NATURAL PRESERVATIVES OF PLANT ORIGIN – ANTIMICROBIAL ACTION AGAINST FOOD SPOILAGE MICROORGANISMS IN VITRO AND IN FOODSTUFFS

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ABSTRACT - Natural preservatives of plant origin – antimicrobial action against food spoilage microorganisms in vitro and in foodstuffs
The in vitro antibacterial and antifungal effect of plant-derived compounds; berry juices, berry extracts and essential oils (EOs); were investigated on selected food-spoilage microorganisms. All compounds showed antimicrobial properties to various extents. In contrast to the insensitivity of yeast against berry juices and extracts, the Gram positive bacteria *B. cereus* and *B. subtilis* proved to be more sensitive to these agents than to EOs. The EO from tarragon showed the best antibacterial and antifungal effect, inhibiting the growth each investigated species. The combination of wild thyme and tarragon EO led to antagonism in the case of *S. cerevisiae* and *B. subtilis* whereas the combination of black currant and yarrow EO resulted in additive effect. All other combinations of the investigated EOs or the EO – bog bilberry combinations showed indifference. The EO of Chinese red pine had no effect on the growth of *S. cerevisiae* in apple juice while the EO of *Ribes nigrum* led to a two-stage growth of the yeast. The investigated plant compounds are potential natural food preservatives.

Keywords: berry juices, berry extracts, essential oils, food spoilage

INTRODUCTION

The growing concern of consumers about artificial compounds in their food has lead to a renaissance of application of natural substances as colors, antioxidants and preservatives in foodstuffs. Plants have a natural defense mechanism against microbial infections. Antimicrobial peptides, lectins, phenolic compounds, terpenoids, essential oils and various other compounds are involved in this phenomenon (COWAN 1999). Phenolic compounds present in plants (phenolic acids, flavonoids, stilbenes, lignans and complex phenolic polymers) have antioxidant and antimicrobial activity (KAHKÖNEN et al, 1999). Essential oils (EOs) are hydrophobic liquids extracted mainly by steam distillation from herbs, spices and various other plants. They can contain more than 60 ingredients with 1-3 main compounds (80-95 % of the whole EO). Most EOs has antibacterial, antifungal, antiviral properties and can be successfully used to inhibit or stop microbial growth (BURT 2004). The main target of antibacterial action both for plant phenolics and for EOs is usually the cell membrane where destabilization and/or permeabilisation can occur (COX et al. 2000; BENNIS et al. 2004; PUUPPONEN-PIMIA et al, 2004). Phenolics and EOs can also inhibit extracellular enzymes. Their possible use as food preservatives has been studied from the

eighties and is under intensive research also today. In our experiments the in vitro antibacterial and antifungal effect of sea-buckthorn and bog bilberry extracts, and some selected EOs and their combinations, was investigated against food spoilage bacteria, yeast and molds. The preservative effect of EOs in apple juice was also determined.

MATERIALS AND METHODS

Plant extracts and essential oils

Essential oils and berry extracts used in our experiments and their main constituents are summarized in *Table 1*.

Table 1. Essential oils and berry extracts and their main constituents

Species	Type of extract	Main constituents (%)
Achillea asiatica yarrow	EO	β-pinene (28.8) 1.8-cineole (11.7) myrcene (7.2) α-thujone (6.4),
Artemisia dracunculus tarragon	EO	sabinene (18.6) terpinen-4-ol (14.2) camphene (7.5) p-cymene (7.3)
Hippophae rhamnoides sea-buckthorn	Juice and water extract	carotenoids (16-28 mg%) flavonoids 120-1000 mg%) Vitamin-C (360 mg%)
Juniperus sabina savin juniper	EO	sabinene (39.0) trans-sabinenehydrate (17.5) cedrol (15.8) myrcene (4.2)
Pinus sinensis chinese red pine	EO	α-pinene (17-39) carene (27) terpinolene (18)
Ribes nigrum black currant	EO	Carene (18.67) B-caryophyllene (17. 7) Sabinene (11.6) Cis-β-ocimene (10.6)
Thymus serpyllum wild thyme	EO	terpinen-4-ol (29) carvacrol (14.94) α-pinene (12.2) thymol (7.39)
Vaccinium uliginosum bog bilberry	Juice and water extract	anthocyanidins (360-500 mg%) flavonols (18 mg%)

Microorganisms:

Bacteria: The Gram positive *Bacillus cereus* SZMC 0042 and *Bacillus subtilis* SZMC 0209, and the Gram negative *E. coli* SZMC 0582 were grown on T1 medium (10g glucose, 4g beef extract, 4g peptone, 1g yeast extract, 1L H_2O) at 37 or 30^oC.

Yeasts: *Saccharomyces cerevisiae* MB 021, *Pichia anomala* MB 102 were grown on malt extract medium (ME; 0.4% malt extract, 1% glucose, 0.1% yeast extract) at 30°C.
Molds: *Fusarium sporotrichioides* FEIC 06 was grown also on ME medium at 28°C.
SZMC: Szeged Microbial Collection, University of Szeged, Department of Microbiology, Szeged, Hungary; **MB**: Microbial Collection, Mongolia Academy of Science, Institute of Biology, Ulaanbaatar, Mongolia; **FEIC**: Food Engineering Institute Collection, University of Szeged, Institute of Food Engineering, Szeged, Hungary.

Berries and extraction methods:
Fresh fruits were harvested in Mongolia. Fruit juices were freshly pressed and stored at -20°C. The remaining pomace was dried at 60°C in an oven for 12 h and then ground to powder. One gram of each powdered pomace was extracted 3 times with 10 ml of distilled water per cycle. The extracts were combined and evaporated to dryness at 100°C in an oven. The dry material was redissolved in 4 ml distilled water and frozen in 1 ml aliquots. Juices and extracts were diluted in the appropriate media for the tests.
Well test bioassay:
Agar plates were inoculated with suspensions from each bacterium, yeasts and *Fusarium* spores (> 10^6 cells/ml). After drying, 8-mm-diameter wells were cut in the agar with a sterile cork borer. Each well was filled with 100 µl of plant extract or EO dissolved in 50 % DMSO. DMSO (50 % v/v) and distilled water was used as controls. After incubation at the appropriate temperature for 24 and 48 h, the size of the inhibition zones formed around each well was measured. Tests were made in triplicate.
Checkerboard method
The checkerboard method was performed by macro dilution assay. The twofold dilutions of EOs (or the bog bilberry extract) in the growth medium (from 0.0625 µl/ml to 1 µl/ml) were combined with each other in all possible combinations. Erlenmeyer flasks containing the combinations were inoculated with 1 ml of 10^5 CFU/ml suspensions of the microorganisms, and were incubated for 24 h. The FIC indices were calculated as the sum of FIC_A and FIC_B for EO "A" and EO "B". The FIC for an individual EO was calculated by dividing the MIC for the EO in combination by the MIC of the EO alone. Results were interpreted as synergy ($FIC<0.5$), addition ($0.5 \leq FIC \leq 1$), indifference ($1<FIC \leq 4$) or antagonism ($FIC>4$) (Gutierrez et al., 2008). Experiments were repeated three times.
Effect of pine and black currant EO on the growth parameters of yeasts in apple juice
Pasteurized clear apple juice was inoculated with 1 ml of 10^5 CFU/ml yeast suspension, and the EO was then added to give a final concentration of 0.25 µl/ml. Every 2 h, samples were taken and the CFU was determined by plate count. Lag phases and growth rates were calculated by determining the slopes and intercepts in the logarithmic phase of time versus log CFU growth curves.

RESULTS AND DISCUSSION

Antimicrobial effect of berry juices and extracts

In the agar diffusion tests yeasts showed no sensitivity to juices or water extracts. Based on the diameter of inhibition zones, the Gram positive *B. cereus* proved to be more sensitive than *B. subtilis*. The 1/8 dilution was the lowest value with detectable inhibition. Sea-buckthorn showed slightly better antibacterial properties than bog bilberry resulting in broader inhibition zones (*Table 2*.).

Antimicrobial effect of essential oils

Best results were achieved with savin juniper and tarragon EO, all of the investigated microorganisms showed sensitivity to them. Wild thyme containing phenolic compounds in medium concentration had only slight inhibitory effect on *B. cereus* and *S. cerevisiae* and no effect on the other microorganisms (*Table 3.*), although thyme species with carvacrol and thymol as main constituents are among the best growth inhibitors (DORMAN AND DEANS, 2000).

Table 2. Antibacterial activities of sea-buckthorn and bog bilberry juices and water extracts Inhibition halos ± SD are given in mm.

	Bacillus cereus					
		1	1/2	1/4	1/8	1/16
Hypophae rhamnoides	Juice	11.0 ± 0.0	8.0 ± 0.0	5.7 ± 0.4	4.0 ± 0.0	-
	Water extract	10.0 ± 0.0	7.3 ± 0.89	3.7 ± 0.4	2.83 ± 0.2	-
Vaccinium uligonosum	Juice	8.83 ± 0.2	6.0 ± 0.0	3.67 ± 0.4	-	-
	Water extract	8.0 ± 0.0	6.0 ± 0.0	3.67 ± 0.4	0.17 ± 0.2	-
	Bacillus subtilis					
Hypophae rhamnoides	Juice	5.0 ± 0.0	3.7 ± 0.4	2.0 ± 0.0	0.17 ± 0.2	-
	Water extract	4.0 ± 0.0	2.0 ± 0.0	0.5 ± 0.0	-	-
Vaccinium uligonosum	Juice	4.0 ± 0.0	3.0 ± 0.0	1.0 ± 0.0	-	-
	Water extract	3.3 ± 0.4	2.0 ± 0.0	1.0 ± 0.0	-	-

Table 3. Antimicrobial activity of essential oils. Inhibition halos ± SD are given in mm.

Species/EO	*Thymus serpyllum*		*Juniperus sabina*		*Artemisia dracunculus*		*Achillea asiatica*	
	24h	48h	24h	48h	24h	48h	24h	48h
B. subtilis	-	-	1.5±0.0	1.0±0.0	5.0±0.0	5.0±0.0	-	-
B. cereus	0.5±0.0	0.5±0.0	2.0±0.0	2.0±0.0	6.0±0.0	3.3±0.4	1.5±0.3	1.0±0.0
E. coli	-	-	1.0±0.0	-	2.7±0.4	2.0±0.0	-	-
S. cerevisiae	0.5±0.0	0.5±0.0	3.0±0.0	3.0±0.0	7.2±0.2	7.0±0.0	-	-
P. anomala	-	-	2.0±0.0	1.0±0.0	7.0±0.0	3.3±0.4	7.0±0.0	-
F. sporotrichioides	-	-	-	2.0±0.0	-	5.0±0.0	-	6.0±0.0

Effect of essential oil combinations

The combination of wild thyme and tarragon EO led to antagonism in the case of *S. cerevisiae* and *B. subtilis* whereas the combination of black currant and yarrow EO resulted in additive effect. All other combinations of the investigated EOs or the EO – bog bilberry combinations showed indifference (data not shown).

Growth reduction of *S. cerevisiae* in apple juice

The growth curve of *S. cerevisiae* treated with 0.25 µl/ml Chinese red pine oil showed a similar shape to the untreated control. The growth rates were 0.119/h and 0.147/h, respectively. There was no difference in the length of the lag phase. *S. cerevisiae* treated with black currant oil showed a two-stage growth curve (*Fig. 1.*). There was a quick

growth in the first log phase with a growth rate of 0.36/h followed by a stationary phase of 8 hours. After this, a new, slower growth occurred with a growth rate of 0.18/h. The maximum viable cell number after 20 hours incubation was almost the same in all cases. It seems that the used concentration was too low to exert real inhibition effect against the proliferation of the yeast in apple juice. In a previous experiment, the lag phase of *S. cerevisiae* in clear apple juice increased more than fivefold in the presence of 0.25 µl/ml lemon EO (TSERENNADMID, 2011).

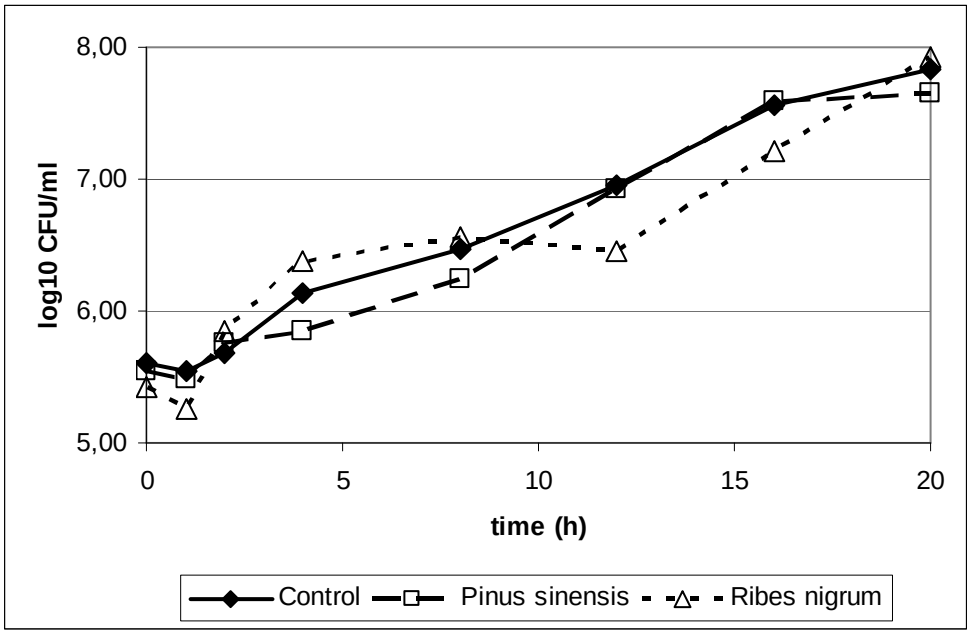

Figure 1. Effect of Chinese red pine and black currant EO on the growth of *S. cerevisiae* in apple juice. The EOs were added in 0.25 µl/ml concentration.

CONCLUSION

All of the investigated plant-derived compounds showed antimicrobial activity against the food spoilage microorganisms. They are potential candidates for the protection of foodstuffs from microbial deterioration. Used in appropriate concentrations, they can extend the shelf life of various foods and beverages.

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