Effect of goji berry ethanol extract (*Lycium barbarum* L.) on *Streptococcus mutans* and *Porphyromonas gingivalis* biofilms

ABSTRACT

Background: Caries and periodontitis are commonly found in the Indonesian population. *Streptococcus mutans* and *Porphyromonas gingivalis* in the form of biofilms play a major role in causing caries and chronic periodontitis. Chlorhexidine mouthwash can be used to prevent and treat periodontitis; however, due to its many side effects, an alternative treatment, using natural ingredients that have antibacterial effects, is needed. *Lycium barbarum* L. fruit, which contains flavonoids and phenolic acids, has antibacterial properties that are expected to inhibit bacterial growth and the formation of *S. mutans* and *P. gingivalis* biofilms. **Objective:** To determine the antibacterial and antibiofilm effects of *L. barbarum* -fruit ethanol extract against *S. mutans* and *P. gingivalis*. **Methods:** An in vitro laboratory experiment was performed with a post-test control group design. The extract of *L. barbarum* -fruit was obtained by maceration using 96% ethanol as a solvent. The test solutions were *L. barbarum* -fruit ethanol extract at a concentration of 100%, 50%, 25%, 12.5%, and 6.25%, chlorhexidine gluconate 0.2% as a positive control, and sterile distilled water as a negative control. The antibacterial assay was performed by microdilution and plate count methods. The antibiofilm effect was performed using a biofilm assay method. **Result:** The results of the microdilution and plate count methods showed that the most effective concentration with antibacterial properties against *S. mutans* and *P. gingivalis* was 100% when compared with the negative control ($p < 0.05$). In the biofilm assay, the most effective concentration against *S. mutans* was 100% at the 3-hour incubation time, while for *P. gingivalis*, the most effective concentration was 100% at the 24-hour incubation time when compared with the negative control ($p < 0.05$). **Conclusion:** The ethanol extract of *L. barbarum* -fruit was demonstrated to have antibacterial and antibiofilm effects against *S. mutans* and *P. gingivalis*.

Keyword:

1. Introduction

According to the World Health Organization (WHO), optimal dental and oral health is free of dental caries, periodontal disease, oral cancer, infections and sores in the mouth, noma~~noma~~, cleft lip and palate, tooth decay, tooth loss, and diseases that cause biting disorders, all of which negatively impact chewing, smiling, talking, and psychosocial well-being (WHO, 2020). Oral health positively affects the appearance, as well as the physical, mental, and interpersonal well-being, of an individual. Thus, oral health, which is part of overall health, contributes to quality of life (Katge et al., 2015).

Basic Health Research Data (Riskesdas ~~Riskesdas,~~ 2018) shows that 57.6% of the Indonesian population experiences dental and oral health problems. The prevalence of dental caries in Indonesia in 2018 was 88.8%, with an average DMF-T index of 7.1, which is a very high severity of dental caries. Periodontitis is experienced by 74.1% of the Indonesian population (Kemenkes, 2018).

Caries is the process of the demineralization of inorganic material and the dissolution of organic material, leading to bacterial invasion through the dentin layer until it reaches the pulp (Heng, 2016; Chenicheri et al., 2017). The process of dental caries depends on the presence of fermentable sugars (substrates), the type of tooth and saliva (host), cariogenic microbial flora (biofilm), and time (Conrads and About, 2018). Periodontitis is a disease caused by inflammation of the tooth-supporting tissue, caused by microorganisms, that causes progressive destruction of the periodontal ligament and alveolar bone, with the formation of pockets, recessions, or both (Hinrichs and Kotsakis, 2015). Periodontitis in adults is caused by numerous local factors, such as biofilms or calculus, classified as chronic periodontitis (Kumar and Sengupta, 2011).

The formation of biofilms begins when microorganisms in the planktonic state merge into bacterial colonies and wrap themselves in a self-produced extracellular polymer matrix (~~Ollie Yiru~~ Yu et al., 2017). At the beginning of the formation of biofilms, there is an increase in the activity of

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Ggram-positive *cocci*, one of which is *Streptococcus mutans*, which is able to adhere to the tooth surface through the formation of extracellular polysaccharides that cause the biofilm matrix to have a gelatin-like consistency that facilitates the attachment of bacteria to the tooth's surface (Arul and Palanivelu, 2014; Chenicheri et al., 2017; Abranches et al., 2018). *Porphyromonas gingivalis*, which is a secondary bacterium, is an anaerobic Ggram-negative bacterium found in periodontal pockets that causes chronic periodontitis. Various virulence factor of *P. gingivalis* such as gingipains, fimbriae, and lipopolysaccharides, plays an important role in ~~regulating virulence, such as gingipains, fimbriae, and lipopolysaccharides, which furthers~~ the development of periodontal disease and induces dysbiosis in biofilms (Bao et al., 2014; Mysak et al., 2014).

Chlorhexidine mouthwash is used to prevent caries and treat periodontitis and is considered the gold standard for controlling dental plaque and gingivitis due to its efficacy against a wide variety of bacteria, fungi, and viruses. However, chlorhexidine has various side effects, including taste disturbances, discoloration of the teeth and mucosa, mucosal desquamation, salivary stone formation, irritation, dry oral cavity, and allergic reactions, such as contact stomatitis. The WHO recommends finding new natural ingredients to overcome the side effects of chemical agents (Rezaei et al., 2016; Jeddy et al., 2018).

The use of natural ingredients as antimicrobial agents has become an alternative because of their low cost and lower toxicity (Martianez et al., 2017). Various herbal mouthwashes that have been tested successfully ~~used by the community~~ include *mimba* (*Azadirachta indica*), aloe vera (*Aloe perfoliata var. Vera L.*), and tea tree oil (*Melaleuca alternifolia*) (Manipal et al., 2016).

Goji berry (*Lycium barbarum* L.) ~~L.)~~ has been widely used as a traditional medicine by people in Asia, especially in the northwestern part of China, for more than 2000 years. Recently, *L. barbarum* has been gaining popularity as ~~and is referred to as a superfruit, which is a~~ highly nutritious food used to improve health in North America, Europe, and Asia (Ma et al., 2019). *L. barbarum* has a red, oblong fruit with a length of 6–20 mm and a diameter of 3–10 mm. *L.~~ycium barbarum L.~~* fruit is harvested when it is ripe and is then dried for later use (Alassadi ~~Fatima S Sabah and Alrubaie~~ et al.~~,~~ 2015). The fruit, roots, tree bark, and flowers of *L. barbarum* have been shown to be used as medicine (Byambasuren et al., 2019).

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The polysaccharides of *L. barbarum* have exhibited properties that improve eye health and reproductive system health, reduce fat and blood sugar, and regulate immunity; they have also been shown to have anticancer, anti-tumor, antioxidant, anti-fatigue, antiviral, anti-aging, hepatoprotective, neuroprotective, and cardioprotective properties (Cheng et al., 2015; Ma et al., 2019). The flavonoids and phenolic acids of *L. barbarum* have potential as antioxidants and antimicrobials (Skenderidis~~, Mitsagga,~~ et al., 2019).

Lycium~~.~~ barbarum fruit has been shown to be effective in inhibiting gram-negative bacteria, such as *Escherichia coli*, and gram-positive bacteria, such as *Staphylococcus aureus* (Skenderidis~~, Mitsagga,~~ et al., 2019). However, there have been no studies regarding the antibacterial effect of *L. barbarum*~~-~~. fruit against *S. mutans* and *P. gingivalis* as bacteria that cause caries and chronic periodontitis. Thus, the aim of this study was to determine the antibacterial and antibiofilm effects of goji berry (*L. barbarum*~~))~~ethanol extract against *S. mutans* and *P. gingivalis*.

2. Material and methods

2.1 Ethanol extract of *L. barbarum* fruit

Dried *L. barbarum* ~~f~~fruit (100 g) from Chinese medicine store "Lancar Jaya" at Teluk Gong Raya No. 43, Jakarta Utara (produced in Zhongning, Ningxia, China) was ground in a blender until it became ~~a~~ powder. It was then immersed in 96% ethanol with a ratio of 1:8 for 72 hours, stirring every 15 minutes. Furthermore, filtration was performed using Whatman No. 1 filter paper and evaporated with a rotary evaporator at 40°C temperature, a speed of 60 rpm, and a pressure of 20 atm so that a thick and solvent-free extract was obtained with a concentration of 100%. The extracts were then diluted using sterile distilled water until concentrations of 50, 25, 12.5, and 6.25% were obtained.

2.2 Phytochemical assay

Phytochemical assays were performed qualitatively to determine whether the ethanol extract of *L. barbarum* fruit contained flavonoids, phenols, quinones, steroids, terpenoids, and alkaloids.

2.3 Bacterial cultures

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Streptococcus~~.~~ *mutans* ATCC 25175 and *P. gingivalis* ATCC 33277 from MiCORE Laboratory, Faculty of Dentistry, Trisakti University, were cultured on BHI-B medium and incubated at 37°C for 24 hours in an anaerobic atmosphere. Furthermore, the absorbance measurements were performed to reach the McFarland standard of 0.5 = 1.5 × 10^8 CFU/mL (OD_{600} = 0.132).

2.4 Microdilution

Each well of a 96-well plate was distributed 100 µL of either an *S. mutans* or *P. gingivalis* culture. Subsequently, 100 µL of the following solutions was used as a treatment: ethanol extracts of *L. barbarum* ~~-~~fruit at 100, 50, 25, 12.5, and 6.25% concentrations, 0.2% chlorhexidine gluconate as a positive control, and sterile distilled water as a negative control. The measurement of bacterial cell density was performed using a microplate reader at a 600 nm wavelength before and after the 96-well plates were incubated for 24 hours.

2.5 Plate count

The microdiluted contents in the 96-well plates were re-diluted 10,000 times and cultured on BHI-A medium and incubated for 24 hours at 37°C to measure bacterial growth. The total bacterial number was calculated by the following formula:

2.6 Biofilm assay

B~~A b~~acterial culture (200 µL) was dispensed into each well of a 96-well plate and incubated at 37°C for 48 hours in an anaerobic atmosphere. Furthermore, the supernatant was removed until a thin layer of biofilm was left on the bottom surface of the well. The wells were rinsed with a solution of phosphate-buffered saline (PBS). The ethanol extracts of *L. barbarum* fruit at a concentration of 100, 50, 25, 12.5, and 6.25%, 0.2% chlorhexidine gluconate as a positive control, and sterile distilled water as a negative control were added 200 µL to the wells ~~, as much as 200 µL,~~ using a micropipette and incubated at 37°C for 1, 3, or 24 h in an anaerobic atmosphere. The well was rinsed twice using PBS and then fixated over a flame. Crystal violet dye (200 µL; 0.05% w/v) was added to each well and left for 15 minutes. The well was rinsed twice using PBS and left for 15 minutes. Then, 200 µL of 96% ethanol was inserted, and OD measurements were performed using a microplate reader at a 595 nm wavelength.

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2.7 Statistical analyses

Statistical Product and Service Solution (SPSS) software version 25.0 was used to process the collected data. The Shapiro-Wilk method was used to test normality. Data that was normally distributed ($P > 0.05$) was analyzed by a one-way analysis of variance (ANOVA) test. Significant data ($P < 0.05$) were analyzed with a post-hoc test using Tukey's test ~~HSD with~~ a significance level of $P < 0.05$ to determine which groups were significantly different.

3. Results

The phytochemical test qualitatively showed that the ethanol extract of *L. barbarum* ~~–~~fruit contained flavonoids, phenols, steroids, and terpenoids.

The results of this study indicated that the ethanol extract of *L. barbarum* ~~-~~fruit has antibacterial and antibiofilm effects against *S. mutans* and *P. gingivalis*. The ethanol extract of *L. barbarum* fruit with a concentration of 100% had the most effective antibacterial effect against *S. mutans* and *P. gingivalis*, with a total number of *S. mutans* colonies of 3 ± 3.46 x 10^6 CFU/mL (Figure 1) and an OD value of 0.358 ± 0.002 (Figure 3). The total number of *P. gingivalis* colonies was 41 ± 4.58 x 10^6 CFU/mL (Figure 2), with an OD value of 0.458 ± 0.024 (Figure 4).

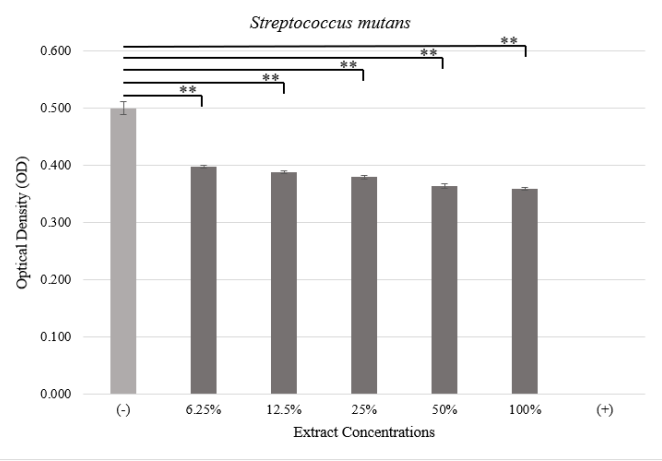

Figure 1. Optical Density (OD) ~~OD~~ value of *Streptococcus~~.~~ ~~-~~mutans* based on the concentration of ethanol extract of *Lycium ~~-~~barbarum ~~L.~~* L. fruit. **Significant difference at $P < 0.01$.

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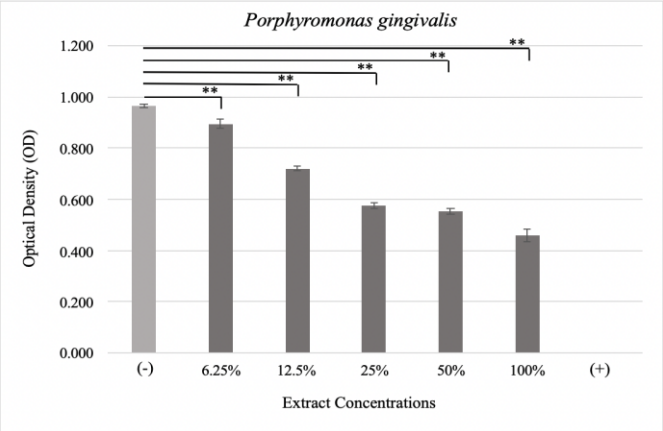

Figure 2. Optical Density (OD) value ~~OD value~~ of *Porphyromonas~~.~~ gingivalis* based on the concentration of ethanol extract of *Lycium~~.~~ barbarum* L.~~ L.~~ fruit. **Significant difference at P < 0.01.

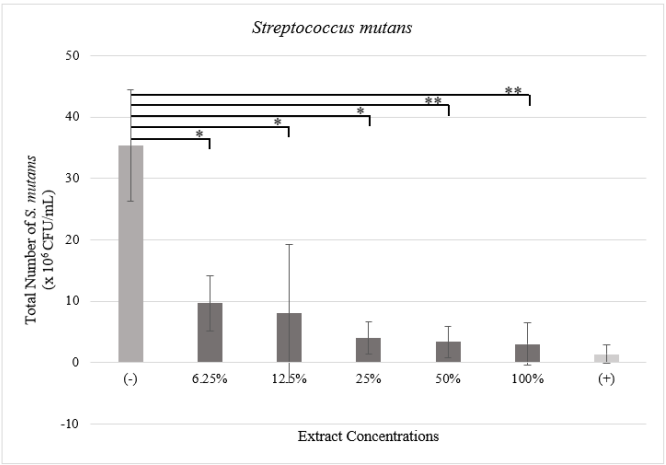

Figure 3. Total number of *Streptococcus~~.~~ mutans* based on the ethanol extract concentration of *Lycium~~L.~~ barbarum* L.~~ L.~~ fruit. *Significant difference at P < 0.05; **Significant difference at P < 0.01.

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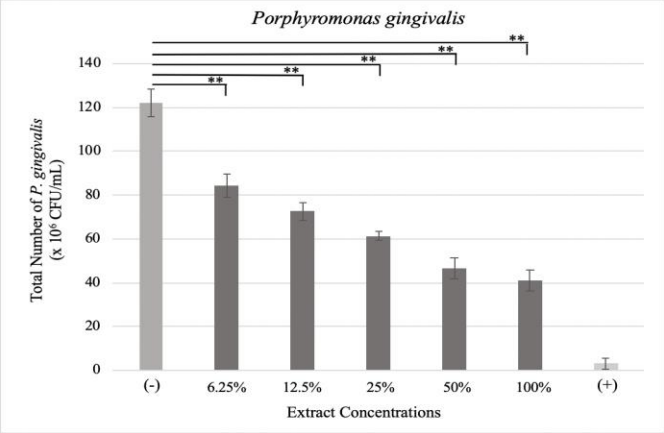

Figure 4. Total number of *Porphyromonas gingivalis* based on the concentration of ethanol extract of *Lycium barbarum* L. fruit. ~~*P. gingivalis* based on the ethanol extract concentration of *L. barbarum* L. fruit.~~
**Significant difference at $P < 0.01$.

In ~~the~~ biofilm assay, the 100% concentration at a 3 h incubation time was the most effective in inhibiting the formation of *S. mutans* biofilm with an OD value of 0.042 ± 0.002 (Figure 6), whereas for *P. gingivalis* biofilm, the 24 h incubation time at a concentration of 100% was the most effective (OD value: 0.007 ± 0.003; Figure 10). Statistical analysis showed that all ethanol extract concentrations of L. barbarum fruit were significantly different from the negative control ($P < 0.05$).

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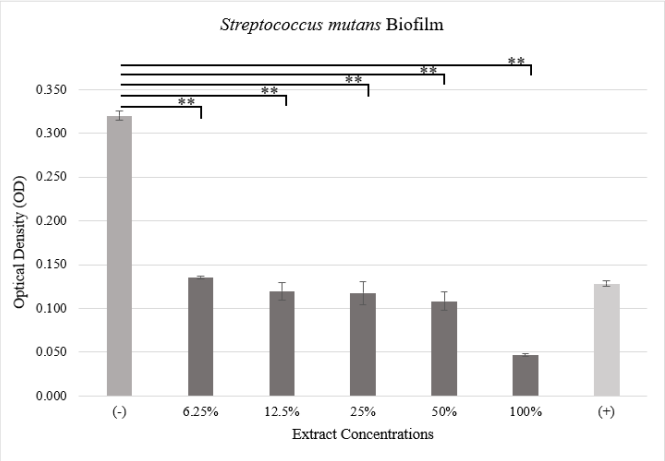

Figure 5. Optical Density (OD)~~D~~ value of *Streptococcus~~.~~ mutans* biofilm at a 1 h incubation time. **Significant difference at P < 0.01.

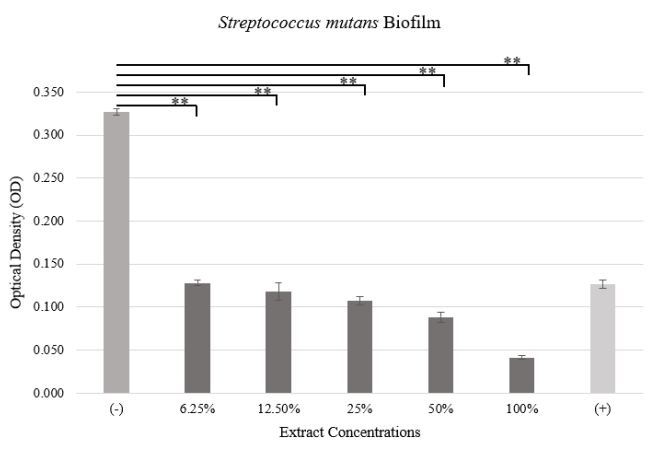

Figure 6. Optical Density (OD)~~D~~ value of *Streptococcus~~.~~ mutans* biofilm at a 3 h incubation time. **Significant difference at P < 0.01.

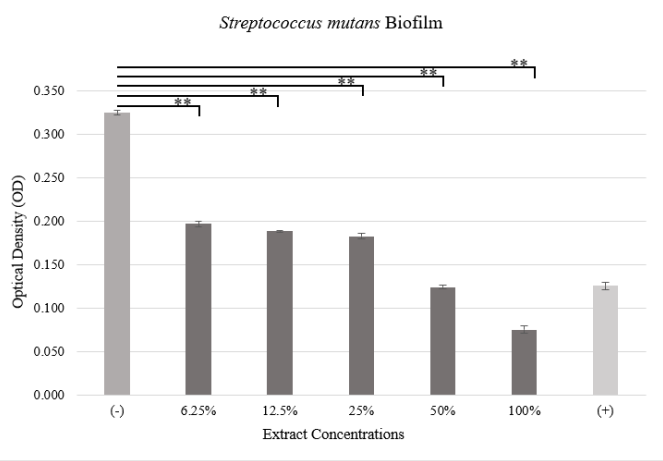

Figure 7. Optical Density (OD) value of *Streptococcus mutans* ~~OD value of *S. mutans*~~ biofilm at a 24 h incubation time. **Significant difference at P < 0.01.

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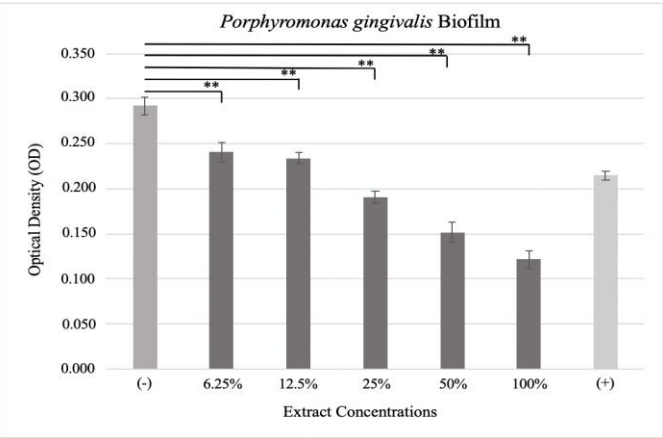

Figure 8. Optical Density (OD) value of *Porphyromonas gingivalis* ~~OD value of *P. gingivalis*~~ biofilm at a 1 h incubation time. **Significant difference at P < 0.01.

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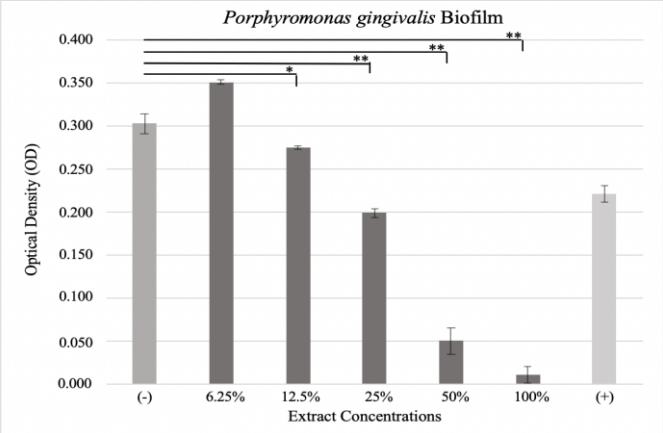

Figure 9. Optical Density (OD) value of *Porphyromonas gingivalis* ~~OD value of *P. gingivalis*~~ biofilm at a 3 h incubation time. *Significant difference at P < 0.05; **Significant difference at P < 0.01.

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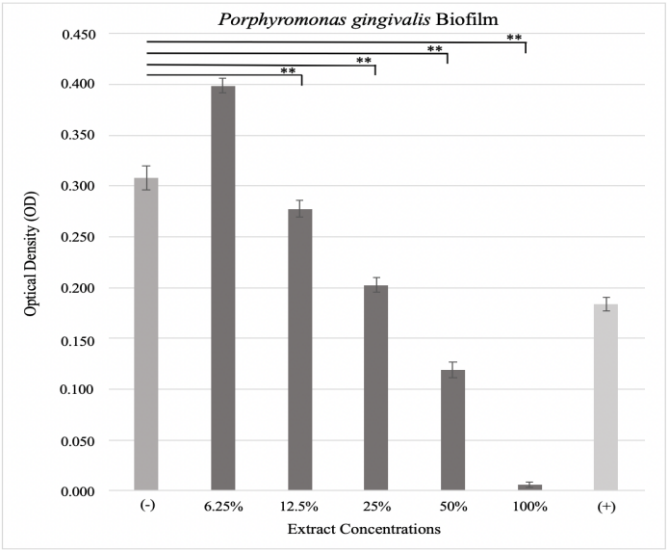

Figure 10. Optical Density (OD) value of *Porphyromonas gingivalis* ~~OD value of *P. gingivalis*~~ biofilm after 24 h of incubation. **Significant difference at P < 0.01.

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~~Statistical analysis showed that all ethanol extract concentrations of *L. barbarum* fruit were significantly different from the negative control ($P < 0.05$).~~

4. Discussion

The ethanol extract of *L. barbarum* fruit has various secondary metabolites, such as flavonoids, phenols, steroids, and terpenoids, which play a role in inhibiting bacterial growth and biofilm formation of *S. mutans* and *P. gingivalis*. Flavonoid compounds are able to damage bacterial cell walls by removing substances such as proteins, nucleic acids, and nucleotides so that bacterial cell lysis occurs (Dewi et al., 2015). Flavonoids can also interfere with the quorum sensing mechanism, causing inhibition of bacterial adhesion and biofilm formation on the tooth's surface. The formation of biofilms is inhibited by the reduction of glucans, which are a medium for bacterial attachment, due to the inactivity of the glucosyltransferase enzyme by flavonoids (Loresta~~Sonya Loresta and Sri Murwani~~ et al.~~,~~ 2015).

The ability of bacterial cell protein denaturation by phenol compounds through the formation of bonds between phenols and proteins causes damage to protein structures. The disruption of permeability in the cell wall and cytoplasmic membrane, which is composed of these proteins, causes irreversible damage and leads to lysis of the bacterial cell (Bontjura et al., 2015; ~~Bouarab-~~Chibane et al., 2019).

Steroid compounds, through their interaction with cell phospholipid membranes, are also capable of causing lysosome leakage for the lysis of bacterial cells. Terpenoids are lipophilic and can bind to carbohydrates and fats, causing disruption of the permeability of bacterial cell walls, denaturation of cytoplasmic proteins, and inactivation of cellular enzymes, causing lysis of bacterial cells (Bontjura et al., 2015; Shinde and Mulay, 2015; Ludwiczuk et al., 2017).

The results of this study are in accordance with previous studies regarding the inhibition effect of *L. barbarum* extracts against *S. aureus* and *E. coli* by the disc diffusion method. Based on the results of these studies, there is an antibacterial effect against *E. coli* (Fiţ et al., 2013). The results of other studies using the well diffusion method have stated that the ethanol extract of *L. barbarum* fruit at concentrations of 10% and 20% had inhibitory effects against *S. aureus* and *E. coli* (Skenderidis et al., 2019).

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This study used three different incubation times in the biofilm assay, namely 1, 3, and 24 hours, to determine the most effective phase for inhibiting *S. mutans* and *P. gingivalis* biofilms. The difference in incubation time was similar to the biofilm formation phase, starting with the pellicle formation phase in a few minutes to 1 hour, the initial adhesion phase at 2 to 4 hours, and the maturation phase after 24 hours (Widyarman and Lazaroni, 2019).

The ethanol extract of *L. barbarum* ~~–~~fruit at a concentration of 100% was the most effective concentration in inhibiting *S. mutans* and *P. gingivalis* bacteria and biofilms. The results of this study are in accordance with the research of Alassadi ~~Iqbal~~ et al (2015)~~et al.~~ toward *L. barbarum* fruit, which showed that the presence of the alcohol group (-OH) in the flavonoid structure increased the ability of the extract to inhibit microbial growth by increasing the permeability of bacterial cell membranes, and the highest concentration, at 100%, was the most effective antibacterial compared to other concentrations, due to less flavonoid content at lower concentrations (Alassadi et al~~(Alassadi Fatima S Sabah and Alrubaie~~, 2015).

The results of the antibiofilm assay showed that the most effective incubation time for inhibiting the formation of *S. mutans* biofilms was at 3 hours of incubation time, and for *P. gingivalis*, it was at ~~a~~ 24~~-~~-hour incubation time. The most effective times for inhibiting biofilm formation were at the initial adhesion and maturation phases, respectively. The antibiofilm effect depends on the inhibition of the polymer matrix formation and quorum sensing, or communication, between bacterial cells in biofilms by inhibiting autoinducer peptides, signaling molecules in gram-positive bacteria, and acylhomoserine lactones (AHLs) in gram-negative bacteria so bacterial virulence factors and biofilm development may be inhibited (Lu et al., 2019).

This is proven by the lowest OD value found at a 100% concentration in *S. mutans* (0.042 ± 0.002) and *P. gingivalis* (0.007 ± 0.003). This antibiofilm assay also showed that a 100% concentration had a lower OD value and was significantly different from the positive control, which means that at a 100% concentration, the antibiofilm effect was more effective than the positive control.

5. Conclusions

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The ethanol extract of *L. barbarum* fruit, containing flavonoids, phenols, steroids, and terpenoids, had antibacterial and antibiofilm effects against *S. mutans* and *P. gingivalis*. However, further research is needed, using toxicity, preclinical, and clinical tests, to determine if *L. barbarum* fruit ethanol extract can be used as an alternative mouthwash in preventing caries and treating chronic periodontitis.

Reference

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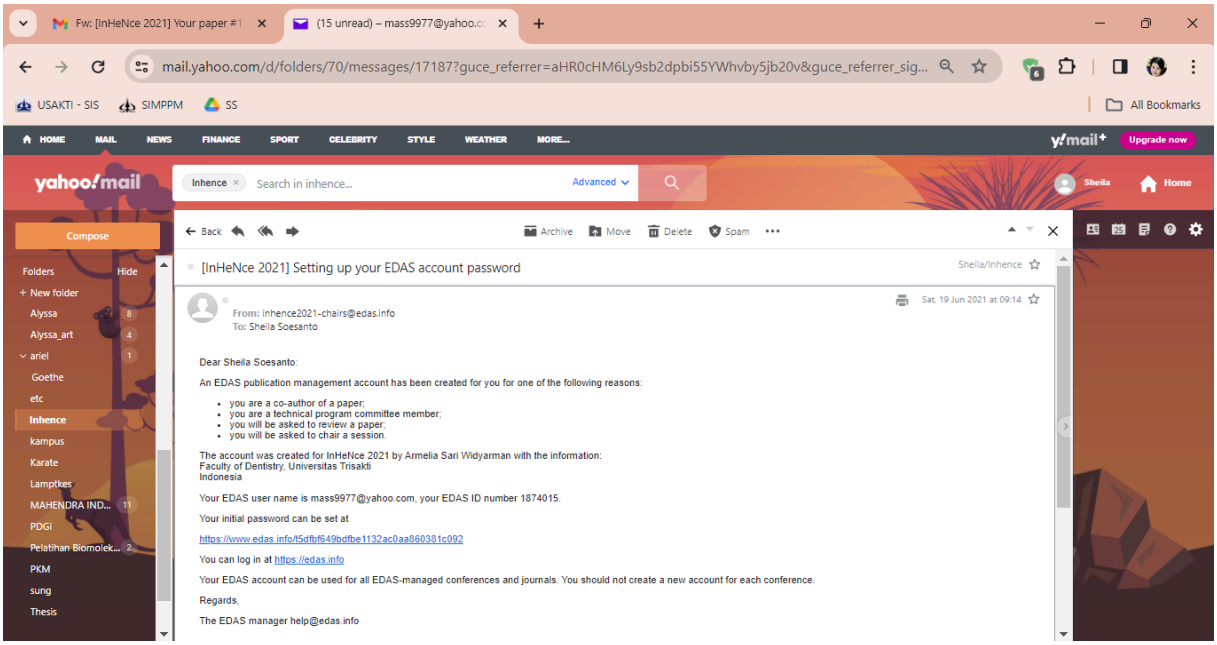
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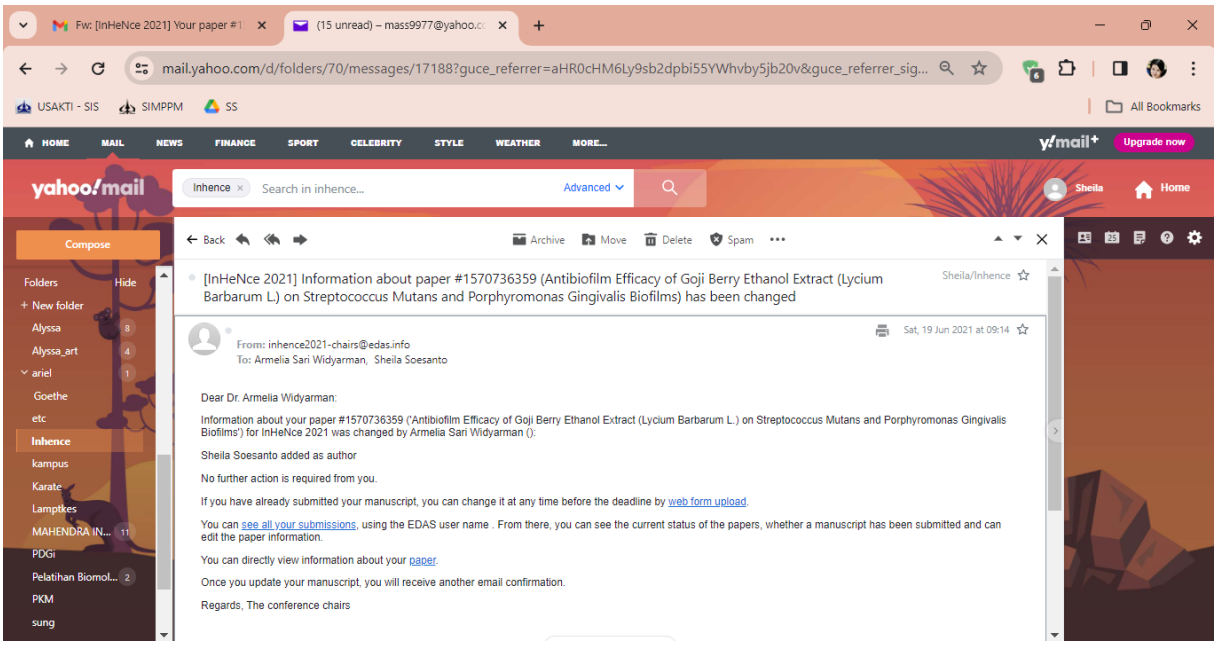
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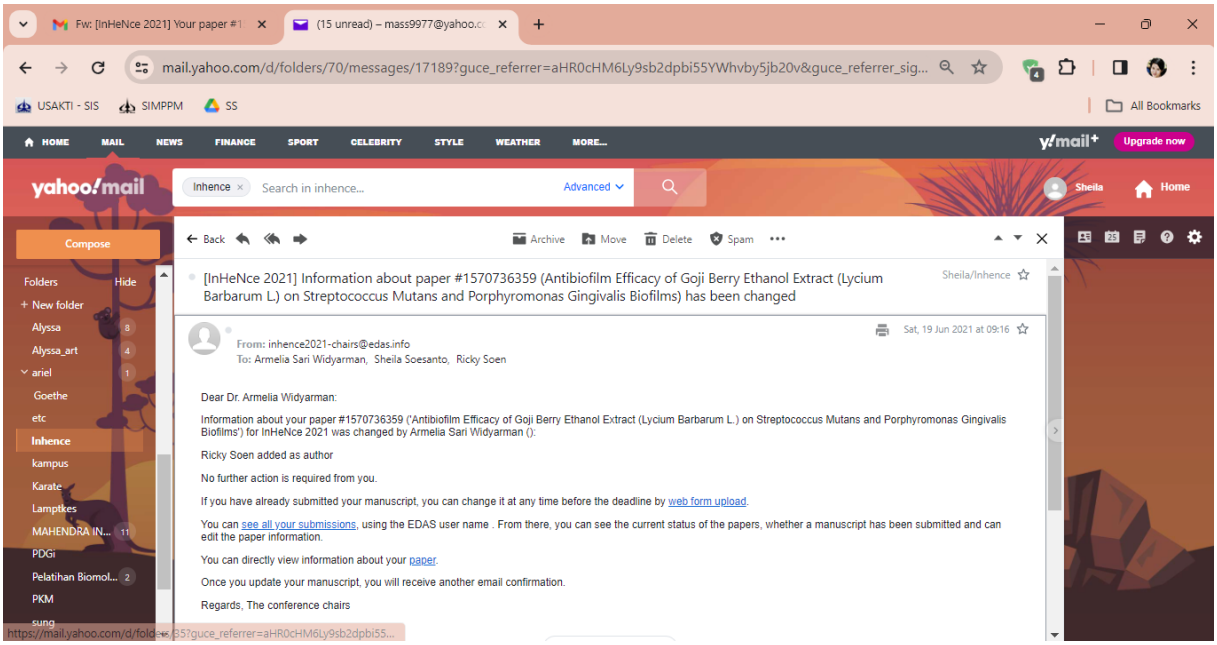
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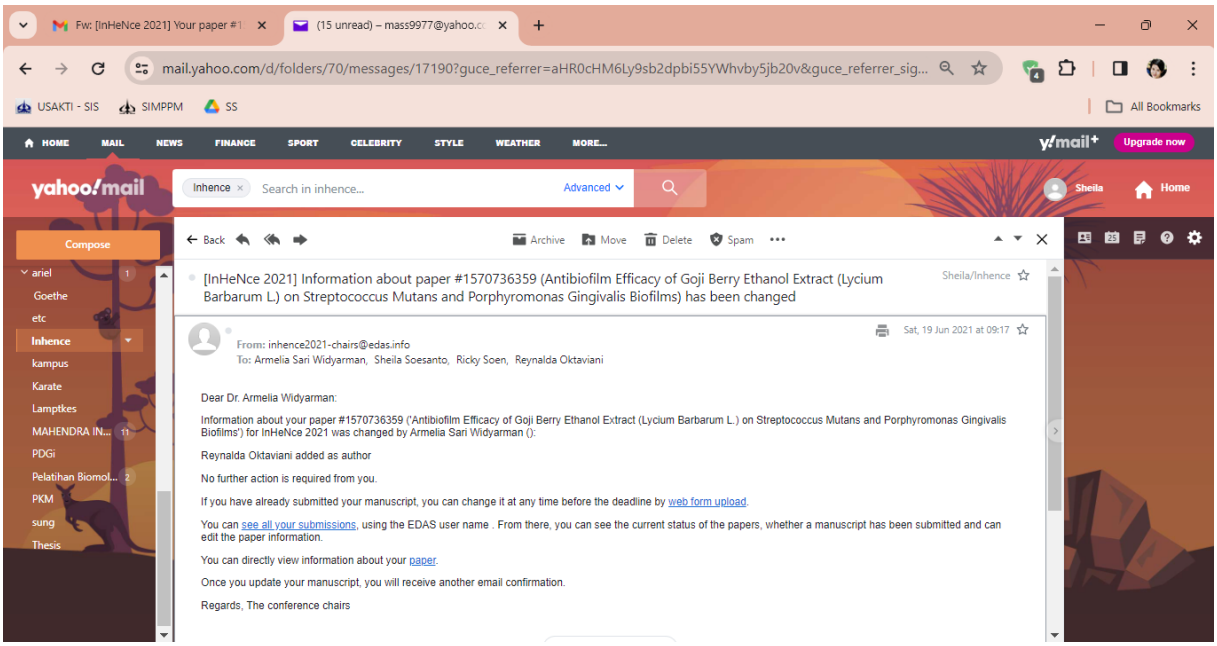
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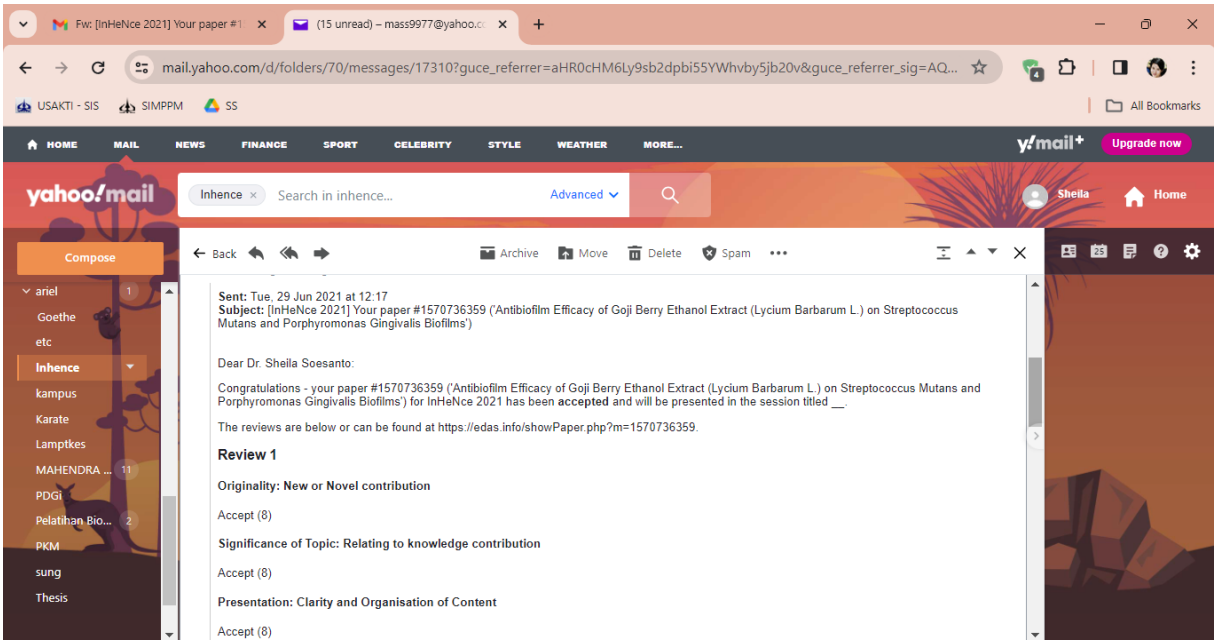

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Dear Dr. Sheila Soesanto:

Congratulations - your paper #1570736359 ('Antibiofilm Efficacy of Goji Berry Ethanol Extract (Lycium Barbarum L.) on Streptococcus Mutans and Porphyromonas Gingivalis Biofilms') for InHeNce 2021 has been **accepted** and will be presented in the session titled __.

The reviews are below or can be found at https://edas.info/showPaper.php?m=1570736359.

Review 1

Originality: New or Novel contribution

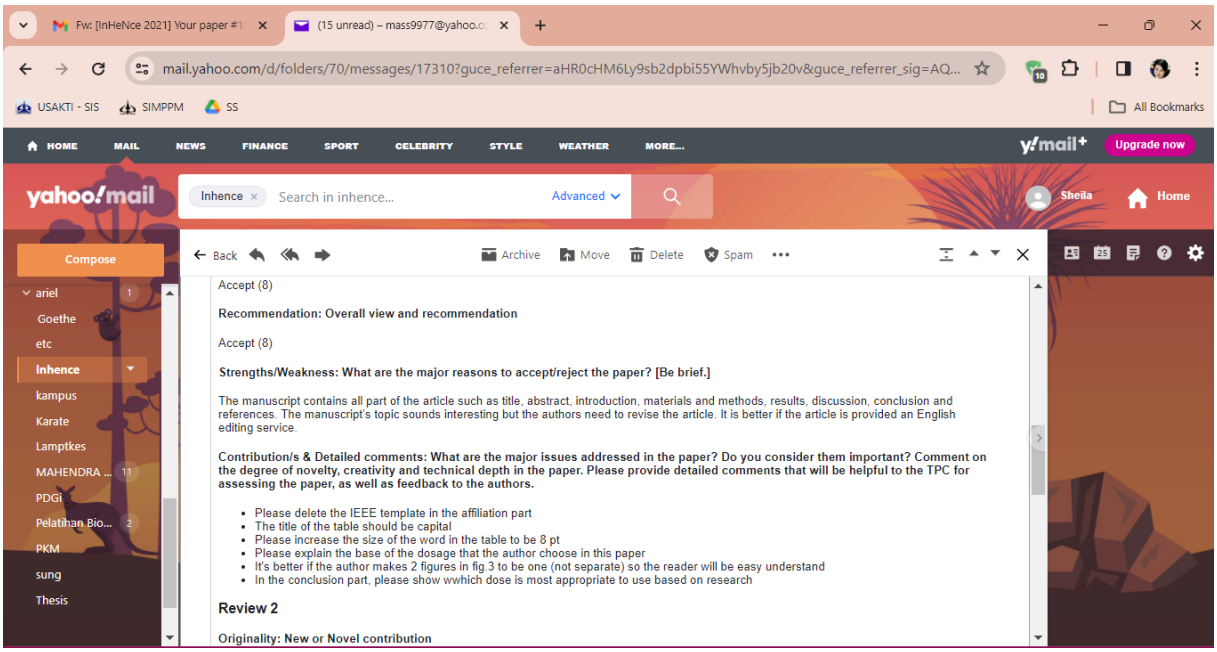
Accept (8)

Significance of Topic: Relating to knowledge contribution

Accept (8)

Presentation: Clarity and Organisation of Content

Accept (8)



Accept (8)

Recommendation: Overall view and recommendation

Accept (8)

Strengths/Weakness: What are the major reasons to accept/reject the paper? [Be brief.]

The manuscript contains all part of the article such as title, abstract, introduction, materials and methods, results, discussion, conclusion and references. The manuscript's topic sounds interesting but the authors need to revise the article. It is better if the article is provided an English editing service.

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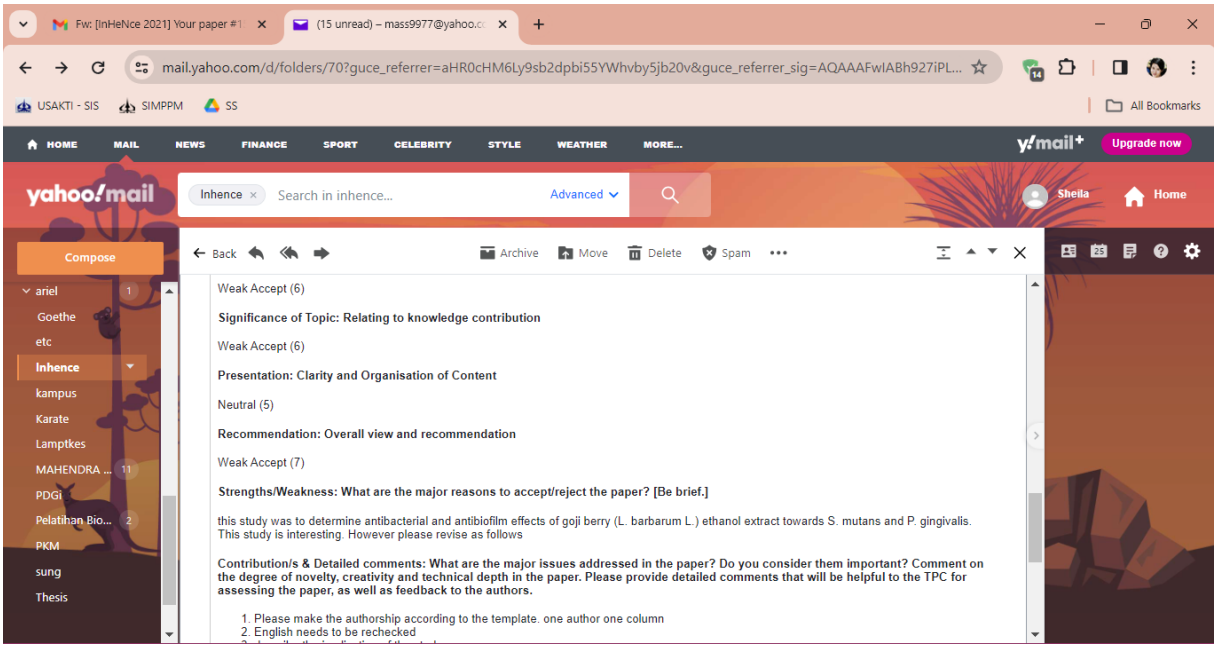
Review 2

Originality: New or Novel contribution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Weak Accept (6)
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Presentation: Clarity and Organisation of Content
Neutral (5)
Recommendation: Overall view and recommendation
Weak Accept (7)
Strengths/Weakness: What are the major reasons to accept/reject the paper? [Be brief.]
this study was to determine antibacterial and antibiofilm effects of goji berry (L. barbarum L.) ethanol extract towards S. mutans and P. gingivalis.
This study is interesting. However please revise as follows
Contribution/s & Detailed comments: What are the major issues addressed in the paper? Do you consider them important? Comment on the degree of novelty, creativity and technical depth in the paper. Please provide detailed comments that will be helpful to the TPC for assessing the paper, as well as feedback to the authors.
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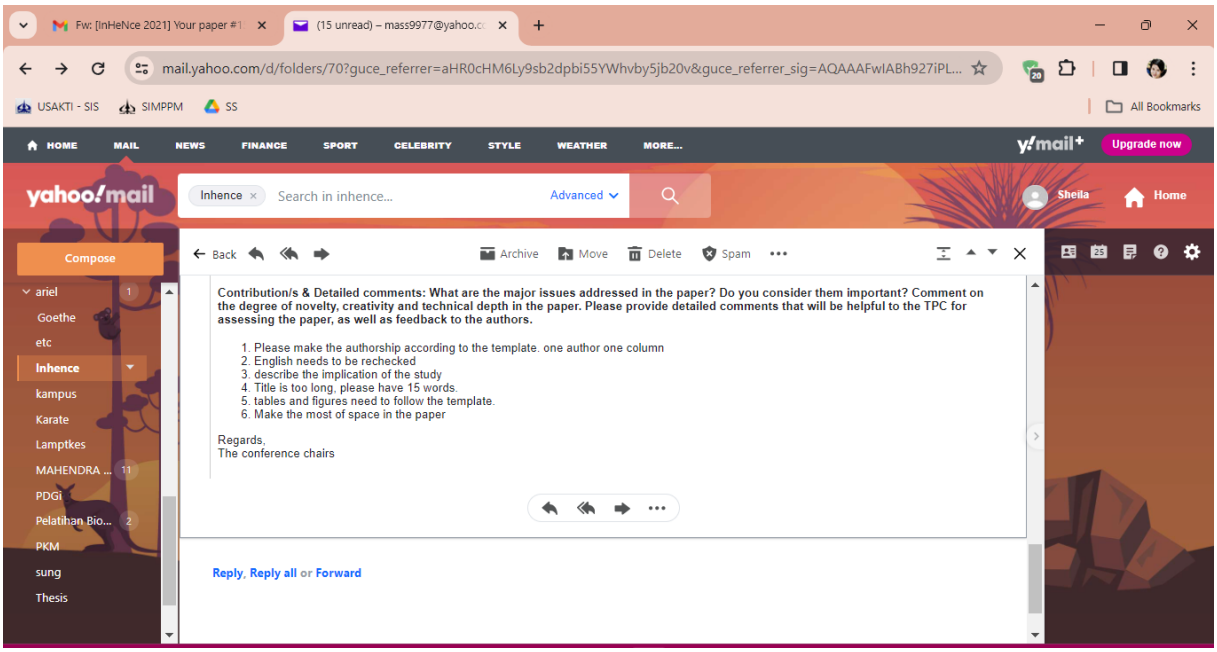
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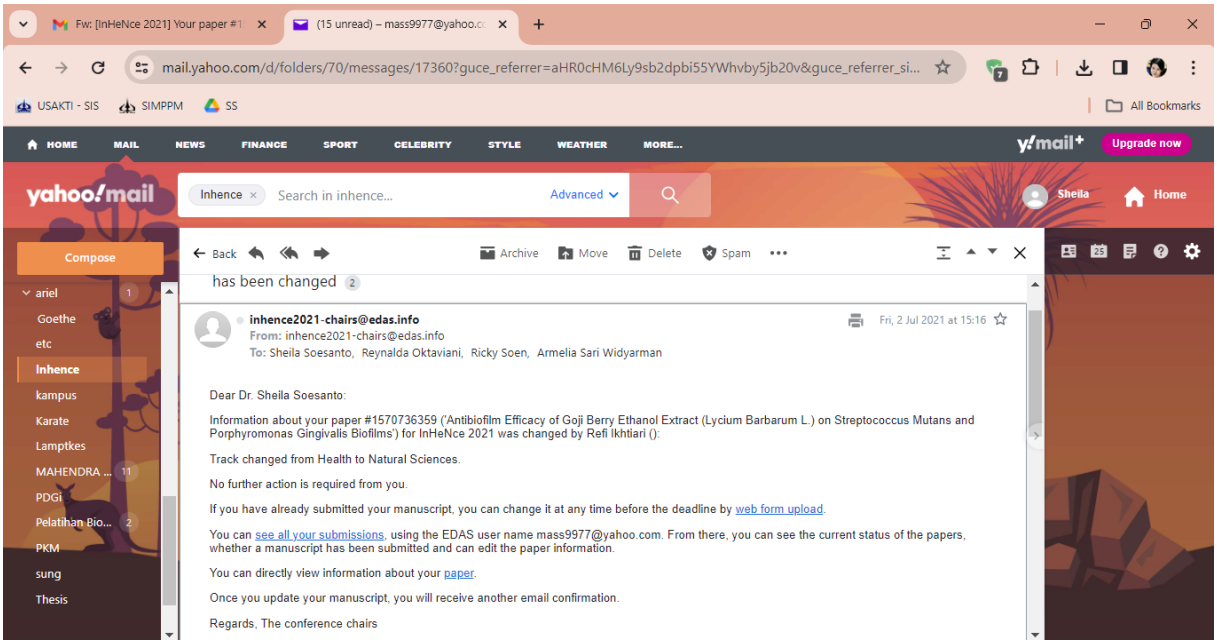
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Information about your paper #1570736359 ('Antibiofilm Efficacy of Goji Berry Ethanol Extract (Lycium Barbarum L.) on Streptococcus Mutans and Porphyromonas Gingivalis Biofilms') for InHeNce 2021 was changed by Refi Ikhtiari ():
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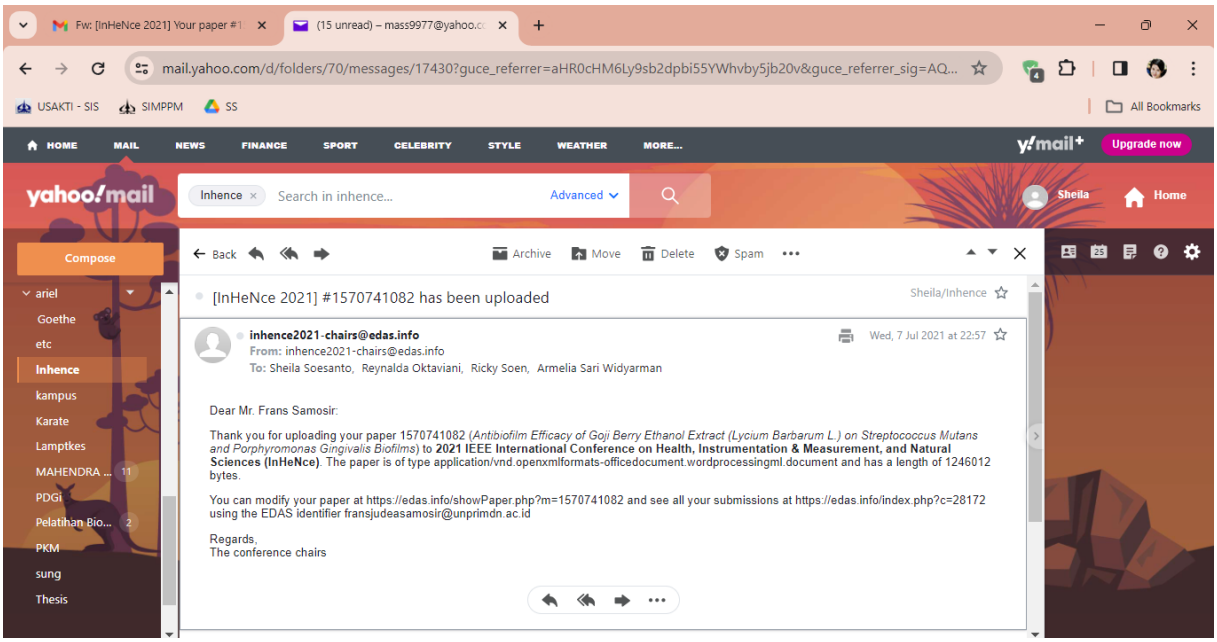
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Thank you for uploading your paper 1570741082 (*Antibiofilm Efficacy of Goji Berry Ethanol Extract (Lycium Barbarum L.) on Streptococcus Mutans and Porphyromonas Gingivalis Biofilms*) to **2021 IEEE International Conference on Health, Instrumentation & Measurement, and Natural Sciences (InHeNce)**. The paper is of type application/vnd.openxmlformats-officedocument.wordprocessingml.document and has a length of 1246012 bytes.
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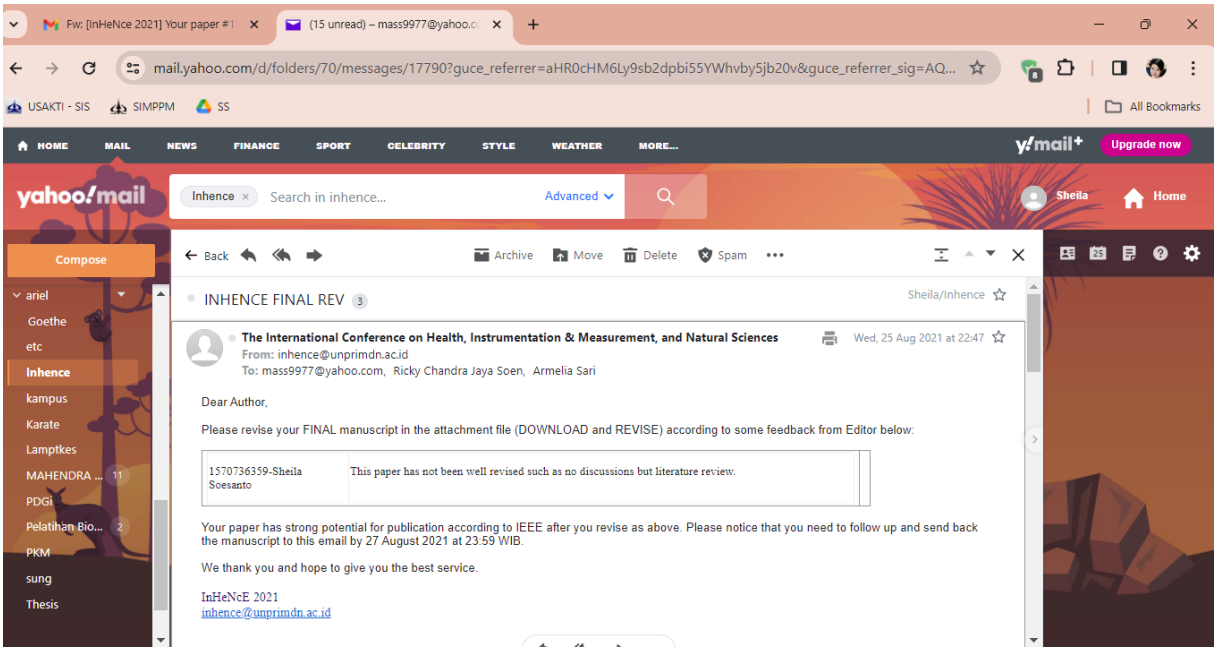
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