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Antibacterial Activity of Spice Extracts against *Pseudomonas fluorescens*: Application of Clove and Thyme Extracts in Decontamination of Raw Chicken

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Abstract

Antimicrobial activity of 6 spice methanolic extracts against *Pseudomonas fluorescens* using disc diffusion assay and minimum inhibitory concentration (MIC) determination was studied. Extract of clove (*Syzygium aromaticum*) was the most effective against the growth of *P. fluorescens* (3.2 mg/ml MIC), followed by the extracts of thyme (*Thymus vulgaris*), star anise (*Illicium verum*), galanga (*Alpini agalanga*), caraway (*Ocimum gratissimum*) and licorice (*Glycyrrhiza glabra*). Clove extract also possessed the strongest antioxidant activity (3.63 mmol Fe (II)/ g extract) by ferric reducing antioxidant power method, followed by the extracts of thyme, caraway, galanga, licorice and star anise. Therefore, clove and thyme extracts were selected for further study. Their effect on microbial load and lipid oxidation in raw chicken thighs has investigated. Total viable counts and total *Pseudomonas* counts on raw chicken thigh surface after dipping in aqueous solution of 1-2% clove extract, 1-2% thyme extract or distilled water were compared. Addition of 2% clove extract in dipping solution was the most effective to reduce the microbial number on the chicken thigh surface and delayed the lipid oxidation in the raw chicken thigh meat. The treatment of naturally contaminated chicken thighs by dipping in aqueous solution of 2% clove extract resulted in immediate reduction of total viable counts and total *Pseudomonas* counts on the chicken thigh surface by 0.56 and 0.28 log CFU/ 25 cm^2, respectively. Moreover, this treatment also resulted in 0.53 log CFU/ 25 cm^2 reduction of total *Pseudomonas* counts on the chicken thigh surface, and delayed lipid oxidation in the raw chicken thigh meat after 7-day refrigerated storage.

Keywords: Chill storage, Psychrotroph, Lipid oxidation, Raw chicken thigh

1. Introduction

Raw chicken meat is recognized as one of the most perishable foods due to its chemical composition that favours microbial growth. It is often spoiled by psychrotrophic bacteria such as *Pseudomonas fluorescens*and other bacteria [1]. Moreover, raw meat deterioration can occur by lipid oxidation as it contains unsaturated fatty acids and prooxidants which are prone to oxidation [2]. Lipid oxidation in meat occurs in two forms, hydrolytic and oxidative rancidity. Hydrolytic rancidity is important in some situation. This type of rancidity starts to occur where enzyme is present and microbial growth exists. Oxidative rancidity occurs more frequently in meat than the hydrolytic rancidity. This type of rancidity is auto-oxidation which occurs where atmospheric

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oxygen is present. Oxygen reacts with double bond of unsaturated fatty acid. Finally, hydroxyl peroxides and off-flavor are formed [3]. Therefore, chicken meat deterioration should be delayed in order to increase its shelf life during refrigerated storage.

Decontaminating meat relys on the activity of physical treatments and others to either remove or destroy the microorganisms. Washing meat with water alone usually reduces the microbial numbers by 1-2 log units. New method in decontamination of raw chicken meat have been developed [4]. Recently, trisodium phosphate solution and water containing lactic, acetic acid or chlorine have been applied for microbial decontamination of poultry meat [4-6]. Although washing poultry carcass with these solutions was reported to be effective to reduce the microbial load, each method had some disadvantages including cost, adverse sensory change or health concern. Thus, there is growing interest in the use of natural antimicrobials. Some spice extracts or their active compounds have been reported to be effective to decontaminate raw meat [7, 8]. Therefore, it is interesting to study the antibacterial activity of some spices including galanga, licorice, star anise, caraway, clove and thyme against *P. fluorescens* and their application as natural antimicrobials in the dipping solution for decontamination of raw chicken.

2. Materials and Methods

2.1 Extraction of spices

Six spices were used in this study (Table 1). These plant materials were washed, dried, cut into small pieces and ground to a fine powder. Then, 10 g of each spice were soaked in 100 ml methanol and shaken at 150 rpm for 48 h at 30°C. The mixtures were filtered. The filtrates were evaporated using vacuum rotary evaporator and air dried. The dried extracts were diluted with 10% dimethyl sulphoxide (DMSO; Carlo Erba, Italy) to prepare the stock solution (200 mg/ml).

Table 1. Spices used in this study

Botanical name	Common name/ Thai name	Family	Plant part
Alpiniagalanga (Linn.) Swartz.	Galanga/ Khar	Zingiberaceae	Rhizomes
Glycyrrhizaglabra Linn.	Licorice/ Cha-aimted	Leguminosae	Roots
Illiciumverum Hooker.f.	Star Anise/ Poyguck	Illiciaceae	Fruits
Ocimumgratissimum Linn.	Caraway/ Yira	Lamiaceae	Seeds
Syzygiumaromaticum (L.) Merrill & Perry	Clove/ Karnpu	Myrtaceae	Flowers
Thymus vulgaris	Thyme	Labiatae	Leaves

2.2 Culture preparation

Pseudomonas fluorescens DMST 20076 was used in this study. This bacterium was obtained from the culture collection of the Department of Medical Science, Ministry of Public Health, Thailand. To prepare this bacterial culture, a loopful of 24-hour old *P. fluorescens* was inoculated into 5 ml Brain Heart Infusion (BHI) broth (Difco Laboratories; Detroit, MI, USA). After incubation at 30 °C for 24 h, cells were harvested by centrifugation at 3000 ×g for 15 min, washed twice and resuspended in 0.1% peptone solution. Turbidity was adjusted to match the turbidity of 3 McFarland standard to obtain 10^8 CFU/ml cell concentration [9].

2.3 Antimicrobial susceptibility testing

Antimicrobial activity of all spice extracts was tested against *P. fluorescens* by disc diffusion assay and minimum inhibitory concentration determination.

2.3.1 Disc diffusion assay

The disc diffusion test against *P. fluorescens* was performed using the procedure as described by Collins *et al.* [10]. Briefly, the bacterial cell suspension (100 µl) was swabbed onto the surface of Mueller Hinton Agar (MHA; Difco Laboratories; Detroit, MI, USA). Sterile 6-mm filter paper discs (Whatman; GE Healthcare UK Limited; UK) were aseptically placed on the surfaces of the MHA plate. Each spice extract (15 µl) was immediately added to the paper disc. Ampicillin (1 µg/ml) was used as a positive control, while 10% DMSO was used as a negative control. The plates were incubated at 30°C for 24 h. Antimicrobial activity was evaluated by measuring the inhibition zone diameters. The experiments were done in triplicate.

2.3.2 Determination of minimum inhibitory concentrations (MICs)

The MICs of all spice extracts were determined by agar dilution method against *P. fluorescens* using the procedure as described by Collins *et al.* [10]. Each spice extract at 0.2 - 6.4 mg/ml final concentration in BHI agar slant was prepared. After surface drying, a loopful of *P. fluorescens* suspension was streaked onto the surface of each agar slant. After incubation, the growth of *P. fluorescens* at each spice concentration was recorded. Ampicillin (0.05-5 mg/ml) was used as a positive control. The lowest concentration of the spice extract that completely inhibited visible growth of *P. fluorescens* was recorded as the MIC.

2.4 Antioxidant activity assay by Ferric reducing antioxidant power (FRAP) method

Antioxidant activity of the spice extracts by the FRAP method was analyzed according to the procedure as previously described by Lado *et al.* [11]. To do FRAP assay, 1 mg/ml extract (100 µl) was mixed with 3.0 ml FRAP reagent (25 ml of 300 mM acetate buffer, 2.5 ml of 10 mM 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ, Fluka, Sigma-Aldrich, Switzerland) in 40 mM HCl and 2.5 ml of 20 mM $FeCl_3$. $6H_2O$), and incubated at 37°C for 5 min. The absorbance was measured at 594 nm using UV-visible spectrophotometer (UV1601, Shimadzu Scientific Instruments (Oceania) Pty. Ltd., Australia) against blank (FRAP reagent without the sample). The concentration of Fe^{2+}-TPTZ (reducing capacity) was calculated by comparing the absorbance at 594 nm with the standard curve of the Fe (II) standard solutions (ferrous sulfate heptahydrate) at the concentration of 0.0469-3mM. The reducing power of each extract was expressed as mmol Fe(II)/g extract.

2.5 Determination of total flavonoid content

Total flavonoid content of spice extracts was determined according to the method as described by Kathirvel and Sujatha [12]. Briefly, 250 µl spice extract (1 mg/ml in 30% methanol), 1.25 ml distilled water, 75 µl of 5% $NaNO_2$ solution were mixed together. The mixture was allowed to stand for 5 min. Then, 150 µl of 10% $AlCl_3$ solution was added and the mixture was allowed to stand for 6 min before adding with 500 µl of 1M sodium hydroxide and 275 µl distilled water. After mixing well, the absorbance was measured at 510 nm using UV-visible spectrophotometer (UV1601, Shimadzu Scientific Instruments (Oceania) Pty. Ltd., Australia). Standard curve of catechin (Sigma-Aldrich, Switzerland)at the concentration of 10-1000 µg/ml was prepared using similar procedures. The results were expressed as mg catechin equivalents (CE) /g extract.

2.6 Application of clove and thyme extracts in decontamination of raw chicken

The extracts of clove and thyme with strong antibacterial and antioxidant activities were selected for use in decontamination treatments of raw chicken thigh. Fifteen raw chicken thighs were purchased from a local market at Ladkrabang district, Bangkok, Thailand. These chicken thighs were randomly divided into 5 groups and determined for the original number of natural microflora such as total viable counts and total *Pseudomonas* counts. These samples were performed the decontamination treatments using the modified method as described by Capita *et al.* [5] and Sakhare *et al.* [6]) by dipping into the dipping solutions as follows: 1) 1% clove extract aqueous

solution, 2) 2% clove extract aqueous solution, 3) 1% thyme extract aqueous solution, 4) 2% thyme extract aqueous solution and 5) sterile distilled water (control). Each chicken thigh sample was separately dipped into each of these dipping solutions (1-min contact time). Then, the samples were left to drain the excess liquid for 5 min before determining total viable counts and total *Pseudomonas* counts. Then, all chicken thigh samples were stored at 4°C in the refrigerator for 7 days. After storage, they were evaluated for total viable counts, total *Pseudomonas* counts and thiobarbituric acid reactive substances (TBARS) values using the method of Kirk and Sawyer [13].

To determine total viable counts (TVC) and total *Pseudomonas* counts on the chicken thigh surface, 25 cm^2 surface of the chicken thigh sample was swabbed using sterile cotton swab. Then, the cotton head of the cotton swab was cut into the sterile test tube with 9 ml of sterile peptone water. This solution was serially diluted and plated onto plate count agar (PCA) for analysis of total viable counts and *Pseudomonas* isolation agar (PIA, Difco Laboratories, Detroit, MI, USA) for analysis of total *Pseudomonas* counts by spiral plate technique using the Spiral Plater (Autoplate 4000, Spiral Biotech company, USA). All PCA plates were incubated at 37°C for 24 h, while all PIA plates were incubated at 30°C for 24 h. Then, all colony counts were recorded, calculated and reported as total viable counts/ 25 cm^2 and total *Pseudomonas* counts/ 25 cm^2 for the number of the total aerobic microorganisms and total *Pseudomonas* on 25 cm^2 of the chicken thigh surface, respectively.

For statistical analysis, data were analyzed by using analysis of variance to determine if significant differences ($P<0.05$) existed between mean values and using Duncan multiple range test to compare between treatment means.

3. Results and Discussion

3.1 Antibacterial and antioxidant activities of spice extracts

Clove extract was the most effective spice to inhibit the growth of *P. fluorescens* (21.41 mm inhibition zone diameter) and 3.2 mg/ml MIC, followed by the extracts of thyme, star anise, galanga, caraway and licorice (Table 2). Among all spice extracts tested, clove extract possessed the strongest antioxidant activity (3.63 mmol Fe (II)/ g extract by FRAP method and highest flavonoid content (209.52 mg CE/g extract). Thyme extract showed relatively strong antioxidant activity (1.95mmol Fe (II)/ g extract) and high flavonoids (199.03 mg CE/g extract) as compared to other spice extracts (Table 3). Therefore, clove and thyme extracts were selected for use in decontamination treatments of raw chicken.

Antibacterial activity of clove extract against *P. fluorescens* has been reported by other researchers [14, 15]. Strong antioxidant activity of clove extract has also been reported [16]. This may be due to its high flavonoid content. Clove extract was found to contain high amount of some active compounds such as eugenol (49.85%), eugenol acetate (22.59%), caryophyllene (14.39%) and other compounds [15]. Antibacterial activity of clove extract may be because of the action of eugenol. Pure eugenol was reported to inhibit the growth of some bacteria including *Escherichia coli* and *Staphylococcus aureus* [17].

Similarly, thyme extract has been reported to possess antibacterial activity against several bacterial strains [18]. Essential oil extracted from leaves and flowers of thyme has been found to contain high amount of carvacrol (45 mg/g), followed by thymol (24.7 mg/g), β-phellandrene (9.7 mg/g), linalool (4.1 mg/g), humuline (3.1 mg/g), α-phellandrene (2.3 mg/g) and myrcene (2.1 mg/g) as well as small amount ofα-pinene, β-pinene, α-thyjone, tricyclene, 1,8-cineole and β-sabinene (0.93 – 1.3 mg/g) [19]. It has been reported that thymol and carvacrol, the main compounds in thyme possessed strong antibacterial activity against several bacterial strains such as *Shigellasonnei, Shigellaflexneri, Escherichia coli* and *Bacillus cereus* [20, 21]. The strong antioxidant activity of thyme extract may be due to the action of its active compounds. Vallverdú-

Queralt *et al.* [22] found that thyme extract contained rosmarinic acid (84.04 µg/g dried extract), caffeic acid (6.56 µg/g dried extract) and *p*-hydroxybenzoic acid (4.38 µg/g dried extract).

Table 2. Antibacterial activity of spice extracts against *Pseudomonas fluorescens* by disc diffusion assay and minimum inhibitory concentration determination

Spice extracts	Diameter of Inhibition zone (mm)[a] ± SD	Minimum Inhibitory Concentration, MIC(mg/ml) [a]
Galanga	7.44±0.58	>6.4
Licorice	-[b]	>6.4
Star Anise	9.11±1.85	>6.4
Caraway	6.69±0.11	>6.4
Clove	21.41±0.71	3.2
Thyme	9.55±1.36	>6.4
Ampicillin	18.80±0.90	0.05

[a]Data are mean of three replications.
[b]No inhibition zone was observed.

Table 3. Antioxidant activity and total flavonoid content of spice extracts

Spice extracts	Antioxidant activity (mmol Fe (II)/ g extract) [a]± SD	Total flavonoid content (mg CE/ g extract) [a]± SD
Galanga	0.37±0.00	16.18±1.35
Licorice	0.15±0.00	8.80±0.29
Star Anise	0.02±0.01	34.14±0.44
Caraway	0.55±0.01	87.65±0.07
Clove	3.63±0.02	209.52±1.64
Thyme	1.95±0.01	199.03±0.74

[a]Data are mean of three replications.

3.2 Use of clove and thyme extracts for decontamination of natural microflora on chickhen thigh surface

3.2.1 Change of total viable counts

Total viable counts on raw chicken thigh surface of all treatments decreased from 6.26-7.61 log CFU/ 25 $cm^2$to 5.76-6.92 log CFU/ 25 cm^2after dipping. The total viable counts on the chicken thigh samples dipped in aqueous solution of 2% clove and 2% thyme extracts decreased by 0.5-0.56 log CFU/ 25 cm^2. However, the number of total viable counts in all treatments of the chicken thigh samples increased by 0.6-1.41 log CFU/ 25 cm^2 after 7-day refrigerated storage, compared to the original number (Table 4).

3.2.2 Change of total *Pseudomonas* counts

Dipping of the chicken thigh samples in aqueous solution of 2% clove and 1% thyme extracts resulted in decrease of total *Pseudomonas* counts (0.03-0.82 log CFU/ 25 cm^2), but not for the control treatment. After 7-day refrigerated storage, total *Pseudomonas* counts on the chicken thigh samples dipped in aqueous solution of 1 and 2% clove extract significantly decreased by 0.19-0.53log CFU/ 25 cm^2, compared to the original number ($P<0.05$), but the total *Pseudomonas* counts on the chicken thigh surface of other treatments increased. The 2% clove extract dipping solution was the most effective to reduce the microbial load on the chicken thigh surface after 7-day refrigerated storage. At the end of storage, the chicken thigh samples dipped in aqueous solution of 1 and 2% clove extract had good appearance (no off odor and color), but not for the control sample which had color change from pink to green and had unacceptable odor (Table 4).

Table 4. Change of total viable counts and total *Pseudomonas* on raw chicken thigh surface dipped in different aqueous solutions of clove and thyme extracts during refrigerated storage

Storage time (days)	The number of microbial cells on chicken thigh surface (log CFU/25 cm^2)[x]				
	Distilled water (control)	1% clove extract	2% clove extract	1% thyme extract	2% thyme extract
Total viable counts					
Before dipping	7.61±0.1[a]	6.61±0.04[b]	6.41±0.08[cd]	6.54±0.06[bc]	6.26±0.05[d]
After dipping					
day0(4°C)	6.92±0.16[a]	6.52±0.05[b]	5.85±0.15[c]	6.36±0.11[b]	5.76±0.08[c]
day7(4°C)	7.52±0.07[a]	7.46±0.11[a]	7.38±0.1[ab]	7.27±0.06[bc]	7.17±0.05[c]
Total *Pseudomonas* counts					
Before dipping	6.26±0.02[a]	5.95±0.1[ab]	5.72±0.07[bc]	5.72±0.08[c]	5.45±0.12[c]
After dipping					
day0(4°C)	6.83±0.08[a]	6.27±0.11[b]	5.44±0.07[c]	5.69±0.05[bc]	5.63±0.03[bc]
day7(4°C)	7.00±0.08[a]	5.76±0.09[b]	5.19±0.09[c]	6.68±0.13[d]	6.43±0.14[e]

[x]Data are mean of three replications.
[a,b,c,d]Different letter in different column of the same row indicates significant difference ($P<0.05$).

This was probably because dipping the raw chicken thigh in aqueous solution of these natural antimicrobials helped to decontaminate the natural microflora. Jay *et al.* [1] stated that washing can reduce the number of microorganisms up to 5 log CFU. However, washing the poultry meat in water may not be effective enough. The addition of some synthetic chemicals or phytochemicals into washing water can help to remove microbe from raw meat surface more effectively. Sakhare *et al.* [6] reported that addition of lactic or acetic acids into washing solution could effectively reduce microbial load in fresh meat. In addition, decontamination by using washing solution supplemented with spice extract or some active compounds isolated from spice has been demonstrated to be more effective to reduce the number of microorganisms in fresh meat than using pure water alone [7, 8].

In the current study, total *Pseudomonas* counts in raw chicken thigh surface were analyzed. *Pseudomonas* spp., a Gram-negative psychrotrophic bacterium is commonly found in spoiled fresh meat stored at chilling temperature. The optimum, minimum and maximum temperatures for growth of psychrotrophic bacteria are 10-15°C, -5-5°C and 20-35°C, respectively [1]. Change of chicken thigh appearance during refrigerated storage was probably due to the growth of this bacterium. The reason that aqueous solution of 2% clove extract could effectively reduce the number of *Pseudomonas* may probably be due to the action of the main compound in clove such as eugenol [8].

3.2.3 Change of TBARS value in raw chicken meat

The original TBARS values of all chicken thigh meat samples were similar (0.56-0.58 mg malonaldehyde/kg). After 7 days of storage, the TBARS values of all chicken thigh meat samples decreased by 0.02-0.04 mg malonaldehyde/kg. The chicken thigh meat sample dipped in aqueous of 2% clove extract had lowest TBARS value (0.52 mg malonaldehyde/kg) (Table 5). This indicated that 2% clove extract in dipping solution may help to delay lipid oxidation in chicken thigh meat. This results are in agreement with the results reported by Naveena *et al.* [7]. They demonstrated that addition of clove oil in washing solution could delay lipid oxidation in raw buffalo meat.

Table 5. Change of thiobabituric acid reactive substances in chicken thigh meat dipped in different aqueous solutions of clove and thyme extracts during refrigerated storage

Treatments	Thiobabituric acid reactive substances (TBARS)±SD (mg MAD/kg)[x]	
	Day 0	Day 7
Distilled water (control)	0.58[a]±0.03	0.56[a]±0.02
1% clove extract	0.58[a]±0.01	0.56[a]±0.02
2% clove extract	0.56[a]±0.01	0.52[b]±0.01
1% thyme extract	0.56[a]±0.01	0.53[ab]±0.01
2% thyme extract	0.56[a]±0.01	0.53[ab]±0.01

[x]Data are mean of three replications.
[a,b] Different letters in different row of the same column indicate significant difference ($P<0.05$).

4. Conclusions

Clove and thyme extracts showed strong antibacterial activity against *P. fluorescens* as well as strong antioxidant activity. It is possible to add clove extract into washing solution to decontaminate raw chicken meat for extending the shelf life during refrigerated storage.

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