Food Chemistry 278 (2019) 692–699

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Dietary antioxidants as a source of hydrogen peroxide

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ARTICLE INFO

Keywords:
Antioxidant
Autoxidation
Ascorbic acid
Hydrogen peroxide
Polyphenols
Tea

Chemical compounds studied:
N-Acetylcysteine, CID: 12035
Aminoguanidine hydrochloride, CID: 2734687
Apigenin, CID: 5280443
L-Ascorbic acid, CID: 54670067
Betanin, CID: 54600918
2,6-di-*tert*-Butyl-4-methylphenol, CID: 66609
tert-Buthylhydroquinone, CID: 16043
Buthylhydroxyanizole, CID: 24667
Caffeic acid, CID: 689043
β-Carotene, CID: 5280489
(+)-Catechin, CID: 9064
Chlorogenic acid, CID: 1794427
Citric acid, CID: 311
p-Coumaric acid, CID: 637542
Curcumin, CID: 969516
L-cysteine, CID: 5862
Daidzein, CID: 5281708
(−)-Epicatechin, CID: 72276
(−)-Epicatechin gallate, CID: 107905
(−)-Epigallocatechin, CID: 72277
(−)-Epigallocatechin gallate, CID: 65064
Ethoxyquin, CID: 3293
trans-Ferulic acid, CID: 445858
Gallic acid, CID: 370
Genistein, CID: 5280961
Gentisic acid, CID: 3469
L-Glutathione, CID: 124886

ABSTRACT

Studies of 54 antioxidants revealed that 27 of them, mainly polyphenols, generated hydrogen peroxide (H_2O_2) when added to Dulbecco's modified Eagle's medium (DMEM), other media used for culture of mammalian and yeast cells and phosphate-buffered saline. The most active antioxidants were: propyl gallate (PG), (−)-epigallocatechin gallate (EGCG) and quercetin (Q). Chelex treatment and iron chelators decreased H_2O_2 generation suggesting that transition metal ions catalyze antioxidant autoxidation and H_2O_2 production. Green tea also generated H_2O_2; tea prepared on tap water generated significantly more H_2O_2 than tea prepared on deionized water. Ascorbic acid decreased H_2O_2 production although it generated H_2O_2 itself, in the absence of other additives. Lemon added to the tea significantly reduced generation of H_2O_2. Hydrogen peroxide generated in the medium contributed to the cytotoxicity of PG, EGCG and Q to human prostate carcinoma DU-145 cells, since catalase increased the survival of the cells subjected to these compounds *in vitro*.

Abbreviations: ABTS˙, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) radical; ARE, Antioxidant Response Element; BHA, butylated hydroxyanisole; C, (+)-catechin; DETAPA, diethylenetriaminepentaacetic acid; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulfoxide; EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechin gallate; H_2O_2, hydrogen peroxide; MEM, Eagle's Minimal Essential Medium; ESR, Electron Spin Resonance; NBT, Nitro Blue Tetrazolium; PBS, Phosphate-buffered saline; PG, Propyl Gallate; Q, quercetin; ROS, reactive oxygen species; SM, Yeast synthetic Minimal Medium; SOD, superoxide dismutase; YNB, Yeast Nitrogen Base; YPD, Yeast Peptone Dextrose; TAC, Total Antioxidant Capacity; TBHQ, *tert*-Butylhydroquinone; $O_2^{\cdot -}$, superoxide radical anion

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https://doi.org/10.1016/j.foodchem.2018.11.109
Received 6 September 2018; Received in revised form 19 November 2018; Accepted 22 November 2018
Available online 30 November 2018
0308-8146/

Glycitein, CID: 5317750
Hesperetin, CID: 72281
Hesperidin, CID: 10621
Hydrocinnamic acid (3-Phenylpropionic acid), CID: 107
D-Isoascorbic acid, CID: 54675810
Mangiferin, CID: 5281647
Melatonin, CID: 896
Metformin hydrochloride, CID: 14219
L-methionine, CID: 6137
Morin, CID: 5281670
Naringenin, CID: 932
Naringin, CID: 442428
Oxaloacetic acid, CID: 970
D-pantothenic acid hemicalcium, CID: 11306073
Propyl gallate, CID: 4947
Pyrogallol, CID: 1057
Pyruvic acid, CID: 1060
Quercetin, CID: 5280343
trans-Resveratrol, CID: 445154
Rutin, CID: 5280805
Sinapic acid, CID: 637775
Sodium ascorbate, CID: 23667548
Sodium succinate, CID: 9020
Trolox, CID: 40634
Vanillic acid, CID: 8468

1. Introduction

From a chemical point of view, an antioxidant can be defined as a substance which, when present at a low concentration with respect to that of an oxidizable substrate, prevents or significantly delays oxidation of this substrate (Halliwell, 1990). Although in biological systems several other mechanisms of action of antioxidants can be distinguished, including inhibition of oxidant-producing enzymes and chelation of metal ions catalyzing oxidation reactions, the main way of an antioxidant action is its sacrificial oxidation instead of the substrate. Such a reaction generates an oxidized form of the antioxidant compound and other reaction product(s). Under aerobic conditions, antioxidants are subject to oxidation by oxygen or reactive oxygen species (ROS), and this reaction may protect other substrates from oxidation. Other products of the reaction of antioxidant oxidation are reduced forms of oxygen: superoxide radical anion ($O_2^{\cdot -}$) in the case of one-electron oxidation or hydrogen peroxide (H_2O_2) in the case of two-electron oxidation. Eventually, dismutation of superoxide produces H_2O_2 so this product of oxygen reduction can be expected to accumulate as a result of oxidation of antioxidant compounds.

Generation of H_2O_2 due to oxidation of phenolic compounds, including gallic acid (Wee, Long, Whiteman, & Halliwell, 2003), (−)-epigallocatechin (EGC), (−)-epigallocatechin gallate (EGCG), (+)-catechin (C), and quercetin (Q) (Long, Clement, & Halliwell, 2000; Halliwell, Clement, Ramalingam, & Long, 2000), hydroxytyrosol, delphinidin and rosmarinic acid (Long, Hoi, & Halliwell, 2010), ascorbic acid (Wee et al., 2003) as well as thiol compounds (cysteine, glutathione, N-acetylcysteine, gamma-glutamylcysteine, cysteinylglycine, cysteamine, homocysteine) in commonly used cell culture media, especially Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 Medium and Eagle's Minimal Essential Medium (MEM) (Hua Long & Halliwell, 2001) has been documented. Green and black tea, coffee and red wine, beverages rich in catechins, also produce H_2O_2 when added to cell culture media (Chai, Long, & Halliwell, 2003; Akagawa, Shigemitsu, & Suyama, 2003; Long, Lan, Hsuan, & Halliwell, 1999). Tea and coffee but not cocoa were shown to generate H_2O_2 to achieve levels of over 100 μM. Milk decreased net H_2O_2 production by beverages and showed some ability to remove H_2O_2 itself (Long et al., 1999). The production of H_2O_2 in these fluids was in good agreement with the content of phenolic compounds, suggesting that polyphenols are responsible for the generation of H_2O_2 in beverages (Akagawa et al., 2003).

Generation of H_2O_2 *in vivo* due to oxidation of antioxidant compounds has also been demonstrated. Holding green tea solution in the mouth or chewing green tea produces micromolar concentrations of H_2O_2 in the mouth (Lambert, Kwon, Hong, & Yang, 2007). Higher levels of H_2O_2 were found in urine of coffee drinkers and attributed to excretion of hydroxyhydroquinone from coffee and its oxidation in urine, resulting in H_2O_2 production (Hiramoto, Kida, & Kikugawa, 2002; Halliwell, Long, Yee, Lim, & Kelly, 2004). The H_2O_2 generated by autoxidation of antioxidants may not only introduce artefacts in cell culture experiments (Halliwell et al., 2000), but also contribute to bactericidal action and to paradoxical genotoxic and mutagenic activities of these substances, generally assumed to have beneficial effects on human health (Lluís et al., 2011; Gomes et al., 2018).

In spite of literature reports, the generation of H_2O_2 in culture media is still an underappreciated problem in model studies of biological effects of food components and antioxidants. In many cases it is unclear if the effects observed *in vitro*, contributed by H_2O_2 generated by antioxidant autoxidation, are relevant to *in vivo* conditions where this autoxidation is absent or strongly attenuated. Moreover, data on autoxidation and H_2O_2 production are available only for a limited number of antioxidants and data on a broader spectrum of antioxidants are lacking. Similarly, our knowledge of the H_2O_2 in food, beverages and our body due to autoxidation of food components is still limited. The aim of this study was to compare the propensity of over 50 of commonly used antioxidants, especially of natural origin (present in food and beverages) for autoxidation and generation of H_2O_2, and to get an insight into the mechanism of this effect.

2. Materials and methods

2.1. Materials

BD™ Difco™ Yeast Nitrogen Base and Bacto™ Peptone were purchased from Becton Dickinson Poland (Warsaw), D-(+)-glucose and Xylenol Orange were obtained from Polish Chemical Reagents (POCh, Gliwice, Poland), perchloric acid ($HClO_4$) was purchased from Chempur (Piekary Śląskie, Poland), phosphate-buffered saline (PBS) and dimethyl sulfoxide (DMSO) were obtained from Lab Empire (Rzeszów,

Poland) and Dulbecco's Modified Eagle's Medium (DMEM), DMEM + GlutaMax Medium and DMEM/F12 Medium were purchased from Thermo Fisher Scientific (Warsaw, Poland). Nitro Blue Tetrazolium (NBT) was obtained from BioShop Canada Inc. (Burlington, Ontario, Canada). Stock solutions of NBT was freshly prepared in PBS before each experiment. Distilled water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Selected antioxidants and all other reagents, if not mentioned otherwise, were purchased from Sigma (Poznan, Poland) and were of analytical grade.

Yeast Peptone Dextrose (YPD) medium had the following composition: Yeast Extract 1%, Bactopeptone 1%, glucose 2% in deionized water. Yeast synthetic minimal medium (SM) contained 0.67% Yeast Nitrogen Base and 2% glucose in deionized water. The media components were sterilized by autoclaving before use (glucose solution separately) and complete media were made by mixing of sterilized components.

Fluorometric and absorptiometric measurements were done in a Spark multimode microplate reader (Tecan Group Ltd., Männedorf, Switzerland). All measurements were performed in triplicate and repeated at least three times on different preparations. The compounds studies were dissolved in PBS or in DMSO/PBS (ECG, EC, C) (0.5 ml of 10 mM antioxidant solution was obtained by dissolving an antioxidant in 10 µl DMSO and then 490 µl PBS was added). Stock solutions of less water-soluble compounds were prepared in DMSO. The effect of DMSO was subtracted from the effects of antioxidants introduced in this solvent. Dimethyl sulfoxide at the concentration used did not generate detectable amounts of H_2O_2.

2.2. Assay of hydrogen peroxide generation

Standard protocol for evaluation of H_2O_2 generation by antioxidants consisted in addition of 18 µl of 10 mM antioxidant to 162 µl of DMEM or other media. The samples were incubated for various times (in the kinetic studies), and routinely for 3 h at 37 ± 1 °C with shaking and the peroxide content was estimated before and after incubation by the ferric-Xylenol Orange method (Gay & Gebicki, 2003). Catalase (10 µg/ml) was added to additive set of samples 15 min before end of incubation in order to check if the reaction product is H_2O_2. Then, to 180 µl samples, 20 µl of Xylenol Orange reagent was added [2.5 mM Xylenol Orange/2.5 mM Mohr's salt ($Fe_2(NH_4)_2SO_4$; purity of 99.997%) in 1.1 M perchloric acid]. After 30-min incubation at room temperature, absorbance of the samples was measured at 560 nm.

When estimating H_2O_2 production in the tea, one bag of green tea (Le speciale) purchased in a local food store was added with 200 ml of boiling water (deionized or tap water) and incubated for 3 h, without or in the presence of a slice of lemon (11.2 g).

2.3. Semiquinone detection

The semiquinone radical can be detected and quantified by electron paramagnetic resonance (EPR) spectrometry. For detection of semiquinone radical formation, 100 mM solutions of propyl gallate (PG) and EGCG were prepared in PBS containing 0.2 M zinc sulfate (to stabilize the semiquinone radicals) (Metodiewa, Jaiswal, Cenas, Dickancaité, & Segura-Aguilar, 1999). The Bruker multifrequency and multiresonance FT-EPR ELEXSYS E580 spectrometer (Bruker Analytische Messtechnik, Rheinstetten, Germany) operating at the X-band (9.837530 GHz). The following settings were used: central field, 3505.6 G; modulation amplitude, 1 G; modulation frequency, 100 kHz; microwave power, 94.64 mW; power attenuation, 2.0 dB; scan range, 80 G; conversion time, 25 ms; and sweep time, 25.6 s. The spectra were recorded in 1024 channels; number of scans: 15.

2.4. Detection of superoxide formation

Superoxide formation during polyphenol autoxidation was assesses by superoxide dismutase (SOD)-inhibitable reduction of NBT and SOD-inhibitable oxidation of dihydroethidine. 1 mM PG, EGCG and Q were incubated in PBS in the presence of 100 µM NBT or 5 µM dihydroethidine, in the absence or in the presence of 10 µg/ml Cu,Zn-superoxide dismutase. Absorbance of formazan formed by NBT reduction was measured at 540 nm. Fluorescence of the reaction products of dihydroethidine oxidation was measured at the excitation/emission wavelengths of 405/570 nm, to maximize the fluorescence share of 2-hydroxyethidium (Nazarewicz, Bikineyeva, & Dikalov, 2013).

2.5. $ABTS^{\cdot}$ scavenging assay

Total antioxidant capacity (TAC) of the media was determined by reduction of the 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) radical ($ABTS^{\cdot}$) (Grzesik, Naparło, Bartosz, & Sadowska-Bartosz, 2018), slightly modified with respect to the time of measurement. Briefly, 5-µl aliquots of the media were added to wells of a 96-well plate containing 245 µl of $ABTS^{\cdot}$ solution having absorbance of 1.00 at 734 nm. Absorbance decrease was measured after 1 and 30 min. The decrease in absorbance after 1 min was assumed to reflect the "*fast scavenging*" of $ABTS^{\cdot}$ while the decrease in absorbance between 1 min and 30 min to reflect "*slow scavenging*". Standard curve obtained for the scavenging of Trolox enabled to express the $ABTS^{\cdot}$ scavenging activity in Trolox equivalents.

2.6. Scavenging of superoxide and hydrogen peroxide by the media

Scavenging of superoxide by the media was estimated by a modified method of Minami and Yoshikawa (1979). The assay system consisted of 167 µM pyrogallol, 50 mM Tris-HCl buffer containing 100 µM diethylenetriaminepentaacetic acid (DETAPA) and various volumes of the media in a final volume of 300 µl. Absorbance was measured at 540 nm every minute in a plate reader for 10 min. One unit of superoxide scavenging activity was defined as the amount of the medium decreasing the rate of NBT reduction (0.005/min in the control) by 50%.

To estimate scavenging of H_2O_2 by the media, H_2O_2 was added to the media to the final concentration of 30 µM. The peroxide concentration was estimated immediately and after 3 h incubation at 37 °C in the dark (unless stated otherwise), according to Gay and Gebicki (2003).

2.7. Fe^{2+}-binding capacity of the media

Fe^{2+}-binding capacity of the media was estimated by their competition for Fe^{2+} with ferrozine. Briefly, various amounts of the media were added to a solution containing 60 µM (final concentration) $Fe_2(NH_4)_2SO_4$ (Mohr's salt) in 20 mM sodium phosphate buffer, pH 7.0. Then ferrozine was added to a concentration of 200 µM, the solution was mixed and after 3 min absorbance of the solution was measured at 562 nm. From the dependence of absorbance of the volume of the medium, the medium volume decreasing absorbance of ferrozine to 50% of a value obtained without medium added. For some media, this method did not work due to interference with absorbance of the medium.

2.8. Assay of cytotoxicity of H_2O_2-generating antioxidants

Human prostate carcinoma DU-145 cells (ATCC® HTB-81™) were seeded into wells of a 96-well plate and cultivated in DMEM-F12 HAM (Sigma, St. Louis, MO) medium supplemented with 10% fetal bovine serum (FBS) and 100 IU/ml penicillin and 10 µg/ml streptomycin in humidified atmosphere with 5% CO_2 at 37 °C for 24 h. Then, the medium was removed and replaced with a medium containing PG, EGCG or Q at various concentration, without or with 10 µg/ml catalase. The cells were cultivated for 24 h, then the medium was removed and replaced by 100 µl/well of a 2% solution of Neutral Red in the medium.

The cells were kept in an incubator for 1 h, washed twice with PBS, added with 100 µl/well of 50% ethanol/49% H_2O/1% glacial acetic acid and shaken for 15 min. For Q, the control contained 0.2% DMSO (concentration identical to that introduced with Q at the maximal concentration). Such a concentration of DMSO did not have any discernible effect on cell survival. Then, absorbance in the wells was measured at 540 nm vs 620 nm. All experiments were done in triplicate on different preparations.

2.9. Statistical analysis

The data are presented as mean ± standard deviation. The significance of differences of the examined parameters among samples was checked using a two-tailed Student's t test. Kruskal-Wallis test was performed to estimate differences between antioxidant treated and non-treated cells. Differences between antioxidant and antioxidant with catalase treated cells were tested using the U Mann-Whitney test. $P \leq 0.05$ was considered as statistically significant in both cases. Every test was performed in triplicates.

Statistical analysis of the data was performed using STATISTICA software package (version 13.1, StatSoft Inc. 2016, Tulsa, OK, USA, www.statsoft.com).

3. Results

Incubation of 54 various antioxidants in DMEM medium (37 °C, 3 h) revealed generation of a product reactive in the peroxide assay by 27 antioxidants, mainly natural food components. No generation of H_2O_2 was detected for other 27 antioxidants (Table 1). The kinetics of peroxide generation was not linear; the highest rate was observed in the initial phase of incubation; then the rate of generation decreased gradually and the H_2O_2 level reached practically a plateau (Fig. 1), probably as a result of equilibrium between the rate of generation and decomposition (see below). Since the kinetics differed for different compounds we decided to present data for 3 h when reaching a plateau level was probable for all compounds studied. Incubation of the reaction mixture with catalase (10 µg/ml) for 15 min resulted in the disappearance of this product demonstrating that the product is H_2O_2 (not shown). PG, EGCG and Q were the most active antioxidants generating H_2O_2 in the DMEM media; therefore, these substances were used in further comparative studies. Ascorbic acid and sodium ascorbate generated substantial amounts of H_2O_2; no H_2O_2 was generated, however, when not neutralized solution of ascorbic acid was added to the medium, decreasing the final pH.

Autoxidation of antioxidants produced H_2O_2 also in other media used for culture of mammalian cells (DMEM + GlutaMAX, DMEM/F12) and yeast cells (YPD and SM media) as well as in PBS, as demonstrated for three antioxidants most active in the DMEM medium. Much less peroxide was generated in media used for yeast culture (Table 2). The polyphenols studied produced in PBS amounts of H_2O_2 comparable to those DMEM media, so PBS was mainly used in further experiments. Inclusion of 10% fetal calf serum (FCS) to the media to imitate fully cell culture conditions increased the generation of H_2O_2 (Table S1).

In order to check the effects of possible interactions of antioxidants in the autoxidation process, we checked the effect of ascorbate on the H_2O_2 production by the three most active polyphenol antioxidants. Increasing concentrations of ascorbate decreased the production of H_2O_2, to zero for PG and EGCG, and to about 1/3 at 1 mM ascorbate for Q (Fig. 2). Ascorbic acid (neutralized to pH 7.0) or sodium ascorbate generated significant amounts of H_2O_2, but aqueous solution of ascorbic acid without neutralization did not generate detectable amounts of hydrogen peroxide.

Light did not accelerate the autoxidation of polyphenols; production of H_2O_2 by PG, EGCG and Q incubated in the DMEM medium amounted to 139 ± 11, 96 ± 12 and 123 ± 9%, respectively, of that observed upon incubation in a daylight.

Removal of the transition metal ions from PBS by Chelex treatment before incubation, as well as the presence of iron chelators, desferoxamine (DFO) and DETAPA decreased H_2O_2 production (Table S2).

Upon autoxidation of PG and EGCG, semiquinone radicals of these substances were detected by electron spin resonance (ESR) (Fig. S1). Formation of superoxide radicals was demonstrated by reduction of NBT and oxidation of dihydroethidine upon autoxidation of EGCG and PG, partly inhibited by SOD (Fig. S2). Measurement for NBT reduction accompanying Q autoxidation was not reliable, because of change of absorbance of the solution absorbing at 540 nm.

In order to get an insight into the factors responsible for differences in the amount of H_2O_2 generated in various media, TAC of the media, their capacity of iron binding, as well as scavenging of superoxide and scavenging of H_2O_2 was studied. Total "fast" antioxidant capacity of the YPD medium was higher in comparison to the DMEM media but that of the SM medium was significantly lower. The "slow" scavenging capacity of the medium was lower than those of the DMEM media and higher than that of the SM medium. Fe^{2+} binding was comparable for the DMEM and YPD media, nevertheless it was also significantly lower for the SM medium (Table S3).

DMEM showed the highest superoxide scavenging activity, while the superoxide scavenging activity of the YPD medium was the lowest. Interestingly, the media, except for the SM medium, decomposed added H_2O_2, the activity being comparable for all the media studied. None of the parameters studied could be correlated with the differences in the H_2O_2 generation in different media, and especially with the low H_2O_2 production in the media used for yeast culture. Another factor which could influence antioxidant autoxidation and H_2O_2 generation in

Table 1
Generation of hydrogen peroxide in DMEM medium [(mean ± SD; n ≥ 3 (independent samples)]. The following substances did not produce detectable amounts of H_2O_2: *N*-acetylcysteine, apigenin, betanin, *tert*-buthylhydroquinone, 2,6-di-*tert*-butyl-4-methylphenol, buthylhydroxyanizole, β-carotene, citric acid, p-coumaric acid, curcumin, L-cysteine, daidzein, ferulic acid, glutathione, glycitein, hesperetin, melatonin, metformin, morin, naringenin, naringin, oxaloacetic acid, D-panthotenic acid, pyruvic acid, sodium succinate, Trolox, vanillic acid.

Compound	H_2O_2 [µM]
Propyl gallate (PG)	95.2 ± 1.9***
Pyrogallol	94.5 ± 1.7***
(−)-Epigallocatechin gallate (EGCG)	90.2 ± 2.6***
Quercetin (Q)	76.4 ± 5.9**
(−)-Epicatechin	44.9 ± 2.2***
Isoascorbic acid	38.6 ± 1.3***
Ascorbic acid#	33.6 ± 0.9***
Sodium ascorbate	28.4 ± 5.1**
Kaempferol	23.9 ± 1.4***
Mangiferin	21.7 ± 0.7***
(−)-Epigallocatechin (EGC)	21.2 ± 3.0**
Caffeic acid	19.9 ± 0.5***
Resveratrol	13.7 ± 0.5***
Gentisic acid	14.7 ± 0.4***
Chlorogenic acid	11.3 ± 2.2**
(−)-Epicatechin gallate	11.0 ± 2.7**
Hydrocinnamic acid	7.1 ± 3.6*
Sinapic acid	7.0 ± 3.0*
Methionine	6.1 ± 0.9**
Gallic acid	5.2 ± 0.7**
Rutin	4.3 ± 2.0*
Genistein	3.3 ± 1.1*
Hesperidin	2.7 ± 0.3**
(+)-Catechin	2.6 ± 0.8*
Ethoxyquin	2.2 ± 0.3**
Aminoguanidine	1.6 ± 0.5*
p-Coumaric acid	0.6 ± 0.2*

#No H_2O_2 was generated when not neutralized ascorbic acid was used. Generation significantly different from 0: *P < 0.05, **P < 0.01, ***P < 0.001 (paired Student's "*t*" test).

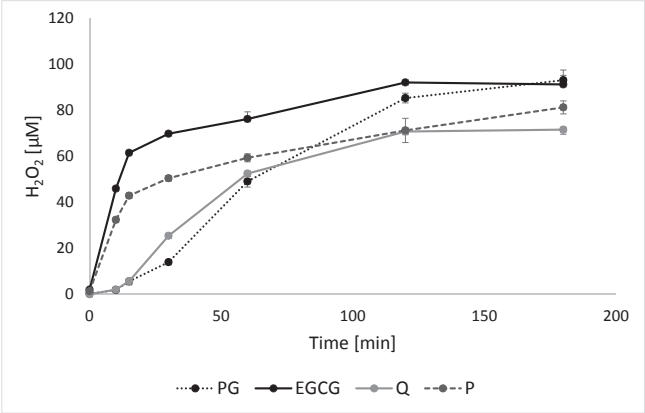

Fig. 1. Kinetics of hydrogen peroxide generation in DMEM medium by 1 mM compounds (propyl gallate, PG, epigallocatechin gallate, EGCG, quercetin, Q and pyrogallol P).

various media is their pH which, was lower for yeast culture media (YPD: 5.85, SM: 5.92) than for media used for mammalian cell culture (DMEM: 7.42, DMEM/F12: 7.25, DMEM + Glutamax: 7.33).

Since ECGC and other flavanols are present in high amounts in the tea, we studied H_2O_2 production in freshly prepared tea. Tea prepared on tap water generated much more H_2O_2 than that prepared on Milli-Q-filtered water. Ascorbic acid induced a concentration-dependent decrease in H_2O_2 production in tea prepared on deionized water. With tea prepared on tap water, low concentrations of ascorbic acid slightly increased H_2O_2 production, but 1 mM ascorbic acid decreased it. Addition of lemon, known to be rich in ascorbic acid, significantly decreased H_2O_2 production in the tea (Fig. 3).

In order to examine the effect of H_2O_2 generated by polyphenols in cell culture medium on cells, we compared the cytotoxicity of PG, EGCG and Q to cultured DU-145 cells. In all cases the survival of cells subjected to increasing concentrations of these compounds was significantly higher in the presence of catalase, a H_2O_2-decomposing enzyme (Fig. 4).

4. Discussion

Production of H_2O_2 due to autoxidation of some antioxidants, especially polyphenols, in cell culture media have been reported previously (Halliwell et al., 2000; Long et al., 2000, 2010) and regarded as one of the mechanisms of prooxidant effects of polyphenols (Kim, Quon, & Kim, 2014). The aim of this study was to extend the check of H_2O_2 production on a range of antioxidants studied by us within a framework of a project on, "*Antioxidant Power Series as a tool for rational design and assessment of health promoting properties of functional food based on antioxidant phytochemicals*". Results of this study indicate that, although antioxidants of various groups, including ascorbic acid, can produce H_2O_2, this feature is typical, first of all, for polyphenols. Interestingly, ethoxyquin, approved in some countries as a food and feed additive, generated relatively small but significant amounts of H_2O_2.

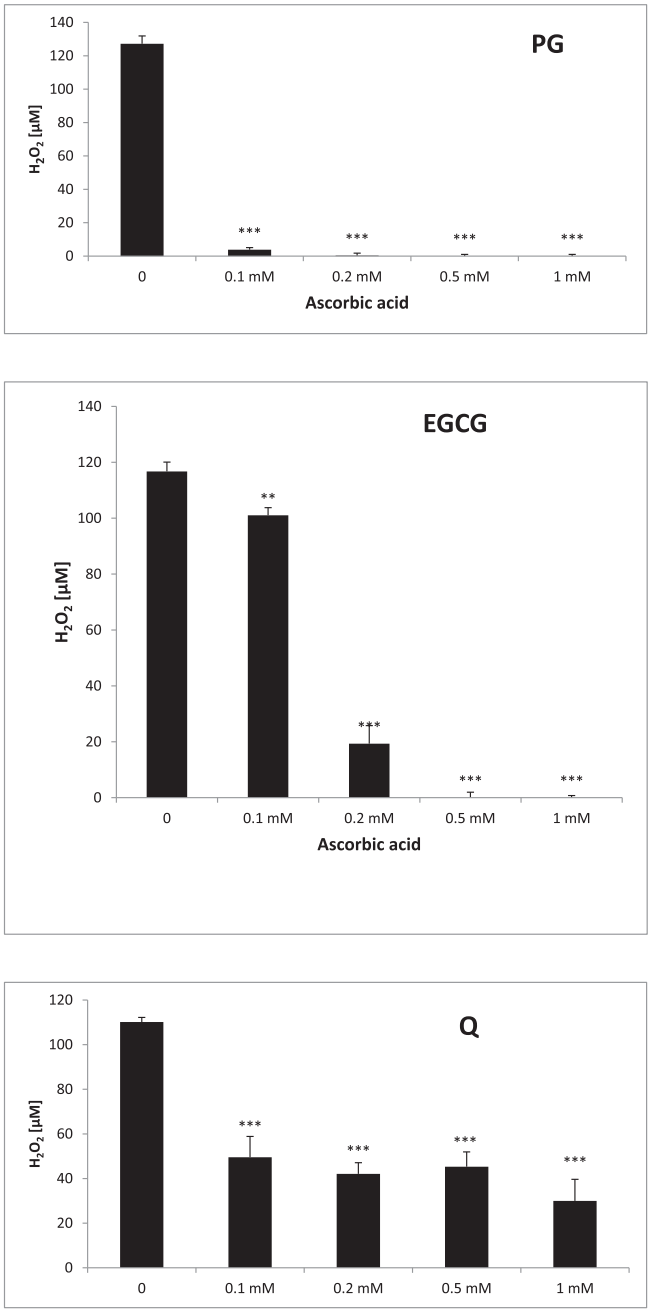

Fig. 2. Effect of ascorbate on the hydrogen peroxide production by autoxidation of PG, EGCG and Q in PBS. $^{**}P < 0.01$, $^{***}P < 0.001$ (with respect to PBS without ascorbic acid).

Table 2
Production of hydrogen peroxide in various media.

Compound	H_2O_2 [μM]				
	DMEM/F12	DMEM + Glutamax	YPG	SM	PBS
PG	90.4 ± 7.1	113.9 ± 7.5*	11.8 ± 0.9***	3.1 ± 0.2***	127.2 ± 4.7**
EGCG	140.9 ± 7.8**	157.9 ± 8.8**	0$^{**\#}$	0$^{**\#}$	116.7 ± 3.3**
Q	46.2 ± 9.7	41.8 ± 3.4	0$^{**\#}$	1.4 ± 1.2**	110.2 ± 2.0***

YPG medium: 0.5% Yeast nitrogen base (YNB), 1% yeast extract, 2% glucose: SM: 0.5% YNB, 2% glucose; PBS: phosphate-buffered saline; $^{\#}$ difference in absorbance between final and initial assay < 0. Peroxide generation significantly different from that in DMEM: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (paired Student's "*t*" test).

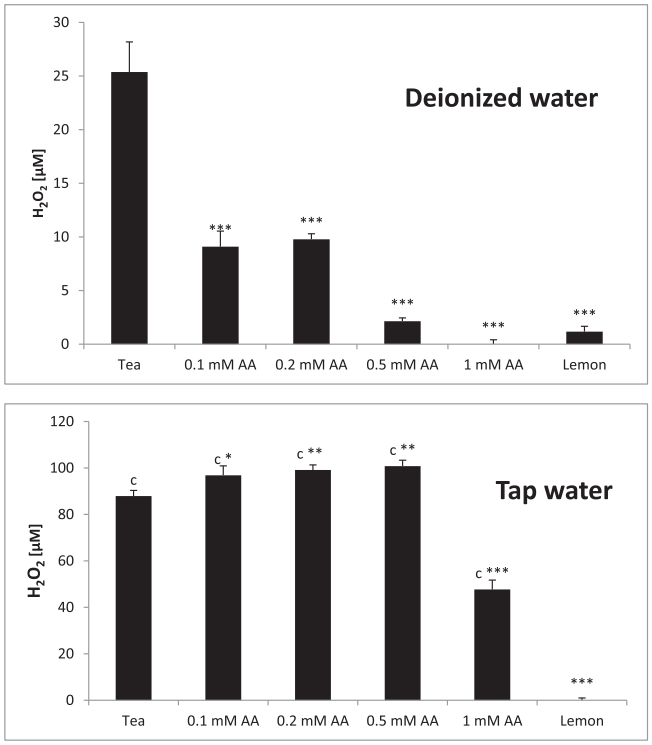

Fig. 3. Effect of ascorbic acid (AA) and lemon on generation of hydrogen peroxide in tea prepared on distilled and tap water. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ with respect to tea without additives; $^{c}P < 0.001$ with respect to analogous samples in deionized water.

The cell culture media containing FBS generated more H_2O_2 than media without FBS (Table S1). We hypothesize that this effect may be due to release of iron from iron-binding proteins (mainly transferrin), perhaps during freezing/thawing of the serum. This question deserves further study.

No correlation between the structure of the compounds studied and generation of H_2O_2 was apparent. As transition metal ions seem to catalyze the autoxidation of antioxidants (see below) the differences between different compounds observed may be affected by such a simple factor as the level of transition ion contamination of the commercial preparations. pH of the media may be another important factor, as the rate of autoxidation of many compounds increases with increasing pH. The case of ascorbic acid is especially instructive: addition of ascorbic acid from a not neutralized 10 mM stock solution did not induce generation of detectable amounts of H_2O_2, but addition of a solution neutralized to pH 7.0 or of sodium ascorbate resulted in production of significant amounts of H_2O_2. This factor could account for the lower rate of H_2O_2 generation in media used for yeast cell culture.

The most active antioxidants in our study were: PG, pyrogallol, EGCG and Q. The antioxidant compounds generated H_2O_2 in DMEM cell culture media and also in media used for yeast cell culture albeit at much lower yields, if any (pyrogallol was not studied in this respect since it is not generally employed as an antioxidant).

We have reported light-dependent production of ROS, which could contribute to the process of antioxidant autoxidation, in cell culture media (Grzelak, Rychlik, & Bartosz, 2001). However, this mechanism does not contribute to autoxidation of exogenous antioxidants since incubation in the darkness did not attenuate H_2O_2 formation by these compounds. H_2O_2 formation was comparable in PBS and in DMEM cell culture media, further suggesting that production of H_2O_2 by antioxidants is not due to interaction of these compounds with organic components of cell culture media, but rather to their autoxidation. Transition metal (presumably mainly iron) ions seem to catalyze this reaction as demonstrated by decrease in H_2O_2 production induced by pretreatment of PBS with Chelex X-100 and addition of iron chelators (DFO and DETAPA). Dulbecco's Modified Eagle's Medium contain ferric nitrate (0.1 mg/l) while DMEM/F12 and DMEM + GlutaMAX media contain 0.0012 mg/l cupric sulfate, 0.05 mg/l ferric nitrate and 0.417 mg/l ferric sulfate. Yeast Peptone Dextrose medium apparently contains iron ions but they may be tightly bound to other components of this medium. Yeast nitrogen base medium contains 0.04 mg copper sulfate, 0.2 mg ferric chloride and 0.4 mg manganese sulfate/l. Furthermore, all media may contain transition metal ions as trace contaminants of other components used to prepare the media. These ions may interact with polyphenols, catalyze their autoxidation and participate in the formation of H_2O_2. Probably the differences in the concentrations of transition metals, able to catalyze polyphenol autoxidation, are the main factors determining the rate of H_2O_2 generation due to autoxidation of polyphenols. Other parameters of the media studied (TAC, ability of Fe^{2+} binding, superoxide scavenging activity and hydrogen peroxide scavenging activity) did not correlate with the H_2O_2 generation. It is worthwhile to note that the cell culture media are able to scavenge H_2O_2 produced. This phenomenon may lead to underestimation of the amount of H_2O_2 formed by exogenous substances added to the media. Pyruvate was identified as one compound scavenging H_2O_2 (Long & Halliwell, 2009) and protecting cultured cells against the cytotoxic action of hydrogen peroxide (Rodemeister & Hill, 2017).

Polyphenol autoxidation is apparently a two-step reaction. First, polyphenol is oxidized to a semiquinone free radical $HQ^{\cdot}$ in a reaction coupled to reduction of molecular oxygen to the superoxide anion radical $O_2^{\cdot -}$ and then semiquinone is oxidized to quinone Q producing second superoxide radical:

$$H_2Q + O_2 \rightarrow HQ^{\cdot} + O_2^{\cdot -} + H^+$$

$$HQ^{\cdot} + O_2 \rightarrow Q + O_2^{\cdot -} + H^+$$

Semiquinone radicals can have extremely long half-lives (up to days at 37 °C) and tend to be neither reactive nor toxic. Problems can arise because of their propensity to donate the excess electron to molecular oxygen, thereby generating superoxide. The semiquinone radical is more susceptible to oxidation with O_2 than fully reduced catechins (Mochizuki, Yamazaki, Kano, & Ikeda, 2002). Dismutation of superoxide radical produces H_2O_2:

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$

Autoxidation of polyphenols is pH-dependent and is accelerated by alkaline pH (Mochizuki et al., 2002). In agreement with this postulated mechanism, formation of $O_2^{\cdot -}$ during autoxidation of Q at alkaline pH has been demonstrated and used for determination of activity of superoxide dismutase (Kostyuk & Potapovich, 1989). As pH is an important factor in the polyphenol autoxidation, it should be expected that their autoxidation can be quite high in the intestine.

Autoxidation of polyphenols produces H_2O_2 in the tea, as reported previously (Long et al., 1999). Much higher H_2O_2 production was observed in tea prepared on tap water, containing transition metal ions, than in tea prepared on deionized water. It further suggests the role of contaminating transition metal ions as catalysts of polyphenol autoxidation. Interestingly, while ascorbate decreased H_2O_2 formation in cell culture medium, in PBS (prepared on deionized water) and in tea prepared on deionized water, low ascorbate concentrations augmented H_2O_2 production in tea prepared on tap water, containing transition metal ions. It has been reported that mixtures of ascorbate and phenolic compounds led to less H_2O_2 generation than it would be expected from the rates observed with ascorbate or phenolic compounds alone; this effect was ascribed to reduction of semiquinone radicals by ascorbate (Wee et al., 2003). Taking into account higher propensity for transition metal binding of polyphenols, compared to ascorbate (Perron & Brumaghim, 2009; Tamilmani & Pandey, 2016), such a transition requires higher concentrations of these ions as those required to catalyze

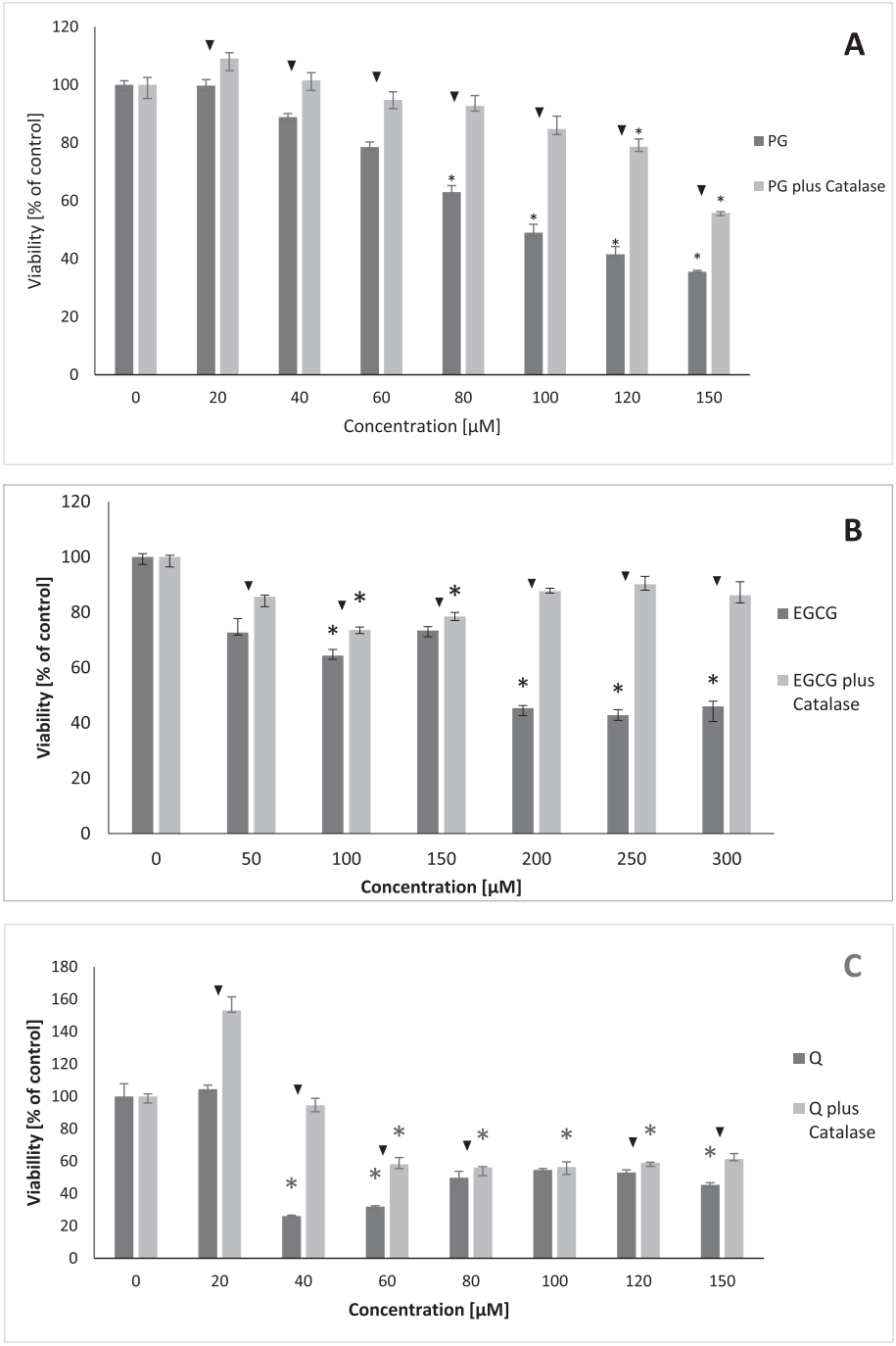

Fig. 4. Cytotoxicity of PG (A), EGCG (B) and Q (C) toward DU-145 cells after 24 h treatment in the absence and in the presence of catalase. *p ≤ 0.05 Kruskal–Wallis test against non-treated control. ▼ p ≤ 0.05 U-Mann-Whitney test (samples without vs in the presence of 10 µg/ml catalase). The whiskers are lower (25%) and upper (75%) quartile ranges.

polyphenol oxidation.

A practical conclusion from the study is that although tea generates and contains hydrogen peroxide, addition of lemon prevents H_2O_2 formation in the tea, an effect apparently due to reduction of semi-quinone radicals by and lowering of pH.

Hydrogen peroxide generated by PG, EGCG and Q contributes to the cytotoxicity of these compounds as the presence of catalase in the medium increased the survival of cells. The contribution of H_2O_2-mediated cytotoxicity is an artefact of cell culture conditions ex-aggerating the cytotoxicity of cells with respect to the *in vivo* conditions, where significant autoxidation of the compound studies does not seem to take place, due both to the lower oxygen level and to the presence of many reducing compounds. Interestingly, the cytotoxic and clastogenic action of EGCG on Chinese Hamster Ovary (CHO) cells correlated with the rate of H_2O_2 generation by EGCG in these media (Long, Kirkland, Whitwell, & Halliwell, 2007) while cytotoxicity of ascorbate is the higher, the higher is its autoxidation with concomitant H_2O_2 generation in various media (Clément, Ramalingam, Long, & Halliwell, 2001; Halliwell, 2018). Moreover, as H_2O_2 is involved in many intracellular signaling pathways (Rhee, 2006), activating, i. a., the redox-sensitive antioxidant response element (ARE) (Ho, Siu-wai, Siu, & Benzie, 2013), its penetration from the cell culture medium may alter cell behavior under *in vitro* conditions, though not necessarily *in vivo*.

5. Conclusions

A number of natural antioxidant compounds, mainly polyphenols, produce H_2O_2 upon autoxidation. This phenomenon may induce artefacts in cell culture experiments employing polyphenols as H_2O_2 produced in vitro, but most probably not *in vivo*, contributes to *in vitro* cytotoxicity of antioxidants and may affect cellular signaling. Autoxidation of polyphenols present in the tea also produces H_2O_2 in the tea which is diminished by lemon.

Authors' contributions

I. S.-B. was responsible for the concept of the study, design of experiments and supervision of experimental work, performed part of experiments as well as had a leading role in the analysis of the results and preparation of the manuscript. M. G. performed part of experiments in cell-free systems and their statistical evaluation as well as contributed reagents/materials/analysis tools. She further contributed to data acquisition and interpretation as well as wrote parts of the manuscript. I. S. carried out EPR measurements and interpreted the data. M. P. performed experiments in the cellular system and their statistical evaluation. G. B. critically discussed the research goals and participated in the revision and improvement of the manuscript. J. N. was responsible for providing the funding for the study. All authors have approved the final manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgements

This study was performed within the project, "Antioxidant Power Series as a tool for rational design and assessment of health promoting properties of functional food based on antioxidant phytochemicals" (2014/14/A/ST4/00640) financed by National Science Centre, Poland within a programme, MAESTRO 6 and research fund of the University of Rzeszów (Grant WBR/ZBA/PB/1/2017). We are grateful to Ms. Natalia Pienkowska (University of Rzeszów), M.Sc., for her excellent technical help.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2018.11.109.

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