In vitro and in vivo activity of citronella oil for the control of spoilage bacteria of semi dried round scad (Decapterus maruadsi)

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Article History: Received 12th September 2011, Revised 25th October 2011, Accepted 26th October 2011.

Abstract: The inhibitory effect of citronella oil against major species of spoilage bacteria including Staphylococcus aureus, Klebsiella spp. and Pseudomonas spp. found on the surface of Decapterus maruadsi (semi-dried round scad) was investigated using the broth dilution method. Citronella oil and its main components d-limonene and linalool were introduced into a nutrient broth at volume concentrations (v/v) between 0.5% and 10% to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentrations (MBC) for the bacteria evaluated. The main components of citronella oil were determined by gas chromatography-mass spectrometry (GC–MS) analysis. The oil exhibited activity against all bacteria with a MIC of 1% v/v, while d-limonene showed activity against Pseudomonas spp. at 0.7% v/v and both Staphylococcus aureus and Klebsiella spp. at 0.9% v/v. No MBC was found in this study. Antimicrobial activity of the citronella oil at the concentration of 1% v/v was further examined on dried fish under storage conditions of 4°C and 30°C. Citronella oil was found to be able to extend the shelf life of the semi-dried fish for up to 7 days at 4°C. Both major constituents, d-limonene (86.0%) and linalool (3.2%), represented 89.2% of the citronella oil. These findings showed that the required shelf life of semi-dried fish could be achieved by manipulating the concentration of the citronella oil.

Keywords: Citronella oil; semi-dried fish; Staphylococcus aureus; Klebsiella spp.; Pseudomonas spp.

Introduction

In Thailand, the round scad are confined in the south. From 2005-2009, the amount of dried fish exported from Thailand to the international market was valued at ₱2,495.76-194.41 million (Fisheries foreign affairs division 2011). Fish are naturally dried in the sun by spreading them on mats or trays. Considerable losses can occur during sun drying due to various causes such as rodents, birds, insects and microorganisms (Patterson et al., 2003). Normally, the dried fish is susceptible to spoilage diseases caused by various species of bacteria such as Salmonella spp. (Corry 1976), Staphylococcus aureus (Vilhelmsson 1997), Klebsiella spp. and Pseudomonas spp. (Rodriguez-Jerez et al 1994). Furthermore, Klebsiella spp. and Pseudomonas spp. (V. da Silva et al 2002) are capable of producing histamine, which possess a risk to human health. Although chemical controls have been the main means to inhibit microorganism growth on fish products for many years, concerns over the uses of chemicals have been growing tremendously in many countries. Therefore, alternative methods to preserve dried fish should be explored. Application of harmless natural preservatives extracted from herbs or plants has been one of the most interesting research topics (Nielsen and Rios 2000; Soliman and Badeaa 2002; Arora and Kaur 1999).

Citronella oil has been widely studied as a possible natural mosquito repellent (Sakulku et
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Jaroenkit et al 2009) and as a volatile flavor compound (Baranauskienė et al. 2006). There have been a limited number of reports on some substances in citronella oil which inhibit the growth of bacteria. The main compound of citronella oil is d-limonene (Sakulku et al. 2009). Dambolena et al. (2008) described the underlining of d-limonene to inhibit the growth of mold and their toxin. In this study, the application of citronella oil in food products was looked at to investigate the potential for the preservation of semi-dried round scad.

Materials and Methods

Essential oil

The food-grade citronella oils derived by steam distillation were provided by the Thai China Flavors & Fragrances Industry Co (Thailand). Linalool and d-limonene, the major components of citronella oil, were purchased from Sigma-Aldrich Pte. Ltd. (Singapore).

GC-MS analysis

This analysis was carried out on a gas chromatograph (Hewlett-Packard Model 7890A, USA) equipped with a DB-5 column (J&W Scientific, USA) at dimensions of 30 m × 0.25 mm ID and 0.25 μm film thickness. The average helium carrier gas flow rate was 1ml/min; the split ratio of the column was 50:1 and the injector and detector temperatures were set at 250°C and 260°C, respectively. The column oven temperature was held at 60°C for 30 seconds, then programmed to 150°C at 40°C/min and then to 260°C at 2°C/min. Citronella oil (1.0 ml) was injected manually. The identification of the constituents was based on comparison of the retention times with those of authentic samples comparing their Kovats indices, and on computer matching with the NIST 08.L (database/chem-station data system).

Cultures

Three strains of bacteria (Staphylococcus aureus, Klebsiella spp. and Pseudomonas spp.) were identified from the semi-dried round scad. Codes refer to strains held in the culture collection of the Food Technology Laboratory at Walailak University.

Preparation of inoculum

Spores of test bacteria were grown and obtained from a nutrient agar medium (Merck Ltd, Thailand) at 35°C for 48 hours, and then were collected by flooding the surface of the plates with ~5 ml sterile saline solution (NaCl, 8.5 g l⁻¹ water) containing Tween 80 (0.1% v/v). After the spores were counted using a haemocytometer, the suspension was standardized to concentrations of 10⁷ spores ml⁻¹ by dilution with sterile water before use. The viability of all strains was checked using quantitative colony counts at 10⁷ CFU ml⁻¹.

Inhibition of bacteria (in vitro test)

Determination of MICs and MBCs of citronella oil and its main components were performed by the broth dilution method in test tubes. Fifty μl of citronella oil, d-limonene, and linalool at a concentration of 0.5% to 10% v/v was added to 5 ml of yeast extract sucrose broth tubes containing 10⁷ spores ml⁻¹. The vegetable oil was used as a control. Different dilutions of the oils (including controls) were made with methanol. The preliminary work revealed that methanol had no effect on mold growth. The tubes were then incubated at 25°C for 3 days on an incubator shaker (Gallenkamp, Loughborough, England) to evenly disperse the oil throughout the broth. The lowest concentration that showed no visible growth was regarded as the MIC. Cells from the tubes showing no growth were subcultured on malt extract agar plates to determine if the inhibition was reversible or permanent. The MBC was determined as the lowest concentration at which no growth occurred on the plates.

Storage of the dried fish (in vivo test)

The round scad caught in the gulf of Thailand, were obtained from the Tha Sala district of Nakhon Si Thammarat. The average weight of these fish was 100±10 g or 10-12 pieces/kg.

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Samples were frozen in a vessel and transported to the laboratory. After thawing, the fish samples (25±5 g) were then placed individually in sterile glass bottles containing 225 ml of sterile deionized water with vegetable oil (control) or at 0.5%, 1% and 2% v/v citronella oil water. The solution of the citronella oil water was amended with 1.2 ml of Teepol® solution (Sherwood Chemicals PCL, Thailand), which was a soapy detergent used as a surfactant (Sivakumar et al. 2002). Bottles were placed in an incubator and shaken gently (100 rpm) at a room temperature of 30±2 °C for 10 minutes. The fish were then dried at 425 watts in a controllable microwave (developed at plasma technology for agricultural application laboratory, Walailak University, Thailand) for 3 hours. A 25 g piece of dried fish was placed inside a polypropylene bag and was kept at 4 °C and 30 °C for 7 days. At 0, 3, 5 and 7 days, the viable cells in treatments were assayed through serially diluting in 9 ml of sterile 0.1% peptone water and then directly plating 0.1 ml of each dilution in duplicate on plate count agar (Merck Ltd, Thailand).

**Results and Discussions**

**Chemical composition of the citronella oil**

The GC-MS analysis of the citronella oil to the identification and quantification of d-limonene and linalool accounted for 89.2% of the total oil (Table 1). D-limonene was found at 21.940 minutes and was selected as a marker due to a high content at 86.0% of the total peak area. Linalool was also found at 3.2% for this test. Sakulku et al. (2009) reported that citronella oil was constituted by d-limonene at 40.48% of its total peak. In our test, the content of d-limonene was found to be much higher than that whereas the amount of linalool (3.9%) was nearly at the 4.0% reported by Baranauskiene et al. (2006). As mentioned by Sakulku et al. (2009) and Beneti et al. (2011), one of the main compositions of citronella oil was citronellal, which was at about 40% of the total peak. However, the citronellal was not found in this test. Compositional differences may be explained in terms of genetic variability, geographic localization, harvest time and climate conditions.

**Table 1: Chemical composition of citronella oil**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Compound</th>
<th>Composition (%)</th>
<th>Retention time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>d-Limonene</td>
<td>86.0</td>
<td>21.940</td>
</tr>
<tr>
<td>2</td>
<td>Linalool</td>
<td>3.2</td>
<td>40.207</td>
</tr>
</tbody>
</table>

**Inhibition of bacteria**

The inhibitory effects of citronella oil and its main components on *Staphylococcus aureus*, *Klebsiella* spp. and *Pseudomonas* spp., the major spoilage bacteria found on the surface of semi-dried round scad, are shown in Table 2. It is evident that citronella oil, d-limonene and linalool were bacteriostatic at 1%, 0.9% and greater than 10% v/v respectively. No bactericidal activities against the test bacteria were observed within a range of concentration examined.

**Table 2: MICs and MFCs of citronella oil, d-limonene, and linalool against Staphylococcus aureus, Klebsiella spp. and Pseudomonas spp.**

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Citronella oil</th>
<th>d-Limonene</th>
<th>Linalool</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (%) (v/v)</td>
<td>MBC (%) (v/v)</td>
<td>MIC (%) (v/v)</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>1.0% &gt;10.0%</td>
<td>0.9% &gt;10.0%</td>
<td>9.0% &gt;10.0%</td>
</tr>
<tr>
<td><em>Klebsiella</em></td>
<td>1.0% &gt;10.0%</td>
<td>0.9% &gt;10.0%</td>
<td>&gt;10.0% &gt;10.0%</td>
</tr>
<tr>
<td><em>Pseudomonas</em></td>
<td>1.0% &gt;10.0%</td>
<td>0.7% &gt;10.0%</td>
<td>&gt;10.0% &gt;10.0%</td>
</tr>
</tbody>
</table>

The effectiveness of citronella oil was also reported by Lertsatitthanakorn et al. (2006) and Moraes et al. (2009). It was expected to arise mainly from its main component, d-limonene. The d-limonene (0.047%) studied had a significant anti- *Listeria monocytogenes* (Moureay and
Canillac 2002). Comparison of the MICs obtained with the studied citronella oil on Staphylococcus aureus, Klebsiella spp. and Pseudomonas spp. and the MICs obtained with the d-limonene (citronella oil MIC = 1.0% v/v; d-limonene MIC = 0.9% v/v) showed that d-limonene components were more bacteriostatic than the citronella oil. Therefore, d-limonene was the most effective compound against bacteria in this test (because the citronella oil contained 86% limonene or d-limonene). Therefore, it was likely that Bagamboula (2004) linalool components had a limited effect on the antimicrobial activity; it was more than 10% in this test. The synergistic effects among various minor constituents of essential oils containing d-limonene as the main component were also indicated by Mário et al. (2009) which could, to a certain extent, contribute to the bacteria inhibition of citronella oil. This, however, warrants further study.

Storage of dried fish

Growth of bacteria on dried fish at temperatures of 4°C and 30°C are shown in Figures 1 and 2. According to the Thai Industrial Standard for dried fish, the total bacteria on the dried fish surface must be ≤10^5. Citronella oil at concentrations of 1% and 2% v/v at a storage temperature of 4°C was effective enough to preserve dried fish from bacteria for up to 7 days. The dried fish preserved under other conditions examined at 30°C, including the controls, were found to be spoiled within 3 days.

![Figure 1: The total bacteria on the semi-dried round scad stored at 4°C with 0.5%, 1% and 2% v/v of citronella oil.](image-url)

The results of the in vivo study are in agreement with in vitro findings. This study also evaluated the potential application of citronella oil on semi-dried round scad as a food model. Comparing the in vitro test with the in vivo test, normally it has been reported that higher levels of essential oil are usually necessary to inhibit microbial growth in foods compared to culture media (Bagamboula et al. 2004), but these results showed didn’t agree.
Conclusions

In this study, citronella oil showed antibacterial activities against pathogenic bacteria. It would be interesting to study the effect of citronella oil against other important bacteria and fungi for developing new antimicrobial agents to control serious bacteria diseases in semi-dried fish. Thus, it can be concluded that the use of citronella oil could be an alternative to the synthetic inhibitors used by the dried fish industry. It is suggested that the concentration of citronella oil at 1% v/v in this work could be employed to extend the shelf life of dried fish products for up to 7 days at 4°C.

Acknowledgements

This study was supported by the Walailak University Fund and the Thailand Center of Excellence in Physics through the Plasma Agricultural Application Laboratory of Walailak University.

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Figure 2: The total bacteria on the semi-dried round scad stored at 30°C with 0.5%, 1% and 2% of citronella oil.


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