Phenolic Extracts from Meadowsweet and Hawthorn Flowers Have Antioxidative Properties

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From the flowers of meadowsweet and inflorescence of hawthorn the whole set of phenolic acids and flavonoids was analysed by TLC. Phenolic compounds were determined both as free ones and those liberated by hydrolysis. Moreover, ethyl ether and ethyl acetate extracts obtained from the analysed plants before and after alkaline and enzymatic hydrolyses were evaporated under reduced pressure and residues were analysed for their antioxidative properties. The weakest antioxidative activity was observed with the remaining residue after evaporation of ethyl ether extract obtained from enzymatically (β -glucosidase) hydrolysed hawthorn inflorescence water extract. The strongest antioxidative activity was noticed with the remaining residues after evaporation of ethyl ether extracts obtained from non-hydrolysed and hydrolysed in alkaline conditions of meadowsweet flower water extracts.

The residues from meadowsweet flowers exhibited stronger antioxidative properties than residues obtained from hawthorn inflorescence and can be recommended as margarine preservatives.

Introduction

Meadowsweet (*Filipendula ulmaria* (L.) Maxim.) is a medicinal plant growing in moist meadows, on water banks from north part of Siberia, Altai, Mongolia to the north part of the Balkans, as well as in northern Italy, France, Spain, England and Poland. Its therapeutic features include diaphoretic, anti-rheumatic and anti-inflammatory. Hawthorn (*Crataegus oxyacantha* L.) is a medicinal plant growing in Europe and North Africa. It is used as a mild, completely non-toxic medicine for heart diseases.

A lot of polyphenolic compounds have been found in both species which can strongly influence their biological activity (Ficcaro *et al.*, 1984; Fisel, 1966; Budzianowski *et al.*, 1991; Wagner and Bladt, 1996).

Margarines are fats with highly unsaturated, essential fatty acids, which are known as effective antiarteriosclerotic agents (Worm, 1995; Mortensen *et al.*, 1992). Margarines containing unsaturated fatty acids are very sensitive against many oxidative agents, which may produce toxic sub-

stances such as ketones, aldehydes and fatty acid peroxides (Hammer and Wills, 1979; Pokorny *et al.*, 1976; Halliwell and Gutteridge, 1989a). At present manufacturers protect margarines against oxidative processes by adding lipid-soluble substances such as vitamin E and A or β -carotene (van het Hof *et al.*, 1998; Halliwell and Gutteridge, 1989b). Another group of antioxidants (soluble in water) are plant phenolics such as flavonoids, tannins, catechins or phenolic acids (Halliwell and Gutteridge, 1999; Fauconneau *et al.* 1997; Benov and Georgiev, 1994; Bajorun *et al.*, 1994; Robak *et al.*, 1986). The therapeutic potential of plant phenolics is continually under study. More data on their clinical efficacy is needed. It is known that flavonoids and their derivatives affect the function of the immune system (Middleton and Kandaswami, 1986). Moreover flavonoids and their derivatives are also known as anti-inflammatory agents. They are considered to reveal vitamin-like activity (Hughes and Wilson, 1977; Kuhnau, 1976), acting on capillary permeability and can be used for the treatment of vascular problems. According to the literature it is encouraging to test plant phenolic

extracts as possible antioxidant “preservatives” in food.

In this work extracts from hawthorn inflorescence and meadowsweet flowers containing flavonoids and phenolic acids were obtained. These extracts were evaporated under reduced pressure and residues were investigated for their antioxidative properties during margarine oxidation. Moreover, the qualitative analysis of phenolic acids, flavonoids and their glycosides in these extracts was also performed.

Materials and Methods

Preparation of extracts

Dried blooms of hawthorn with 2–5 leaves and dried blooms of meadowsweet were used as material. The raw materials (75 g of each species), obtained from Kawon (Herb Factory, 63–800 Gostyń, Poland), after defatting with petroleum ether (750 ml), were extracted three times with 50% ethanol/water (3 × 750 ml) by boiling in a water bath. The extracts were stored in a refrigerator for 7 days and then separated from the precipitate formed. Then ethanol was removed under reduced pressure from the combined ethanol/water extracts and the formed precipitate filtered and discarded to obtain water extracts (1100 ml of each extract) from hawthorn inflorescence (HI) and meadowsweet flowers (MSF).

Two methods of extraction were performed from this step in order to obtain phenolic acids and flavonoid aglycones fraction (method I) and flavonoid glycosides fraction (method II).

Method I. Extracts HI and MSF (2 × 820 ml) were exhaustively extracted with ethyl ether (10 × 50 ml – each extract). The pooled ethyl ether extracts were dried under reduced pressure to obtain residues respectively HA (0.51 g) and MA (0.72 g). The remaining water layer was hydrolysed under alkaline conditions (pH 12, 1 hr) and extracted with ethyl ether (8 × 50 ml – each extract). The ethyl ether extracts were combined and dried under reduced pressure to obtain residues HB (0.41 g) and MB (0.75 g). The water layers (remaining after alkaline hydrolysis and ethyl ether extraction) were further hydrolysed by β -glucosidase for 10 hr at 37 °C, pH 5.0, (13 μ g/ml, Fluka, Switzerland ~6U/mg). The hydrolysates were separated from precipitate formed and sub-

sequently extracted with ethyl ether (10 × 50 ml – each extract). The pooled ethyl ether extracts were concentrated to dryness under reduced pressure to obtain residues HC (0.22 g), MC (0.41 g).

Method II. Extracts HI and MSF (2 × 280 ml) were extracted with ethyl acetate (8 × 50 ml – each extract). The pooled ethyl acetate extracts were dried under reduced pressure to obtain residues HD (0.31 g), MD (1.35 g).

All residues were further investigated for phenolic compounds composition and antioxidative activity.

Thin layer chromatography

Residues HA, MA, HB, MB, HC, MC, HD, MD were dissolved in methanol and examined by one dimensional thin layer chromatography on silica gel plates (Merck, Kieselgel 60 F₂₅₄, DC-Fertigplatten, layer thickness 0.25 mm). Phenolic acids and flavonoid aglycones were analysed after migration in toluene-ethyl formate-formic acid, 5:4:1 (Dey and Harborne, 1989a) and isopropyl ether-cyclohexane-formic acid, 4:1:0.1 and visualised by spraying with 1% FeCl₃ in methanol, diazotised sulphanilic acid amide (preparation of reagent see below), 0.1% AlCl₃ in methanol and under UV at 365 nm. Flavonoid glycosides were analysed with the solvent acetone-chloroform–water, 8:2:0.48 (Dey and Harborne, 1989b) and visualised by 1% AlCl₃, diazotised sulphanilic acid amide and UV at 365 nm. All phenolic compounds were identified by TLC analysis against standards.

Preparation of diazotised sulphanilic acid amide

Solution A: 0.5 g of sulphanilic acid was dissolved in 70 ml of water, 6 ml of 6 N HCl was added and adjusted to 100 ml with water. Before use solution A was mixed with 5% (w/v) water solution of sodium nitrite in voluminal relation 1:1.

Solution B: 20% (w/v) sodium acetate in water.

Plates were developed first with solution A and then with solution B.

Measurement of margarine oxidation

Thiobarbituric acid reagent (TBA) was prepared by dissolving 375 g of thiobarbituric acid in a small amount of water. Then 15 g of trichloroacetic acid and 2.1 ml of 10 N HCl were added. The solution was adjusted to 100 ml with distilled water.

The measurement of antioxidative activity was performed by determining TBARS (thiobarbituric acid reactive substances) according to Buege and Aust (1978) modified after Sroka *et al.* (1994). Margarine containing plant residues as the only antioxidative agent was prepared in our laboratory according to the method of Sroka and Cisowski (1999).

Six samples of margarines were prepared – one with no plant residue (control sample) and five with 0.002, 0.008, 0.03, 0.1 and 0.5% (w/w) residue (HA, MA, HB, MB, HC, MC, HD and MD) in solid margarine. The margarine peroxidation was induced by ultraviolet radiation UV at 254 nm – bactericidal lamp intensity 80 $\mu\text{W}/\text{cm}^2$. The margarines were sampled after 0, 3, 6 and 9 h of UV exposure. Then the samples were dissolved in ethyl ether at the concentration of 0.17 mg/ml. 1 ml of ethyl ether solution of each sample was evaporated under nitrogen and 2 ml of TBA reagent added. All samples were put into a boiling water bath for 20 min, then centrifuged for 5 min at $1000 \times g$ and the supernatant was measured at 535 nm.

All measures were repeated three times and standard deviations were calculated approximately 0.05.

Results and Discussion

The whole set of phenolic acids and flavonoids identified in our experiments in HI and MSF extracts are demonstrated in Tables I and II. The TLC qualitative analysis fully confirmed literature data on the presence of a rich set of phenolic compounds in the analysed plant materials.

Hawthorn inflorescence contain many quercetin derivatives such as quercetin 3-galactoside (hyperoside) (Ficcara *et al.*, 1984), quercetin 3-rutinoside (rutin) (Fisel, 1966), quercetin 3-glucoside (isoquercitrin) (Bahorun *et al.*, 1994), quercetin 3-rhamnogalactoside (Fisel, 1966), kaempferol and its derivatives (Nikolov *et al.*, 1982), luteolin, apigenin (Ficcara *et al.*, 1990a) and their C-glycosides such as apigenin 8-C glycoside (vitexin), isovitexin (Budzianowski *et al.*, 1991). Among other simple phenolic compounds chlorogenic (Ficcara *et al.*, 1984), caffeic (Ficcara *et al.*, 1990b) and gallic (Bahorun *et al.*, 1994) acids were identified.

Table I. Phenolic acids identified in residues obtained from hawthorn inflorescence and meadowsweet flowers.

Identified compound	Hawthorn inflorescence residues			Meadowsweet flower residues		
	HA	HB	HC	MA	MB	MC
Protocatechuic	+	+	+	+	+	+
Caffeic	+	+	+	+	+	+
Chlorogenic	+	+	+	–	–	–
Salicylic	+	+	–	+	+	+
Gallic	+	+	+	+	+	+
<i>p</i> -Hydroxybenzoic	+	+	+	–	–	–
<i>m</i> -Hydroxybenzoic	–	–	–	+	–	–
2,3-dihydroxybenzoic	+	–	+	+	+	+
3,5-dihydroxybenzoic (α -resorcylic)	–	–	–	+	+	–
2,4-dihydroxybenzoic (β -resorcylic)	+	+	–	–	–	–
Veratric	+	+	+	–	+	+
Ferulic	+	+	+	+	+	+
Vanillic	–	–	–	+	–	–
Syringic	+	+	+	–	–	–
<i>p</i> -Coumaric	–	+	+	+	+	–
<i>o</i> -Coumaric	+	+	+	+	–	–
<i>m</i> -Coumaric	+	–	–	–	–	–
Rosmarinic	+	+	–	–	–	–
Elagic	+	–	+	–	+	+

Legend: (+) compound present; (–) compound absent.

Abbreviations: HA, MA, HB, MB, HC, MC stand for residues after reduced pressure evaporation of ethyl ether extracts obtained from hawthorn inflorescence (HI) and meadowsweet flowers (MSF) water extracts, alkaline hydrolysis HI and MSF and enzymatically hydrolysed HI and MSF respectively.

Table II. Flavonoids identified in residues obtained from hawthorn inflorescence and meadowsweet flowers.

Identified compound	Hawthorn inflorescence residues				Meadowsweet flower residues			
	HA	HB	HC	HD	MA	MB	MC	MD
Quercetin	+	+	+	–	+	+	+	–
Hyperoside (quercetin-3-galactoside)	–	–	–	+	–	–	–	+
Luteolin	+	+	+	–	+	+	+	–
Kaempferol	+	+	+	–	+	+	+	–
Vitexin	–	–	–	+	–	–	–	–
Isovitexin	–	–	–	+	–	–	–	–
Avicularin (quercetin-3-arabinoside)	–	–	–	+	–	–	–	+
Apigenin	+	+	+	+	+	+	–	–
Myricetin	–	–	–	–	+	+	+	–
Rutin (quercetin-3-rutinoside)	–	–	–	+	–	–	–	+
Quercetin-3-rhamnogalactoside	–	–	–	+	–	–	–	–

Legend: (+) compound present; (–) compound absent.

Abbreviations: HA, MA, HB, MB, HC, MC stand for residues after reduced pressure evaporation of ethyl ether extracts obtained from hawthorn inflorescence (HI) and meadowsweet flowers (MSF) water extracts, alkaline hydrolysis HI and MSF and enzymatically hydrolysed HI and MSF respectively. HD, MD denotes residues after evaporation of ethyl acetate extracts obtained from HI and MSF.

Phenolic contents identified in meadowsweet flowers are flavonoids (3–5%) mainly spiraeoside (Pouknes-Renwart, 1992), quercetin, hyperoside, avicularin, rutin, kaempferol and their glycosides (Wagner and Bladt, 1996). Important simple phenolics identified in meadowsweet flowers are derivatives of salicylic alcohol, aldehyde and acid (Hegnauer, 1973).

Our TLC analysis showed the presence of quercetin and its derivatives which are one of the strongest antioxidants among flavonoids in HI extract. Strongly antioxidative phenolic acids such as gallic, caffeic, chlorogenic were also observed in HI.

Myricetin (strong antioxidant) which was not yet mentioned in literature was determined in MSF extract. Among phenolic acids with strong antioxidative properties gallic, caffeic, chlorogenic, and protocatechuic acids were identified too.

The results of our phytochemical analyses (Tables I and II), together with literature reports indicate that selected HI and MSF extracts may be considered for use as antioxidants in margarine.

The oxidation results, expressed as absorbance at 535 nm, for margarine containing HA, HB, HC and HD residues are demonstrated in Table III, while MA, MB, MC and MD residues are demonstrated in Table IV.

The HA residue only weakly inhibited oxidation at maximal concentration of 0.5%. HB residue in-

hibited the oxidation process weakly at 0.1% and strongly at 0.5%. HC residue was a poor antioxidant, no inhibition of oxidation of margarine was observed in concentrations of 0.1 and 0.5% of this residue. HD residue stimulated the oxidation process at 0.002, 0.008 and 0.03%. There was no effect at the concentration of 0.1% and strong inhibition was observed at 0.5% of HD.

The MA residue did not influence oxidation at the concentration of 0.002, 0.03%, and it increased margarine oxidation at 0.008%. At the concentration of 0.1 and 0.5% this residue caused strong inhibition of the oxidation process. MB residue stimulated margarine oxidation strongly at 0.002% and weakly at 0.008 and 0.03%. At 0.1 and 0.5% almost complete inhibition was observed. When MC was investigated, the stimulation of the oxidation process was observed at 0.002, 0.008, 0.03 and 0.1%. However, only at 0.5% weak oxidation of margarine was observed. MD residue did not influence oxidation at 0.002, 0.03%, while strong oxidation inhibition took place at 0.1% and 0.5%, respectively.

Our results show a stronger effect of oxidation inhibition when residues obtained from MSF were used. These extracts can be recommended as antioxidants effective in margarine. Residues obtained from HI appear to be weak antioxidant agents but they may be very useful as margarine additives to prevent arteriosclerosis and heart diseases.

Residues	Residues concentration in solid margarine % (w/w)	Time of reaction [h]		
		3	6	9
		Absorbance A 535		
HA	0	0.18	0.52	0.76
	0.002	0.17	0.68	0.59
	0.008	0.08	0.43	0.45
	0.03	0.23	0.41	0.47
	0.1	0.17	0.44	0.51
	0.5	0.12	0.26	0.47
HB	0	0.13	0.84	0.99
	0.002	0.28	0.64	0.98
	0.008	0.08	0.57	0.97
	0.03	0.23	0.44	0.91
	0.1	0.09	0.47	0.49
	0.5	0.11	0.05	0.06
HC	0	0.16	0.51	1.14
	0.002	0.53	0.94	0.98
	0.008	0.51	1.09	0.85
	0.03	0.55	0.68	1.14
	0.1	0.48	1.1	1.0
	0.5	0.39	0.68	1.09
HD	0	0.41	0.53	0.82
	0.002	0.21	0.65	1.13
	0.008	0.24	0.90	1.08
	0.03	0.21	0.78	1.09
	0.1	0.27	0.53	0.79
	0.5	0.19	0.44	0.31

Table III. Margarine oxidation in the presence of residues obtained from hawthorn inflorescence.

Abbreviations: HA, HB, HC stand for residues after reduced pressure evaporation of ethyl ether extracts obtained from hawthorn inflorescence water extract (HI); alkaline hydrolysed HI and enzymatically hydrolysed HI respectively. HD denotes residues after evaporation of ethyl acetate extract obtained from HI.

Residues	Residues concentration in solid margarine % (w/w)	Time of reaction [h]		
		3	6	9
		Absorbance A 535		
MA	0	0.22	0.40	0.73
	0.002	0.20	0.66	0.60
	0.008	0.32	0.83	0.80
	0.03	0.09	0.57	0.61
	0.1	0.06	0.10	0.09
	0.5	0.06	0.12	0.03
MB	0	0.22	0.41	0.73
	0.002	0.23	0.79	1.27
	0.008	0.40	0.76	0.67
	0.03	0.26	0.52	0.84
	0.1	0.06	0.11	0.16
	0.5	0.06	0.06	0.06
MC	0	0.45	0.58	0.74
	0.002	0.36	0.78	0.98
	0.008	0.30	0.86	1.29
	0.03	0.30	0.46	1.12
	0.1	0.08	0.55	1.10
	0.5	0.14	0.07	0.23
MD	0	0.15	0.91	1.03
	0.002	0.43	0.68	1.04
	0.008	0.12	1.04	1.24
	0.03	0.23	0.86	1.01
	0.1	0.06	0.27	0.36
	0.5	0.06	0.21	0.32

Table IV. Margarine oxidation in the presence of residues from meadowsweet flowers.

Abbreviations: MA, MB, MC stand for residues after reduced pressure evaporation of ethyl ether extracts obtained from meadowsweet flowers water extract (MSF); alkaline hydrolysed MSF and enzymatically hydrolysed MSF respectively. MD denotes residues after evaporation of ethyl acetate extract obtained from MSF.

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