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Note

Effects of ice containing three ethanolic plant extracts on formation and accumulation of biofilm communities on fish contact surfaces

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ABSTRACT

The effect of ice containing thyme (0.04% w/v), oregano (0.03% w/v) and clove (0.02% w/v) extracts on formation and accumulation of biofilm on high density polyethylene coupons during chilling storage of anchovy was investigated. The results showed that ice containing plant-extracts had no antibiofilm activity ($p>0.05$) against adherent mesophilic and *Pseudomonas* spp. bacteria during the early stages (< 3 days) of film formation and did not prevent biofilm adhesion. After 6 days of storage, significant inhibition on biofilm formation and accumulation ($p<0.05$) on high density polyethylene coupons was observed in the presence of plant extracts in ice; however, none of these plant extracts was able to inhibit cell adhesion completely. A lower biofilm inhibition ($p<0.05$) was observed with ice incorporated with thyme and clove-extracts after 9 and 12 days of storage compared to biofilm control, when a biofilm-reduction in adherent cell number (0.4 to 0.9 log cfu per coupon) was obtained for both microorganism indicators. Moreover, there was no significant difference in the effect of ice containing oregano-extract on biofilm formation and accumulation.

Keywords: Antibiofilm activity, Fish, Icing, Plant extracts, Total phenolic content

The initial number of spoilage bacteria in fresh fish is one of the most critical factors affecting shelf-life of fish products. Therefore, efforts should be made to minimise initial contamination level to prevent fresh fish from rapid microbial deterioration (Samelis, 2006). The formation of microbial biofilm on the fish contact surfaces increases the threat of a crossover contamination of the fish product (Kusumaningrum *et al*., 2003). The shelf life of fish products is severely diminished if measures for preventing contamination are unsatisfactory, especially if the specific spoilage organisms become dominant in the biofilm (Guðbjörnsdóttir *et al*., 2004).

Biofilms are multicellular matrices of bacteria surrounded by extracellular polymeric substances called glycocalyx. These polymers, especially exopolysaccharide, designated as EPS frequently, are strongly anionic thereby protecting the bacterial microcolony from antibiotics, detergents or biocides (Jeyasekaran *et al*., 2000). EPS frequently serve as a multipurpose functional element for adhesion on the surface, protection, recognition and facilitating spatial arrangement of different species within the biofilm (Allison *et al*., 1998).

Research for new substances in biofilm eradication and prevention is therefore an important area of focus. The interest in natural antimicrobial agents has been growing due to the changes of consumer attitude toward the use of artificial synthetic chemicals which have negative impacts on the environment (Davidson, 1997). Thyme, oregano and clove extracts are normally used as antioxidant and antimicrobial additives to preserve foods and to control the growth of both spoilage and pathogenic microflora. They contain high concentrations of phenolic compounds including carvacrol, thymol and eugenol (Tajkarimi *et al*., 2010). The antibacterial activity is most likely due to the combined effects of adsorption of phenolic compounds to bacterial membranes with membrane disruption and subsequent leakage of cellular contents (Negi, 2012). Previous studies have demonstrated the antimibiofilm properties of several plant-derived compounds. Eugenol, carvacrol, and thymol have been shown to exert antibiofilm effects against *Pseudomonas aeruginosa*, *Listeria monocytogenes* and *Escherichia coli* O157: H7 (Pérez-Conesa *et al*., 2006; El abed *et al*., 2011). Biofilm prevention is therefore a priority in the fish processing industry, and this industry should be stimulated to invest in

research on developing novel control strategies by killing early colonising bacteria or by preventing further growth of established biofilms. For this reason, this study was conducted to investigate whether ice containing thyme, oregano and clove extracts can prevent the formation of microbial biofilm and inhibit further growth of established biofilms on high density polyethylene (HDPE) coupons during chilled storage of anchovy.

The buds of clove (*Syzygium aromaticum*) were purchased from local retail markets. The aerial parts of oregano (*Origanum glandulosum*) were collected from the mountains surrounding Djelfa, Algeria, while the aerial parts of thyme (*Thymus vulgaris*) originated from cultivation. Plant materials were identified by the experts at Saad Dahlab University in Blida, Algeria. After drying in the dark at ambient temperature, the plant materials (clove, oregano and thyme) were ground separately in a grinding machine in the laboratory.

The extracts of the plants were prepared according to the methods described by Erturk *et al*. (2003), with some modifications. Each dry sample was soaked in ethanol absolute (1:5 w/v) for 24 h. The extract was filtered through Whatman No.1 filter paper. After filtration, activated carbon was added to filtrate (10 g of active carbon per 50 g of plant material) and removed immediately from the filtrate using filter paper. Subsequently, all ethanol was evaporated in a vacuum evaporator at 50°C. All the dried extracts were weighed and exposed to UV-light (30 W, 50 cm irradiation distance) for 30 min. Then, they were dissolved in ethanol absolute and stored in amber flasks in the dark at 4 °C until use. The extraction yield of ethanolic extract is presented as % weight (g of ethanolic extract for each 100 g of herb powder).

High density polyethylene (HDPE) was the surface material chosen for biofilm development, as it is extensively used in fish-holds of fishing vessels and fish boxes. HDPE coupons (2 × 1 × 0.1 cm) were cleaned with 70% ethanol and sterilised by autoclaving at 121°C for 15 min before use. Anchovies (*Engraulis encrasicholus*), caught from the Black Sea were iced in boxes and brought to the laboratory. After removing head, gills and intestine, they were washed with tap water.

Four different ice treatments were used for this experiment: Ice containing distilled water (control) and three different plant extracts. Thyme, oregano and clove extracts (400, 300 and 200 mg, respectively) were dissolved in 1 l distilled water, sealed in polyethylene bags and kept frozen until use. Ice used in all treatments were crushed before use. The concentrations of plant extracts yielding best appearance of treated fish and less presence of odour and colour in fish were chosen according to preliminary trials (100 –1000 mg plant extract per litre of distilled water).

The fish were divided into four lots. The HDPE coupons were placed horizontally in each self-draining polystyrene box. The fish samples were then placed in the boxes and covered with a layer of respective ice treatments at a fish-to-ice ratio of 2:1 (w/w). One lot was treated with normal ice (control) and the remaining three lots were treated with 0.04% (w/v), 0.03% (w/v) and 0.02% (w/v) ice with thyme, oregano and clove extracts, respectively. All boxes were then stored in a refrigerator with controlled temperature (3 ± 1°C). Ice was added to the boxes during storage, as and when required. Coupons from the different icing treatments were taken for analysis on days 3, 6, 9, and 12 post-treatment.

The speed of melting was determined for each of the ice treatments according to the method of Feliciano *et al*. (2010) with a slight modification. Ice cubes (dimensions: 1.5 × 1.5 × 2 cm; volume: 1 l) were placed into plastic trays (20 × 35 cm) that were left uncovered at 25ºC. At 30 min intervals, the volume of liquid from the melting ice for each ice treatment was measured until the ice melted completely. The tests were repeated three times.

For microbiological analysis, the HDPE coupons were removed aseptically and were washed with sterile distilled water three times, in order to remove residual plant extracts and planktonic cells. Then, they were transferred to test tubes containing 10 ml of ringer solution. Biofilm bacteria were recovered by subjecting coupons to three alternating cycles (30 sec) of sonication at a frequency of 35 kHz, followed by vortex agitation for 1 min. Serial decimal dilutions were prepared from the 10^{-1} dilution and samples (0.1 ml) of serial dilutions were inoculated on the surface of appropriate dry microbiological media in Petridishes by spread plate method.

Mesophilic aerobic bacteria were determined using plate count agar, after incubation for 48 h at 30°C (Harrigan and McCance, 1976). *Pseudomonas* spp. were enumerated on CFC medium (*Pseudomonas* Agar Base, supplemented with selective supplement consisting of cetrimide, fucidin and cephaloridine) after incubation at 25°C for 48 h. Results are expressed as a logarithm of colony forming units (log cfu) per coupon. Triplicate samples were taken from each of four different groups.

The total phenolic content in the plant extracts was determined by spectrophotometry using the Folin-Ciocalteau method and expressed as gallic acid equivalent (mg GAE per g extract). as per Singleton *et al*. (1999) with some modifications. Briefly 0.8 ml of undiluted Folin-Ciocalteu reagent was added to , to 0.2 ml sample (1 g l^{-1}). After 5 min, 3.6 ml of 20% (w/v) aqueous

Na_2CO_3 were added, and the volume was made up to 12 ml with HPLC water. After 2 h of incubation at 20°C, the absorbance of the reaction mixtures was measured at 765 nm and compared to a gallic acid calibration curve (0–500 mg l^{-1}). Measurements of every sample were taken in triplicate and the results were expressed as mg gallic acid equivalents (GAE) per g extract.

All statistical analyses were carried out using the SPSS package (SPSS 17.0 for Windows, SPSS Inc., Chicago, IL, USA). Data from the different analyses were subjected to one-way ANOVA in order to assess differences ($p<0.05$) among the different icing conditions. Duncan's Multiple Comparison Test was used to assess further differences between the treatment means.

The effect of ice containing thyme, oregano and clove extracts on formation and accumulation of biofilm communities on HDPE coupons during chilled storage of anchovy is shown in Table 1. After 3 days of storage, no detectable differences were found in the numbers of adherent mesophilic bacteria and *Pseudomonas* spp. in biofilms formed on HDPE coupons under different icing conditions (Table 1). The extracts showed no inhibitory effect on the attachment of cells and were not, however, effective in preventing the formation of biofilms on HDPE coupon surfaces. The failure in inhibiting cell attachment can be attributed to several causes.Treated ice takes few hours to melt and reach HDPE coupons and in the absence of exposure to antimicrobials, formation of the biofilm begins. On the other hand, microbial attachment to food processing surface is a rather fast process (Van Houdt and Michiels, 2010). *Pseudomonas* spp. requires 4 h attachment period for 78% of the total bacteria attached on plastic to become irreversibly attached (Marshall, 1992). After cells have adhered, the production of extracellular matrix begins; it should be noted that the slimes are thought to be important for intercell connection during surface colonisation (Hussain *et al*., 1992). Thus, high density polyethylene creates a favourable material for bacterial attachment. The degree of hydrophobicity of the surface has often been cited as determining cell attachment, suggesting that adhesion is favoured by hydrophobic surfaces (Brooks and Flint, 2008; Simões *et al*., 2010). Marine *Pseudomonas* spp. have been reported to attach more rapidly to hydrophobic surfaces such as polyethylene rather than hydrophilic surfaces, such as metal (Fletcher and Loeb, 1979). Secondly, when melted ice containing plant extracts flows across the fishes, its concentrations reach low levels before contact with HDPE coupons. Therefore, initial attachment occurs rapidly and growth of the film begins in the absence of antimicrobials exposure.

After 6 days of storage, the use of ice prepared with plant extracts to inhibit biofilm formation and accumulation (both mesophilic and *Pseudomonas* spp. bacteria) on HDPE coupons was found effective with all extracts showing reduced biofilm cell number ($p<0.05$) compared to the control biofilm. However, none of the used extracts was able to inhibit cell adhesion completely (Table 1). The marked inhibitory effect of used plant extracts is mostly attributed to the action of their principal phenolic components, which prevent further growth of established biofilms; presumably, viable cells were killed prior to the cell-biofilm attachment. Thyme, oregano and clove are rich in phenolic compounds, including thymol, carvacrol and eugenol that are likely to be responsible for the anti-bacterial activity of extracts derived from these plants (Tajkarimi *et al*., 2010). These phenolic compounds interact with the lipid bilayer of bacterial cytoplasmic membranes causing loss of integrity and leakage of cellular material such as ions, ATP and nucleic acids (Nostro *et al*., 2007).

Biofilms of 6 days old were found difficult to be removed completely. Biofilms in nature are generally multispecies; several workers have suggested that mixed species biofilms are frequently more stable and resistant than biofilms consisting of a single species (Brooks and Flint, 2008). In addition, microorganisms are much more protected from plant extracts components when embedded

Table 1. Inhibition of formation and accumulation of biofilm on high density polyethylene surfaces, under different icing conditions.

Biofilm forming bacteria	Treatments	Storage time (days)			
		3	6	9	12
Mesophilic aerobic bacteria (log cfu per coupon)	Control	3.14±0.32^{a}	5.78±0.13^{a}	6.17±0.22^{a}	7.03±0.35^{a}
	Thyme	3.21±0.18^{a}	4.47±0.01^{c}	5.37±0.11^{b}	6.31±0.10^{b}
	Oregano	3.17±0.76^{a}	5.32±0.18^{b}	6.21±0.48^{a}	7.37±0.08^{a}
	Clove	2.68±0.25^{a}	4.35±0.14^{c}	5.53±0.36^{b}	6.18±0.29^{b}
Psychrotrophic bacteria (log cfu per coupon)	Control	3.03±0.19^{a}	5.91±0.38^{a}	6.19±0.13^{a}	7.32±0.43^{a}
	Thyme	2.42±0.49^{a}	4.25±0.15^{c}	5.31±0.08^{c}	6.55±0.27^{b}
	Oregano	3.06±0.33^{a}	5.14±0.61^{b}	6.47±0.24^{a}	7.40±0.03^{a}
	Clove	2.90±0.30^{a}	4.30±0.26^{c}	5.77±0.23^{b}	6.19±0.13^{b}

The values are expressed as mean ± standard deviation, n = 3. Values bearing different superscripts in the same column differ significantly ($p<0.05$).

in biofilms (El abed *et al*., 2011). Biofilm cells can be 10–1000 times more resistant to antimicrobial agents than their planktonic counterparts (Simões, 2011). On the other hand, exopolymeric matrix could reduce the penetration of the antimicrobial agents or cause its inactivation (Stewart and Costerton, 2001). Stewart and Costerton (2001) reported that protection from antimicrobial agents in biofilms is due to physiological changes in the cells due to reduced growth rates and production of enzymes which are able to degrade the antimicrobial substances. The reduced effectiveness may also be attributed to the use of crude extracts which contain flavonoids in glycosidic form, where the sugar present in them decreases the effectiveness against some bacteria (Negi, 2012).

Thyme and clove-extract ices elicited a significant biofilm-reduction ($p<0.05$) of mesophilic bacteria and *Pseudomonas* spp. (1.3 to 1.7 log cfu per coupon) compared to the control biofilm within 6 days of film development. In addition, oregano-extract ice caused significant ($p<0.05$) decrease (0.5 to 0.8 log cfu per coupon), but was less effective than thyme and clove-extract ices (Table 1). The difference found between the actions of thyme, oregano and clove extracts on biofilm formation and accumulation (Table 1) may be attributed mainly to their distinct chemical composition. Differences in the antibiofilm activity existing between plant extracts are related to the concentration and nature of their chemical constituents, such as composition, functional groups, structural configuration of the components and possible synergistic interactions (Chang *et al*., 2001).

At day 9 and 12 of storage, thyme and clove-extract incorporated ice showed minimal effect ($p<0.05$) on the development and accumulation of a preformed biofilm compared to the control biofilm, when a biofilm-reduction in number (0.4 to 0.9 log cfu per coupon) of mesophilic bacteria and *Pseudomonas* spp. was obtained (Table 1). In contrast, ice containing oregano-extract differed from others, as it did not show antibiofilm activity ($p>0.05$). Possible reasons for the increased resistance of biofilm cells to plant extracts include the age of biofilm and the production of polysaccharide, which is probably an adaptive response. Many studies have indicated that resistance of food-borne biofilm bacteria against various antimicrobials increases with biofilm age (Frank and Koffi, 1990; Sommer *et al*., 1999). In addition, prolonged exposure to low concentration of antimicrobial agents may also provoke the adaptation of microorganisms, which secrete excess exopolysaccharide in the presence of plant extracts (De Laubenfels, 2001).

The results of our study also indicated that ice containing selected plant extracts did not enhance biofilm growth throughout the storage period as evidenced by the decrease in biofilm cell numbers compared to the control biofilm. The enhanced attachment observed upon exposure to some plant extracts may be due to the presence of certain compounds within the extracts that provided a conditioning film promoting microbial adhesion. Sandasi *et al*. (2008) showed that treatment of listerial biofilms with some plant extract components enhanced biofilm growth.

Results for the extraction yields indicated that the clove extract showed the highest extraction yield of 9.05 % (w/w) followed by thyme extract which was 1.72 % (w/w). Low extraction yield was found for oregano extract (0.71 %). In this study, the total phenolic contents in extracts are obtained from the aerial part of thyme and oregano, and the buds of clove. The highest contents (637.35±24.29 mg GAE per g extract) were observed in extract of clove buds followed by oregano (325.56±19.49 mg GAE per g extract) and thyme (139.74±9.71 mg GAE per g extract). The amount of total phenolics that can be extracted from a plant material is mainly affected by the vigour of the extraction procedure and the efficiency of the extracting solvent to dissolve endogenous compounds (Siddhuraju and Becker, 2003; Sultana *et al*., 2007). Solvents, such as methanol, ethanol, acetone, and ethyl acetate have been used for the extraction of polyphenols from plant materials (Xu and Chang, 2007). In our research, ethanol was chosen for polyphenol extraction because it is safe for human consumption (Shi *et al*., 2005).

The content of total phenolics showed siginificant correlation with most of the antibiofilm assays, such as biofilm-reduction of mesophilic aerobic bacteria at 6, 9 and 12 days ($r = 0.77$, $r = 0.5$ and $r = 0.58$, respectively), and biofilm-reduction of *Pseudomonas* spp. at 6 and 12 days ($r = 0.8$ and $r = 0.73$, respectively). This result was in accordance with previous reports suggesting that antimicrobial and antioxidant activities of plant extracts increase significantly with the presence of high concentration of total phenolics (Lim *et al*., 2009; Edziri *et al*., 2012).

The results obtained from this work show that the buds of clove, which gave the highest extraction yield and total phenolic recovery may be exploitable as a potential source of phenolic compounds for possible use as antimicrobial agents.

Fig. 1. shows the volume of liquid collected from the ice treatments at 30 min intervals. Results showed that initially, the distilled water ice appeared to melt faster than the plant-extract containing ice ($p<0.05$) during the first 120 min. After 30 min storage time at 25°C, the volume of liquid collected from the control ice was 130.33±9.07 ml whereas 42.33±5.13, 68±1, and 71±4.58 ml were collected from thyme, oregano and clove-extract treated ice respectively. However, for most

storage times, the volume collected for all treatments appeared to be relatively similar ($p>0.05$). The statistical analyses revealed that in general, the used plant extracts did not have significant effect on ice melting rate when compared with the control.

Various approaches are currently under investigation to reduce the adhesion of microorganisms on inert surfaces. These include inhibiting initial attachment on the surface or impeding the growth of an established biofilm. In this study, we have shown that ice prepared

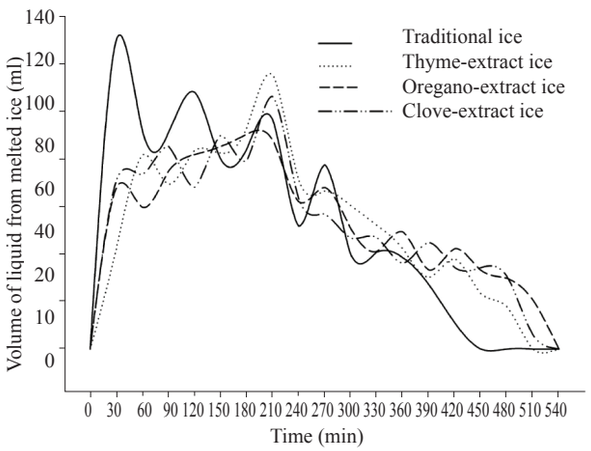

Fig. 1. Volume of liquid collected from the ice treatments

with thyme, oregano and clove extracts was less effective in preventing biofilm formation and in removing mature biofilms on HDPE surfaces. However, the application of these extracts at the doses used in this study did not inhibit cell adhesion completely. It would be possible to control biofilm formation and accumulation using high concentrations, but the practical application of these plant extracts may still be hampered by certain limitations (bitter taste and strong odour of fish flesh). In addition, the ethanolic extract of clove possessed the highest total phenolic content value followed by oregano and thyme. Moreover, there was a good correlation between the antibiofilm capacity and phenolic contents. We suggest further research using other natural plant extracts against bacterial biofilms, as there are few works on this perspective and the works already conducted have shown promising results.

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