In vitro antimicrobial activity of hydrosol from Litsea cubeba (Lour.) Pers. against Helicobacter pylori and Candida albicans

Tran Thanh Hung¹,², Pham Thu Trang¹, Hoang Viet¹, Nguyen Thi My Lan¹, Luong Thi My Ngan¹,² ¹, Tran Trung Hieu¹,²*

ABSTRACT

Introduction: Helicobacter pylori and Candida albicans are classified as the most common pathogenic agents in humans. H. pylori is responsible for gastroduodenal diseases and greatly associated with gastric carcinogenesis, while C. albicans is the main cause of fungal urinary tract, genital yeast, and fungal skin infections. The increasing appearance of drug-resistant strains of H. pylori and C. albicans has made the treatment of the infections more serious. Hydrosols from plant steam distillation have been traditionally used in medicine, cosmetics, and culinary uses. They have been recently suggested as antimicrobial agents owing to their safety and ability to reduce the potential of resistance. The aim of the present study is to assess antibacterial and antifungal activities of hydrosols extracted from the fresh fruits of Litsea cubeba against H. pylori and C. albicans.

Methods: The L. cubeba fruit hydrosol was obtained by steam distillation method. Evaluation of the growth-inhibiting and microbicidal effects of the hydrosol towards the H. pylori ATCC 43504 and C. albicans ATCC 10231 was determined through MIC (minimal inhibitory concentration), MBC (minimal bactericidal concentration), and MFC (minimal fungicidal concentration) measurements using broth dilution assays. Compositions of the dissolved essential oil (dEO) from the hydrosol were analyzed by GC-MS (gas chromatography-mass spectrometry).

Results: The results indicated that the L. cubeba fruit hydrosol exhibited strong antimicrobial ability towards the bacterium H. pylori (MIC of 10%, MBC of 30%) and the yeast C. albicans (MIC of 10%, MFC of 40%). The cells of H. pylori and C. albicans were killed completely after 24 and 18 hours of treatment with 30% and 40% of the hydrosol, respectively. The major constituents of the dEO were geranial (32.92%), neral (27.12%), p-menthan-8-yl acetate (8.45%), 2-cyclopropyl-2-methylspirol[2.2]pentane-1-carboxylic acid (8.09%), linalool (4.24%), and methyl heptenone (4.15%).

Conclusion: The results of the study suggest that L. cubeba fruit hydrosols could be used as potent natural antibacterial and antifungal preparations in the global effort to discover safe alternatives to toxic antimicrobial agents.

Key words: Antimicrobial activity, Candida albicans, Helicobacter pylori, Hydrosol, Litsea cubeba, steam distillation

INTRODUCTION

Hydrosol is an aqueous layer (or aromatic water) located just below the essential oil (EO) layer during steam distillation of aromatic and medicinal plant materials. Hydrosol is the byproduct of steam distillation of EOs. Almost all hydrosols contain small quantities of dissolved essential oil (dEO). However, some plants contain a high amount of dEO. Since hydrosols possess a wide range of beneficial properties, such as pH 5.5, pleasant scents, relaxing fragrance and antimicrobial ability, they are traditionally used in cosmetics, food applications, and medicinal purposes in many countries around the world. These days, hydrosols have attracted an increasing number of researchers because of their eco-friendly, health-benefitting and safe characteristics. Several recent studies have demonstrated that hydrosols from different plants exhibit strong antimicrobial activities against a wide variety of fungi and bacteria. Therefore, research on hydrosols would bring promising solutions for controlling pathogenic fungi and bacteria. Helicobacter pylori and Candida albicans are two of the most common human pathogens that cause a wide range of serious diseases for humans. H. pylori is a Gram-negative spiral bacterium with the ability to produce urease, persistently colonizing in the gastric mucosa of at least half of the world’s population.

In some developing countries, the rate of infection with H. pylori can reach up to 80% or higher. The bacterium has been proven to be responsible for gastroduodenal diseases and is greatly associated with gastric carcinogenesis. C. albicans is a polymorphic fungus that is the common infectious agent of the skin, oral cavity and esophagus, gastrointestinal
tract, vagina, and vascular system of humans. The fungus, which is uniquely adapted to its human host, often exists as a harmless organism at various mucosa. However, the pathogen can cause serious conditions under certain circumstances, including life-threatening systemic infection. Antibiotic and antifungal therapies were successful in controlling the H. pylori and C. albicans infections, greatly contributing to reduction of diseases caused by these pathogens. However, the side effects which resulted from the drugs and the rapid emergence of drug-resistant strains have made the therapies for the treatment of H. pylori and C. albicans lose their effectiveness. Hence, there is an urgent need for new antimicrobial agents for more effective management of these infectious agents.

Increasing public concern about healthy and natural products nowadays has promoted much research focusing on developing new medicines from plant sources. Numerous extracts and EOs were demonstrated to exhibit strong antimicrobial activities towards H. pylori and C. albicans without any resistance development of these microbes. Fruit of Litsea cubeba, a member of Lauraceae family, has been used for stimulating the digestive system and in treatment of stomach ache in Vietnam and Malaysia. The EO from the fruit of Litsea cubeba was found to have a strong activity against both Gram-negative and Gram-positive bacteria. In addition, the EO and terpenoid extraction from fruits of L. cubeba exhibited effective activity against several pathogenic fungi in plants and humans, including C. albicans. However, no information has been obtained related to the constituents and the potential of the hydrosol from the L. cubeba fruit to control H. pylori and C. albicans. In the present study, the growth-inhibiting, bactericidal, and fungicidal effects of the hydrosol extracted from steam distillation of the L. cubeba fruit against H. pylori ATCC 43504 and C. albicans ATCC 10231 were assessed. Chemical constituents of dEO extracted from the hydrosol were also determined by gas chromatography-mass spectrometry (GC-MS).

MATERIALS AND METHODS

Plant collection

The fresh samples of Litsea cubeba (Lour.) Pers. (1806) were collected from the Mang Den Medicinal Plants Garden, in Kon Tum province (the Central Highlands of Vietnam) in June 2018. The identification to species of the specimen was conducted by the method of morphological descriptions. A voucher specimen has been deposited in the PHHH Herbarium of Vietnam National University Ho Chi Minh City.

Preparation of hydrosol

An amount of 800 gram of fresh fruit of L. cubeba was finely ground in a mixer and then subjected to steam distillation at 100°C using a Clevenger-type apparatus with 3000 mL of water for 3 hours. The hydrosol was separated by a separation funnel; afterwards, the hydrosol (~1800 mL) was obtained and stored in dark bottles at 4°C until used.

Reagents

Brucella broth (BB), brain heart infusion broth (BHB), sabouraud dextrose agar (SDA), and sabouraud dextrose broth (SDB) were purchased from Becton Dickinson, Inc. (Sparks, MD, USA). Newborn bovine serum (NBS) was obtained from Hyclone (Longan, UT, USA). Amoxicillin (~98%) was purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Nystatin was provided by Merck (Kenilworth, NJ, USA). All other chemicals and reagents used in this study were of analytical grade quality and available commercially.

Microbial strains and culture conditions

The reference strains of H. pylori ATCC 43504 and C. albicans ATCC 10231 were provided by the Oxford University Clinical Research Unit Vietnam (OUCRU - VN) and Department of Plant Biotechnology and Biotransformation, Faculty of Biology and Biotechnology, University of Science, VNU - HCM, respectively. Authentication of these strains was done using MALDI-TOF MS (OUCRU - VN). The bacterial and the yeast strains were stored in BHB and SDB media, respectively, supplemented with 25% glycerol and placed in a nitrogen liquid container until use. The H. pylori strain was cultured on Brucella agar added with 10% NBS, and then incubated at 37°C for 3 days under a microaerophilic condition created by a 2.5 L Oxoid Anaerojar and Oxoid CampyGen sachet (Thermo Fisher Scientific, Waltham, MA, USA). The bacterial suspensions (~ 5x10^7 CFU/mL) used for the bioassay were made in Brucella broth using a 72-h subculture of H. pylori on Brucella agar. The C. albicans strain was activated in SDB medium at 37°C for 48 h. The yeast suspensions for bioassay (~ 5x10^6 CFU/mL) were prepared in SDB medium from a 48-h subculture of the activated C. albicans. Microbial density was determined using McFarland turbidity standards.

Separation and analysis of chemical constituents of dissolved essential oil

Dissolved essential oil (dEO) was extracted by vigorously shaking the hydrosol (900 mL) with hexane.
The dissolved essential oil (dEO) extracted from the *L. cubeba* fruit hydrosol had a yield of 0.18% (w/w).
on fresh weight basis). The constituents of the dEO were identified by GC-MS and presented in Table 1. The results showed that the main components of the dEO were as follows: geranial (32.92%), neral (27.12%), p-menthan-8-yl acetate (8.45%), 2-cyclopropyl-2-methylspiro[2.2]pentane-1-carboxylic acid (8.09%), eucalyptol (4.24%), methyl heptenone (4.15%), (R)-(−)-citronellal (2.10%), α-terpineol (1.04%), and terpinen-4-ol (1.02%), which accounted for 89.13% and 0.1604% of the total dEO content and the hydrosol, respectively (Figure 1). The minor and unidentified compounds of the dEO accounted for 2.76% and 8.11%, respectively. Out of 15 determined compounds, there were 14 oxygenated components (91.24%) and one hydrocarbon only (0.65%) (Table 1).

Antimicrobial effects of the L. cubeba fruit hydrosol on H. pylori and C. albicans

The MIC and MBC values of the hydrosol towards the H. pylori strain ATCC 43504 are shown in Table 2. The hydrosol displayed a high growth inhibitory activity with a MIC value of 10% against the H. pylori strain. The hydrosol also exhibited a relatively strong bactericidal activity towards the bacterial strain with an MBC value of 30%.

The growth-inhibiting and fungicidal effects of the hydrosol on the C. albicans strain ATCC 10231 are shown in Table 1. The hydrosol inhibited the growth of the yeast with the same MIC value (10%), in comparison with its effect on H. pylori. These results also indicate that the hydrosol had a potential fungicidal activity on the C. albicans strain with an MFC value of 40%.

Microbicidal time-kill of the L. cubeba fruit hydrosol against H. pylori and C. albicans

The growth curves of H. pylori ATCC 43504 were performed in order to evaluate the H. pylori bactericidal activity of the hydrosol over time. The results provided in Figure 2 indicated that the bacterial cells grew in a log phase from the start to 18 hours of the culture (5.92 – 8.15 Log_{10} CFU/mL) and in a stationary phase (8.48 – 8.62 Log_{10} CFU/mL) after 18 – 48 hours in the negative control culture. In the cultures with the hydrosol, the viable count of the bacteria decreased in a concentration- and time-dependent manner. The H. pylori population treated with 10%, 20% and 30% of the hydrosol decreased significantly (p<0.05) compared with the negative control after 12 - 48 hours. In the cultures treated with 10% hydrosol, the H. pylori strain was inhibited and could survive for 48 hours with no significant change in cell density (5.92 – 6.08 Log_{10} CFU/mL). The populations of the test strains declined significantly in the treatments of H. pylori with the hydrosol (20%) with cell density ranging from 3.74 – 3.25 Log_{10} CFU/mL in comparison with 8.48 – 8.62 Log_{10} CFU/mL for the controls after 24 – 48 hours of treatment. In the cultures of 30% hydrosol, the microbial strains had sharp decreases in the viable count- from 5.92 to 2.61 Log_{10} CFU/mL after 18 hours- and completely killed after 24 hours of treatment.

Fungal time-kill curves of C. albicans after exposure to the hydrosol at various percentages were also determined and are shown in Figure 3. In the negative control cultures, the yeast population increased steadily (5.09 – 7.81 Log_{10} CFU/mL) during the period of 48 hours. In the cultures treated with the hydrosol, the growth of the yeast populations reduced in a concentration- and time-dependent manner. The C. albicans population gradually increased (5.09 – 6.92 Log_{10} CFU/mL) for 48 hours treatment with 10% of hydrosol, but the growth was significantly slower (p<0.05) than that in the control culture at 48 h (7.81 Log_{10} CFU/mL). The C. albicans population treated with 20%, 30% and 40% of the hydrosol decreased significantly (p<0.05), compared with the negative control after 12 - 48 hours. When treated with the hydrosol 20% and 30%, the number of the yeast cells had a significant fall (p<0.05) after 24 – 48 hours of treatments with cell density ranging 3.51 – 2.94 and 2.86 – 2.48 Log_{10} CFU/mL, in comparison with 7.19 – 7.81 Log_{10} CFU/mL of the controls, respectively. The exposure to the hydrosol 40% caused the yeast population to obviously drop, ranging from 5.09 to 2.60 Log_{10} CFU/mL after 12 hours and completely killed after 18 hours of treatment.

DISCUSSION

Hydrosols from some plants were identified chemical constituents, but there was no information about the composition of the L. cubeba fruit hydrosol. The previous studies of EOs from L. cubeba fruits collected in Vietnam, China, and India indicated that neral and geranial were the two main components of the EOs (66.3 – 83.9%) . Both of the two compounds were also the predominant constituents accounting for 60.04% of the dEO from the hydrosol in our study. Furthermore, other compounds, including methyl heptenone, eucalyptol, linalool, isopulegol, citronellal, terpinen-4-ol, and α-terpinol, were found present in the fruit EOs in several previous studies. Interestingly, they were also found in the hydrosol in our study herein. In addition, the
Table 1: The percentage of chemical components (%) of dissolved essential oil (dEO) from the *L. cubeba* fruit hydrosol

<table>
<thead>
<tr>
<th>No.</th>
<th>Chemical Compound</th>
<th>Molecular formula</th>
<th>Retention time (minute)</th>
<th>dEO (%)</th>
<th>Hydrosol (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Methyl heptenone</td>
<td>C₈H₁₂O</td>
<td>7.118</td>
<td>4.15</td>
<td>0.0075</td>
</tr>
<tr>
<td>2</td>
<td>3,4-pentadienal</td>
<td>C₅H₁₀O</td>
<td>8.609</td>
<td>0.20</td>
<td>0.0004</td>
</tr>
<tr>
<td>3</td>
<td>Eucalyptol</td>
<td>C₁₀H₁₈O</td>
<td>8.707</td>
<td>0.85</td>
<td>0.0015</td>
</tr>
<tr>
<td>4</td>
<td>Linalool</td>
<td>C₁₀H₁₈O</td>
<td>11.691</td>
<td>4.24</td>
<td>0.0076</td>
</tr>
<tr>
<td>5</td>
<td>Octanol acetate</td>
<td>C₁₀H₂₀O₂</td>
<td>12.553</td>
<td>0.15</td>
<td>0.0003</td>
</tr>
<tr>
<td>6</td>
<td>Isopulegol</td>
<td>C₁₀H₁₈O</td>
<td>13.578</td>
<td>0.82</td>
<td>0.0015</td>
</tr>
<tr>
<td>7</td>
<td>(R)-(+)citronellal</td>
<td>C₁₀H₁₈O</td>
<td>14.016</td>
<td>2.10</td>
<td>0.0038</td>
</tr>
<tr>
<td>8</td>
<td>Terpinen-4-ol</td>
<td>C₁₀H₁₈O</td>
<td>14.987</td>
<td>1.02</td>
<td>0.0018</td>
</tr>
<tr>
<td>9</td>
<td>Santolinsyl acetate</td>
<td>C₁₂H₂₀O₂</td>
<td>15.372</td>
<td>0.65</td>
<td>0.0012</td>
</tr>
<tr>
<td>10</td>
<td>2,5-octadiene</td>
<td>C₄H₁₄</td>
<td>15.732</td>
<td>0.65</td>
<td>0.0019</td>
</tr>
<tr>
<td>11</td>
<td>α-terpineol</td>
<td>C₁₀H₁₈O</td>
<td>15.640</td>
<td>1.04</td>
<td>0.0019</td>
</tr>
<tr>
<td>12</td>
<td>Neral</td>
<td>C₁₀H₁₆O</td>
<td>17.975</td>
<td>27.12</td>
<td>0.0048</td>
</tr>
<tr>
<td>13</td>
<td>Geranial</td>
<td>C₁₀H₁₆O</td>
<td>19.348</td>
<td>32.92</td>
<td>0.0593</td>
</tr>
<tr>
<td>14</td>
<td>2-cyclopentyl-2-methylspirol[2.2]pentane-1-carboxylic acid</td>
<td>C₁₂H₁₄O₂</td>
<td>20.009</td>
<td>8.09</td>
<td>0.0146</td>
</tr>
<tr>
<td>15</td>
<td>-menthan-8-yl acetate</td>
<td>C₁₂H₂₂O₂</td>
<td>21.380</td>
<td>8.45</td>
<td>0.0152</td>
</tr>
</tbody>
</table>

Total 91.89 0.1654

Table 2: MIC and MBC/MFC of the *L. cubeba* fruit hydrosol toward *H. pylori* and *C. albicans* using broth dilution assay

<table>
<thead>
<tr>
<th>Organism</th>
<th>Sample</th>
<th>MIC</th>
<th>MBC/MFC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. pylori</em> strain ATCC 43504</td>
<td>Hydrosol</td>
<td>10 %</td>
<td>30 %</td>
</tr>
<tr>
<td></td>
<td>Amoxicillin</td>
<td>0.04 μg/mL</td>
<td>0.10 μg/mL</td>
</tr>
<tr>
<td><em>C. albicans</em> strain ATCC 10231</td>
<td>Hydrosol</td>
<td>10 %</td>
<td>40 %</td>
</tr>
<tr>
<td></td>
<td>Nystatin</td>
<td>4 μg/mL</td>
<td>16 μg/mL</td>
</tr>
</tbody>
</table>

dEO in the hydrosol had hydrophilic properties because it mainly contained oxygenated compounds (91.24%) yet with less hydrocarbons (0.65%), whereas the EO extracted from the fruit in Thua Thien - Hue Province, Vietnam showed a lower amount of oxygen-containing components (78.8%) and a higher amount of hydrocarbons (21%) [44]. Studies on hydrosols of Indian oregano (*Origanum vulgare*), caraway (*Carum carvi*), bergamot-mint (*Mentha citrata*) also led to similar results [19,40,41,45,46]. Hydrosols have been widely used in many regions of the world for food flavoring and medicinal purposes for a long time [2,3]. Moreover, the hydrophilic property makes hydrosols safe for skin. Since hydrosols are well-tolerated by the skin, they have thus been used in cosmetic products [47]. Hydrosols of several spices and aromatic plants were demonstrated to possess high antifungal and antibacterial properties against a variety of bacteria and fungi [48–51]. For example, hydrosols extracted from cardamom (*Elettaria cardamomum*), thyme (*Thymus schimperi*), and cinnamon (*Cinnamomum zeylanicum*) showed the complete growth inhibition against *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhi* [49]. The *Satureja hortensis* hydrosol 15% (v/v) was shown to have fungicidal effects, inhibiting 100% of mycelial growth of *Rhizoctonia solani*, *Botrytis cinerea* and *Alternaria citri* [48]. However, hydrosol of *Epilobium parviflorum* exhibited...
weak antimicrobial activities towards tested Gram-negative bacteria (E. coli and Pseudomonas aeruginosa) and yeast (C. albicans), with MIC values of 70% (v/v); towards Gram-positive bacteria (S. aureus and Enterococcus faecalis), the MIC values were greater than 90%47. In the present study, the L. cubeba fruit hydrosol exhibited strong antimicrobial activities towards the bacterium H. pylori (MIC of 10%, MBC of 30%) and the yeast C. albicans (MIC of 10%, MFC of 40%). The cells of H. pylori and C. albicans were killed completely after 24 and 18 hours of treatment with the 30% and 40% hydrosol, respectively.

There have been no data of antibacterial and antifungal activities of the hydrosol extracted from the L. cubeba fruits, yet the information of those of the L. cubeba fruit EO have been well-known. The L. cubeba fruit EO showed strong inhibitory effects on Gram-positive bacteria (Bacillus subtilis, E. faecalis, S. aureus) and Gram-negative bacteria (E. coli and P. aeruginosa), and Monilia albicans33, and high growth-inhibiting activity towards methicillin-resistant S. aureus47. In addition, the EO exhibited good fungicidal activities against Sclerotinia sclerotiorum, Thanatephorus cucumeris35, Aspergillus flavus52, and C. albicans42. Bacteriostatic and bactericidal activities of the L. cubeba EO significantly depend on concentration and exposure time32. The EO at concentration of 0.0625% (v/v) was able to prolong the growth lag phase of E. coli cells to approximate 12 hours, whereas the 0.125% (v/v) concentration killed the cells completely within 2 hours32.

The antibacterial and antifungal effects of plant EOs and their hydrosols have been attributed to their com
Figure 2: Bacterial growth inhibitory and bactericidal activities of *L. cubeba* fruit hydrosol against *H. pylori* ATCC 43504 over time at MIC (hydrosol 10%), 2 x MIC, and 3 x MIC (= MBC) compared with the control (without hydrosol). The mean values (± SD) for the log number of colony forming unit (CFU)/mL were plotted.

Figure 3: Fungal growth inhibitory and fungicidal activities of *L. cubeba* fruit hydrosol against *C. albicans* ATCC 10231 over time at MIC (hydrosol 10%), 2 x MIC, 3 x MIC and 4 x MIC (= MFC) compared with the control (without hydrosol). The mean values (± SD) for the log number of colony forming unit (CFU)/mL were plotted.
positions. The richness in oxygenated monoterpenes, oxygenated sesquiterpenes, and phenolic compounds contributed to the strong antimicrobial activities of hydrosols. Phenolic compounds can interfere with microbial membranes, cell walls, or the action of microbial enzymes. Terpenoid compounds may disrupt the lipid structure and thus cause loss of membrane integrity, membrane protein functions (proton pumps and enzymes), and synthesis of cellular metabolites, leading to cell death. The antimicrobial effects of the *L. cubeba* fruit hydrosol may be mainly due to the presence of citral (geranial and neral). Neral and geranial were found to be the two main components of lemongrass EO, which was demonstrated to have strong antibacterial activity against Gram-negative and Gram-positive bacteria and resulted in a considerable decrease in the density of *H. pylori* in mouse stomach. Citral and sabinene were reported to exhibit strong anti-*H. pylori* activities. Citral was also shown to exhibit excellent activities against dermatophytes, thereby suggesting its potency as a fungicidal agent. Furthermore, citral showed strong anti-fungal abilities towards pathogenic microbes *E. coli*, *P. aeruginosa*, *S. aureus*, and *C. albicans*, and was considered as a potential agent to control skin and mucosal infections. The synergistic effects among major and minor components of the *L. cubeba* fruit hydrosol against *H. pylori* and *C. albicans* need to be further studied.

**CONCLUSION**

The present study revealed that the *L. cubeba* fruit hydrosol exhibited pronounced inhibitory and microbiocidal effects against *H. pylori* and *C. albicans*. The results suggest that the *L. cubeba* fruit hydrosol warrants further study and could be developed as a potent antimicrobial product for treating *C. albicans* and *H. pylori* infections.

**ABBREVIATIONS**

ATCC: American Type Culture Collection  
EO: Essential oil  
GC-MS: Gas chromatography-mass spectrometry  
MALDI-TOF MS: Matrix-assisted laser desorption ionization-time of flight mass spectrometry  
MBC: Minimal bacterial concentration  
MFC: Minimal fungicidal concentration  
MIC: Minimal inhibitory concentration  
MFC: Minimal fungicidal concentration

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**AUTHOR’S CONTRIBUTIONS**

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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**AVAILABILITY OF DATA AND MATERIALS**

Data and materials used and/or analysed during the current study are available from the corresponding author on reasonable request.

**ETHICS APPROVAL AND CONSENT TO PARTICIPATE**

Not applicable.

**CONSENT FOR PUBLICATION**

Not applicable.

**COMPETING INTERESTS**

The authors declare that they have no competing interests.

**REFERENCES**


