Metabolite Profile and Antibacterial Potential of Leaf And Stem Extract *Castanopsis tungurrut* (Blume) A.DC. against *Escherichia coli* and *Staphylococcus aureus*

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Abstract. Giving antibiotics or antibacterial drugs is one of the therapeutic strategies used to overcome the problem of infectious diseases caused by microorganisms. The emergence of bacteria that are resistant to various types of antibacterials can delay the recovery period from infectious disorders and cause treatment with antibiotics to become ineffective to use. For this reason, research to find new alternative antibacterial drugs from natural materials needs to be carried out. This study aims to determine the potential of *Castanopsis tungurrut* leaf and stem extracts as natural antibacterials against *Escherichia coli* and *Staphylococcus aureus* bacteria. The research method was extraction of samples by maceration with ethanol, ethyl acetate and aquadest. Antibacterial test with Kirby-Bauer method or disc diffusion. Identification of secondary metabolite compounds by TLC method and continued with metabolite profiling by spectrophotometric method. The results showed the highest antibacterial activity was found in 70% ethanol extract of stem at a concentration of 400 mg/mL with an inhibition zone diameter of 9.06 mm. The 70% ethanol leaf extract was detected to contain phenolics and flavonoids and the ethyl acetate leaf extract was detected to contain triterpenoid and phenolic compounds evaluated by TLC. The metabolite profile showed that ethyl acetate extract had maximum absorbance at 400-600 nm. The 70% ethanol extract has maximum absorbance at 270-350 nm. The distilled water extract had maximum absorbance at 205-250 nm.

Keywords: *Castanopsis tungurrut,* Antibacterial, Secondary Metabolite, Spectrophotometry, Thin Layer Chromatography, Chemometric

1 Introduction

Gram-positive bacteria like *Staphylococcus aureus* and gram-negative bacteria like *Escherichia coli* are the most frequent bacteria that infect and cause health issues in the population. In order to reduce the incidence of serious diseases caused by microorganisms, the administration of antibiotics or antibacterial drugs is one of the therapy strategies used to combat the problem of infectious diseases. Antibiotics in the forms of amoxicillin, penicillin, tetracycline, and other antibiotics are extensively produced and widely used[1].

The onset of numerous side effects, such as diarrhea, vomiting, allergic responses, and other digestive diseases, is a common concern among[2]. The emergence of bacteria that are resistant to various types of antibacterials can delay the recovery period from infectious disorders and cause treatment with antibiotics to be no longer effective for use [3]. According to Jaja et al. (2020)[4] *E. coli* and *S. aureus* bacteria have high antibiotic resistance in clindamycin, ampicillin, rifampicin, streptomycin, and amoxicillin antibiotics.

Antibiotic resistant microorganisms are a key issue in the development of antibiotic medications today, necessitating the use of alternative therapies. One of these is the use of antibacterial agents derived from plant secondary metabolites. The use of secondary metabolites from plants has been extensively used in traditional medicine. Traditional medicine is recognized as having less negative effects than synthetic drugs and uses materials that are widely available [5]. Secondary metabolites found in plants include tannins, terpenoids, alkaloids, and flavonoids which have been shown to provide antibacterial activity [6].

Castanopsis genus is one recognized plant with antibacterial properties. According to the findings of Khan et al. (2001)[7], the leaves and bark of *Castanopsis acuminatissima* contain many secondary metabolites with antibacterial activity, including flavonoids, tannins, triterpenoids, and saponin. Some Indonesian *Castanopsis* species, such as *Castanopsis tungurrut* and *Castanopsis argentea*, have not been examined extensively about their antibacterial properties.

Based on the background described, this study aims to determine the potential of *C. tungurrut* extract as a

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natural antibacterial in *E. coli* and *S. aureus*. Followed by characterization of the extracts used in this study using Thin Layer Chromatography (TLC) and metabolic fingerprinting using UV/Vis spectrophotometric scanning.

2 Methodology

2.1 Materials

The materials used in this study were leaves and stems of *C. tungurrut* obtained from Kebon Raya Cibodas, West Java. The solvents used for extraction of samples were ethyl acetate, ethanol, and aquadest. All solvents used were analytical grades. The bacteria including *E. coli* (FNCC 0091) and *S. aureus* (FNCC 0047) were obtained from PAU UGM.

2.2 Methods

2.2.1 Extraction of leaves and stems of Castanopsis tungurrut

Powder samples were weighed as much as 250 g. Samples were macerated with several polarity solvents, including ethyl acetate, 70% ethanol, and water with a sample to solvent ratio of 1:10 (w/v). Maceration with ethanol and ethyl acetate solvents was carried out for three days and 4 nights with occasional shaking. Extraction with distilled water was carried out by mixing the dry ingredients in distilled water and heating it at 50 °C for 50 minutes in a water bath. The mixture was incubated in the refrigerator for 24 hours. All the mixture is then filtered using a funnel and filter paper. The filtrate was evaporated using a rotating vacuum evaporator at 40 °C for ethyl acetate and ethanol solvents and 50 °C for water solvents to form a thick filtrate.

2.2.2 Antibacterial assay

Antibacterial assay was performed by Kirby-Bauer method (Filter Paper Disk Agar Diffusion Method) using Agar disk diffusion. The sample is diluted to 100 - 400 mg/mL with 10% DMSO as the sample to be tested. 1 ml of 10% DMSO without sample was used as a negative control. Amoxicillin was dissolved in 10% DMSO to 1 mg/mL as a positive control. Pure isolates were subculture on MHB medium and turbidity levels were measured using a 600 nm OD spectrophotometer in the absorbance range of 0.1 – 0.5 A. Bacteria *E. coli* and *S. aureus* were inoculated into MHA media in petri dishes using the swab method. Each of the 6 paper discs was dripped with 20 µL of 1 mg/mL amoxicillin; DMSO 10%; sample solution 100 mg/mL; sample solution 200 mg/mL; and 400 mg/mL sample solutions were tested in bacterial culture. The bacterial culture was incubated for 24 hours at 37 °C.

2.2.3 Thin layer chromatography (TLC)

The sample selected for the detection of secondary metabolites is a leaf sample with 70% ethanol and ethyl acetate solvent. Samples were diluted to 0.5 mg/mL in each solvent. The content of active compounds was identified using TLC with a stationary phase of GF254 silica gel plates (Merck). The mobile phase with n-hexane and ethyl acetate eluents = 7:3 for the ethyl acetate and toluene, ethyl acetate and glacial acetic acid extract samples = 7:2:0.5. A silica plate with a size of 2 cm 10 cm was added with an upper mark of 0.5 cm and a lower mark of 1 cm. The plate was then activated using an oven with a temperature of approximately 80°C - 100°C for 15 minutes. Eluent saturation was carried out by inserting a rectangular filter paper measuring 18 cm x 2 cm into the TLC vessel. The sample is placed on the right of the plate and the reference compound is placed on the left of the plate. TLC plates were run until the separated sample reached the upper limit mark. The TLC plate was baked for 5 minutes at 80°C - 100°C. The TLC plates were sprayed with reagent ($FeCl_3$, vanillin sulfate, citroborate, dragendorff) to detect phenolic compounds, terpenoids, flavonoids and alkoloids. Observed under UV light with a wavelength of 254 nm, 366 nm and visible light.

2.2.4 Compound profile of secondary metabolites

Identification of compound content in ethyl acetate extract, ethanol, and aqueous leaves and stems *of C. tungurut* was carried out with a UV-Vis spectrophotometer (Genesys 150). The wavelength is set at 200 nm-800 nm. The sample solution is diluted to 0.5 mg/mL as much as 10 mL inserted into the test tube and vortex for approximately 5 minutes. Solution blanks are used with customized individual sample solvents. A total of approximately 3 mL of sample is inserted into the quartz cuvette and the absorbance is read with a UV-Vis spectrophotometer until a chromatogram appears.

2.2.5 Data analysis

Antibacterial activity data were analyzed using ANOVA (Analysis of Variance) method and post hoc analysis of DMRT (Duncan's Multiple Range Test) Test at a signification level of 0.05 to determine the difference in significance between data. Profile analysis of secondary metabolite compounds was carried out using the MetaboAnalyst website (www.metaboanalyst.ca)

3 Result and discussion

3.1 Extraction of leaves and stems of *Castanopsis tungurrut*

The solvent used in this study was ethyl acetate, 70% ethanol and aquadest because all three have different polarity properties. Soluble compounds will follow the principle of "Like Dissolved Like", polar compounds will dissolve in polar solvents and vice versa nonpolar compounds will dissolve in nonpolar solvents. Ethyl acetate polarity level is 0.228; ethanol 0.654; and aquadest 1,000. The higher the level of polarity of the solvent, the more polar the solution will be. It can be seen that aquadest have a very polar level of polarity, ethanol is semipolar, and ethyl acetate is nonpolar[8].

Table 1. Results of Sample Extracts of *Castanopsis tungurrut* Leaves and Stems

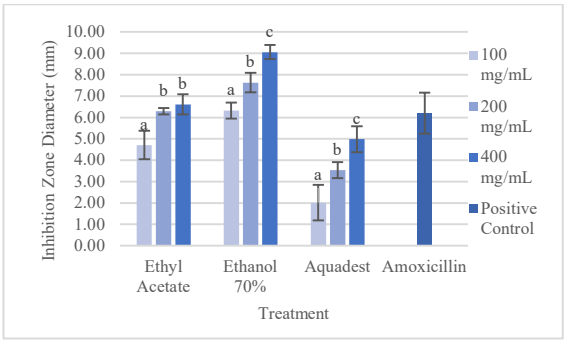
Sample	Solvent	Simplisia Weight (g)	Extract Weight (g)	Yield (%)
Leaves	**Ethyl Acetate**	25,00	2,36	9,44
	Ethanol 70%	25,00	5,06	20,24
	Aquadest	25,00	3,24	12,96
Stems	**Ethyl Acetate**	25,00	2,23	8,92
	Ethanol 70%	25,00	3,53	14,12
	Aquadest	25,00	3,24	12,96

The extraction results obtained using 70% ethanol solvent have the highest yield of around 20.24% and 14.12% of the other three solvents, and ethyl acetate has the lowest yield of 9.44% and 8.92% as seen in the (Table 1). Alcohol has a high enough level of polarity to dissolve polar and nonpolar compounds. Aquades also have high polarity properties and can dissolve more polar compounds. Ethyl acetate is more likely to be nonpolar so that it only extracts secondary metabolite compounds that are nonpolar [9].

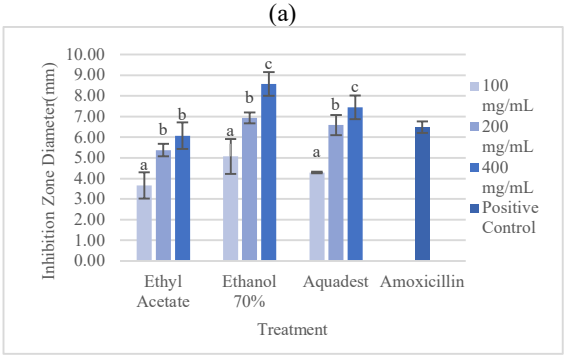
Leaf and stem samples extracted with ethyl acetate solvent have wet properties and tend to be green in color rather than extracts with ethanol and aqueous solvents. Ethyl acetate tends to be nonpolar so it can extract more nonpolar compounds such as essential oils. Based on research conducted by Lohani *et al*. (2015)[10] essential oil is found in ethyl acetate extract of Canola (*Brassica napus*). Extraction samples with 70% ethanol solvent have the same color as aquadest but the ethanol texture is slightly wet compared to aqueous with a dry texture. According to research by Yulianti *et al*. (2020)[11] ethanol solvents include semipolar solvents that can extract more phenolic compounds in cherry fruit (*Mutingia calabura*) leaf samples. Polar aquadest that can extract more polar compounds such as tannins and glycosides [12]

3.2 Antimicrobial activity

Antibacterial assay in this study used the Kirby-Bauer method to order disk diffusion. The samples tested were made at 3 different concentrations, namely concentrations of 100 mg/mL, 200 mg/mL and 400 mg/mL. Antibacterial activity is characterized by the formation of a clear zone around the disc. The wider the clear zone, the higher the antibacterial activity [13].



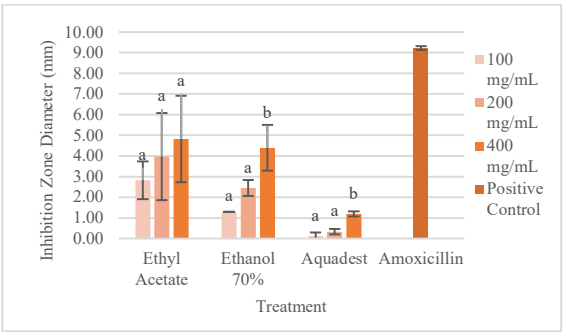
(a)



(b)

Fig. 1. Antibacterial Activity against *E. coli* Bacteria in *C. tungurrut* Leaf (a) and *C. tungurrut* Stems samples (b)

The results of antibacterial assay samples against *E. coli* bacteria are seen in (Figure 1). The difference in letters shows a real difference based on Duncan's post hoc ANOVA analysis with a significance value of $p < 0.05$. The solvent with the highest activity was recorded in 70% ethanol solvent, the concentration of 400 mg/mL leaf samples had an inhibitory zone diameter of 9.06±0.33 mm and the stem sample had an inhibitory zone diameter of 8.58±0.57 mm. Extracts of compounds with alcohol solvents showed the presence of secondary metabolite compounds that more actively inhibited bacterial growth compared to other solvents.



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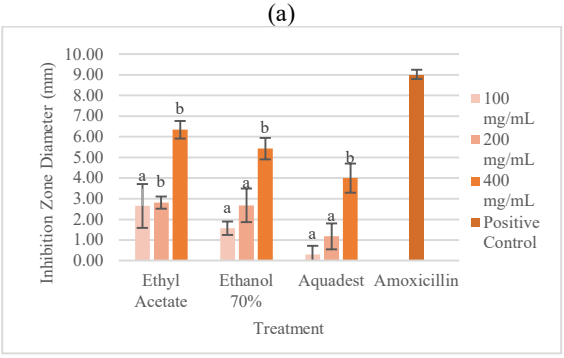


(b)

Fig. 2. Antibacterial Activity against *S. aureus* Bacteria in *C. tungurrut* Leaf (a) and *C. tungurrut* Stems samples (b)

The results of antibacterial assay samples against *S. aureus* bacteria are seen in (Figure 2). The results showed a significant difference in each concentration in 70% ethanol solvent and aquadest in stem organs. The difference in letters shows a real difference based on Duncan's post hoc ANOVA analysis with a significance value of $p < 0.05$. The solvent with the highest activity was recorded in 70% ethanol solvent, 400 mg/mL concentration, leaf samples had an inhibitory zone diameter of 4.39±1.11 mm, and stem samples had an inhibitory zone diameter of 5.43±0.52 mm. In ethyl acetate solvent concentration of 400 mg/mL, leaf samples have an inhibitory zone diameter of 4.82±2.09 mm and stem samples have an inhibitory zone diameter of 6.35±0.43 mm. But the increase in concentration in ethyl solvent does not show any significant difference. Extracts of compounds with alcohol and ethyl acetate solvents showed the presence of secondary metabolite compounds that more actively inhibited the growth of *S. aureus* bacteria compared to other solvents.

The difference in antibacterial activity results between *E. coli* and *S. aureus bacteria* can be influenced by microbial defense mechanisms against antibacterial compounds. Based on a scientific article by Ebbensgaard *et. al.* (2018) [14] gram-negative bacteria have an outer membrane (OM) equipped with a lipopolysaccharide structure (LPS). This structure allows gram-negative bacteria such as E. *coli* to block the entry of compounds toxic to cells. Thick peptidoglycan in *S. aureus* bacteria can decrease the permeability of toxic compounds to enter and damage cells [15].

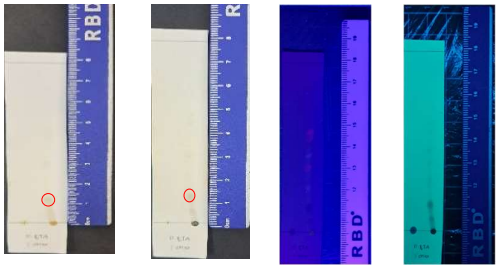
3.3 Thin layer chromatography

Thin-layer chromatography is performed to detect the presence of compounds that have antibacterial activity such as alkaloids, phenolics, flavonoids, and triterpenoids [16]. The sample used for the thin layer chromatography test was selected which had the highest antibacterial activity found in the sample using 70% ethanol and ethyl acetate solvent. TLC Rf value can be seen in (Table 2).

Based on the results of TLC testing in (Figure 3 and Figure 4), 70% ethanol leaf extract samples showed positive results on flavonoid and phenolic compounds. Flavonoid compounds were found to be positive for yellow fluorescence at Rf 0.10 and 0.27 after spraying with citroborate reagents. Phenolic compounds were also found at rf 0.18 after spraying marked with a blue-black color after spraying with $FeCl_3$ reagent. TLC test results on ethyl acetate leaf extract samples showed positive results on triterpenoid and phenolic compounds in (Figure 5 and Figure 6). The triterpenoid compound was found positive in Rf 0.97 purple spots in visible light after spraying with vanillin sulfate reagent. Phenolic compounds are found at rf 0.83 and 0.91 in visible light marked with a blue-black color.

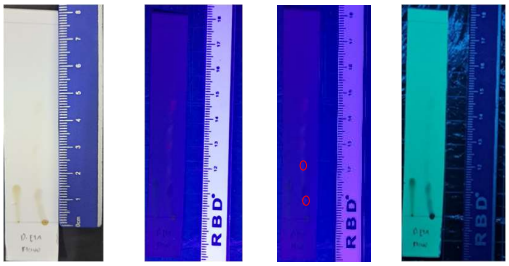
Table 2. Rf TLC Value

Sample	Target Compunds	Rf Value
Ethanol 70% Leaf Extract	Alkaloid	-
	Phenolic	0.18
	Flavonoid	0.10
		0.27
	Triterpenoid	-
Ethyl Acetate Leaf Extract	Alkaloid	-
	Phenolic	0.83
		0.91
	Flavonoid	-
	Triterpenoid	0.97



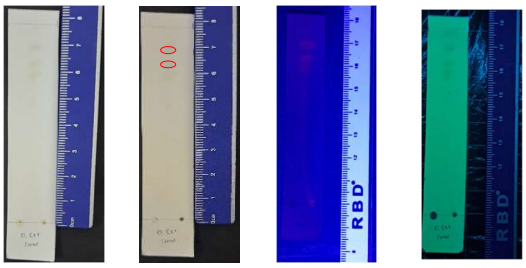
(a) (b) (c) (d)

Fig. 3. TLC Visualization of Phenolic Compounds of 70% Ethanol Leaf Samples in Visible Light Before Spraying (a), After Spraying (b); UV light 366 nm After Spraying (c); and UV Light 254 nm After Spraying (d)



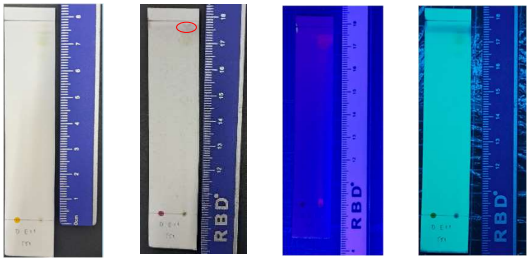
(a) (b) (c) (d)

Fig. 4. TLC Visulization of Flavonoid Compounds Ethanol Leaf Samples 70% on Visible Light (a), UV Light 366 nm Before Spraying (b); UV light 366 nm After Spraying (c); and UV Light 254 nm After Spraying (d)



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

Fig. 5. Visulization of TLC Phenolic Compounds of Ethyl Acetate Leaf Samples in Visible Light Before Spraying (a), After Spraying (b); UV light 366 nm After Spraying (c); and UV Light 254 nm After Spraying (d)



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

Fig. 6. TLC Visulization of Triterpenoid Compounds of Ethyl Acetate Leaf Samples in Visible Light Before Spraying (a), After Spraying (b); UV light 366 nm After Spraying (c); and UV Light 254 nm After Spraying (d)

3.4 Profile of Secondary Metabolites of *Castanopsis tungurrut*

Profiling of secondary metabolite compounds of *C. tungurrut* leaf and stem extracts with ethyl acetate, ethanol, and aqueous solvents was carried out using UV-Vis Spectrophotometry and chromatograms were obtained from each sample. The chromatogram is analyzed by the chemometric method. A commonly used chemometric method is Principal Component Analysis (PCA) [17].

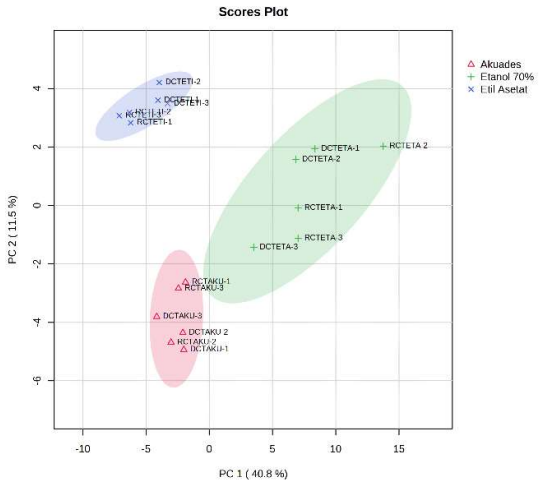


Fig. 7. Visualization *Scores Plot* between Solvent Ethyl Acetate (Blue), Ethanol 70% (Green), Aquades (Red)

The results of the analysis using the PCA method showed a grouping of data on the content of secondary metabolite compounds found in leaf samples and *C. tungurrut* stem is based on solvents in the form of ethyl acetate, ethanol and aquadest. Based on Figure 7, the results of Visualization Scores Plot of *C. tungurrut* leaf and Stem extracts based on the solvent were obtained. The results showed that PC 11 had a value of 40.8% and PC 2 had a value of 11.5%. Each sample is separate and grouped based on its own solvent, showing that each

solvent can extract different metabolite compounds depending on their respective polarities.

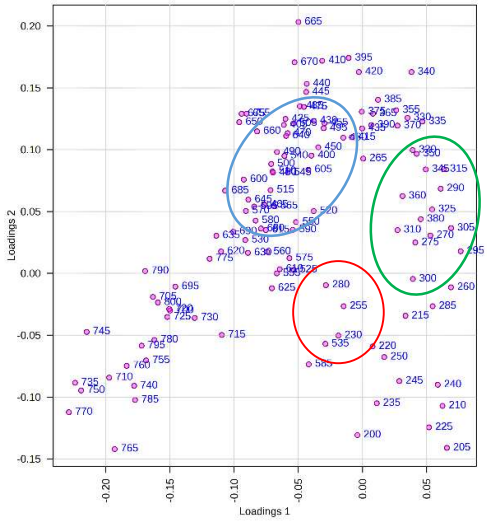


Fig 8. Visualization of Loading Plot between Ethyl Acetate Solvent (Blue), 70% Ethanol (Green), and Aquadest (Red)

Figure 8 shows the results of loading *C. tungurrut* leaves and Stems on each solvent. Each solvent groups and separates based on its spectrum. The first group of ethyl acetate solvents marked with a blue circle showed secondary metabolite compounds capable of absorbing UV-Vis light at wavelengths of 400-600 nm. Based on the absorbance of compounds at wavelengths of 400-600 nm, sample extracts with ethyl acetate solvent contain alkaloids and terpenoids. It is known that alkaloids have absorbance values at wavelengths of 400 – 650 [18]. Terpenoids have wave absorbance at lengths of 520-530 nm [19].

The group marked with a green line shows a grouping of secondary metabolite compounds with 70% ethanol solvent. Grouping based on absorbance wavelength 270 – 350 nm. Based on the absorbance at the wavelength, the sample extract with 70% ethanol solvent contains metabolite compounds in the form of tannins, phenolics and flavonoids. Known Flavonoids and Tannins have absorbance at wavelengths of 210 – 280 nm. Phenolic compounds have absorbance at wavelengths of 200 nm – 400 nm.

Groups marked in red indicate the grouping of secondary metabolite compounds with aqueous solvents. Grouping occurs at wavelength absorbances of 205-230 and 535 nm. Based on absorbance at these wavelengths, sample extracts with aqueous solvents contain secondary metabolite compounds in the form of phenolics, tannins, alkaloids, and triterpenoids [18].

4 Conclusion

The results of this study showed the highest antibacterial activity observed in leaf extracts with 70% ethanol solvent in *E. coli* bacteria. The highest activity was in stem extract with ethyl acetate solvent in *S. aureus* bacteria. Identification of secondary metabolite compounds from ethyl acetate leaf extract found triterpenoid compounds and 70% ethanol leaves found flavonoid compounds. The results of profiling of secondary metabolite compounds of *C. tungurrut* leaf and Stem extracts found alkodaloid and terpenoid compounds with maximum absorption at wavelengths of 400-600 nm. Leaf and Stem extracts from ethanol and aqueous solvents found compounds in the form of flavonoids, phenolics and tannins with maximum absorption at successive wavelengths of 270-350 nm and 205-250 nm.

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