In vitro glucuronidation of xanthohumol, a flavonoid in hop and beer, by rat and human liver microsomes

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Abstract **Xanthohumol (XN) is the major prenylated flavonoid of hop plants and has been detected in beer. Previous studies suggest a variety of potential cancer chemopreventive effects for XN, but there is no information on its metabolism. The aim of this study was to investigate in vitro glucuronidation of XN by rat and human liver microsomes. Using high-performance liquid chromatography, two major glucuronides of XN were found with either rat or human liver microsomes. Release of the aglycone by enzymatic hydrolysis with β-glucuronidase followed by liquid chromatography/mass spectrometry and nuclear magnetic resonance analysis revealed that these were C-4′ and C-4 monoglucuronides of XN.** **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Xanthohumol; Flavonoid; Hop; Glucuronidation; Rat; Human

1. Introduction

Flavonoids are a group of phenolic compounds that naturally occur in fruits, vegetables, nuts, seeds, flowers, and bark. More than 4000 different flavonoids have been described. They have been considered inert and non-essential for human health; however, in the last few years it has been shown that flavonoids affect a wide variety of biological systems in mammals, exhibiting antioxidant, anti-inflammatory, antiviral, antiproliferative, and anticarcinogenic effects [1]. Studies show that several flavonoids can inhibit or activate the cytochrome P450 (CYP) enzyme system in vitro and in vivo [2,3]. Recently in animal studies much attention has been paid to the antioxidant properties and the inhibitory role of flavonoids in various stages of tumor development. This improved understanding of the biological and pharmacological properties of individual flavonoid compounds has led to the development of flavonoid drugs which contain either naturally occurring flavonoids or their chemically modified derivatives [4].

Hop (*Humulus lupulus* L.) cones are known not only as brewing materials but also in Europe as a medicinal plant. Even today, hop is used as a tranquilizer in folk medicine [5]. Xanthohumol (XN), which has a prenylated chalcone structure (Fig. 1B), is the principal flavonoid present in hop flower (cone) extracts [6] and is present in beer [7]. Studies have shown that XN is an effective antiproliferative agent in human breast cancer cells (MCF-7), colon cancer cells (HT-29) and ovarian cancer cells (A-2780) [8]. It also was shown that XN inhibited bone resorption and has been patented as a drug for osteoporosis treatment [9].

Although flavonoids are abundant, little is known about their fate in animals. Flavonoid biotransformation is primarily catalyzed by the liver and the gut microflora [10]. In a recent study, we showed that XN was metabolized to four different metabolites by isosafrole/β-naphthoflavone (ISF/BNF) -induced rat liver microsomes [11]. Numerous studies have been published demonstrating that many types of flavonoids are excreted as glucuronides by humans or other mammals [12,4]. Glucuronidation is the main pathway of the Phase II detoxification processes for most xenobiotics, including flavonoids, and is catalyzed by UDP-glucuronosyl transferases which are membrane-bound and located mainly in the liver endoplasmic reticulum [13]. It is also known that flavonoids may be conjugated in vivo yielding metabolites that exhibit antioxidant properties [1].

The glucuronidation of flavonoids such as diosmetin, quercetin, genistein, rutin, and kaempferol has been investigated in rat and human, but there is no information about glucuronidation of prenylated flavonoids. This study is the first one to demonstrate that XN, a prenylated flavonoid, produced glucuronides by liver microsomes from human or rats untreated or rats pretreated with various CYP-inducing agents, including phenobarbital (PB), ISF, and BNF. The glucuronides were characterized by high-performance liquid chromatography (HPLC), UV spectroscopy, liquid chromatography/mass spectrometry (LC/MS), and proton nuclear magnetic resonance (^{1}H-NMR).

2. Materials and methods

2.1. Chemicals

XN was isolated and purified from hop as described [6]. Formic acid, β-glucuronidase (Type B-1: from bovine liver), PB, BNF and uridine 5′-diphosphoglucuronic acid (UDPGA) were purchased from Sigma Chemical Company (St. Louis, MO, USA). ISF was from Aldrich Co. (Milwaukee, WI, USA). Acetonitrile, ethanol and meth-

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Abbreviations: XN, xanthohumol; IX, isoxanthohumol; PB, phenobarbital; ISF, isosafrole; BNF, β-naphthoflavone; G1, glucuronide 1; G2, glucuronide 2; G3, glucuronide 3; G4, glucuronide 4; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; ^{1}H-NMR, proton nuclear magnetic resonance; UDPGA, uridine 5′-diphosphoglucuronic acid

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anol were HPLC grade from Mallinckrodt Baker, Inc. (Paris, KY, USA). Dimethyl-d_6 sulfoxide (100%) and $MgCl_2$ were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA) and Kodak (Rochester, NY, USA), respectively. Human liver microsomes were purchased from Genetest.

2.2. Isolation of rat liver microsomes

Sixteen male Sprague–Dawley rats (165–185 g body weight) were purchased from Simonsen Company (Gilroy, CA, USA). Animals were divided into four groups with four animals in each cage. Microsomes were prepared as described [14] from pooled livers or rats that had been treated intraperitoneally with 0.9% NaCl, PB (80 mg/kg) in NaCl, ISF (150 mg/kg) in corn oil, or BNF (40 mg/kg) in corn oil daily for 3 days. On the fourth day after exposure, preceded by a 24-h fasting period, the rats were anaesthetized using CO_2 and killed by asphyxiation. The washed liver microsomes, resuspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol and 1 mM EDTA, were frozen at −80°C before use. Protein concentrations of liver microsomes were determined by Coomassie Plus™ Protein Assay from Pierce Chemical Co. (Rockford, IL, USA). P450 contents were determined by the method of Omura and Sato [15].

2.3. XN glucuronidation by rat or human liver microsomes

A typical 0.5-ml incubation mixture consisted of 0.5 mg protein of microsomes from untreated or PB-, ISF-, or BNF-treated rats or human liver microsomes, 52 mM HEPES/NaOH buffer pH 7.4 containing 10 mM $MgCl_2$, 0.25 mM Triton X-100, 52 mM UDPGA, and 100 μM XN (dissolved in 3 μl ethanol) as a substrate [12]. The reaction was initiated by adding UDPGA. Incubations were carried out at 37°C for 60 min with continuous shaking in a Dubnoff incubator. Control incubations were performed without the addition of UDPGA or without microsomes. Reactions were terminated by adding 1.5 ml ice-cold methanol, followed by centrifugation at 4°C. The supernatants were evaporated to dryness under nitrogen gas. The residues were redissolved in 100 μl 25% CH_3CN containing 1% formic acid, and subsequently analyzed on the same day by HPLC for XN metabolites. All experiments were carried out in duplicate. Sets of experiments also were done with ethanol alone or without microsomes.

2.4. Time course of glucuronidation of XN

Liver microsomes from untreated rats were used in incubations as described above with a final volume of 250 μl. Reactions were stopped at 0, 10, 20, 30, 40, 50, and 60 min by adding ice-cold MeOH. The procedure described above was followed to analyze in HPLC.

2.5. Hydrolysis with β-glucuronidase

The dried fractions or incubation samples were incubated in 50 mM phosphate buffer (pH 5.5) with the presence of 1000 units/ml of β-glucuronidase for 1.5 h at 37°C with a final volume of 250 μl. Controls were run simultaneously under the same conditions without β-glucuronidase. All reactions were carried out in duplicate. The reactions were stopped by adding 500 μl of ice-cold MeOH and centrifuged for 20 min. The supernatant was analyzed by HPLC.

2.6. HPLC and isolation of metabolites by HPLC

A waters 2690 HPLC system with a 996 diode-array detector and a 4 μm Nova-Pak C_{18} column (3.9×150 mm, Waters) were used to separate metabolites. Detection was at 370 nm. Samples dissolved in 100 μl 75% aqueous acetonitrile containing 1% formic acid were analyzed using a slight modification of the method of Nielsen et al. [2]. Metabolites were eluted with acetonitrile and water containing 1% formic acid at a flow rate of 0.8 ml/min. The initial 29% CH_3CN was increased to 60% over 18 min and then to 84% over the next 10 min with a linear gradient function. At 34 min, the CH_3CN was returned to 29% in 4 min. The metabolites were identified by LC/MS and ^{1}H-NMR after running a large-scale incubation with 1.27 mg XN and collecting fractions of this incubation by preparative HPLC with an Alltech Econosil C_{18} (250×22 mm) column at a flow rate of 11 ml/min. All isolated metabolite fractions were lyophilized and redissolved in 25% CH_3CN containing 1% formic acid before LC/MS analysis.

2.7. LC/MS

LC/MS was performed with Waters 6000A pumps using a 5 μm C_{18} column (250×4.0 mm) at a flow rate of 0.8 ml/min. XN metabolites were separated with a linear solvent gradient starting from 40% CH_3CN to 100% CH_3CN in 1% aqueous formic acid over 30 min. At 35 min, the percentage CH_3CN was returned to 40% in 2 min, and the column was equilibrated for 15 min prior to the next injection. Mass spectra were recorded on a PE Sciex API III+ triple quadrupole mass spectrometer using atmospheric-pressure chemical ionization in positive mode, with an orifice voltage of +55 V, source temperature of 60°C, and scanning from *m/z* 110 to 800. Samples were introduced by loop injection or by HPLC via the heated nebulizer interface set at 500°C. After data acquisition, multiple-ion monitoring was employed for selective detection of metabolites. Daughter-ion scanning in the MS–MS mode was used to obtain structural information. The target gas in the collision cell was argon:nitrogen (9:1) at a density of ca 1.8×10^{14} atoms cm^{-2}. The collision energy was set at 15 V.

2.8. NMR

^{1}H-NMR spectra of metabolite glucuronide 1 (G1) and G2 fractions were recorded in DMSO-d_6 at room temperature on a Bruker DRX 600 spectrometer at 600 and 150.9 MHz. DMSO resonances at 2.5 and 39.51 ppm were used as internal shift references. ^{1}H–H COSY was performed using standard pulse sequences.

3. Results and discussion

XN is the major prenylated chalcone in hop and beer. It is of general interest to humans because it represents a dietary flavonoid with significant biological activities [8]. In previous work, the oxidative metabolism of XN was studied using rat liver microsomes [11]. The present study deals with glucuronidation of XN investigated by incubation of XN with UDPGA and liver microsomes from rats or humans. The glucuronides were characterized by HPLC, LC/MS and NMR analysis. Untreated male rat liver microsomes metabolized XN to two major and two minor glucuronides, designated G1, G2, G3, G4 (Fig. 1). The retention times for XN and its glucuronides were as follows; XN -17.6 min; G1 -9.6 min; G2 -11.2 min; G3 -3.4 min; and G4 -5.0 min. Liver

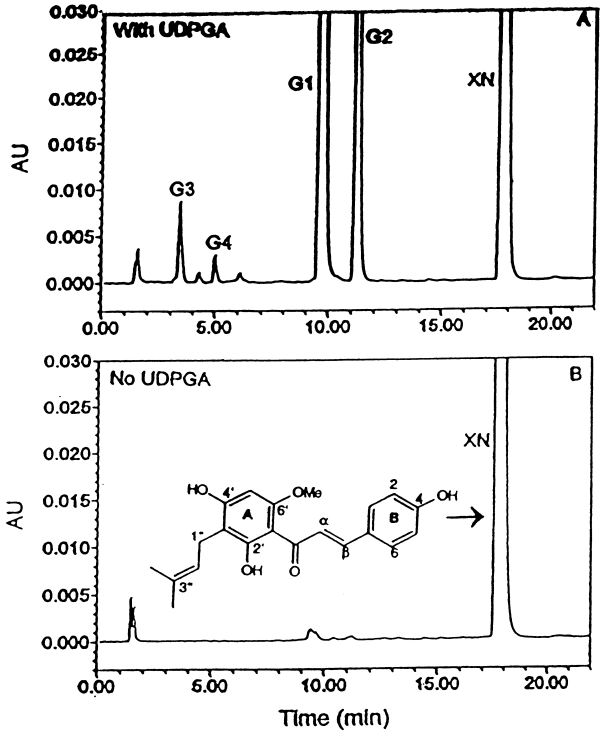

Fig. 1. A typical HPLC chromatogram of XN glucuronidation mediated by liver microsomes from untreated rats with UDPGA (A) and without UDPGA (B).

microsomes from rats pretreated with PB (an inducer of CYP2B1 and CYP2B2), ISF (a CYP1A inducer) or BNF (a CYP1A inducer) [16], yielded the same four glucuronides. The relative proportions of these glucuronides was not affected by the different CYP inducers which is in agreement with previous studies and which shows that the microsomal UGTs responsible for the conjugation of flavonoids are not inducible by CYP1A and CYP2B inducers [12]. Based on integrated peak areas, G1 and G2 represented approximately 89% and 10% of total glucuronides formed in incubations, respectively. G3 or G4, however, only accounted for about 1% of total glucuronides. Human liver microsomes (H056) that were rich in CYP1A2 formed only the G1 and G2 glucuronides. However, human liver microsomes (H112) that were rich in CYP3A4 produced a small amount of the third glucuronide, G3, in addition to G1 and G2. This may be a result of different concentrations of UDP-glucuronosyl transferase isozymes in the two human liver microsomal samples. Control incubations were carried out without UDPGA or microsomes. No glucuronidation of XN occurred in the absence of UDPGA, and neither were glucuronides formed when liver microsomes (rat or human) were absent (Fig. 1B).

β-Glucuronidase treatment of the mixture of four glucuronides caused a substantial decrease of amounts of G1, G2, G3 and G4 (95, 91, 96, and 92%, respectively) and a 30% increase of XN. The hydrolytic cleavage experiments with bovine liver β-glucuronidase confirms that metabolites G1 through G4, are glucuronide conjugates. The time course of glucuronidation of XN by untreated male rat liver microsomes showed an increase of glucuronide formation and a corresponding decrease of parent compound, XN, over a 1.0-h incubation period.

The treatment of the G1 and G2, obtained by preparative HPLC, with β-glucuronidase led to the disappearance of G1

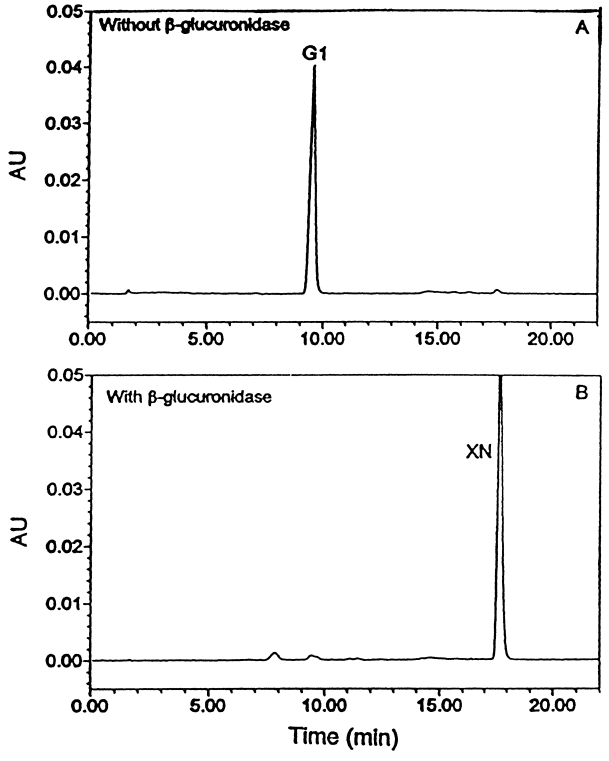

Fig. 2. HPLC chromatogram of G1 before (A) and after (B) β-glucuronidase treatment.

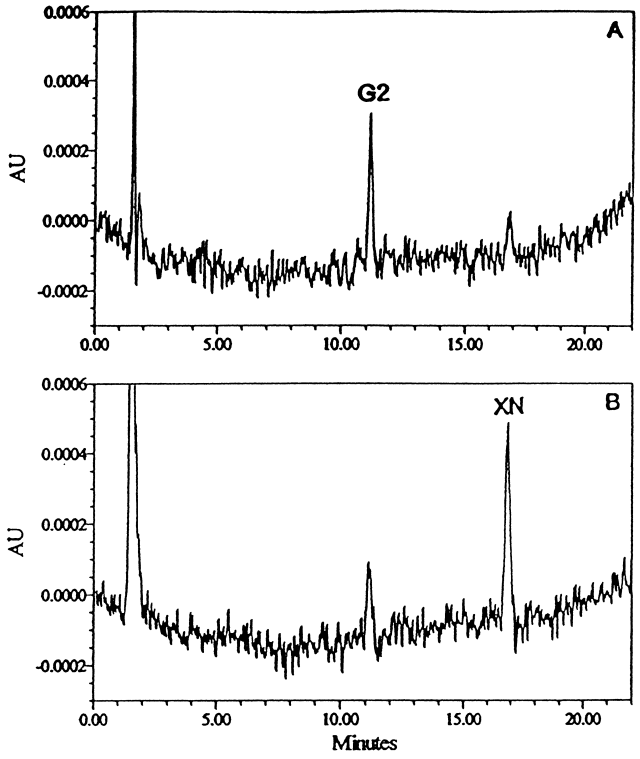

Fig. 3. HPLC chromatogram of G2 before (A) and after (B) β-glucuronidase treatment.

and G2 and the concomitant appearance of XN (Figs. 2 and 3, respectively).

The structures of two major glucuronides, G1 and G2, were identified as monoglucuronides of XN by UV, LC/MS and ^{1}H NMR analysis after preparative HPLC isolation of the glucuronides. G1 showed a maximum UV absorption at 370 nm similar to the chalcone flavonoid XN. In LC/MS studies the molecular weight, 530 Da, of G1 suggested that the conjugate contained a glucuronide group attached to XN (Fig. 4A). Upon MS–MS fragmentation of the MH^+ ion, loss of the prenyl moiety from glucuronide, and loss of glucuronide from the parent compound, were most prominent, giving rise to fragments with *m/z* 475 $[531–56]^+$, and 355 $[531–176]^+$, respectively. The intensity of the fragment with *m/z* 355 was greater than that of *m/z* 475 indicating that C″–C2″ cleavage of the prenyl group was more difficult in G1 than in G2 (compare Fig. 4A and B). This suggested that in G1 the glucuronide residue was attached to OH-4′ because cleavage of the 1″–2″ bond of the prenyl substituent leaves an Ar-CH_2^+ fragment ion which is stabilized by a free *ortho* hydroxyl group [6]. Apparently, such a free hydroxyl group was not available in G1, rendering loss of the prenyl substituent less favorable. In G1, the hydroxyl group at C-4′ was connected to the glucuronide residue while OH-2′ was chelated with the keto function of XN. Further fragments of G1 were observed at *m/z* 299 $[355–56]^+$ and 179 $[A\text{-}C_4H_8]^+$. Proton NMR analysis of G1 showed an OMe resonance at δ 3.91 (singlet) and two olefinic protons, H-α and H-β (broad singlet at δ 7.71, integrating for two protons). The B-ring protons, H-2/H-6 and H-3/H-5, appeared as a set of doublets at δ 7.59 and 6.85 ($J = 10$ Hz). The aromatic ring proton H-5′ gave a singlet at δ 6.47. Other signals were attributed to 2′-OH (δ 14.61), H-2″ (δ 4.99), and H-1″ (δ 3.17). Compared to XN ($\delta_{H5'}$ 6.0) and

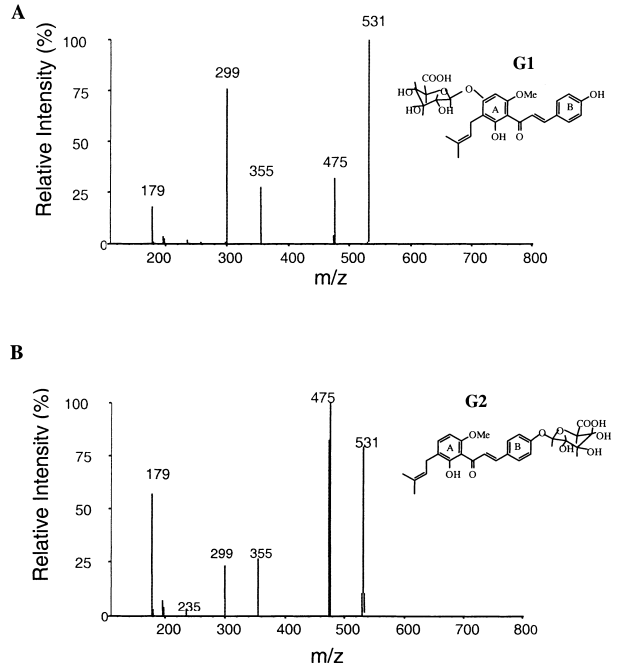

Fig. 4. LC/MS–MS spectrum of G1 (A) and G2 (B).

G2 ($\delta_{H5'}$ 6.14), the H-5′ resonance of G1 ($\delta_{H5'}$ 6.47) resonated at lower field. This strongly suggested that the glucuronide residue was connected *ortho* to C-5′, that is, to the oxygen atom at C-4′.

G2 was formed at lower concentrations than G1 by both rat or human liver microsomes. The UV spectrum of G2 showed maximum absorption at 359 nm similar to the chalcone flavonoid XN. Its molecular weight, 530 Da, suggested that G2 contained a glucuronide group attached to XN (Fig. 4B). MS–MS fragmentation of the MH^+ ion yielded the same fragments as G1. The most conspicuous difference between the fragmentation patterns of G1 and G2 was the relative intensity of the fragment ions. In G2, loss of the prenyl substituent was more favorable than cleavage of the glucuronic acid residue judging from the greater intensity of the $[531–56]^+$ ion. This suggested that the glucuronic acid molecule was connected to the B-ring hydroxyl at C-4 in G2, leaving the OH-4′ available for stabilization of the Ar-CH_2^+ fragment with *m/z* 475. Proton NMR analysis of G1 showed an OMe resonance at δ 3.85 (singlet) and two olefinic protons, H-α and H-β (broad singlet at δ 7.84, integrating for two protons). The B-ring protons, H-2/H-6 and H-3/H-5, appeared as a set of doublets at δ 7.66 and 7.08 ($J = 10$ Hz). The aromatic ring proton H-5′ gave a singlet at 6.14. Other signals were at δ 14.60 (2′-OH) and δ 4.98 (H-2″). The observation that the B-ring protons resonated at 0.1–0.2 ppm lower field in G2 than in G1 suggested the position of the glucuronide residue at OH-4 in G2. The formation of *O*-glucuronides was expected as shown in other studies of flavonoids [17,18].

G3 and G4 produced UV maxima at 262/362 nm and 262/358 nm, respectively. In LC/MS studies both conjugates gave pseudo-molecular ions with *m/z* 531, confirming that both minor metabolites were monoglucuronides. β-Glucuronidase-catalyzed hydrolysis of G3, obtained by preparative HPLC, gave rise to a mixture of XN and its flavanone isomer, isoxanthohumol (IX). These data suggest that G3 may be a glucuronide of IX with the glucuronide portion attached to either one of the two free hydroxyls, OH-4 or OH-4′ (in the flavanone, IX, OH-2′ is fixed in a γ-pyranone system). Since IX can be formed from XN by non-enzymatic isomerization, it is possible that the IX formed by hydrolysis of its glucuronide conjugate could reisomerize to form XN. Alternatively, the IX glucuronide could co-chromatograph with a glucuronide of XN (possibly the 2′-OH glucuronide) so that both IX and XN are released upon β-glucuronidase hydrolysis. Another possibility is that G3 is a different positional glucuronide isomer of IX.

G4 was obtained in insufficient amounts for further characterization. However, since the UV and MS–MS data showed that it was monoglucuronide, there was a possibility that G4 was a monoglucuronide of a XN metabolite formed non-enzymatically in these incubation conditions. For example, the involvement of the non-enzymatic oxidations in XN metabolite formation, such as M1, has been shown [11].

Glucuronidation of XN at position C4′ (G1) appears to be favored above glucuronidation at C4 (G2) in these experiments. This suggests that the presence of a prenyl group in the A ring does not preclude glucuronyl transferase-catalyzed *O*-glucuronidation of XN as expected due to limited acceptability of the substrate as a results of steric hindrance.

In this study, we have shown that rat and human liver microsomes readily convert XN to glucuronides. Flavonoids are also conjugated in vivo, yielding flavonoid glucuronides that exhibit a variety of biological activities, such as antioxidant [1] and estrogenic activities, and activation of human natural killer cells [19]. Most previous research has not been conducted with non-prenylated flavonoids. It is not yet known if dietary prenylated flavonoids (0.4–4.0 mg/l in hopped beers) are conjugated in vivo and whether or not the resulting glucuronides have biological activity.

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