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Inactive Inhibitory Activity of 0.025% Oregano (*Plectranthus amboinicus*) Essential Oil Lozenge Against Tonsillar Microbiota

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Abstract

Lozenges have long been employed as a vehicle for drug administration, particularly for addressing oral health issues like sore throats, typically containing plant-based drugs with potent medicinal properties, such as oregano leaves. However, studies regarding its inhibitory activity, especially in a lozenge form, remain insufficient. Thus, this study focuses on evaluating the effect of 0.025% oregano lozenges on inhibiting tonsillar microbial growth. The sample of tonsillar microbial growth was collected from a 23-year-old 5th year dentistry student of the University of the East - Manila through an oropharyngeal swab, after meeting specific criteria. Steam distillation extraction method was used to obtain the *Plectranthus amboinicus* essential oil (PAEO). In the production process, researchers considered the inert ingredients, heating or cooking temperature, and time of oregano essential oil extract incorporation. A posttest control group design was employed. To ascertain the causal relationship, an agar well diffusion test was utilized wherein fifteen agar plates were divided into half containing the oregano essential oil lozenge (OEOL) and peppermint essential oil lozenge (PEOL). After 18 hours of incubation, both groups exhibited a distinct halo-like appearance with a 6 mm zone of inhibition (ZOI), indicating ineffectiveness against tonsillar microbiota. Guevarra's index was employed to interpret the ZOI where <10 mm diameter indicates inactive inhibition, concluding that PAEO at 0.025% concentration is ineffective as a primary ingredient in lozenge production for inhibiting tonsillar microbiota.

Keywords: oregano, lozenge, Plectranthus amboinicus essential oil (PAEO), oregano essential oil lozenge (OEOL), peppermint essential oil lozenge (PEOL)

Traditional healers in the Philippines utilize around 1500 medicinal herbs, with 120 of them having been scientifically confirmed for safety and efficacy (Dapar *et al.*, 2020). Among these is *Plectranthus amboinicus,* also known as oregano (Manlubatan *et al.*, 2021). The inhibitory properties of *P. amboinicus* depend on the amount of the active ingredient present in the essential oil extract (Arumugam *et al.*, 2016). Phenolic compounds such as carvacrol and thymol remain the most responsible for their antioxidative, antimicrobial, and antifungal effects, yielding the minimum inhibitory concentration (MIC) needed against different microorganisms (Jaber *et al.,* 2012) living in varying environmental conditions.

One specific environment that supports different microorganisms is the human tonsillar area located near the oropharynx and nasopharynx. A study by Babaiwa *et al.* (2013), obtained 966 bacteria from the tonsil cores of 824 patients with recurrent tonsillitis and tonsillar hypertrophy. Among these bacteria, *Staphylococcus aureus* was the most frequently isolated pathogen, accounting for 29.3 percent of all isolates, followed by *Streptococcus pyogenes* with 23.4 percent. Additionally, Vasconcelos *et al.* (2017) revealed that a MIC of $0.25 mg/mL^{-1}$, using *P. amboinicus,* is effective in inhibiting the proliferation of *Staphylococcus aureus,* the most common pathogenic microorganism in the tonsillar area.

Although numerous studies have demonstrated the antimicrobial properties of *P. amboinicus* and the efficacy of lozenges as a medicinal means of delivery, there is currently a lack of understanding surrounding the retention of its antimicrobial properties in the form of a lozenge, considering that there are a lot of factors to take into account when it comes to the production process. One of which is the cooking temperature wherein Hrncic *et al.* (2020) revealed that in the production of lozenges, the temperature must be kept below 70°C during mixing. This is congruent with the study of Pothu and Yamsani (2014) indicating the need to control the temperature of phytochemicals in the process of production at 40–50°C, to avoid the degradation and oxidation of polyphenols when exposed to temperatures above 70°C. Other factors, such as the inert ingredients and time of oregano essential oil extract incorporation, remain unknown as to how they affect the inhibitory activity of lozenge production.

To assess the inhibitory activity, the researchers used the agar well diffusion technique as the official method of antimicrobial susceptibility testing. The proven potential antimicrobial property of *P. amboinicus* offers researchers the idea of utilizing its essential oil extract and developing a lozenge that would test its inhibitory activity against the tonsillar microbiota.

The production process itself has not been established including the retention of the 0.25 mg/mL^{-1} MIC value of *P. amboinicus*' inhibitory activity in a lozenge form. The researchers therefore aim to further explore and provide a thorough understanding of how the manufacturing process affects the therapeutic inhibitory activity of 0.025% oregano lozenges against tonsillar microbiota.

The specific question in this study is "How does the production of 0.025% oregano lozenge affect its inhibitory activity against tonsillar microbiota?"

This study focused on the production of 0.025% oregano lozenge and an evaluation of its inhibitory activity against tonsillar microbiota. The production of lozenge utilized the essential oil from the oregano leaves that served as the main ingredient in the production of lozenge with a given MIC of $0.25 mg/mL^{-1}$. The oregano leaves were extracted using the steam distillation method. The study also included the production of a hard candy oregano lozenge which has a dimension of 6mm in diameter and 3mm in height.

A tonsillar microbial sample was obtained by an oropharyngeal swab from a consenting 23 year-old 5^{th} year UE Dentistry student. The participant met the criteria of having a good oral hygiene index score, no systemic illness, and not having undergone any antimicrobial therapy 2 weeks before swabbing. The inhibitory activity of the produced lozenge was evaluated using the agar well diffusion method. Only oregano leaves were used in the extraction process.

The study did not establish the presence of bioactive compounds of PAEO and their actual amount per mL because the college does not have the necessary facilities for this procedure.

Review of Literature

Plectranthus amboinicus, also known as oregano in the Philippines, is used as a medicinal plant with a wide variety of uses. Several studies have supported the claims of its nutritional, antiepileptic, antioxidant, and antimicrobial properties. The antimicrobial property of *P. amboinicus* is mostly due to its high levels of bioactive compounds (Arumugam *et al.*, 2016). Carvacrol and thymol have been identified to be most responsible for oregano's antimicrobial effects, yielding the MIC needed against different microorganisms (Jaber *et al.*, 2012) living in different environmental conditions.

To determine its MIC value, Vasconcelos *et al.* (2017) used a polystyrene plate with 96 wells and two strains to test the antibacterial susceptibility of PAEO against *Staphylococcus aureus* using the microdilution method in Tryptone Soy Broth (TSB). The two strains used were *S. aureus* ATCC 6538 and *S. aureus* resistant to oxacillin and vancomycin aureus (OVRSA). *P. amboinicus* essential oil was tested on 20 samples of each strain at concentrations of 4 mg/mL, 2 mg/mL, 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, and 0.0625 mg/mL. The concentration of both strains was adjusted in TSB to 1.25 107 Colony Forming Units (CFU mL1). Based on the findings, an inhibition zone of 16-38 mm was identified in all of the tested strains revealing a MIC value of 0.25 mg/mL^{-1} for *P. amboinicus* against *S. aureus* (Vasconcelos *et al.*, 2017). This is equivalent to an active to very active interpretation of the zone of inhibition using Guevarra's index. *S. aureus* is one of the predominant bacteria detected on the tonsillar surface and core (Buname *et al.*, 2021).

Based on current scientific literature, oregano has consistently exhibited antibacterial effects against a wide range of microorganisms (Fournomiti, 2015). Lu *et al.* (2018) also claimed that both essential oils and extracts from oregano demonstrate potential growth-inhibitory effects toward gram-positive and gram-negative bacteria, yeast, and fungi. Moreover, Cui *et al.* (2019) concluded in their study that oregano essential oil can inhibit Methicillin-resistant *Staphylococcus aureus* (MRSA) by making the cell membrane permeable causing irreversible damage and also by inhibiting the respiratory metabolism of MRSA by affecting the metabolites and key enzymes of the tricarboxylic acid (TCA) cycle.

Nazliniwaty and Laila in 2019 used the ethanolic extract of *P. amboinicus* as the active ingredient in making an herbal mouthwash. The diffusion agar method was used to test the antibacterial activity of both the extract only and the mouthwash against *S. aureus* and *Streptococcus mutans* after 24 hours of incubation. Results showed successful inhibition of the extract at 0.3% (*S. aureus*) and 0.4% (*S. mutans*), and inhibition of the mouthwash at 1% (*S. aureus*) and 2% (*S. mutans*). The formulation in mouthwash dosage form did not show antibacterial effect.

Oregano essential oil can be obtained through various methods of extraction. Extraction is the main procedure by which bioactive compounds are obtained from biomass materials. The objective of this process is to maximize the number of needed compounds and obtain the highest biological activity of these extracts (Truong *et al.*, 2019). Different factors are found to affect the yield and bioactive compounds of oregano extract such as the drying method, the anatomical part of the plant used for extraction, and the extraction technique (Lopez *et al.*, 2017). Steam distillation is a method for extracting bioactive compounds from plants where the steam is injected directly into the plant sample. To obtain the plant extract, condensation through indirect cooling and separation from water were performed in the vapor mixture. It is crucial not to use high temperatures in steam distillation as it may cause the reduction of heat-sensitive phenols (Hasbay and Galanakis, 2018).

The tonsils are two lymphoid epithelial tissue balls found near the oropharynx and nasopharynx that are usually associated with infection management in humans. These tissues are susceptible to infection by viruses, bacteria, *Chlamydia,* and fungus, resulting in tonsillitis (Babaiwa *et al.,* 2013). Oral bacteria can spread throughout the digestive system and are linked to a variety of disorders. Due to their location, microbiota from both the mouth cavity, especially the saliva, and the alimentary tract may affect the tonsillar tissue (Choi *et al.*, 2020). Further investigation by Babaiwa *et al.* (2013) yielded aerobic bacteria in tonsillar tissues and antibacterial sensitivity patterns in patients who had tonsillectomy in a teaching hospital. This investigation discovered a significant prevalence of 70% for *S. aureus* compared to 14% for *S. pyogenes* the typical and commonly observed causative agents in tonsillar infection (Babaiwa *et al.*, 2013). Their findings were consistent with other previous studies which found *S. aureus* to be the most commonly isolated pathogen accounting for 29.3% of the infections, followed by *S. pyogenes* with 23.4%.

The agar well diffusion is a popular method for determining the antibacterial activity of plants or microbial extracts. Agar plates are inoculated with a specific volume of microbial inoculum spread over the entire agar surface. Using a sterile cork borer or tip, a hole with a diameter of 6 mm is punched aseptically, and a volume (20–100 L) of the antimicrobial agent or extract solution at the necessary concentration is put into the well. The agar plates are then incubated at the appropriate conditions for the test microorganism. Antimicrobial drugs permeate into the agar and inhibit the test microorganism's germination and development, after which the diameters of inhibitory growth zones are determined. The antibacterial and antifungal properties of various essential oil extracts were screened using the agar well diffusion method, as shown by Daoud *et al.* (2015).

Hard candy lozenges are commonly regarded as solid syrups of sugars because it is a mixture of sugar and carbohydrates in a noncrystalline state. Their moisture content should be between 0.5 to 1.5% and their weight should be at least 1.5 to 4.5g. These should be dissolved slowly and uniformly in 5-10 minutes without disintegrating. Since the temperature required for this preparation is usually high, heat-labile materials cannot be used during the production process (Mulla and Salunkhe, 2021). The study of Pothu and Yamsani (2014) stated that the initial phase in the manufacturing of medicated lozenges is the fabrication of candy base, followed by the addition of medicament, acidulants, colors, and other ingredients, and ultimately lozenge formation. The steps involved in making hard-candy-based oral pharmaceutical lozenges are heating the candy base, mixing, batch forming, rope size, weight modification, manufacturing of lozenges, cooling, and storing them.

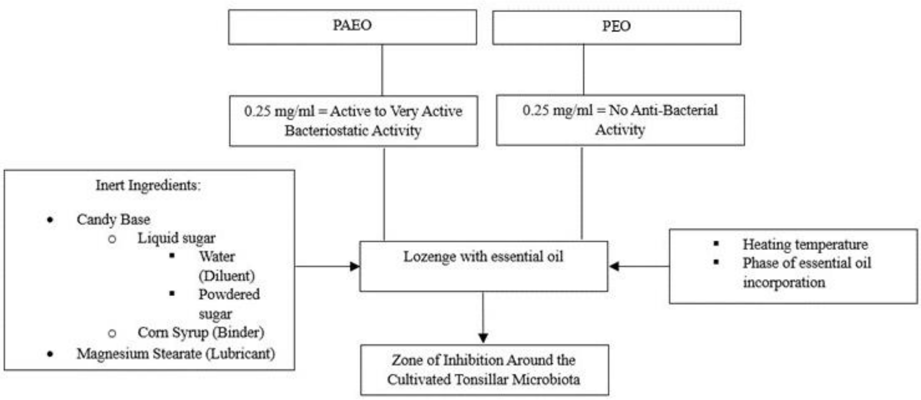
The first step is making the candy base, which involves mixing both the 67% liquid sugar and corn syrup comprised of liquid glucose and 80% solids in a 60:40 ratio. The pre-cooked solution is then injected into a steam-heated coil after being precooked at a specific temperature under a vacuum. The precooked solution is boiled and moved to the intermediate chamber where the final mixture is produced. The moisture level of the candy base should be around 1%, the temperature should be around 135°C, and the consistency should be plastic-like. The candy foundation is placed in a kettle with an appropriate scale and the batch weight is adjusted as needed. At this point, heat-stable colors can be added as cubes or paste and then transferred to a 40–50°C cold stainless steel cooling plate for manual or mechanical mixing.

Flavor, medicament, acidulant, and ground salvage can now be added to the candy base. Now considered a medicated candy base, it is placed on a heated slab and allowed to cool to a consistent temperature. After obtaining the appropriately-sized lozenges, they are gathered and stored in a climate-controlled chamber at a temperature of 15-20°C and relative humidity of 5-35% until the end products are approved for packing by the quality control unit.

The current investigation utilized the use of 1 gram of medication, 4 grams of powdered sugar, 16 grams of corn syrup, 24 mL of water, 1.2 mL of mint extract, and color Quantum satis (q.s) for the formulation of the hard lozenge.

According to the study of Jawale *et al.* (2012), peppermint extract oil (PEO) exhibited no antibacterial activity against the microbial strain *Streptococcus mutans*, thus PEO was used as the control.

Theoretical Framework



In the above theoretical framework, two essential oils, PAEO and PEO, were used in producing lozenges to assess the antimicrobial property against tonsillar microbiota. The 0.025% PEO served as the control group with no antibacterial activity. The inert ingredients of lozenges, such as the candy base and lubricant, may influence the zone of inhibition because of the properties of sugar, water, corn syrup, and magnesium stearate. Likewise, the heating temperature and the phase when the essential oil was incorporated may also be variables which can affect the zone of inhibition against tonsillar microbiota.

Methodology/Research Design

The research protocol was submitted to the University of the East - Ethical Review Committee for review and approval before the conduct of the study. After obtaining ethical clearance, the researchers secured a letter of approval from the head of the College Research Committee and the Dean of the University of the East - College of Dentistry to commence the study and use the undergraduate laboratory. The collection of samples from the participant began by obtaining the participant's willful consent to participate. Furthermore, pertinent information such as the participant's right to withdraw at any time during the study was given including a discussion of the handling, storage, and disposal of their specimen.

All specimens were stored appropriately while considering the temperature, time, and volume of samples to be collected. All materials and instruments used were considered biohazard materials. The bacterial suspension was disinfected by using 1 chlorine tablet for every liter before it was discarded in the sink. All instruments including specimens were autoclaved at 121°C for 30 minutes. Wastes were segregated properly using the color-coded bags – yellow for biohazard wastes. These bags were sealed properly with tape to prevent leaks. Unused materials were disposed of properly following the standard storage and transportation protocols. The researchers were under the direct supervision of the laboratory technician on duty to ensure adherence to all rules and biosafety regulations of the institution.

Phase I: *Authentication of Oregano Leaves*

The Bureau of Plant Industry (BPI) verified the identification of oregano based on leaves of plants collected from P. Campa St. Sampaloc, Manila submitted to it.

Extraction of Oregano Essential Oil

The extraction method was adopted from Hasbay and Galanakis (2018) which uses steam distillation method. Three kg of oregano leaves were separated from plants collected at P. Campa St., Sampaloc, Manila, and sent to the Industrial Technology Development Institute for essential oil extraction. Through the steam distillation method, pressurized steam is driven through the oregano leaves separating the active chemicals from the plant material and enabling them to evaporate into the steam. High temperatures were used to to allow the essential oil to be released without destroying the plant material. The steam containing essential oil flowed via a cooling system, where it condensed into a liquid containing the oregano essential oil and water. Three kilograms of oregano leaves yielded 2.6 mL of oregano essential oil which were transferred into an amber-colored vial for storage at room temperature.

Production of Hard Candy Lozenge

The production of medicated candy lozenges was based on the study of Pothu and Yamsani (2014), which included heating the candy base, mixing, molding, cooling, and storing.

The first step in lozenge production was making the candy base, which involved mixing 24 mL of distilled water and 42 grams of powdered sugar in a bowl to create a liquid sugar. This was then precooked in a non-stick pan with a controlled temperature of 135℃ together with the 16 g corn syrup. A candy thermometer was used to monitor the temperature throughout the cooking process. Continuous mixing was done until a plastic-like consistency was achieved. The candy base was then removed from direct heat and allowed to cool down from 135℃ until it reached the desired temperature of 40-50℃. Then, 1 g of magnesium stearate was added as lubricant to keep the produced candy from sticking to the teeth and improve the flow of the final mixture. The final ingredient added after completely incorporating the magnesium stearate into the mixture was the 20.75 mL PAEO. The theoretical yield of the mixture was 103.75 g and the researchers' estimate of actual yield was 77.81g accounting for a percent yield of 75%, considering all the inert ingredients that have been evaporated during the production process.

In formulating the lozenge without treatment, the same inert ingredients were used but a substitute oil, in the form of peppermint oil, was used in place of the PAEO. This was to make sure that there will be no difference in the formulation of the two lozenge groups. After mixing, the final composition was placed onto a 6mm x 3mm molder, allowed to cool and solidify for storage. The lozenges were kept out of direct sunlight and away from excessive humidity when being stored. Either room temperature or refrigerator temperature is typically recommended depending on the storage needs, the OEO, and the base (Pokale *et al.*, 2019).

Phase II: ***Oropharyngeal Swab, Inoculation, Incubation, and Cultivation of Tonsillar Microorganisms***

An oropharyngeal swab of one participant, aged 23 years old, a 5th year dentistry student from the University of the East - Manila was obtained. The participant met the expected criteria which included: (1) "Good" oral hygiene index (OHI) score, (2) no systemic illness, (3) physical health, and (4) no antimicrobial therapy for 2 weeks before the swab procedure. A simplified OHI with debris and calculus component (Greene and Vermillion, 1964) was used to calculate the OHI score of the participant. A plaque disclosing solution was used as adjunct to standard OHI to make the supragingival plaque visible. The participant was instructed to brush and floss before placement of disclosing solution in the mouth for 30 seconds. The maxillary and mandibular arches were divided into three segments and examined for debris and calculus present. From each segment, one tooth identified was used for the calculation that has the greatest area covered by debris or calculus. The debris and calculus scores were added and divided by the number of segments scored. The debris and calculus scores may range from 0 (absence of debris or calculus) to 3 (presence of debris or calculus on more than two-thirds of the exposed tooth surface). The debris and calculus scores were combined to attain the OHI score, which ranges from 0-6 (0-1.2 = Good, 1.3-3 = Fair, 3.1-6 =

Poor). The participant's OHI score of 1.16 suggests a good state of oral hygiene.

To minimize vagal stimulation, the participant was instructed to breathe slowly and concentrate on breathing. The anterior tonsillar pillar was swabbed twice with two sterile cotton swabs in a sweeping motion. Afterward, the cotton swabs were inoculated in a test tube containing nutrient broth. It was then incubated for 24 hours to promote proliferation of microbes.

Serial Dilution

A 1:100 serial dilution was performed to correct the turbidity of the suspension and reduce the concentration of the cells to gather an adequate quantity of microbial colonies.

Inoculation of Tonsillar Microbiota

In inoculating the agar plate, the researchers followed the protocol of the American Society for Microbiology. The researchers used a metal spreader or reusable glass that has been sterilized by soaking in 70% isopropyl alcohol or ethanol and then flamed. To evenly distribute 0.5mL of inoculum in the 20 agar plates, the spread plate technique was utilized, wherein the spreader was positioned to allow the inoculum to run evenly along the length of the spreader, and on the agar plate, a loop of sample was spread back and forth.

Phase III: *Placement of the Lozenge*

The 20 agar plates were divided into two halves. Each half was punched with a 6mm x 3mm hole using a sterile cork borer, where the lozenges were placed. Randomization was utilized as each plate was labeled with two consecutive numbers – the first half was labeled as an odd number where the lozenge with almond oil was placed, and the second half was labeled as an even number that had PAEO lozenge as the treatment group. The agar plates were then incubated for 48 hours at 37°C.

Measuring the Zone of Inhibition

The agar plates were inverted to accurately measure the zone of inhibition using a vernier caliper. The following points were considered in measuring: (1) The zone margin is the area that is not growing. If a faint growth or small single colonies are seen, the colony-free inner zone shall be measured. (2) The tonsillar microbiota's swarming growth is ignored and the evident zone of inhibition is measured. (3) The measured diameter is compared to the breakpoint diameter.

To reduce inter-rater bias, 2-3 examiners were assigned to measure the zone of inhibition of all agar plates. Examiners compared each other's gathered measurements in all agar plates to ensure that any potential errors were addressed immediately.

Interpretation of Zone of Inhibition

The diameters of the zones of inhibition gathered from the experimental group and control group were compared for interpretation through Guevarra’s Index (2005).

The statistical tool employed was a T-test since the sample population is less than 30. The T-test is one of the most used statistical methods in medical research publications, and it is employed when the samples consist of an independent population. In this research study, the variable is the production of 0.025% oregano lozenge with factors affecting it such as the cooking temperature and phase of PAEO incorporation. Moreover, the T-test belongs to the category of parametric test, wherein the assumptions of the parametric test, including independence, normality, and homogeneity of variance, must be met to ensure the correct use of the T-test. In addition, according to the theoretical deduction of the T-test, it can only be applied to the quantitative data of single factor design (Liang *et al.* 2019), so the researchers thought it would be appropriate to perform a T-test for this research design, which compares the means for only two groups - the control group and the experimental group.

Presentation and Discussion of Results

The objective of this study was to evaluate the effect of the 0.025% oregano essential oil lozenge in inhibiting the growth of tonsillar microbiota by agar well diffusion test. During observation after 18 hours of incubation, all fifteen agar plates showed a distinct halo-like appearance surrounding the lozenge, both in the control and treatment group which is not considered a true zone of inhibition. Hence, both groups indicate a 6 mm or inactive ZOI in all the agar plates. Thus, the ineffectiveness of PAEO at a concentration of 0.025% was observed when utilized as a primary ingredient in the production of lozenges against tonsillar microbiota. In addition, the researchers did not utilize the planned statistical tool which is the T-test, since the results obtained from this study revealed a 6 mm ZOI, indicating an inactive inhibitory activity of OEOL against the tonsillar microbiota.

P. amboinicus, commonly known as oregano, contains high levels of bioactive compounds that contribute to its antimicrobial properties (Arumugam *et al.*, 2016). According to Vasconcelos *et al.* (2017), *P. amboinicus* is effective in inhibiting the growth of most common pathogenic microorganisms in the tonsillar area at a minimum inhibitory concentration (MIC) value of 0.25mg/ml^{-1}. In the present study, the researchers evaluated the effect of 0.025% OEOL in inhibiting the growth of tonsillar microbiota using the agar well diffusion test. The result revealed a distinct halo-like appearance around the lozenge in both control and treatment groups on all fifteen agar plates. Nevertheless, the presence of microbial growth within these halo-like appearances was also observed, indicating an inactive zone of inhibition (ZOI).

This finding prompted the researchers to reconsider possible factors that may have influenced the obtained outcome. The researchers have considered the lozenge formulation, microbial sample, oregano essential oil concentration, choice of agar medium, and time of incubation. The formulation, which included inert ingredients and PAEO, failed to retain the inhibitory property against the tonsillar microbiota. The lozenge formulation utilized in this study was derived from Pothu and Yamsani (2014), where the formula recommended was for 30 lozenges. The ingredients were: medication (1 g), powdered sugar (42 g), corn syrup (16 g), water (24 mL), mint extract (1.2 mL), and color (q.s).

For this study, the formulation however was modified, incorporating 1 g of magnesium stearate as a lubricant, powdered sugar 42 g as a candy base, 16 mL of corn syrup as a binder, 24 mL of distilled water as a diluent, and 1.2 mL of oregano essential oil as the medication. The modified formulation may have resulted in the degradation of oregano essential oil concentration during the cooking process, reducing its inhibitory effect. It is plausible that the remaining concentration of PAEO after lozenge production was insufficient to inhibit the tonsillar microbiota.

Additionally, the present study employed the use of commercialized powdered sugar to serve as a base, following a precedent in the literature. Such powdered sugar might have supplied enough nutrients for the microorganisms to flourish, causing microbial overgrowth and the absence of true zones of inhibition in the agar plates.

Previous investigations have shown the antimicrobial activity of *P. amboinicus* against a wide range of microorganisms such as *Candida krusei*, *Candida albicans, Proteus mirabilis,* and *Escherichia coli* (Manlubatan *et al.*, 2021). Vasconcelos *et al.* (2017) also proved that*P. amboinicus* provides an excellent source of antimicrobial inhibition against the most common pathogenic microorganism found in the tonsillar area, *Staphylococcus aureus*.

In the current study, the focus was on the inhibitory effect of oregano on a broad spectrum of microorganisms within the tonsillar area. No specific colony of microorganisms was distinguished and isolated during the experiment. The agar well diffusion test was adopted because of its versatility in supporting the growth of various microorganisms. Since the microbial samples were not isolated per colony, the researchers could not ascertain which among the microorganisms had antimicrobial resistance against *P. amboinicus* and which were susceptible.

In addition, microorganisms require different incubation conditions where some need to be in an aerobic environment, while others thrive more under anaerobic conditions. This might have also affected the result, causing variations of microbial growth on the agar plates, with certain areas displaying possible overlapping of growth while others showed no signs of growth.

Microbial tolerance and time of incubation might have also played a role. In the current investigation, the researchers incubated both the treatment and control group for 18 hours, following the standard protocol, before observation. Upon checking, the researchers immediately noticed the difference in the quantity of microbial growth within the halo-like appearance surrounding the lozenge, from the growth outside the proximity of the circle. Initially, some microorganisms might have been suppressed in the early stages of the treatment process. However, the capacity of *P. amboinicus* to inhibit microbial growth may have weakened over time, possibly decreasing the antimicrobial activity of the agent. These findings are in line with the previous investigation done by Gefen *et al.* (2017) where the growth of microorganisms inside the inhibition zone was observed after a few hours.

Conclusions

The study was not able to prove the antimicrobial activity of 0.025% oregano lozenge extract against tonsillar microbiota. Possible reasons include the use of commercial sugar in the preparation of the lozenge and the conduct of the agar medium test itself. Identification and isolation of tonsillar microorganisms are critical to determine the compatible agar medium and the specific duration of incubation of agar plates.

Recommendations

Future studies should replace conventionally used powdered sugars, glucose, and sucrose with lower calorie sweetener. The effect of each ingredient on the entire lozenge formulation should be determined to avoid incompatibility when combined with the other ingredients. Isolation of tonsillar microorganisms is highly recommended to distinguish one organism from another. Identification of microorganisms is needed so that an appropriate and clear zone of inhibition can be achieved by the oregano essential oil lozenge produced. From the isolated tonsillar microorganisms, future researchers should determine the compatible agar medium and the specific duration of incubation of agar plates.

It might be necessary to increase the concentration of the oregano extract from 0.025% used in the current study and found insufficient to inhibit the tonsillar microbial growth. Lastly, a swab test on the produced lozenges can be done to ensure the absence of contamination that might affect the accuracy of results from the agar well diffusion test.

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