ANTI - BACTERIAL POTENTIAL OF HAND SANITIZER INCORPORATED WITH - *Alpinia malaccensis* - CRUDE EXTRACT

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INTRODUCTION

Hand sanitization and hygiene have become vital practices among the general public to overcome cross-contamination of various microorganisms. Different sanitizers including ethanol-based liquid spray, foam, gels, and soap have been heavily used to sanitize hands, to control the cross-contamination of COVID-19 viral infection.
Alcohol-based hand sanitizers contain 60-95% (v/v) alcohol in water and has the ability to denature proteins of cell walls of microorganisms (Akash *et al.*, 2021). Furthermore, the alcohol-based hand sanitizers in the market have claimed the ability to destroy 99.99% of microorganisms (Surwase *et al.*, 2021). However, alcohols are highly volatile; as a result, alcohols can evaporate from the skin surface; hence, there is no residual antibacterial activity (Bondurant, Duley and Harbell, 2019).

Plants produce naturally derived antimicrobials as secondary metabolites and these are accompanied by anti-infective mechanisms against a broad spectrum of pathogenic microorganisms (Zhang *et al.*, 2019). The combination of non-toxic concentrations of different chemical compounds may insert a synergistic antimicrobial activity by minimizing side effects (Shintre, Gaonkar and Modak, 2007). *Alpinia malaccensis* (Ran keeriya) is a perennial plant, which is native to Indonesia and Malaysia. This plant is one of the 230 species of the Zingiberaceae family. This plant is rhizome-producing and grows in the tropical and subtropical regions of Asia (Juwitaningsih, Juliawaty and Syah, 2016). Previous studies have identified 1' Acetoxy chavicol acetate (1'ACA) as the bioactive chemical compound in the hexane extract of *A. malaccensis* rhizome (Somarathna *et al.*, 2018). 1'ACA has strong antibacterial activities against microorganisms specifically against *Staphylococcus aureus* (Weerakkody *et al.*, 2011), and *Listeria monocytogenes* (Somarathna *et al.*, 2018, 2020). 1' ACA has demonstrated efficiency in the elimination of multi-drug resistant bacteria such as *Salmonella* Typhi, *Pseudomonas aeruginosa*, *Escherichia coli*, Vancomysin resistant *Enterococcus* (Latha *et al.*, 2009). In addition, 1'ACA is a very effective phytochemical for inhibiting the function of HIV-1 virus activity (Ye and Li, 2006). Eye irritation toxicity levels were evaluated with Hens Egg Test ChoriAllantoic Membrane (HET-CAM) assay and results showed that 5 mg/ml and 20 mg/ml concentrations of crude galangal extract were non-irritant on CAM surface (Karunarathna *et al*.,2018). Moreover, oral toxicity studies in the rat model showed that *A. malaccensis* n-hexane extract 2000 mg/ kg body weight did not produce any adverse effect on behavior, body weight, feed intake, biochemical parameters, and organ histology (Somarathna *et al.*, 2021).

There is a requirement to develop a sanitizer having a multiple mode of action in controlling a broad spectrum of microorganisms. Therefore, it was speculated that adding the *A. malaccensis* crude extract could exert synergistic antimicrobial activity than solely using Isopropyl Alcohol (IPA). *A. malaccensis'* active chemical compound 1'ACA is not volatile. There is no published data on the development of hand sanitizer using *A. malaccensis* crude extract. The main objective of this study is to determine the efficacy of the alcohol-based hand sanitizer with *A. malaccensis* crude extract compared to the WHO formulation (75% isopropyl alcohol) and commercial product.

MATERIALS AND METHODS

Extraction of Herbs

Fresh *A. malaccensis* was purchased from Nature's Secrets (Pvt) Ltd, Millewa, Horana, Sri Lanka. Fresh *A. malacccensis* rhizomes were cleaned using running water and the outer skin was removed.

The cleaned rhizomes were sliced and oven-dried at 40ºC for 10 hours (Model NB-7500E, Japan). The slices were ground (Prestige PMG 02, India) for 2 hours at 3-minute intervals. The ground powder was stored at -20ºC until use. Ethanol was used as the solvent for extraction. The extract was prepared by adding 20 g of *A. malaccensis* powder to 200 ml of 96% ethanol. The content was agitated (140 rpm) for 24h at 28ºC in a rotary shaker (Bibby scientific limited, Stone, Staffordshire, ST15 OSA, UK). The mixture was filtered using a Buncher funnel with No 1 Whatman filter paper under a vacuum. The filtrate was evaporated to dryness by using a rotary evaporator (Bouchi Labortechnik AG 9230 Flawil, Switzerland) under a vacuum at 40ºC water bath. Finally, the concentrated extract was redissolved in ethanol (96%) to make a 0.5 g/ml stock solution, and was stored at 4 ºC until use (Weerakkody *et al.*, 2011).

Formulation of hand sanitizer

Hydrogen peroxide (3%) was added to a flask containing Isopropyl alcohol (99.8%). Next, Glycerol (98%) was added gradually and a uniform mixture was prepared. From the *A. malaccensis* 0.5 g/ml stock solution, 1 ml and 2 ml were added after that, to make 5 mg/ml and 10 mg/ml formulations respectively. The final volume is made up to 100 ml using deionized water and the mixture was vortexed to get a homogenous solution (WHO, 2010).

Testing for micro-organisms

Antibacterial activities of test sanitizers were determined against gram-positive *S. aureus* 113, *L. monocytogenes,* and gram-negative *E. coli, S.* Typhimurium. Source of culture collection for *S. aureus* 113 and *L. monocytogenes* V7 were obtained from the University of Queensland, Brisbane, Australia. *E. coli* ATCC 1858 and *S.* Typhimurium were obtained from the American Type culture collection (Manassa, USA).

Bacterial strains were confirmed using the Gram staining method and Biochemical methods (Catalase test). Baird Parker Agar with Egg Yolk Tellurite Emulsion was used for the identification of *S. aureus* 113. *E. coli* was identified using violet red bile glucose agar medium. *S.* Typhimurium was identified using xylose lysine deoxycholate agar medium. All bacterial strains were maintained in 80% glycerol at -20 ºC as frozen stock cultures. Working cultures were maintained in Nutrient Agar.

Disc diffusion assay

The antibacterial activity of sanitizers was checked by using the disc diffusion method (Somarathna *et al.*, 2018). A single colony of bacteria was grown for 18± 2h. The content was centrifuged at 9000 g for 10 min to obtain a bacterial pellet. The supernatant was removed and the bacterial pellet was re-suspended in 1 ml sterile 0.85% NaCl and serially diluted in 9 ml of sterile 0.85% NaCl to obtain $5x10^5$ CFU/ml. This procedure was carried out for all bacterial strains separately. 100 µl of each diluted bacterial suspension was spread on Mueller-Hinton agar plates. An aliquot of 10 µl of test sanitizer was pipetted onto sterile paper discs (5.5 mm diameter, Whatman no.1) on the agar surface. Incubation was carried out for 18 h at 37 ºC. Each experiment was repeated in triplicate. The antibacterial activity was analyzed by measuring the Diameter of the Inhibition Zone (DIZ) in millimeters.

Finger Imprint method

The study was performed on 7 healthy volunteers without any clinical signs. They were asked to rub both hands thoroughly before the experiment. Under the aseptic condition, four fingers (thumb wasn't used) of both hands were firmly pressed on the surface of the nutrient agar plates and incubated at 37 ºC for 24 hours as the control. Test sanitizers 2 ml of each with 10 mg/ml *A. malaccensis* was applied on the right hand and WHO formulation (without *A. malaccensis*) was applied on the left hand. The volunteers were asked to rub each palm with fingers in both hands

separately for 30 seconds. As earlier, finger imprints were taken on agar plates at 0 minutes and incubated at 37 ºC for 24 hours. A similar test was performed at 2, 5, 10, and 15 minutes. After incubation, colonies were observed and counted using a colony counter. Percentage reduction in the bacterial load was calculated as, % R = [(BBW – BAW)/BBW] × 100, Where BAW is bacterial load after sanitizer use at 0, 2, 5, 10, and 15 minutes respectively and BBW is the bacterial load before sanitizer use.

pH determination

A digital pH meter was used to determine the pH measure for eight weeks at room temperature (27ºC) (Aodah *et al.*, 2021).

Statistical analysis

The results were expressed as mean ± standard deviation (SD) for analysis performed in triplicates for Disk diffusion assay. Statistical analysis of the data was performed by analysis of variance and mean comparison using the Tukey test utilising the general linear model procedure in software Minitab version 17. $P<0.05$ was considered statistically significant.

Statistical analysis of the data was performed by analysis of variance and mean comparison using the Tukey test utilising the general linear model procedure in software Minitab version 17 for Finger Imprint method.

RESULTS AND DISCUSSION

Disc diffusion assay

Antimicrobial activity (Diameter Inhibition Zone) of treatments is shown in Table 1. There was a significant difference among treatments. The commercial sanitizer showed significantly ($p<0.05$) lower DIZ 14.33±0.58 for *S. aureus* 113 compared to other treatments. Commercial hand sanitizers may not have used the standard formula or perhaps the antimicrobial properties have degraded while in storage. Therefore, the addition of *A. malaccensis* crude extract could enhance the efficacy of the available commercial sanitizer.

In addition, there was no significant difference between *A. malaccensis* containing sanitizers and 75% Isopropyl alcohol standard hand sanitizers. This may be due to the incompatibilities of polar ethanol solvent to perform *A. malaccensis* activity. Zhang *et al*., (2021) found that the main antibacterial compounds (1'ACA) of *A. galanga* have low polarities because the highest DIZ values are exhibited with non-polar solvents such as n-hexane and chloroform. It can be concluded that to exhibit the antibacterial properties of 1'ACA phytochemical needs a solvent with low polarity because, in a polar solvent, 1'ACA becomes an unstable form. Furthermore, there was no significant difference ($p>0.05$) in DIZ observed for sanitizers against *E.coli*, *L. monocytogenes* V7, and *S.* Typhimurium. Therefore, further studies are needed with a higher concentration of extract, to see synergistic antimicrobial activity on the broad spectrum of microorganisms including fungus and viruses.

Table 1: Antimicrobial activity (Diameter Inhibition Zone) of treatments

Micro-organism	Diameter of Inhibition zone (mm)			
	T1	T2	T3	T4
S. aureus 113	$23.33\pm1.15^{a*}$	23.67 ± 0.58^{a}	23.67 ± 0.58^{a}	**14.33 ± 0.58^{b}**

E. coli	13±1[a]	13.67±1.15[a]	14±2.65[a]	13±1[a]
L. monocytogenes	12±0 [a]	13.67±0.58 [a]	12.33±0.58 [a]	13.33±0.58 [a]
S. Typhimurium	12.67±0.58 [a]	13.67±0.58 [a]	13.33±0.58 [a]	13±1 [a]

T1-5 mg/ml+75% Alcohol hand sanitizer, T2-10 mg/ml+75% Alcohol hand sanitizer, T3-75% Alcohol hand sanitizer, T4-Commercial hand sanitizer
*Within a row mean values with the same lowercase letter are not significantly different (p >0.05).

Finger imprint method

The reduction percentage of microorganisms on fingers by the WHO formula and 10 mg/ml *A. malaccensis* sanitizer over time is shown in fig 1. There was no significant difference (p>0.05) in reduction % between WHO and 10 mg/ml *A. malaccensis* hand sanitizer with the time.

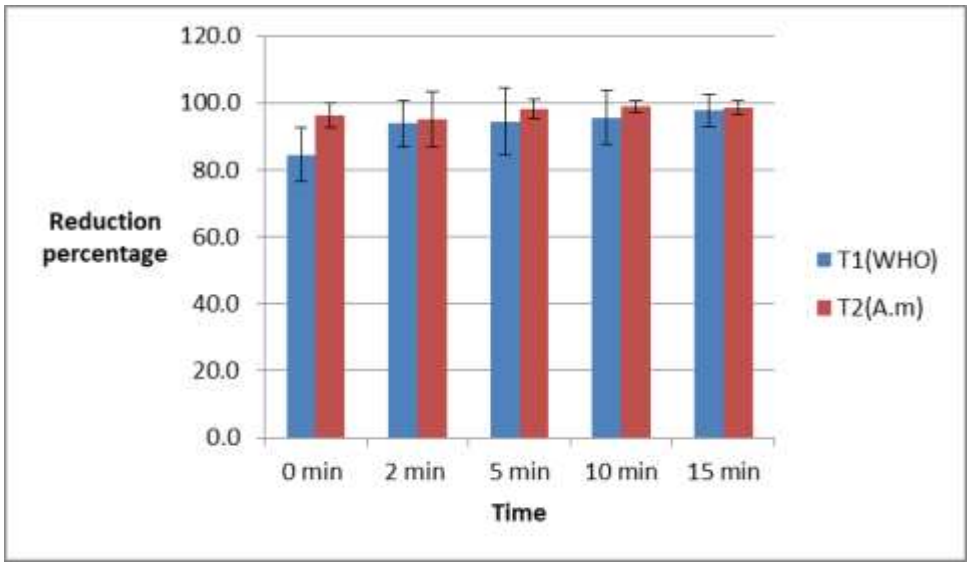

Figure 1: Reduction percentage with time

Previous studies showed higher DIZ (40±0.5 mm) against *S. aureus* 113 for *A. malaccensis* hexane extracted crude extract dissolved with DMSO (Somarathna *et al.*, 2018). However, our study showed DIZ (23.67±0.58 mm) against *S. aureus* 113 ethanol extracted crude extract 10 mg/ml with 75% alcohol sanitizer. According to the polarity of the used solvents, this could happen. It is not safe to use hexane as a solvent to obtain crude extract for hand sanitizer. In addition, the efficacy can change due to various factors such as provenience of the plant material, age, variety, time of harvesting, time of the day, stage of development, freshness, or dryness of the plant material, and isolation technique (Janssen, Scheffer and Baerheim Svendsen, 1987).

pH

Changes in the pH of 10 mg/ml of *A. malaccensis* hand sanitizer did not show a significant difference (P<0.05) over time. The pH was found in the range of 5.21-5.99 which is within a mild acidic and neutral range.

CONCLUSION

The *A. malaccensis* crude extract 10 mg/ml with 75% Isopropyl alcohol sanitizer and standard WHO-recommended Isopropyl alcohol sanitizer had a strong, as well as the same bacterial inhibitory effect than the available commercial sanitizer. This can be due to commercial hand sanitizers not using standard formula or degeneration of their antimicrobial properties while in storage.

This alcohol-based herbal hand sanitizer has a good color and fragrance, hence there was no need to add synthetic colors or fragrances. Therefore, this alcohol-based hand sanitizer which has herbal phytochemical, can be used effectively as a hand sanitizer against the *S. aureus* 113 bacteria.

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