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Original Article

ETHYL ACETATE FRACTION OF *NIGELLA SATIVA* HAS ANTIBACTERIAL ACTIVITY AND THE PROFILE OF ITS BIOAUTOGRAPHY

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ABSTRACT

Objective: Pathogenic microorganisms, including bacteria, viruses, fungi, and parasites, are sources of infectious diseases. It is known that *Nigella sativa* contains nigellimine, nigellimine-N-oxide, nigellidine, and nigellicine, as well as other active alkaloid compounds. The purpose of this study was to examine the antibacterial activity and bioautography of the ethyl acetate fraction of *Nigella sativa* against *Bacillus cereus, Shigella sonnei, Streptococcus mutans,* and *Klebsiella pneumoniae.*

Methods: Thin Layer Chromatography (TLC) was used in the bioautography analysis, and the disc diffusion technique was used to test the antibacterial activity. The concentrations of the ethyl acetate fractions were 20%, 40%, 60%, and 80%.

Results: The ethyl acetate fraction of *Nigella sativa* showed strong antibacterial activity against a number of microorganisms studied. According to the data, the inhibition zone was the best at a concentration of 80%. The diameters of the inhibition zones for the following bacteria were measured: 11.08±0.38 mm for *Klebsiella pneumoniae*, 12.50±0.50 mm for *Shigella sonnei*, and 17.67±0.58 mm for *Bacillus cereus*, covering a disc diameter of 6.00 mm. The findings of the bioautography test indicated that the alkaloid component is most likely the active ingredient causing the antibacterial activity.

Conclusion: Based on this research, the ethyl acetate fraction of *Nigella sativa* can be used as a natural antibacterial agent.

Keywords: Antibacterial, Bioautography, *Nigella sativa*, Ethyl acetate fraction

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INTRODUCTION

Infection is one of the main health problems in Indonesia. Infection is caused by microorganisms such as bacteria, viruses, fungi, or parasites [1]. Antimicrobial drugs, such as drugs containing antibacterial/antibiotics, antifungals, antivirals, and antiprotozoal are the best drugs to overcome this problem. Inappropriate use of antibiotics can cause resistance problems. Using antibiotics more wisely means considering the effects of the emergence and spread of resistant bacteria [2].

The main therapy for bacterial infections is the use of antibiotics. The use of antibiotics has increased significantly due to their ability to treat and prevent disease. Antibiotics are sometimes used excessively or carelessly, especially for diseases that are not needed, and buying antibiotics without a doctor's prescription is common. This is what causes bacteria to multiply and become resistant to antibiotics [3]. Excessive use of antibiotics can cause side effects that can be harmful to the human body. Likewise, reducing the dose from the normal dose will prevent bacteria from being completely destroyed and provide space for bacteria to develop resistance to the antibiotic. The same thing applies to the time of use; stopping the use of antibiotics prematurely because feel healthy will also prevent bacteria from dying completely and potentially becoming resistant [4].

There are several things that can be done, such as controlling antibiotic use, increasing research into the mechanisms underlying genetic resistance, and encouraging the development of new natural products [5]. An alternative way to overcome this resistance problem is to find new antibacterial compounds that have not caused resistance. The compounds can be obtained from plants. Plants contain compounds that have new mechanisms of action and are not yet resistant, making them promising antibacterials [6]. *Nigella sativa* (NS) is one of the plants that has antibacterial properties.

NS has been identified and reported to have various active compounds. The main active compounds in this plant are thymoquinone (30%-48%), thymohydroquinone, dithymoquinone, p-cymene (7%-15%), carvacrol (6%-12%), 4-terpineol (2%-7%), t-anetol (1%-4%), sesquiterpene longifolene (1%-8%), α-pinene and thymol. *NS* contains two different types of alkaloids; namely isoquinoline alkaloids such as nigellicine and nigellimine-N-oxide, and pyrazole alkaloids or indazole ring alkaloids, including nigellidine and nigellicine [7, 8].

This is proven by the results of previous studies, namely in the antibacterial test of ethanol extract of *NS* seeds, an inhibition zone was formed on *Klebsiella pneumoniae* (*Kp)* bacteria with concentrations of 5%, 7.5%, 10%, 12.5%, and 15%, respectively, which were 4.5, 6.7, 10.1, 11, and 12.8 mm [9]. Methanol extract of *NS* seeds has antibacterial activity on the growth of *Streptococcus mutans (Sm)* bacteria at concentrations of 3%, 4%, 5%, 6% and 7% [10].

The previous research was in ethanol extract, so this study was conducted on ethyl acetate fraction of *NS* against *Kp, Shigella sonnei (Ss), Sm*, and *Bacillus cereus* (*Bc)* bacteria because there has been no previous study showing such activity. This study was conducted to determine the class of compounds carried in the ethyl acetate fraction method. The disk diffusion method was used in this study for antibacterial testing, and the bioautography TLC method was used to identify the class of antibacterial compounds.

MATERIALS AND METHODS

Materials

The equipment used in this study were an incubator (Memmert), Laminar Air Flow (LAF), autoclave (Hirayama), oven (Memmert), refrigerator, shaker incubator, analytical balance (Ohaus), vortex, petri dish, bunsen, test tube, spreader glass, erlenmeyer flask, measuring cup, beaker, 1000 µl** micropipette (Socorex), 10 µl** micropipette (Socorex), 254 nm UV lamp, 366 nm UV lamp, and Poco F4 cellphone camera with 64 megapixel resolution for documentation.

The materials used are *NS* Fraction, distilled water, sterile 0.9% sodium chloride (NaCl), Brain Heart Infusion (BHI), Dimethyl sulfoxide (DMSO), Mueller Hinton Agar (MHA), cotton, aluminum foil, methanol,

chloroform, standard 0.5 Mc Farland (1.5 x 108 CFU/ml), blank disc (Oxoid TM), silica gel GF 254, blank disc (Liofilmchem), Tetracycline antibiotic disc 30µg, Erythromycin 15µg, Vancomycin 30µg, Ampicillin 10µg (Oxoid TM), bacteria *Kp, Ss, Sm* and *Bc* from the Microbiology Laboratory of the UMS Faculty of Pharmacy, and spray reagents Liebermann-Burchard, Dragendorff, $FeCl_3$ and $AlCl_3$.

Preparation of bacterial suspension

Three to five colonies from a pure culture of the test bacteria are collected using a circular loop to prepare a bacterial suspension, which is subsequently injected into 5 ml of BHI media.

The culture is thereafter incubated for 18 to 24 h in a shaker incubator. The test bacteria in BHI are obtained with a sterile loop and inoculated onto MHA media with the streak plate technique. Subsequently, they were incubated for 18 to 24 h at 37 °C, and the culture outcomes were preserved as bacterial stock in the refrigerator. Utilizing a micropipette, 200 µl** of the BHI test bacterial suspension was obtained, diluted in a 0.9% NaCl solution, and its turbidity was calibrated to correspond with the 0.5 McFarland standard solution (1.5 x 10^8 CFU/ml).

Bacterial sensitivity test

Utilizing a micropipette, 200 µl** of a 0.9% NaCl bacterial solution was introduced into the MHA medium, followed by the application of a glass spreader for uniform distribution. Upon the suspension's uniform drying, the antibiotic disc was positioned on the MHA medium with the test bacteria, adequately spaced, and incubated for 24 h at 37 °C. The width of the clear zone surrounding it demonstrates that the sample can inhibit bacterial growth [11]. The antibiotics utilized for testing included 30 µg tetracycline discs, 15 µg erythromycin, 30 µg vancomycin, and 10 µg ampicillin.

Preparation of test solution concentration

Ethyl acetate fraction of *NS* was made at concentrations of 20, 40%, 60%, and 80%. Stock solution with a concentration of 1 g/ml and a volume of 5 ml was made by weighing and diluting 5 gs of fraction with 100% DMSO solvent. DMSO solvent was used to dilute the stock solution to concentrations of 20%, 40%, 60%, and 80% [12].

Antibacterial activity test

The disk diffusion method was obtained to evaluate antibacterial efficacy. Following the preparation of a bacterial suspension including *Kp, Ss, Sm,* and *Bc*, 200 µl of MHA medium was incorporated, evenly disseminated using a glass spreader, and permitted to dry. The *NS* fraction was applied to the surface of the MHA media using sterile tweezers after dispensing up to 10 µl into a sterile empty plate at concentrations of 20%, 40%, 60%, and 80%. Up to 10 µl of DMSO was employed as a negative control, while antibiotics chosen based on prior sensitivity test outcomes served as positive controls. Subsequently incubated at 37 °C for duration of 18 to 24 h. additionally, the inhibition zone of the experimental material was documented [13, 14].

Thin layer chromatography (TLC) test

The ethyl acetate fraction of *NS* was isolated using a 1x7 cm GF254 silica gel plate as the stationary phase. The initial spot location and the final position post-elution were marked with a distance of 1 cm on the upper and lower edges of the plate. The silica plate underwent activation for ten minutes at 105 °C in an oven. Additionally, the filter paper was saturated with the mobile phase within the chamber. The mobile phase comprised a chloroform: methanol mixture at a 0.5:9.5 v/v ratio, prepared in 2 ml of solution. The saturated mobile phase was employed to elute the *NS* fraction, which was applied in a 1 µl** volume on the GF254 silica gel plate. Following the elution, the plate was examined under UV light at 254 and 366 nm to calculate the Rf value. Subsequently, various elution products were identified by spraying with $AlCl_3$, $FeCl_3$, Liebermann-Burchard, and Dragendorff reagents and were observed under UV light at 254 and 366 nm.

Bioautography test

Subsequent to the compound separation process, the chromatogram was introduced into MHA media infected with test microorganisms. After 30 min, the sample was permitted to diffuse into the solid medium, after which the chromatogram plate was extracted from the medium. The media was subsequently incubated for 24 h at 37 °C. The Rf clear zone from the TLC elution findings is observable in the petri dish.

Data analysis

The inhibitory zone diameter values from the NS ethyl acetate fraction were subsequently analyzed using SPSS for statistical testing. The conducted study involved a data homogeneity test, followed by the application of the Kruskal-Wallis and Mann-Whitney tests to ascertain the significance of the differences in inhibition zones across various concentrations.

RESULTS AND DISCUSSION

Bacterial sensitivity test results

Bacterial sensitivity testing is analyzed based on the diameter of the largest inhibition zone of antibiotics against the test bacteria and this result serves as a positive control. The results of the sensitivity test are obtained based on the comparison of the width of the largest inhibition zone obtained with the antibiotic sensitivity standard.

Based on the sensitivity test findings in table 1, in *Kp* bacteria, 30 µg tetracycline antibiotic produced the highest inhibition zone diameter of 28.3 mm (sensitive). It was proven that 30 µg tetracycline antibiotic had the largest inhibition zone width in *Ss* bacteria, which was 23.5 mm (sensitive). The test findings showed that 15 µg erythromycin antibiotic had the largest inhibition zone width or 20.2 mm in *Sm* bacteria. This shows that 30 µg tetracycline antibiotic with the largest inhibition zone diameter in Bc bacteria, which was 25.1 mm (sensitive). Tetracycline is a broad-spectrum bacteriostatic antibiotic and can stop the growth of both Gram-positive and Gram-negative bacteria [15]. Tetracycline works in the protein synthesis process by attaching itself to the 30S ribosome subunit and inhibiting the aminoacyl-tRNA bond in the ribosome, thereby breaking the peptide bond [16]. The macrolide antibiotic group, including erythromycin, is the most widely used group to treat Gram-positive infections. By interfering with the translocation process and the formation of the initiation complex, erythromycin inhibits protein synthesis [17]. Based on these findings, the positive control for the antibacterial activity test of *Kp, Ss*, and *Bc* was 30 µg tetracycline antibiotic and for the antibacterial activity test of *Sm,* which had the largest inhibition zone was 15 µg erythromycin antibiotic.

Table 1: Results of antibiotic sensitivity tests against *Kp, Ss, Sm* and *Bc* bacteria. The largest inhibition zone was erythromycin antibiotics and chosen as positive control

Antibiotics	Mean diameter of inhibition zone (mm)±SD						
	S*	I*	R*	*Kp*	*Ss*	*Sm*	*Bc*
Tetracycline 30µg	≥19	15-18	≤14	28.3±2.93	23.5±0.29	15.7±0.58	25.1±1.77
Erythromycin 15µg	≥23	14-22	≤13	8.8±2.54	11.3±0.76	20.2±1.04	7.8±1.75
Vancomycin 30µg	≥12	10-11	≤9	9.0±0.43	6.5±0.87	8.1±0.52	6.0±0
Ampicillin 10µg	≥17	14-16	≤13	20.8±2.54	21.8±0.76	10.5±0	13.8±0.95

*S=sensitive; I=intermediate; R=resistance

Antibacterial activity test results

The disk diffusion method is used to conduct antibacterial activity tests, using paper disks to test bacterial sensitivity to certain antibacterial active chemicals [18]. This test is carried out on the basis that the surface of the medium forms an inhibition zone that prevents bacterial growth through the fraction inoculated with bacteria. The disk diffusion method was chosen because it does not require special equipment and is suitable for liquid fraction samples [19]. The *NS* ethyl acetate fraction was tested for antibacterial activity at concentrations of 20%, 40%, 60%, and 80%. The concentration of the fraction in this study was determined by testing antibacterial activity using 100% DMSO as a negative control. Because of its ability to dissolve polar and nonpolar substances, DMSO was chosen as a solvent [20]. To ensure whether the solvent affects the growth ability of the test bacteria, a negative control was used, meaning that the inhibition zone formed came from the active components contained in the ethyl acetate fraction of *NS*, not from the solvent [21]. The findings of the activity test of the ethyl acetate fraction of *NS* against the test microorganisms were repeated three times because it can reduce variability, increase data reliability, and allow valid statistical analysis to ensure accurate results. The result of antibacterial test showed that ethyl acetate fraction of *NS* inhibit the growth of *Kp, Ss and Bc*, but not to *Sm*.

Table 2: Results of antibacterial activity test of ethyl acetate fraction of *NS* showed the activity of fraction to the sample of bacteria

Bacteria	Mean diameter of inhibition zone (mm)±SD					
	Fraction concentration				Positive control	Negative control
	20%	40%	60%	80%		
*Kp**	9.17±0.39	9.75±0.25	10.33±0.29	11.08±0.38	29.33±1.26	6
*Ss**	6.67±0.29	8.33±0.29	10.08±0.80	12.50±0.50	24.83±0.76	6
*Sm**	6.00	6.00	6.00	6.00	20.17±1.04	6
*Bc**	13.58±0.14	14.58±0.38	16.00	17.67±0.58	25.08±1.77	6

*Kp=Klebsiella pneumoniae; Ss=Shigella sonnei; Sm=Streptococcus mutans; Bc=Bacillus cereus

Based on the research results in table 2, in *Kp* bacteria, the largest inhibition zone was at a concentration of 80% with an average diameter of 11.08±0.38 mm, *Ss* bacteria at a concentration of 80% with an average inhibition zone diameter of 12.50±0.50, and *Bc* bacteria at a concentration of 80% with an average diameter of 11.08±0.38 mm, while in *Sm* bacteria there was no inhibition zone. The inhibitory effect analyzed based on the clear zone surrounding the disk. Normality of inhibition zone data was assessed by the Shapiro–Wilk test ($p>0.05$ for Bc, $p<0.05$ for Kp and Ss), justifying use of non-parametric Kruskal–Wallis and Mann–Whitney tests.

The results of the statistical test are shown in the *NS* ethyl acetate fraction test data on *Kp* bacteria with a significance value of 0.01<p (0.05), which means it is not normally distributed. A significant score of 0.032<p (0.05) in the data homogeneity test indicates that the data is not homogeneous. The *NS* ethyl acetate fraction is proven to have different concentration effects on the size of the inhibition zone in the Kruskal Wallis test, with a significance value of 0.006<P (0.05) on the growth of *Kp* bacteria. Then the Mann-Whitney test was carried out after finding the results of the Kruskal-Wallis test. The test results showed that the concentration groups of 20% and 60%, 20% and 80%, and 60% and 80% had differences between concentrations obtained a significance value of<P (0.05). Meanwhile, there was no difference (p>0.05) between the concentration groups of 20% and 40%, 40% and 60%, and 40% and 80%.

The statistical test results obtained data from the ethyl acetate fraction of NS against *Ss* bacteria at a significance value of 0.01<p (0.05), which means it is not normally distributed. The results of the homogeneity test showed that the data was not homogeneous, with a significance value of 0.049<p (0.05). In the Kruskal Wallis test, the ethyl acetate fraction of *NS* was proven to have different concentration effects on the size of the inhibition zone with a significance value of 0.005<P (0.05) on the development of *Ss* bacteria. The Mann-Whitney test was then carried out after finding the results of the Kruskal-Wallis test. The test results showed that all relationships between concentration groups had a large average difference with a significance value of<P (0.05) except for the 40% and 60% concentration groups, which showed no difference (p>0.05) between the two.

The statistical test results were obtained on the ethyl acetate fraction test data of *NS* against *Bc* bacteria at a significance value of 0.159>p (0.05), which means it is normally distributed. In the homogeneity test, a significance value of 0.006<p (0.05) was obtained, indicating that the data was not homogeneous. Furthermore, the Post hoc Games-Howell was done. The test results show that all relationships between concentration groups have a large average difference with a significance value<P (0.05) except for the concentration groups of 20% and 40% and 60% and 80%, which showed no difference (p>0.05).

TLC (Thin layer chromatography) test results

TLC can be used to qualitatively analyze the ethyl acetate fraction of *NS*. TLC has a separation principle by eluting the analyte through a chromatography plate then observing the components of the compound separated by spraying. TLC analysis was carried out to identify active ingredients that have antibacterial activity in the ethyl acetate fraction of *NS*. The TLC method was chosen because of its low cost, limited material and solvent requirements, and fast analysis time [22].

Table 3: TLC test results of ethyl acetate fraction of *NS*

Spray reagent	Rf	Visualization	Color/fluorescence	Information
Dragendorff	0.43	Visible light	Orange	(+) Alkaloid
$FeCl_3$	0.41	Visible light	Fixed/unchanged	(-) Tannin
$AlCl_3$	0.50	UV366	Light blue	(-) Flavonoid
Liebermann-Burchard	0.43	UV366	Fluorescent yellow	(-) Terpenoid
			Turquoise	(-) Sterol

Based on thin layer chromatography (TLC) analysis of the ethyl acetate fraction of *NS* (fig. 1), it shows a faint yellow color in visible light, black when exposed to UV light at 254 nm, and turns light blue when exposed to UV light at 366 nm. The presence of alkaloids in the ethyl acetate fraction of *NS* is indicated by a reddish-brown hue with an Rf of 0.43 when Dragendorff's reagent is sprayed under visible light. Dragendorff's reagent is known to react with alkaloids, which often causes test results to appear red or brown. According to previous studies, nigellamine and nigellidine, two alkaloids found in *NS*, may be responsible for this color change [23].

Furthermore, when spraying $FeCl_3$ reagent, the color remains faint yellow with Rf 0.41 under visible light. This reaction indicates that the phenolic or tannin compounds in this fraction are not enough, or the concentration is not high enough to cause a significant color change. Usually, these compounds react with $FeCl_3$ to produce a blue or green color. *NS* contains phenolic compounds, including thymoquinone and carvacrol, but their interaction with $FeCl_3$ may not be sufficient in this fraction so that a significant color change occurs [24]. The presence of flavonoid compounds is indicated by a slightly dark light blue color with Rf 0.50 obtained by spraying $AlCl_3$ reagent and observing it under 366 nm UV light. $AlCl_3$ reagents are widely used in the identification of flavonoids because they show significant UV light fluorescence. This color shift indicates that flavonoids from the ethyl acetate fraction of *NS* react with $AlCl_3$. *NS* contains flavonoids, including quercetin and kaempferol, which are known to emit a lot of fluorescence when combined with $AlCl_3$ reagent [23].

Spraying with Liebermann-Burchard reagent still shows a bright light blue color with Rf 0.43 when exposed to UV light 366 nm. This indicates that the compounds in the fraction do not react with Liebermann-Burchard reagent. As a result, it is likely that the substance in the ethyl acetate fraction of *NS* that gives the plant a bright light blue color is not a sterol or triterpenoid. Although *NS* contains sterols such as β-sitosterol, these sterols do not appear to react significantly with Liebermann-Burchard in the fraction in this study [24].

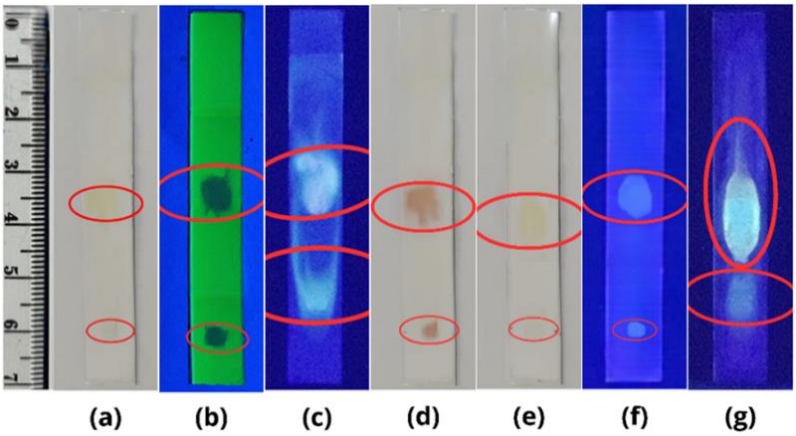

Fig. 1: TLC test results of ethyl acetate fraction of *NS* with mobile phase of methanol: chloroform (95:5) v/v and stationary phase of silica gel 254 (a) Visible light (b) UV light 254 (c) UV light 366 (d) Dragendorff in visible light (e) $FeCl_3$ in visible light (f) $AlCl_3$ in UV light 366 (g) Liebermann in UV 366

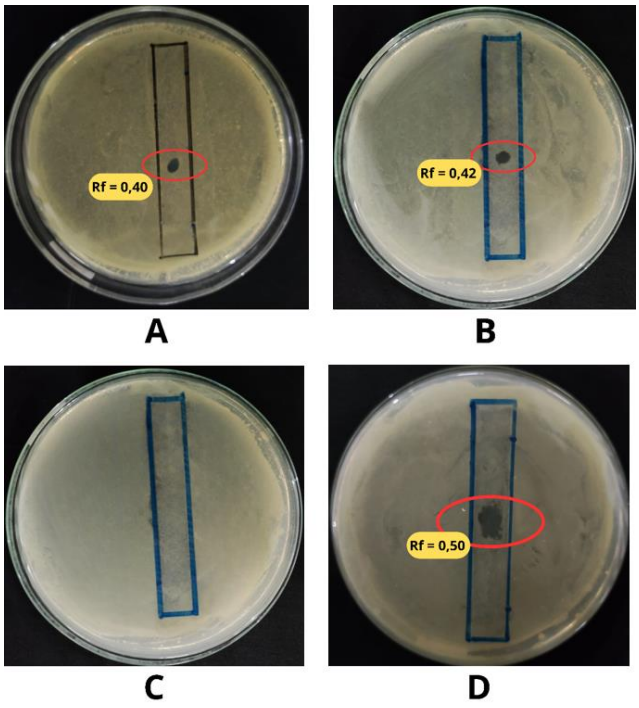

Fig. 2: Results of the bioautography test of the ethyl acetate fraction of *NS* against bacteria (A) *Kp (B) Ss (C) Sm (D) Bc.* There were an inhibition zone in *K. pneumoniae, S. sonnei and B. cereus*, which is indicate that the active compounds of ethyl acetate of *NS* has antibacterial activity *NS* as a natural antibacterial agent that can be used to treat certain bacterial diseases is strengthened by this study. *NS* may be a viable substitute for treating diseases caused by *Ss, Bc,* and *Kp* due to its efficacy against all three bacteria. However, the lack of efficacy against *Sm* suggests that further research or combination with other antibacterial agents is needed to expand the scope of antibacterial activity. This research showed that NS has antibacterial activity besides another activity [32]. Further development, such as formulation [33] and clinical trials are needed to confirm the safety and effectiveness of *NS* in medical applications

Bioautography test results

Bioautography test can be used to identify active antibacterial substances based on chromatogram results. Bioautography test is conducted to determine the content of active ingredients of *NS* ethyl acetate fraction, which has antibacterial activity against *Kp, Ss, Sm,* and *Bc*. The TLC plate is attached directly to the surface of the MHA that has been spread with bacteria to perform the bioautography procedure. The observation results show a clear zone with a cloudy background in the TLC plate placement area.

The results of the bioautography test showed inhibition zone data with an Rf value of 0.40 found in *Kp* bacteria, Rf 0.42 in *Ss*, and Rf 0.50 in *Bc* (fig. 2). These findings indicate that *NS* contains active compounds that are effective against these bacteria. While in *Sm* bacteria did not show an inhibition zone, indicating that the *NS* fraction was not effective against these bacteria. This group of substances is called the alkaloid group if the Rf from thin-layer chromatography (TLC) analysis is compared with the Rf from bioautography. Additional testing is necessary for subsequent research on the composition of the active chemicals in the NS isolate, particularly for the determination of its alkaloid content, utilizing methods such as HPLC or LC-MS. The first identification using TLC possesses limitations, such as occasionally inadequate resolution in compound separation, variability in Rf values influenced by ambient circumstances, and the necessity for a proficient operator to achieve best results.

Alkaloids work as antibacterial [25, 26] by breaking down the peptidoglycan of bacterial cells. Bacterial cell death is caused by the formation of an imperfect bacterial cell wall layer. In the other mechanism, alkaloid also has activity to inhibit the topoisomerase enzyme and acts as a DNA interchelator [27]. The alkaloids contained in *NS* are nigelicine, nigellamine, nigellidine, nigellimine, and nigellimine N-oxide [28-30]. Based on Zielinska *et al.* [28], NS seeds contain saponins such as alpha-hederin, a water-soluble pentacyclic triterpene with potential anti-cancer properties. Studies have also examined the content of flavonoids; coumarins; tannins; and (in trace amounts) other compounds, including carvone, limonene and citronellol. NS mainly contains alpha-hedrein, carvacrol, nigellimine, N-oxide, nigellicine, p-cymene, carvacrol, 4-terpineol, t-anethole, sesquiterpene, α-pinene, thymol, TQ, and alkaloids [31].

CONCLUSION

In conclusion, this study showed that the ethyl acetate fraction of *NS* has significant antibacterial activity against *Kp, Ss,* and *Bc*, with the highest potency seen at a concentration of 80%. Alkaloid compounds are thought to be the main contributors to the antibacterial activity of the ethyl acetate fraction of *NS*. These results indicate *NS* as a potential natural antibacterial agent. However, further research is needed to understand the in-depth mechanism of action, potential side effects, and its safe and effective clinical application.

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AUTHORS CONTRIBUTIONS

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution and data acquisition, analysis and interpretation, drafting, revising and reviewing the article.

CONFLICT OF INTERESTS

Declared none

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