Antimicrobial and Antibiofilm Activities of the Methanol Extracts of *Rosa Damascena* against *S. aureus.*

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Objectives: The aims of this study are to investigate antibacterial and antibiofilm activity of *Rosa damascena (R.damascena)* flower extract against *Staphylococcus aureus (S. aureus).* **Method:** The antibacterial activity was assessed with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The potential of *R.damascena* flower extract as an antibiofilm agent was evaluated using biofilm inhibition assay and biofilm eradication assay. Chlorhexidine (CHX) 0.12% was used as a positive control. The experiment was performed in triplicate and repeated four times independently. All the data obtained were analysed using SPSS version 23. A Kruskal-Wallis test followed by post-hoc Mann-Whitney U test and level of significance was set at $P < 0.05$. **Results:** From this study, *R.damascena* extract shows antibacterial activity with MIC 50 mg/ml and MBC 100 mg/ml. Treatment with 0.5 MIC of extract inhibited 43.2% of biofilm formation. The biofilm eradication assay shows that 60.9% ($p<0.001$) of biofilm was eradicated by the extract upon 5 minutes treatment and 50.65% by 0.12% CHX. **Conclusion:** The result of this *in-vitro* study suggests a possible utilisation of *R.damascena* flower extract in treating *S. aureus* biofilm-associated infection in the oral environment.

1. Introduction

Denture-induced stomatitis (DIS) is an infectious condition affecting denture wearers. It is an inflammatory process underlies removable dentures and can affect from 15% up to 70% denture wearers [1]. This condition appears to cause redness, pain and eventually burning sensation. It usually affects a hard and soft palate of the mouth but may be spread to the cheeks and mouth corner [2]. DIS is commonly seen in elderly who wear dentures. The occurrence of DIS in edentulous patients has been reported up to 62%, 39% and 23% respectively by different researchers [3-5].

DIS is a multifactorial condition and usually caused by *Candida* yeast infections and bacterial infections due to poor oral and denture hygiene. Besides, denture trauma, allergic reaction to denture materials and immunological factors are other examples that also lead to DIS [6]. Common bacterial species that can be found in these conditions are *Streptococcus aureus (S. aureus) and Streptococcus mutans (S. mutans)* [7]. Few studies have been done regarding pathogenic colonisation causing DIS. Two studies observed the colonisation on mucosal surface and denture prosthesis. They reported that the colonisation on mucosal surface *Candida albicans (C. albicans)* is about 86%, *S. aureus* is 84 % and *S. mutans* is 16%-20%. However, based on prosthesis colonisation, *C. albicans* is only 26%-28%, *S. aureus* is 36%-40% and *S. mutans* is 40%-43% [8, 9]. Therefore, in our study we used *S. aureus* as the opportunistic pathogen that is commonly isolated together with *C. albicans* and *S. mutans* in denture-induced stomatitis cases.

Chlorhexidine (CHX) is one of the antimicrobial agents that commonly used and prescribed widely in dentistry. It is used as an antiseptic mouthwash and can be used as denture disinfectant. CHX 0.2% has been successfully used as a mouth rinse in treatment of *Candida*-associated denture stomatitis [10] and also widely considered as antiseptic of choice for decontaminating dentures infected by *C. albicans* [11]. CHX is available in the dental clinic with prescription and over-the-counter (OTC) forms at the pharmacy and local store. Denture cleanser is used to clean dentures outside from mouth. This is to minimise microbial accumulation on denture surfaces and eventually to prevent denture-induced stomatitis. Denture cleansers also used to remove stains and debris from food such as tea and coffee and tobacco. Denture cleansers can be classified into two main classes which are mechanical and chemical. An ideal denture cleanser is those that have antimicrobial activity, simple to use, effectively remove an organic and inorganic matter from denture surface and compatible with all denture base materials [12]. The mechanical type of denture cleanser includes brushing using a soft toothbrush with tap water. It is the most popular, simple, inexpensive and effective method. However, there is a limitation of this method as the elderly patients have deficient in motor function that may cause difficulties to them. Thus, chemical denture cleanser is used to compensate for the situation and make it easier and effective as well.

In this new era, researchers are keen to study the therapeutic agents originated from a natural source such as plant extracts. These natural sources can be developed as one of the treatments and prevention choices for DIS. *Rosa damascena (R.damascena)* is proven to have antimicrobial

activity, anti-inflammatory, antioxidant and analgesics effects [13]. Since DIS main causative agents are yeast and bacterial infections, *R.damascena* flower extract could be a potential compound as antibacterial and antibiofilm against *S. aureus* infection.

Therefore, the objectives of this study are i) to investigate the antibacterial activity of *R.damascena* flower extract and ii) to investigate the ability of *R.damascena* flower extract to eradicate *S. aureus* biofilm formed on microtiter plate compared to CHX.

2. Methods

2.1 Preparation of Extract

R.damascena flower extract was prepared following the methodology proposed by Sánchez et al. 2010, with minor modifications [14].

2.2 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC analysis was performed in BHI broth (BHIB) via broth microdilution techniques according to Clinical and Laboratory Standards Institute (CLSI, 2012) [15].

2.3 Determination of Biofilm Inhibition

The plant extract at subMIC concentrations were evaluated for their inhibition potential against cell attachments (antiadhesion test) according to the method described by Bazargani et al., 2106 [16]. The percentage of biofilm formation inhibition was calculated using the following formula:

Biofilm inhibition %=	$OD_{negative\ control}$-OD_{test}/ $OD_{negative\ control}$	X 100

2.4 Determination Eradication of Biofilm

The eradication of biofilm formation of the extract was performed according to methods described by Bazargani et al., 2106 [16]. Percentage eradication was calculated by using the following equation:

Biofilm eradication %=	1- $OD_{negative\ control}$-OD_{test}/ $OD_{negative\ control}$	X 100

3. Results

3.1. Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The MIC of *R.damascena* flower extract against *S. aureus* is 50 mg/mL meanwhile for the MBC is 100 mg/mL. The MIC/MBC ratio was calculated as 0.5.

Minimum Inhibition Concentration (mg/ml)	Minimum Bactericidal Concentration (mg/ml)
50	100

Table 1. Result shows MIC and MBC of *R. damascena* flower extract against *S. aureus.*

3.2 Biofilm Eradication Assay

Based on the results of the biofilm eradication assay (Figure 1), the effect of *R. damascena* extract had significantly increasing the percentage of biofilm eradication, as compared to the CHX 0.12%. Percentage of biofilm eradication by *R. damascena* extracts at 25 mg/ml is 60.90% whereas for biofilm treated with CHX 0.12% is 50.65%.

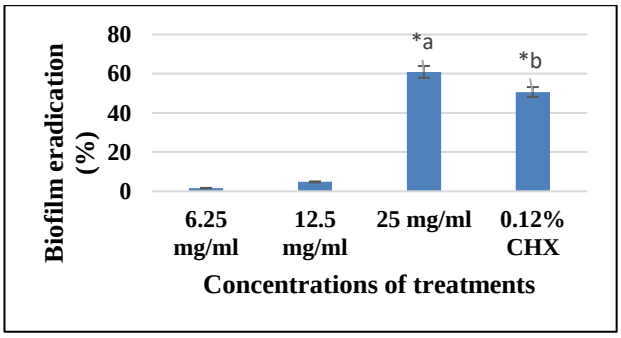

Fig. 1. Percentage of biofilm eradication after treated with *R. damascena* extracts of 6.25 mg/ml, 12.5 mg/ml and 25 mg/ml concentrations and CHX 0.12%. The overall percentage of biofilm obtained from 4 sets experiments in triplicate (n=12) is presented in a bar graph. (*) indicates significant differences in mean percentages were compared to the untreated control ($p<0.001$) according to the non-parametric Kruskal Wallis test with Mann Whitney.Different letters indicate statically significant differences between groups.

3.3 Biofilm Inhibition Assay

Based on the results of the biofilm eradication assay (Figure 2), the adherence of bacteria on *R. damascena* extract treated surface was significantly reduced. Percentage of biofilm inhibition by *R. damascena* extracts

at 25 mg/ml is 43.24% whereas for biofilm treated with CHX 0.12% is 53.83%.

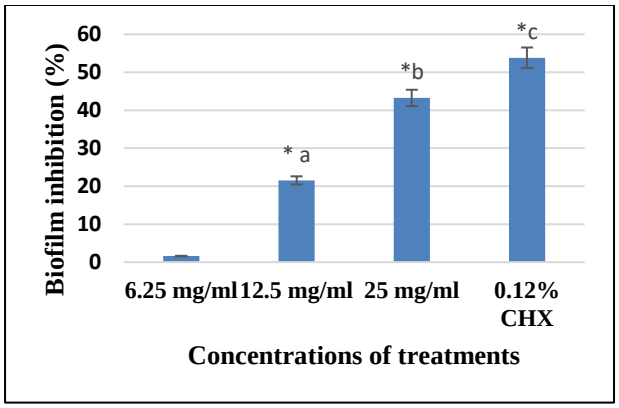

Fig. 2. Percentage of biofilm inhibition after treated with *R. damascena* extracts of 6.25 mg/ml, 12.5mg/ml and 25mg/ml concentrations and CHX 0.12%. The overall percentage of biofilm obtained from 4 sets experiments in triplicate (n=12) is presented in a bar graph. (*) indicates significant differences in mean percentages were compared to the untreated control ($p<0.001$) according to the non-parametric Kruskal Wallis test with Mann Whitney.Different letters indicate statically significant differences between groups.

4. Discussion

R. damascena has anti-microbial effects which are found to be highly effective *in vitro* against a broad spectrum of bacteria such as *S. aureus*, *E. coli*, *S. bovis* and *C. albicans* [17]. Previously, researchers had reported that extraction methods for *R. damascena* are varied which include aqueous, alcoholic, essential oil and petroleum ether. Different types of extract have its efficacy against bacteria, fungal or even virus [17]. Alcohol and aqueous flower extract of *R. damascena* showed higher antibacterial activity compared to petroleum ether [18]. The study done by Hirulkar NB is to test the sensitivity of alcohol, petroleum ether and aqueous extract of *R. damascena* against ten types of bacteria which are *Escherichia coli, Streptococcus pneumoniae, Salmonella thyphimurium, Enterobacter aerogens, Proteus vulgaris, Staphylococcus aureus, Staphylococcus epidermis, Bacillus subtilis, Citrobacter frundii* and *Pseudomonas aeruginosa* showed the highest zone of inhibition upon exposure to *R. damascena* alcohol flower extract at 1:1 dilution while *S. aureus* showed 20 mm of inhibited zone upon exposure to the alcohol flower extract [18]. According to the study, *E. coli* was resistant to alcohol flower extract of *Rosa damascena* but showed higher sensitivity to aqueous extract of the flower. The alcohol flower extracts of *R. damascena* also showed antimicrobial activity against methicillin-resistant *S. aureus, S. typhimurium, B. cereus* and *C. albicans* [19].

A number of previous studies reported that *R. damascena* contains citronellol, geraniol, nerol, phenyl ethyl alcohol, nonadecane, nonadecene, eicosane, heneicosane, tricosane, α-guaiene, geranyl acetate and eugenol [17]. A study done by Aridoğan BC *et al.* found that treatment with *R. damascena* essential oil showed inhibition activity against *S. aureus* with a zone of inhibition detected was 8 mm [20]. The main components in the essential oil are citronellol, geraniol and nerol. These compounds showed a different zone of inhibition against *S. aureus*. The treatments of citronellol showed the inhibition zone of 20 mm followed by geraniol at 21 mm and nerol at 19 mm [20].

In the present study, the antibiofilm activity of *R. damascena* flower extracts was dose-dependent. In this study, we are using methanol flower extract of *R. damascena.* The flower extract was found to exhibit antibacterial activity against *S. aureus* with a concentration of 50 mg/mL for MIC and 100 mg/ml for MBC. The flower extract also was observed to exhibit bactericidal effects on the *S. aureus* upon 24 hours treatment. The potential antibiofilm activity of *R. damascena* flower extracts against *S. aureus* was evaluated using biofilm eradication assay and biofilm inhibition assay using CHX 0.12% as a positive control. The results showed that *R. damascena* flower extract has the potential to treat *S. aureus* infection in the oral cavity. Referring to Figure 1, biofilm eradication assay shows that 60.9% *S. aureus* was dispersed by *Rosa damascena* flower extracts at a concentration of 25 mg/ml which was higher and significantly different compared to CHX 0.12% (50.65%) ($p<0.001$). As for Figure 2, in biofilm inhibition assay, only 43.24% of *S. aureus* was inhibited by *R. damascena* flower extract at a concentration of 25 mg/mL which is significantly lower compared to CHX 0.12% with the percentage of 53.83% ($p<0.001$). These results show that *R. damascena* flower extracts have the potential activity as an antibacterial agent against *S. aureus.*

5. Conclusion

This preliminary study had successfully identified the antibacterial activity of *R. damascena* flower extracts against *S. aureus*. It shows that the methanol flower extracts have the potential as an antibacterial agent. Further study on *R. damascena* flower extract needs to be done by looking on the underlying inhibition mechanism at the molecular level. It is believed that, *R. damascena* could be a good candidate as an alternative treatment of *S. aureus* infection in the oral cavity. Thus, it also could be therapeutically used as an antibacterial agent which cause less side effect as it is from the natural source.

6. References

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