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The inhibitory effect of plant infusions on selected bacteria

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The present study determined the inhibitory patterns of the water and ethanol infusions of garden rhubarb, sea buckthorn, bilberry, blue-berried honeysuckle, black currant, and tomato on targeted bacterial species. The larger inhibition zones against more than four different bacterial species were measured in case of ethanol infusions of garden rhubarb root, blue-berried honeysuckle, and sea buckthorn. In case of water infusions, a stronger inhibitory effect was found for garden rhubarb root, black currant and blue-berried honeysuckle. *L. acidophilus, K. rhizophila* and *B. subtilis* were the most susceptible bacteria in relation to ethanol infusions of the study plants, whereas the growth of *K. rhizophila, B. subtilis,* and *C. jejuni* were most inhibited by water infusions. In accordance with the present *in vitro* study, we conclude that the garden rhubarb and blue-berried honeysuckle are attractive candidates for food industries as natural antimicrobial additives in foods.

Introduction

Food-borne diseases, the spread of multiresistance patterns on pathogenic bacteria, concerns regarding safety of synthetic antimicrobial agents and of other chemical food additives have increased consumers demand for the use of plant extracts as natural antimicrobials in foods [7, 16, 19]. In nature, there are a number of different types of antimicrobial compounds that play an important role in the natural defense of living organisms, and this was the main reason to study compounds naturally derived from local plants in many countries [3, 11, 15, 17, 23].

Among the pathogenic bacteria often associated with foods, *Listeria monocytogenes* is a special concern in ready-to-eat (RTE) processed foods, and since 1987 the United States Department of Agriculture (USDA) has established a strict "zero" tolerance for *L. monocytogenes* in refrigerated foods [21]. In the European Union (EU) countries, *Listeria* is seldom detected above the legal safety limit from RTE foods, which is in most cases above 100 CFU/g during the shelf-life of a RTE product, but is recognized by most RTE food producers as the most unwanted food-borne pathogenic bacterium because this organism can survive in relatively extreme physico-chemical conditions. Therefore, the authors of the present article agree that it is very important to study the antibacterial effect of natural food additives on *L. monocytogenes* and on other well-known food-borne pathogenic bacteria like *Campylobacter* spp., *Escherichia coli* and others. Additionally to natural plant-derived compounds, the functionality of the food can be increased by using well-known probiotic lactic acid bacteria, bifidobacteria as well as certain yeasts and bacilli [5, 14].

For testing the inhibitory effects of natural antimicrobial agents, different microbiological methods have been used, but mostly these are based on agar macrodilution, broth microdilution, agar disc-diffusion or on agar well-diffusion assays [1, 4, 9, 22, 24].

Effective antibacterial natural compounds have been previously found in various plant materials such as cranberry, willow herb, meadowsweet, Argentinean green tea, propolis, pomegranate, blackberry, tomato seeds, and in many other plants [6, 10, 12, 15, 19, 23]. Still, there are many natural compounds from various plants to study, and some of them could be very attractive candidates as natural food additives able to eliminate hazardous bacteria but not showing bactericidal effects against useful bacterial species used in the modern food technology.

In the present study, we are reporting the antimicrobial effect of the infusions of tomato, bilberry, sea buckthorn, black currant, garden rhubarb petioles, garden rhubarb roots, and blue-berried honeysuckle on the growth of food-borne pathogenic and useful bacteria as well as of bacterial species often used in microbiological sensitivity tests.

Materials and methods

Plant material

The plant materials were the roots and petioles of the garden rhubarb (*Rheum rhaponticum* L.), tomato (*Lycopersicon esculentum*), black currant (*Ribes nigrum* L.), bilberry (*Vaccinium myrtillus* L.), sea buckthorn (*Hippophae rhamnoides* L.), and blue-berried honeysuckle (*Lonicera caerulea* L.). The plant material was collected by the reserachers of the Estonian University of Life Sciences in 2011 from the collection of genetical resources of the Polli Horticultural Research Centre. The plant material was freeze-dried, except rhubarb petioles which were thermally dried and prepared using the decoction method. To the dry material, the aqueous phosphate buffer (pH = 7) was added in the ratio 1:10 (w/v), and the

solutions were heated at 95 °C for 10 min. The obtained infusions were cooled down and diluted for the measurements up to 1:80 (w/v). For ethanol infusions, the plant material was macerated in a 10-fold excess (w/v) of 30 % ethanol at room temperature for 24 hours with periodical by shaking (18 r/min) on a rotating shaker. The obtained infusion was centrifuged on the Eppendorf 510R cooling centrifuge. The obtained supernatant was centrifuged once more and diluted for the measurements up to 1 : 80 (w/v).

Bacterial species and strains

The inhibitory effect was tested on selected bacteria such as *Listeria monocytogenes* (ATCC 19115), *Escherichia coli* (NCCB 100282), *Campylobacter jejuni* (ATCC 33291), *Bacillus subtilis* (obtained from the Veterinary and Food Laboratory, Tartu), *Kocuria rhizophila* (ATCC 9341), *Lactobacillus acidophilus* (ATCC 4356), and *Bifidobacterium bifidum* (Bb12). Bacterial strains were obtained from the strain collections of the laboratories that participated in the present research.

Antimicrobial activity test

The inhibitory activity of plant material on selected bacteria was measured using the slightly modified agar well-diffusion method similar to that of Al-Zoreky [3] and Kalogeropoulos et al. [10]. For *L. monocytogenes*, *E. coli*, *C. jejuni*, *B. bifidum*, *L. acidophilus*, 1 µl loopful of bacterial mass was subcultivated in ten milliliters of the Mueller–Hinton broth (Oxoid) for *L. monocytogenes*, *E. coli*, *C. jejuni* or of MRS broth (Oxoid) for *L. acidophilus*, *B. bifidum* and then incubated at 37 ºC for 20 hours. The Mueller–Hinton broth with *C. jejuni* was incubated in microaerobic conditions. Four milliliters of incubated bacterial suspension was mixed with 400 ml sterilized at 45 ºC Mueller–Hinton agar (Oxoid) to the final density of 10^6 cfu/ml and then poured into Petri dishes for solidification at room temperature. The adequacy of the density of the bacterial suspensions was randomly controlled by conventional bacterial counting tests. Test-agar pH 7 (Merck) and Test-agar pH 8 (Merck) were used for testing *B. subtilis* and *K. rhizophila*, respectively. Wells in solidified agar (6 mm in diameter) were made using a sterilized stainless steel borer and finally filled with 30 µl of certain plant infusions of different concentrations such as 1 : 10, 1 : 20, 1 : 40, 1 : 80 (w/v). Appropriate incubation temperatures for each bacterial species were followed. Chloramphenicol (1000 mg/l) was used as a positive control and is indicated as C (+) in Fig. 1 and 2. The ethanol and phosphate buffer (pH = 7) was used as a negative control. After 24 h of incubation, the clear inhibition zones in mm (including the well diameter) were measured using a ruler to an accuracy of 0.5 mm, and the antibacterial effect was calculated as a mean of duplicate tests. The antibacterial activity of plant infusions against bacteria was statistically analysed using Student's t test.

Results and discussion

Fig. 1 and 2 show the inhibitory effect of different concentrations of plant water and ethanol infusions against the test bacterial species, respectively. All tested plant infusions showed an inhibitory effect against selected bacteria, except tomato water infusions. We found very a limited antibacterial effect of tomato infusions, but Taveira et al. [20] concentrated on tomato seeds and found an antibacterial effect against gram-positive gastrointestinal bacteria. Additionally, gram-negative bacteria were found [20] to be not susceptible to different tomato seed extracts; this was explained by a different structure of the cell wall of these bacteria.

In our study, the larger inhibition zones against more than four different bacteria were measured for ethanol infusions of garden rhubarb root, blue-berried honeysuckle, and sea buckthorn. In case of water infusions, the most significant inhibition of the test bacterial strains was obtained by using garden rhubarb root, black currant and blue-berried honeysuckle. Practically, all the plant materials studied showed antimicrobial properties against selected bacteria, and it was mainly dependent on the concentration and the basis of the infusions. The inhibitory effect of plant infusions can mostly be explained by biologically active compounds found in plants, which may show antimicrobial properties [12].

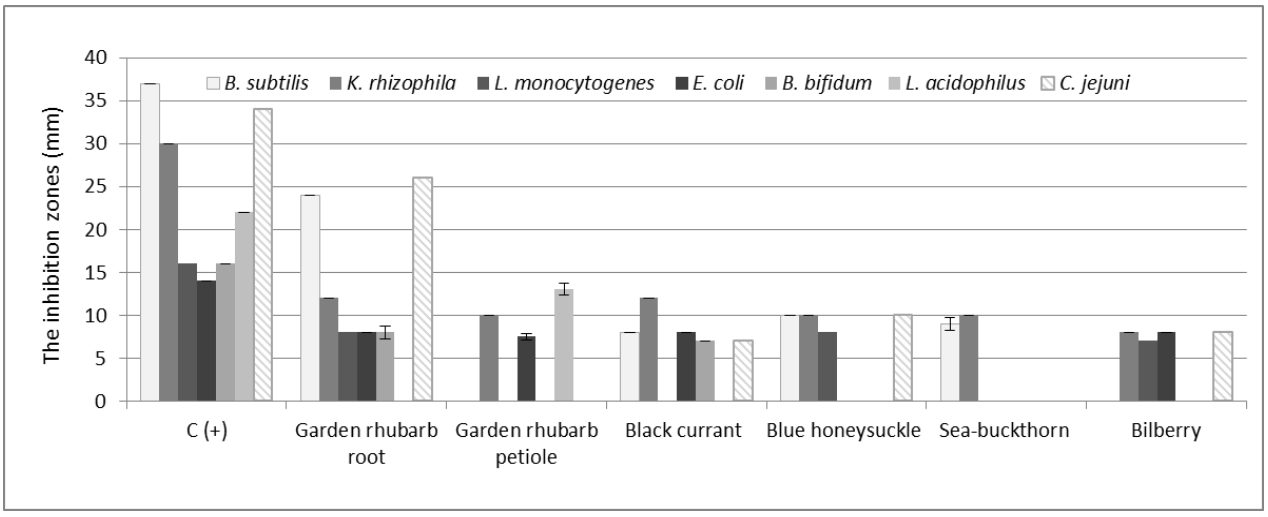

Fig. 1. Inhibitory effect of 1 : 10 concentration of the study plant water infusions on selected bacteria

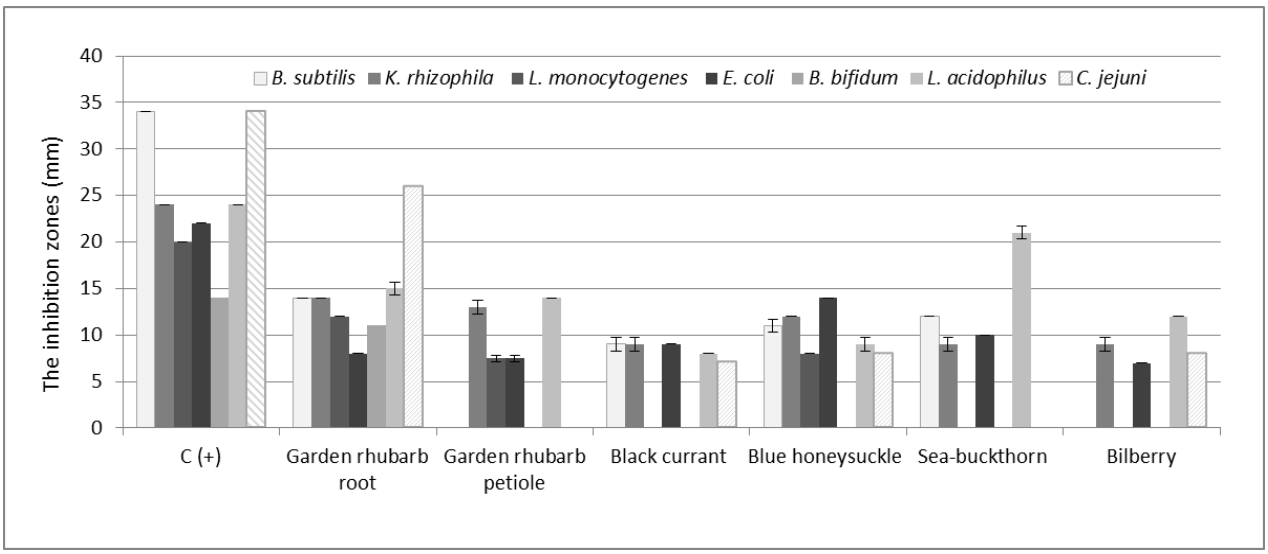

Fig. 2. Inhibitory effect of 1 : 10 concentration of the study plant ethanol infusions on selected bacteria

In our study, *L. acidophilus*, *K. rhizophila* and *B. subtilis* were the most sensitive bacteria against all plant ethanol infusions studied. Water infusions were most effective against the growth of *K. rhizophila*, *B. subtilis*, and *C. jejuni*. The strongest inhibitory activity by different berry extracts against *Micrococcus luteus* (*K. rhizophila*) and *B. subtilis* was detected also by Rauha et al. [15]. *M. luteus* was found to be the most sensitive bacterial species against tea extracts in the study of Almajano et al. [2].

In our study, *L. monocytogenes* and *B. bifidum* were found to be more resistant to most tested plant infusions. Contrary to our study, Al-Zoreky [3] found that *L. monocytogenes* was effectively inhibited by a methanolic extract of fruit peels. *L. monocytogenes* is one of the most important food-borne pathogenic bacteria, which is often related to the food production environment and could lead the contamination of various food products, especially RTE-foods. Therefore, *L. monocytogenes* was chosen for the present susceptibility study. We found that *L. monocytogenes* was not susceptible to most of the tested plant infusions, except the infusions of blue honeysuckle as well as the root and petiole of the garden rhubarb.

E. coli and *C. jejuni* were selected in our study because these bacteria are associated with many food-borne illnesses and outbreaks in all over the world. It is well-known that *E. coli* is an important component of human gut microbiota, and this is the beneficial effect of *E. coli*. On the contrary, some verotoxigenic strains of *E. coli* have recently caused serious food-borne poisoning cases in the European Union (EU). In our study, the blue honeysuckle and sea buckthorn ethanol infusions showed the highest antibacterial activity against *E. coli*. Additionally, a strong or moderate antimicrobial activity was measured for garden rhubarb root, blue honeysuckle, and black currant infusions.

One more important food-borne pathogenic bacterium, *Campylobacter jejuni*, was studied in the present study. *Campylobacter* spp. is well-known and widely spread as the gastroenteritis-causing bacterium in the EU. *Campylobacter jejuni* human infections are mainly associated with the consumption of contaminated chicken meat [13]. In our study, *C. jejuni* was the most susceptible food-borne pathogenic bacterium, which indicates the possible use of natural plant additives in broiler chicken meat to eliminate pathogenic bacteria and to prolong the shelf-life of the products.

Generally, as compared with plant water infusions, the inhibitory effect of ethanol infusions on the test bacteria were significantly ($p < 0.001$) more effective, forming the larger inhibition zones in our study; this is in good agreement with the results of the study of Sebiomo et al. [18]. The difference between water and ethanol infusions was conditioned by the different types of plant material, which is in accordance with the studies of Rauha et al. [15].

In our study, the inhibitory effect of both water and ethanol infusions was significantly ($p < 0.01$) different in cases of 1:10 (w/v) and 1:40 (w/v) dilutions. Accordingly, stronger antimicrobial effect was measured for the 1:10 (w/v) dilution and weaker for the a 1:40 (w/v) dilution. Generally, no inhibitory effect of plant infusions was detected at the dilution of 1:80 (w/v), except for garden rhubarb root and sea-buckthorn.

Compared with tomato and bilberry, the garden rhubarb and garden rhubarb root showed a statistically significant ($p < 0.001$) inhibitory effect on the growth of the test bacteria. The garden rhubarb root ethanol and water infusions showed the largest inhibition zones against the test bacteria as compared to other plant infusions. Therefore, garden rhubarb can be an attractive plant material for industrial food production as an effective natural antimicrobial in foods if further studies with different food matrixes will prove that its chemical, physical and sensory attributes are reliable. The garden rhubarb root ethanol infusions were found to have maximum inhibition zones against *C. jejuni* (26 mm), followed by *L. acidophilus* (15 mm), *B. subtilis* (14 mm) and *K. rhizophila* (14 mm) at the dilution of 1:10 (w/v). The water infusions of the garden rhubarb root showed the strongest inhibitory effect against *C. jejuni* (26 mm), followed by *B. subtilis* (24 mm) and *K. rhizophila* (12 mm)

at the dilution of 1:10 (w/v). However, compared with the garden rhubarb root, garden rhubarb petioles had a significantly ($p < 0.01$) weaker inhibitory effect on the test bacteria.

Among ethanol infusions, the inhibition zones for blue-berried honeysuckle were maximum for *E. coli* (14 mm), followed by *K. rhizophila* (10 mm) and *B. subtilis* (11 mm) at the dilution of 1:10 (w/v). In addition, sea-buckthorn ethanol infusion at the dilutions of 1:10 (w/v) had inhibitory effects on *L. acidophilus*, *B. subtilis* and *E. coli*, while the inhibition zones were measured from 10 to 21 mm. At the dilution of 1:10 (w/v), the largest inhibition zone formed by black currant water infusion was 12 mm for *K. rhizophila*, followed by *E. coli* (8 mm) and *B. subtilis* (8 mm).

The maximum inhibition zones by blue-berried honeysuckle were measured against *B. subtilis* (10 mm), *K. rhizophila* (10 mm) and *C. jejuni* (10 mm) in the presence of water infusions at the dilution of 1:10 (w/v). For comparison, the sea-buckthorn water infusion showed inhibitory effects on only *K. rhizophila* (10 mm) and *B. subtilis* (9 mm) at the 1:10 (w/v) dilution.

Tomato water infusion had no antimicrobial activity against any test bacteria. Neither did tomato ethanol infusion have any inhibitory effect on the selected bacteria, except for *L. acidophilus* (inhibition zone 10 mm).

Conclusions

In the present study, among all the plant infusions test, the root of garden rhubarb showed the highest antimicrobial activity against all the test bacteria except *L. acidophilus* in water infusions. In the light of the current study, it can be concluded that the garden rhubarb and blue-berried honeysuckle are good candidates for the use in food industry as natural antimicrobials in foods. Additionally, the antimicrobial effect of black currant and sea-buckthorn was also satisfactory.

We have found that in spite of the present study, there is a need for the further studies with real food matrixes under different processing conditions to estimate the possible factors that may influence the antibacterial effect of plant infusions or extracts.

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AUGALŲ UŽPILDŲ INHIBITORINIS POVEIKIS ATRINKTOMS BAKTERIJOMS

S a n t r a u k a

Tirta rabarbarų, šaltalankio, vaivorų, sausmedžio, juodųjų serbentų ir pomidorų vandeninių ir etanolinių užpilų įtaka mikroorganizmų augimui. *L. acidophilus*, *K. rhizophila* ir *B. subtilis* bakterijos buvo jautriausios etanoliniams užpilams, o vandeniniai užpilai stipriausiai slopino *K. rhizophila*, *B. subtilis* ir *C. jejuni* augimą. Taip pat nustatyta, kad rabarbarų ir sausmedžio užpilai turėjo stipriausių antimikrobinių savybių.