

The Effect of Landscape Altitude on Antibacterial activities in Ethanolic Extract of Cocoa leaf (*Theobroma cacao*)

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Abstract : Cocoa is one of the leading plantation commodities in Indonesia. The part of cocoa trees that can be found in abundance is the leaf. The leaves cut out of cocoa trees are utilized by some farmers in composting, when in fact cocoa leaves have a great potential to be processed into health care products. The present study aims to determine the effect of high altitude landscape on antibacterial activities in ethanolic extract of cocoa leaves (*Theobroma cacao*, L). For the experiment, we employed a Completely Randomized Design (CRD) that is divided into 3 treatments—Aqua Dest (Control), ethanolic extract of high-altitude cocoa leaves (P1), and ethanolic extract of low-altitude cocoa leaves (P2)—repeated 5 times. The results show that the largest resistance zones of *Eschericia coli* and *staphylococcus aureus* are created by ethanolic extract of high-altitude cocoa leaves with the size of 19.96 mm and 20.52 mm, respectively. Our ANOVA test shows that extracts of both high- and low-altitude cocoa leaves have an effect on the growth of *S.aureus* ($P = 0,000$) and *E. coli* ($P = 0,000$) bacteria. From Duncan's test we can conclude that ethanolic extract of high-altitude cocoa leaves differs significantly ($P < 0.05$) from its lower altitude counterpart in inhibiting the growth of *E.coli* and *S.aureus* bacteria.

Keywords : cocoa leaf, *Theobroma cacao*, highlands, lowlands, antibacterial.

Introduction

Cocoa (*Theobroma cacao*) is one of plantation commodities in Indonesia. This species continues to be cultivated for its high economic value and serves as a source of income for the community. Cocoa tree main parts consist of stems, leaves, flowers, fruit and seeds. The part of the tree widely used to make chocolate is the seed, or cocoa bean. As for the leaves, they constitute the part of the tree that can be found in abundance. The pruning of cocoa trees is generally done every six months to maintain a healthy growth of the pod. While some farmers have been using leaves cut out of cocoa tree for making compost, still more farmers considered them as nothing more than leaf litters.¹ In fact, cocoa leaves have the potential to be processed into more valuable products, such as healthcare products. This has been confirmed by a number of studies, such as Sigh (2015), which reported that methanolic extract of cocoa leaves inhibits the growth of *Staphylococcus aureus*,

Klebsiella pneumonia and *Shigella dysenteriae* bacteria. Sigh also reported that methanolic extract of cocoa leaves contain chemical compounds such as saponin, flavonoid and tanin.² This result is in line with that of Baharum's (2014) who reported that the extract of cocoa leaf contains secondary metabolites, such as saponins, flavonoids, tannins, triterpenoids and steroids.³

The quality of secondary metabolite produced by plants is affected by ecological factors, such as the altitude of landscape.⁴ This was in agreement with a number of studies, one of them is Fatchurrozaq et al (2013), which reported that the content of vitamin C and antioxidant in *Carica pubescens* increases with the higher altitude.⁵ Another study by El-Jalel et al (2017), which reported that antibacterial activities in *Thymus capitatus* L. essential oils of lower altitude landscape of Abu-draa have larger zone of inhibition than that of *Thymus capitatus* L essential oil from higher altitude landscape.⁶ Cocoa can be found in both higher and lower altitude. For that reason, we are interested in assessing "The Effect of Landscape Altitude on Antibacterial Activities in Ethanolic Extract of Cocoa Leaf (*Theobroma cacao*)."

Materials and Methods

Equipments:

Blender, digital scales, maceration container, stirrer, beaker glass, *vacum rotary evaporator*, Erlenmeyer, hot plate, petri dish, test tube, tube rack, round ose, Bunsen burner, pincher, autoclave, incubator.

Materials:

Cocoa leaves from Saree highlands and Sigli lowlands, ethanol 96%, aqua dest, *Staphylococcus aureus* and *Escherichia coli* bacteria, *Nutrient Agar* (NA), NaCl 0.9%, BaCl and H₂SO₄, empty disc.

Methods

Extraction of Cocoa Leaves

Each of highland and lowland cocoa leaf powders are prepared in 100 gram of weight. They are subsequently macerated with 1000 mL of ethanol 96% for 24 hours, and then filtrated. The remaining dregs are then remacerated using 500 mL of the same solvent. The resulting filtrat is evaporated using *vacuum rotary evaporator* to yield a thick extract.

Phytochemical Test

Test for Alkaloids is conducted by adding 5 mL of distilled water, 10 ml of CHCl₃, and ammonia 0.05 N into the sample, stirs it up until separation occurs. 0.5 mL of H₂SO₄ 2 N is then added to the CHCl layer, stirred up until separation occurs. The acid layer is removed. One drop of Mayer or Dragendroff reagent is added. Positive alkaloid is characterized by white deposit (Mayer) or orange-coloured deposit (Dragendroff).

Test for flavonoids is conducted by adding thick hydrochloric acid and propanolol into the sample, let it stands for 15 to 30 minutes. Red color indicates the presence of flavonoids.

Test for saponnins is performed by vertically shaking the test tube. The presence of saponin is indicated by the foaming that last for ± 15 minutes.

Test for steroids and terpenoids is conducted by adding Lieberman-Burchard reagent to the residual sample. Red color indicates the presence of terpenoids, blue and green color for steroid.

Test for tannins is performed by adding gelatin to the sample. Formation of white or cloudy precipitate indicates a positive result.

Antibacterial Testing

Cotton buds were dipped into bacterial suspension, innoculated evenly with bacterial suspension on a hardened surface of NA media. Place a dish containing aqua dest (P0), ethanolic extract of high altitude cocoa

leaves (P1), and ethanolic extract of low-altitude cocoa leaves (P2) on a medium that already divided into 3 different areas (P₀, P₁, P₂). Incubate at 37°C for 2×24 hours. Observe clear zones developed along the dish lining and measure the inhibiting zone developed.

Data Analysis

Data obtained in this study were statistically analyzed using F-test (one-way Anova test) and Duncan's Multiple Range test.

Results and Discussion

Phytochemical test revealed that ethanolic extract of high-altitude cocoa leaves contains alkaloids, tannins, flavonoids and triterpenoids. As for the low-altitude cocoa leaves, their ethanolic extract contains saponins, tannins, steroids, and flavonoids (Table 1).

Table 1. Phytochemical test results of ethanolic extracts of high-altitude and low-altitude cocoa leaves.

Phytochemical test	Ethanolic extract of high-altitude cocoa leaves	ethanolic extract of low-altitude cocoa leaves
Alkaloid		
a. Wagner	-	-
b. Dragendrof	+	-
c. Bouchardat	+	-
Saponin	-	+
Tannin	+	+
Steroid	-	+
Flavonoid	+	+
Triterpenoid	+	-

The results of this study showed that the extracts of both high- and low-altitude cocoa leaves had antibacterial activities that affect the growth of *Staphylococcus aureus* and *Escherichia coli* bacteria, as indicated by the development of inhibiting zones around the sample discs. The average diameter of inhibiting zones of high- and low-altitude cocoa leaf extracts for *Staphylococcus aureus* is 19.96 mm and 14.36 mm, respectively. As for *Escherichia coli*, the average diameter of inhibiting zones of high- and low-altitude cocoa leaf extracts is 20.52mm and 15.08 mm, respectively.

The results of the Anova indicate that the extracts of cocoa leaf from high-altitude and low-altitude landscapes have a significant (P=0.000) effect on the growth of *Staphylococcus aureus* (Table 2) and *Escherichia coli* (Table 3).

Table 2. Inhibition zone diameter of of ethanolic extracts of high-altitude and low-altitude cocoa leaves of *Staphylococcus aureus*.

Treatment	Inhibition zone diameter ± SD	p-value
Aquadest (control)	0,00 ^a ± 0,000	0,000
Ethanolic extract of high-altitude cocoa leaves	19,96 ^c ± 0,52	
Ethanolic extract of low-altitude cocoa leaves	14,36 ^b ± 1,03	

Table 3. Inhibition zone diameter of of ethanolic extracts of high-altitude and low-altitude cocoa leaves of *Escherichia coli*.

Treatment	Inhibition zone diameter \pm SD	p-value
Aquadest (control)	0,00 ^a \pm 0,000	0,000
Ethanolic extract of high-altitude cocoa leaves	20,52 ^c \pm 1,09	
Ethanolic extract of low-altitude cocoa leaves	15,08 ^b \pm 0,70	

What makes high- and low-altitude cocoa leaves capable of inhibiting the growth of *Staphylococcus aureus* and *Escherichia coli* is the content of chemical compounds that serve as antibacterial agents. Phytochemical test revealed that ethanolic extract of high-altitude cocoa leaves contains alkaloids, tannins, flavonoids and triterpenoids. As for the low-altitude cocoa leaves, their ethanolic extract contains saponins, tannins, steroids, and falvonoids (Table 1).

The antibacterial mechanism of flavonoids works by destroying the permeability of bacterial outer cell wall.⁷ Tannins are phenolic compounds with an antibacterial capability that works by inhibiting the extracelullar enzyme of bacteria.⁸ As for the alkaloids, they have a quaternary aromatic group capable of interacting with DNA.⁹ Alkaloids can also interfere with the constituent components of peptidoglycan in bacterial cells, so that the cell wall layer will not grow fully and this leads to cell death in bacteria.⁷

Duncan's Multiple Range test revealed that the average diameter of inhibiting zones of high-altitude cocoa leaf extracts differs significantly from that of low-altitude cocoa leaf extracts in inhibiting *Staphylococcus aureus* (Table 2) and *Escherichia coli* (Table 3) bacteria. The average diameter of inhibiting zones indicates that high-altitude cocoa leaf extracts have a stronger ability to inhibit the growth of *Staphylococcus aureus* and *Escherichia coli*, as can be seen in figure 1.

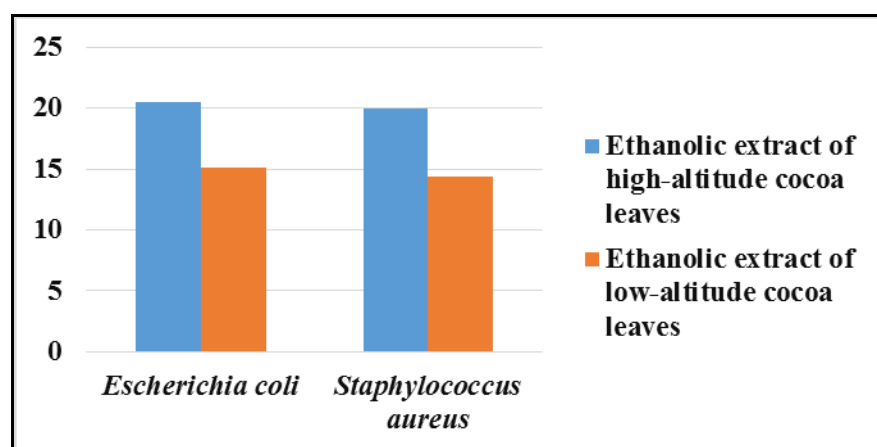


Figure 1. Inhibition zone diameter of high and low altitude cocoa leaf extract on the growth of *S. aureus* and *E.coli*

The difference in ability to inhibit bacterial growth is thought to be due to differences in chemical levels of cocoa leaves from different landscapes. The altitude of the landscape affects plant metabolic process, such as secondary metabolite synthesis. The higher the landscape elevation, the higher the ecological stresses, such as lower temperature, higher humidity level, lower solar intensity, and shorter sunshine duration. Stress, temperature, sunlight, humidity, and other factors affect the production of plant secondary metabolites. Under a stressful condition, the production of plant secondary metabolites will increase. Our results are in agreement with those of Fatchurrozak's (2013), which stated that the content of antioxidant in the flesh of *C.pubescens* fruits increases with landscape elevation.⁵ The difference in bacterial inhibition is also affected by the composition of active compounds in the cocoa leaf extracts.¹⁰

From the development of inhibition zone, we can see that the extract of cocoa leaves from high-altitude landscape has larger diameter of inhibition zone for gram-negative bacteria (*Escherichia coli*). This could be

due to differences in the composition of secondary metabolites found in the extract and to differences in cell wall structure of each bacterium. *Escherichia coli* has a lipid membrane on the cell wall. Lipid itself is non-polar in nature, so that the antibacterial compounds, such as alkaloids—which also non-polar in nature and can be found in the extract of cocoa leaves from high-altitude landscape—can easily penetrate bacterial cells to inhibit the growth of *Escherichia coli*.¹¹ The present study, however, has a limitation that future researchers and other interested party need to consider, i.e. it has not conducted a quantitative test to determine the level of antibacterial compounds in cocoa leaves (*Theobroma cacao*).

Conclusion

1. Extracts of cocoa leaves grown in both high- and low-altitude landscapes have a significant effect on growth rate of *S.aureus* and *Escherichia coli* bacteria ($P = 0,000$).
2. Ethanolic extract of high-altitude cocoa leaves has the average-largest diameter of the inhibition zone and significantly differs from ethanolic extract of low-altitude cocoa leaves in inhibiting the growth rate *Staphylococcus aureus* and *Escherichia coli* bacteria.

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