IJAMBR 8 (2020) 73-80 ISSN 2053-1818

International Journal of Applied Microbiology and Biotechnology Research
www.bluepenjournals.org/ijambr

Antibacterial and antibiofilm activity of *Alpinia malaccensis* and *Terminalia catappa* extract combinations on *Staphylococcus aureus* and *Listeria monocytogene* strains

doi.org/10.33500/ijambr.2020.08.008

T. Somarathna[1, 5], W. M. A. D. B. Fernando [2,3], K. K. D. S. Ranaweera[1], G. A. S. Premakumara[4] and N. S. Weerakkody[5]*

[1]Department of Food Science and Technology, Faculty of Graduate Studies, University of Sri Jayewardenepura, Nugegoda, Sri Lanka.
[2]Centre of Excellence for Alzheimer's Disease Research and Care, School of Medical and health Sciences, Edith Cowan University, 270 Joondalup Drive, Joondalup, Western Australia 6027.
[3]Australian Alzheimer's Research Foundation, 8 Verdun St., Nedlands WA 6009.
[4]Industrial Technology Institutes, 393, Bauddhaloka Mw, Colombo, Sri Lanka.
[5]Department of Agricultural and Plantation Engineering, Faculty of Engineering Technology, The Open University of Sri Lanka, Nawala, Sri Lanka.

Article History
Received 17 August, 2020
Received in revised form 14 October, 2020
Accepted 20 October, 2020

Keywords:
Antimicrobial activity, Scanning electron microscopy, Minimum inhibition concentration.

ABSTRACT

A significant antibacterial effect of *Alpinia malaccensis* (Ran-kiriya) against foodborne bacteria *Staphylococcus aureus* and *Listeria monocytogenes* was reported. However, the effect on biofilm formation and impact of the combination of *A. malaccensis* with other plant extracts on foodborne bacteria is unknown. *In-vitro* antibacterial and antibiofilm activity of both plant extracts were determined using micro broth dilution method and 96 well plate respectively. Antibiofilm activity was further confirmed by scanning electron microscopy (SEM). The highest antibacterial activity and biofilm inhibition effect of *A. malaccensis* against *S. aureus* and *L. monocytogenes* were observed at 20 mg ml^{-1} when used alone. The combined plant extract of *A. malaccensis* (2.5 mg ml^{-1}) and *Terminalia catappa* (20 mg ml^{-1}) showed significant (P<0.05) synergistic antibacterial activity against *S. aureus* and *L. monocytogenes* whilst 5 mg ml^{-1} of *A. malaccensis* with 20 mg ml-1 of *T. catappa* showed the highest antibiofilm activity. The strong antibiofilm activity of *A. malaccensis* was further confirmed by morphological deformities of cells, release of cytoplasmic constituents and lower number of attached bacteria cells obtained in SEM analysis. The results suggest that combination of these plant extracts may provide a novel approach for the control of biofilms produced by foodborne *S. aureus* and *L. monocytogenes*. The synergistic effect of crude extracts on microbial growth and biofilm formation could be potentially developed as a natural food preservative or natural sanitizer.

Article Type:
Full Length Research Article

INTRODUCTION

Biofilm-associated foodborne contaminants outbreaks caused by *Listeria monocytogenes* and *Staphylococcus aureus* are a serious public health problem in many countries. National Institute of Health of USA (1997)

reported that 80% of the bacterial infections have been associated with biofilms. Biofilms are sessile microbiological communities where cells are attached to a surface with aggregation substances and extracellular surface proteins which enhance increased antibiotic resistance (William et al., 2018).

The main extracellular surface proteins secreted by *S. aureus* are staphylococcal enterotoxins (SEs), which are highly stable, resistant to environmental conditions like drying, freezing variations (Alžbeta et al., 2017). These toxins are also resistant to proteolytic enzymes like pepsin or trypsin, which allows them to survive in the gastrointestinal tract for a longer period. *L. monocytogenes* is gram-positive and cause both invasive and non-invasive infections such as listeriosis (Galié et al., 2018; Sandasi et al., 2010). The ability of *S. aureus* and *L. monocytogenes* to form biofilms improves their survival and growth in food processing plants, providing an additional physiological advantage to be less sensitive to antibacterial agents (Lakicevic et al, 2017; Rakholiya and Chanda, 2012). On food processing surfaces and medical devices, the bacterial resistivity could increase as biofilms act as a barrier for the available sanitizers or antibacterial agents to penetrate through the extracellular polymeric substances. Therefore, there is a need to envisage novel and safe biomolecules as antimicrobial agents to control biofilms on food processing surfaces and medical devices.

*Alpinia malaccensis*is a perennial plant from Zingiberaceae family, growing widely in the subtropical and tropical regions. The rhizome of *A. malaccensis* is used as a traditional medicine to cure nausea, vomiting and certain wounds, and is also used also as a seasoning ingredient in processed meat (Bhuiyan et al., 2010). In our previous study we identified the chemical constituents in the hexane extract of *A. malaccensis* rhizome and confirmed the bioactive chemical compound as 1'-acetoxychavicolacetate (1'ACA). It is the most abundant (82.87%) chemical compound of the crude extract. Crude *A. malaccensis* extract as well as the pure active compound 1'ACA showed strong antibacterial activities against *L. monocytogenes* and *S. aureus* (Somarathna et al., 2018). *Terminalia catappa* (Combretaceae) is native to Southeast Asia. It is abundantly grown as a shady tree but in ancient time it has been used for the preparation of traditional medicine (Anand et al., 2015). In our previous study, *T. catappa* also showed pharmacological properties and strong antibacterial activity against gram-positive bacteria, due to the presence of many phenolic chemical compounds including abundantly 2, 5-Furandione (Somarathna et al., 2017).

Literature indicates that the use of combined plant extracts may exert multiple modes of inhibition on microbes, which may reduce the development of resistance to antimicrobials (Moussaoui and Alaoui, 2016). Further, relatively scarce information is available on the synergistic effect of *A. malaccensis* and *T. catappa* on antibiofilm activity (Letícia et al., 2019). It is therefore essential that further research is undertaken to determine the combined effects of plant extracts against bacteria in planktonic and sessile forms. In this context, the objective of this study was to investigate the synergistic antibacterial and antibiofilm potentials of *A. malaccensis* and *T. catappa* extracts against *L. monocytogenes* and *S. aureus*.

MATERIALS AND METHODS

Plant materials

A. malaccensis rhizomes were collected from Nature Secret (Pvt) Ltd, Millewa, Horana, Sri Lanka, authenticated using a key and the description (Dassanayake and Fosberg, 1983) and a reference sample was kept at the herbarium of the Department of Agricultural and Plantation Engineering, The Open University of Sri Lanka. *T. catappa* red fruits were collected from the Open University of Sri Lanka. Fresh *A. malaccensis* rhizomes were cleaned under running water and peeled off and sliced. Fresh red color outer pericarp of *T. catappa* fruits were oven-dried and ground (National super blender, Taiwan, MX-TIIOPN) for 1 min to make a fine powder and stored at 4°C until use.

Plant extraction

Two different solvents, hexane and ethanol were used to extract *A. malaccensis* and *T. catappa* plant extracts respectively. Hexane or ethanol extracts were prepared by adding 10 g of *A. malaccensis* or *T. catappa* powder in to 100 ml of hexane and agitated for 24 h at 30°C in a rotary shaker (Stuart® SSL1, Staffordshire, UK). Mixture was vacuum filtered using a Buchner funnel with No 1 Whatman filter paper. The filtrate was evaporated under vacuum at 40°C using a rotary evaporator (KIA RV 5, Switzerland) and concentrated extract was sterilized through a 0.45 µm filter unit (Millex® HA, Germany). Concentrated extract was evaporated to dryness through nitrogen fluxing and dissolved in dimethyl sulfoxide (DMSO) to make a 0.5 gml^{-1} stock solution. Stock solutions were stored at 4°C until use.

Test microorganism

Bacterial strains, *S. aureus* 113 and *L. monocytogenes* Scott A serotype 4b were provided by the University of

*Corresponding author. E-mail: nweer@ou.ac.lk. Tel: +94112881396.

Queensland, Brisbane, Australia. Both organisms were tested for synergistic antibacterial activity and antibiofilm activity. Glycerol stock cultures of each organism were prepared and kept at -20°C prior to use. Unless otherwise stated, bacteria were incubated at 37°C, maintained on tryptic soy agar (TSA) (Basingstoke, UK) slants and sub cultured weekly. A single colony of bacteria was grown in 2 ml tryptic soy broth (TSB) at 37°C for 18 h. The content was centrifuged (Centurion scientific Ltd, UK) at 10000 g for 10 min to obtain the bacterial pellet. Supernatant was removed and the bacterial pellet was re-suspended in 1 ml of sterile 0.85% NaCl solution to obtain 5 x 10^8 cfu ml^{-1}.

Antibacterial effect of plant extracts

An aliquot 50 µl (5 × 10^8 cfu ml^{-1}) of bacterial suspensions was added to each 5 ml Mullen Hilton broth (MHB) tube to give ~10^5 cfu ml^{-1}. The previously determined (Somarathna et al., 2018) minimum inhibition concentration (MIC) values of, *A. malaccensis* and *T. catappa* extracts against *S. aureus* 0.625 and 5 mg ml^{-1} respectively were used in this study. Two fold *A. malaccensis* different MIC concentrations such as (1.25, 2.5 and 5 mg ml^{-1}) with two times of MIC (10 mg ml^{-1} and 20 mg ml^{-1}) of *T. catappa* were evaluated according to the method Somarathna et al. (2018). Three drops (81.56 mg) of Tween 20 were added as emulsifier to the MHB media. Inoculated test tubes were vortexed to solubilize the content and incubated at 37°C for 24 or 48 h. An aliquot of 100 µl was obtained from each treatment after 24 and 48 h incubation and serially diluted in to 900 µl of 0.85% NaCl and 100 µl was plated onto TSA. Inoculated plates were incubated at 37°C for 18 h and visible colonies were counted. MHB, 1.75 % DMSO and Tween 20 were used as negative controls.

Antibiofilm activity of *A. malaccensis*

Inhibition of initial cell attachment

The two fold *A. malaccensis* different MIC concentrations such as 1.25, 2.5, 5, 10 and 20 mg ml^{-1} were evaluated for potential anti-adhesion properties against *S. aureus* and *L. monocytogenes* using 96 well plate (Grotenier, Germany) method (Sandasi et al., 2010). Plant extract 100 µl from each concentration was added to 96-well plate in three replicates. Equal volumes of commercial disinfectant containing active ingredients of alkyl *di*-methylbenzyl ammoniumchloride alkyl alcohol ethoxylate and sodium carbonate in (AAS) and sodium hypochlorite were added as standards.

Negative control was maintained without adding plant extract. A 100 µl of the standardized culture (1.0 × 10^6 cfu ml^{-1}) was pipetted into the same wells to yield a final volume of 200 µl in each well. The plates were wrapped loosely with parafilm and incubated at 37°C for 8 h without shaking allowing cells to attach to the surface. Following incubation, supernatant of each well was removed. Wells were rinsed three times with sterile distilled water to remove loosely attached cells and non-adherent planktonic cells. The plates were oven-dried at 60°C for 45 min. Then wells were stained with 50 µl crystal violet (0.01%) for 3 min. The plates were rinsed three times with sterile distilled water to remove unabsorbed stain. The wells were de-stained by adding 200 µl of 95% absolute ethanol and transferred to a new 96 well plate. Then the absorbance of 96 well plates was measured at optical density 595 nm using a micro plate reader (model s/n MPN 5405, UK). Experiment was repeated independently three times with triplicates. Percentage of the inhibition was obtained using the following formula.

$$\text{Percentage inhibition} = \frac{\text{OD Negative control} - \text{OD Experimental}}{\text{OD Negative control}} \times 100$$

Inhibition of biofilm formation and development

Biofilm formation was achieved after adding 100 µl of a standardized 1 × 10^6 cfu ml^{-1} of *S. aureus or L. monocytogenes* cultures into a 96 well plate. The plate was incubated for 4 h at 37°C without shaking allowing the cells to attach the surface. Then, 100 µl of each plant extract at different MIC concentrations was added in triplicates. Also equal volumes of MHB, streptomycin, and commercial disinfectant were added in to separate wells to maintain negative and positive standards controls, respectively according to the method Sandasi et al. (2010). The plates were further incubated at 37°C for 24 h. Inhibition of biofilm growth and development was determined by crystal violet assay and percentage inhibition was calculated as described above in inhibition of initial cell attachment method.

Inhibition of biofilm formation

Similar to the above method, *S. aureus* or *L. monocytogenes* biofilms were allowed to form for 18 h at 37°C incubation (Sandasi et al., 2010). Then, 100 µl of each plant extract at different MIC concentrations was added in triplicates. The negative, positive and media control were maintained. The plates were incubated for another 24 h at 37°C with shaking. Inhibition of biofilm formation was determined using crystal violet assay, and percentage of inhibition was calculated using the formula as given above in inhibition of initial cell attachment method.

Combination antibiofilm effect of *A. malaccensis* and *T. catappa* plant extracts

Similar to the method described as above in inhibition of biofilm formation *S. aureus* or *L. monocytogenes* biofilm were allowed to form for 18 h at 37°C incubation. Then, 100 µl of *A. malaccensis* extracts at different concentrations (A1: 1.25, A2: 2.5, and A3: 5 mg ml^{-1}) and *T. catappa* (B: 20 mg ml^{-1}) were pipetted to a 96- well plate in triplicate wells respectively. Then, combination of A1B, A2B and A3B extracts were made using 100 µl of *A. malaccensis* (A; 1.25, 2.5 and 5 mg ml^{-1}) in a separate Eppendorf tubes and each concentrations were mixed with 4 µl of 0.5 g ml^{-1} of *T. catappa* (20 mg/ml) extracts. Three drops of Tween 20 (81.56 mg) was added as emulsifier and vortexed before transferring 100 µl to wells. Assessment of biofilm formation was done according to the method described above in the inhibition of initial cell attachment method.

Scanning electron microscopy of antibiofilm activity of *A. malaccensis*

Three sterilized 0.5 mm^2 stainless steel pieces were aseptically placed in a sterilized three separate McCartney bottles. One ml of standardized 1 × 10^5 cfu ml^{-1} of *S. aureus* or *L. monocytogenes* culture were added and placed at 37°C for 24 h for biofilm formation. The content of each bottle was aspirated and washed three time using 0.85% NaCl to remove planktonic cells. Then, 1 ml of 20 mg/ml of *A. malaccensis* extract, 0.04% v/v of AAS commercial disinfectant and MHB broth were transferred to each McCartney bottles as test sample, standard and media control, respectively. The McCarthy bottles were incubated for another 24 h at 37°C. Then, content was aspirated and stainless steel surfaces were washed using distilled water and dehydrated using 30, 40, 50, 60, 70 and 90% ethanol solution (Salimena et al., 2014). The stainless steel surfaces were transferred to sterile Petri dishes and sublimation was performed using freeze dryer at −105°C under a vacuum of 10^{-7} mbar (Leica EM MED020) for 16 h full sublimation. The samples were coated with gold 1 mm^2 under plumbing machine for 1 min before SEM imaging. SEM was carried out using a Zeiss Auriga Dual-Beam FIB-SEM (Carl Zeiss Microscopy) on accelerating voltage of 1 kV using an Everhart–Thornley secondary electron detector. Furthermore, ten images at 15000x magnification were randomly selected for each sample and cell counting was performed manually in each image where only enumerating cells that were either clearly rod-shaped or round.

Statistical analysis

Antibacterial and antibiofilm activity experiments were independently replicated three times. The complete randomized triplicate data were subjected to analysis of variance of the general linear model using SPSS statistical software, version 16 for Windows (MinitabInc; Stateee College, PA, USA). Dunnet simultaneously test was used to compare the difference with the corresponding control at the level of $P< 0.05$.

RESULTS AND DISCUSSION

Synergistic antibacterial activity

The results of the effect of *S. aureus* and *L. monocytogenes* by single and combined *A. malaccensis* and *T. catappa* plant extracts are presented in Table 1. In our study we considered a combination of medicinal plant extracts as synergistic, if it resulted in a >2 log reduction in a viable count of bacteria after 24 or 48 h, as compared with the most active single plant extract alone (Wagner, 2011; Moellering and Weinberg, 1971).

A. malaccensis extract 1.25 and 2.5 mg/ml showed a significant ($p<0.05$) reduction in *S. aureus* viability at 24 and 48 h as compared to the control. With respect to the synergistic activity, the combination A2B showed a significantly ($p<0.05$) higher inhibition 2.25 log reduction against *S. aureus* at 48 h compared to respective extract alone. Similarly, the combination A3B showed significantly higher inhibition of 3.43 log reduction against *S. aureus*. However, individual plant extracts did not show significant ($p>0.05$) inhibition on *L. monocytogenes* at 24 h. The combination A2B and A3B showed > 2 log reduction 2.12, 2.46 log reduction after 24 h respectively and 3.26, 2.46 log reduction after 48 h against *L. monocytogenes* respectively, compared to their extracts alone.

A. malaccensis showed higher antibacterial activity at a concentration of 2, 4 and 8 times of MIC level. The addition of *T. cattapa* extract synergistically enhanced the antibacterial activity of *A. malaccensis.* This may be due the presence of phenolic compounds of *T. cattapa* with the major none phenolic 1'ACA chemical compounds of *A. malaccensis*. We enumerated the viable counts from broth dilution assay and observed that the viable counts are much more reliable as a result than the physical observation of presence or absence of bacterial growth in determination of MIC value. The visibility of the presence or absence of bacterial growth was impaired by the pigments of plant extracts.

In this study, synergistic antibacterial activity (>2 log reduction of cfu ml^{-1}) was observed when *A. malaccensis* 2.5 or 5 mg ml^{-1} was combined with *T. catappa* 20 mg ml^{-1} against *L. monocytogenes* at both 24 and 48 h, and against *S. aureus* at 48 h compared with their respective extract alone. Similar synergistic effect was reported by Weerakkody et al. (2011a) when combined *Alpinia galanga* (1.25 mg ml^{-1}) with *Rosmarinus officinalis* (2.5 mg

Table 1. Antibacterial activity of *A. malacensis* and *T. catappa* extracts.

Bacteria	Plant	24 h(log CFU mL^{-1})	48 h(log CFU mL^{-1})
S. aureus 113	*A1(1.25 mg/ml)	8.11±0.49^{a**}	7.76±0.16^{a}
	A2(2.5 mg/ml)	6.73±0.49^{bc}	7.59±0.89^{a}
	A3(5 mg/ml)	5.67±0.43^{c}	5.78±2.76^{b}
	B(20 mg/ml)	8.01±0.71^{a}	8.24±0.14^{a}
	A1B	6.82±0.23^{bc}	7.30±0.35^{a}
	A2B	6.45±0.35^{c}	5.34±0.84^{b}
	A3B	4.65±0.52^{d}	2.45±0.76^{c}
	Control(MHB)	8.61±0.31^{a}	8.19±0.34^{a}
	Control (1.75% DMSO)	8.37±0.09^{a}	8.34±.032^{a}
	Control(Tween 20)	8.77±0.04^{a}	8.54±0.15^{a}
	Initial Count	5.84±0.41^{c}	Nd
L. monocytogenes	A1(1.25 mg/ml)	8.98 ± 0.13^{a}	7.15±0.04^{b}
	A2(2.5 mg/ml)	8.37±0.37^{a}	8.36±0.18^{a}
	A3(5 mg/ml)	7.93±0.46^{a}	6.49±0.00^{b}
	B(20 mg/ml)	8.83±0.90^{a}	6.94±0.46^{b}
	A1B	8.51±0.03^{a}	8.34±0.00^{a}
	A2B	6.25±0.45^{b}	5.10±1.33^{c}
	A3B	5.47±0.00^{b}	4.47±0.00^{c}
	Control(MHB)	9.25±0.48^{a}	7.87±1.23^{a}
	Control (1.75% DMSO)	8.30±0.58^{a}	8.24±0.15^{a}
	Control(Tween 20)	8.62±0.42^{a}	8.35±0.18^{a}
	Initial Count	6.55±0.51^{b}	Nd

1, A2, A3, A. malaccensis*, B, *T. catappa*; ND, Not determined. **Means of inhibition of each bacteria with different letters in a colomn differ significantly (P<0.05) compared to its corresponding control.

ml^{-1}) against *S. aureus* 113. According to Wagner (2011), lower concentrations of agents are necessary to achieve synergism as compared with single plant extract alone. Therefore, in our study the lowest concentration A2B was selected as the best combination for inhibiting *S. aureus* and *L. monocytogenes*. We speculate that the activity of *A. malaccensis* extract against gram-negative bacteria may have been enhanced by the addition of polyphenols present in the *T. catappa* ethanol extracts. The mechanism of synergism may be due to cell wall damage cause by few compounds which could then facilitate the penetration of other compounds leading to the damage of intracellular targets (Galié et al., 2018; Elaine et al., 2006).

Antibiofilm activity of *A. malaccensis*and*T. catappa*

Antibiofilm activity of *A. malaccensis* and/or *T. catappa* against *S. aureus* and *L. monocytogenes* is shown in Table 2. *A. malaccensis* showed the highest (p<0.05) inhibition of cell attachment 98.5 and 97.6% and biofilm formation 73.5 and 67.3% at 20 and 10 mg ml^{-1} concentrations, respectively, while the inhibition was 81.1% at 20 mg ml^{-1} when applied alone against *S. aureus*. Similar percentage of biofilm inhibition in cell attachment, growth and formation were observed with commercial sodium hypochlorite and AAS commercial disinfectant. On the other hand, 20 mg ml^{-1} of *A. malaccensis* extract showed significantly higher (p<0.05) biofilm inhibition of adhesion (81.4%), growth (76.8%) and formation (73.4%) for *L. monocytogenes*. In addition, commercial sodium hypochlorite and AAS showed somewhat lower inhibition in cell attachment, growth and formation respectively for *L. monocytogenes* compared to *S. aureus* biofilm inhibition.

The synergistic antibiofilm effect against *S. aureus* of *A. malaccensis* and *T. catappa* A3B combination showed significantly higher inhibition on initial cell attachment, growth and development and biofilm formation 98, 91 and 69% respectively than their single extracts alone. Similarly, A3B combination showed significantly higher inhibition on initial cell attachment, growth and development and biofilm formation 70, 75 and 71% respectively for *L. monocytogenes* than single plants extracts alone. It confirms that the lower concentration of the *A. malaccensis* (5 mg ml^{-1}) with *T. catappa* (20 mg ml^{-1}) exert synergistic biofilm activity higher than *T. catappa* (20 mg ml^{-1}) extract alone. However, *L. monocytogenes* biofilm inhibition was comparatively low.

Table 2. The effect of plant extracts on biofilm-adhesion, growth and formation.

Plant extract	Concentration	% Inhibition of *S. aureus*113			% Inhibition of *L. monocytogenes*		
		Initial cell attachment	Growth and development	Biofilm formation	Initial cell attachment	Growth and development	Biofilm formation
A. malaccensis	A0*	90.3^{c}	65.3^{e}	52.4^{d}	50.8^{k}	52.4^{j}	42.1^{j}
	A1	94.2^{b}	70.2^{d}	57.5^{c}	57.8^{i}	59.2^{h}	52.0^{h}
	A2	95.7^{b}	74.0^{c}	61.0^{c}	69.7^{f}	66.2^{g}	50.4^{i}
	A3	96.1^{b}	74.0^{c}	63.6^{c}	65.8^{g}	66.4^{f}	62.8^{f}
	A4	97.6^{a}	75.4^{c}	67.3a^{b}	70.5^{e}	71.3^{e}	64.1^{e}
	A5	98.5^{a}	81.1^{b}	73.5^{a}	81.4^{b}	76.8^{b}	73.4^{b}
T. catappa	B	95.8^{b}	32.1^{g}	12.5^{e}	56.1^{j}	51.2^{i}	55.2^{g}
A. malaccensis× *T. catappa*	A1B	97.2^{b}	56.8^{e}	42.1^{d}	55.0^{j}	44.1^{k}	64.4^{e}
	A2B	97.4^{b}	61.1^{f}	48.8^{d}	62.4^{h}	54.1^{i}	66.9^{d}
	A3B	97.9^{a}	90.7^{a}	68.8a^{b}	69.7^{f}	74.8^{c}	70.5^{c}
Control -MHBonly		0.0	0.0	0.0	0.0	0.0	0.0
Control -MHBwith MO		0.0	0.0	0.0	0.0	0.0	0.0
Streptomycin(100 µg/ml)		98.0^{a}	70.0^{d}	74.0^{a}	79.0^{c}	ND	73.5^{b}
Sodium hypochlorite	0.01%	98.3^{a}	81.6^{b}	74.0^{a}	78.0^{d}	72.9^{d}	73.0^{b}
AAS disinfectant	0.04%	99.5^{a}	82.2^{b}	72.0^{a}	99.2^{a}	82.3^{a}	78.0^{a}

**A. malaccnesis*; A0= 0.625, A1 = 1.25, A2= 2.5, A3= 5.0, A4=10, A5= 20 mg/ml; *T. catappa*; B= 20 mg/ml; ND: not determine. **Means of inhibition of each bacteria with different letters in a colomn differ significantly (P<0.05).

Scanning electron microscopy of biofilm inhibition

The effects of *A. malaccensis* on *S. aureus* and *L. monocytogenes* biofilm inhibition are shown in Figures 1 and 2 respectively. Lower number of attached cells appeared in 20 mg/ml *A. malaccensis* treated stainless steel surfaces compared to the control. Similarly, AAS disinfectant (0.04%) treated surface leaded to the lower number of attached cells. *Staphylococcus aureus* cells treated with *A. malaccensis* and AAS showed multi cellular clumps (Figure 1a and b). In addition, *A. malacccensis* treated surface appeared with deformed cells where cellular content had released through damaged cell wall (Figure 1a). Similarly, less number of *L. monocytogenes* cells appeared in both treated surfaces while *A. malaccensis* treated cells appeared damaged (Figure 2a) with extra cellular content (Figure 2b). Significant biofilm formation inhibition 80.68% was reported when 20 mg/ml *A. galanga* crude extract treated against *S. aureus* developed biofilm on stainless steel surfaces (Karunarathne et al., 2020). Where same major chemical compound 1´acetochavicol acetate was found to be present in both *A. galanga* and *A. malaccensis* extracts.

It is reported that bacterial cell death occurs mainly due to increased permeability of cell membrane followed by the loss of cytoplasmic content (Weerakkody et al., 2011a). These results are in agreement with Weerakkody et al. (2011a) and showed 1'ACA primarily damages the cell envelop. This leads to irreversible change to the morphology of *S. aureus* cells that appear roughand/or deflated. Further, 1'ACA either directly or indirectly has effects on cellular membrane integrity. Moreover, according to Weerakkody et al. (2011b), 1' ACA has a specific mode of action where 1'ACA do not induce general stress response leading to concurrent virulence gene up regulation in bacteria. White square particles

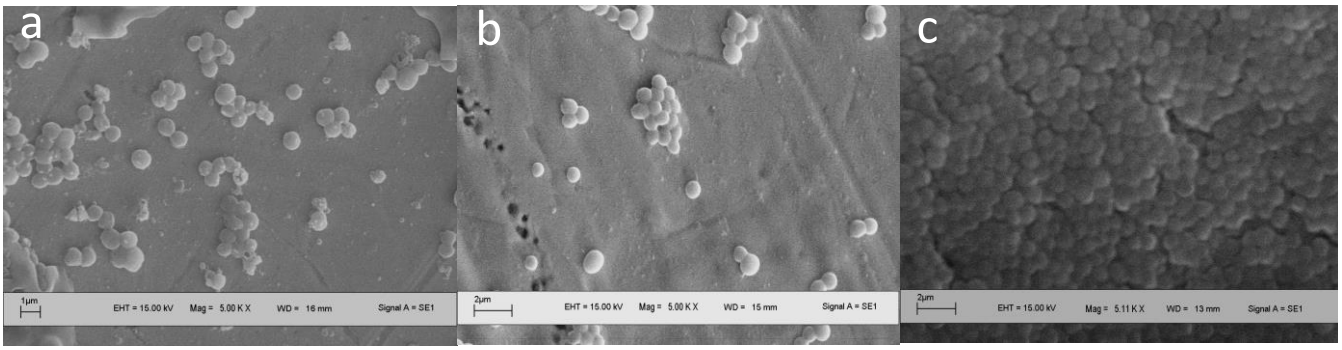

Figure 1. SEM image of *S. aureus* 113 biofilm on stainless steel surfaces. **a,** *A. malaccensis* concentration at 20 mg/ml; **b,** AAS commercial disinfectant (0.04%); **c,** control (TSM with bacteria only).

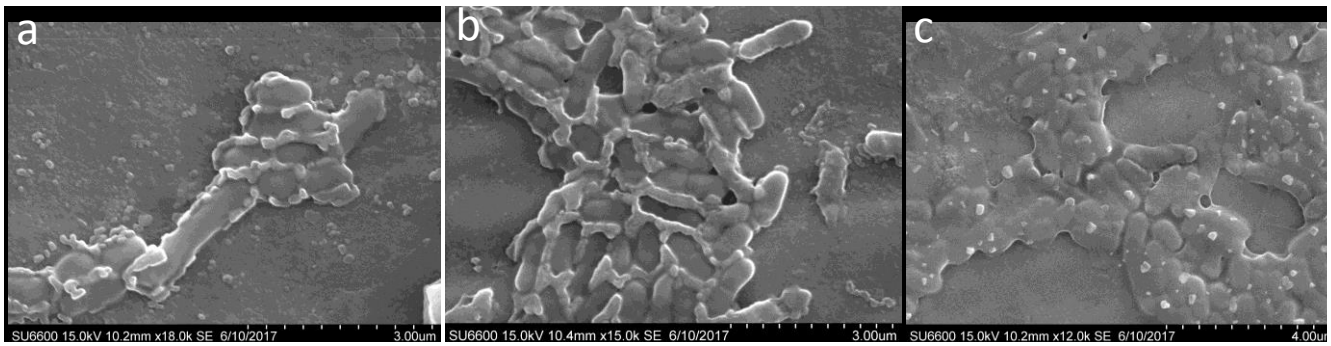

Figure 2. SEM image of *Listeria monocytogenes* biofilm on stainless steel surfaces. **a,** *A. malaccensis* concentration at 20 mg/ml; **b,** AAS commercial disinfectant (0.04 %); **c,** control (TSM with bacteria only).

observed in the samples (Figure 2) are remnants of sodium saline solution used to wash the cells in preservation of SEM method. This confirms the requirement of further washings to remove sodium remnants.

The 96 well plate assay method is the most well-known assay method for the detection of biofilm formation (Peter et al., 2008). Biomass of the biofilm structures of cell attachment, growth and formation were different with the time duration for the growth of biofilm after 0, 4 and 18 h. We observed the inhibition of cell attachment, growth and formation against for *S. aureus* and *L. monocytogenes* when increasing the *A. malaccensis* concentration. The highest biofilm inhibition percentage for cell attachment, growth and formation was recorded on 20 mg ml^{-1} concentration of *A. malaccensis* for both bacteria tested. It can therefore be postulated that extracellular polymeric substance (EPS) of *S. aureus* or *L. monocytogenes* protected the bacterium from bactericidal effect of plant extract. The other factor could be the negative charge on the EPS that restricts the penetration of molecules by charge attraction, thereby imparting resistance to the biofilm. Further, it could be due to the degradation or inactivation of the antimicrobial agent and efflux pumps that expel drugs or antimicrobial agents. On the other hand, enhanced biofilm formation was observed due to conditioning film effect of some extracts which may promoting microbial adhesion (Sandasi et al., 2008).

The biofilm inhibition effect of single extracts *A. malaccensis* was enhanced by combination with *T. catappa.* We observed that combination of 5 mg ml^{-1} of *A. malaccensis* with 20 mg ml^{-1} of *T. catappa* inhibition is much higher than the highest concentration of 20 mg ml^{-1} of *A. malaccensis* when used alone. According to our previous study, major chemical compound (82.87 %) and bioactive compound of *A. malaccensis* hexane extract is 1'ACA which is a non-phenol responsible for exerting strong antimicrobial activity. *Terminalia catappa* contains 2, 5-Furandione, as the major chemical compound with other phenolic compounds (Somarathna et al., 2017). It can be suggested that synergistic inhibitory effect of the non-phenolic active compound of *A. malaccensis* will be enhanced by phenolic compounds of *T. catappa*. However, it is important to further investigate the mechanism of action of *A. malaccensis* when combined with *T. catappa* in antimicrobial activity.

Conclusion

The combination effect showed the highest antibacterial and biofilm inhibition against *S. aureus* and *L. monocytogenes* at lower concentrations of *A. malaccensis* combined with of *T. catappa*. Therefore, it is found that *T. catappa* extract enhanced the antibacterial and antibiofilm activity of *A. malaccensis* against *S. aureus* or *L. monocytogenes*. The strong antibiofilm activity of *A. malaccensis* was further confirmed by cell shape deformation, release of cytoplasmic constituent, damaged cell membrane and lower number of attached bacteria cells determined in SEM analysis. Therefore, the results indicate that *A. malaccensis* rhizome extract and *T. catappa* ethanol extract can be used as the potential sources of biopreservative. Further investigations are needed to identify the mode of action of synergistic antibiofilm activity of these plant combinations.

ACKNOWLEDGMENT

The authors wish to thank National Research Council of Sri Lanka for financial support with Grant No 12-054.

Conflicts of interests

The authors and planners have disclosed no potential conflicts of interest, financial or otherwise.

REFERENCE

Alžbeta M., Havlíková A. & Valík Ľ. (2017). *Staphylococcus aureus* enterotoxin production in relation to environmental factors. The rise of virulence and antibiotic resistance in *Staphylococcus aureus*, Shymaa Enany and Laura E. Crotty Alexander, IntechOpen. doi: 10.5772/66736.

Anand A., Divya N. & Kotti P. (2015). An updated review of *Terminalia catappa*. Pharmacogn. Rev. 9(18):93.

Bhuiyan M. N. I., Chowdhury J. U., Begum J. & Nandi N. C. (2010). Essential oils analysis of the rhizomes of *Alpinia conchigera* Griff. and leaves of *Alpinia malaccensis* (Burm.f.) Roscoe from Bangladesh. Afr. J. Plant Sci. 4:197-201.

Dassanayake M. D. & Fosberg F. R. (1983). A revised handbook to the flora of Ceylon. Fourth ed. Washington D.C: Amerindian Publishing Co. Pvt. Ltd. New Delhi.

Elaine B. J. C., Mantovani R. P., Barbosa L. N., Di Stasi L. C. & Fernandes Jr. A. (2006). Synergism between plant extract and antimicrobial drugs used on *Staphylococcus aureus* diseases. Memórias do Instituto Oswaldo Cruz. 101(4):387-390. https://doi.org/10.1590/S0074-02762006000400007

Galié S., García-Gutiérrez C., Miguélez E. M., Villar C. J. & Lombó F. (2018). Biofilms in the food industry: Health aspects and control methods. Frontiers in microbiology. 9: 898. https://doi.org/10.3389/fmicb.2018.00898

Karunarathne P. U. H. S., Thammitiyagodage M. G. & Weerakkody N. S. (2020). Antibiofilm activity of Galangal (*Alpinia galanga*) against *Staphylococcus aureus*. Food Process. Nutr. Sci. 1:123-131.

Lakicevic B. & Nastasijevic I. (2017). *Listeria monocytogenes* in retail establishments: Contamination routes and control strategies. Food Rev. Int. 33:247-269. doi: 10.1080/87559129.2016.1175017

Letícia M. G., Petrus L. B. M., Rafael S. D., Rammon F. N., Fabiana S. F. S., Eduardo M. S., David C. S. F., Flaviane M. G. R., Carlos H. L. R., Andréa D. N. L. & Cristina de Andrade M. (2019). Effect of *Terminalia catappa* Linn. on biofilms of *Candida albicans* and *Candida glabrata* and on changes in color and roughness of acrylic resin. Evidence-based complementary and alternative medicine. Vol. 2019, Article: 7481341. 8p. https://doi.org/10.1155/2019/7481341

Moellering R. C. & Weinberg A. N. (1971). Studies on antibiotic synergism against Enterococci 4C-labeled streptomycin by enterococci. J. Clin. Invest. 50:2580-2584.

Moussaoui F. & Alaoui T. (2016). Evaluation of antibacterial activity and synergistic effect between antibiotic and the essential oils of some medicinal plants. Asian Pac. J. Trop. Biomed. 6(1):32-37.

National Institutes of Health, USA. (1997). Minutes of the National Advisory Dental and Craniofacial Research Council—153rd Meeting, Bethesda, MD.

Peter E., Nelis H. J. & Coyene T. (2008). Comparison of multiple methods for quantification of biofilm growth on micro tier plates. J. Microbiol. Methods. 72:157-165.

Rakholiya K. & Chanda S. (2012). *In vitro* interaction of certain antimicrobial agents in combination with plant extracts against some pathogenic bacterial strains. Asian Pacc. J. Trop. Biomed. 2(3 SUPPL.): S876-S880. S876-S880 http://doi.org/10.1016/S2221-1691(12)60439-0

Salimena A. P. S., Santos A. C., Cardoso M. D. G., Alves E. & Piccoli R. H. (2014). Scanning electron microscopy of biofilm formation by *Staphylococcus aureus* on stainless steel and polypropylene surfaces. Afr. J. Microbiol. Res. 8:3136-3143.

Sandasi M., Leonard C. M. & Viljoen A. M. (2008). The effect of five common essential oil components on *Listeria monocytogenes* biofilms. Food Control. 19:1070-1075.

Sandasi M., Leonard C. M. & Viljoen A. M. (2010). The *in vitro* antibiofilm activity of selected culinary herbs and medicinal plants against *Listeria monocytogenes*. Lett. Appl. Microbiol. 50:30-35.

Somarathna T., Fernando W. M. A. D. B., Ranaweera K. K. D. S., Premakumara G. A. S., Abeysinghe T. & Weerakkody N. S. (2018). Antimicrobial activity and phytochemical screening of *Alpinia malaccensis* (Ran-kiriya) against food-borne bacteria. J. Appl. Microbiol. 125:1276-1285.

Somarathna T., Premakumara G. A. S., Akbarsha M. D., Balamuthu K. & Weerakkody N. S. (2017). *Invitro* antibacterial activity of selected underutilized plants and cytotoxic property of *Terminalia catappa*. Int. J. Pharm. Pharm. Sci. 9:218-225.

Wagner H. (2011). Synergy research: Approaching a new generation of phytopharmaceuticals. Fitoterapia. 82:34-37.

Weerakkody N. S., Caffin N., Lambert L. K., Turner M. S. & Dykes G. A. (2011a). Synergistic antimicrobial activity of galangal (*Alpinia galanga*), rosemary (*Rosmarinus officinalis*) and lemon iron bark (*Eucalyptus staigerana*) extracts. J. Sci. Food Agric. 91:461-468.

Weerakkody N. S., Caffin N., Lambert L. K., Turner M. S. & Dykes G. A. (2011b). Purified 1'acetoxychavicol acetate (1' ACA) from galangal spice affects membrane fatty acid composition and triggers a cell envelope stress response in *Staphylococcus aureus.* Int. J. Antimicrob. Agents. 39:263-272.

William H. B., Robert A. B., Hui W., Hyun K. & Oral B. (2018). Pathogens, matrix, and polymicrobial interactions in microenvironments. Trends in Microbiology. 8:877-886. https://doi.org/10.1016/j.tim.2017.09.008.