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Antibacterial activity of red chili fruit (*Capsicum annuum* L.) extract against *Xanthomonas oryzae pv oryzae*, the causative agent of bacterial leaf blight

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A B S T R A C T

Red chili fruit (*Capsicum annuum* L.) contains bioactive compounds with antimicrobial properties. The bacterium *Xanthomonas oryzae pv oryzae* (*Xoo*) is the causal agent of bacterial leaf blight (BLB) in rice. The use of chili as an antibacterial agent could serve as an alternative to control this disease. This study aimed to determine the antibacterial activity of red chili fruit extract against *Xoo*. The red chili fruits were macerated using acetone, ethanol, and chloroform as solvents. Antibacterial activity was tested using diffusion and dilution methods. The diffusion assay was conducted using extract concentrations of 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%, 0.78%, and 0.39%. Antibacterial activity was observed for all extracts, with the highest inhibition zone found at a 25% concentration. There was no significant difference in inhibition zone based on the type of solvent ($p > 0.05$), but a significant difference was found based on concentration ($p < 0.05$). The minimum inhibitory concentrations (MICs) from the diffusion test for acetone, ethanol, and chloroform extracts were 3.12%, 12.5%, and 12.5%, respectively. The dilution method was conducted by comparing the absorbance values of the test cultures before and after incubation. The MICs obtained from the dilution test were 0.78% for acetone, undetectable for ethanol, and 12.5% for chloroform extract. The best antibacterial activity observed in this study was from the 25% acetone extract.

Keywords: Capsicum annuum; antibacterial; Xanthomonas oryzae pv oryzae

1. INTRODUCTION

Red chili (*Capsicum annuum* L.) is known as a food crop commonly used as a kitchen spice due to its pungent taste. It is also utilized in the food industry and for health products. Several studies have demonstrated the use of chili extracts to inhibit the growth of foodborne bacteria and fungi (Dorantes et al., 2000; Moreira et al., 2004; Kunasakdakul and Suwitchayanon, 2012). The fruit of *C. annuum* contains bioactive compounds with antimicrobial properties, such as flavonoids, steroidal alkaloids, triterpenoids, tannins, saponins (Sapitri et al., 2021), glycosides (Mahendrasari, 2014), and capsaicin (Tiandora et al., 2017).

Several studies have reported the antimicrobial activity of chili extracts against foodborne pathogens such as *Salmonella typhimurium, Pseudomonas aeruginosa, Listeria monocytogenes, Staphylococcus aureus, Bacillus cereus* (Dorantes, 2000; 2003; Santos et al., 2012), *Escherichia coli, Vibrio cholerae, Helicobacter pylori*, and *Listeria monocytogenes* (Omolo et al., 2014). The antimicrobial activity of *C. annuum* against plant pathogens has also been demonstrated by Kunasakdakul and Suwitchayanon (2012), who showed that C. annuum extract could inhibit the fungi *Alternaria brassicicola* (the causative agent of leaf spot) and the bacterium *Xanthomonas campestris pv. campestris* (Xcc), which causes black rot.

Xanthomonas oryzae pv. oryzae (*Xoo*) is a pathogenic bacterium that causes bacterial leaf blight (BLB) in rice. *Xoo* and Xcc are important plant pathogens that are closely related phylogenetically. Both bacteria share similar colonization strategies, such as forming aggregates in the xylem system (An et al., 2020).

Rice (*Oryza sativa*) is the primary staple food in Indonesia. According to the Food Security Agency (2020), rice consumption in Indonesia was projected to reach 91.2 kg per capita per year by 2024. This high demand for rice must be balanced with adequate supply; however, rice production often fluctuates due to various challenges such as climate change and disease. BLB is one of the major diseases affecting rice crops. Infection by BLB can reduce yields by 21–36% during the rainy season and by 18–28% in the dry season (Wahyudi, 2011). This disease starts with lesions on the leaf margins in the form of blister-like lines, which then expand and cause waviness.

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These lesions damage the stomata and chlorophyll, turning the leaves yellow and eventually pale gray. This disrupts the photosynthesis process and hampers plant growth (Patihong, 2012).

Synthetic pesticides are commonly used to control BLB, but their use poses risks to both consumer safety and the environment. Furthermore, they can lead to resistance and secondary pest outbreaks. Therefore, environmentally friendly alternatives are needed. Botanical pesticides may serve as such alternatives because they are derived from natural ingredients, decompose easily in the environment, leave negligible residues, and are effective in managing plant pests and diseases. Based on previous research, *C. annuum* fruit has potential antibacterial activity against plant pathogens. However, antibacterial activity of *C. annuum* against *Xoo* has not yet been studied, making this research necessary.

2. MATERIAL AND METHODS

2.1 Research location and materials

This research was conducted at the Biotechnology Laboratory, Department of Biology, Faculty of Science and Mathematics, Universitas Diponegoro, and the Central Microbiology Laboratory of the National Diponegoro Hospital. The test materials used in this study were red chili fruits (*Capsicum annuum*) of the brand Barkah Fresh Superindo 365 and the bacterium *Xanthomonas oryzae* pv. *oryzae*, obtained from the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (BB-Biogen), Bogor.

2.2 Extraction of red chili fruit

The extraction process was carried out using the maceration method, adapted from Khalil (2012). The red chili fruits were washed with clean water and then dried in an oven at 45°C until a constant weight was achieved. The dried samples were ground into powder using a blender. Extracts of *C. annuum* were prepared using three different solvents: acetone, ethanol, and chloroform. A total of 30 g of chili powder was placed into three glass jars and mixed with 300 mL of each solvent, then stirred.

The mixtures were stored in closed dark glass jars at room temperature for three days, away from direct sunlight. During storage, they were stirred twice a day. After three days, the solutions were filtered using filter paper, and the filtrates were evaporated with a rotary evaporator to obtain concentrated extracts. According to Bintoro et al. (2017), evaporation continued until no more solvent droplets were visible in the rotary evaporator. The concentrated extracts were stored in sample bottles. The extract yield of *C. annuum* was calculated using the following formula:

$$\text{Yield (\%)} = (\text{Weight of Extract} / \text{Weight of Sample}) \times 100 \quad (1)$$

2.3 Revival of test bacteria

Revival of *Xoo* was performed by taking one inoculating loop of *Xoo* from the stock culture and inoculating it into nutrient broth (NB) medium, followed by incubation using a rotary shaker for 24 hours. Then, 1 loop of the culture was streaked onto a petri dish containing nutrient agar (NA) and incubated at room temperature for 24 hours.

2.4 Preparation of test bacterial suspension

The bacterial suspension was prepared according to Rundengan et al. (2017) by transferring one loop of bacteria from the NA medium into a test tube containing 5 mL of 0.9% (w/v) NaCl solution and homogenizing it. The turbidity of the solution was adjusted to match the 0.5 McFarland standard.

2.5 Antibacterial diffusion assay

The antibacterial test used acetone, chloroform, and ethanol extracts at 100% concentration, diluted with 10% and 100% DMSO. Negative controls were 10% and 100% DMSO without extract, while the positive control was bismerthiazol at 0.05 g/mL. The disk diffusion method used was a modified Kirby-Bauer protocol (Hudzicki, 2016). A sterile swab was dipped into the bacterial suspension and the excess liquid was removed by pressing the swab against the wall of the tube. The swab was then evenly streaked over the surface of NA plates. Paper disks (6 mm in diameter) were each loaded with 20 µL of extract and allowed to absorb fully before being placed on the inoculated agar using forceps, then gently pressed. The plates were incubated for 24 hours at room temperature. A clear zone around the disk indicated antibacterial activity. The inhibition zone

diameter was measured in millimeters by subtracting the disk diameter (6 mm) from the total diameter. All tests were performed in triplicate. The antibacterial activity index (AAI) was calculated using the formula:

AAI = (Inhibition Zone of Extract) / (Inhibition Zone of Positive Control) (2)

Further disk diffusion tests were conducted using extract concentrations of 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%, 0.78%, and 0.39% (w/v). The minimum inhibitory concentration (MIC) was determined as the lowest concentration that produced a clear zone around the paper disk.

2.6 Antibacterial dilution assay

The dilution method was adapted from Nuraina (2015) and Situmorang et al. (2016), using NB medium in test tubes. Each test required seven tubes of equal size, labeled accordingly. Tubes 1 to 5 were for the test treatments, tube 6 served as the positive control, and tube 7 as the negative control. A 25% stock solution was made by dissolving 1.25 g of *C. annuum* extract in 5 mL DMSO. The test concentrations were chosen based on MIC results from the diffusion test and lower concentrations, prepared via two-fold serial dilutions (w/v) as shown in Figure 1.

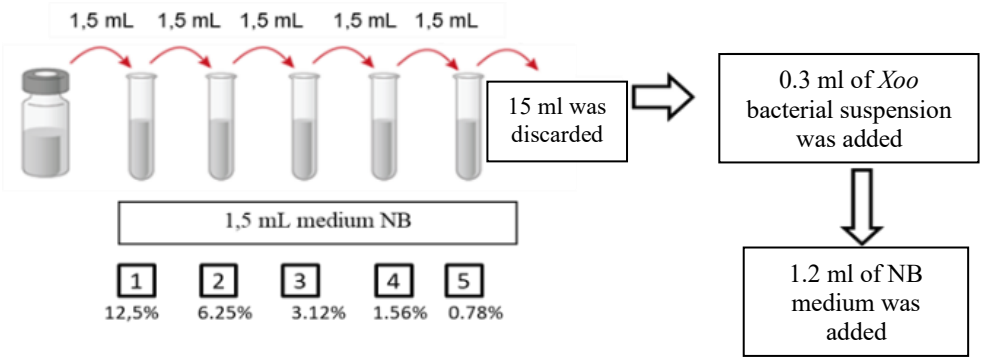


Figure 1. Serial dilution with concentrations ranging from 12.5% to 0.78%

The positive control was made by mixing 1.5 mL of 0.05 g/mL bismerthiazol solution with 1.5 mL NB medium, homogenized, and 1.5 mL discarded. Then, 0.3 mL of bacterial suspension and 1.2 mL NB were added. The negative control was made by mixing 1.5 mL NB with 1.5 mL DMSO, homogenized, and 1.5 mL discarded, followed by the addition of 0.3 mL bacterial suspension and 1.2 mL NB (Wardani et al., 2012; Nuraina, 2015).

All test solutions were measured for absorbance using a spectrophotometer at 500 nm wavelength before and after 24-hour incubation at room temperature. If the final absorbance was higher than the initial value, bacterial growth was not inhibited. If the final absorbance remained the same or decreased, it indicated bacterial growth inhibition by the extract (Situmorang et al., 2016). The lowest extract concentration that showed a reduction or no change in absorbance was defined as the Minimum Inhibitory Concentration (MIC).

2.7 Statistical analysis

The inhibition zone diameter data of red chili fruit extracts against *Xanthomonas oryzae* pv. *oryzae* were statistically analyzed using SPSS version 26. Normally distributed data were analyzed with ANOVA, while non-normally distributed data were analyzed with the Kruskal-Wallis test.

3. Results and Discussion

3.1 Red chili fruit extract

The three extracts displayed different shades of red (Table 1 and Figure 2), due to variations in solvent polarity. Chloroform is capable of extracting pigments from red chili such as capsanthin (Arimboor et al., 2015), capsorubin, β-carotene, zeaxanthin (Popova, 2017), and lutein (Craft and Soares, 1992; Ashenafi et al., 2023). Capsanthin and capsorubin are the main pigments responsible for the red color in chili (Arimboor et al., 2015).

Acetone can dissolve pigments like capsanthin, capsorubin, capsolutein, violaxanthin, zeaxanthin, β-carotene, β-cryptoxanthin (Topuz and Ozdemir, 2007; Hasanah and Fatmawati, 2022), and lycopene (Amaya,

2005; Puspita et al., 2018). Capsanthin and capsorubin give red coloration, while zeaxanthin, β-carotene, and β-cryptoxanthin provide yellow-orange hues (Campos et al., 2013). These pigments give the acetone extract an orange-red appearance.

The ethanol extract in this study had a dark red color, likely due to high carotenoid content. Generally, darker colors indicate higher carotenoid levels (Britton et al., 2009; Schieber and Weber, 2016). Carotenoids that are soluble in ethanol include capsanthin (Dang et al., 2014), β-carotene (Popova, 2017), lutein, zeaxanthin, and violaxanthin (Jaeschke et al., 2016). Oxidation during storage could also darken the chili extract. According to Pinem (2010) in Wahyuni et al. (2020), carotenoids are easily oxidized due to their many conjugated double bonds. Dried chili may darken during storage (Kwanhathai et al., 2012).

Table 1. Extraction results of *Capsicum annuum* using acetone, ethanol, and chloroform solvents

Solvent	Sample Weight (g)	Extract Weight (g)	Solvent Volume (mL)	Yield (%)	Texture
Acetone	30	4.03	300	13.43	Liquid paste
Ethanol	30	4.59	300	15.30	Paste
Chloroform	30	4.37	300	14.56	Liquid paste

The highest yield was obtained from the ethanol extract, suggesting a greater abundance of polar compounds in red chili. According to Irsyad (2013), the yield reflects the amount of chemical compounds in the extract. These yield values are consistent with Soldan et al. (2021), which reported a 15.5% yield for ethanol extract and 9.2% for acetone extract. All extracts had yields above 10%, meeting the Ministry of Health's quality standard for good extracts (Depkes, 2000, in Luginda et al., 2018).

(1) (2) (3)

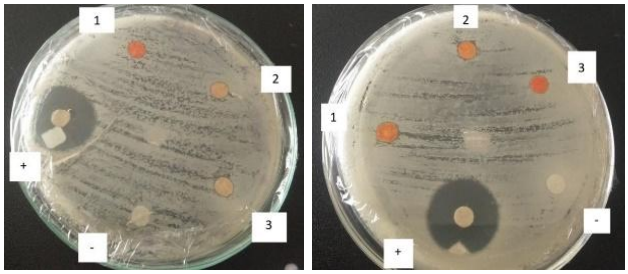
Figure 2. Red Chili Extracts
(1) Acetone extract, (2) Ethanol extract, (3) Chloroform extract

3.2 Antibacterial diffusion assay

The preliminary antibacterial assay was conducted using acetone, chloroform, and ethanol extracts at 100% concentration, diluted with 10% and 100% DMSO, against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Due to the oily and poorly water-soluble nature of chili extract, specific solvents were needed to prepare the concentrations. DMSO is a solvent with wide solubility capacity, capable of dissolving both organic and inorganic components, with low toxicity, remaining liquid over a wide temperature range (19°C–189°C), and is miscible with water and various organic solvents (Mi et al., 2016). Chili extracts were more soluble in 100% DMSO than in 10%, but according to Mahmud (2018), high-concentration DMSO may exhibit antibacterial properties; thus, both DMSO concentrations were used to assess dilution effects.

Negative controls used were 10% and 100% DMSO. According to Niswah (2014), the solvent used to dilute the extract should serve as the negative control to ensure it does not affect the test results. No inhibition zones were observed in the negative controls of this study, indicating that DMSO, at either concentration, had no antibacterial activity, and any observed inhibition was solely due to the extract.

The positive control used was bismerthiazol at 0.5 g/10 mL, a bactericide for controlling BLB caused by *Xoo* (Zhu et al., 2013). The positive control served as a comparison for the inhibition zone diameter produced by the chili fruit extracts.



(A) (B)

Figure 3. Diffusion test results of red chili fruit extracts against *Xoo*
A: Extracts diluted in 10% DMSO; B: Extracts diluted in 100% DMSO
1: Chloroform extract, 2: Ethanol extract, 3: Acetone extract; (+): Positive Control, (–): Negative Control

The diffusion test of 100% chili fruit extract (Figure 3, Table 2 and 3) demonstrated antibacterial activity against *Xoo*, indicated by clear zones around the paper disks. The acetone extract, whether diluted in 10% or 100% DMSO, produced inhibition zones in all replicates. The interaction between DMSO concentration and inhibition zone diameter showed a significance value greater than 0.05 (0.728 > 0.05), indicating no significant difference due to DMSO concentration. Thus, both 10% and 100% DMSO-diluted extracts had similar antibacterial activity.

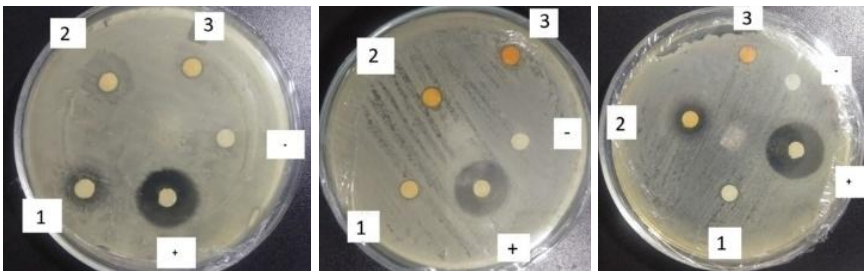
Table 2. Diffusion test results of 100% red chili fruit extract diluted with 10% DMSO after 24h incubation against *Xoo*

Solvent	Avg. Inhibition Zone (mm)	Antibacterial Activity Index
Acetone	0.43	0.030
Ethanol	0.40	0.029
Chloroform	0.27	0.018
Positive Control	14.13	–
Negative Control	0	–

Table 3. Diffusion test results of 100% red chili fruit extract diluted with 100% DMSO after 24h incubation against *Xoo*

Solvent	Avg. Inhibition Zone (mm)	Antibacterial Activity Index
Acetone	0.47	0.032
Ethanol	0.37	0.025
Chloroform	0.37	0.024
Positive Control	15.03	–
Negative Control	0.00	–

Subsequent antibacterial tests were conducted using acetone, ethanol, and chloroform extracts diluted with 100% DMSO at concentrations of 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%, 0.78%, and 0.39%. Results are shown in Figure 4.



(1) (2) (3)

Figure 4. Diffusion assay results of red chili fruit extract
Solvents: A: Acetone, B: Ethanol, C: Chloroform
Concentrations: 1 = 12.5%, 2 = 25%, 3 = 50%; (+): Positive control, (–): Negative control

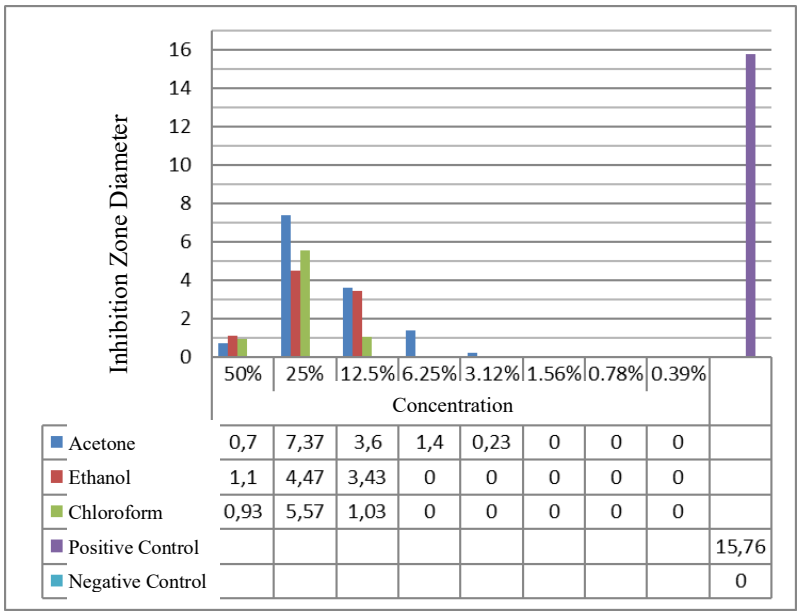


Figure 5. Average inhibition zone diameters of red chili extract (50–0.39%) against *Xoo*

Table 4. Antibacterial activity index of red chili extract at various concentrations

Solvent	50%	25%	12.5%	6.25%	3.12%
Acetone	0.050	0.520	0.260	0.078	0.015
Ethanol	0.076	0.275	0.210	0.000	0.000
Chloroform	0.058	0.349	0.068	0.000	0.000

The highest antibacterial activity was observed with 25% acetone extract, showing a 7.37 mm inhibition zone (Figure 5). This aligns with findings by Demirkol and Erturk (2019), who reported inhibition zones of 8.33 mm, 8.33 mm, and 5.6 mm for *Proteus vulgaris*, *Escherichia coli*, and *Salmonella typhimurium* using acetone extract. All antibacterial activity index values were below 1, indicating they were not stronger than the standard antibacterial agent, as per Abass et al. (2021). Kruskal-Wallis analysis showed no significant difference ($p > 0.05$) in inhibition zone diameters between different solvents ($p = 0.367$), indicating similar antibacterial effects among the extracts.

Overall, the 25% concentration yielded the highest inhibition zones across all solvents. A significant difference ($p < 0.05$) was found between concentration and inhibition zone diameters. Interestingly, the 25% concentration produced larger zones than 50% and even 100% in earlier tests, contrary to Pelczar and Chan's principle (1998 in Trisia et al., 2018) that higher antimicrobial concentrations typically yield stronger inhibition. The reduced diffusion of higher concentrations may be due to their viscosity, limiting agar penetration (Dewi et al., 2018; Mokhtar et al., 2017).

At concentrations of 12.5% to 0.39%, lower inhibition zones were observed, aligning with the theory that higher concentrations generally exhibit stronger inhibition (Pelczar and Chan, 1998). The 100% and 50% extracts were likely too viscous to diffuse effectively. Post-hoc Kruskal-Wallis tests showed that the 25% concentration did not significantly differ from 50% and 12.5%, but did differ significantly from 6.25% and lower.

The acetone extract had the lowest MIC at 3.12%, indicating the strongest inhibition, while ethanol and chloroform extracts had MICs of 12.5%. MIC was further confirmed via liquid dilution assays.

3.3 Antibacterial dilution assay

The concentrations used in the dilution test were selected based on the MIC results from the diffusion assay: 12.5%, 6.25%, 3.12%, 1.56%, and 0.78%. According to the spectrophotometric measurements (Table 5), the MICs for acetone and chloroform extracts were 0.78% and 12.5%, respectively, while ethanol extract showed no detectable inhibitory effect.

Table 5. Results of red chili fruit extract dilution test against *Xanthomonas oryzae* pv. *oryzae* measured with a spectrophotometer

Extract Solvent	Extract Concentration (%)	Absorbance (Before)	Absorbance (After)	Growth Inhibition
Acetone	12.50	0.474	0.298	Yes
	6.25	0.469	0.064	Yes
	3.12	0.180	0.040	Yes
	1.56	0.313	0.033	Yes
	0.78	0.215	0.068	Yes
Ethanol	12.50	0.112	0.182	No
	6.25	0.133	0.257	No
	3.12	0.022	0.056	No
	1.56	0.046	0.110	No
	0.78	0.128	0.623	No
Chloroform	12.50	0.527	0.086	Yes
	6.25	0.042	0.087	No
	3.12	0.021	0.036	No
	1.56	0.039	0.242	No
	0.78	0.040	0.070	No
Positive Control	–	1.31	0.74	Yes
Negative Control	–	0.03	0.63	No

The dilution test results for acetone and ethanol extracts differed from those of the diffusion test. The acetone extract showed a lower MIC in the dilution assay than in the diffusion assay. While ethanol extract exhibited antibacterial activity at 12.5% in the diffusion test, it had no inhibitory effect in the dilution assay. This discrepancy may be attributed to the solubility characteristics of the antibacterial compounds. As noted by Santas et al. (2009) in Mokhtar et al. (2017), diffusion assays are highly dependent on compound solubility and diffusion in agar media. Antibacterial compounds in acetone extract are likely semi-polar, which hampers their diffusion through agar but enhances activity in liquid media.

The growth medium used in the dilution test was less compatible with chloroform and ethanol extracts dissolved in DMSO, as it was prepared in water, a non-polar solvent. Additionally, the cultures were incubated in a shaker incubator.

The increased absorbance values for ethanol extract at 12.5% concentration might also be due to the solution's density, which can affect light absorption measurements. According to Warokka et al. (2016), increased absorbance does not always indicate bacterial growth but may also be due to high solution concentration interfering with light transmission. UV-Vis spectrophotometers also cannot differentiate between light absorbed by live and dead bacteria.

These dilution assay results are consistent with Wystaputra (2020), who found a MIC of 25% for red chili ethanol extract against *Klebsiella pneumoniae*, a gram-negative bacterium. Compared to Tiandora et al. (2017), who reported a MIC of 0.11% for red chili extract against *Streptococcus viridians* (a gram-positive bacterium), the MICs found here are higher. Gram-negative bacteria, such as *Xoo*, tend to be more resistant than gram-positive bacteria due to the presence of an outer membrane that limits the penetration of antimicrobial agents (Mokhtar et al., 2017). According to Wang et al. (2021), resistance in gram-negative bacteria may arise through mechanisms such as enzymatic antibiotic degradation, alteration of antimicrobial targets, efflux pumps, and changes in membrane permeability.

Based on the results of both diffusion and dilution assays, the acetone extract exhibited the most effective antibacterial activity compared to ethanol and chloroform extracts. Although statistical analysis showed no significant differences among the extract types, the acetone extract consistently produced the largest inhibition

zone and the lowest MIC values in both methods. This suggests that the active antibacterial components in red chili fruit are more readily soluble in acetone.

Compounds in red chili fruit that can be dissolved by acetone include phenols, flavonoids (Soldan et al., 2021), capsaicin (Ichim and Blaga, 2021), quinine, steroids, terpenes (Kouassi et al., 2010), tannins (Artati and Fadilah, 2007; Sibuea, 2015), saponins, and phlobatannins (Ali et al., 2018). Compounds that dissolve in ethanol include capsaicin (Chin et al., 2011), tannins (Pandey and Tripathi, 2014), steroids/triterpenoids (Sapitri et al., 2021), and polar flavonoids such as glycosides (Suryani et al., 2015). Compounds soluble in chloroform include capsaicin (Handoko et al., 2017), terpenoids (Abubakar et al., 2020), saponins, and non-polar flavonoids like flavonols, isoflavones, flavanones, and flavanols (Alsuhendra et al., 2007; Hendryani et al., 2015).

Soldan et al. (2021) studied the total phenolic and flavonoid content of *C. annuum* extracts using ethanol, acetone, and hexane, and found that acetone extract yielded the highest values in both tests. Chili flavonoids include aglycones and glycosides, which can dissolve in polar solvents like acetone. According to Hendra (2011) in Rijayanti (2014), flavonoids inhibit membrane function, metabolism, and nucleic acid synthesis.

Chin et al. (2011) compared capsaicin extraction using ethanol, acetone, and acetonitrile, and found acetone to yield the highest capsaicin concentration. Amaliah (2018) also noted that capsaicin can dissolve in polar solvents like alcohol due to its polar nature. Capsaicin exerts its antibacterial effect by inducing osmotic stress and disrupting the bacterial cell membrane (Kurita, 2002; Adaszek et al., 2019).

Ali et al. (2018) demonstrated that acetone can also dissolve saponins. According to Rijayanti, saponins act by lysing bacterial cell walls and disrupting membrane permeability. Saponins can be both polar and non-polar, allowing them to dissolve in acetone (Vilcheze et al., 2013; Ayuningtyas et al., 2021).

4. CONCLUSION

Extracts of *Capsicum annuum* fruit, whether prepared with acetone, ethanol, or chloroform, exhibited antibacterial activity against *Xanthomonas oryzae* pv. *oryzae*. The 25% concentration produced the largest inhibition zones, while concentrations below 25% (12.5–0.39%) showed reduced inhibitory effects. Based on the minimum inhibitory concentration (MIC) values, the most effective antibacterial activity was found in the acetone extract, with MICs of 3.12% in the diffusion test and 0.78% in the dilution test. Further research on the antibacterial activity of *Capsicum annuum* fruit using different methods or solvents is recommended, as well as studies on its antimicrobial activity against other microorganisms.

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