Inhibition of microbial biofilm formation by *Cydonia oblonga* Mill. fruit peel and leaf ethanolic extracts

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Biological activities of *Sorbus aucuparia* L. leaves extract were tested in our study. This study aimed to investigate the activity of *Cydonia oblonga* Mill. peels and leaf ethanolic extracts on biofilm formation of different microbial strains, including pathogenic bacteria and food poisoning strains. It was shown that both of the investigated extracts inhibited biofilm formation in a dose dependent manner with sub-minimal inhibitory concentrations. The percentage of biofilm formation inhibition varied for each bacterial strain and was in the range of 10-100%, for both of the tested extracts. The ability of extracts to inhibit already formed biofilms presented as minimal concentration necessary to disrupt biofilms and concentrations was in the range of 10-100 μg/mL for the leaf extract and 5-75 μg/mL for the peel extract. The investigated extracts showed a promising antimicrobial effect comparable to, or even higher than the used reference compounds, which make these plant parts attractive for pharmaceutical and food industry.

Key words: *Cydonia oblonga; peel; leaves; biofilm; antibacterial*

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1. INTRODUCTION

Cydonia oblonga Mill. (quince), a plant of Rosaceae family is well known for its medicinal, nutritional, and ornamental uses (Ashraf et al., 2016). As a rich source of secondary metabolites (phenolic compounds, steroids, terpenoids, sugars, and organic acids) it possess a wide range of bioactivities (antioxidant, antibacterial, antifungal, anti-inflammatory, hepatoprotective, cardiovascular, antidepressant, antidiarrheal, hypolipidemic, diuretic, and hypoglycaemic). Although the ripe fruit of quince have a pleasant, lasting, and powerful flavour, consumption of fresh quince fruits is not widespread mainly due to excessive and aggressive astringency and sourness as well as woodiness (Griñán et al., 2019). Due to its hardness and acidity, it is not edible without prior processing (Griñán et al., 2019), but it is generally used for preparation of jam, jelly, liqueur, and marmalade, and it is applied in canning and for aromatic distillation (Baroni et al., 2018).

In nature, mostly of microorganisms, are organized in the form of biofilms, and about 80% of human infections are connected with the development of biofilms (Zijnge et al., 2010). The formation of biofilm may contain one or more species. It is often formed on organic or inorganic surfaces. The major component of biofilm is water (95%) then extracellular matrix (1-2%) and microorganisms (2-5%). Biofilm's forming can be organized in three phases: annexation, maturation and dispersion (Williams and Cámara, 2009). Also depending on a number of factors, like a physical environmental factor or available food, there are three types of biofilm: heterogenous-mosaic type, and porous type which is intersected by water channels and thick-confluent type (Tolker-Nielsen, 2014). Infections caused by biofilms are considerably complicated, because these infections are asymptomatic.

This work aimed to investigate the activity of *Cydonia oblonga* Mill. peels and leaf ethanolic extracts on biofilm formation of different microbial strains, including pathogenic bacteria and food poisoning strains.

2. MATERIALS AND METHODS

2.1. Sample collection and extract preparation

Cydonia oblonga Mill. leaves and fruits were collected in Vranje, southern Serbia. Peels were separated from the fruits and further used for the experiments. The powdered fruit peels and leaves (~10 g) were extracted by stirring with 300 mL of ethanol, at room temperature, 150 rpm, for 24 h. The extract was filtered through Whatman No. 4 paper. The residue was then re-extracted twice with additional portions (300 mL)

Table 1. Minimal inhibitory (MIC), minimal bactericidal (MBC) and fungicidal concentrations (MFC) of *C. oblonga* extracts (μg/mL) that influenced biofilms

MO strain	Peel extract		Leaf extract		Ampicilin		Streptomycin		Miconazole	
	MIC	MBC (MFC)	MIC	MBC (MFC)	MIC	MBC	MIC	MBC	MIC	MFC
S. aureus	10	20	5	10	100	150	250	500	-	-
L. monocytogenes	20	20	10	20	150	300	150	300	-	-
Ps. aeruginosa	80	100	60	75	100	200	50	100	-	-
E. coli	40	80	20	20	300	500	50	100	-	-
S. typhimurium	100	100	50	75	150	200	50	100	-	-
C. albicans	60	80	40	60	-	-	-	-	1	1

of ethanol. The combined extracts were evaporated at 45 °C (rotary evaporator Büchi R-210, Flawil, Switzerland) to remove ethanol.

2.2. Microorganisms

The microorganisms tested included 5 bacterial: Gram-negative bacteria *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), and *Salmonella typhimurium* (ATCC 13311), Gram-positive bacteria *Listeria monocytogenes* (NCTC 7973) and *Staphylococcus aureus* (ATCC 6538), and a yeast *Candida albicans* (ATCC 10231). The species were maintained in Mueller Hinton Agar and Trptone Soy Agar (MHA, TSA, Torlak, Serbia). The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia.

2.3. Biofilm susceptibility test

The effect of peels and leaves ethanolic extracts of *C. oblonga* on the biofilm were determined as previously described by Pierce et al. (2008), with some modifications. Briefly, fresh overnight cultures of bacteria and yeast were harvested from liquid cultures for cells and re-suspended for suspensions of 1 × 106 cells/mL final density. Biofilms were formed in 96-well polystyrene flat bottom TC treated microtitre plates (Sarstedt, North Carolina, USA). The plates were incubated at 37 °C for 48 h to allow biofilm formation. The culture media was removed, and the wells were washed three times with saline solution to remove the non-adhered cells. The extracts solution (200 μL) was added to the wells. The microplates were incubated at 37 °C for 24 h. Three repetitions were performed. After 24 h at 37 °C, biofilm reduction was determined by following staining process and measuring the UV absorbance of stains at 620 nm using a plate reader. MIC was defined as the minimum concentration of antimicrobial agent that inhibited further growth of the initial biofilm, and MBC was defined as the concentration that resulted in level of luminescence presenting no bacterial growth (empty well).

2.4. Inhibition of biofilm formation

Biofilm was grown as described previously. The effect of different concentrations of the extract (ranging from 5 to 125 μg/mL of MIC) on biofilm forming ability was tested on polystyrene flat-bottomed microtitre 96 well plates as described (Pierce et al., 2008), with some modifications. Briefly, 100 μL of overnight cultures were added to each well of the plates in the presence of 100 μL sub-inhibitory concentrations (subMIC) of the extracts (0.5, 0.25 and 0.125 mg/mL MIC) or 100 mL medium (control). After incubation for 24 h at 37 °C, each well was washed twice with sterile PBS (pH 7.4), dried, and stained for 10 min with 0.1% crystal violet in order to determine the biofilm mass. After drying, 200 μL of 95% ethanol (v/v) was added to solubilize the dye that had stained the biofilm cells. The excess stain was washed off with dH_2O. After 10 min, the content of the wells was homogenized and the absorbance at λ = 625 nm was read on a Sunrise™– Tecan ELISA reader.

3. RESULTS AND DISCUSSION

It is well known that biofilm forming pathogens are essential for severe infections which are commonly very difficult to treat. This is due to the high resistance of biofilms to antibiotics, antiseptics and disinfectants as well as to host defence mechanisms (Rozalski et al., 2013). Therefore, biofilms present severe problems for food and healthcare industries.

In our study the ability of extracts to inhibit already formed biofilms was presented as minimal concentration necessary to disrupt biofilms and these concentrations were in the range of 10-100 μg/mL for the leaf extract and 5-75 μg/mL for the peel extract (Table 1). For most of the bacterial strains investigated extracts showed significantly higher activity compared to the used standard compounds. Namely, commercial antibiotics ampicillin and streptomycin showed bacteriostatic activity in the range of 50 to 250 μg/mL, and bactericidal activity in the range of 100 to 500 mg/mL.

In test of microplate biofilm formation of microorganisms, leaf extract was more effective among the two tested extracts, and its MIC and MBC values were lowest against *Staphylococcus aureus* (MIC=5 μg/mL and MBC=10 μg/mL). The most resistant microbial biofilm was the one formed by *Pseudomonas aeruginosa* (MIC and MBC values were 60 μg/mL and 75 μg/mL, respectively). As for the ethanolic extract obtained from the peel of *C. oblonga*, the most resistant biofilm was the one formed by *Salmonella typhimurium*, while once again *Staphylococcus aureus* was the most sensitive strain (results in Table 1).

The effects of peel and leaf extracts on the biofilm growth of *S. aureus*, *L. monocytogenes*, *P. aeruginosa*, *E. coli* and *C. albicans* were tested with concentrations of the extracts equal to 0.500, 0.125, 0.062 and 0.031 parts of the determined MIC values. It was shown that both of the tested extracts inhibited biofilm formation with sub-minimal inhibitory concentrations. The percentage of biofilm inhibition was variable for each species and was in range of 5-100%, for both of the extracts (Tables 2 and 3). Biofilm formation was in both cases completely inhibited at MIC concentration, while in cases of applied concentrations of 1/16 MIC and in all cases 1/32 MIC the biofilm was not inhibited in comparison to the control. Among these two extracts, again sub-MIC concentrations of leaf extract were more effective and had higher percentages of inhibition. The antibacterial effects of the leaves of *C. oblonga* extracts have been poorly investigated to date. Fattouch et al. (2007) stated that chlorogenic acids and rutin are dominant phenolic compounds in the peel of quince and these compounds showed

Table 2. Inhibition of biofilm formation by *C. oblonga* leaf extract

	mic[a] [%]	1/2mic [%]	1/4mic [%]	1/8mic [%]	1/16mic [%]	1/32mic [%]
S. aureus	NB	67.0000	74.9000	78.7400	55.0600	NI
L. monocytogenes	NB	74.8691	76.6143	73.4729	24.6073	NI
Ps. aeruginosa	NB	86.8201	89.7490	90.1674	74.6862	NI
E. coli	NB	84.1450	87.3160	86.7497	NI	NI
S. typhimurium	NB	83.1254	79.2145	43.2510	NI	NI
C. albicans	NB	37.6106	31.4159	5.3097	NI	NI

[a]NB – no biofilm is formed; NI – no inhibition

Table 3. Inhibition of biofilm formation by *C. oblonga* peels extract

	mic[a] [%]	1/2mic [%]	1/4mic [%]	1/8mic [%]	1/16mic [%]	1/32mic [%]
S. aureus	NB	62.0000	72.4200	42.1000	24.0620	NI
L. monocytogenes	NB	80.2514	82.3578	74.6582	44.2517	NI
Ps. aeruginosa	NB	84.2144	88.2143	88.4579	72.4795	NI
E. coli	NB	87.6316	89.4148	89.7689	30.2145	NI
S. typhimurium	NB	78.2142	72.2145	41.2153	20.6457	NI
C. albicans	NB	46.5617	21.4164	11.6792	NI	NI

[a]NB – no biofilm is formed; NI – no inhibition

significant antimicrobial activity against *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*. Karar et al. (2014) showed that *C. oblonga* fruit is a rich source of polyphenols including chlorogenic acids, proanthocyanidins and flavonol *C*- and *O*-glycosides. Also, same authors showed that *C. oblonga* fruits possess significant antibacterial activity against *E. coli* which is in accordance with its traditional use as a food preservative. According to previous results the content of polyphenols in quince leaves is higher compared to some other plant parts, and tannins can make 17% of the total content. This group of compounds possess significant antibacterial activity against certain microorganisms such as *S. aureus*, *E. coli* and *S. epidermids*, due to ability to precipitate microbial proteins and to make nutritional proteins inaccessible for them (Cerempei et al., 2016).

CONCLUSION

In the present study we have demonstrated for the first time antibiofilm potential of the quince leaf and peel extracts. The investigated extracts showed a promising antimicrobial effect comparable to, or even higher than the used reference compounds, which makes these plant parts also attractive for pharmaceutical and food industry.

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