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Electrochemical screening of genoprotective and antioxidative effectiveness of *Origanum vulgare* L. and its functionality in the prevention of neurodegenerative disorders

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ABSTRACT

A new way of electrochemical DNA sensor using as a screening tool for the determination of phytochemicals with high genoprotective functionality is proposed. The biosensor's detection layer was prepared with double stranded deoxyribonucleic acid (ds DNA) that were subjected to oxidative stress induced by •OH radicals generated by Fenton reaction. The oxidized guanine derivative, 8-oxo-7,8-dihydro-2′-deoxyguanosine, was treated as an indicator of DNA oxidative damage. This derivative may cause mutation through its ability to pair with adenine. The abnormalities of DNA structure and DNA repair system are known to be directly related to progressive neurodegeneration. The present study showed that during oxidative stress, the 2.5% oregano extract protected guanine from undergoing oxidation to 8-oxoguanine. The results revealed that this genoprotective effectiveness can make oregano a very efficient protective barrier against oxidative stress. Due to these unique properties of oregano we propose the recipe of a functional bread with its addition. It was found that the functionality of the prepared bread was not limited to antioxidative activity but also is expressed in the inhibition of cholinesterases. These findings indicate that oregano can act as an important component in the therapeutic diet recommended in neurodegenerative disorders.

1. Introduction

Dysfunctions in the nervous system and the brain are the cause of particularly troublesome ailments, which significantly reduce the quality of life. Literature data indicate that in the pathophysiology of neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease, higher levels of oxidative stress biomarkers and lower levels of antioxidant defense biomarkers are found [1,2]. To avoid the fluctuations in the levels of these biomarkers, it is very important to use a diet rich in bioactive components. Therefore, there is a need for a continuous search for phytochemicals with high functionality in terms of antioxidative and genoprotective activity for the prevention of neurodegenerative diseases [3].

In this area electrochemical DNA sensors are very useful tools, because they allow obtaining unique information about the effects of the interaction of particular molecules or their mixtures with nucleic acids [4]. In model systems, these sensors are prepared with nucleic acid molecules in the detection layer immobilized on the surface of the working electrode. Interaction with hazardous compounds causes damage in the structure of nucleic acids. Voltammetric measurement makes it possible to determine the degree of these damages on the basis of the changes in the DNA signals [5]. Commonly considered are the guanine and adenine signals, and less frequently is taken into account the peak of 8-oxoguanine (8-oxo-7,8-dihydro-2′-deoxyguanosine,

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8-oxodG), treated as an indicator of DNA oxidative damage [4,6]. This guanine derivative pairs with cytosine, but it can also pair with adenosine leading to mutation [7]. It should be noted that in addition to the undisputed effect of mutations on the development of cancer, the abnormalities of DNA structure and DNA repair are directly related to progressive neurodegeneration [8–10].

Electrochemical biosensors have a fundamental advantage over the other methods used for the determination of 8-oxodG levels as they allow direct examination of DNA samples without hydrolysis, a process that is necessary for other methods and causes additional damage, leading to false-positive results [6,10,11]. In turn one of the most reactive forms of reactive oxygen species (ROS), that can cause oxidation of guanine, is the hydroxyl radical. $^{\bullet}OH$ can be generated from H_2O_2 by the Fenton-type reaction in the presence of transition metal cations such as Fe^{2+}. The data on the effect of $^{\bullet}OH$ on nucleobases damage and the evaluation of the protective effect of antioxidant compounds using electrochemical sensors can be found in the literature [12–14].

Intracellular imbalance between oxidant and antioxidant status might facilitate numerous disorders. Various cellular factors, including glutathione, α-tocopherol, and ascorbic acid, induce protection against oxidative lesions [15,16]. However, these endogenous antioxidants cannot always provide complete protection. Therefore, additional antioxidants that can limit these oxidative lesions need to be acquired from dietary sources [3,13,17]. Plants exhibit a very broad spectrum of effects on the human body. An important aspect that should be considered is that certain food ingredients can slow down the neurodegenerative processes, including those related to AD [17–19]. In the AD patients, the levels of various neurotransmitters are found to be lower in the brain, one of which is acetylcholine [20]. It has been proven many years ago that the occurrence of clinical symptoms of AD and their severity are related to the degree of damage to the cholinergic system [21]. A breakthrough in the AD treatment was the introduction of cholinesterase inhibitors (ChEI) that can inhibit acetylcholine catabolism. Acetylcholine, as one of the key neurotransmitters of the central, peripheral, and autonomic nervous system, plays an essential role in the regulation of various factors such as heart function, blood pressure, memory processes, muscle contractions, functioning of the gastrointestinal tract, and so on [18,21]. The cholinergic anti-inflammatory pathway is also associated with the suppression of inflammatory response, especially the prevention of neuroinflammation [22].

Origanum vulgare L. is a rich source of natural antioxidants as phenolic acids, including rosmarinic acid and caffeic acid, flavonoids, tocopherols, and essential oils such as carvacrol, thymol, and phytosterols. Numerous data on the exophthalmic, disinfecting, antidiarrheal, diuretic, antispasmodic, carminative, and detoxifying properties of oregano are available in the literature [23]. These data also indicate the high antimicrobial and antioxidant properties of the essential oils and water extracts of oregano [24].

Considering all these properties, and especially the high antioxidant activity, oregano was chosen for the present study aiming at the construction of an electrochemical DNA sensor that can be useful in the rapid screening of phytochemicals with genoprotective functionality. Subsequently, by preparing a bread with the addition of oregano, an attempt was made to show the practical applications of the electrochemical screening tests. For various types of bread containing oregano leaves or oregano leaf extract the antioxidant and cholinesterases inhibitory activity was determined.

2. Material and methods

2.1. Chemicals

Graphite powder, mineral oil, DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), and enzymes were purchased from Sigma. All other chemicals were purchased from POCH (Poland). Oligonucleotides of the sequence 5′GTCAACTTCCGTACCGAGC and 5′GCTCGGTACGGAAGTTGAC were acquired from Tib Molbiol (Poland). dsDNA was obtained by incubating the above oligonucleotides at 40 °C for 30 min in a stirred 0.05 *M* phosphate buffer solution with 0.5 *M* KCl (pH 7.4). Oligonucleotide containing 8-oxoguanine of the sequence 5′GTCAACTTCC-8oksoG-TACCGAGC was obtained from Future Synthesis (Poland).

2.2. Oregano extract preparation

The test material, oregano leaves (*O. vulgare* L.), was ground in the Retsch GM200 mill (Retsch, Germany) at a pulse of 5000 rpm for 10 *s*. Water extract was obtained from the leaves by extraction. For this, the raw material (leaf powder) weighing 50 *g* was mixed with 1000 mL of water at 85 °C and extracted. The extraction was repeated three times, each time for 4 min. During each extraction, the extract was centrifuged and the supernatant was filtered (Whatman 1:11 μm). The obtained supernatants were combined and freeze-dried.

2.3. Electroanalytical assays

Electrochemical measurements were performed using the potentiostat PGSTAT12 with GPES version 4.9 software (Eco Chemie, Utrecht, the Netherlands) and a three-electrode system consisting of a carbon paste working electrode (CPE), Ag/AgCl (3 M KCl) reference electrode, and a platinum wire counter electrode. The carbon electrode (1-mm diameter) was made from carbon paste prepared by mixing the graphite powder with mineral oil at a ratio of 70:30 (w/w) using a method described previously [25]. The surface of the working electrode was renewed before use by removing the outer layer of carbon paste and polishing it to a smooth finish on a frosted glass microscope slide. Before electrochemical measurement, the surface of CPE was treated with 0.05 M phosphate buffer mixed with 0.01 M KCl (pH 7.0) at a potential of +1.7 V for 60 *s*.

The content of electroactive compounds in the oregano extract was determined by analyzing the redox signals on the voltammograms from cyclic voltammetry, CV (scan rate of 0.1 V/s) and square-wave voltammetry, SWV (step potential of 0.005 *V*, amplitude of 0.04 *V*, frequency of 50 Hz). The analysis was performed using 5% (CV) and 2.5% (SWV) oregano extract in phosphate buffer. The relative standard deviation (RSD) value calculated for 3 repetitions of measurements was in the range 5–9% (CV) and 4–7% (SWV), depending on the considered peak.

2.4. DNA biosensor to assess genoprotective effectiveness of oregano extract

The protective effectiveness of the oregano extract on nucleic acids against oxidative stress was evaluated using a DNA biosensor and the SWV technique (Fig. 1). The biosensor was prepared by immobilizing dsDNA on the surface of the CPE electrode through potential-assisted adsorption (+0.5 *V*, 120 *s*). The SWV measurement obtained on the detection layer after 120 *s* of rinsing in phosphate buffer at 200 rpm was treated as the DNA biosensor output signal. Then, using new detection layers, dsDNA was interacted with a 1% aqueous solution of H_2O_2 containing 0.1% $FeCl_2$ and the same solution prepared with 2.5% oregano extract. The time of interaction with DNA was 60 *s*, after which the electrode was washed in a pure buffer for 30 *s* and the SWV measurement was taken. The degree of oxidative damage to dsDNA was determined by analyzing the guanine and 8-oxoguanine signals current before and after the interaction of dsDNA with the solutions containing oxidizing agents. The RSD value calculated for 3 repetitions of measurements was in the range 5–8% (for guanine signal) and 6–11% (for 8-oxoguanine signal), depending on the performed measurement.

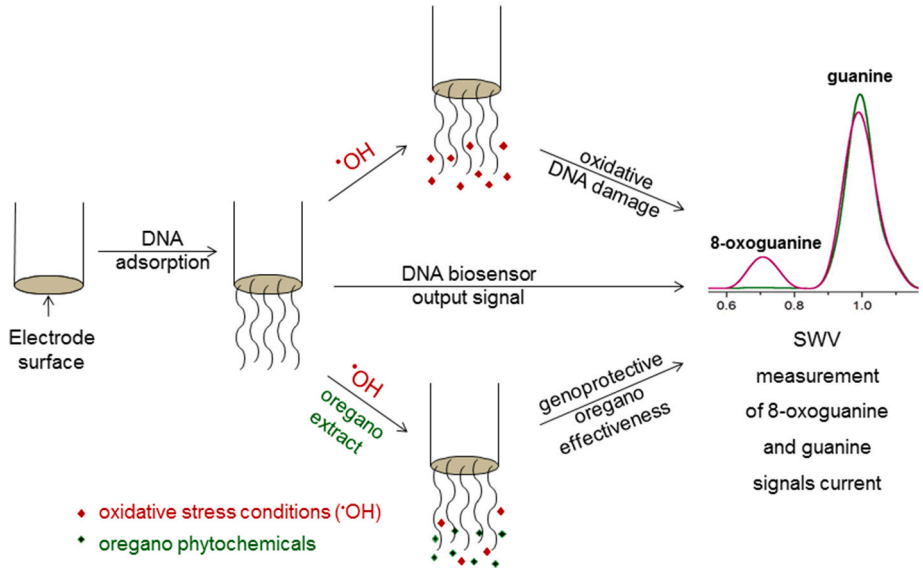

Fig. 1. Diagram illustrating the electrochemical DNA biosensor application to assess the genoprotective effectiveness of oregano extract.

2.5. Preparation of bread with oregano

To prepare the dough, 1000 g of the appropriate mixture (Table 1) was mixed with 600 mL of drinking water. Oregano leaf extract was added to the dough at an amount of 0.1%, 0.4%, and 0.7% in relation to the dry matter of ingredients in the dough, and the prepared samples were labeled as E1, E2, and E3, respectively. In addition, bread samples were prepared using crushed oregano leaves added at an amount of 2%, 3%, and 4%, and were labeled as L2, L3, and L4, respectively. The control sample prepared without the addition of oregano was labeled as KC.

Bread samples were examined on the first day after production and after 5 days of storage in closed foil packaging. The stored bread samples were labeled as E1S, E2S, and E3S (samples with the addition of oregano extract) and as L2S L3S, and L4S (samples with dried oregano leaves). The stored bread control was labeled as KCS. Samples were dried in a dryer at 60 °C to constant mass and then ground in the Retsch GM200 laboratory mill at a pulse of 5000 rpm for 10 s. The samples prepared in this way were extracted for a total of three times: the first time with 60% methanol at 20 °C and then twice with distilled water at 85 °C. The extraction was carried out using the Dionex ASE 350 apparatus (Thermo Fisher Scientific, USA). This apparatus works on the principle of accelerated solvent extraction at an elevated temperature and pressure. Following extraction, the content of biologically active compounds and antioxidant activity were determined in the obtained extracts.

2.6. Antioxidative potential analysis of bread with oregano

The antioxidant effect of bread extracts was determined by analyzing the DPPH-scavenging activity, ABTS-scavenging activity, chelating activity and indirectly by a method that allows evaluating the total content of compounds reacting with Folin-Ciocalteu reagent (POCH, Poland). The DPPH procedure was based on the reduction of absorbance of the DPPH solution at a wavelength of 517 nm in the presence of free radicals [26]. Measurements were performed using the SP-830 Plus apparatus (Metertech, Taiwan). The percentage of DPPH radical scavenging was evaluated on the basis of the standard curve y = 321.54x+21.54 (R^2 = 0.95), and the results were presented as Trolox equivalent (TE) in 1 *g* of the product (μM Tx/*g* d.m.). The ABTS cation radical-scavenging activity was measured according to the TEAC (Trolox Equivalent Antioxidant

Table 1
Composition of bread ingredients (%).

Ingredients	KC	E1	E2	E3	L2	L3	L4
Oregano extract	0.0	0.1	0.4	0.7	0.0	0.0	0.0
Oregano leaves	0.0	0.0	0.0	0.0	2.0	3.0	4.0
Wheat flakes	2.7	2.7	2.7	2.7	2.7	2.7	2.7
Corn flour	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Millet	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Broken soya beans	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Soybean flour	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Wheat malt flour	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Lactic acid	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Citric acid	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Sugar	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Emulsifier E472—mono- and di-fatty acid glycerides	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Rapeseed lecithin	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Stabilizer E466—sodium carboxymethylcellulose	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Ascorbic acid	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Wheat flour	49.1	49	48.7	48.4	47.1	46.1	45.1
Rye flour	26.0	26.0	26.0	26.0	26.0	26.0	26.0
Yeast	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Low-sodium salt	1.0	1.0	1.0	1.0	1.0	1.0	1.0

KC—control bread; E1, E2, and E3—bread samples with addition of oregano extract at an amount of 0.1%, 0.4%, and 0.7%, respectively; L2, L3, and L4—bread samples with addition of ground oregano leaves at an amount of 2%, 3%, and 4%, respectively.

Capacity) test using the method described by Kulczyński et al. [27]. Spectrophotometric measurement of the ability to scavenge $ABTS^{\bullet+}$ formed from ABTS by oxidation with potassium persulfate was carried out at a wavelength of 414 nm using the SPECORD® 40 spectrophotometer, Analytik Jena. The percentage rate of $ABTS^{\bullet+}$ scavenging was calculated from the standard curve $y = 121.63x+26.33$ ($R^2 = 0.97$), and the results were expressed as TE in 1 *g* of the product (*μM* Tx/*g* d.m.).

Chelating activities were determined according to the method of Tang et al. [28]. The colorimetric assay used involved the determination of the amount of unchelated Fe^{2+} in the crude asparagus extract after reaction with 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine monosodium salt (i.e., ferrozine). For this assay, test tubes were filled with 1 mL sample, 0.1 mL of 2 mM $FeCl_2$, and 0.2 mL of ferrozine reagent. The mixture was vortexed for ~60 *s* and left to stand for 20 min at room temperature. Subsequently, absorbance values were recorded at λ = 562 nm using the Metertech SP-830 apparatus (Taiwan). The control consisted of deionized water instead of the extract aliquot, and ferrozine was used as a reference. The chelating activity was calculated according to the following formula:

$$\text{Chelating activity} = 1-[(\text{Abs of sample}-\text{Abs of reference})/\text{Abs of control})] \times 100$$

2.7. Inhibitory effect of bread extract on cholinesterases activity

The activity of extracts as acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitors was determined by the spectrometric method described by Ellman et al. [29] with the modifications proposed by Kobus-Cisowska et al. [26]. Measurements were carried out with the POLARstar Omega (BMG LABTECH) plate reader on 96-well plates using a maximum volume of 300 μL. Hydrolysis of acetylthiocholine/butyrylthiocholine carried out by the examined enzymes caused a change of color, which was determined by measuring the absorbance at a wavelength of 412 nm, 10 min after pipetting onto a microtiter plate. The solution that was used for spectrophotometric analysis contained 1 mL of phosphate buffer (0.1 *M*, pH 8.0), 50 μL of DTNB dye (0.01 *M*), 50 μL of acetylthiocholine iodide (or butyrylthiocholine iodide), 10 μL of AChE enzyme (or BChE), and 50 μL of the test sample. The enzyme solutions were prepared by dissolving 2 U/*mL* in 2 mL of phosphate buffer. Eserine solution was used as a reference. The results were expressed as *μM* eserine/*g* dw of extracts from bread fortified with oregano.

2.8. Statistical analysis

The mean values of the examined traits were compared using the analysis of variance for factor systems with a different number of observations, and the intergroup differences were evaluated using Tukey's test or Spjotvoll's test (extended Tukey's test for an uneven number of samples). It was also checked whether the assumptions of this analysis were met. Pearson's correlation coefficients were calculated to assess the strength of the relationships between the tested samples. STATISTICA PL 13.1 (StatSoft, Inc., Krakow, Poland) software was used for the principal component analysis (PCA). The significance of the correlation coefficient was checked using Student's *T*-test. Statistical analysis was carried out at a significance level of $\alpha = 0.05$.

3. Results and discussion

3.1. Evaluation of the electrochemical activity of oregano extract

The content of electroactive compounds in oregano extract was evaluated on the basis of voltammetric measurements using cyclic (CV) and square wave voltammetry (SWV). CV allows obtaining qualitative information about the chemical reaction and checking its reversibility. The dependence of oxidation and reduction signals of the studied analytes on their position on the potential axis was obvious in the voltammograms. Four signals were recorded for the anode current and two for the cathode current, which are marked in red and blue, respectively, on the voltammogram obtained for the oregano extract (Fig. 2A).

The oxidation signals determined on the CV voltammogram indicated the high electrochemical activity of the oregano extract. Anode signals 3 and 4 had a current above 10^5 A. The lowest oxidation potential within the determined range was found for the signal located at the region of −0.16 ***V***. The extract was also evaluated using the SWV technique (Fig. 2B). This very sensitive technique makes it possible to determine analytes at a very low concentration (10^{-8} *M*). The high sensitivity of this method results from the fact that the influence of capacitive current on the obtained results is eliminated by sampling the current in the form of pulses and measuring the current at the end of each pulse. In this study, the SWV voltammogram also showed four signals confirming the presence of four active centers undergoing redox reactions in the applied potential range. The clearest signals were located at +0.40 and + 0.94 *V*. In addition, two signals were visible in the region of negative potential at −0.59 and −0.31 *V*. This indicated the high ability of compounds present in the oregano extract to yield electrons and reduce other molecules, including ROS, and the ability of these compounds to regenerate endogenous antioxidants, such as tocopherols, vitamin C, or glutathione [15,16].

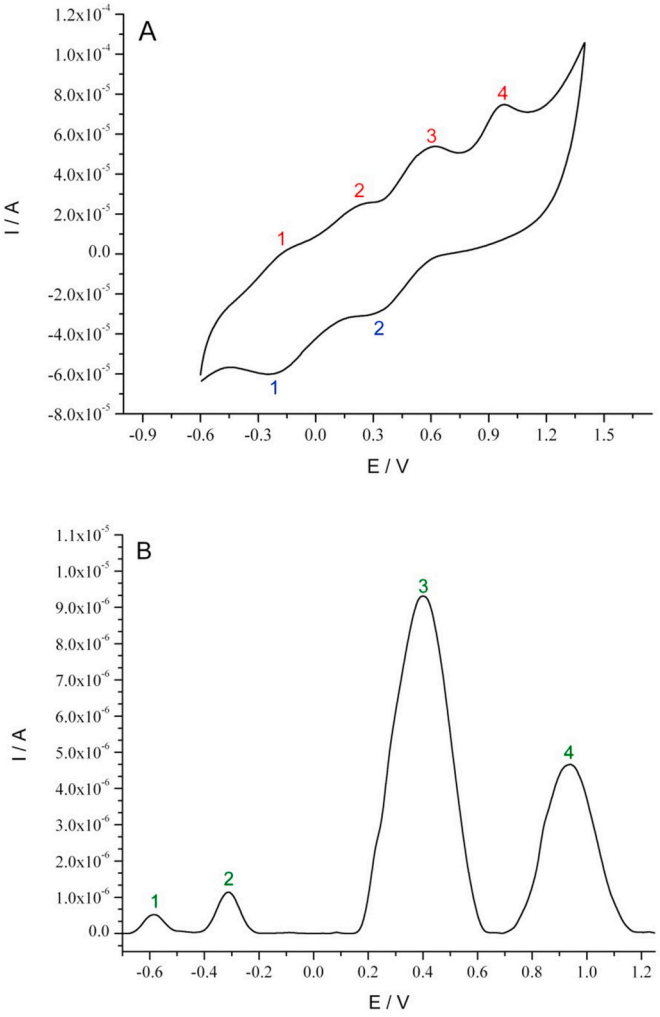

Fig. 2. A: CV voltammogram of 5% oregano extract (scan rate of 0.1 V/s). **B:** SWV voltammogram of 2.5% oregano extract (step potential of 0.005 V, amplitude of 0.04 V, frequency of 50 Hz). Measurements were performed in 0.05 M phosphate buffer with 0.01 M KCl (pH 7.0).

3.2. Protection of nucleic acids during oxidative stress by the oregano extract

Hydroxyl radical is the most reactive molecule that can be formed in cells and body fluids. It is formed when hydrogen peroxide reacts with Fe^{2+} ions. The redox potential of OH radicals, which are strong oxidants, depends on pH and usually varies between +2.1 and + 2.3 *V* [30]. This means that the hydroxyl radicals can oxidize compounds that have a lower redox potential, which practically include all the components of living cells. The resulting radicals may cause oxidative damage to DNA bases [12,31,32]. In this study, the undamaged nucleotide bases, bound in the dsDNA, generated two signals that were clearly visible on the SWV voltammograms in the region at about +1.0 and + 1.3 *V*. The first and the most intense signal originated from the oxidation reaction of guanine, and the second from adenine (Fig. 3). In previous studies, guanine signal was treated as an indicator of dsDNA oxidative damage induced by hydroxyl radicals. An additional marker of DNA damage that has been considered in the studies is the 8-oxoguanine signal. The guanine derivative 8-oxoguanine can pair with adenine. Its presence in the DNA chain may be a cause of mutagenic changes, and its accumulation with age may trigger many diseases [7,31,33]. Therefore, it is extremely important to evaluate the protection of genetic material against the formation of this oxidized guanine derivative. However, the 8-oxoguanine signal is relatively rarely considered when evaluating the antioxidant properties of plant extracts, and as has already been shown, it may be an important indicator of the health-enhancing properties of the analyzed compounds [13,34–36]. The results of the present study showed that the degree of damage of DNA immobilized in the detection layer of the sensor was significantly lower in the presence of 2.5% oregano leaf extract.

Fig. 3 shows an oligonucleotide signal containing 8-oxoguanine to determine the potential of its position under the applied measurement conditions. The signal of this derivative was also recorded after the interaction of dsDNA with the solution containing H_2O_2 and $FeCl_2$. The oxidative stress caused a significant decrease in the current of guanine signal. However, if the solution containing additionally 2.5% oregano leaf extract, the 8-oxoguanine signal was not observed, whereas the guanine signal was much intense than that observed with the solution lacking oregano extract. These results indicate the high ability of the components contained in oregano to protect the genetic material against strong oxidative stress.

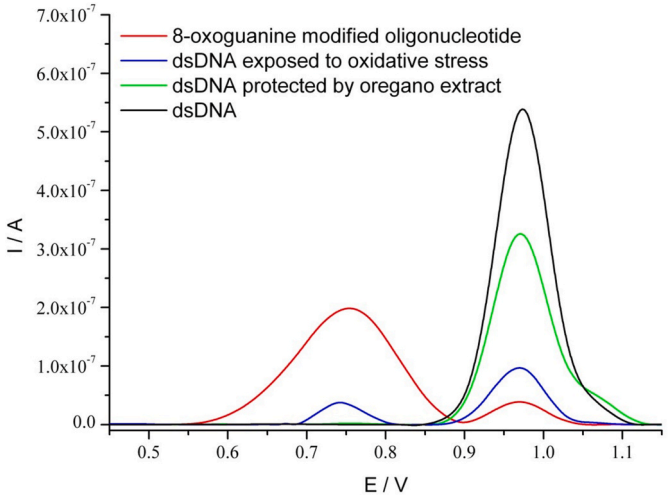

Fig. 3. SWV voltammograms of 10 μM oligonucleotide containing 8-oxoguanine and 10 μM dsDNA before and after interaction with 1% H_2O_2 containing 0.1% $FeCl_2$ or with 1% H_2O_2 containing 0.1% $FeCl_2$ and 2.5% oregano extract. SWV parameters are the same as described in Fig. 2B.

3.3. Antioxidant activity of the bread with oregano

Taking into account the high electroactivity and antioxidative potential of compounds contained in the oregano extract, which were determined on the basis of voltammetric measurements, their ability to bind free $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radicals was evaluated using the bread samples prepared with the addition of oregano. Bread is an excessively complex matrix to be used for electrochemical measurements; therefore, the antioxidant properties of this product were determined using chemical methods. The antioxidant capacity of the extracts was expressed in *μM* Trolox/*g* d.m. of the bread. In addition, the chelating activity and total content of compounds reacting with Folin-Ciocalteu reagent were determined in the bread samples, and the data are presented in Table 2.

It was observed that the addition of oregano had a statistically significant effect on the antiradical activity of the baked bread. Moreover, it was found that after storage the chelating activity against $DPPH^{\bullet}$ was higher, whereas the activity against $ABTS^{\bullet+}$ was lower. The addition of oregano increased the chelating activity of the prepared bread samples, but the activity was also affected by other ingredients, which probably had a synergistic effect. A statistically significant increase in the chelating activity was found in the bread samples stored for 5 days. In addition, the study showed that the addition of oregano extract and oregano leaves increased the content of polyphenols in the bread samples in the range of 50–68%. It was also observed that the polyphenols contained in the samples remained stable during the storage period.

The antioxidant effect of oregano in the prepared bread samples was found to be related to the presence of phenolic compounds. The results of statistical analysis (not shown in the paper) showed a positive correlation between total polyphenols and the level of inactivation of DPPH radicals ($r = 0.77$ and $r = 0.91$), ABTS-scavenging activity ($r = 0.79$ and $r = 0.91$), and chelating activity ($r = 0.81$ and $r = 0.95$).

The antioxidant effect of polyphenols in oregano has been documented in a previous study, which determined this parameter in the prepared samples of bread [37]. In the study, bread prepared without oregano also showed antioxidant activity which was measured using the same methods as that used for oregano-containing bread. The bread samples were made of wheat flour (49%) and rye flour (26%). The presence of polyphenols in kernels was noted mainly in fruit and seed cover, aleurone cells, husk, and rarely in the embryo. The basic raw material used for the production of bread was whole-grain wheat flour, in which the main polyphenolic compound present was tricin (5,7,

Table 2
Antioxidant properties, chelating activity, and total phenolic content of oregano leaves and extracts and fortified bread.

Sample	DPPH (μM Tx/g d.m.)	ABTS (μM Tx/g d.m.)	Chelating activity 50 μg/mL (%)	Total content of phenolic compounds (μg GAE/g d.m.)
KC	$5.75^{a}\pm1.45$	$2.07^{a}\pm0.21$	$2.54^{a}\pm0.02$	$3.65^{a}\pm0.09$
E1	$7.56^{c}\pm1.22$	$2.45^{b}\pm0.07$	$2.77^{b}\pm0.05$	$5.52^{b}\pm0.07$
E2	$7.98^{d}\pm1.31$	$2.76^{b}\pm0.44$	$3.22^{c}\pm0.04$	$5.87^{b,c}\pm0.04$
E3	$8.43^{e}\pm1.39$	$2.81^{b}\pm0.31$	$3.53^{c}\pm0.07$	$5.99^{b,c}\pm0.21$
L2	6.84 ± 1.22	$2.22^{a}\pm0.26$	$2.97^{b}\pm0.06$	$5.12^{b}\pm0.28$
L3	$6.98^{b}\pm1.08$	$2.54^{b}\pm0.22$	$2.99^{b}\pm0.03$	$5.32^{b}\pm0.31$
L4	$7.11^{c}\pm1.03$	$2.62^{b}\pm0.08$	$3.26^{c}\pm0.02$	$5.54^{b}\pm0.09$
KCS	$5.84^{a}\pm1.15$	$1.87^{a}\pm0.11$	$3.02^{b,c}\pm0.05$	$3.55^{a}\pm0.11$
E1S	$7.76^{c,d}\pm1.63$	$2.44^{b}\pm0.15$	$3.16^{c}\pm0.07$	$5.63^{b}\pm0.22$
E2S	$8.12^{e}\pm1.19$	$2.52^{b}\pm0.18$	$2.34^{a}\pm0.01$	$5.97^{b,c}\pm0.12$
E3S	$8.22^{e}\pm1.22$	$2.79^{c}\pm0.04$	$3.43^{c}\pm0.03$	$6.12^{c}\pm0.31$
L2S	$6.71^{b}\pm1.15$	$2.03^{a}\pm0.10$	$2.78^{b}\pm0.04$	$5.11^{b}\pm0.20$
L3S	$6.55^{b}\pm1.41$	$2.25^{a}\pm0.11$	$2.99^{b}\pm0.02$	$5.38^{b}\pm0.22$
L4S	$6.78^{b}\pm1.47$	$2.38^{b}\pm0.05$	$3.21^{c}\pm0.04$	$5.49^{b}\pm0.09$

Results are presented as mean value ± standard deviation (n = 4); mean values designated by different lowercase (a-e) letters and placed in the same column differ statistically ($p \leq 0.05$).

4′-trihydroxy-3′,5′-dimethoxy flavone). Tricin and other compounds could interact with oregano polyphenols. Active compounds in bread, which exhibited the antioxidants activity, were identified to function through different mechanisms.

3.4. Inhibitory activity of bread on cholinesterases

Bread samples were evaluated for the inhibitory effect on cholinesterases, AChE and BChE, and the results of the analyses are presented in Fig. 4. It was found that depending on the form used (extract or leaves), oregano influenced the inhibitory activity of bread on cholinesterase. At

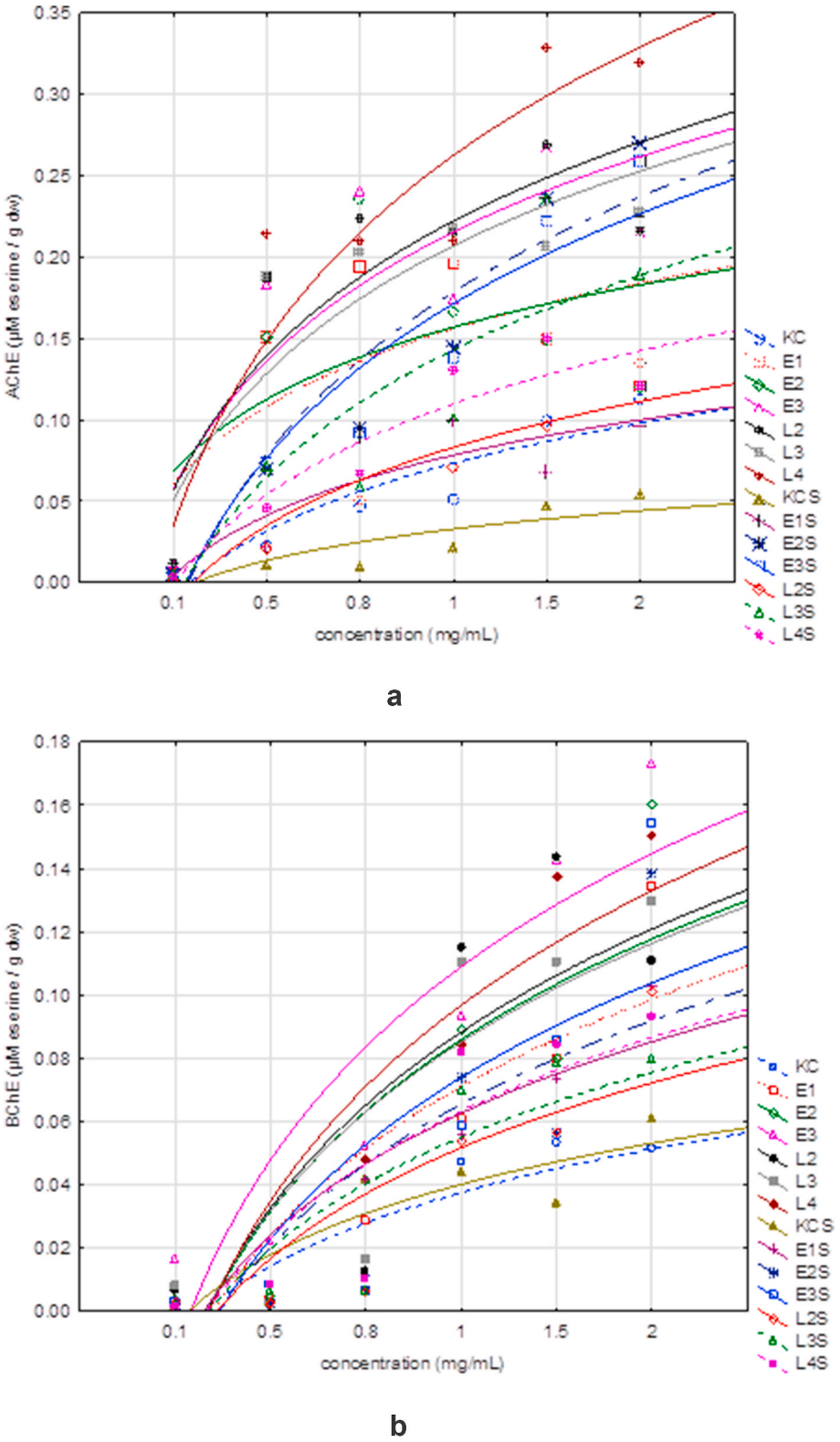

Fig. 4. Inhibitory activity of extracts from bread fortified with oregano on cholinesterases.

the same time, it was observed that both the leaf extract and whole leaves had a higher influence on AChE inhibition compared to the inhibitory effect on BChE.

All samples of bread prepared with oregano leaves showed a statistically significantly higher inhibitory activity compared to the bread samples produced with oregano leaf extract. In the case of all the analyzed extracts, the inhibitory activity was noticeable only at the second analyzed concentration (0.5 mg/mL), and the measured inhibitory activity of bread prepared with whole leaves was on average 15–25% higher compared to the samples prepared with leaf extracts. Concurrently, it was found that the inhibitory activity of the bread samples toward the enzymes studied was decreased during the 5-day storage and was on average 23–52% lower than the activity observed on the first day after baking.

Currently, numerous studies on the use of plant extracts as functional ingredients that can act as inhibitors of AChE and BChE in neurodegenerative diseases are in progress [26,38]. An example of plants studied for this purpose is *O. vulgare*, the extract of which was reported to be as effective as donepezil, rivastigmine, and galantamine in behavioral tests in animal and human studies and was additionally devoid of major side effects [39,40]. The present study also explains one of the mechanisms of action of the compounds present in oregano, the inhibition of cholinesterases. Oregano has two important fractions of biologically active compounds. One is the water fraction, constituted by flavonol and phenolic acids, which may affect the functions of the central nervous system. The second fraction is formed by essential oils, the health-enhancing effect of which has been documented in some studies [39,41]. The results of the present study indicated, however, that cholinesterase-inhibiting activity of oregano depends on the type of method used for extraction. Higher inhibitory activity was found on AChE, especially with the use of oregano leaves, while lower activity was observed with water extracts. As shown by the results, despite the fact that the bread samples prepared with extract showed higher antiradical activity, the higher activity against enzymes was observed only in the samples prepared with whole leaves. The ability of chlorogenic acid and 3- and 4-hydroxybenzoic acid to inhibit the activity of cholinesterases was documented in the study of Szwajgier [42]. It was found that the inhibitory activity is influenced by the distribution and number of –OH and/or $–OCH_3$ groups substituted in the phenyl ring. Moreover, the methyl or ethyl esters of phenolic acids were found to be more effective inhibitors of cholinesterases than the corresponding free acids. Therefore, the use of oregano as a spice and as a component of the diet recommended for patients treated for neurodegenerative diseases may influence the course and development of these conditions.

4. Conclusions

The health-enhancing effects of food components are an interesting research topic. Their protective effect on cellular structures against free-radical damages deserves a special mention because these damages accelerate the broadly understood aging process of an organism. The protective aspect of the functional diet components in relation to the genetic material is particularly important in the prevention of a number of chronic diseases related to the neurodegenerative processes. The mutations of genetic material caused by oxidative stress processes, including an increased level of 8-oxoguanine which is considered as an indicator of oxidative DNA damage, are associated with a growing risk of serious diseases, such as cancer, atherosclerosis, or neurodegenerative or metabolic diseases. Increased oxidative stress in people of childbearing age not only contributes to fertility problems but also leads to the transfer of inferior-quality genetic material to offspring. Premature aging, which significantly reduces the quality of life and is associated with the chemicalization of the environment and consumed food, has led to the search for natural sources of ingredients that could prevent these unfavorable changes. Oregano is an herb and a spice that has been used for a long time. However, its health-enhancing effects have not been thoroughly studied. The present study revealed that the ingredients contained in oregano can protect the genetic material against oxidative damage that leads to the accumulation of 8-oxoguanine. The compounds present in oregano were found to exhibit antioxidant activity, even after technological processing associated with bread production. Another important result of the study was that oregano has an inhibitory effect on cholinesterases, which is important in the prevention and treatment of many diseases, including AD. The demonstrated properties of oregano prove that it can serve as a significant ingredient in functional food with a broad spectrum of health-enhancing properties, which are especially helpful in the prevention and treatment of neurodegenerative diseases.

Author contribution

Conceptualization: Marta Ligaj and Joanna Kobus-Cisowska; Data curation: Marta Ligaj and Maciej Jarzębski; Formal analysis: Marta Ligaj, Oskar Szczepaniak, Anna Mikołajczak-Ratajczak and Monika Przeor; Funding acquisition: Maciej Jarzębski and Joanna Kobus-Cisowska; Investigation: Joanna Kobus-Cisowska, Anna Mikołajczak-Ratajczak and Patryk Bykowski; Methodology: Marta Ligaj; Project administration: Joanna Kobus-Cisowska; Resources: Marta Ligaj and Joanna Kobus-Cisowska; Software: Oskar Szczepaniak; Supervision: Maciej Jarzębski, Krzysztof Polewski; Validation: Daria Szymanowska and Dariusz Kikut-Ligaj; Visualization: Marta Ligaj and Dariusz Kikut-Ligaj; Writing – original draft: Marta Ligaj, Joanna Kobus-Cisowska and Monika Przeor; Writing – review & editing: Oskar Szczepaniak and Maciej Jarzębski.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] E. Mariani, M.C. Polidori, A. Cherubini, P. Mecocci, Oxidative stress in brain aging, neurodegenerative and vascular diseases: an overview, J. Chromatogr. B 827 (2005) 65–75, https://doi.org/10.1016/j.jchromb.2005.04.023.
[2] E. Niedzielska, I. Smaga, M. Gawlik, A. Moniczewski, Oxidative stress in neurodegenerative diseases, Mol. Neurobiol. 53 (2016) 4094–4125, https://doi.org/10.1007/s12035-015-9337-5.
[3] Z. Sabahi, F. Soltani, M. Moein, Insight into DNA protection ability of medicinal herbs and potential mechanisms in hydrogen peroxide damages model, Asian Pac. J. Trop. Biomed. 8 (2018) 120–129, https://doi.org/10.4103/2221-1691.225616.
[4] V. Diculescu, A.M. Paquim, A.M. Brett, Electrochemical DNA sensors for detection of DNA damage, Sensors 5 (2005) 377–393, https://doi.org/10.3390/s5060377.
[5] M. Fojta, A. Daňhel, L. Havran, V. Vyskočil, Recent progress in electrochemical sensors and assays for DNA damage and repair, TrAC Trends Anal. Chem. (Reference Ed.) 79 (2016) 160–167, https://doi.org/10.1016/j.trac.2015.11.018.
[6] V.I. Bruskov, L.V. Malakhova, Z.K. Masalimov, A.V. Chernikov, Heat-induced formation of reactive oxygen species and 8-oxoguanine, a biomarker of damage to DNA, Nucleic Acids Res. 30 (2002) 1354–1363, https://doi.org/10.1093/nar/30.6.1354.
[7] C.L. Simms, H.S. Zaher, Quality control of chemically damaged RNA, Cell. Mol. Life Sci. 73 (2016) 3639–3653, https://doi.org/10.1007/s00018-016-2261-7.
[8] L.J. Martin, DNA damage and repair, J. Neuropathol. Exp. Neurol. 67 (2008) 377–387, https://doi.org/10.1097/NEN.0b013e31816ff780.
[9] R. Madabhushi, L. Pan, L.H. Tsai, DNA damage and its links to neurodegeneration, Neuron 83 (2014) 266–282, https://doi.org/10.1016/j.neuron.2014.06.034.
[10] M. Fojta, Detecting DNA damage with electrodes, in: E. Palecek, F. Scheller, J. Wang (Eds.), Electrochemistry of Nucleic Acids and Proteins - towards Electrochemical Sensors for Genomics and Proteomics, Elsevier, Amsterdam, 2005, pp. 385–431.
[11] S. Kumari, R.P. Rastogi, K.L. Singh, S.P. Singh, R.P. Sinha, DNA damage: detection strategies, EXCLI J 7 (2008) 44–62, https://doi.org/10.17877/DE290R-8293.

[12] A. Hájková, J. Barek, V. Vyskočil, Electrochemical DNA biosensor for detection of DNA damage induced by hydroxyl radicals, Bioelectrochemistry 116 (2017) 1–9, https://doi.org/10.1016/j.bioelechem.2017.02.003.
[13] L. Hlavatá, V. Vyskočil, K. Beníková, M. Borbélyová, J. Labuda, DNA-based biosensors with external nafion and chitosan Membranes for the evaluation of the antioxidant activity of beer, coffee, and tea, Cent. Eur. J. Chem. 12 (2014) 604–611, https://doi.org/10.2478/s11532-014-0516-4.
[14] M.F. Barroso, C. Delerue-Matos, M.B.P.P. Oliveira, Electrochemical evaluation of total antioxidant capacity of beverages using a purine-biosensor, Food Chem. 132 (2012) 1055–1062, https://doi.org/10.1016/j.foodchem.2011.10.072.
[15] M. Kondolot, E.N. Ozmert, A. Ascı, P. Erkekoglu, D.B. Oztop, H. Gumus, et al., Plasma phthalate and bisphenol a levels and oxidant-antioxidant status in autistic children, Environ. Toxicol. Pharmacol. 43 (2016) 149–158, https://doi.org/10.1016/j.etap.2016.03.006.
[16] C. Guo, P. Ding, C. Xie, C. Ye, M. Ye, C. Pan, et al., Potential application of the oxidative nucleic acid damage biomarkers in detection of diseases, Oncotarget 8 (2017) 75767–75777, https://doi.org/10.18632/oncotarget.20801.
[17] L. Mello, L. Kubota, Biosensors as a tool for the antioxidant status evaluation, Talanta 72 (2007) 335–348, https://doi.org/10.1016/j.talanta.2006.11.041.
[18] N. Hu, J.T. Yu, L. Tan, Y.L. Wang, L. Sun, L. Tan, Nutrition and the risk of Alzheimer's disease, BioMed Res. Int. 524820 (2013) 1–12, https://doi.org/10.1155/2013/524820.
[19] D. Sani, N.I.O. Khatab, B.P. Kirby, A. Yong, S. Hasan, H. Basri, et al., A standardised *Andrographis paniculata* Burm. Nees aqueous extract prevents Lipopolysaccharide-induced cognitive deficits through suppression of inflammatory cytokines and oxidative stress mediators, J. Adv. Res. 16 (2018) 87–97, https://doi.org/10.1016/j.jare.2018.11.005.
[20] L. Canniatti Brazaca, I. Sampaio, V. Zucolotto, B.C. Janegitz, Applications of biosensors in Alzheimer's disease diagnosis, Talanta 210 (2020) 120644, https://doi.org/10.1016/j.talanta.2019.120644.
[21] P.T. Francis, A.M. Palmer, M. Snape, G.K. Wilcock, The cholinergic hypothesis of Alzheimer's disease: a review of progress, J. Neurol. Neurosurg. Psychiatry 66 (1999) 137–147, https://doi.org/10.1136/jnnp.66.2.137.
[22] A. Kalb, C. von Haefen, M. Sifringer, A. Tegethoff, N. Paeschke, M. Kostova, et al., Acetylcholinesterase inhibitors reduce neuroinflammation and degeneration in the cortex and Hippocampus of a surgery stress rat model, PloS One 8 (2013) 1–13, https://doi.org/10.1371/journal.pone.0062679.
[23] N. Leyva-Lopez, E.P. Gutierrez-Grijalva, G. Vazquez-Olivo, J.B. Heredia, Essential oils of oregano: biological activity beyond their antimicrobial properties, Molecules 22 (2017) 1–24, https://doi.org/10.3390/molecules22060989.
[24] C. Lee, J. Jeong, H. Kim, Y. Kim, Enhanced superoxide sensitivity in organic field-effect transistor sensors by introducing nanoclay-polyphenol-polymer hybrid sensing channels, J. Hazard Mater. 374 (2019) 159–166, https://doi.org/10.1016/J.JHAZMAT.2019.04.013.
[25] M. Ligaj, M. Tichoniuk, M. Filipiak, Detection of *bar* gene encoding phosphinothricin herbicide resistance in plants by electrochemical biosensor, Bioelectrochemistry 74 (2008) 32–37, https://doi.org/10.1016/j.bioelechem.2008.03.003.
[26] J. Kobus-Cisowska, D. Szymanowska, P. Maciejewska, D. Kmiecik, A. Gramza-Michałowska, B. Kulczyński, et al., *In vitro* screening for acetylcholinesterase and butyrylcholinesterase inhibition and antimicrobial activity of chia seeds (*Salvia hispanica*), Electron, J. Biotechnol. 37 (2019) 1–10, https://doi.org/10.1016/j.ejbt.2018.10.002.
[27] B. Kulczyński, J. Kobus-Cisowska, D. Kmiecik, A. Gramza-Michałowska, D. Golczak, J. Korczak, Antiradical capacity and polyphenol composition of asparagus spears varieties cultivated under different sunlight conditions, Acta Sci. Pol. Technol. Aliment. 15 (2016) 267–279, https://doi.org/10.17306/J.AFS.2016.3.26.
[28] S. Tang, J. Kerry, D. Sheehan, D. Buckley, Antioxidative mechanisms of tea catechins in chicken meat systems, Food Chem. 76 (2002) 45–51, https://doi.org/10.1016/S0308-8146(01)00248-5.
[29] G.L. Ellman, K.D. Courtney, V. Andres, R.M. Featherstone, A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem. Pharmacol. 7 (1961) 88–95, https://doi.org/10.1016/0006-2952(61)90145-9.
[30] A. Ohara, M. Sayuri, Oxygen radicals and related species, in: K. Pantopoulos, H. M. Schipper (Eds.), Principles of Free Radical Biomedicine, Nova Science Publishers, Inc., New York, 2012, pp. 1–23.
[31] L. Hlavata, K. Benikova, V. Vyskocil, J. Labuda, Evaluation of damage to DNA induced by UV-C radiation and chemical agents using electrochemical biosensor based on low molecular weight DNA and screen-printed carbon electrode, Electrochim. Acta 71 (2012) 134–139, https://doi.org/10.1016/j.electacta.2012.03.119.
[32] Z. Zhao, Iron and oxidizing species in oxidative stress and Alzheimer's disease, Aging Med. 2 (2019) 82–87, https://doi.org/10.1002/agm2.12074.
[33] T. Finkel, N.J. Holbrook, Oxidants, Oxidative stress and the biology of ageing, Nature 408 (2000) 239–247, https://doi.org/10.1038/35041687.
[34] D. Faust, T. Nikolova, W. Wätjen, B. Kaina, C. Dietrich, The *Brassica*-derived phytochemical indolo[3,2-b]carbazole protects against oxidative DNA damage by aryl hydrocarbon receptor activation, Arch. Toxicol. 91 (2017) 967–982, https://doi.org/10.1007/s00204-016-1672-4.
[35] R. Lagoa, D. Marques-da-Silva, M. Diniz, M. Daglia, A. Bishayee, Molecular mechanisms linking environmental toxicants to cancer development: significance for protective interventions with polyphenols, Semin. Canc. Biol. (2020), https://doi.org/10.1016/j.semcancer.2020.02.002 (In press).
[36] O. Szczepaniak, M. Ligaj, J. Kobus-Cisowska, M. Tichoniuk, M. Dziedziński, M. Przeor, P. Szulc, The genoprotective role of naringin, Biomolecules 10 (2020) 700, https://doi.org/10.3390/biom10050700.
[37] M. Hao, T. Beta, Development of Chinese steamed bread enriched in bioactive compounds from barley hull and flaxseed hull extracts, Food Chem. 133 (2012) 1320–1325, https://doi.org/10.1016/j.foodchem.2012.02.008.
[38] J. Kobus-Cisowska, D. Szymanowska, P. Maciejewska, O. Szczepaniak, D. Kmiecik, A. Gramza-Michałowska, et al., Enriching novel dark chocolate with *Bacillus coagulans* as a way to provide beneficial nutrients, Food Funct 10 (2019) 997–1006, https://doi.org/10.1039/C8FO02099J.
[39] M.R. Loizzo, F. Menichini, F. Conforti, R. Tundis, M. Bonesi, A.M. Saab, et al., Chemical analysis, antioxidant, antiinflammatory and anticholinesterase activities of *Origanum ehrenbergii* Boiss and *Origanum syriacum* L. essential oils, Food Chem. 117 (2009) 174–180, https://doi.org/10.1016/j.foodchem.2009.03.095.
[40] I.E. Orhan, R. Belhattab, F.S. Şenol, A.R. Gülpinar, S. Hoşbaş, M. Kartal, Profiling of cholinesterase inhibitory and antioxidant activities of *Artemisia absinthium, A. herba-alba, A. fragrans, Marrubium vulgare, M. astranicum, Origanum vulgare* subsp. *glandulossum* and essential oil analysis of two *Artemisia* species, Ind. Crop. Prod. 32 (2010) 566–571, https://doi.org/10.1016/j.indcrop.2010.07.005.
[41] C. Sarikurkcu, G. Zengin, M. Oskay, S. Uysal, R. Ceylan, A. Aktumsek, Composition, antioxidant, antimicrobial and enzyme inhibition activities of two *Origanum vulgare* subspecies (subsp. *vulgare* and subsp. *hirtum*) essential oils, Ind. Crop. Prod. 70 (2015) 178–184, https://doi.org/10.1016/j.indcrop.2015.03.030.
[42] D. Szwajgier, Anticholinesterase activity of phenolic acids and their derivatives, Z. Naturforsch. C Biosci. 68 (2013) 125–132, https://doi.org/10.1515/znc-2013-3-408.