Food Microbiology 72 (2018) 176–184

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Antimicrobial efficacy of *Syzygium antisepticum* plant extract against *Staphylococcus aureus* and methicillin-resistant *S. aureus* and its application potential with cooked chicken

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ARTICLE INFO

Article history:
Available online 5 December 2017

Keywords:
Staphylococcus aureus
MRSA
Syzygium antisepticum
Cooked-chicken
Membrane damage

ABSTRACT

For the past decades, there has been a growing demand for natural antimicrobials in the food industry. Plant extracts have attracted strong research interests due to their wide-spectrum antimicrobial activities, but only a limited number have been investigated thoroughly. The present study aimed at identifying a novel anti-staphylococcal plant extract, to validate its activity in a food model, and to investigate on its composition and antimicrobial mechanism. Four plant extracts were evaluated against *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) *in vitro*, with *Syzygium antisepticum* leaf extract showing the strongest antimicrobial activity (MIC = 0.125 mg/mL). Relatively high total phenolic content (276.3 mg GAE/g extract) and antioxidant activities (90.2–138.0 mg TE/g extract) were measured in *S. antisepticum* extract. Food validation study revealed that higher extract concentration (32 mg/mL) was able to inhibit or reduce staphylococcal growth in cooked chicken, but caused color change on meat surface. By GC-MS, β-caryophyllene (12.76 area%) was identified as the dominant volatile compound in extract. Both crude extract and pure β-caryophyllene induced membrane damages in *S. aureus*. These results suggested good anti-staphylococcal properties of *S. antisepticum* plant extract, identified its major volatile composition and its membrane-damaging antimicrobial mechanism.

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1. Introduction

Food safety remains as a major global concern facing the food industry nowadays. The estimated global burden of foodborne illnesses and their associated social and economic impacts remained unacceptably high. It was estimated that, annually, 9.4 million illnesses, 56,000 hospitalization cases and 1300 deaths resulted from foodborne diseases (WHO, 2016). Among the reported foodborne pathogens, *Staphylococcus aureus* is a significant source of foodborne diseases, causing approximately 241,000 illnesses, and an estimated annual cost-of-illness of $167 million in the United States (US) (Scharff, 2012). The European Food Safety Authority (EFSA, 2015) reported that infections associated with staphylococcal toxins increased substantially from 2008 to 2013 in 32 European countries. Similarly, foodborne disease cases were observed to increase in Singapore since 2006, with *S. aureus* being the most often isolated pathogen from food samples (MOH, 2015). Foods associated with staphylococcal food poisoning differ widely among countries, with poultry, red meat, egg, seafood and dairy products being the most commonly reported (Le Loir et al., 2003).

Many attempts have been made to enhance food processing hygiene and preservation efficacy. However, cases such as incomplete microbial inactivation and post-processing re-contamination were frequently reported (Negi, 2012). Meanwhile, consumers have shown increasing unease towards the use of synthetic chemicals in foods, due to concerns on their potential toxicity and side effect (Gyawali and Ibrahim, 2014). As a result, there has been a constant search for efficient natural antimicrobials for food application.

Plant essential oils (EOs) and extracts have received considerable research attention as a potential source of natural antimicrobial substances. For centuries, plants have been used as spices, food preservatives, and/or for disease treatment, and a large number of them have historical record of safe use in humans (Akhtar and

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https://doi.org/10.1016/j.fm.2017.12.002
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Mirza, 2015). It is well-known that plant extracts are valuable sources of phytochemicals, and studies have demonstrated wide-spectrum antimicrobial activities of some plant extracts against food spoilage and pathogenic microorganisms, including *Escherichia coli* O157:H7 (Al-Reza et al., 2010), *Salmonella* Typhimurium (Tornuk et al., 2011) and *S. aureus* (Mehrotra and Srivastava, 2010). A number of plant-based food antimicrobial products, such as DMC Base Natura (Alhendin, Granada, Spain), BioVia™ YM10 (Dupont, Denmark) and Citrox® (Citrox, Auckland), have been commercialized in response to market demand.

Despite intensive research activities carried out in the past decades, it was estimated that less than 10% of the known plant species in the world has been studied for antimicrobial activities, and data is lacking regarding their compositions and detailed antimicrobial mechanisms (Negi, 2012). Furthermore, most studies performed to date were conducted in laboratory media, the results of which were known to differ drastically from those tested in food systems (Gyawali and Ibrahim, 2014). Hence, to evaluate the food application potential of plant extracts, it is crucial to validate their antimicrobial efficacies in real food matrix. The present study was performed to explore antimicrobial activities of novel plant extracts, to validate its efficacy in cooked chicken, and to investigate its antimicrobial mechanism.

2. Material and methods

2.1. Preparation of plant extract

Freeze-dried powders of plants *Atuna racemosa* leaf, *Xanthostemon verticillatus* leaf, *Syzygium antisepticum* leaf and stem were kindly provided by Dr. Huang Dejian from the National University of Singapore. Extraction was performed according to Othman et al. (2011), with some modifications. Briefly, plant powders were extracted in acetone:methanol:water (AMW, 2:2:1) for 24 h at room temperature. The extractant was separated using Whatman Grade 1 filter paper (Sigma-Aldrich, St. Louise, MO, USA), and solids were re-extracted with fresh solvent for another 24 h. Dried extracts were obtained by evaporating solvents at 40 °C using rotary evaporator (Büchi, Flawil, Switzerland), followed with vacuum dryer (Shellab, Cornelius, OR, USA). Stock solutions of dried extract were prepared using dimethyl sulfoxide (DMSO; QReC, Singapore), and stored at −18 °C in the dark until use.

2.2. Bacterial strain and culture conditions

S. aureus (ATCC 6538) and methicillin-resistant *S. aureus* (MRSA) (ATCC 33591) were purchased from the American Type Culture Collection (Manassas, VA, USA). Microbial stock cultures were stored in cryoinstant vials with porous beads (DeltaLab, Barcelona, Spain) at −80 °C. Frozen stock cultures were activated by two consecutive transfer (24 h, 37 °C) in sterile tryptic soya broth (TSB) (Oxoid, Hampshire, England). Microbial cultures were centrifuged (5000×*g*, 4 °C, 5 min), washed twice with 1x phosphate buffered saline (PBS) (Vivantis Inc., Oceanside, CA, USA), and diluted to appropriate concentration before use.

2.3. Determination of MIC and MBC

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of plant extracts were determined using the Clinical and Laboratory Standards Institute (CLSI) microdilution method (Othman et al., 2011). Briefly, using a 96-well plate, plant extract was two-fold serially diluted in TSB, and 100 μL of the diluted extract was mixed with 100 μL microbial culture, to achieve final extract concentrations of 0.02–2 mg/mL, and a final inoculum level of 10^5 colony forming unit (CFU)/mL. Tetracycline (128–0.06 μg/mL) was included as positive control. The highest DMSO concentration was 4%, which has been shown to have no influence on microbial growth (data not shown). The 96-well plate was sealed and incubated at 37 °C for 24 h in a Multiskan FC microplate photometer (ThermoFisher Scientific, Waltham, Massachusetts, USA). Aliquot from wells without visible microbial growth was spread plated onto tryptic soya agar (TSA; Oxoid), and incubated at 37 °C for 24 h to determine the number of surviving cells. MIC was defined as "the minimum concentration that inhibited microbial growth, as determined by plating", while MBC was defined as "the minimum concentration that resulted in ≥3-log CFU/mL reduction of the inoculum" in this study.

2.4. Phytochemical analysis

2.4.1. Total phenolic content

Total phenolic content (TPC) of plant extract was measured using the Folin-Ciocalteu (FC) method (Sui et al., 2015). A 40 μL of plant extract, diluted in DMSO, 3.16 mL deionized water and 200 μL FC phenol reagent were mixed and incubated in the dark for 5 min. The mixture was added with 600 μL sodium carbonate solution (0.7 M), and further incubated in the dark at room temperature for 2 h. TPC was determined by measuring absorbance of the mixture at 765 nm, using a UV-VIS spectrophotometer (UV-1650PC; Shimadzu, Kyoto, Japan). Absorbance values were compared against a gallic acid calibration curve, and final results were expressed as milligram gallic acid equivalent per gram of dry extract (mg GAE/g DE). Appropriate blank (deionized water) and solvent control (DMSO + reagents) were included for each trial.

2.4.2. DPPH antioxidant assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) antioxidant capacity of the plant extract was measured as previously reported (Sui et al., 2015). Briefly, 0.1 mL plant extract, diluted in DMSO, was added into 3.9 mL DPPH (Sigma-Aldrich) stock solution (60 μM, in methanol), and incubated in the dark for 2 h at room temperature. As DPPH radicals have an absorbance maximal at 515 nm, which decrease after reduction by antioxidant compounds, the incubated mixture was measured at 515 nm using a UV-VIS spectrophotometer, and its absorbance value was compared against a Trolox calibration curve. Final sample antioxidant capacity results were expressed as milligram Trolox equivalent per gram of dry extract (mg TE/g DE). Appropriate blank (methanol) and solvent control (DMSO + DPPH) were included in each trial.

2.4.3. ABTS antioxidant assay

ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate)) antioxidant capacity of the plant extract was determined as reported (Re et al., 1999). Briefly, ABTS radicals ($ABTS^{\bullet+}$) were generated by reacting ABTS (Sigma-Aldrich) stock solution (7 mM, in deionized water) with potassium persulfate (2.45 mM) in the dark for 16 h at room temperature. Before use, $ABTS^{\bullet+}$ solution was diluted in ethanol to an absorbance of 0.7 ± 0.02 at 734 nm, and 1 mL of diluted $ABTS^{\bullet+}$solution was incubated with 10 μL plant extract for in the dark for 10 min at room temperature. As $ABTS^{\bullet+}$ radicals have an absorbance maximal at 734 nm, which decrease upon reduction by antioxidant compounds, the incubated mixture was measured at 734 nm, and its absorbance value was compared against a Trolox calibration curve. Final sample antioxidant capacity results were expressed as mg TE/g DE. Appropriate blank (ethanol) and solvent control (DMSO + $ABTS^{\bullet+}$) were included in each trial.

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