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Assessing Antimicrobial Activity of Aloe Vera against *Colletrotrichum Capsici* and *Bacillus Subtilis*

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ABSTRACT

Nowadays, overuse of chemical fungicide has a side effect which is harmful to human, cause environmental problem and destroys the antagonist microorganisms. Therefore, it is important to develop natural fungicide and bactericide from the plant extract. For this study, chilli anthracnose fungus, Colletotrichum capsici and a pathogenic bacterium Bacillus subtilis were used for assessing the antimicrobial activity. C. capcisi was isolated from the chilli plant and its morphology was identified. Antifungal activity was demonstrated by using different concentrations Aloe vera extract (from 1000 to 5000 ppm). Aloe vera has a lower inhibition of C. capsici at each concentration, which was 1.17 % at 1000 ppm and 7.20 % at 5000 ppm. As for the antibacterial activity, different concentrations of Aloe vera extracts (from 1.25 to 200 mg/ml) were tested on B. subtilis by disc diffusion method. Aloe vera has a higher inhibition of B. subtilis at 200 mg/ml which was 21.33 mm while there is no inhibition zone at 1.25 mg/ml. B. subtilis proved that MIC is 2.5 mg/ml. In conclusion, Aloe vera can be used as the biological fungicide to replace the chemical fungicide. On the other hand, Aloe vera is suitable to be the commercialized as a natural bactericide.

Keywords: *Aloe Vera*, *Colletotrichum Capsici*, *Bacillus Subtilis*, Antimicrobial, Anthracnose Disease.

1. INTRODUCTION

Capsicum Annuum (Chili) is an important economic crop and widely consumed by the people in the worldwide. In this situation, the quality and quantity of the chili must be maintained in order to fulfill the demand. However, chili is severely infected by anthracnose disease that caused by *Colletotrichum* species which losses up to 50% yield (Than *et al*., 2008). *Colletotrichum* species was voted as the most important plant pathogenic fungi in ranking eighth based on the perceived scientific and economic importance (Irieda & Takano, 2016). Moreover, *Colletotrichum* species, a common group of a plant pathogen that is responsible for the diseases on many plant species (Dean *et al*., 2012).

Symptoms of chilli most obvious on riped fruits and leave. Anthracnose generally appears on leaves with small and irregular yellow or black spots at the first stage. The spots are then expand and merge to cover the whole affected area. The infected area become darkens, the center of an older spot becomes blackish and emits gelatinous pink spore masses. Typical anthracnose symptoms on

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chilli fruit include sunken necrotic tissues, with concentric rings of acervuli (Than *et al*., 2008). The disease can also produce cankers on petioles and on stems that cause severe defoliation and rotting of fruits and roots. Anthracnose spread faster in the rainy period which resulting in higher infection rate among the plants because the *Collectotrichum* spores are splashed or washed onto other fresh fruits (Oo & Oh, 2016). Plant extracts are well known with continuous studies to find a new compound against pathogens (Bele *et al*., 2016). The use of plant extracts that have antimicrobial properties to treat various microbial infections is highly recommended (Ramasubramaniam *et al*., 2010). There are almost 20% of the plant found in the world have been present to pharmacological or biological test, and a significant number of new antibiotics presently available are obtained from characteristic or semisynthetic assets (Bele *et al*., 2016).

Various studies on *Aloe vera* are being done to portray the antiviral, antibacterial, and also its other uses as a pain relieving, anti-inflammatory, and wound healing properties. *Aloe vera* is one of the plants that have antimicrobial properties. Nowadays, *Aloe vera* gel are using as a edible coating material for fruits and vegetables because the research proved that it has antifungal and antimicrobial activity that able to prevent loss of moisture and firmness of fruits, control respiratory rate and maturation development, delay oxidative browning and reduce microorganism proliferation in fruits such as oranges, grapes, sweet cherries and papaya (Kumar & Bhatnagar, 2014).

2. MATERIAL AND METHODS

2.1 Collection of Crude Plant

Fresh leaves of *Aloe vera* were collected. The plants were washed thoroughly in running water to remove the dust particles and debris. It was then rinsed in distilled water. The leaves were dried in the oven at 80 ˚C for 48 hours and after that used for the extraction.

2.2 Extraction of Plant

Whole leaves of *Aloe vera* were cut into thin pieces and crushed by using Mortar and Pestle and then it will be pulverized in an electric blender into a fine powder. 10 g of fine powder was weighted by using an analytical balance and filled in the thimble. 100 mL of ethanol (HmbG Chemical, Germany) was poured into a round bottom flask which is attached to the Soxhlet extractor and condenser on a heating mantle. The thimble was placed inside the Soxhlet extractor and the solvent was heated by the heating mantle. The extraction complete when the solvent in the siphon tube become colourless. Then, the Whatman no. 2 filter paper (GE Healthcare Life Sciences, UK) was used to filter the mixture. The filtrate was then transferred to the glass and the solvent was evaporated by the rotary vacuum evaporator (Fischer Scientific, Selangor, Malaysia). After that, the extract was cooled to room temperature by sealing with aluminium foil. The extract was then stored in a refrigerator at 4 ˚C for further use (Karpagam & Devaraj, 2011; Stanley *et al*., 2014).

2.3 Isolation of Anthracnose Fungi, *Colletotrichum Capsici*

The infected region of chili was cut into small pieces for about 5 mm in diameter. It was then surface sterilized in 0.1% mercury chloride for 30 seconds and then continuously washed with distilled water to remove the mercury before transfer to the Potato Dextrose Agar (PDA). The PDA plates were seized with paraffin film and transferred to an incubator at 30˚C in dark condition for 7 days. Isolated colonies were sub-cultured into fresh plates several times until the pure culture was obtained.

2.4 Preparation of Extract Dilutions

Aloe vera extract was used as an agent for antifungal and antibacterial tests. For the antifungal test, the crude extract was diluted into 1000 ppm, 2000 ppm, 3000 ppm, 4000 ppm and 5000 ppm, respectively by mixing with the sterilized distilled water. To prepare 1000 ppm of plant extract, 1 g of crude extract was diluted with 1.0 L sterilized distilled water. For the antibacterial tests, the crude extract was diluted into 10 different concentrations which were from 1.25 mg/ml to 200 mg/ml. For example, to prepare 200 mg/ml of plant extract, 2 g of crude extract was mixed with 10 mL of sterilized distilled water.

2.5 Antifungal Assay

Seven conical flasks were used to prepare the PDA media; 50 mL of PDA media were prepared in each conical flask. All conical flasks were autoclaved for 15 minutes at 121˚C. The flasks were transferred to the water bath and maintained at 50˚C to prevent solidification. Then, 5 ml of each extract from different concentrations was added to the flasks by using micropipette. The flask was shaken for few minutes to ensure the proper mixing between PDA and plant extract. Chloramphenicol was added to each medium to prevent the bacterial growth. Media cultures were then poured into Petri dishes and let it solidify. After that, 50 µL of *C. capsici* spore suspensions was pipetted onto the centre of the PDA media. The Petri dishes were then sealed with parafilm carefully to prevent spreading of spore suspension on PDA. The plates were incubated at 30˚C for 7 days. The negative and positive controls were DMSO and Mancozed respectively.

2.6 Antibacterial Assay

The antibacterial activity was carried out by a disc diffusion technique. The needle was sterilized by placing it in the alcohol and then it was flamed to remove the alcohol. 100 µL of *B. subtilis* bacteria suspension was pipetted onto NA plate and spread over by using sterilized 'hockey stick'. Then, 6 mm paper disk was then placed on the surface of the inoculated NA plate. The discs were placed and pressed gently with the tips of the forceps to allow the discs fully contact with the agar. 20 µL of each concentration plant extract was pipetted onto the disk. The plate was then incubated at 37 ˚C for 24 hours. The diameter of the minimum zone of inhibition was measured in millimeter (mm) by using millimeter ruler. The positive and negative controls used in this test were Chloramphenicol and dimethylsulfoxide (DMSO) (Stanley *et al.*, 2014). The Minimum Inhibitory Concentration (MIC) was used to determine the concentration that giving the least inhibition activity and below which there is no further inhibition.

2.7 Phytochemical Test

Phytochemical screening for terpenoids, flavonoids, saponins, tannins and carbohydrate were carried out as described below. The procedure of these tests was modified from Dharajiya *et al.* (2017) and Raphael (2012).

2.7.1 Test for Terpenoids

0.05 g of crude extract was mixed with 2 mL of chloroform. 2 mL of concentrated sulphuric acid (H_2SO_4) was carefully added to form a layer. A reddish brown colouration of the interface indicates the presence of terpenoids.

2.7.2 Test for Flavonoids

0.05 g crude extract was added to 5 mL of diluted ammonia solution followed by 1 mL of concentrated sulphuric acid. A yellow colouration that appears on standing indicates the presence of flavonoids.

2.7.3 Test for Saponins

0.05 g of crude extract was shaken vigorously with 5 mL of distilled water in a test tube. The formation of stable foam, honeycomb in shapes, was taken as an indication of the presence of saponins.

2.7.4 Test for Tannins

0.05 g of crude extract was mixed with 5 mL chloroform and 1 mL acetic anhydride. 1 mL of concentrated sulphuric acid was added slowly. The formation of a green precipitate was an indication of the presence of tannins.

2.7.5 Test for Steroids

0.05 g of crude extract was mixed with 2 mL of acetic anhydride. Few drops of concentrated sulphuric acid were added. Greenish colour indicates the presence of steroids.

2.7.6 Test for Carbohydrate

0.05 g of crude extract was measured in a test tube and 1 mL of iodine solution was added. A purple colouration at the interphone indicates the presence of carbohydrate.

3. RESULTS AND DISCUSSION

3.1 Sample Preparation

Before drying in the oven, *Aloe vera* leaves were rinsed with distilled water and then cut into small pieces. The weights of *Aloe vera* used before drying and after drying were 317.40 g and 22.5 g respectively. From the calculation, the moisture content of *Aloe vera* was 92.9 %. Therefore, 141.07 g of *Aloe vera* was needed to obtain 10 g of *Aloe vera* powder.

3.2 Isolation and Identification of *Colletotrichum Capsici*

The isolate produces a colour of greyish-white to dark grey on the ventral surface with cottony colonies on potato dextrose agar [PDA] and the reverse of the colonies was black colour. Compared to the research done, the colony colour of *C. capsici* is white to grey colour with cottony mycelium and then it will develop into dark grey to graphite grey on PDA media (Kumar *et al*., 2015; Shenoy *et al*., 2007). Besides, the colony was observed for colony reverse in terms of concentric ring and zones formed by acervuli. The figures 1 and 2 showed the isolated fungus with greyish-white to dark grey colony colour with cottony colonies and concentric rings on PDA.

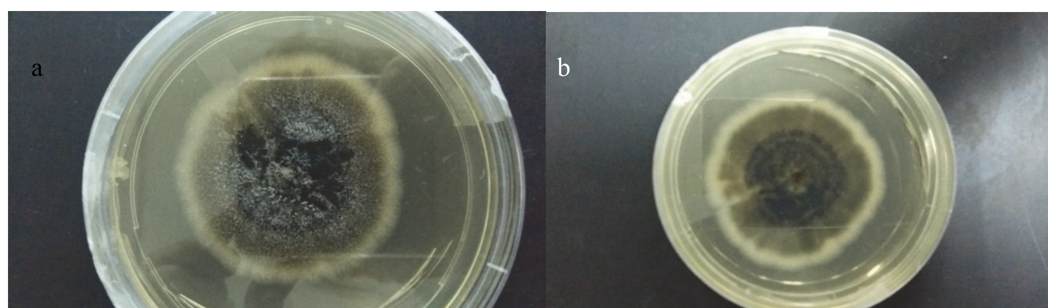

Figure 1. (a) Ventral surface of *C.capsici* on PDA plate after 7 days at 30°C (b) Reverse surface of *C. capsici.*

Morphology of conidia also has been observed under the microscope. The main characteristic of *C. capsici* is falcate or curved moon shape. The Plate 3 below showed the result of falcate shape conidia under microscope observation.

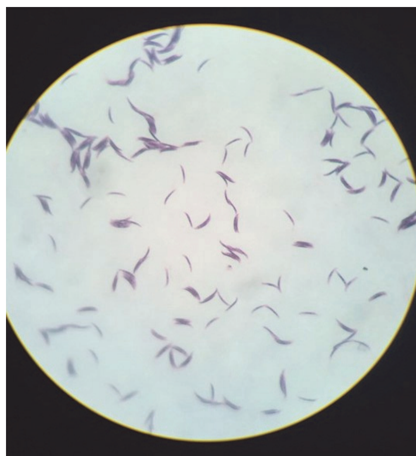

Figure 2. Conidia under microscope (60 x magnifications).

3.3 Antifungal Activities against *C. Capsici*

Poisoned food technique was performed to investigate the antifungal efficiency of *Aloe vera* extract. This method is reversed by disc diffusion method, where the larger zone of inhibition indicates the lower inhibition of extract and vice versa. Besides, the preparation of agar was also different with disc diffusion method, the plant extract with various concentrations was added into agar solution before solidified. The zones of inhibition for *C. capsici* were measured by using ruler which is shown in Table 1.

Table 1 Zone of inhibition of different concentrations of *Aloe vera* extract

Concentration of extract (ppm)	*C. capsici*	
	Inhibition zone (mm)	**Percentage of inhibition (%)**
1000	50.33	1.31
2000	50.00	1.96
3000	49.67	2.61
4000	49.00	3.92
5000	47.33	7.20
Positive control (Mancozed)	51.00	100.00
Negative control (DMSO)	0	-

According to Table 1, it showed a significant inhibitory activity in all concentrations of *Aloe vera* extract compared to the negative control. From 1000 ppm to 5000 ppm, the zone of inhibition was decreased when the concentration of plant extract increased. This analysis shows that there has inhibitory activity towards fungi but with a lower percentage of inhibitory which from 1.31 % to 7.20 %. The percentage of inhibitory was determined by comparing negative control with each concentration of plant extract. Figure 3 below showed the comparison of the inhibition activity of different concentrations of *Aloe vera* extract with negative control DMSO. From ANOVA analysis, the p-value is smaller than 0.05. This means that there is a significant difference among the data.

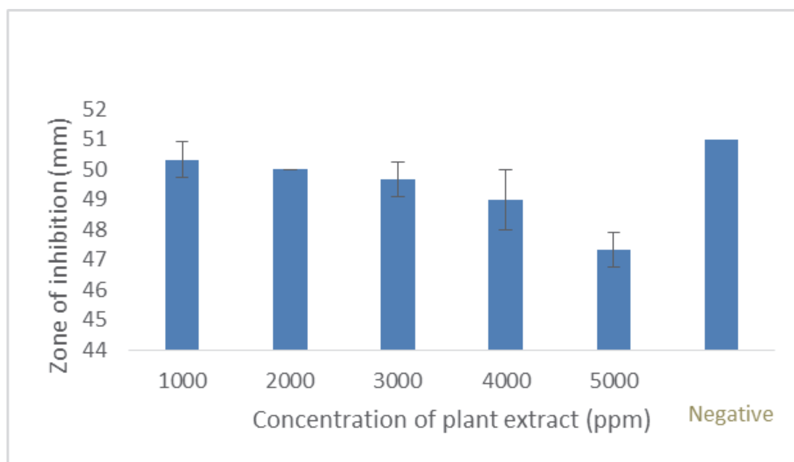

Figure 3. Zone of inhibition in different concentration of plant extract for antifungal activity.

The positive control used was a commercial fungicide, Mancozed to test the effectiveness of *Aloe vera* extract towards *C. capsici* while DMSO used as the negative control. There have a huge different between antifungal activity by plant extract with the antifungal activity by Mancozed. Mancozed showed no fungal growth on PDA, this means that this fungicide is very effective to treat anthracnose disease and it showed 100% inhibitory towards *C. capsici.* For the plant extract, the zone of inhibition was 47.33 mm at the highest concentration of plant extract. The smaller inhibition zone indicates the stronger inhibition against *C. capcisi*.

Based on the research from Nidiry *et al*. (2011), inhibition zone for antifungal activity against *C. capsici* by using methanol extractive of *Aloe vera* was 10.6 %, while for the ethanol extract it was 7.2 %. Ethanol and methanol are polar solvents as the presence of OH group. However, ethanol is lesser polar than methanol because of the alkyl chain of ethanol is longer than methanol. Therefore, methanol is more effective for plant extraction so the antifungal activity of methanol extract showed the higher inhibition zone than the ethanol extract.

From this result, it can be concluded that Mancozed is effective to inhibit the growth of *C. capsici* while *Aloe vera* extracted from ethanol gives a lower inhibition against *C. capsici*. Figure 4 below showed the inhibition zone of *C. capsici* with different concentrations of *Aloe vera* extract and the positive and the negative control on PDA.

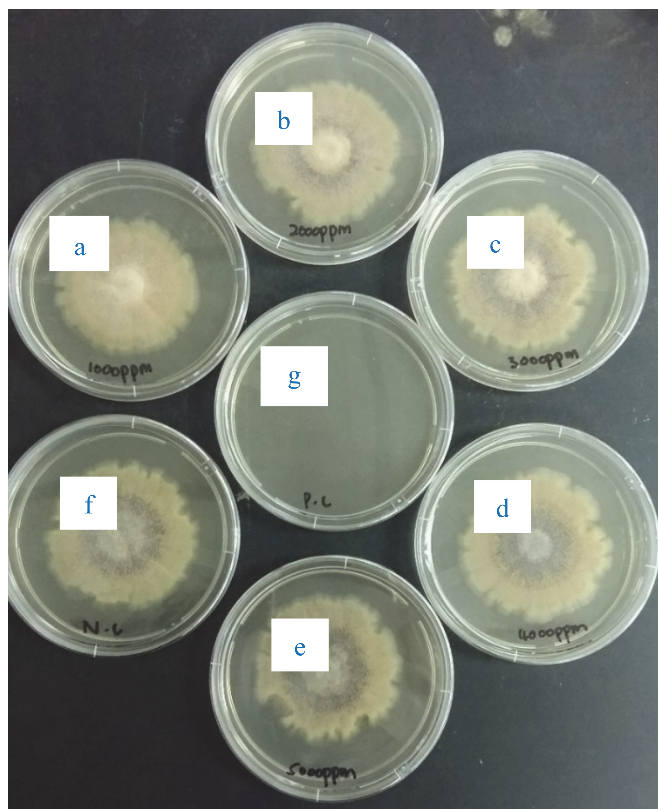

Figure 4. Antifungal activity with different concentrations of *Aloe vera* extract and positive and negative control (a) 1000 ppm (b) 2000 ppm (c) 3000 ppm (d) 4000 ppm (e) 5000 ppm (f) Negative Control (g) Positive Control.

3.4 Antibacterial Activities against *B. Subtilis*

Antibacterial activity was carried out by using disc diffusion method. The zone of inhibition in millimeter was measured by using ruler according to different concentrations. Minimal inhibitory concentration (MIC) was determined by preparing different concentrations of plant extract from the higher concentration to lower concentration which was from 200 mg/ml to 1.25 mg/ml. The larger zone of inhibition indicated that the higher inhibitory effect of plant extract. Table 2 showed the zone of inhibition with different concentrations of *Aloe vera* extract.

Table 2 Zone of inhibition with different concentrations of *Aloe vera* extract

Concentration of plant extract (mg/ml)	Zone of inhibition (mm)
	Average *
200	17.67
100	13.33
80	12.00
60	9.33
40	9.00

20	8.00
10	7.67
5	7.33
2.5	7.00
1.25	-
Positive control (Chloramphenicol)	11.00
Negative control (DMSO)	6.00

According to Table 2, the zone of inhibition against *B. subtilis* increase when the concentrations of *Aloe vera* extract increase. The zone of inhibition was increased from 7.00 mm at 2.5 mg/ml to 21.33 mm at 200 mg/ml. The result showed a strong inhibition at 200 mg/ml because it showed a larger inhibition zone than the positive control. From ANOVA analysis, the p-value is smaller than 0.05. This means that there is a significant difference between the data. Figure 5 showed the antibacterial activity with different concentrations of a plant extract with a zone of inhibition against *B. subtilis.*

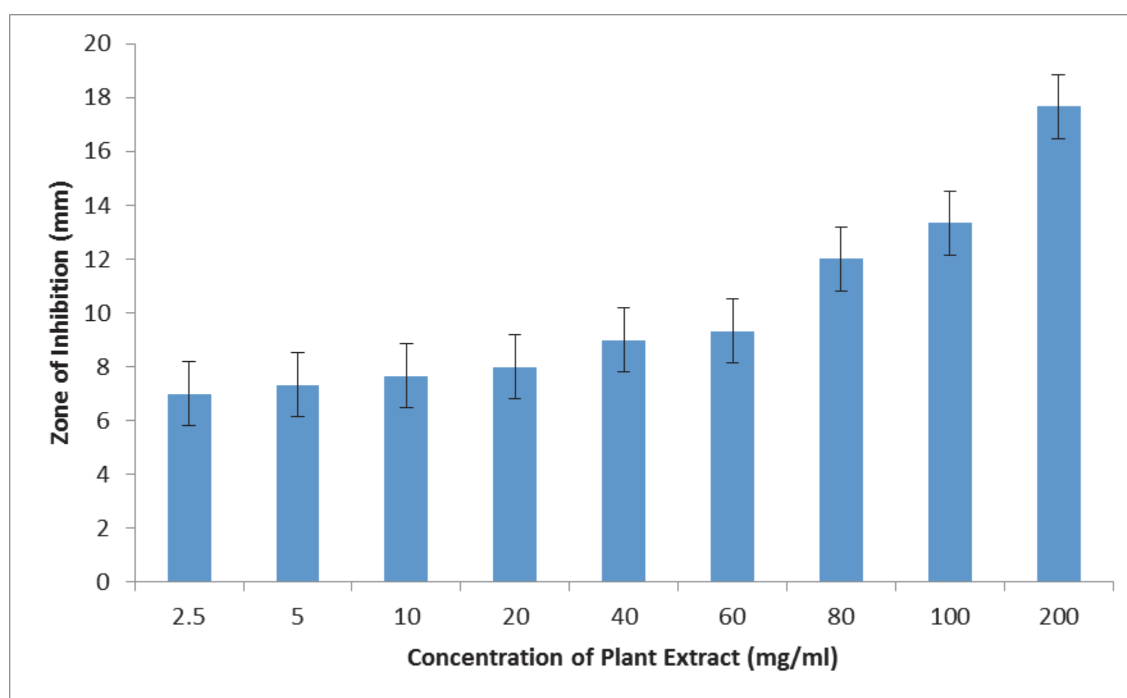

Figure 5. Zone of inhibition in different concentrations of plant extract for antibacterial activity.

The positive control used was chloramphenicol and its inhibition zone showed 11 mm. Based on the previous report by Racadio *et al*, 2008, the positive control has inhibition zone of 18 mm which is larger than this experiment. However, the inhibition zones of positive control are also smaller than 200 mg/mL of *Aloe vera* extract. This indicated that *Aloe vera* extract is effective than chloramphenicol. Negative control (DMSO) showed no inhibition of *B. subtilis. Aloe vera* extract at the concentration of 1.25 mg/ml does not show any inhibition zone against *B. subtilis*. This indicates that the minimum inhibitory concentration (MIC) is 2.5 mg/ml. MIC is the lowest concentration of plant extract that inhibits the bacterial growth. Overall, *Aloe vera* has shown the antibacterial activity and to be very useful in the discovery of natural bactericide.

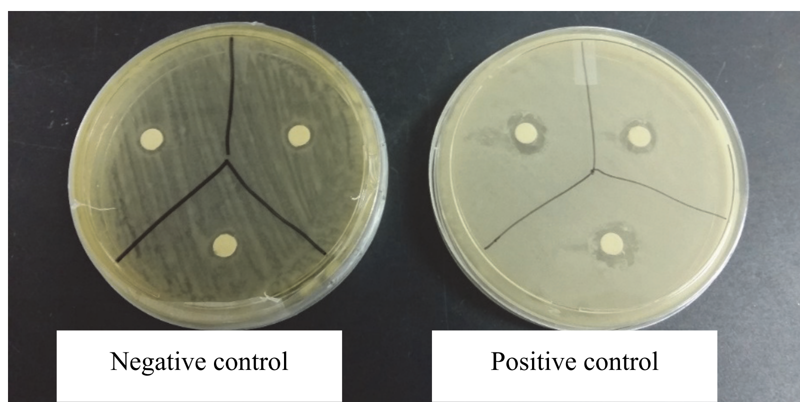

Figure 6. Zone of inhibition with positive control and negative control.

3.5 Phytochemical Properties

The results from phytochemical examination were shown in Table 3. The phytochemical screening of the extract of *Aloe vera* indicated that the presence of terpenoids, flavonoids, steroids, and tannins. Saponins and carbohydrate showed the negative result on the phytochemical test. According to the research done by Thu *et al*. (2013), the result showed the presence of saponins in *Aloe vera* extract but they were absent in this project. The result did not show frothing after shaken where phytochemical analysis from Raphael (2012) has shown that absence of tannins in aqueous *Aloe vera* extract.

Phytochemicals which reported to have antimicrobial activities were tannins, terpenoids, flavonoids and alkaloids (Hayriye, 2011; Sulaiman *et al*., 2011). Terpenoids are active against fungi, bacteria, protozoa, and viruses. Terpenoids produced from an ethanol-soluble fraction of purple prairie clover showed an excellent activity against *Staphylococcus aureus* and *Bacillus subtilis.* For flavonoids, it has been found as effective antimicrobial substances against a wide range of microorganisms. This is due to their ability to complex with the extracellular and soluble protein and to complex with the bacterial cell walls. Besides that, tannins are able to inactive the microbial adhesins, enzymes, cell envelope transport proteins as their antimicrobial actions (Doughari *et al*. 2009; Thu *et al*. 2013).

Table 3 Qualitative analysis of the phytochemicals in *Aloe vera*

Sample No	Test	Result
1	Terpenoids	Presence
2	Flavonoids	Presence
3	Saponins	Absence
4	Tannins	Presence
5	Steroids	Presence
6	Carbohydrate	Absence

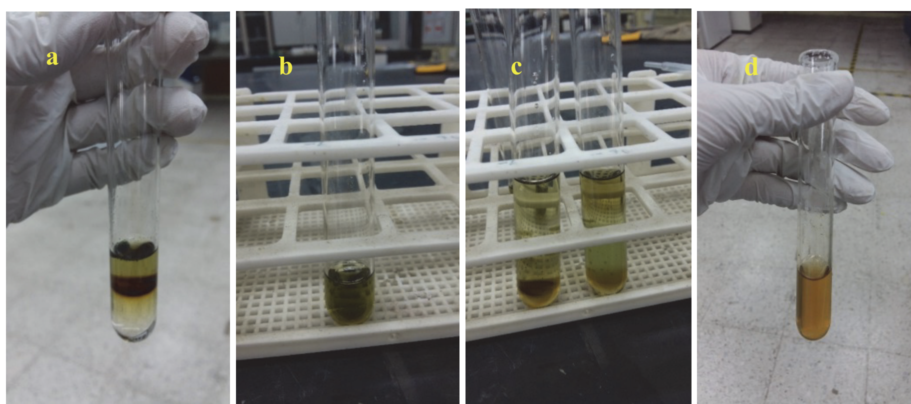

Figure 7. Phytochemical test (a) Presence of Terpenoids; (b) Presence of Steroids; compound (c) Presence of Tannins; (d) Presence of Flavonoid.

4. CONCLUSIONS

In this project, *Aloe vera* was studied for its antifungal activity against chili anthracnose fungus, *C. capsici* and its antibacterial activity against *B. subtilis*. Firstly, *C. capsici* was successfully isolated from the chili. The main characteristics of the isolated fungus to be identified as *C. capsici* are its greyish-white colony color, curved conidia, the presence of rings of acervuli and fluffy cottony look on PDA. Next, five concentrations (1000 ppm, 2000 ppm, 3000 ppm, 4000 ppm and 5000 ppm) were used in an antifungal experiment to inhibit the growth of causative fungus of chili anthracnose, *C. capsici*. From the result, the zone of inhibition decreases when the concentration of plant extracts increases. The inhibition zone of the negative control is smaller than the inhibition zone of plant extracts. The inhibition zone of 1000 ppm was 1.13% while 5000 ppm was 7.2%. This showed a lower inhibition activity against anthracnose disease. In antibacterial activity, ten concentrations of *Aloe vera* extract (1.25 mg/ml, 2.5 mg/ml, 5.0 mg/ml, 10 mg/ml, 20 mg/ml, 40 mg/ml, 60 mg/ml, 80 mg/ml, 100 mg/ml and 200 mg/ml) were used for antibacterial activity. The results showed that 200 mg/ml exhibit a strong inhibition while 100 mg/ml and 80 mg/ml exhibit the moderate inhibitions towards *B. subtilis*. As a conclusion, *Aloe vera* can be used to inhibit *B. subtilis* to grow and has potential to act as antibiotic in future. *Aloe vera* also can be an alternative fungicide to replace chemical fungicide but still need to do some research to enhance the inhibitory activity.

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