingredients from complex mixtures such as botanicals and to evaluate their health benefits and mechanisms of action. The Purdue–UAB Botanicals Research Center for Age Related Disease joins scientists from Purdue University and the University of Alabama at Birmingham with participation from the University of Illinois, Rutgers University, and the Indiana University School of Medicine. Scientists with expertise in nutrition, plant physiology, horticulture, pharmacy, veterinary medicine, physics, chemistry, statistics, and medicine are part of our team. Our center targets age-related diseases because many of them have an underlying etiology of oxidative damage, and the polyphenolics in many botanicals have potent antioxidant properties. The incidence of bone loss, cognitive loss, hypertension, cardiovascular disease, type 2 diabetes, and stroke increases with age and dramatically in women after menopause, and each of these components appears to be related to some common mechanisms that synergize and thereby accelerate morbidity and mortality. Because the use of estrogen to offset these effects has been strongly questioned after the findings of the Women’s Health Initiative, it is clear that a better understanding of the basis for these postmenopausal symptoms and their alleviation is needed. This brief overview presents examples of studies conducted by the center to screen botanical dietary supplements for their efficacy for bone anti-resorption capacity in postmenopausal women, neuron protection in animal models, anticancer potential in cell culture and animal models, and vascular effects and glucose tolerance in animal models.

## BIOAVAILABILITY OF BIOACTIVE COMPOUNDS FROM COMPLEX MIXTURES

The study of individual or mixtures of compounds from complex plant materials is a challenge. Our center is capitalizing on several novel technologies developed or adapted by our investigators that involve transferring samples from laboratory to laboratory across institutions. The general plan is shown in **Figure 1**. Starting plant materials of interest are selected or produced in a greenhouse at Rutgers University or the University of Illinois. At the University of Illinois, <sup>14</sup>C-enriched polyphenolics are biosynthesized in plant cell suspension cultures or root cultures grown in specially designed chambers as described for flavonoids (1). Polyphenolics in the plants and fractions are characterized at one of the several participating universities depending on the need. Crude extracts or fractions resulting from vacuum liquid chromatography are transferred to Purdue University for animal studies. Rats receive fractions by gavage; and pharmacokinetics, tissue distribution, and metabolism of <sup>14</sup>C-labeled or unlabeled bioactive compounds are followed in serum or intestinal fluid from inserted membrane probes connected to a programmable automatic sampling station machine developed at Purdue Research Park (2). The biological samples are analyzed for label by standard scintillation counting or for very low concentrations by accelerator mass spectrometry (AMS) at Purdue University (3) or for polyphenols and metabolites by mass spectrometry at UAB. The ability of AMS to detect <sup>14</sup>C with  $1 \times 10^3$ – $10^5$  greater efficiency than liquid scintillation counting and developments in mass spectrometry to measure femtomole quantities in microliter samples allows absorption and transfer to tissues to be characterized even for poorly absorbed compounds and across the blood-brain barrier.

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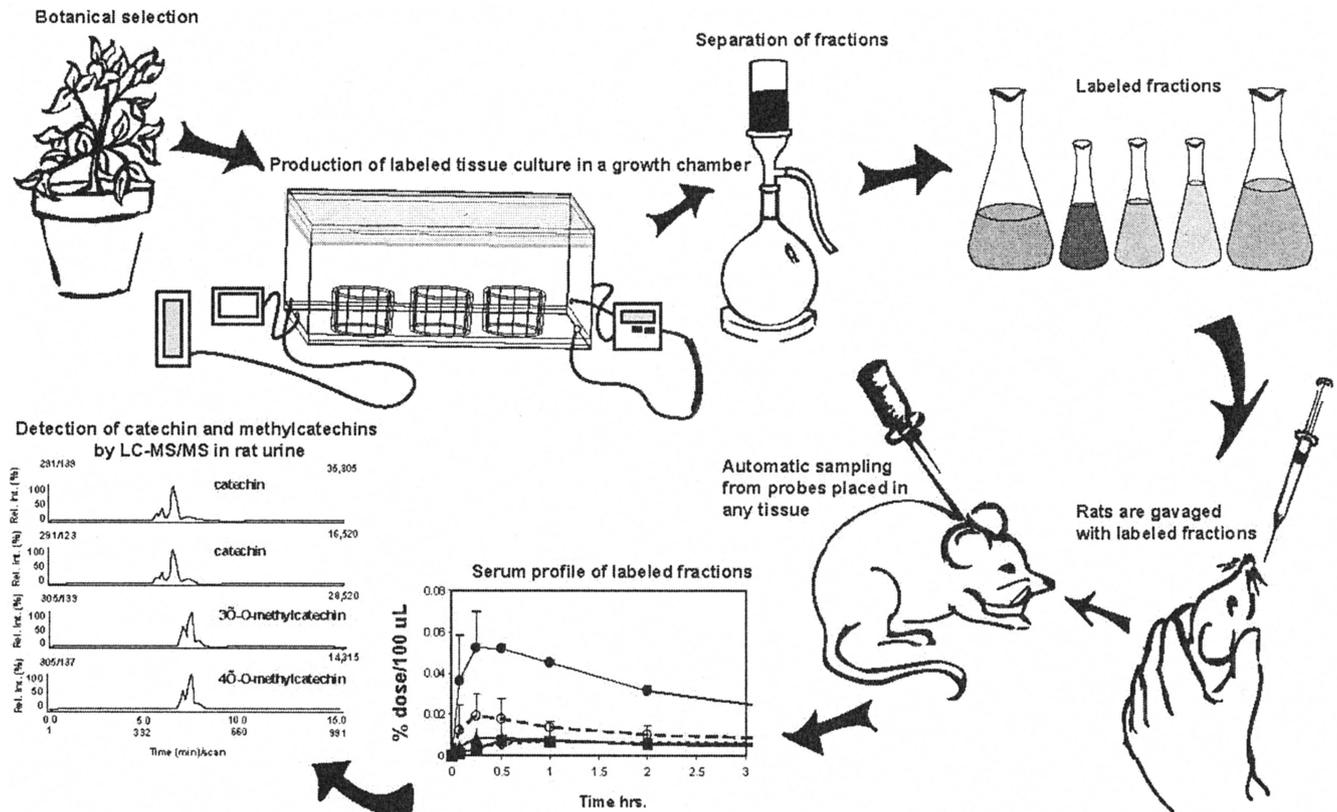


FIGURE 1. Schematic of determining the bioavailability of bioactive polyphenolics from botanicals. Rel. Int., relative intensity.

### ISOFLAVONES AS BONE ANTIRESORPTIVE AGENTS

Several commercially available botanical dietary supplements claim to be substitutes for estrogen therapy for preventing the bone loss associated with menopause. Because the phytoestrogens in certain botanicals, including soy, red clover, and kudzu, are chemically similar to estrogen and bind to the estrogen receptor, albeit weakly, much attention has been given to their ability to attenuate the accelerated bone resorption associated with estrogen deficiency accompanying menopause. A concern has been raised that these bioactive compounds may affect reproductive tissues similarly to estrogen (4). The barrier to evaluating effective interventions for preventing bone loss is that bone has a very slow turnover rate and it takes several years to properly monitor changes in bone mineral density, the classic approach and the US Food and Drug Administration–approved method. Thus, it is very expensive to measure dose-response effects of a candidate therapy or to compare multiple interventions.

Our center contributed to the development of a novel method for rapidly and sensitively monitoring bone resorption by following  $^{41}\text{Ca}$  losses in urine from prelabeled bone measured by AMS. Our study design screens multiple interventions per year in the same subjects with the use of 50-d interventions administered in a randomized, crossover design. Development of statistical methods was also required to analyze data when  $^{41}\text{Ca}$  levels change independent of treatment as bone remodels. We recently reported the first intervention trial using  $^{41}\text{Ca}$  (5). A dose-response study of soy protein containing 0–135.5 mg isoflavones/d showed no effect on bone resorption in postmenopausal women. In contrast, estrogen therapy and a bisphosphate reduced

bone resorption with sufficient power to see significant effects in 4 and 6 women, respectively.

### GRAPE SEED EXTRACT AND COGNITIVE FUNCTION

Botanical ingredients such as grape seed extract (GSE) enriched in proanthocyanidins (oligomeric polyphenols) had been suggested to have multiple health benefits due to antioxidant and other beneficial activities of the proanthocyanidin, but a systematic analysis of the molecular basis of these benefits has not been done. Because the brain is particularly vulnerable to age-related oxidative damage as well as inflammatory insults, we hypothesized that ingestion of GSE would affect specific proteins in the brains of animals in a manner consistent with neuroprotection.

We were the first to identify and quantify specific proteins in mammalian tissues that were modulated by a dietary supplement ingredient and were the first to demonstrate a link of ingesting any complex botanical ingredient with any disease (6). Healthy adult female rats were fed defined (AIN-76A; Teklad Diets, Madison, WI) diets supplemented with 5% GSE (Kikkoman Corporation, Chiba, Japan) for 6 wk. The brains were then removed and the homogenates were subjected to proteomics analysis (two-dimensional electrophoresis and mass spectrometry). Image and statistical analysis of the two-dimensional gel images determined that 13 proteins were altered in amount, charge, or both, which is qualitatively consistent with GSE being neuroprotective. It is unknown whether the effects of GSE were due to the polyphenols or their metabolites in the brain directly or indirectly to the metabolism of endogenous compounds in the gut

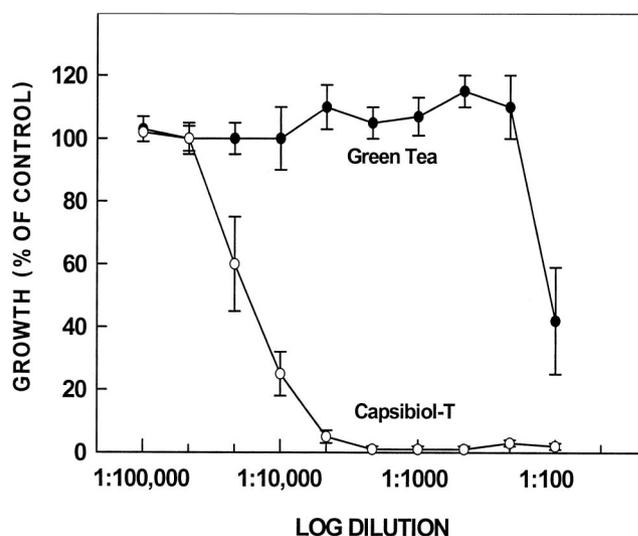
during their enterohepatic circulation. Another possibility is regulation of signaling through the gut-brain axis via the nervous system.

### GREEN TEA AND CANCER PREVENTION

Epidemiologic and animal data support a potential role for tea and tea catechins in the prevention of several chronic diseases, including cancer, cardiovascular disorders, and obesity. Poor oral bioavailability of catechins is believed to minimize the potential efficacy of these tea polyphenols by limiting concentrations at specific target tissues. Factors believed to contribute to poor bioavailability of catechins include digestive instability, poor transcellular efflux in intestinal cells, and rapid metabolism and excretion (7). A further complication to understanding physiologic catechin profiles is matrix effects associated with consumption of tea. Adjuncts affecting catechin stability in food systems are present in milk, juice, and antioxidants added to beverages. We are using a coupled *in vitro* digestion Caco-2 intestinal cell culture model to evaluate digestive stability and intestinal uptake of green tea catechins from various beverage blends (8). Formulation affects the intestinal catechin profile; *in vivo* investigations are underway on specific formulations that positively influence catechin bioavailability.

Our center has studied the relation between green tea catechins and cancer mediated through a cell surface protein, tNOX (GenBank accession no. AF207881) (9). tNOX appears to be a specific target for low-dose apoptosis of cancer cells by green tea catechins (10), and its expression is restricted to cancer cells. Cancer specificity resides in its expression of a splice variant (11) that is uniquely drug inhibited and uniquely associated with human cancer (9). When the tNOX of cancer cells is inhibited, the cells fail to enlarge after division, cease to divide, and after a few days undergo apoptosis.

The availability of a specific target provided a mechanism-based approach to rapid evaluation and development of botanicals with anticancer activity. Key ameliorating polyphenols that target tNOX were identified along with combinations based on botanical sources that often duplicated the key ameliorating polyphenols and interacted synergistically. Botanical preparations that most inhibited cancer cell growth involved synergies between a decaffeinated green tea concentrate and vanilloid-containing *Capsicum* preparations (Figure 2) (12). A 25:1 ratio of green tea concentrate to *Capsicum* preparation killed cancer cells in culture 100-times greater, by weight, than did green tea. Assuming 2 g green tea per cup in preparing a usual green tea infusion, our findings suggest that a 350-mg capsule of the commercially available green tea extract-*Capsicum* preparations (Capsibiol-T or Capsol-T; BND Service Company, West Union, IL) is equivalent to drinking 16 cups of green tea on the basis of the comparative responses of growth of 4T1 and HeLa cells in culture (12). (-)-Epigallocatechin-3-gallate (EGCg), capsaicin, vanillylamine, and green tea mixtures alone or combined with vanilloids did not inhibit the growth of noncancer cell lines in culture nor did they inhibit the activity of the corresponding NOX protein constitutive to normal cells. This differential activity suggests cancer specificity and safety of the green tea-*Capsicum* mixtures. tNOX transfection and overexpression together with antisense results showed that functional cell surface expression of tNOX was both necessary and sufficient for the cancer-specific inhibition of cell growth attributed to EGCg (13). As a



**FIGURE 2.** Dose response of HeLa cells at 72 h in cell culture to a 25:1 decaffeinated green tea concentrate-Capsibiol-T mixture prepared on an equivalent weight basis. Points are the mean  $\pm$  SD of 3–5 determinations. Growth responses to mixture dilutions were analyzed by using a two-way analysis of variance and were considered to be significant at  $P < 0.05$ . One 350-mg capsule of Capsibiol-T is equivalent to drinking 16 cups of green tea. Modified from Morr  and Morr  (12).

logical next step, transgenic mice overexpressing tNOX were generated. By expressing the tNOX protein, transgenic animals exhibited a level of unregulated cell enlargement and sensitivity to EGCg similar to cancer cells. The transgenic mice experiments provided an important test of the hypothesis that tNOX represents a necessary and sufficient molecular target as the basis for the cancer therapeutic activity of EGCg (14).

In a series of open-label sequential trials, 36% of 50 cancer participants with active cancer receiving Capsol-T had a significant prolongation of life or remained alive, 32% improved, and the rest had a normal course of disease (15). A Phase II/III clinical study (toxicology and pharmacokinetics) of Capsol-T with renal cancer and melanoma directed by Theodore Logan at the Indiana University School of Medicine is underway, and a more extensive clinical study to evaluate efficacy is under development at the Goshen Cancer Center, Goshen, IN. A favorable interaction between a tea catechin-*Capsicum* preparation and radiation therapy was previously indicated from compassionate intervention studies with 5 cancer patients (16).

### VASCULAR EFFECTS OF POLYPHENOLICS AND INTERACTIONS WITH ANTIOXODANTS

Polyphenols in botanicals, including the bioflavonoids and stilbenoids, have a role in regulating oxidative stress in cardiovascular disease and other chronic diseases of aging. These diseases are marked by the production of reactive nitrogen species (RNS), including peroxynitrite ( $\text{ONOO}^-$ ) and nitrogen dioxide, and reactive oxygen species (ROS), including superoxide, hydrogen peroxide, hypobromous acid, and hypochlorous acid (HOCl), by eosinophils, macrophages, and neutrophils. Although RNS and ROS modify DNA and proteins and disrupt gene expression and protein function in targeted cells, they also damage neighboring uninvolved normal cells. Because many of the protein modifications are on tyrosine (3-nitrotyrosine,

3-chlorotyrosine, and 3-bromotyrosine), an aromatic group, we investigated how the RNS and ROS reacted with dietary polyphenols. In vitro, both HOCl and ONOO<sup>-</sup> formed chloro and nitro derivatives with the soy isoflavones daidzein and genistein (17). These experiments were extended to incubation with activated neutrophil-like HL-60 cells (which produce HOCl when stimulated with a phorbol ester) and freshly isolated human polymorphonuclear cells. In these studies, >90% of a bioflavonoid (10 μmol/L) in the medium was converted to chlorinated products within 30 min (18, 19). The nature of the chloro and nitro derivatives was determined by <sup>1</sup>H-NMR and liquid chromatography–tandem mass spectrometry. The nitration occurred at the 3'-position in the phenolic B-ring as has been observed for nitrotyrosine in proteins. Chlorination was more complex, occurring at the 6- and 8-positions on the A-ring of genistein and at the 3'-position in the B ring (20).

The isoflavones, as do other polyphenols, increase the lag time for Cu<sup>2+</sup>-induced oxidation of human LDL and reduce the propagation rate of lipid peroxidation. However, their effect is weak. We hypothesized that polyphenols undergo a cycle: they react with lipid peroxide free radicals and are converted to free radicals themselves. These attack new lipid molecules and restart the process of lipid radical formation. Adding ascorbic acid in molar excess to the reaction markedly improves overall antioxidant activity to 2–3 times greater than the sum of the individual antioxidant species (21). This is particularly relevant to the work being carried out on the prevention of lens cataract disease. Genistein administered in the diet enters the aqueous humor that flows in front of the lens (22). Because the aqueous humor contains 5–10 mmol ascorbic acid/L, the high ratio between ascorbic acid and genistein may create a strong antioxidant environment. It also suggests that the study of polyphenol effects in vivo has to consider the whole botanical and not the individual components (23).

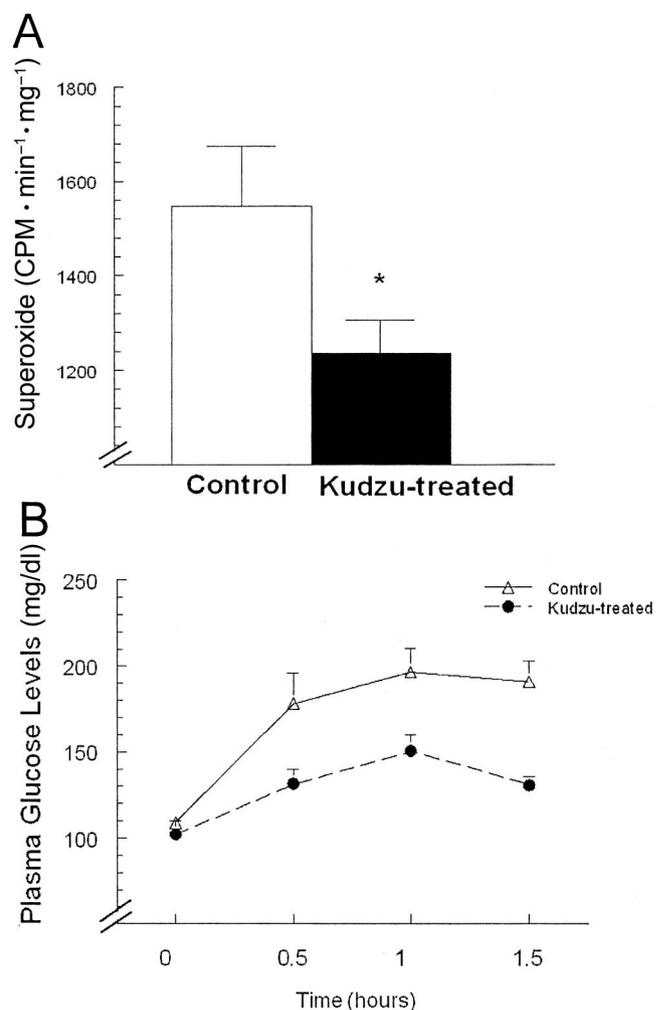
Isoflavones, particularly genistein, prevent tumor necrosis factor-α-induced inhibition of the rolling of neutrophils to the blood vessel wall in a flowing system (24). Intriguingly, chlorination of daidzein and genistein reverses this protective effect (25), which suggests that these products from the reaction of isoflavones and HOCl have their own biological activities that should not be ignored.

Polyphenols also appear to delay or partially prevent several aspects of the metabolic syndrome, but the mechanisms underlying these effects remain to be elucidated. A model that demonstrates these beneficial effects is in middle-aged, ovariectomized spontaneously hypertensive rats, which are used as a model for menopause, hypertension, insulin resistance, and stroke. In these rats, administration of genistein and grape seed extract independently blunts hypertension, and grape seed extract decreases cognitive decline. Recently, we have focused on the effects of another polyphenol, puerarin, which is present in kudzu root. Puerarin is a C-glucoside and in contrast with most other polyphenols that are O-glucosides, puerarin does not require deglycosylation to cross from the intestines into the circulating blood. This attribute has allowed us to track the kinetics, sequestration, and elimination of this polyphenol (compared with other polyphenols) with much greater accuracy.

In the initial long-term feeding studies, 3-mo-old female spontaneously hypertensive rats were fed casein-based AIN-76 diets either containing 0.3% kudzu root extract or no polyphenols for 3 mo. Kudzu prevented 15 ± 2 mm Hg of the 30 ± 3 mm Hg

blood pressure rise that occurred in control (non-kudzu-treated) rats. Heart rate was not affected by the treatment, but when the ganglionic blocker hexamethonium was administered, the decrease in arterial pressure was significantly greater in the control than in the treated rats, which suggests that sympathetic nervous system activity is required to maintain the higher arterial pressure in the control animals. Superoxide production was significantly reduced in the kudzu-treated compared with the control animals (Figure 3A). Kudzu root extract reduced resting blood glucose, insulin, serum cholesterol, and leptin. Glucose tolerance and glucose sensitivity were improved in the kudzu-supplemented groups by ≈20%. Acute studies in rats and mice showed that glucose tolerance was improved by ≈50% when puerarin and glucose were administered simultaneously to otherwise naive spontaneously hypertensive rats (26) (Figure 3B).

Safety assessment trials showed that kudzu root extract does not cause adverse side effects in rats even when administered at



**FIGURE 3.** Effect of kudzu on superoxide anion formation and glucose tolerance in spontaneously hypertensive rats. (A) In vitro measurement of superoxide anion formation in aortic ring segments. The assay was calibrated by monitoring the chemiluminescence signal of known amounts of superoxide generated by xanthine + xanthine oxidase (mean ± SEM; \*  $P < 0.05$  by  $t$  test;  $n = 5$ /group). (B) Dietary kudzu improved glucose tolerance in spontaneously hypertensive rats. Glucose was given by gavage (2 g glucose/kg) at time 0. Note that the plasma glucose concentration was decreased by kudzu extract treatment at all times (mean ± SEM;  $P < 0.05$  for main effect of treatment by one-way ANOVA;  $n = 5$ /group).

1% of the diet for 2 mo. No treated or control animal showed significant weight loss during the 3 mo feeding, but the rats on the 1.0% and 0.3% kudzu diets gained weight less rapidly than did the rats on the 0.1% or 0.0% diets. No obvious pathology was noted in any of the animals; blood pressure was reduced by  $\approx 15$  mm Hg, and heart rate was reduced by the 1.0% and 0.3% diets. Thus, we have seen no adverse effects of the kudzu root extract on the health of the animals.

To better understand the mechanisms by which kudzu root extract affected health, we developed a highly sensitive mass spectrometry method to assess puerarin in the body (27). Male spontaneously hypertensive rats received a single-dose gavage of puerarin (50 mg/kg body wt), and serial blood samples were collected. The main pharmacokinetic parameters for puerarin after oral administration were as follows:  $C_{\max}$  ( $3.54 \pm 2.03$  mg/L),  $T_{\max}$  ( $0.68 \pm 0.37$  h),  $AUC_{0-1}$  ( $7.29 \pm 3.79$  mg h/L),  $AUC_{0-\infty}$  ( $9.17 \pm 4.87$  mg h/L),  $T_{1/2}$  ( $1.7 \pm 0.6$  h),  $CL/F$  ( $7.24 \pm 4.27$  L/h/kg), and  $V/F$  ( $17.88 \pm 13.55$  L/h/kg), which indicates that puerarin is rapidly absorbed into the blood and is relatively rapidly cleared from the body. We also quantified puerarin in bile and urine and body tissues by the use of liquid chromatography–mass spectrometry/mass spectrometry. Intact puerarin was detected in kidney, liver, lungs, pancreas, and eyes. Puerarin was detected in bile samples within 10 min after oral administration. Detection of puerarin in kidneys in a significant amount supports the hypothesis that puerarin may inhibit the renal sodium-dependent glucose transporter 2 and thereby decrease reabsorption of glucose to the blood.

## CONCLUSIONS

Our center has used a multidisciplinary approach to develop a model for studying the bioavailability of bioactive compounds from complex mixtures. We have a long distance to go to make this a routine procedure. Relating bioactive compounds in botanicals to the aging process and disease development through multiple mechanisms presents great challenges but rewarding opportunities.

CMW is on the advisory boards of Pharmavite and Wyeth. She receives grant support from Wyeth and reviews research proposals for the United Soybean Board. None of the other authors identified a potential conflict of interest.

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