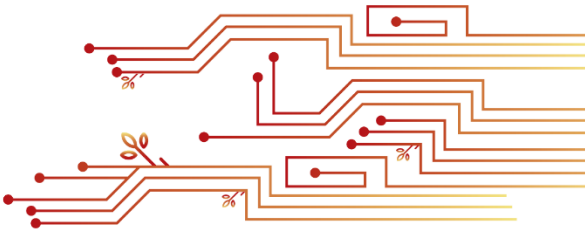

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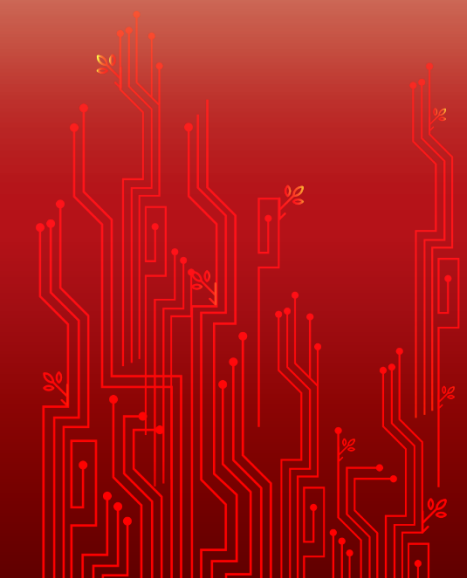

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B2_011_PF: ANTIBACTERIAL AND ANTIBIOFILM ACTIVITIES OF RAMBUTAN (*Nephelium lappaceum* L.) PEEL EXTRACT ON *Vibrio parahaemolyticus* and *Escherichia coli* ISOLATED FROM FOODS

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Abstract: *Vibrio parahaemolyticus* and *Escherichia coli* are significant food-borne pathogens associated with acute diarrhea in human. The formation of biofilms by these bacteria plays various roles in nature and also food safety. The aim of this study were to investigate the antibacterial and antibiofilm activities of rambutan peel extract (RPE) on food isolates of *V. parahaemolyticus* and *E. coli*. RPE had the minimum inhibitory concentrations (MICs) values of 0.5 to 1 mg/ml for *V. parahaemolyticus* and ≥4 for *E. coli* isolates. Meanwhile results of minimal bactericidal concentrations (MBCs) values were in the range of 2 to 4 and >4 mg/ml for *V. parahaemolyticus* and *E. coli*, respectively. Moreover, RPE inhibited the biofilm formation and caused the reduction of pre-formed biofilm of both *V. parahaemolyticus* and *E. coli* at the sub-MIC concentrations (0.5 MIC and 0.25 MIC). The results revealed that RPE exhibits antibacterial and antibiofilm activities against *V. parahaemolyticus* and *E. coli*, which can be considered as an alternative substance for inhibit biofilms or removal of these pathogens on food contact surfaces.

Introduction: *Vibrio parahaemolyticus* and *Escherichia coli* are common causative agents of diarrhea diseases worldwide, especially in developing countries. *V. parahaemolyticus* is a halophilic bacteria found in marine environments and can be transmitted to human by consumption of seafood [1]. *E. coli* is normally found as a normal microbiota in worm-blooded animals including humans [2]. Some of this species are pathogens causing food-borne diarrhea such as enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and enteroaggregative *E. coli* (EAEC) [3]. Department of Medical Sciences, Ministry of Public Health, Thailand, regulated *V. parahaemolyticus* should not be detected in 25 g of raw seafood and for *E. coli* should not be detected higher than 100 MPN in 1 g of raw meat. For food safety reason, various antibiotics and chemical substances were used during food production and processing to reduce number of these pathogen. There are several reports demonstrated the ability of these bacterial to form biofilms on different types of food contact surfaces which make it difficult to eliminate them and the resistant to sanitizer sodium hypochlorite has been reported [4],[5],[6]. Several natural extracts derived from plants demonstrated antibacterial and antibiofilm properties [7],[8],[9]. In contrast to chemical synthesis substances, they are more acceptable and have been proposed as the alternative way to avoid the problem of some chemical residues which will be harmful to consumers [10]. Rambutan (*Nephelium lappaceum* L.) is commonly grown in every part of South East Asia, including Thailand and rambutan peel is considered as fruit waste. Rambutan peel contains many phytochemical compounds such as saponin and tannin which exhibit various biological activities [11]. Tadtong *et al*. revealed that rambutan peel extract (RPE) contains antibacterial activity against *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA) and *Streptococcus mutans* [12]. Moreover, inhibition of *Enterococcus faecalis*, *S. epidermidis*, *Pseudomonas aeruginosa*, and *Vibrio cholerae* has also

been reported [13]. The aim of this study was therefore to evaluate the antibacterial and antibiofilm activities of RPE against *V. parahaemolyticus* and *E. coli*.

Methodology:

Bacterial strains and culture conditions: V. parahaemolyticus and *E. coli* isolated from food samples were used in this study (Table 1). Species identification of all isolates were confirmed by PCR targeted to *toxR* gene for *V. parahaemolyticus* and *uidA* gene for *E. coli* [14],[15]. Bacteria were grown on Mueller Hinton Broth (MHB; beef extract powder, acid digest of casein, starch) and incubated overnight at 37°C under aerobic conditions. Medium supplemented with 1% of NaCl were used for *V. parahaemolyticus*.

Table 1. Bacterial strains used in this study.

Strain	Source
Vibrio parahaemolyticus	
PSU 166, PSU 476, PSU 5382	Clam
PSU 360	Mussel
PSU 513, PSU 582, PSU 4413, PSU 4415	Bloody clam
PSU 3819	Crab
PSU 3831	Fish
Escherichia coli	
PSU 5026, PSU 5027, PSU 5028, PSU 5029 PSU 5030, PSU 4169, PSU 4170	Thai Beef
PSU 4153, PSU 4159, PSU 4164	Malaysian Beef

Preparation of rambutan peel extract (RPE): Fresh rambutan peels were obtained from a market in Hat Yai, Songkhla, Thailand during June to September 2018. The peels were washed under flowing tap water and dried by sun light. A 100 g of dried rambutan peels were soaked for 5 days in 500 ml of methanol, filtered through double layers of muslin, and evaporated using rotatory vacuum evaporator. The crude extract powder was kept protected from light and stored under 4°C for further experiments [16].

Evaluation of Antibacterial activity of RPE: Antibacterial activity of RPE was preliminary performed using the agar-well diffusion technique. Briefly, bacteria lawn was prepared on Mueller-Hinton agar (MHA) and wells (diameter=6 mm) were made with sterile Pasteur pipette. Then, 50 µl of the desired concentrations of RPE (100, 50, 25 mg/ml) were pipetted into the wells. After incubated for 18 hours at 37°C, the diameters of the growth inhibition zone surrounding the well was measured [17].

Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs): The minimum inhibitory concentrations (MICs) and the minimum bactericidal concentrations (MBCs) of RPE were determined in *V. parahaemolyticus* and *E. coli* isolates using broth microdilution method. Briefly, RPE was dissolved in 100% dimethyl sulfoxide (DMSO) and diluted with MHB to obtain concentrations of 1-4 mg/ml. The bacterial culture was adjusted to 0.5 McFarland and was diluted to 10^5 cfu/ml. A hundred µl of bacterial culture was added into each prepared RPE dilution well and incubated for 18 hours. Bacterial growth was determined by 0.1% resazurin solution and the results were recorded after 4 hours [18].

The minimum bactericidal concentrations (MBCs) of RPE were determined at MIC and higher MIC concentration by subculturing on agar plate which do not contain the extract and incubated for 24 hours. The MBCs were recorded by determining the lowest concentration of RPE that reduces the viability of the initial bacterial inoculum by ≥99.9% [19].

Biofilm formation assay of bacterial isolates: Quantitative biofilm measurement was performed in 96-well microtiter plate assay. A 100 µl of each overnight bacterial culture (10^8 cfu/ml) was incubated for 16 hours. To measure biofilm formation, bacterial cultures were poured out, wash three times with distilled water, stained with 0.1% crystal violet solution, and determined the OD_{570} [20].

Evaluation of antibiofilm effect of RPE: The inhibition of biofilm formation and the degradation of pre-formed biofilm were evaluated at subinhibitory concentrations of RPE (0.5 MIC and 0.25 MIC) by a cover slip-based technique.

Inhibition of biofilm was performed using RPE coated glass cover-slip in 6-well microtiter plate. To coat the cover-slips, each well was inoculated with a desired concentration of RPE and left at room temperature for 2 hours. The RPE-coated cover-slips were introduced into new plates containing bacterial culture and were then incubated for 6 hours. A cover-slip from each well was removed, unattached bacterial cells were rinsed off with phosphate buffered saline (PBS), and biofilms were stained with 0.1% crystal violet.

The effect of RPE on the degradation established biofilms was tested as described previously [21] with some modifications. Briefly, a sterile cover-slip in each well was inoculated with 3 ml of overnight culture of a representative isolate of *V. parahaemolyticus* or *E. coli* and incubated for 16 hours in 6 well plate. The cover-slips with biofilm were washed in PBS to remove the unattached cells, placed in new wells, and stained with 0.1% crystal violet.

The stained biofilms in each experiment were observed by light microscope.

Results and Discussion:

Antibacterial activity of RPE: The agar-well diffusion technique is mainly used for screening antimicrobial activity of plant extracts [22]. In this study, RPE was preliminary tested for its antibacterial activity against *V. parahaemolyticus* (n=10) and *E. coli* (n=10) by this method. The results showed that RPE was active against all isolates and the activity against *V. parahaemolyticus* strains were higher than *E. coli* isolate (Table 2). MIC results of RPE were comparable to those obtained in the agar-well diffusion technique. RPE had the MIC value ranging from 0.5 to 1 mg/ml for *V. parahaemolyticus* and ≥4 for *E. coli* isolates. MBC results showed that *V. parahaemolyticus* was more sensitive to RPE than *E. coli* (Table 2). Previous study has been reported about the antibacterial activity of agricultural by-products [23],[24]. The result showed that rambutan peels extracted with water or 95% ethanol were failed to inhibit *V. parahaemolyticus*. However, the MIC of pomegranate peels extracted with ethanol against *V. parahaemolyticus* was 2.5 mg/ml [25]. Another study also showed that the rambutan peels extracted with ether, methanol, and aqueous at the concentration of 2.5 mg/disc had no antibacterial activity against *E. coli* [11]. These might be due to the reasons that the antibacterial activity of extracts was a result of active phytochemical substances contained in the extract. Moreover, extracted solvent, extraction method, or different rambutan varieties can affect the presence of these compounds in the extracts [12],[26].

Table 2. Antibacterial activity of the RPE against *V. parahaemolyticus* and *E. coli* isolates.

Strain	Diameter of inhibition zone (mm)			MIC (mg/ml)	MBC (mg/ml)
	RPE 100 mg/ml	RPE 50 mg/ml	RPE 25 mg/ml		
Vibrio parahaemolyticus					
PSU 166	14.1	12.4	10.5	1	2
PSU 360	17.8	13.6	13.5	0.5	2
PSU 476	15.3	15.2	15.1	1	2
PSU 513	15.0	14.7	14.5	1	2
PSU 582	18.1	17.2	14.4	1	2
PSU 3819	13.9	11.9	11.2	1	2
PSU 3831	15.6	13.6	12.7	1	2
PSU 4413	15.8	15.3	14.1	1	2
PSU 4415	14.9	14.4	12.8	1	4
PSU 5382	15.0	14.2	12.8	1	2
Escherichia coli					
PSU 5026	8.9	NI	NI	4	>4
PSU 5027	9.5	NI	NI	4	>4
PSU 5028	9.6	NI	NI	4	>4
PSU 5029	8.8	NI	NI	4	>4
PSU 5030	9.4	NI	NI	>4	>4
PSU 4153	9.3	NI	NI	4	>4
PSU 4159	9.1	NI	NI	4	>4
PSU 4164	9.8	NI	NI	4	>4
PSU 4169	9.0	NI	NI	4	>4
PSU 4170	9.1	NI	NI	4	>4

NI: no inhibition.

Biofilm production and effect of RPE on bacterial biofilms: All bacterial isolates were capable to form biofilms on 96-well microtiter plate (data not shown). Several studies have reported *V. parahaemolyticus* and *E. coli* isolated from foods had ability to form biofilms [5]. This study evaluated the different concentrations of RPE (0.5 MIC and 0.25 MIC) to affect the adhesion ability and disrupt pre-formed biofilms and found a biomass reduction on the cover-slip of both conditions compared to that of the control. However, RPE was more active against *V. parahaemlyticus* biofilms than that of *E. coli* (Figure 1 and 2). Increasing the exposure time or the concentration of RPE is therefore required to obtain more effective results. Previous studies evaluated the effect of plant-based extracts on biofilm of clinical isolates [25],[27]. Analyses of phytochemical contents revealed the presence of flavonoids, tannins, and coumarines which are capable of reducing biofilm formation [28]. Rambutan peel contains saponins, flavonoids, and tannins [29]. Tannins have been reported to contain anticarcinogenic activity and antimicrobial activity against *E. coli*, *Enterobacter cloacae*, *Clostridium perfringens*, *Salmonella* Typhimurium, and *Bacteroides fragilis* [30]. Flavonoids contain broad spectrum of chemicals and biological activities including antioxidant and free radical scavenging properties. Furthermore, flavonoids have proven to be antibacterial agents which include ability against multi-drug resistant bacterial isolates including *S. aureus*,

B. cereus, *S. enterica*, Enterohemorrhagic *E. coli* O157:H7 and MRSA [31]. Saponin is a surfactant agent which act as a biofilm dispersant by disintegrate bacterial bond in biofilm, degrading biofilm matrix by disturbing biofilm metabolism then release the bond between the bacteria on the biofilm. When the biofilm dispersed, the other active compound such as xanthone, flavonoid, and tannin will come in and damage the bacteria inside biofilm [32].

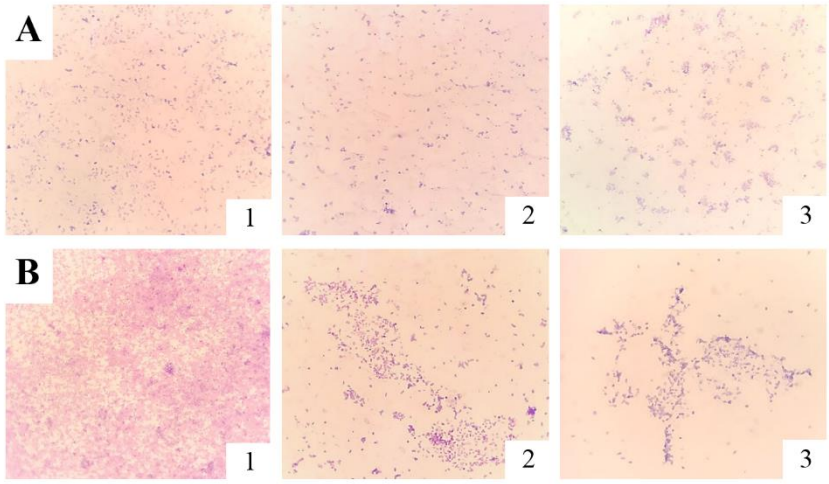

Figure 1. Microscopic analysis of *V. parahaemolyticus* biofilm on glass coverslips in the absence (1) or the presence of RPE at 0.5MIC (2) and 0.25MIC (3). The inhibition of biofilm formation (A) and the degradation of pre-formed biofilm (B) were evaluated.

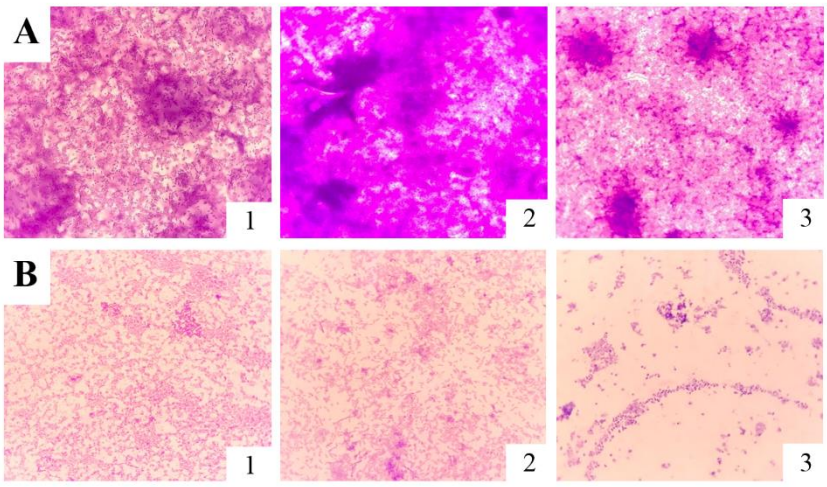

Figure 2. Microscopic analysis of *E. coli* biofilm on glass coverslips in the absence (1) or the presence of RPE at 0.5MIC (2) and 0.25MIC (3). The inhibition of biofilm formation (A) and the degradation of pre-formed biofilm (B) were evaluated.

Conclusion: RPE exhibited antibacterial and antibiofilm activities against *V. parahaemolyticus* and *E. coli.* Therefore, this plant-based compound may be an alternative to chemical for inhibit biofilms or removal of bacteria in food processing environments. Further analyses of the active compounds presence in extract and its mechanisms is needed.

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