Applied Computer Science, vol. 15, no. 4, pp. 49–62
doi:10.23743/acs-2019-29

Submitted: 2019-09-21
Revised: 2019-11-25
Accepted: 2019-12-06

herb extract, phenolic compounds, antioxidant capacity, VisionPro™ software

Karolina FERYSIUK[*], *Karolina M. WÓJCIAK*[0000-0002-4202-433X]*

THE SPECTROPHOTOMETRIC ANALYSIS OF ANTIOXIDANT PROPERTIES OF SELECTED HERBS IN VISION-PRO™ UV-VIS

Abstract

The aim of the study was to evaluate the influence of type of the solvent (water, aqueous ethanol and ethanol) on the antioxidant properties of four various herbs: couch grass (A. repens), milk thistle (S. marianum), dandelion (T. officinale) and fireweed (E. angustifolium) measurement by three common UV-VIS methods (TPC, ABTS^{+}, DPPH). The results were collected through the Vision-Pro™ UV-VIS spectrophotometer software. Aqueous ethanol was the most effective solvent for extraction for all type of herbs. Fireweed contains the highest amount of polyphenol compounds (0.625 μg GA/ml). The lowest antioxidant capacity was presented by extracts from couch grass (0.019 μg GA/ml).

1. INTRODUCTION

Meat products are exposed to the oxidation process, which is responsible, among others, for deterioration of nutritional value, shortening of shelf-life and creation of off-flavors. To avoid those processes, a synthetic antioxidant (e.g. sodium nitrite) is added to meat products. Unfortunately, generally speaking, consumers associate food chemical additives as a negative factor for their health – while the naturalness of the products is linked with a positive effect on health (Rodríguez-Rojo, Visentin, Maestri & Cocero, 2012; Hung, de Kok & Verbeke, 2016). For that reason, alternatives for typical food additives are needed. One of the substances that could partially or even fully replace synthetic additives is phenolic compound.

[*] University of Life Sciences in Lublin, Faculty of Food Science and Biotechnology, Department of Animals Raw Materials Technology, Skromna 8 Street, 20-704 Lublin, Poland, karolina.ferysiuk@student.up.edu.pl, karolina.wojciak@up.lublin.pl

Phenolic compounds (polyphenols) are secondary plant metabolites; depending on their chemical structure, several classes are highlighted: phenolic acids, tannins, flavonoids, lignans and stilbenes. Polyphenols show good antioxidant activity and can be used as a food preservative. The relations between carboxyl group and numbers and positions of the –OH are factors which determine their antioxidant activity. Moreover, secondary plant metabolites also presented a positive influence on human health by reducing the risk of some pathological disturbances (e.g. reduce the incidence of coronary diseases, present anti-atherosclerotic and anti-carcinogenic effects). They also present antimicrobial properties and can inhibit the growth of pathogenic microorganisms (Falowo, Fayemi & Muchenje, 2014; Ignat, Volf & Popa, 2011; Oroian & Escriche, 2015; Martillanes, Rocha-Pimienta, Cabrera-Bañegil, Martín-Vertedor & Delgado-Adámez, 2017; Wendakoon, Calderon & Gagnon, 2012). As Al-Snafi (2015) pointed out, secondary metabolites found in the plant have a therapeutic and pharmacological effect. Herbs and spices are a rich source of phenolic compounds and they are often applied to the food products – except for the prolonging of the durability of the food they also carry flavor. Antioxidant substances applicated in food industry must be effective at low concentrations, inexpensive, highly stable, non-toxic, colorless, tasteless and odourless. In order to avoid the too intense taste of plant additives in the product, the extraction process is used (Hinneburg, Dorman & Hiltunen, 2006; Shahidi & Ambigaipalan, 2015). But this is not the only reason – in general, this process is considered as one of the best sustainable methods of the biological components extraction (Gupta, Naraniwal & Kothari, 2012). In general, extraction is a process in which the main purpose is the isolation and separation of the specific components through the application of an appropriate, adequate solvent. It is important to remember there is no one, standard method of extraction (Ignat et al., 2011). Various extraction solvents can be used: water, ethanol, methanol, acetone and their mixtures with water (Wendakoon, Calderon & Gagnon, 2012). The choice of reagent depends on, among others, chemical nature of polyphenols and their solubility in the solvent (e.g. methanol or even acidified methanol is usually applied for anthocyanin extractions) (Naczka & Shahidi, 2004). Therefore, compared with other solvents, water and ethanol are recommended as an extraction solvents for food industry due to their safety for human consumption (Wendakoon et al., 2012; Ignat et al., 2011).

As it was mentioned earlier, the lipid oxidation is a very negative process for food, especially for meat products. The oxidation process consists of the following stages: initiation (the lipid-free radical occurs), propagation and termination (occurs of non-radical products). Antioxidant substances can stop this process through the scavenging of initial radicals, braking chain reactions, intercepting singlet oxygen or decreasing concentration of oxygen. Phenols present strong antioxidant activity and should be added to food products in low concentrations; in higher concentrations they can lose their activity and become prooxidants (Shahidi & Ambigaipalan, 2015).

The aim of this article is to compare the influence of the three various solvents on the antioxidant properties of herb extracts from dandelion, couch grass, milk thistle and fireweed, extracted at 40°C by using a different UV-VIS antioxidant measurement methods. The data were collected through the Vision-Pro™ UV-Vis spectrophotometer software version 2.03.

2. MATERIAL AND METHODS

2.1. Material

Plant material was dry, shredded herbs: milk thistle (*S. marianum*), dandelion (*T. officinale*), couch grass (*A. repens*) and fireweed (*E. angustifolium*). Three variants of the extracts were created: aqueous, ethanolic and their mixture (50:50). 30 ml of fresh solvent was added to herbs (5 g) and shaken at 150 rpm for 3 hours at 40°C. Solvent was then changed every hour. The extract was filtered through filter paper Whatman No 1 and prepared for analysis.

2.2. Methods

2.2.1. Antioxidant activity

The analyzes of antioxidant properties included total phenolic content (TPC) which determined by a modified Folin–Ciocalteau method, described by Singleton and Rossi (1965) using a Folin–Ciocalteu reagent. Samples were measured after 30 min of storage at room temperature, in the dark. The TPC values were calculated from a standard curve of gallic acid equivalent and expressed as mg GA/ml. The absorbance was measured by using a UV-VIS spectrophotometer (Nicolet Evolution 300).

2.2.2. Radical scavenging activity

The radical scavenging activity (DPPH and $ABTS^+$ methods) was measured according to Jung et al. (2010) with some modifications. For both methods absorbance was measured after 3 min. Volume of $ABTS^+$ reagent was reduced to 1.8 ml, volume of extracted sample was reduced to 12 µl. The absorbance was measured by using a UV-VIS spectrophotometer (Nicolet Evolution 300) at 734 nm for the ABTS assay and at 517 nm for DPPH assay.

2.3. Statistical analysis

The results were statistically analyzed using KyPlot statistical program and presented as mean±standard deviations using a T-Tukey's range test. The three research series were created and samples were measured in duplicate.

3. THE ANTIOXIDANT MEASUREMENT METHODS

How was mentioned earlier, the polyphenols compounds occurring in plant materials, are considered to be a substances of antioxidant properties. For those reason they can be applied as additives to food products and therefore extend their durability. It should be keep in mind that the antioxidant capacity of plants can be various and this determine their application as food additives. For the measurement of antioxidant potential various methods can be applied, but the most common are: TPC, DPPH and TEAC/$ABTS^+$. All those assays are based on the reaction between substances of antioxidant properties and a special reagent. The common features connecting these methods are simplicity, inexpensive, reproducibility and no need for specialized equipment except ultra violet visible (UV–VIS) spectrophotometer (Karadag, Ozcelik & Saner, 2009; Moniruzzaman, Khalil, Sulaiman & Gan, 2012; Shahidi & Zhong, 2015). UV–VIS spectrometry is considered as a sensitive, fast, environment friendly and simple method for antioxidant potential measurement (Biswas, Sahoo & Chatli, 2011; Yu, Wang, Zhan & Huang, 2018). How explain Yu et al. (2018) in spectroscopic techniques the concentration of the chemical component is predicted through the calibrating a predicting models which, through the proper chemometrics, correlates collected spectral data and reference values of chemical concentrations. The wavelength range for the UV–Vis method is between 200 and 780 nm which corresponded to the X–rays and NIR (near-infrared) range. The spectrophotometer apparatus consist of a proper, dedicated optical spectrometer (light source, detector, sample compartment, monochromator) and a control unit (PC).

In general speaking spectrophotometer methods for antioxidant measurements are based on the measurement of changing the absorbance spectrum of the tested sample against the blank sample (Moniruzzaman, Khalil, Sulaiman & Gan, 2012; Wojdyło, Oszmiański & Czemerys, 2007). Depending on the method, various wavelength are chosen: DPPH reagent have a characteristic, strong absorbance at 515–517 nm (Wojdyło, Oszmiański & Czemerys, 2007; Moon & Shibamoto, 2009) however other authors also pointed out that the reducing ability can be also measured at 518, 520 to 528 nm (Kedare & Singh, 2011; Karadag, Ozcelik & Saner, 2009). Moreover, for the $ABTS^+$ radical scavenging activity measurement, Karadag et. al. (2009) divided wavelength in two groups, depending on the solvent type: for aqueous: 414, 752, 842 nm, for ethanolic: 414, 730 and 873 nm.

The data from measurement are collected through the proper software, which can be built into the device (a computer unit is not needed) or installed separately on the computer. The recommended progremm for the Nicolet Evolution 300 spectrophotometer is the Vision-Pro™ program (Vision-Pro Thermo Electron UV-Visible Spectrometry, version 2.03; Math Version 24.00). The main view of Vision-Pro™ program is presented on Fig. 1. On the toolbar are presented a standards options: file, application, command data store etc. Before the measurement, the user sets the wavelength, performs zeroing of the device and prepares the sample.

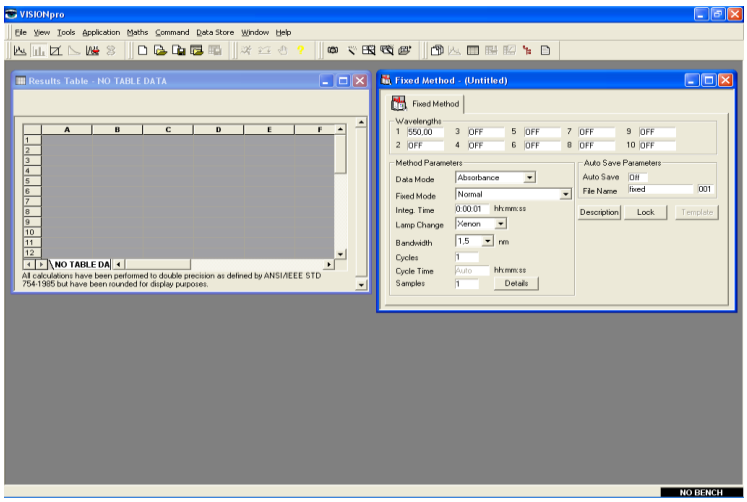

Fig. 1. Main menu of Vision-Pro™

This program allows to perform many different types of measurements e. g. sample measurement in selected cycles and time intervals (method applied e. g. for radical scavenging assays for the Inhibition Concentration IC_{50} determination) or for the calibration graphs drawing (Fig. 2).

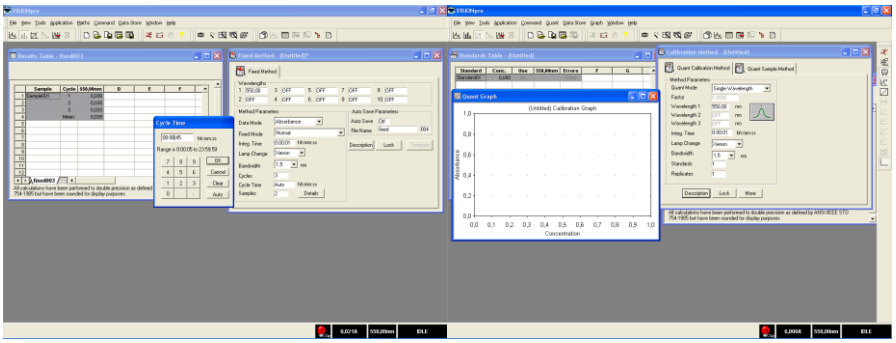

Fig. 2. Various analyses from program

The effect of the carried out measurements are a certain absorbance values. To the standardize the results the absorbance values are recalculated through the proper mathematic formula or equations from the standard calibration curve. For example, the results of DPPH and $ABTS^+$ measurements can be recalculated and presented as a percentage radical scavenging activity according to the formula e.g. for DPPH:

$$\% = \frac{A_{control} - A_{sample}}{A_{control}} \cdot 100 \quad (1)$$

where: *A* – absorbance.

Or can be show as an Inhibition Concentration (IC_{50} or EC_{50}) – concentration of the antioxidant substances which can inhibit of free radical by 50% (Moniruzzaman, Khalil & Sulaiman, 2012; Kedare & Singh, 2011). Although most scientists prefer the presents results as an equivalent of amount of selected antioxidant – standard – per volume (μL or mL). A calibration curve is constructed by reference values of measurement of the absorbance of selected, diluted standard (Trolox, gallic acid) to the standard's concentration. On the basis of the equation obtained, the antioxidant activity of tested sample is calculated (Sochor et al., 2010; Shirazi, Khattak, Shukri & Nasyriq, 2014). Usually, for comparison the degree of the $ABTS^+$ and DPPH radical scavenge activity the Trolox Equivalent Antioxidant Capacity (TEAC or TE) is applied. Trolox is a commercial vitamin E analogue; results are expressed as amount of Trolox equivalents per volume (Badarinath, Mallikarjuna, Chetty, Ramkanth, Rajan & Gnanaprakash, 2010; Sochor et al., 2010; Moon & Shibamoto, 2009**).** For the TPC assay the results are usually presented as a gallic acid equivalent (e.g. as a mgGA/g). However, as Shahidi and Zhong (2015) noted, caffeic acid, ferulic acid and catechins are also popular (Shahidi & Zhong, 2015).

For the statistical analysis also various computer programs can be applied – in this trial it was decided to used KyPlot – a simple program created by KyensLab Incorporated. For this experiment important is to find differences or similarities between samples (various herbs) and between type of extraction solvents (water, ethanol, and aqueous ethanol). The T-Tukey test is a perfect tool for this analysis. An important factor is also a comparison between herbs and extraction solvents and assays. The tests listed above are all spectrophotometric methods based on a absorbance measurement during a discolored reactions between reagent and potential antioxidant substances. Those assays based on the different chemical reaction – the radical scavenging, the ion metal reduction etc. and therefore the response to antioxidant substances can be slightly different. But together, those methods allows to determine how strong the antioxidant properties has the tested compound and whether depends on the selected solvent.

4. RESULTS AND DISCUSSION

In this chapter the results of the experiment will be discussed. Due to the application of four different herbs (couch grass, dandelion, milk thistle, fireweed) each of them will be briefly characterized. Also, the applied methods for the antioxidant properties measurement will be characterized from the chemical point of view.

The antioxidant activity of extracts from herb materials are presented in Table 1 and the radical scavenging activity for $ABTS^{+}$ and DPPH methods are presented in Table 2. As it can be seen in general, the total polyphenolic content (TPC) was significantly ($P<0.05$) higher in all samples treated with aqueous ethanol as a solvent (Table 1). As it was mentioned earlier, the amount of extracted polyphenol compounds depends on solvent selection. Grujic et al. (2012) noted that mono-component solvent is not as much effective as their mixture. Addition of water to ethanol facilitates extraction through polar medium creation.

Tab. 1. Antioxidant and radical scavenging activity of herb extracts

PARAMETER	SAMPLE	SOLVENT		
		WATER	AQUOEUS ETHANOL (50:50)	ETHANOL
TPC [μg GA/ml]	C	0.005 ± 0.00^{Cb}	0.019 ± 0.01^{Da}	0.00 ± 0.01^{Cb}
	D	0.09 ± 0.00^{Bb}	0.244 ± 0.04^{Ba}	0.023 ± 0.00^{Bc}
	M	0.009 ± 0.01^{Cc}	0.083 ± 0.00^{Ca}	0.05 ± 0.00^{Ab}
	F	0.468 ± 0.01^{Ab}	0.625 ± 0.01^{Aa}	0.057 ± 0.00^{Ac}

C – couch grass extract (*A. repens*), D – dandelion (*T. officinale*) extract, M – milk thistle (*S. marianum*) extract, F – fireweed (*E. angustifolium*) herb. Means with different capital letters are significantly different ($p<0.05$) in the same column. Means with different small letters are significantly different ($p<0.05$) in the same row. Means ± standard error.

Agropyron repens (couch grass or quack grass) is a plant with highly branched, long yellowish-white rhizomes. In folk medicine it is usually used for treating various symptoms of urinary disease – prostatic disease, urinary infections, it's also used for calming spasms and pains in the urinary tract and as a soothing diuretic remedy. Couch grass contains phenol substances, carbohydrates, pectins, saponins and essential oils. Moreover, *A. repens* herb is a rich source of minerals especially silica (Al-Snafi, 2015). Furthermore, couch grass is an aggressive herb, presenting allelopathic effects on higher plants (due to the tricin presence) (Friebe, Schultz, Kock & Schnabl, 1995). Due to the lack of information

about polyphenol substances in couch grass extract, it could be suspected that *A. repens* is not a rich source of biologically active compounds. It was noted that couch grass shows the lowest values of antioxidant properties compared to the other herbs (Tab. 1).

Silybum marianum (milk thistle) is a herb used as a medicine for various liver diseases (e.g. removing excess bile from gallbladder, protecting the organ from poisoning, as intoxication after *Amanita* spices consumption). The main antioxidant substance in milk thistle is silymarin. Silymarin is a mixture of flavonolignans (silibinin, silybin A and B, isosilybin A and B) which ensure strong hepatoprotective effects (Soleimani, Delghandi, Moallem & Karimi, 2019). Elwekeel, Elfishawy & AbouZid (2013) found that part of the plant can determine the polyphenol amount – mature fruit and fruit heads of milk thistle contain the highest concentration of silymarin. Moreover, as Chambers et al. (2017) observed sylimarin is usually extracted from seeds (seeds cake or whole pulverized seeds) by hexagene, petroleum, ethyl acetate, acetone or methanol extraction (in conventional methods). However, in the experiment carried out by Barreto, Wallace, Carrier & Clausen (2003) hot water as solvent in extraction process was applied. Hot water presented the solubility characteristic similar to methanol and ethanol, which increase along with the temperature. Authors noted, that depending on temperature of the extraction process (85°C and 100°C), different amounts of polyphenol compounds were identified (taxifolin, silychristin and silybin A and B respectively). This situation was explained by the various polarity of those substances. Authors also pointed out that extraction at 50°C was not so effective compared to the other temperatures. In our experiment, herb extractions were carried out in 40°C so it could be concluded, that this ratio of temperature was too low for milk thistle flavonolignan extractions.

Taraxacum officinale L. (dandelion) was used in folk medicine as antidiabetic, diuretic medicine and as a substance enhancing the immune response. Extract from dandelion also presents hepatoprotective and antioxidant effects. The main component of dandelion is chicorcic acid (dicaffeyltartaric acid) but the plant also contains other polyphenol compounds (e.g. saponins, phenols, flavonoids). For the extraction of antioxidant substances from dandelion aqueous ethanol mixture was the best solvent. The obtained data agree with the results presented by Ivanov (2014), which carried out an extraction process at 80°C, although the extract from *T. officinale* did not present the strongest antioxidant properties. As Ghaima, Hashim & Ali (2013) noted, most of the polyphenol substances are concentrated in flower, root and steam. This could explain lower antioxidant activity of dandelion extract in comparison to the extract from fireweed. However, Sengul et al. (2009) pointed out that the Folin-Ciocalteu method for TPC measurement is not an unlimited, perfect method. Differences between samples and results from other assays could be an effect of various chemical structure of phenolic compounds which affected their antioxidant activity. There is always a risk that antioxidant substances could react between

themselves, so for these reasons, several methods for antioxidant property measurements should always be applied. Moreover, solvent type and the method used boh have a significant influence on the values of antioxidant activity of tested samples (Skotti, Anastasaki, Kanellou, Polissiou & Tarantilis, 2014), which are in agreement with our study.

The antioxidant activity is the ability to inhibit the oxidation process (Shalaby & Shanab, 2013). The amount of the polyphenol substances is usually correlated with antioxidant properties of the extract and with the radical scavenging ability (Ivanov, 2014; Sengul et al., 2009). This dependence was observed for samples measured by using $ABTS^+$ method (Table 2). For samples tested by DPPH assay, it was noted that lower amount of the polyphenol substances in extract from couch grass did not result in lower percentage of radical inhibition values. Extract from milk thistle and fireweed also presented similar data. This dependence was found for ethanolic extract.

Tab. 2. The radical scavenging activity of herbs extracts

PARAMETER	SAMPLE	SOLVENT		
		WATER	AQOUEOUS ETHANOL (50:50)	ETHANOL
$ABTS^+$ [%]	C	$1,23\pm0,17^{Cc}$	$4,85\pm0,22^{Da}$	$3,6\pm0,15^{Db}$
	D	$11,08\pm1,2^{Bb}$	$23,95\pm2,52^{Ba}$	$12,15\pm2,72^{Cb}$
	M	$9,11\pm3,87^{Bb}$	$17,8\pm2,12^{Ca}$	$18,5\pm2,75^{Ba}$
	F	$92,22\pm0,63^{Aa}$	$93,37\pm0,08^{Aa}$	$27,73\pm2,55^{Ab}$
DPPH [%]	C	$1,77\pm0,41^{Dc}$	$2,92\pm0,18^{Db}$	$4,42\pm0,3^{Ca}$
	D	$7,62\pm1,3^{Bc}$	$37,45\pm2,76^{Ba}$	$21,41\pm1,74^{Bb}$
	M	$2,95\pm0,38^{Cc}$	$5,82\pm0,21^{Cb}$	$7,29\pm1,03^{Ca}$
	F	$84,85\pm0,12^{Ab}$	$85,36\pm0,1^{Ab}$	$118,6\pm3,56^{Aa}$

C – couch grass extract (*A. repens*), D – dandelion (*T. officinale*) extract, M – milk thistle (*S. marianum*) extract, F – fireweed (*E. angustifolium*). Means with different capital letters are significantly different ($p<0.05$) in the same column. Means with different small letters are significantly different ($p<0.05$) in the same row. Means ± standard error.

Epilobium angustifolium (fireweed) is a herb commonly used in an alternative medicine for gastrointestinal disorder, rectal bleeding, sleeping disorders, bladder, prostate and kidney diseases. The herb can be applied as an extract or as a tea. According to literature data, *E. angustifolium* is a very rich source of various polyphenol substances (e.g. tannins, phenolic acids, flavonoids, steroids) (Onar, Yusufoglu, Turker & Yanardag, 2012; Granica, Piwowarski, Czerwińska & Kiss, 2014; Schepetkin et al., 2016). Dudonne, Vitrac, Coutiere, Woillez

& Merillon (2009) pointed out that usually strong correlations between ABTS$^+$ and DPPH methods and TPC values are observed. Also, Onar et al. (2012) have found a positive relationship between the amount of polyphenol substances in fireweed water extract and radical scavenging capacity. Moreover, Schepetkin et al. (2016) pointed out that an aqueous extract has a stronger anti-proliferative activity than ethanol extracts. Also, in the experiment carried out by Ostrovska et al. (2017) it was found that ethanol extract from fireweed contains very high amount of polyphenols (26.95 g as gallic acid equivalent per 100 g of dry weight). In our experiment the highest amount of substances of antioxidant activity was found for aqueous ethanol as a solvent. This could be connected with the increase of the solvent polarity which increases the amount of extracted polyphenols (Grujic et al., 2012). According to our experiment, both water and aqueous ethanol extracts from *E. angustifolium* presented strong antioxidant activity. In that case, higher activity percentage (118.6%) of ethanol extract could be connected with the conditions of the method applied. Pérez-Jiménez & Saura-Calixto (2006) observed, that the values of tested samples depended on the polarity of the solvent. For the ABTS$^+$ method, along with the increase of the polarity of the solvent, values of ABTS$^+$ increased, which is in the agreement with our results.

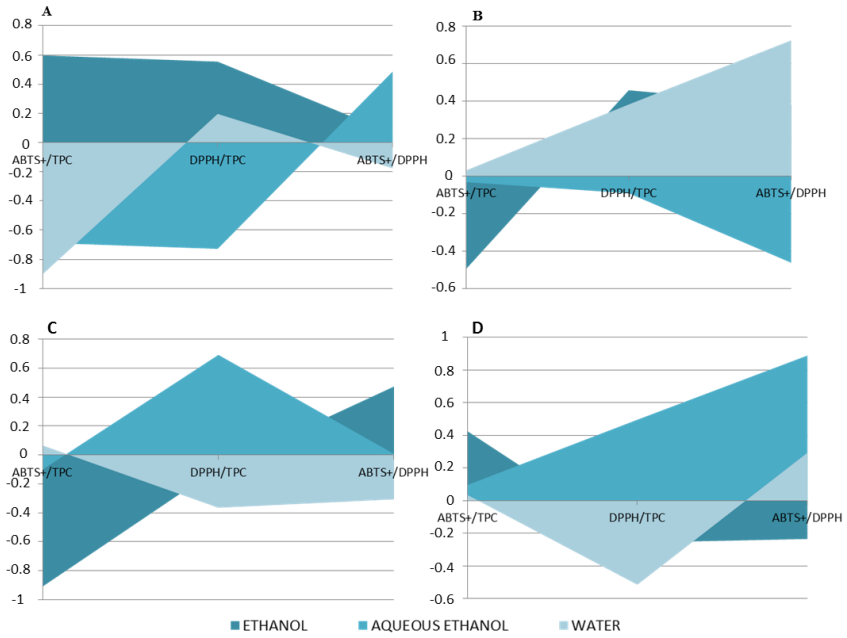

Fig. 3. Correlation between of type of solvent and antioxidant and radical scavenging parameters: A – Dandelion extracts, B – Fireweed extracts, C – Milk thistle extracts, D – Couch grass extracts

Both, $ABTS^+$ and DPPH methods are based on a degree of the color change which is correlated with antiradical scavenging ability of the sample (Dudonne et al., 2009). DPPH solution has a deep, violet color; $ABTS^+$ is characterized by blue-green color. During reactions with an antioxidant substances, radicals in the solutions are reduced and the color is loss, which allows to evaluate the antioxidant potential of tested samples (Alam, Bristi & Rafiquzzaman, 2013; Sahalaby & Shanab, 2012). Neverthless, some differences between methods occur. Preparing of the $ABTS^+$ takes more over 12 hours due to the chemical or enzymatic reactions which allow to free radical generations. For this reasons, the $ABTS^+$ working solution can give slightly different results along with the time. $ABTS^+$ is soluble in organic media and in water which allows to measure lipophilic and hydrophilic substances in tested sample. In the DPPH method, the 2,2-diphenyl-1-picrylhydrazyl is ready to dissolve and can be used directly after preparation. However, this radical is soluble only in organic, alcoholic media (Sahalaby & Shanab, 2012; Arnao, 2000; Thaipong, Boonprakob, Crosby, Cisneros-Zevallos & Bryne, 2006). Furthermore, how Singh & Singh (2008) notes for $ABTS^+$ and DPPH methods various wavelength can be applied (415, 660, 734, 820 and 515 to 528 nm respectively). In the experiment carried out by Arnao (2000) fresh fruits juices and wines were examined by using two antioxidant measurements methods: DPPH (515 nm) and $ABTS^+$ (for $ABTS^+$ two wavelengths – 730 and 414 nm – were applied). For the juices, wavelength 730 nm for $ABTS^+$ method and wavelength 515 nm for DPPH method gives convergent results. Author pointed out that the biggest differences between antioxidant capacities concerned red wines samples. The differences depending not only on the methods, but also depending on wavelength – in general, samples measured at 720 nm presented higher antioxidant activity (216.20 and 234.06 mg TEAC/100 ml) than measured at 414 nm (183.65 and 195.40 mg TEAC /100 ml). Fig. 3 shows the correlations between solvents and between method applied. The strongest, positive correlations were found for dependence between DPPH and TPC. No correlation was observe between ABTS+ and DPPH for dandelion (ethanol solvent) and milk thistle (aqueous ethanol solvent).

5. CONCLUSIONS

The data collected through the Vision-Pro™ UV-VIS software by different spectrophotometric method applied allows to state that aqueous ethanol as a solvent gives best results for phenolic compound extractions for all examined herbs. Extract from fireweed was characterized by the highest antioxidant and radical scavenging activities compared to the other herbs. However, it must be added, that extract from dandelion also showed strong antioxidant properties. As it was also noted, extracts from couch grass presented the lowest antioxidant potential compared to the analyzed extracts. In general, the antioxidant activity of the herb

extracts decreased in the following order W>D>M>C. Results of the research indicate that herb extracts can be applied in the food industry to extend the durability of the product, which allows to make product more attractive for the potential customers. Due to the strong antioxidant properties, extracts from *E. angustifolium, T. officinale* and *S. marianum* should be applied.

ACKNOWLEDGEMENTS

The research was financed under the program of the Minister of Science and Higher Education under the name "Regional Initiative of Excellence" in 2019–2022 project number 029/RID/2018/19 funding amount 11 927 330.00 PLN".

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