



Antibacterial Activity of Flavonoid-Rich Fractions of *Citrus maxima* Peel Extract

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ABSTRACT

Natural products can be used as an alternative for the treatment of various diseases such as infectious diseases due to the bioactive compounds contained therein. Moreover nowadays, there are many antibiotic resistance in the treatment of infectious diseases. *Citrus maxima* is one of the natural products. *Citrus maxima* have been used for many diseases in traditional medicine. The aim of this study was to investigate the antibacterial activity of flavonoid-rich fractions of *Citrus maxima* peel extract. The bioactive compounds contained in *Citrus maxima* peel were extracted by maceration method using 96% ethanol solvent. Fractionation was conducted using liquid-liquid extraction using a solvent of water and ethyl acetate obtained ethyl acetate fraction. In this fraction, the TLC test was carried out to confirm the presence of phenolic and flavonoid compounds. The antibacterial activity testing for ethyl acetate fraction against *Staphylococcus aureus* and *Escherichia coli* was determined by disk diffusion method with concentration of 25 ppm, 50 ppm, 75 ppm and 100 ppm. The ciprofloxacin and distilled water were used as positive and negative control, respectively. The result of this study showed that ethyl acetate fraction (flavonoid-rich fractions) of *C. maxima* has potential as antibacterial for bacterial *S.aureus* and *E.coli* with medium inhibitory ability in all of concentration ranges. The highest inhibition zone for *S.aureus* was found at a concentration of 100 ppm while for *E.coli* was at a concentration of 75 ppm.

Keywords: Ethyl acetate fraction, Antibacterial activity, Phytochemical screening, Flavonoids

INTRODUCTION

Antibiotic resistance has become a major issue around the world, and has affected patients sincerely and financially (Subramaniam et al., 2020). The large number of antibiotics used in hospitals has made the hospital as a place was first discovered of drug-resistant strains. Penicillin resistant *Staphylococcus aureus* stood up to London civilian hospitals exceptionally before long after

the presentation of penicillin within the 1940. Then, *Mycobacterium tuberculosis* with resistance to streptomycin emerged in the community before long after the disclosure of this antibiotic (Levy & Bonnie, 2004). In arrange to overcome the antibiotic resistnce, alternative healing agents should be investigated.

Medicinal plants play an important role in the refinement and advancement of contemporary studies by serving as a start line

to discover the novelty of the drug. (Wright, 2005) and various modern drugs were extracted from medicinal plants based on the benefits that have been obtained from the used the materials as native cure in lore or ancient system of drugs (Verma & Singh, 2008). Medicinal plants will continue to play a serious role within the primary health care as therapeutic remedies in several developing countries. Medicinal plants have also been known for a long time to treat several infectious diseases throughout human history. The discovery of medicinal plants as a supply of antimicrobial agents is very helpful in increasing the variety of antibiotics that can be obtained (Zaidan et al., 2005).

Citrus maxima commonly known as Pomelo is one of the citrus species, has a place to family *Rutaceae*, developed broadly in tropical and subtropical climates of Southeast Asia, Indonesia, Taiwan, China, India and Philippines (Thielen et al., 2013). *C. maxima* has a medium sized tree. Its leaves have the smell and winged petioles. The flowers are bisexual and sweet scent (Abirami et al., 2013). *C. maxima* have been used for many diseases in traditional medicine. Hot leaf decoction is added to swellings and ulcers. The juice of the fruit is used as a febrifuge. Seeds are used against coughs, dyspepsia and lumbago. Fruit requires the care of cough, fever, heart disease, cancer and gastrointestinal disorders (Kalidhar & Kaur, 2013).

According to studied of Nogata et al. (2006) showed that the flavonoid content in citrus was more abundant in the peels in flavonoids than the in juice and pulp. Flavonoids are one of the most important classes of secondary metabolites that have been reported to possess antimicrobial and antioxidant activity (Akroum et al., 2010; Arokiyaraj et al., 2018; Zengin et al., 2011). The studied of Hasim et al. (2018), showed that fractionation of red yeast ethanol extract with ethyl acetate obtained highest of total flavonoid compared crude ethanol extract, n-hexane, dicloromethane and water fraction (Hasim et al., 2018).

Similar with the result studied of Wijaya et al. (2017), showed that ethyl acetate as a semi polar solvent was the best solvent to obtained flavonoid content (Wijaya et al., 2017). The aim of this study to investigate antibacterial activity of flavonoid-rich fractions of *C. maxima* peel extract.

EXPERIMENTAL SECTION

Materials

The following analytical grade chemicals were used namely methanol, sodium hydroxide, potassium acetate, ethyl acetate, ferric chloride, nutrient agar, muellerhinton agar (Merck) and 96% ethanol. Then, two bacteria strains were used in this study, namely *S. aureus* (gram-positive bacteria) and *E. coli* (gram-negative bacteria). These bacterial strains were obtained from Microbiology Laboratory, FMIPA-USU, Medan, Indonesia.

Instrumentation

The equipment used in this study were TLC Silica gel 60 F₂₅₄ Plates, Analytical Balance (Shimadzu, Model AU220), Micro-pipet, Rotary evaporator (Heidolph), Incubator (Mettler), Laminar Air Flow, Refrigerator, Vials and spray bottle.

Procedure

Plant material and preparation of extracts

The fresh *C. maxima* fruit samples were collected from supermarket in Medan city. Samples were then washed by clean water to remove the impurity. After that, the samples peel was separated from the flesh by knife and cut into smaller size around 1x2 cm. The whole peels of *C. maxima* were fresh dried for one weeks and blended until it forms into powder. The extraction process was carried out by the maceration method where 300 gr of the were macerated by 96 % ethanol at room temperature for 72hour. After that, the extract was filtered using a funnel covered by cotton and the filtrate was concentrated using a rotary evaporator to evaporate the solvent. Furthermore, the extract from rotary evaporator was evaporated again using hot plate to obtained a viscous extract.

Phytochemical screening

The viscous extract of *C. maxima* were qualitative tested using reported methods to determine the presence of important phytochemicals such as flavonoid, tannins, alkaloids, steroid, terpenoid and phenolic compounds, etc (Tiwari et al., 2011). Briefly, flavonoids presence were determined by mixing extract with few drops of lead acetate solution. Formation of yellow colour precipitate showed the presence of flavonoids. Meanwhile, ferric chloride reagent was used to prove the presence of phenols, extracts were mixed with 3-4 drops of ferric chloride solution and the presence of

phenols were indicated by bluish black colour formation. Presence of alkaloids were tested by dissolving 1 g of extract in aquadest and mixed with 5 ml of 1 % HCl for acidification. After that, 2 ml of filtrate were mixed with several drops of Meyer's reagent. The presence of alkaloids were indicated by yellow coloured precipitate. Presence of saponins were determined by foam testing, 0,5 mg of extract was mixed with 2 ml of water and shaken for a few minutes. The presence of saponins were indicated by the foam produced persists for ten minutes.

Fractionation

Fractionation of *C. maxima* peels was performed by liquid-liquid extraction method. The viscous extract obtained was dissolved in 300 ml of distilled water and then transferred into a separating funnel and mixed with ethyl acetate in an equal volume with the starting solvent. After that, It was shaken gently by occasionally opening the lid of the separating funnel to remove the air inside the funnel. Then, two layers were produced, namely the water layer and the ethyl acetate layer which further be separated. Extraction was repeated to get a fraction of ethyl acetate was almost colorless. The ethyl acetate fraction that has been separated from water, then concentrated by rotary evaporator (Mustarichie et al., 2017).

Thin Layer Chromatography

TLC was carried out according to Kumar et al (2013) (Kumar et al., 2013) with a little modification. Briefly, the ethyl acetate fraction was spotted on a silica plate size 10 cm x 2 cm by the above limit and under limit of 1 cm. A blend of chloroform: methanol: (7:3) was prepared as a mobile phase. Mobile phase was added into chamber and let the mobile phase saturate. After saturated, silica plate was added and allowed the mobile phase to touch bottom base. Then plat silica was evaluated with spray reagent (FeCl₃ 5%).

Antibacterial testing

Antibacterial activity of flavonoid-rich fractions of *Citrus maxima* extract were carried out by the disc diffusion method (Lim et al., 2009)(Bereksi et al., 2018). The ethyl acetate fractions concentration used for antibacterial test were 25 ppm, 50 ppm, 75 ppm and 100 ppm. The ethyl acetate fractions were dissolved using dimethylsulfoxide (DMSO).

Disc diffusion method

The bacterial strains were used this study were *S. aureus* (gram positive) and *E. coli* (gram negative) species were cultured in nutrient agar for 24 hours at 37°C. Suspensions of the bacterial strains were made in isotonic sodium chloride solution refers to turbidity level of McFarland 0.5. Mueller-Hinton agar were prepared according to the manufacturer's description for antibacterial screening. The agar was autoclaved at 121°C and allowed to cool in laminar air flow. Sterile, 6 mm diameter filter paper disc were impregnated with the ethyl acetate fractions with variation concentrations, gently tapped to remove excess liquid, and positioned on seeded plates. The blank disk was impregnated in distilled water and it was used as negative control. The ciprofloxacin was used as positive control. The plates were incubated for 24 h at 37°C. The diameters of inhibition zones were measured in millimeters.

RESULTS AND DISCUSSION

Extraction and Fractionation

The extraction processed of *C. maxima* (300g) peel powder by maceration method with 96% ethanol was obtained as viscous extract of 41.4 gr. Fractionation ethanol extract of *C. maxima* was carried out by liquid-liquid extraction method. The weight result of viscous ethyl acetate fractions namely 28.2 gr. Based on some literature showed that the fractionation process with ethyl acetate was the best solvent to obtained high flavonoid content (Gupta et al., 2015; Hasim et al., 2018; Singh et al., 2007; Wijaya et al., 2017).

Phytochemicals screening

Phytochemicals screening test was performed to discover the content of secondary metabolite such as terpenoids/steroids, saponins, alkaloids, flavonoids, and tannins in the *C. maxima* peel extracts. All results of phytochemical screening are showed in the Table 1.

TLC Analysis

TLC results of the ethyl acetate fraction of *Citrus maxima* peel was observed by spraying the TLC plates with FeCl₃ spray reagents to detect phenolics. After that, the spots were also exposed with ammonia vapour to identify flavonoids compounds. The TLC results are shown in Figure 1.

Table 1. Phytochemical screening of *C maxima* peel extract

No.	Secondary metabolite	<i>C. maxima</i> peel Extract	Indicator
1	Alkaloids	+	Yellow coloured precipitate with Meyer's reagent Black coloured precipitate with FeCl ₃ 5%
2	Tannins	+	(sample was dissolved by methanol) Yellow coloured precipitate with lead acetate reagent
3	Flavonoids	+	Redish-brown with Liebermann-Bouchard's Reagent
4	Terpenoids	+	There wasn't foam produced when shaken and wait until 10 min.
5	Saponins	-	

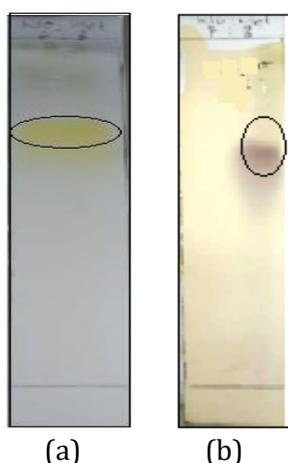


Figure 1. a. TLC results with ammonia vapour, b. TLC Results with 5% FeCl₃ reagent spray. Sillica Gel 60 F₂₅₄ as a stationary phase wa and mixture of chloroform and methanol (7: 3) as a mobile phase.

Antibacterial activity

Evaluation of the antibacterial activity of ethyl acetate fraction of *C. maxima* peel extract was performed by the disc diffusion method against different bacteria. These bacterial strains were *S. aureus* (gram positive) and *E. coli* (gram negative) species frequently found in

infectious diseases. The antibacterial activity of the ethyl acetate fraction of *C. maxima* was observed by the presence or absence of inhibition zone, then the diameters of inhibition zones around each disk were measured in millimeters. Table 2 and Figure 2 showed the results of the diameters of inhibition zones.

Table 2. Bacterial inhibition zones (mm) of ethyl acetate fraction of *C. maxima* peel extract

No	Concentration	Antibacterial activity (zone of inhibition, mm) against	
		<i>S. aureus</i>	<i>E. coli</i>
1	25 ppm	7.7	7.0
2	50 ppm	7.9	7.6
3	75 ppm	8.1	9.0
4	100 ppm	8.4	7.3
5	Positive control (Ciprofloxacin)	28.3	26.05
6	Negative control	-	-

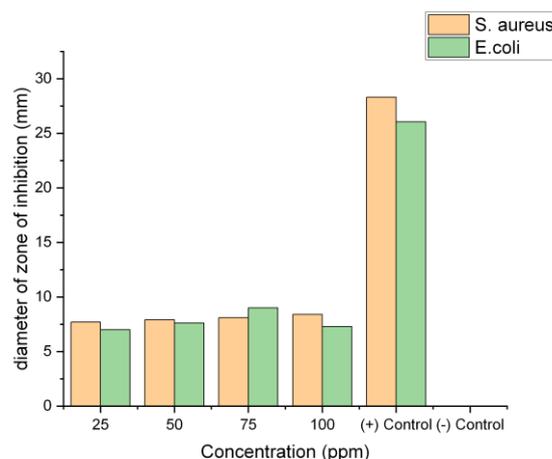


Figure 2. Comparison of zones inhibition produced using concentration variation of ethyl acetate fraction, positive control and negative control on gram positive and gram negative bacterial

From Table 2 and Figure 2 showed that the different of antibacterial inhibition zone diameters of ethyl acetate fraction of *Citrus maxima* peel extract against *S. aureus* (gram-positive) and *E. coli* (gram-negative). These differences can be caused by the differences in the arrangement of the cell walls between the

two bacteria. The cell wall of gram-negative bacteria is more complex than gram-positive. Gram-positive bacteria consist of a single layer, meanwhile the gram-negative cell wall is multi-layer structure (Essawi & Srour, 2000).

The mechanism of antimicrobial of bioactive flavonoids is depend on the interaction between the hydrophilic region of the phospholipids on the cell membranes and penetration of the hydrophobic core at increased flavonoid concentrations (He et al., 2014).

The average diameter of inhibition zone from this present study towards *S. aureus* and *E. coli* for concentrations (25 ppm, 50 ppm, 75 ppm and 100 ppm) showed diameter of inhibition zone in range of 7-9 mm. According to Davis & Stout (1971) (Davis & Stout, 1971), the category antibacterial inhibition zone with diameter in range of 5-10 mm means medium. Based on the inhibition zone obtained, ethyl acetate fraction of *C. maxima* peel extract had a medium antibacterial activity in all concentration treatments (25 ppm, 50 ppm, 75 ppm, and 100 ppm). Although the highest zone of inhibition for *S. aureus* was found at a concentration of 100 ppm while for *E. coli* was at a concentration of 75 ppm. According to literature, it was found that the antibacterial activity is depends on the type of secondary metabolite compounds present in plant extract such as flavonoids, triterpenoids, and other phenolic compound, which are these compounds are categorized as compounds that play a role in antimicrobial (Rojas et al., 1992).

CONCLUSION

This present study showed that the ethyl acetate fraction of *C. maxima* peel extract has potential as antibacterial for bacterial *S. aureus* and *E. coli*. The highest zone of inhibition for *S. aureus* was found at a concentration of 100 ppm while for *E. coli* was at a concentration of 75 ppm. This ethyl acetate fraction possesses flavonoid content according to phytochemical screening and TLC test result.

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