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RESEARCH PAPER

Antibacterial Activities of the Essential Oils of Some Aromatic Medicinal Plants to Control Pathogenic Bacteria and Extend the Shelf-Life of Seafood

Nabil Qaid M. Al-Hajj[1,3], Algabr MN[2], Husnain Raza[1], Riyadh Thabi[1], Al-Farga Ammar[1], Waleed Aboshora[1], Hongxin Wang[1,*]

[1] Jiangnan University, School of Food Science and Technology, Wuxi 214122, P.R. China.
[2] Hajjah University, Department of chemistry, Faculty of Applied Science, P.O. Box 80004, Yemen.
[3] Marine Science and Biological Research Authority, Aden, P.O. Box-1231, Aden, Yemen.

* Corresponding Author: Tel.: +8651085917795; Fax: +8651085876799 ;
E-mail: whx1960@126.com

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Abstract

The antibacterial activity of *Pulicaria inuloides* and *Pulicaria crispa* essential oils was tested against *Listeria monocytogenes, Escherichia coli* and *Staphylococcus aureus* using agar disc diffusion and microtiter broth microdilution assays. PIEO, the essential oil from *Pulicaria inuloides*, inhibited all tested microorganisms with a minimum inhibitory concentration (MIC) of 5.7, 0.12 and 5.11 mg/ml against *Listeria monocytogenes, Escherichia coli* and *Staphylococcus aureus* respectively. PCEO, the essential oil from *Pulicaria crispa*, showed a lower inhibition for all of the tested microorganisms. The present study evaluated the impact of PIEO on *L. monocytogenes, E. coli, S. aureus* and tap water (control) inoculated in fish fillets. Results indicated that PIEO eliminated most of *L. monocytogenes, E. coli, S. aureus* and SPC immediately after treatment at time zero (25°C) and during storage at 10°C for 12 days, and it increased the shelf -life of seafood during storage at 10°C compared with the tap water, whereas SPC counts were high than 7 log CFU/g at the end of storage.

The sensory evaluation of fillet fish treated with *Pulicaria inuloides* essential oil were acceptable by consumers at the level of 0.1 g/100g EO. Fish purchased from supermarkets in Wuxi province, Jiangsu, China, were maintained with good hygiene practices and health conditions, whereas fish purchased from local markets were not.

Keywords: Pulicaria inuloides, Pulicaria crispa, fillet fish, pathogenic bacteria, microbial loads.

Introduction

Fresh fish are among the most common processed meat products worldwide. These products have very high microbial load and water activity, which leads to a short shelf life (Georgantelis, Ambrosiadis, Katikou, Blekas, and Georgakis, 2007). Food-borne infections are among the most serious and costly public health concerns worldwide (Dadkhah *et al.,* 2012). The natural habitat of fish is extremely prone to pollution that may originate from domestic, industrial and agricultural discharges; therefore, fish and other aquatic life forms are vulnerable to many environmental hazards. The microbiological safety of seafood has been a source of concern to consumers, industries and regulatory agencies all over the world. *L. monocytogenes* can grow at temperatures ranging from 1 to 45°C, a pH range of 4.6 to 9.6, in high salt concentrations and can survive on the surfaces of food and seafood processing equipment by forming a biofilm (Castellano, Belfiore, Fadda and Vignolo, 2008). More recently, some studies have demonstrated the antimicrobial capacity of bay leaf essential oil to reduce the population of *Campylobacter jejuni* by 3.5 log CFU/g in fresh chicken after four days (Djenane, Yangüela, Gomez and Roncales, 2012) and control the growth of *Listeria monocytogenes* and *Escherichia coli* in ground chicken (Irkin and Esmer, 2010). Enterohaemorrhagic *Escherichia coli* O157:H7 is an exceptionally potent pathogen that causes food poisoning even at doses as low as 10 log CFU (Magiorakos *et al.,* 2012). This leads to severe abdominal pain and bloody stool and sometimes even haemolytic uremic syndrome or encephalopathy, which may occasionally lead to death. In Japan, a food poisoning outbreak occurred in April 2011 due to enterohaemorrhagic *E. coli* contamination at a grilled meat chain restaurant (Dadkhah et al., 2012). *Staphylococcus aureus* may produce enterotoxins and cause food poisoning or staph infections, such as cellulitis and osteomyelitis (Sears and McCarthy, 2003). In contrast to *E. coli* O157:H7 and *L. monocytogenes, S. aureus* food poisoning characteristically results from the ingestion of between 0.2 and 1.0 mg toxin present in contaminated food (Roberts *et al.,* 1979). To produce these quantities of toxins requires the growth of

Staphylococcus aureus in the food to densities of 10 /g or greater (Emswiler *et al.,* 1976). Staphylococcal food poisoning has been linked to the consumption of a variety of foods, including both dairy and meat products (Venkitanarayanan *et al.,* 1999). Amongst foods, seafood is the most perishable of all. It is harvested and processed under a wide array of circumstances, often in remote, under-equipped and unsanitary conditions. Therefore, juvenile kachlan fish and shrimp, whether wild-caught or cultured, is subjected to a wide range of safety hazards (Kanduri and Eckhardt, 2008). The genus Pulicaria, which belongs to the family Asteraceae (tribe Inuleae), consists of more than 77 species found throughout the world. Members of this genus contain various bioactive compounds such as monoterpenes, flavonoids, acetylenes, isocomene, and sesquiterpene lactones (Al-Hajj *et al.,* 2014). The leaves of *Pulicaria inuloides* and *Pulicaria crispa* are used to repel insects, to reduce influenza and common cold symptoms, and to treat back pain, intestinal disorders and inflammation. In addition, members of this genus have antibacterial and antispasmodic activities (Al-Hajj *et al.,* 2014). The use of natural preservatives in foods has been widely accepted by consumers, who increasingly seek natural and healthier products, free of synthetic additives (Sacchetti *et al.* 2005; Viuda-Martos *et al*., 2010a). In addition, consumers are accustomed to the presence of herbs and spices commonly added to provide flavour and aroma in meats. Therefore, essential oils (EOs) can be considered as a good choice of natural preservatives for fish, meat products (Militello *et al.,* 2011).

The objectives of the present study were to evaluate the in vitro effect of *Pulicaria inuloides* and *Pulicaria crispa* essential oils against *L. monocytogenes, E. coli* and *S. aureus*. In addition, the efficacy of essential oil of *P. inuloides* on the inhibition of *L. monocytogenes, E. coli* and *S. aureus* was tested by inoculating raw fillet fish during storage at 10°C for 12 days, in addition to collecting sensory evaluations of fish fillets treated with *Pulicaria inuloides* essential oil.

Materials and Methods

Plant Material and Essential Oil

The aerial parts of *P. inuloides* and *P. crispa* (leaves) were collected in August 2014 during the flowering stage in the Sana'a areas of Yemen. The plant specimens were air-dried and taxonomically identified by Prof. Abdellah Amine (College of Agriculture, Sana'a University). A voucher specimen (SAN BI. 222, BC. 223) of the plant material was deposited in the Department of Biology in the College of Agriculture at Sana'a University. The essential oils of *P. inuloides* and *P. crispa* were obtained by steam-distillation of the plants using a pilot-scale system (Khuzwayo, J. K. 2011) and (Ren *et al.,* 1989). Briefly, approximately 10 kg of fresh botanical material was loaded in the still. Steam was produced by a boiler and forced through the plant material to release the aromatic content. Steam containing the EOs was put through a cooling system for condensation. The EOs were spontaneously separated from the water solution and collected in a 50-mL Falcon tube containing 0.5 g anhydrous sodium sulphate (Na_2SO_4). The sample was vortexed for 30 s and centrifuged at 5000x g for 10 min at 5 °C. The EOs were then transferred to a screw-capped amber flask and stored at 10°C±1.

Preparation and Maintenance of Bacteria

Cultures of *Listeria monocytogenes* (ATCC7644), *Escherichia coli* (ATCC10536) and *Staphylococcus aureus* 25923 were purchased from the China General Microbiological Culture Collection Center (CGMCC) (Beijing, China). Cultures were activated with 10 ml Brain Heart Infusion Broth (BHIB) (Oxoide, United Kingdom), incubated overnight at 35°C, streaked on to Brain Heart Infusion Agar (BHIA) (Oxoid, Basingstoke, United Kingdom) plates and incubated for 24h at 35°C (Hsiao and Siebert, 1999). Working cultures were kept on BHIA slants at 5°C±1 and subcultured every two weeks. Inoculums were prepared by transferring (0.1 ml) a loopful of culture from the slants to10 ml BHIA and incubated for 18 h at 35°C. The optical density (OD) of the growth of cultures was measured at 625 nm (1 cm disposable curette) using non-inoculated broth as blank.

Antimicrobial Assay: Disc Diffusion Assay

The antimicrobial activity of the *P. inuloides* and *P. crispa* essential oils were tested against the *L. monocytogenes, E. coli* and *S. aureus* using the disc diffusion assay (Zhu et al., 2005) with some modifications. Mueller-Hinton agar (Merck, Germany) was inoculated with bacteria 4 log CFU/ml. A 6-mm paper filter disc loaded with 90mg/ml essential oils were dissolved in dimethylsulphoxide (DMSO) and diluted to the highest concentration 20-0.1 mg/ml for bacterial strains, was placed on the agar, and the oil was allowed to diffuse into the medium for 30 min at room temperature. The plates were then incubated at 37 °C for 24 h., and the diameter of the resulting zone of inhibition was measured in mm with a digital caliper (0.001 mm, Mitutoyo, Mizonokuchi, Japan). The zone of inhibition was recorded as the mean ± standard deviation (SD) of triplicate experiments. Ampicillin (10 µg) and gentamicin (10 µg) were used as reference antibiotics for bacteria. The total number of cells was reported as log CFU/ml (colony forming unites/ml).

Determination of Minimal Inhibitory Concentration (MIC)

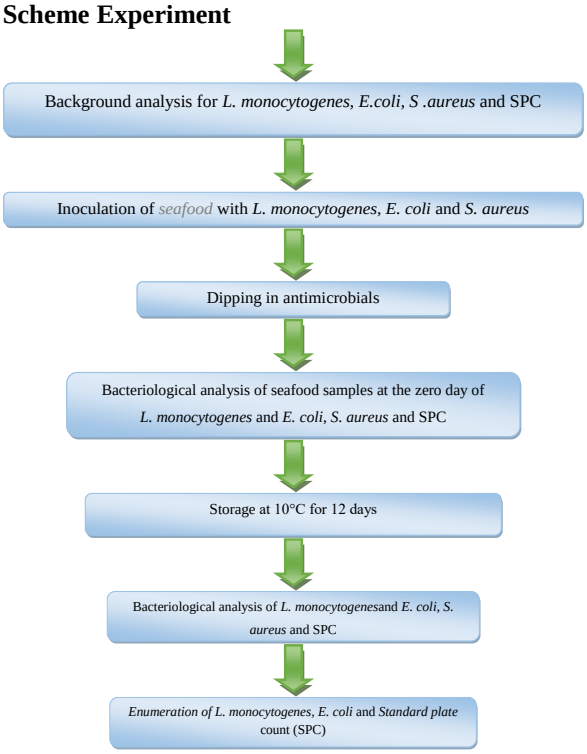
A broth microdilution method was used to determine the minimum inhibitory concentration (MIC) according to the National Committee for Clinical Laboratory Standards (NCCLS, 1999). The MIC is defined as the lowest concentration of the essential oil at which the microorganism does not demonstrate visible growth.

The MIC of the essential oils was determined using the microtiter broth micro dilution assay described by (Amsterdam, 1996). The essential oils were diluted to 90 mg/mL and subjected to a serial dilution (20 -0.1 mg/ml) in amicrotiter plate containing tryptic soy broth (TSB) for bacteria. The bacteria were suspended in the liquid culture medium at a final concentration of 4 log CFU/ml. After incubation at 37 °C for 24 h, the optical density was measured at 520 nm using a spectrophotometer.

Fish Preparation

This study was performed on beheaded fish fillets of juvenile kachlan (*Trachinotus ovatus*, Linnaeus) (6 kg), which are economically important in China. Juvenile kachlan were bought from supermarkets (three) and local markets in Wuxi province, Jiangsu, China in September 2014. The fish was cut into fillets under hygienic conditions. The standard methods of (AOAC, 2005), and the International Commission for the Microbiological Specifications of Foods (ICMSF, 2002) were adopted for preparation and microbiological analyses of seafood samples.

Scheme Experiment



Treatment Preparation: Fillet Fish

The vacuum-packed bags were opened according to (AOAC, 2005). Approximately six representative samples (25 g in duplicates) of chilled fish were separately homogenized in a wiring blender for 1 min with 225 ml of sterile peptone water (0.1%) under aseptic conditions. Serial dilutions of 1/10 ($1/10^2$ to $1/10^4$) were further prepared in peptone water. Samples were subjected to microbiological analysis after the treatment procedure (day0) and after storage for 12 days at 10 ±1°C.

Fish Inoculation and Treatment Application

Fresh chilled, peeled juvenile kachlan fillets were surface spread with 0.1 ml of diluted active cultures of *L. monocytogenes* (ATCC 7644), *E. coli* (ATCC 10536) and *S. aureus* (25923) with a sterile glass rod. Inoculations were done using 10^4 colony forming units/ml of either *L. monocytogenes* (ATCC 7644), *E. coli* (ATCC 10536), and *S. aureus* (25923). After inoculation, the fillets were divided into four groups of six fillets each, dipped in cold solutions of plant extraction plant 0.1, 0.2 and 0.3 g per 100 ml (w/g) water for 30 min to enable attachment (ratio of 1:2) After, the fillets were then drained to remove excess solution and kept in plastic bags, under vacuum and stored at 10±1°C for 12 days.

Enumeration of *Listeria monocytogenes*

The presence of *L. monocytogenes* and other *Listeria spp*. was confirmed according to (Alişarli *et al.*, 2005). Briefly, 1 ml of each dilution of prepared samples was streaked on Palcam Agar Base plates (Oxoid, U.K.) supplemented with Listeria Selective Supplement (PALCAM) (FD061). The plates were incubated at 37°C for 48 h and analysed for the presence of *Listeria* colonies. The colonies were examined by Gram staining. Black colonies were selected as presumptive *L. monocytogenes*.

Enumeration of *Escherichia coli*

Escherichia coli was enumerated on Eosin Methylene Blue Agar according to (Gonzalez-fandos *et al.*, 2005). One ml of each dilution of prepared samples was streaked on Eosin Methylene Blue Agar plats (Oxoid, U.K.).The plates were incubated at 37°C for 48 h and analysed for the presence of *Escherichia* colonies. The colonies were examined by Gram staining. Fluorescent green colonies were confirmed as *E. coli.*

Enumeration of *Staphylococcus aureus*

Staphylococcus aureus Tryptone Soya Agar with Sodium Chloride (TSAS) (Lancette *et al.*, 1986) was

used for preparing *S. aureus* cultures, with some modifications. One ml of each dilution of prepared samples was streaked on TSAS plates (Oxoid, U.K.).The plates were incubated at 37°C for 48 h and analysed for the of *Staphylococcus* colonies. Yellow colonies with a yellow halo were confirmed to be *S. aureus.*

Enumeration of Standard Plate Count (SPC)

Microbial loads SPC were determined by surface spreading homogenate dilutions (0.1 ml) on Plate Count Agar (Difco, USA) according to Gram *et al.* (1996). Inverted plates were incubated at 35-37°C for 24-48 h. Mean values of colony forming units (CFU) were calculated as the average of two dilutions and choosing the plates with (25 - 250) colonies.

Determination of Ph

The pH of the fish was measured at each sampling day using Delta 320 PH meter (Mettler-Toledo, Shanghai, China). Ten grams from each sample was homogenized with 100 ml of distilled water. The pH was measured during the storage period. Measurements were made in triplicate for each experiment (n=9).

Sensory Evaluation

Sensory evaluation was performed only on uninoculated fillet fish samples. Sensory evaluation of fillet fish treated with PIEO (0.1, 0.2 and 0.3 g/100 g of EO) was performed using anine-member trained panel. Panellists were recruited from among the students of Jiangnan University, China. Each experimental batch was prepared with 4 kg of fish batter and then cooked in boiling water for 20 min and served warm to panellists for sensory evaluation. The fish fillet was coded with its own three-digit random number in evaluation control. The panellists scored the sensory colour, odour and overall acceptability. A hedonic scale of 1 to 9 was used: 1: extremely bad, 2: very bad, 3: bad, 4: fairly bad, 5: satisfactory, 6: fairly good, 7: good, 8: very good and 9: excellent (Meilgaard, *et al.,* 2007). Sensory evaluation was accomplished at day 0 and at two-day intervals up to the end of the 12 days of storage at 10°C (Govaris *et al.,* 2010).

Statistical Analysis

One-way ANOVA (SPSS 17.0) was performed to analyse all treatment effects of plant extractions and the control. When the results were significant, the Duncan test was also used (Yin *et al.,* 2007). Sensory data were also analysed by analysis of variance in the general linear model (univariate) and one-sample t-test for separation of mean differences.

Results

Microbiological Composition of Samples

Data of the microbial composition of juvenile kachlan fish from supermarkets A, B and C compared with the local market in Wuxi province, Jiangsu, China, are shown in Figure 1. The Standard Plate Count (SPC) ranged between 1.6, 2 and less than 1 log $_{CFU}$/g, from supermarkets A, B and C; while the SPC was 4 log CFU/g in fish from the local market. *L. monocytogenes*, *E. coli* and *S. aureus* were less than 1 log CFU/g in juvenile kachlan fish from supermarkets while *E. coli* was detected in juvenile kachlan fish from local markets as shown in Figure 1. Depending on the source of fish, bacterial growth is primarily responsible for fish spoilage.

Antimicrobial Activity of *P. inuloides* and *P. crispa* Essential Oils

The antimicrobial activity of the *P. inuloides* and *P. crispa* essential oils was tested against *L. monocytogenes*, *E. coli* and *S. aureus* by the agar disc diffusion methods (Table1) and by the microtiter broth microdilution assay (Table 2). *P. inuloides* essential oil showed the greatest antimicrobial activity against tested bacteria, while *P. crispa* was a lower antimicrobial activity. Overall, the intensity of antimicrobial efficacy ranked as follows: *P. inuloides* >*P. crispa* essential oils.

Antimicrobial Activity in Fish Fillets

The antimicrobial effects of *P. inuloides* essentials oil at 0 (tap water), 0.1, 0.2 and 0.3 g/100g

Figure 1. Juvenile kachlan, *Trachinotus ovatus*- Linnaeus.

of EO on fish fillet samples during storage at the 10 °C±1for 12 days are shown in Table 3. At 10 °C, the initial population of *L. monocytogenes*, *E. coli*, and *S. aureus* in the control group increased during the storage period. Samples treated with *Pulicaria inuloides* essential oil PIEO at 0.2 and 0.3 g/100g resulted in that populations of *L. monocytogenes*, *E. coli*, and *S. aureus* were significantly lower than the control samples (P<0.05), during the storage period. However, samples treated with PIEO at 0.1 g/100g exhibited the lower population of *L. monocytogenes*, *E. coli*, and *S. aureus* until day 4, when compared with the untreated group (P<0.05). The addition of 0.2 and 0.3 g/100g PIEO resulted in decreased populations of *L. monocytogenes*, *E. coli*, and *S. aureus*, ranging below the acceptable level less than 2 log CFU/g from day 6 until the end of storage (Gudbjörnsdóttir *et al.*, 2004). Severity of the decrease of the initial *L. monocytogenes*, *E. coli* and *S. aureus* population in fish fillets at 1.2 g/100g was more than 1 g/100g *Pulicaria inuloides* oil.

The average value of initial microbial load SPC of juvenile kachlan samples (control group) was 2 log CFU/g. The SPC in PIEO-treated samples at 0.2 and 0.3 g/100g were significantly (P<0.05) increased to more than 7 log CFU/g from day 10 until the end of storage.

Results represent control (no *P. inuloides* essential oil added) and treatments of PIEO at 0.1, 0.2 and 0.3 g/100 g of EO.

Determination of pH

Fish treated with or without essential oil from *P. inuloides* had an initial pH of 6.1 and 6.2, respectively. These values increased during the storage period, reaching an average of 6.7 in the control and 6.4-6.7 in the treated samples after 12 days (Figure 3).

Sensory Evaluation

The results of the sensory evaluation (odor, taste and Overall acceptability) of fish fillet samples are presented in Figure 4a, b and c, respectively. PIEO at 0.1 g/100g concentration significantly influenced (P<0.05) all attributes evaluated. The overall acceptability and taste of fish fillets treated with PIEO at 0.2 and 0.3 g/100g was unacceptable. The overall acceptability of the control group after day 8 was significantly reduced below the rejection limit (P<0.05). Odor and taste scores for filleted fish samples stored at 10 ^{0}C for 12 days with essential oil at 0.2 and 0.3 g/100g showed a similar pattern of decreasing acceptability. On day 4 of storage fish fillets had a pleasant odor and taste, characteristic of fresh fish at 0.1 g/100g EO. However, preliminary sensory studies indicated that the application of PIEO at concentrations higher than 1 g/100 g would be unacceptable. For this reason, the concentrations of 0.1 g/100 g of PIEO were applied to the fish.

Discussion

The antibacterial activity of the *P. inuloides* and *P. crispa* essential oils was evaluated against *L. monocytogenes, E. coli* and *S. aureus*. Potency was assessed qualitatively and quantitatively by observing the presence or absence of inhibition zones and measuring zone diameters. Most published results demonstrate that the observed variation in antibacterial activities of extracts of plants is due to at least two factors: 1) the major components of the plant extract and 2) the type of bacterium tested (Rather *et al.*, 2012). The strong antibacterial activity of PIEO is possibly due to its major constituents, i.e.,

Table1. Antimicrobial activity of *Pulicaria inuloides* and *Pulicaria crispa* essential oils by agar disc diffusion assay

Test microorganism	*Pulicaria inuloides* (zone of inhibition, mm)	*Pulicaria crispa* (zone of inhibition, mm	Standard antibiotic[b] (zone of inhibition, mm)
Gram-positive bacteria			Ampicillin
L. monocytogenes	19.3±0.00	4.33±0.14	25.2 ± 0.18
S. aureus	24.00±0.34	4.27±0.12	26.0 ± 0.0
Gram-negative bacteria			Gentamicin
E. coli	7.1 ± 0.13	3.04±0.55	25.0 ± 0.9

[b] Standard antibiotics used as positive control and extract has antimicrobial activity against this microorganism**.**

Table 2. Minimal inhibitory concentration (MIC) of the essential oils of *Pulicaria inuloides* and *Pulicaria crispa* against pathogenic bacteria.

	MIC (mg/mL)	
Microorganism	*P.inuloides*	*P. crispa*
Listeria monocytogenes	5.7	2
Escherichia coli	0.12	≤0.5
Staphylococcus aureus	5.11	2

[a]Tests were performed in triplicate and modal values are presented

Table 3. The effect of *Pulicaria inuloides* essential oil on pathogenic bacteria and total viable counts (SPC) in fish fillets samples during storage at the 10 °C for 12 days

Microorganism [b]	Storage days	Tap water	PIEO (0.1g/100g) Log cfu/g	PIEO (0.2 g/100g)	PIEO (0.3 g/100g)
	0	4.60±0.00[a]	4.60±0.00[a]	4.60±0.00[a]	4.60±0.00[a]
	2	4.88±0.00[a]	4.11±0.00[b]	2.78±0.00[c]	2.29±0.00[d]
	4	5.90±0.00[a]	5.30±0.00[b]	2.45±0.00[c]	2.00±0.00[d]
	6	5.60±0.00[a]	5.25±0.21[b]	<2[c]	<2[c]
L. monocytogenes	8	5.88±0.01[a]	5.80±0.00[a]	<2[b]	<2[b]
	10	5.90±0.1a	4.90±0.03b	<2c	<2c
	12	5.95±0.01[a]	4.90±0.15[b]	<2[c]	<2[c]
	0	4.70±0.00[a]	4.70±0.00[a]	4.70±0.00[a]	4.70±0.00[a]
	2	4.94±0.00[a]	4.15±0.00[b]	3.85±.00[c]	3.82±0.00[c]
E. coli	4	5.90±0.2[a]	4.20±0.00[b]	4.18±0.00[b]	3.99±0.00[b]
	6	4.20±0.00[a]	3.97±0.01[a]	3.93±0.00[a]	3.90±0.00[a]
	8	5.80±0.01[a]	4.14±0.01[b]	3.98±0.02[c]	4.19±0.12[b]
	10	4.90±0.04a	4.17±0.00b	4.19±0.01b	4.14±0.01b
	12	4.98±0.11[a]	4.11±0.02[b]	4.15±0.03[b]	4.18±0.01[b]
	0	4.60±0.00[a]	4.60±0.00[a]	4.60±0.00[a]	4.60±0.00[a]
	2	4.88±0.00[a]	4.60±0.00[a]	2.87±0.00[b]	2.29±0.00[c]
S.aureus	4	5.90±.0.00[a]	5.30±0.01[a]	2.45±0.00[b]	2.00.0.00[c]
	6	4.25±0.21[a]	5.60±0.00[b]	<2[c]	<2[c]
	8	5.88±0.01[a]	5.80±0.00[b]	<2[c]	<2[c]
	10	4.90±0.03[a]	5.95±0.10[b]	<2[c]	<2[c]
	12	4.96±0.15[a]	5.00±0.01[a]	<2[b]	<2[b]
	0	2.32±0.04[a]	2.19±0.03[a]	2.11±0.07[a]	1.09±0.04[b]
	2	3.83±0.16[a]	3.28±0.00[b]	3.01±0.00[b]	2.21±0.00[c]
SPC	4	5.88±0.02[a]	5.31±0.01[b]	4.70±0.00[c]	4.11±0.00[d]
	6	6.62±0.02[a]	6.14±0.05[b]	6.11±0.00[b]	5.57±0.03[c]
	8	6.88±0.01[a]	6.34±0.02[b]	6.11±0.01[b]	6.07±0.00[b]
	10	7.78±0.02a	7.70±0.1a	7.40±0.00a	6.40.00b
	12	8.62±0.03[a]	7.62±0.03[b]	7.50±0.02[b]	7.30±0.00[b]

[a]Counts without common letters (A-C) within a row are significantly different (P<0.05).
[b]Each group of microorganisms was tested separately.

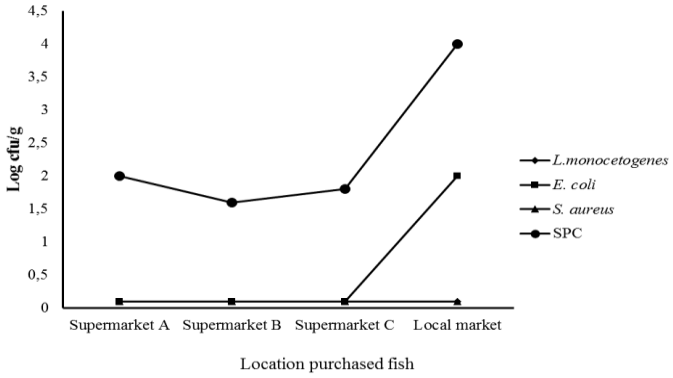


Figure 2**.** Means of *L. monocytogenes, E. coli, S. aureus* and SPC counts of juvenilekachlan from seafood consumed in Wuxi province, Jiangsu, China.

thymol and carvacrol. Thymol and carvacrol are structurally very similar. Both of these components increase bacterial cell permeability and membrane fluidity and inhibit medium acidification. Moreover, Thymol and carvacrol are thought to be inducing alterations in cell permeability by entering between the fatty acyl chains that make up the membrane lipid bilayers, thus disrupting lipid packing and causing changes to membrane properties and functions of gram-negative bacteria (Burt, 2004). In general, PIEO is more effective against gram-positive than gram-negative bacteria (Delaquis, Stanich *et al.,* 2002; Demetzos, Perdetzoglou and Tan, 2001). The essential oil of *P. crispa* exhibited lower antibacterial activity against all tested bacteria (Table1), which is consistent with a previous report (Chah *et al.,* 2006). That study reported that extracts derived from another Ocimum species, *O. gratissimum,* showed lower antibacterial activity against 11 tested bacterial strains including *S. aureus* (four strains), *E. coli* (two

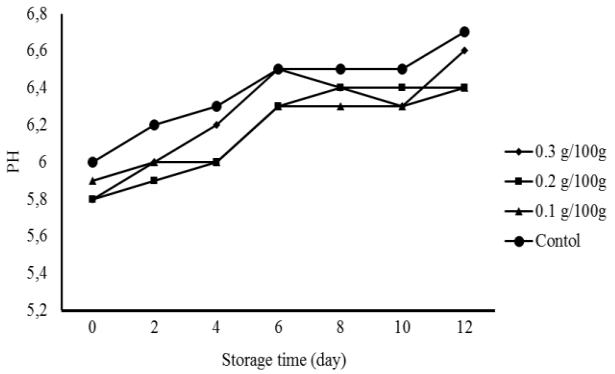


Figure 3. Changes in pH values of fresh fish stored at 10 ±1 °C. Results represent control (no *P. inuloides* essential oil added) and treatments of PIEO at 0.1 g/100 g and 0.2 g/100 g and 0.3 g/100 g.

strains), *Pseudomonas aeruginosa* (one strain), Proteus spp. (three strains) and Shigella (one strain). Similarly, the essential oils of *O. basilicum* were less effective against gram-positive and gram-negative bacteria tested in another study (Algabr *et al.*, 2010).

MIC values indicate that *P. inuloides* essential oil has greater inhibitory action against gram-positive bacteria than gram-negative bacteria. A previous study showed that the antimicrobial effects of spices and herbs against *E. coli*, and other gram-negative bacteria were due to their complex chemical composition, which included compounds such as thymol, carvacrol, methyl eugenol, linalool, α-pinene, 1,8-cineole, camphor (Nanasombat and Lohasupthawee, 2005). This discrepancy in antibacterial potential may be caused by variations in chemical composition, which may be influenced by the distillation and extraction techniques, as well as geographical origin. In our study, *P. crispa* essential oil was less active against all tested microorganisms. This result may have resulted from evaporation of the oil during the boiling process.

The SPC provides a good index of freshness quality as well as degree of contamination (Pedrosa-Menabrito and Regenstein, 1990). Grossly unhygienic fish handling and cleaning practices at the local retail markets caused significant recontamination in SPC counts, ranging from 2-7 log CFU/g on juvenile kachlan fish, whereas Standard Plate Counts (SPCs) in supermarkets were within the recommended microbiological limits is no more than 5 log CFU/g (ICMSF, 2002). *E. coli* counts exceeded the recommended microbiological limits of more than 2 log CFU/g in local markets in Wuxi, China, indicating poor hygiene practices. Fish with SPC counts exceeding 7 log CFU/g begin to spoil, showing notable sensory and microbiological changes (Kim and Mershall 1999). It was reported that such types in SPC could be due to variation of bacterial flora amongst fish in different seasons (De Silva *et al.*, 2004). The recommended microbiological limits of faecal coliform bacteria counts is no more than 2.6 log CFU/g; *E. coli* must not exceed 2 log CFU/g in fish and fish products (ICMSF, 2002).

Principle methods used to extend the shelf life of fish and fishery products involve good hygiene practice, health conditions, rapid freezing, in addition to the use of natural substance inhibition of *L. monocytogenes*, *E. coli* and other microbial loads (Al-Dagal and Bazarra, 1999).

The antibacterial activity of PIEO against *L. monocytogenes*, *E. coli* and *S. aureus* was previously determined through in vitro experiments (Desai *et al.*, 2012; Millezi *et al.*, 2012; Al-Hajj *et al.*, 2014). However, a literature review revealed that the activity of PIEO against *L. monocytogenes*, *E. coli* and *S. aureus* had not been investigated in fish fillet (Al-Hajj *et al.*, 2014). PIEO at 0.2 or 0.3 g/100g in fish fillets showed strong antimicrobial activity against bacteria tested during storage at 10°C. PIEO at 0.3 g/100g was more effective than 0.2 g/100 during storage against *L. monocytogenes*, *E. coli*, *S. aureus* and SPC. These observations are in agreement with the results of (Solomakos *et al.*, 2008) in minced beef at 0.6 g/100g and in low-fat cheese at 1% thyme oil, respectively. The antimicrobial efficacy of thyme oil at 1 g/100g in fish fillets is strong against the tested pathogen during the initial days of storage. Our findings were consistent with the results of these studies analysing *P. inuloides*. To our knowledge, no studies have evaluated the antimicrobial activity of essential oils and/or plant extracts from *P. inuloides* on fish. Therefore, to our knowledge, our study is the first to report on this topic, thus making a significant contribution to the scientific field. However, some research on PIEO has concluded that Gram positives are more susceptible and that it can be safe food additives are classified as "Generally Recognized as Safe" (GRAS) (AL-Hajj *et al.*, 2014).

Recommended microbiological limits of SPC for fish and fish products range between 6 to 7 log CFU/g (ICMSF, 2002). Fish with SPC counts exceeding 7 log CFU/g starts to spoil, showing notable sensory and microbiological changes (Kozempel *et al.*, 2001). It was reported that such types in SPC could be due to variation of bacterial flora amongst fish in different seasons (Davis, 1993).

Comparing the first and last day of storage, the

pH of control samples was significantly higher than those with *P. inuloides* PIEO addition. This pH increase might have resulted from microbial growth during the storage period. When the supply of simple carbohydrates is depleted, species such as Pseudomonas, as well as psychrotrophic gram-negative bacteria, such as Moraxella, Alcaligenes, Aeromonas and Serratia, use proteinaceous compounds as an energy source, producing ammonia, and thereby increasing the food's pH (Gabriel, 2008).

Sensory evaluation of fish fillet samples was performed with consideration of the most acceptable parameters including odor, taste and the overall acceptability was taken and used as a control (Figure 4a, b and c). According to our findings of sensory evaluation, the sensory properties of fish fillets treated with *P. inuloides* oil were acceptable by the panellists at the level of 0.1 g/100g but unacceptable at the levels of 0.2 and 0.3 g/100g . Similarly, (Solomakos *et al.*, 2008) reported that the addition of thyme oil at 0.6 g/100g was organoleptically acceptable. Other studies have also demonstrated the sensory viability of adding essential oils to meat products. Marjoram EO added to fresh sausages at 0.11 ml/100 g obtained the same acceptability as the product with no essential oil (Busatta *et al.*, 2008). The addition of oregano EO (0.01 ml/100 g) to chicken promoted desirable odor, according to a panel of trained evaluators (Chouliara *et al.*, 2007). Addition of oregano, rosemary and thyme essential oils at 0.02 ml/100 g in mortadella obtained similar or higher scores than the samples free of essential oil (Viuda-Martos, Ruiz-Navajas, Fernandez-Lopez, and Perez-Alvarez, 2009; Viuda-Martos, RuizNavajas, *et al.*, 2010a; Viuda-Martos, Ruiz-Navajas, Fernandez-L opez, and Perez - Alvarez, 2010b, 2010c).

Conclusions

These results suggest that *P. inuloides* can be used as an effective natural source of antimicrobial, an antioxidant and food additives. Additionally, it

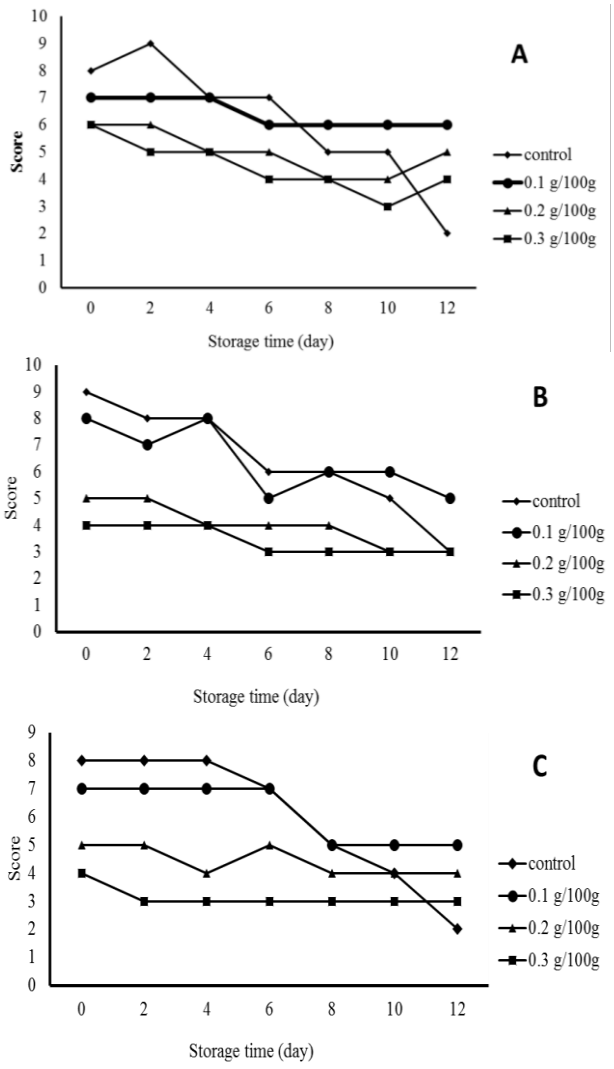


Figure 4. Sensory evaluation of fish fillets treated with PIEO at 0 (control), 0.1, 0.2 and 0.3 g/100g during storage at 10 °C for 12 days. (**A**) = odour, (**B**) = Taste and (**C**) = Overall acceptability.

could be a good candidate for phytochemical and pharmacological investigations to discover new broad spectrum bioactive compounds.

The treatment of fillet fish *P. inuloides* of essential oil at 0.1, 0.2 and 0.3 g/100g of EO showed higher activity against tested bacteria compared to the treatments of the microbial loads (SPC) and control (tap water). Our findings suggest that *P. inuloides* oil at 0.2 and 0.3 g/100g is an efficient means of controlling *L. monocytogenes*, *E. coli* and *S. aureus* in fillet fish meat. Furthermore, this oil reduced the inoculated pathogens by 2 log CFU/g up to the end of the storage period. The sensory properties of this treatment were acceptable to the panellists. Finally, the use of PIEO to wash or sanitize fish will not eliminate the pathogenic aerobic total count completely but will reduce the number of most harmful pathogens and microbial loads on fish. As a result, the fish quality and shelf-life will be increased. The use of PIEO to wash meat fish is generally recognized as safe (GRAS).

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