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RESEARCH NOTE

REVISED Preliminary study on the inhibitory effect of seaweed *Gracilaria verrucosa* extract on biofilm formation of *Candida albicans* cultured from the saliva of a smoker [version 3; referees: 3 approved]

Zaki Mubarak [1], Adintya Humaira[1], Basri A. Gani[1], Zainal A. Muchlisin [2]

[1]Faculty of Dentistry, Syiah Kuala University, Banda Aceh, 23111, Indonesia
[2]Faculty of Marine and Fisheries, Syiah Kuala University, Banda Aceh, 23111, Indonesia

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Abstract

Background: *Candida albicans* is an opportunistic fungus that might infect the oral cavity. Increased colony numbers of *C. albicans* in the mouth can be caused by multiple factors, such as smoking, weakened immune system, antibiotics use and immune-compromised condition. Smoking can increase expression of virulence factors of *C. albicans* and make it stronger. One virulence factor of *C. albicans* is biofilm formation. The ability of creating biofilm makes *C. albicans* more tolerant to commercial antifungal agents. The objective of this preliminary study was to examine the ability of the seaweed *G. verrucosa* extracts to inhibit the formation of biofilm by *C. albicans* isolated from the saliva of a smoker.

Methods: The extract of *G. verrucosa* was prepared by maceration using 96% methanol and subjected for phytochemical analysis. *C. albicans* was isolated from the saliva of a smoker who voluntarily participated in the study after providing informed consent. In triplicate, the fungus was cultured in the growth medium containing increased concentrations of *G. verrucosa* (6.25, 12.5, 25, 50, 75 and 100%).The same reaction using fluconazole 0.31 µg/ml *C. albicans* was prepared as positive control. Biofilm formation was accessed based on optical density of cell mixtures using an ELISA reader. The data obtained were subjected to Kruskal-Wallis test at a significance limit of 0.05.

Results: Methanol extract of seaweed *G. verrucosa* contained three bio-active compounds namely steroids, terpenoid, and tannins. Inhibitory activity of seaweed extracts on *C. albicans* biofilm formation increased as their concentration increased. The highest inhibitory effect was recorded at fungus culture treated with seaweed concentration of 25% at 24 hours of time exposure.

Conclusions: Seaweed *G. verrucosa* extract contained steroids, terpenoids and tannins that were able to effectively inhibit the formation of biofilm by *C. albicans* at the concentration of 25% after 24 hours of time exposure.

Keywords

Candida albicans, oral candidiasis, seaweed Gracilaria verrucosa

Open Peer Review

Referee Status: ✓ ✓ ✓

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version 1 published 31 May 2018	? report	? report	? report

1 **Heni Susilowati**, Gadjah Mada University, Indonesia

2 **Shahida Mohd Said**, Universiti Kebangsaan Malaysia, Malaysia

3 **Cristiane Y. Koga-Ito**, São Paulo State University - UNESP, Brazil

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Comments (1)

Corresponding author: Zaki Mubarak (zakimubarak54@yahoo.com)

Author roles: Mubarak Z: Conceptualization, Formal Analysis, Funding Acquisition, Investigation, Methodology, Supervision, Validation; **Humaira A**: Data Curation, Investigation, Project Administration, Software, Visualization, Writing – Original Draft Preparation; **Gani BA**: Conceptualization, Formal Analysis, Investigation, Methodology, Resources, Writing – Original Draft Preparation; **Muchlisin ZA**: Software, Visualization, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

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REVISED **Amendments from Version 2**

Revisions, corrections and details addition have been done throughout the manuscript as a response to the last comments/questions of reviewers. Significant improvements made are including an explanation about the reasons why fluconazole 0.31 mg/ml did not show good antimycotic properties as expected. References supported this explanation were also provided. Some parts of this explanation also answers a question from reviewer about the reason for determining dose of fluconazole. Correction and enrichment in methods were also performed to provide better clarity and readability. The method used was a modification of those previously used by two different researchers in analyzing biofilm formation by bacteria. Further details, especially those related to the low respondent involved, could not be made because research presented was still in the preliminary stage.

See referee reports

Introduction

Smoking is a common problem in both developed and developing countries, including in Indonesia. Based on a survey by the Tobacco Atlas in 2015, Indonesia has the highest number of smokers in Asia, with 66% of men in Indonesia being active smokers[1]. Smoking can lead to addiction owing to the nicotine contents, and harm due to the presence of toxic compounds such as carbon monoxide, ammonia and tar contents in tobacco[1]. Substances in cigarettes can also contribute for the occurrence of oral candidiasis, infection of in the mouth cavity caused by the fungus *Candida albicans*[2]. This fungus is part of the normal flora of the human mouth, but it can become pathogenic in certain conditions, for example, due to nicotine exposure[3].

The *C. albicans* that infects human tissues generally form biofilm[3], an extracellular matrix consisting of *C. albicans* colonies[4]. The size of the biofilm increases when the fungus was exposed to substances in cigarette smoke because cigarette contains chemicals that can initiate the growth of and nourish *C. albicans*[5,6].

Currently, fluconazole and nystatin are the most effective drugs for treating oral candidiasis. Unfortunately, these drugs could result in undesired side effects. Prolonged use of fluconazole, for example, can leads to resistance[7] whereas high dosages of nystatin give gastrointestinal discomfort and increase plaque formation[8]. Therefore, plant-derived antifungals may be a viable oral treatment option for candidiasis. One of these potential plants is *Gracilaria verrucosa.* This seaweed contains several bioactive compounds, including alkaloids, flavonoids, phenolics, saponins, steroids and terpenoids[9]. Aceh Province, Indonesia, has large *G. verrucosa* resources, although this aquatic plant has not been commonly used for medicinal purposes. Hence, the objective of the present study was to examine the ability of seaweed extract to inhibit the biofilm formation of *C. albicans* isolated from the saliva of smoker.

Methods

Time and site

The study was conducted in August 2017 at the Laboratory of Microbiology, Veterinary Faculty, Syiah Kuala University. The *G. verrucosa* seaweeds were collected from a farmer in Pulo Aceh, Aceh Province.

Ethics

All research protocols used in this study were approved by the Research Ethics Committee of the Dentistry Faculty of Syiah Kuala University No. 1741/UN11.1.21/TU/2017.

Saliva collection

The saliva was collected from an active smoker who worked as administrative staff at the Faculty of Dentistry Medicine of Syiah Kuala University and voluntarily participated in this study after completing informed consent. Inclusion criterion of the volunteer was active smoker who smoked 20 cigarettes per day. Saliva was collected once by spitting into a glass jar (15 ml) right after the subject finished smoking, and added with 10 ml of PBS (0.01 M, pH 7.2). The jar was centrifuged at 10,000 rpm for 10 minutes. The precipitate was stored for the microbiological examination.

Candida albicans isolation and preparation

Precipitate was cultured in ChromAgar *Candida* medium and incubated at 37°C for 2 days. The *C. albicans* fungus grew as green colonies. One colony of *C. albicans* was mixed with 5 ml of peptone in a tube and incubated at 37°C for 24 hours. Turbidity of medium was compared to a 0.5 McFarland solution standard, which was equivalent to 1.5×10^8 CFU/ml.

Seaweed extraction

Seaweed extraction was performed based by maceration using 96% methanol[10]. In brief, a total of 500 g of fresh seaweeds were washed with tap water then with distilled water. Seaweed samples were at 25°C for 24 hours, chopped into small pieces (2 mm), and soaked in 1.5 liters of 96% methanol. Macerate was filtered using Whatman filter paper No. 42. Filtrate collected was concentrated using a vacuum rotary evaporator (Laborta 4003 control, Heildolph) 60°C and at a speed of 80–90 rpm for 3 days. Concentrated extract was put in a sealed dark bottle and stored at 4°C.

Phytochemical tests

Flavonoid test. A 0.5 cm magnesium plate was rinsed in 5 ml of seaweed extract, mixed with two drops of HCl, and heated by passing it over a Bunsen flame. Red or purple coloration formed on the heating indicated the presence of flavonoids[11].

Alkaloid test. Seaweed extract (5 ml) was mixed with 8 ml of HCl and filtered. Filtrate was subjected to Mayer, Wagner and Dragendroff tests for alkaloids[11]. This was done by mixing 2 ml of filtrate with 5 g potassium mercuric iodide (Mayer test), 2 ml of Wagner reagent, or 2 ml of bismuth potassium iodide solution (Dragendroff test). The formation of white or pale precipitates (Mayer test), brown or reddish-brown precipitates (Wagner test) and red precipitates (Dragendroff test) indicated the presence of alkaloids.

Tannin/phenolic test. Two drops of 1% $FeCl_3$ was added to 1 ml seaweed extract. The change in the color to a blackish green indicated the presence of tannin/phenolic[12].

Saponin test. Seaweed extract, 1 ml, was diluted in 20 ml of distilled water and shaken vertically for 15 seconds. Persistent foaming indicated the presence of saponin content.

Steroid test. Two milliliters of seaweed extract was added with 2 ml of $CHCl_3$, 2 drops of H_2S and 1 ml of CH_3COOH. The formation of green or blue precipitates indicated the presence of steroid[11].

Terpenoid test. Seaweed extracts (5 ml) was mixed with 2 ml of chloroform. Concentrated H_2SO_4, 3 ml, were carefully added. The formation of reddish brown layer at the interface of extract and chloroform solution indicated the presence of terpenoids[13].

Examination of biofilm formation

Casein-peptone lecithin polysorbate broths (Merck-1117230500), 100 µl, were poured in each well of a 96-well plate, incubated at room temperature for 5 minutes and discarded by blotting the plate on paper towels 2–5 times. Fifty microliter *C. albicans* culture had turbidity was equal to 0.5 McFarland standard were added to each well and incubated for 5 minutes to attachment of fungal cells on casein. Cell mixtures were washed by aspiration. In triplicate, 50 µl of decreased concentrations of seaweed extracts (100, 75, 50, 25, 12.5 and 6.25%) were added. The same reaction using fluconazole 0.31 µg/ml was prepared as positive control. The plates were then incubated at 37°C for 24, 48 or 72 hours. Each well was added with 200 µl of 0.1% violet crystal, incubated at 25°C for 15 min, and washed three times with 200 µl of 0.01 M PBS. The crystal violet in each well was then removed by adding 100 µl of 96% ethanol for 2 min. The biofilm formation was analyzed by reading optical density of mixture using an ELISA reader at 620 nm[14,15].

Data analysis

The data obtained were subjected to Kruskal-Wallis test using SPSS software v20.0 for windows.

Results

The results of phytochemical tests in Table 1 show that methanol extract of seaweed *G. verrucosa* contained bioactive compounds belonged to steroids, terpenoids, and tannins/polyphenols.

In general, the inhibitory effect was reduced as seaweed concentration increased (Figure 1). The highest inhibition after 24 and 48 hours time exposures was recorded from *C. albicans* culture treated with 25% seaweed extract, followed by those with 50% and 75%. The best inhibition after 75 hours time exposure, however, was found in *C. albicans* culture treated with 25% seaweed extract. In all time exposures, the inhibitory effect caused by fluconazole (control) was the worst, followed by those caused by 100% seaweed extracts, but there was no difference between these two groups. Results of Kruskal-Wallis analysis showed that seaweed extract significantly ($p<0.05$) inhibited the formation of biofilm by *C. albicans,* indicated potential of the extract to inhibit the growth of the fungus.

Dataset 1. The raw data of the Triplo anti-Biofilm seaweed to *C. albicans* for 24, 48 and 72 h at a wavelength 620 nm

http://dx.doi.org/10.5256/f1000research.14879.d204270

Discussion

The study showed that 25–75% seaweed extracts are promising for inhibiting the growth of *C. albicans* as shown by much lower biofilm formation after 24, 48 and 72 hours exposure compared to those caused by positive control (fluconazole) and 100% seaweed extract. This indicated potential of seaweed extract as natural anti-fungus to treat oral candidiosis caused by against *C. albicans* in smokers.

C. albicans is a normal micro-organism in the human mouth. This fungus, however, can be pathogenic in certain circumstances[3] such as in the mouth of smokers[2]. Smoking can stimulate synthesis of HWP1, EAP1 and SAP2 proteins in *C. albicans*, causing higher virulence of the fungus. This can lead to increased formation of biofilm and finally cause oral candidiasis[6]. Smoking also cause a decreased immune function, making individuals more susceptible to oral infections, including candidiasis[4,6].

The low effectiveness of fluconazole 0.31 µg/ml, the leading choice of therapy against *C. albicans*, against this fungus was probably caused by several factors such as low therapeutic dose and resistance of the fungus to the drug. Pfaller *et al.*, who

Table 1. Phytochemical contents of seaweed *Gracilaria verrucosa* extract.

Substance	Reagent	Result	Indication
Alkaloid	Mayer	-	White deposit
	Wagner	-	Brown deposit
	Dragendroff	-	Red deposit
Steroid	Uji Lieberman-Burchard	+	Green or blue colors
Terpenoid	Uji Lieberman-Burchard	+	Red or purple colors
Saponin	Shuffling method	-	Stable foams
Flavonoid	0.5 Mg and HCl	-	Red or purple colors
Tannin/Phenolic	$MgCl_3$	+	Dark green

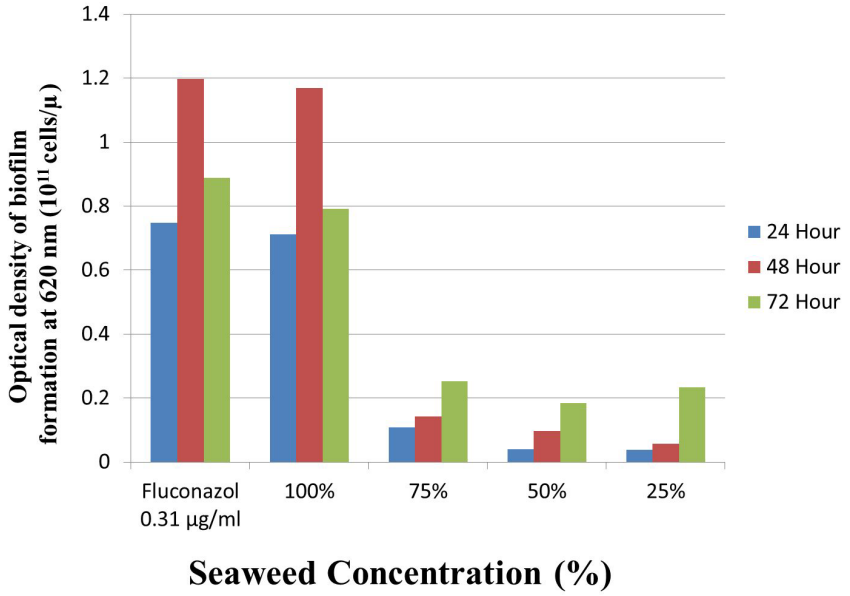

Figure 1. The formation of biofilm by *Candida albicans* exposed to seaweed *Gracilaria verrucosa* extract on different time exposures and concentrations.

complement CLSI guidelines with the EUCAST guidelines about species-specific interpretative breakpoints for fluconazole susceptibility suggested that the breakpoints for *C. albicans* are <2 mg/ml, 4 mg/ml and >8 mg ml for susceptible, susceptible dose-dependent, and resistant isolates, respectively[16]. Dosage criteria for analyzing candida species using MIC test were susceptible (MIC < 8 µg/ml; range, 0.25 to 4 µg/ml), susceptible dose-dependent (MIC 8 – 16 µg/ml; range, >4 to <16 µg/ml), and resistant (MIC > 16 mg/ml; range, 16 to >128 mg/ml)[17]. Fluconazole dose of 0.31 µg/ml used in this study was slightly higher than minimum dose ideally used to test antifungal resistance of the antifungal. This also meant that *C. albicans* strain isolated from smoker respondent involved in this study already developed resistance to the dose used. Increasing resistance of *C. albicans* against this fluconazole, one of the most azole antimycotics have been reported[18,19]. This resistance, that is assumed related to prolonged or repeated exposure to low-dose of the antifungus[20], must be confirmed by further study.

The stronger ability of 25–75% seaweed extracts to inhibit the formation of *C. albicans* biofilm significantly compared to those of fluconazole (positive control) was probably caused by bioactive compound presence in the extracts. These included steroids, terpenoids, and tannins/polyphenols, all of which have been known their benefit for human health. According to Sampaio *et al.*[21], antifungal activity of a substance strongly depends on the composition of its bioactive compounds. Steroids can kill *C. albicans* through their lypophilic properties, interfering with the formation of fungal spores and mycelium[22]. This activity weakens *C. albicans*, inhibiting the formation of the biofilm. To function optimally, steroids require oligosaccharides that are also present in the seaweed content[23]

Terpenoids are derivatives of saponins that may act as an antifungals by damaging organelles of the fungus and by inhibiting secretion of enzymes, leading to growth the inhibition *C. albicans* cells. Terpenoids can also damage the morphology of *C. albicans*[24]. Tannins may inhibit chitin synthesis in *C. albicans* cell walls, leading to lost of membrane cell protection and disrupted cellular metabolism. Tannins also can inhibit ergosteron activity of *C. albicans*[25].

The effectiveness of seaweed extracts in inhibiting fungal growth is influenced by at least three factors, namely concentration, exposure time, and contact surface media[26]. The present study showed that diluted concentration (25–75%) of extracts showed better inhibitory effect on the growth of *C. albicans* than the more concentrated 100% extract. Concentrated extract usually has lesser effectiveness *in vitro* due to solubility and import problems. In more aqueous condition plant extracts generally show better medicinal properties with increased concentration as shown by a study testing antifungal activity of ethanol extracts of *Syzygium jombolanum*, *Cassia siamea*, *Ordina wodier*, *Momodica charantia* and *Melia azedarach* as well as two algal species *Sargassum wightii* and *Saulerpa scalpellformis* against 25 *C. albicans* isolates *in vitro*[27].

This study also showed the best inhibitory effect of seaweed extracts was recorded at 24 hour of exposure. This is probably because the farnesol, a quorum-sensing molecule that has the potency to inhibit *C. albicans* growth[28], works effectively after 48–72 hour of exposure.

This study, however, was still preliminary due to limited number of subject and unavailability of non-smoker, control. Virulence

factor of *C. albicans* analyzed was also only biofilm formation. Since more virulence factors are possibly synthesized by the fungus under certain environmental condition, further studies are need to be conducted to investigate effect of individual bioactive compound contained in the seaweeds on the formation of biofilm and on the expression other virulence factors of *C. albicans* isolated form both smoker and non-smoker individuals.

Conclusion

Extract of *Gracilaria verrucosa* seaweed could inhibit the growth of *C. albicans* isolated from the saliva of a smoker. The inhibitory effect decrease with the increase of concentration, and reached the highest at concentration of 25% and time exposure of 24 hours.

Data availability

Dataset 1. The raw data of the Triplo anti-Biofilm seaweed to *C. albicans* for 24, 48 and 72 h at a wavelength 620 nm. DOI: 10.5256/f1000research.14879.d204270[29].

Grant information

The author(s) declared that no funding was involved in supporting this work.

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http://www.doi.org/10.5256/f1000research.14879.d204270

Open Peer Review

Current Referee Status: ✔ ✔ ✔

Version 3

Referee Report 26 September 2018

doi:10.5256/f1000research.17646.r38461

✔ **Cristiane Y. Koga-Ito**
Oral Biopathology Graduate Program, Institute of Science and Technology, São Paulo State University - UNESP, São José dos Campos, Brazil

The revised manuscript has improved. I recommend indexing of the revised manuscript as a Research Note.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Referee Report 21 September 2018

doi:10.5256/f1000research.17646.r38462

✔ **Heni Susilowati**
Department of Oral Biology, Faculty of Dentistry, Gadjah Mada University, Yogyakarta, Indonesia

I have read the revised version and can accept the revisions that have been made by the author insofar as this research is still an initial research.

Competing Interests: No competing interests were disclosed.

Referee Expertise: Molecular biology and oral microbiology

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 2

Referee Report 23 August 2018

doi:10.5256/f1000research.17357.r37284

Shahida Mohd Said
Periodontology Unit, Centre for Restorative Dentistry, Faculty of Dentistry, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia

Improvements made are acknowledged and approved.

Competing Interests: No competing interests were disclosed.

Referee Expertise: Periodontology, natural product drug discovery

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Referee Report 20 August 2018

doi:10.5256/f1000research.17357.r37282

Heni Susilowati
Department of Oral Biology, Faculty of Dentistry, Gadjah Mada University, Yogyakarta, Indonesia

I really appreciate the efforts of the authors who have revised the report, but I would like to add suggestions to the authors to explain why the optical density value of the treatment group treated with Fluconazole and extracts of 100% was even greater than the extracts with smaller concentrations.

Competing Interests: No competing interests were disclosed.

Referee Expertise: Molecular Biology and Oral Microbiology

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Version 1

Referee Report 27 June 2018

doi:10.5256/f1000research.16195.r34560

Cristiane Y. Koga-Ito
Oral Biopathology Graduate Program, Institute of Science and Technology, São Paulo State University - UNESP, São José dos Campos, Brazil

The manuscript by Mubarak et al. aimed to evaluate the effect of G. verrucosa extract on Candida albicans biofilm formation. The rationale of the study should be clearer. Also, there is lack of essential information throughout the text. The English should be revised.

Specific comments

Introduction
The period “Smoking is a common problem in most developing countries, including Indonesia.”. A reference should be added. Also, smoking is not a problem only or mainly in developing countries. Please, consider revising.
The authors stated that “This fungus is part of the normal flora of the human mouth, but it can become pathogenic in certain conditions, for example, due to nicotine exposure.”. The exposure to nicotine has been correlated with increasing in C. albicans virulence factor expression. The predisposing conditions for candidiasis are much more related to immunologic state of the host and imbalance in microflora. Please, consider revising.
Revise the period “Infection with C. albicans will increase the formation of a biofilm of the fungus”, it is confusing.
Revise the period “The biofilm is an extracellular matrix consisting of C. albicans colonies”, it is confusing.
The authors stated that “high dosages of nystatin give gastrointestinal discomfort and increase plaque formation”. What do authors mean by “increase plaque formation”?
The rationale of the study is not clear. Why did the authors select G. verrucosa? Is this plant commonly used? Why did the authors decide to use saliva from a smoker individual?

Methods
Revise the period “C. albicans was extracted from the saliva”, C. albicans was isolated from saliva.
The authors reported that “G. verrucosa seaweed was collected from a farmer in Pulo Aceh, Aceh Province.”. More information on the plant, the exact location it was collected from, the period of the year, identification procedure (how and who did the identification?), registration in herbarium, number of voucher should be included in the text.
Please, revise the period “Saliva was collected by spitting into a glass jar (15 ml), then 1 ml PBS (0.01 M, pH 7.2) was added to the jar.” Why did 1 ml of PBS added to the saliva? What was the final volume of saliva collected? Was saliva stimulated?
The authors stated that “If the colour of a colony was green, this indicated that the colony was C. albicans.”. However, the color of the colony in CHROMagar is only a presumptive test and phenotypic or genotypic definitive identification should be done.
The inclusion of a reference strain is highly needed.

Include the number of experiments/replicates performed.

The inclusion of more clinical isolates from non-smokers patients is needed.

The methodology of activity of extract on biofilm formation is not clear. Why and how peptone was removed from the wells? Why did the fungal suspension left in the wells for 5 min? How 100% extract concentration was obtained in the well (you already has the broth inside the well)? Why the concentration of 0.31 was chosen for fluconazole?

Figure 1 should be revised. Note that optical density at 620 nm is higher after treatment with 100% extract when compared to the other concentrations.

Discussion and Conclusion sections should be revised after the revision of the aforementioned points.

Is the work clearly and accurately presented and does it cite the current literature?
No

Is the study design appropriate and is the work technically sound?
No

Are sufficient details of methods and analysis provided to allow replication by others?
No

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
No

Are the conclusions drawn adequately supported by the results?
No

Competing Interests: No competing interests were disclosed.

Referee Expertise: Microbiology

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Referee Report 19 June 2018

doi:10.5256/f1000research.16195.r34561

? **Shahida Mohd Said**
Periodontology Unit, Centre for Restorative Dentistry, Faculty of Dentistry, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia

Overall fair manuscript with sound finding and relevant area of study. Can much be improved with grammar check and essential scientific writing reorganisation especially in Introduction and Discussion section. Inclusion of results for negative control (untreated biofilm) would critically improve Results presentation and appreciation of findings. Lacks relevant information on findings between smoker and non-smoker in the study that may not strongly support the conclusion on the anti-fungal effect of agent on smoker.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
No

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Referee Expertise: Periodontology, natural product drug discovery

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 05 Aug 2018

Zaki Mubarak, Syiah Kuala University, Indonesia

Some improvement in grammar, sentences and writing reorganization have been done as a response to reviewer Dr Shahida M. Said. Some details in methods and analysis have also been provided to make better readibility and potential reproducibility of the work. As a result the work in this study is much better presented. These improvements can be found in track changes.

As this study is still preliminary comparison between smoker and non-smoker has not been performed yet, but the finding clearly show potential of use of seaweed extract to treat oral candidiasis.

Competing Interests: No competing interests were disclosed.

Referee Report 12 June 2018

doi:10.5256/f1000research.16195.r34557

Heni Susilowati
Department of Oral Biology, Faculty of Dentistry, Gadjah Mada University, Yogyakarta, Indonesia

This preliminary research is interesting enough to be developed but there are some things that need to be reconsidered:

1. ABSTRACT
- Research background that written on the abstract (lines 6 and 7) does not match the purpose of the study. The sentence can be interpreted in contrast to the antifungal potential possessed by Garcinia verrrucosa. It is dubious to investigate the potency of biofilm inhbition effect if Candida albicans more tolerant to Gracilaria extract.

2. METHODS

Methods need to explain the following:

- Systemic conditions and state of teeth and oral soft tissue volunteers,
- The predestined determination of the Gracilaria verrucosa plant should be mentioned.
- Were the culture washed after the incubation period on treatment?
- How many times an experiment produces a representative result?

3. RESULTS

- Interpretation of the results is confusing; as far as I know the higher the optical density value the more biofilms are formed. The results in Fig. 1 show that 100% and Fluconazole extracts have higher optical densities rather than the lower concentration of extracts, as far as I know this shows that the mass of biofilms formed in the group is higher. Please observe the methods and results of research reported by Sebaa et al 2016.
- The statistical method used was only Kruskal-Wallis, is there a multiple difference analysis? Researchers need to discuss the effect of antibiofilm extract at lower concentrations, because of course 100% extract is not a good recommendation for subsequent experimental use.

References

1. Sebaa S, Hizette N, Boucherit-Otmani Z, Courtois P: Dosedependent effect of lysozyme upon Candida albicans biofilm.*Mol Med Rep*. 2017; **15** (3): 1135-1142 PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
No

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
No

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 05 Aug 2018

Zaki Mubarak, Syiah Kuala University, Indonesia

Abstract

Comments from reviewer :

The objective has been clarified
Action done:
We think aims of study still need to be stated
Rearrangement and sentences improvement were done in some sections of abstract

Introduction
Comments from reviewer :
No comments
Action done:
Grammar check, revise and improvement were done to provide more concise and clear sentences/statements

Material and method
Comments from reviewer :
Need clarification on sentence:
“This volunteer has a bad OHIS4” (time and place)
“.Then microtiter wells plate wells were washed three times with 200µl of PBS buffer and dried for 15 minutes.”(biofilm examination)
“This examination was repeted three times with triplo method”(biofilm examination)
Action done:
Sentences improvement, grammar check and correction had been done throughout this section to provide better readability, clarity and possible reproducibility

Results
Comments from reviewer :
No comments
Action done:
Rewritten this part due to misinterpretations we did in the first previous manuscript about which treatment gave best inhibition effect on *C. albicans*

Discussion
Comments from reviewer :
No specific comments
Action done:
Rewritten has been done to this part in realtion to due to changes in results presentation
Sentences improvement, grammar check and reorganization had been done throughout this section to provide better readability and clarity

Conclusion
Comments from reviewer :
No specific comments
Action done:
This part had been rewritten in relation to the revision performed in result and discussion.

Competing Interests: No competing interests were disclosed.

Discuss this Article

Version 1

Author Response 05 Aug 2018

Zaki Mubarak, Syiah Kuala University, Indonesia

Some revisions and improvements have been done throughout the manuscript as responses to the good comments and suggestions from reviewers. Major revisions performed include grammar check and corrections; sentence improvements; and reorganization and adding appropriate details in each section of the material and methods part. Grammar issues, were concerned by all reviewers, had been addressed. The ways to present results were also changed as response to comment from Indonesia reviewer about the meaning of OD values in presenting the growth of *C. albicans* and how to interpret them. Restructure of result and discussion, therefore, was also performed. These changes became the basis for synthesizing more appropriate conclusion sentences. One reference was also added to support discussion about the importance of extract concentration for resulting in expected medicinal effects, in this study was growth inhibition that reflected by biofilm formation. Since this study is still in preliminary step, no non-smoker participant was participated. As consequence, no comparison could be made between smoker and non-smoker groups. Yet the study was still able to confirm potential of seaweed *Gracia verrucose* extract as anti candidiasis in smokers. Potential of doing further studies was mentioned in relation to this weakness of our study.

Competing Interests: No competing interests were disclosed.

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