

Pinostrobin from Honey and Thai Ginger (*Boesenbergia pandurata*): A Potent Flavonoid Inducer of Mammalian Phase 2 Chemoprotective and Antioxidant Enzymes

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Over 60 different samples comprising 35 distinct honeys were evaluated for their ability to induce mammalian phase 2 detoxication enzymes using a microtiter plate assay of quinone reductase (QR) induction with murine hepatoma cells in microtiter plates. This assay has been used extensively to identify and isolate a variety of natural and synthetic inducers from plants. All 35 honeys examined induced elevations of mammalian QR activity ranging from 153 to 2155 units/g with a mean of 630 and a median of 417 units/g. The concentrations for doubling the QR activity (CD) of certain of the prominent flavonoids found in honey were also assessed (pinostrobin, 0.5 μ M; pinocembrin, 110 μ M; chrysin, 25 μ M) and compared to those of related, more commonly described flavonoids such as quercetin (2.7 μ M) and myricetin (58 μ M). On the basis of the extremely high QR inducing potency of one of these compounds, pinostrobin (5-hydroxy-7-methoxyflavanone), a bioassay-guided search was conducted which revealed a dietary source of pinostrobin, *Boesenbergia pandurata* (fingerroot), with extraordinarily high ability to induce mammalian phase 2 detoxication enzymes. Although the QR inducing activity of buckwheat honeys was 2155 ± 951 units/g ($n = 8$ samples), which is less than 10% of the average values obtained from fresh broccoli, the potency of fingerroot rhizomes (ca. 110 000 units/g) is even higher than that of broccoli and the potencies of fingerroot oil and powdered rhizome (ca. 500 000 units/g) rival that of broccoli sprouts.

KEYWORDS: Cancer; chemoprotection; fingerroot

INTRODUCTION

Massive increases in the consumption of sugar in soft drinks and highly processed sweets, coupled with reduced physical activity and a more sedentary lifestyle, have led directly to a dramatic increase in obesity, with comorbidity from chronic, noncommunicable conditions such as diabetes and hypertension (1, 2). This dramatic increase in obesity has been paralleled by advances in modern medicine and a sharp reduction in contagious diseases as a cause of death. This has resulted in persons in economically advantaged regions living longer, so that chronic and degenerative diseases such as cancer are ultimately responsible for the vast majority of deaths in these countries (3).

Per capita calorific sweetener consumption in the United States is now estimated to be 158 lb/person/year (ca. 194 g/person/day) with the majority attributed to refined sugar and most of the balance from high fructose corn syrup (4).

Americans, however, eat much less honey than, for example, people in Saudi Arabia, where families consume over 2 lb of honey a month (5). If the consumption of refined sugar (cane and beet sugar [sucrose] and high fructose corn syrup) was reduced and in part replaced by a more healthful sweetener with other beneficial properties, such as antioxidative and cancer chemoprotective, it could potentially address the two major public health concerns of obesity and cancer prevention at the same time.

Flavonoids are a large class of phytochemicals which are omnipresent in human diets, found for example in fruit, vegetables, tea, chocolate, and wine, and to which a number of beneficial effects on human health, such as antioxidant, anti-inflammatory, antiallergic, antiviral, and anticarcinogenic activities, have been ascribed (reviewed in 6 and 7). Estimates of total dietary flavonoid intake range from as little as 23 mg/day in The Netherlands to as much as 1 g/day in economically advantaged areas, with the latter figure generally considered to be at the very high end of a broad range of intakes (6, 8). The elevation of phase 2 detoxication and antioxidant enzymes by isothiocyanates, carotenoids, flavonoids, and other phytochemicals is now recognized as one of the mechanisms by which fruits

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and vegetables, in particular, cruciferous vegetables, exert their chemoprotective effects (9). Certain of these phytochemicals are also found in dietary ingredients that are produced either commercially or from plants or plant parts. Thus, the presence in honey of similarly acting phytochemicals, such as the flavonoids pinocembrin, pinostrobin, pinobanksin, and chrysin, makes this natural sweetener a logical source of dietary chemoprotective activity.

We thus sought to determine whether honey, a natural, botanically derived product with manifold well-documented medical benefits, such as antibiotic, antiulcerogenic, anti-inflammatory, and angiogenic properties (10–12), might, in addition, have chemoprotective activity due to its documented antioxidant action (10, 13) and its flavonoid content. The quinone reductase microtiter plate bioassay (14, 15) has now been widely used to identify both natural and synthetic potentially cancer chemoprotective compounds as well as complex mixtures from plants. We have used this methodology to compare honeys and to conduct a bioassay-guided search for flavonoids and for potent dietary sources of these compounds.

MATERIALS AND METHODS

Raw Materials. All solvents (dimethyl sulfoxide, acetonitrile, dimethyl formamide, methanol) were HPLC grade from J. T. Baker (Phillipsburg, NJ). Cell culture medium and serum were from Gibco-BRL (Grand Island, NY). Bioassay and other reagents were from Sigma/Aldrich (St. Louis, MO). Flavonoids were purchased from Indofine Chemical Company, Inc. (Sommerville, NJ) and Sigma/Aldrich (St. Louis, MO).

Honeys were obtained as gifts or purchased from the following sources: Alberta Honey Producers Cooperative, Spruce Grove, Alberta, Canada; Dutch Gold Honey, Lancaster, PA; Honeylands Naturally Limited, Waiatarua, Auckland, New Zealand; National Honey Board (Marcia Cardetti); P. Molan, Department of Biological Sciences, University of Waikato, Waikato, New Zealand; R. B. Swan & Son, Brewer, ME; McCutcheon's Apple Products, Frederick, MD; Trader Joe's, So. Pasadena, CA; Waitemata Honey Co., Ltd., Auckland, New Zealand. Brown and white sugar was Domino brand, and molasses was Brer Rabbit brand (B & G Foods, Roseland, NJ). Honey moisture content and color intensity were measured using refractive index (16) and absorbance at 560 nm (17) of a 10-fold dilution of honey in distilled water, respectively.

Plant samples were as follows: buckwheat (*Fagopyrum esculentum* Moench; Polygonaceae) hulls (Caudill Seed Co., Louisville, KY), seeds (Johnny's Select Seeds, Albion, ME), and flour (Arrowhead Mills, Hereford, TX); fresh ginger (*Zingiber officinale* Rosc.; Zingiberaceae) rhizomes (Brazil-grown, Fresh Fields, Baltimore, MD); live galangal (*Alpinia galanga* L. Wild.; syn. *Languas galanga* L. Stuntz.; Zingiberaceae) plants (Thai Herbs & Spices, Austin, TX); live fingerroot (*Boesenbergia pandurata* (Roxb.) Schltr.) plants (Thai Herbs & Spices, Austin, TX); fingerroot oil (Haldin International, Inc., Closter, NJ), fingerroot rhizome powder (Wayang Brand Teukunci Halus Moulo, Holland's Best, San Jose, CA); galangal rhizome powder (Cominex Kentjur, Holland's Best, San Jose, CA); grated fresh fingerroot rhizome (Surachai Vongvight—Thai Classic, Eldersburg, MD).

Extracts. Both fresh and dried plant material was boiled for 3 min in 10 volumes of 80% methanol/20% water and homogenized (Brinkmann Polytron, Westbury, NY). In all cases, homogenates were centrifuged to remove particulates and stored at -20°C until analyzed.

Cell Culture and Bioassay. Quinone reductase (QR) induction determinations were made (a) of plant extracts prepared in 80% boiling methanol; (b) of pure compounds diluted from a 20 mM "triple solvent" (equal volumes of dimethyl formamide, acetonitrile, and dimethyl sulfoxide) stock solution; and (c) of honeys, sugars, and molasses dissolved directly in cell culture medium immediately prior to assay. QR induction was determined by a coupled tetrazolium dye assay performed on digitonin lysates of Hepa 1c1c7 murine hepatoma cells

or their mutants defective in the Ah (aryl hydrocarbon) receptor (bp^r-c1) (18), grown in microtiter plates as described by Prochaska and colleagues (14, 15) and modified by Fahey et al. (19). Protein content of digitonin lysates was determined using a bicinchoninic acid assay (20). Just prior to dosing the bioassay plates, plant extracts, stock solutions of pure compounds in triple solvent (DMSO/CH₃CN/DMF), were diluted 1:200 directly into cell culture medium to attain the highest concentration to be examined in the bioassay. Serial dilutions were made into wells containing equivalent volumes of the appropriate solvent to control for possible solvent toxicity (21, 22). Flavonoids were tested over a concentration range from 0.78 to 100 μM , and the final concentration of organic solvent was 0.5 vol % (which was not cytotoxic). Activity is reported as follows: One unit of inducer activity is the amount required to double the quinone reductase activity in a microtiter plate well, initially seeded with 10 000 cells and containing 0.15 mL of α -MEM culture medium amended with 10% charcoal treated fetal calf serum, 1 $\mu\text{g}/\text{mL}$ streptomycin, and 1 unit/mL penicillin. Limits of detection were 2.5 units/g for honey, sugars, or molasses and 500 units/g for plant extracts.

RESULTS AND DISCUSSION

A variety of honeys, both of unifloral and mixed origin, were evaluated in vitro for their potential to induce quinone reductase, a representative phase 2 chemoprotective enzyme. Samples of over 35 different types of honey as well as representative sugars and molasses were evaluated for their quinone reductase (QR) induction. The microtiter plate assay employed to determine QR induction has been used extensively to characterize the inducer potency of a variety of natural and synthetic compounds as well as crude plant extracts (reviewed in 9 and 21).

In general, buckwheat honey and other darker colored honeys were more potent inducers than the light colored or "unifloral" honeys which we examined (Table 1 and Figure 1). Moisture content of all honeys examined ranged from 13.6% to 19.3% (mean 16.2%), and there was no correlation between moisture content and either color or QR induction. Frankel and colleagues have demonstrated that the water soluble antioxidant capacity of 19 honeys was positively correlated with honey color (10). When we contrasted QR induction to their published results for honeys with matching descriptions, there was a good correlation between antioxidant capacity (10^{-4} μequiv) and QR induction (linear regression $r^2 = 0.79$). Dark honeys were not only higher in direct antioxidant capacity, but they were better inducers of mammalian phase 2 enzymes than light colored honeys. The QR induction of the most potent honey (buckwheat) was only about 10% of the average levels found in fresh broccoli (35 000 units/g) (19), but when considered in the context of the amount of sugar consumed in economically advantaged countries, it gains significance. The potential chemoprotective benefit to be realized by replacing even a small portion of the 194 g/day of sugar eaten by Americans with honey might be substantial and could potentially augment the health benefits to be derived from fruit and/or vegetable consumption. Whereas refined white sugar had no detectable phase 2 enzyme inducing activity, there was very low induction by brown sugar (~ 100 units/g). The QR induction of molasses was similar to that of buckwheat honey (Table 1) and merits further investigation, since some molasses has been shown to contain significant quantities of flavonoids (22).

Although flowers of buckwheat (*Fagopyrum esculentum* Moench) are expected to be the source of the phase 2 enzyme inducer activity in buckwheat honey, the portions of the plant which are used in commerce are reported to be good sources of certain flavonoids (23). Thus seeds, hulls, and flour were extracted and evaluated for QR induction activity but did not yield any measurable activity (limits of detection, 500 units/g).

Table 1. Mammalian Phase 2 Enzyme (QR) Induction by Honeys

honey sample ^a	QR induction (units/g) ^{b,c}
buckwheat	2155 ± 951; n = 8
wildflower	2014 ± 800; n = 3
soybean	1786
manuka	1667
ling heather	1613
gallberry	1220
borage	1000
raw organic	1000
tupelo	984 ± 601; n = 3
avocado	562
comb honey	556
safflower	556
saw palmetto	455
orange blossom	454 ± 329; n = 3
oilseed rape	417
eucalyptus	417 ± 197; n = 2
christmas berry	417
blueberry	417
fireweed	417
blackberry	400
rewarewa	392 ± 267; n = 3
sunflower	278
"wild flora"	278
star thistle	278
clover	255 ± 81; n = 5
alfalfa	255 ± 95; n = 4
mesquite	250
wild raspberry	250
sage	210 ± 25; n = 2
sourwood	208
pohutukawa	200
kamahi	200
cotton	179
tawari	168 ± 46; n = 2
clover/canola	153 ± 73; n = 5

^a The "unifloral" honeys are labeled as coming from one dominant floral source. The highest value, 3400 units/g, was from one of the buckwheat honey accessions, and the lowest value, 50 units/g, was from one of the clover honey accessions. *Brassica* sp. honeys, to our surprise, were not particularly strong QR inducers, although a pollen sample obtained from *Brassica napus* was as potent as any of the honey samples tested (4550 units/g). ^b QR induction (in units per gram of honey) as measured in a microtiter plate assay with Hepa 1c1c7 cells (14, 15, 19). Limit of detection was 2.5 units/g. ^c Confidence intervals are presented as ±1 standard deviation; the number of separate honey samples examined follows this number. Single values are from an assay performed on a single honey accession.

In addition to evaluating the QR induction of raw and processed honeys, we evaluated the inducer potency of some of the primary flavonoid constituents of honey (Figure 2). Pinostrobin (5-hydroxy-7-methoxyflavanone) was an extremely potent inducer (CD = 0.5 μM), and there were progressive and substantial reductions in activity for its 5,7-dimethoxy-, 5-methoxy-7-hydroxy-, and 5,7-dihydroxy- analogues, respectively (Figure 2). The former two analogues have not been reported to occur in honey, but the latter (pinocembrin) has been found. The mutant Hepa1c1c7 cell line, bp^rc1 (18), which is defective in the Ah (aryl hydrocarbon) receptor, can distinguish monofunctional inducers (which only induce phase 2 enzymes) from bifunctional inducers (which elevate both phase 1 and phase 2 enzymes) (25, 26). When pinostrobin and pinocembrin were tested with the bp^rc1 mutant, there was no induction of quinone reductase, thus indicating that they are bifunctional inducers. Likewise, neither molasses nor buckwheat honey induced quinone reductase significantly in the bp^rc1 cell line whereas sulforaphane, a monofunctional inducer, induced at levels comparable to those in Hepa 1c1c7 (wild type) cells.

Related flavones and flavanones were much less active, and quercetin, a widely distributed tetrahydroxyflavonol, was a good

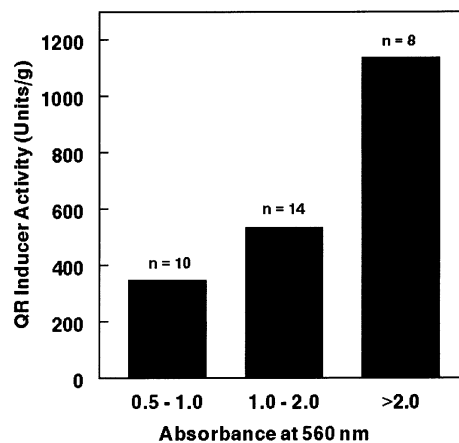


Figure 1. Correlation between honey color as determined by spectrophotometry (absorbance at 560 nm) and quinone reductase (QR) induction. Note that the darkest honey, buckwheat, had an Abs₅₆₀ of 6.8 and a corresponding QR induction of 2155 units/g. Colors were not determined for 3 of the 35 honey types in Table 1 (raw organic, wild flora, and comb honey), because they were high in particulate matter and were not suitable for direct comparison.

Structure	Compound	CD ^a
	Pinocembrin (5,7-dihydroxyflavanone)	110 μM
	5-methoxy-7-hydroxyflavanone ^b	11 μM
	5,7-dimethoxyflavanone ^b	2.0 μM
	Pinostrobin (5-hydroxy-7-methoxyflavanone)	0.5 μM
	Chrysin (5,7-dihydroxyflavone)	25 μM
	Myricetin (3,5,7,3',4',5'-hexahydroxyflavone)	58 μM
	Quercetin (3,5,7,3',4'-pentahydroxyflavone)	2.7 μM

^aCD = concentration required to double the QR induction.

^bThese compounds have not been reported to occur in honey; the others all have been.

Figure 2. Quinone reductase (QR) induction of selected flavonoids.

inducer of phase 2 enzymes (CD = 2.7 μM), as has already been demonstrated (25, 26). Both of these authors report a CD of about 20 μM in a 24 h Hepa1c1c7 cell bioassay, following exposure to quercetin, whereas we report an almost 10-fold lower CD on the basis of the more commonly used 48 h exposure to inducing agent. These induction potentials can be contrasted with that of sulforaphane, CD = 0.2 μM, from broccoli (19) or resveratrol, CD = 21 μM, from grape skins (27). Other flavonoids and related compounds, such as those found in chocolate, wine, tea, and various fruits, nuts, and berries, were much less potent inducers or were essentially inactive (CD ≫ 1000), and these are not shown in Figure 2. For example, caffeic acid (3,4-dihydroxycinnamic acid) and gallic acid (trihydroxybenzoic acid), and the flavan-3-ols

Table 2. Quinone Reductase Induction of Extracts Made from Plants in the Ginger Family

plant preparation	QR induction ^a (units/g)
fresh galangal (<i>A. galanga</i>) leaves	ND ^b
fresh galangal (<i>A. galanga</i>) rhizomes	ND
dry galangal (<i>A. galanga</i>) rhizome powder	42 700
fresh ginger (<i>Z. officinale</i>) rhizomes	750
fresh fingerroot (<i>B. pandurata</i>) leaves	ND
fresh fingerroot (<i>B. pandurata</i>) rhizomes	110 000
frozen, grated fingerroot (<i>B. pandurata</i>) rhizomes	83 300
fingerroot (<i>B. pandurata</i>) oil	625 000
dry fingerroot (<i>B. pandurata</i>) rhizome powder	490 000

^a QR induction in units per gram of plant (fresh or dry weight) refers to quinone reductase induction as measured in a microtiter plate assay with Hepa 1c1c7 cells (14, 15, 19). ^b ND = not detected. Limit of detection was 500 units/g.

catechin and epicatechin, had CD's of $\gg 1000$, 290, $\gg 1000$, and $\gg 1000 \mu\text{M}$, respectively.

Although various phenylpropanoid metabolites from plants have been found to have quite potent phase 2 enzyme inducing potential (28), many of these are not suitable for dietary consideration. In addition, much remains to be learned about how substituents on the flavonoid nucleus act to mitigate inducer potency. For example, for reasons that are unclear, the position of a substituent aromatic ring on the "A" ring of a basic flavone nucleus profoundly affects the inducer activity of the resulting molecule; the CD's of β -, α -, and γ -naphthoflavone are 15, 80, and 500 nM, respectively (28). Potent chemoprotective flavonoids such as 4'-bromoflavone (CD = 10 nM) have also been synthesized (29), but they may not be suitable for long-term dietary prophylaxis against cancer.

Certain flavonoids such as pinocembrin, pinobanksin, and chrysin, obtained primarily from propolis or honey, as well as other more omnipresent phytochemicals, including quercetin, kaempferol, myricetin, ellagic acid, and hesperetin (30), are likely to be the agents responsible for much of the antioxidant activity of honeys. Pinocembrin, a flavanone and a potential inducer of phase 2 enzymes, was originally identified in 1970 by Villanueva and colleagues on the basis of its antibacterial activity (31). Chrysin is an inhibitor of xanthine oxidase and as such may be effective in combating the symptoms of gout (32). Pinocembrin, chrysin, pinobanksin (3,5,7-trihydroxy flavanone) and related flavonoids are purported to have antibacterial, ovicidal, and larvicidal activity and local anaesthetic activity on mammals (33–35). Quercetin, a common flavonoid in foods, has been shown to have antioxidant properties (36), to enhance both phase 1 enzyme transcription activation (37) and phase 2 enzyme induction (26, 38), as we have shown herein for pinostrobin and pinocembrin (Figure 2), and to possess cancer chemoprevention activity (39).

Since pinostrobin is such a potent inducer, we examined the QR induction of other edible plants that have been previously identified as sources of this compound. Although it has been identified in propolis and from the wood and flowers of a number of plants, the most promising edible source appears to be the rhizomes of *B. pandurata* (syn. *Kaempferia pandurata*; Zingiberaceae), which is commonly known as fingerroot, Thai ginger, gkra-chai, Chinese key, and temu kunci and is widely consumed (40–43). Both dried and fresh samples of fingerroot rhizome, as well as fingerroot oil, were obtained from multiple sources, and they were very potent inducers of quinone reductase (Table 2). Leaves of this plant and leaves and rhizomes of

closely related members of the ginger family, galangal (*A. galanga*) and ginger (*Z. officinale*), that are substituted for fingerroot in some Asian cuisine have only negligible phase 2 inducer activity. The highest activity was found in fingerroot oil and in the powdered rhizomes (Table 2), both of which rivaled that shown previously for fresh 3-day-old broccoli sprouts (19).

We have thus identified a commodity sweetener and a flavoring or spice, as well as one of the flavonoids that is common to both of them, as potent inducers of phase 2 chemoprotective and antioxidant enzymes. Furthermore, the capacity of a range of honeys to induce mammalian quinone reductase roughly parallels their color, darker honeys being consistently more active. This observation is complementary to those of published studies correlating the antioxidant capacities of honeys with their color. Further studies focusing on the direct chemoprotective action of these compounds and their botanical sources, and efforts to determine the nature of the protective activity in molasses, could lead to specific dietary recommendations addressing the incorporation of little-used sweeteners and spices into healthy and protective diets.

ABBREVIATIONS USED

CD, concentration for doubling; QRIP, quinone reductase inducer potential

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