Chemical Composition and Antibacterial Activity of Kaempferia galanga Essential Oil

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Abstract

Kaempferia galanga L. is a traditional herbal medicine with high essential oil content. Gas chromatography-mass spectrometry (GC-MS) detected 25 different compounds in K. galanga essential oil, representing 95.98% of the total oil. The antibacterial activity of the essential oil was measured by the microtiter broth dilution method, and minimum inhibitory concentrations (MICs) against the three pathogenic bacterial strains Escherichia coli, Salmonella typhimurium and Staphylococcus aureus were 5, 1, and 2 mg/mL, respectively. The antibacterial mechanisms of K. galanga essential oil were further explored, and massive nucleotide and protein leakage were confirmed that it caused membrane disruption in the pathogens. SDS-PAGE confirmed the disruptive action of the essential oil on the cytoplasmic membrane. Moreover, the essential oil-induced cell wall and membrane damage was observed by scanning electron microscopy. These findings indicated K. galanga essential oil might constitute a candidate for developing new food preservatives and pharmaceuticals. © 2018 Friends Science Publishers

Keywords: Antibacterial activity; Essential oil; GC/MS; Kaempferia galanga L.; SDS-PAGE; SEM

Introduction

Kaempferia galanga L. (Zingiberaceae) is a perennial aromatic plant employed as traditional herbal medicine. It is mostly found in tropical and subtropical areas of Asia such as China, Indonesia, Malaysia and India (Holltum, 1950). K. galanga has demonstrated wide spectrum biopharmacological attributes, e.g., larvicidal (Choochote et al., 1999; Satoto et al., 2011), nematicidal (Choi et al., 2006), antibacterial (George and Pandalai, 1949), vasorelaxant (Othman et al., 2006), antinoceptive (Sulaiman et al., 2008), anti-inflammatory (Umar et al., 2014) and antineoplastic properties (Liu et al., 2010). In addition to its medicinal uses, K. galanga is also served as non-staple food and condiment. In cooking, its rhizome is used as a seasoning with meat products to give food a distinctive odor. The K. galanga is rich in essential oil, which is widely used in perfume, cosmetics, flavor and food industries in China.

In recent years, a plethora of essential oils have been isolated from multiple organs of various aromatic plants by hydro-distillation or supercritical CO₂ fluid extraction (Meng et al., 2016). Nowadays, an increasing amount of attention has been cast on essential oils and their various components, due to their various biological activities used in healthcare, food, or medicine, especially antioxidant, antimicrobial (Ud-Daula et al., 2016), antifungal (Mehparvar et al., 2016), anxiolytic (Zhang et al., 2016a), antimycotoxicogenic (Rasooli et al., 2008), and antitumor activities (Gong et al., 2016). Essential oils also display antispasmodic (Blanco et al., 2013) and antiseptic effects, and constitute efficient food preservatives (Yuan et al., 2016). Synthetic chemical preservatives, including sodium benzoate and nitrate, used in food processing and storage, are toxic to the lung and enhance liver tumor incidence (Nair, 2000). Meanwhile, multiple infectious diseases have become hard to treat due to antibiotic resistance that is rising at a terrifying rate. Hence, to fight against the increasing antibiotic resistance and suit the growing demand for food security, the development of natural broad spectrum antimicrobial preservatives has gained considerable interest.

This report analyzed the chemical composition and antibacterial activities of K. galanga essential oil against multiple bacterial organisms. In addition, the antibacterial mechanism was evaluated by scanning electron microscope (SEM), cell membrane integrity assessment and SDS-PAGE. Our results provide a reference for the development and application of K. galanga essential oil.
Materials and Methods

Plant Material and Bacterial Strains

*Melaleuca leucadendron* rhizomes were purchased from Anguo Medicine Company of Hebei province, China in 2014, and identified by senior engineer Tian Shangyi. *Escherichia coli* (ATCC35218), *Salmonella typhimurium* (ATCC13311) and *Staphylococcus aureus* (ATCC25923) were obtained from School of Life Sciences, Northeast Normal University, China.

Isolation of the Essential Oil

The rhizomes of *K. galanga* were air dried and submitted to 6 h of hydro-distillation, using a Clevenger-type apparatus on the basis of the normative procedure (Clevenger, 1928). The obtained essential oil was dehumidified with anhydrous Na2SO4, and stored in sealed brown vials under cold storage conditions.

Gas Chromatography and Mass Spectrometry Analysis

The components of *K. galanga* essential oil was assessed with gas chromatography–mass spectrometry (GC-MS), performed on an Agilent 6890 GC-5973 MSD system coupled to a DB-5MS (30 m × 0.25 mm × 0.25 μm) capillary column. Helium was employed as the carrier gas and was delivered at a flow rate of 1.0 mL/min. The initial temperature of GC oven was maintained 50°C for 5 min, afterwards was increased to 230°C at 6°C/min, and then held for 10 min at 230°C before increasing to 280°C at 10°C/min. The temperature of the injector was set to 250°C. The split mode was used at a ratio of 3:1. Mass spectra were obtained in the electron impact mode at 70 eV with mass ranging from m/z 29 to 500. Linear retention index (RI) values were determined using a homologous series of n-alkanes (C3-C30) under the same temperature-programmed conditions. The compositions were identified by comparison of their RI and mass spectra values to those in the NIST 21, NIST107 and PAW-TOX2 libraries, and reviewing the literature. Compound percentages in the essential oil were computed as GC peak areas to the total oil peak area (Adams, 2007; Cannon et al., 2015).

Evaluation of Minimum Inhibitory Concentration for Bacteria

Minimum inhibitory concentrations (MICs) were assessed by the microtiter broth dilution method (Andrews, 2001) with slight modifications. Briefly, after two consecutive transfers, test strain inocula were obtained in the mid-logarithmic phase by centrifugation, and resuspended at 1×10^5 CFU/mL in fresh Luria-Bertani (LB) culture medium. *K. galanga* essential oil was dissolved in 2.0% (v/v) Tween-80 (pH 7.2) to achieve concentrations of 0.1–10 mg/mL. Aliquots (100 μL) of twofold serial dilutions of *K. galanga* essential oil in 1% peptone were mixed with 100 μL of bacterial cultures in 96-well plates, and cultured overnight in incubator. Controls were prepared without essential oil but contained 2.0% Tween-80. Bacterial growth inhibition was evaluated by optical density measurements at 600 nm on a microplate reader (Thermo, Shanghai, China). MIC was indicated as the lowest essential oil concentration that completely inhibited bacterial growth.

Cell Morphology Observation with SEM

SEM was carried out to evaluate the effects of *K. galanga* essential oil on the morphology and surface structure of the bacterial test cells. SEM imaging of samples was accomplished according to a previously established method (Rasool et al., 2016). The bacterial suspension after one night of incubation in LB at 37°C, added the essential oil at the final concentration of 2 × MIC, controls were prepared without essential oil. Then the suspensions were incubated at 37°C for 18 h. The bacterial samples were centrifuged at 6,000rpm for 5 min, and washed gently with 0.1M phosphate buffer solution. Afterwards, the cells were submitted to fixation with 2.5% glutaraldehyde overnight at 4°C, followed by dehydration in graded ethanol series (30, 50, 80, 95 and 100%). For preparation for scanning electron microscopy, samples were allowed to dry completely at room temperature and were then coated with gold by sputtering (5 nm).

Nucleic Acid and Protein Leakage

Bacterial cell membrane integrity was checked by assessing the release of intracellular components. The leakage of nucleic acids and proteins into the supernatant was ascertained according to a previously reported method (Bradford, 1976; Chen and Cooper, 2002; Zhao et al., 2015) with slight modifications. Logarithmic phase *E. coli, S. typhimurium* and *S. aureus* were obtained by centrifugation (6,000 rpm; 5 min) and resuspended in sterile PBS (pH7.4) to keep them at a final density of 10^7 CFU/mL. Then, the bacterial suspensions were exposed to essential oil at concentrations of 2 × MIC at 37°C. Control cells treated with 2% Tween-80 vehicle were tested under the same conditions. Samples were taken out after being treated for 1, 2, and 6 h, and then centrifuged at 6,000 rpm for 5 min. Afterward, 200 μL of supernatant liquid was immediately added to a sterile 96-well microplate, and determined at 260 nm and 280 nm, respectively using the microplate reader.

SDS-PAGE of Whole-cell Proteins

Logarithmic phase *E. coli, S. typhimurium* and *S. aureus* were collected as described above and resuspended in sterile PBS to achieve a final density of 10^7 CFU/mL. Then, the bacterial suspensions were exposed to *K. galanga* essential
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oil at a concentration of 2 × MIC for 24 h, followed by centrifugation (6000 rpm, 5 min). The resulting cell pellets were rinsed and re-suspended in PBS. Control samples were logarithmic phase bacterial cells. Before SDS-PAGE analysis, bacterial cells were disrupted by homogenization in liquid nitrogen, followed by the addition of protein lysis solution. Protein amounts in lysates were assessed by the BCA technique. Equal quantities of protein were resolved by SDS-PAGE according to Laemmli (1970), with 3% and 12% stacking and separating gels, respectively. The gels were subjected to staining with 0.1% (w/v) Coomassie Brilliant Blue R-250, and destained in 10% (v/v) acetic acid and 40% (v/v) ethanol for visualization.

Statistical Analysis
All experiments were replicated at least three times, and results were showed as means ± standard error. Microsoft Excel 2007 and Origin were used for data analysis. Assessments were analyzed at a significance level of p<0.05.

Results

Chemical Composition of K. galanga Essential Oil

The GC/MS analysis of K. galanga essential oil is presented in Table 1, and a total number of 25 components were identified (95.98% of total essential oil), among which the master compounds found in the essential oil were ethyl-p-methoxycinnamate (75.83%) and ethylcinnamate (17.48%).

Antimicrobial Activity

K. galanga essential oil showed a variable degree of antibacterial activity against different tested strains. The MIC values of the essential oil showed variable antimicrobial activity against three pathogenic strains (Table 2). Comparatively speaking, the essential oil illustrated weaker activity against E. coli, with a MIC of 5 mg/mL, whereas, it displayed increased antimicrobial activity against S. typhimurium and S. aureus was more sensitive to the essential oil, with a MIC of 2 mg/mL. The essential oil has the strongest inhibitory activity against S. aureus (MIC 1 mg/mL).

Structural Changes in Bacterial Cells

SEM was employed to assess the morphological changes in E. coli, S. typhimurium and S. aureus. As shown in Fig. 1, SEM micrographs of untreated E. coli, S. typhimurium and S. aureus displayed regular and intact cells, with a smooth surface. In contrast, after treatment with K. galanga essential oil, the cells adhered to each other and membrane lysis was visible; in addition, the cells changed morphologically, underwent severe damage, and became deformed, pitted and shriveled.

<table>
<thead>
<tr>
<th>Compound</th>
<th>RI</th>
<th>RT (min)</th>
<th>PA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Furfural</td>
<td>836</td>
<td>5.93</td>
<td>0.02</td>
</tr>
<tr>
<td>1R-α-pinene</td>
<td>937</td>
<td>8.65</td>
<td>0.01</td>
</tr>
<tr>
<td>Carphene</td>
<td>952</td>
<td>9.10</td>
<td>0.01</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>964</td>
<td>9.40</td>
<td>0.01</td>
</tr>
<tr>
<td>3-Carene</td>
<td>1009</td>
<td>10.69</td>
<td>0.03</td>
</tr>
<tr>
<td>1,8-Cineole</td>
<td>1034</td>
<td>11.34</td>
<td>0.21</td>
</tr>
<tr>
<td>Terpinene</td>
<td>1056</td>
<td>12.02</td>
<td>0.01</td>
</tr>
<tr>
<td>4-ethyl-1,2-dimethyl-benzene</td>
<td>1100</td>
<td>12.86</td>
<td>0.01</td>
</tr>
<tr>
<td>Borneol</td>
<td>1163</td>
<td>15.01</td>
<td>0.35</td>
</tr>
<tr>
<td>(−)Terpine-4-ol</td>
<td>1178</td>
<td>15.19</td>
<td>0.17</td>
</tr>
<tr>
<td>2-(4-Methylphenyl)propan-2-ol</td>
<td>1187</td>
<td>15.33</td>
<td>0.11</td>
</tr>
<tr>
<td>α-Terpineol</td>
<td>1190</td>
<td>15.55</td>
<td>0.33</td>
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<tr>
<td>Eucarvone</td>
<td>1223</td>
<td>15.93</td>
<td>0.11</td>
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<tr>
<td>p-Methoxybenzaldehyde</td>
<td>1239</td>
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<td>0.39</td>
</tr>
<tr>
<td>5-Isopropyl-2-methylphenol</td>
<td>1287</td>
<td>17.56</td>
<td>0.03</td>
</tr>
<tr>
<td>Thymol</td>
<td>1299</td>
<td>17.77</td>
<td>0.05</td>
</tr>
<tr>
<td>3-Methoxyacetophenone</td>
<td>1321</td>
<td>19.21</td>
<td>0.03</td>
</tr>
<tr>
<td>Methylcinnamate</td>
<td>1397</td>
<td>19.92</td>
<td>0.04</td>
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<tr>
<td>Ethylcinnamate</td>
<td>1464</td>
<td>21.77</td>
<td>17.48</td>
</tr>
<tr>
<td>Cyclohexanemethanol</td>
<td>1520</td>
<td>23.44</td>
<td>0.13</td>
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<tr>
<td>Cubenol</td>
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<td>0.13</td>
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<td>Seleninol</td>
<td>1652</td>
<td>25.11</td>
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<tr>
<td>α-Bisabolol</td>
<td>1691</td>
<td>26.10</td>
<td>0.06</td>
</tr>
<tr>
<td>Ethyl-p-methoxycinnamate</td>
<td>1760</td>
<td>27.49</td>
<td>75.83</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>1960</td>
<td>30.78</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Table 1: Major chemical components of K. galanga essential oil

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Gram</th>
<th>MIC (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>−</td>
<td>5</td>
</tr>
<tr>
<td>S. typhimurium</td>
<td>−</td>
<td>1</td>
</tr>
<tr>
<td>S. aureus</td>
<td>+</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 2: MIC of K. galanga essential oil against tested bacteria

Nucleic Acid and Protein Leakage into the Extracellular Environment

It was obvious that nucleic acids (Fig. 2A) and proteins (Fig. 2B) were released into the cell supernatants, at levels markedly increasing with time after exposure to K. galanga essential oil. Nucleic acid and protein leakage increased significantly in the first hour of treatment; and in the following 5 h, it continued to increase to varying degrees. Meanwhile, the control groups showed almost no increase.

Analysis of Protein by SDS-PAGE

Protein bands in E. coli, S. typhimurium and S. aureus treated with K. galanga essential oil were significantly different from their respective control groups (Fig. 3). E. coli, S. typhimurium and S. aureus had more intense protein bands in the control groups. After 24 h of the essential oil treatment, reduction of bacterial protein bands in E. coli, S. typhimurium and S. aureus became overt and much fainter, with some even disappearing.
Discussion

*Kaempferia galanga* L. is one of the most abundant species of genus *Kaempferia*. Ethyl-p-methoxycinnamate was identified as the principal constituents of the essential oil of *Kaempferia galanga* (75.83%). The other ingredients were mainly terpenoids, but they were present in smaller amount than the major components. This is in agreement with previous work by Sukari et al. (2008). Past studies also found that ethyl-p-methoxycinnamate and ethyl cinnamate were found to be the predominant compositions, in *K. galanga* and other compound were found to be ethyl cinnamate (29.48%), ethyl-p-methoxycinnamate (18.42%), γ-cadinene (9.81%), 1, 8-cineole (6.54%), δ-carene (6.19%), and borneol (5.21%) (Kumar, 2014). Liu et al. (2014) identified the other compounds were composed by ethyl-p-methoxycinnamate (38.6%), ethyl cinnamate (23.2%), 1, 8-cineole (11.5%), transcinnamaldehyde (5.3%), and borneol (5.2%).

The *K. galanga* essential oil showed stronger inhibitory activity against *S. typhimurium* and *S. aureus* than against *E. coli*. This may be because of the different cellular organization of bacteria. There are a few reports on the antimicrobial activity of the oil of *K. galanga* species (Indrayan et al., 2007; Kumar, 2014), but there was no in-depth study on its antibacterial mechanism. In future work, we will study the antibacterial mechanism of the essential of *K. galanga*.

Multiple studies have confirmed the antibacterial activities of different essential oils, but the related antibacterial mechanisms remain relatively undefined. The antimicrobial activity could result from membrane disruption, cell wall perturbation, and the destruction of electron transport systems (SOLECZEK et al., 2015). It could also result from cytoplasmic membrane coagulation, breakdown of the proton motive force, and electron flux or active transport impairment, which elicits a rapid depletion of intracellular ATP (Dimroth et al., 2000; Odhav et al., 2002).

**Fig. 1:** SEM images of the *E. coli* (top panel), *S. typhimurium* (middle panel) and *S. aureus* (bottom panel) treated with 0 mg/mL [control] (A), 2MIC (B) of *K. galanga* essential oil, respectively

**Fig. 2:** Leakage of nucleic acids and proteins into the extracellular environment during treatment. E−, *E. coli* for control; E-T−, *E. coli* with treatment; Sa−, *S. typhimurium* for control; Sa-T−, *S. typhimurium* with treatment; St−, *S. aureus* for control; St-T−, *S. aureus* with treatment. Error bars indicate standard error of the mean; where error bars are not visible, they are smaller than the symbol

**Fig. 3:** SDS-PAGE profiles of bacterial proteins treated with *K. galanga* essential oil or no essential oil. M−, Marker; E−, *E. coli* for control; E-T−, *E. coli* with treatment; Sa−, *S. typhimurium* for control; Sa-T−, *S. typhimurium* with treatment; St−, *S. aureus* for control; St-T−, *S. aureus* with treatment. Molecular weight markers are in lane M and the molecular weights are indicated
Cell membrane integrity was evaluated by measuring the relative amounts of intracellular substances leaking into the cell culture medium. Intracellular components, including small ions, ATP, nucleic acids, and proteins, would be released in case of bacterial membrane alteration after exposure to antibacterial agents. Therefore, release of cytoplasmic components, such as nucleic acids and proteins, is a good indicator for membrane integrity (Chen and Cooper, 2002; Aronsson et al., 2005). Nucleic acids are in the nucleus, and proteins in the cell membrane, cytoplasm, and nucleus; their release indicated severe damages of both the cell wall and membrane. The longer the oil treatment duration, the more intense the damage is. Release of cell constituents was assessed by optical density measurements at 260 and 280 nm in culture supernatants from the three tested strains (Zhang et al., 2016b). Nucleic acids and proteins have strong UV-absorption at 260 nm and 280 nm, respectively. Cell membrane integrity is one of the main factors affecting the normal growth and metabolism of bacteria. Macromolecules, such as nucleic acids and proteins, are present throughout the entire membrane and cytoplasm, and constitute important structural units. Therefore, nucleic acid and protein release showed that cell membrane integrity was impaired, affecting the growth of cells, thereby inhibiting bacterial replication.

Degradation of bacterial proteins was also observed in bacteria treated with other antimicrobials, such as lactic acid (Wang et al., 2015) and the essential oil from Juniperus rigida leaves (Meng et al., 2016). The present findings suggested K. galanga essential oil has remarkable effects on bacterial proteins, decreasing cellular protein amounts by permeating and disturbing the cell membrane. Additionally, K. galanga essential oil could potentially affect cellular proteins either by disrupting them or suppressing their synthesis, resulting in bacterial death (Zeng et al., 2010; Zhao et al., 2015).

Conclusion

In summary, K. galanga essential oil showed antibacterial activity against E. coli, S. typhimurium and S. aureus. The antibacterial activity of K. galanga essential oil was further proven by scanning electron microscopy, nucleic acid and protein leakage experiments and SDS-PAGE. These results indicate that this oil is a potential candidate with broad development prospects for food preservation or application in the pharmaceutical industry.

Acknowledgments

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