ORIGINAL ARTICLE

Citrus aurontifolia and Cymbopogan flexuosus against Staphylococcus aureus and Escherichia coli

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ABSTRACT

Introduction: Good hand hygiene is an important measure to avoid the transmission of infectious agents in the hospital and in the community. This study aimed to investigate the antimicrobial effects of *Citrus aurantifolia* (key lime) juice and the aqueous extract of *Cymbopogan flexuosus* leaves on the growth of *Staphylocuccus aureus* and *Escherichia coli*. **Methods:** Antimicrobial activity was quantitatively evaluated by spectrophotometry and viable cell count versus bacterial growth time. The efficacy of the plant extracts and the commercial hand wash product was also compared by measuring the number of bacterial colonies before and after using *C. aurantifolia* juice and commercial hand wash product. **Results:** In the kinetic growth study, *C. aurantifolia* juice and the aqueous extracts of *C. flexuosus* leaves effectively eliminated *S. aureus* and *E. coli*. *C. aurantifolia* juice also efficiently removed most of the microorganisms on the volunteers' hand, indicating a significant finding in human trial compared to *in vitro* study. The percentage of microorganisms left after hand washing with *C. aurantifolia* juice was significantly reduced to 91.72 %, which was comparable with that of the commercial hand wash product (82.87 %). **Conclusion:** This study showed that these plant materials are promising alternatives for antibacterial agents in hand wash products. Further studies should be conducted on the use of *C. aurantifolia* juice as hand sanitiser given its antibacterial activities against endemic microbes.

Keywords: Antimicrobial; *Citrus aurantifolia*; *Cymbopogan flexuosus*; *Escherichia coli*; *Staphylocuccus aureus*

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INTRODUCTION

Hands play a major part in transmitting infectious agents in hospitals (1) and in the community (2). The human skin typically harbours bacteria between 102 and 106 colony forming units (CFU)·cm^{-2} (3). Microorganisms that reside on the hands can be divided into two types: resident flora (*Corynebacterium diphtheriae, Staphylococcus aureus, Staphylococcus epidermidis* and *Staphylococcus viridans*) and transient flora (*S. aureus*, Gram-negative *Bacilli* and *Candida species*) (4). The resident flora has lower pathogenic potential and is more resistant to mechanical removal than the transient flora. Moreover, the resident flora can be found in deep skin layers and does not usually cause infection, except when introduced during invasive procedures (e.g. line insertion or surgical procedures). Meanwhile, the transient flora is found in superficial skin layers for short periods. This flora is not part of the normal flora and can usually be acquired through contact with patients or contaminated environment. In addition, the transient flora can be easily removed by mechanical means (e.g. hand washing). Microorganisms belonging to this flora are resistant to antibiotics and cause health-care-associated infection.

Many kinds of antimicrobial soaps have been formulated and sold for hand sanitisation and disinfection. A commercial antimicrobial soap typically consists of antiseptic agents, such as alcohols, chlorhexidine, iodine compounds, triclosan, phenol derivatives and quaternary ammonium compounds (5). Based on the characteristics of antiseptic agents, they bind to skin stratum corneum and cause a persistent activity by removing microbial flora after hand washing (6). Although these antiseptic agents can help to control the transmission of contagious diseases, they present several limitations or adverse effects. The frequent use of these agents can cause unwanted skin reactions, such as irritant contact dermatitis or allergic contact dermatitis (7). Frequent and repeated use of hand hygiene products may cause dryness, irritation, itching, cracking and bleeding. Such occupational skin disease is common among healthcare workers.

The use of plant product as a source of antimicrobial agents dates to prehistoric periods (8). Plant-based products are important in the treatment of many diseases (9), especially infectious diseases (10) due to their abundant source of medicinally active compounds (11). Plant also provides a broad spectrum of antimicrobial

properties and has fewer side effects than synthetic antimicrobials. *Citrus aurantifolia* (key lime), which belongs to the Rutaceae family, is an evergreen tree that can reach up to 5 m tall. The leaves are medium sized (6–9 cm long), ovate and bluntly pointed at tips, with rounded to cunate base. It has white flowers, which are solitary or in a short raceme and are small yet fragrant. The fruits become yellow when ripe with globose diameter of approximately 4–5 cm and thinner rind. On the other hand, *Cymbopogon flexuosus* (lemongrass) is a type of perennial grass that is fast growing, tall and lemon scented. Its height can reach up to 1.5 m. *C. flexuosus* essential oils are commonly used in aromatherapy, flavouring, pharmaceutical and perfumery industries (12). These plants could be used as alternatives to hand sanitisers given the potential health risks posed by commercial hand wash products. This study aimed to investigate the antimicrobial effects of *C. aurantifolia* juice and the aqueous extract of *C. flexuosus* leaves on the growth of *S. aureus* and *E. coli*. The efficacy of *C. aurantifolia* juice was also evaluated in comparison with a commercial antimicrobial hand wash product.

MATERIALS AND METHODS

Plant Materials

C. aurantifolia fruits and *C. flexuosus* leaves were harvested from Bertam, Kepala Batas, Malaysia. The plants were submitted to a qualified plant taxonomist for identification and authentication. The plant samples were pressed and mounted on herbarium paper to create voucher herbarium specimens, which were then kept in the Integrative Medicine Cluster Herbarium Collection for future cross-reference.

Juice and Aqueous Extraction

For cleaning purpose, the *C. aurantifolia* fruits were wiped with 70 % ethanol and then rinsed with distilled water prior to extraction. The fruits were sliced into two with a sterilised knife and the juice was obtained by aseptically squeezing the fruits juice into a sterile beaker. For *C. flexuosus* leaves, 20 g dried plant was boiled in 50 mL sterilised water (50 mL) until one-fifth of the original volume. The extract was then sterilised using autoclave machine at 100 °C for 15 min. The juice and the extracts were centrifuged at 3500 x g for 20 min. The supernatant was collected carefully and stored at –20 °C until the next experiments (13).

Bacteria Susceptibility Test

S. aureus (ATCC 25923) and *E. coli* (ATCC 25922) were obtained from the American Type Culture Collection and were maintained accordingly to the instructions. A colony was inoculated into freshly prepared nutrient broth. The broth was vortexed to prevent aggregation. The bacteria colony was allowed to propagate on a shaker overnight at 37 °C. For each bacterium species, 103 cells·mL^{-1} were prepared for the test and control samples. *C. aurantifolia* juice (2 mL) and the aqueous extract of *C. flexuosus* leaves (0.4 g·mL^{-1}) were evaluated in the susceptibility tests (total volume is 10 mL). Finally, 50 µL aliquot of each test samples were aseptically inoculated onto a freshly prepared agar plate to obtain viable counts. OD readings and plating analysis were conducted using a UV-VIS spectrophotometer (Perkin Elmer, Lambda 25, Norwalk, CT, USA) at 0, 1, 2 and 3 h (13).

Hand Antisepsis Protocol

The experimental procedures were approved by the Human Research Ethics Committee USM (FWA Reg. No: 00007718; IRB Reg. No: 00004494). Seventeen non-clinical volunteers without visible skin injuries, eczema or apparent skin disease were recruited (14). The volunteers were requested not to use an antibacterial soap 8 h prior to sampling, and the wearing of rings and wristwatches was not allowed during washing and sampling. The experiment was performed in two different days. The commercial hand wash product was applied on the first day, and *C. aurantifolia* juice was applied on the second day.

The subject's dominant hand was wet with distilled water (10 s). The pre-washed sample was collected for culture by pressing the wet hand on the agar plate for 15 s. The pre-washed sample was collected to establish a baseline enumeration of bacteria. The hand was then rinsed with running water for 10 s. The commercial hand wash product or *C. aurantifolia* juice was applied on the hand. The hand was lathered by rubbing them together with the soap. The back of the hand, between the fingers, and under the nails were lathered thoroughly for 1 min. The hand was rinsed with running water for 30 s. Post-wash sample for culture was collected by pressing the dominant hand on another agar plate for 15 s. The hand was washed with running water and dried with clean tissue paper.

The number of CFUs per plate was evaluated. The reduction percentage of pre- and post-washing was calculated using the following formula (1) (15):

$$\% \text{ Reduction} = \frac{\text{Before wash-After wash}}{\text{Before wash}} \times 100\ \% \quad (1)$$

Statistical Analysis

The significant differences among the samples at different time points was determined using Statistical Package for Social Sciences (IBM SPSS version 22.0, Armonk, NY, USA). One-way ANOVA Dunnett T3 test and post-hoc Tukey HSD test were used. Statistically significance of the obtained results was considered when $p < 0.05$.

RESULTS

Preliminary Screening of Plant Extracts

The antibacterial effects of *C. aurantifolia* juice and

the aqueous extract of *C. flexuosus* leaves on 103 cells·mL^{-1} *S. aureus* and *E. coli* were determined using a spectrophotometer [for optical density (OD) values]. The standard method of viable counts on agar was used as reference. As shown in Figure 1A and B, the graph pattern of OD and CFU of untreated *S. aureus* increased from OD (600 nm) 0.133 and 4780 CFU·mL^{-1} at 0 h to 0.155 and 6420 CFU·mL^{-1} after 3 h. The number of the *S. aureus*, as an untreated control group, increased with time in the normal growth condition showing healthy kinetic growth of bacteria.

When *C. aurantifolia* juice was added to the culture, the growth of *S. aureus* sharply decreased over time. Figure 1C and D showed the OD and CFU number of the *S. aureus* co-cultured with the *C. aurantifolia* juice were declined significantly from OD 0.087 and 4060 CFU·mL^{-1} at 0 h to OD 0.04 and no viable cell was counted after 3 h. *S. aureus* was susceptible to *C. aurantifolia* juice at 3 h, at which the OD value was 0.0401 and no viable cells were counted. Meanwhile, the aqueous extract of *C. flexuosus* leaves demonstrated better bactericidal activity against *S. aureus* than *C. aurantifolia* juice, as indicated by the constant decrease in absorbance and the elimination of viable counts at 1 h (Figure 1E and 1F). The OD and viable count values decreased dramatically from 0.121 and 320 CFU·mL^{-1} at 0 h to 0.09 and 0 CFU·mL^{-1} at 1 h, respectively. The aqueous extract of *C. flexuosus* leaves was more effective in inhibiting *S. aureus* than *C. aurantifolia* juice. The antibacterial effects of the aqueous extract of *C. flexuosus* leaves on *S. aureus* maintained thereafter.

The *E. coli* OD values of the untreated control increased linearly, and the viable count also increased (Figure 2A and B). The growth of *E. coli* increased with time. The OD and the viable cell count of *E. coli* exposed to *C. aurantifolia* juice exhibited a constant decline (Figure 2C and D). The juice eliminated the *E. coli* colony at 1 h (0 CFU·mL^{-1}) and sustained its bacteriostatic effects thereafter. The aqueous extract of *C. flexuosus* leaves was also effective at inhibiting *E. coli*. The viable counts decreased from 2060 CFU·mL^{-1} at 0 h to 100 (1 h), 20 (2 h) and 0 CFU·mL^{-1} at 3 h. *E. coli* was susceptible to *C. aurantifolia* juice and the aqueous extract of *C. flexuosus* leaves.

Preliminary Studies of Plant Extracts as Potential Hand Sanitiser

Table I shows that the total reduction percentage of bacteria for *C. aurantifolia* juice was 91.72 % on the hands of 17 volunteers. This value was marginally higher than that of the commercial hand wash product (82.87 %). The juice produced a better reduction percentage than the commercial hand wash product and this could be a competent hand sanitiser. The student t-test result indicated no significant difference between the commercial hand wash product and *C. aurantifolia* juice.

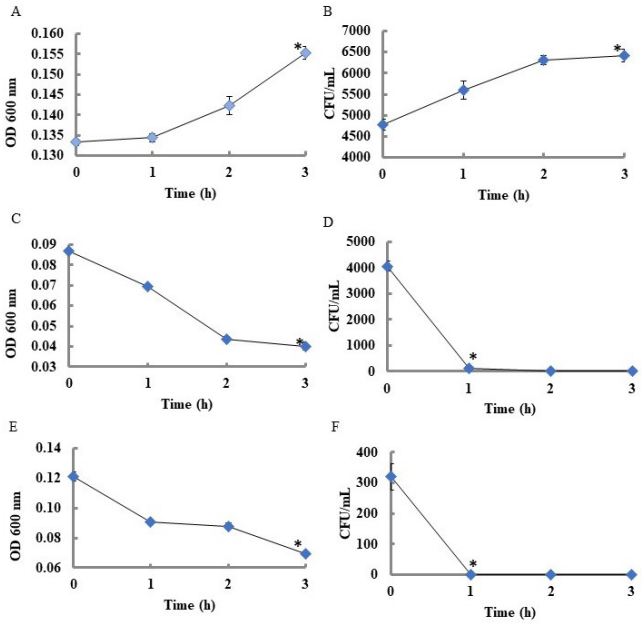

Figure 1: S. aureus growth curve for susceptibility test after exposure to extracts from 1 h to 3 h. A. control OD values, B. control viable count value, C. *C. aurantifolia* juice OD values, D. *C. aurantifolia* juice viable count value, E. aqueous extract of *C. flexuosus* leaves OD values, F. aqueous extract of *C. flexuosus* leaves viable count value. Results are expressed in OD (600 nm) and 103 CFU·mL^{-1}, respectively, with n = 3. The Dunnett T3 test indicated that the OD and viable count values differed significantly from one another (* $p < 0.05$).

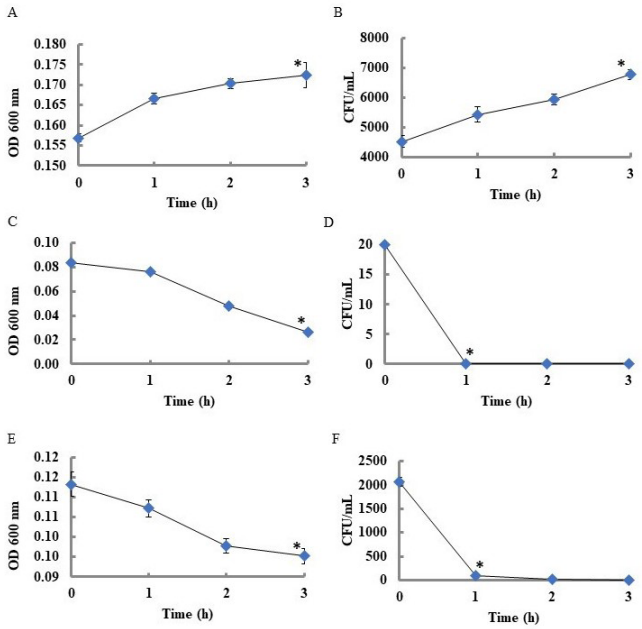

Figure 2: E. coli growth curve for susceptibility test after exposure to extracts from 1 h to 3 h. A. control OD values, B. control viable count value, C. *C. aurantifolia* juice OD values, D. *C. aurantifolia* juice viable count value, E. aqueous extract of *C. flexuosus* leaves OD values, F. aqueous extract of *C. flexuosus* leaves viable count value. Results are expressed in OD (600 nm) and 103 CFU·mL^{-1}, respectively, with n = 3. The Dunnett T3 test indicated that the OD and viable count values differed significantly from one another (* $p < 0.05$).

Table I: Effectiveness of the commercial hand wash product and *C. aurantifolia* juice (%) as hand sanitiser

	Min (CFU per plate)		
	Pre-washing	Post-washing	Mean % Colony Reduction[a]
Commercial hand wash (n = 17)	1128.47	211.24	82.87
C. aurantifolia juice (n = 17)	1480.59	101.18	91.72

[a] The mean percentage of colony reduction of each individual CFU value

DISCUSSION

Many studies have proven the relationship between chemical constituents of plants with antimicrobial activities and capable to control microbial growth (16-19). The phytochemicals from secondary metabolites can act as plant defense mechanism to protect them from herbivores, insects and microorganisms (20). Tannins, saponin, phenolic compounds, essential oils and flavonoids exert antimicrobial properties (21). In the present study, *S. aureus* and *E. coli* were sensitive to *C. aurantifolia* juice and the aqueous extract of *C. flexuosus* leaves.

Many studies have been conducted on the antimicrobial activities of *C. flexuosus* extracts (22-25). The major components of *C. flexuosus* oil are neral (citral-b), geranial (citral-a) and myrcene (26), and its water extract contains linalool oxide and epoxy linalool oxide (27). Linalool possesses antimicrobial activity against *S. aureus*, *E. coli* (0157:H7), *Bacillus subtilis, Enterobacter cloacae, Micrococcus flavus, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella enteritidis and Salmonella typhimurium* (28). *C. aurantifolia* juice is also an antimicrobial agent (29, 30) and mainly contains citric acid (31). Citric acid, which belongs to the flavonoid group of plant phytochemicals, exerts antimicrobial activities against various microorganisms, such as *S. aureus*, *Enterococcus faecalis, P. aeruginosa, E. coli* and *Salmonella spp.*, in *in vitro* studies (30).

In this study, *C. aurantifolia* juice and the aqueous extracts of *C. flexuosus* leaves effectively eliminated *S. aureus* and *E. coli*. *C. aurantifolia* juice also efficiently removed most of the microorganisms on the volunteers' hand. The percentage of microorganisms after hand washing with *C. aurantifolia* juice was significantly reduced to 91.72 %, which was comparable with that of the commercial hand wash product (82.87 %). Organic acids possess antimicrobial activity, and citric acid is abundant in *C. aurantifolia* juice. Moreover, citric acid, which provides lime juice its acidic pH, is thought to possess an antibacterial activity (32). The mechanism of citric acid inhibition towards Gram-negative bacteria is that it can potentiate the effect of monolaurin against the bacteria (33). The mechanism of Gram-positive bacteria is not clearly known, but their activity is probably caused by their complex relationship to extracellular and soluble proteins, which bind to bacterial cell walls (34-37).

CONCLUSION

This study showed that *C. aurantifolia* juice is a promising alternative for antibacterial agents in hand wash products. The application of plant-based materials in disinfectant product provides a sustainable and environmental friendly to the mother nature. Nevertheless, further studies could be conducted to substantiate the results for mechanism of action in bacterial membrane integrity, membrane potential, internal pH and ATP synthesis. This may include the inhibition studies for other microorganisms, such as fungi.

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