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Xanthine Oxidase Activity in Vitro: Effects of Food Extracts and Components

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There is significant interest in the direct antioxidant activities of dietary polyphenols, due to associations between consumption of polyphenol-rich foods, such as fruits and vegetables, and decreased incidence of oxidative-stress related disease. However, indirect antioxidant action, such as the inhibition of ROS-producing enzymes, may be equally relevant to health benefits through a general reduction in oxidative stress in vivo. To this end, the effects of food extracts and individual compounds on the in vitro activity of xanthine oxidase (XO) were assessed, many for the first time. Several compounds were shown to be potent inhibitors in vitro, including hesperetin and theaflavin-3,3′-digallate with IC_{50} values of 39 and 49 μM, respectively. Of the extracts, cranberry juice, purple grape juice, and black tea were the most potent, with IC_{50} values of 2.4, 3.5, and 5.8% of extracts, respectively. Some samples were shown to promote XO activity over the concentration ranges tested, including orange juice and pink grapefruit juice. Certain "inhibitors", such as purple grape juice and black tea, promoted XO activity at low concentration. The possible role of dietary inhibitors of XO in reducing oxidative stress in vivo is discussed.

KEYWORDS: Xanthine oxidase; polyphenols; phytochemicals; diet and health; hyperuricemia; antioxidant activity

INTRODUCTION

The consumption of fruits and vegetables has been associated with reduced risk of various oxidative-stress related diseases (for example, ref *1*). Such information led the World Health Organization in 1990 to set a daily target for consumption of at least 400 g of fruit and vegetables (including 30 g of nuts, pulses, and seeds), which has been interpreted by various countries and health groups as the "five portions of fruits and vegetables daily" advice. Although the protective effects of fruits and vegetables may be associated with their micronutrient and fiber content, these foods also contain many other potentially beneficial non-nutrient components, including the polyphenols (*1*). The term "oxidative stress" is applied in vivo to situations in which elevated levels of free radicals or other reactive oxygen species (ROS) can cause either direct or indirect damage to the body. Oxidative stress-related illnesses have been reported to include cancer (*2*), coronary heart disease (*3*), Parkinson's disease (*4*), and possibly Alzheimer's disease (*5*). Research on dietary polyphenols has intensified over the past decade, mainly due to the direct radical scavenging properties of many such compounds. More recently, however, it has become evident that polyphenols may also decrease oxidative stress through indirect antioxidant action, such as the inhibition of ROS-producing enzymes such as myeloperoxidase, lipoxygenase, cyclooxygenase (*6*), and xanthine oxidase (*7*).

Xanthine oxidase (XO; EC 1.1.3.22) is a member of the xanthine oxidoreductase (XOR) group, found in mammals at highest concentration within the liver and intestine (*8*). Under normal conditions, the predominant mammalian XOR is xanthine dehydrogenase (XDH; EC 1.1.1.204), but ~10% of the group is found as XO. However, under ischemic conditions (where oxygen is limited), XDH is converted to XO via limited proteolysis. Both XDH and XO convert hypoxanthine to uric acid via xanthine. However, although the operation of XDH is predominantly associated with the reduction of NAD^+ to NADH, the operation of XO is predominantly associated with the reduction of oxygen (**Figure 1**). Superoxide and hydrogen peroxide are created as byproducts of the reaction, with XO activity being the major source of ROS in the ischemic small intestine (*9*). Although superoxide and hydrogen peroxide are relatively inactive, both compounds are easily converted to more reactive species such as hydroxyl and peroxyl radicals or may interact with other species (e.g., ROS can react with nitrite oxide to create peroxynitrite). Therefore, the activity of XO may contribute significantly to levels of oxidative stress in vivo, particularly within ischemic tissues undergoing reperfusion (*10*).

Excessive levels of uric acid in vivo may also lead to a state of hyperuricemia, which is associated with ailments such as gout and renal stones (*11*). Furthermore, serum levels of XO are also elevated during hepatitis (and mild hepatotoxicity; *12*) with the concentration of XO dictating the degree of brain edema and injury (*13*). Various studies have also associated the involvement of XO with "thermal stress, respiratory syndrome, viral infection, and hemorrhagic shock" (*14*). It could therefore be hypothesized that a decreased activity of xanthine oxidase may be considered to be beneficial to health.

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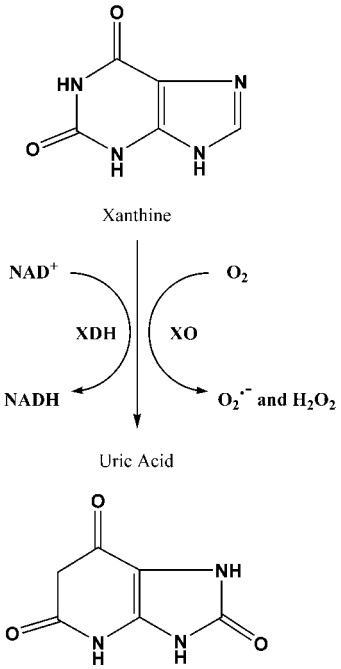

Figure 1. Conversion of xanthine to uric acid by xanthine oxidoreductase enzymes.

After water, tea is the most highly consumed beverage in the world with a history of use dating back over 5000 years. Tea consumption may be associated with health benefits (for reviews, see refs *15*−*17*). The mechanisms associated with protective effects are not firmly established, although tea is rich in plant secondary metabolites including significant amounts of polyphenols. It is the secondary metabolites that are believed to be likely sources of bioactivity. Tea components including the catechins (*18*) and theaflavins (*9*) have been shown to inhibit XO in vitro.

Other plant foods, including herbs, spices, fruits, and vegetables, might also inhibit XO in vitro. A twice-daily 2.5 g dose of "Xiao-Chai-Hu-Tang" (made from several herbs including ginseng and ginger) over 5 days promoted 25 and 20% decreases in XO activity on days 1 and 5, respectively, compared to baseline in 26 volunteers (*19*). Several traditional herbal remedies used to treat gout in China (*20*) and as anti-inflammatory agents in Australia (*21*) have also been shown to inhibit XO in vitro to various degrees. The aim of the present work was to extend present knowledge by assessing and comparing the potential to inhibit XO of various teas, single compounds, fruit and vegetable juices, and aqueous herb and spice extracts in vitro.

MATERIALS AND METHODS

Reagents. Xanthine oxidase (grade 1 from buttermilk), allopurinol, caffeine, catechin (C), (+)-epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), (−)-epigallocatechin gallate (EGCG), naringin, naringenin, retinyl acetate, rutin, and all other reagents where a source is not mentioned specifically were purchased from Sigma-Aldrich (Gillingham, Dorset, U.K.). Eriodictyol, equol, daidzin, hesperetin, and isoquercitrin were purchased from Extrasynthèse (Genay, France). Ascorbic acid was purchased from BDH (Poole, Dorset, U.K.). Theaflavin, theaflavin-3-gallate, and theaflavin-3,3′-digallate were purchased from Chromadex (Hatfield, Hertfordshire, U.K.). All water used was purified using a "Direct Q" demineralization system combined with a Millipak 40 0.22 μm filter, supplied by Millipore (U.K.) Ltd. (Watford, U.K.). Black tea, rooibus (Worlee), and yerba mate (Alicia) were produced by the Tetley Group Ltd. (Greenford, Middlesex, U.K.). Silver Needle and White Peony white teas were produced by Ming Cha (Quarry Bay, Hong Kong). Fennel, peppermint, camomile, and echinacea herbal teas were produced by Health and Heather (Lincolnshire, U.K.). *Ginkgo biloba* and ginseng herbal teas were produced by Double Dragon Co. (Essex, U.K.). Devil's claw herbal tea was produced by Salus Haus (Bruckmühl, Germany). Raspberry leaf herbal tea was produced by Cotswold Health Products Ltd. (Gloucestershire, U.K.). Cranberry (Ocean Spray) and purple grape (Welch's) juices were both produced by Gerber Foods Soft Drinks Ltd. (Bridgwater, Somerset, U.K.). Organic carrot and tomato juices (Rabenhorst) were both produced by Brewhurst Healthfood Supplies Ltd. (Surrey, U.K.). Cinnamon, whole dried ginger, cardamom, black cohosh root, whole nutmegs, and cloves were produced by English Herbal (Leeds, U.K.). Florida pink grapefruit, Florida orange juice, pineapple juice, apple juice, sage, rosemary, and thyme were purchased from Morrisons Supermarkets PLC (Merrion Centre, Leeds, U.K.). All herbal teas, herbs, spices, and fruit juices were purchased locally.

Preparation of Samples. Catechins and theaflavins (2 mM) were dissolved in a phosphate buffer (0.2 M, pH 7.5). If not immediately soluble, samples were sonicated for 10 min (machine number PUL55, Kerry Ultrasonics Ltd., Hertfordshire, U.K.). Other flavonoids (1 mM) were dissolved in a small amount of dimethyl sulfoxide (DMSO) and then diluted with phosphate buffer to give a final assay concentration of 0.5% DMSO. Ascorbic acid, allopurinol, and caffeine (1 mM) were dissolved in water. All teas, herbal teas, and herb and spice infusions were prepared according to manufacturers' instructions (where applicable) as follows: black tea, 3.18 g (average contents of a tea bag); herbal teas, 1 bag; white teas, 2 heaped tablespoons; rooibus and mate teas, 2 heaped teaspoons; raspberry leaf tea, 1 heaped teaspoon; herbs and spices, 0.88 g. Portions of test materials were placed into three separate 230 mL aliquots of boiling water for the maximum recommended time without stirring. Black tea was infused for 45 s; herbs and spices were infused for 10 min. Cloves, cinnamon, and cardamom were crushed before use, whereas ginger and nutmeg were grated to a coarse powder. Fruit juices, loose leaf teas, and herb and spice extracts were all filtered through filter paper (Whatman no. 1) before the triplicate infusions were combined and cooled to room temperature.

Assessment of XO Activity. XO activity was determined using a modified version of the method of Cos et al. (*22*). Briefly, the assay determines the amount of uric acid produced through the action of XO on xanthine substrate within a set time. Changes in the levels of uric acid produced through the presence of test samples allow the potency of inhibitors/promoters to be assessed. Each test solution (0.8 mL) comprised xanthine (150 μM), hydroxylamine (0.2 mM), and EDTA (0.1 mM), all in sodium phosphate buffer (0.2 M, pH 7.5) and a test sample (various concentrations). Both xanthine and xanthine oxidase working solutions were stored at 5−8 °C. The reaction was initiated through the addition of 0.2 mL of XO (23.42 milliunits/mL, 0.2 M phosphate buffer) and incubated for 30 min (37 °C). The reaction was stopped with HCl (0.1 mL, 5 M). The production of uric acid was determined spectrophotometrically (at room temperature) at a wavelength of 290 nm. Sample tests were performed in triplicate. Many flavonoids also have maxima close to 290 nm; thus, flavonoid-rich samples would often significantly contribute to optical density. Therefore, control solutions were prepared in the same way as test solutions, except enzyme addition was delayed until after solutions had been acidified. IC_{50} values were determined as the concentration of sample required to inhibit XO activity by 50%. Similarly, compounds found to promote XO activity were compared in terms of PC_{50} values (concentration of sample required to promote XO activity by 50%). However, the concentration range of promoters was limited, with theoretical PC_{50} values exceeding the upper limits of ranges tested. Therefore, stated PC_{50} values were treated as estimates only.

RESULTS AND DISCUSSION

Figure 2 shows examples of inhibitors, promoters, and inhibitors showing promotional effects at low concentration, from both single compound and extract test groups. A summary of results can be seen in **Tables 1** and **2**, which list the compounds and extracts tested, whether they were found to be

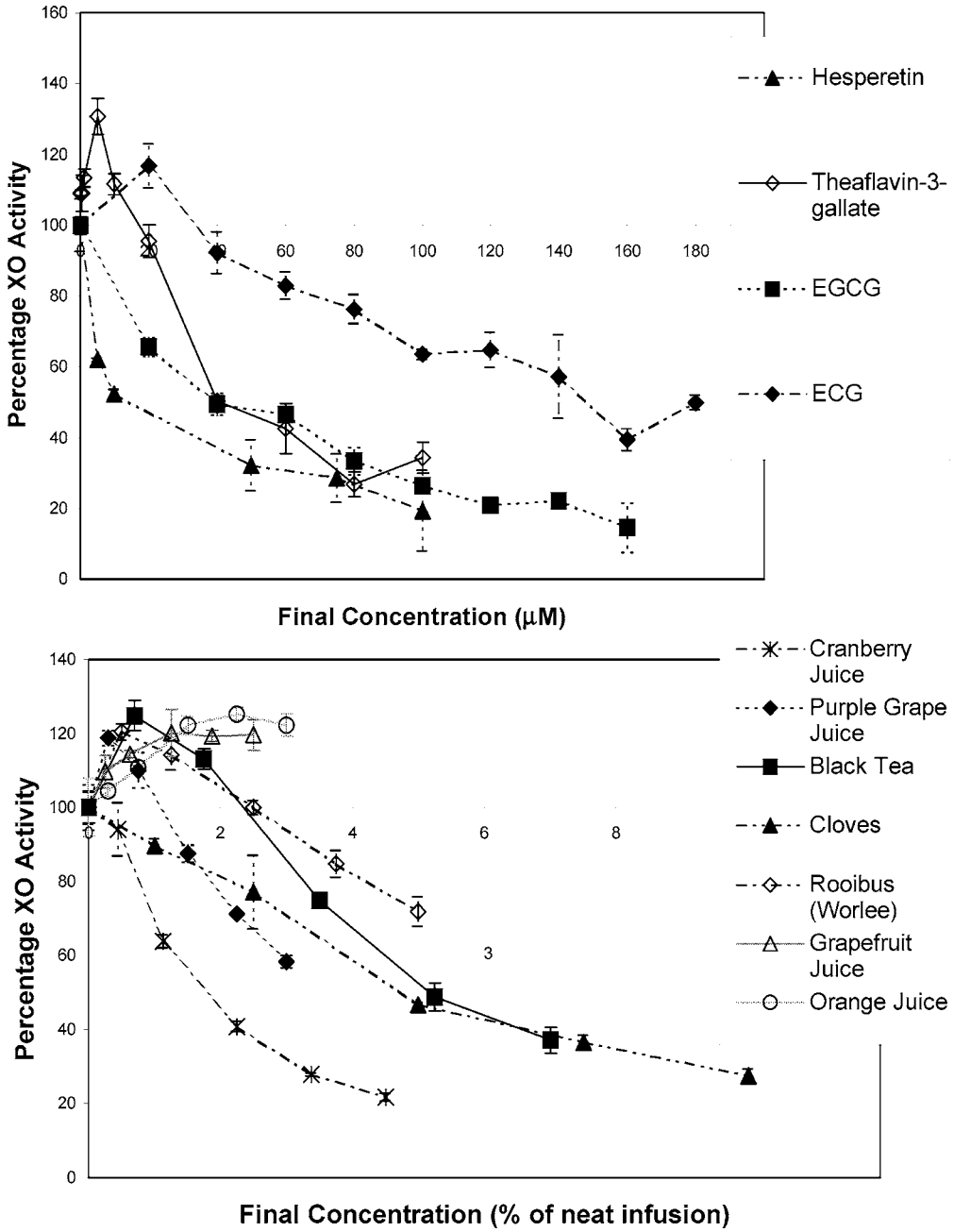

Figure 2. Modulation of XO by (**a**, top) individual compounds and (**b**, bottom) food extracts. Points are means ($n = 3$) ± standard deviation.

inhibitors or promoters, and the IC_{50} or PC_{50} values as appropriate. Although DMSO was found to be a mild promoter of XO activity, it was determined that inclusion of DMSO in the assay at final concentrations of 0.5% did not affect results.

Catechins. ECG and EGCG were found to have IC_{50} values of 155.0 ± 11.6 and 63.6 ± 5.4 μM, respectively. C, EC, and EGC did not show inhibition over the concentration range tested (0−180 μM), instead exhibiting a mild apparent promotion of XO activity, although the effect was non-dose-dependent. Aucamp et al. (*18*) also report a similar order of behavior for tea catechins as observed here (although nongallated catechins showed mild inhibition activity). Potency for inhibition was reported in the order EGCG > ECG > EGC > EC > C, with Aucamp et al. noting that the type of inhibition differed among the compounds. For example, whereas catechin (the least potent) showed uncompetitive inhibition, epigallocatechin gallate exhibited competitive inhibition, with all others showing a mixed type.

Theaflavins. Theaflavin, theaflavin-3-gallate, and theaflavin-3,3′-digallate were found to act as XO inhibitors, with IC_{50} values of 66.4 ± 4.2, 64.0 ± 4.4, and 49.1 ± 9.7 μM, respectively. Once again, potency was associated with the presence of galloyl groups. Lin et al. (*9*) also found that theaflavins and EGCG inhibited XO, with mono- and digallated theaflavins showing more potent activity compared to EGCG (IC_{50} values of 7.6 and 4.5 μM, respectively, compared to 12.5 μM).

Other Flavonoid Compounds. All but two of the flavonoids tested (equol and daidzin) were shown to inhibit XO. The most potent were hesperetin, isoquercitrin, naringenin, and naringin, with IC_{50} values of 38.5 ± 8.1, 63.4 ± 3.4, 77.4 ± 20.9, and 94 ± 9.9 μM, respectively. Eriodictyol and rutin were found to have mild inhibitory effects, although IC_{50} values could only be approximated at 116 ± 12 and 284 ± 55 μM, respectively, as values were outside the concentration range tested. Equol and daidzin were shown to have no effect on XO activity in

Table 1. Summary of Results—Effects of Single Compounds on Xanthine Oxidase Activity

sample	concn range tested (μM)	effect on XO	IC_{50}/PC_{50} (μM)
catechins			
catechin	0–180	none	
epicatechin	0–180	none	
epicatechin gallate	0–180	inhibitor	155.12 ± 1.6
epigallocatechin	0–180	none	
epigallocatechin gallate	0–180	inhibitor	63.6 ± 5.4
theaflavins			
theaflavin	0–100	inhibitor	66.4 ± 4.2
theaflavin-3-gallate	0–100	inhibitor	64 ± 4.4
theaflavin-3,3′-digallate	0–92	inhibitor	49.1 ± 9.7
other flavonoid compounds			
eriodictyol	0–100	mild inhibitor	116 ± 12 (estimated)
equol	0–100	none	
daidzin	0–100	none	
hesperetin	0–100	inhibitor	38.5 ± 8.1
isoquercitrin	0–100	inhibitor	63.4 ± 3.4
naringenin	0–100	inhibitor	77.4 ± 20.9
naringin	0–100	inhibitor	94 ± 9.9
rutin	0–100	mild inhibitor	284 ± 55 (estimated)
nonflavonoid compounds			
ascorbic acid	0–100	promoter	384 ± 35 (estimated)
allopurinol	0–100	inhibitor	3.68 ± 0.3
caffeine	0–100	none	
retinyl acetate	0–100	promoter	250 ± 1 (estimated)

Table 2. Summary of Results—Effects of Extracts upon Xanthine Oxidase Activity

sample	concn range tested (%)	effect on XO	IC_{50}/PC_{50} (%)
teas/herbal teas			
black	0–7	inhibitor	5.8 ± 0.26
camomile herbal	0–10	none	
devil's claw herbal	0–8	none	
echinacea herbal	0–10	none	
fennel herbal	0–10	promoter	19 ± 0 (estimated)
Ginkgo biloba herbal	0–10	none	
ginseng herbal	0–10	promoter	20 ± 6 (estimated)
yerba mate herbal	0–2.5	none	
peppermint herbal	0–7	inhibitor	13 ± 1 (estimated)
raspberry leaf herbal	0–6	inhibitor	10 ± 1 (estimated)
rooibus herbal	0–5	inhibitor	8 ± 0 (estimated)
Silver Needle white	0–10	mild inhibitor	17 ± 1 (estimated)
White Peony white	0–10	mild inhibitor	26 ± 0 (estimated)
fruit and vegetable juices			
apple	0–3.5	none	-
carrot	0–2	none	-
cranberry	0–4.5	inhibitor	2.4 ± 0.1
orange	0–3	promoter	7 ± 1 (estimated)
pineapple	0–4.5	none	-
pink grapefruit	0–2.5	promoter	9 ± 1 (estimated)
purple grape	0–3	inhibitor	3.5 ± 0 (estimated)
tomato	0–8	mild inhibitor	24 ± 1 (estimated)
aqueous herb and spice extracts			
black cohosh root	0–10	none	
cardamom	0–10	promoter	29 ± 4 (estimated)
clove	0–10	inhibitor	6.1 ± 0.38
cinnamon	0–10	mild inhibitor	17 ± 1 (estimated)
nutmeg	0–10	none	
rosemary	0–10	mild inhibitor	20 ± 1 (estimated)
sage	0–10	mild inhibitor	13 ± 0 (estimated)
thyme	0–10	mild inhibitor	18 ± 0 (estimated)
whole dried ginger	0–10	promoter	20 ± 8 (estimated)

the concentration range tested (0−100 μM). The results contrast slightly with the work of Cos et al. (*22*), as whereas they report a similar inhibitory activity for naringenin ($IC_{50} > 50$ μM), rutin was found to have a much higher potency (IC_{50} 52.2 μM) than reported here. However, other workers have reported rutin as having only mild inhibitory effects (*23*), with Russo et al. (*24*) stating that rutin produced just over 50% inhibition when present at 200 μM. Nagao et al. (*25*) reported hesperetin as having a potency similar to that found during the current investigation ($IC_{50} = 27.4$ μM).

Nonflavonoid Compounds. Several nonflavonoid compounds were tested. As was expected, allopurinol (a XO inhibitor used to treat hyperuricemia; *8*) was shown to be the most potent XO inhibitor tested, with an IC_{50} value of 3.7 ±

0.3 μM. The IC_{50} value agrees with that of Orallo et al. (*26*; 3.63 μM), although 0.24 μM was reported by Cos et al. (22). Ascorbic acid was shown to slightly promote XO activity, with an approximated PC_{50} value of 384 ± 35 μM. A water-soluble version of vitamin A (retinyl acetate) promoted XO activity in a more pronounced manner, with an approximated PC_{50} value of 250 ± 1 μM. Similarly, Schimpl et al. (*27*) reported that dietary supplementation with vitamins C and E did not reduce hepatic XO activity in chronic cholestatic rats (in which the common bile duct was ligated to increase hepatic XO and XD levels). Caffeine was found to have no effect on XO activity over the concentration range tested (0−100 μM), which was expected as XO is among several enzymes involved in caffeine clearance in vivo.

Black, White, and Herbal Teas. Black tea was the most potent "tea" to inhibit XO, with an IC_{50} value of 5.8 ± 0.26% of original infusion concentration. Rooibus, raspberry leaf, and peppermint herbal teas were also found to inhibit XO, with estimated IC_{50} values of 5.8 ± 0.26, 8 ± 0, 10 ± 1, and 13 ± 1% of original infusion concentrations, respectively. Silver Needle and White Peony white teas were found to be mild inhibitors of XO, with approximate IC_{50} values of 16.9 ± 0.94 and 25.9 ± 0.01% of original infusion concentrations, respectively. Camomile, devil's claw, echinacea, *Ginkgo biloba*, and mate herbal teas were found to promote XO activity to a small extent in a non-dose-dependent manner. Fennel and ginseng herbal teas were found to promote XO activity slightly, with approximate PC_{50} values of 19 ± 0 and 20 ± 6% of original infusion concentrations, respectively.

Fruit and Vegetable Juices. Cranberry and purple grape juices were found to inhibit XO, with IC_{50} values of 2.4 ± 0.1 and 3.5 ± 0% (estimated) of original concentrations, respectively. Tomato juice was found to mildly inhibit XO, with an approximate IC_{50} value of 24 ± 0.55% of original concentration. Apple, carrot, and pineapple juices were found to promote XO activity slightly in a non-dose-dependent manner (data not shown). Orange and pink grapefruit juices were found to promote XO activity, with approximate PC_{50} values of 7 ± 1 and 9 ± 1% of original concentration, respectively. Various health benefits associated with the consumption of certain fruit juices have been widely publicized. For example, cranberry juice has been reported to protect against urinary tract infection and may also be of use in the prevention of kidney stone formation (*28*). However, little research concerning the inhibition of XO by fruit juices has been reported to date. Further research is certainly needed to establish the in vivo activities of both promoters and inhibitors.

The potent inhibitory behavior of cranberries and purple grapes may be derived in part from anthocyanins, which are responsible for the deep red and blue colors in many plants. Anthocyanins are a subclass of flavonoid that are glycosides of anthocyanidins. Anthocyanidins have been shown previously to exert a moderate inhibitory effect on XO, with Nagao et al. (*25*) reporting IC_{50} values between 21.9 and 29.1 μM for apigenidin > cyanidin > peonidin > pelargonidin. Anthocyanin-rich foods have also shown potentially beneficial behavior in vivo with respect to gout. For example, consumption of a 280 g portion of Bing sweet cherries (in which anthocyanins represented 23% of total phenolics) led to a significant decrease in plasma urate concentration within a group of 10 healthy women over a 5 h period (*29*). It is possible that part of the observed decrease in plasma urate may have been due to XO inhibition, mediated by the phenolic component of cherries. Potential inhibition of XO by anthocyanins should be investigated further. Grapefruit juice has been reported as a dietary risk factor with regard to the formation of urinary stones (*30*). Dakovic-Svajcer et al. (*31*) also report that although a single large oral dose of grapefruit juice (administered 90 min before sacrifice) significantly decreased the activity of XO in the livers of male mice, the activity of XOD was increased in mice that had been regularly fed smaller doses of grapefruit juice over 10 days, which would agree with the potent promotion of XO observed during the present study.

Herb and Spice Infusions. Clove infusion was found to inhibit XO, with an IC_{50} value of 6.1 ± 0.38% of original infusion concentration. Sage, cinnamon, thyme, and rosemary infusions were found to mildly inhibit XO, although potency could be only approximated with IC_{50} values of 12.9 ± 0.19, 17.4 ± 0.85, 17.9 ± 0.38, and 20.4 ± 0.64% of original concentrations, respectively, as values were outside the concentration range tested. Black cohosh root and nutmeg infusions were found to mildly promote XO activity in a non-dose-dependent manner. Whole dried ginger and cardamom infusions were found to mildly promote XO activity, with approximate PC_{50} values of 20 ± 8 and 29 ± 4% of original infusion concentrations, respectively.

The antioxidant properties of herbs and spices are well documented (for example, ref *32*), but considerably less work has been performed regarding the inhibition of XO. Eugenol (from cloves) was shown to inhibit XO activity potently in vitro, with an IC_{50} value of 2.2 μM (*14*). Interestingly, the action of eugenol was reduced by the addition of some metal chelators.

In conclusion, several flavonoids, fruit juices, teas, and herb and spice extracts were shown, many for the first time, to inhibit XO in vitro, including hesperetin, theaflavin-3,3′-digallate, cranberry juice, purple grape juice, black tea, cloves, and rooibus as among the most potent. Other samples, for example, orange juice and grapefruit juice, were shown to promote XO activity over the concentration ranges tested. It is of interest that these juices are significant dietary sources of the potent XO inhibitor hesperetin, highlighting the requirement to consider potentially beneficial dietary ingredients as a whole rather than on the activity of a single component. It should be noted that the concentrations of vitamin C in the juices would not be sufficient to explain all of the observed activities. For example, orange and grapefruit juices have high vitamin C contents and were shown to be strong promoters, yet cranberry juice has a similar vitamin C content and was the most potent inhibitor (among the beverages) tested. However, the limited concentration range of promoter samples should be remembered, especially when it is considered that some inhibitors showed promotional behavior at low concentrations (**Figure 2**). Therefore, some "promoters" might display inhibitory behavior when assayed at higher concentration.

The effects of XO inhibitors/promoters in vivo should be determined. Metabolites may well have activities different from those of their parent compounds. Therefore, the prediction of in vivo activity using in vitro data is difficult, owing to the potential for dietary compounds to undergo metabolism that may consequently alter functionality. For example, *G. biloba* was shown to limit increases in brain tissue XO activity within rats occurring as a result of exposure to EMR radiation from mobile phones (6.39, 4.31, and 3.74 units/g of protein for non-ginkgo-treated, ginkgo-treated, and control rats, respectively; *33*). The present results showed that *G. biloba* had no effect on XO activity. Future work should aim to assess XO activity in vivo within human populations using appropriate biomarkers such as the urinary caffeine metabolites, 1-methylurate and 1-meth-

ylxanthine. However, the exact concentrations of XO inhibitors required to yield a beneficial effect in vivo remain to be determined. Potent dietary XO inhibitors could potentially decrease XO activity in vivo, giving rise to the possibility of reducing hyperuricemia and overall oxidative stress.

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