Advances in Kinetics and Mechanism of Chemical Reactions

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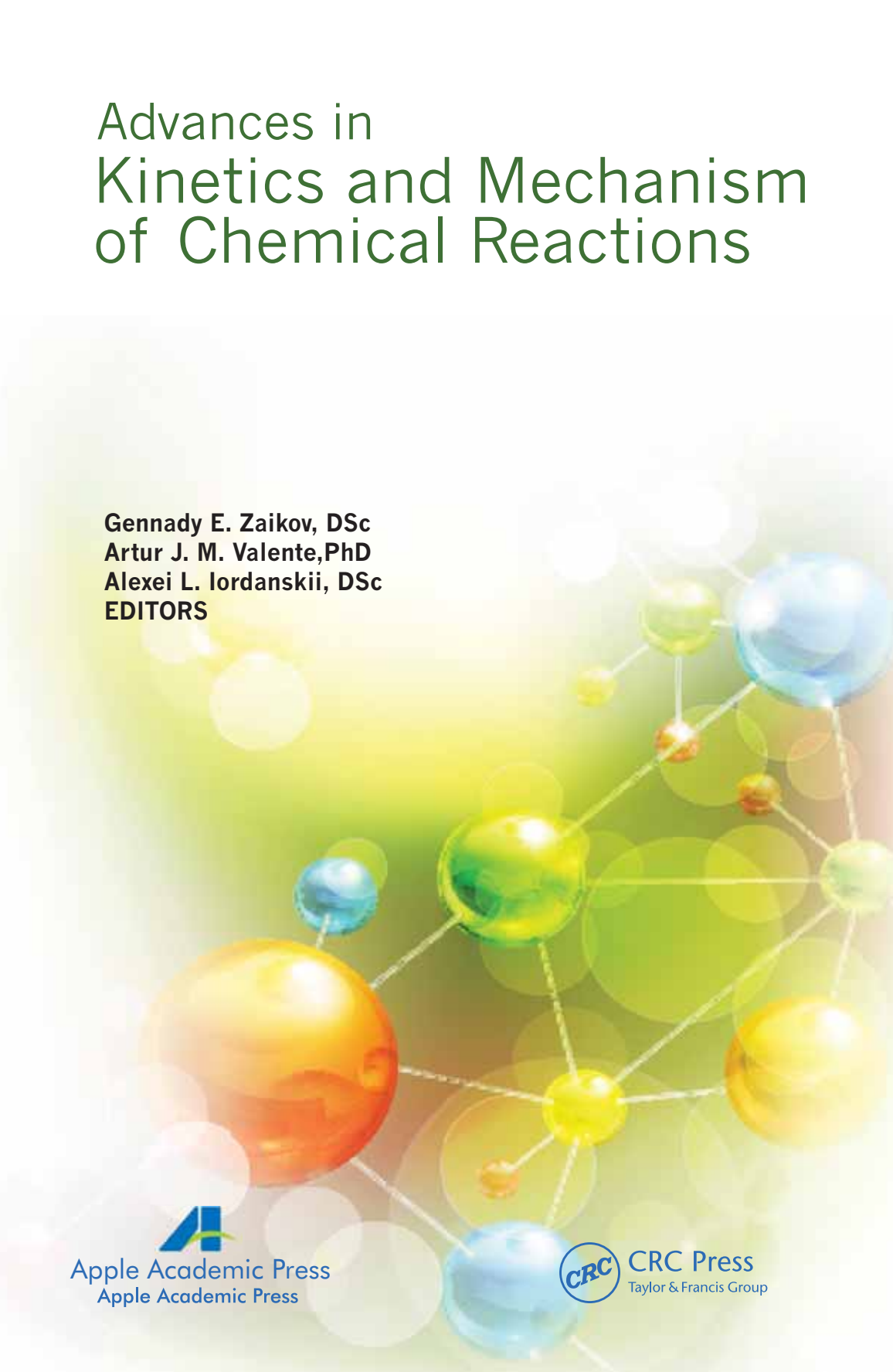

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1 Radical Degradation of High Molar Mass Hyaluronan Induced by Ascorbate Plus Cupric Ions Testing of Arbutin in the Function of Antioxidant

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1.1 INTRODUCTION

Hyaluronan (HA, Figure 1) is a linear polysaccharide consisting of repeating disaccharide units of β-1,3-*N*-acetyl-D-glucosamine and β-1,4-D-glucuronic acid. The HA molar sizes in the human organism vary between 2×10^5 and 10×10^6 Da. An adult person weighing 70 kg has about 15 g of HA in the body, yet about one-third of this amount turns over daily.

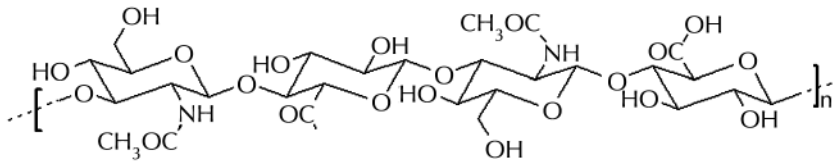


FIGURE 1 The HA acid form of the macromolecule.

In the human organism, HA is present in two forms: associated with certain glycosaminoglycans (GAGs) and proteins for example in the skin and cartilage, or free/unassociated in the synovial fluid (SF) and vitreous humor of the eye. The highest contents of (associated) HA in the human body occurs in the skin in which HA has a rapid turnover rate with a half-life of 1.5 days. Unassociated high molar mass HA in SF confers its unique viscoelastic properties required for maintaining proper functioning of the synovial joints. The half-life of HA within SF is approximately 12 hr [1].

The fast HA turnover in SF of the joints of healthy individuals may be attributed to the oxidative/degradative action of reactive oxygen species (ROS), generated among others by the catalytic effect of transition metal ions on the autoxidation of ascorbate [2]. Uninhibited and/or inhibited HA degradation by the action of various ROS has been studied on applying several *in vitro* models. In these studies, the change of the HA molar mass or a related parameter, such as the HA solution dynamic viscosity, was used as a marker of inflicted damage [3].

Arbutin (termed also hydroquinone-β-D-glucopyranoside, Figure 2), a substance used in the function of skin lightening and depigmentation is a popular drug especially in Japan and other Asian countries, also due to its lower toxicity compared to hydroquinone [4, 5]. Two anomeric forms, namely α- and β-arbutin, exist from which the latter occurs naturally in plants, for example in leaves of bearberry (*Arctostaphylos uva-ursi* Spreng., Ericaceae) and pear trees (*Pyrus communis* L., Rosaceae).

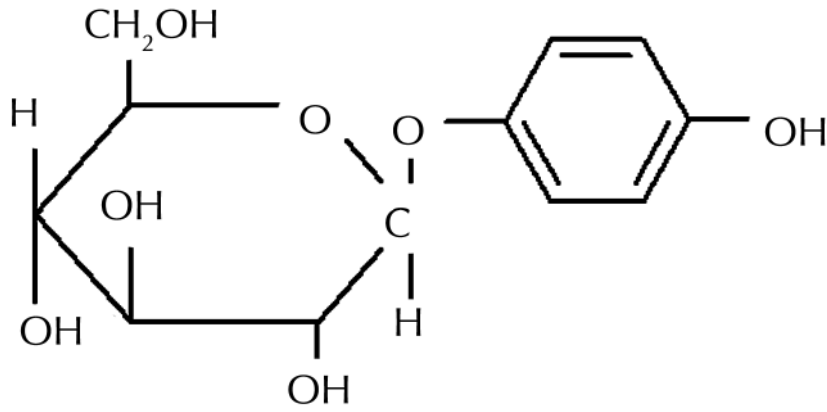


FIGURE 2 Arbutin structure.

The primary mechanism of arbutin action is in inhibiting tyrosinase activity in the skin, resulting in the significantly diminished formation of the brown colored melanin pigment. Further, arbutin acts as an "anti-aging" agent and a UVB/UVC filter which protects the skin against deleterious effects caused by photon generated free radicals. Beyond their cosmetical actions, products containing arbutin are therapeutically used for treating cystitis and kidney stones. Arbutin, a very hygroscopic substance, readily hydrolyzes for example by diluted acids yielding D-glucose and hydroquinone in a 1:1 mole ratio [6]. Arbutin based products are successfully applied also as diuretics: it is anticipated that the drug inhibits/retards the proliferation of pathogens within the urinary tract according to its high electrochemical potential, which within the pH range of 7.5 and 2.0 lies between +466 and +691 mV, respectively [7].

In the body, arbutin is decomposed to D-glucose and hydroquinone. It is a well known fact the latter substance bears strong antimicrobial and disinfectant properties. The greater the pH values of urine, the stronger the bacteria killing efficiency of free (not glucuronided) hydroquinone. However, in more acidic urine, hydroquinone undergoes extensive glucuronidation, which is believed to lead to the formation of the antibacterially ineffective hydroquinone glucuronide [7, 8].

So far no reports have been published devoted to action(s) of arbutin under conditions simulating ROS damaging effects on HA macromolecules. Since the HA content is so high in various tissues/body fluids–the skin/SF–the presented study is focused on investigating the ability of arbutin to act anti and/or prooxidatively by monitoring the kinetics of free radical degradation of high molar mass HA. As an effective $^{\bullet}OH$ radical inducer, the system comprising ascorbate plus Cu(II) – the so-called Weissberger's oxidative system was used. Along with monitoring the dynamic viscosity of HA solution by the method of rotational viscometry, further methods such as size exclusion chromatography (SEC), cyclic voltammetry (CV), EPR spectrometry, and standardized decolorization 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)

assay were applied at testing arbutin in the function of preventive and chain breaking antioxidant.

Arbutin was tested in the function of a potential anti or prooxidant in Cu(II) plus ascorbate induced degradation of high molar mass HA. The time and dose dependences of dynamic viscosity changes of the HA solutions were investigated by the method of rotational viscometry. First, the HA solution was exposed to degradation induced by Cu(II) ions (1 μM) with various concentrations of ascorbic acid (10, 50, or 100 μM). Further the action of arbutin (100 μM) addition into the reaction system, preceding that of ascorbic acid (100 μM), was inspected. The results obtained clearly indicated prooxidative properties of arbutin in relation to HA free radical degradation. Both the reduction of the dynamic viscosity of the HA solution and the decrease of the polymer mean molar mass as revealed by the method of SEC proved the tenet that on using the Cu(II) ions plus ascorbate, that is the Weissberger's oxidative system, the degradation of HA macromolecules is pronounced by added arbutin. Cyclovoltammetric studies of arbutin both in aqueous (0.15 M NaCl) and nonaqueous (0.15M TBAPF6 in DMSO) solutions confirmed a substantial protonation and hydrolysis reactions of arbutin in aqueous solutions. By applying the method of Electron Paramagnetic Resonance (EPR) spectroscopy, the reaction mixture comprising HA, Cu(II) ions, ascorbate, and arbutin indicated the presence exclusively of ascorbyl radicals. A remarkable radical scavenging activity of arbutin was observed on using the ABTS decolorization assay.

1.2 MATERIAL AND METHODS

1.2.1 Biopolymer

The high molar mass HA sample P9710-2A (M_w = 808.7 kDa; M_w/M_n = 1.63) used was obtained from Lifecore Biomedical Inc., Chaska, MN, U.S.A. The declared contents of transition metals in the HA sample given by the Certificate of Analysis are 4 ppm Cu and 13 ppm Fe.

1.2.2 Chemicals

Analytical purity grade NaCl and $CuCl_2 \cdot 2H_2O$ were purchased from Slavus Ltd., Bratislava, Slovakia. Dimethyl sulfoxide (DMSO), L-ascorbic acid, and $K_2S_2O_8$ were the products of Merck KGaA, Darmstadt, Germany. Trolox, arbutin, and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) were from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Tetrabutylammonium perchlorate (TBAP), $LiClO_4$, and ABTS (purum, >99%) were from Fluka, Chemie GmbH, Steinheim, and Germany. Redistilled deionized high quality grade water, with conductivity of <0.055 mS/cm, was produced by using the TKA water purification system from Water Purification Systems GmbH, Niederelbert, Germany.

1.2.3 Preparation of Stock and Working Solutions

The HA sample solutions (2.5 mg/ml) were prepared in the dark at room temperature in 0.15M aqueous NaCl in two steps: First, 4.0 ml of the solvent was added to 20 mg of dry HA powder. Then 3.80, 3.85, or 3.90 ml of the solvent was added after 6 hr. The stock solutions of L-ascorbic acid (1.6, 8, and 16 mM), arbutin (16 mM), and $CuCl_2$ (160 μM prepared from 16 mM $CuCl_2$) were also prepared in 0.15M aqueous NaCl.

1.2.4 Study of Uninhibited/Inhibited HA Degradation

Uninhibited

The HA degradation was induced by the oxidative system comprising $CuCl_2$ (1.0 μM) with altering concentrations of L-ascorbic acid (10, 50, and 100 μM). The procedure was as follows: the volume of 50 μl of 160 μM $CuCl_2$ solution was added to the HA solution (7.90 ml) and after 30 s stirring the reaction solution was left to stand for 7.5 min at room temperature. Then 50 μl of L-ascorbic acid (1.6, 8, or 16 mM) were added to the reaction vessel and the solution was gently stirred for 30 s. The reaction mixture was then immediately transferred into the viscometer Teflon cup reservoir.

Inhibited

The HA degradation: The procedures to test arbutin in the function of (i) preventive and (ii) chain breaking antioxidant were as follows:

(i) A volume of 50 μl of 160 μM $CuCl_2$ solution was added to the HA solution (7.85 ml) and after 30 s stirring the reaction solution was left to stand for 7.5 min at room temperature. Then 50 μl of arbutin (16 mM) were added to the solution and stirred again for 30 s. Finally, 50 μl of L-ascorbic acid (16 mM) were added to the reaction vessel and the solution was gently stirred for 30 s. The reaction mixture was then immediately transferred into the viscometer Teflon cup reservoir.

(ii) A similar procedure as described (i) was applied, however, 50 μl of L-ascorbic acid (16 mM) were added to the 7.5 min equilibrated reaction solution (7.90 or 7.85 ml)–comprising HA plus $CuCl_2$–and 30 s solution stirring followed. After 1 hr, finally 50 or 100 μl of arbutin (16 mM) were added to the solution and stirred again for 30 s. The solution mixture was then immediately transferred into the viscometer Teflon cup reservoir.

1.2.5 Rotational Viscometry

The resulting reaction mixture (8.0 ml) was transferred into the Teflon cup reservoir of a Brookfield LVDV-II+PRO digital rotational viscometer (Brookfield Engineering Labs., Inc., Middleboro, MA, U.S.A.). The recording of viscometer output parameters started 2 min after the experiment onset. The changes of the dynamic viscosity (η) values of the reaction mixture were measured at 25.0 ± 0.1°C in 3 min intervals for up to 5 hr. The viscometer Teflon spindle rotated at 180 rpm that is at a shear rate of 237.6 s^{-1}.

1.2.6 SEC Analyses

Evaluation of HA molar mass changes was performed with Shimadzu apparatus using a packed HEMA-BIO 1,000 column of dimensions 7.8 mm × 250 mm, the packing particle size was 10 μm. The mobile phase 100 mM phosphate buffer (pH 7.0) containing 0.15M NaCl was pumped by the LC-10AD device at a flow rate of 0.5 ml/min. For calibration of the SEC system used reference HAs (M_w = 90.2–1380 kDa) with broader molar mass distributions (M_w/M_n = 1.60–1.88) were applied. The samples of the volume of 20 μl with the polymer concentrations 1 mg/ml or lower were injected by a 7725i-type Rheodyne valve. The SEC analyses performance was monitored online by UV (SPD-10AV, set at 206 nm) and refractive index (RID-10) detectors.

1.2.7 Cyclic Voltammetry

All cyclovoltammetric experiments were performed with HEKA PG 284 (Germany) potentiostat under argon using a standard three-electrode arrangement of a platinum wire as working electrode, a platinum coil as counter electrode, and a Ag/AgCl as reference electrode. The scan rate used was 100 mV/s.

1.2.8 EPR Spectroscopy

The generation of free radicals during HA degradation was examined by spin trapping technique using an EPR X-band EMX spectrometer (Bruker, Rheinstetten, Germany) at ambient temperature [9, 10]. The sample preparation conditions were identical to those described under the paragraph "Study of uninhibited/inhibited HA degradation"–see the section "Uninhibited" as well as that of (i) within the section "Inhibited HA degradation". Yet the prepared total sample volume was only 800 μl and the addition of L-ascorbic acid solution (the last reactant) when appropriate was replaced by addition of the aqueous NaCl diluent (5 μL).

The volume of 250 μl of each sample solution was thoroughly mixed with 5.0 μl of DMPO spin trap prior to each experimental set carried out in a thin flat EPR quartz cell. The operational parameters of the equipment were adjusted as follows: Centre field 3354 G, sweep width 100 G, time constant 81.92 ms, conversion time 20.48 ms, receiver gain 5e + 5, microwave power 10 mW, and modulation amplitude 2 G.

1.2.9 ABTS Assay–Determination of the TEAC Value

The $ABTS^{\bullet+}$ radical cations were performed by the reaction of an aqueous solution of $K_2S_2O_8$ (3.3 mg) in water (5 ml) with ABTS (17.2 mg). The resulting bluish green radical cation solution was stored overnight in the dark below 0°C. Before experiment, the solution (1 ml) was diluted into a final volume (60 ml) with water [11].

Arbutin solution was prepared as specified under the paragraph "Preparation of stock and working solutions". The investigated samples comprised 2 ml of the diluted $ABTS^{\bullet+}$ solution with addition of 50 μl of working arbutin solution (1 mM).

A modified ABTS assay was run applying a UV/VIS S2000 spectrophotometer (Sentronic, Germany). The UV/VIS spectra were taken up to 120 min. The relative differences (ΔA) in the light absorbance at 731 nm in the 10th and 120th min compared to the reference experiment using Trolox were used to calculate the values of Trolox Equivalent of Antioxidant Capacity (TEAC).

1.2.10 ABTS Assay–Determination of the IC_{50} Value

The $ABTS^{\bullet+}$ radical cation solution (250 μl) prepared from 7 mM ABTS and 2.45 mM $K_2S_2O_8$, 1:1 v/v ratio of water solutions was added to 2.5 μl of the arbutin solution and the absorbance of the sample mixture was measured at 734 nm after 6 min. The radical scavenging capacity of arbutin was investigated at the drug concentration range 2–1000 μM.

The light absorbance was measured quadruplicately in 96-well Greiner UV-Star microplates (Greiner-Bio-One GmbH, Germany) with Tecan Infinite M 200 reader (Tecan AG, Austria). The IC_{50} value was calculated with CompuSyn 1.0.1 software (ComboSyn, Inc., Paramus, USA).

1.3 DISCUSSION AND RESULTS

1.3.1 Establishing a Proper HA Oxidative Degradation System

The HA macromolecules were exposed to free radical degradation induced by Cu(II) ions (1 μM) and ascorbate at various concentrations (10, 50, or 100 μM) where the primary variable—the concentration of cupric ions – was set to such a value which may occur at the early stages of acute joint inflammation. The second variable, the concentration of ascorbate, covers that range which is characteristic in SF of a human being. As shown in Figure 3, the curve coded 1, the reaction system Cu(II):ascorbate equaling 1:10 was during the whole time period of 5 hr ineffective to induce a relevant HA degradation. The observed minor growing of the solution η value represents the well known effect called rheopexy–which is characterized by orientations and attractive self-interactions of dissolved HA macromolecules in the space between the viscometer reservoir wall and the rotating spindle [2].

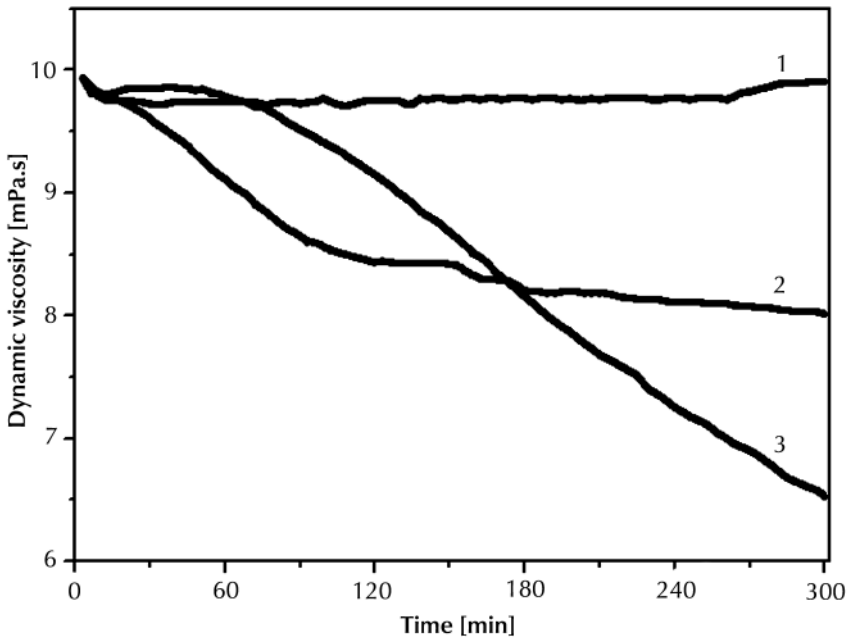


FIGURE 3 Time dependences of dynamic viscosity values of the HA solution in the presence of 1.0 μM $CuCl_2$ plus ascorbate at concentrations 10 (1), 50 (2), or 100 (3) μM.

By increasing the ascorbate concentration to 50 μM (compare Figure 3, curve coded 2), a gradual decrease of the dynamic viscosity of HA solution was evidenced during the first 90 min. Then a slower HA degrading process was observed, which continued up to the end of monitoring the solution η values 5 hr. The attained decrease of the HA dynamic viscosity from its initial value of 9.93 to 8.01 MPa·s was, however, classified as not sufficiently effective for further investigations.

Increasing the ascorbate concentration to 100 μM resulted in an approximately 60 min time interval during which "no degradation" of HA macromolecules was registered. Yet as evident from the curve coded 3 in Figure 3, this stationary/lag phase was continued by a stepwise decrease of the solution dynamic viscosity, achieving at 5 hr the value of 6.53 MPa·s. As proven by using the method of EPR spectroscopy [12] under the experimental conditions represented in Figure 3, curve coded 3, the signal detected during the earlier time intervals (≤ 60 min) belonged exclusively to ascorbyl radical. However as confirmed by applying the DMPO spin trapping method, at later time intervals, the generated $^{\bullet}OH$ radicals "exceeded" the scavenging effectivity of the actual ascorbate ($AscH^-$) level and thus they represented the radical species which reacted with the HA chains leading to polysaccharide fragmentation. As seen in Figure 3, curve coded 3, during the time interval between 60 and 300 min the continual stepwise decrease of the solution η values covers a range equaling to 3.25 MPa·s.

Earliest Time Interval of the Reaction

Let us first analyze the potential chemical processes which resulted in the observations represented by the curve coded 3 in Figure 3 namely the time interval up to approximately 60 min: Under aerobic conditions, the bicomponent system comprising ascorbate and a trace amount of cupric ions is a well known Weissberger's oxidative system generating the strong oxidant hydrogen peroxide. The following flow chart can be primarily used to characterize the individual reaction steps which however take place simultaneously in a concerted action.

$$AscH^- + Cu(II) \rightarrow Asc^{\bullet -} + H^+ + Cu(I) \text{ (reduction of cupric to cuprous ions)} \quad (1)$$

Note 1: It is a well known fact that $AscH^-$ donates a hydrogen atom ($H^{\bullet}$ or $H^+ + e^-$) yielding the resonance stabilized tricarbonyl ascorbate free radical. Since the latter has a pK_a value of 0.86, it is not protonated and thus in aqueous solution it will be present as $Asc^{\bullet -}$ [13].

As the cuprous ions are unstable, namely they can quickly undergo a disproportional reaction $Cu(I) + Cu(I) \rightarrow Cu(0) + Cu(II)$, they are charge stabilized by an excess of the present ascorbate as follows $AscH^- + Cu(I) \rightarrow [AscH^{-}\cdots Cu(I)]$. This reaction intermediate participates in the next bielectron reduction of the dioxygen molecule (O = O)

$$[AscH^{-}\cdots Cu(I)] + O{=}O + H^+ \rightarrow Asc^{\bullet -} + Cu(II) + H_2O_2 \text{ .(hydrogen peroxide formation)} \quad (2)$$

and one may suppose that the nascent H_2O_2 molecule is decomposed immediately by the "uncomplexed"/complexed cuprous ion(s) according to a Fenton-like reaction:

$$H_2O_2 + [Cu(I)] \rightarrow {}^{\bullet}OH + HO^- + Cu(II) \text{ (generation of hydroxyl radicals)} \quad (3)$$

In summary, we could express the following final/net reaction:

$$AscH^- + [AscH^{-}\cdots Cu(I)] + O{=}O \rightarrow 2Asc^{\bullet -} + Cu(II) + HO^- + {}^{\bullet}OH \text{ .(net reaction)}$$

Yet the conditions of the flow chart should be taken into account: Since within the reaction system there is a real ascorbate excess, the nascent hydroxyl radicals are continually quenched and due to these facts the EPR spectrometer monitors exclusively the ascorbyl radicals.

$$AscH^- + {}^{\bullet}OH \rightarrow Asc^{\bullet-} + H_2O \text{ (quenching the hydroxyl radicals)} \qquad (4)$$

According to our findings obtained by using the method of EPR spectroscopy [12] under the experimental conditions represented also in Figure 3, curve coded 3, during the earlier time intervals (≤ 60 min) the monitored ascorbyls – $Asc^{\bullet-}$–indicate that any potentially generated hydroxyl radical is quenched immediately at its phase of nascence.

The informed reader knows that (two) ascorbyls undergo a redox disproportional reaction yielding back a molecule of ascorbate and a not charged dehydroascorbate (DHA). The DHA molecule however hydrolyzes yielding an intermediate, namely 2,3-diketo-L-gluconic acid, and/or several final products such as L-xylose, and L-xylonic-, L-lyxonic-, L-threonic-, as well as oxalic acids [13]. Study of the participation of the mentioned intermediate as well as of the final products within the reaction flow charts (compare reactions 1–4) was however beyond the scope of the present investigation.

Later Time Interval of the Reaction

Let us continue the analysis of the potential chemical processes which could result in the observations represented by the curve coded 3 in Figure 3 namely the result within the time interval from approximately 60 to 300 min: As anticipated according to the sequential depletion of the ascorbate the nonquenched hydroxyl radicals can react with the HA macromolecule by abstracting a hydrogen radical ($H^{\bullet}$), resulting in the formation of a *C*-macroradical represented in Figure 4 and further denoted as $A^{\bullet}$.

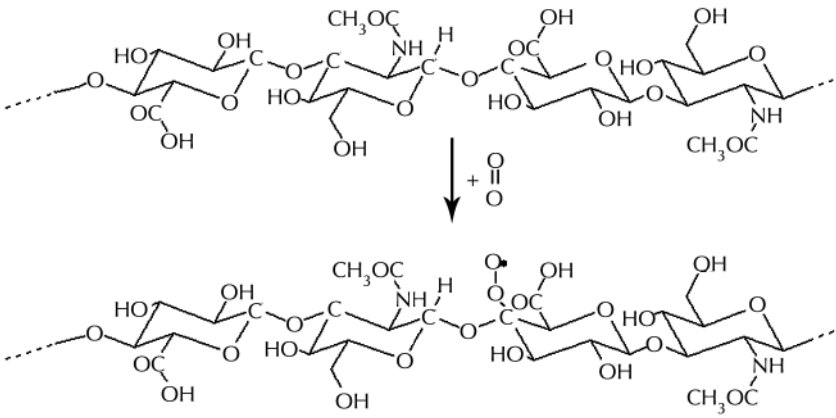
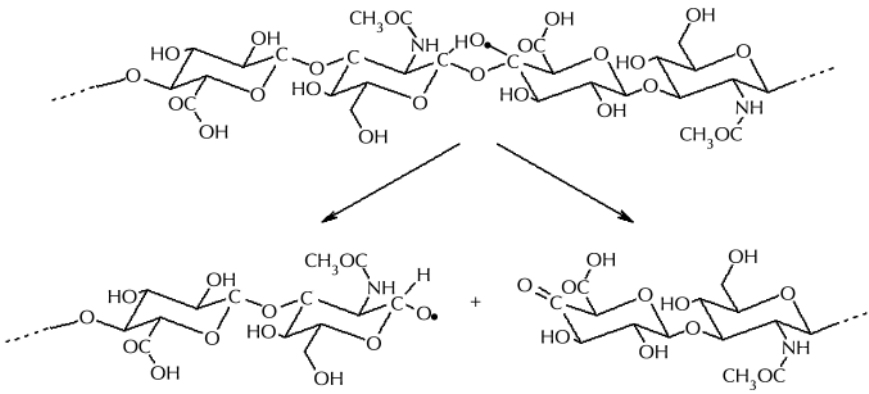


FIGURE 4 The HA *C*-type macroradical, due to its high reactivity, immediately traps a molecule of dioxygen yielding a peroxyl-type macroradical.

Under aerobic conditions, the alkyl-type macroradical – A$^{\bullet}$ – reacts rapidly with the molecule of dioxygen (O_2) yielding a peroxyl type macroradical, hereafter denoted as AOO$^{\bullet}$. The intermediate peroxyl type macroradical formed may react with an adjacent HA macromolecule (HA), and thus the radical chain reaction propagates quickly.

$$AOO^{\bullet} + HA \rightarrow AOOH + A^{\bullet} \text{ (propagation of the radical chain reaction)} \qquad (5)$$

After its "collision" with an HA macromolecule (compare reaction 5), the generated peroxyl type macroradical yields a high molar mass hydroperoxide which subsequently, mostly induced by the present Cu(I) ions {AOOH + Cu(I) → AO$^{\bullet}$ + HO^- + Cu(II)}, yields an alkoxyl type macroradical (AO$^{\bullet}$). This is a presumed intermediate of the main chain splitting, resulting in biopolymer fragments whose solution is characterized by a reduced dynamic viscosity (compare Scheme 1 [14]).



SCHEME 1 Fragmentation of the alkoxy type macroradical (AO$^{\bullet}$).

The attack of hydroxyl radicals on D-glucuronate or *N*-acetyl-D-glucosamine moieties of HA can also lead to the ''opening'' of rings without breaking the polymer chain [15, 16].

1.3.2 Investigating Pro and Antioxidative Action of Arbutin

Based on its chemical structure (compare Figure 2), the natural compound arbutin can be grouped among O-alkyl derivatives of hydroquinone which are generally classified as efficient antioxidants [17]. To assay arbutin action in its function as a preventive antioxidant, the drug was loaded into cupric ions containing HA solution, directly before ascorbic acid admixing as described in procedure (i) under the paragraph "Study of uninhibited/inhibited HA degradation". As indicated in Figure 5, contrary to the

expected inhibition/retardation of the degradation of HA macromolecules, on introducing the drug – resulting in its concentration of 50 or 100 μM (compare curves coded 2' or 3' in Figure 5) – a remarkable reduction of the η value of the solution monitored was recorded at the earliest time interval. While the shape of curve 3 (compare Figure 5), relating to the reaction mixture without arbutin, indicates a stationary/lag phase of the HA degradation process during the first 60 min, addition of the arbutin drug tested (100 μM) shows both elimination of the reaction lag phase and significant acceleration of the decay of HA macromolecules.

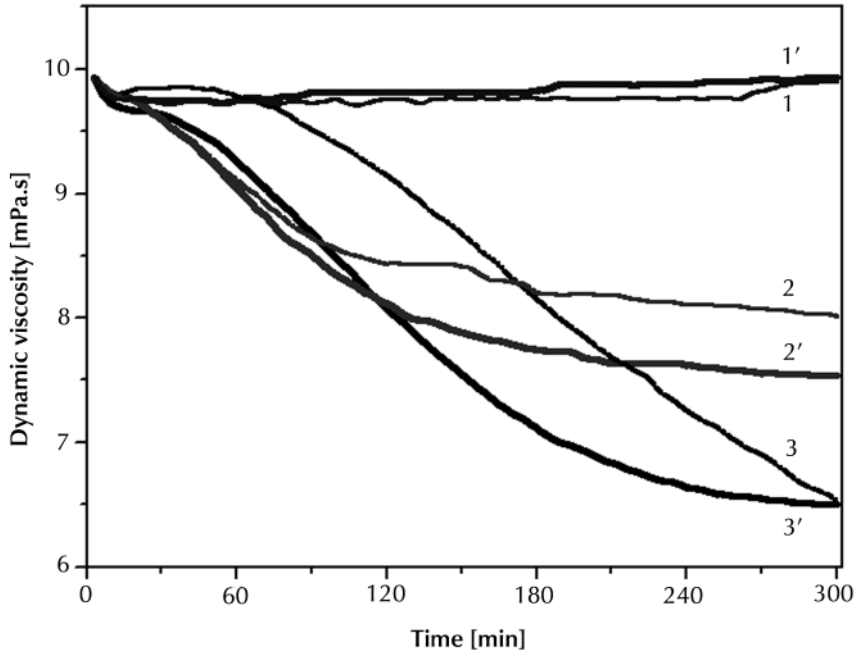


FIGURE 5 Time dependences of dynamic viscosity values of the HA solution in the presence of 1 μM $CuCl_2$, 100 μM arbutin, plus ascorbate at the concentrations 10 (1'), 50 (2'), or 100 (3') μM, as well the dependences observed under similar experimental condition without any arbutin – curves 1, 2, and 3 – relate to ascorbate concentrations of 10, 50, and 100 μM, respectively.

The degradation of the native HA sample after 5 hr treatment by the system comprising Cu(II):arbutin:ascorbate in the molar concentration ratio of 1:100:100 was unequivocally proved by SEC analysis (compare Figure 6, UV and RI traces). As evident from Figure 6, the polymer sample at 5 hr had a lower molar mass ($M_{peak} \approx 695$ kDa) as compared to that of the original HA.

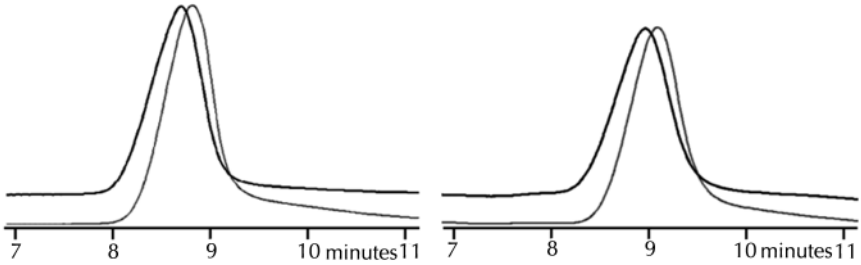


FIGURE 6 The SEC analyses of the native HA sample and of that degrading over 5 hr. (The native HA peaked earlier.) Left panel – UV traces at 206 nm; right panel – RI records.

To explain the observations, the reader's attention is to be switched to the reversible redox processes known for the pair of hydroquinone and quinone (denoted hereafter shortly QH_2 and Q), which can be described as follows [18]:

$$QH_2 \leftrightarrow Q + 2H^+ + 2e^- \text{ (hydroquinone two-electron oxidation)}$$

As claimed, arbutin is readily hydrolyzed by diluted acids yielding QH_2 and D-glucose in a 1:1 mole ratio [6]. The latter tenet was supported also by cyclic voltammetric experiments performed both in DMSO/TBAPF6 and aqueous 0.15M NaCl solution, as shown in Figure 7. Clear differences are seen on comparing DMSO and water solutions. In water solution, the products of hydrolysis as well as protonation of arbutin are observable already during the first CV scan, in contrast to DMSO where only a small increase of arbutin degradation products is seen. In water solution, the strong increase of the CV peak, characteristic of Q/QH_2 redox couple during repetitive redox cycling, indicates that compared to non-oxidized arbutin, the oxidized form of arbutin strongly hydrolyzes (decomposes). Thus due to the addition of ascorbic acid to arbutin, rapid drug hydrolysis occurred.

Reaction Chemistry Investigated by Spin-trapping EPR Spectroscopic Method

Figure 8 represents typical records registered on applying DMPO in the function of radical trapping within the reaction system comprising (a) HA and Cu(II), (b) HA, Cu(II), and arbutin, as well as (c) HA, Cu(II), arbutin, and ascorbate, where the reactants were applied at such concentration ratios which were identical to those described under the paragraph "Study of uninhibited/inhibited HA degradation".

(a, b): By admixing DMPO into the reaction vessel containing already the two components – HA and Cu(II) – a signal characteristic for the $^{\bullet}$DMPO-OH adduct was recorded, however the following arbutin application diminished somewhat the signal abundance (compare Figure 8, traces coded **a** and **b**). The simplest explanation of these observations could be that DMPO added in a great excess reduced Cu(II) ions to cuprous ones, which subsequently reacted with dioxygen, yielding superoxide anion radicals ($O_2^{\bullet -}$). These ROS spontaneously dismutate and the dismutation product H_2O_2 is decomposed by the reduced copper ions in a Fenton-like reaction.

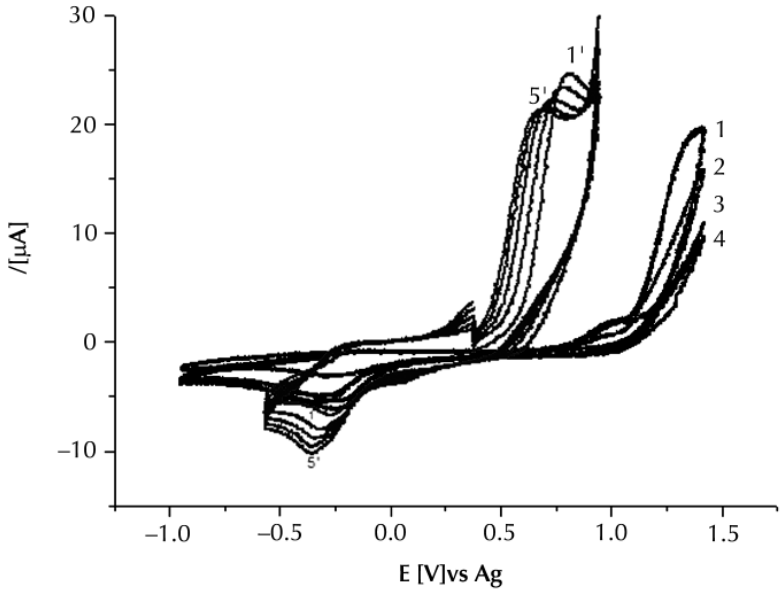


FIGURE 7 The CV (repetitive cycling, CV scans are indicated with numbers) of arbutin in DMSO/TBAPF6 (black lines 1–4) or in H_2O/NaCl (gray lines 1'–5').

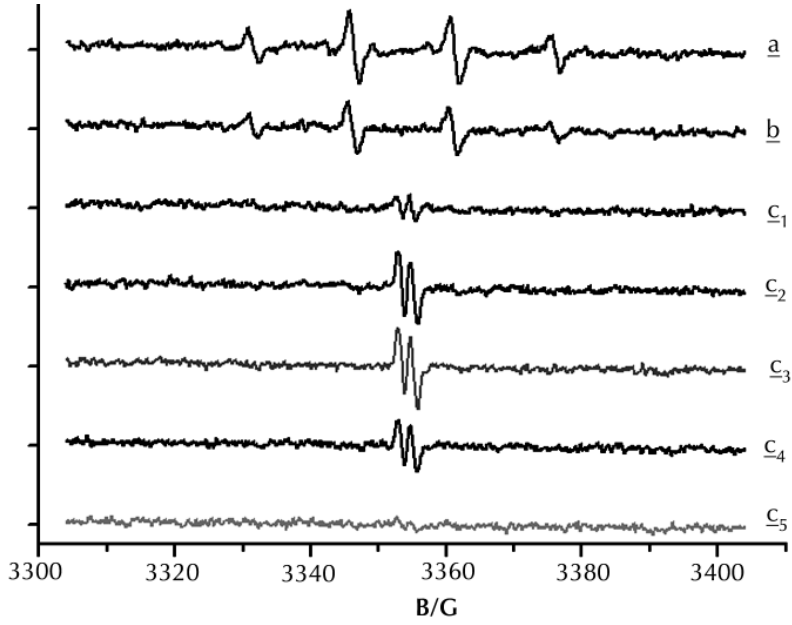


FIGURE 8 The ˙DMPO-OH signals registered 1 min after mixing the DMPO spin trap with the sample solution containing HA and Cu(II) – trace **a**; and with the solution comprising HA, Cu(II), and arbutin – trace **b**.

Note 2: It should be pointed out, however, that systems comprising HA and Cu(II) or HA, Cu(II), and arbutin did not induce any significant degradation of HA macromolecules (not shown).

The signals for ascorbyl detected at time intervals of 10, 30, 60, 90, and 120 min are represented by the respective traces from $\underline{\mathbf{c}}_1$ to $\underline{\mathbf{c}}_5$.

(c): It should be pointed out here that the mixing of all the reaction components, namely DMPO with the sample solution containing HA, Cu(II), arbutin, and ascorbate, took but approximately 1 min during which time the sample was not scanned by the EPR spectrometer. However, after a short time, a signal related to $Asc^{\bullet-}$ emerged, whose intensity varied: During the first time interval up to approximately 60 min the intensity of the recorded signal rose, then it declined and after 120 min the spectrometer monitor was "silent" (compare Figure 8, traces $\underline{\mathbf{c}}_1$–$\underline{\mathbf{c}}_5$). The EPR spectroscopic observations at a later time interval, namely the less and less abundant signal related to $Asc^{\bullet-}$ between 60 and 120 min, as well as later on the signal disappearance, coincide well with the results represented in Figure 5, curve coded 3' at similar time intervals.

By admixing DMPO into the reaction vessel containing already the two components – HA and Cu(II) – a signal characteristic for the $^{\bullet}$DMPO-OH adduct was recorded, however the following arbutin application reduced the EPR signal of $^{\bullet}$DMPO-OH adducts by about 20–30% (compare Figure 9).

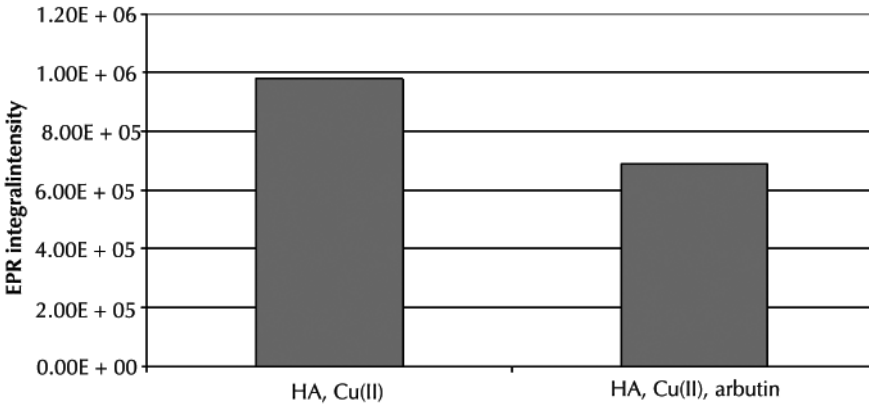


FIGURE 9 Integral EPR intensity of $^{\bullet}$DMPO-OH adducts registered 1 min after mixing the DMPO spin trap with sample solution containing HA and Cu(II) and with the solution comprising HA, Cu(II), and arbutin.

The simplest explanation of these observations could be that hydroperoxides already present in the initial HA solution [9, 19, 20] rapidly decompose due to the presence of Cu(II)/Cu(I) ions (compare also Scheme 1), as already well established in the field of peroxidation of lipids (denoted here as L)

$$LOOH + Cu(II) \rightarrow LOO^{\bullet} + Cu(I) + H^{+}$$
$$LOOH + Cu(I) \rightarrow LO^{\bullet} + Cu(II) + HO^{-}$$

Note 3: It is well known that the presence of peroxyl and alkoxyl radicals as well as their DMPO spin adducts in water solutions leads mostly to the formation of $^{\bullet}OH$ radicals generating the signal of $^{\bullet}$DMPO-OH adduct. Arbutin is probably able to eliminate reactive alkoxy and peroxyl radicals before their spin trapping.

In the case of the system comprising a trace amount of oxidized HA, or more precisely the HA sample containing already a preformed macrohydroperoxides (AOOH), the two electrons gained according to reaction (6) could reduce Cu(II) ions to Cu(I). The generated cuprous ions are then involved in the following reaction

$$AOOH + Cu(I) \rightarrow AO^{\bullet} + Cu(II) + HO^{-}$$

Thus a short-living (ca. 1 μs) alkoxy type macroradical was only present in our experimental system. This macroradical was extremely unstable and degraded quickly to fragments with lower molar mass (compare Scheme 1).

Note 4: It should be pointed out, however, that systems comprising HA and Cu(II) or HA, Cu(II), and arbutin did not induce any significant degradation of HA macromolecules (not shown).

ABTS Assay of Arbutin

The determined TEAC values that is those for the 10th min 1.22 mmol Trolox/L and for the 120th min 1.59 mmol Trolox/L (compare Figure 10) prove a really high reductant power of arbutin in aqueous solutions.

The arbutin scavenging activity represented by its established IC_{50} value 5.43 mM should be classified as relatively high, comparable to that value of quercetin (IC_{50} = 2.86 mM) – the substance used as a standard effective natural antioxidant [21, 22].

The reaction of decolorization of the $ABTS^{\bullet+}$ radical cation solution is based on a simple reaction during which a proper reductant provides an electron to the acceptor – the $ABTS^{\bullet+}$ radical cation. Although the ABTS assay is still one of the primary choices, the reader should take into account that the results described by either the TEAC or IC_{50} values represent just values determined at the experimental conditions used by applying for example another electron acceptor (such as that of $DPPH^{\bullet}$ – the 1,1-diphenyl-2-picrylhydrazyl radical) different IC_{50} values are established.

Note 5: On applying the DPPH assay, the determined IC_{50} value for arbutin (≈ 768 mM) represents a 176 times lower antioxidant capacity than the one determined for quercetin (4.36 mM). Although the arbutin IC_{50} value correlates well with those published by other investigators applying similar DPPH-assay conditions [23], it should be pointed out that there is an experimental "conflict": The DPPH assay, which uses alcohols (methanol) as the 1,1-diphenyl-2-picrylhydrazyl-radical dissolvent, is preferably used to investigate the antioxidant capacity of lipophilic (alcohol

soluble) substances. Yet arbutin is classifiable as a hydrophilic substance, perfectly soluble in water. As calculated, the arbutin *versus* quercetin solubility in water, 39.1 *versus* 0.26 g/l, closely correlates to the logP - lipophilicity values of these substances, namely -1.36 *versus*. + 1.07 [24], respectively. Thus, the IC_{50} value for arbutin (≈ 768 mM) determined by using the DPPH assay does not primarily indicate the very low reduction power of the drug, but most plausibly, this value reflects also a much poorer arbutin solubility compared to that of quercetin when methanol was the solvent used.

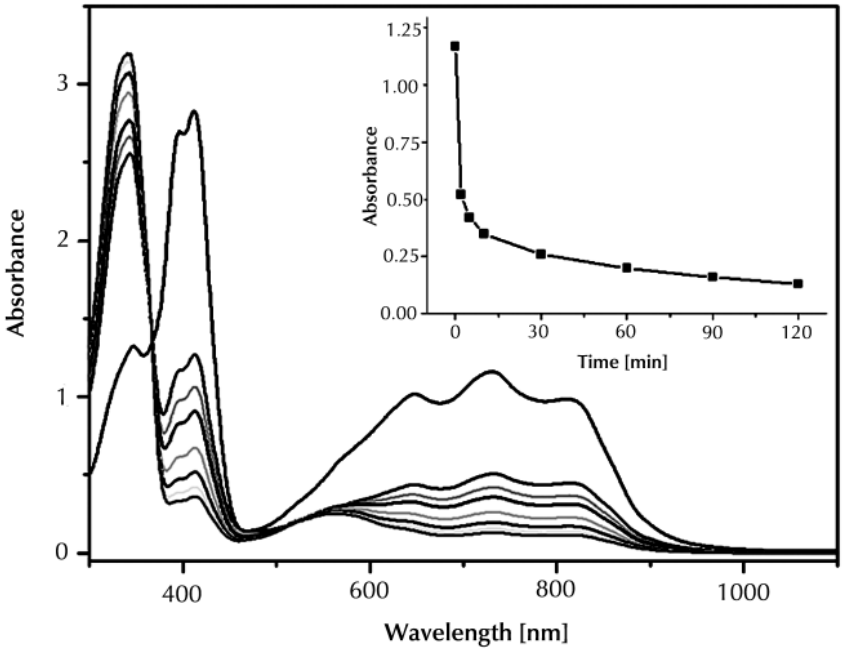


FIGURE 10 The UV/Vis records documenting arbutin scavenging of the $ABTS^{\bullet+}$ radical cation. Inset: Time dependence of the absorbance at 731 nm.

1.3.3 Investigating Arbutin in the Function of a Chain Breaking Antioxidant

There is a discrepancy of arbutin classification as an efficient antioxidant/reductant based on the ABTS assays and on the contrary a pro-oxidant investigated by the drug action when introduced into the running free radical HA degradation during the phase of the reaction propagation. Under the experimental conditions specified under the paragraph "Study of uninhibited/inhibited HA degradation" (ii) the results represented in Figure 11 should be assessed as biphasic. As evident (compare Figure

11, curves coded 100 and 200), addition of the arbutin solution after 1 hr of the reaction onset, immediately increased the degradation rate of HA macromolecules. This process however continued by a stepwise slowing down of the solution dynamic viscosity with the final values 8.43 and 7.33 MPa·s, proving the inhibitory action of arbutin compared to uninhibited free-radical HA degradation represented by the curve coded 0 in Figure 11.

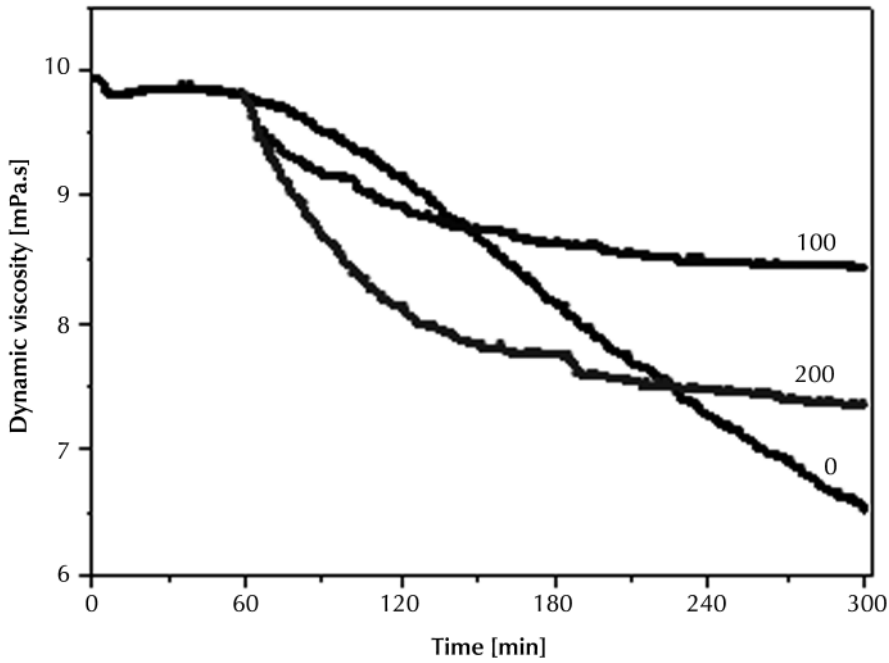


FIGURE 11 Time dependences of dynamic viscosity values of HA solution in the presence of 1 μM $CuCl_2$ plus 100 μM ascorbate (0) and after addition of arbutin in the concentration of 100 (100) and 200 (200) μM.

1.4 CONCLUSION

Based on the results (compare Figure 11), one may conclude that arbutin functions as a chain-breaking antioxidant. Yet it should be pointed out again that such a statement is valid only under the experimental conditions used. On applying the drug for skin lightening, one should take care since the pro-oxidative action of arbutin may result in damaging the tissues involved. The skin contents of HA, ascorbate, and the ubiquitous redox-active (transition) metals [18] including Cu ions predispose the processes documented by the observations.

KEYWORDS

- **ABTS assay**
- **Cyclic voltammetry**
- **EPR spectroscopy**
- **Hydroquinone derivatives**
- **Quinone**
- **Rotational viscometry**
- **Size exclusion chromatography**
- **Weissberger's oxidative system**

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