Article

Hydrogen Peroxide Is a Component of Infusions of Medicinal Herbs

Anna Tama [1], Natalia Pieńkowska [1], Ireneusz Stefaniuk [2], Grzegorz Bartosz [3] and Izabela Sadowska-Bartosz [1,*]

[1] Laboratory of Analytical Biochemistry, Institute of Food Technology and Nutrition, College of Natural Sciences, University of Rzeszow, Rzeszow, Poland; aniatama@gmail.com (A.T.), natalia.pien@gmail.com (N.P.)
[2] Institute of Materials Engineering, College of Natural Sciences, University of Rzeszow, Rzeszow, Poland; istefaniuk@ur.edu.pl
[3] Department of Bioenergetics, Food Analysis and Microbiology, Institute of Food Technology and Nutrition, College of Natural Sciences, University of Rzeszow, Rzeszow, Poland; gbartosz@ur.edu.pl
* Correspondence: isadowska@ur.edu.pl; Tel.: +48 17 785 5408

Abstract: The aim of this study was to examine whether H_2O_2, generated in such beverages as tea and coffee, is formed also in infusions of medicinal herbs and propose the possible 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*, and of the inflorescence of *Tilia*. These low amounts of H_2O_2 should not have a harmful effect and may even exert beneficial action, perhaps contributing to the health effects of the herbs. Generation of semiquinone and superoxide radicals was detected in herbal infusions, indicating that oxidation of phenolic compounds is the main source of H_2O_2 in these infusions. 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 hydrogen peroxide to the cytotoxic action of herbal extracts.

Keywords: hydrogen peroxide; FOX assay; medicinal herbs; ovary cancer cells; polyphenols

1. Introduction

Apart from the most popular beverages of plant origin such as tea and coffee, infusions of medicinal herbs are also often consumed as recommended by traditional or complementary medicine or as tea substituents. The commonly consumed beverages such as tea [1-4] and coffee [5-7] generate H_2O_2 upon brewing and subsequent standing. Hydrogen peroxide concentration in freshly brewed coffee was found to be 20-80 μM, depending on the type of coffee [6] while in the green and black tea it exceeded 200 μM and 700 μM after standing for 1 and 12 hours, respectively [2]. Lately, we documented the generation of H_2O_2 in cooked vegetables [8,9]. Wines, especially red wines, generate H_2O_2 [10,11] although wine components may also react with this compound [11]. Generation of hydrogen peroxide in these beverages is due to the autoxidation of antioxidants, mainly phenolic compounds contained in the plant material.

Hydrogen peroxide is a physiologically relevant compound, important in the defense against pathogenic microorganisms at micromolar up to millimolar concentrations and participating in intra- and intercellular signaling at nanomolar up to low micromolar concentrations [12,13]. Thus, not only endogenous generation but also exogenous sources of H_2O_2 in the human body are of interest.

Infusions of medicinal herbs are also rich in antioxidants including phenolic compounds and are an important source of dietary antioxidants. Part of the beneficial effects of medicinal herbs is attributed to the presence of their antioxidants, especially phenolics [14-17]. Phenolic compounds may be expected to generate hydrogen peroxide also in these infusions.

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The aim of this study was to check whether H_2O_2 is generated in infusions of chosen medicinal herbs and inquire about the mechanism of H_2O_2 formation in the infusions. Sixteen various herbs were examined.

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 [10]. 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. Results and Discussion

2.1. Methodology of hydrogen peroxide assay

The generation of hydrogen peroxide in the herbal infusions was assayed using the Ferrous Oxidation-Xylenol Orange method [18]. Although results obtained by 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, and their oxidation products, which can be present in plant infusions, can oxidize ferrous ions [19]. Therefore, we used two procedures, which should at least partly eliminate the artifacts but are also not free from drawbacks.

Procedure (i) using a Reagent Blank should eliminate the effect of an 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 able to oxidize Fe^{2+}. Procedure (ii) based on the use 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 sample components with Fe^{2+}. However, this procedure may lead to the underestimation of 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 [20]. As the strong inhibitors of catalase may be present in the infusions at various concentrations, the extent of underestimation of the H_2O_2 concentration may be different for various infusions, depending on their composition.

2.2. Hydrogen peroxide is generated in herbal infusions

Both applied procedures demonstrated the presence of hydrogen peroxide in herbal infusions (Figure 1). With Procedure (i), all the infusions showed the presence of hydrogen peroxide albeit at different concentrations; with Procedure (ii), the vast majority of the infusions contained significant amounts of H_2O_2, except for infusions of *Artemisia*, *Taraxacum*, *Tussilago*, and *Urtica*. The highest generation occurred during brewing and within the first hour but further incubation for up to 3 hours augmented the level of hydrogen peroxide in most cases, except for *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 that found in the green tea used (up to 35 μM). Generally, Procedure (ii) yielded lower H_2O_2 concentrations than Procedure (i).

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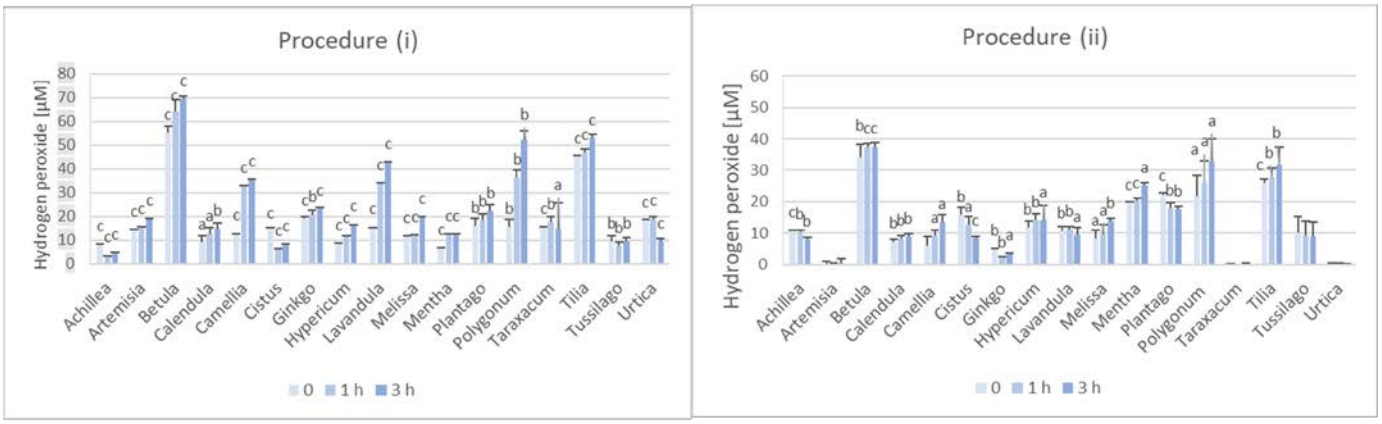

Figure 1. The concentration of hydrogen peroxide in the infusions of medicinal herbs and of green tea immediately after brewing and after 1 hour and 3 hours standing at ambient temperature. H_2O_2 concentration was estimated by two procedures, based on the use of a reagent blank (i) and on the decomposition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

It should be taken into account that the cultivar, location, growth conditions and time of collection, and even the extent of fragmentation may considerably affect the composition of phenolics and other compounds generating H_2O_2 so the values obtained in this study may be not representative of other batches of medicinal herbs. In any case, this study demonstrates that herbal infusions contain some amounts of H_2O_2.

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 catalase and peroxidase activities present in the saliva and in gastric juice [21,22]. The residual H_2O_2 can exert bactericidal and virucidal action and thus may contribute to mouth hygiene and health [23,24] and act against *Helicobacter pylori* in the stomach [25]. Hydrogen peroxide promotes gastric motility [26]. 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 [27]. Reaction with available iron in the intestine forms the hydroxyl radical and other free radicals, which facilitate digestion, since proteins subjected to free radical action may show enhanced susceptibility to proteolytic enzymes [28]. One can speculate whether the generation of H_2O_2 in the infusions of medicinal herbs can contribute to the prophylactic and therapeutic effects of at least some of these herbs.

2.3. Mechanism of hydrogen peroxide generation

The generation of H_2O_2 in tea and coffee has been ascribed to the autoxidation of phenolic compounds present in these beverages such as epigallocatechin gallate, catechin, epicatechin, quercetin, delphinidin, hydroxytyrosol, rosmarinic acid and a range of other plant polyphenols [1-4, 6]. As polyphenols are secondary metabolites ubiquitous in plants, they are also present in other products of plant origin and may be expected to generate H_2O_2, especially upon cooking/heating, which inactivates enzymes decomposing this compound *in vivo* (mainly catalase and peroxidases). 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 of molecular oxygen to the superoxide anion radical $O_2^{\bullet-}$ (1) and then semiquinone is oxidized to quinone Q producing second superoxide radical (2):

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

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

Dismutation of superoxide radicals (3) or 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.

EPR spectra of pulverized dry herbs and freshly prepared infusions demonstrated the presence of a singlet corresponding to the semiquinone radical. In spectra of dry herbs, This signal was superimposed on complex broad manganese and perhaps Fe signal, especially in dry samples; examples of signals of infusions are reported in Figure 2. The intensity of the signal varied for different herbs. EPR signals of dry samples of teas [29-31] and *Salvia officinalis* [32]; no signals of semiquinone radicals in herb extracts were published, to our best knowledge.

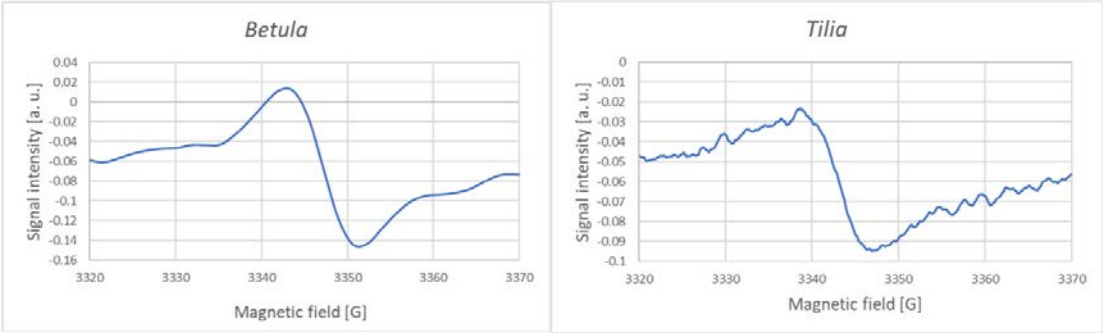

Figure 2. EPR signals of the semiquinone radical measured in infusions of selected herbs.

The plant material used in this study contained also other compounds producing hydrogen peroxide upon autoxidation, e.g. ascorbic acid [3] though the content of ascorbate is expected to be rather low in herbal infusions, and no signal of ascorbyl radical was detected in the ESR spectra.

Formation of superoxide radicals in herbal infusions was demonstrated by superoxide-dismutase (SOD) inhibitable reduction of Nitro Blue Tetrazolium (NBT) and SOD-inhibitable oxidation of dihydroethidium (DHE). Both probes are not specific in their reactions. Reduction of NBT is often used to quantify superoxide production in animal and plant tissues [33,34] but other compounds may also reduce NBT so 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 [35,36]. The conditions of fluorescence measurements employed [37] 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 so SOD-inhibitable DHE oxidation is more reliable.

The presence of SOD-inhibitable components of NBT reduction and DHE oxidation in herbal infusions is demonstrated in Figure 3.

The concentration of NBT formazan formed in a SOD-inhibitable manner during 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 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.

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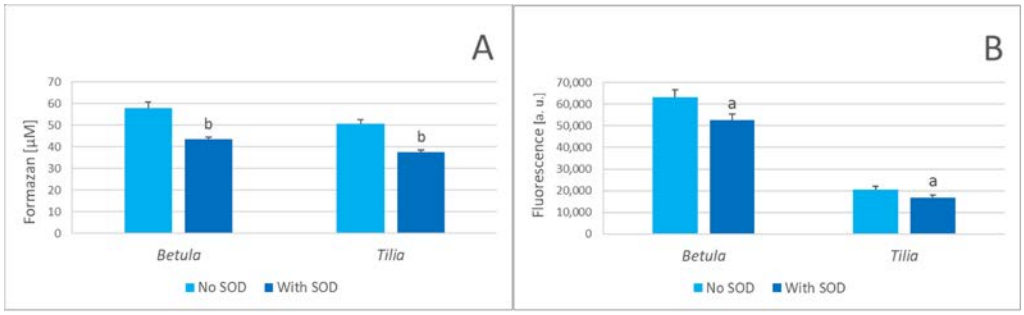

Figure 3. Superoxide dismutase inhibition of NBT reduction (A) and DHE oxidation (B) in plant infusions. $^{a}p < 0.05$, $^{b}p < 0.01$ (with SOD vs no SOD).

3.3. Herbal infusions scavenge hydrogen peroxide

In most cases, the H_2O_2 concentration increased upon incubation of herbal infusions but in some cases, it decreased in time. It suggests that herbal infusions may not only generate but also scavenge H_2O_2. To test this possibility, some herbal extracts were added with H_2O_2, and the time course of the H_2O_2 concentration was monitored in infusions supplemented and not supplemented with hydrogen peroxide. Figure 4 shows that in all cases the H_2O_2 concentration measured in herbal infusions was significantly lower than the sum of concentration measured without the addition of exogenous H_2O_2 and the concentration of added H_2O_2 (finally 25 µM). 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 supplemented samples supplemented with H_2O_2 and not supplemented samples had a tendency to decrease in time (with the exception of *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 minutes after the addition of H_2O_2).

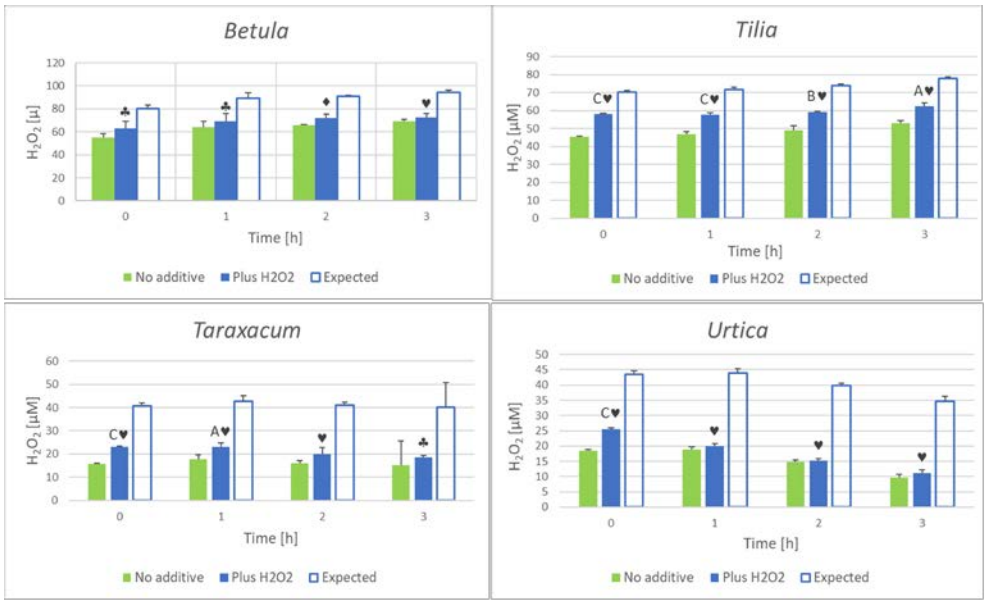

Figure 4. Time course of hydrogen peroxide concentration in herbal infusions not supplemented with H_2O_2 and added with 25 µM (final) H_2O_2. Expected, H_2O_2 concentration calculated on the basis of concentration in non-supplemented infusions plus 25 µM. $^{A}P < 0.05$, $^{C}P < 0.001$ (supplemented vs

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not supplemented infusions), ♣P < 0.05, ♦P < 0.01, ♥P < 0.001 (supplemented with H_2O_2, measured vs expected).

There are components of herbal infusions, especially polyphenols, that can react with H_2O_2 [38,39] and transition metal ions so a decrease in the H_2O_2 concentration can occur in these infusions. Thus, the H_2O_2 measured in herbal infusion represents a net outcome of the rate of production and scavenging of this compound.

2.4. 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 effect against malignant cells often performed as a measure of their potential anticancer activity. The H_2O_2 formed in these infusions may contribute to the cytotoxicity of these infusions, green tea, and wines [10], like isolated flavonoids such as epigallocatechin gallate and quercetin [3]. 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 [40]. *Plantago* extracts were cytotoxic for human leukemia cells [41], MCF-7 breast cancer cells, A431 epidermal cells, and U87-MG glioma cell line [42].

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 the infusions studied except for 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 slightly enhanced by infusions of *Taraxacum* (Figure 5).

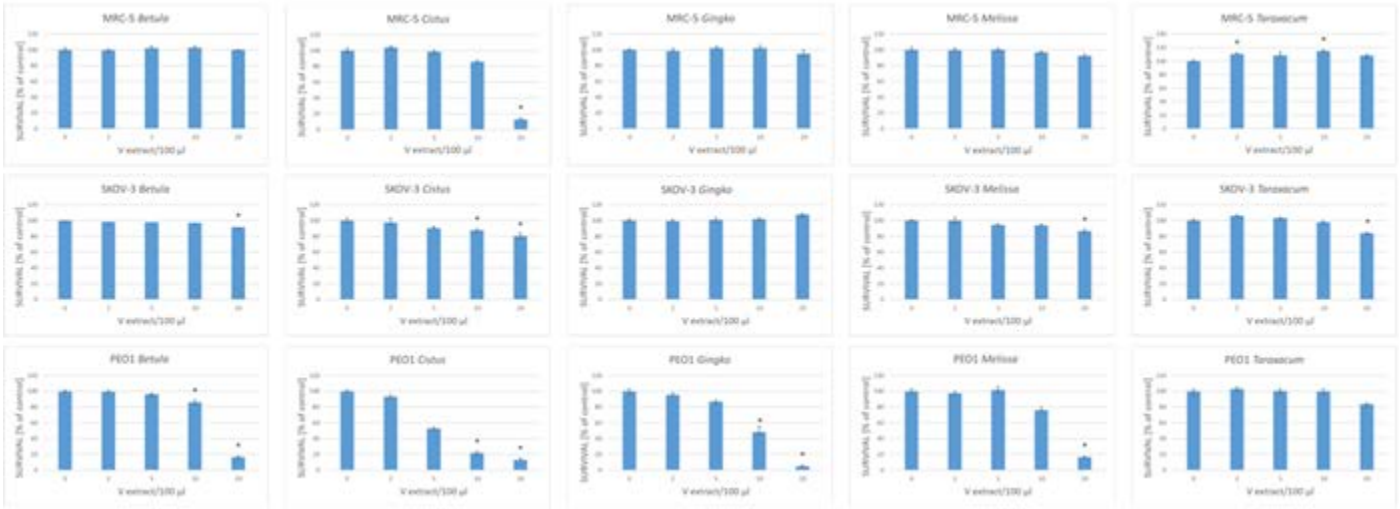

Figure 5. Survival of SKOV-3 and PEO1 ovary cancer cells, and MRC-5 fibroblasts treated with various volumes of herbal infusions after 24-h incubation; [a]P <0.05.

We checked whether the toxicity of three infusions, most effective with respect to the more sensitive PEO1 cells, is dependent on the production of H_2O_2. The presence of catalase, decomposing H_2O_2, significantly attenuated the cytotoxicity of all infusions (Figure 6). The toxic effects of the extracts included an H_2O_2-independent component, which was prevailing, but the effect of H_2O_2 also contributed to their toxicity as demonstrated by the protective action of catalase on cell survival. This effect is an artifact of *in vitro* experiments since *in vivo* conditions do not allow for an easy autoxidation of polyphenols due to lower oxygen concentration, and hydrogen peroxide, if formed, is efficiently disposed of, mainly by catalase and glutathione peroxidase. This difference between the *in vitro* and *in vivo*

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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 it 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.

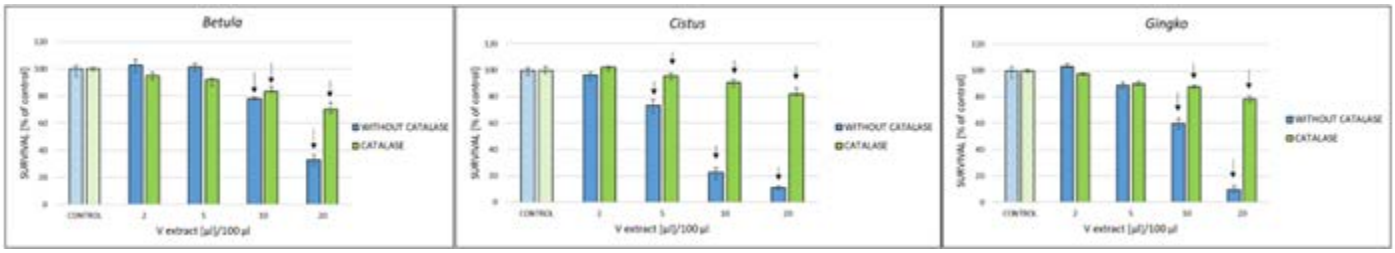

Figure 6. Effect of catalase (10 μg/mL) on the survival of PEO1 cells treated with various volumes of infusions of *Betula, Cistus*, and *Gingko* infusions. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ (catalase vs no catalase).

3. Materials and Methods

3.1. Materials

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), Nitrotetrazolium Blue (NBT; cat. no. N6876), and dihydroethidium (cat. no. 309800) were purchased from Merck (Poznań, Poland).

75 cm^2 flasks (cat. no. 156499) were provided by Thermo Fisher 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 human lung normal fibroblast cell line (MRC-5) were obtained from American Type Culture Collection (ATCC). We also used a second ovarian cancer cell line derived from human ovarian cancer (PEO1) purchased in the European Collection of Authenticated Cell Cultures (ECACC).

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

3.2. Plant 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 in a local herbal store in Rzeszów, Poland. Leaves of *Ginkgo biloba* were collected from a tree grown in the garden of the corresponding author.

3.3. 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. Immediately and after standing for 1 and 3 hours aliquots of

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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).

3.4. Determination of hydrogen peroxide

Hydrogen peroxide was estimated with Xylenol Orange [35] using two procedures:

Procedure (i): The infusion (180 µL) was added to two wells of a 96-well plate. One was added 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 another 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 added 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. 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): The infusion (180 µL) was added to two wells of a 96-well plate. One well was added with 2 µL of water and another 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, and after 30-min incubation, 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 of 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.

3.5. Detection of semiquinone radicals

Electron paramagnetic resonance (ESR) measurements were performed on 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 deposited in quartz glass capillary tubes (Bruker, ER 221TUB/4 CFQ).

3.6. Detection of superoxide generation

Pulverized herbs were (10%) herbal extracts were added with NBT solution to a final concentration of 250 µM or dihydroethidium to a final concentration of 10 µM, SOD to a final concentration of 10 mg/ml (if present), and water to a final volume of 1 ml), 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 was calculated in the supernatants using a millimolar absorption coefficient of 25.4 mM^{-1} cm^{-1} [43]. The fluorescence of ethidium was measured at the excitation wavelength of 405 nm and the emission wavelength of 570 nm to increase its specificity or the detection of the superoxide reaction product [37].

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3.7. Cell culture

SKOV-3 cells were cultured in McCoy's 5A medium, PEO1 cells were cultured in RPMI+GlutaMAX and MRC-5 cells were cultured in DMEM+GlutaMax. Media used in the experiment were supplemented with 1% v/v penicillin and streptomycin solution and 10% heat-inactivated fetal bovine serum (FBS). 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 microscope with phase contrast Zeiss Primo Vert (Oberkochen, Germany). Ovarian adenocarcinoma cells and fibroblast viability was estimated by the Trypan Blue exclusion test; cells were counted in a Thoma hemocytometer (Superior Marienfeld, Lauda-Königshofen, Germany).

3.8. 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. The infusions were prepared by treating 250 mg of the herbs with boiling phosphate-buffered saline (to provide isotonicity with the cell culture medium). After a 30-minute incubation, the infusions were centrifuged and sterilized using a syringe filter (0.2 µM). Cells were treated with different volumes of the infusions (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 (700 rpm) 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.

3.9. 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 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 cytotoxicity was estimated with Neutral Red as described above.

3.10. Statistical analysis

To estimate differences between cells treated by chosen herbs and vegetable infusions and non-treated control Kruskal-Wallis test was performed. $p \leq 0.05$ was considered as statistically significant. Differences between catalase-treated and non-treated trials were assessed by using the U Mann Whitney test with the same significance level ($p \leq 0.05$). Statistical analysis of the data was performed using STATISTICA software package (version 13.1, StatSoft Inc. 2016, Tulsa, OK, USA).

4. Conclusions

Hydrogen peroxide is generated in herbal infusions and may be ingested with the infusions. These small amounts of H_2O_2 are unlikely to have adverse health effects; on the contrary, they may contribute to the beneficial effects of the infusions. Hydrogen peroxide contributes to the cytotoxic effects of herbal infusions found by *in vitro* studies.

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