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Antioxidant, metal-binding and DNA-damaging properties of flavonolignans: A joint experimental and computational highlight based on 7-*O*-galloylsilybin

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ABSTRACT

Besides the well-known chemoprotective effects of polyphenols, their prooxidant activities *via* interactions with biomacromolecules as DNA and proteins are of the utmost importance. Current research focuses not only on natural polyphenols but also on synthetically prepared analogs with promising biological activities. In the present study, the antioxidant and prooxidant properties of a semi-synthetic flavonolignan 7-*O*-galloylsilybin (7-GSB) are described. The presence of the galloyl moiety significantly enhances the antioxidant capacity of 7-GSB compared to that of silybin (SB). These findings were supported by electrochemistry, DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity, total antioxidant capacity (CL-TAC) and DFT (density functional theory) calculations. A three-step oxidation mechanism of 7-GSB is proposed at pH 7.4, in which the galloyl moiety is first oxidized at $E_{p,1}$ = +0.20 V (*vs.* $Ag/AgCl_{3M\ KCl}$) followed by oxidation of the 20-OH ($E_{p,2}$ = +0.55 V) and most probably 5-OH ($E_{p,3}$ = +0.95 V) group of SB moiety. The molecular orbital analysis and the calculation of O–H bond dissociation enthalpies (BDE) fully rationalize the electrooxidation processes. The metal (Cu^{2+}) complexation of 7-GSB was studied, which appeared to involve both the galloyl moiety and the 5-OH group. The prooxidant effects of the metal-complexes were then studied according to their capacity to oxidatively induce DNA modification and cleavage. These results paved the way towards the conclusion that 7-*O*-galloyl substitution to SB concomitantly (i) enhances antioxidant (ROS scavenging) capacity and (ii) decreases prooxidant effect/DNA damage after Cu complexation. This multidisciplinary approach provides a comprehensive mechanistic picture of the antioxidant *vs.* metal-induced prooxidant effects of flavonolignans at the molecular level, under *ex vivo* conditions.

1. Introduction

Polyphenolic compounds exhibit number of biological activities including antioxidant capacities; some of these activities are related to their chemoprotective effects [1–4]. Simultaneously with the chemoprotection, adverse effects must be taken into a consideration, mainly due to prooxidant actions. The prooxidant effects are involved after polyphenol complexation with transition metals (predominantly Fe and Cu), which could be facilitated in the presence of molecular oxygen and other prooxidant components, *e.g.* H_2O_2 [5–7]. Most of the biological activities of polyphenols have been evidenced under *in vitro* conditions using cell model systems. However, there is insufficient information on the polyphenol behavior at the molecular level,*i.e.* their reactivity and interaction abilities with macromolecules – DNA, proteins or lipid membranes.

This study aims at the investigation of antioxidant and prooxidant properties of flavonolignans. The main representative of the natural flavonolignans is silybin (SB) (Fig. 1). Within a commercial sphere, SB is used in its pure form and/or as an integral part of a plant extract (termed silymarin) prepared from milk thistle seeds (*Silybum marianum*). Flavonolignans from silymarin are used as nutraceutics (with proven hepatoprotective effects) and they are used in the treatment of death cap (*Amanita phalloides*) intoxications [8,9]. In addition to SB, silymarin contains other

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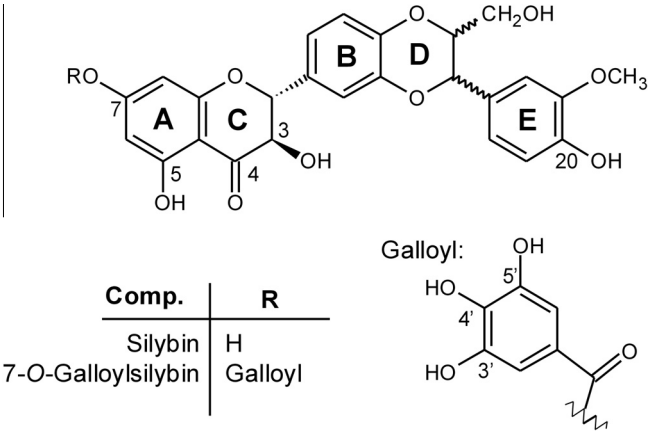


Fig. 1. Chemical structures of SB and 7-GSB.

flavonolignans, flavonols, taxifolin and about 30% of unidentified polymeric phenolic fraction [9].

Due to the excellent chemoprotective activities of SB, the current research is focused on the pharmacological potential of SB derivatives prepared semi-synthetically. In this context, we have recently prepared a series of galloyl esters as 7-*O*-galloylsilybin (7-GSB, Fig. 1) [10]. The substance was prepared following the hypothesis that 7-GSB should exhibit better solubility in aqueous media and higher antioxidant capacity compared to SB, which can be considered as a weak antioxidant with limited solubility. The C-7 position for the substitution of SB molecule was chosen based on the fact that the 7-OH group has considerable prooxidant activity [11,12], therefore, its substitution by gallate has a push–pull effect. Galloyl substitution also induced other new biological activities as, *e.g.*, antiangiogenic activity under *in vitro* conditions [10].

Antioxidant properties of SB and its congeners were fully rationalized from both experimental and theoretical aspects. The individual role of hydroxyl groups in the radical-scavenging activity of SB and 2,3-dehydrosilybin was studied using the corresponding selectively methylated derivatives [11] and dimers [13]. The 20-OH group of SB and both 3-OH and 20-OH groups of 2,3-dehydrosilybin exhibited a major role in the capacity to scavenge free radicals. These findings were in good agreement with experimental oxidation potentials (E_p) of SB and of the methyl derivatives [12,14]. The antioxidant activities were determined also for various SB derivatives including glycosides [15], derivatives substituted with sulfate, phosphodiester or amine [16], fatty acid conjugates [17], oxidized derivatives [18], and flavanone and flavone SB analogs [19].

Despite the fact that free radical scavenging by flavonolignans have been thoroughly studied, the knowledge concerning their prooxidant action, DNA-damage and interactions with biomacromolecules is rather limited. We have recently studied Cu-SB complex formation and examined its prooxidant effects in presence of DNA using electrochemical approaches and fluorescence spectroscopy [14]. Under *in vitro* conditions, the prooxidant effects (suppression of cell proliferation and DNA-synthesis) were studied for Fe-SB complex in Jurkat cells and compared to desferrioxamine, a well characterized potent iron chelator [20]. Antioxidant, DNA cleavage, and cellular effects of SB and its oxovanadium(IV) complex were also shown [21].

According to our best knowledge, no study about antioxidant and prooxidant effects of flavonolignan galloyl esters and their reactivity with DNA and proteins has been published so far.

This work aims at (i) studying the oxidation of 7-GSB, (ii) describing its antioxidant capacity and Cu/7-GSB complex formation using a joint experimental and theoretical approach, (iii) analyzing interactions of 7-GSB Cu-complexes with DNA in the presence or absence of albumin, and (iv) proposing and discussing the general mechanism for prooxidant and DNA-damaging effects of flavonolignans.

2. Material and methods

2.1. Chemicals

SB was kindly provided by Dr. L. Cvak (TAPI Galena, IVAX Pharmaceuticals, Opava, CZ) and 7-GSB was prepared as described previously [10]. Both compounds tested were diastereomeric mixtures (A and B, 1:1). It was previously shown [11,12] that both silybin A and B exhibit very similar redox behavior. Methanol was obtained from Merck (Darmstadt, DE). Buffer components, hydrogen peroxide, EDTA and $CuCl_2$ and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (*St. Louis*, MO, USA). All solutions were prepared using reverse-osmosis deionized water (Ultrapur, Watrex, CZ). The Cu/flavonolignan complexes were prepared by mixing $CuCl_2$ with SB and/or 7-GSB in 0.2 M NaCl solution (pH 6.5) for 15 min at 25 °C. All sample preparations, reactions and analyses were performed under aerobic conditions.

2.2. DNA samples

Single-stranded (ss) DNA strands (strand*I*: 5′-CCG CGC GCC ACG CTG GGG GAC CTC GGG GCC and strand*II*: 5′-GGC CCC GAG GTC CCC CAG CGT GGC GCG CGG*) were prepared synthetically (VBC-Biotech, AT). For double surface technique (DST) experiments, strand *II* contained A_{25} sequence attached to * (see strand *II* above) was used. The DNA duplexes were prepared by mixing both strands (*I* and *II*) in molar ratio 1:1 in 0.2 M NaCl after heating procedure at 85 °C for 3 min followed by slow cooling down to room temperature. For all experiments, with exception of DST and PAGE, the ssDNA (strand *I*) was used.

2.3. Electrochemical measurement

The polyphenols were analyzed using *in situ* voltammetry with the working electrode (PGE: pyrolytic graphite electrode) dipped in the supporting electrolyte containing the analytes and/or *ex situ* voltammetric (adsorptive transfer, AdT technique) analysis. For this purpose, PGE was first dipped into 5-μL aliquot of the studied sample. After an accumulation period, the electrode was washed by deionized water and placed in an electrochemical cell containing supporting electrolyte. Cyclic voltammetry (CV) and square-wave voltammetry (SWV) were performed at the room temperature with a μAutolab III analyzer (EcoChemie, NL) in a three-electrode setup ($Ag/AgCl_{3M\ KCl}$ electrode as a reference and platinum wire as an auxiliary electrode). Individual settings for CV and SWV, as well as concentrations of the compounds and accumulation times, are given in the Figure legends. The flavonolignans analyzed were dissolved in methanol as a stock solution of 1 mg mL^{-1} (stored in a fridge in dark), and diluted with the supporting electrolyte and/or 0.2 M acetate buffer (pH 5) for *in situ* and/or *ex situ* voltammetry.

2.4. DPPH assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) solution was prepared in methanol (1 mg per 50 mL). The flavonolignans were dissolved in methanol and mixed with DPPH solution in the ratio 1:2 (v/v). The reaction mixture was incubated for 30 min at the laboratory temperature and the absorbance was measured at 515 nm against methanol with DPPH. For other details see [22].

2.5. Chemiluminescence measurement of total antioxidant capacity (CL-TAC)

CL-TAC was performed on Luminoskan Ascent (Thermolabsystems, SF) using modified protocol [23,24]; for the original one see [25,26]. SB, 7-GSB and/or Trolox solution (20 μL) was mixed in the microplate well together with the 20 μL of 0.1 mg/mL horse-radish peroxidase solution (Sigma, cat. No. P8250-25KU) before the start of the measurement. The chemiluminescent (CL) mixture (160 μL) containing hydrogen peroxide (0.01 M) and luminol (0.04 mM) in a phosphate buffer (pH 7.4) was injected using autosampling device of the luminometer to trigger the luminol chemiluminescence reaction. In the absence of the antioxidant species tested the light emission occurs immediately after the injection of the CL mixture into the well. If the antioxidant species is present in the sample, the light signal is inhibited for a certain period of time and then increases abruptly up to the values close to those observed in the absence of the antioxidant in the sample. The time period (in seconds) between the injection and the return to the maximum emission is a linear function of the antioxidant capacity of the examined sample, expressed as CL delay.

2.6. Magnetoseparation and application of double-surface technique (DST) for DNA-oxidation products isolation and analysis

Magnetoseparation procedure was modified according to our previously published protocol [27]. The detailed experimental design for DST is shown in the Fig. S1 in the Supplementary Material. Paramagnetic microparticles Dynabeads Oligo$(dT)_{25}$ and magnetic concentrator were supplied by Dynal A.S. (Oslo, Norway).

2.7. UV/VIS absorption spectroscopy

UV/VIS absorption spectra were recorded on the spectrometer Specord 250 Plus (Analytik Jena, DE) with scan-speed 5 nm/s and step 0.5 nm, the bandwidth was 1 nm. Aqueous solutions of 10 μM SB or 7-GSB with 0.2 M NaCl were titrated in quartz cuvettes with increasing concentrations of $CuCl_2$ and incubated for 5 min before the absorption spectrum was scanned. Cuvette with aqueous solution of 0.2 NaCl and corresponding $CuCl_2$ concentration was used as a reference.

2.8. Gel electrophoresis of DNA

Samples containing 50 μg of DNA duplex (see Section 2.2) per mL were mixed with 6X Orange DNA loading dye (Fermentas, Thermo Fisher Scientific Inc.) in the ratio 5:1 (v/v) and heated for 2 min at 90 °C. After the heating procedure, the samples were separated in 15% polyacrylamide gel. The gel was composed of 4 mL 40% monomer solution (acrylamide/bisacrylamide in ratio 29:1), 3.7 mL distilled water, 2.6 mL 10 × TBE (pH 8.3), 100 μL 10% ammonium persulfate and 10 μL tetramethylethylendiamin. Electrophoresis was performed at 150 V in TBE buffer. The gels were stained with 0.02% methylene blue and decolorized in distilled water. Gels were scanned and analyzed using Image J software (ver. 1.4). For DNA-damage (fragmentation) analysis the intensity of stained native DNA (100%) bands was measured, the loss in the intensity indicated DNA-damage.

2.9. Statistical evaluation

The statistical significance of observed differences in antioxidant effects of flavonolignans estimated by DPPH assay (Section 2.4) and prooxidant effects estimated by DNA-electrophoresis (Section 2.8) was evaluated using one-way Anova and multiple comparison tests (HSD method) at 0.01 significance level.

2.10. Calculation methods

The most stable conformers of 7-GSB were obtained within the DFT (density functional theory) formalism using the B3P86/6-31+G(d,p)//B3P86/6-31G(d) methodology. Hybrid functional and in particular the B3P86 functional have appeared well-adapted to evaluate the redox properties of polyphenols [12,28]. Ground-state geometries were confirmed by a vibrational frequency analysis that indicated the absence of imaginary frequency. Enthalpies (H) were calculated at 298 K for the polyphenol (ArO–H) and the corresponding radical (ArO$^{\cdot}$) formed after H atom abstraction from a given OH group. The O–H bond dissociation enthalpy (BDE) of each OH group was calculated as follows:

$$\mathbf{BDE(ArO{-}H)} = \mathbf{H^{298K}(ArO^{\cdot})} + \mathbf{H^{298K}(H^{\cdot})} - \mathbf{H^{298K}(ArOH)}$$

Solvent effects were taken into account implicitly using the IEFPCM (integral equation formalism polarizable continuum model) method coupled to UA0 radii was used. In this method the solute is embedded in a shape adapted cavity surrounding by a dielectric continuum characterized by its dielectric constant ε. To allow an optimum comparison with the experimental data (mainly of DPPH scavenging capacity) methanol was used (ε = 32.6).

The Cu-7-GSB complexes were performed at the same level of calculation (*i.e.*, B3P86/6-31+G(d,p)//B3P86/6-31G(d)) except that the LANL2DZ basis set and pseudopotential was used to describe the copper ions. Explicit water molecules were added to favor the coordination of the metal. The molecular system was then solvated implicitly using PCM-type water (ε = 78.3).

All calculations were performed with Gaussian 09 [29].

3. Results and discussion

3.1. 7-GSB oxidation and correlation with its antioxidant capacity

The mechanism for the oxidation of 7-GSB and its related antioxidant capacity[1] was studied with respect to SB, using *ex situ* cyclic voltammetry (CV, Fig. 2A) and square-wave voltammetry (SWV, Fig. 2B).[2] In such experiments and as previously described [14] SB is oxidized in two steps at $E_{p,1}$ + 0.45 V and $E_{p,2}$ + 0.85 V (*vs.* Ag/$AgCl_{3M\ KCl}$, other potentials mentioned further are referred to this electrode) (Fig. 2). The first oxidation ($E_{p,1}$) occurs at relatively high potential indicating the relatively low antioxidant capacity of SB in comparison to *e.g.*, flavonol antioxidants as quercetin, which are oxidized at around +0.15 V under the same conditions [14].

A three-step oxidation mechanism was observed for 7-GSB at potentials around +0.20, +0.45, and +0.95 V. This clearly indicates that the first peak (peak G at +0.20 V) is attributed to the galloyl moiety (Fig. 2). This was confirmed by the two model systems *i.e.*, gallic acid and gallic acid methyl ester exhibiting peaks around +0.2 V. The other two peaks (peaks 1 and 2 in Fig. 2) were assigned to the SB moiety *i.e.*, involving electron abstraction from the molecular orbital located at the 20-OH and probably 5-OH group, respectively [14]. Peak G is assigned to the oxidation of the galloyl moiety precisely to electron abstraction from HOMO-2 of 7-GSB, which is delocalized on this moiety (Fig. 3). The oxidation mechanism for galloyl moiety of 7-GSB could be similar to the recently described gallic acid oxidation at glassy carbon electrode [32].

It is remarkable that the structure–activity relationship of 7-GSB with respect to SB differs from that one observed in the oxidation of *epi*-catechin gallate with respect to *epi*-catechin. Indeed, in

[1] For a better understanding of the electrochemistry applications in the evaluation of antioxidant capacity of bioactive compounds see reviews [30,31].

[2] All presented results (peaks and related E_p values) obtained by *ex situ* approach (with respect to low-consumption of samples) are fully consistent with *in situ* voltammetric analyses that were measured simultaneously.

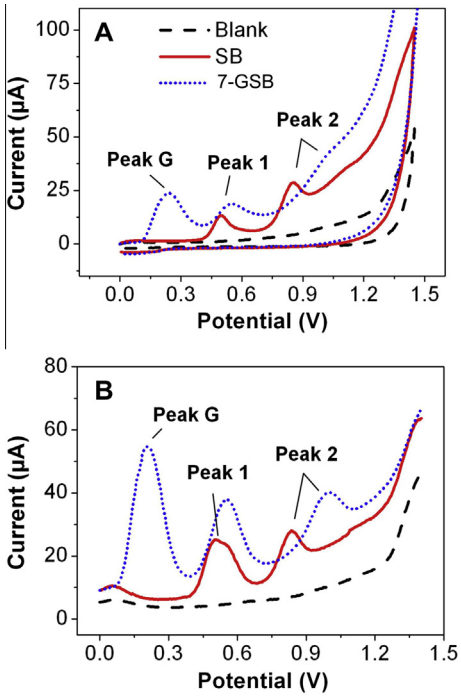


Fig. 2. Adsorptive transfer cyclic (A) and square-wave (B) voltammograms of 50 μM SB and 7-GSB. AdT voltammetry; working electrode: PGE, t_A = 30 s, medium for adsorption: 0.2 M acetate buffer (pH 5.0), supporting electrolyte: 0.2 M phosphate buffer (pH 7.4), scan rate (for A) 1 V s^{-1} and frequency 200 Hz (for B).

epi-catechin gallate the first anodic peak was assigned to the catechol 3,4-dihydroxyl moiety, while the galloyl moiety oxidation probably corresponds to the second peak [33,34]. It follows that, unlike *epi*-catechin, galloylation of SB leads to the significant improvement of its antioxidant capacity at the qualitative level.

3.1.1. DPPH free radical scavenging and total antioxidant capacity of 7-GSB

To further investigate the antioxidant capacity of 7-GSB *vs.* SB not only electrochemistry but also DPPH scavenging activity (Fig. 4A) and CL-TAC (Fig. 4B) was measured. These two assays confirmed much better antioxidant capacity of 7-GSB with respect to SB. The 7-GSB compound exhibits double Trolox capacity to scavenge DPPH (Fig. 4A). This compound has the same CL-TAC activity as Trolox until saturation is reached at 10 μM (Fig. 4B). Such a non-linear evolution of CL delays *vs.* concentration is probably attributed to the oxidatively induced dimerization and/or oligomerization of 7-GSB during time-consuming experiment [35]. The statistical significance of all mentioned differences is described in the legend to Fig. 4.

The increase of free radical scavenging capacity of 7-GSB compared to SB is unambiguously attributed to the OH groups of the galloyl moiety. As extensively described in the literature, polyphenols scavenge DPPH free radicals by H-atom transfer (HAT) *via* either (i) coupled proton electron transfer (PCET), or (ii) electron transfer proton transfer (ET–PT) or (iii) sequential proton loss electron transfer (SPLET) [36–38]. Whatever the mechanism, the oxidation is accompanied by proton release and the global process is mainly governed by the intrinsic O–H BDE parameter; the lower the BDE, the higher the antioxidant capacity of the corresponding OH group. In 7-GSB, the 4′-OH group appears as the most active *i.e.*, lowest BDE (79.1 kcal mol^{-1} in Table 1). The 3′- and 5′-OH groups exhibit higher BDE comparable to that of the 20-OH group of 7-GSB and SB (Table 1), and may also play a minor role in the antioxidant activity. The lower BDE obtained for the 4′-OH group

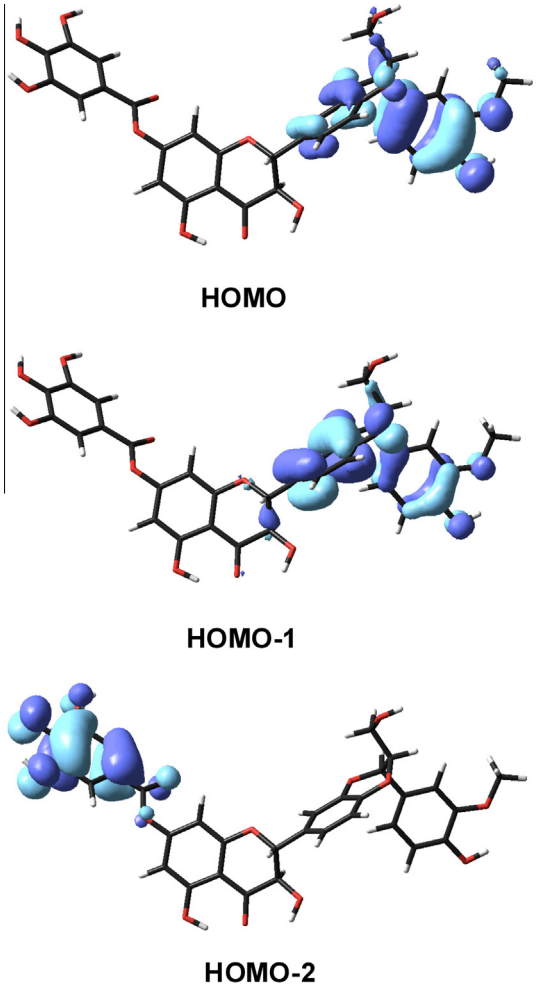


Fig. 3. 3D distribution of the highest three occupied molecular orbitals namely HOMO, HOMO-1 and HOMO-2. The first two are delocalized on the B, D and E-rings of the SB moiety, while HOMO-2 is delocalized on the galloyl moiety.

is attributed to a better π-conjugation in the corresponding ArO· radical (formed after HAT) and thus a better spin density delocalization, with respect to the other groups *e.g.*, 3′-OH (Fig. 5). The other groups exhibit high BDEs making these sites poorly active as free radical scavengers. The low BDE obtained for the 4′-OH group, is comparable to the BDE of very active antioxidant including quercetin [12,28]; this clearly shows an efficient HAT capacity, making 7-GSB an efficient DPPH free radical scavenger and a powerful antioxidant.

3.2. The mechanism of 7-GSB-metal complexation

Flavonolignans were shown as effective transition metal chelators allowing several biomedical applications [20,39,40]. The chelation activity was shown for SB with iron [39] and copper [14], the metal-binding being primarily attributed to the hydroxyl and oxo groups [14].

The formation of flavonolignan–metal complexes may occur in the organism after ingestion of flavonolignans. These complexes can directly interact with DNA inducing the formation of reactive oxygen species (ROS), which in turn may induce oxidative DNA degradation and fragmentation [7]. We recently reported on such prooxidant effects for the SB-Cu complexes [14]. In this case, the 5-OH group and the oxo-group at C-4 (5-OH/O-4 binding site) appeared the most probable site for Cu^{2+} coordination.

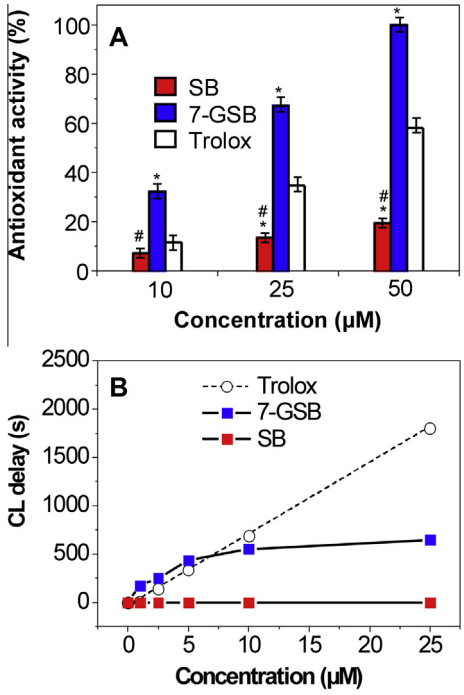


Fig. 4. DPPH (A) and CL-TAC (B) assay of SB and 7-GSB. Relative antioxidant activity is expressed for panel A, 100% = 50.9% inhibition; $n = 6$. R.S.D. ⩽ 10% for panel B. For details see Experimental section. The values that were significantly different from values obtained with Trolox are denoted by *($p < 0.01$), significant difference between SB and 7-GSB values is denoted by # ($p < 0.01$).

Table 1
Bond dissociation enthalpies (BDE in kcal mol^{-1}) for the different OH groups of 7-GSB and SB in PCM-type methanol, calculated with B3P86/6-31+G(d,p).

	7-GSB	SB
3-OH	109.1	106.8
5-OH	97.5	99.1
7-OH	–	92.3
20-OH	84.6	84.8
23-OH	110.6	–
3′-OH	88.1	–
4′-OH	79.1	–
5′-OH	84.8	–

In the present study we are dealing with the Cu-7-GSB complex formation and the related prooxidant effects involving DNA-damage. The results are compared to the prooxidant action of SB (see Section 3.3). The interaction of Cu^{2+} with 7-GSB was studied by using *ex situ* SWV (Fig. 6A) following the decrease of the 7-GSB oxidation peaks *vs.* Cu^{2+} concentration (Fig. 6B). Both peaks G and 2 dramatically decreased from 100% to 0% (Fig. 6), which was attributed to the complexation at the galloyl moiety or at the 5-OH/O-4 site, respectively. During titration experiment, the peaks (10 μM 7-GSB) completely disappeared at 10 μM Cu^{2+}. DFT calculations confirmed that the most stable complexes were formed at both the metal coordination 4′-OH/5′-OH and 5-OH/O-4 sites (Table 2). Peak 1 of 7-GSB did not completely disappear during titration by Cu^{2+}, only a partial decrease was observed, which is in good agreement with our previously published findings that metal coordination at the 20-OH group is less likely [14].

For a detailed characterization of Cu-7-GSB complexes, the UV/Vis spectra were thoroughly analyzed (Fig. 7). The absorption spectrum of 7-GSB exhibit two overlapping peaks at 206 nm and 225 nm, and one peak at 286 nm (Fig. 7A). These three peaks gradually decreased with increasing $CuCl_2$ concentration. The decrease was non-linear (Fig. 7B) indicating that interaction of Cu^{2+} and 7-GSB is more complex process. No further changes in absorption spectrum were observed at concentrations higher than 20 μM Cu^{2+}, suggesting that the maximal chelating capacity is two Cu^{2+} ions per one 7-GSB molecule.

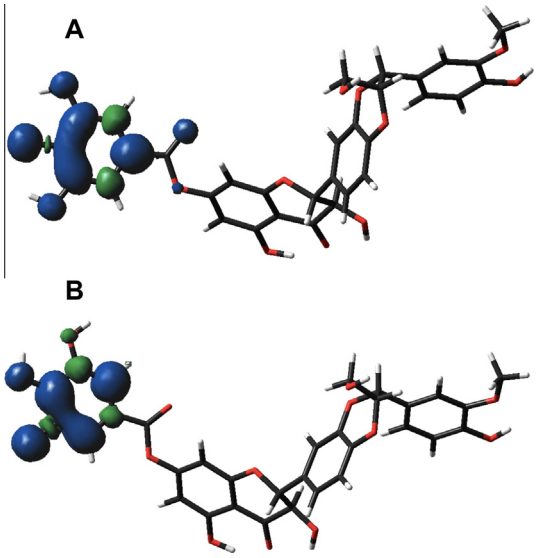


Fig. 5. 3D spin density distribution for the ArO· radical formed after H atom transfer from the 4′-OH (A) and 3′-OH (B) groups.

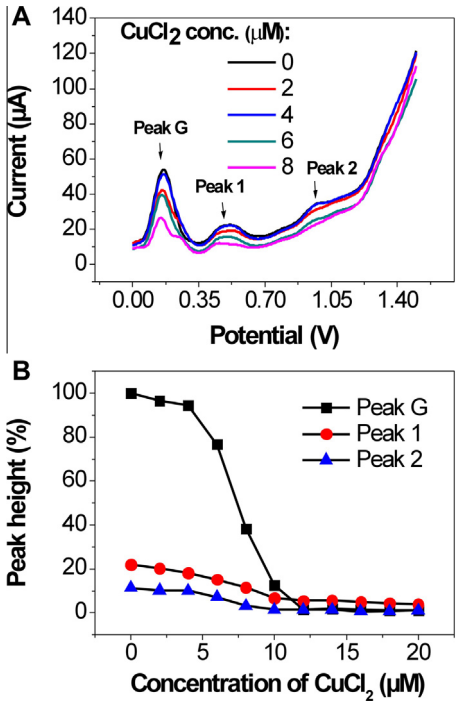


Fig. 6. SW voltammograms (A) and changes in heights of voltammetric peaks (B) of 7-GSB (10 μM) after 15 min incubation with Cu^{2+}. The reaction (incubation) was performed in 0.2 M NaCl for 15 min at 25 °C under aerobic experimental conditions. For AdT SWV, the same conditions as in Fig. 2 were used, with exception of $t_A = 60$ s. R.S.D. ⩽ 15% ($n = 6$).

3.3. Interaction of 7-GSB with DNA and DNA degradation in the presence of metal-complexes

The model GC-rich deoxyribonucleotide duplex (fully described in Section 2.2) was used to study prooxidant effects of

Table 2
Relative (with respect to the most stable complex) electronic energies of complexation (kcal mol^{-1}) for the different sites of 7-GSB chelation.

Chelation site	Relative energy of complexation
3-OH/O-4	15.1
5-OH/O-4	0.0
3′-OH/4′-OH	12.6
4′-OH/5′-OH	3.5

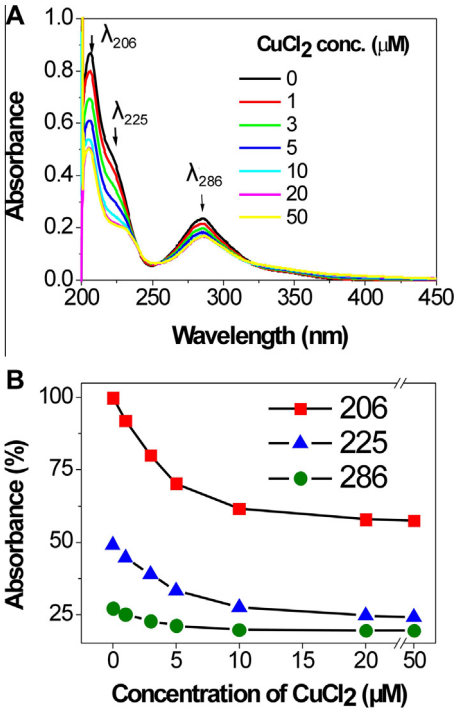


Fig. 7. Absorption spectra (A) and changes in absorbance intensity at 206, 225 and 286 nm (B) of 7-GSB (10 μM) after 15 min incubation with Cu^{2+}. The reaction (incubation) was performed in 0.2 M NaCl for 15 min at 25 °C under aerobic experimental conditions. For experimental details, see Section 2.7. R.S.D. ⩽ 5% (n = 6).

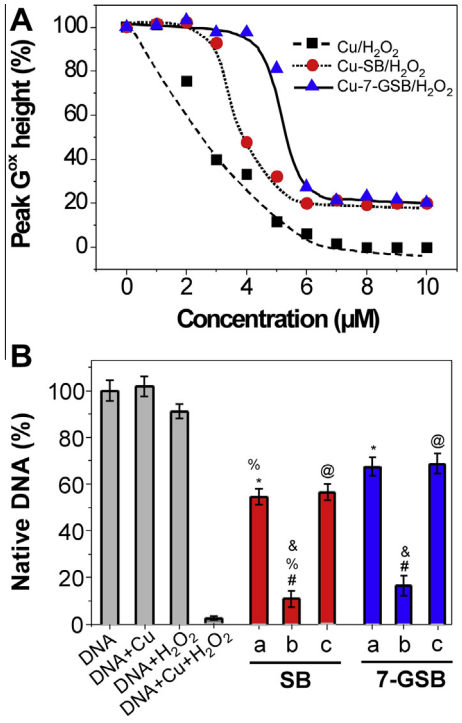
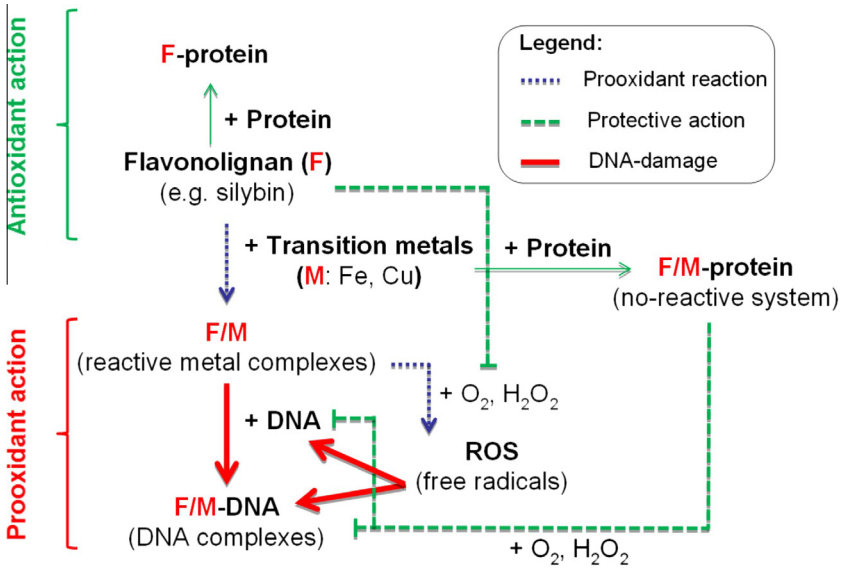


Fig. 8. (A) Changes in electrochemical peak G^{ox} height after DNA treatment by different Cu^{2+}-complexed species in presence H_2O_2. (B) The electrophoretically detected depletion of native DNA after treatment by (a) flavonolignan/Cu complex, (b) flavonolignan/Cu complex in H_2O_2 presence, and (c) flavonolignan/Cu complex in H_2O_2 and BSA presence. Concentration of SB/7-GSB: 10 μM, DNA: 20 μg/mL (for A) and 50 μg/mL (for B), BSA: 10 μM, H_2O_2: 2%. The reaction (incubation) was performed in 0.2 M phosphate buffer (pH 7.4) for 10 min at 37 °C; n = 6 (R.S.D. ⩽ 10% for panel A). For AdT SWV, the same conditions as in Fig. 2 were used. The statistical significance of differences on $p < 0.01$ level is quoted * for comparison of DNA in the presence of Cu^{2+} and DNA in the presence of flavonolignan/Cu^{2+} complex, # for comparison of DNA in the presence of H_2O_2 and DNA in the presence of flavonolignan/Cu^{2+} complex and H_2O_2, & for comparison of DNA in the presence of Cu^{2+} and H_2O_2 and DNA in the presence of flavonolignan/Cu^{2+} and H_2O_2, @ for comparison of DNA in the presence of flavonolignan/Cu^{2+} and H_2O_2 and DNA in the presence of flavonolignan/Cu^{2+}, H_2O_2 and albumin (protective effect of albumin), and for comparison of the corresponding values for SB and 7-GSB by %.

Cu-7-GSB complexes on DNA. To analyze DNA modification oxidatively induced, the electrochemical approach based on the observation of oxidation of the guanine (G^{ox}) and adenine (A^{ox}) residues was applied [41,42]. After oxidative modification of DNA by Cu-7-GSB complexes (in the presence of H_2O_2) the decrease in DNA oxidation peaks (G^{ox} = +1.10 V and A^{ox} = +1.40 V) was observed, especially for the G^{ox} peak because guanine residues have high reactivity and are easily oxidized [43] (Fig. 8). The prooxidant effects of Cu-7-GSB were compared to the prooxidant effects of Cu^{2+} (Fenton-like reaction) and Cu-SB complex in the presence of H_2O_2. The data on oxidative modification of guanine residues and DNA fragmentation by *ex situ* SWV and PAGE are shown in Fig. 8A and B, respectively.

The prooxidant action of 7-GSB is significantly lower than that of SB, which could be connected to the formation of several complexes with different prooxidant potential and/or to the substitution of the 7-OH group, which blocks its possible prooxidant action as observed for SB [11]. From the point of view of real situations in the organism, the presented prooxidant action can be found only in the absence of proteins. If a protein (BSA) is present in the samples with Cu–flavonolignan complexes, the prooxidant effects were significantly suppressed (Fig. 8B, column c). This effect is attributed to antioxidant properties [44] and multi-metal binding capacity [45], including Cu, of the serum albumins. The protection by albumin against the prooxidant actions of phenolic compounds was previously shown [46] and our results well agrees with this finding. The statistical significance of all differences discussed here is described in the legend to Fig. 8.

All above-mentioned electrochemical results concerning DNA damage were performed in most complex samples without the purification of the DNA-damage (oxidation) products. For this reason, the magnetoseparation technique was used to isolate DNA-damage products. The combination of magnetoseparation with *ex situ* voltammetry (double-surface technique, DST) was used to avoid artifacts [27] (Fig. S1 in Supplementary Material). First, we subjected native DNA to DST where two well-resolved oxidation peaks G^{ox} and A^{ox} were found (Fig. S2A). The A^{ox} peak exhibits a higher current response with respect to the A_{25} tail required for immobilization of the DNA duplex onto the T_{25} attached paramagnetic microparticles. After DNA-damage mediated by the Cu-flavonolignan complexes, the two samples were collected (sample "A" and "B"), see Fig. S1. Sample "B" corresponded to the pure oxidation DNA products, which were released from the surface of the paramagnetic microparticles after washing of all interfering compounds and analyzed by *ex situ* SWV (Fig. S2A). No G^{ox} peaks were observed in the purified sample "B", which definitely



Scheme 1. Simplified scheme of antioxidant and prooxidant (DNA-damaging) action of flavonolignans in absence and in presence of proteins. The scheme is proposed according the joint experimental and computational results acquired at molecular level, namely under *ex vivo* conditions.

confirmed that Cu–flavonolignan complexes were involved in DNA oxidative modification (Fig. S2B).

In addition to the decrease of the DNA oxidation peaks, the decrease of the oxidation peaks of flavonolignans and their Cu-complexes was observed. These effects correspond to the oxidation and cleavage of flavonolignans by the free radicals generated and interaction of flavonolignan complexes with DNA resulting in the decrease of their ability to interact with electrode surface. This statement is in a good agreement with our previous results [14].

4. Conclusion

Based on our new results presented here for 7-GSB and those published previously for SB [14,47], we drew up a mechanism for the prooxidant action of metal–flavonolignan complexes (M/F) on DNA (Scheme 1). The first step is the coordination complex formation. These reactive complexes may participate in ROS generation in the presence of molecular oxygen, inducing oxidative DNA modification and cleavage. Another possibility is the direct interaction of M/F complexes with DNA, yielding the ternary M/F–DNA complexes. Such a more sophisticated reaction can also participate in the ROS production and M/F-DNA binding can modulate and/or prevent the DNA replication and translation processes. The prooxidant action of M/F complexes is facilitated in the presence of other oxidants as hydrogen peroxide. In contrast, the prooxidant effects can be strongly suppressed by proteins; indeed most of the polyphenols binds to the albumin fraction in blood [48], and their antioxidant capacity can be maintained [49]. The subtle balance between antioxidant and prooxidant actions of flavonolignans is a crucial aspect to elucidate many of the biological activities of these compounds (e.g., antiproliferative effects or adverse effects). The present joint experimental and theoretical work provides new highlights on the mechanisms of the antioxidant *vs.* prooxidant effects of flavonolignans at the molecular level under *ex vivo* conditions, which could be extended to other polyphenolic substances in future. This work provides a solid guideline for further *in vivo* investigations of anti- vs. pro-oxidant effects of this series of derivatives. Our conclusions can be extrapolated and adapted to flavonolignan metabolites, which would target proteins and DNA in the organism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cbi.2013.07.006.

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