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Investigation of Anti-biofilm Effect of *Syzygiumaromaticum* Plant Extracts against Oral Pathogens

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Abstract: ***Given the incidence of oral disease, increased resistance by microorganisms to antibiotics, adverse effects of some antimicrobial agents currently used in dentistry.Therefore, this work was performed to estimate the antimicrobial activity of both the aqueous and ethanol extracts of Syzygiumaromaticum against oral pathogens. The activity of the S. aromaticum was evaluated by broth microdilution method determining minimal inhibitory concentration and biofilm adopting microtitre plate crystal violet assay. The results showed that the MIC values of the aqueous extracts against all oral species ranged between 12.50-25mg/ml, while the MIC value of ethanol extracts was ranged between 6.25-12.50mg/ml.All clinical isolates were capable of biofilm formation in various amounts.The aqueous and ethanol extracts of the S. aromaticum presented anti-biofilm activity against the oral pathogens, the best inhibitory effect on Staphlococcus. epidermidis which was (68.3%), as compared to the inhibition percentage of aqueous extract which was (59.2%) Also, Candida albicans was demonstrated the better results in ethanol extracts 67.61% as compared to aqueous extract which was 60.31%. Finally, the ethanol extract of S. aromaticum appeared the better results of antimicrobial activity and ability to control biofilm formation.***

Keywords: Antimicrobial Activity, *Syzygiumaromaticum*, Biofilm, and Oral Pathogens

1. Introduction

The oral cavity is the natural environment for various species of microorganisms, which form compound society structure that can adhere to the teeth surface to forming biofilms [1]. Biofilms are multicellular society in which cells are establish in an extracellular matrix in close proximity to one another .the Surface adhesion of bacterial and fungi are thought to be the essential first step to the formation of dental plaque .When acidogenic oral bacteria were present in sufficient number due to Fermentation of sucrose and it evenly results in development of dental caries [2,3].

Many species of oral cavity are known to form biofilm the *Streptococcus sanguis, S. mutans, S. mitis and S. sobrinus* and *Lactobacillus* species. Also they have been principal etiology of development dental plaque, causative agents of caries and periodontal diseases [4]. *Candida* species are an important component of the dental plaque biofilm, which are regular communities of fungal cells in a matrix of extracellular polymeric substances [5]. The process of mechanical removal to the dental plaque biofilm is an important factor to prevention of tooth decay and gingivitis, it is important to associated both chemical and mechanical procedures in order to control its formation [6]

The antibiotics commonly used to treat oral infections i.e. Penicillin, Erythromycin, Cephalosporin, Tetracycline and derivatives, and Metronidazole have been documented [7]. These chemicals have undesirable side effects such as diarrhea, vomiting, and tooth staining [8].Other antimicrobial agents used in the prevention and treatment of oral diseases including Chlorhexidin,Cetypridinium chloride, amine fluoride , cause staining of teeth or in the case if ethanol (commonly found in mouth washes) have been linked to oral cancer [9].

The biofilm forms those are more resistant to antimicrobial agents and therefore more difficult to control is a matter of concern. Their inherent resistances to antimicrobial agents are at the root of many persistent and chronic bacterial infections.In recent years, much of research has been focused in identifying various alternative medicines to treat infections caused by the drug resistant microbial [10].These alternative medicines must be safe, effective and economical. Natural phytochemicals isolated from plants used in traditional medicine are considered as good alternatives to synthetic chemicals [11].

Syzygiumaromaticum has a long ago record in dentistry as anesthetic, toothache, antiseptic as well as general hygiene, and as an antimicrobial for oral and skin infections [12, 13]. *S.aromaticum* generally recognized as clove, from the Myrtaceae family native from the Maluku islands in east Indonesia [14]. Clove is wealthy source of phenolic compounds; especially Eugenol is the major bioactive compound. The activities of clove have been demonstrated against different strains from bacterial and fungal [15]. This research was aimed to evaluate the effectiveness of aqueous and ethanol plant extracts for *Syzygiumaromaticum* (clove) against a biofilm of the clinical isolates of oral cavity.

2. Materials and Methods

Microorganisms

Thirteen clinical isolates were collected from Al-elawiya specialized dental center. The pure cultures of three Gram-positive bacterial species (*Staphylococcus aureus* ,*Staphylococcus epidermidis,Streptococcus spp*.) and five-negative bacterial isolates (*Escherichia coli, Klebsiellapneumoniae, Proteus sp., Pseudomonas aeruginosa, and Pseudomonas monili*), and five different strains of the *Candida spp. Candida albicans, Candida krusie, Candida guillermondii, Candida glabrata,* and

Candida parapsilosis were isolated from patients with dental caries, dental plaque and gingivitis.

The bacterial isolates were sub-cultured on Nutrient agar (NA) media, while the fungal isolates were sub-cultured on Potato dextrose agar (PDA) media, then all the plates were incubated at 37 °C for 24 hours and stored at 4 °C in refrigerator to maintain stock culture.

Preparation of Plant Extracts

Dry clove flower *Syzygiumaromaticum* were homogenized to fine powder form using the grinder mixer, then stored in glass container at room temperature to be used later.

Preparations of Aqueous Extract

Twenty gram of flower powder was added into beaker with 100 ml of sterile distilled water. The mix was heated in water bath with shaking for 3hrs at 60°C .The solvent was first filtered through double layer cloth then with Whatman filter paper No.1 and concentrated in oven. Concentrated crude extract was reconstituted in 10ml of sterile distilled water to make a stock solution and stored at 4°C until used [16].

Preparation of Ethanol Extract

Ethanol extract was prepared by putting 20gm of flower powder in thumble, and extracted with 400 ml of ethanol by using a soxhlet apparatus. The solvent was boiled for 6hrs and evaporated to use a rotary evaporator at 40°C. Then the concentrated crude extract was reconstituted in 10ml of dimethylsulfoxide (DMSO) to make a stock solution and stored in refrigerator at 4°C until used [17].

Antimicrobial Assay

Minimum Inhibitory Concentrations Assay

Antimicrobial activity was tested by determination the minimum inhibitory concentration (MIC) by using microtiterplates (96 wells) as recommended by the clinical and laboratory standard institute [18]. The inoculum of bacterial isolates was prepared in tryptone soy broth, and for *Candidaspp.* in Sabouraud dextrose broth. The suspension turbidity was adjusted to 0.5 on the McFarland's standard. The stock solution of the *S.aromaticum* extract was dissolved in DMSO to give a final concentration of 200mg/ml. Serial dilutions of the stock solution were prepared in a microtitre plate with Mueller-Hinton broth to obtain serial concentrations ranging from 100mg/ml to 0.390mg/ml. A 10µl of diluted cell suspension was added to each well to give a final concentration of 10^6CFU/ml. The inoculated plates were incubated at 37°C for 24h. The MIC value was determined as the lowest concentration of the plant extracts that didn't permit any visible growth of the microorganism.

Biofilm Formation Assay

The biofilm formation of all tested isolates was performed as mentioned by Djordjevic *et al*., [19] and Agarwal *et al.*, [20] with some modification.The steps were as following,

1) 200µl cell suspension of all tested isolates 1.0×10^6cells/mL for yeasts and 1.0×10^8 cells/mL for bacteria were added into 96 well microtitreplatesand incubated at 37◦C for 24 hnegative control contained media only.
2) The medium was discard and washed three times with distilled water then the plates were dried at room temperature for 1hr.
3) 200µL crystal violet 0.1% for bacteria and 0.3% for yeasts were stained in each wells for 15min and the wells were washed three times with distilled water and distained with 200µL of 95% ethanol for bacteria and ethanol/acetone in the ratio 80:20% for yeasts for 15min. The OD wave length of 595nm was measured using microtiter plate ELISA reader.
4) After comparing the optical density (O.D) of biofilm with control and according to the readings, the isolates were classified as follows: O.D≤O. Dc no biofilm producer, O. Dc <O.D ≤ 2×O. Dc weak biofilm, 2×O. Dc<O.D≤4×O. Dc moderate and 4×O. Dc<O.D strong biofilm [21].

Biofilm Inhibition Assay

The anti-biofilm activity of the *Syzygiumaromaticum* extracts was tested on all isolates biofilm using 96 well microtitere plates as described by Regev-Shoshani et al. [22]. double serial dilutions for *S. aromaticum* extracts were made in sterile 96-well tissue culture plates containing 50µl of Mueller-Hinton broth per well to obtain concentrations ranging from 100mg/ml to 0.390mg/ml, A 100µl cell suspension of all tested isolates 1.0×10^6cells/mL for yeasts and 1.0×10^8cells/mL for bacteria were added into each well and incubated at 37ºC for 48 h. The medium without extract was used as growth control and the biofilm formed was determined by using the crystal violet staining assay as described above .The percentage of biofilm inhibition was calculated using the following formula:

% inhibition=[(OD_{595} in control-OD_{595} in treatment)/OD_{595} in control]× 100

Statistical Analysis

The data acquired were undergo to analysis of variance (ANOVA), and Significant difference between means was analyzed by least significant difference (LSD) at $p<0.05$ using program 2010.

3. Results and Discussion

The detection of antimicrobial activity determined by using the minimum inhibitory concentration (MIC) of the both aqueous and ethanol extracts for *S. aromaticum* plant against the oral microorganisms are illustrated in Table (1). The minimum inhibitory concentration (MIC) values of the aqueous extracts were ranged between 12.50-25mg/ml,while the MIC value of ethanol extracts was ranged between 6.25-12.50mg/ml. The gram positive bacterial species showed more susceptibility than gram negative species to the aqueous extract, the lowest MIC was 12.50mg/ml against gram positive bacteriawhile the highest MIC was 25mg/ml against gram negative bacteria only *K. spp.* and *E.coli*. The effect of aqueous extract of *S. aromaticum* were studied by Chavan*et al.*[23], and have showed that the best effect of *S. aromaticum* against *S. mutans* as it contain eugenol which is an antimicrobial agent. All *Candida spp*. were resistant towards the aqueous extract; MIC was 25mg /ml compared with ethanol extracts was 6.25mg/ml.

Table 1: The minimum inhibitory concentration mg/ml values of both aqueous and ethanol extracts of*S. Aromaticum* against the bacterial and candida species by broth microdilution technique

Microorganisms	Aqueous extract MIC (mg/ml)	Ethanol extract MIC (mg/ml)
Staphylococcus aureus	12.5	12.5
Staphylococcus epidermidis	12.5	12.5
Streptococcus spp.	12.5	12.5
Pseudomonsmonili	25	12.5
Pseudomonsaeruginosia	25	12.5
Escherichia coli	12.5	12.5
Protus spp.	25	12.5
Klebsiella spp.	12.5	12.5
Candida albicans	25	12.5
Candida glabrata	25	6.25
Candida krusie	25	6.25
Candida guillermondii	25	6.25
Candida parapsilosis	25	6.25

Results of the biofilm production revealed that all clinical isolates were capable of biofilm formation in various amounts. There were four bacterial isolates, *Staph. aureus*, *Staph. epidermidis*, *Streptococcus spp*. ,and *E.coli* that produce strong biofilm, whereas the four other bacterial species were classified as moderate biofilm producer as shown in figure 1. In current study all clinical isolates showed significant differences ($P \leq 0.05$) in biofilm formation. *Staph.aureus* bacteria showed higher biofilm production with an OD value of 0.371. The results were similar to Chamdit*et al.*,[24], they used ten clinical isolates and two reference strains were evaluated by crystal violet staining technique, in their study the *Staph. aureus* are biofilm-producing isolates.

Also, the results showed that the OD values that obtained by *Staph. epidermidis* isolates were lower than the OD values that obtained by *Staph. aureus* isolates. This was disagreed with Al-Hasani [25] who demonstrated that the OD values obtained by *Staph. epidermidis* (MRSE) isolates were higher than the OD values obtained by *Staph. aureus* (MRSA) isolates but this difference was statistically non-significant. Rachid*et al.*, [26] mentioned that biofilm formation seemed to be highly variable among *Staph.* spp.One of the important mechanisms that enable *Staphylococcus spp* to persist in the diseases is the ability to adhere and form biofilms on host tissue and other surfaces [27]. An association was observed between multi resistance and biofilm production. The environment of biofilm appeared to increase genetic exchanges and hence may subscribe to multi resistance phenotypes [28].

The specified results were indicated that the bacteria *Streptococcus* spp. had the ability to form biofilms; its absorbance value was 0.267. The ability of formation a stubborn biofilm was considered major virulence attributes of this organism. The results of current study was agreed with other studies, Bersan*et al.*, [29] who demonstrated Many species of oral streptococci such as *Strept.sanguis* and *Strept.mitis* have the ability to form biofilms; especially the *Strept. mutans* have the ability to form biofilm by using microtiter plates as recorded by [30]. Kolembrander [31]stated that oral biofilms have been obtained by several oral species which could harm the enamel and gum tissue due their pathogenicity.In the present study the biofilms were formed by gram negative bacteria *Pseudomonas spp.*, *Escherichiacoli*, *Protus spp*. and *Klebsiella spp*. the absorbance values shown in Figure (1). Current results are consistent with the findings of Agrawal [32] who stated that different species of gram negative bacterial have the ability to form biofilms.

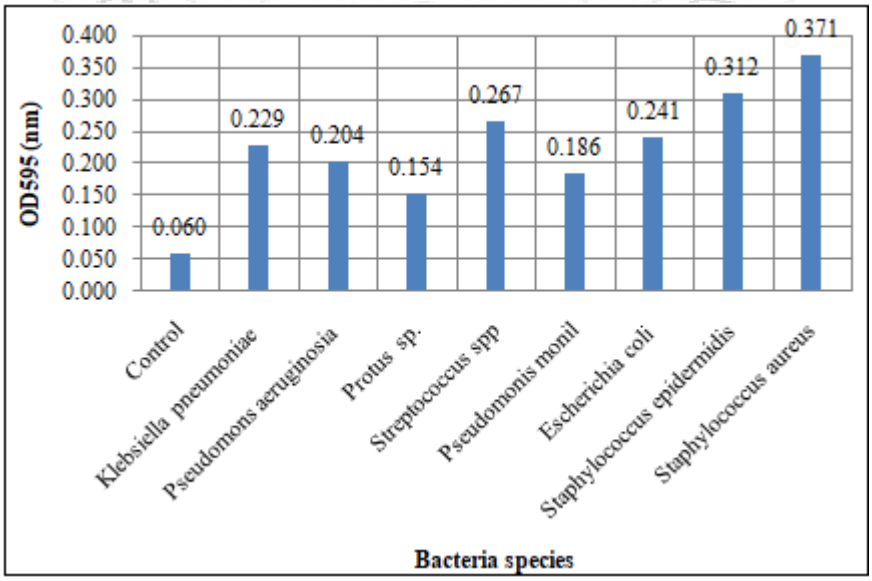

Figure 1: The Absorbance value (OD_{595}) of biofilm formation for bacterial species

Furthermore, Data showed that the strains of *Candida spp*. were strong biofilm formation as illustrated in Figure (2). The reports from other workers revealed that most of *Candida* infections are associated with biofilm forming ability of *Candida spp*. The ability of *Candida* strains to form biofilms in this study was in agreed with published study by Khan *etal*., [33]who mentioned that candida spp. that were isolated from several clinical conditions including urinary tract infections, vaginitis and blood infections have

the ability of formation a tenacious biofilm, 77% of *Candida albicans* strains were strong biofilm formation.

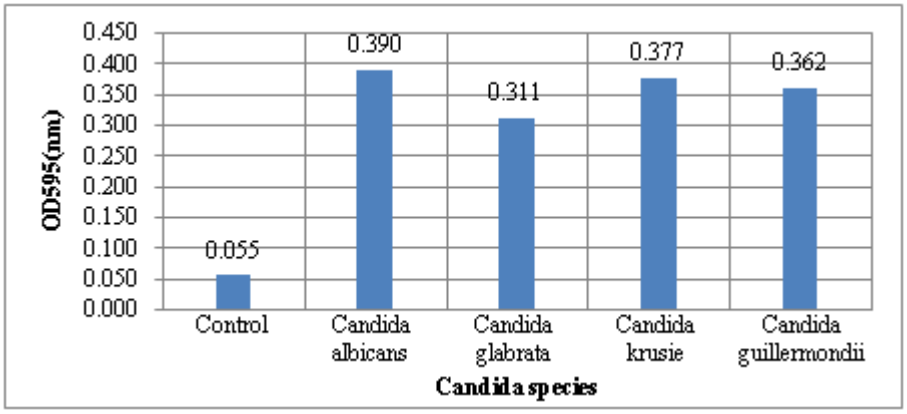

Figure 2: The Absorbance value (OD_{595}) of biofilm formation for Candida species

Biofilm inhibition studies carried out using various extracts of a plant *Syzygiumaromaticum*. In present study it was found that the extract from *S. aromaticum* showed strong activity and prevents the formation of biofilm by all clinical isolates. The results illustrated in figure 3&4, clearly indicate that ethanol extract of *S. aromaticum* showed anti-biofilm activity, and the best inhibitory effect on *Staph. Epidermidis* which was (68.3%), as compared to the inhibition percentage of aqueous extract which was (59.2%)with have significantly differences ($p \leq 0.05$), Using (LSD test). Similarly, *Candida albicans* was demonstrated the better results in ethanol extracts (67.61%) as compared to aqueous extract which was (60.31%). Also, all isolates were demonstrated the better results in ethanol extracts due to that ethanol extract resolves more major compounds resulting in the freeing of greater amount of antimicrobial components. Thepresent study is agreement with Kumar D. and Sidhu P.(34) who reported that clove extracts produce antibacterial activity against gram positive strains and multiple antibiotic resistant bacteria and can be used for maintaining oral hygiene.

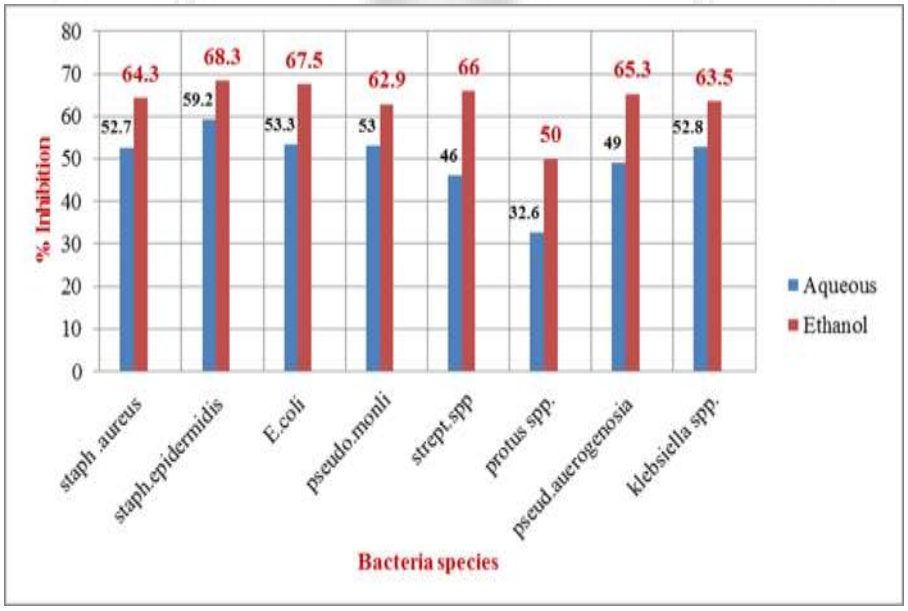

Figure 3: Effect of *S. aromaticum* onbiofilm formation against bacterial species

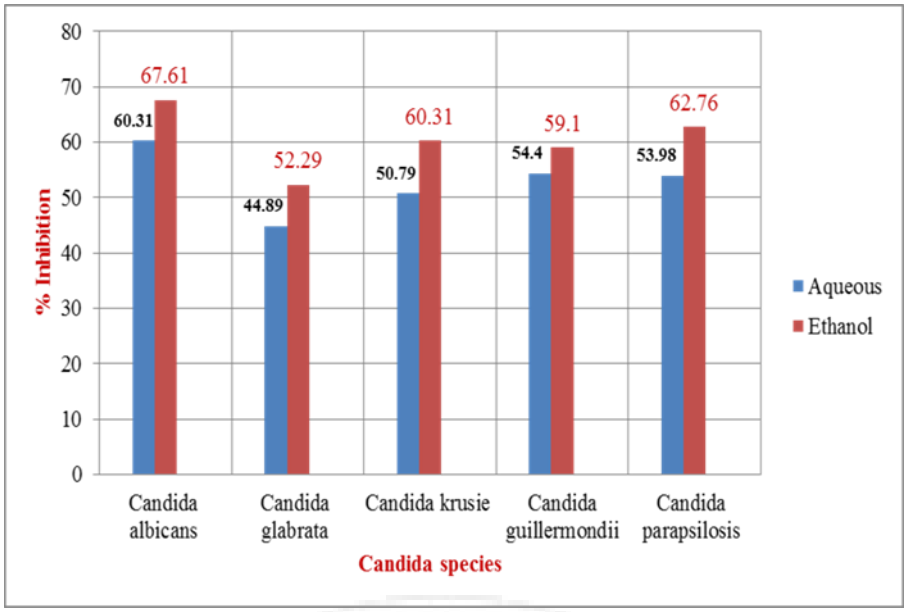

Figure 4: Effect of *S. aromaticum*on Biofilm formation against candida species

4. Conclusion

Currently, the Particular attention is oriented towards the need for antimicrobial that are able to eliminate infections that caused by antibiotic-resistant strains of oral cavity, especially the biofilm formation is the one of the major virulent factor of most pathogenic microorganism. Therefore, researchers have been encouraged to identify another option for the treatment of infections;Plant extracts have gained widespread interest in this regard.The present study demonstrated that *Syzygiumaromaticum* inhibit the growth of oral pathogens, reduce the rate of biofilms and dental plaque can serve as alternatives in prevention and treatment of dental caries.

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