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Effectiveness of a nasal spray containing essential oil from *Citrus microcarpa* Bunge on the SEM profile of *Aspergillus fumigatus* mycelium

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A B S T R A C T

Deteriorating air quality during seasonal transitions contributes to the increased growth of pathogenic microorganisms, including *Aspergillus fumigatus*, which may lead to adverse health impacts. Synthetic antifungal agents may induce resistance and undesirable side effects, thereby promoting interest in safer natural alternatives. This study aimed to evaluate the antifungal effectiveness of a nasal spray formulated with kasturi orange (*Citrus microcarpa*) peel essential oil against *A. fumigatus*. The essential oil was extracted through steam distillation and tested at five concentrations to identify the most effective range, after which three concentrations exhibiting the strongest antifungal activity were formulated into a nasal spray. Antifungal effectiveness was assessed using the disc diffusion method, morphological alterations of fungal hyphae were observed using Scanning Electron Microscopy (SEM), and statistical analysis was performed using Analysis of Variance (ANOVA). The results showed that the nasal spray demonstrated significant antifungal activity ($p < 0.05$), with inhibition zone diameters of 12.8 mm, 16.1 mm, and 20.5 mm at essential oil concentrations of 10%, 20%, and 30%, respectively. Although the highest concentration produced the largest inhibition zone, excessively high essential oil levels do not necessarily improve antifungal performance and may trigger adaptive responses approaching resistance. SEM observations confirmed notable cellular damage, including hyphal lysis, cell wall thinning, and structural shrinkage following exposure to the nasal spray formulation. In conclusion, kasturi orange peel essential oil is effective when formulated as a nasal spray to inhibit *A. fumigatus*, with 20% identified as the most suitable concentration for further product development.

Keywords: *Aspergillus fumigatus*; *Citrus microcarpa* Bunge; nasal spray; scanning electron microscopy.

1. INTRODUCTION

Rhinosinusitis is an inflammatory condition affecting the nasal cavity and paranasal sinuses. It is classified into two types: acute and chronic rhinosinusitis (Riskia, 2022). This condition can be influenced by environmental pollution, particularly vehicle emissions and humid air during seasonal transitions, both of which deteriorate air quality. Poor air quality promotes the growth of microorganisms that may threaten human health, including respiratory infections (Luh, 2019). National data indicate that respiratory tract infections remain a significant health concern in Indonesia. Nasal and sinus diseases rank 25th among the 50 major diseases (Depkes RI, 2003), and upper respiratory tract infections (URTIs) have a prevalence of 9.3% based on the 2018 Basic Health Research (Riskesdas). At Cipto Mangunkusumo Hospital, 300 cases of rhinosinusitis were recorded in 2005, with an incidence proportion of 69% (Nugraha, 2022). One of the microorganisms that thrive under these conditions is the fungus *Aspergillus fumigatus* (Suharsono, 2019).

Aspergillus fumigatus belongs to the class Ascomycetes and exhibits long, branching filamentous structures that form mycelia and conidiospores. The spores are freely dispersed in open air and can easily enter the respiratory tract through inhalation (Luh, 2019). However, the use of chemical-based medications may cause long-term side effects with prolonged administration**.** Moreover, prolonged use of chemical-based antifungal agents may lead to long-term side effects. A potential solution to these issues is the development of traditional or plant-based therapies, one of which utilizes the peel of kasturi orange (*Citrus microcarpa* Bunge).

The peel of *C. microcarpa* is well known for its essential oil content. Minyak atsiri (EO) telah menunjukkan efek anti inflamasi dan potensi dalam mengobati pasien rinitis alergi (Caimmi,2021). This essential oil contains secondary metabolites, primarily D-limonene (Jian, 2023). D-limonene has been identified as the major compound responsible for antifungal activity, exhibiting an inhibition zone diameter of up to 13.39 mm at a concentration of

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15%. The peel contains antifungal constituents and bioactive compounds with potential health benefits. Consistent with the findings of Li (2021), limonene is effective in inhibiting the growth of pathogenic fungi such as *Aspergillus* spp. Given that limonene is known to possess antifungal activity, the essential oil derived from kasturi orange peel has the potential to be utilized as an active ingredient in natural nasal spray formulations. Based on the study conducted by Corazza (2024), nasal sprays formulated from citrus peel extracts may be applied in traditional treatments for respiratory infections, including otitis media, chronic rhinosinusitis, and acute sinusitis.

This study focuses on evaluating the antifungal effectiveness of a nasal spray containing kasturi orange peel essential oil against *A. fumigatus*. Observations of morphological changes in fungal mycelia using Scanning Electron Microscopy (SEM) were conducted to understand how the fungal morphology is altered. As described by Shobham (2025), the morphology and cell wall characteristics of *Aspergillus terreus* in biosorption processes can reflect the organism's response to external substances. By integrating information on bioactivity, formulation characteristics, and microscopic findings, this study aims to determine the potential of kasturi orange peel essential oil as an active ingredient supporting the development of natural nasal spray preparations to inhibit *A. fumigatus*.

2. Material and Methods

2.1 Research location and materials

The study was carried out across several laboratories to accommodate the different stages of analysis. Essential oil extraction from kasturi orange peel took place at the Bioprocess Laboratory of Politeknik Kimia Industri Medan. Subsequent GC–MS analysis of the extracted oil was performed at the Biosciences Laboratory, Politeknik Negeri Jember. Antifungal activity testing was then undertaken at the Microbiology Laboratory, Universitas Islam Negeri Sumatera Utara. Finally, morphological observations using scanning electron microscopy (SEM) were conducted at the Laboratory of the Department of Chemical Engineering, Politeknik Negeri Lhokseumawe.

The materials used in this study included kasturi orange (*Citrus microcarpa* Bunge) peel, a pure culture of *Aspergillus fumigatus*, Sabouraud dextrose agar (SDA), Potato dextrose agar (PDA), Potato dextrose broth (PDB), 1% NaCMC, demineralized water, nasal NaCl, Tween 80, paper discs, aquades, 2% glutaraldehyde, cacodylate buffer, 1% osmium tetroxide, t-butanol, alcohol, Lactophenol Cotton Blue, and Itraconazole (Sporacid).

2.2 Essential oil extraction from kasturi orange peel

The essential oil was obtained using the steam distillation method. Fresh kasturi orange peel was cut into small pieces, weighed to 120 g and placed into a 1000 mL distillation flask. Subsequently, 650 mL of distilled water was added until the flask reached two-thirds of its volume. Petroleum jelly was applied to the mouth of the distillation apparatus to prevent steam leakage. The distillation assembly was then set up and operated at approximately 100 °C for 5 hours. The mixture of distilled steam and essential oil was condensed and collected, after which the oil was separated from the water using hydrodistillation or hydro-steam distillation, and purified with anhydrous sodium sulfate (Herlina, 2020).

The collected essential oil was stored in glass vials and its characteristics were observed visually. Subsequently, density determination of the essential oil was performed using a pycnometer based on the mass ratio of the oil to distilled water at a constant volume and at a temperature of 25 °C. The procedure included weighing the empty pycnometer (m), followed by weighing the pycnometer filled with distilled water at 25 °C (m_1). After being emptied and dried, the pycnometer was filled with essential oil at the same temperature and reweighed to obtain the mass (m_2) (Lestari, 2023). According to Ayuni et al. (2021), solubility testing was performed by measuring 1 mL of essential oil into a 25 mL graduated cylinder and adding ethanol dropwise with constant shaking until a clear solution was obtained.

2.3 GC–MS analysis of kasturi orange peel essential oil

GC–MS analysis of the essential oil was performed using a Shimadzu GCMS-QP2020 NX instrument. Samples were automatically injected into the column at 280 °C, with helium as the carrier gas. The gaseous sample was passed through a 30 m SH-50 capillary column maintained at 320 °C, allowing the chemical

components of the essential oil to separate. The resulting fragments were detected to determine the relative ratios of the analytes present in the oil (Ayyandurai et al., 2022).

2.4 Morphological identification of *Aspergillus fumigatus*

Fungal identification was carried out following the method of Prakash and Bhargava (2016). A square (1 × 1 cm) piece of SDA medium was placed on a glass slide inside a sterile Petri dish lined with filter paper and supported by aluminum foil. *A. fumigatus* colonies were inoculated on all four sides of the medium using an inoculation needle. A coverslip was placed on top, and sterile distilled water was added to maintain humidity. The plates were incubated at 37 °C for 72 hours with daily observations. The resulting colonies were then transferred onto microscope slides containing 2 µL of Lactophenol Cotton Blue and examined under a light microscope at 100× magnification.

2.5 Antifungal activity testing of kasturi orange peel essential oil against *Aspergillus fumigatus*

Antifungal activity testing was performed using the disc diffusion method. *A. fumigatus* suspension was uniformly inoculated onto the medium using a sterile cotton swab. Essential oil at various concentrations was dissolved in 5 mL of DMSO and vortexed until homogeneous. Sterile paper discs were soaked in the solution for 15 minutes, then placed on the agar surface. The plates were incubated at 37 °C for 4 × 24 hours in an inverted position (Purwaningsih, 2020).

Antifungal activity testing was performed using the disc diffusion method. A suspension of *A. fumigatus* was uniformly inoculated onto the medium using a sterile cotton swab. Essential oil solutions were prepared at concentrations of 10% v/v, 20% v/v, 30% v/v, 40% v/v, and 60% v/v. The disc diffusion solutions were prepared by mixing 10% v/v (500 µL EO + 1000 µL DMSO), vortexed until homogeneous, and each sterile paper disc was immersed in 1500 µL of the test solution for 15 minutes. The discs that had absorbed the solution were then placed onto the surface of the *Potato Dextrose Broth* medium and incubated at 37 °C for 4 × 24 hours in an inverted position (Purwaningsih, 2020).

2.6 Formulation of nasal spray containing kasturi orange peel essential oil

The essential oil extracted from kasturi orange peel was subsequently formulated into nasal spray preparations using additional ingredients based on the Indonesian National Standard (SNI) and relevant literature. The formulations are presented in Table 1

Table 1. Formulation of nasal spray

Ingredients	K^-	F1	F2	F3	F4	F5
Essential Oil	0%	10%	20%	30%	40%	60%
Nassal NaCl	15%	15%	15%	15%	15%	15%
NaCMC 1%	0,8%	0,8%	0,8 %	0,8 %	0,8 %	0,8 %
Tween 80	0,5%	0,5%	0,5%	0,5%	0,5%	0,5%
Demineralized Water	60%	60%	60%	60%	60%	60%

The nasal spray formulations were prepared by mixing all ingredients in a beaker glass according to each formula. The mixtures were homogenized using a magnetic stirrer for one hour at 30 °C. The formulations were then transferred into nasal spray bottles and subjected to antimicrobial activity testing as well as physical evaluations, including visual inspection, homogeneity, pH, and spray distribution characteristics of the nasal spray (Wylie, 2021).

2.7 Activity testing of nasal spray containing kasturi orange peel essential oil against *Aspergillus fumigatus*

Fungal suspensions were uniformly inoculated onto PDA medium using a sterile inoculating loop. Paper discs were soaked for 15 minutes in the test solutions, which included distilled water (negative control), commercial nasal spray (positive control), and kasturi orange peel nasal spray at the three most effective concentrations. After draining, the discs were placed on the surface of the agar and incubated at 37 °C for 4 × 24 hours. The resulting inhibition zones were measured using a caliper (Meliyana, 2022).

Fungal suspensions were uniformly inoculated onto PDA medium using a sterile inoculating loop. Kasturi orange peel nasal spray was prepared at the three most effective concentrations, namely 10% v/v, 20% v/v, and 30% v/v, calculated as 10% v/v = 2000 µL nasal spray + 1000 µL distilled water. Sterile paper discs were immersed for 15 minutes in 3000 µL of the test solution. For the control groups, 1000 µL of distilled water was used as the negative control and 1000 µL of commercial nasal spray was used as the positive control. After draining, the discs were placed on the surface of the agar and incubated at 37 °C for 4 × 24 hours. The resulting inhibition zones were then measured using a caliper (Meliyana, 2022).

2.8 Morphological damage assessment of *Aspergillus fumigatus* using scanning electron microscopy

SEM sample preparation began by inoculating an actively growing *A. fumigatus* culture into 80 mL of PDB in a 250 mL Erlenmeyer flask, followed by incubation at 28 °C for 5 × 24 hours in a shaker incubator set at 150 rpm under dark conditions (Shobham, 2025). After incubation, 40 mL of the fungal suspension was transferred into a 50 mL Falcon tube, followed by the addition of 2 mL of nasal spray containing 20% (v/v) kasturi orange peel essential oil, and the mixture was left to stand for 4 hours. The suspension was then centrifuged at 3000 rpm for 10 minutes at 4 °C, the supernatant was discarded, and the pellet was washed with 5 mL of sterile distilled water. The washing and centrifugation steps were repeated twice. Subsequently, 2% glutaraldehyde was added to the pellet and allowed to fix for 1–2 hours. The pellet was further fixed in cacodylate buffer for 20 minutes, followed by post-fixation with 1% osmium tetroxide for 1 hour. Dehydration was carried out sequentially using 50% ethanol for 20 minutes, then 70%, 80%, and 95% ethanol for 10 minutes each, and finally absolute ethanol for 20 minutes. The sample was then centrifuged at 3000 rpm for 10 minutes. t-Butanol was added and allowed to stand for 20 minutes, and this treatment was repeated twice. The resulting pellet was suspended in t-butanol and centrifuged at 3000 rpm for 15 seconds at 4 °C. The fungal pellet was then spread onto a watch glass and air-dried inside a biosafety cabinet for 6 hours. The dried sample was subsequently examined using a JSM-6510 Scanning Electron Microscope at 4000× magnification (Poejiani, 2018).

2.9 Statistical analysis

The inhibition zone diameter data for *Aspergillus fumigatus* from each treatment were presented in both qualitative and quantitative form and subsequently analyzed using SPSS software with a one-way ANOVA test. If the ANOVA results showed a p-value < 0.05, the analysis was continued using Duncan's post hoc test.

3. Results and Discussion

3.1 Characteristics of kasturi orange peel essential oil

The extraction of essential oil from kasturi orange peel yielded an essential oil with a density of 0.858 g/mL. This value is consistent with the density standard for citrus peel essential oils as specified in SNI ISO 3519:2005 at 20 °C, which ranges from 0.858 to 0.866 g/mL. Density determination serves as one of the analytical methods for evaluating the purity level of essential oils (Ikarini, 2021).

The essential oil obtained from *C. microcarpa* peel exhibited a pale greenish-cream color and a strong characteristic citrus aroma. It did not leave any oily stains on filter paper, indicating its purity and volatile nature, making it suitable for further testing. The solubility test showed that the essential oil was completely soluble and produced a clear solution at a ratio of 1:6 in ethanol. This result complies with ISO 3519:2005, which states that citrus peel essential oils typically exhibit ethanol solubility in the range of 1:6 to 1:8 (Latifah, 2023).

3.2 GC–MS analysis of kasturi orange peel essential oil

GC–MS analysis conducted at the Biosciences Laboratory, Politeknik Negeri Jember, produced the following results.

Table 2. GC–MS analysis results

Retention Time (Min)	Concentration (%)	Compound
1.552	13.45	dl-Limonene
3.906	9.60	beta-Eudesmol (CAS)
2.465	8.49	Germacrene-D
1.481	7.56	beta-Myrcene

GC–MS analysis revealed that the essential oil extracted from *Citrus microcarpa* peel contains a relatively complex mixture of compounds, with a total of 43 identified components. As shown in Table 3.1, four compounds were present at the highest concentrations, representing the major constituents: dl-Limonene (13.45% v/v), β-Eudesmol (9.60% v/v), Germacrene-D (8.49% v/v), and β-Myrcene (7.56% v/v). Among these, dl-limonene was the most dominant compound. dl-Limonene is a key determinant of the characteristic aroma of essential oils and is known to exhibit antifungal activity against various fungal species, including *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Penicillium digitatum*, *Fusarium* sp., and *Aspergillus* sp.

β-Eudesmol is an oxidized sesquiterpene classified as a sesquiterpenoid alcohol. Terpenoids are widely recognized for their antimicrobial properties and their ability to inhibit fungal growth (Li, 2021). Germacrene-D is an organic compound belonging to the Germacrene group of sesquiterpenoids. Germacrene sesquiterpene hydrocarbons exist as five isomers (A–E), with Germacrene-D known to possess antimicrobial activity (Pérez, 2018). β-Myrcene has been reported to disrupt fungal cells and alter their growth patterns, making it a promising candidate for use as an antifungal agent (Albayrak, 2023).

3.3 Identification of *Aspergillus fumigatus*

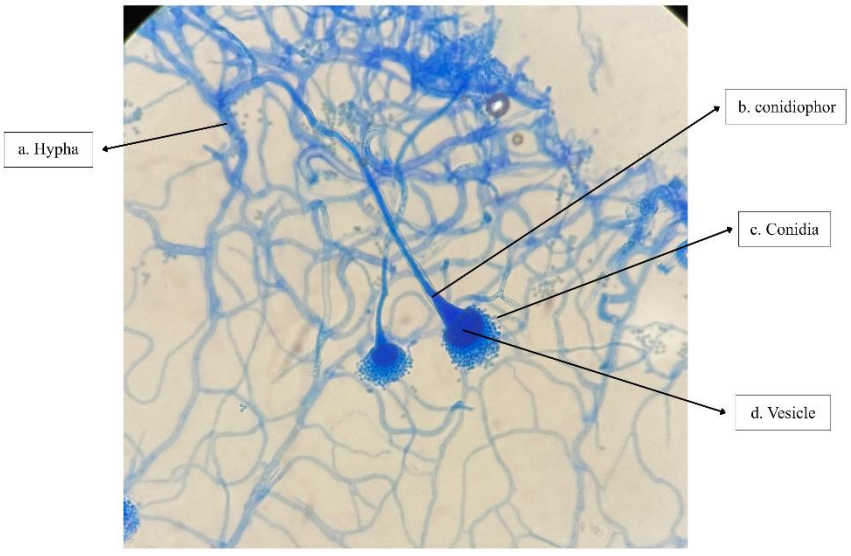


Figure 1. Identification of *Aspergillus fumigatus*.

Aspergillus fumigatus exhibits highly specialized morphological attributes, characterized by extensively branched, filamentous hyphae that constitute the primary vegetative structure and facilitate tissue penetration during host invasion. These hyphae are septate and multinucleated, reflecting compartmentalization through regularly spaced transverse septa. Moreover, the hyphae are classified as hyaline due to their transparent, non-pigmented appearance, a typical feature of many pathogenic Aspergillus species. Conidia are also produced,

appearing as small, spherical to ovoid asexual spores typically arranged in basipetal chains or compact clusters (Schoen, 2023).

This description aligns with the findings of Latgé (2020), who emphasized that the taxonomic identification of *A. fumigatus* is predominantly based on conidial morphology. The conidia are extremely small, measuring approximately 2.5–3 μm in diameter, airborne, haploid, and generally anucleate. Each conidium possesses a bilayered wall consisting of an outer melanin-rich layer with fine rod-like ornamentations and an inner structural layer composed primarily of chitin and β-glucan. The melanin layer plays a critical role in immune evasion by suppressing phagolysosomal acidification within macrophages.

Conidiophores form the specialized stalk structures that support conidiogenesis, culminating in the formation of a distinct conidial head. As a key element of the asexual reproductive apparatus, conidiophores mediate efficient aerosolized spore dispersal. In mycological diagnostics, microscopic characterization of conidiophores—particularly the morphology of the vesicle and surface architecture of the conidia—is routinely employed for species-level identification (Rajković, 2019). The vesicle, located at the terminal region of the conidiophore, functions as a membrane-bound nanostructure measuring approximately 100–200 nm and contains diverse biomolecular constituents such as proteins, lipids, and nucleic acids (Brauer, 2020).

3.4 Antifungal activity of kasturi orange peel essential oil against *Aspergillus fumigatus*

The antifungal activity of kasturi orange peel essential oil against *Aspergillus fumigatus* was evaluated using five concentration variations: 10% v/v, 20% v/v, 30% v/v, 40% v/v, and 60% v/v, employing the Kirby–Bauer disc diffusion method. In this method, paper discs containing the antifungal agent itraconazole were placed on the surface of agar plates previously inoculated with the test fungus. The antifungal agent diffuses radially into the agar, creating a gradient until its concentration is no longer sufficient to inhibit fungal growth. The effectiveness of the antifungal compound is determined by measuring the diameter of the inhibition zone, which appears as a clear area surrounding the disc where active substances have diffused (Purwatiningsih et al., 2021).

Table 3. Antifungal activity of kasturi orange peel essential oil against *Aspergillus fumigatus*

Inhibition zone diameter (mm)							
Kasturi orange peel essential oil							
n = 3	**10%**	**20%**	**30%**	**40%**	**60%**	**K+**	**K-**
1	11,85	16,5	19,7	22,4	22,1	23,2	0
2	11,5	15,9	16,7	18,9	20,1	27,1	0
3	13,9	17,9	17,3	17,5	20,6	28,25	0
Mean ± SD	12,4 ± 167,6 [d]	16, 7 ± 179,0 [d]	17, 9 ± 196,0 [d]	19, 6 ± 209,3 [d]	20, 9 ± 479,6 [d]	26,1±1 109,6 [b]	0,0 ± 0,0 [e]

Notes: *) Different superscript letters indicate significant differences according to Duncan's test.
SD = Standard deviation; Significant at $p < 0.05$

The clear zone measurements in the antifungal assay were carried out at concentrations of 10%, 20%, 30%, 40%, and 60%, and were compared with both positive and negative controls. The results showed that increasing the concentration of *Citrus microcarpa* essential oil led to a larger inhibition zone diameter against *Aspergillus fumigatus*. For comparison, the positive control using itraconazole produced an inhibition zone of 26.1 mm, whereas the negative control using DMSO showed no inhibition zone. These findings indicate that the inhibition zones produced by the essential oil at concentrations of 10%, 20%, 30%, 40%, and 60% were able to inhibit the growth of *A. fumigatus*, although the diameters were smaller than those produced by the itraconazole positive control.

Following data analysis using ANOVA, which showed a significant difference, further testing was performed using Duncan's multiple range test. The mean values for each treatment, as shown in Table 3, indicated that the negative control group differed significantly from all treatment concentrations. This was attributed to the use of DMSO in the negative control group, which does not contain any bioactive antifungal compounds. Meanwhile, treatments with concentrations of 10%, 20%, 30%, and 40% did not differ significantly from each other, but all showed significant differences when compared to the 60% concentration and the positive control group.

The observed antifungal effectiveness is related to the presence of secondary metabolites such as d-limonene and β-myrcene in the essential oil of kasturi orange (*Citrus microcarpa*) peel, both of which are known for their antimicrobial and antifungal properties. These compounds act by disrupting fungal cell membranes and inhibiting ergosterol biosynthesis, a crucial component in fungal cell wall formation (Dewajani, 2024). These findings are consistent with previous research by Retnaningsih et al. (2019), which reported that citrus essential oils exhibit antifungal activity against *Aspergillus* spp., including *A. fumigatus*, through mechanisms involving membrane permeability disruption.

3.5 Antifungal activity of nasal spray against *Aspergillus fumigatus*

Based on the antifungal activity test of nasal spray containing kasturi orange (*Citrus microcarpa*) peel essential oil against *Aspergillus fumigatus*, the results are presented in the following table:

Tabel 4. Antifungal activity data of the essential oil nasal spray against *A. fumigatus*

Inhibition Zone Diameter (mm)					
	Essential Oil Nasal Spray				
n = 3	**10%**	**20%**	**30%**	**K+**	**K-**
1	14,0	14,5	20,6	15,7	0
2	12,5	17,5	19,9	15,8	0
3	12,0	16,5	21,1	16,2	0
Mean ± SD	12,8 ± 128,3 [a]	16, 1 ± 159,0 [a]	20,5 ± 161, 6 [a]	15,9 ± 205, 3 [a]	0,0 ± 0,0 [e]

Notes: *) Different superscript letters indicate significant differences according to Duncan's test.
SD = Standard deviation; Significant at $p < 0.05$

In the positive control (K^+), a commercial nasal spray was used as a reference and demonstrated effective activity against *Aspergillus fumigatus*. Meanwhile, three concentrations of nasal spray containing kasturi orange (*Citrus microcarpa*) peel essential oil were tested, and the negative control (K^-) consisted of the basic formulation without essential oil. The negative control produced an inhibition zone of 0 mm, indicating that the nasal spray formulation without kasturi orange peel essential oil exhibited no antifungal activity. This confirms that the observed fungal inhibition was not influenced by the negative control.

Based on the data presented in Table 4, the 30% concentration exhibited strong antifungal activity, the 10% concentration showed significant activity, and the 20% concentration demonstrated the highest effectiveness as a nasal spray formulation. These findings are consistent with previous research by Husni et al. (2021), which reported that essential oil derived from kasturi orange peel possesses antimicrobial activity against various fungal species, with a minimum inhibitory concentration of 0.39% for the peel extract. The antifungal activity of *C. microcarpa* essential oil is attributed to its secondary metabolites, such as limonene, which act by disrupting fungal cell membranes and interfering with chitin synthesis in the cell wall (Ustna et al., 2023).

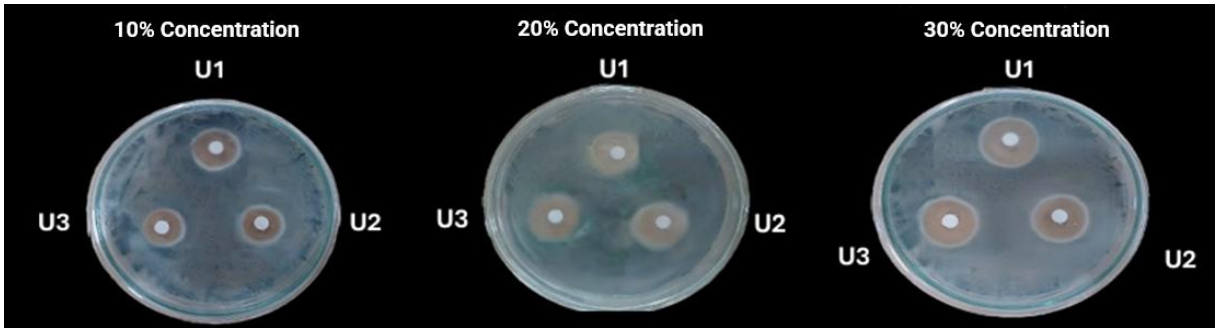


Figure 2. Diffusion test results of nasal spray containing kasturi orange peel essential oil against *Aspergillus fumigatus*

These results are consistent with the findings of Lestari et al. (2023), which demonstrated that nasal sprays containing kasturi orange peel essential oil exhibit significant antifungal activity, with inhibition zone diameters increasing proportionally with higher concentrations. A study conducted by Kartika *et al*. (2022) further revealed that sesquiterpene compounds possess pharmacological antifungal properties, disrupting membrane permeability and causing fungal cell damage. Therefore, nasal spray formulations containing *C. microcarpa* essential oil have strong potential as a natural antifungal active ingredient, particularly against *A. fumigatus*.

3.6 Characteristics of nasal spray formulations containing kasturi orange peel essential oil

Physical characterization of the nasal spray formulations was conducted to ensure product quality, stability, and user comfort. Physical evaluations are essential to confirm that the formulation meets pharmaceutical standards, ensuring safety and effectiveness during application. The evaluation included visual appearance, homogeneity, pH measurement, and spray distribution.

Table 4. Characteristics of nasal spray formulations

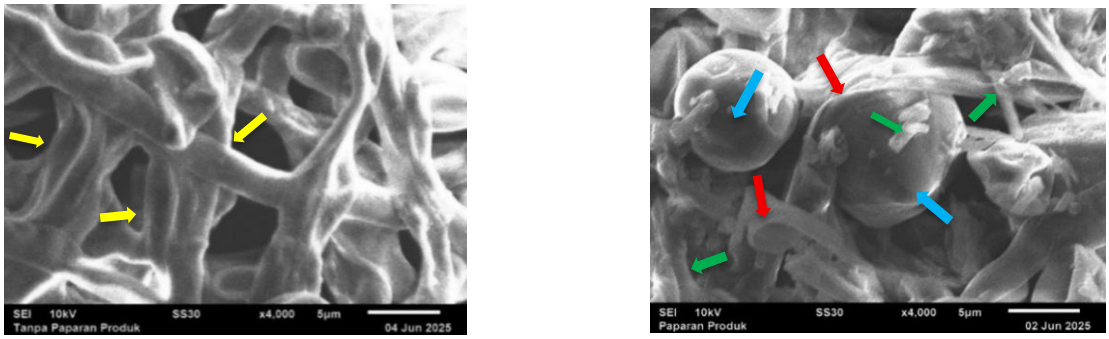
Physical Evaluation	Basic	10%	20%	30%
Visual Appearance	Clear	White	White	White
Homogeneity	Homogeneous	Homogeneous	Homogeneous	Homogeneous
pH	5,4	4,4	4,5	4,7
Spray Distribution (cm)	6,5	5,9	6,1	7

Based on Table 4, the visual evaluation of the nasal spray formulations shows that the basic formulation appeared clear due to the absence of essential oil. The addition of essential oil at concentrations of 10% to 30% resulted in a white appearance, indicating that the presence of essential oil influenced the visual characteristics of the formulation. The homogeneity evaluation revealed that all ingredients in the nasal spray formulations were well mixed and evenly distributed, with no sediment or coarse particles visible on the surface.

The pH values of the nasal spray formulations were within the permissible range established by BPOM Regulation No. 9 of 2023, which is 4.5–6.5. This indicates that the addition of essential oil did not significantly alter the pH of the formulation. These findings are consistent with Caimmi (2021), who reported that the incorporation of essential oils into liquid formulations does not cause pH changes due to their hydrophobic properties. In addition, the spray distribution results showed an increase in spray distance with higher essential oil concentrations. This suggests that increasing the amount of essential oil can influence the physical characteristics of the solution.

3.7 Morphological damage of *Aspergillus fumigatus* observed using scanning electron microscopy

Observations of *Aspergillus fumigatus* using scanning electron microscopy revealed significant morphological differences between untreated mycelia and those exposed to nasal spray containing kasturi orange (*Citrus microcarpa*) peel essential oil.



(a) (b)

Figure 5. Scanning electron microscopy results of *Aspergillus fumigatus* mycelia: (a) fungus grown in potato dextrose broth medium; (b) fungus exposed to nasal spray containing kasturi orange (*Citrus microcarpa*) peel essential oil.

Observations presented in Figure 5 show that the red arrows indicate lysed and shrunken hyphae, while the green arrows point to damaged cell wall structures and intracellular organelles following treatment with the nasal spray formulation. The blue arrows indicate the presence of particles from the nasal spray containing 20% kasturi orange (*Citrus microcarpa*) peel essential oil, and the yellow arrows highlight intact fungal hyphae. Figure 5 depicts *Aspergillus fumigatus* grown in Potato Dextrose Broth medium, observed under a acanning electron microscope (SEM) at 4000× magnification.

The morphological damage to *A. fumigatus* hyphae was evident from structural alterations following exposure to the nasal spray formulation containing antifungal compounds derived from kasturi orange essential oil. SEM observations at 4000× magnification revealed clear signs of cell leakage, indicating hyphal lysis and subsequent disruption of the fungal cell wall. In contrast, untreated *A. fumigatus* mycelia appeared intact and morphologically normal. Damage to the fungal hyphae was characterized by wrinkling and collapse of the cell wall surface. Poejiani (2018) reported that essential oils can penetrate fungal cells and disrupt membrane integrity, thereby affecting membrane fluidity and cellular metabolism. This effect is primarily attributed to active compounds such as D-limonene present in kasturi orange peel essential oil. D-limonene damages the fungal cell wall structure, disrupts active transport and proton movement across the cytoplasmic membrane, and denatures or inactivates proteins, particularly enzymes, leading to decreased membrane permeability. These changes impair the transport of organic ions and fungal cell metabolism, ultimately resulting in fungal cell death (Miyazawa, 2022).

Terpenoid compounds such as limonene, β-myrcene, and β-eudesmol are known to possess antimicrobial properties and inhibit fungal growth. Previous studies have shown that β-eudesmol can disrupt fungal cell wall integrity and block metabolic pathways by inhibiting the activity of key fungal enzymes, including acid phosphatase, chitinase, and protease, thereby exerting antifungal effects. Terpenoid compounds such as β-eudesmol found in citrus peel essential oils have been proven to play a significant role in antifungal activity (Li, 2025).

Fungal vacuoles are membrane-bound organelles that play key roles in degradation processes, primary storage, osmoregulation, and homeostatic regulation, including the control of cytosolic ions, basic amino acid concentrations, and intracellular pH (Takahashi et al., 2020). Jian (2023) demonstrated that limonene inhibits fungal growth by damaging vacuoles, destroying hyphal cell membranes, and disrupting osmotic regulation, which leads to the depletion of cellular energy metabolism required for growth, ATP synthesis, and fungal proliferation, ultimately damaging fungal cell wall structures.

The surface of *A. fumigatus* hyphae grown in Potato Dextrose Broth medium without exposure to the nasal spray formulation appeared smooth and intact, indicating the absence of contact with antifungal compounds. Consequently, both the hyphal surface and fungal cell membranes displayed normal morphology.

4. CONCLUSION

Katsuri orange *Citrus microcarpa*) peel essential oil contains D-limonene as its major compound, which demonstrates antifungal activity as evidenced by the inhibition zones formed against *Aspergillus fumigatus*. The nasal spray formulated with kasturi orange peel essential oil proved effective in inhibiting the growth of A. fumigatus, with the most prominent inhibition zone measuring 16.1 mm at the 20% concentration. Furthermore, Scanning Electron Microscopy (SEM) revealed significant structural damage to the fungal cell wall and hyphae following treatment with the essential oil-based nasal spray. These findings confirm that the study objective was achieved and support the potential application of a 20% kasturi orange peel essential oil nasal spray as a natural antifungal agent for fungal infection control.

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