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SCREENING OF ANTIBACTERIAL ACTIVITY OF RASPBERRY (*Rubus idaeus* L.) FRUIT AND POMACE EXTRACTS

Aleksandra S. Velićanski, Dragoljub D. Cvetković and Siniša L. Markov*

University of Novi Sad, Faculty of Technology, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia

Antibacterial activity of fruit and pomace extracts (concentration 50 mg/ml) of two raspberry (Rubus idaeus L.) cultivars (Meeker and Willamette) was tested against selected Gram-positive and Gram-negative bacteria (reference and wild strains). Disc diffusion method with 15 µl of extracts and agar-well diffusion method with 50 and 100 µl were used. Antibiotic (cefotaxime/clavulanic acid) was used as a control. Both raspberry fruit extracts showed the strongest antibacterial activity against Pseudomonas aeruginosa (wild strain) and Bacillus cereus, where the largest clear zones (without growth) appeared. Escherichia coli was the most resistant strain, with only zone of reduced growth. The highest antibacterial activity of pomace extracts was against Staphylococcus aureus and Staphylococcus saprophyticus. There were no differences in the antibacterial activity between cultivars for both fruit and pomace extracts.

KEY WORDS: *Rubus idaeus* L., raspberry fruit and pomace extracts, antibacterial activity, agar diffusion method

INTRODUCTION

Rubus fruit have long been collected and consumed worldwide, regardless of whether they were recognised for their possible health benefits from their natural phytochemicals or simply because they tasted good. Today, rubus fruit are considered a healthy and nutritious food, containing phenolics, vitamin C, dietary fibre, α-tocopherol, tocotrienol, calcium, potassium, magnesium, carotenoids, linoleic acid (1). Serbia is one of the largest producers and exporters of raspberries (*Rubus idaeus* L.) in the world. Between 90 and 95% of cultivated raspberries in Serbia are North American Willamette cultivar, which is characterized by the excellent taste and a dark red colour. Besides the Willamette, the Meeker cultivar is also popular (2,3).

Berries contain a variety of phenolic compounds (phenolic acids, flavonoids, lignans and polymeric tannins) located in plant tissues, often in the surface layer of the plant or berry, which is in connection to their main natural function, to protect the plant against environmental stress and pathogens. The main phenolic compounds in raspberries are fla-

* Corresponding author: Aleksandra S. Velićanski, University of Novi Sad, Faculty of Technology, 21000 Novi Sad, Bulevar cara Lazara 1, Serbia, e-mail: sanja@tf.uns.ac.rs

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vonoid anthocyanins (coloured substances), ellagic acid and ellagitannins, complex water-soluble phenolic polymers. From the phenolic acids, hydroxycinnamic or hydroxybenzoic acid derivatives are the most common in berries (1, 4).

Various phytochemical constituents of berry fruits exhibit a wide range of biological effects, including antioxidant, anticarcinogenic, antiinflammatory, antineurodegenerative, antiviral, and antibacterial activities which are attributed to the phenolic compounds, such as flavonoids, phenolic acids and tannins. Due to a high content and wide diversity of phenolic compounds and their health-promoting properties, berries are often regarded as natural functional products properties (4-6). Not only the fruit, but also the raspberry leaves and roots have long tradition as medicinal agents. Infusion from the leaves are traditionally used for easing childbirth-related muscle spasms, morning sickness, for colds, sour throats, diarrhoea, threat wounds, colic pain, uterin relaxant, etc. The roots of the plant are traditionally used for wound cleaning and relief from sore throats (7, 8).

Berries have been used as natural antimicrobial pharmaceuticals. For example, cranberry has been reported to control the growth of *Listeria monocytogenes* and to possess compounds suppressing adhesion and growth of *Helicobacter pylori* and bacteria causing urinaty tract infections (4). Phenolic berry extracts inhibit the growth of selected Gram-negative intestinal bacteria, while they are not active against Gram-positive benefitial probiotic lactic acid bacteria. Cloudberry and raspberry phenolic extracts were shown to be strong inhibitors of a nonvirulent *Salmonella* strains (9).

By-products of plant food processing represent a major disposal problem for the industry concerned, but they are also promising sources of compounds which may be used because of their favourable technological or nutritional properties (10). These phytochemicals from waste materials deriving from agro-industrial production may be used as functional food ingredients and as natural antioxidants to replace their synthetic equivalents. So, pomace (peels, seeds, and flesh remaining after juice pressing) extracts of 20 cultivated and wild fruits and berries showed antioxidant and antimicrobial activity (11).

Taking into account biological activities of raspberry as well as possible potential of plant by-products, in this study, fruit and pomace extracts from two raspberry (*Rubus idaeus* L.) cultivars (Meeker and Willamette) were used to screen antibacterial activity to eight reference cultures and wild strains. Two methods were used: disc diffusion method with the limited capacity of discs and agar-well diffusion method with much higher volume of holes.

EXPERIMENTAL

Plant material

Two raspberry (*Rubus idaeus* L.) cultivars (Meeker and Willamette) were obtained from "Alfa RS", Lipolist, Serbia. Samples of the raspberry were stored at -20^{o}C until analysis. Raspberry pomaces from both cultivars was obtained after juice separation. The yields of the Meeker and Willamette pomaces were 126.89 g and 101.19 g, respectively.

Preparation of raspberry fruit and pomace extracts

Samples of the raspberry cultivars (70 g) and their pomaces (70 g) were extracted two times: 60 minutes (560 ml) and 30 minutes (280 ml) at room temperature using a homogenizer, Ultraturax, DIAX 900 (Heidolph Instruments GmbH, Kelheim, Germany). The extraction was performed with an 80% methanol aqueous solution containing 0.05% acetic acid. The obtained extracts were combined and evaporated to dryness under reduced pressure and lyophilisated (Alpha 2-4 LSC Martin Christ, Osterode, Germany).

The yields of the lyophilisated fruit extracts from Meeker and Willamette cultivars were: 8.39 g and 7.74 g, and of the lyophilisated pomace extracts were 6.99 g and 6.6 g, respectively.

Samples for the determination of antibacterial activity

Samples for the determination of antibacterial activity were two raspberry (*Rubus idaeus* L.) fruit and pomace extracts, from Meeker and Willamette cultivars, which were dissolved in sterile distilled water to a concentration of 50 mg/ml.

Bacterial strains

Bacterial strains for the determination of antibacterial activity were reference and wild strains from foodstuffs and drinking water. Gram-negative bacteria were: *Salmonella typhymurium* (ATCC 14028), *Escherichia coli* (ATCC 10536), *Pseudomonas aeruginosa* (ATCC 27853) and *Pseudomonas aeruginosa* (isolated from drinking water). Gram-positive bacteria were: *Staphylococcus aureus* (ATCC 11632), *Bacillus cereus* (ATCC 10876), *Staphylococcus saprophyticus* (isolated from the confectionery product) and *Listeria monocytogenes* (isolated from minced meat). Wild strains were identified using Vitek®2 Compact System (bioMérieux, France).

Antimicrobial assay

Antibacterial activity was determined by disc diffusion and agar-well diffusion method (12). Bacterial strains were grown on Müeller–Hinton slant (Himedia, Mumbai, India) for 24 h at 37°C, except *Bacillus cereus*, which was grown at 30°C. Cells were then suspended in a sterile 0.9% NaCl solution. The suspension for inoculation was adjusted to a concentration of 1×10^6 cfu/ml, which was estimated by Densichek (Biomerieux, France). A volume of 2 ml of the suspensions for inoculation was homogenised with 18 ml of melted (45°C) Müeller–Hinton agar and poured into Petri dishes.

For disc diffusion method, sterile 6 mm discs (Himedia, Mumbai, India) were placed on the inoculated agar plates and impregnated with 15 µl of extracts solution. The antibiotic (30 µg cefotaxime /10 µg clavulanic acid per disc, Bioanalyse®, Ankara, Turkey) was used as control. For the agar-well diffusion method, wells of 9 mm diameter were made with a sterile metal tube by means of a vacuum pump. The extracts solution (50 and 100 µl) was then transferred into the wells of inoculated agar plates. For both methods, the test plates were refrigerated at 8°C for 1 hour to allow the extracts to diffuse into the medium, and then were incubated for 24 hours at 37°C or 30°C (*Bacillus cereus*). After the incubation, the diameters of the inhibition zones were measured and recorded in millimeters (mm). The evaluation of antibacterial activity was carried out in triplicate.

RESULTS AND DISSCUSION

The results of antibacterial activity of two raspberry fruit and pomace extracts are presented in Tables 1 and 2.

Table 1. Antibacterial activity of raspberry fruit extracts (mean of diameter of the inhibition zone (mm) including disc (6 mm) or well (9 mm) with standard deviation in parentheses)

Group	Tested strains	Cultivar	Method: Disc diffusion	Method: Agar-well diffusion		Control(cefotaxime/ clavulanic acid)
			15μl	50 μl	100 μl	
G (–) bacteria	*Escherichia coli* ATCC 10536	M	nd	12.58** (0.0)	15.5** (0.58)	34.87* (0.35)
		W	nd	14.0** (0.82)	16.5** (0.58)	
	Salmonella typhymurium ATCC 14028	M	nd	16.5* (1.73)	16.5* (0.58)	35.6* (0.85)*
		W	nd	13.5** (1.73)	15.0* (0.0)	
	Pseudomonas aeruginosa ATCC 27853	M	7.75** (0.5)	11.75** (0.5)	14.25* (0.5)	20.17* (0.76)
		W	nd	12.25** (0.5)	15.25* (0.5)	
	*Pseudomonas aeruginosa****	M	7.0* (0.0)	11.75* (0.5)	16.0* (2.31)	15.33* (0.58)
		W	7.0* (0.0)	13.0* (0.0)	18.0* (0.0)	
G (+) bacteria	*Staphylococcus aureus* ATCC 11632	M	7.5** (0.55)	14.5* (0.58)	16.0* (0.0)	37.3* (0.75)
		W	8.5** (0.55)	14.5* (0.5)	16.0* (0.0)	
	*Staphylococcus saprophyticus****	M	nd	nd	13.5* (1.0)	27.0* (0.5)
		W	nd	nd	13.5* (0.58)	
	Bacillus Cereus ATCC 10876	M	7.5* (0.58)	13.5* (0.58)	16.25* (0.5)	35.5* (1.5)
		W	9.0* (0.5)	13.5* (0.58)	17.0* (1.0)	
	*Listeria monocytogenes****	M	nd	9.5* (0.58)	12.5* (0.58)	12.25* (0.55)
		W	nd	10.0* (0.0)	12.5* (0.58)	

M – Meeker cultivar; W – Willamette cultivar; nd - not detected inhibition zone; * - clear zone around the disc/well; ** - zone of reduced growth; *** - wild strain.

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Table 2. Antibacterial activity of raspberry pomace extracts (mean of diameter of the clear inhibition zone (mm) including disc (6 mm) or well (9 mm) with standard deviation in parentheses)

Group	Tested strains	Cultivar	Method		
			Disc diffusion	Agar-well diffusion	
			15μl	50 μl	100 μl
G (–) bacteria	*Escherichia coli* ATCC 10536	M	21.75 (0.5)	37.33 (0.58)	34.32 (1.54)
		W	20.0 (0.85)	25.67 (0.58)	31.67 (0.58)
	Salmonella typhymurium ATCC 14028	M	22.0 (0.82)	29.5 (1.0)	35.5 (1.0)
		W	23.0 (1.73)	30.0 (1.0)	34.0 (1.63)
	Pseudomonas aeruginosa ATCC 27853	M	18.25 (0.5)	22.67 (0.58)	25.0 (1.0)
		W	16.75 (0.5)	22.0 (1.0)	24.67 (0.58)
	Pseudomonas aeruginosa[*]	M	15.75 (1.26)	20.67 (0.58)	24.0 (1.0)
		W	15.5 (1.0)	21.33 (1.15)	24.67 (0.58)
G (+) bacteria	*Staphylococcus aureus* ATCC 11632	M	34.75 (0.96)	41.5 (4.43)	48.5 (1.0)
		W	33.25 (0.96)	39.0 (1.15)	46.0 (1.63)
	Staphylococcus saprophyticus[*]	M	11.75 (0.96)	23.0 (1.0)	39.0 (1.0)
		W	12.25 (0.96)	21.0 (0.5)	38.0 (1.0)
	Bacillus cereus ATCC 10876	M	22.5 (0.58)	32.0 (0.58)	36.0 (1.0)
		W	24.25 (0.96)	28.75 (2.5)	36.0 (1.63)
	Listeria monocytogenes[*]	M	10.0 (0.05)	16.67 (0.58)	30.0 (1.0)
		W	10.75 (0.5)	17.33 (0.58)	30.33 (0.58)

M – Meeker cultivar; W – Willamette cultivar, [*] - wild strain

The smallest inhibition zones of raspberry fruit extracts for all tested bacterial strains were observed by disc diffusion method (Table 1). Clear zones (not higher than 9 mm) were found only for *Pseudomonas aeruginosa* (wild strain) and *Bacillus cereus.* Similar clear zones of reduced growth were found for *Pseudomonas aeruginosa* and *Staphylococcus aureus*. All strains except for *Staphylococcus saprophyticus* were susceptible to 50 μl of extracts, and higher zones appeared with using 100 μl of both Meeker and Willamette fruit extracts. The most susceptible strains tested by agar-well diffusion method, were *Pseudomonas aeruginosa* (wild strain) and *Bacillus cereus*. From all tested strains, *Escherichia coli* was the most resistant strain because only zone of reduced growth appeared, even when 100 µl of extracts were used. This result indicate bacteriostatic activity of tested extracts against *Escherichia coli*. The smallest clear zones of 12.5-13.5 mm are appeared for *Listeria monocytogenes* and *Staphylococcus saprophyticus* by applying agar-well diffusion method (Table 1).

As for raspberry pomace extracts (Table 2), for both methods and extracts volumes (15 µl, 50 µl and 100 µl) only clear zones appeared, indicating bactericidal activity of the pomace extracts. Both Meeker and Willamette pomace extracts showed the highest antibacterial activity against *Staphylococcus aureus* and *Staphylococcus saprophyticus*, while the smallest zones appeared for *Pseudomonas* strains (both reference and wild strains).

The inhibition zones of tested control (cefotaxime/clavulanic acid disc) were significant, and for all bacteria clear zones around the discs appeared. The antibiotic showed the most pronounced activity against *Escherichia coli, Salmonella typhymurium, Staphylococcus aureus* and *Bacillus cereus* (more than 30 mm). The least clear zone (less than 15 mm) appeared for *Listeria monocytogenes*, the strain with the smallest clear zone in the case of action raspberry fruit extracts. Raspberry pomace extract showed similar or higher activity against tested strains compared with control.

Rauha et al. (13) tested the antimicrobial activity of *Rubus idaeus* L. extract (extracted with 70% aqueous acetone) by cylinder diffusion method (500 µl of extract, concentration of 1 mg/ml). Only slight antibacterial activity was obtained against *Staphylococcus aureus, Staphylococcus epidermidis, Micrococcus luteus* and *Escherichia coli*, and moderate activity for *Bacillus subtilis*. Antibacterial activity of raspberry extract in concentration of 1 mg/ml (extracted with acetone:water 70:30 v/v), on the growth of microbial strains in liquid culture was tested by Nohynek et al. (4). Results showed the strong activity of raspberry extract, as it caused death of the *Helicobacter pylori*, very strong inhibition of *Bacillus cereus*, *Staphylococcus aureus* and *Staphylococcus epidermidis*, and strong inhibition of *Campylobacter jejuni* and *Clostridium perfringens*. Strong antibacterial activity was attributed to phenolic compounds, especially ellagitannin fraction (4).

Study of Ryan et al. (7) showed absence of antimicrobial activity of water and ethanol raspberry leaf extracts. On the other hand, raspberry juice and cordial displayed growth inhibition (at 10% and 20% concentration) of *Staphylococcus aureus*, *Escherichia coli*, *Mycobacterium phlei*, *Clostridium perfringens*, *Alcaligenes faecalis*, *Enterococcus faecalis*, *Shigella soneri* and three *Salmonella* serovars (7). Similarly, using 100 µl of raspberry leaf methanolic extract (concentration of 20 mg/ml), Bonjar (14) obtained inhibition zone of 10-14 mm only for *Bordetella bronchiseptica*, which was generally (taking into account all 48 plant species tested) the most sensitive strain among eleven strains tested.

Ördögh et al. (11) tested antibacterial activity of the raspberry juice, as well as water and methanol extracts of the pomace against acne-inducing bacteria by broth microdilution assay. Raspberry juice showed antibacterial activity only against *Staphylococcus epidermidis* (minimal inhibitory concentration at the pH 7 was 12.35 mg/ml, and 9.18 mg/ml at the pH 5.5). Juice did not affect the growth of *S. aureus*, *Streptococcus pyogenes* and *Propionibacterium acnes*, while pomace extracts did not show any antibacterial activity. The difference in the antibacterial activity of raspberry juices and pomace extracts tested in this study (from 20 cultivated and wild fruits) may be due to their different components; soluble in aqueous and alcoholic media. Water extract contains the majority of anthocyanins, tannins, starches, saponins, polypeptides and lectins, while methanol extracts also contain polyphenols, lactones, flavones, and phenons (11).

Pupponen-Pimiä et al. (9) tested antimicrobial activity of Nordic berries including raspberry (2 and 10 mg of lyophilized berry powders/ml) and phenolic berry extracts (concentration of 1 and 5 mg/ml) against eight human pathogens in liquid culture by plate count method. *Salmonella enterica* sv. Typhimurium and *Staphylococcus aureus* were strongly affected by lyophilized raspberry. However, no effects on the growth of *Listeria monocytogenes* was obtained. Phenolic raspberry extracts showed strong inhibitory activity against *Staphylococcus* spp. *Salmonella* spp. were only partly inhibited by the raspberry phenolic extrscts (9).

Phenolic compounds affected the growth of different bacterial species by different mechanisms, yet not well understood. It is assumed that not only phenolic compounds are responsible for the antimicrobial activity. The fruits of the genus *Rubus* are rich in ellagitannins, which can permeate the outer cell membrane of Gram-negative bacteria (9, 11). So, antimicrobial activity of berries is likely to be caused by multiple mechanisms and synergies because they contain various compounds, for example, weak organic acids, phenolic acids, and tannins and their mixtures of different chemical forms (4). Additionally, the effect of pH is very important in case of microbicidal acids of the fruits. These are membrane-active substances which damage the inner cell membrane in their undissociated form. They alter the membrane permeability of the microbial cell and acidify the cytoplasm (11). In the study of Ördögh et al. (11), the antibacterial effect of juices and pomace extracts was more or less independent of the pH, suggesting that other, non-dissociable compounds were responsible for the growth inhibition.

It is obvious that there are differences between the results shown in Tables 1 and 2, and other mentioned studies. The observed differences are probably caused by the different methods and concentrations of tested extract solutions, but also, results depend on the susceptibility of tested wild strains, as well as the composition and amount of active components extracted from tested materials originated from different geographic areas, growth conditions of plant material as well as seasonal variations (7).

CONCLUSION

In this study, the antibacterial potential of a *Rubus idaeus* L. (raspberry) fruit and pomace extracts against selected Gram-positive and Gram-negative organisms has been demonstrated. These findings can form the basis for further studies to isolate active compounds, elucidate their structures, and also evaluate them against wider range of bacterial strains with the goal to find new therapeutic principles.

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СКРИНИНГ АНТИБАКТЕРИЈСКЕ АКТИВНОСТИ ЕКСТРАКАТА ВОЋА И ТРОПА МАЛИНЕ (*Rubus idaeus L.)*

Александра С. Велићански, Драгољуб Д. Цветковић и Синиша Л. Марков

Универзитет у Новом Саду, Технолошки факултет, Булевар цара Лазара 1, 21000 Нови Сад, Србија

Антибактеријска активност екстраката воћа и тропа две сорте *(Meeker* и *Willamette)* малине (*Rubus idaeus L.)*, концентрације 50 mg/ml, испитана је на одабране Грам–позитивне и Грам–негативне бактерије (референтни и дивљи сојеви). За испитивање су коришћене диск-дифузиона метода (са 15 μl екстраката) и метода бунарчића у подлози (са 50 и 100 μl). Екстракти воћа од обе сорте показали су највећу активност према сојевима *Pseudomonas aeruginosa* и *Bacillus cereus*, док је *Escherichia coli* показала најмању осетљивост. Екстракти тропа обе сорте малине дали су зоне без раста (показатељ бактерицидног деловања) за све тест микроорганизне, што указује на значајан антибактеријски потенцијал тропа малине. Највећу антибактеријску активност екстракти тропа су показали према *Staphylococcus aureus* и *Staphylococcus saprophyticus*. Сорта малине није утицала на антибактеријску активност испитаних екстраката.

Кључне речи: *Rubus idaeus* L., екстракти воћа и тропа малине, антибактеријска активност, агар дифузиона метода

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