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Re-evaluation of superoxide scavenging capacity of xanthohumol

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Abstract
The chemopreventive chalcone xanthohumol (Xh) has been reported to decrease xanthine oxidase (XOD) catalysed formation of formazan from nitroblue tetrazolium (NBT) and is discussed as a potent scavenger of superoxide. Re-evaluation of the scavenging capacity indicated that Xh disturbed detection of superoxide with NBT, in case of an insufficient NBT/Xh ratio. Xh lacked superoxide scavenging activity in contrast to the Xh-derivative 3´-hydroxy-Xh with catechol substructure, used as positive control. This was shown by the use of sufficient concentration of NBT and other detectors such as hydroxylamine, XTT, cytochrome c and hydroethidine. HPLC analysis of reaction products in a xanthine/XOD/peroxidase system demonstrated beside enhanced inhibition of NBT-formazan by Xh that NBT even prevented oxidation of Xh. *p*-coumaric acid or ferulic acid could replace Xh in that system, indicating that superoxide detection using NBT is likely jeopardized by interference of phenoxyl-radicals. Furthermore, this study provides evidence that Xh can moderately generate superoxide via auto-oxidation.

Keywords: *Superoxide, xanthohumol, 3´-hydroxy-xanthohumol, bovine serum albumin, xanthine oxidase, NBT, XTT, cytochrome c, hydroxylamine, hydroethidine, horseradish peroxidase*

Abbreviations: *BSA, bovine serum albumin; EtOH, ethanol; Xh, xanthohumol; 3´-OH-Xh, 3´-hydroxy-xanthohumol; XTT, 3´-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzenesulphonic acid hydrate; NBT^{2+}, nitroblue tetrazolium; NH_2OH, hydroxylamine; cyto c, cytochrome c; pCa, p-coumaric acid; Fa, ferulic acid; X, xanthine; XOD, xanthine oxidase (EC 1.17.3.2); SOD, superoxide dismutase (EC 1.15.1.1); CAT, catalase (EC 1.11.1.6); HRP, horseradish peroxidase (EC 1.11.1.7).*

Introduction

The prenylated chalcone xanthohumol (Xh) is the principal flavonoid found in the female inflorescences of the hop plant, *Humulus lupulus* L. (Cannabaceae). Hops have been used since ancient times to add bitterness and flavour to beer. However, Xh (see Figure 1) is largely converted into its isomeric flavanone, isoxanthohumol (IXh), during boiling of the wort. In contrast to IXh, studies have shown that Xh exerts remarkable activities as an anti-proliferative agent in human cancer cell lines and exhibits pro-apoptotic, anti-mutagenic and antioxidant effects [1–11]. Gerhäuser et al. [12] published superoxide scavenging activity of Xh [13], whereas IXh was inactive. In terms of these biofunctions of Xh, successful attempts have been made to maintain a considerable high Xh content in beer by special brewing procedures [14] in order to improve potential health effects of beer.

Superoxide is far less reactive than $^{\bullet}OH$-radical or peroxyl- or alkoxyl-radicals. It is biologically formed by NADPH oxidases, but also in other enzyme catalysed redox processes, or without participation of

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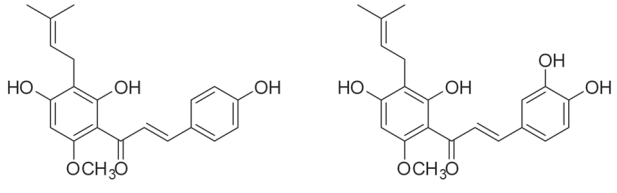


Figure 1. Molecular structures of xanthohumol (Xh) and 3'-hydroxy-xanthohumol (3'-HO-Xh).

enzymes, in auto-oxidation events. As an outcome of aerobic metabolism most of these reactions are undesired, but under control of the antioxidant system. The harm of superoxide, which is at neutral pH only a weak oxidant, and thus the high demand for its removal by superoxide dismutase (SOD), is on the one hand its fast reaction with radicals, for example with NO, leading to the strong oxidant peroxynitrite. On the other hand, superoxide can reduce metal ions such as Fe^{3+} of Cu^{2+} very fast, thereby driving superoxide assisted Fenton reactions leading to generation of $^{\bullet}OH$ and, *in vivo* even more important, superoxide can inactivate iron-sulphur enzymes such as aconitase [15]. Furthermore its protonated form, the hydroperoxyl radical, is discussed to occur in the microenvironment of membranes where it can initiate lipid peroxidation.

Xh has been reported by Gerhäuser et al. [12] to scavenge superoxide in two model systems. First, they used xanthine oxidase in the presence of hypoxanthine and measured the influence on formazan formation from NBT^{2+}. Furthermore, the influence of Xh on superoxide radical production after TPA (12-O-tetradecanoylphorbol-13-acetate) stimulation of differentiated HL-60 cells was measured via cytochrome c (cyto c) reduction. In addition to its direct superoxide scavenging potential, the effect of Xh was explained by inhibition of the TPA signal transduction cascade [12].

In the reported set-up [12] we observed interference between Xh and the NBT-based superoxide detector reaction. After excluding this experimental pitfall, superoxide radical scavenging activity was not longer attributable to Xh. This was neither the case in the xanthine oxidase model in the presence of xanthine applying hydroxylamine oxidation to nitrite, nor in the cyto c reduction assay. For understanding of the mechanism, we looked closer to the reactions of Xh in the xanthine oxidase model with several detectors of superoxide. Furthermore, we compared Xh with its metabolite 3′-hydroxy-xanthohumol (3'-OH-Xh), which we expected to scavenge superoxide more efficiently, due to the catechol sub-structure. Further studies gave evidence that Xh is not stable under biochemical assay conditions and that detection of superoxide by reduction of NBT to formazan is impaired by Xh-radicals. As a general consequence, superoxide scavengers being able to form phenoxyl radicals have to be carefully evaluated for interference of detector and scavenger chemistry.

Materials and methods

Chemicals

Bovine serum albumin (BSA), glycine, NaOH, xanthine sodium salt, hydroxylamine, superoxide dismutase, p-coumaric acid, ferulic acid, hydroethidine, cytochrome c, NBT and XTT were from Sigma (Deisenhofen, Germany). Methanol and acetonitril of HPLC gradient grade were from J.T. Baker (Deventer, Netherlands). Ethanol (99.9%), trifluoroacetic acid (TFA), sodium dihydrogen phosphate, disodiumhydrogen phosphate, sodium tetraborate (Borax), sodium hydrogen carbonate and sodium carbonate were from Merck (Darmstadt, Germany). Xanthine oxidase, horseradish peroxidase and catalase were from Roche (Mannheim, Germany). Xh and 3′-hydroxy-xanthohumol were synthesized according to Vogel et al. [16]. Xanthohumol was also purchased from Phytoplant (Heidelberg, Germany). All chemicals used were of high or analytical purity. Xh, 3'-OH-Xh, *p*-coumaric acid and ferulic acid were dissolved in ethanol (EtOH) or dimethyl sulphoxide (DMSO).

Assays for detection of superoxide

Xanthine in the presence of xanthine oxidase was used to generate superoxide radicals. To establish homogenous, aqueous solutions of lipophilic compounds, such as NBT-formazan and Xh, the assay comprised BSA. The superoxide generating system consisted of (in 1.0 ml): Buffer 25–100 mM as indicated, xanthine 100 μM, bovine serum albumin (BSA) 0.05% or, where indicated, 0.5% w/v, div. concentration of xanthohumol 1–100 μM in EtOH (finally 10% or 1% v/v, as indicated) or DMSO (finally 1% v/v). The following buffers were used: phosphate buffer, borate buffer, carbonate buffer, glycine/NaOH buffer at pH 7.4, 8.9 and 9.4. The reaction was started with xanthine oxidase at finally 0.01 U/ml. Both kinetics and end-point measurements were performed. Kinetics of indicator development was recorded in a Kontron Uvikon 922 spectrophotometer. In a 96-well (final volume of the assay was 250 μl) microplate reader MS 212 from ICN, equipped with 550nm filter end-points were measured after 30 min of reaction at pH 7.4 and after 45 min for pH 9.4. The following detectors [17] where used: (a) NBT 25 μM and 100 μM (NBT-formazan measured at $A_{560\ nm}$); (b) XTT 25 μM and 100 μM (XTT-formazan measured at $A_{510\ nm}$); (c) hydroxylamine 25 μM, 100 μM and 1 mM (nitrite quantified by Griess reaction as azo dye at $A_{540\ nm}$); (d) cytochrome c 25 μM and 50 μM (reduced cyto c, measured as $\Delta A_{550\ nm}$). To keep the reduced cytochrome c stable, catalase (100 U/ml) was present to remove

hydrogen peroxide. To generate xanthohumol-radicals horseradish peroxidase (HRP) was added to the X/XOD/NBT set-up: to keep superoxide scavenging by HRP at low level, HRP was used at 0.01U/ml. BSA was present in the XOD-system at 0.05% and 0.5% (w/v, equals to 7.5 μM or 75 μM BSA). The latter concentration of BSA is equivalent to a thiol-content of 30 μM (determined by Ellman reagent [18,19], calibrated with cystein) and thus slightly decreased the sensitivity/range of the superoxide-indicating detectors compared to 0.05% BSA.

Detection of NBT, NBT-formazan, xanthohumol and oxidation products of xanthohumol by HPLC

For HPLC measurements waters 600 controller and pump, waters 717plus autosampler, column oven and waters 996 PDA detector was used. The column was a Merck LichroCART 125 × 4, filled with LichroSphere 60 RP select B (5 μm) with additional guard column 4 × 4 of the same type, kept at 35°C. The eluents were 50 mM phosphate pH 2.1 (A) and methanol (B), HPLC gradient grade. The injection volume of the samples was 20 μl and the following elution-programme was used with linear gradients at a flow rate of 1.0 ml/min: 0–15 min 60% B, 15–20 min linear gradient to 100% B, 20–25 min 100% B, 25–30 min linear gradient to 60% B, 30–40 min 60% B equilibration. Typical retention times (RT) were 1.9 min for NBT^{2+}, 12.0 min for Xh and 25.0 min for the NBT-formazan. Products of xanthohumol oxidation (Xh_{ox}) were detected at R_t 3.3 min, R_t 5.3 min, R_t 6.4 min, R_t 8.0 min, R_t 9.3 min and R_t 22.5 min.

HPLC-quantification of 2-OH-ethidium formed from hydroethidine by xanthine/xanthine oxidase

Hydroethidine (HE) stock solution of 15 mM HE was prepared in deoxygenated DMSO as described [20] and stored after bubbling with molecular nitrogen (N_2) at 4°C in the fridge protected from light. Just before use, stock was diluted in 10 mM HCl to 1 mM HE. In the assay, the final concentration of HE in phosphate buffer 50 mM (pH 7.4) was 100 μM. Superoxide was detected by formation of 2-hydroxy-ethidium (2-OH-E^+) from HE and quantified after separation by HPLC [20–22] via absorbance at 290 nm and 370 nm, additionally by fluorescence (Waters 474 fluorescence detector set-up: excitation 270 nm, emission 596 nm, positioned behind the Waters PDA 996). Except the fluorescence detector and the length of the column (250 mm), the same HPLC equipment was used as already described for quantification of Xh. The sample volume was 50 μl and the flow rate was adjusted to 0.5 ml/min with the mobile phases A (aqua bidest., 0.1% TFA) and B (acetonitrile (HPLC gradient grade), 0.1% TFA). From 0–46 min gave a linear gradient from 10% B to 70% B, increasing B to 100% at 50 min, from 55–60 min there was a linear gradient to 10% B. From 60–75 min the column was equilibrated. The X/XOD samples (± Xh or test compound) were incubated at 37°C for 60 min and thereafter transferred into HPLC vials and analysed.

In general, data shown are expressed as mean ± standard deviation (of at least three repetitions).

Results and discussion

Xh exerts cancer chemopreventive activities [1–5, 12,13] and was reported to *in vitro* scavenge superoxide radicals besides other ROS such as hydroxyl and peroxyl radicals [12,13]. However, evaluating the antioxidant potential of Xh in several *in vitro* model systems, we observed scavenging activity of xanthohumol only towards peroxynitrite, hydroxyl- and peroxyl-radicals, as well as towards the synthetic DPPH- and ABTS-radicals (results not shown). No or only low scavenging activity of Xh towards superoxide anion radicals was detectable using oxidation of hydroxylamine (Figure 2) to nitrite by X/XOD as model system [23–25]. In contrast, the hydroxylated metabolite 3'-HO-Xh exerted significant superoxide scavenging activity and served as a positive control (Figure 2). This is in-line with the enediol and catechol sub-structure of 3'-HO-Xh. Enediols such as ascorbic acid or catechols such as caffeic acid, quercetin, etc. are relevant scavengers of superoxide [15,26,27]. The discrepancy of the published [12] and our results prompted us to take a closer look on the superoxide scavenging property of Xh, due to the

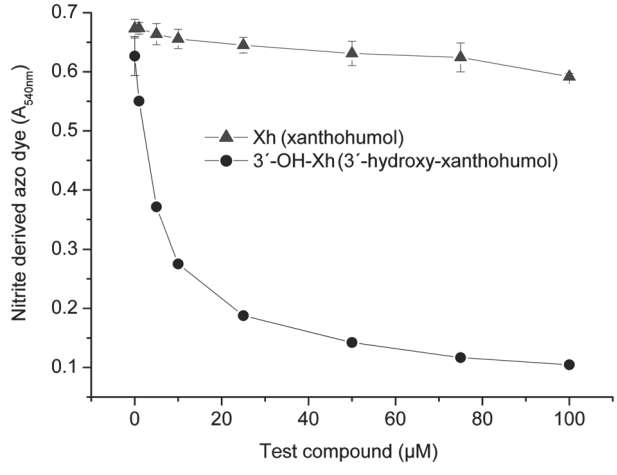


Figure 2. Detection of superoxide from X/XOD by oxidation of hydroxylamine into nitrite. Influence of the chalcones xanthohumol (Xh) and 3'-hydroxy-xanthohumol (3'-HO-Xh). In 1.0 ml: 50 mM phosphate buffer pH 7.4, BSA 0.5% (w/v), hydroxylamine 1 mM, xanthine 500 μM, EtOH 10% (v/v); or Xh, final EtOH 10% (v/v); or 3′-OH-Xh, final EtOH 10% (v/v); XOD 0.01U/ml. Incubation for 30 min in the water bath at 37°C in the dark. Accumulated nitrite was quantified as azo-dye (Griess reaction) by $A_{540\ nm}$.

fact that real scavenging of superoxide should not be limited to only one specific assay set-up.

Formation of NBT-formazan in the presence of Xh

Under the published [12,28] conditions (0.5% BSA, 100 μM EDTA, 25 μM NBT^{2+} and 50 mM phosphate pH 8.0 or carbonate buffer pH 9.4 or 25 mM glycine/NaOH buffer pH 9.4) we proved that 100 μM Xh could almost completely inhibit formazan formation by xanthine oxidase in the presence of xanthine. Additionally, we reproduced the reported IC_{50} value of ~ 27 μM Xh (a value equimolar to NBT^{2+} in this set-up). However, as Xh was added in excess compared to NBT^{2+}, this set-up lacks the pre-requisites for an antioxidant [15], e.g. the antioxidant should exert its activity at low concentration compared to the target. Furthermore, when NBT^{2+} was raised equimolar to Xh (100 μM), Xh was only able to decrease a small amount of the NBT-formazan. Moreover, varying NBT^{2+} from 25 μM up to 1 mM clearly showed a limitation of superoxide detection at low concentration of NBT^{2+}. When NBT^{2+} (500 and 1000 μM) was in excess, 100 μM Xh did not inhibit formation of NBT-formazan; moreover, NBT-formazan values were even slightly higher in the presence of Xh as compared to its absence (Figure 3). At more neutral conditions (pH 7.4), xanthohumol (1–100 μM) was completely ineffective in preventing NBT-formazan formation by X/XOD in the presence of 100 μM NBT^{2+} (Figure 4), but was still active with 25 μM NBT^{2+}. These results implied a set-up-dependent phenomenon concerning scavenging of superoxide by Xh. Additionally, the Xh-metabolite 3'-HO-Xh, as a positive control, could clearly inhibit formation of NBT-formazan in the low μM range (Figure 4) in contrast to Xh, when NBT was used at sufficient concentration and at the physiologically more relevant pH 7.4 (Figure 4).

To explain the set-up-dependent activity of Xh, we supposed formation of a reactive intermediate of Xh under the according condition. First, we assumed that NBT^{2+} at 25 μM does not quantitatively scavenge the generated superoxide radicals (as indicated in Figure 3). In such a scenario, superoxide would be able to contribute to other reactions such as metal-catalysed formation of $^{\bullet}OH$ (superoxide assisted Fenton reaction). Catalytic ferric ions are present in phosphate buffer [29] and in the chemicals, especially in the XOD-suspension containing ethylene-diamine-tetra-actetate (EDTA, 5 μM in 0.01 U/ml XOD). Thus, $^{\bullet}OH$ may be formed and trapped by Xh, generating Xh-radicals which might scavenge superoxide more efficiently than Xh. However, catalase (100 U/ml) as well as the iron-chelator di-ethylene-triamine-penta-actetate (DTPA, 100 μM), in order to replace EDTA, was not able to lower the effect of Xh in the NBT^{2+}-limited X/XOD-system (results not shown). In contrast to EDTA, which accelerates superoxide assisted Fenton reactions, DTPA slows them down. DTPA-Fe^{3+} is reduced to DTPA-Fe^{2+} by superoxide by the factor 220 slower than EDTA-Fe^{3+} to EDTA-Fe^{2+} (EDTA, $k = 1.3 \times 10^6$ M^{-1} s^{-1}; DTPA, $k = 5.9 \times 10^3$ M^{-1} s^{-1} [15,29,30]). These results suggested that the superoxide assisted Fenton reaction did not contribute to the effect of Xh in the NBT-limited set-up by generation of Xh-radicals. Furthermore, the solubilizers BSA, ethanol or DMSO in this set-up would certainly scavenge $^{\bullet}OH$ formed more likely than Xh, due to their concentration. For

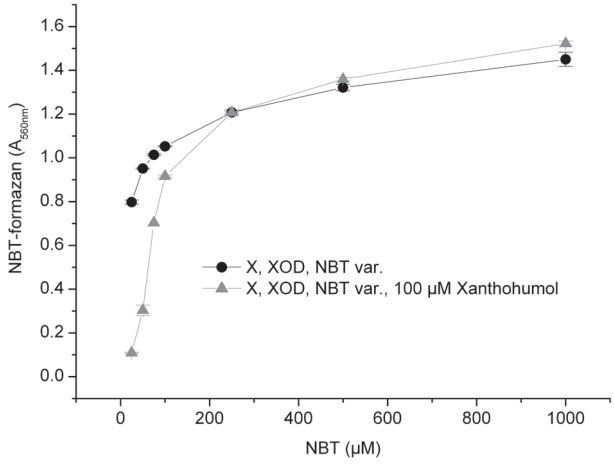


Figure 3. Detection of superoxide from X/XOD by formazan formation of NBT^{2+} at pH 9.4. Influence of raising the concentration of NBT^{2+} in the presence and absence of 100 μM xanthohumol. In 1.0 ml: 50 mM phosphate buffer or 25 mM glycine/NaOH pH 9.4, BSA 0.05% (w/v), xanthine 100 μM, NBT^{2+} 25-1000 μM, EtOH 10% (v/v); or Xh 100 μM, final EtOH 10% (v/v), XOD 0.01 U/ml. Incubation for 45 min at room temperature in the dark. NBT-formazan formed was measured at $A_{560\ nm}$.

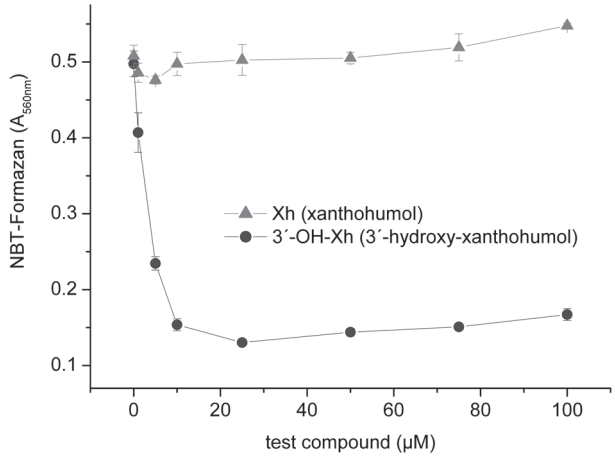


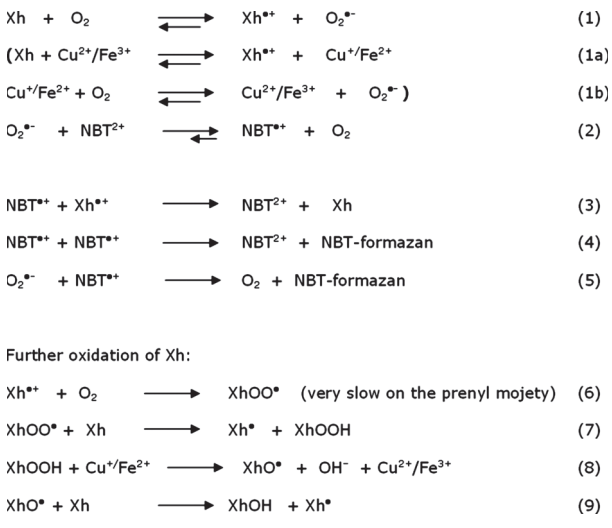
Figure 4. Formazan-formation of NBT^{2+} (100 μM) catalysed by xanthine oxidase (0.01U/ml) in the presence of 100 μM xanthine and 0.5% BSA in 50 mM phosphate buffer pH 7.4. Influence of the chalcones xanthohumol and 3´-hydroxy-xanthohumol. In 1.0 ml: 50 mM phosphate buffer pH 7.4, BSA 0.5% (w/v), xanthine 100 μM, NBT^{2+} 100 μM, EtOH 10% (v/v) or Xh 0–100 μM (final EtOH 10% v/v); or 3'-HO-Xh 0-100 μM (final EtOH 10% v/v); XOD 0.01 U/ml. Incubation for 45 min at room temperature in the dark. NBT-formazan formed was measured at $A_{560\ nm}$.

example, EtOH was used at final 10% or 1% (v/v), which is equal to 1.71 M (79 mg/ml) or 171mM (7.9 mg/ml). BSA (average molecular weight: 66 kdal) was used at two concentrations, finally at 0.5% (m/v) and 0.05%(m/v), which is equal to 76 µM (5 mg/ml) or 7.6 µM BSA (0.5 mg/ml). Interestingly, the latter did not lower the detection of superoxide, whereas 76 µM BSA slightly decreased the detectable amount of superoxide as measured either as nitrite formed from NH_2OH or as formazan formed from NBT^{2+} (data not shown). Nevertheless, the concentration of BSA did not influence the activity of Xh in the NBT-limited set-up.

Secondly, we analysed the stability of Xh in the chosen set-up by HPLC to assay its consumption and product formation ($A_{370\ nm}$). Xh was stable in pure ethanol but not stable in the buffered solution, as indicated by HPLC of auto-oxidation products of Xh. We further observed that gas bubbling of the phosphate buffered Xh-solution with molecular nitrogen decreased oxidation of Xh (data not shown), whereas bubbling with synthetic air (20% O_2, 80% N_2) increased loss of Xh. Importantly, HPLC data revealed that increasing concentration of NBT protected Xh from auto-oxidation (Table I). One might explain this protective effect of NBT by scavenging of superoxide from auto-oxidation of Xh, assuming that in turn $NBT^{\bullet+}$-radical reduced Xh-radical back to Xh (see proposed reactions 1–5 in Scheme 1 available online at www.informahealthcare.com/fra). Further oxidation of Xh is speculated to proceed via reactions 6–9 in Scheme 1. Involvement of superoxide could be detected by some formation of formazan, outcompeting Xh-radicals (Table I). The amount of NBT-formazan approximately corresponded to the amount of lost Xh via auto-oxidation. In addition, SOD prevented formation of the marginal amounts of NBT-formazan during auto-oxidation of Xh (data not shown). Accordingly, the generation of Xh-radicals and superoxide via auto-oxidation is likely to occur under the used conditions. Oxidation (or subsequent auto-oxidation) of Xh may be catalysed by traces of transition metal ions (see assumed reactions 1a and 1b in Scheme 1 available online). As EDTA and

Table I. HPLC-data (peak area $A_{370\ nm}$) of xanthohumol in phosphate buffer and its oxidation products (only two of several products are listed). Oxidation products of Xanthohumol (Xh_{ox}) decreased with increasing amounts of NBT (0, 25, 100 µM) indicating protection of xanthohumol by NBT. In 1.0 ml: 50 mM phosphate buffer pH7.4, BSA 0.05%, NBT^{2+} 0, 25 µM or 100 µM as indicated; Xh 100 µM, final EtOH 10% (v/v). Incubation for 60 min at room temperature in the dark after bubbling with synthetic air for 1 min.

NBT (µM)	$Xh_{ox.}$ Peak area R_t 8 min ($A_{370\ nm}$)	Xh Peak area R_t 8 min ($A_{370\ nm}$)	$Xh_{ox.}$ Peak R_t 22.5 min ($A_{370\ nm}$)	NBT-formazan Peak R_t 25 min ($A_{370\ nm}$)
0	393199	313673	166685	–
25	194414	1446840	110981	16810
100	61538	2049610	50681	26212



Scheme 1. Assumed radical reactions in the xanthine/xanthine oxidase model in the presence of NBT^{2+} and xanthohumol.

DTPA showed no influence, the metal ions were probably chelated by BSA [31,32]. Crucial for our interpretation of the protective effect of NBT is that Xh and NBT^{2+}, Xh-radical, $NBT^{\bullet+}$-radical and NBT-formazan are lipophilic compounds. They may associate in aqueous solution or group close together with BSA. Hence, reaction of $NBT^{\bullet+}$-radical with Xh-radical should be favoured. As already mentioned, $NBT^{\bullet+}$-radical could be formed by trapping of superoxide (auto-oxidation product of Xh) by NBT^{2+}. Interestingly, a big difference was observed between the tetrazolium dyes XTT and NBT in combination with Xh. On the one hand, additional formazan of XTT was observed in the presence of Xh, compared to control in the X/XOD assay (see Figure 5). On the other hand, NBT stabilized Xh in the absence of

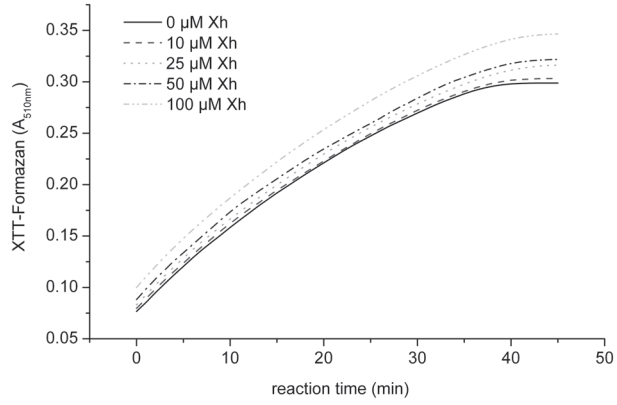


Figure 5. Formazan-formation of XTT (25 µM) catalysed by xanthine oxidase (0.01 U/ml) in the presence of 100 µM xanthine and 0.5% (w/v) BSA in 50 mM phosphate buffer pH 7.4. Influence of the chalcone xanthohumol (Xh). In 1.0 ml: 50 mM phosphate buffer pH 7.4, BSA 0.5% (w/v), xanthine 100 µM, XTT 25 µM, EtOH 10% (v/v) or Xh 0–100 µM (EtOH 10% v/v); or 3'-HO-Xh 0–100 µM (final EtOH 10% v/v); XOD 0.01 U/ml. The reaction was started by addition of XOD and incubated for 45 min at room temperature in the dark. XTT-formazan formed was measured at $A_{510\ nm}$.

X/XOD, likely protecting it from auto-oxidation (Table I), whereas XTT catalysed oxidation of Xh. However, the latter process proceeded only slowly and was observed by transformation of Xh to auto-oxidation products as well as by formation of XTT-formazan. Only low amounts of XTT-formazan were detected during the first 30–60 min of incubation of phosphate buffered Xh with a concomitant loss of Xh. Incubation of Xh for longer time periods (hours) caused strong transformation of Xh to auto-oxidation products with concurrently strong formation of XTT-formazan. Similar results were obtained when cyto c was used to detect superoxide (see supplement data available online). When SOD (100 U/ml) was also present, XTT-formazan or reduced cyto c was not or only marginally formed. The effect of XTT on oxidation of Xh could be lowered by SOD, but oxidation of Xh was increased by SOD when cyto c was used as a detector of superoxide. However, in this case most likely hydrogen peroxide from dismutation of superoxide drives the pseudoperoxidase activity of cyto c, generating additional Xh radicals (data not shown). Vyas et al. [33] reported that certain phenoxyl radicals (in particular semiquinones of catechins and catechols) are able to reduce XTT to its formazan. In the example given by these authors, SOD enhanced formation of XTT-formazan, due to increased oxidation of the corresponding phenols. Because SOD did not stimulate but inhibited formation of XTT-formazan, we conclude that Xh-radicals were not involved in an analogous interference, but SOD removed most likely superoxide deriving from oxidation of Xh.

The $NBT^{\bullet+}$-radical is known to act as a reducing agent, especially in reducing molecular oxygen to superoxide to a certain extent (auto-oxidation [17,34–36]). The potential of the $NBT^{\bullet+}$-radical do reduce electron acceptors is in support of our hypothesis. Interestingly, both the corresponding $XTT^{\bullet+}$-radical (of the tetrazolium dye XTT) as well as reduced cytochrome c (cyto c Fe^{2+}) did not show a similar extent of auto-oxidation. In line with our hypothesis, neither XTT (Figure 5) nor cyto c (results not shown) indicate Xh as a potent superoxide scavenger. In fact, Xh slightly lowered the reduction rate of cyto c by XOD, but Xh surprisingly did not lower the amount of total reduced cyto c at the end of the reaction. In the absence of XOD we observed that cyto c enhanced oxidation of Xh, forming a small amount of reduced cyto c. Hence, the marginal inhibiting activity of Xh observed during kinetics of cyto c reduction by X/XOD may be explained by Xh-radicals oxidizing reduced cyto c (results not shown). Nevertheless, according to our hypothesis, superoxide should be produced at the same amount as Xh-radicals by auto-oxidation (see Scheme 1). This could explain why the amount of total reduced cyto c was not influenced by Xh. Although reduction of cyto c by Xh was extremely slow (in the order of hours, see supplement data available online at www.informahealthcare.com/fra) in the absence of XOD, it might be of importance in cellular test systems with longer incubation times (e.g. cytotoxicity assays).

Important role of Xh-radicals in suppressing formation of NBT-formazan

Xh-radicals might play a central role as reaction partners of $NBT^{\bullet+}$-radicals (equation 3 of Scheme 1). To elucidate their function in the X/XOD-system, we increased the steady state concentration of Xh-radicals by addition of peroxidase in the presence of hydrogen peroxide. Xanthine oxidase produces both hydrogen peroxide and superoxide, while oxidizing xanthine to uric acid. In controls without Xh, peroxidase could act as a superoxide scavenger, too, as it is known for heme compounds, forming oxy-ferroperoxidase [15]. Therefore, horseradish peroxidase (HRP) was used at low concentration (0.01 U/ml), not obviously decreasing the amount of NBT-formazan. As expected, we observed that HRP could activate the formazan suppressing activity of Xh, at pH 7.4 and at pH9.4 with both 25μM and 100μM NBT^{2+} Figure 6). Moreover, *p*-coumaric acid or ferulic acid could replace Xh, demonstrating the potential of phenoxyl radicals to interfere with formazan formation of NBT (Figure 6), although superoxide was present. In addition, NBT was able to lower HRP-mediated oxidation of Xh in a dose-dependent manner as shown by HPLC, e.g. for ± NBT 25 μM (Figure 7). This was in perfect agreement with our hypothesis that NBT^{2+}, if reduced to the NBT-radical by X/XOD, is able to regenerate Xh from the intermediate Xh-radical.

If we assume that Xh actually acts as a superoxide scavenger, it should be consumed during the reaction. However, even in the NBT^{2+}-limited X/XOD-system, where Xh decreased formation of formazan from NBT^{2+} in a dose-dependent manner, there was no considerable corresponding decrease of Xh as measured by HPLC. However, there is the possibility that superoxide is dismutated by Xh (buffer would provide the H^+-ions). Thereby, Xh would first reduce superoxide to hydrogen peroxide and the resulting Xh-radical is then reduced back by the second superoxide in analogy to the reaction of tyrosyl radical with superoxide [37–39]. Nevertheless, dismutation or scavenging of superoxide by Xh should be visible also with other detectors of superoxide, and hence can be excluded, because Xh was neither obviously active in the cyto c nor in the XTT-assay for superoxide.

As a further important point in our studies, HPLC chromatograms revealed that Xh was oxidized by X/XOD in the absence of NBT^{2+} and that NBT^{2+} was able to prevent oxidation of Xh dose-dependently, similar to in the experiments with HRP. In the NBT-limited X/XOD-system no (auto)oxidation product of Xh was detectable and Xh was fully recovered after the reaction. In contrast, (auto)oxidation of Xh in the

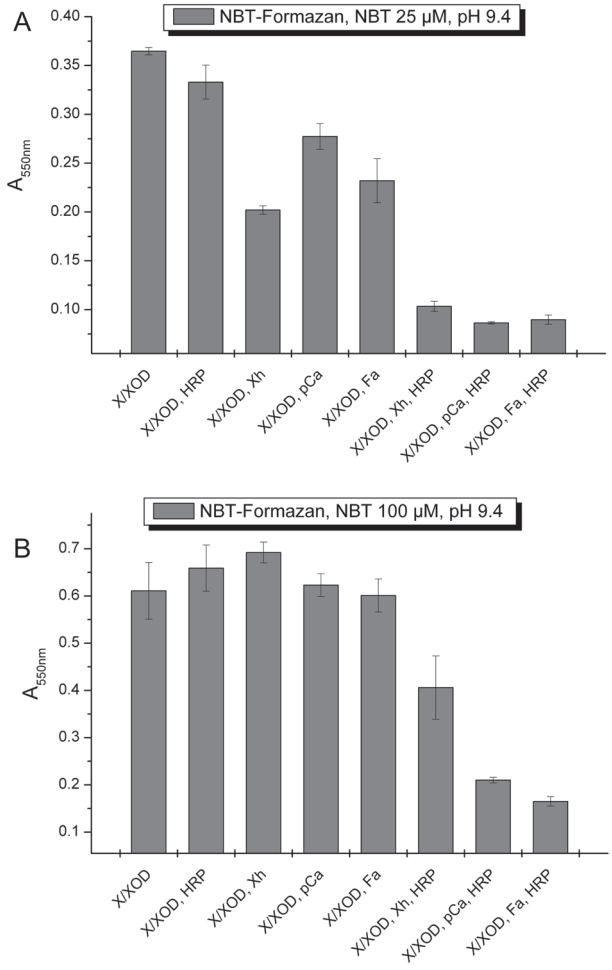


Figure 6. Effect of 0.01 U/ml horseradish peroxidase (HRP) and 25 µM xanthohumol (Xh) or *p*-coumaric acid (pCa) or ferulic acid (Fa) and the combination of both, peroxidase and phenolic, on the amount of produced NBT-formazan by X/XOD: (A) NBT^{2+} 25 µM; (B) NBT^{2+} 100 µM. In 1.0 ml: 50 mM phosphate buffer pH 9.4, BSA 0.05% (w/v), NBT 25 µM or 100 µM as indicated, xanthine 100 µM; EtOH, final 1% (v/v); or Xh 25 µM or *p*-coumaric acid 25 µM or ferulic acid 25 µM (all phenolics final 1% v/v EtOH); horseradish peroxidase (HRP) 0.01 U/ml, XOD 0.01 U/ml. Incubation for 45 min at room temperature in the dark. NBT-formazan formed was measured at $A_{550\ nm}$.

absence of X or XOD or X/XOD was not completely prevented by NBT^{2+} (Table I). Thus, we conclude that the steady state concentration of $NBT^{\bullet+}$-radicals formed in the beginning of the X/XOD-reaction is sufficient to reduce the Xh-radical back to Xh during the whole reaction, avoiding formation of further Xh-auto-oxidation products. In the absence of X/XOD the $NBT^{\bullet+}$-radicals can be formed by superoxide from the (auto)oxidation of Xh. It is conceivable that here the concentration of superoxide and of NBT-radicals is much lower. In consequence, a certain amount of Xh is oxidized to further products and hence auto-oxidation of Xh is not prevented completely.

To gain more information about superoxide scavenging of Xh we further checked set-ups with the chemiluminescent probes lucigenin and luminol. In accordance with Hartkorn et al. [7], chemiluminescence could be

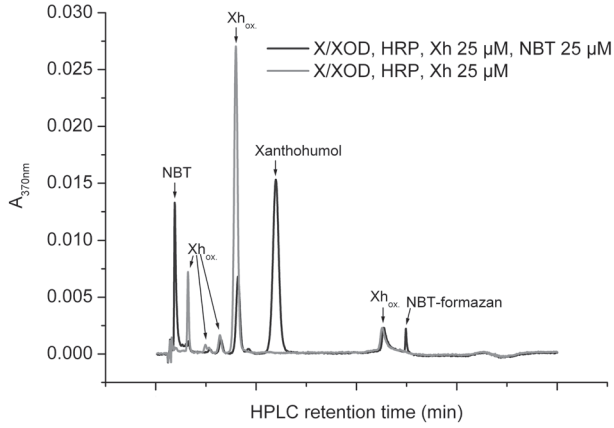


Figure 7. HPLC-chromatograms of xanthohumol ± NBT in the presence of X/XOD/HRP. Protection of xanthohumol (decrease of oxidation products) by NBT. In 1.0 ml: 50 mM phosphate buffer pH 7.4, BSA 0.5% (w/v), NBT 0 or 25 µM as indicated, xanthine 100 µM, Xh 25 µM, final EtOH 1% (v/v), horseradish peroxidase (HRP) 0.01 U/ml, XOD 0.01 U/ml. After incubation for 30 min at room temperature in the dark xanthohumol and its auto-oxidation products were detected at $A_{370\ nm}$ by HPLC.

diminished by Xh dose-dependently (data not shown). However, due to the absorbance of Xh in the wavelength range where these chemiluminescent probes emit light, it cannot clearly be distinguished whether the effect of Xh is due to scavenging of superoxide or due to absorbance of the light emitted. Furthermore, it should be considered that the luminol radical is as important as superoxide in forming the light emitting intermediate [40]. Thus, it can not be excluded that Xh inhibited the luminol chemiluminescene by reducing the luminol radical instead of scavenging superoxide.

Effect of Xh in the X/XOD assay for superoxide generation detected via oxidation of Hydroethidine (HE) into 2-OH-Ethidium (2-OH-E^+)

To get clear information about the behaviour of Xh in our X/XOD assay we tried the oxidation of hydroethidine to 2-hydroxy-ethidium [20–22,41] (and detection by HPLC), which is thought to be the most specific detection of superoxide. As shown in Figure 8, the amount of 2-OH-E^+ formed in the X/XOD assay was not diminished in the presence of Xh. Quite the contrary, Xh increased formation of 2-OH-E^+ dose-dependently, whereas in the presence of both Xh and SOD, formation of 2-OH-E^+ was blocked (Figure 8). Moreover, 3'-HO-Xh was able to decrease the amounts of 2-OH-E^+ (Figure 8) in accordance with the experiments using sufficient NBT^{2+} or NH_2OH as detectors of superoxide (see Figures 2 and 4). The higher amount of 2-OH-E^+ in the presence of Xh can be explained by Xh-radical catalysed formation of the intermediate $HE^{\bullet+}$, the limiting factor for superoxide scavenging and formation of 2-OH-E^+, as well as by Xh derived superoxide from auto-oxidation.

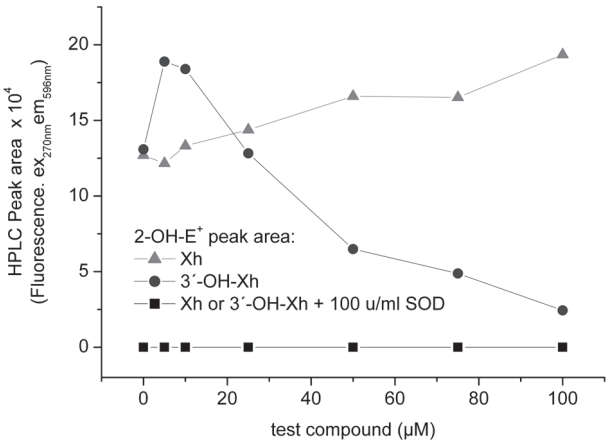


Figure 8. Formation of 2-OH-E^+ from HE catalysed by xanthine oxidase (0.01 U/ml) in the presence of 100 µM xanthine and 0.05% (w/v) BSA in 50 mM phosphate buffer pH 7.4. Influence of the chalcones xanthohumol (Xh) and 3´-OH-Xh with catechol sub-structure. In 1.00 ml: 50 mM phosphate buffer pH 7.4, BSA 0.05% (w/v), xanthine 100 µM, HE 100 µM (final 0.6% DMSO), EtOH 10% (v/v) or Xh 0–100 µM (final EtOH 10% v/v); or 3'-HO-Xh 0–100 µM (final EtOH 10% v/v); XOD 0.01 U/ml. The reaction was started by addition of XOD and incubated for 60 min at 37°C in a gently shaking water bath. HE, 2-OH-E^+, E^+ and further oxidation products were determined and quantified by HPLC with PDA and fluorescence detection.

Conclusions

Superoxide scavenging activity of Xh could neither be visualized with cyto c, nor with XTT, nor with hydroxylamine in contrast to NBT, when used at limited concentration (25µM, as with NBT^{2+}). This discrepancy disappeared when NBT was applied in sufficient concentration, indicating that Xh is not a scavenger of superoxide. This was clearly confirmed by the superoxide indicator 2-OH-ethidium where Xh was shown to produce additional superoxide. The effect of Xh in the NBT-limited X/XOD-system can be explained by interference of Xh-radicals with the assay scavenging $NBT^{\bullet+}$-radicals. Thus, superoxide formation was not indicated, although NBT^{2+} was reduced by superoxide. The crucial role of Xh-radicals in this system was demonstrated by peroxidase. Data revealed evidence that Xh-radicals are formed mainly via (auto)oxidation. Provided that Xh is in excess over NBT^{2+}, we postulate that Xh interferes by blocking NBT^{2+}, depending on the ratio of Xh-to-NBT^{2+} and due to the lipophilicity of both. In addition, it is very likely that all players (NBT^{2+}, $NBT^{\bullet+}$, NBT-formazan, Xh and traces of Fe^{3+}) associate with BSA, the solvent of the sparingly water-soluble compounds NBT-formazan and Xh. This may be one reason why these reactions preferentially occur and may also be the reason why catalase and iron chelators are without effect on these reactions. Similar effects were reported concerning lipid peroxidation, although $^{\bullet}OH$-radicals have been detected ([15], pp. 243–246). Our data revealed that $^{\bullet}OH$, if at all, play only a minor role in generating Xh-radicals under the assay conditions.

In contrast to Xh, the metabolite 3'-HO-Xh showed strong superoxide scavenging activity. Moreover, our data suggest that Xh acts even as a moderate generator of superoxide anion radicals, especially under conditions used in pharmacological assays, when Xh was diluted into buffer solution or cell culture media. Only in one publication stability of Xh has been considered: Mendes et al. [6] checked stability of Xh (5 µM and 20 µM) in their used cell culture media containing 10% heat inactivated FCS (incubated at 37°C for 24, 48, 72 h). They concluded no significant loss of Xh as quantified by HPLC (values within normal variation), although they measured a Xh-loss after 24 h (11.4 ± 2.7% for 5 µM Xh and 5.1 ± 1.9% for 20 µM Xh [6]). We also observed better stability of Xh in the presence of higher amounts of BSA using 0.5% (w/v) instead of 0.05% (w/v).

Consequences, relevance and perspectives

Based on our findings, cancer protective activity of Xh as an antioxidant is likely not due to scavenging of superoxide. However, this mode of action can be mediated by Xh metabolites of phase I such as 3'-HO-Xh in addition to peroxyl radical scavenging activity of Xh. Interestingly, phase-I metabolites of Xh can have significantly higher activity [16,42] than Xh itself. It should be noted that Xh may be not stable in the media or buffered solution used in pharmacological assays. (Auto)oxidation of Xh to alkyl, alkoxyl, peroxyl radicals and organic hydroperoxides may be one mode of action responsible for cytotoxicity in cell culture assays. For example, Plochmann et al. [43] reported on Xh as cytotoxic in human leukaemia cells and suggested that cytotoxicity of Xh may be due to several properties; high lipophilicity, interaction of the prenyl group with membranes, auto-oxidation processes in cell culture media and other unknown mechanisms. Hence it should be carefully checked whether the effect of Xh could be due to pro-oxidant activity, induced via auto-oxidation processes. This aspect deserves particular attention for interpretation of results and can be controlled for example by HPLC analysis of Xh. As a further possibility, organic peroxides formed by auto-oxidation processes may be detected by the FOX (ferric xylenol orange [44,45]) assay.

NBT as superoxide detector should be applied in sufficient concentration to exclude interfering side reactions. For the use of NBT for superoxide detection in plants [46], the potential of phenoxyl radicals to interfere with formazan formation is of relevance. In host/pathogen-interactions, the apoplast of plants often contains superoxide, hydrogen peroxide, peroxidases and phenolics simultaneoulsy. In the presence of hydrogen peroxide cell wall peroxidases catalyse oxidation of phenolics to according phenoxyl radicals. However, in many cases superoxide, although present, may not be detectable as NBT-formazan.

Hence, the reason for the failure of detection of superoxide as NBT-formazan might be simply the amount of present phenolics (phenoxyl radicals as scavenger of $NBT^{\bullet+}$-radicals and phenols with catechol substructure as direct scavenger of superoxide).

As an outlook for further investigation of the mode of action of Xh inducing apoptosis in cancer cells, interaction of Xh with cyto c in the respiratory chain should be in focus. In case Xh can reach the mitochondrial membrane, we speculate that this interaction might be able to slowly induce oxidative stress and eventually cyto c release, and in consequence promotes apoptosis.

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Supplementary material available online

Figures S1–S4

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