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EFFECT OF VARIOUS pH CONDITIONS SIMULATED *IN VIVO* ON THE ACTIVITY OF LIPOPHILIC ANTIOXIDANTS ISOLATED FROM SELECTED SPICES

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Key words: spices, antiradical activity, reducing power, chelating power, lipid peroxidation, phenolic compounds, *in vitro* digestion

Extracts of rosemary (*Rosemarinus officinalis*), thyme (*Thymus vulgaris*) and marjoram (*Origanum majorana*) before and after simulated digestion were investigated for their antiradical activity, iron chelation, iron reduction and inhibition of lipid peroxidation. The changes of total phenolic compounds, phenolic acids, quercetin, kaempferol and luteolin contents were measured as well. Digestion *in vitro* caused a significant increase in the content of total phenolics in all samples. The rosemary extract showed the highest ability to scavenge free DPPH radicals (91.97%). The activities of marjoram and thyme samples were much lower (32.55 and 24%, respectively). Digestion *in vitro* caused a decrease of activity in all samples. The highest decrease (88.47%) occurred after rosemary extract digestion. The activity of thyme and marjoram extracts decreased about 71.05% and 29.24% respectively. The extracts obtained from marjoram and thyme showed significant chelating power (>76%), whereas the rosemary extract was less active (19.26%). No interchangeable effect of hydrolysis under variable pH conditions on the chelating power was noticed in the study. *In vitro* digestion had no significant effect on the ability of the rosemary extract to inhibit linoleic acid autooxidation, the activity of this sample was high (about 87%). In the case of the other samples a significant decrease in their activity was observed after the *in vitro* digestion. The highest losses of activity were observed in the case of the marjoram extract. Reducing power of thyme and marjoram extracts was lower than the activity of rosemary extract but the differences were not truly significant. After digestion the activity of all samples decreased dramatically. In the case of rosemary and marjoram extracts digestion caused about 83% decrease of their activity. The thyme extract after hydrolysis retained 12.26% of its reducing power.

INTRODUCTION

A great number of aromatic, spicy, medicinal and other plants contain chemical compounds exhibiting antioxidant properties. Antioxidants can be defined as compounds that can delay or prevent the oxidation of lipids or other molecules by inhibiting the initiation or propagation of an oxidizing chain reaction [Zheng & Wang, 2001]. Plant secondary metabolites are an enormously variable group of phytochemicals in terms of their number, structural heterogeneity and distribution. Among the most studied diet-related phytochemicals are the hydroxycinnamic acids and flavonoids [Kosar *et al.*, 2005]. Despite the epidemiological evidence that consumption of various herbs and spices is associated with positive health benefits, there is little information on their changes in the gastrointestinal tract. In this study we have taken extracts of selected, normally consumed, spices and subjected them to a simulated gastric and intestinal digestion *in vitro* and monitored changes of antioxidant activity and concentration of total phenolics.

MATERIALS AND METHODS

The following spices purchased at a local supermarket were used for investigations: rosemary (*Rosemarinus officinalis*), thyme (*Thymus vulgaris*) and marjoram (*Origanum majorana*). Ten grams of spice were extracted with 100 mL of boiled methanol. Simulated gastric digestion was carried out essentially as described by Yoshino *et al.* [1999]. Samples of spices were incubated at 37°C for 1 h after acidification with HCl to pH 2.0. The samples were then brought to pH 7.5 with sodium bicarbonate and the incubation was continued for up to 60 min. No digestive enzymes such as pepsin in gastric juice and pancreatin in intestinal juice were added to the mixture in this study, because the methanolic spice extracts contain a very low amount of proteins, so these effects on the activity of phenolic compounds were neglected.

Total phenolic assay. Total polyphenols concentration was determined with the Folin-Ciocalteau reagent. The absorbance was measured at 725 nm and calculated as gallic acid equivalents (mg GAE/mL). The content of phenolic acids and selected flavonoids was analysed following the HPLC method [Li *et al.*2005] with some modification. A Varian HPLC separation module (Varian, Palo Alto, CA) equipped with Varian ChromSpher C18 (25 mm x 4.6 mm) column and Pro star 325 UV-Vis Detector was used. The mobile phase contained solvent A (1% acetic acid) and solvent B (100% methanol). The solvent gradient was programmed as follows: at 0 min, 5% B; 5 min, 5% B; 15 min, 15% B; 30 min, 30% B; 40 min, 35% B; 50 min, 70% B; 55 min, 100% B. At the end of gradient the column was washed with 100% methanol and

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TABLE 1. Qualitative and quantitative analysis of the extracts by HPLC (290 nm).

Compounds	RT (min)	Crude non-treated extract (μg/mL extract)			Treated extract (μg/mL extract)		
		Marjoram	Rosemary	Thyme	Marjoram	Rosemary	Thyme
Caffeic acid	28.00	1.716	2.668	0.577	0.927	0.144	nd
p-Coumaric acid	36.79	0.704	2.085	1.472	0.042	0.108	nd
Ferulic acid	39.43	0.581	0.308	0.633	0.0442	nd	nd
Luteolin	53.62	nd	0.642	0.889	nd	nd	nd
Quercetin	52.97	7.058	6.318	12.232	nd	5.866	99.059
Kaempferol	54.33	0.615	0.230	0.358	nd	0.982	0,997
Total phenolics (mgGAE/mL)		0.49	0.88	0.72	1.75	2.34	1.60

nd – not detected

equilibrated to initial condition. Detection was performed at 290 nm. Phenolic compounds in sample were identified by comparing their retention times with those of the standard compounds.

Antioxidant assay. The antiradical activity was measured in terms of hydrogen donating or radical scavenging ability using the stable DPPH method [Brand-Williams *et al.*, 1995]. The Fe(III)-Fe(II) reduction activity of spice extracts was determined according the method of Oyaizu [1996]. The chelation of iron (II) ions by the samples was carried out as described by Guo *et al.* [2001]. Inhibition of linoleic acid peroxidation was determined according to the method of Lingnert *et al.* [1979].

Statistical analysis. All analyses were run in triplicate. Analysis of variance was used to evaluate differences between various treatments at a significance level of $\alpha=0.05$.

RESULTS AND DISCUSSION

Herbs are known to produce a wide array of secondary metabolites such as hydroxybenzoates, hydroxycinnamates and flavonoids, among others; all these phytochemicals can occur either as aglycones or glycosides. The highest content of phenolics (gallic acid equivalent) was observed in both samples of rosemary, and the lowest in the non-treated marjoram extract. In all cases the *in vitro* digestion resulted in a significant increase in the total phenolics content (Table 1). Kosar *et al.* [2005] analysed the influence of pH on the composition and the activity of methanolic extracts from herbs and confirmed that acid hydrolysis had caused a significant increase of polyphenols content in thyme, sage, rosemary and oregano extracts; however, the decrease of phenolics content was observed in the case of basil extract. Yet in the reported study no re-alkalization was carried out. Investigations of Record & Lane [2001] show that various catechin components of green tea had different stabilities at alkaline pH. The results obtained in this work show that phenolic compounds released after gastric digestion were more stable under these conditions.

Various mechanisms may contribute to oxidative processes in complex systems such as nourishment and food preparation, therefore, it is important to characterise the extracts by a variety of antioxidant assays. Herbs and spices are added to food freshly chopped, or ground into a paste, either individually or as a mix. In some cases they are consumed fresh, without any processing but in most dishes they are cooked. Shobana & Akhilender Naidu [2000] showed that when boiled (at 100°C for 30 min) garlic, ginger, cloves, cinnamon and pepper extracts did not only retain the antioxidant activity but also revealed significantly higher antioxidant activity – indicating that the herb and spice constituents were resistant to thermal denaturation. The possible release of bound antioxidant compounds during the heat treatment could be responsible for the higher antioxidant activity compared to a fresh herb extract.

Rosemary extract showed the highest ability to scavenge free DPPH radicals (91.97%). The activities of marjoram and thyme samples were much lower (32.55 and 24%, respectively). Dorman *et al.* [2003] analysed the antiradical activity of hydrophilic antioxidants isolated from selected *Lamiaceae* species; they found that thyme and rosemary extracts showed the highest activity. Digestion *in vitro* caused a decrease of the activity in all samples. The highest decrease (88.47%) occurred after rosemary extract digestion. The activity of thyme and marjoram extracts decreased by about 71.05% and 29.24%, respectively (Figure 1). Analysing the ability of methanolic extracts obtained from species to neutralize free radicals Kosar

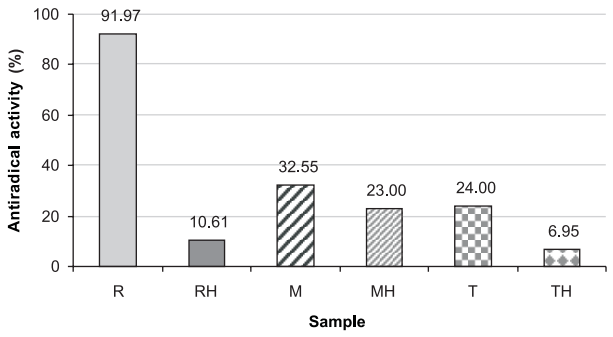

FIGURE 1. Antiradical activity of non-treated and treated spice extracts. R – rosemary extract, RH – hydrolysed rosemary extract; M – marjoram extract, MH – hydrolysed marjoram extract; T – thyme extract, TH – hydrolysed thyme extract.

et al. [2005] affirmed that the most effective were thyme and rosemary extracts, and the least effective was basil extract. The available literature on the topic mentions little similar researches. In the research by Kosar *et al.* [2005] acid treatment significantly increased radical-scavenging properties of basil, rosemary and thyme extracts, but the activity of sage extract appears to decrease after the treatment, yet in the reported study no re-alkalization was carried out. It is possible that the compounds responsible for the antiradical activity were not stable in the alkaline pH and lost their properties after alkalization, but the explanation of this problem still requires further investigation.

Food is often contaminated with transition metal ions that may be introduced by various processing methods. Bivalent transition metal ions play an important role as catalysts of oxidative processes, leading to the formation of hydroxyl radicals and hydroperoxide decomposition reactions *via* Fenton reaction [Hallivell, 1997]. These processes can be delayed by iron chelation and deactivation. The extracts obtained from marjoram and thyme showed significant chelating power (>76%), whereas the rosemary extract was the least active (19.26%). No interchangeable effect of hydrolysis under different pH conditions on the chelating power was shown in the study. The activity of the rosemary extract increased significantly after digestion. Digestion *in vitro* revealed no significant effect on the activity of marjoram extract, yet its activity decreased in the case of thyme sample (Figure 2).

Lipid peroxidation leads to rapid development of rancid and stale flavors and it is considered a primary mechanism of quality deterioration in lipid foods and oils [Mathew & Abraham, 2006]. Herbs are well-known to exert this effect on lipid peroxidation by scavenging reactive oxygen species or chelate metal ions needed for initiation of lipid peroxidation. Herbal extracts might contain more than one antioxidant and, thus, they may scavenge the hydroxy radical or quench the fatty acid radicals by inhibiting the propagation of lipid peroxidation [Shobana & Akhilender Naidu, 2000]. *In vitro* digestion had no significant effect on the ability of rosemary extract to inhibit linoleic acid autooxidation; the activity of this sample was high (about 87%). In the case of the other samples a significant decrease in their activity was observed after the *in vitro* digestion. The highest losses of activity were observed in the case of the marjoram extract (Figure 3). The extracts of rosemary were the first marketed natural antioxidants. As compared with the commercial antioxidants BHA and BHT, the phenolic antioxidants from rosemary provide desirable flavors in frying operations [Zheng & Wang, 2001]. Rosemary extracts have been added to lipid products to preserve or retard lipid oxidation.

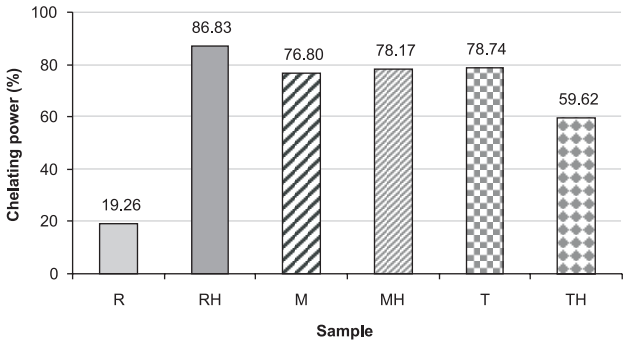

FIGURE 2. Chelating power of non-treated and treated spice extracts. R – rosemary extract, RH – hydrolysed rosemary extract; M – marjoram extract, MH – hydrolysed marjoram extract; T – thyme extract, TH – hydrolysed thyme extract.

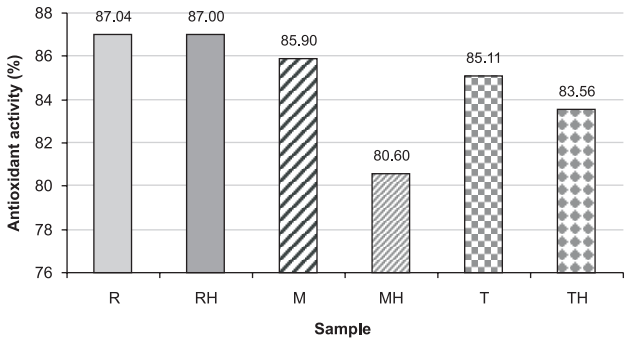

FIGURE 3. Ability of non-treated and treated spice extracts to inhibit lipid peroxidation. R – rosemary extract, RH – hydrolysed rosemary extract; M – marjoram extract, MH – hydrolysed marjoram extract; T – thyme extract, TH – hydrolysed thyme extract.

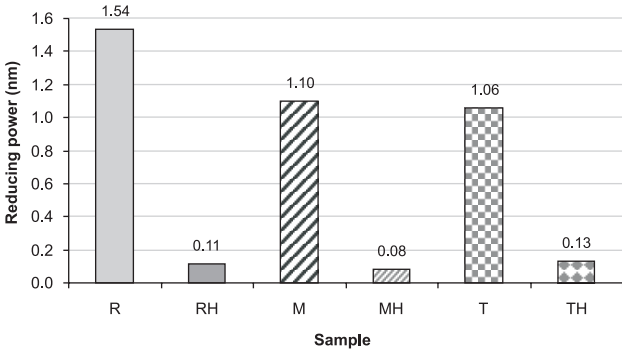

FIGURE 4. Reducing power of non-treated and treated spice extracts. R – rosemary extract, RH – hydrolysed rosemary extract; M – marjoram extract, MH – hydrolysed marjoram extract; T – thyme extract, TH – hydrolysed thyme extract.

Fe (III) reduction is often used as an indicator of electron-donating activity, and this is an important mechanism of phenolic antioxidants action [Kosar *et al.*, 2005]. In the research by Dorman *et al.* [2003] a rosemary water extract showed high reduction power in reference to ascorbic acid – 0.188 RRE (relative reductive efficiency). The activity was lower by half in the case of a thyme extract (0.099 RRE). The assumed relation was not confirmed after the analysis of lipophilic antioxidants in this work. Thyme and marjoram extracts activity was lower than that of the rosemary extract but the differences were not truly significant (Figure 4). After digestion, the activity of all samples decreased dramatically. In the case of rosemary and marjoram extracts digestion caused about 83% decrease of their activity. The thyme extract after hydrolysis retained 12.26% of its chelating power (Figure 4).

In the research by Kosar *et al.* [2005] acid treatment significantly increased the reducing power of extracts obtained from rosemary, sage and thyme. The data obtained in this work points straightforward to the negative influence of reaction mixture alkalization on the antioxidant activity of lipophilic antioxidants isolated from herbs.

CONCLUSIONS

The results obtained show the complexity of changes that biologically-active compounds undergo under changeable pH conditions. Apart from the interactions between compounds, changes in the gastrointestinal tract can significantly influence the activity of these compounds as well. Future research will be oriented on the explanation of these problems.

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WPŁYW SYMULOWANYCH *IN VIVO* WARUNKÓW pH NA AKTYWNOŚĆ LIPOFILNYCH PRZECIWUTLENIACZY WYIZOLOWANYCH Z WYBRANYCH PRZYPRAW

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W ekstraktach z rozmarynu (*Rosemarinus officinalis*), tymianku (*Thymus vulgaris),* i majeranku (*Origanum marjorana*) przed i po symulowanym trawieniu określono aktywność przeciwrodnikową, zdolność do chelatowania jonów żelaza, zdolność do redukcji żelaza i inhibicji peroksydacji lipidów a także zmiany zawartości związków fenolowych ogółem, fenolokwasów, kwercetyny, kempferolu i luteoliny. Trawienie *in vitro* spowodowało znaczący wzrost zawartości związków fenolowych we wszystkich próbach. Najwyższą zdolnością do neutralizacji wolnych rodników DPPH charakteryzował się ekstrakt z rozmarynu (91,97%). Aktywność prób z rozmarynu i majeranku była znacznie niższa (odpowiednio 32,55 i 24%). Trawienie *in vitro* spowodowało spadek aktywności wszystkich prób. Najwyższy spadek aktywności (88,47%) nastąpił po trawieniu ekstraktu z rozmarynu. Aktywność ekstraktów z tymianku i majeranku spadła odpowiednio o 71,05% i 29,24% (rys. 1). Ekstrakty otrzymane z majeranku i tymianku wykazywały znaczącą zdolność do chelatowania (>76%), ekstrakt z rozmarynu był mniej aktywny (19,26%). Nie stwierdzono jednoznacznego wpływu hydrolizy w zmiennych warunkach pH na zdolność do chelatowania (rys. 2). Trawienie *in vitro* nie wpłynęło na zdolność do hamowania samoutleniania kwasu linolowego przez ekstrakt z rozmarynu; jego aktywność była wysoka i kształtowała się na poziomie około 87% (rys. 3). W przypadku pozostałych prób po trawieniu *in vitro* nastąpił znaczący spadek aktywności. Największe straty aktywności zaobserwowano w przypadku ekstraktu z majeranku. Siła redukcji ekstraktów z tymianku i majeranku była niższa niż aktywność ekstraktu z rozmarynu, ale różnice nie były znaczące (rys. 4). Po trawieniu aktywność wszystkich prób drastycznie zmalała. W przypadku ekstraktów z majeranku i tymianku trawienie spowodowało około 83% spadek aktywności. Ekstrakt z tymianku po hydrolizie zachował 12,26% zdolności do redukcji.