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Original Research Article

Formulation of Lozenges Containing Menthol and *Citrus hystrix* DC. Essential Oil for Pharyngitis Support Treatment

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ABSTRACT

This study aimed to develop antibacterial lozenges using kaffir lime fruit peel oil (*Citrus hystrix*) and assess its efficacy against bacteria responsible for sore throats. The essential components of the lozenges included *Citrus hystrix* DC essential oil and menthol. The lozenges were fabricated through the direct compression method. Several parameters, such as color, taste, odor, time for oral disintegration, and adherence to in-house specifications, were also assessed. Additionally, the stability of the lozenges was examined following ICH guidelines under accelerated conditions for a duration of six months (at 40°C and relative humidity of 70%). The antibacterial properties of menthol and *Citrus hystrix* DC essential oil were evaluated against common bacterial strains, including *Streptococcus pneumoniae* ATCC 29212, *Haemophilus influenzae* ATCC 33533, *Staphylococcus aureus* ATCC 29213, *Streptococcus mutans* ATCC 25175, *Streptococcus faecalis* ATCC 29212, and *Moraxella catarrhalis* ATCC 25238. The development of lozenges incorporating menthol and *Citrus hystrix* essential oil was successfully achieved. The preferred combination ratio was determined to be 2:1 of calcium silicate to menthol and essential oils, as it effectively masked the bitter taste. These lozenges adhered to the specifications outlined in the USP 38 monograph for menthol lozenges and exhibited notable antibacterial efficacy. Based on accelerated stability testing, the estimated expiration date for these lozenges was approximately 26 months. The results suggested that the lozenge tablet containing menthol and *Citrus hystrix* DC essential oils may offer an alternative functional supplement for the prevention of pharyngitis.

Keywords: *Citrus hystrix*, essential oil, lozenges, pharyngitis .

Introduction

Sore throat, medically known as pharyngitis, is a prevalent ailment with both infectious and non-infectious origins. Infectious causes are predominant in the majority of sore throat cases. Pharyngitis can arise from various infectious agents, including viruses, streptococcus bacteria, mononucleosis (caused by the Epstein-Barr virus), mycoplasma (a type of bacteria), gonorrhea (caused by the bacterium *Neisseria gonorrhoeae*), and diphtheria (caused by *Corynebacterium diphtheriae*).[1] In cases where the origin of sore throat is unclear and the condition is in a mild to moderate stage that does not necessitate the prescription of antibiotics, healthcare providers often recommend the use of a mild antiseptic and anti-inflammatory throat spray. This approach aims to alleviate pain and discomfort while addressing any mild infection that may be present.[2] Such throat sprays typically contain ingredients with antiseptic properties to help reduce the growth of bacteria or other microorganisms in the throat. Additionally, they may contain anti-inflammatory agents to reduce swelling and relieve pain in the throat.

There are many effective drugs for pharyngitis treatment and more than half of them are synthetic products.

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Nevertheless, synthetic compounds may have the risk of antimicrobial resistance or adverse drug reaction (ADR). Meanwhile, herbal drugs are reported to be less toxic and less harmful than synthetic ones. [3]Kaffir lime is one of the plants that has been used as herbal medicine.[2] Kaffir lime, with the scientific name *Citrus hystrix* DC, is a member of the genus Citrus, a part of the family Rutaceae. It is widely grown in An Giang province in the Mekong Delta region of Vietnam, where the soil is suitable for many types of medicinal herbs.[4] According to previous studies, *Citrus hystrix* has antioxidant, antibacterial, and antimicrobial,[5-8] anticancer,[9-10] and anti-inflammatory effects.[11] Furthermore, this plant is also used as a natural insecticide (botanical insecticide),[12-13] an essential ingredient in aromatherapy,[14-15] and also as an antidandruff.[16] The antimicrobial activities observed in *Citrus hystrix* DC can be attributed to the presence of essential oils found in various parts of the plant, including the leaves, fruits, and seeds. However, it is worth noting that the majority of essential oil production is typically derived from the fruit of *Citrus hystrix*. Previous research findings have indicated that essential oils extracted from both the leaves and peel of *Citrus hystrix* possess antimicrobial properties.

Most production of essential oil was from the fruit. Previous results showed that essential oils from the leaf and peel of *Citrus hystrix* possessed antimicrobial activities against *Salmonella typhi*, *Escherichia coli*, *Streptococcus mutans*, *Staphylococcus aureus*, *Saccharomyces cerevisiae*, and *Candida albicans*.[17-19] Similarly, research involving ethanolic extracts and the combination of extracts from the peels of various citrus fruits revealed that two or three extracts of peel powder exhibited significant antibacterial activity against common pathogens such as *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa.* [20]

A prior work used GC-MS to determine the chemical composition of Kaffir lime fruit peel oil, a volatile oil derived from the fruit peel of *Citrus hystrix* DC. L-limonene, -terpineol, 2--pinene, terpinene-4-ol, -terpinene, and -terpinolene were the main components.[4] *Salmonella typhimurium* ATCC 13311, *Escherichia coli* ATCC 25922, *Bacillus*

subtilis ATCC 6633, and *Staphylococcus aureus* ATCC 25923 all had minimum inhibitory concentrations (MICs) of 0.1, 0.3, 0.4, and 0.6 mg/mL, respectively. Essential oils from *Citrus hystrix* DC were used in another study. In addition, 411 isolates of group A, B, C, F, and *G streptococci*, *S. pneumoniae*, *H. influenzae*, *S. aureus* (methicillin-resistant and methicillin-sensitive *S. aureus*), and *Acinetobacter baumannii* obtained from patients with respiratory tract infections were reported to be resistant to kaffir lime fruit peel oil, with MIC ranges of 0.03-17.40 mg/mL. Terpinene-4-ol, -terpineol, and l-limonene were the primary ingredients of Kaffir lime fruit peel oil.[21]

Multiple test results involving the ethanol extract of lime peel have consistently demonstrated antibacterial effects when compared to positive controls. This underscores the potential of formulations containing kaffir lime fruit peel oil as a natural product with antibacterial properties for the treatment of sore throat (pharyngitis). However, it is noteworthy that there is limited prior research on products incorporating lime fruit peel oil. The primary objective of the present study was to develop antibacterial lozenges utilizing kaffir lime fruit peel oil and subsequently assess the efficacy of these lozenges against the bacteria responsible for causing a sore throat (pharyngitis). This investigation sought to bridge the gap in knowledge regarding the utilization of lime peel oil-based products for such therapeutic purposes.

Materials and Methods

Materials

Kaffir lime peels were harvested in Nha Bang town, Tinh Bien district, An Giang province, Vietnam (16° 0' N and 106° 0' E) in June 2020. The plant samples were identified by the Biology Laboratory of the Faculty of Pharmacy, Can Tho University of Medicine and Pharmacy, Vietnam. A specimen has been reserved in the Department of Botany-Traditional Medicine Pharmacognosy of Can Tho University of Medicine and Pharmacy (PBTMD/2020/001). Chemicals used for analysis included Mueller Hinton agar (Merck-Germany), defibrillated sheep blood agar (Vietnam), sodium sulfate anhydrate, and sodium chloride anhydrate (Guangdong-China). Standard samples used for analysis included sabinene (ChromaDex-United States), limonene (Dr. Ehrenstorfer-United Kingdom), citronellal (Sigma Aldrich-Switzerland), β-pinene (Dr. Ehrenstorfer-United Kingdom), internal standard camphor (Sigma Aldrich-Switzerland), and menthol (Sigma Aldrich-Switzerland). Ingredients used for lozenges formulation included menthol (Bhagat-India), calcium silicate (Florite-R, Tomita-Vietnam) sponsored by Asia Shine Ltd, pea green color (Mayur-India), talc (HTMC group-India), Aerosil (Evonik-United States), and alpha tocopherol (Basf-Germany).

Extraction of essential oil

The process began by grinding *Citrus hystrix* peels into pieces measuring 2-3 mm. Subsequently, 20 grams of these ground peels were placed into a distillation flask. A mixture of water, maintaining a ratio of 4 milliliters per gram, was added to the flask along with a few pumice stones. To complete the setup, a condenser was affixed. Next, a funnel on the return arm of the apparatus was used to introduce 5 milliliters of water. The system was sealed by closing the lock, ensuring that the vents were properly aligned. Water was then circulated through the condenser to sustain a condenser temperature of approximately 20°C. The hydro distillation process was carried out over a period of 2 hours, with strict temperature control maintained at around 100°C. Following this, the system was allowed to cool for 15 minutes. To measure the volume of distilled oil, the upper level of the essential oil layer in the receiver was adjusted to the 0 mark. The collected essential oils underwent a drying process utilizing anhydrous sodium sulfate to remove residual moisture. These oils were subsequently stored in sealed vials, shielded from light, at a temperature of 4°C in preparation for Gas Chromatography-Flame Ionization Detector (GC-FID) analysis.

Identification of components by GC-FID analysis

An Agilent Technologies 7890B Gas Chromatograph (GC) equipped with an Agilent Flame Ionization Detector (FID) and an Agilent DB-5MS Column (30 meters in length, 250 micrometers in inner diameter, and a film thickness of 0.25 micrometers) was employed for Gas Chromatography-Flame Ionization Detector (GC-FID) analysis. The injector and detector temperatures were precisely controlled at 250°C and 280°C, respectively. The column temperature was programmatically ramped, initiating at 60°C and subsequently increasing at a rate of 5°C per minute until reaching 126°C, followed by a rapid temperature increase at a rate of 60°C per minute until reaching 250°C. Helium served as the carrier gas with a flow rate of 1 mL per minute. The sample introduction was achieved through a split injection with a splitting ratio of 10:1, using 4 µL of essential oils. The quantification of individual components was conducted without any correction, relying on the Gas Chromatography peak area as measured by the Mass Selective Detector (MSD) response.

Preparation process of lozenges containing menthol and Citrus hystrix essential oil

The essential formulation of the lozenges developed in this study is outlined in Table 1. The lozenges were manufactured using the direct compression method, employing a rotary compressor equipped with 25 pestles, each measuring 11 mm in diameter, and the tablets had a target weight of 500 mg. Following were the steps in the production process:

The filler and sweetener excipients were initially mixed.

A 0.1% solution of green pea extract in 50% ethanol was used as a solvent and was sprayed into the mixture from step 1 until a uniform color was achieved.

Menthol and an antioxidant were dissolved into the essential oil.

Florite-R was introduced to absorb all of the essential oil and this mixture was subsequently blended with the mixture from step 2, following the principle of achieving uniformity in quantity.

The resulting tablets, each weighing 500 mg and measuring 11 mm in diameter, were then packaged and stored for further use.

Evaluation of sweetener and filler excipients

Table 2 illustrates the formulations for exploring sweetener and filler options. In accordance with Table 1, *Citrus hystrix* essential oil, antioxidant excipient, lubricant, and absorbent were included in the quantities specified. Additionally, menthol was incorporated at a concentration of 0.8%, and sorbitol was utilized at a dosage of 500 mg. To ensure the suitability of the powder before compression, it was necessary to meet specific parameters related to moisture content, flow rate, and apparent density. The lozenges underwent a series of tests, including assessments of hardness, uniformity of mass, friability, and oral disintegration time. Subsequently, evaluations of sweet and bitter taste were conducted to identify the optimal formulation for menthol and calcium silicate probing. These systematic evaluations and tests were integral to the formulation and quality control process, facilitating the selection of the most suitable formula for the desired taste and functional attributes of the lozenges.

Evaluation of menthol content

The percent of menthol was assessed at four concentrations: 0.3%, 0.6%, 0.8%, and 1% (F12, F13, F14, and F15, respectively). The aroma of lozenges was evaluated after compression and 4 weeks after storing at 40ºC and 75% humidity.

Probing of calcium silicate content

The amount of calcium silicate was examined at the ratios 1:1, 1.5:1, and 2:1 compared to the total amount of *Citrus hystrix* essential oil and menthol (F16, F17, and F18). The appropriate ratio of calcium silicate was chosen based on the results of the bitterness examination.

Taste assessment

The evaluation of the taste of the lozenges was conducted by a consistent panel of six trained testers with expertise in discerning sweetness, bitterness, and aroma characteristics. The Can Tho University of Medicine and Pharmacy Committee accepted the experimental procedure (decision number 406/DHYDCT, April 28th, 2020). During each assessment session, a range of standard and test solutions was presented to the testers, along with information about the flavor profile of each solution. The taste assessment procedure involved the following steps:

Testers sucked on a standard solution for a duration of 10 seconds.

Subsequently, they drank 20 milliliters of water to cleanse their palates.

Taste intensity was promptly recorded within 10 seconds of holding the solutions in their mouths and after consuming water.
Testers were assigned a score to indicate the perceived intensity of the taste for each sample.
The remaining four samples were then tasted in succession, and each sample was given a score proportional to the taste intensity of the standard sample. Allowable score coefficients for errors were 1/2, 1, 2, and 4, but position errors were not tolerated.
Each formulation's taste score was determined by the testers based on the perceived taste intensity compared to the standard solutions. Each tester evaluated up to three samples per day, with a minimum 15-minute gap between samples after rinsing their mouths with water.
The results were documented on a preparation form, where the assessed scores for each sample were recorded, tallied by both column and row and the means were calculated. Statistical analyses, including ANOVA and F-tests, were applied to ascertain differences between the various samples. Additionally, the T-test was utilized to identify which sample exhibited differences in taste intensity compared to the others.
This rigorous taste assessment methodology ensured a comprehensive evaluation of the taste attributes of the lozenge formulations, allowing for objective comparisons and statistical analysis to determine variations among the samples.

Upgrading the batch size to the pilot scale
Upon determining the optimal formulation for the lozenges containing menthol and Citrus hystrix essential oil, the production scale was increased from 1000 tablets (totaling 500 grams) to 5000 tablets (equivalent to 2500 grams). To ensure consistency in the production process, several critical steps and parameters were evaluated, including production conditions and equipment, during this scale-up. Here is a detailed description of the scale-up process and evaluation:
Ingredient Preparation: All ingredients were subjected to sieving through a 0.3 mm sieve to ensure uniform particle size.
Green Pea Solution Preparation: A 0.1% green pea solution in 50% ethanol was prepared.
This solution was then sprayed onto sorbitol using a fluidized bed dryer. The drying process in the fluidized bed dryer involved a compressed air supply at a rate of 70-80 m^3/h, cleaning of the filter bag by air pulsing for 5 minutes, a spray gun pressure of 1 bar, and a drying temperature of 30°C within a specified time frame (t minutes).
Mixing and Blending: The sorbitol coated with the green pea solution was mixed with sweetness excipients. This mixture was carefully blended with a combination of menthol, Citrus hystrix essential oil, antioxidant excipients, and calcium silicate, following the principle of achieving uniform quantity. Lubricants were gently added to this mixture and mixed in a cube mixer at 200 rpm within a specific time frame (t minutes).
Quality Assessment: The uniformity of the color of the excipients was observed at time intervals of 3, 5, 10, and 15 minutes after spraying the green pea solution onto 200 grams of sorbitol. Similarly, samples were taken from five different positions within a cube mixer to evaluate the uniformity of the color of the mixture at time intervals of 5, 10, 15, and 20 minutes after blending. Three batches of tablets, each weighing 500 mg and measuring 11 mm in diameter, were prepared. The powder before compression was analyzed for moisture content, flow rate, and apparent density. The finished lozenges were subjected to tests for weight uniformity, hardness, friability, and oral disintegration time. These evaluations and adjustments in the production process ensured that the lozenges produced on a larger scale retained the same essential properties and quality as those prepared in the laboratory-scale batches.

Stability testing
After upgrading the batch size to 5000 tablets, lozenges were examined for stability according to ICH under accelerated aging conditions at 40°C ± 2°C and 75% ± 5% relative humidity.[22] The experiment was performed on three (3) consecutive batches. The lozenge appearance, hardness, content of menthol, and identification of *Citrus hystrix* essential oil at the beginning and after 1, 3, and 6 months were monitored. Each sample was determined 3 times to record the average result. The shelf life of lozenges was calculated by Van't Hoff principle.

Quantification of menthol
The content of menthol was determined according to the menthol lozenges monograph of USP 38 using the GC-FID system.[23] 25% sodium chloride solution in water (solution A) was prepared. 20 lozenges were weighed, and the average mass was lightly ground to a homogenous powder, accurately weighted the amount of powder equivalent to 2 lozenges into a 100 mL separatory funnel, added 20 mL of water, 26 mL of solution A, shaken in 30 minutes. 10 mL of hexane was added and continued shaking. The hexane layer was used as the test solution. Standard solution was prepared by weighing exactly 60 mg of menthol into a 100 mL volumetric flask and hexane was added to the line marked.
The gas chromatograph was equipped with a flame-ionization detector, a split injection system with a split ratio of 10:1, and a DB-5MS column (30 m × 250 µm × 0.25 µm). The injection port and the detector block were maintained at about 250°C. The injection volume was 1 µL and the flow rate of carrier gas was about 10 mL per minute. The content of menthol was calculated according to the formula:

$$X(mg) = \frac{A_{test}}{A_{standard}} \times \frac{m_{standard}}{m_{test}} \times \frac{D_{test}}{D_{standard}} \times P \times A\%$$

where A_{test} and $A_{standard}$ were the peak areas of menthol in the test solution and standard solution, respectively.
m_{test} and $m_{standard}$ were the amount of menthol used to prepare the test solution and standard solution, respectively.
D_{test} and $D_{standard}$ were the dilution of the test solution and standard solution, respectively.
P: the average weight of lozenges
A%: standard concentration

Identification of Citrus hystrix essential oil
Lozenges were ground to a homogenous powder and dissolved in 2 g of powder in 5 mL of water. The solution was transferred into a separatory funnel, and then 2 mL of petroleum ether was added, shaken, and let sit for 15 min. Then, the upper layer was used for chromatography analysis. Reference solutions including menthol or *Citrus hystrix* essential oil in petroleum ether were prepared. Development chromatography was performed on silica gel F254 TLC aluminum sheet (2.5 × 10 cm) using solvent systems including petroleum ether: ethyl acetate (85:15), petroleum ether-ethyl acetate (85:15), and benzene-ethyl acetate (9:1). Spots were detected by spraying VS reagent and heated on an electric stove.

Table 1: Basic formulation of lozenges

Ingredients	Amount (%)
Citrus hystrix essential oil	0.6
Menthol	Surveyed
Talc	1
Aerosil	1
Calcium silicate	Surveyed
Sweetener (surveyed)	Surveyed
Pea-green solution 0.1%	q.s
Alpha-tocopherol antioxidant	0.5%
Sorbitol	q.s 100%

Table 2: Formulas for sweetener and filler exploration

Excipients	Amount (%)										
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11
Aspartame	0.6	0.8	0	0	0.8	0.6	0	0	0	0	0.2
Acesulfame K	0.6	0.8	0	0	0	0	0	0	0	0	0
Mannitol	0	0	40	20	0	0	0	0	0	0	0
Isomalt	0	0	0	0	0	0	30	20	0	0	0
Rebaten	0	0	0	0	0	0	0	0	0.8	1	0.8

Antibacterial activities of lozenges containing menthol and Citrus hystrix essential oil

The antibacterial activity of lozenges was evaluated by agar diffusion method using *Streptococcus pneumoniae* ATCC 29212, *Staphylococcus aureus* ATCC 29213, *Haemophilus influenzae* ATCC 33533, *Streptococcus mutans* ATCC 25175, *Streptococcus faecalis* ATCC 29212, and *Moraxella catarrhalis* ATCC 25238. 10 g of lozenge powder was dissolved in 5 mL of DMSO with 0.05% Tween 80. 250 µL of this solution was pipetted on top of the agar. The solution of corresponding excipients in DMSO with 0.05% Tween 80 was used as control samples. The diameter of inhibition zones in mm was recorded after incubation of the plates at 37°C for 18-24 h.

Statistical analysis

Tests of the specification were evaluated at least three times, expressing the results as means or means ± standard deviations (SDs). ANOVA and the F-test were applied to determine the differences between samples. Simultaneously, a T-test was used to identify which sample is different from any others.

Results and Discussion

To promote the utilization of valuable medicinal plants, it is imperative to identify convenient dosage forms that can be feasibly employed in practical applications. A key facet in this pursuit involves standardizing the quality and assessing the stability of herbal-based medications, with an emphasis on not only retaining the inherent properties of medicinal plants but also satisfying the stringent quality criteria applicable to novel dosage formulations. This study centers on the exploration of the antibacterial properties exhibited by the essential oil derived from Citrus hystrix peels. Additionally, we have successfully devised a formulation for lozenges that incorporates menthol and Citrus hystrix essential oil, thereby rendering them potentially efficacious in the treatment of pharyngitis.

The significance of this investigation is underscored by its contribution to the development and preservation of the invaluable tenets of Vietnamese traditional medicine. By elucidating the antibacterial attributes of Citrus hystrix essential oil and formulating lozenges tailored for pharyngitis management, this study not only bolsters the scientific understanding of medicinal plants but also furnishes a practical avenue for integrating these traditional remedies into contemporary healthcare practices.

Identification of components by GC-FID analysis

The results of GC-FID analysis of *Citrus hystrix* peels are shown in Figure 1. It was demonstrated that the essential oil obtained consisted of 3 major constituents namely β-pinene + Sabinene (54.96%), limonene (20.24%), citronellal (11.58%), and other constituents.

Preparation process of lozenges containing menthol and Citrus hystrix essential oil

Lozenges containing Citrus hystrix essential oil represent a suitable option for a broad spectrum of individuals, particularly those adhering to sugar-free dietary preferences. To establish a rational preparation process for these lozenges, it is imperative to possess a comprehensive understanding of the fundamental attributes of the raw materials and their transformations throughout the manufacturing process.

The chosen formula must impart a balanced sensory experience, characterized by a mild cooling sensation immediately upon placement in the oral cavity and a distinctive aroma emanating from the Citrus hystrix essential oil. This sensory profile should persist throughout the duration of lozenge consumption.

The concentration of sorbitol within the formulation plays a pivotal role in shaping the organoleptic qualities of the lozenges. Sorbitol, owing to its suitability for direct compression, is favored for this purpose. However, it is important to note that sorbitol exhibits high hygroscopicity, which could pose challenges to the product's stability. Furthermore, if the sorbitol concentration falls below an optimal threshold, the lozenges may become brittle and excessively moist, failing to meet the stipulated disintegration time. This challenge can be mitigated by incorporating a higher concentration of lubricants and employing tableting processes under reduced temperature and humidity conditions. To this end, a notable feature of this study is the utilization of lubricants at a substantial concentration of 1% to address these concerns.

Probing of sweetener and filler excipients

The lozenges must be delicately sweet but not sharp, less bitter, do not cause lumpiness while disintegrating, and must create a cool sensation with the typical aroma of *Citrus hystrix* essential oil during sucking and holding in the mouth. For sweetener and filler exploration, we tested five (5) different types of sugar. They are all new generation and low-calorie sweeteners. However, the Vietnam Ministry of Health classified them as food additives, so their use needs to comply with regulations. The acceptable daily intake of aspartame is 0-40 mg/kg, acesulfame K and isomalt is 0-15 mg/kg, sorbitol is 10 g/kg, and mannitol is not yet determined.[24] The study's findings indicate that most of the sweeteners and fillers exhibited favorable flowability and compressibility characteristics, rendering them suitable for the tablet manufacturing process, as detailed in Tables 4, 5, and 6. Notably, there were significant differences observed among the various formulations.

One of the key objectives was to create lozenges with a balanced level of sweetness while preserving the essential properties of menthol and Citrus hystrix essential oils. In this regard, both aspartame and acesulfame K were considered for their sweetness-enhancing properties. Aspartame contributed a mildly sweet and aromatic taste, whereas acesulfame K provided sweetness but left a bitter aftertaste. Consequently, aspartame was selected for inclusion in the formula, while acesulfame K was omitted due to its bitter aftertaste.

Additionally, mannitol and isomalt were incorporated into some formulations (F3, F4, F7, and F8). However, these sugar types possessed small particle sizes and relatively low sweetness levels. Insufficient sweetness at lower concentrations and the potential to affect powder fluidity at higher concentrations prompted the removal of mannitol and isomalt from the formula.

Rebaten, a natural sugar derived from the Stevia rebaudiana plant, offered a pleasant natural sweetness. When combined with sorbitol, it created an agreeable taste. Nevertheless, it was noted that this combination did not effectively mask the bitterness associated with essential oils.

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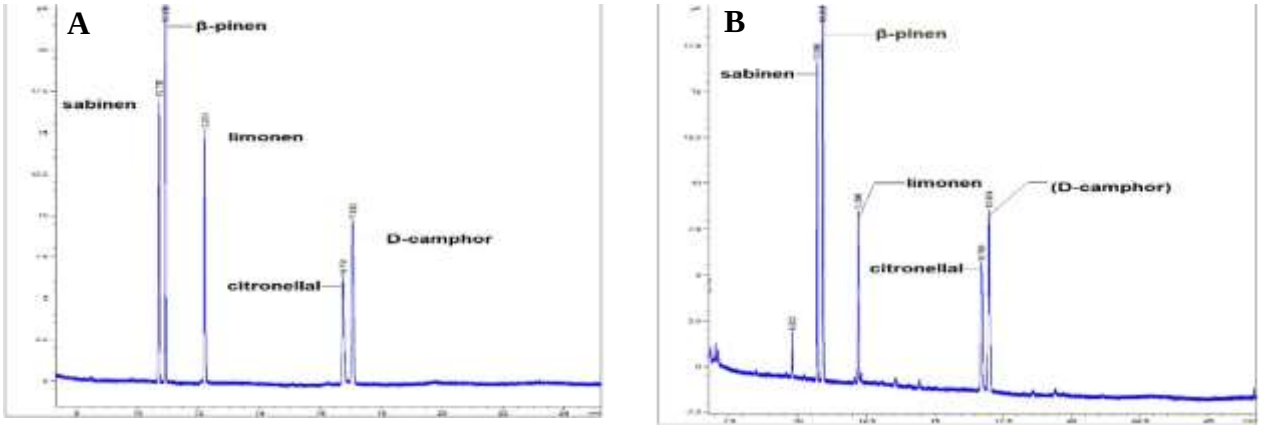

Figure 1: GC chromatogram of *Citrus hystrix* peel essential oils.
A: Standard sample; B: *Citrus hystrix* peel essential oils.

Table 3: Solutions for taste assessment

Tastes	Standard (g/L)	Test (g/L)			
Sweetness (Saccharose)	10	5	10	20	40
Bitterness (*Citrus hystrix* essential oil)	0.5	0.25	0.75	1,5	3.0
Aroma (Menthol)	0.2	0.1	0.2	0.4	0.8

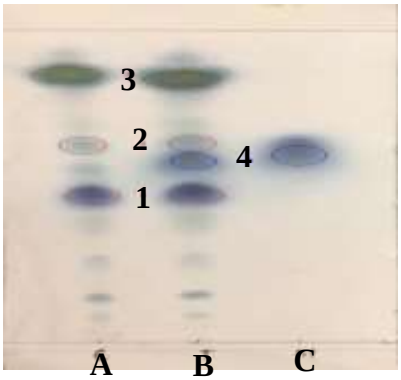

Figure 2: TLC results of *Citrus hystrix* essential oil (A), lozenges (B), and menthol (C) under visible light.

The inclusion of aspartame in formula F6, characterized by its fine powder consistency, proved advantageous. When combined with sorbitol at a concentration of 0.6-0.8%, the minute aspartame particles filled the voids created by sorbitol, resulting in improved powder compressibility. Consequently, formula F6 met the specified requirements, producing lozenges with a moderate level of sweetness while retaining the refreshing quality of menthol and the aroma of Citrus hystrix essential oils.
In summary, the selection and optimization of sweeteners in the formulation played a crucial role in achieving the desired taste and sensory attributes of the lozenges, ensuring that they met the intended criteria for sweetness, cooling effect, and aroma retention.

Probing of menthol content
Menthol was used in the formulation to give the lozenges a cool sensation. On the other hand, menthol also has antibacterial and antiviral properties that cooperate with the antibacterial effects of *Citrus hystrix* essential oils. The research by Mahboubi and Kazempour (2014) revealed that menthol was effective against *Staphylococcus aureus* and *Streptococcus mutans* with a MIC of 15.6 µg/mL, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Streptococcus faecalis* with MICs of 0.21 mg/mL, 0.35 mg/mL, and 31.2 mg/mL, respectively. Menthol showed no activity against *Streptococcus pneumoniae*. Menthol is classified as a food additive by The Vietnam Ministry of Health with a daily intake allowance of 0-0.2 mg/kg.[22] Lozenges containing menthol must give a moderate cooling sensation immediately after taking them by mouth, and the coolness must remain throughout the sucking process. The value of Fm was 23.09 which is more than F = 2.42 (0.05) (Table 7). It was concluded that the aroma taste scores were significantly different between these formulas. The aroma test score of formula 13 was closest to the standard, thus we chose this formula for calcium silicate probing.

Probing of calcium silicate content
Citrus hystrix essential oils possess natural aromas but also a pronounced bitter taste due to their extraction from the peels. Consequently, their concentration in the formula had to be carefully regulated. A concentration of 0.6% of *Citrus hystrix* essential oils was selected, which not only met the requirements for maintaining antibacterial properties but also retained the desired aroma in the lozenges. However, this level of essential oils could impart a slight bitterness to the lozenges.
To mitigate the bitter taste, calcium silicate was incorporated into the formulation. Calcium silicate possesses a unique crystal structure with hollow internal cavities capable of retaining significant amounts of liquid. Consequently, calcium silicate absorbed the essential oils into these cavities at a rate of up to 4.6 mL per gram. This property of calcium silicate was instrumental in masking the bitterness of the lozenges.
Importantly, the inclusion of calcium silicate did not compromise the structural integrity of the lozenges. It prevented the lozenges from becoming excessively soft, moist, or prone to friability, which could occur when essential oils are present at higher concentrations and may lead to difficulties in meeting the disintegration time test requirements.
The study also sought to determine the optimal combination ratio of calcium silicate with menthol and essential oils. The objective was to identify the lowest ratio that effectively masked the bitter taste while preserving the desired characteristics of the lozenges.[16]
The value of Fm was 16,17 which is more than F = 3.68 (p=0.00018) (Table 8). The results were similar to the aroma taste assessment in which the bitterness scores of all formulas were significantly different from each other. The bitter test score of Formula 18 was closest to the standard. Then we chose the ratio of calcium silicate in this formula (2:1) for establishing the optimal formulation which is shown in Table 9.
Upgrading the batch size to the pilot scale
Upgrading the batch size is a process where the composition ratios in the formula remain constant, but various process parameters are explored and defined. An effective manufacturing process necessitates meticulous control from the input of materials to the production phase,

encompassing drug quality testing before the final output. This stringent control ensures that all products meet the specified quality and uniformity standards consistently throughout the entire batch and from one batch to another. During the course of this study, investigations into the mixing times of excipients were conducted. It was observed that the minimum spraying time for the green pea solution required to achieve uniformity in the color of the excipients was 10 minutes. Similarly, the mixing times in the cube mixer required to attain uniformity in the powder before compression was identified as 15 and 20 minutes. To optimize energy and machinery usage, a mixing time of 15 minutes was selected.

The color solution was exclusively sprayed onto sorbitol, as this excipient constituted a significant portion of the formula, approximately 95%. Consequently, the addition of the remaining ingredients had a negligible impact on the appearance of the pellets. Table 10 presents the test results of the powder before the tableting process, indicating that the semi-finished products met the necessary criteria for compression. The final products were subjected to assessments related to organoleptic properties, uniformity of mass, and other quality control criteria for tablets.

Table 4: Powder parameters before compression

Formulas	Moisture (%)	Flow rate (s/100g)	Apparent density (g/mL)
F1	2.14	8.5	0.81
F2	2.15	8.5	0.80
F3	2.17	-	0.79
F4	2.16	16.3	0.80
F5	2.16	8.5	0.79
F6	1.67	8.5	0.81
F7	2.12	19.7	0.80
F8	2.16	16.1	0.82
F9	1.67	5.9	0.80
F10	1.82	6.3	0.81
F11	1.86	7.1	0.80

Table 5: The test results of lozenges

Formulas	Hardness (N)*	Uniformity of mass	Friabiliy (%)	Disintegration time (min)
F1	113 ± 5.6	Passed	0.67	13
F2	113 ± 3.5	Passed	0.53	14
F3	75 ± 4.7	Failed	1.54	5
F4	62 ± 9.7	Failed	1.23	6
F5	78 ± 4.2	Passed	0.67	17
F6	87 ± 2.7	Passed	0.72	25
F7	91 ± 1.3	Failed	0.78	26
F8	86 ± 2.7	Failed	0.73	19
F9	103 ± 6.1	Passed	0.76	22
F10	111 ± 5.6	Passed	0.68	25
F11	109 ± 4.9	Passed	0.72	23

*Values are represented as mean ± SD from 10 times experiments

Table 6: Sweet and bitter taste scores of lozenges

Formulas	Sweet taste score	Bitter taste score
F1	3.33	1.67
F2	2.83	2.17
F3	1.33	2.83
F4	1.00	2.17
F5	3.33	1.83
F6	2.00	1.83
F7	1.50	2.50
F8	1.50	3.33
F9	2.33	2.33
F10	3.33	2.00
F11	2.83	2.50
Statistical analysis	Fm = 17.89 > F = 2.1 (0.05)	Fm = 63.53 > F = 2.1 (0.05)

Table 7: Aroma taste scores of lozenges

Tester	F12		F13		F14		F15	
	After compression	After 4 week-storage	After compression	After 4 week-storage	After compression	After 4 week-storage	After compression	After 4 week-storage
1	2	0	2	1	4	3	4	3
2	1	0	2	2	3	2	4	3
3	1	0	2	2	3	3	4	4
4	2	0	3	3	4	3	4	3
5	1	0	2	2	3	3	4	3
6	1	0	2	2	4	2	4	2
Total	8	0	13	17	21	16	24	18
Average	1.33	0.00	2.17	2.00	3.50	2.67	4.00	3.00

Table 8: Bitter taste scores of lozenges

Tester	F16	F17	F18
1	3	2	1
2	4	4	2
3	3	4	2
4	4	3	2
5	4	3	2
6	4	3	2
Total	22	19	11
Average	3.67	3.17	1.83

Specifically, the weight uniformity was required to fall within the range of 500 mg ± 25 mg, and the friability of the lozenges should be less than 2%. The results demonstrated that the quality of the end products remained consistent across batches and fulfilled all the tablet testing standards, as shown in Table 11. Consequently, this study successfully established the optimal formulation and manufacturing conditions for the production of 5000 lozenges. The rigorous control and testing processes implemented ensure the uniformity and quality of the final products, meeting the specified standards batch after batch.

Stability testing

The Association of Southeast Asian Nations

Lozenges were monitored for stability under accelerated aging conditions following the Association of Southeast Asian Nations (ASEAN) regulations. As mentioned above, because the amount of *Citrus hystrix* essential oils in lozenges was quite low, the stability test results were calculated based on the menthol content and recorded in Table 12. The menthol quantification method was referred to menthol lozenges monograph of USP 38.[20] It was shown that, after 6 months of storage at 40ºC ± 2ºC and 75% ± 5% relative humidity, all three batches of lozenges met the criteria of organoleptic properties, identification of *Citrus hystrix* essential oil, menthol content, and hardness. Besides, the shelf life of lozenges was calculated as 790 days at storage conditions of 30ºC ± 2ºC and 75% ± 5% relative humidity.

In the contemporary pharmaceutical industry, the advancement of chromatographic techniques has significantly enhanced both quantitative and qualitative analyses. To streamline the quality control of raw materials and plant-based medicines, the European Medicines Agency (EMA) has advocated an approach that centers around establishing the chemical fingerprint of these phytomedicines and monitoring specific chemical markers. The chromatographic analysis, as shown in Figure 2, has unveiled the presence of various chemical groups. This analytical approach allows for a comprehensive understanding of the chemical composition of the substances under investigation, aiding in quality control and ensuring the consistency and reliability of pharmaceutical products, particularly those derived from plant sources.

In the analysis of Citrus hystrix essential oil, three distinct spots were observed, each associated with a specific Retention Factor (Rf) value. These Rf values were as follows: Rf1 = 0.259 (calculated as 2.2 divided by 8.5), Rf2 = 0.318 (calculated as 2.7 divided by 8.5), and Rf3 = 0.647 (calculated as 5.5 divided by 8.5). In the case of menthol, a characteristic blue-violet stain was the only visual observation, and its corresponding Rf value was 0.4 (calculated as 3.4 divided by 8.5). Meanwhile, lozenge samples showed four (4) clear spots that are equivalent to *Citrus hystrix* essential oil and menthol. It was concluded that, after 6 months of storage, the content of essential oil and menthol in lozenges remains stable and detectable. This figure could be used as a chromatographic fingerprint. The blue fluorescence that appeared after spraying the VS reagent characterized the presence of phenolic compounds in the extracts. These compounds could be used as markers for the quality control of extracts and finished products. [25]

Antibacterial activities of lozenges

The antibacterial activity of essential oils from *Citrus hystrix* leaves and fruits was investigated by Srisukh et al. (2012) against 411 isolates of group A, B, C, F, and *G streptococci*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Staphylococcus aureus* (methicillin-resistant and sensitive to *Staphylococcus aureus*) that were obtained from patients with respiratory tract infections. The outcomes showed that *Citrus hystrix* fruit and leaf essential oils both had antibacterial activity with MICs of 0.03-17.40 mg/mL and 0.06-68 mg/mL, respectively.[18]

In a recent study, the antibacterial ability of lozenges was evaluated by using *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Streptococcus mutans*, *Haemophilus influenzae*, *Streptococcus faecalis*, and *Moraxella catarrhalis* which are common agents that cause upper respiratory tract infections, especially superinfections after sore throats by viruses or cold and flu. The results of the diameter of inhibition zones against some bacteria by agar disk diffusion method are shown in Table 13.

The test samples expressed significant effects against bacteria such as *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Streptococcus mutans*, *Haemophilus influenzae*, and *Moraxella catarrhalis* but not *Streptococcus faecalis*. The antibacterial property of test samples was more pronounced on *Haemophilus influenzae* and *Moraxella catarrhalis* species. This is also consistent with previous studies on the antibacterial activities of *Citrus hystrix* essential oils against these two bacteria. In addition, Domagoj (2016) confirmed the antibacterial abilities of menthol against *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Streptococcus mutans*, and *Haemophilus influenzae*.[26-27] Therefore, it was concluded that lozenges possess better antibacterial

effects than essential oils because of the synergistic actions of *Citrus hystrix* essential oils and menthol.

Conclusion

This study was centered on the development of lozenges incorporating menthol and Citrus hystrix essential oil obtained from An Giang province, Vietnam. The research successfully formulated these lozenges and assessed their stability using the accelerated stability method. According to Van't Hoff's principle, the determined shelf life of the lozenges was approximately 26 months when stored at 30°C. Furthermore, the combination of Citrus hystrix essential oil and menthol in the form of lozenges demonstrated notable antibacterial effects against various bacteria, including *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Streptococcus mutans*, *Haemophilus influenzae*, and *Moraxella catarrhalis*. Consequently, this formulation holds promise as a potential functional supplement for the prevention of pharyngitis.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Table 9: Formula of a lozenge containing menthol and *Citrus hystrix* essential oil

Ingredients	Amount (mg)
Citrus hystrix essential oil	3
Menthol	3
Aspartame	3
Calcium silicate	12
Talc	5
Aerosil	5
Alpha-tocopherol	0.03
0.1% green pea solution	qs
Sorbitol	qs 500 mg

Table 10: Powder parameters before tableting

Lot	Moisture (%)	Flow rate (s/100 g)	Apparent density (g/mL)
1	1.67	8.5	0.81
2	1.65	8.5	0.80
3	1.67	8.5	0.81

Table 11: Parameters of lozenges

Lot	Weight uniformity	Hardness (N)*	Friability (%)	Oral disintegration time (min)
1	Passed	83 ± 3.7	0.53	> 20
2	Passed	86 ± 2.5	0.52	> 20
3	Passed	87 ± 4.2	0.53	> 20

*Values are represented as mean ± SD from 10 times experiments

Table 12: Shelf-life calculation of lozenges

Day	Lot 1		Lot 2		Lot 3	
	[C%]	k	[C%]	k	[C%]	k
0	100.2		99.7		100.1	
30	99.6	0.00020	98.8	0.00030	99.2	0.00030
60	98.2	0.00034	97.9	0.00030	98.3	0.00030
90	97.6	0.00029	97.3	0.00027	97.4	0.00030
180	96.1	0.00023	96.8	0.00016	96.7	0.00019
Mean		0.00027		0.00026		0.00027
t_{90} (40°C)		397.18		404.74		383.16
t_{90} (30°C)		794.37		809.49		766.32

Table 13: The diameter of inhibition zones results (mm)

	Streptococcus pneumoniae	*Staphylo-coccus aureus*	*Streptococcus mutans*	*Haemophilus influenzae*	*Streptococcus faecalis*	*Moraxella catarrhalis*
Test sample	13	21	22	24	-	26
Control	-	-	-	-	-	-

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