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Antimicrobial Effects of Herb Extracts and Their Applications in Edible Films

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ABSTRACT

Crude extracts from dried fingerroot, garlic, cloves and cinnamon were studied for their antimicrobial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus luteus* and *Pseudomonas aeruginosa*. Extract from cinnamon showed the highest activity with minimum inhibitory concentration (MIC) ranging from 31.25 to 100 mg/ml, followed by extracts from cloves and fingerroot with MICs of 100-250 and 200-500 mg/ml, respectively. Garlic extract showed no inhibitory effect against these bacteria. The inhibitory activity of the extracts decreased over the storage period of 8 weeks at 4°C. However, cinnamon and cloves extracts still possessed considerable inhibitory activity with slightly higher MICs of 100-250 and 200-500 mg/ml, respectively. Carrageenan films incorporated with cinnamon and cloves extracts of one five and ten times their MICs were tested for their antimicrobial effects against the test microorganisms and those taken from dried salted fish, called cocktail microorganisms. Higher concentrations than their MICs were observed and cinnamon extract gave better results than cloves extract. The inhibitory effects were promising against cocktail microorganisms, evidenced by the fact that neither bacterial nor yeast and mold growth was shown with cinnamon extract of five times and at their MICs, respectively. Additionally, cloves extract could inhibit the growth of cocktail yeast and mold at its MIC.

Key words: antimicrobial film, carrageenan, herb extract, minimum inhibitory concentration (MIC), dried salted fish

INTRODUCTION

The increasing demand for high quality foods with less synthetic chemicals added means a continuing search for alternative sources of antimicrobial compounds. Spices and herbs used as seasoning agents in foods and beverages have been extensively studied for their antimicrobial activities. Garlic, onion, leek, cinnamon, allspice, cloves, oregano, thyme, savory, celery, parsley and angelica have been investigated (Beuchat and Golden, 1989; Conner, 1993; Elgayyar *et al.*, 2001). The latest trend in food packaging includes the incorporation of antimicrobial substances in packaging materials (Hotchkiss, 1995; Brody *et al.*, 2001; Appendini and Hotchkiss, 2002). Incorporation of plant extracts as a source of antimicrobial compounds is being widely investigated because not only does it decrease the use of synthetic antimicrobial chemicals, but it also provides added functions, such as provision of nutraceuticals and flavors (Han, 2002).

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Furthermore, the use of edible coatings and films as carriers of antimicrobial compounds from plants would provide additional advantages, such as biodegradability, edibility, biocompatibility, aesthetic appearance and barrier properties (Han, 2000). Dried salted fish (*Trichogaster pectoralis* Regan, Sepat–Siam) is one of the most popular preserved foods in Thailand, but it is susceptible to microbial spoilage. Therefore, synthetic preservatives have been extensively used to extend the product's shelf life. The aim of this study was to select herb extracts with respect to their antimicrobial activity and subsequently to incorporate them into edible coatings for further application as antimicrobial coatings for dried salted fish in order to replace the use of synthetic preservatives.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains used in this study were *Staphylococcus aureus* DMST 8840, *Staphylococcus epidermidis* TISTR, *Micrococcus luteus* DMST 15503 and *Pseudomonas aeruginosa* DMST 4739, obtained from the Department of Medical Sciences, Ministry of Public Health, and the culture collection at Thailand Institute of Scientific and Technological Research, Thailand. They were kept in 40% (v/v) glycerol and nutrient broth, and stored at -18°C. A working culture was prepared by inoculating a loopful of each culture into 10 ml of nutrient broth, which was subjected to successive 24 h transfers before use. The initial concentration of stock culture was approximately 10^6-10^8 CFU/ml.

Preparation of herb extracts

Four selected herbs in powder form (moisture content 10% dry basis, size 40/60 mesh), namely fingerroot (*Boesenbergia pandurata* Holtt.), garlic (*Allium sativum* Linn.), cloves (*Eugenia caryophyllus*) and cinnamon (*Cinnamomun cassia* Ness.), were collected from C. R. Pure Light Co., Ltd., Thailand. Fifty grams of each sample was extracted in 500 ml of both ethyl alcohol 95% and distilled water at 50°C for 24 h, then filtered and concentrated by vacuum evaporation at 50°C, 150 mbar to obtain 50 ml of crude extract (Jaturapornchai, 2003). Thus, the concentration of crude herb extract was 1,000 mg/ml. The extracts were kept in amber glass bottles and stored at 4°C.

Determination of minimum inhibitory concentration (MIC) and stability of extracts

Antimicrobial activities of crude extracts were assayed using the agar well diffusion method (Rauha *et al*., 2000). Four wells (diameter 5 mm) were made on each nutrient agar plate using a sterile cork borer and inoculated with test bacteria of 10^6-10^8 CFU/ml. Crude extracts were serially diluted to yield dilutions of 31.25, 62.5, 100, 125, 200, 250, 500, 750 and 1000 mg/ml. Forty microliters of the dilution was transferred to each well and sterile water served as a control. Then, the plates were incubated at 37°C, for 24 h. MICs (the lowest concentration of extract that resulted in the zones of inhibition with a diameter greater than 7 mm (Nascimento *et al.,* 2000)), were determined. The tests were repeated at week 1, week 2, week 4 and week 8 to investigate the stability of the extracts during storage at 4°C.

Determination of antimicrobial effect of carrageenan films incorporated with crude extracts

κ-carrageenan solutions (2.5 w/v) were mixed with one, five and ten times the MIC value of each extract and then cast into film on glass plates (20 × 20 cm) with a 1 mm spacer. Films without extract added served as a control. The films were dried at 25°C for 2 h. Antimicrobial activities of films were assayed using the agar disc diffusion method. Film samples of 8 mm diameter were placed on nutrient agar plates inoculated with the test bacteria of 10^6-10^8 CFU/ml. In addition, the films were tested for their inhibitory activity

against stock cultures, which were taken from dried salted fish samples (*Trichogaster pectoralis* Regan, Sepat–Siam) and serially diluted to yield 10^{-1}, 10^{-2} and 10^{-3} dilutions, called cocktail stocks. After incubation for 24 h at 37°C for bacteria, and for 48 h at 30°C for yeast and mold (Downes and Ito, 2001), MICs were determined with reference to the presence of an inhibition zone.

Statistical analysis

The results were statistically analyzed using one-way analysis of variance (ANOVA). Differences among means were determined using Duncan's new multiple range test, with a p value < 0.05 considered as significant. SPSS for Windows was used to carry out statistical analyses for the study.

RESULTS AND DISCUSSIONS

Determination of minimum inhibitory concentrations (MICs) and stability of extracts

The results in Table 1 were very useful for selecting herb extracts for further study. Garlic was screened out, as no inhibitory effect against any test bacteria was observed. This result was in accordance with a previous report (Onyeagba *et al*., 2004). It was also evident that the preparation of crude extracts using ethyl alcohol was preferable to water. Therefore, only extracts with ethyl alcohol were considered for further studies. Cinnamon had the most pronounced antimicrobial effect, followed by cloves and fingerroot, with MICs of 31.25-100, 100-250 and 200-500 mg/ml, respectively. It is important to note that extracts of cinnamon and cloves could inhibit the growth of all test bacteria (Figures 1 and 2), while fingerroot extract could inhibit only *S. aureus* and *S. epidermidis*. In the case of cloves extract, the diameters of the inhibition zone varied in the range of 11 to 17 mm for the gram-positive bacteria, *S. aureus* and *S. epidermidis*, and *M. luteus*; and from 9 to 15 mm for the gram-negative bacterium, *P. aeruginosa*. Cinnamon produced a bigger zone, for instance, 13-19 mm for the three gram-positive bacteria and 13-17 mm for the one gram-negative bacterium. This finding corresponded well to previous reports (Kanika, 2001; Jaturapornchai, 2003; Petinaki *et al*., 2006), though small variations of MIC values were observed. Cinnamic aldehyde, linalool (E)-nerolidol and perpineol from cinnamon have all been found to suppress bacterial growth (Petinaki *et al*., 2006). Cloves contain eugenol compounds, triterpenes and many organic acids, which can disturb cell activities in many ways, such as food absorbability and cell

Table 1 MICs of crude herb extracts.

Herb	Solvent	MIC (mg/ml)			
		S. aureus	*S. epidermidis*	*M. luteus*	*P. aeruginosa*
Fingerroot	Water	–	750	–	–
	Ethanol	500	200	–	–
Garlic	Water	–	–	–	–
	Ethanol	–	–	–	–
Cloves	Water	500	500	250	250
	Ethanol	100	200	125	250
Cinnamon	Water	62.5	200	100	31.25
	Ethanol	31.25	62.5	100	62.5

– = no inhibition zone

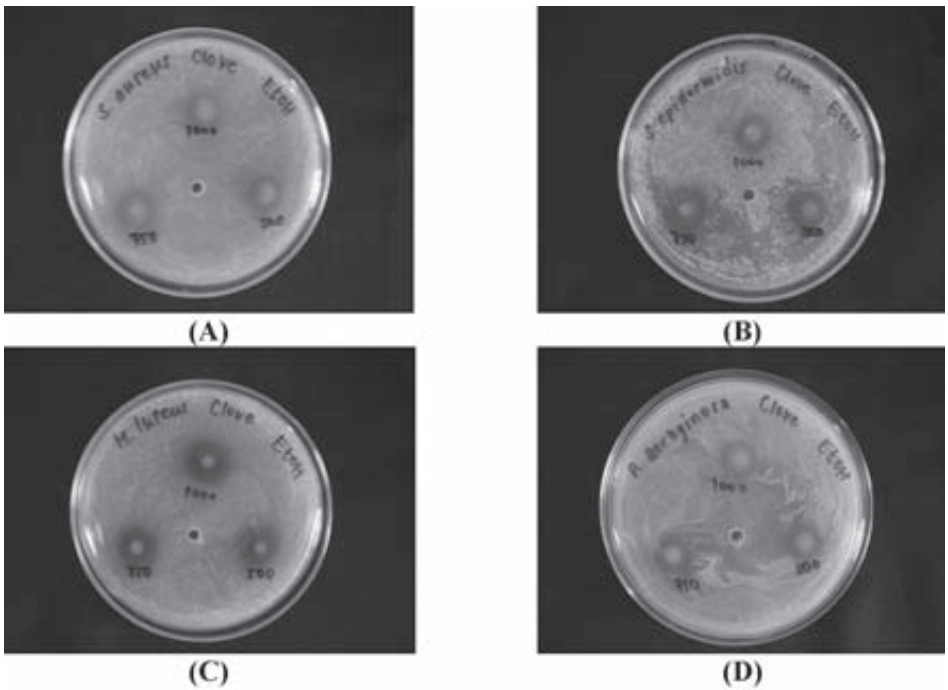

Figure 1 Zone of inhibition of cloves extracts with: **(A)** *Staphylococcus aureus*; **(B)** *Staphylococcus epidermidis*; **(C)** *Micrococcus luteus*; **(D)** *Pseudomonas aeruginosa*.

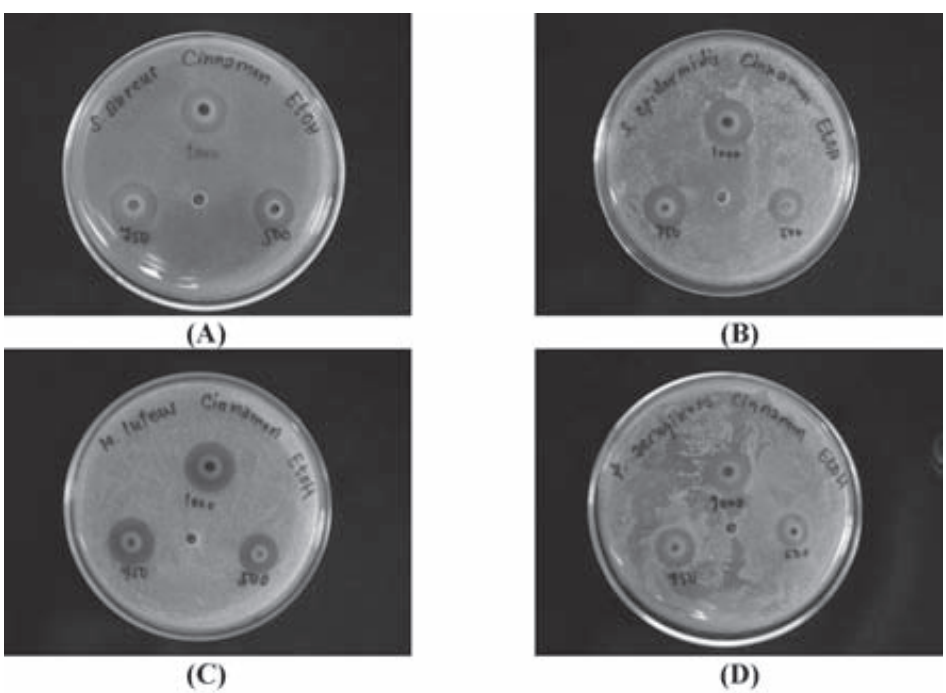

Figure 2 Zone of inhibition of cinnamon extracts with: **(A)** *Staphylococcus aureus*; **(B)** *Staphylococcus epidermidis*; **(C)** *Micrococcus luteus*; **(D)** *Pseudomonas aeruginosa*.

membrane disruption. Thus, cloves were capable of inhibiting both gram-positive and gram-negative bacteria (Kanika, 2001; Jaturapornchai, 2003). Fingerroot could inhibit *S. aureus* and *S. epidermidis* with MICs of 200-500 mg/ml, similar to the results reported by Jaturapornchai (2003) and Petinaki *et al*. (2006). This inhibition is due to chromene compounds in fingerroot, which are able to modify the groups of protein and nucleic acid of bacteria, subsequently preventing bacterial growth (Kanika, 2001; Jaturapornchai, 2003). The inhibitory effect of crude extracts marginally decreased over the storage period of 8 weeks at 4° C as shown in Table 2. Therefore, MICs slightly increased, as can be seen in Table 3. However, cinnamon and cloves extracts still possessed promising inhibitory activity against the test bacteria with MICs of 100-250 and 200-500 mg/ml, respectively. Therefore, it is essential to take into account the stability of antimicrobial agents during storage when studying their MICs and their suitability in various applications.

Determination of antimicrobial effect of carrageenan films incorporated with crude extracts

Preliminary study (data not presented) revealed that carrageenan solution of 2.5% (w/v) was best suited for coating on fatty skin of dried salted fish and could be peeled off easily before

Table 2 Changes in MICs of crude herb extracts during storage at 4°C.

Herb	Storage period	MIC (mg/ml)			
		S. aureus	*S. epidermidis*	*M. luteus*	*P. aeruginosa*
Fingerroot	1 day	500	200	–	–
	1 week	500	500	–	–
	2 weeks	500	750	–	–
	4 weeks	500	1,000	–	–
	8 weeks	500	1,000	–	–
Cloves	1 day	100	200	125	250
	1 week	200	200	125	250
	2 weeks	200	200	200	250
	4 weeks	200	200	200	500
	8 weeks	200	250	200	500
Cinnamon	1 day	31.25	62.5	100	62.5
	1 week	100	100	100	100
	2 weeks	100	100	100	100
	4 weeks	100	100	100	100
	8 weeks	100	250	100	100

– = not detected

Table 3 MICs of herb extracts after storage at 4°C for 8 weeks.

Herb	MIC (mg/ml)			
	S. aureus	*S. epidermidis*	*M. luteus*	*P. aeruginosa*
Fingerroot	500	1,000	–	–
Cloves	200	250	200	500
Cinnamon	100	250	100	100

– = not detected

cooking, if required. Therefore, carrageenan films were selected for further investigation of their antimicrobial activities. The results in Table 4 show that carrageenan films incorporated with cinnamon extract gave slightly better results than those with cloves extract. To inhibit the growth of test bacteria, concentrations of ten times their MICs were required, with the exception of *S. epidermidis*, for which there was no inhibitory effect observed. The study also demonstrated that the effects were relatively promising with cocktail microorganisms, evidenced by the fact that neither bacterial nor yeast and mold growth was observed with cinnamon extract of five times and at its MIC, respectively. In addition, cloves extract could inhibit the growth of cocktail yeast and mold at five times its MIC but it had no effect against cocktail bacteria even at ten times its MIC. This finding was in accordance with some previous observations. It was suggested that an antimicrobial packaging for food applications should contain the active agent at levels about ten times or more its MIC, as some might be lost during the process (Davidson and Branen. 1993). It was also reported (Burt, 2004) that the effectiveness of antimicrobial agents in their liquid phase was normally higher than as solids, due to the larger diffusion coefficients in the liquid phase. Furthermore, the migration rate of antimicrobial agents through films, which should be fast enough to inhibit the growth of target microorganisms, depends directly on their concentration, solubility and diffusivity. Consequently, a high concentration of extracts, such as five or ten times their MICs as reported in this study, would be recommended in practice, unless there was an adverse effect on the sensorial qualities of products.

CONCLUSIONS

The experimental findings indicated that cinnamon extract showed the highest inhibitory effect, with MICs ranging from 32.5 to 100 mg/ml, against the growth of *S. aureus, S. epidermidis,*

Table 4 Inhibitory activity of carrageenan films incorporated with crude herb extracts.

Herb	Microorganisms	Zone of inhibition[1] ± Standard deviation		
		1xMIC	5xMIC	10xMIC
Cloves	*S. aureus*	–	–	11.71 ± 0.10^{B}
	S. epidermidis	–	–	–
	M. luteus	–	–	10.83 ± 0.14^{A}
	P. aeruginosa	–	–	11.81 ± 0.05^{B}
	Cocktail bacteria	–	–	–
	Cocktail yeast and mold	–	14.43 ± 0.05^{a}	14.91 ± 0.05^{bC}
Cinnamon	*S. aureus*	–	-	11.99 ± 0.07^{B}
	S. epidermidis	–	-	–
	M. luteus	–	12.77 ± 0.18^{aB}	14.90 ± 0.07^{bD}
	P. aeruginosa	–	–	12.20 ± 0.06^{C}
	Cocktail bacteria	–	10.68 ± 0.08^{aA}	11.88 ± 0.14^{bA}
	Cocktail yeast and mold	10.94 ± 0.04^{a}	14.72 ± 0.04^{bC}	15.15 ± 0.03^{cE}

[1] = means of three replicates

– = not detected

[a-f] = lower case alphabetical characters for rows indicate significant differences among the formulation means at the 95% level

[A-G] = upper case alphabetical character for columns indicate significant differences among the formulation means at the 95% level

M. luteus and *P. aeruginosa*; followed by cloves and fingerroot extracts. No inhibitory effect was observed in the case of garlic extract. In addition, regarding the inhibitory effect, the preparation of crude extracts using ethyl alcohol was preferable to water. The inhibitory activity of the extracts decreased over the storage period of 8 weeks at 4°C. However, cinnamon and cloves extracts still possessed considerable inhibitory activity against the test bacteria with slightly higher MICs of 100-250 and 200-500 mg/ml, respectively. The inhibitory effects of cinnamon and cloves extracts incorporated into carrageenan films were evident with higher concentrations (for instance at levels five or ten times) than their MICs. Moreover, the effect was promising against cocktail microorganisms, evidenced by the fact that neither bacterial nor yeast and mold growth was shown with cinnamon extract at levels of five times and at their MICs, respectively. In addition, cloves extract could inhibit the growth of cocktail yeast and mold at five times its MIC but it had no effect against cocktail bacteria even at levels of ten times. These findings would encouragingly lead to further study on the use of these antimicrobial films for preserving the quality of dried salted fish (*Trichogaster pectoralis* Regan, Sepat–Siam), while reducing the use of synthetic preservatives.

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