The antibiofilm activity of peel extracts of *Mangifera indica* L. (Carabao mango) at different ripeness stages against *Staphylococcus aureus* biofilm

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Abstract

Staphylococcus aureus biofilm is a matrix composed of extracellular polymeric substances (EPS) that protect bacteria and is responsible for antibiotic resistance and many nosocomial infections. It can be broken down by phytochemicals such as tannins, flavonoids, alkaloids, quinones, and saponins found in *Mangifera indica* L. These compounds are influenced by ripening of the fruit. The study focused on determining the ripeness stage that produced the peel extract with the most effective antibiofilm properties. Phytochemical screening and biofilm assay were conducted and absorbance values and percent reduction were obtained. At 50% concentration, none of the samples showed antibiofilm activity. At 25% concentration, all except the extract from ripeness stage 2 fruits exhibited antibiofilm activity. At 10% concentration, all extracts exhibited antibiofilm activity. In conclusion, no trend for antibiofilm activity was observed for ripeness stages. Decreased concentration of extracts yielded greater antibiofilm activity which was contradictory to many cited studies.

Keywords: *antibiofilm, Staphylococcus aureus, Mangifera indica L., ripeness stages, peel extracts*

Introduction. Many nosocomial infections and hospital-acquired diseases persist because some bacteria can form a biofilm, a matrix of hydrated extracellular polymeric substances such as polysaccharides, proteins, and extracellularly released nucleic acids [1,2]. *Staphylococcus aureus* is a commensal bacterium and human pathogen with some strains that are resistant to the antibiotic methicillin [2]. Their bacterial colonies form biofilms protecting them from external factors such as temperature, humidity, and antimicrobial substances. Some antibiotics cannot penetrate the biofilm because of mechanisms such as restricted diffusion into the matrix, expression of multidrug efflux pumps, type IV secretion systems, decreased permeability, and the action of antibiotic-modifying enzymes that form a multi-layered defense [3]. With a biofilm, bacteria can adhere to the surface of medical devices and consequently, be able to spread inside the human body [3,4]. However, studies on the antibiofilm activity have shown that plant extracts are promising alternatives for antibiotics and can be a source of active ingredients in the synthesis of pharmaceutical drugs [5,6,7].

Phytochemicals are antimicrobial agents that have been repeatedly proven to yield significant results in inhibiting biofilm formation and dislodging the biofilm matrix [5]. Such potential antibiofilm agents include tannins, flavonoids, alkaloids, quinones and saponins. Tannins can inhibit and impair biofilm formation and growth such as when tannic acid increases autolytic proteins to make *S. aureus* strain susceptible to its antibiofilm properties [8,9]. Alkaloids also exhibit antibiofilm activity by breaking down complex polysaccharides in the biofilm matrix into simpler oligosaccharides and disaccharides [10]. These polysaccharides serve as the structural component of the biofilm matrix, therefore, breaking them down would make it easier to remove biofilm. These secondary metabolites, as well as the others mentioned, interact with bacterial proteins and cell wall structures by causing damage to cytoplasmic membranes, reducing membrane fluidity, and inhibiting nucleic acid synthesis, cell wall synthesis, or energy metabolism [11]. They can also manipulate certain factors of the environment around bacteria such as glucose, osmolarity, ethanol, hemoglobin, temperature and anaerobiosis which affect biofilm formation and the biochemical processes of the biofilm [9].

In the present study, *Mangifera indica* or carabao mangoes were used because of their abundance and value of production in the Philippines. Flavonoids, saponins, tannins, quinones, and alkaloids are some of the phytochemicals present in the fruit. They are most especially abundant in its peel where total phenolic content and flavonoid content were significantly higher than that of the pulp [10,12,13]. However, the ripening process directly influences the total phenolic content of *Mangifera indica* [14]. Major polyphenolics collectively increased in concentration as the fruit ripened [15]. The phytochemical content of the *Mangifera indica varied* as the fruit ripened.

This study aimed to determine the ripeness stage of *Mangifera indica* (Carabao mango) at which its peel extract exhibits the most antibiofilm activity against *S. aureus* biofilm. It specifically aims:

(i) To determine the phytochemicals present in the peel extracts from *Mangifera indica* at each ripeness stage

(ii) To determine the absorbance values of the negative control and experimental wells after biofilm assay with each peel extract from *Mangifera indica* from each ripeness stage

(iii) To compare the absorbance values of the negative control and experimental wells after biofilm assay with each peel extract from *Mangifera indica* from each ripeness stage

(iv) To calculate the percent reduction of the treatments after biofilm assay with each peel extract from *Mangifera indica* from each ripeness stage

(v) To compare the percent reduction values of negative control and treatments of each peel extract from *Mangifera indica* from each ripeness stage

Methods. This section includes the collection of plant materials, plant extraction, phytochemical screening, biofilm assay, biofilm quantification, data analysis, and safety procedures. The duration of the implementation was three non-consecutive months in the year 2018. Preliminary tasks included obtaining the *Mangifera indica* fruits and the *S. aureus* culture dilution and preparing the requirements for use of laboratories.

Plant Extraction. *Mangifera indica* fruits were monitored daily to determine ripeness stage using a ripeness chart. Peels were separated from the fruit and oven-dried at 37°C for 24 hours. After pulverization, the plant material was macerated for six days with 80% ethanol. Pure extract was obtained after filtered crude extract underwent rotary evaporation at 38°C. It was then used for stock solutions in the following concentrations: 50% (v/v), 25% (v/v), and 10% (v/v) in dimethyl sulfoxide (DMSO). This procedure was repeated for each ripeness stage.

Phytochemical Screening. Crude peel extracts from each ripeness stage underwent phytochemical screening for tannins, flavonoids, alkaloids, quinones, and saponins. Standard protocol for phytochemical tests was followed.

Biofilm Assay. A 96-well microtiter plate was inoculated with 195 µl of *S. aureus* culture dilution and was incubated at 37°C for 20 hours for biofilm formation. Then the plate was washed, air dried and incubated at 60°C for 60 minutes to fix the biofilm. Each well was added with 125 µl of the peel extract stock solutions from different ripeness stages and in three concentrations (50%, 25%, and 10%). After 17 hours of incubation at 37°C, 125 µL of 0.1% crystal violet (CV) solution was added to each well to stain the remaining biofilm. The plate was incubated for 15 minutes, washed four times and turned upside-down to dry overnight.

Biofilm Quantification. After the addition of 125 µL of 30% acetic acid to each well, the plates were incubated for 15 minutes at room temperature. Absorbance was measured at 570 nm using a microplate reader with 30% acetic acid as a test plate to ensure accuracy.

Percent Reduction. Measurement for antibiofilm efficacy is called Percentage Reduction [16]. It was calculated using the absorbance values obtained from the microtiter plate and substituting them into the following equation (Let B denote the average absorbance per well for the blank wells, C denote the average absorbance per well for the control wells, and T denote the average absorbance per well for the treated wells):

$$PR = \frac{((C - B) - (T - B))}{(C - B)}$$

Data Analysis. The data was assumed to have a normal distribution. One-way analysis of variance (ANOVA) statistical analysis with a 95% level of confidence was performed to compare the means of the optical density of the untreated cells in different treatments. Least significant difference post-hoc was done to determine which pairs significantly differ in each set. The same procedures were done for the percent reduction from each ripeness stage to determine biofilm mass reduction.

Safety Procedure. Leftover peels from *Mangifera indica* were placed in a separate bag and thrown into the compost bin. All the chemicals used were disposed in a chemical waste container provided by the laboratory. Leftover media and plates containing bacterial specimens were washed and properly disposed, following the protocol of the Medical Technology Laboratory of University of San Agustin.

Results and Discussion. The aim of the study was to determine the ripeness stage of *Mangifera indica* that was most effective against biofilm. Phytochemical screening for each ethanol peel extract from all ripeness stages and biofilm assay with each extract at 50%, 25%, and 10% concentration were conducted. The results and findings of these tests are presented.

Phytochemical Screening. In table 1, the phytochemical screening of the crude ethanolic peel extracts of *Mangifera indica* showed that tannins, alkaloids, and quinones were present in all ripeness stages. This agrees with the phytochemical screening results of crude methanolic peel extracts [10].

Table 1. Phytochemical screening of crude ethanolic extracts from each ripeness stage.

Phytochemicals	1	2	3	4	5
Tannins	+	+	+	+	+
Flavonoids	+	+	+	+	-
Alkaloids	+	+	+	+	+
Quinones	+	+	+	+	+
Saponins	-	-	-	-	-

"+" denotes presence and "-" denotes absence of the phytochemical in the ethanolic peel extract

Flavonoids, which should have been present, were not found in the last ripeness stage. The phytochemical content might have decreased but this only occurs during the senescent stages of the fruit [17]. Furthermore, flavonoid content of methanolic pulp extract was reported to not significantly differ between ripeness stages or even increased as the fruit ripened [18,14]. The absence of flavonoids in ripeness stage 5 could be attributed to degradation of the

phytochemical during storage. Saponins were not identified in the peel extract from any of the ripeness stages when they should have been present [3]. The leaves of the *Mangifera indica* were also reported to have saponins present in them but their crude methanolic and ethanolic extracts yielded negative test results for phytochemical screening [16,19]. It could not have been that saponins were not extracted as ethanol would be able to and saponins were found to be present in both the peel and leaf extracts [10,20]. The presence of alcohol in the crude extracts could have been interfering with the foam test. The solvent could have dissolved the saponins and disallowed the formation of micelles during the interaction of the phytochemical and water. Hence, the negative results for saponins in the crude ethanolic extracts of *Mangifera indica.*

Biofilm Quantification. The absorbance values indicated the amount of CV dye that has stuck unto the S. aureus biofilm and corresponded to the biofilm mass left in the wells after treatment. In the One-Way ANOVA statistical analysis, the calculated p-value of the absorbance value of the biofilms from each concentration group was less than p=0.05. This indicated that there is a significant difference in biofilm mass within each concentration group. The LSD post hoc was done to compare each treatment within each set and to determine which pairs significantly differ, as seen in Table 2 and Figure 1.

Low absorbance values denoted low amount of remaining biofilm mass. This could imply antibiofilm activity. At 50% concentration, results from treatments from ripeness stages 1, 3, 4, and 5 showed no significant difference from the negative control. They also did not significantly differ from each other. Treatment from ripeness stage 2 was significantly different but its absorbance value implied an increase in biofilm mass. At 25% concentration, treatments from ripeness stage 1, 3, 4, and 5 were effective against the biofilm. The peel extract from ripeness stage 5 was significantly different from the other treatments which were only comparable with each other. Treatments from stage 2 still exhibited higher absorbance values than the others. It showed no significant difference from the negative control and was ineffective in reducing biofilm. At 10% concentration, all treatments were significantly different from the negative control and were effective against the biofilm. Extract from ripeness stage 3 was significantly less effective than the others.

All treatments at 10% concentration and treatments 1, 3, 4, and 5 at 25% concentration were effective in reducing *S. aureus* biofilm. Only a trend where lower concentration of extracts resulted in lesser amount of remaining biofilm mass was observed. This implied that lower extract concentrations would yield lower amount of biofilm mass caused by the antibiofilm activity of the extract. However, this contradicted the linearly increasing trend between extract concentration and antibiofilm activity in extracts containing tannins, alkaloids, quinones, and saponins [3,7,10,21]. Flavonoids and tannins cause bacterial death by inhibiting DNA or RNA synthesis and microbial enzymes, respectively [3]. Increased concentrations of extracts would indicate a greater amount of these phytochemicals

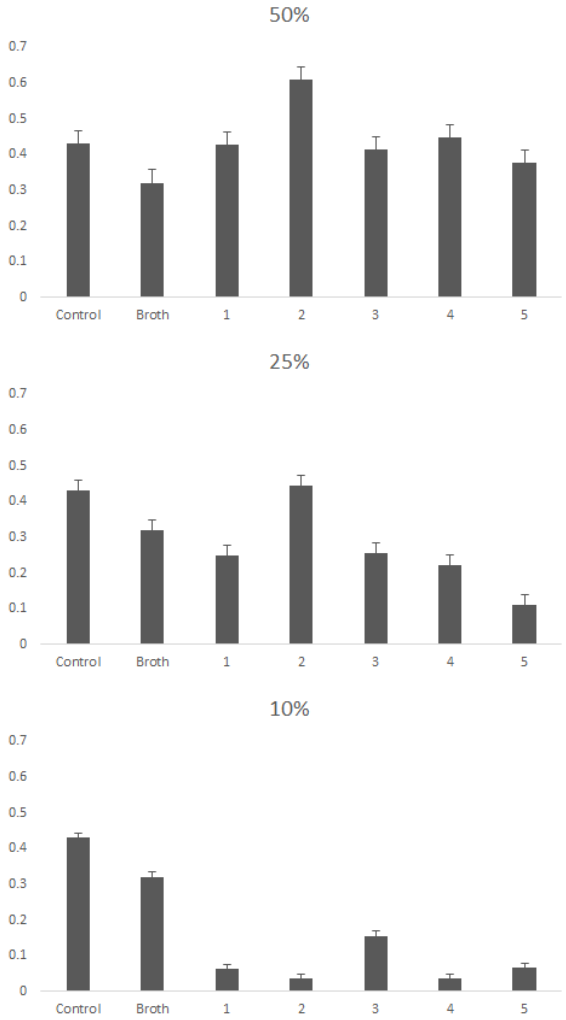

Figure 1. Absorbance readings of the negative control, and experimental wells at 50%, 25%, and 10% treatment; Different bar superscripts indicate significant difference at p<0.05.

which could inhibit biofilm synthesis and formation. Higher extract concentrations would contain more phytochemicals, thus, exhibiting stronger antibiofilm activity and consequently, yielding lesser amount of biofilm mass in biofilm assays.

Percent Reduction. The percent reduction values indicated the percent of biofilm reduced by the treatments. Higher percent reduction denoted stronger antibiofilm activity. In the One-Way ANOVA statistical analysis, the calculated p-value of the percent reduction of each biofilms from each concentration group was less than p=0.05. This indicated that there was a significant difference in biofilm mass within each concentration group. The LSD post hoc was done to compare each treatment within each set and to determine which pairs significantly differ, as seen Table 3 and Figure 2.

The LSD post-hoc results of percent reduction were similar to the results of the absorbance values. At 50% concentration, the p-values of the percent reduction from ripeness stages 1, 3, 4, and 5 were shown to be greater than p=0.05 when compared to the negative control indicating no significant difference. This implied that the percent reductions

of the treatments were comparable to that of the negative control. Treatments from ripeness stages 1, 3, 4, and 5 were not significantly different from each other. On the other hand, percent reduction of treatment from ripeness stage 2 was significantly different from that of the negative control and the other ripeness stages. The negative mean difference indicated that the percent reduction was negative and implied an increase in biofilm mass. At 25% concentration, the p-values of the percent reduction from ripeness stages 1, 3, 4, and 5 were shown to be less than p=0.05 when compared to the negative control These treatments were significantly different from the results of the negative control. However, they were not significantly different from each other.

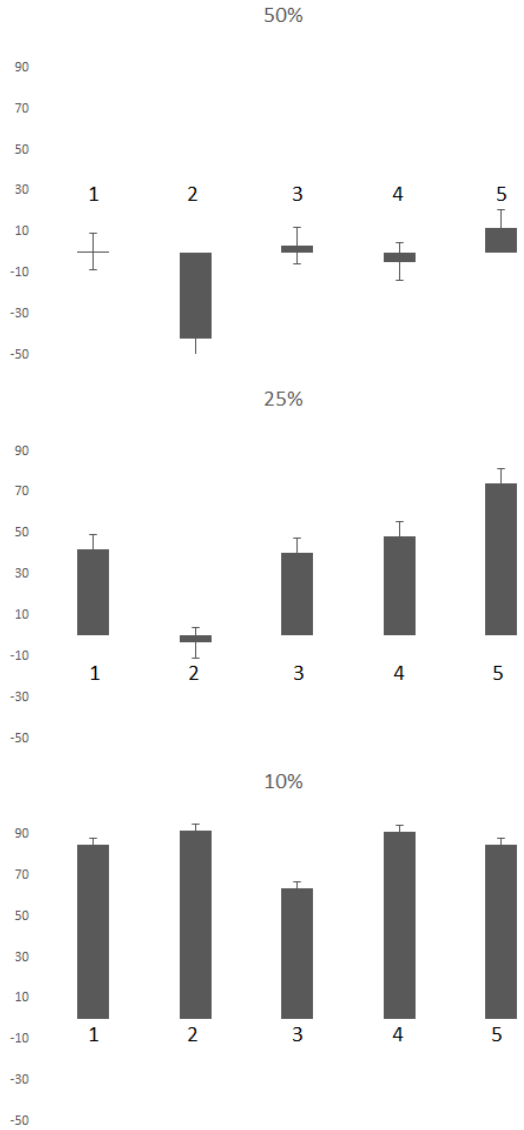

Figure 2. Average percent reduction of each treatment at 50%, 25%, and 10% concentration; Different bar superscripts indicate significant difference at p<0.05.

The exception being the peel extract from ripeness stage 5 where its percent reduction was significantly different with a positive mean difference. This implied that ripeness stage 5 had a peel extract with a percent reduction significantly greater than that of the others. No significant difference between the percent reduction of the ripeness stage 2 treatment and the negative control was observed. At 10% concentration, the percent reduction of all treatments was significantly different from the negative control. Treatments from ripeness stages 1, 2, 4, and 5 had comparable percent reductions. Ripeness stage 3 had a percent reduction significantly different from the others. Its negative mean difference indicated that its percent reduction was significantly lower than that of the other treatments.

As with the absorbance values, percent reduction was used as a measurement for antibiofilm efficacy. Percent reduction represented the amount of biofilm removed by the treatments when compared to the negative control, which represented the biofilm at its full form. It was calculated using absorbance values of the negative control and the treatments. High percent reduction denoted high antibiofilm efficacy while low percent reduction denoted low antibiofilm efficacy. Negative percent reduction denoted an increase in biofilm mass and a promotion of biofilm production. LSD post hoc was used to compare the treatments to the negative control and to each other. Treatments from different ripeness stages and at different concentration levels showed varying effectiveness against *S. aureus* biofilm.

This contradicted with results reporting that higher percent reduction values resulted from higher extract concentration of samples containing flavonoids and phenolic acids [22,23]. A linear trend between extract concentration and antibiofilm efficacy was observed. This could be attributed to the expected higher phytochemical content in higher concentrations of the plant extracts. However, this did not occur with the *Mangifera indica* peel extract despite having the same phytochemicals found in the previously cited articles. Further studies must be conducted to investigate this trend.

Error Analysis. The absorbance value of the broth or blank was shown to be significantly lower than the negative control group but significantly higher than some treatments with lower extract concentrations. This suggested that there might have been an issue with technique such as error in pipetting and possibility of contamination. The environmental conditions inside the laboratory where the experiment was conducted may have influenced the results. Another factor could be the prolonged period between the plant extraction and the biofilm assay. Samples were stored in cool temperature before being used in the biofilm assay.

Conclusion. The antibiofilm activity of the *Mangifera indica* peel extracts from five ripeness stages in three concentrations (50%, 25%, 10%) was determined against *S. aureus* biofilm. At 25% concentration, extracts from ripeness stage 2 and stage 5 showed no and the greatest antibiofilm activity, respectively. Other treatments were not significantly different. At 10% concentration, all treatments showed antibiofilm activity. Extract from stage 3 had the least antibiofilm activity when compared to the other treatments. There was no noticeable trend in antibiofilm activity between the ripeness stages. However, it was observed that lower

extract concentrations yielded lower absorbance values, and therefore, greater antibiofilm activity.

Recommendations. Ripeness stage must be identified through quantitative rather than qualitative measures to avoid imprecise categorization. Phytochemical quantitative determination, alongside qualitative, should be conducted on the peel extract. The two results may be correlated. Further practice of microbiology procedures must also be done in order to reduce the errors that occurred. In the study, only a negative control was used. A positive control should be included to further assess the antibiofilm activity of the peel extracts. This is to ensure that the treatment is effective in dislodging the biofilm matrix.

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