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Original Article

The Antibacterial Activity of Palm Oil (*Elaeis guineensis*) Leaf Extracts Against *Staphylococcus aureus*

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ABSTRACT

Plants offer a rich source of antimicrobial agents and bioactive compounds. In this study, aqueous palm oil leaves extracts (POLE) have been used as an alternative antibacterial agent against oral infections mainly caused by S. aureus. Many previous studies report the potential use of palm oil leaf extracts in treating bacterial infections such as Escherichia coli, Salmonella species, Pseudomonas aeruginosa and Bacillus species. However, few studies have been reported on the effect of palm oil leaves extract on oral microbes. Agar diffusion method, minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) assay were conducted to observe the antibacterial activity of aqueous palm oil leaves extract. The crystal violet assay was used to determine the anti-biofilm activity of the extracts. For agar diffusion method, the diameter of inhibition zone was measured. The inhibition zone of the tested bacteria was observed between 0-20 mm. The MIC and MBC values for the tested bacteria were 3.906 mg/mL and 7.813 mg/mL respectively. While for anti-biofilm assays, aqueous POLE extract acts as a potent anti-biofilm agent with dual actions, preventing and eradicating the biofilm of the tested bacteria. In conclusion, we suggest that the aqueous POLE extract may serve as alternative natural antibacterial and anti-biofilm agent against oral infections.

Keywords: *Antibacterial, Palm oil (Elaeis guineensis) leaf, Staphylococcus aureus*

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INTRODUCTION

Recently, there is a substantial increase in the global need for alternative prevention and treatment options that are safe, effective, and economical due to the rise in resistance by pathogenic bacteria to currently used antibiotics and chemotherapeutics (Torwane et al., 2014). *Staphylococcus aureus* (ATCC strain from oral cavity) is defined as circular, golden yellow colonies, coagulase-positive, and exhibits β-haemolysis on blood agar. *Staphylococcus aureus*, a Gram positive that usually found as normal flora on human skin and sometimes occurs in the nose but in certain condition it may breached the barriers thus penetrate the skin or other mucous membranes to invade a range of tissues that may leads to various infections.

The infections associated with *S. aureus* led to many diseases, notably periodontitis and peri-implantitis, osteomyelitis, chronic wound infection, chronic rhinosinusitis, endocarditis, and ocular infection (Cogen et al., 2008). The formation of *S. aureus* biofilm in the oral cavity may function as passage for oral infections as the frequency of *S. aureus* inhibiting the periodontal pocket and oral cavity is 13.4% and 15.8% respectively. Besides, *S. aureus* may dwell in biofilms and tend to be highly resistant towards the action of antibiotics. In oral cavity, *S. aureus* was associated with soft tissues infections such as angular cheilitis, staphylococcal mucositis and parotitis (McCormack et al., 2015).

The failure of conventional therapies indicates that biofilm treatments need auxiliary upgradation (Zhang et al., 2020). Whiles some of the synthetic chemicals that are available in market for the treatment of dental diseases were found to develop bacterial resistance and discoloration of the teeth. Therefore, there is a greatest need to develop an effective antibiofilm therapeutic strategies against *S. aureus*. Research for alternative products continue and natural phytochemicals isolated from plants used as traditional medicines are considered as good alternatives (Palombo, 2011). Nowadays, natural products have been used widely in pharmaceutical field as it is considered as safe, with minimal adverse effects.

The palm oil industry produces several wastes during harvesting, pruning, replanting, and processing in the mills, and at least 53% of the dry weight of these wastes is from palm oil leaves. Besides, it has been reported that the palm oil leaves contain bioactive agents such as antioxidants and antihyperglycemic as well as hypertensive (Tahir et al., 2012). Various studies have been conducted on the potentials of *Elaeis guineensis* against human pathogens. *Elaeis guineensis* was found to have activity against *Escherichia coli*, *Pseudomonas aeruginosa, and Staphylococci aureus* (Aziz et al., 2015). Although previous studies have demonstrated the antimicrobial properties of this *Elaeis guineensis* leaf extracts against human pathogens, only a few studies on oral microbes have been conducted. Infection rate can be reduced by inhibiting the growth of *S. aureus* (Archer et al., 2011). Therefore, in this study, the palm oil leaf (*Elaeis guineensis*) was used to determine the antibacterial and antibiofilm activity of aqueous palm oil leaf extract against *S. aureus* (ATCC strain from oral cavity).

METHODS

Preparation of Extracts

The aqueous palm oil leaves extract (POLE) was purchased from Nova Laboratories Sdn Bhd. The aqueous POLE extract was then re-dissolved in distilled water to yield a solution containing 500 mg of extract per mL. The mixture of aqueous palm oil leaf extract was autoclaved prior further analysis.

Phytochemical Screening

Phytochemical analysis was carried out on the aqueous extract using standard procedures to identify the constituents.

Test for flavonoid (Alkaline reagent test) (Gul et al, 2017).

A volume of 2 mL of 2% Sodium hydroxide (NaOH) was mixed with 2 mL of aqueous plant crude extract, concentrated yellow was produced. Then, 2 drops of diluted hydrochloric acid (HCL) were added to the mixture. The mixture should become colourless due to the presence of flavonoids.

Test for alkaloids (Wagner's reagent test) (Solomon et al., 2013).

Wagner's reagent was prepared by adding an amount of 1.27 g of iodine (I_2) and 2 g of potassium iodide (KI). A few drops of Wagner's reagent were added into 5 mL of aqueous plant extract. The presence of alkaloids was demonstrated by the formation of reddish-brown precipitate.

Test for terpenoids (Edeoga et al., 2005).

A volume of 5 mL aqueous plant extract and 2 mL of chloroform was mixed. Then, 3ml of concentrated sulphuric acid (H_2SO_4) was added carefully to the mixture forming a reddish-brown layer indicating the presence of terpenoids.

Test for steroids (Gul et al, 2017).

A volume of 2 mL concentrated sulphuric acid (H_2SO_4) was added into 2ml of chloroform ($ChCl_3$). Then, the mixture was added to 5ml of aqueous plant extract. The red colour of the lower layer of chloroform represented the presence of steroids.

Test for glycoside (Liebermann's test) (Gul et al, 2017).

A volume of 2 mL of acetic acid (CH_3COOH) was mixed with 2 mL of chloroform ($ChCl_3$). The mixture was then added to a portion of aqueous palm oil leaf extract. The mixture was cooled, and concentrated sulphuric acid (H_2SO_4) was added slowly showing the green colour formation.

Test for tannins (Abdullah et al., 2013).

An amount of 0.5 g of powdered sample of plant leaves is boiled in 20 mL of distilled water, and then the mixture was filtered. A few drops of 0.1% Iron (III) chloride ($FeCl_3$) were added, and the formation of blue-black colour indicated the presence of tannins.

Test for phenols (Solomon et al., 2013).

An aqueous 5% Iron (III) chloride ($FeCl_3$) was added into 5 mL of the aqueous extract. There is formation of deep blue, showing the presence of phenols.

Test for saponin (Solomon et al., 2013; Edeoga et al., 2005; Abdullah et al., 2013).

An amount of 2 g of the powdered sample was boiled in 20 mL of distilled water then being filtered. 10 mL of the filtrate was mixed with 5 mL of distilled water. Next, the mixture was shaken vigorously for a stable persistent froth. The frothing was mixed with three drops of olive oil and shaken vigorously. The formation of emulsion was observed.

Preparation of Bacterial Suspension

Staphylococcus aureus taken from glycerol stock (- 80°C) was inoculated on Mueller-Hinton (MH) agar and incubated at 37˚C for 24 hours. On the next day, the isolated colony was subculture into MH broth. Overnight culture was then used to standardise the bacterial cell number. The cell number was standardized to OD 0.1 using spectrophotometer (625nm) which is equivalent to approximately $1x10^8$ cell/mL for experimental purpose.

Control Experiment. Positive and negative control were prepared to compare the reaction and ensure the reliability of the result. 0.12% Chlorohexidine (mouthwash) were used as positive control and sterile distilled water were used as a negative control.

Antibacterial Susceptibility Testing (AST) By Agar Diffusion

A volume of 100 µL of the standardized inoculum suspension of the organisms were spread on the MH agar by using sterile cotton swab. Then, four wells were made on the MH agar for the test using the opening of sterilised tips (~6 mm in diameter). The wells were labelled, 500 mg/mL and 250 mg/mL of the aqueous POLE extracts were added in well 1 and 2, 0.12% chlorhexidine (as control positive) was added in well 3 and sterilised distilled water (as control negative) were added well 4. The plates were allowed to dry for 30 minutes prior to incubation at 37˚C for 24 hours. The experiment was conducted in triplicate. The diameter of zone of inhibition were measured and recorded on the next day. The method was taken from Xu *et al.*, 2016 with slight modification (Xu et al, 2016).

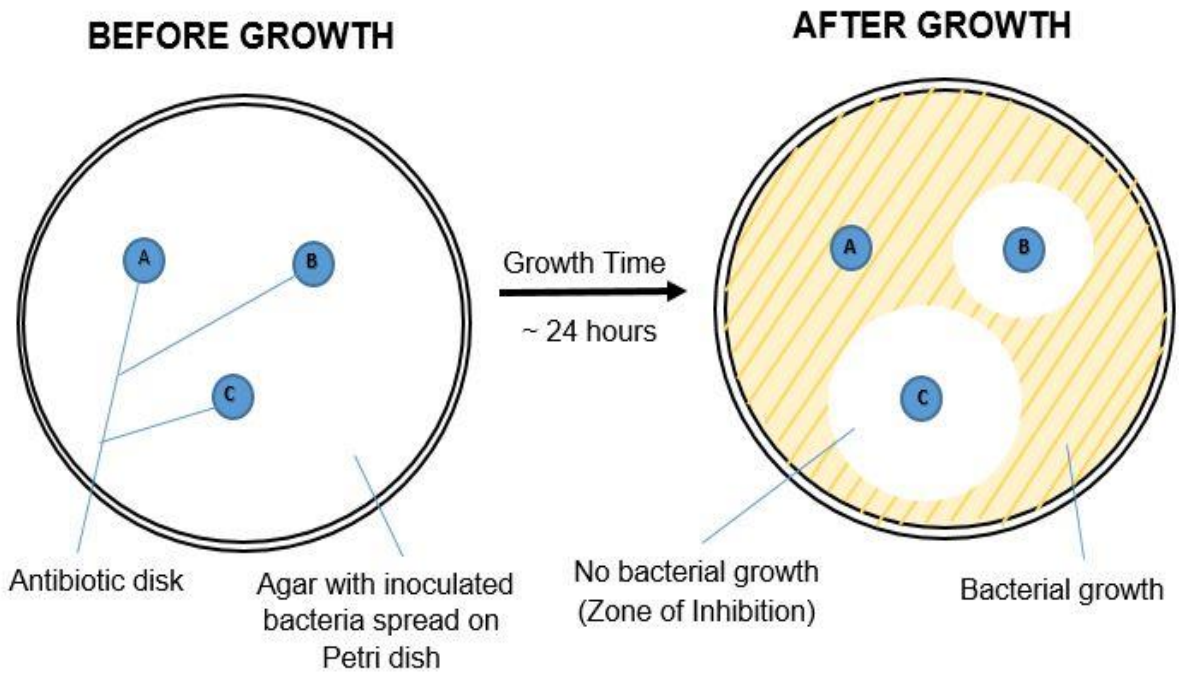

Figure 1: Schematic diagram of antibacterial susceptibility test

Determination Of Minimum Inhibitory Concentration (MIC) And Minimum Bactericidal Concentration (MBC)

MIC and MBC values of aqueous POLE extract were determined by micro-broth dilution assay method. The standardized inoculum was prepared using the direct colony suspension method. The stock solution of plant extract was prepared by dissolving 500 mg of aqueous extract into 1 mL of sterile distilled water. Each well consisting of 100 µL of broth with 10 µL of inoculum. The final number of bacteria cells in each well was approximately $5x10^5$ cell/mL. Chlorhexidine and deionised distilled water were added in well number 11 and 12 respectively. Then, it was incubated at 37˚C for 24 hours. For the MBC, the mixture from well 1 to well 8 were sub-cultured on MH agar to observe the lowest concentration of the extract tested that led to bacterial death. The plate was incubated at 37˚C for 24 hours. The MBC was observed on the next day (CLSI, 2010).

Antibiofilm Assay

The antibiofilm assay of the aqueous POLE extract on *S. aureus* was conducted using 96-wells plate (Lee et al., 2013). The *S. aureus* was subculture into 10 mL of MH broth and standardized to OD 0.1 using spectrophotometer (600nm). The standardised bacterial suspension was used for microdilution. A volume of 100 µL of MH broth was added into each well. Aqueous extract from the stock (500mg/mL) was added in the first well for each row. The extracts were then serially diluted by pipetting 100 µL of the mixture from first well into the second well. This step was repeated until well number 8 (For the first three rows: A, B, C). Chlorohexidine (100 µL) as positive control was added into first well of D, E, F. Then, 10 µl of bacterial suspension was added into all tested wells. The 96 well plate was incubated for 24 hr at 37˚C. On the next day, the treated bacteria culture was discarded. A volume of 100 µL of phosphate buffer saline (PBS) was used to wash the remaining unattached bacteria. A volume of 100 µL of crystal violet (0.4%) was added into each well and incubated for 15 minutes at 37˚C. After 15 minutes, the crystal violet was discarded in the sink and washed with slow tap water to remove the excess crystal violet in the plate. Then, the plate was dried in an oven for 30 minutes. Finally, 100 µL of 95% of ethanol was added to solubilize the crystal violet. The absorbance of the final mixture was read at 570 nm.

$$\textbf{Percentage of Inhibition (\%)} = [(OD_{\text{negative control}} - OD_{\text{experimental}}) / OD_{\text{negative control}}] \times 100\%$$

RESULTS

Phytochemical Screening

The presence of the phytochemicals in aqueous palm oil leaves extract (POLE) was determined using standard procedure (Table 1). The phytochemical analysis revealed the presence of steroids, glycosides, tannins, phenols and sapronin.

Table 1: Screening of The Compound Present in Aqueous POLE Extract

Compound	Presence (+/-)
Flavonoid	**-**
Alkaloids	**-**
Terpenoids	**-**
Steroids	**+**
Glycoside	**+**
Tannins	**+**
Phenols	**+**
Saponin	**+**

Antibacterial Susceptibility Testing (AST) By Agar Diffusion

Agar diffusion assay was used to test the antibacterial susceptibility of oil palm leaf aqueous extract against *Staphylococcus aureus*. Chlorhexidine (0.12%) and distilled water were used as positive and negative controls, respectively. In the presence of oil palm leaf aqueous extracts at concentrations of 250 mg/mL and 500 g/mL, a clear zone of inhibitions was observed.

Figure 2 and Table 2 depict the zone of inhibition surrounding the wells, as well as the diameter of the zone of inhibition for all tested samples. There were no clear zones for negative control. However, clear inhibition zones were observed for all three wells with extracts at 250 mg/mL and 500 mg/mL and chlorhexidine (0.12%) against the bacterium (Figure 2 and Table 2).

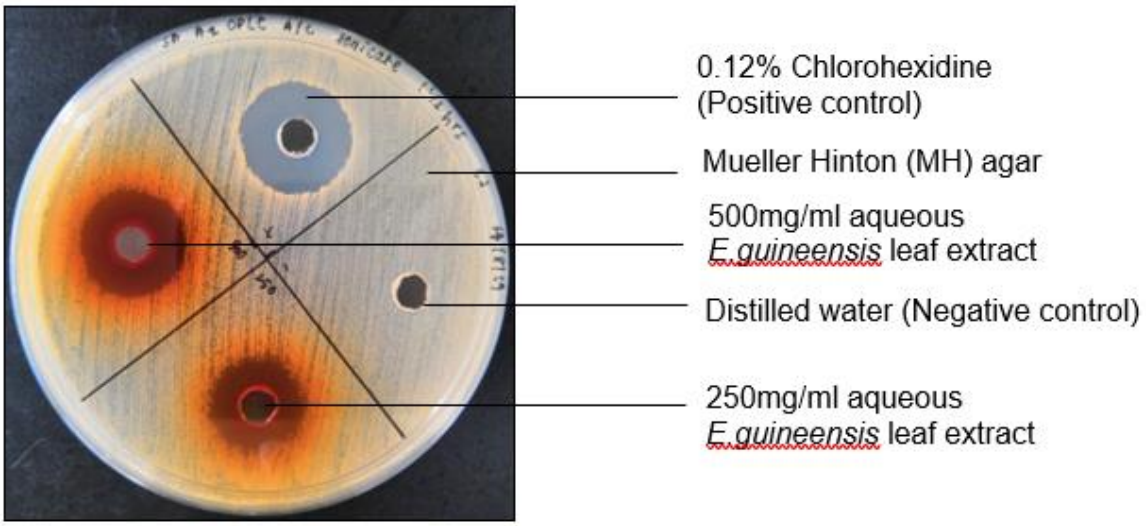

Figure 2: Antibacterial susceptibility test of aqueous POLE against *S. aureus*

Table 2: Antibacterial Activity of Aqueous POLE Extract Against *S. aureus*

Test	Aqueous palm oil leaves extract (mg/mL)		Positive control	Negative control
	Diameter of zone of inhibition (mm)			
	250	500	Chlorohexidine (0.12%)	Distilled water
1.	13.2	16.3	18.8	0.00
2.	13.3	16.7	19.5	0.00
3.	13.8	18	19.2	0.00
Avg ± SD	13.43±0.32	17±0.89	19.17±0.35	0.00±0.00

Minimum Inhibitory Concentration (MIC) And Minimum Bactericidal Concentration (MBC)

The minimal inhibitory (MIC) concentration of the extracts tested against *S. aureus* was determined based on the turbidity using naked eyes. The MIC value of the extracts was found to be 3.906 mg/mL (Table 3). At this concentration, no visible growth was observed in the broth. Sample from MIC test (well 1 – 8) were inoculated on MH agar and incubated for 24 hours. According to the finding, the lowest concentration of the extracts (MBC) that inhibited and killed the growth of *S aureus* is 7.813 mg/mL (Figure 3).

Table 3: The MIC of Aqueous POLE Extract Against *S. aureus*

Bacterial species	Row	Concentrations of extract in mg/mL											
		250	125	62.5	31.25	15.63	7.81	3.91	1.95	0.98	0.49	P	N
S. aureus	1	-	-	-	-	-	-	-	+	+	+	+	-
	2	-	-	-	-	-	-	-	+	+	+	+	-
	3	-	-	-	-	-	-	-	+	+	+	+	-

(**+**): cloudy; (-): clear; P = positive control (0.12%); N = negative control (dH_2O)

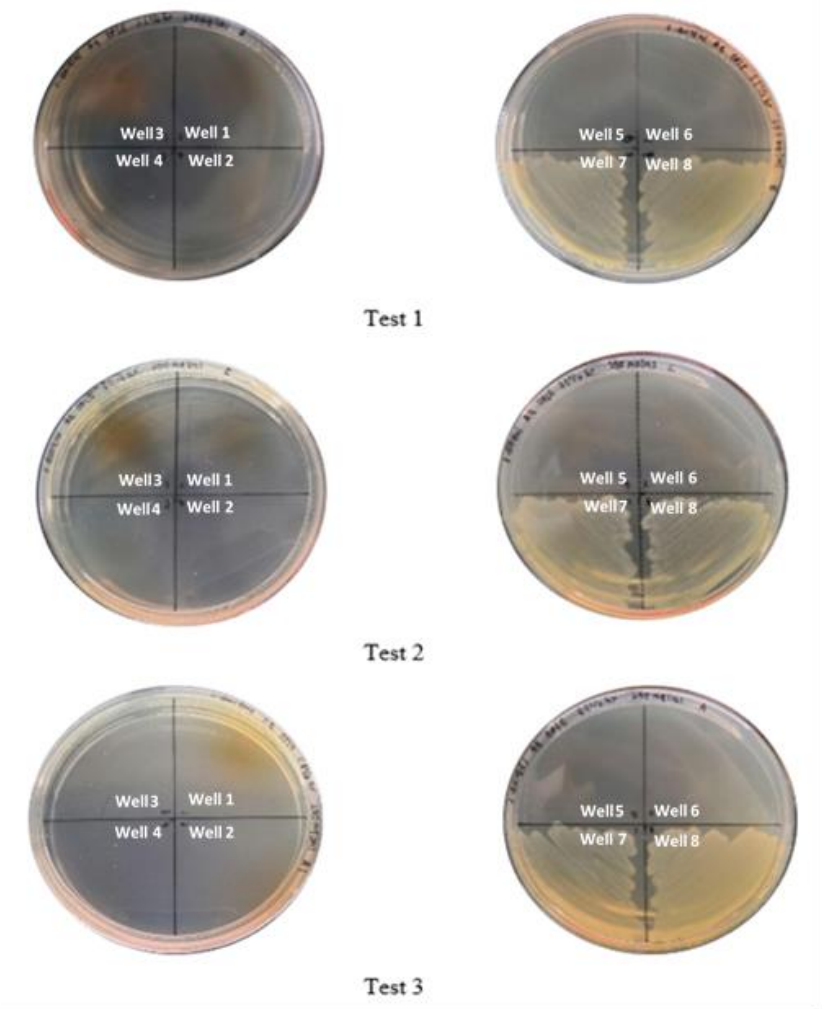

Figure 3: The minimal bactericidal concentration (MBC) of aqueous palm oil leaf extract (mg/mL) on *S. aureus*

Antibiofilm Assay

Antibiofilm activity of aqueous POLE extract and control positive (0.12% CHX) against *S. aureus* are presented in Figure 4 and Figure 5 respectively. It demonstrated that the higher the concentration of extracts, the greater the percentage of inhibition / antibiofilm (Figure 4). In the presence of 250 mg/mLof aqueous POLE extract, the biofilm that attached to the bottom of the plate was inhibited by approximately 97.82%. At MIC and MBC value, about 79 – 80% of inhibiton were observed. Control positive showed a similar inhibition trand. At 0.12%, chlorhexidine inhibited approximately 94.5% which is slighly lower than the initial concentration used for the aqueous POLE extract.

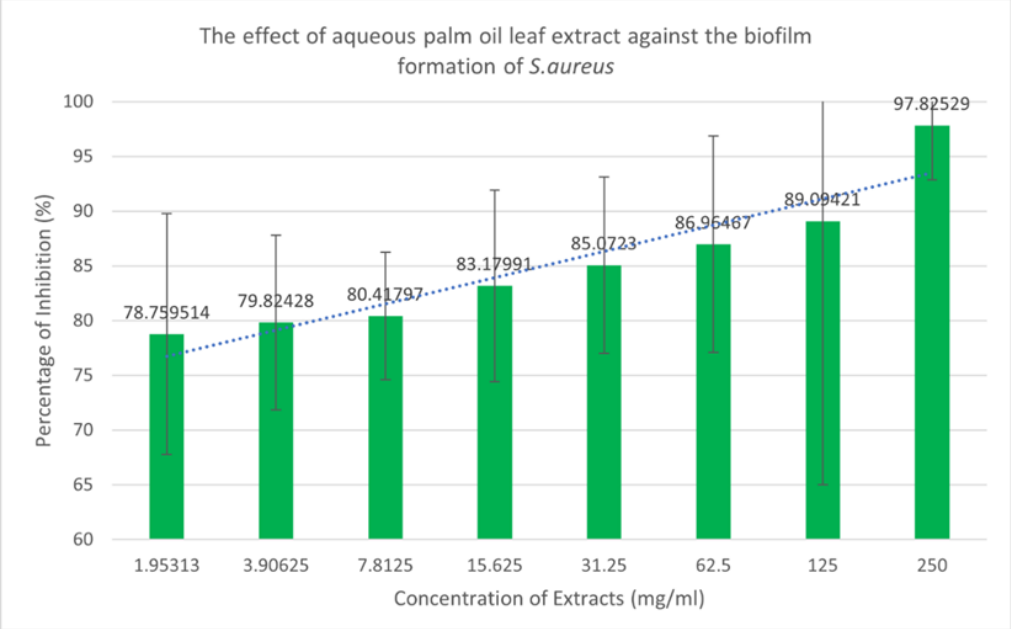

Figure 4: A graph depicting the effect of aqueous POLE extract against the biofilm formation of *S. aureus*

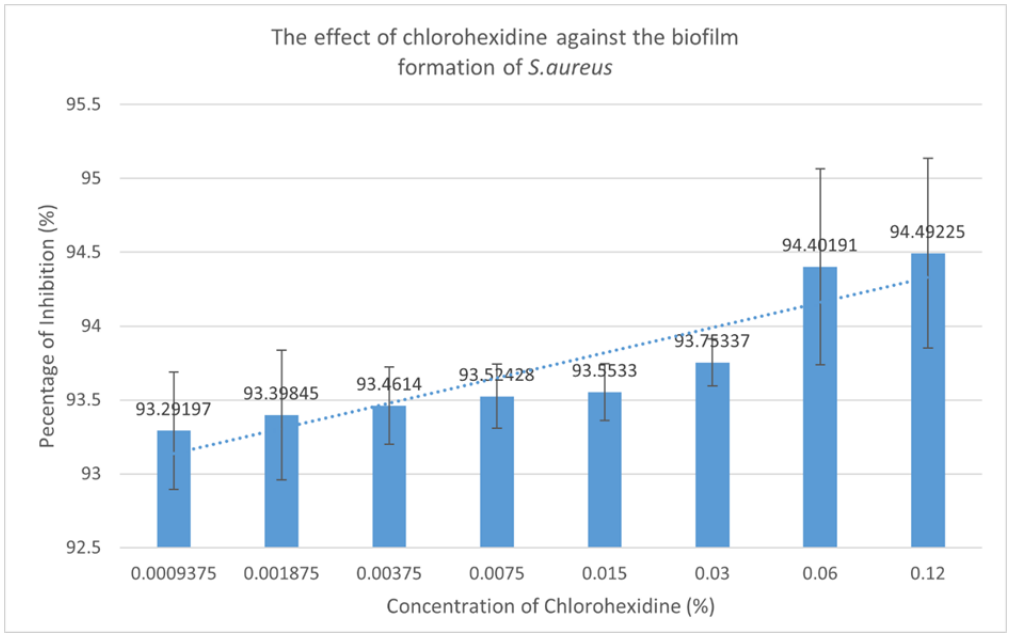

Figure 5: A graph shows the effect of chlorohexidine against the biofilm formation of *S. aureus*

DISCUSSION

The phytochemical screening revealed that the aqueous palm oil leaves extract (POLE) contained steroids, tannins, saponins, glycoside and phenol but was devoid of alkaloid, flavonoid and terpenoids. Different solvent used for extraction, yielded different bioactive compounds. Water commonly used as a universal solvent in the extraction of antimicrobial agent products. High Performance Liquid Chromatography (HPLC) or Liquid Chromatography and Mass Spectrometry (LCMS) can be used to confirm the present and the quantity of this various bioactive compound. Gram staining was used to examine the cell morphology, which revealed Gram positive cocci in cluster cells.

The antimicrobial activity of aqueous POLE extract against *S. aureus* was tested using AST, MIC and MBC. The diameter of inhibition zone around the well of different controls was used to determine antibacterial activity. In AST, chlorohexidine (0.12% CHX) and distilled water were used as positive and negative controls, respectively. The diameter of the zone of inhibition for chlorohexidine, ranges between 18.8-19.5 mm. This is thought to have potent antibacterial activity against *S. aureus.* According to one study, using chlorhexidine in mouthwash may cause undesirable side effects, such as tooth staining and taste alteration (16). The negative control showed no inhibitory zone. The diameter of zone of inhibition for 250 mg/mL and 500 mg/mL aqueous POLE extracts was 13.2-13.8 mm and 16.3-18 mm, respectively. However, a lower concentration of aqueous POLE extract was required to inhibit and completely killed the growth of *S. aureus*. Based on the MIC and MBC results, this is justified. The MIC and MBC values of aqueous POLE extracts against *S. aureus* were 1.953 mg/mL and 3.906mg/mL, respectively.

The antibacterial effects exhibited by aqueous POLE extract on *S. aureus* could be attributed to the presence of active compounds such as tannin and saponin in the plant or due to the presence of a combination of these bioactive compounds with diverse mechanism of actions. A study stated that the antimicrobial agents such as saponin and tannin inhibits the progression of microorganisms by precipitating the microbial proteins and thus disrupting the essential protein that bacteria require to survive (Abdullah et al., 2013). More research is needed to determine the underlying mechanism by which the extract affects the bacteria. Scanning Electron Microscopy (SEM) can be used to examine the morphological changes of bacteria.

Futhermore, *S. aureus* is known to form a multi-layered biofilm embedded with a glycocalyx either on host tissues or medical devices. Biofilms are the extracellular secretion of microbes which composed of extracellular polymeric substances mainly polysaccharides, proteins, and DNA. Biofilms formed by bacterial and fungal pathogens are a major concern because they confer broad spectrum resistance to the underlying microbes. Bacteria and fungi growing as biofilms are up to 1000 times more resistant to antibiotics than their planktonic counterparts. This is a significant issue in the medical field because biofilm has been shown to form on the medical device surfaces, allowing pathogens to persist as reservoirs, and dispersal of single and clustered cells implies a significant risk of microbial dissemination within the host and increased risk of infection. Figure 4 shows that as the concentration of aqueous palm oil leaf extract increases, so does the percentage of inhibition of *S. aureus* biofilm formation. According to Figure 5, the optimum concentration of the positive control (CHX) is at 0.12% and 0.06%. The aqueous palm oil leaf extract can act as a potent anti-biofilm agent, preventing and eradicating the tested bacteria's biofilm. Furthermore, anti-cancer activities (Jaffri et al., 2011), wound healing, hepatoprotective effects (Sasidharan et al., 2012), anti-diabetic effects and anti-inflammatory activities (Owoyele et al., 2014) have been reported for palm oil leaf extract.

CONCLUSION

In conclusion, we suggest that aqueous palm oil leaves extract has a significant potential to serve as an alternative natural antibacterial and anti-biofilm agent against oral infections. Thus, when compared to synthetically derived antibacterial agent for oral pathogens, this naturally derived, cost-effective and environmentally friendly aqueous palm oil leaves extract that is safe to use as an oral health care product and produces fewer side effects.

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