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Antibacterial Activity of Pyroligneous Extract from *Rhizophora Apiculata* against *Escherichia Coli, Staphylococcus Aureus,* and *Salmonella Cholerasuis*: New Insights on their Feasibility as a Natural Disinfectant

Chee Loong Teo*

Department of Research and Development, Agri Season Sdn. Bhd. No 30, Lot 2718 Jalan Kejayaan 1, Batu 24, 81900 Kota Tinggi, Johor, MALAYSIA.

*Email: anthony1109@hotmail.my

Abstract: The increasing number of varieties of infections, contaminations, and diseases caused by bacteria, even multidrug-resistant bacteria appear to become a critical issue. Besides, artificial chemical-based disinfectant also brings adverse effects to humans and animals. This study aims to investigate pyroligneous extract as the alternative green potential disinfectant. A quantitative suspension test (EN 1040) was used to evaluate the bactericidal activity. On the obtaining results, the bioprocess kinetics of different bacteria strains to pyroligneous extract-based disinfectants were further investigated. Also, the disinfectant's physicochemical characteristics were analysed. This study resulted that the bioactivity reduction percentage and mean log reduction: *Escherichia coli* (ATCC 8739) (100%, 6), *Staphylococcus aureus* (ATCC 6538) (100%, 5.9243), and *Salmonella cholerasuis* (ATCC 10708) (100%, 5.9868) respectively. For bioprocess kinetics analysis, the specific reduction rate and halve rate: *E.coli* (2.7631, 3.9872), *S.aureus* (2.7282, 3.9368), and *S.cholerasuis* (2.7570, 3.9784) respectively. Physicochemical properties are acidic (pH 2.7), low density (1.0019 $g.ml^{-1}$), and low viscosity liquid (10.6 cps). These findings showed that the ability of pyroligneous extract-based disinfectants to reduce the bacteria population with applied appropriately.

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*Corresponding Author:

Chee Loong Teo,
Department of Research and Development,
Agri Season Sdn. Bhd. No 30,
Lot 2718 Jalan Kejayaan 1, Batu 24,
81900 Kota Tinggi, Johor, MALAYSIA
Email: anthony1109@hotmail.my

1. Introduction

Infections, diseases, and contaminations by bacteria such as *Salmonella sp*., *E. coli* or *Staphylococcus aureus* already become a critical problem to human health and animal life. Every year, around 450 deaths, 23,000 hospitalizations, and 1.2 million illnesses caused by *Salmonella sp*. in the United States (USA) due to the consumption of contaminated food [1]. Besides, *E. coli* which found and exists in the food, environment and humans and animals' intestine [2] caused a global morbidity and mortality in animals and humans including gut and extraintestinal infections illness [3, 4]. *Staphylococcus aureus* is part of the human resident microbiota, generally colonizing mucosa and skin of healthy people, being a versatile pathogenic microorganism capable of causing a lot of human diseases. This pathogen is gram-positive cocci, about 1.0 mm in diameter, catalase-positive, non-endospore forming, immobile, and usually non-encapsulated [5]. The majority of pathogen caused by nosocomial infections and transmitted by insects is *Staphylococcus aureus*. In the infection process, the pathogen must adhere to epithelial cells, allow the pathogen to receive the nutrients for multiplication, and secrete molecules that can adversely influence infected tissues of individuals [6].

Disinfectants are commonly used in majority countries and are essential to achieve health, hygiene, and quality standards. Normally, disinfectant is applied in the agricultural field, food processing, domestic households, health care industry even pharmaceutical and cosmetics products [7]. Also, the increasing of awareness to the role of contaminated surrounding surfaces within the health care settings and house as a potential disease transmission vector [8, 9]. The application of disinfectant has been used to solve the contaminated environment and has been proven significantly in infectious disease spreading prevention [10, 11]. The efficacy of these standard disinfection methods on product application is well-characterized [12, 13].

Unfortunately, numerous pathogenic microorganisms that have undergone significant changes in antimicrobial susceptibility [14]. Although utilization of disinfectants and antiseptics is an important way to stop the pathogenic microorganism spreading in the hospital, a lot of the findings found that antiseptic susceptibility to the pathogen is decreasing. Those pathogenic microorganisms have been isolated from a clinical sample which proved that required minimal inhibitory concentration against antiseptics increased [15-19] These pathogenic microbes resistant are the main causes for nosocomial cross-infection in hospital and some infection is fatal [20]. Kearns et al. (1995) also reported that gram-positive bacteria resistance to disinfectants and antiseptics. Three *Enterococcus spp.* can survive in five minutes in 100 ppm chlorine present, while only can survive two minutes for non-resistant Enterococcusin in 0.5 ppm chlorine present [21]. The number of multidrug-resistant bacteria issues increased extremely since the last decade, including the prevalence, the bacterial number, and the proportion population (Roca et al., 2015). Antibiotic and multidrug-resistant bacteria have been considered as a critical worldwide disease [22]. Multidrug-resistant (MDR) bacterial could cause forming a post-antibiotic era and a lot of antibiotics still under development process [23].

Due to disinfectant and antiseptic resistance, these issues already triggering people begin emphasis the utilization of disinfectant [24, 25]. One of the reasons, the unexpected emergence of resistance of disinfectant issues can be attributed to the misuse or abuse of disinfectants along with insufficient knowledge regard biosecurity principles [22, 24]. The possibility of rising cell resistance when using antimicrobial agents, including disinfectants [26]. So, the selection of disinfectant and antiseptic to avoid rising bacterial resistance is crucial [27].

Besides, some disinfectants and antiseptics have some common characteristics such as urticaria, irritation, and contact dermatitis must carefully apply at the minimal bactericidal concentration in controlling pathogenic microorganism contamination, although in clinical practice, the hygiene concern is very important to prevent pathogenic bacteria spreading in any potentially contaminated area such as utensils, room, and hands [20]. Thus, the discovery of alternative disinfectant agents with cost-effective and green chemical are important challenge for public health and the human community.

Pyroligneous acid, also called pyroligneous extract is produced from the condensation process of liquid vapor during charcoal production [28]. Generally, pyroligneous acid contains 10-20% of water, formic acid, syringols, methanol, acetone, isoeugenol, vanillin, ketones, ester, and more than 200 hundred compounds [29] depending on the wood species. In several studies reported that pyroligneous extract tested as an antifungal agent [30], anticandidal agent [31], and anti-bacterial agent [32].

However, in recent researches regard bactericidal efficacy of plant-based disinfectants against *Escherichia coli*, *Staphylococcus aureus* and *Salmonella cholerasuis* has not been investigated further. Therefore, in this study the three strains that major caused infections, contamination, or illness (*Escherichia coli* ATCC 8379, *Staphylococcus aureus* ATCC 6538 and *Salmonella cholerasuis* ATCC 10708) and dilute pyroligneous extract as potential disinfectant were selected. The antimicrobial effects of pyroligneous extract-based disinfectant on the selected microbial strains' percentage of reduction and its bioprocess kinetics were explored. Also, the pyroligneous extract as potential disinfectant's physicochemical characteristic such as pH value, viscosity value, and its density were analysed.

2. Materials and Methods

2.1 Test Strains and Inoculum preparation

Escherichia coli (ATCC 8739), *Staphylococcus aureus* (ATCC 6538), and *Salmonella cholerasuis* (ATCC 10708) were selected. The bacteria had been incubated on suitable agar until it was ripe and fruiting at 36-37ºC.

2.2 EN 1040:2005 analysis

A quantitative suspension test (EN 1040:2005) was selected in this testing. Dilute pyroligneous extract as a potential disinfectant is added to a test suspension of bacteria. The mixture is maintained at (20 ± 1) °C for 5 minutes ± 10 seconds. At the end of this contact time, an aliquot is taken; the bactericidal activity in this portion is immediately neutralized. The numbers of surviving bacteria are determined, and the reduction is calculated.

2.3 Bacterial activity analysis

2.3.1 Mean number of cells, mean log, percentage of reduction, log reduction, and log growth

The bacterial activity was determined by the plate count method with the colony counter (Funke Gerber, Colony Star 8502-3952). Each experiment was performed in duplicates to ensure reproducibility of results. Then, bacterial strains' bioactivity and its bioprocess kinetics were calculated with the Eq. 1 -7:

$$Mean\ nuumber\ of\ cell\ (m), CFU/g = \frac{Sample\ 1 + Sample\ 2}{2} \quad (1)$$

$$Mean\ log = log_{10}\, m \quad (2)$$

$$Percentage\ of\ reduction, \% = \frac{Initial\ value - Sample\ value}{Initial\ value}\ X\ 100\% \quad (3)$$

$$log\ growth = \log_{10} Sample\ value - \log_{10} Initial\ value \quad (4)$$

$$log\ reduction = \log_{10} Initial\ value - \log_{10} Sample\ value \quad (5)$$

$$Specific\ growth\ rate\ (\mu) = \frac{\ln(N_2 - N_1)}{t_2 - t_1} \quad (6)$$

$$Specific\ reduction\ rate\ (v) = \frac{\ln(N_1 - N_2)}{t_2 - t_1} \quad (7)$$

where N_2 and N_1 represent bacterial number concentrations at time t_2 and t_1, respectively whereas k_1 represents the time taken to duplicate the bacterial division rate for control and k_2 represents the time take to halve the bacterial halve rate for sample, evaluated according to the Eq. 8 and 9.

$$k_1 = \frac{\mu}{0.693} \quad (8)$$

$$k_2 = \frac{v}{0.693} \quad (9)$$

2.4 Physicochemical test

The acidity was measured using a pH meter (Trans instrument, Bench Top Professional pH meter BP 3001, Singapore) calibrated in standard buffer solutions (pH 4.0, 7.0, and 10.0). The viscosity was measured using a digital viscometer (Brookfield, RVDVE, Canada). The weight of the sample was measured by electronic balance (Shimadzu, TX323N, Japan) and converted to density according to Eq. 10.

$$Density, p = \frac{m}{v} \quad (10)$$

where m and v represent sample mass and sample volume respectively.

3 Results and Discussions

3.1 Effect of plant-based disinfectant against different bacterial strains

Antibacterial activity of pyroligneous extract-based disinfectant with a variety of bacteria strains within 5 minutes contact time showed in figure 1. The pyroligneous extract-based disinfectant achieved 100% of reduction to *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (ATCC 6538), and *Salmonella cholerasuis (*ATCC 10708). The control showed a negative percentage of reduction (*Escherichia coli* ATCC 8739: -5%, *Staphylococcus aureus* ATCC 6538: -2 and *Salmonella cholerasuis* ATCC 10708: -2%) represented that the bacteria strains test in this study is healthy and valid used in the antibacterial analysis. In similar result reported that using silver-ionized pyroligneous extract able to prevent disease caused by pathogenic bacteria *Escherichia coli*, *Salmonella sp*., *Bacillus sp*., *Staphylococcus sp*., *Vibrio sp*., Aspergillus sp., Fusarium sp., *Tricoderma sp*., and *Candida sp*. [33]. Besides, this strong antibacterial activity disinfectant due to its highly phenolic compositions in pyroligneous extract [34].

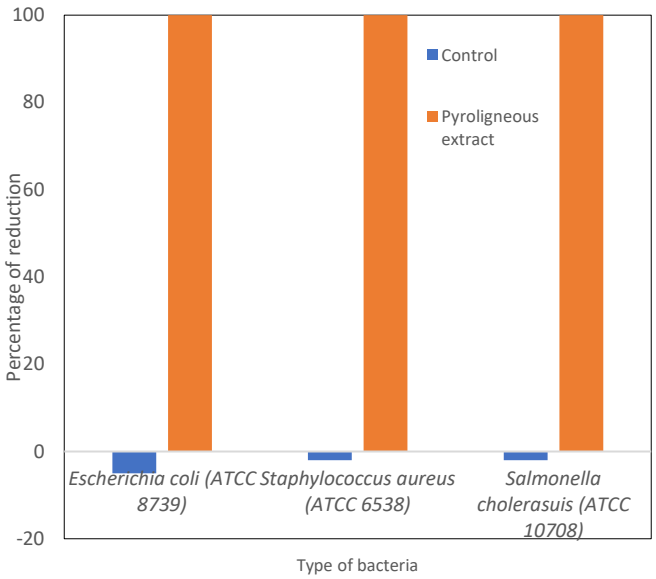

Fig. 1 - Reduction percentage of pyroligneous extract-based disinfectant against *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (ATCC 6538) and *Salmonella cholerasuis* (ATCC 10708).

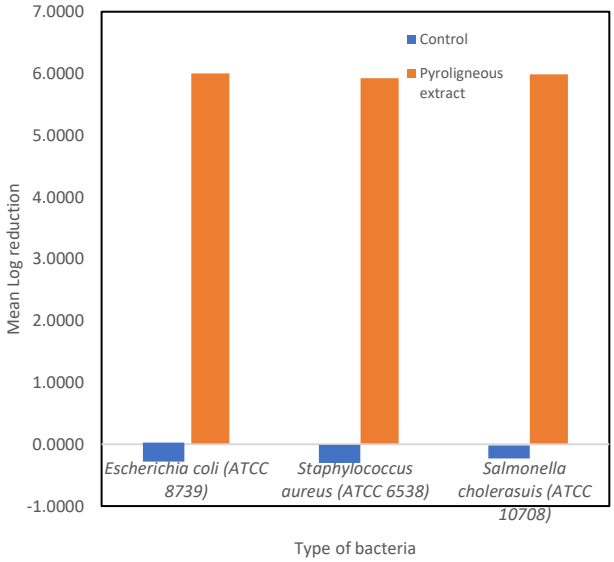

Fig. 2 - Mean log reduction of pyroligneous extract-based disinfectant against *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (ATCC 6538) and *Salmonella cholerasuis* (ATCC 10708).

Figure 2 represented the mean log reduction of pyroligneous extract-based disinfectant against *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (ATCC 6538), and *Salmonella cholerasuis* (ATCC 10708). The mean log reduction to *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (ATCC 6538), and *Salmonella cholerasuis* (ATCC 10708) are 6.0000, 5.9243 and 5.9868 respectively which proved that this disinfectant can reduce bacterial population effectively. In research of Brady et al. (2003) to antimicrobial activity, ≥3.0 log reduction to control consider significant and equivalent [35] and achieved 99.9% effective against a certain bacterium. 6-log reduction represents the number of bacteria on the surface has been reduced by 1×10^6 times.

3.2 Antibacterial bioprocess kinetics analysis to plant-based disinfectant

Table 1 summarizes the antibacterial bioprocess kinetics of pyroligneous extract-based disinfectants against selected strains. The pyroligneous extract showed the promising reduction rate and halve rate to each bacterial strain within 5 minutes exposure time: *Escherichia coli* ATCC 8739 (2.7631 and 3.9872), *Staphylococcus aureus* ATCC 6538 (2.7282, 3.9368) and *Salmonella cholerasuis* ATCC 10708 (2.7570, 3.9784) respectively. This might because pyroligneous extract brings the deleterious effect as one of the reasons for the reduction in the rate of bacterial growth. Among the specific reduction rate and halve rate, *Escherichia coli* ATCC 8739 showed the highest rate compare to the other bacterial strains. It is because pyroligneous extract-based disinfectant able to reduce *Escherichia coli* ATCC 8739 population faster than *Staphylococcus aureus* ATCC 6538 and *Salmonella cholerasuis* ATCC 10708 within the same contact time and area. Table 2 showed the physicochemical properties of pyroligneous extract-based disinfectant. From the analysis, this disinfectant agent is acidic (pH 2.7), low density (1.0019 $g.ml^{-1}$), and low viscosity liquid (10.6 cps).

Table 1 - Antibacterial bioprocess kinetics of pyroligneous extract-based disinfectant against *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (ATCC 6538) and *Salmonella cholerasuis* (ATCC 10708)

		***Escherichia coli* (ATCC 8739)**		***Staphylococcus aureus* (ATCC 6538)**		***Salmonella cholerasuis* (ATCC 10708)**	
	Time, min	**Specific growth rate [a]**	**Division rate [c]**	**Specific growth rate [a]**	**Division rate [c]**	**Specific growth rate [a]**	**Division rate [c]**
Control	0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	5	2.1640	3.1226	2.0043	2.8921	1.9232	2.7751
		Specific reduction rate [b]	**Halve rate [d]**	**Specific reduction rate [b]**	**Halve rate [d]**	**Specific reduction rate [b]**	**Halve rate [d]**
Pyroligneous extract	0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	5	2.7631	3.9872	2.7282	3.9368	2.7570	3.9784

[a] Specific growth rate, min^{-1}
[b] Specific reduction rate, min^{-1}
[c] Division rate, min^{-1}
[d] Halve rate, min^{-1}

Table 2 - Pyroligneous extract-based disinfectant ‘s physicochemical analysis

Physical and chemical parameter	**Value**	**Unit**
pH	2.7000	-
Viscosity	10.600	cps
Density	1.0019	$g.ml^{-1}$

4 Conclusion

The bioactivity reduction of pyroligneous extract as potential plant-based disinfectant determined by a quantitative suspension test (EN1040). In this research, pyroligneous extract from *Rhizophora apiculata* showed significant and equivalent antibacterial effect to *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella cholerasuis*. Also, within only 5 minutes of contact time, the pyroligneous extract able to reduce the number of bacteria to zero. These in vitro test studies showed the pyroligneous extract-based disinfectant consider an effective antimicrobial agent that provides environmental control of harmful bacteria in the disinfectant treatment area.

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