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ANTIOXIDATIVE EFFECT OF PLANT EXTRACTS AND FLAVONES ON LIPOSOME AND ERYTRHOCYTE MEMBRANES

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The paper describes the use of liposomes and erythrocytes membrane as a real membrane models to evaluate the potential benefits of several plants extracts and two flavones in relation to lipid peroxidation. The antioxidant behaviour of the plant extracts from pine (*Pinus silvestris* L), hawthorn (*Crataegus oxyacantha* L, two extracts: from hawthorn's leaves-l and bark-b), evening primrose (*Oenothena paradoxa* Hudziok – three extracts differ in procyanidins content P1, P2 and P3) and rosemary (*Rosmarinus officinalis,* as a standard for extracts) and flavones of baicalin and rutin have been studied. The results obtained showed that the studied extracts exhibited differentiated, dose-dependent antioxidant activity against phospatidylcholine liposomes (rosemary>pine≈hawthorn-l>hawthorn-b≈P1≈P2≈P3; statistically significant differences were observed between the extracts at $p \leq 0.05$) and erythrocyte membranes (rosemary≥hawthorn-b≈hawthorn-l>P1≈pine>P2≈P3) when the oxidation was induced by UV-C radiation. They also reduce the oxidation of liposomes and erythrocyte membrane when its oxidation was induced by 2,2'-azobis(2-amidinopropane) dihydrochloride (pine≥P1≈P2≈rosemary≈P3 in the case of liposomes and rosemary>> pine≥P1≈P2≥P3 in a case of erythrocyte). Moreover, the results of the study show that baicalin is characterised by high inhibition ability towards liposome PC peroxidation, as well as towards erythrocyte ghosts, when oxidation was initiated by UV radiation. However, at the same experimental conditions, the inhibitory capacity of rutin was about 7-8 times weaker. The presence of cholesterol in liposome membrane decreased the level of membrane peroxidation but do not influenced on the antioxidant activity of hawthorn extract.

INTRODUCTION

Free radicals and active forms of oxygen, involved in physiological and biochemical processes, are formed due to continuous use of oxygen by aerobic cells and their levels are kept under control by different endogenous antioxidants. The consequence of the continuous exposition of biological systems to an uncontrolled oxygen radical flux are damages, aging and age-related physiological disorders, and, ultimately cell death [Piotrowski *et al*., 1990; Bonina *et al*., 1996; Jellinger, 1999; Osawa, 1999; Christen, 2000; Sastre *et al*., 2000]. The oxidative stress mainly affects the membrane systems of cells, being the phospholipids the most unstable molecules of living organisms. Thus, the lipid peroxidation is the common consequence of free radicals-mediated chain reactions [Buege & Aust, 1978; Bose & Chatterjee, 1995]. The products of peroxidation such as hydroperoxides and various species that contain unpaired electrons possess protein oxidation, DNA damaging potential and carcinogenic activity [Yang & Schaich, 1996; Wieseman & Halliwel, 1996; Mc Vean & Lieber, 1997; Skupień *et al*., 2006]. It is known the protection of organisms against free radical toxicity by exo- and endogenous compounds and that dietary antioxidant reduce the risk of many diseases such cardiovascular disorder, cancer, chronic inflammation and others [Hertog *et al.*, 1995; Wiseman & Halliwel, 1996, Bonina *et al*., 1996; Kamei *et al*., 1998]. Moreover, an intensive search for novel natural antioxidants from plant extracts is now in course since endogenous antioxidant in plants must play an important role in their antioxidant defence [Peterson & Dwyer, 1998; Rice-Evans *at al*., 1996; Osawa, 1999; Gabrielska & Oszmiański, 2005]. Natural antioxidants, principally, do not express any toxicity towards living organisms in comparison to synthetic one like *i.e.* 2,6-di-tert-butyl-4-methylphenol (BHT) [Kupfer *at al*., 2002]. Thus, nowadays they are very often used in inhibition of lipids compound oxidation processes, especially in healthy daily products of human diets. Products, which are especially prone to oxidation are butter, margarine, lard, mayonnaise, meat and meat products.

The objective of the study was evaluation of antioxidant activity of chosen plant extracts, *i.e*. hawthorn, pine, evening primrose and rosemary, as well as flavones of rutin and baicalin, in relation to model lipids biological membrane *in vitro.* Rosemary extract, which is widely used in many ford products as a highly effective antioxidant [Lindberg-Madsen & Bertelsen, 1995], was regarded in this study as a standard of antioxidant activity in relation to other extracts. Skullcap (baicalin) flavones and rutin were analysed in the experiment due to widen the spectrum of those antioxidative compounds, whose molecular structure are already known, for checking their abilities in model study.

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The results collected in this study should give information about the possibility of utilisation of plant extracts in meat industry in order to prolong the shelf life of meat and meat products. This study was focused on the use of phosphatidylcholine liposomes and erythocytes membranes, as the most appropriate model for real biological membrane (they content phospholipids as a main lipid of the biological membranes) to evaluate the potential benefits of several antioxidant extracts in relation to lipid peroxidation. The paper studied the protective effect of chosen extracts and flavonoids on liposome and erythrocyte peroxidation induced by the UV induced generation of hydroxyl radicals and by the carbon centered radicals derived from water-soluble azo-compound 2.2'-azobis(2-amidinopropane) dihydrochloride (AAPH). A measure of this activity is the concentration of a compound (in mg/L) that causes 50% reduction in phospholipid membrane oxidation (IC_{50}).

MATERIALS AND METHODS

Materials. Phosphatidylcholine from eggs was purchased from Lipids Product (South Nutfield, U.K.). The lipid stock solution in pure ethanol was maintained at -20°C in glass vials that were layered with nitrogen. 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was obtained from Sigma (St. Louis, MO). AAPH and plant extracts were stock solution prepared immediately before the use in double-distilled water or in ethanol/water solution (1:1), respectively. The remaining chemicals were of the highest analytical grades from Sigma (Deisenhoffen, Germany). Light-induced lipid oxidation was carried out by the exposure of liposome suspension to UV radiation (3.0 mW/cm^2 at the sample). Light quanta (90%) were emitted at 253.7 nm.

Plant extracts preparations. The bark and leaves of hawthorn (*Crataegus oxyacantha)* and pine (*Pinus silvestris)* was collected from Botanical Garden of the Medical Academy of Wrocław. Extracts from both plants were prepared according to the method proposed by Oszmiański & Bourzeix [1995] and determined with HPLC. Procyanidins from the bark of hawthorn contained (-)-epicatechin (10.1%) and their polymers: B2 (24.2%), B4 (14.0%) and other compounds B4+B5 (10.5%). The content of procyanidins in hawthorn's leaves was as follows: (-)-epicatechin (11.2%) and polymers: B2 (21.0%) and B4 (12.9%). The extracts of evening primrose (P1, P2, P3) (*Oenothera paradoxa* Hudziok) were purchased from AGROPHARM (Tuszyn, Poland). Extracts P1, P2 and P3 differ in total procyanidins content. The percentage of procyanidins content was: P1 – 78.9%, P2 – 53.0% and P3 – 44.5%. Flavones of skullcap (*Scutellaria baicalensis* Georgi) containing 95% of baicalin were purchased from Wimex Beijng (China). Rutin was obtained from Sigma (Deisenhoffen, Germany). The extract of rosemary containing 56.20% of carnosic acid was purchased from TKI PINUS Rače d.d. (Czech Republic).

Liposome and erythrocyte membranes preparation and induction of peroxidation. A ethanol solution of egg yolk phosphatidylcholine (or with appropriate amount of cholesterol) was dried in vacuum under nitrogen atmosphere [Gabrielska *et al*., 2005]. A portion of 50 mmol/L Tris:HCl [(hydroksymethyl) aminomethane] buffer of pH 7.4 was added and the sample was vortexed to obtain a milky suspension of multilamellar vesicles. The final concentration of lipids in the vesicle suspension was 1.5 g/L. Such a suspension was then sonicated for 10 min with a 20 kHz sonicator. The plant extracts or flavonoids was then added from a concentrated ethanol solution (39.3 mg/L) to the stirred sample of suspended vesicles. The concentration of ethanol never exceeded 2% of the final 6 ml volume of the sample. The amount of the plant extracts studied changed in the range from 0.0025 g/L to 0.2500 g/L. Lipid peroxidation in the egg phospholipid liposomes or erythrocytes membranes was induced by ultraviolet radiation – using a bactericidal lamp or by incubation with 10 mmol/L AAPH at 37°C or with 20 μmol/L of Fe(II)/ascorbate mixture. The accumulation of phospholipid peroxidation products was estimated by determination of 2-thiobarbituric acid reactive substances (TBARS) in the incubation medium [Ohkawa *et al.,* 1979; Huang *et al.,* 2005]. This test have allowed us to establish the order of effectiveness of those extracts to scavenge the hydroxyl radical ($^{\cdot}OH$) and the radicals derived from AAPH thermal decomposition ($A^{\cdot}$). The measure of thiobarbituric acid reactive substances gives the concentration of malondialdehyde (MDA) and related compounds, generated from cyclic peroxides or endoperoxides. A pink chromofor than can be detected at 535 nm was formed as a consequence of their reaction with thiobarbituric acid at high temperature. The percentage of phosphatidylcholine (PC) liposome oxidation induction or inhibition was calculated based on the relation:

$$\%\ INHIBITION = \{(1- \Delta A_A) / \Delta A_0 \} \times 100\%$$

where ΔA_A – absorption increase (at $\lambda = 535$ nm) after 30 minutes of light exposure (or 60 min of incubation with AAPH) with an antioxidant added, ΔA_O – absorption increase (at $\lambda = 535$ nm) after 30 min of light exposure of PC liposomes (or 60 min of incubation with AAPH) without antioxidant added. From dependence of the% of oxidation inhibition after 30 min of induction of oxidation (or 60 min of incubation with AAPH) on concentration of the extracts studied the value of IC_{50} was determined. On this basis for y=50% the value of x=IC_{50} was calculated. IC_{50} denote the antioxidant concentration (in g/L) causing 50% inhibition of liposome/erythrocyte membrane oxidation after 30 min UV irradiation or after 60 min incubation with AAPH or after 60 min incubation with Fe(II)/ascorbate. The erythrocyte membrane was prepared by the method written by Dodge *et al*. [1963].

Statistical analysis. The fitting by least squares method was applied to an initial linear dependence of the% of oxidation inhibition after 30 min of induction of oxidation (or 60 min of induction of oxidation in the case of AAPH or Fe(II)/ascorbate inducers) on concentration of the compound studied for at least 4-6 determinations. The error of IC_{50} was calculated by a complete differential method. The maximal errors of the fitted parameters were taken as their mean square errors. All results

were analysed statistically by using STATISTICA ver. 7.0 with the Duncan test with probability level of p(0.05.

RESULTS AND DISCUSSION

Summing up, it can be stated that the results presented in Table 1 show a protective role of studied extracts and flavones towards PC liposome membranes and erythrocyte membranes against the consequences of the free-radical action on membranes exposed to UV radiation and AAPH incubation. The values of IC_{50} are several times higher in case of extracts from evening primrose and pine when oxidation was induced by UV radiation in comparison to AAPH chemical induction. Antioxidation mechanism of analysed plant extracts towards free radicals, *i.e*. hydroxyl radical, which are formed during water hydrolysis induced by UV radiation [Kruk, 1998], can be explained by electrons donation during scavenging processes. Probably, quantum yield of hydroxyl radical formation is higher than the rate of the velocity of AAPH thermal decomposition, which can be caused the differences in IC_{50} values. There are no significant differences in IC_{50} values for rosemary extracts despite induction method. There are many literature data concerning very high antioxidative activity of rosemary extract at different experimental conditions [Foti *et al.,* 1996; Frankel *et al.,* 1996; Basaga *et al.*, 1997; Perez--Fons *et al.,* 2006], what was confirmed also by the results of our study in which model biological membranes were used. Antioxidation mechanism of rosemary extract towards model lipids membranes was proposed by Perez-Fons *et al.* [2006], on the basis on the experiments carried out with main biologically active compounds of rosemary (carnosal, carnosic acid, rosmadial, genkwanin and rosmarinic acid). They suggested that diterpenes and genkwanin show membrane-rigidifying effects, which may contribute to their antioxidant capacity through hindering diffusion of free radicals.

For baicalin and rutin flavones IC_{50} values were higher when liposomes oxidation was induced by AAPH, but low in case of extract protection. All analysed extracts were effective in slowing down the liposome peroxidation (no statistical differences, $p \leq 0.05$). Both analysed flavones are characterised by quite difficult protons donation in AAPH scavenging processes in comparison to all the extracts used. Higher antioxidative activity of plant extracts in relation to pure Skullcap flavones and rutin (antioxidants with defined molecular structures), can be explained by the possible interaction between active components present in extracts. The phenomenon of synergism (or antagonism)is explained can be explained by the differences in paths of activity of single compounds. There is no single compound able to react with all kinds of radicals, or witch could optimally act on lipid oxidation products; hence the mixture of antioxidants molecules acting on various radicals and oxidation products may be more efficient than are individual compounds [Saucier & Waterhouse, 1999; Sokół--Łętowska *et al.,* 2007].

When analysed the sequences of antioxidant activity of plant extracts (Table 1), for UV oxidation induction of membranes, can be stated their good compability, either for liposomes membranes, or for erythrocytes, for extracts from pine, evening primrose and rosemary. Moreover, it can be suggested that antioxidants have similar partition coefficients. Protection of PC liposomes and erythrocyte membranes against AAPH free radicals (Table 1 and Figure 1), which was more effective by extracts can be explained by their membrane surface localisation in relation to deeper penetration by flavones. This favour of better acceptability of those particles during AAPH free radicals scavenging solved in water.

The results presented in Table 1 suggested potential possibilities of practical utilisation of all studied plant extracts (hawthorn, pine, evening primrose and rosemary) for prolongation of meat and sausages durability. However, all analysed plant extracts expressed a few times lower antioxidant activity than rosemary when oxidation was induced by UV radiation, thus when analysed lipids membrane oxidation induced by AAPH comparable activity of rosemary was observed for pine and

TABLE 1. Comparison of IC_{50} parameters (in g/L) for the plant extracts and flavonoids studied obtained for phosphatidylcholine membranes (PC) or erythrocyte membranes (ERYT). Phosphatidylcholine liposome oxidation was induce by UV radiation {IC_{50}^{PC} (UV)} or by AAPH incubation {IC_{50}^{PC} (AAPH)}. Results are means ± SE. Statistically homogeneous groups ($p \leq 0.05$) are designed with the same letters.

Plant extracts or flavones	IC_{50}^{PC}(UV) (g/L)	IC_{50}^{ERYT}(UV) (g/L)	IC_{50}^{PC}(AAPH) (g/L)
Pine – bark	0.026±0.002 c	0.024±0.002 c	0.005±0.0005 a
Evening primrose P1	0.036±0.003 a	0.024±0.002 c	0.008±0.0008 a
Evening primrose P2	0.038±0.003 a	0.035±0.004 d	0.009± 0.001 a
Evening primrose P3	0.040±0.004 a	0.037±0.004 d	0.010± 0.001 a
Hawthorn – leaves	0.027± 0.002 c	0.014±0.001 a	-
Hawthorn – bark	0.035± 0.003 a	0.010±0.001 ab	-
Rosemary	0.008±0.001 b	0.005±0.0005 b	0.009±0.001 a
Baicalin	0.014±0.001 b	0.012±0.001 a	0.120±0.012 b
Rutin	0.110±0.010 d	0.087±0.009 e	0.141± 0.011 c

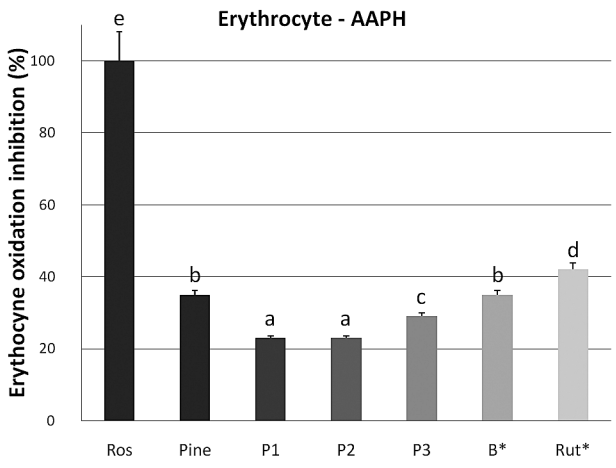

FIGURE 1. Erythrocyte oxidation inhibition (in percent) in the presence of plant extracts (Ros-rosemary, Pine, P1, P2 and P3 -evening primrose extracts which differ in total procyanidins content at 0.0236 g/L concentration. Concentration of baicalin (B*) and rutin (Rut*) was 0.142 g/L. Oxidation was induced during incubation of erythrocytes with 10 mmol/L AAPH at 37°C. Values represent the means of for to six measurements±SE. Standard deviation not exceeds 8%. Statistically homogeneous groups (p≤0.05) are designed with the same letters.

evening primrose extracts (no statistical differences, p≤0.05). There was a tendency observed in relation in antioxidative activity of three analysed evening primrose extracts (P1≥P2≥P3), however, no statistical differences between IC_{50} values (for all methods of oxidation induction), excluding IC_{50}^{ERYT}(UV) for P1 and P2 (P1>P2, groups c and d). This tendency can be explained by increasing content of procyanidins in analysed extracts (P1>P2>P3, see in Materials and Methods).

Figure 2 showed the results of percentage inhibition of peroxidation measured in PC liposome with different level of cholesterol (PC:Ch=100:30, 100:40, and 100:50) after hawthorn extract (0.0236 g/L) exposition. Figures 2A, 2B and 2C presented the results obtained for liposomes membrane when oxidation was induced by UV radiation, AAPH free radical and Fe(II)AA ions, respectively. High bars on Figure 2 represented PC liposomes containing respectively PC:Ch= 100:30, 100:40, and 100:50 of cholesterol without hawthorn addition, whereas lower bars represented the same liposomes but with hawthorn addition. Collected results showed that in case of oxidation induced by UV (Figure 2A), AAPH (Figure 2B) or Fe(II)AA (Figure 2C) the level of oxidation decreased along to increased cholesterol content in membranes. The highest rate of the inhibition the oxidation processes (even of 50%) was observed for PC/CHol liposomes induced by AAPH (Figure 2B), whereas it did not exceed 20% in two other cases (Figures 2A and 2C). Observed in the experiment the level of reduction of PC membranes oxidation processes can be caused by condensation which is induced by cholesterol in bilayers [Parassasi *et al.,* 1995]. This obstructed penetration of free radicals into the hydrophobic part of the membrane. There were no influence of cholesterol on effectiveness of oxidation inhibition by hawthorn extract, which is represented by the same level of membrane oxidation (Figures 2A, 2B and 2C). It can be explained by the possibility to formed hydrogen bridges between cholesterol and hawthorn constituents.

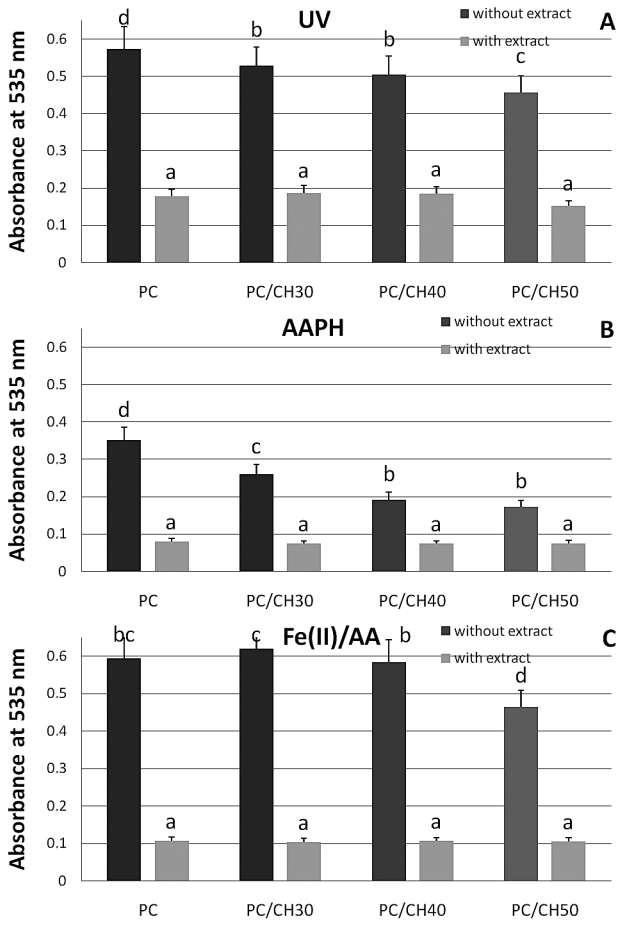

FIGURE 2. Level of liposome oxidation (proportional to absorbance at 535 nm) content different amount of cholesterol (30, 40 and 50 mol%) in the presence of hawthorn extract (leaves) at 0.0236 g/L concentration. Liposome oxidation was induced by: A) UV radiation; B) by incubation with AAPH; C) by incubation with Fe(II)/ascorbate. Values represent the means of triple measurements±SE. Standard deviation not exceeds 10%. Statistically homogeneous groups (p ≤ 0.05) are designed with the same letters.

There are many literature data concerning antioxidative activity of plant extracts, however, most of the studies are conducted *in vitro* using emulsions formed with synthetic fatty acids or natural oils [Zandi &Gorgon, 1999; Duffy & Power, 2001; Sokół-Łętowska *et al.,* 2007]. Moreover, extracts are analysed only in water solutions with presence of stable free radicals, such as $DPPH^{\bullet}$ or $ABTS^{+\bullet}$ [Malterud *et al.,* 1996; Arts *et al.,* 2004; Samaniego *et al.,* 2007]. There are no research papers concerning antioxidative activity of plant extracts tested on model cell membranes, such as liposomes or erythrocyte membranes. That is why, the results of our study are difficult to compare with those obtained on emulsions. Research is going to be continued, especially for explaining the interaction's mechanism of plant extracts and cell membranes.

CONCLUSIONS

The results obtained show that plant extracts and also baicalin exhibited high dose-dependent antioxidant activity against model lipid membrane and suggested potential pos-

sibilities of practical utilization of all studied plant extracts (hawthorn, pine, evening primrose and rosemary) in meat industry (for prolongation of meat and sausages durability).

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DZIAŁANIE ANTYOKSYDACYJNE EKSTRAKTÓW ROŚLINNYCH I FLAWONÓW NA BŁONY LIPOSOMÓW I ERYTROCYTÓW

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W pracy zastosowano liposomy oraz błony erytrocytów jako modele błon biologicznych do opisu procesu utleniania lipidów. Zbadano antyoksydacyjne działanie ekstraktów roślinnych z sosny (*Pinus silvestris* L), głogu (*Crataegus oxyacantha* L,dwa ekstrakty: z liści i kory głogu), wiesiołka (*Oenothena paradoxa* Hudziok, trzy ekstrakty różniące się zawartością procyjanidyn oznaczone P1, P2 and P3) and rozmarynu (*Rosmarinus officinalis,* jako standardu dla ekstraktów) oraz flawony bajkaliny i rutyny. Uzyskane wyniki badań wykazały zależną od stężenia, zróżnicowaną aktywność antyoksydacyjną ekstraktów w stosunku do błon liposomów (rozmaryn>sosna≈głóg-l>głóg-b≈P1≈P2≈P3; oraz błon erytrocytów (rozmaryn≥głóg-b≈głóg-l>P1≈sosna>P2≈P3) w sytuacji gdy utlenianie indukowano promieniowaniem UV-C. Redukują one także utlenienie liposomów i błon erytrocytów indukowane 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH) (sosna≥P1≈P2≈rozmaryn≈P3, w przypadku liposomów and rozmaryn>>sosna≥P1≈P2≥P3, w przypadku erytrocytów). Bajkalina posiada wysoką zdolność inhibicji peroksydacji zarówno liposomów PC jak i cieni erytrocytów w sytuacji utleniania indukowango promieniami UV, natomiast rutyna w tych warunkach jest ok. 7-8 krotnie słabszym inhibitorem procesu. Obecność cholesterolu w błonach liposomów w zasadzie zmniejsza poziom peroksydacji liposomów (różnice istotne statystycznie) lecz nie wpływa na aktywność antyoksydacyjną ekstraktu z liści głogu.