Antibacterial activity of strawberry fruit extract against *Streptococcus sanguinis* (ATCC 10556)

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ABSTRACT

Introduction: *Streptococcus sanguinis* is a facultative anaerobic Gram-positive bacteria known as a pioneer that plays a role in creating the biofilm in the oral cavity. Strawberry fruit (*Fragaria x ananassa*) is an edible fruit widely used for the study as their active compound synergy to improve health. This study aims to analyse the antibacterial activity of strawberry fruit against *S. sanguinis* (ATCC 10556). **Methods:** An explorative study was conducted with high concentration methanol extract from strawberry fruit. Antibacterial activity was tested on the methanol extract; afterwards, the extract was fractionated and divided into three fractions: water, ethyl acetate, and hexane. Zone of inhibition was used to assess the most effective fraction among those three, then continued by testing for Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). **Results:** Inhibitory zone of the ethyl acetate fraction from strawberry fruit 1%, 2%, 3% ,4% and 5% sequentially were 7.3 mm,10.2 mm, 12.3 mm, 16.3 mm and 16.1 mm. Ethyl acetate fraction of 4% was the most effective to create the zone of inhibition with a size of 16.3 mm compared to the others. The Minimum Inhibitory Concentration (MIC) value was 0.25%. It was obtained by diluting a 4% ethyl acetate fraction on a microplate. The Minimum Bactericidal Concentration (MBC) was 2%. **Conclusion:** Ethyl acetate fraction was an effective fraction from strawberry fruit and had antibacterial activity against *S. sanguinis* with the inhibitory zone in the concentration of 4%, MIC of 0.25%, and MBC of 2%.

Keywords: antibacterial; strawberry fruit; *Streptococcus sanguinis*

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INTRODUCTION

Streptococcus sanguinis, previously known as *Streptococcus sanguis*, is a commensal bacteria that is widely distributed in the oral cavity. This bacteria is abundant on the surface of teeth, oral mucosal surfaces and saliva.[5,6,7] As a facultative anaerobic species, *S. sanguinis* is also abundant in

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supragingival and subgingival plaque.[8] At different tooth locations, *S. sanguinis* biomass may differ significantly despite the similarity in plaque mass. It is present in a high proportion in the lower incisors/canines but a low proportion in the upper molars. *S. sanguinis* tend to be involved in interspecies interactions with *S. mutans*, known as competition/coexistence in dental biofilm. Biofilm is important in developing caries and periodontal diseases.[1,2,3]

Biofilm can be controlled by mechanical cleaning such as brushing, dental flossing or rinsing using chemical product such as mouthwash. In general, mouthwashes widely used to prevent and treat early caries contains hydrogen peroxide, chlorhexidine, and alcohol.[9] A previous study showed that brushing teeth and rinsing the mouth with the antiseptic compound can change the pH into alkali, thus controlling the acid formation in the oral cavity.[10] However, there are side effects of synthetic products such as tooth discolouration, taste buds sensation alteration, toxicity to the oral tissue, dryness and soreness in the oral cavity and exfoliation of the mucosa in children.[11,12]

Several studies have been conducted to explore the efficacy of natural herbs contained in a number of plants, both fruits and vegetables in an effort to replace the use of synthetic drugs that have many side effects.[13,14] Strawberry fruit is one of the herbal plants that is often used as the object of research.

Strawberry fruit (*Fragaria x ananassa*) is an edible fruit (safe for consumption) and is rich in nutrients (vitamins, fiber, minerals and folic acid) as well as non-nutritive compounds (polyphenols). Several studies have been discovered that strawberry fruit contains many phytochemical compounds, which are secondary metabolites. These compounds consist of a wide variety of polyphenols important for plants against bacteria and fungi. The antimicrobial activity of these polyphenols can be divided into simple phenolic acids (Pcresol, 3-ethylphenol, vanillic, gallic, ellagic acids, hydroquinone), hydrocinnamic acid derivatives (p-coumaric, caffeic acid, ferulic, sinapic acids), flavonoids including flavonols, flavones, isoflavones, anthocyanidins (fruit's pigment colour) Moreover, flavanols (catechin-monomers, proanthocyanidins-polymers known as condensed tannins) and tannins. These compounds provide a synergistic and cumulative effect in improving human health and preventing disease.[13,14,15,16,17,18]

There have been no studies investigating the effect of strawberry fruit, specifically on *S. sanguinis*. Therefore, this study aims to analyse the antibacterial activity of strawberry fruit against *S. sanguinis* (ATCC 10556).

METHODS

Exploratory research was conducted to analyse the antimicrobial activity of strawberry fruit extract on the growth of *S. sanguinis* strain ATCC 10556 by determining the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the extract. Extracts were made using the maceration method, namely the cold extraction method and the simplest, where the solvent will penetrate the plant cell wall and enter the cell cavity containing the active substance so that the active substance, which is a concentrated solution, will be pushed out of the cell due to the difference in concentration between the solution outside and inside the cell.[19] The strawberry fruit plant used in this study was obtained from a plantation in Rancabali Village, Ciwidey District, Bandung Regency, West Java. The plant has been identified as strawberry fruit by the Plant Taxonomy Laboratory of Jatinangor Herbarium, Department of Biology, Faculty of Mathematics and Natural Sciences Universitas Padjadjaran.

Extraction of strawberry fruit

Extraction was made by cutting 20 kg of fresh Strawberry fruit into small pieces. Then, the maceration method immersed the samples in 10 litres of methanol solvent for 3 x 24 hours. Next, the immersion results were filtered using a funnel filter paper to produce a filtrate. Finally, the filtrate was evaporated using a rotary evaporator to obtain a concentrated methanol extract.

The concentrated methanol extract was put into a separating funnel by adding 100 ml of distilled water and 200 ml of hexane, then allowed to form two layers. The aqueous phase was below the hexane phase and then concentrated using a rotary evaporator to obtain the hexane fraction. The aqueous phase was then put into a separating

funnel by adding 200 ml of ethyl acetate solution to form the ethyl acetate phase above the water layer. The aqueous and ethyl acetate phases were then concentrated using a rotary evaporator to obtain the water and ethyl acetate fractions.

Inhibitory zone tests

Inhibition zone testing were carried out by incubating *S. sanguinis* on Mueller Hinton Agar for 48 hours at a temperature of 35-37°C. Samples in the form of a solution of each fraction (water, ethyl acetate and hexane), each as much as 20 µl, dripped on a paper disk with a diameter of 6 mm, then inserted into Mueller Hinton agar medium containing *Streptococcus sanguinis*. The inhibitory zone tests were performed at the concentrations of 1%, 2%, 3%, 4% and 5%. The inhibition zone diameter seen on the media was then measured using a calliper on a millimetre scale. The best measurement results are used as the active fraction to test MIC and MBC further.

MIC test

The MIC value test was carried out using a microplate reader. For every 96 sterile microplate wells, 100µl of Brain Heart Infusion (BHI) was added. In rows A and C, 100 µl of the active fraction (ethyl acetate fraction of strawberry fruit) was added, while 100 µl of solvent was added to rows B and D (pipetted up and down to obtain a homogeneous mixture). The dilutions were carried out serially up to the 12th well in each row (A, B, C and D) by taking 100 µl from each of the first wells to adjacent wells. In columns 5 to 8 (rows C and D), *S. sanguinis* were added with turbidity McFarland 0.5 as much as 5 µl. The microplate was then closed and incubated at 37°C for 48 hours, after which it was scanned to measure its turbidity using a Microplate reader with a wavelength of 620 nm. Minimum Inhibitory Concentration was indicated by the well containing the lowest concentration that still inhibits the growth of bacteria in the C-line (media, sample, bacteria).

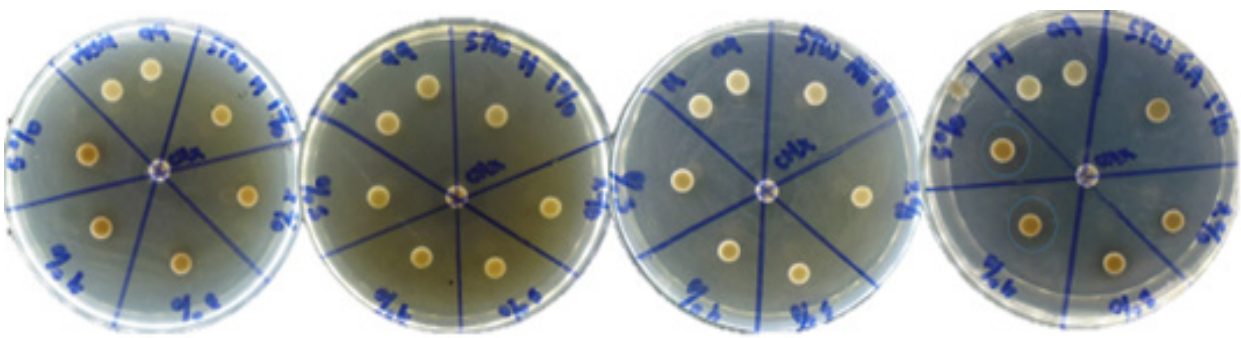
MBC test

The MBC test was conducted after determining the lowest concentration solution of the *S. sanguinis* inhibition (MIC) test. Then, it tests two more full concentrations and two more dilute concentrations. Subsequently, subcultures were performed on Mueller Hinton Agar media. The media was incubated at 37°C for 48 hours to determine the decrease in the number of colonies. Agar media that does not contain bacterial colonies was designated as MBC.

RESULTS

Petri dishes that had been incubated for 48 hours (Figure 1 and Table 1) showed that the ethyl acetate fraction of strawberry fruit extract from a concentration of 1% to 5% was the most effective fraction in inhibiting the growth of *Streptococcus sanguinis* compared to other fractions, with ethyl acetate fraction 4 % shows the highest inhibition zone compared to other concentrations.

Minimum inhibitory concentration (MIC) was obtained by testing the ethyl acetate fraction of 4% of strawberry fruits using a microplate reader. The MIC values are shown in Table 2. The MIC value



(a) (b) (c) (d)

Figure 1. Inhibitory test results: (a) Methanol extract; (b) Hexane fraction; (c) Water fraction; (d) Ethyl acetate

Table 1. Inhibitory value zone of strawberry fruits against *Streptococcus sanguinis*

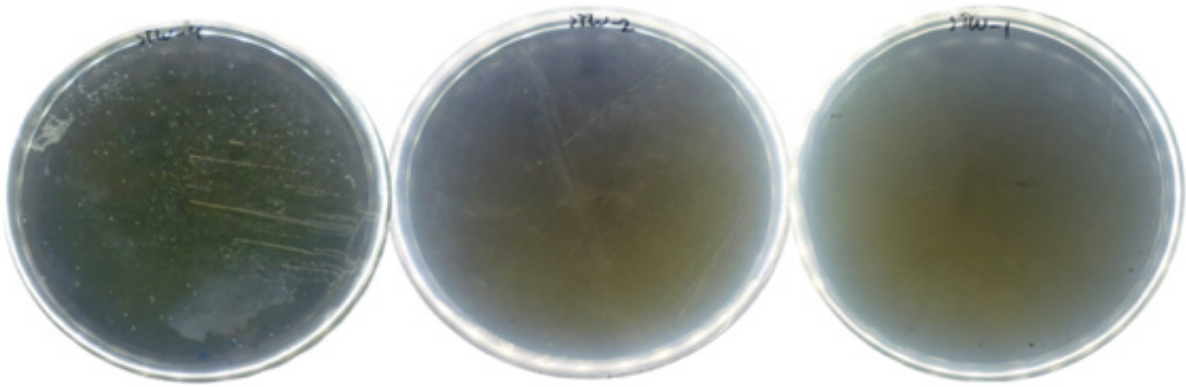
No	Sample	Inhibitory zone (mm)				
		1%	2%	3%	4%	5%
1	Methanol extract	0	7.4	10.5	10.5	11.4
2	Hexane fraction	0	0	0	8.2	9.8
3	Ethyl acetate fraction	7.3	10.2	12.3	16.3	16.1
4	Water fraction	0	0	0	0	10.0

Table 2. Microplate reader result of the MIC of the ethyl acetate fraction of strawberry fruits

Microplate content	Inhibitory zone (mm)											
	20,000	10,000	5,000	2,500	1,250	625	312.5	156.25	78.125	39.0625	19.53125	9.765625
MS	0.368	0.271	0.235	0.107	0.082	0.072	0.070	0.069	0.065	0.064	0.064	0.064
	0.369	0.247	0.220	0.104	0.075	0.069	0.074	0.062	0.060	0.059	0.059	0.059
MP	0.047	0.050	0.053	0.056	0.058	0.059	0.059	0.060	0.061	0.061	0.061	0.065
	0.050	0.052	0.054	0.056	0.056	0.055	0.055	0.056	0.056	0.058	0.058	0.058
MSB	0.329	0.290	0.182	0.118	0.221	0.299	0.366	0.331	0.337	0.318	0.330	0.313
	0.315	0.295	0.196	0.121	0.307	0.282	0.318	0.332	0.328	0.342	0.352	0.322
MPB	0.225	0.273	0.284	0.274	0.282	0.272	0.271	0.296	0.295	0.310	0.308	0.351
	0.209	0.303	0.298	0.312	0.304	0.289	0.265	0.275	0.290	0.265	0.297	0.326

Notes: MS = Media with sample added; MP = Media with solvent added; MSB = Media with sample and bacteria added; MPB = Media with solvent and bacteria added

of ethyl acetate fraction of strawberry fruits was 2500 ppm or 0.25%. It was confirmed in Table 2 that there was decreasing value in the well containing the mixture of media, samples, and bacteria (MSB) in the fourth column at the concentration of 2500 ppm. The Minimum Inhibitory Concentration (MIC) value were 0.118 and 0.121 consecutively. The Minimum Bactericidal Concentration (MBC) was obtained by culturing the bacteria from the MIC result at Mueller Hinton agar for 48 hours. There was decreasing bacterial growth in concentrations between 1250 ppm and 20000 ppm. Then it was observed under a Light Emitting Diode (LED) lamp. It was clear at the concentration of 20000 ppm (Figure 2). Therefore, the MBC of ethyl acetate fraction of strawberry fruit was 20000 ppm or 2%.



2500 ppm 10000 ppm 20000 ppm

Figure 2. Minimum Bactericidal Concentration test result observed under an LED lamp

DISCUSSION

Antibacterial activity of strawberry fruit can be seen by forming a clear area around the paper disk when the bacterial inhibition zone was tested using the disc diffusion method (Kirby-Bauer method). This observation result indicated that *S. sanguinis* was inhibited due to the antimicrobial substance contained in the fruit extract. The diameter scale obtained from the results of the

inhibition zone measurement is divided into several groups, namely the resistant group with a diameter of the inhibition zone formed less than 14 mm, the intermediate and dose-dependent susceptible groups having a diameter of 15-19 mm and the very susceptible group with the diameter of the inhibition zone formed above 20 mm.[20] Strawberry fruit extract with the most effective antibacterial activity was seen in the ethyl acetate fraction with a concentration of 4%, which forms an intermediate inhibition zone diameter of 16.3 mm.

Ethyl acetate is a semi-polar solvent with low toxicity that can extract many biological compounds (polar and non-polar) contained in strawberry fruit extracts, such as alkaloids, aglycons, glycosides, sterols, terpenoids, and flavonoids compounds. Water is a common solvent often used to dissolve polar compounds that are soluble in water, such as glycosides, amino acids, sugars, vitamin C and aglycon compounds. Studies have shown that water solvents used in plants containing bioactive compounds such as strawberry fruit can only dissolve anthocyanins that tend to be antioxidants and are not effective in dissolving phenolic compounds such as flavonoids' antibacterial effects. Hexane solvent is a non-polar solvent that can only dissolve non-polar compounds such as lignin, wax, fat, aglycon, sterol, and terpenoids. It cannot dissolve polar compounds in Strawberry fruit, making it less effective in inhibiting bacterial growth.[21,22]

The MIC of ethyl acetate fraction of strawberry fruit was checked using the dilution method in 96 wells, where the values were evaluated using a microplate reader. The MIC value was 2500 ppm or 0.25%. The result from the microplate reader showed a linear value from the lowest to the highest dilution concentration. Decreasing value on the well containing a mixture of media, samples and bacteria (MSB) occurred in the fourth column at a concentration of 2500 ppm with the value of 0.118 and 0.121. This value means the lowest concentration in inhibiting *S. sanguinis*. The MIC value below 0.5 g/ml is declared as strong and susceptible to *S. sanguinis*.[20]

In the present study, the results of the MBC test for the ethyl acetate fraction of strawberry fruit was 20000 ppm or 2%. It was more effective in killing the growth of *S. sanguinis* compared to concentrations of 10000 ppm and 2500 ppm. Plants that have an antibacterial ingredient will effectively inhibit the growth of *Streptococcus sanguinis* because this gram-positive bacterium only has a simple cell structure consisting of a single layer with a low lipid content (1-4%) so that antibacterial ingredients can easily penetrate the cell.[23]

Strawberry fruit has bioactive compounds divided into five essential groups: phenolic acids, stilbenes, flavonoids (flavonols or catechins, flavones, flavonones), isoflavonoids anthocyanins), tannins, and lignans. Strawberry fruit extract contains high levels of flavonoids (proanthocyanidins, anthocyanins, ellagic acid and catechins), 50.52 ppm and 0.69% catechins. These flavonoid compounds and tannins are reported to act as antimicrobials that can control pathogens.[13] The United States Department of Agriculture's released data reported that Strawberry fruit contains 40 mg of flavonoids per 100 gram.[13] Flavonoid is the main polyphenolic compound in strawberry fruit with antibacterial abilities to inhibit or even kill the growth of pathogenic bacteria.

The present study showed that the MIC value of strawberry fruit extract against S. sanguinis was obtained at a concentration of 12.5%. The results of this study were similar to research conducted by Erycesar.[16] Strawberry fruit extract has antibacterial power against *S. mutans* with a MIC value of 12.5%. In another study, the MIC and MBC values of strawberry fruit extract against *S. epidermidis* were at the concentration of 1.5%. Meanwhile, the study of Widyaman et al.[24] suggested that flavonoids in this fruit extract were also reported to inhibit the formation of monospecies and multispecies biofilms from *P. gingivalis* and *E. faecalis*.

Although many studies explain that strawberries are flavonoid-containing antibacterial activity, Sitorus et al.[17] contradictory prove that the content of strawberry fruit extract does not have an effective antibacterial activity. This phenomenon would probably be due to the sensibility of the bacteria used, the different procedures of making extracts, or flavonoids and other active substance that is antibacterial in strawberry fruit may be damaged during manufacturing the extracts.[17,25]

The mechanism of flavonoid action is by denaturing protein molecules and nucleic acids from bacteria as their ability to form complete compounds with proteins through hydrogen bonds. Phenolic compounds such as ellagitannins and flavonoids (proanthocyanidins and catechins) in strawberry fruit have a strong antibacterial effect of preventing colonisation and infection of several pathogenic bacteria.[14,15,16] The mechanism of action of flavonoids is divided into three main targets, namely inhibiting nucleic acid synthesis, inhibiting energy metabolism by interfering with nutrient exchange and metabolism, and inhibiting bacterial cell membrane synthesis.[26]

In general, the antibacterial activity contained in fruit can cause damage to bacterial membranes, suppress several virulence factors, including bacterial enzymes and toxins and inhibit the formation of bacterial biofilms. Recent studies reported that in addition to having antibacterial activity, compounds from phytochemicals could also inhibit quorum sensing bacteria as targets to attract or control their pathogenicity, thereby inhibiting the development of oral biofilms.[26,27]

CONCLUSION

Ethyl acetate fraction was the most effective fraction from strawberry fruit with antibacterial activity against *S. sanguinis* with the inhibitory zone in the concentration of 4%, MIC of 0.25%, and MBC of 2%.

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