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THE ANTIBACTERIAL ACTIVITY OF A COMBINATION OF YOGURT AND SAFFRON EXTRACT AGAINST *Staphylococcus aureus*

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ABSTRACT

Background: Yogurt is a fermented milk product that has a positive influence on health because of its bioactive proteins, vitamins, minerals, improved bioavailability, and hydrolyzed carbohydrates. Yogurt contains lactic acid bacteria that can inhibit the growth of *Staphylococcus aureus*. Objectives: This study aimed to determine the addition of Saffron Extract to yogurt against Staphylococcus aureus and to test its antibacterial activity against *Staphylococcus aureus*. Material and Methods: The study was an experimental study with various concentrations of Saffron extract (25%; 50%; 75%; and 100%) that were added to the cow milk-based yogurt. The mixed saffron extract (MSE) was centrifuged to obtain the supernatant. The antibacterial activity was evaluated using the disc diffusion method. based on the strength of the zone of inhibition. The negative control was plain yogurt and the positive control was chloramphenicol. Results: Phytochemical screening was used to observe flavonoids, steroids, phenols, saponins, and tannins in the saffron extract. MSE showed antibacterial activity against S. aureus with inhibition zones of 5.19 ± 0.39 mm, 5,77± 0.78 mm, 7,98± 1.43 mm, 9,01± 1.48 mm for, concentration of 25%, 50%, 75%, and 100%, respectively. The positive control inhibition zone was 46.61 mm and the negative control was 4.98± 0.39 mm. Conclusions: Saffron extract added to yogurt at concentrations of 75% and 100% inhibited the growth of S. aureus.

Keywords: Saffron extract, S. aureus, Yogurt

INTRODUCTION

Pneumonia is an acute infectious disease of the respiratory tract that attacks the lung parenchyma (Lathifah et al., 2021). The prevalence of pneumonia in Indonesia increased from 2.1% in 2007 to 2.7% in 2013 (Andayani 2021). It is estimated that by 2050, there will be a spike in the death rate of up to 10 million people due to pneumonia caused by bacteria that cannot be treated. In 2012, at the Jember Lung Hospital, pneumonia was the top 10 disease that frequently occurred in outpatients, while in inpatients, there were 2,343 cases. This figure has continued to increase every year until it reached 3,856 cases in 2014 (Lathifah et al., 2021). Pneumonia is caused by bacteria, viruses, fungi, or parasites, with the highest prevalence being caused by bacteria (Hashemi & Jafarpour, 2020; Zara et al., 2021a). The most common bacteria that cause pneumonia are *Streptococcus pneumonia*, *Haemophilus influenzae*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Legionella sp.*, *Pseudomonas aeruginosa*, and *Acinobacter sp.* (Shoar & Musher, 2020). In patients with pneumonia, this pathogen is easily transmitted through direct contact with sputum and through droplets that come from coughing or sneezing. *Staphylococcus aureus* (*S. aureus*) is one the Gram-positive bacteria that often causes pneumonia. In research by (Tang et al., 2023). *S. aureus* was ranked first as the cause of pneumonia, namely 51 of 119 samples were detected as Gram-positive. Of these 51 strains, they have high resistance to penicillin, erythromycin, tetracycline, and clindamycin (Lathifah et al., 2021). This indicated that antibiotic resistance occurred in *S. aureus.*

Lactic acid bacteria are Gram-positive, rod-shaped bacteria that do not form spores, produce lactic acid, and produce antimicrobial substances, including organic acids, bacteriocins, hydrogen peroxide, carbon dioxide, and alcohol (Hashemi & Jafarpour, 2020; Zara et al., 2021a). Lactic acid bacteria are generally used in the food sector, particularly in fermented foods, which are used as a substitute for chemical preservatives (preservatives) in food ingredients to extend the shelf life of products (Fitriany et al., 2023; Zara et al., 2021b). Lactic acid bacteria can also be used in the health sector as a probiotic drink. Lactic acid bacteria isolated from various vegetable sources and milk-based materials have antagonistic activities against the pathogenic bacteria *Staphylococcus aureus, Bacillus cereus, and Escherichia coli* (Fazeli-nasab, 2019; Jafari-Sales & Pashazadeh, 2020). Several studies have shown that the addition of plant extracts to yogurt can increase its ability to inhibit the growth of pathogenic bacteria. The addition of saffron extract to yoghurt can inhibit the growth of *Pseudomonas aeruginosa* bacteria (Widhyasih et al., 2022). Saffron is the most expensive spice, called "Red Gold". Based on ethnomedicine, saffron can be used to inhibit mucous membrane inflammation, depression, cough, lactation, and constipation. Saffron has a distinctive bitter taste because of the presence of the monoterpene glycoside, picrocrocin. The antioxidant content of saffron includes phenolic and carotenoid compounds. Saffron also has antibacterial properties owing to the presence of flavonoids, tannins, and saponins. Flavonoids have anti-inflammatory, anticancer, and antioxidant effects because they can inhibit the formation of free radicals (Horozić et al., 2019). In addition to being useful as a herbal medicine, saffron can also be used in fermented products such as yogurt. Yogurt is a dairy product that utilizes lactic acid bacteria. The lactic acid bacteria used as starters in yogurt production are *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (Astarina et al., 2013; Rasyid et al., 2021). Probiotics are live microorganisms that provide health benefits for those who consume them when consumed in sufficient quantities. Prebiotics are carbohydrates or food sources for microbes in yogurt. Natural prebiotics are found in grains, vegetables, fruits, and cooking spices such as saffron. The addition of saffron is expected to improve the quality of yogurt and provide a natural taste and color. Based on literature studies, this research will develop yogurt, which will be added to saffron extract, and will phytochemically study the chemical content of saffron and its antibacterial activity against *S. aureus*, which is one of the causes of pneumonia.

RESEARCH METHODS

Materials

The materials used in this study were saffron (Crocus sativus), UHP Water (Merck), and the bacterial strain *Staphylococcus aureus* ATCC 6538. Pure bacterial cultures were grown on Mueller Hinton Agar (MHA) (Merck) and then stored through regular subcultures on the same medium at 4°C before further research was carried out. Chloramphenicol antibiotics (Hexpharm Jaya), NaCl solution 0,9% (Otsuka), NaOH (Merck), Mg powder (Bratachem), FeCl3, Meyer's reagent, anhydrous acetic acid (Merck), chloroform (Merck), yogurt starter, and pure cow milk.

Methods

Preparation of Saffron Extract

Saffron extract was prepared by selecting saffron. The saffron used in this study was dry saffron and had a reddish-orange color. 2,5 grams of saffron was weighed and then heated in water at a temperature of 70^{o}C as much as 100 mL of UHP water. After adding UHP, an infusion was administered. Heating was performed for 30 minutes at a temperature of 70°C, the solution was stirred continuously during preparation. The final infusion was then filtered using Whatman No. 2 filter paper (6 mm). The infusion was continued until the temperature approached the room temperature.

Phytochemical Screening

Phytochemical screening was used to detect the presence of saponins, flavonoids, tannins, phenols, alkaloids, and steroids in saffron extracts. This was a qualitative test using a color reagent.

Detection of flavonoid

The flavonoid test was carried out by placing the saffron extract in 2 different test tubes. Each test tube was filled with 5 mL of the extract. In the first test tube, 5 mL of concentrated HCl and Mg powder was added. The second test tube was added with concentrated H_2SO_4 solution. In the third reaction tube, 5 mL of the extract was added to 5 mL NaOH (Shaikh & Patil, 2020). Based on the results of the analysis, test tubes 1 and 2 produced a red color, which indicated positive flavonoids, whereas the third test tube produced a yellow solution. It can be concluded that all three tests showed positive for flavonoids (Shaikh & Patil, 2020).

Detection of tannin

The tannin test was carried out by adding 5 mL of saffron extract into a test tube, followed by the addition of $FeCl_3$ reagent, producing a blue-black precipitate that indicated positive tannin (Shaikh & Patil, 2020).

Detection of saponin

The saponin test was performed by placing 5 mL of extract in a test tube, followed by vigorous shaking for 30 seconds. The results showed that a foam with a height of 1 cm was formed which was stable for 30 seconds (Shaikh & Patil, 2020).

Detection of alkaloid

The alkaloid test is carried out by inserting 5 mL of the extract into a test tube, and then adding Mayer's reagent, the color change and sediment indicate that the sample is positive for containing alkaloids (Shaikh & Patil, 2020).

Detection of phenol

The phenol test was carried out by placing 5 mL of the extract into a test tube and then adding 10 drops of hot UHP water and 3 drops of $FeCl_3$ reagent. A color change to dark blue indicated positive phenol (Shaikh & Patil, 2020).

Detection of steroid

The steroid test was carried out by adding 5 mL of extract into a test tube, 2 mL of chloroform, 10 drops of anhydrous acetic acid, and 2 drops of concentrated H_2SO_4, and the color changed to bluish-red, indicating that it was positive for steroids (Shaikh & Patil, 2020).

Preparation of Yogurt combined with saffron extract

Pure cow milk (250 mL) was heated to 70 °C for 30 minutes to initiate the preparation process. This stage kills germs, bacteria, and other microorganisms in milk to maximize the growth of lactic acid bacteria. The next step was to wait for the temperature of the heated milk to reach 45 °C. After the temperature reached 45 °C, a combination of saffron extract and yogurt was added to the milk (the formulation was attached).

Table I. Formulation of Yogurt Combined With Saffron Extract

MSE Concentration (v/v)	25%	50%	75%	100%
Pure cow's milk (mL)	100,0	100,0	100,0	100,0
Starter yogurt (mg)	100,0	100,0	100,0	100,0
Saffron extract (mL)	12,5	25	37,5	50,0
Aquadest (mL)	37,5	25,0	12,5	0,0

Antibacterial activity

Making Muller Hinton Agar (MHA) media begins by weighing 19 grams of MHA and dissolving it in an Erlenmeyer flask with distilled water until it reaches a volume of 500 mL, and then heating until homogeneous. The media was sterilized using an autoclave at 121oC for 15 minutes. Approximately 25 mL of the medium was poured into a petri dish and left until it solidified. A suspension of *S. aureus* test colonies was prepared by transferring one cycle of colonies from solid NA media to a test tube containing 5 mL of physiological NaCl. Turbidity in the test colony suspension was standardized to the 0.5 McFarland standard (approximately 1.5 x 10^8 CFU/mL). The suspension was used as an inoculum for 15 minutes. The test bacterial suspension was inoculated into 0.1 mL MHA media, leveled with a plate spreader, and allowed to sit until dry. Wells were made using a perforator, and 40 µL of yogurt starter was placed into the well that has been made, which was then incubated for 24 hours at 37^{o}C. The clear zone around the well was observed. The test bacterial suspension was inoculated into 0.1 mL MHA medium, leveled with a hockey stick, and allowed to sit until dry. Paper discs soaked in the yogurt starter at each concentration for 15 minutes were then aseptically placed on the surface of the media. A clear zone around the disc paper was observed.

RESULTS AND DISCUSSION

Preparation of Saffron Extract

The saffron extract was prepared by soaking saffron in ultra-high pressure (UHP) water at a temperature of 70 °C for 30 minutes. The use of distilled water as a solvent also has considerations for filtering polar phytochemical compounds, such as phenols and flavonoids, because these compounds are known to have antioxidant and antibacterial activities. Occasionally, the solution was stirred so that the results obtained were the maximum. The solution was then filtered using Whatman filter paper until a clear extract was obtained. The extract was then placed in a measuring flask, which was then wrapped in aluminum foil to prevent oxidation due to light and temperature factors (Ikalinus et al., 2015; Krisridwany et al., 2022).

Figure 1. **Saffron Extract**

Phytochemical Screening

Phytochemical screening aims to determine the presence of secondary metabolite compounds in saffron extract. This research confirms the presence of secondary metabolites in the form of flavonoids, saponins, tannins, alkaloids, and steroids in the saffron extract. Based on the results of phytochemical screening tests, saffron extract contains flavonoids, saponins, alkaloids, phenols, tannins, and steroids (Januarti et al., 2019).

Flavonoids are polyphenolic compounds that have various effects, including antioxidant, anti-tumor, anti-inflammatory, antibacterial, and antiviral effects. Flavonoids act as antibacterial agents by denaturing bacterial cell proteins and irreparably damaging the cell membranes. Based on the results of the phytochemical screening test, the saffron extract contains flavonoid compounds that are high in antioxidants (Nomer et al., 2019; Parubak, 2013). Several studies have shown that flavonoids have antibacterial properties. In this study, it was proven that the flavonoid compounds contained in saffron can damage the defense mechanisms of *S. aureus*. Based on previous studies, saponins, such as flavonoids, also have antibacterial properties. Saponin reduces permeability of cell membranes and cell walls. With the ability to penetrate cell membranes, saponins can damage cell walls and penetrate defenses, damaging the cytoplasm will be damaged and causing bacterial death. Phenols and tannins also have antibacterial properties by damaging bacterial walls and accelerating the protein denaturation process which leads to a decrease in the ability of cell membranes to withstand attacks. Based on the phytochemical screening test, the secondary metabolite content in saffron has antibacterial properties; therefore, in this study, an antibacterial test was also performed against S. aureus (Mudaningrat et al., 2023; Rahmawati et al., 2014).

Yogurt combined with saffron extract

Yogurt is the result of fermented cow's milk. Yogurt contains lactic acid bacteria that convert lactose into milk, thereby increasing the acidity of the milk. Fermentation is a microbial metabolic process that produces products with high sales value, such as organic acids, single-cell proteins, antibiotics, and biopolymers. Fermentation in yogurt is the result of bacteria that produce lactic acid, which is beneficial for intestinal health, for example, the bacteria *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. Lactic acid bacteria are beneficial bacteria in the digestive system. The mechanism of action of these bacteria involves the inhibition of the growth of pathogenic bacteria in the digestive system. Yogurt is a bacterial fermentation product that combines the effects of *Lactobacillus delbrueckii*, *the subspecies bulgaricus*, *Streptococcus salivarius*, and the subspecies thermophilus. These lactic acid bacteria are referred to as "yogurt cultures". (Mastuti, 2022; Yerlikaya, 2023). *L. bulgaricus* produces a distinctive taste, acetaldehyde, whereas *S. thermophilus* produces fresh acid (Dan et al., 2023). Yogurt is rich in calcium and contains other micronutrients such as potassium, zinc, phosphorus, magnesium, vitamin A, riboflavin, vitamin B5, vitamin B12, and vitamin D, as well as other nutrients. Low-fat yogurt contains approximately 25% more potassium, calcium, and magnesium per 8-ounce serving than the equivalent serving of low-fat milk. The nutritional profile of yogurt is unique because it is a combination of real milk nutrients and a fermentation process. Yogurt combined with saffron extract has a thick texture, and yellowish-white color (the greater the concentration of saffron extract added, the yellower the resulting color), the resulting pH is 4.5001, and the resulting aroma is like yogurt. Based on the results of organoleptic tests and pH tests, the developed yogurt met the FDA requirements.

Figure 2. **Yogurt Combined Saffron Extract**

Antibacterial activity

Lactic Acid Bacteria (LAB) are bacteria that make a major contribution to the world of food. The widespread use of LAB is believed to be able to suppress the growth of pathogenic bacteria and improve taste, aroma, and color so that it can be used to maintain digestive tract health and can be isolated from various natural ingredients such as fruits, vegetables, fermented products, and the digestive tract of both humans and animals (Fachrial et al., 2024). In this study, the antibacterial activity of the developed yogurt was tested using Staphylococcus aureus. Staphylococcus aureus is a Gram-positive, round pathogenic bacterium with a diameter of 0.5-1μm and in clusters. These bacteria cannot move or are nonmotile. Biochemical tests on this bacterium yielded positive results for catalase and the Voges-Proskauer test and could ferment glucose and mannitol. The pathogenic characteristics of *S. aureus* are that it produces positive coagulase and thermostable nuclease. Infection with these bacteria can cause mild skin infections and food poisoning (Dewi et al., 2024).

Table II. **Diameter Zone of Inhibition (mm)**

Sample (% v/v)	Inhibition zone (mm)
Positive cotrol (chloramphenicol 30 mg/ mL)	46.61
Negative control (yogurt without saffron extract)	4.98
Yogurt + saffron extract 100%	9.01 ± 1.48
Yogurt + saffron extract 75%	7.98 ± 1.43
Yogurt + saffron extract 50%	5.77± 0.78
Yogurt + saffron extract 25%	5.19 ± 0.39

Yogurt added with saffron extract showed a greater zone of inhibition than yogurt without extract. This is because saffron contains secondary metabolites such as flavonoids, tannins, saponins, phenols, alkaloids, and steroids, which have high antibacterial activity. These secondary metabolites inhibited the growth of S. aureus bacteria so that, resulting in a higher zone of inhibition.

CONCLUSION

The addition of saffron extract to yogurt can inhibit the growth of *Staphylococcus aureus*.

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