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Utilization of neem leaf extract (*Azadirachta indica* A. Juss.) in inhibiting the growth of acne-causing bacteria *Cutibacterium acnes*

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Abstract
Background: Acne is an infection that occurs on the surface of the skin in the form of inflammation of the oil glands in the epidermal layer of the skin, when infected by the bacteria *Cutibacterium acnes*, blackheads will develop into inflammation and cause acne vulgaris. One of the plants that can be used as a natural alternative in acne treatment is neem (*Azadirachta indica A. Juss*). *Objectives*: This research aims to identify the inhibitory effect of neem leaf extract on *C. acne*. *Materials and Methods*: Neem leaves samples were obtained from the Sumenep district. The research was conducted at the PIPOT-UBAYA Laboratory, the Pharmacy Laboratory of PGRI Adi Buana University Surabaya and the Microbiology Laboratory of Stikes Ngudia Husada Madura. The research design used is experimental, testing of the inhibition power of neem leaves using the diffusion method by observing the inhibition zone around the disc. This research used concentrations of 25, 50, 75, and 100%. The positive and negative controls used are sterile aquades and Clindamycin. *Results*: The results showed the inhibition zones of neem leaf extract against *C. acnes* at concentrations of 25% and 50% were 6.3 mm and 9.3 mm, while at concentrations of 75% and 100% were 12.3 mm and 16 mm. *Conclusions*: The results of the one-way ANOVA test showed p-value is 0.000, which means that neem leaves can inhibit the growth of *C. acnes*.

Keywords
Antibacteria, Leaves Extract, Neem (*Azadirachta indica A. Juss*), *Cutibacterium acnes* Bacteria

1. Introduction

Cutibacterium acnes is a gram-positive bacterium and a microbiota commonly found on the facial skin and scalp, which have many sebaceous glands. *C. acnes* is a relatively slow-growing bacterium and has air-tolerant properties. This bacterium produces enzymes that weaken the skin and proteins, which can activate the immune system (Mclaughlin, J., et al., 2019). Colonization *of C. acnes* is one of the factors that causes acne, which plays a role in the process of acne formation by producing lipase which can break down free fatty acids (Bashar, A.M., et al., 2020).

Acne begins with sebum clumping in the epidermal layer of the skin, leading to blackheads protruding to the skin's surface; if the blackhead becomes infected with *C. acnes* bacteria, it will develop into inflammation (Platsidaki and Dessinioti, 2018).

Acne vulgaris is a dermatological condition that impacts about 9.4% of the global population. A study conducted at Abdul Moelok hospital in 2019 on 66 patients found that 30.3% of men were affected by acne, while in women, the figure was 69.7%. In Indonesia, the prevalence of acne among adolescents is between 80–85%, with the highest occurrence at ages 15 to 18., 12% of women over the age of 25 years old, and 3% at individuals aged 35-44 years are also detected. The number of Acne vulgaris sufferers increases by 40% to 80% every year (Sibero et al., 2019).

Treatment for acne vulgaris can be done with topical medications that are directly applied to the acne-affected area or oral medications taken by mouth. Topical therapies used directly on the affected areas contain ingredients such as sulfur, sodium, sulfacetamide, resorcinol, alpha hydroxy acids, and beta hydroxy acids. Oral treatment for acne vulgaris commonly involves the antibiotics like trimethoprim, sulfamethoxazole, erythromycin, clindamycin, and tetracycline (Teresa, 2020). Prolonged use of antibiotics will cause *C. acnes* to become resistant to antibiotics (Dessinioti et al., 2022).

The use of antibiotics must be rational, understanding various types of infectious diseases that consider the pharmacokinetics and pharmacodynamics of the antibiotics to be used, as well as factors such as individual resistance, virulence, and microorganisms. Irrational use of antibiotics leads to the development of bacteria resistant to antibiotics, rendering antibiotics ineffective. Therefore, alternative treatments are needed, one of which is utilizing plants (Edwards et al., 2021).

Indonesia has the second highest biodiversity in the world with 30,000 types of medicinal plants. One type of plant that can be an alternative source of medicine with potential as an antibacterial is Neem (*Azadirachta indica A. Juss*) (Fahrurin et al., 2023). Generally, neem contains 135 different active chemical compounds. Neem leaves contain bioactive compounds such as terpenoids, flavonoids, alkaloids, saponins, and tannins that function as antibacterials. Empirically, people have used neem leaves to treat itching by crushing 5-7 leaves and applying them to the itchy area. Another method is to boil the leaves, then drink the water from the boiling to treat itching (Fatmawati, 2019).

Studies have demonstrated that neem leaf extract exhibits antibacterial effects against *Staphylococcus aureus* bacteria showed antibacterial activity at concentrations of 50% to 90%, producing an average inhibition zone of 9.8 mm, indicating that the formed inhibition zone is classified as medium activity. The present research seeks to identify the inhibitory potential of neem leaf extract on *C. acnes* bacteria using the in vitro disc diffusion method by observing the formation of inhibition zones (Fatmawati, 2019).

This study aims to determine the antibacterial effectiveness of neem leaf extract (*Azadirachtaindica A. Juss*.) against the growth of *C. acnes* bacteria.

2. Materials and Methods

2.1 Materials and Tools

The tools and materials used include neem leaves fresh and green, etanol 96% (Cat No.: E9600, Merck, Darmstadt, Germany), aluminium foil, beaker glass, rotary evaporator DLAB RE100-PRO, filter paper, analytical balance, dragendorff (Cat No.: DRG001, Sigma-Aldrich, St. Louis, USA), concentrated HCl (Cat No.: H1758, Sigma-Aldrich, St. Louis, USA), Mg powder (Cat No.: M7406, Sigma-Aldrich, St. Louis, USA), aquadest (Cat No.: AQ123, OneMed, Surabaya, Indonesia), $FeCl_3$ 1% (Cat No.: FC100, Merck, Darmstadt, Germany), kloroform (Cat No.: C2432, Sigma-Aldrich, St. Louis, USA), acetic anhydride (Cat No.: A6404, Sigma-Aldrich, St. Louis, USA), DMSO 10% (Cat No.: D8418, Sigma-Aldrich, St. Louis, USA), *Propionibacterium acnes* (Cat No.: ATCC 6919, ATCC, Manassas, USA), MHA (Cat No.: 70191, Sigma-Aldrich, St. Louis, USA), incubator, ose loop, NaCl 0,9% (Cat No.: NS090, Otsuka, Jakarta, Indonesia), *McFarland* (Cat No.: MF050, Bio-Rad, Hercules, USA), disk paper, petri dish, caliper.

2.2 Method

The type of research in this study is quantitative and the research design used is experimental. This research was conducted to test the inhibitory power of neem leaves (*Azadirachta indica A. Juss*) in vitro by observing the occurrence of inhibition zones. The population used is neem leaves (*Azadirachta indica A. Juss*) from Sumenep regency, Indonesia. The samples used are fresh green neem leaf extracts (*Azadirachta indica A. Juss*) with concentration variations of 25%, 50%, 75%, and 100%, *C. acnes* bacteria, positive control Clindamycin 20 mg, and negative control sterile aquadest, then the sample repetitions were calculated using the Federer formula and resulted in a total of 4 repetitions.

The research was conducted from March to May 2024 at the PIPOT Laboratory - University of Surabaya, Indonesia for plant determination, and the results showed that the plant used was neem (*Azadirachta indica A. Juss*), as proven by certificate No. 1567/D.T/I/2024. The Pharmacy Laboratory of PGRI Adi Buana University Surabaya was used for the extraction process, and the Microbiology Laboratory of Stikes Ngudia Husada Madura, Indonesia was used for antibacterial testing. This research has passed the ethical feasibility test from the Research Ethics Commission of Stikes Ngudia Husada Madura, Indonesia No: 2127/KEPK/STIKES-NHM/EC/V/2024.

2.3 Work Steps

1. Preparation of maceration extract

   The extraction process begins by taking 1 kg of fresh neem leaves, which are then cleaned of dirt by washing them in running water. The leaves are drained to remove excess water and cut into smaller pieces, then dried by airing them at room temperature. Once the leaves are dry, they become simplicia, which is then crushed using a blender to form a powder. The simplicia powder is then filtered using a sieve, placed in a beaker glass, and weighed using an analytical scale, resulting in 327.5 g of simplicia powder. This powder is then macerated with 96% ethanol solvent at a ratio of 1:10. It is tightly closed with aluminum foil and left to sit for 24 hours. After 24 hours, the maceration results are filtered, and the filtrate is collected in another beaker glass. The residue from the filtration is macerated again by adding 96% ethanol until submerged, sealed tightly with aluminum foil, and left for 24 hours. The re-maceration process was carried out three times. The extract was evaporated in a rotary evaporator branded Rotary Evaporator DLAB RE100-Pro until all the solvent evaporated and a thick extract was obtained. The thick extract was then weighed using an analytical balance to determine the total mass of the extract obtained, resulting in 37 g of extract (Riskiyani et al., 2020).

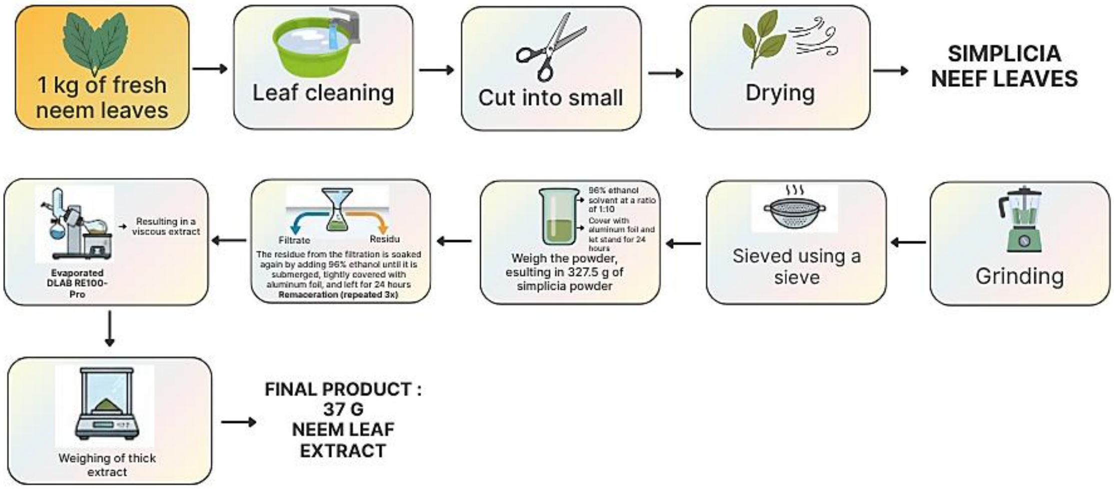

Figure 1. Process Maceration Extract

2. Phytochemical tests

The extraction of neem leaves is then subjected to phytochemical testing, with qualitative phytochemical screening that can be performed through color reactions using specific reagents. The tests conducted consist of tests for alkaloids, flavonoids, saponins, tannins, and terpenoids. The alkaloid test is performed using Dragendorff's reagent, the flavonoid test is conducted using concentrated HCl and Mg powder, the saponin test is done using hot distilled water until stable foam is formed, the tannin test is performed using 1% $FeCl_3$ reagent, and the terpenoid test uses chloroform, anhydrous acetic acid, and concentrated sulfuric acid (Vifta et al., 2018).

3. Preparation of variation in extract concentration

The concentrations of the leaf extract used are 25%, 50%, 75%, and 100%. Each concentration is made by weighing and diluting the concentrated neem leaf extract (100% concentration) with 10% DMSO. The preparation of the concentration variations is done in a sample cup with a total volume of 1ml. The sample solution is made by weighing the thick extract: 0.25 g of extract + 1ml of 10% DMSO solution (25%). 0.50 g of extract + 1ml of 10% DMSO solution (50%). 0.75 g of extract + 1ml of 10% DMSO solution (75%).

4. Preparation of bacterial suspension

Pure culture of *C. acnes* bacteria was obtained at the Microbiology Laboratory of STIKes Ngudia Husada Madura with the bacteria strain number ATCC-6919. The *C. acnes* bacterial culture was inoculated into solid MHA agar media. It was then incubated in an incubator at 37° C for 24 hours. The preparation of bacterial suspension was carried out by taking bacterial colonies using an inoculating loop and suspended in a tube containing 5ml of sterile 0.9% NaCl solution until a concentration of 0.5 x 10^8 *McFarland* was achieved. The turbidity of the bacterial suspension was measured using a *McFarland* 0.5 x 10^8 standard; McFarland equivalent to 1.5 x 10^8 (Colony Forming Unit) CFU/ml. If the suspension is more turbid than *McFarland*, 0.9% NaCl is added; if less turbid, more bacterial suspension is added (Sibero et al., 2019).

5. Test of antibacterial activity

Antibacterial activity testing was conducted using the diffusion method, with discs of 6 mm in diameter. Sterilized and heated MHA medium was poured into a petri dish with 15 ml and then allowed to solidify. *C. acnes* was spread using the streak method, and then discs that had been treated with the solution concentration were placed on the MHA inoculated with bacteria, incubated at 37° C for 24 hours. Subsequently, observations were made, and the clear zone formed around the holes was measured using calipers and entered into the formula for bacterial inhibition diameter (Susanti et al., 2016).

$$\frac{(Dv - Dc) + (Dh - Dc)}{2}$$

In which Dv is the vertical diameter, Dh is the horizontal diameter, and Dc is the disc diameter (Rini et al., 2018).

2.4 Data analysis

The data from the bacterial resistance tests obtained were analyzed parametrically using one-way ANOVA statistical test to determine differences in variation between groups with IBM SPSS Statistics 25.0 software.

3. Results and Discussion

3.1 Neem Leaves Extract

The following is the result of extracting neem leaves carried out using the maceration process and using 96% ethanol as the solvent.

Table 1. Results of Neem Leaf Extraction

Sample	Neam Leaf
Simplisia	327,5 g
Volume of solvent	3275 ml
Soaking Time	3x24 hours 3x solvent replacement after 24 hours
Concentrated Extract	37 g
Yield	11,29%

Neem leaf extraction is performed using the maceration method with 96% ethanol as the solvent. This technique involves repeated stirring or shaking at room temperature. One of the main benefits of maceration is its simplicity and the absence of heat application, which minimizes the risk of damaging the natural compounds. Additionally, the extended duration and static conditions of the process enhance the extraction of a wide range of compounds (Adhariani et al., 2018). In this study, 96% ethanol was used as the sample solvent. One reason for using ethanol is that it is relatively non-toxic compared to acetone and methanol, cost-effective, can be used in various extraction methods, and is safe for use in the production of medicines and food. Another reason is that ethanol is an easily obtainable solvent, effective, environmentally safe, and has a high extraction rate (Dianda, 2022). 96% ethanol solvent can increase the color intensity of the leaf extract to become concentrated. This is due to ethanol's ability to dissolve phenolic and flavonoid compounds (Haideri et al., 2024). Dissolved phenolic compounds can undergo oxidation resulting in a darker extract color. Apart from that, the high content of phytochemicals extracted due to the use of organic solvents also influences changes in the color characteristics of the extract (Chatepa et al., 2024).

In the research results, a concentrated extract of 37 g was obtained and the extract yield value was 11.29%. Yield refers to the ratio between the dry weight of the obtained extract and the weight of the original raw material; a higher yield suggests a greater concentration of bioactive compounds. This value reflects the level of bioactive substances present in the plant, where a higher yield indicates that more active compounds have been successfully extracted from the material (Putri, 2022).

Phytochemical tests

Phytochemical testing was conducted to ensure the presence of compounds contained in the neem leaf extract. Below are the results of the qualitative phytochemical tests of neem leaf extract (*Azadirachta indica A. Juss*) using color reaction.

Table 2. Results of Phytochemical Tests

Test	Reactant	Result
Alkaloids	Dragendorff	(+) Orange precipitate
Saponins	Warm Aquadest	(+) Stable foam
Flavonoids	Mg + HCl	(+) Yellowish green
Tanins	$FeCl_3$ 1%	(+) Brownish green
Terpenoids	Liebermen Burchard	(+) Reddish brown

The results of phytochemical tests show that neem leaf extract contains antibacterial compounds namely alkaloids, saponins, flavonoids, tannins, and terpenoids which serve to inhibit bacterial growth.

Phytochemical screening is conducted as an initial step to provide an overview of the compound content in the material to be studied, and is usually performed qualitatively using color reactions. The tests conducted include alkaloid, flavonoid, saponin, tannin, and terpenoid tests.

Alkaloid detection was carried out using Dragendorff's reagent, which produced an orange precipitate as a positive indication. This reaction likely occurs due to the formation of a covalent bond between the nitrogen atom in the alkaloid and the metal ion K^+ (Senduk et al., 2020). Alkaloids exhibit antibacterial properties by interfering with the peptidoglycan structure in bacterial cell walls, causing defects in cell wall synthesis and ultimately leading to cell death (Ergina, 2014).

The reagents used were concentrated HCl and Mg powder. The result shown by this method is positive because there is a color change to yellowish green. Theoretically, the color that arises in this test is yellow-orange to red, which can be stated as positive for flavonoids [18]. The yellowish green color obtained is due to flavonoids, which include phenolic compounds that are polar, allowing them to be extracted in a polar solvent. Flavonoids exhibit antibacterial effects by binding with extracellular proteins of bacteria, leading to disruption of the plasma membrane and the subsequent leakage of intracellular contents (Ergina, 2014).

The saponin test was conducted using hot aquadest until a stable foam was formed. The results obtained from the saponin test on the neem leaf extract showed a foam formation of 1cm high and stable for a duration of 10 minutes. The foam formed is due to saponin having the property of reducing surface tension and containing hydrophilic and lipophilic groups. Saponin has antibacterial activity with a mechanism that causes leakage of proteins and enzymes from within the cells, resulting in cell death (Donadio et al., 2021).

The tannin test was conducted using 1% $FeCl_3$ reagent. The tannin test yielded a positive result, indicated by a brownish-green color change in the solution. This reaction occurs because tannins are soluble in water, alcohol, and acetone, and the color shift is a result of a reduction process. Tannin belongs to the class of polyphenolic compounds, and polyphenols can reduce iron (III) to iron (II). This is also a classical method for detecting phenolic compounds, by adding 1% ferric chloride solution in water or ethanol to the sample solution, resulting in green, red, purple, blue, or black coloration. Tannins possess antibacterial properties by blocking the activity of enzymes such as reverse transcriptase and DNA topoisomerase, which in turn hinders the formation of bacterial cells (Surahmaida, 2020).

The terpenoid test using chloroform, anhydrous acetic acid, and concentrated sulfuric acid showed a positive result with a reddish-brown color. This occurs due to the oxidation reaction of the terpenoid compound that produces a chromophore group (conjugated unsaturated carbon). Terpenoids act as antibacterial agents by interacting with porins, which are transmembrane proteins located in the outer membrane of bacterial cell walls. This interaction forms strong polymeric bonds that damage the porins, decreasing cell wall permeability and ultimately causing bacterial cell death (Supriyanto, 2017).

3.2 Antibacterial test

This study employed the disk diffusion method to assess antibacterial resistance. Test concentrations included 25%, 50%, 75%, and 100%. Clindamycin served as the positive control, while sterile distilled water (aquadest) was used as the negative control.

Table 3. Results of the measurement of the inhibition zone diameter

Concentration	Average	Referance Value (David-Stoud)	Description
25%	6,3 ± 0,79	5-10 mm	Intermediate
50%	9,3 ± 0,17	5-10 mm	Intermediate
75%	12,3 ± 0,34	10-20 mm	Sensitive
100%	16 ± 0,35	10-20 mm	Sensitive
Control (+)	17,2 ± 0,58	10-20 mm	Sensitive
Control (-)	0	0 mm	Resistant
p-value 0,000			

The neem leaf extract inhibition test revealed that a 100% concentration produced an average inhibition zone of 16 mm, indicating a sensitive response. At 75% concentration treatment, the average inhibition zone was 12.3 mm, categorized as sensitive. At a 50% concentration treatment, the average inhibition zone was 9.3 mm, categorized as intermediate. At a 25% concentration treatment, the average inhibition zone was 6.3 mm, categorized as intermediate. The positive control clindamycin showed an average value of 17.2 mm, categorized as sensitive, while the negative control showed a result of 0 mm, meaning no inhibition zone was formed.

The resistance test used in this study is the disk diffusion method. The concentrations used in this study are 25%, 50%, 75%, and 100%. Clindamycin was used as the positive control, while sterile distilled water served as the negative control. The results show the presence of an inhibition zone formed by the neem leaf extract with a solvent of 96% ethanol, which is indicated by the formation of a clear zone on the growth media of Propionibacterium acnes. The results of the antibacterial test of 100% neem leaf extract showed an average inhibition zone of 16 ± 0.35, which falls into the strong category. For the treatment of 75% concentration, the average inhibition zone was 12.3 ± 0.3, also classified as strong. For the 50% concentration treatment, the average inhibition zone was 9.3 ± 0.17, categorized as moderate. In the 25% concentration treatment, the average inhibition zone was 6.3 ± 0.79, also in the moderate category. The positive control with clindamycin showed an average value of 17.2 ± 0.58, classified as strong, while the negative control showed 0 mm result or no inhibition zone formed.

Clindamycin is used as a control because clindamycin is a broad-spectrum antibiotic used in the treatment of infections caused by gram-positive bacteria and also anaerobic bacteria. Clindamycin is bacteriostatic, meaning it inhibits the growth of microbes but does not kill them. The mechanism of action of clindamycin is by inhibiting protein synthesis in microorganisms by affecting the 50s ribosomal subunit, thereby disrupting the formation of the bacterial peptidoglycan chain (Gerung et al., 2021). Sterile aquadest is used as a negative control, as the use of aquades as a negative control is because the compounds from aquades are neutral and will not have an effect on bacterial growth or do not possess antibacterial activity (Gerung et al., 2021).

Based on the average results of the inhibition test, it shows that the largest inhibition zone occurs at the highest concentration, which is 100%. Theoretically, as the concentration of an antimicrobial substance increases, the diameter of the inhibition zone formed also increases. This occurs due to the increased concentration of the antimicrobial agent, the more active substances it contains, thereby increasing its effectiveness in inhibiting bacteria, resulting in a wider inhibition zone. With an increasing concentration of an antimicrobial, diffusion occurs more rapidly, thus enhancing its antibacterial power and enlarging the diameter of the inhibition zone produced (Hasanuddin, 2020).

Research on the extract of neem leaves conducted on *Staphylococcus aureus* bacteria showed antibacterial activity at concentrations of 50% to 90% with an average inhibition zone of 9.8 mm, which means the formed inhibition zone falls into the moderate inhibition category. In the inhibition test conducted on Staphylococcus epidermidis bacteria at concentrations of 15% to 50%, antibacterial activity was observed with an average inhibition zone of 11.18 mm to 16.30 mm, which falls into the strong inhibition category (Sayekti et al., 2020). Thus, it is known that the extract of neem leaves contains compounds that function as antibacterial agents.

The bacterium *Cutibacterium acnes* can be inhibited using extracts from neem leaves due to the effectiveness of alkaloid, saponin, flavonoid, tannin, and terpenoid compounds. These phytochemical compounds generally play a role as antibacterial by interfering with the peptidoglycan in bacterial cells, forming complex compounds that damage the bacterial cytoplasmic membrane, leading to inhibition and leakage of proteins and enzymes within the cell, leading to the disruption of porins and a subsequent reduction in the bacterial cell wall's permeability, thereby causing bacterial death.

The initial statistical analysis involved testing for normality and homogeneity using the Shapiro-Wilk and Levene's methods. The Shapiro-Wilk normality test produced a p-value > 0.05, indicating that the data were normally distributed. Subsequently, the homogeneity test using Levene's method also yielded a p-value > 0.05, signifying that the mean diameter of the inhibition zones was uniformly distributed.

After the average data of the inhibitory zone diameter is normally and homogeneously distributed, an analysis using one way ANOVA will be continued to see the variation differences, and it was found that the p-value is 0.000, which means it has an effect on the inhibition of *Propionibacterium acnes* bacteria, this indicates that the alternative hypothesis (H1) is accepted while the null hypothesis (H0) is rejected. The Post hoc test results reveal significant differences among nearly all groups with a p-value of 0.000, except between the 100% concentration group and the positive control, which showed no significant difference with a p-value of 0.24.

4. Conclusions

Neem leaves extract (*Azadirachta indica A. Juss*) can inhibit the growth of *Cutibacterium acnes* bacteria. The inhibition effect, which is classified as moderate, at concentrations of 25% and 50%. The inhibition effect, which is classified as strong, at concentrations of 75% and 100%. The most effective concentration to inhibit the growth of *C. acnes* bacteria is 100% (sensitive category).

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Author Contributions: DPS: Data curation, Methodology, Formal analysis, investigation, Resources, Data Curration, Writing - Original Draft, Visualization; CSR: Conceptualization, Validation, formal analysis, Writing - Review and Editing, Supervision, Project administration

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