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Is Hydrogen Peroxide Generated in Infusions of Medicinal Herbs?

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Abstract: Hydrogen peroxide is an important intra- and inter-cellular messenger. It has been reported that beverages such as tea and coffee generate H_2O_2. The aim of this study was to examine whether H_2O_2 is formed in infusions of medicinal herbs and to explain the mechanism of its formation. Infusions of sixteen herbs were studied and the presence of H_2O_2 was found in all cases. The highest concentrations of H_2O_2, exceeding 50 μM, were found in the infusions of leaves of *Betula* and *Polygonum*, as well as of the inflorescence of *Tilia*. Considerable amounts of diverse phenolic compounds were detected in the infusions. Two successive one-electron oxidations of phenolics are the main source of H_2O_2 in these infusions; as expected, the generation of semiquinone and superoxide radicals was confirmed. The infusions of medicinal herbs were also able to scavenge H_2O_2. The herbal infusions were cytotoxic to human ovary cancer SKOV-3 and PEO1 cells; this cytotoxicity was compromised by catalase added to the growth medium, demonstrating the contribution of H_2O_2 to the cytotoxic action of herbal extracts.

Keywords: hydrogen peroxide; Xylenol Orange; catalase; semiquinone radical; cytotoxicity; SKOV-3; PEO1

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1. Introduction

Hydrogen peroxide (H_2O_2) is an oxidizing compound mainly used in industry for bleaching cotton, other textiles and wood pulp, as a rocket propellant and for cosmetic and medicinal purposes [1]. Hydrogen peroxide is a bactericidal and virucidal agent [2–4]. Solutions (1–6%) of H_2O_2 are used as a debriding agent for wound care [5]. In 1% formulations, H_2O_2 is used to treat acne; it is studied in concentrations of 25% to 40% to treat skin tumors and in concentrations of 40–45% to treat common warts [6]. Hydrogen peroxide is also used for dental whitening [7,8] and as a component of mouthwash [9]. However, H_2O_2 should be used with caution, as exposure to this compound can cause adverse effects due to its oxidant capabilities. Hydrogen peroxide solutions containing more than about 8 percent hydrogen peroxide are corrosive to the skin [6]. The accidental ingestion of 3% (i.e., ca 0.88 M) hydrogen peroxide may lead to gastric injury in children [10].

Hydrogen peroxide is a physiologically relevant compound; it is important in the defense against pathogenic microorganisms at micromolar up to low millimolar concentrations and participates in intra- and inter-cellular signaling at nanomolar up to low micromolar concentrations [11,12]. However, higher H_2O_2 concentrations are also cytotoxic to mammalian cells. The sensitivity of cells to hydrogen peroxide differs substantially,

depending mainly on the activity of catalase [13–15]. We found H_2O_2 concentrations half-inhibiting the proliferation of two fibroblast cell lines, which differed considerably in their catalase activity, to be 528 and 33.5 μM, respectively [15].

Interestingly, humans are permanently ingesting H_2O_2, from the first days of life, albeit in micromolar concentrations. Human milk contains H_2O_2; average H_2O_2 concentrations of 27 μM and 25 μM were reported in human milk collected 1–5 days post-partum [16] and in the first week after birth, respectively [17]. It has been convincingly demonstrated that commonly consumed beverages such as tea [18–21] and coffee [22–24] generate H_2O_2 upon brewing and subsequent standing. The hydrogen peroxide concentration in freshly brewed coffee was found to be 20–80 μM, depending on the type of coffee [23], while in green and black tea, it exceeded 200 μM and 700 μM after standing for 1 and 12 h, respectively [19]. We recently documented the generation of H_2O_2 in cooked vegetables [25,26]. Wines, especially red wines, generate H_2O_2 [27,28], although wine components may also react with this compound [28].

In addition to the most popular beverages from plants, infusions of medicinal herbs are also often consumed as they are recommended by traditional or complementary medicine or as tea substituents. To the best of our knowledge, there is no information on the presence of H_2O_2 in infusions of medicinal herbs. The aim of this study was to check whether H_2O_2 is generated in infusions of the chosen medicinal herbs, like in tea, and to investigate the mechanism of H_2O_2 formation in the infusions.

It has been reported that the cytotoxic effects of tea and wine in vitro were contributed by the hydrogen peroxide generated by them in the cell culture media [27]. The cytotoxicity of medicinal herbs has been also the subject of numerous in vitro studies, e.g., [29–33]. Another aim of this study was thus to examine whether H_2O_2 also contributes to the cytotoxicity of several herbal infusions to human cancer cells in vitro.

2. Materials and Methods

2.1. Materials and Equipment

Cell culture medium (McCoy's 5A (cat. no 22330-021)), RPMI + GlutaMAX (cat. no 72400-021), DMEM + GlutaMax (cat. no. 21885-025) and Dulbecco's Phosphate Buffered Saline (DPBS) (cat. no. 14040-117) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Fetal Bovine Serum (cat. no. S1813), Penicillin-Streptomycin solution (cat. no. L0022), Trypsin-EDTA solution (10×) (cat. no. X0930) and Phosphate-Buffered Saline without Ca^{2+} and Mg^{2+} (cat. no. P0750) were obtained from Biowest (Nuaillé, France). Ethanol (96%, cat. no. 396420113), glacial acetic acid (cat. no. 568760114) and Xylenol Orange were obtained from Avantor Performance Materials (Gliwice, Poland). Other reagents including catalase (cat. no. C40; ≥10,000 units/mg protein), superoxide dismutase (cat. no. S9697), Nitrotetrazolium Blue (NBT; cat. no. N6876) and dihydroethidium (cat. no. 309800) were purchased from Merck (Poznań, Poland).

Additionally, 75 cm^2 flasks (cat. no. 156499) were provided by ThermoFisher Scientific (Waltham, MA, USA). Transparent 96-well culture plates (cat. no 655180) were obtained from Greiner (Kremsmünster, Austria). Other sterile cell culture materials were provided by Nerbe (Winsen, Germany).

Human ovarian cancer cell lines (SKOV-3) and the human lung normal fibroblast cell line (MRC-5) were obtained from the American Type Culture Collection (ATCC). We also used a second ovarian cancer cell line (PEO1). purchased from the European Collection of Authenticated Cell Cultures (ECACC).

Spectrophotometric and fluorimetric measurements were conducted using a Spark multimode microplate reader (Tecan Group Ltd., Männedorf, Switzerland).

2.2. Plant Material

Sixteen different herbs were examined. Their basic characteristics and application in traditional and alternative medicine are given in the Supplementary material. The herbs of *Achillea millefolium*, *Artemisia absinthium*, *Cistus incanus*, *Hypericum perforatum* and

Polygonum aviculare; leaves of *Betula pendula*, *Taraxacum officinale*, *Melissa officinalis*, *Mentha piperica*, *Plantago lanceolata*, *Tussilago farfara* and *Urtica dioica*; inflorescence of *Tilia cordata*; and flowers of *Calendula officinalis* and *Lavandula angustifolia* were purchased from a local herbal store in Rzeszów, Poland. Data on their origin and time of collection were not available. Leaves of *Ginkgo biloba* were collected from a tree grown in the garden of the corresponding author in Rzeszow (podkarpackie region, Poland, in August 2022).

2.3. Preparation of Infusions

The material was crushed in a mortar and 250 mg of the dry material was treated with 25 mL of hot tap water to imitate the standard conditions of preparations of herbal infusions. Immediately and after standing for 1 and 3 h, aliquots of the infusions were withdrawn for the assay of hydrogen peroxide. For the detection of semiquinone radicals and production of superoxide, 10% infusions were prepared (100 mg herb + 900 µL of water). The infusions used for the assay of cytotoxicity were prepared by treating 250 mg of the herbs with 25 mL of boiling phosphate-buffered saline (to provide isotonicity with the cell culture medium). After 30 min incubation, the infusions were centrifuged and sterilized using a syringe filter (0.22 µM).

2.4. Determination of Hydrogen Peroxide

Hydrogen peroxide was estimated using Xylenol Orange [34]. Although the results obtained using this method are generally assumed to reflect the actual level of peroxides, our experience points to the possibility of artifacts when analyzing complex mixtures. Firstly, colored solutions can have basic absorbance at the analytical wavelength used, which may contribute to absorbance readings. Secondly, as the method is based on the oxidation of Fe^{2+} to Fe^{3+} by hydrogen peroxide, it can be affected by the presence of other compounds able to oxidize Fe^{2+}. The presence of significant amounts of peroxides other than H_2O_2 is rather improbable in herbal infusions, but polyphenols—such as epigallocatechin gallate, gallic acid, and epicatechin, as well as their oxidation products, which can be present in plant infusions—can oxidize ferrous ions [35]. Therefore, we used two procedures, which should at least partly eliminate the artifacts, but are also not free from drawbacks.

Procedure (i) using Reagent Blank: The infusion (180 µL) was added to two wells of a 96-well plate. One was supplemented with 20 µL of the Xylenol Orange Reagent (2.5 mM Xylenol Orange/2.5 mM Mohr salt in 1.1 M perchloric acid) and the other with a Blank Reagent containing the Mohr salt and perchloric acid, but no Xylenol Orange. In parallel, a blank for Xylenol Orange was prepared: two wells with 180 µL of water were supplemented with 20 µL of the Xylenol Orange Reagent and Blank Reagent, respectively. After 30 min incubation, the absorbance of the samples was measured at 560 nm. The hydrogen peroxide concentration in the infusions was calculated on the basis of corrected absorbance A_{corr}:

$$A_{corr} = A_{S,XO} - A_{S,B} - (A_{W,XO} - A_{W,B})$$

where $A_{S,XO}$—absorbance of a sample added with the Xylenol Orange Reagent;
$A_{S,B}$—absorbance of a sample added with the Blank Reagent;
$A_{W,XO}$—absorbance of water added with the Xylenol Orange Reagent;
$A_{S,B}$—absorbance of water added with the Blank Reagent.

Procedure (ii) using catalase: The infusion (180 µL) was added to two wells of a 96-well plate. One well was supplemented with 2 µL of water and the other with 2 µL of a 1 mg/mL catalase solution. After 15 min incubation, 20.2 µL of the Xylenol Orange Reagent was added to both wells; after 30 min incubation, the absorbance was read at 560 nm. The difference in absorbance between the sample not treated with catalase and the catalase-treated sample was used as a measure of the hydrogen peroxide concentration. Preliminary experiments showed that the amount of catalase used was sufficient to fully decompose 1 mM hydrogen peroxide present in a 200-µL sample during the incubation time employed. The concentration of hydrogen peroxide was calculated using a calibration curve.

Procedure (i) using a Reagent Blank should eliminate the effect of the endogenous color of the solutions, as well as the basic absorbance of the Xylenol Orange Reagent, but it can be still subject to interference by compounds other than hydrogen peroxide that are able to oxidize Fe^{2+}. Procedure (ii), based on the use of the absorbance difference between a sample not treated with catalase and a sample treated with catalase to decompose H_2O_2, should be specific for H_2O_2, irrespective of reactions of the sample components with Fe^{2+}. However, this procedure may lead to an underestimation of the H_2O_2 concentration as some polyphenols inhibit catalase. The compounds most potent in catalase inhibition are epicatechin gallate and epigallocatechin gallate (IC_{50} values < 1 μM); they are even better inhibitors of the enzyme than the standard inhibitor azide [36]. As the strong inhibitors of catalase may be present in the infusions at various concentrations, the extent of the underestimation of the H_2O_2 concentration may be different for various infusions, depending on their composition.

2.5. Determination of Hydrogen Peroxide Scavenging by Herbal Extracts

Herbal infusions were added with 25 μL (final) of hydrogen peroxide, and the H_2O_2 concentrations were estimated immediately after the addition and after 1, 2 and 3 h of incubation. The difference—(sum of concentrations of H_2O_2 generated in the absence of added H_2O_2 and that of added H_2O_2) minus (the H_2O_2 concentrations determined in the H_2O_2-supplemented extracts)—was assumed to represent the magnitude of H_2O_2 scavenging.

2.6. Determination of Polyphenols by UPLC-PDA-MS/MS

Determination of polyphenolic compounds was carried out using the UPLC equipped with a binary pump, column and sample manager, photodiode array detector and tandem quadrupole mass spectrometer, with the electrospray ionization source working in the negative mode (Waters, Milford, MA, USA), as described elsewhere [37]. Separation was performed using the UPLC BEH C18 column (1.7 μm, 100 mm × 2.1 mm, Waters) at 50 °C, at a flow rate of 0.35 mL/min. The injection volume of the samples was 5 μL. The mobile phase consisted of water (solvent A) and 40% acetonitrile in water, *v*/*v* (solvent B). The following parameters were used: capillary voltage of 3500 V; con voltage of 30 V; con gas flow 100 L/h; source temperature of 120 °C; desolvation temperature of 350 °C; desolvation gas flow rate of 800 L/h. Polyphenolic identification and quantitative analyses were performed on the basis of the mass-to-charge ratio, retention time, specific PDA spectra, fragment ions and comparison of the data obtained from the commercial standards and literature findings. The analyses were performed in two replications.

2.7. Detection of Semiquinone Radicals

Electron paramagnetic resonance (EPR) measurements were performed using a Bruker multifrequency and multiresonance FT-EPR ELEXSYS E580 spectrometer (Bruker Analytische Messtechnik, Rheinstetten, Germany) operating at the X-band (9.378989 GHz). The following settings were used: modulation amplitude, 0.4 G; modulation frequency, 100 kHz; microwave power, 94.64 mW; power attenuation, 2 dB; conversion time, 25 ms; sweep time, 102.4 s; powder sample: central field, 3501 G; scan range, 7000 G; liquid sample: central field, 3353.15 G; scan range, 100 G; accumulation, 10.

The test samples (10% herbal infusions, prepared as below) were measured in quartz glass capillary tubes (Bruker, ER 221TUB/4 CFQ).

2.8. Detection of Superoxide Generation

Pulverized herbs (10%) herbal extracts supplemented with NBT solution to a final concentration of 250 μM or dihydroethidium to a final concentration of 10 μM, superoxide dismutase (SOD) to a final concentration of 10 μg/mL (if present were heated in a thermoblock at 60 °C for 60 min, cooled and centrifuged. NBT reduction was measured at 530 nm; the concentration of the formazan in the supernatants was calculated using a millimolar absorption coefficient of 25.4 mM^{-1} cm^{-1} [38]. The fluorescence of ethidium

was measured at the excitation wavelength of 405 nm and the emission wavelength of 570 nm to increase its specificity for the detection of the superoxide reaction product [39].

2.9. Cell Culture

SKOV-3 cells were cultured in McCoy's 5A medium; PEO1 cells were cultured in RPMI + GlutaMAX; MRC-5 cells were cultured in DMEM + GlutaMax. The media used in the experiment were supplemented with 1% v/v penicillin and streptomycin solution and 10% heat-inactivated fetal bovine serum (FBS). The cells were incubated at 37 °C under 5% carbon dioxide and 95% humidity and were passaged at about 85% confluence. Cell morphology was examined under an inverted Zeiss Primo Vert microscope with phase contrast (Oberkochen, Germany). The viability of ovarian adenocarcinoma cells and fibroblasts was estimated using the Trypan Blue exclusion test. The cells were counted in a Thoma hemocytometer (Superior Marienfeld, Lauda-Königshofen, Germany).

2.10. Estimation of Cytotoxicity of Herbal Infusions

SKOV-3 cells were seeded in wells of a clear 96-well plate at a density of 1×10^4 cells/well in 100 µL of culture medium. PEO1 cells were seeded at a density of 1.5×10^4 cells/well, and MRC-5 cells were seeded at a density of 7.5×10^3 cells/well and allowed to attach for 24 h at 37 °C. Six 1% (w/v) herbal infusions (of leaves of *Betula*, *Cistus*, *Ginkgo*, *Melissa* and *Taraxacum*) were used for determination of their toxicity towards human cancer ovary cells and fibroblasts. After 30 min incubation, the infusions were centrifuged and sterilized using a syringe filter (0.2 µM). Cells were treated with different volumes of the infusions made with PBS (0 µL, 2 µL, 5 µL, 10 µL and 20 µL added to 100 µL of the medium) for 24 h. After 24 h exposure, the medium was removed, replaced with 100 µL of 2% Neutral Red solution, and incubated for 1 h at 37 °C. Then, the cells were washed with PBS, fixed with 100 µL/well 50% ethanol, 49% H_2O and 1% glacial acetic acid, and shaken for 20 min (1000× g) at room temperature. Absorbance was measured at 540 nm against 620 nm in a Spark multimode microplate reader (Tecan Group Ltd., Männedorf, Switzerland). Measurements were performed in sextuplicate.

2.11. Effect of Catalase on the Cytotoxicity of Herbal Infusions

PEO1 cells were seeded in a 96-well clear plate at density 1.5×10^4 cells/well and allowed to attach for 24 h at 37 °C. After incubation, the cells were treated with the three most cytotoxic infusions as above but in the presence of 10 µg catalase/mL. After 24 h exposure, the medium was removed and the cytotoxicity was estimated using Neutral Red, as described above.

2.12. Statistical Analysis

The significance of differences, shown in Figures 1 and 3, was obtained using the two-tailed Student's "t" test. Differences in cellular systems (Figures 5 and 6) were assessed using the U Mann-Whitney test, assuming a significance level of $p \leq 0.05$. Statistical analysis of the data was performed using STATISTICA software package (version 13.1, StatSoft Inc. 2016, Tulsa, OK, USA).

3. Results

3.1. Hydrogen Peroxide Is Generated in Herbal Infusions

Both of the applied procedures demonstrated the generation of hydrogen peroxide in herbal infusions (Figure 1). Under Procedure (i), based on the subtraction of a sample blank to eliminate the background color of the samples, all of the infusions showed the presence of hydrogen peroxide, albeit at different concentrations; under Procedure (ii), based on the measurement of the difference in the absorbance readings between the samples incubated and not incubated with catalase, the vast majority of the infusions contained significant amounts of H_2O_2, with the exception of the infusions of *Artemisia*, *Taraxacum*, *Tussilago* and *Urtica*. The highest generation occurred during brewing and within the first hour;

however, further incubation for up to 3 h augmented the level of hydrogen peroxide in most cases, with the exception of *Cistus*, *Urtica* (both Procedures), *Tussilago* (Procedure (i)), *Gingko* and *Lavandula* (Procedure (ii)). In Procedure (i), the highest concentrations of H_2O_2, reaching or even exceeding 50 µM, were detected in the infusions of the leaves of *Betula* (up to 70 µM) and *Polygonum*, and of the inflorescence of *Tilia* (up to over 50 µM). These concentrations exceeded those found in the green tea used for comparison (up to 35 µM). In general, Procedure (ii) yielded lower H_2O_2 concentrations than Procedure (i), although, in some cases (extracts of *Achillea* and *Mentha*), an inverse relationship was observed.

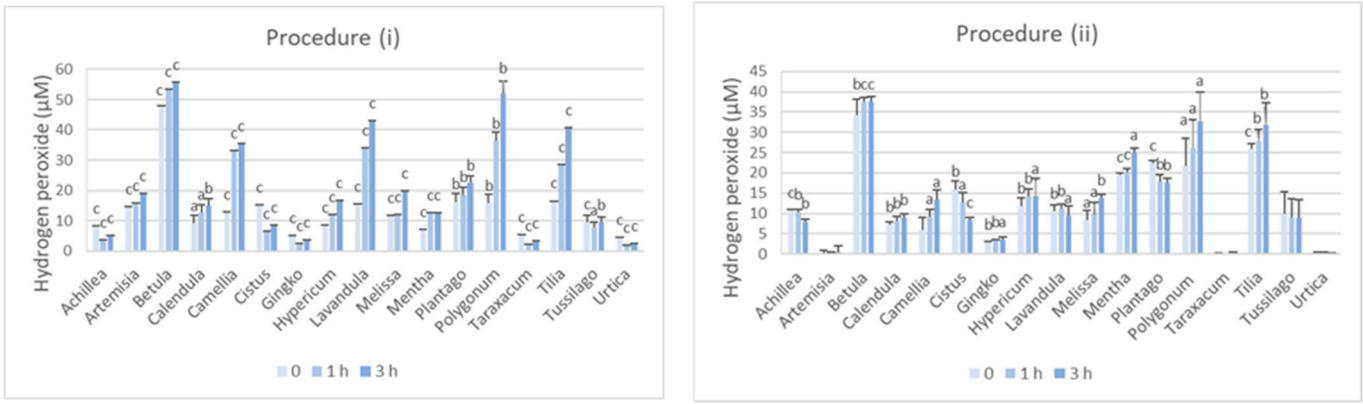

Figure 1. The concentration of hydrogen peroxide in the infusions of medicinal herbs and green tea immediately after brewing and after 1 h and 3 h standing at ambient temperature. H_2O_2 concentration was estimated by two procedures, based on the use of a reagent blank (i) and on the decomposition of H_2O_2 by 10 µg/mL catalase in one of two parallel samples (ii). [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ with respect to zero generation.

3.2. Herbal Infusions Contain Phenolic Compounds

The content of phenolic compounds was analyzed in the infusions of the chosen herbs. The presence of various phenolic compounds was demonstrated in all of the studied extracts (Tables 1–4).

Table 1. Identification of phenolic compounds by UPLC-PDA-MS/MS in the *Betula* extract.

Compound	Rt (min)	λ_{max} (nm)	[M-H] *m/z*	
			MS	MS/MS
Neo-chlorogenic acid	2.27	322	353	191
Betuloside	2.68	276	327	165
Coumaryl-quinic acid I	2.79	310	337	163
Chlorogenic acid	2.88	324	353	191
3-(4-Hydroxyphenyl)-1-methylpropyl	2.99	276	165	-
Feruloyl-quinic acid	3.00	322	357	193
Caffeic acid glucoside	3.21	324	341	179
Coumaryl-quinic acid II	3.54	310	337	163
Myricetin 3-O-glucoside	3.89	253, 354	479	317
Kaempferol 3-O-rhamnoside	3.97	264, 342	431	285
Myricetin 3-O-pentoside	4.37	253, 354	449	317
Quercetin 3-O-glucoside	4.54	255, 355	463	301
Quercetin 3-O-glucuronide	4.57	255, 355	477	301
Quercetin 3-O-pentoside I	4.96	255, 355	433	301
Quercetin 3-O-pentoside II	5.06	255, 355	433	301
Quercetin 3-O-pentoside III	5.16	255, 355	433	301
Quercetin 3-O-rhamnoside	5.26	255, 355	447	301

Table 2. Identification of phenolic compounds by UPLC-PDA-MS/MS in the *Tilia* extract.

Compound	Rt (min)	λ_{max} (nm)	[M-H] *m/z* MS	[M-H] *m/z* MS/MS
Neo-chlorogenic acid	2.28	322	353	191
Ferulic acid glucoside	2.62	324	355	193
Coumarylo-quinic acid I	2.79	310	337	163
Chlorogenic acid	2.88	324	353	191
Coumarylo-quinic acid II	2.95	310	337	163
Coumarylic acid glucoside	310	310	325	163
Procyanidin dimer	3.23	274	577	289
Procyanidin trimer I	3.30	274	865	577, 289
(+)Catechin	3.39	274	289	141
Coumaryl-quinic acid III	3.53	310	337	163
Undefined derivative	3.67	284	401	189
Procyanidin trimer II	3.83	278	865	577, 289
Procyanidin tetramer	3.97	277	1152	865, 577
Kaempferol 3-O-sophoroside	4.29	264, 352	609	285
Quercetin 3-O-rutinoside	4.51	255, 355	609	301
Quercetin 3-O-glucoside	4.66	255, 355	463	301
Quercetin 3-O-(6″-malonyl)-glucoside	4.94	255, 352	549	505, 463, 301
Kaempferol 3-O-rutinoside	5.00	264, 347	593	285
Kaempferol 3-O-glucoside	5.21	264, 347	447	285
Quercetin 3-O-glucuronide	5.44	355, 350	477	301
Chrysoeriol 7-O-rutinoside	5.60	345	607	299
Quercetin 4′-O-glucoside	5.76	255, 355	463	301
Kaempferol 3-O-glucuronide	6.07	264, 342	461	285
Kaempferol 3-O-(6″-acetyl)-rutinoside	7.01	264, 333	637	593, 301

Table 3. Identification of phenolic compounds by UPLC-PDA-MS/MS in the *Taraxaxum* extract.

Compound	Rt (min)	λ_{max} (nm)	[M-H] *m/z* MS	[M-H] *m/z* MS/MS
Caftaric acid	2.25	327	311	179
Chlorogenic acid	2.88	327	353	191
tri-Caffeoyl-tartaric acid	3.35	324	635	473, 311
Coutaric acid	3.66	309	295	163
Chicoric acid	4.32	324	473	311, 179

Table 4. Identification of phenolic compounds by UPLC-PDA-MS/MS in the *Urtica* extract.

Compound	Rt (min)	λ_{max} (nm)	[M-H] *m/z* MS	[M-H] *m/z* MS/MS
Caffeic acid cinnamyl ester	3.68	327	295	179
p-Coumaric acid	4.09	309	163	116
Coumaroyl-malic acid I	4.41	312	279	163
Coumaroyl-malic acid II	4.62	324	279	163
Feruloyl-sinapoyl-tartaric acid	4.85	324	531	309, 193

The concentrations of phenolics in the studied extracts are presented in Tables 5–8. The extract of *Betula* leaves, generating the highest concentrations of hydrogen peroxide, was characterized by an especially high total concentration of phenolic compounds, mainly due to the high content of betuloside (Table 5).

Table 5. The concentrations of polyphenolic compounds in the *Betula* extract.

Compound	Concentration (μg/mL)
Neo-chlorogenic acid	12.08 ± 0.05
Betuloside	394.77 ± 18.87
Chlorogenic acid	46.73 ± 2.02
3-(4-Hydroxyphenyl)-1-methylpropyl	78.38 ± 1.79
Feruloyl-quinic acid	2.85 ± 0.07
Caffeic acid glucoside	2.50 ± 0.01
Coumarylo-quinic acid II	5.58 ± 0.19
Myricetin 3-O-glucoside	66.20 ± 2.19
Kaempferol 3-O-rhamnoside	9.00 ± 0.01
Myricetin 3-O-pentoside	4.64 ± 0.29
Quercetin 3-O-glucoside	78.46 ± 0.96
Quercetin 3-O-glucuronide	62.25 ± 0.55
Quercetin 3-O-pentoside I	15.74 ± 0.40
Quercetin 3-O-pentoside II	8.46 ± 0.08
Quercetin 3-O-pentoside III	5.61 ± 0.13
Quercetin 3-O-rhamnoside	9.81 ± 0.05
Total	808.99 ± 12.51

Table 6. The concentrations of polyphenolic compounds in the *Tilia* extract.

Compound	Concentration (μg/mL)
Neo-chlorogenic acid Ferulic acid glucoside	2.95 ± 0.03
Coumaryl-quinic acid I	1.35 ± 0.07
Chlorogenic acid	3.06 ± 0.01
Coumaryl-quinic acid II	2.22 ± 0.10
Coumarylic acid glucoside	1.30 ± 0.03
Procyanidin dimer	1.68 ± 0.04
Procyanidin trimer I	2.97 ± 0.01
(+)Catechin	0.85 ± 0.03
Coumaryl-quinic acid III	5.07 ± 0.17
Undefined derivative	3.03 ± 0.00
Procyanidin trimer II	2.75 ± 0.18
Procyanidin tetramer	3.70 ± 0.05
Kaempferol 3-O-sophoroside	11.15 ± 0.10
Quercetin 3-O-rutinoside	21.92 ± 0.56
Quercetin 3-O-glucoside	48.33 ± 0.48
Quercetin 3-O-(6″-malonyl)-glucoside	8.88 ± 0.21
Kaempferol 3-O-rutinoside	6.71 ± 0.11
Kaempferol 3-O-glucoside	9.61 ± 0.25
Quercetin 3-O-glucuronide	10.54 ± 0.42
Chrysoeriol 7-O-rutinoside	6.76 ± 0.16
Quercetin 4′-O-glucoside	4.20 ± 0.05
Kaempferol 3-O-glucuronide	3.46 ± 0.06
Kaempferol 3-O-(6″-acetyl)-rutinoside	10.11 ± 0.17
Total	174.56 ± 1.02

Table 7. The concentrations of polyphenolic compounds in the *Taraxacum* extract.

Compound	Concentration (μg/mL)
Caftaric acid	88.19 ± 2.28
Chlorogenic acid	12.57 ± 0.50
tri-Caffeoyl-tartaric acid	5.28 ± 0.12
Coutaric acid	6.20 ± 0.07
Chicoric acid	54.14 ± 0.92
Total	163.24 ± 2.77

Table 8. The concentrations of polyphenolic compounds in the *Urtica* extract.

Compound	Concentration (μg/mL)
Caffeic acid cinnamyl ester	24.57 ± 0.30
p-Coumaric acid	2.32 ± 0.02
Coumaroyl-malic acid I	6.21 ± 0.16
Coumaroyl-malic acid II	9.77 ± 0.10
Feruloyl-sinapoyl-tartaric acid	43.35 ± 1.03
Total	86.22 ± 0.89

3.3. Mechanism of Hydrogen Peroxide Generation

The generation of H_2O_2 in tea and coffee has been ascribed to the autoxidation of the phenolic compounds present in these beverages [18–21,23]. Apparently, this mechanism also operates in infusions of medicinal herbs, which contain polyphenols. Phenolics may generate H_2O_2, especially upon cooking/heating, which inactivates the enzymes that decompose this compound in vivo (mainly catalase, peroxidases and peroxiredoxins). The formation of H_2O_2 due to phenolic oxidation is known to proceed in two steps: a polyphenol H_2Q is oxidized to a semiquinone free radical $HQ^{\bullet}$ in a reaction coupled to the reduction in molecular oxygen to the superoxide anion radical $O_2^{\bullet-}$ (1); then, semiquinone is oxidized to quinone Q, producing the second superoxide radical (2):

$$H_2Q + O_2 \rightarrow HQ^{\bullet} + O_2^{\bullet-} + H^+ \quad (1)$$

$$HQ^{\bullet} + O_2 \rightarrow Q + O_2^{\bullet-} + H^+ \quad (2)$$

The dismutation of superoxide radicals (3) or the oxidation of another molecule by the superoxide radical (4) produces hydrogen peroxide:

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 \quad (3)$$

$$H_2S + O_2^{\bullet-} + H^+ \rightarrow HS^{\bullet} + H_2O_2 \quad (4)$$

where H_2S is an oxidizable substrate and $HS^{\bullet}$ represents a radical product of its one-electron oxidation.

The EPR spectra of pulverized dry herbs and freshly prepared infusions demonstrated the presence of a singlet signal corresponding to the semiquinone radical. In the spectra of dry herbs, this signal was superimposed on the complex broad Mn and perhaps Fe signals, especially in dry samples (not shown). The EPR signals of the infusions of leaves of *Betula* and *Tilia*, which showed the highest H_2O_2 generation (Figure 1), are reported in Figure 2.

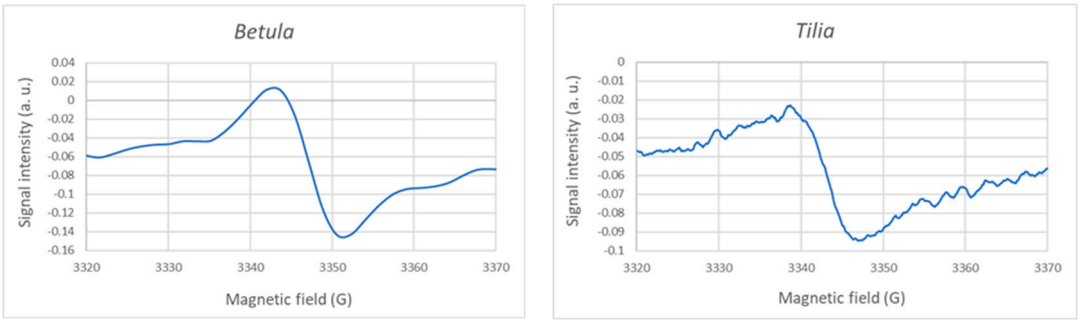

Figure 2. EPR signals of the semiquinone radical measured in infusions of selected herbs.

The plant material used in this study also contained also compounds that produce hydrogen peroxide upon autoxidation; e.g., ascorbic acid [20], although the content of ascorbate is expected to be rather low in herbal infusions, and no signal of ascorbyl radical was detected in the EPR spectra.

The formation of superoxide radicals in herbal infusions was demonstrated through the superoxide-dismutase (SOD) inhibitable reduction in Nitro Blue Tetrazolium (NBT) and SOD-inhibitable oxidation of dihydroethidium (DHE) (Figure 3).

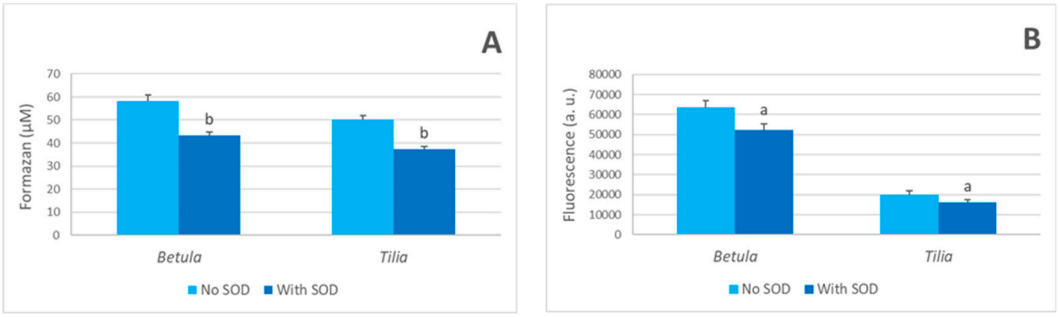

Figure 3. Superoxide dismutase inhibition of NBT reduction (**A**) and DHE oxidation (**B**) in plant infusions. [a] $p < 0.05$, [b] $p < 0.01$ (with SOD vs. no SOD).

The concentration of NBT formazan, formed in a SOD-inhibitable manner during the 15 min brewing at 60 °C, was 23.3 ± 4.6 μM and 20.8.1 ± 3.7 μM for 10% infusions of *Betula* leaves and *Tilia* inflorescence, respectively. These values probably underestimate the real superoxide production as a fraction of the superoxide could still react with the probe or extract components, avoiding dismutation by SOD. Nevertheless, these results document superoxide production—an intermediate in hydrogen formation—in herbal extracts.

3.4. Herbal Infusions Scavenge Hydrogen Peroxide

In most cases, the H_2O_2 concentration increased upon the incubation of the herbal infusions; however, in some cases, it decreased with time. This suggests that herbal infusions may not only generate, but also scavenge, H_2O_2. To test this possibility, some of the herbal extracts were supplemented with H_2O_2, and the time course of the H_2O_2 concentration was monitored in the infusions supplemented and not supplemented with hydrogen peroxide. Figure 4 shows that, in all cases, the H_2O_2 concentration measured in the herbal infusions was significantly lower than the sum of the concentration measured without the addition of exogenous H_2O_2 and the concentration of added H_2O_2 (25 μM final). This difference between the measured and expected hydrogen peroxide concentration was observed from the first measurement ("zero" time), and was higher in the infusion of herbs showing a decrease in the H_2O_2 concentration in the absence of exogenous H_2O_2 (*Urtica* and *Taraxacum*) than in the infusion of herbs in which the H_2O_2 concentration increased with time (exemplified by *Betula* and *Tilia*). The difference between the H_2O_2 concentrations measured in the samples supplemented with H_2O_2 and the not-supplemented samples had a tendency to decrease with time (with the exception of the *Tilia* inflorescence infusions). These results demonstrate that herbal infusions react with H_2O_2. The reaction was apparently the most rapid upon the addition of exogenous H_2O_2 (the "zero" time measurements were in fact performed 4 ± 1 min after the addition of H_2O_2). Thus, the H_2O_2 measured in this herbal infusion represents a net outcome of the rate of production and scavenging of this compound.

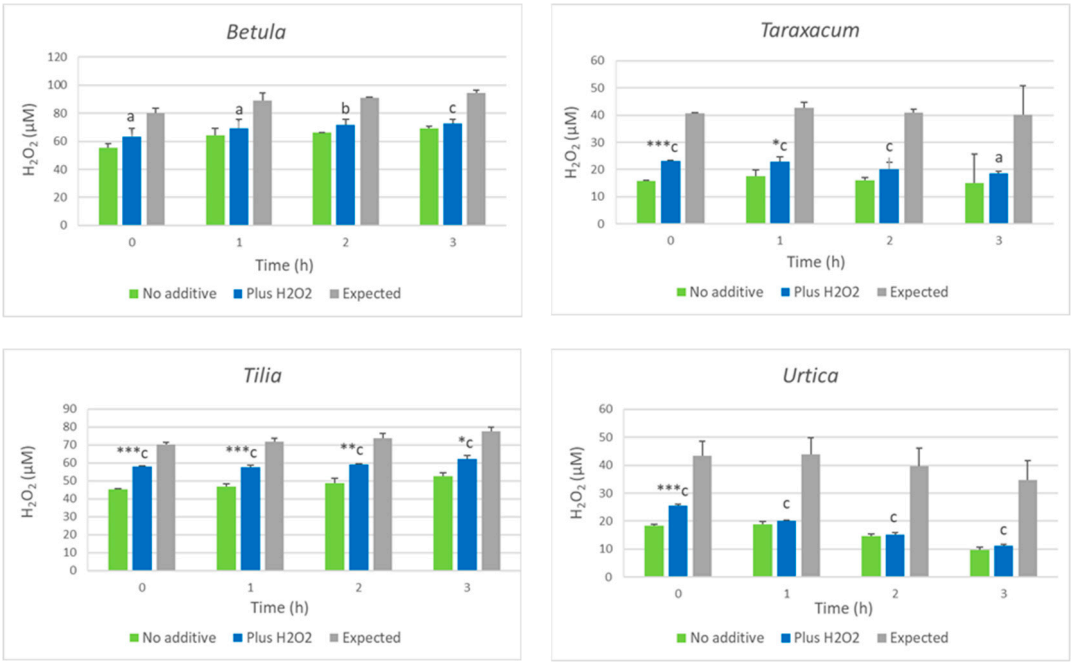

Figure 4. Time course of hydrogen peroxide concentration in herbal infusions not supplemented with H_2O_2 and added with 25 μM (final) H_2O_2. Expected, H_2O_2 concentration calculated on the basis of concentration in non-supplemented infusions plus 25 μM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (supplemented vs not supplemented infusions); [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ (supplemented with H_2O_2, measured vs. expected).

3.5. Hydrogen Peroxide Contributes to the Cytotoxic Action of Herbal Infusions

An important consequence of H_2O_2 formation by herbal infusions concerns in vitro studies of their cytotoxic effects. We compared the cytotoxicity of five herbal infusions to human cancer ovary cells of two lines (SKOV-3 and PEO1) and human MRC-5 fibroblasts used as reference cells. PEO1 is a high-grade serous, and SKOV-3 is a non-serous, ovary cancer cell line. All of the studied infusions, with the exception of the infusion of *Taraxacum* leaves, added at volumes of up to 20 μL (corresponding to infusions of 200 ng of dry herb) per 100 μL medium significantly decreased the survival of PEO1 cells; all the infusions except for those of *Ginkgo* leaves decreased the survival of SKOV-3 cells. The survival of MRC-5 fibroblasts was compromised only by the highest concentration of the infusions of *Cistus*, and was slightly enhanced by the infusions of *Taraxacum* (Figure 5).

In order to check whether the toxicity of the three infusions that were most effective with respect to the more sensitive PEO1 cells, which is indeed dependent on the production of H_2O_2, the effect of catalase on the cell survival treated with these extracts was studied. The presence of catalase, which decomposes H_2O_2, significantly attenuated the cytotoxicity of all the infusions (Figure 6). The toxic effects of the extracts included an H_2O_2-independent component, which was prevailing, but the effect of the H_2O_2 also contributed to their toxicity, as demonstrated by the protective action of catalase on the cell survival.

Figure 5. Survival of SKOV-3 and PEO1 ovary cancer cells, and MRC-5 fibroblasts treated with various volumes of herbal infusions after 24 h incubation; * $p < 0.05$.

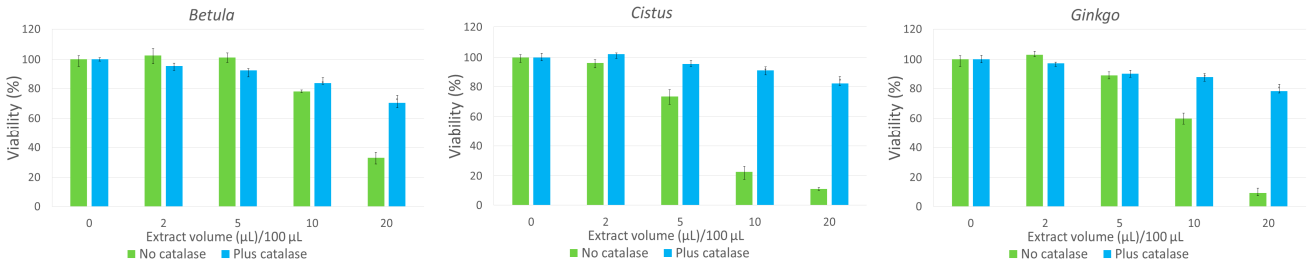

Figure 6. Effect of catalase (10 µg/mL) on the survival of PEO1 cells treated with various volumes of infusions of *Betula*, *Cistus* and *Gingko* infusions. * $p < 0.05$ (catalase vs. no catalase).

4. Discussion

4.1. Formation and Scavenging of Hydrogen Peroxide by Herbal Infusions

The two-step mechanism of hydrogen peroxide formation proposed for tea polyphenols (Reactions (1)–(4)) [18,40] was demonstrated to also be valid for infusions of medicinal plants. The formation of semiquinone radicals—products of reaction (1)—was detected in the studied extracts of *Betula* leaves and *Tilia* inflorescence (Figure 2). Previously, EPR signals of semiquinone radicals were reported for dry samples of teas [41–43] and *Salvia officinalis* [44]; however, to the best of our knowledge, no signals of semiquinone radicals in herb extracts have been published.

This study also confirms the formation of superoxide radicals—another product of reactions (1) and (2)—through the SOD-inhibitable reduction in NBT and oxidation of DHE.

Both probes are not specific in their reactions. A reduction in NBT is often used to quantify superoxide production in animal and plant tissues [38,45,46], but other compounds may also reduce NBT; therefore, the SOD-inhibitable reduction is a more reliable measure of superoxide production. Similarly, superoxide was suggested to be the main, but not the only, reactive oxygen species responsible for DHE oxidation [47,48]. The employed conditions of the fluorescence measurements [39] were claimed to increase the selectivity of the method of DHE oxidation for superoxide; nevertheless, even this version of the method is far from being specific [47,48]. Thus, the SOD-inhibitable DHE oxidation is more reliable.

The herbal extracts were shown to generate H_2O_2, but also to scavenge H_2O_2. Polyphenols are known to react with hydrogen peroxide, although they show varying reactivities. Phenolic acids, especially gallic acid and pyrogallol, were reported to react with hydrogen peroxide [49]. Benzoate derivatives were found to be much stronger H_2O_2 scavengers than cinnamic acids [50]. Among benzoic acid derivatives, vanillic acid (3-hydroxy-4-methoxybenzoic acid) was found to be the most efficient H_2O_2 scavenger, with the hydrogen peroxide scavenging activity of 170.2 μM^{-1}, whereas protocatechuic acid (3,4-dihydroxybenzoic acid) exhibited the weakest activity (5.90 μM^{-1}) [51]. In another study, among six phenolic acids, caffeic acid was found to be the most efficient H_2O_2 scavenger, with the H_2O_2-scavenging activity of 125×10^{-3} μM^{-1}, while *trans*-cinnamic acid exhibited the weakest activity (0.73×10^{-3} μM^{-1}). In that study, the H_2O_2-scavenging activity of various herbal extracts was also estimated, with black and green tea showing activities of about 1.1×10^5 mL/g and 1.3×10^5 mL/g, respectively [52]. Hydrotyrosol, a polyphenol from olive oil, was also found to react with hydrogen peroxide [53]. In an aqueous peppermint extract, eriocitrin showed the highest H_2O_2-scavenging activity, followed by rosmarinic acid, while hesperidin showed a low scavenging activity, and diosmin, narirutin and isorhoifolin exhibited almost no H_2O_2-scavenging activity [54]. Thus, the behavior of various phenolics is different both with respect to the rate of generation and to the rate of scavenging of hydrogen peroxide, which may explain the difference in the rate of generation and the time-dependent changes in the hydrogen peroxide concentration in the extracts of various herbs.

4.2. Biomedical Relevance of the Presence of Hydrogen Peroxide in Infusions of Medicinal Herbs

This study demonstrates that infusions of medicinal herbs contain some amounts of H_2O_2. It should be taken into account that the cultivar, location, growth conditions, time of collection and even the extent of fragmentation may considerably affect the composition of phenolics and other compounds that generate H_2O_2 [55,56]; therefore, the values obtained in this study may not necessarily be representative of other batches of medicinal herbs.

The presence of micromolar concentrations of H_2O_2 in herbal infusions should not have deleterious consequences for health; on the contrary, it may even exert some beneficial effects. In the digestive tract, H_2O_2 may be partly decomposed by the catalase and peroxidase activities present in the saliva and in gastric juice [57,58]; i.a., saliva contains catalase and peroxidases that are important in the defense against the H_2O_2 produced by mouth bacteria [59]. The residual H_2O_2 can exert bactericidal and virucidal action, and thus may contribute to mouth hygiene and health [60,61] and act against *Helicobacter pylori* in the

stomach [62]. Hydrogen peroxide promotes gastric motility [63]. High concentrations of H_2O_2 may damage colon cells, but low concentrations were suggested to stimulate cell divisions in the damaged intestine, thus contributing to epithelial repair [64]. Reaction with available iron in the intestine forms the hydroxyl radical and other free radicals, which facilitate digestion as proteins subjected to free radical action may show enhanced susceptibility to proteolytic enzymes [65]. One can speculate whether the generation of H_2O_2 in the infusions of medicinal herbs in this study can contribute to the prophylactic and therapeutic effects of at least some of these herbs.

4.3. Generation of Hydrogen Peroxide May Introduce Artifacts in Cellular In Vitro Experiments

The cytotoxicity of various herbal extracts in vitro has been reported, e.g.,: *G. biloba* leaf extracts were found to be cytotoxic to carcinoma HSC-2 cells [29]; *Plantago* extracts were found to be cytotoxic for human leukemia cells [30], MCF-7 breast cancer cells, A431 epidermal cells and U87-MG glioma cells [31]; extracts of *Tilia* were found to inhibit the proliferation of AGS stomach adenocarcinoma and SKOV-3 ovarian cancer cells [32]; extracts of *Artemisia absinthium* showed cytotoxicity against HaCaT keratinocytes and HGF-1 gingival fibroblasts [33]. The H_2O_2 formed in the infusions may contribute to the cytotoxicity of infusions of medicinal herbs, green tea and wines [27], like individual flavonoids such as pyrogallol, epigallocatechin gallate and quercetin [20].

This study demonstrated that the cytotoxic effects of herbal extracts are partly dependent on the hydrogen peroxide present in the extracts. This is an artifact of in vitro experiments, which does not occur under in vivo conditions. The difference between the in vitro and in vivo conditions should be kept in mind when evaluating the effect of herbal infusions on malignant cells. These infusions may be more effective in vitro, and one reason for this may be the formation of H_2O_2 under in vitro conditions (availability of oxygen). They may be less cytotoxic under in vivo conditions when the oxygen concentration is much smaller, the formation of hydrogen H_2O_2 due to autoxidation of herb components is much lower (if any) and the contribution of H_2O_2 to the herb cytotoxicity is much lower or absent. Thus, under in vivo conditions, the adsorbed polyphenols may exhibit antioxidant and not prooxidant properties (scavenging of H_2O_2 rather than net H_2O_2 generation).

5. Conclusions

Hydrogen peroxide is generated in infusions of medicinal herbs in micromolar concentrations due mainly to the autoxidation of phenolic compounds. The infusions have also the ability to scavenge hydrogen peroxide so the net concentration of H_2O_2 is the result of the rates of these two processes. Hydrogen peroxide present in the infusions of medicinal herbs contributes to their cytotoxic effects in vitro.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr11102855/s1, short description of the herbs used and their applications. Refs. [66–83] are cited in Supplementary Materials.

Author Contributions: Conceptualization, I.S.-B. and G.B.; methodology, I.S.-B., I.S., I.K. and G.B.; validation, I.S.-B.; investigation, A.T., N.P., I.S.-B., I.S. and I.K.; writing—original draft preparation, I.S.-B., I.S., I.K. and G.B.; writing—review and editing, I.S.-B. and G.B.; supervision, I.S.-B.; project administration, I.S.-B. All authors have read and agreed to the published version of the manuscript.

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