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Antioxidant, Antibacterial, and Degradation *Streptococcus mutans* Biofilms Activities of Black Pepper (*Piper nigrum*) Seed Extract

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Abstract. The phenolic, alkaloids, and essential oil of black pepper are potential as an antioxidant, antibacterial, and expected to be able to degrade *Streptococcus mutans* biofilms. The aim of this study was to determine antioxidant, antibacterial, and *Streptococcus mutans* biofilm degradation activities of extract with different solvents (water, ethanol, chloroform, ethyl acetate) and essential oils of black pepper seeds. All extracts, essential oils, and piperin as component of black pepper were active as antioxidant based on phosphomolybdate method. The most active antioxidant is the essential oils (139.05 mmol α-tocopherol equivalents/g sample) which more active than that of BHT (30.62 mmol α-tocopherol equivalents/g sample). Qualitative identification of the active antioxidant compounds was performed by thin layer chromatography-bioautography. Ethyl acetate extract, chloroform, and ethanol gave 4-5 active bands as antioxidant. The extracts and essential oils of black pepper has low antibacterial activity against *S. mutans*. To degrade the *S. mutans* biofilms, piperin showed the highest capability of 48% at the concentration of 2000 µg/mL followed by the essential oils with capability of 41% at the same concentration. In conclusion, the essential oils of black pepper was the most active as antioxidant and *S. mutans* degradation.

INTRODUCTION

Plants produce various bioactive molecules making it a rich source to developed as raw materials of medicine. One of the medicinal plants that is known to have many benefits is black pepper. Black pepper, known as the king of spices, is needed to be developed into other products [1]. Black pepper is also reported contains 1.0-2.5% volatile oil and 5.0-9.0% piperamide alkaloid which accumulates in fruit skin and pepper seeds, with the main ingredients being piperine, cavicin, piperidine, and piperetin [2-4]. Some researchers reported that black pepper could be used in the treatment of vertigo, asthma, obesity, sinusitis, arthritis, fever [3], and having antimicrobial properties [5] antimutagenic [6], antipyretic, antioxidant, anti-inflammatory, anticancer, anti-quorum-sensing, antidiarrheal, antitumor, antiplatelet, antihypertensive, antithyroid and hepatoprotective [7]. Therefore, black pepper can be developed as antibacterial, biofilm degradation, and antioxidant, especially for oral care product.

Antibacterial and biofilm degradation against *Streptococcus mutans,* an oral microbe, are used to maintain oral and dental hygiene. When bacteria grow, bacteria can make formation of biofilm and caused dental plaque [7]. On the other hand, bacterial attacks on the mouth can stimulate the release of free radicals in the process of destroying these bacteria. Excessive free radicals have the potential to cause disruption of the oral cavity and tongue which results in damage to the periodontal tissues of the teeth, thus requiring antioxidant oral cavity. Therefore, the importance of antioxidant compounds in controlling damage caused by bacterial attacks is necessary. This study aimed to determine the antioxidant, antibacterial, and biofilm degradation against *Streptococcus mutans* activities of black pepper seed extracts with different solvents, and black pepper seed essential oils.

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METHODS

Preparation of Samples

The black pepper sample used in this study was determined at the Biology Research Center, LIPI Cibinong-Bogor and it is reported as *Piper nigrum*. The water content of black pepper seed powder used in this study is 8.37%. A total of 5 g of black pepper seed powder then used for extraction process by adding 100 mL of solvent with different polarity, namely, chloroform, ethyl acetate, ethanol, and water. The mixture is then refluxed for 4 hours. The extract from several solvents was then concentrated using rotary evaporator at a temperature of 50 ºC. To obtain the essential oils of black pepper seed, 200 g of powder is distilled by steam distillation for 5 hours. The essential oil then added sodium sulfate anhydrous. The extracts and essential oils were stored at a temperature of 0 ºC until then used.

Determination of Antioxidant Activity

The phosphomolibdat method [9] was carried out to determine the total antioxidants of black pepper extract in various solvents, essential oils, and piperines (Sigma). A total of 0.50 mL sample solution was mixed with 5.00 mL phosphomolibdate reagent which contains 0.60 M concentrated sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. The samples and phosphomolybdate reagent then incubated at 95 ºC for 90 minutes, cooled at room temperature and the absorption is determined by spectrophotometer at 695 nm. BHT is used as positive control. The antioxidant activity of each sample was expressed as equivalent to α-tocopherol.

TLC-Bioautography of Antioxidant

The antioxidant activity of each extract also determined by TLC-bioautography that was modification of the method from Prieto [10]. Each 8 µL crude extract of black pepper in various solvents and 2 µL of standard piperine was applied to the TLC plate. The stationary phase in the chromatographic system used is silica gel 60 F_{254}, while the mobile phase used is chloroform:dichloromethane (37:3). After separation step, the TLC plate was dryed and observed under UV-Vis light, then dipped in phosphomolibdate reagent, incubated at 95 ºC for 30 minutes. Blue-green spots show compounds that act as antioxidants.

Determination of Antibacterial Activity

The bacteria used in this study is 99.00% as *Streptococcus mutans*. The antibacterial test in this experiment used the 96-well plate microdilution method with the medium is TSB [11]. A total of 100 µL samples (15-2000 µg/mL) were inserted into each well. Each well was added 100 µL TSB medium and $6.0x10^8$ CFU/mL *S. mutans* inoculants. Then incubated at 37 ºC for 24 hours. A clear well after incubation at the lowest concentration was chosen as MIC. The still clear well is piped as much as 100 µL to the new 96-well plate and added 100 µL TSB. Then incubate for 24 hours at 37 ºC. A clear well with the lowest concentration is determined as the minimum kill concentration (KBM). DMSO 20% as a negative control and positive control used tetracycline and commercial mouthwash.

Determination of Biofilm Degradation Activity

The artificial saliva was prepared using Tanuwiria *et al*. [12] methods. Determination of biofilm degradation activity was carried out using microdilution method [13]. Biofilms are formed by putting 100 µL of saliva, 100 µL TSB media with 3% glucose, and $6.0x10^8$ CFU/mL *S. mutans* inoculants into a 96-well plate and incubated for 24 hours at 37 ºC. After the biofilm is formed, the rest of the medium is removed, and 100 µL sample was added (15-2000 µg/mL). Chlorhexidine as positive control and DMSO 20% as negative control. Then incubated for 24 hours at 37 ºC. Biofilms degraded by extracts or essential oils were then removed by washing using phosphate buffer and sterile water. A total of 100 µL of crystal violet 0.01% was added to the well and allowed to stand for 30 minutes, rinsed with sterile water and then added with 200 µL of 95% ethanol. After incubation at room temperature for 45 minutes, 100 µL of the solution was transferred to a new and sterile 96-well plate. The absorbance of the solution in the well is measured using a microplate reader (λ = 595 nm) and percentage of degradation was determined.

RESULTS AND DISCUSSIONS

Black pepper used in this study has varied value of extraction and essential oil yield, antioxidant, antibacterial, and percent degradation of *S. mutans* biofilms (Table 1). The highest yield is found on water extract while the lowest is on essential oils. It is means that the black pepper seed consist more polar component compared to non-polar component. The yield of essential oils on this research is lower than reported by Deshwal [2] which is about 1.00-2.50%. The different of essential oils yield can be caused by post-harvest processing which decrease the essential oils.

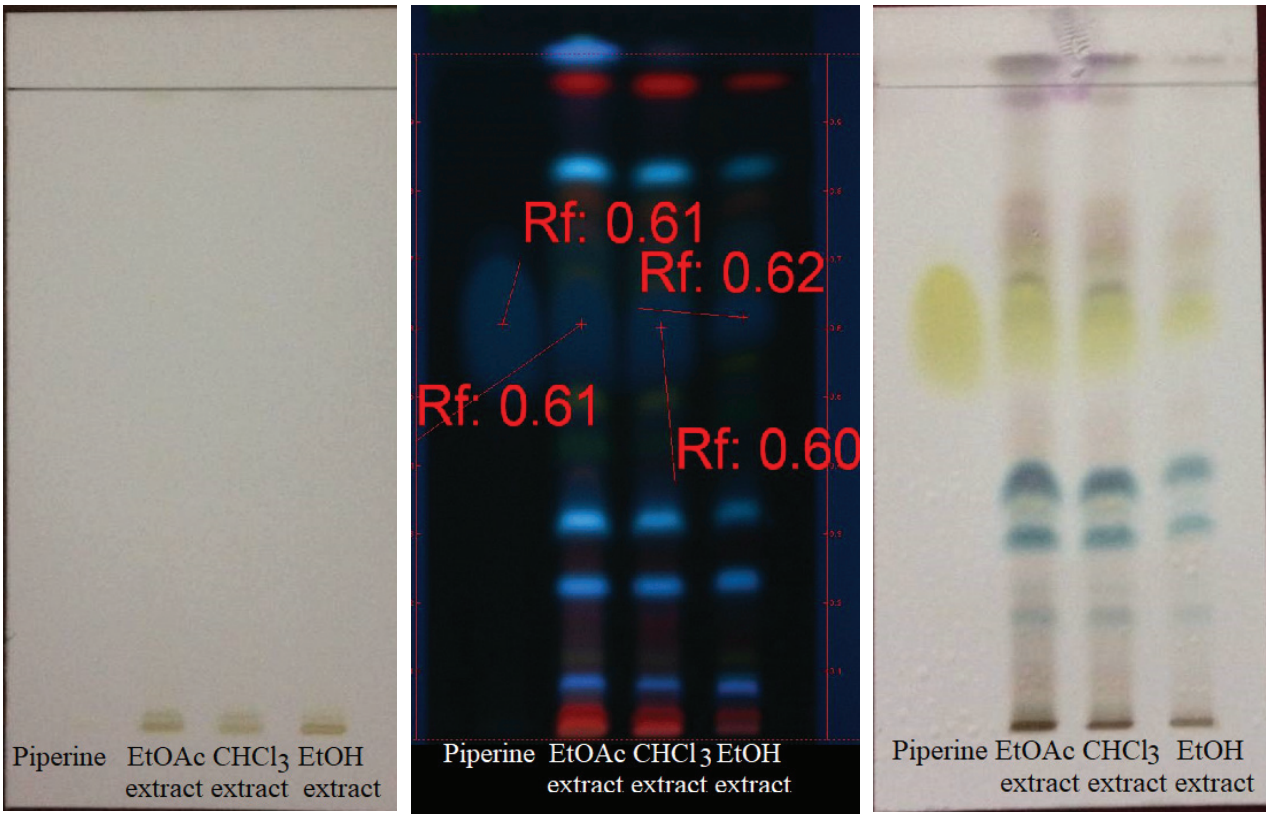
TABLE 1. The yield, antioxidant, antibacterial, and percent biofilm degradation activity of extracts and essential oils of black pepper seeds

Sample name	Yield (%)	Antioxidant activity (mmol α-tocopherol equivalent/g sample)	Antibacterial activity against *S. mutans* (μg/mL)		Biofilm degradation (%)
			MIC	MBC	
Water extract	21.74	20.68	1000	2000	19.73
Ethanol extract	9.20	119.59	>2000	>2000	15.97
Chloroform extract	5.55	113.59	>2000	>2000	37.94
Ethyl acetate extract	7.10	124.08	>2000	>2000	26.99
Essential oils	0.22	139.05	>2000	>2000	41.11
Piperine	-	91.59	2000	>2000	48.53
BHT	-	30.62	-	-	-
Tetracycline	-	-	15.63	15.63	-
Chlorhexidine	-	-	-	-	98.26
Commercial mount wash	-	-	2000	>2000	75.34

The antioxidant activity of black pepper was determined using the phosphomolibdate method. The phosphomolibdat method measures the antioxidant capacity of the sample and it is related to the phenolic content of the sample. The principle is based on the reduction of Mo (VI) to Mo (V) by analyte and the formation of phosphate-Mo (V) green-blue complexes at acidic pH and high temperatures measured at a wavelength of 695 nm. During the reaction, the hydroxyl group in the phenolic compound will react with phosphomolybdate reagent [10].

Black pepper extract in various solvents, essential oils, and piperine had different antioxidant activities (Table 1). Essential oil has the greatest activity as antioxidant agent 139.05 mmol α-tocopherol equivalent/g sample, followed by ethyl acetate extract. Their activities are greater than the positive control of BHT and piperine which is thought to be an active antioxidant compound. BHT is monophenol compound, so only one hydroxyl group was involved in the reduction-oxidation process, besides that the butyl tertiary structure on both sides of the phenol BHT group did not allow BHT to form stable phenolic ions, so it was thought to cause low antioxidant activity of BHT. The antioxidant activity of ethanol extract from this research is equivalent to 51.5 g α-tocopherol/g extract. This result is higher than reported by [9] which only about 2 g α-tocopherol/g extract.

Amide phenolic compounds, polyphenols, flavonoids contained in black pepper are thought to play an important role as antioxidants [13]. Polar solvent such as water was able to dissolve macromolecular component such as flavonoid glycosides which have lower antioxidant activity than free flavonoids, so it cause very low antioxidant activity in water extracts. In addition, based on Kapoor *et al.* [14] extracts and essential oils of black pepper contain β-caryophyllene, limonene, β-pinene, piperine, and piperolein which is contribute to antioxidant activity.



(a) (b) (c)

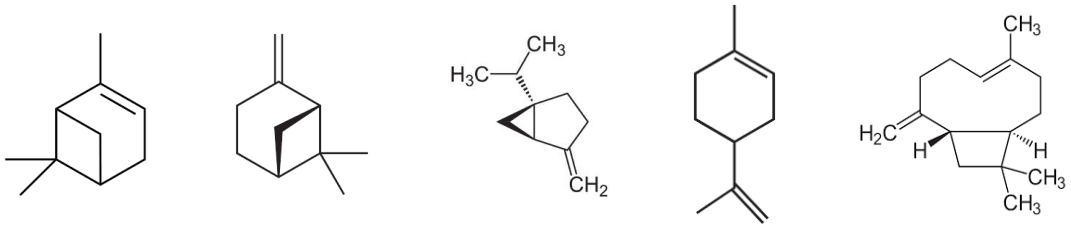
FIGURE 1. Bioautography antioxidant chromatogram on visual light (a) detected at 366 nm (b) and by phosphomolibdate (c), eluent: chloroform:dichloromethane (37:3)

The thin layer chromatography pattern of the three active extracts as antioxidant namely EtOAc, chloroform and EtOH extract, and piperine as the marker compounds is determined. The pattern could not be determined by visual light (Figure 1a) but could be determined using UV 366 nm (Figure 1b). Piperine (Rf 0.60 – 0.62) could be found in all extracts. The EtOAc extract has the highest number of spots (8 spots) detected by UV 366 nm and only different one spot with chloroform extract. Based on TLC-bioautography antioxidant results (Figure 1c), there were 5 active spots for chloroform and ethyl acetate extract, while ethanol extract contained 4 active bands as antioxidants which characterized by blue-green color. The piperine is qualitatively inactive as an antioxidant.

The extract which has antimicrobial activity against *S. mutans* is only at water extract with MIC and MBC of 1000 and 2000 µg/mL respectively and this activity is not as good as tetracycline as positive control (Table 1). Compounds that play a role in the antibacterial activity in water extract probably phenolic and polyphenol groups [15]. The other extracts and essential oils, the MIC and MBC value cannot be determined since until 2000 µg/mL did not inhibit the bacterial growth. This result is different with the review article by Salehi *et al.* [16] which is inform that the MIC and MBC value of black pepper essential oils is around 50-500 ppm including against *S. aureus.* Piperine as marker compound in black pepper and commercial mouthwash are used also as positive controls. The MIC of piperine and commercial mouthwash is 2000 µg/mL, while the MBC value are more than 2000 µg/mL. Commercial mouthwash has lower antibacterial activity than water extract, this can be caused by the low concentration of antibacterial compounds on commercial mouthwash which has been adjusted for oral use. In addition, dilution was carried out in the test which caused an increasingly reduced concentration of commercial mouthwash.

The *S. mutans* biofilm degradation activity of black pepper extracts and essential oils is shown in Table 1. Percentage of degradation displayed at the concentration of 2000 µg/mL. Black pepper essential oil has the best degradation ability compared to all black pepper extracts. The essential oils of black pepper contain mostly monoterpene and sesquiterpene such as α-pinene, β-pinene, sabinene, limonene, and β-caryophyllene (Figure 2)

which can accumulate in membrane lipids of bacteria cells, and cause disruption of the structure and function of cell membranes and changes in permeability of bacterial cell membrane [17-18].



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

FIGURE 2. The Structure of black pepper essential oils components: α-pinene (a), β-pinene (b), sabinene (c), limonene (d), and β-caryophyllene (e)

Chlorhexidine, an antiseptic bactericide, as a positive control has a percent degradation of 98.26% while commercial mouthwash used as a comparison has percent degradation of 75.34% and piperine of 48.53%. As positive control, chlorhexidine causes the breakdown of bacteria cell membrane and release the cytoplasmic which causes bacterial cell death. Chlorhexidine is bound to salivary protein, reduces the formation of glycoproteins on the tooth surface, and inhibits the colonization of plaque-forming bacteria. In addition, it is also bound to bacteria which inhibits its adsorption on the tooth surface [19]. Piperine degradation ability is better than extract because the piperine used is pure compound. Chloroform extract has the best percent degradation among the four black pepper extracts, it is suspected that chloroform extract is better at extracting compounds that play a role in the degradation process of *S. mutans* biofilms including piperine compounds whose polarity is similar to that of chloroform. Biofilm degradation ability of related compound materials is capable of penetrating into biofilms formed in the extracellular layer of polysaccharides (EPS) or mucus layers that envelop the bacteria and can eliminate EPS in formed biofilms [20]. Therefore, it is important to find the biofilm degradation mechanism of the active extract.

CONCLUSION

Black pepper essential oil has the potential as an antioxidant and biofilm degradation, with an antioxidant activity of 139.05 mmol α-tocopherol equivalent/g sample and 41% degradation of *S. mutans* biofilm. The TLC bioautography of ethyl acetate, chloroform, and ethanol extracts is resulting 4-5 active bands as antioxidants based on phosphomolybdate method. Black pepper water extract has a fairly low antibacterial activity against *S. aureus* with MIC and MBC 1000 and 2000 µg/mL, respectively.

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