Inhibition by Salix- Extracts and Phytodolor^R of Copper-Catalyzed Oxidative Destructions*

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Oxidation of low density lipoprotein (LDL) by copper ions is strongly inhibited by different aqueous extracts (Salix spec (SE); Fraxinus-Solidago-Populus (Phytodolor^R)(PD)) in a concentration range between 4 and 7 μg/ml. 10 to 50 μM salicylic acid (SA) stimulate LDL-oxidation whereas higher concentrations (10 to 500 μM) show no effect. Likewise ethene release from 2-keto-4-methylthiobutyrate (KMB) is strongly inhibited by the above extracts in a reaction driven by dihydroxyfumarate (DHF) in the presence of copper ions. This system may represent some features of the diabetic situation where DHF as an endiole may stand for certain Amadori products. In order to find out whether the inhibitory effects are due to copper chelation we tested the copper-dependent conversion of photodynamic ethane release from α-linolenic acid into ethene formation. Copper chelation is apparently only partially involved in inhibition of copper-dependent oxidations and only at a certain concentration of extracts from *Salix* spec (SE) or extracts from *Fraxinus-Solidago-Populus* (Phytodolor^R)(PD).

Introduction

Reactive oxygen species (ROS) are involved in inflammatory diseases, arteriosclerosis, cancer, diabetes, cataract, and many more (Halliwell and Gutteridge, 1989; Elstner, 1990; Sies, 1985; 1991). Major principles for biological generation of ROS such as OH· include xanthine oxidase (XOD), Fenton-type oxidants (FO), activated leukocytes, different NAD(P)H oxidases, decay of peroxynitrite (ONOOH) and the reaction of hypochlorite with superoxide. Lipid peroxidation is one common characteristic of almost all pathological processes involving ROS. During the onset of arteriosclerosis, uptake of oxidized LDL by the so-called "scavenger receptor" of macrophages seems to represent one initial step (Brown and Goldstein, 1979; 1985). LDL- oxidation as a prerequisit for uptake by this not-back-regulated receptor, on the other hand, seems to be driven by diverse oxidants such as chelated iron- or copper- ions, enzymes such as lipoxygenase or ROS produced by activated neutrophils or stimulated endothelial cells and delayed by endogenous antioxidants such as vitamin E (Esterbauer *et al.,* 1988; 1990; Jürgens *et al.,* 1987; Steinbrecher *et al.,* 1989; Kuzuya *et al.,* 1991; Jessup *et al.,* 1990) and other antioxidants such as pyridoxalphosphate-glutamate (Kögl *et al.,* 1994; Selmer *et al.,* 1997). On the basis of our present knowledge, ROS are produced by all types of leukocytes and endothelial cells and are undoubtedly involved in both microbial killing, cytotoxicity and blood vessel damage (Sies, 1985; 1991; Halliwell and Gutteridge, 1989; Elstner, 1990). Damage of endothelial cells and induction of atherogenesis is thus based on a very complex interaction of several cooperative, pathological processes. If ROS are involved in atherogenesis, efficient radical

Abbreviations: DC, diene conjugation; DHF, dihydroxy fumaric acid; EM, electrophoretic mobility; FO, Fenton-type oxidant; KMB, 2- **k**eto-4-**m**ethylthio **b**utyrate; LDL, low density lipoprotein; lin, α-linolenic acid; ONOOH, peroxynitrous acid; ROS, reactive oxygen species; SA, salicylic acid; PD, extracts from Fraxinus-Solidago-Populus (Phytodolor^R); SE, extracts from Salix spec; SE-h, PD-h, derivatized extracts; SOD, superoxide dismutase; XOD, xanthine oxidase.

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scavengers should have protecting effects on LDL in this respect (c.f. Selmer *et al.,* 1997).

Salicylic acid derivatives ("salicylates") are widely used as antiinflammatory drugs. The underlying reaction mechanisms concern inhibitory effects on enzymes such as myeloperoxidase (Pekoe *et al.,* 1982), free radical scavenging (Ahnfelt-Ronne and Nielsen, 1987; Betts *et al.,* 1985) and inhibition of arachidonic acid-induced platelet aggregation (Kagawa *et al.,* 1992), to mention just a few. We have recently shown that SE and PD are effective OH-scavengers (Rohnert *et al.,* 1998) in XOD- or FO-catalyzed oxidative fragmentation of KMB. Further protective mechanisms might concern chelate formation with copper- or iron-ions thus avoiding FO- formation. The activity of copper-ions in this respect has been reported by several authors in context with inflammatory diseases: copper contents increase in mice after endotoxin- (LPS; lipopoly-saccharide-) treatment (Rofe *et al.,* 1996) and plasma copper is increased by approximately 30-fold in the extracellular fluid compartments of rabbit eyes during uveitis (Mc Gahan and Bito, 1982). Focal interstitial granuloma and increased formation of elastic and collagenous fibers is found in the lungs of "vinyard sprayers" inhaling Bordeaux mixture (1–2% $CuSO_4$-solution) for longer times indicating toxic effects of copper ions as responsible for this occupational disease (Eckert and Jerochin, 1982). An outbreak of heliotropic dystrophy of the livers in more than three thousand cases in Tadzhikistan was registered in 1992. In 83 cases thorough investigations showed an increase in plasma-copper, LDL and sialic aicd connected with vascular liver lesions and enhanced lipid peroxidation (Mansurov, 1995) again stressing a correlation between LDL sialylation and susceptibility to Cu-mediated oxidizability (Tertow *et al.,* 1993; Schlüssel and Elstner, 1996).

In this communication we report on the effects of SE or the plant drug, Phytodolor^R on different copper-catalyzed oxidations demonstrating their protective effects in several independent model reactions.

Materials and Methods

Materials

SE and PD were produced and partial HPLC-analysis were a gift from Steigerwald-Arzneimittelwerk GmbH (Darmstadt). PD and SE represent different extract preparations from *Salix* bark or from *Fraxinus*, *Solidago* and *Populus* (Phytodolor^R, Steigerwald-Arzneimittelwerk GmbH), respectively.

1 g dry matter of SE contained (partial analysis by HPLC): 107–124 mg salicin and 1,14–1,19 mg salicylic alcohol.

The aqueous extract of PD contained in 1 g dry matter (partial analysis): 1.38 mg isofraxidine, 0.94 mg rutin, 9.74 mg salicin and 0.44 mg salicylic alcohol. Derivatization was achieved by a special treatment to be published elsewhere (patents pending or in preparation).

KMB and XOD (xanthine oxidase, E. C. 1.2.3.2.) were from SIGMA-Deisenhofen; all other chemicals were either purchased from SIGMA, Boehringer-Mannheim or Merck-Darmstadt. The enzyme activity (units) is defined according to the details given by the distributer.

LDL was isolated from human blood serum (donor: healthy male, 58 years, 88 kg) by ultracentrifugation as recently described (Kögl *et al.,* 1994; Selmer *et al.,* 1997).

Methods

A comparism of the effects of SE and PD on the oxidative capabilities of different ROS-generators as catalyzed by copper ions was made with the following systems:

Photodynamic lipid peroxidation

α-Linolenic acid peroxidation was measured as ethane release and as both formation of ethane and ethene in the presence of copper ions driven by the photodynamic dye, rose bengal (Heiser *et al.,* 1998; in press).

The test solution contained in 2 ml : 0.1M phosphate buffer pH 7.4; 3.55 mM α-linolenic acid; 1.68 μM Cu^{2+}; 20 μM rose bengal; salicylic acid and PD or SE as indicated in the figures. The reaction was conducted for 30 min at 37 °C under illumination (30 Klux) in reaction vessels with gas-tight rubber stoppers. After the reaction 1 ml gas was withdrawn from the head space of the reaction vessels; ethane and ethene were quantified gas-chromatographically as outlined by v. Kruedener *et al.* (1995).

Endiole autoxidation

The reductant dihydroxyfumaric acid (DHF) is an endiole which, similar to ascorbic acid or Amadori-products from the reactions of aldoses with amino acids, monovalently reduces oxygen in the presence of transitions metal ions such as Cu. As indicator for the production of ROS we used the transamination product of the amino acid, methionine, namely the sulfur-ketoacid, KMB, producing ethene in the presence of ROS such as FO, OH-radical-type oxidants or ONOOH (Hippeli *et al.*, 1997).

The test system contained in 2 ml: 0.1 M phosphate buffer pH 7.4; 0.5 mM DHF; 1.5 mM KMB; in addition, SE, SA or diethyl dithio-carbamate were present in concentrations as indicated in the figures. The reaction was conducted in vesseles with gas-tight rubber stoppers for 30 min in the dark. After the reaction 1 ml gas was withdrawn from the headspace of the reaction vessels and ethene was quantified as described under above.

LDL-diene conjugation (DC) and electrophoretic mobility (EM)

LDL oxidation was followed via DC (diene conjugation; the "Esterbauer"-method as modified by Schlüssel and Elstner, 1996) or by agarose gel chromatography (Kögl *et al.*, 1994). Shortly, LDL-oxidation was determined photometrically at 234 nm (37 °C) using 25 µg LDL and 1.67 µM Cu^{2+}- ions and the indicated amounts of SE. The electrophoretic mobility was tested in 0.8% agarose gels after incubation of 30 µg LDL with 3.36 µM Cu^{2+} and the indicated amounts of SE for 24h at 37 °C.

Further experimental details are given in the individual figures. All experiments were conducted with four parallels and were repeated at least twice; standard deviations are given in the figures.

Results

LDL oxidation by Cu^{2+} ions

Diene conjugation (DC) in LDL

Treatment of isolated LDL by low concentrations of copper-ions is a widely used method for testing the oxidizability of LDL or substances delaying it such as α-tocopherol (Esterbauer *et al.*, 1990; Kuzuya *et al.*, 1991). Several methods for monitoring oxidation of LDL have been described where the increase of both light-absorbance at 234 nm (DC; "diene conjugation") and electrophoretic mobility (EM as an indication of the increase in negative charge of the apolipoprotein) have been proven most useful for routine measurements (Kögl *et al.*, 1994; Schlüssel and Elstner, 1996 and refs. therein).

During incubation of LDL with 1.68 µM Cu^{2+}-ions, diene conjugation is inhibited by SE and PD in a charateristic manner: the onset of LDL-diene conjugation i.e. the transition of the lag-time of absorbance into its quasi-linear increase at 234 nm: this increase is shifted from approximately 95 min with LDL in the presence of 1.68 µM $CuSO_4$ to approximately 120 min by SE in concentrations of 0.001 mg/ml test solution. In the presence of 0.007 mg/ml SE the delay of absorbance increase during Cu-catalyzed LDL oxidation equals more than 350 min with all extracts tested (data not shown). With 0.004 mg/ml SE or PD characteristic differences between SE and PD can be seen: As shown in Fig. 1 (a-d), SE retards DC by ca. 50 min and SE-h by ca. 150 min. Even stronger effects are observed with PD which retard by ca. 120 min (PD) and by ca. 220 min (PD-h). Salicylic acid at low (10 and 50 µM) concentrations accelerates DC and has little or no effect at higher (250 and 500 µM) concentrations (Fig. 2).

Electrophoretic mobility (EM) of LDL

EM of LDL is increased by 100% after Cu- oxidation; this increase in EM is completely reversed by SE-h and PD-h, slightly retarded by SE and SE-h while 2 mM salicylic acid slightly enhance EM (Fig. 3).

KMB fragmentation by DHF-Cu^{2+}

As an indicator reaction for ROS-production by DHF-copper we used ethene formation from KMB (v.Kruedener *et al.*, 1995; Hippeli *et al.*, 1997). As shown in Fig. 4 ethene production from KMB is inhibited to the same extent by both SE and PD by approximately 20% by 0,1 mg/ml and by 90% by 1 mg/ml, respectively where no differences after derivatization of the extracts can be observed. Inhibition by the copper chelator DDC present at 20 µM is equivalent to the inhibition by

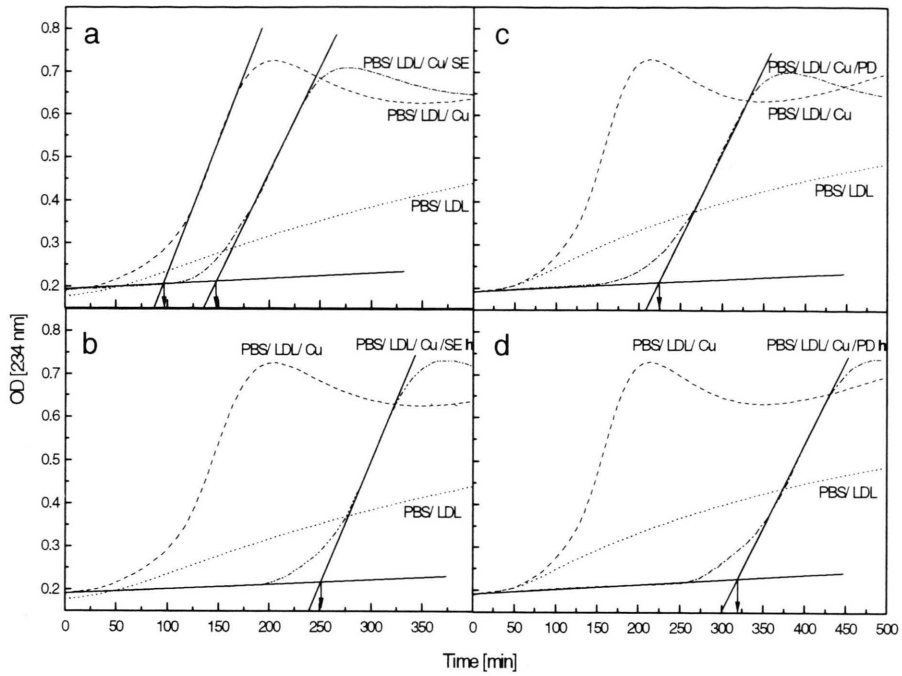

Fig. 1. Effects of different plant-extracts on copper-catalyzed low density lipoprotein (LDL) oxidation.
Time- dependent photometric monitoring of DC measured as absorbance-increase at 234 nm of isolated LDL (50 nM).
The reaction mixtures contained in 1 ml: 20 mM PBS buffer pH 7.4; 25 μg/ml LDL; 1.68 μM $CuSO_4$; SE or PD at the indicated concentrations (0.004 mg/ml).
For experimental details see text and Materials and Methods
a) effects of SE;
b) effects of SE-h;
c) effects of PD;
d) effects of PD-h.

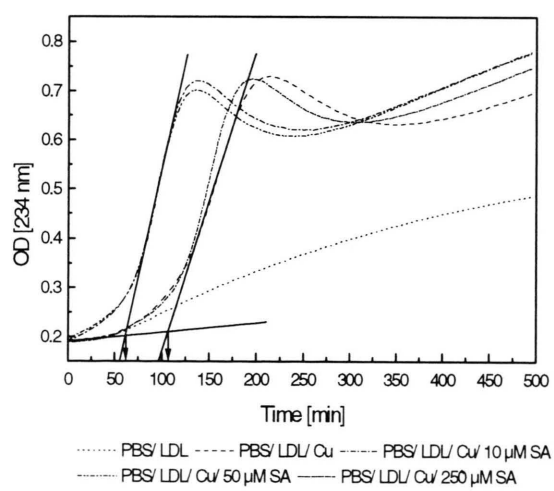

Fig. 2. Effects of salicylic acid on copper-catalyzed low density lipoprotein (LDL) oxidation.
For experimental conditions see Fig. 1 and Materials and Methods.

0.1 mg/ml SE. 1 mM salicylic acid inhibit by approximately 50%.

Photodynamic ethane- and ethene- formation from α-linolenic acid

Illumination of lin in the presence of the photoactivator, rose bengal, results in ethane release from the omega-1,2-fragment of the fatty acid. Ethane production is partially converted into ethene in the presence of copper ions (Heiser *et al.*, 1998; in press). This reaction is a reliable test system for the involvement of copper and thus suitable for testing the copper-chelating properties of compounds or extracts under question.

As shown in Fig. 5 increasing amounts of salicylic acid as a known copper chelator and radical scavenger inhibit both ethane and ethene production where a stronger inhibition of ethene release is observed in the range of 1 mM of the inhib-

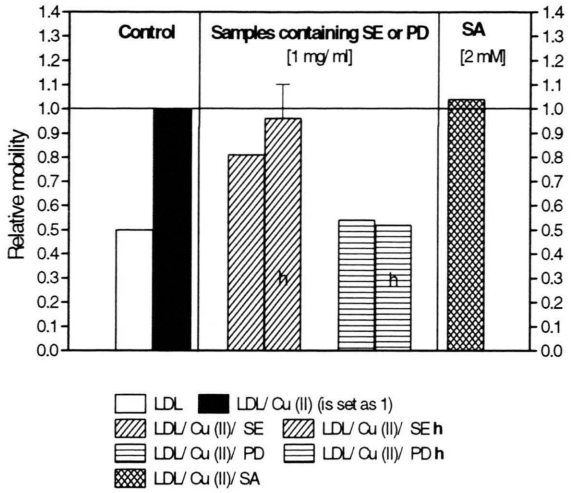

Fig. 3. Increase of electrophoretic mobility after copper-catalyzed low density lipoprotein (LDL) oxidation and effects of different plant extracts.
The reaction mixture contained in 1 ml: 20 mM PBS buffer pH 7.4; 30 μg LDL; 3.36 μM $CuSO_4$; SE, PD and SA at the indicated concentrations.
For further experimental conditions see Materials and Methods.

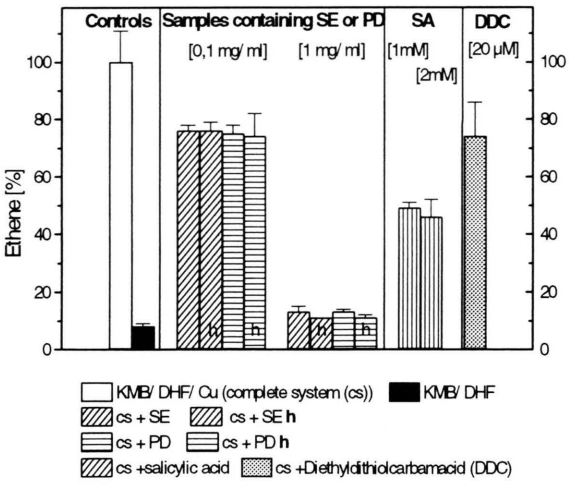

Fig. 4. Ethene formation from 2- keto-4-methylthio butyrate (KMB) driven by dihydroxy fumaric acid (DHF)-copper.
The reaction mixture contained in 2 ml: 0.1 M phosphate buffer pH 7.4; 1.5 mM KMB; 20 μM $CuSO_4$; 0.5 mM DHF and the indicated amounts of SE, PD, SA or DDC.
The reaction was conducted for 30 min at 37 °C and ethene was determined as described in Materials and Methods.

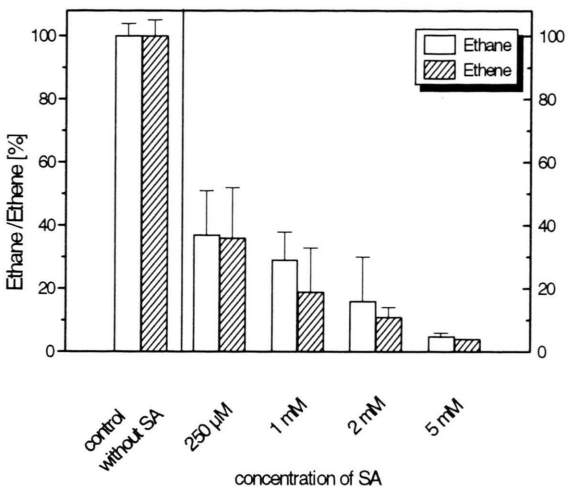

Fig. 5. Photodynamic ethane- and ethene-production from α-linolenic acid: Effects of salicylic acid.
The reaction mixture contained in 2 ml:
3.55 mM lin; 20 μM rose bengal; 1.68 μM $CuSO_4$.
For further details see Materials and Methods.

itor. Likewise, effects of all extracts are seen: where 0.01 mg/ml show little effect (maybe except PD which inhibits by ca. 10 to 20%; large standard deviations, however), 0.5 mg/ml of all extracts completely abolish (inhibition = 100%) photodynamic product formation from lin. At lower concentrations the extracts inhibit between 60 and 90% with a stronger influence on ethene formation as compared to ethane release thus indicating copper-chelation (data not shown).

In this concentration of 0.1 mg/ml, inhibition-values for ethane, and ethene formation, are: 65% and 80% (SE), 80% and 90% (SE-h), 88% and 90% (PD) and 80% and 85% (PD-h), respectively.

Discussion

Plant drugs such as Phytodolor or extracts derived from Salix spec. have been in use as antiinflammatory drugs since the old days (Morton and Meisinger, 1977). It is well known that "salicylates" i.e. derivatives of salicylic acid or generally aqueous extracts from the bark of Salix spec. exhibit radical scavenging properties. Due to the broad spectrum of substances which may generally be addressed as polyphenols and flavonoids a wide spectrum of interference with radical generating reactions and with radicals themselves have been

described (Bors and Saran, 1987; Bors *et al.*, 1992). In a forthcoming report (Rohnert *et al.*, 1998; this volume) we describe experiments on inhibitory SE-and PD activities concerning FO and hydroxylamine oxidation. In this report we show that these plant extracts contain substances functioning as scavengers of FO and others that interact with ROS-generating enzymes such as XOD. We specially investigated on copper-dependent reactions in order to find out whether SE contains transition metal-chelating properties in addition to the mentioned radical-quenching activities.

The lag-time of the appearance of absorbance-increase at 234 nm is an appropriate indicator for diene conjugation (DC) in isolated LDL after oxidation by copper ions. This photometric method is a common and well known mean for determining the intrinsic antioxidative potential of LDL or functions of external antioxidants (Esterbauer *et al.*, 1988; 1990; Jürgens *et al.*, 1987; Steinbrecher *et al.*, 1989; Kuzuya *et al.*, 1991; Jessup *et al.*, 1990). We used this method in comparism to electrophoretic mobility (EM) in order to study LDL oxidation. As shown under results, SE and PD, and here especially the derivatized (PD-h) products drastically retard the onset of diene conjugation, where PD is more active than SE. The best result in this context was the 220 min-retardation of the onset of DC by 0.004 mg/ml PD-h (Fig. 1d).

This result is in contrast to the findings with salicylic acid which accelerated DC at lower concentrations so that the onset of DC was already visible after 60 min instead of 100 min. This effect may be due to intermediary phenoxylradical formation which in turn may drive DC as very recently shown for ferulic acid (Bourne and Rice-Evans, 1997) and for polyalcohols and sugars (Koske and Elstner, 1998; in press).

EM as an indicator for LDL oxidation was doubled by copper treatment and also slightly enhanced by 2 mM salicylic acid. SE inhibites copper-enhanced EM by ca. 20% and the PD by approximately 50%. This result is in perfect agreement with the findings with DC. As far as LDL oxidation is concerned the extract PD-h appears to warrant best protection.

The radical-producing potential of DHF is well known since more than one decade and its destructive reactions have been described using isolated cardiocytes (Barrington *et al.*, 1988) or LDL (Fong *et al.*, 1987) to mention just two reports out of approximately fifty others published on this topic. KMB fragmentation by DHF is an appropriate biochemical model reaction mimicking some properties of the diabetic situation. In this system 0.1 mg/ml test solution, all four tested extracts show the same inhibition (ca. 25%) as 20 μM of the copper chelator, DDC; 1 mM SA inhibit by ca. 50%. The extracts in 1 mg/ml-concentrations inhibit by approximately 90% and thus only allow the rate of ethene formation from KMB already visible without added copper-ions: alltogether, again an indication of copper-chelation.

Ethane formation from lin is, like the thiobarbituric acid assay, an indication for lipid peroxidation. In the presence of increasing copper-concentrations, however, the peroxidation product ethane is converted into ethene formation by oxidation of the intermediary ethyl-radical instead of its reduction (Heiser *et al.*, 1998, in press; Elstner, 1990). Increasing amounts of added SA increasingly suppress both ethane and ethene release from lin. Inhibition of ethene production is stronger as compared to ethane formation (Fig. 5). This is again indicative for copper chelation. The extracts in a concentration of 0.5 mg/ml completely abolish (100% inhibition) both photodynamic ethane and ethene formation from α-lin; already 0.01 mg/ml inhibit by 20–25% (PD; PD-h). At concentrations of 0.1 mg/ml inhibition between 60 and 90% are obtained where ethene production is always less than ethane formation. This result clearly shows that SE contains compounds acting both as radical-chain-breakers blocking lin peroxidation (visualized as ethane production) and also as copper chelators measured as percentually less ethene as compared to ethane. Copper-catalysed ROS formation and oxidations apparently can involve several different ROS such as superoxide as demonstrated in the case of doxorubicin-copper complex (Wallace, 1986) or singlet oxygen as postulated by Duchstein (1987). Whatever ROS is involved in our biochemical model systems driving oxidative destructions, our tested plant extracts seem to possess individual mixtures of several inhibitors concerning all involved oxidants.

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