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ANTIOXIDANT POTENTIAL OF HERBS EXTRACTS AND IMPACT ON HEPG2 CELLS VIABILITY

Anna Gramza-Michałowska[1], Zyta Abramowski[2], Eduardo Jovel[2], Marzanna Hęś[1]

[1]Poznań University of Life Sciences, Poland
[2]University of British Columbia Vancouver, Canada

Abstract. Mercury poisoning is responsible for inducing serious adverse effects in living organisms. One of protection factors could be substances proven to possess high antioxidant and metal chelating activity – plant polyphenols. There are many sources of polyphenols in plant kingdom but the most interesting for food industry could be widely consumed herbs. Aim of the research was to evaluate antioxidative potential of selected plant extracts and its influence on HepG2 cells in different conditions. Ethanolic herbs extracts were characterised by total polyphenol content. Antioxidant activity was estimated with use of $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ radicals scavenging methods and FRAP. Research included cells viability estimation by the MTT assay and cells exposition to $HgCl_2$, chemical agent inducing cell death. Analysis of herbs extracts antioxidative activity showed best potential represented thyme and marjoram, highest FRAP was evaluated in samples with mint and marjoram extracts. On the basis of received results it was found that examined plant extracts showed weak protection against Hg presence in examined cells environment.

Key words: herbs, plant extracts, cytotoxicity, HepG2 cells, FRAP, radicals

INTRODUCTION

Rosemary (*Rosmarinus officinalis*), garlic (*Allium sativum*), thyme (*Thymus vulgaris*), mint (*Mentha piperita*), marjoram (*Origanum majorana*) and cilantro (*Coriandrum sativum*) are popular spices used for centuries as a remedy for many diseases and for cuisine matter. Some of examined herbs are included in European pharmacopoeias and still commercially available [Matkowski and Piotrowska 2006]. Health properties of commonly used herbs are known for centuries, but its activity depends on bioactive components amount. Herbs like marjoram, garlic, thyme or rosemary are widely used in many cooked dishes of European cuisine. Moreover herbs, as a human diet constituent are recognized as important preventive factor of some diseases [Park and Pezzuto 2002,

Corresponding author – Adres do korespondencji: Dr inż. Anna Gramza-Michałowska, Department of Food Service and Catering of Poznań University of Life Sciences, Wojska Polskiego 31, 60-624 Poznań, Poland, e-mail: angramza@up.poznan.pl

Williams et al. 2005, Suhaj 2006]. Antioxidants present in food are very important for human health since the reactive oxygen species are recognised as aging and carcinogenesis factor. Plant components as antioxidants play important role in foods and living organisms because of the radicals scavenging ability and reducing cells degradation in human body [Madsen and Bertelsen 1995, Jin et al. 2004, Matkowski and Piotrowska 2006, Yoo et al. 2008]. Polyphenols are recognised as great scavengers of free radicals, hydroxyl radicals and superoxide anion radicals [Hanasaki et al. 1994, Cao and Cao 1999, Kahkonen et al. 1999].

Environment is widely polluted with heavy metals like mercury or lead. Mercury poisoning is responsible for inducing serious adverse effects in living organisms. Evidence indicates that cellular damage mediated by reactive oxygen species may be involved with heavy metals intoxication [Chen et al. 2002, Hermes-Lima et al. 1991]. Substances that are well proven to possess high antioxidant and metal chelating activity are polyphenols, also present in herbs. There are many results suggesting antioxidants role in the treatment of heavy metals poisoning as metal ions chelators and scavengers of free radicals [Matsingou et al. 2001]. Although plant extracts might protect cell from oxidative stress the mechanism remains unclear.

In present work the antioxidative activity as radical scavengers of herbs extracts had been evaluated. The aim of this study was to estimate total polyphenol content and correlation with antiradical activity. Second aim was the introductory evaluation of herbs extracts activity as cells protectors from oxidative damage in presence of mercury.

MATERIALS AND METHODS

Chemicals. The following chemicals were used: [3-(4,5-dimethylthiazol-2-yl)-2,5--diphenyl-tetrazolium bromide] (MTT); $HgCl_2$ (Sigma); ethanol; HCl; ddH_2O; phosphate-buffered saline – PBS, Ca^{2+} and Mg^{2+} free, purchased from Gibco BRL (Gaithersburg, MD, USA); (+)-sodium L-ascorbate (Sigma); EDTA (Sigma); 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid – Trolox (Aldrich); iron (III) chloride $FeCl_3$ (Aldrich); 2,4,6 Tri (2-pirydyl)-s-triazine (Fluka). 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) (Fluka); 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich); ethanol POCH (Poland). For the extracts sterilization Syringe filters were used (Nalgene 25 mm). All chemicals were of the highest analytical grade and purchased from common sources. Measurements were taken on FluoStar Galaxy (BMG Labtechnologies Ltd.) – multifunctional microplate reader. Cell cultures were grown on Falcon microtest^{TM} tissue culture plates, 96 well (Becton Dickinson Labware).

Plant material. Samples of medicinal plants were purchased at a local pharmacy in Poznan, Poland, while cilantro was cultivated at UBC farm in Vancouver, Canada. For the research the following plants were chosen: rosemary (*Rosmarinus officinalis*), garlic (*Allium sativum*), thyme (*Thymus vulgaris*), mint (*Mentha piperita*), marjoram (*Origanum majorana*) and cilantro (*Coriandrum sativum*). Research was conducted on the ethanol extracts received according to Gramza et al. [2006]. The powder was dissolved to give a final extract concentration ranging from 0.005 to 1.0 $mg \cdot mL^{-1}$. Total polyphenols content in examined plant extracts was determined with Folin-Ciocalteu method [Horwiz 1970]. Results expressed as mg of gallic acid equivalent per gram of the extract's dry weight (mg GAE/g).

Antiradical activity assays. Plant polyphenols antioxidative activity was estimated using $DPPH^{\bullet}$ [Sanchez-Moreno et al. 1998] and $ABTS^{+\bullet}$ [Re et al. 1999] radicals scavenging ability of examined extracts. Results were expressed as mg Trolox per 1 g of extract's dry weight (mg T/g).

FRAP assay. FRAP assay was performed as previously described by Benzie and Strain [1996] with modifications of Griffin and Bhagooli [2004]. After addition of FRAP reagent next readings were performed after 4 min (λ = 600 nm). The changes in absorbance were compared to that of a standard that was run simultaneously. Final results were expressed as μM Trolox equivalents (μMT) per sample concentration.

Cell culture. HepG2 cells (human hepatocellular carcinoma cells – liver tumor) were cultured in 75-cm^2 cell culture flasks to confluence and harvested using a solution containing 0.05% (v/v) trypsin and 0.02% (w/v) EDTA in PBS. For experiments, cells were grown in 96-well plates, and inspected under an inverted system microscope, carried out before starting the experiments.

Cell viability assay. Cell viability was estimated by the MTT assay, which is based on the cleavage of a tetrazolium salt by mitochondrial dehydrogenases in viable cells [Loikkanen et al. 1998]. Data were mean percentages of viable cells versus the respective controls.

Cells exposure to chemical agents and plant extracts. Cells were exposed to chemical agent inducing cell death – $HgCl_2$. The possible protective effect of extracts, when added immediately before toxic compound, was assessed according to Fallarero et al. [2003]. All extracts were dissolved, sterilized by filtering through 0.2-μm filters, and then added to cells. Extract concentrations in all experiments were selected considering that extracts up to 0.1 $mg \cdot mL^{-1}$ are not toxic to HepG2 cells during exposure. Cells were exposed to $HgCl_2$ for 24 hours in darkness at 37°C. Final concentration of ethanol was in all cases lower than or equal to 3%, concentrations which have no effect on cell viability in this cell line.

Statistical analysis. Data were expressed as mean values of three independent experiments (each in triplicate) and analysed by the analysis of variance ($p \leq 0.05$) to estimate the differences between values of compounds tested.

RESULTS AND DISCUSSION

Evaluations of total polyphenols content with Folin-Ciocalteu method showed that examined herbs extracts differed significantly. Highest polyphenols content was evaluated in mint (221.6 mg GAE/g) and thyme extracts (217.1 mg GAE/g). Nearly 20% less polyphenols content was evaluated in samples of cilantro (183.8 mg GAE/g) and rosemary (171.0 mg GAE/g). Significantly lower amount was evaluated in marjoram extract (119.8 mg GAE/g). Garlic extract was found to contain the lowest polyphenol amount among examined extracts (41.1 mg GAE/g).

Plant polyphenols are well characterised with its radicals scavenging potential. For evaluations of antiradical activity of the herbs extracts the most popular $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ methods were used. Research showed that highest $DPPH^{\bullet}$ radicals scavenging activity exhibited extracts of thyme (1.44 mg T/g). Also high antiradical activity was evaluated in samples with marjoram (1.28 mg T/g), cilantro (1.26 mg T/g) and mint (1.19 mg T/g). Lowest activity exhibited garlic (0.65 mg T/g) and rosemary (0.46 mg T/g) extracts (Fig. 2).

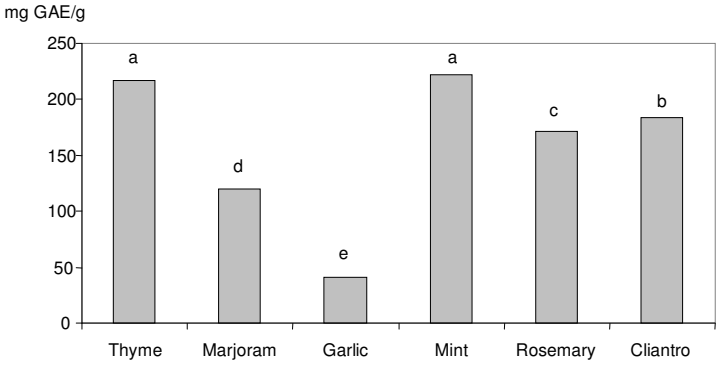

Fig. 1. Total polyphenols content in herbs extracts, mg gallic acid/g dry weight

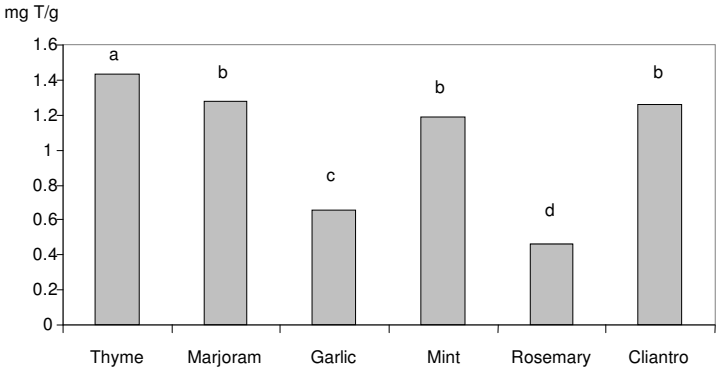

Fig. 2. DPPH radical scavenging activity of selected herbs extracts, mg Trolox/g dry weight

Statistical analysis of received data showed that herbs extracts possessed radical scavenging ability, depending on their concentration. It was evaluated that with higher extracts concentration, also antiradical activity increases, reaching highest at concentration of 0.1%. Analysis of $DPPH^{\bullet}$ scavenging activity calculated on equal Trolox concentration showed that all herbs extracts exhibited activity significantly higher than of garlic and rosemary extracts. As the result of the $DPPH^{\bullet}$ analysis herbs extracts were ranked as follows: rosemary < garlic < mint < marjoram < cilantro < thyme. Statistical analysis evaluations did not confirm the correlations between antiradical activity and total polyphenols in examined herbs extracts ($r = 0.86$, $p < 0.05$).

Second method for the evaluation of antiradical activity of herbs extracts was method using the $ABTS^{+\bullet}$ radical (Fig. 3). On the basis of present research it was evaluated that highest radical scavenging activity possessed equally rosemary (14.53 mg T/g) and marjoram (14.09 mg T/g) extract. Weaker antiradical activity was evaluated in samples

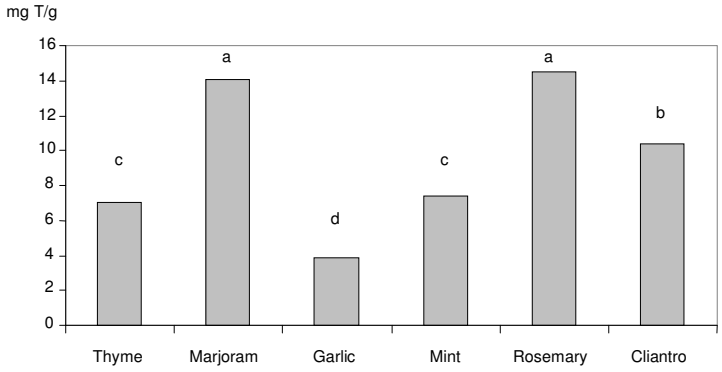

Fig. 3. ABTS radical scavenging activity of selected herbs extracts, mg Trolox/g dry weight

with cilantro (10.34 mg T/g), mint (7.38 mg T/g) and thyme extracts (7.00 mg T/g). Among examined extracts garlic possessed lowest antiradical activity (3.91 mg T/g). Thyme and mint extract did not scavenge the radicals as well as in presence of $DPPH^{\bullet}$ radical, rosemary however, was a better scavenger of $ABTS^{+\bullet}$ radical.

Statistical analysis of received data showed that antiradical activity was dependent from its concentration, higher extracts concentration resulted in higher antiradical activity, reaching highest at concentration of 0.1%. Analysis of $ABTS^{+\bullet}$ scavenging activity calculated on equal Trolox concentration allowed to rank the extracts as follows: garlic < thyme < mint < cilantro < marjoram < rosemary. On the basis of statistical analysis results it was stated that $ABTS^{+\bullet}$ scavenging activity was poorly correlated with total polyphenol content ($r = 0.72$, $p < 0.05$).

Antioxidative activity of examined plant extracts was also measured with Ferric Reducing Antioxidant Power, FRAP method (Fig. 4). Results of the evaluations presented as μM Trolox per dry extract's concentration, showed increasing activity with extracts concentration. Highest FRAP values were evaluated in samples of mint (1665.5 uMT) and marjoram (1614.9 uMT), similarly high activity showed rosemary extract (1586.4 uMT). Other extracts exhibited significantly lower activity, lowest however was found in garlic sample (35.4 uMT).

As the result of the FRAP analysis herbs extracts were ranked as follows: garlic < cilantro < thyme < rosemary < mint < marjoram. Statistical analysis of relationships between ferric reducing antioxidant power of ethanol herbs extracts and total polyphenol content showed high correlations ($r = 0.93$, $p < 0.05$).

Exposure of HepG2 cells to different herb extracts significantly decreased cell viability and in some cases stimulated its apoptosis. It was found that with increasing extracts concentration also the percentage of cells viability changed (Fig. 5). Highest extracts concentration (1 mg/mL) however significantly decreased cell's viability. It was evaluated that rosemary, mint and marjoram extracts strongly influenced cells viability, ranging 27.5 – 3.8%. Thyme and cilantro extracts decreased cell's viability for nearly 60% at highest concentration. Surprisingly rosemary, garlic and cilantro extract addition resulted in cells proliferation, reaching 130.4; 122.2 and 109.7% respectively.

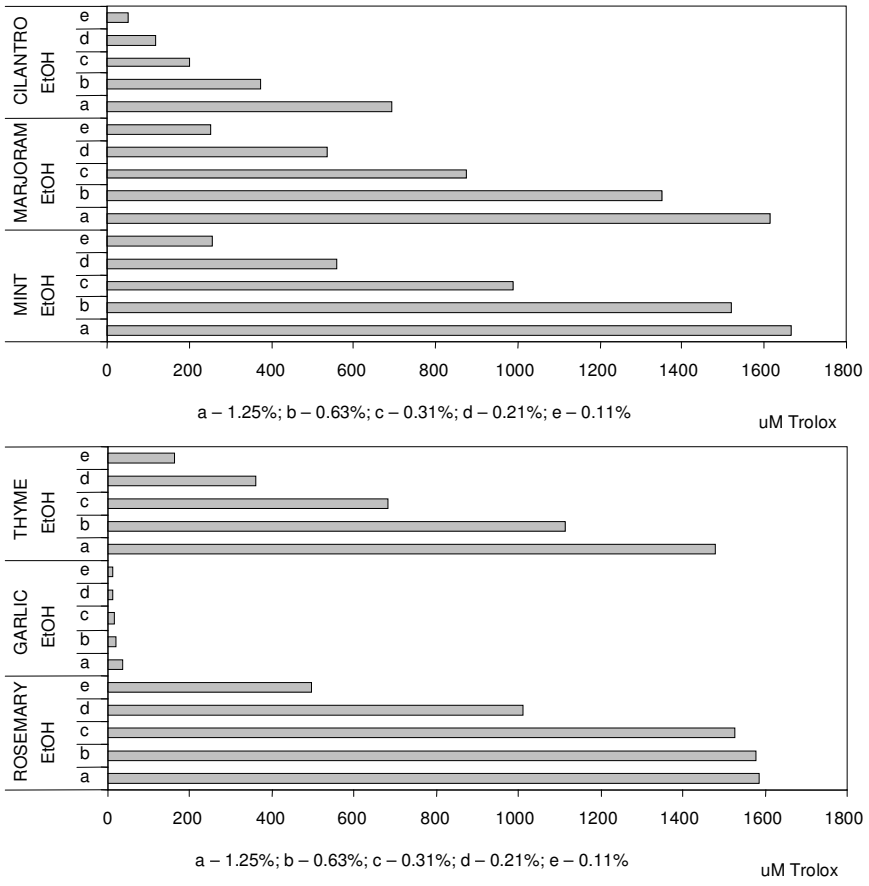

Fig. 4. FRAP assay for selected herbs extracts, uM T

Further research on Hg influence was conducted on the extracts concentration which did not cause the cells apoptosis or proliferation (0.005 mg/mL). No correlations between total polyphenols content and cell viability were evaluated ($r = 0.21$, $p < 0.05$).

Using the MTT method, the toxicity of Hg on HepG2 cells was evaluated (Fig. 6). There was no statistical difference in cells viability when the mercury concentration was 58 (μg Hg/mL) or exceeded to 60 (μg Hg/mL). As seen on Figure 6, it was found that all examined extracts at 0.005 mg/mL concentration caused cells proliferation, except cilantro sample. Results showed that plant extracts did not or weakly protected HepG2 cells from Hg influence. It also must be noticed that HepG2 cells are very unstable and sensitive to reaction conditions. Among herbs extracts best protection offered addition of garlic (3.56%) and rosemary extract (3.39%). Cells apoptosis was evaluated in sample with mint extract. This suggests that no chelating activity was evaluated during cells exposure to Hg in chosen concentration.

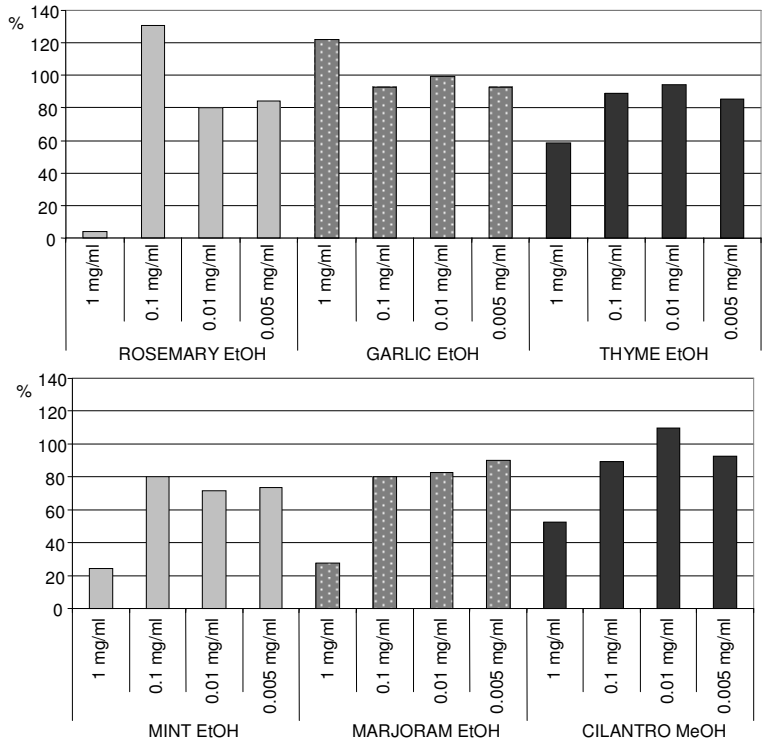

Fig. 5. Viability of HepG2 cells exposed for 24 h to various concentrations of plant extracts (MTT assay; 20 000 cells/well)

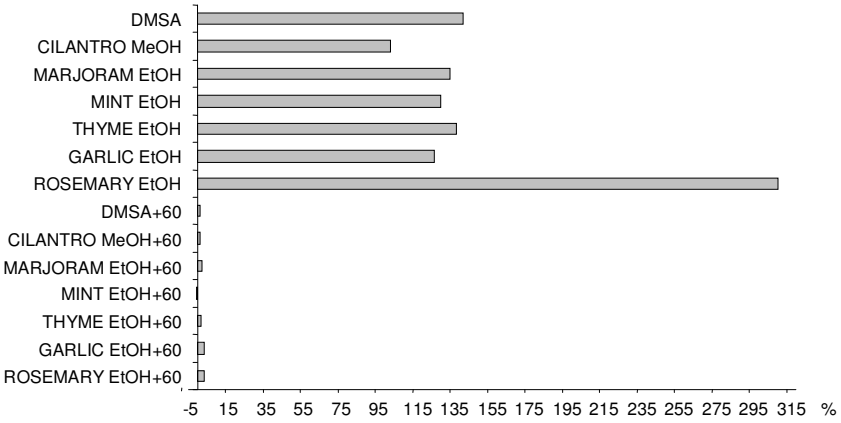

Fig. 6. Viability of HepG2 cells exposed for 24 h to plant extracts and mercury Hg60 (MTT assay; 20 000 cells/well)

Summarizing it was found that HepG2 cells exposure to Hg significantly decreased cell viability. The results pre-suggest that supplementation could play an important role in modulating oxidative stress in HepG2 cells exposed for a mercury influence. According to results of Yoo et al. [2008] it was suggested that antioxidant effect of herbs on cell viability could be explained by two mechanisms. One is direct action as reactive oxygen species ROS scavengers, second is indirect action through induction of antioxidative enzymes and intracellular communications protection.

DISCUSSION

Herbs could be very promising antioxidants sources. Of the six selected herbs used for the research, it is quite difficult to show the best source of natural antioxidants. As the results showed herbs extracts exhibited different antiradical and antioxidant activity, depending on the evaluation method used, as a result of methods complexity of the involved antioxidative mechanism. Example of that could be the cilantro extract that exhibited strong antioxidative activity and low FRAP values.

Wojdyło et al. [2007] examined selected herbs for their total polyphenol content and showed that rosemary possessed higher amount that thyme, what was in agreement with results of Cosio et al. [2006] and Parejo et al. [2002]. There are researches showing extremely high differences in polyphenols content of garlic extracts. Leelarungrayub et al. [2006] evaluated nearly 500 (mg GAE/g) of garlic extract, Nencini et al. [2007] however, is in agreement with the present research, with rage from 0.32-0.64 (mg GAE/g). Results of other research showed that herbs are relatively high in polyphenols, depending genotypic, environmental and sampling differences [Parejo et al. 2002, Shan et al. 2005]. Total polyphenols content also differs with method used. Folin-Ciocalteu procedure does not give the exact picture of the polyphenols quality and quantity in tested extract. Main polyphenols evaluated in herbs were phenolic acids, like hydroxynnamic acid, caffeic acid, rosmarinic, p-coumaric and ferulic acids [Zheng and Wang 2001, Kim and Lee 2004, Wojdyło et al. 2007, Fecka and Turek 2008]. Other flavonoids found in herbs were quercetin, kaempferol, apigenin and luteolin [Justesen and Knuthsen 2001, Shan et al. 2005].

Concerning all herbs used marjoram and thyme were found to be the best antiradical scavengers in $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ methods. However, garlic extract did not show any significant antiradical or antioxidant activity in examined conditions. According to Wojdyło et al. [2007] it was found that rosemary exhibited twice as good activity as thyme in scavenging the $DPPH^{\bullet}$ radical, what is in agreement with work of Cosio et al. [2006]. Results of Aoshima and Ayabe [2007] showed similar $DPPH^{\bullet}$ radical scavenging activity, significantly higher than of mint extract. In presence of $ABTS^{+\bullet}$ radical rosemary extract exhibited slightly higher scavenging activity than of thyme [Wojdyło et al. 2007]. It was evaluated that similarly to results of Wojdyło et al. [2007] $DPPH^{\bullet}$ values were lower than of $ABTS^{+\bullet}$ radical scavenging of examined herbs extracts. Results of both evaluations differed and there was no correlation found between total polyphenols content and antiradical activity. Results of many studies showed that many species of *Labiatae* family possess strong antioxidant activity [Zheng and Wang 2001, Shan et al. 2005].

Present research showed that rosemary extract exhibited best FRAP values, marjoram and thyme extracts however possessed also strong antioxidant activity measured with FRAP. Other authors results showed similar FRAP values for rosemary and thyme extracts [Wojdyło et al. 2007]. In present research all examined herbs extracts reduced the ferric ion. In contrast the weakest ability to reduce the ferric ion exhibited cilantro extract, as in previous results of $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ methods. Rosemary which exhibited the highest scavenging of $ABTS^{+\bullet}$ and ferric ion reducing ability did not reveal the same activity as $DPPH^{\bullet}$ radical scavenger. The weakest activity however, in all evaluations was found in garlic sample.

All examined extracts possessed high polyphenols content but there was no simple correlation between antioxidant capacity and total polyphenol content that would be confirmed in assays used for the research. The present research results are in agreement with other showing poor correlation between total polyphenols content and antioxidant activity [Czapecka et al. 2005, Wong et al. 2005].

Results of Chen et al. [2002] showed that cell's exposure to heavy metals induce oxidative stress and stimulates lipid peroxidation of lipid membrane. As a result of the process radicals and other lipid degradation products (like aldehydes) are formed, being extremely toxic for the cells. Strong chelating activity of herbs extracts made herbs polyphenols good candidates for treatment of mercury toxicity. There are however, results showing prooxidant activity of natural products. It was found that copper and tea catechins presence induced lipid peroxidation and DNA cleavage, resulting cells death [Hayakawa et al. 1997]. Chen et al. [2002] suggested that plant polyphenols could be toxic; so much consideration for safety should be required if used as therapeutic agents or nutrition supplements.

The results of the above analysis suggest a complexity of the antioxidative mechanism involved in different herbs extracts.

CONCLUSIONS

On the basis of the received results the following statements were formed:
- plant extracts differed with total polyphenols content;
- according to antioxidative activity it was found that best radical scavenging activity represented thyme, marjoram and rosemary extracts;
- highest FRAP was evaluated in samples of mint, marjoram and rosemary extracts;
- no significant correlations between total polyphenols content and antiradical activity of examined extracts was evaluated;
- higher extracts concentrations resulted in low HepG2 cells viability in presence of mercury;
- research showed weak herbs extracts protection against Hg presence in examined HepG2 cells environment.

Acknowledgments

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POTENCJAŁ PRZECIWUTLENIAJĄCY EKSTRAKTÓW ZIOŁOWYCH ORAZ WPŁYW NA PRZEŻYWALNOŚĆ KOMÓREK HEPG2

Streszczenie. Zatrucia rtęcią prowadzą do powstania wielu niepożądanych skutków w organizmach żywych. Jednym z czynników ochronnych mogą być polifenole roślinne, których wysoki potencjał przeciwutleniający oraz zdolności wiązania jonów metali potwierdziło wiele badań. Spośród różnych źródeł tych związków powszechnie spożywane zioła mogą być grupą najbardziej interesującą dla przemysłu spożywczego. Celem badań było określenie potencjału przeciwutleniającego wybranych ekstraktów roślinnych oraz ich wpływu na komórki HepG2 w różnych warunkach. Badania obejmowały określenie stopnia przeżywalności komórek w metodzie MTT oraz poddanych działaniu $HgCl_2$, związku indukującego śmierć komórki. Ekstrakty etanolowe ziół scharakteryzowano pod kątem zawartości polifenoli ogółem. Aktywność przeciwutleniająca została określona z wyko-

rzystaniem rodników $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ oraz FRAP. Na podstawie uzyskanych wyników stwierdzono, że badane ekstrakty słabo chroniły komórki przed działaniem Hg. Analiza aktywności przeciwutleniającej ekstraktów ziołowych wskazała najwyższy potencjał tymianku i majeranku, natomiast w metodzie FRAP największą aktywność stwierdzono dla ekstraktów mięty, majeranku i rozmarynu.

Słowa kluczowe: zioła, ekstrakty roślinne, cytotoksyczność, komórki HepG2, FRAP, rodniki

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