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Antibacterial Potential of Ethanolic Extract of Avocado Leaves (*Persea americana* mill.) against Clinical Isolate of *Klebsiella pneumoniae* and *Proteus mirabilis*

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Abstract

Multidrug resistance is a global health problem that is closely related to microbial resistance. *Klebsiella pneumonia* and *Proteus mirabilis* are opportunistic bacteria that cause urinary tract infections, especially in the use of catheters in hospitals in inpatient care. Handling it using commercial antibiotics can increase the effect of antibacterial resistance if the use is irrational and neglected. Avocado leaves have been reported to have antibacterial, antidiabetic and antioxidant activity. The purpose of this study was to observe into the antibacterial activity of avocado leaves ethanol extract against *K. pneumoniae* and *P. mirabilis*. The minimum inhibitory concentration (MIC) was determined using the Kirby-Bauer method, the minimum bactericidal concentration (MBC) was determined using the streaking method of the inhibition zone formed, and the mechanism of bacterial membrane leakage cell was measured using UV-VIS spectrophotometry at wavelengths of 260 and 280 nm. The MIC value was found to be 3.125 mg/mL in both bacteria, with an inhibition zone of 6.43 ± 0.06 mm (*K. pneumoniae*) and 6.73 ± 0.15 mm (*P. mirabilis*). The MBC value at 200 mg/mL with a percent reduction of 98.35 % (*K. pneumoniae*) and at 100 mg/mL with a percent reduction of 98.06 % (*P. mirabilis*). The supernatant of a membrane leakage cell (DNA and protein) was absorbed at wavelengths of 260 and 280 nm. It can be concluded that the ethanolic extract of avocado leaves has antibacterial potential against clinical isolates of *K. pneumoniae* and *P. mirabilis*.

Keywords: Antibacterial, *Persea americana* mill., *Klebsiella pneumoniae*, *Proteus mirabilis*, Membrane leakage cell, DNA and protein

Introduction

The increasing prevalence of multidrug resistant (MDR) infections in the world has become one of the focuses of the health sector to seek treatment related to this problem. Infections that are a scourge in the world are infections caused by *K. pneumoniae* and *P. mirabilis*. *K. pneumoniae* is one of the bacteria that causes opportunistic infections that cause resistance in hospitalized patients [1]. *K. pneumoniae* causes diseases such as pneumoniae, urinary tract infections (UTIs), bloodstream infections, and sepsis [2,3]. Infections caused by *P. mirabilis* are also an important problem to be followed up in the treatment of MDR [4,5]. Infections caused by *P. mirabilis* are catheter-associated urinary tract infections (CAUTI's) [6]. These bacteria are widely found in the urinary environment because they have urease activity, which often causes polymicrobial urinary tract infections [7,8].

Indonesia is a country that is rich in abundant natural resources and has the potential for utilization as a source of new drugs for the treatment of various diseases [9]. One of the plants that can be used as a medicinal plant is avocado leaf. Because it contains secondary metabolites such as flavonoids, tannins, saponins and alkaloids, the avocado leaves has the potential to be used as traditional medicine [10]. Several

previous studies have also reported the antidiabetic activity of several fractions of avocado leaves extract with a decrease in blood glucose levels of 47.87 % [10]. Other studies have also reported the antibacterial activity of ethanolic extract of avocado leaves against *Escherichia coli* (12.37 mm), *Salmonella typhi* (11.60 mm), and *Pseudomonas aeruginosa* (10.87 mm) [11] and tested their antioxidant activity with a free radical inhibition value of 19.83 % [12,13].

Based on the foregoing, the researchers sought to assess the antibacterial potential of an ethanolic extract of avocado leaves against bacteria that cause urinary tract infections, which frequently infect the catheter area during hospitalization. The minimum inhibition concentration (MIC), activity index value, minimum bactericidal concentration (MBC), and membrane leakage cell at 260 nm (DNA) and 280 nm (protein) wavelengths were all assessed as part of the research.

Materials and methods

Materials and apparatus

The materials used in this research are Muller Hinton Agar (Himedia), Muller Hinton Broth (Himedia), Plate Count Agar (Himedia), Phosphate Buffer Saline (Merck) and Ethanol absolute 96 % (Smartlab). *K. pneumoniae* and *P. mirabilis* are clinical isolate bacteria culture collections from the Microbiology Laboratory, Faculty of Pharmacy, Universitas Sumatera Utara. Oven and incubator (Memmert), UV-VIS spectrophotometry (Thermo Fisher Scientific), centrifuge (Eppendorff), and colony counter (Interscience 300®), Amoxicillin antibiotics dics (30 µg), dimethylsulfoxide (Smartlab), rotary vacuum evaporator (Heidolph) all used.

Methods

Sample preparation

Avocado leaves samples were taken from an avocado plantation in the Delitua, Namorambe, Sumatera Utara, Medan, Indonesia. After the leaves were harvested, they are washed and dried in a tumble dryer until they are brittle. Then process it in a blender until it is powdered [14].

Ethanolic extract of avocado leaves preparation

The dried powder leaves were weighed at as much as 500 g and put into a glass container for extraction. The extraction method used is the maceration method, by soaking the dry powder using 96 % absolute ethanol (pa) as much as 75 parts until the powder is submerged. Left for 5 days, protected from sunlight and occasionally stirred. The mixture was then filtered, macerated once more using 25 parts of the residual solvent, and let to stand for 2 days [15]. After that, it was filtered and combined with filtrate 1 and filtrate 2, then concentrated with a rotary vacuum evaporator until a thick extract obtained [11,16].

Phytochemical screening

Examination of secondary metabolites in the ethanolic extract of avocado leaves was examined for alkaloids, flavonoids, tannins, saponins, and terpenoids/steroids [17].

Flavonoid compound identification

Identify Flavonoid Compounds, Ethylacetate-methanol-water (100:13, 5:10) is the mobile phase, and 10 % $AlCl_3$ reagent stain is visible. A golden orange or greenish yellow stain that forms after a positive reaction is an indication of that flavonoid content [18].

Ethanolic extract of avocado leaves various concentration preparation

The concentration of avocado leaf ethanol extract in the test sample was varied to 300, 200, 100, 50, 25, 12.5, 6.25 and 3.125 mg/mL weighed up to 3 g of thick extract, then dissolved in up to 10 mL of DMSO. Then a series of concentration variations are made using the dilution formula [19].

Determination of minimum inhibitory concentration (MIC)

The Kirby-Bauer method [20] was used to determine the MIC. Inserting 100 µL of the test bacterium inoculum onto a petri dish, 15 mL of MHA medium was poured in, mixed, and allowed to harden [21]. The paper disc was immersed in each concentration variation until saturated while waiting for the medium to solidify. Then, on the surface of the solidified material, each test concentration is deposited. The negative control was DMSO, and the positive control was amoxicillin disc antibiotic. Petri dishes were incubated at 37 °C for 24 h to determine the diameter of the inhibitory zone produced. A digital caliper was used to measure the inhibitory zone's diameter (mm) [22]. The work was done 3 times in a row [23].

Activity index value calculation

Calculation of the index activity value by comparing the value of the inhibition zone formed with the value of the inhibition zone in the positive control. It can be calculated using the formula below [24]:

$$\text{Activity Index} = \frac{\text{DIZ each concentration}}{\text{DIZ positive control}}$$

Note: DIZ = Diameter Inhibition Zone.

Determination of minimum bactericidal concentration (MBC)

The streaking method of determining the diameter of the inhibition zone formed was carried out to determine the minimum bactericidal concentration [25]. After streaking, a sterile cotton swab was dipped into the MHB medium to the extent of 1 mL. Then, from the MHB medium, it was pipetted and cultured on PCA [5]. Incubated the samples and counted the number of colonies at each test concentration. The number of colonies was determined using the colony counter. Then the MBC value was calculated using the formula below [26]:

$$\%\ \text{reduction} = \frac{\text{count of each concentration}}{\text{count of negative control}}$$

Membrane leakage cell (DNA and protein)

The test bacteria were inoculated from the culture stock on agar media for 1 night in liquid media. The inoculum was centrifuged at 3,500 rpm for 20 min, then the supernatant was removed and the bacterial cells were washed with PBS to check for cell leakage. The cleaning procedure was carried out twice. After washing, the bacterial cells were mixed with liquid media and treated with various concentrations of avocado leaves ethanolic extract and incubated for 24 h. The sample was then centrifuged again for 20 min at 3500 rpm. The supernatant from the last centrifugation was used to quantify bacterial membrane leakage cells using UV-VIS spectrophotometry at 260 nm for DNA and 280 nm for protein [27,28].

Data analysis

The data were presented in the form of a mean and standard deviation. The findings of identifying the lowest inhibitory concentration were subjected to an analysis of variance test to see whether there was a significant difference between the test concentration treatment and control (both negative control and positive control).

Results and discussion

Phytochemical screening

The results of phytochemical screening showed that the ethanol extract of avocado leaves was positive for several groups of compounds such as flavonoids, tannins, saponins and terpenoids. The results of phytochemical screening are provided in **Table 1**. The results of alkaloid testing using 3 reagents, namely Mayer, Bouchardat and Dragendorff reagents, showed only 1 positive result, namely Meyer reagent. This means that the ethanol extract of avocado leaves is not positive for alkaloids because if it is positive it contains alkaloids, at least 2 of the 3 reactions tested showed positive results [29]. The results of the examination of flavonoid compounds using Mg (powder) + HCl (p) reagent showed a color change to orange [30]. The reaction that occurs after the addition of the reagent is the reduction of the benzo pyran nucleus and the formation of a red or orange flavylium salt. Tannin examination with 1 % $FeCl_3$ reagent showed positive results with the formation of a blackish green color [31]. Saponin compounds by mixing using hot water and adding 2N HCl showed a stable foam for approximately 10 min, indicating the presence of saponin compounds, and terpenoid/steroid examination showed positive results if a purplish red color was formed [32,33].

Table 1 Phytochemical screening of avocado leaves ethanol extract.

No	Metabolites	Reagents	Result
1	Alkaloids	Mayer	+
		Bouchardat	-
		Dragendorff	-

No	Metabolites	Reagents	Result
2	Flavonoid	Mg powder, HCl (p), Amyl Alcohol	+
3	Tannin	$FeCL_3$ 1%	+
4	Saponin	Hot water and HCl 2N	+
5	Terpenoid	Anhydrous acetic acid and H_2SO_4 (p)	+

Note: + (present); - (absent)

Flavonoid TLC identification

Tests using TLC were used to confirm the presence of flavonoid compounds from the phytochemical test results [34]. Isolation of flavonoid compounds was identified by the thin layer chromatography (TLC) method made of silica gel measuring 2×10 cm^2 GF254, which had previously been activated by heating using an oven at 100 °C for 1 h [35]. The TLC results are shown in **Figure 1**. In the flavonoid group identification test, the TLC method produce yellow spots on the chromatogram which are irradiated with 366 nm UV light. The TLC test results for flavonoid compounds in the extract samples had an Rf value of 0.6. This test was carried out using ethanol as a solvent. Flavonoid compounds are polar compounds; therefore the use of polar solvents will further strengthen the withdrawal of flavonoid compounds so that the color produced in the TLC test is more clearly visible. This test was previously carried out by Pujiastuti [36], and related to variations in the use of solvents with a large number of metabolites identified, proving that solvents that have the same polarity as metabolites will have a higher ability to dissolve compounds. Previous tests also proved that avocados contain flavonoids by TLC [37].

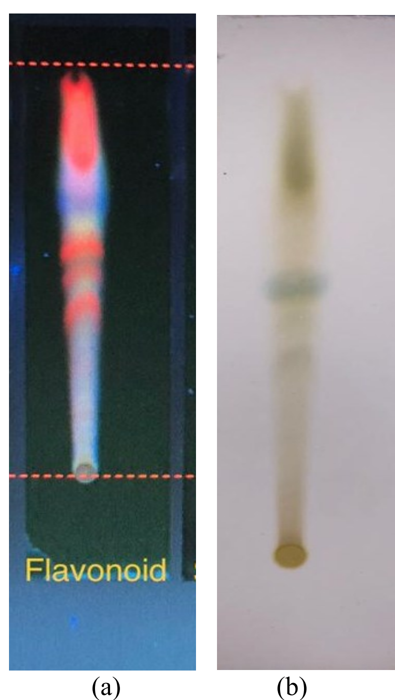


Figure 1 Results of TLC analysis of avocado leaves flavonoids (a) on UV lamp at 366 nm (b) after spraying (without UV lamp).

Determination of Minimum Inhibitory Concentration (MIC)

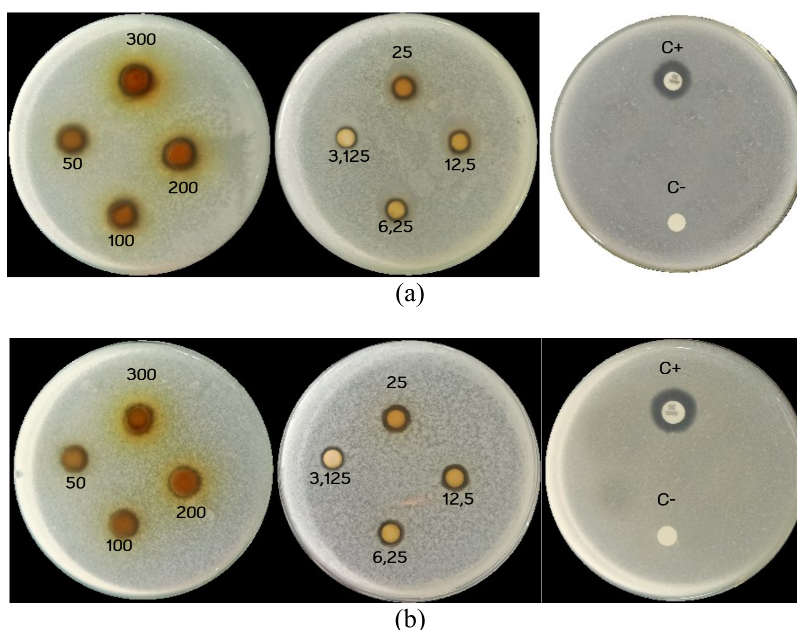
The MIC value of avocado leaves ethanol extract was found to be 3.125 mg/mL in the 2 test bacteria. **Table 2** shows the results of measuring the diameter of the zone where bacterial growth is inhibited. The MIC values for *K. pneumoniae* at 3.125 mg/mL with an inhibition zone diameter of 6.43 ± 0.06 mm and *P. mirabilis* with an inhibition zone diameter of 6.73 ± 0.15 mm were both classified as medium [38]. At a dosage of 200 mg/mL, the bacterium *K. pneumoniae* demonstrated a strong category with an inhibition zone diameter of 10.43 ± 0.29 mm. *P. mirabilis*, on the other hand, demonstrated a strong category at a dose of 100 mg/mL, with an inhibition zone diameter of 10.00 ± 0.10 mm. This is possible because the amount of compound substrate in each concentration changes based on the difference in concentration [38].

The diameter of the inhibitory zone formed is also controlled by the osmosis diffusion power from high to low concentration. In this situation, secondary metabolite chemicals present in the extract conveyed by paper discs diffuse to the media's surface, resulting in an antibacterial mechanism [39]. The MIC is the lowest concentration at which bacterial growth can still be inhibited [39]. The figure of inhibitor zones can see in **Figure 2**. The bacterial inhibition zone category is divided into 3 categories, according to Davis and Stout [40]: very strong if the diameter of the inhibition zone formed exceeds 20 mm, strong if the diameter of the inhibition zone is between 10 and 20 mm, and moderate if the diameter of the inhibition zone is between 5 to 10 mm. If the diameter of the inhibition zone is less than 5 mm, it suggests that there is no activity or that the antibacterial response is ineffective [5].

Table 2 Inhibition zone diameter of avocado leaf ethanol extract against *K. pneumoniae* and *P. mirabilis.*

Conc. (mg/mL)	**Inhibition Zone Diameter (mm)**			
	K. pneumoniae [*]	**Category**	***P. mirabilis*** [*]	**Category**
Positive control	14.33 ± 0.15[2]	Strong	14.87 ± 0.21[2]	Strong
300	11.77 ± 0.15[1]	Strong	11.10 ± 0.20[1]	Strong
200	10.43 ± 0.29[1]	Strong	10.50 ± 0.10[1,2]	Strong
100	9.50 ± 0.10[1,2]	Moderate	10.00 ± 0.10[1,2]	Strong
50	8.70 ± 0.20[1,2]	Moderate	9.20 ± 0.26[1,2]	Moderate
25	8.13 ± 0.12[1,2]	Moderate	8.47 ± 0.06[1,2]	Moderate
12.5	7.77 ± 0.06[1,2]	Moderate	8.00 ± 0.10[1,2]	Moderate
6.25	6.97 ± 0.15[1,2]	Moderate	7.37 ± 0.15[1,2]	Moderate
3.125	6.43 ± 0.06[1,2]	Moderate	6.73 ± 0.15[1,2]	Moderate
Negative control	0.00 ± 0.00[1]	No response	0.00 ± 0.00[1]	No response

Note:
Positive control: Amoxicillin antibiotic disc (30 µg).
Negative control: DMSO.
[*]: data is the average of 3 repetitions
[1]: sig. 0.000: There was a significant difference to the positive control ($p < 0.05$).
[2]: sig. 0.000: There was a significant difference to the negative control ($p < 0.05$).



(a)

(b)

Figure 2 Antibacterial diameter inhibition zones of (a) *K. pneumoniae* and (b) *P. mirabilis.*

Activity index value calculation

The calculation of the activity index value aims to interpret the ability of an antibacterial agent compared to the positive control value. If the calculation result is close to the value of 1.000, it means that the antibacterial activity of the test compound has an ability comparable to that of the positive control [24]. **Table 3** shows the value of the Activity Index computation result. The index activity value calculated at a test concentration of 300 mg/mL was 0.821 (*K. pneumoniae*) and 0.747 (*P. mirabilis*), indicating that the test compound's strength compared to the positive control of amoxicillin antibiotics is approximately 82.1 % (*K. pneumoniae*) and 74.7 % (*P. mirabilis*).

Table 3 Activity index value calculation.

Concentration (mg/mL)	**Index Activity**	
	K. pneumoniae	***P. mirabilis***
Positive control (C+)	1.000 ± 0.00	1.000 ± 0.00
300	0.821 ± 0.05	0.747 ± 0.08
200	0.728 ± 0.12	0.706 ± 0.17
100	0.663 ± 0.06	0.673 ± 0.07
50	0.607 ± 0.10	0.619 ± 0.15
25	0.567 ± 0.11	0.570 ± 0.06
12.5	0.542 ± 0.08	0.538 ± 0.11
6.25	0.486 ± 0.03	0.496 ± 0.09
3.125	0.449 ± 0.15	0.453 ± 0.12
Negative control	0.000 ± 0.00	0.000 ± 0.00

Note:
data is a calculation of the average of 3 repetitions
Positive control: Amoxicillin antibiotic disc (30 μg).
Negative control: DMSO.

Determination of Minimum Bactericidal Concentration (MBC)

The MBC was estimated by subtracting the number of bacteria in each concentration and comparing it to the number of bacteria in the negative control. **Table 4** shows the computation results for determining the MBC value. The MBC in *K. pneumoniae* was exhibited at 200 mg/mL since the MBC calculation results showed a percent reduction value of 98.35 %, whereas the MBC in *P. mirabilis* was shown at 100 mg/mL with a percent reduction value of 98.06 %. If there is a decrease in the percentage reduction value of 98.00 to 99.99 % between the difference in the number of final colonies of each concentration compared to the number of negative control colonies (initial colonies), then the concentration is expressed as MBC [5,41]. The graph of the log reduction is provided in **Figure 3**. The log reduction increases with increasing concentration. This means that the concentration of the test is directly proportional to the number of decreased bacteria. The higher the test concentration, the greater the decrease in the number of bacterial colonies [42,43].

Table 4 Percentage of reduction as determination of MBC for *K. pneumoniae* and *P. mirabilis.*

Concentration (mg/mL)	**Percent Reduction (%)**	
	K. pneumoniae	***P. mirabilis***
Positive control (C+)	100.00 ± 0.00	100.00 ± 0.00
300	99.36 ± 0.07	99.09 ± 0.13
200	98.35 ± 0.14	98.74 ± 0.07
100	92.75 ± 0.12	98.06 ± 0.04
50	83.97 ± 0.04	87.77 ± 0.11
25	70.99 ± 0.11	74.40 ± 0.15
12.5	65.52 ± 0.17	67.31 ± 0.12

Concentration (mg/mL)	Percent Reduction (%)	
	K. pneumoniae	***P. mirabilis***
6.25	57.51 ± 0.07	64.91 ± 0.06
3.125	32.44 ± 0.10	37.26 ± 0.08
Negative control (C-)	0.00 ± 0.00	0.00 ± 0.00

Note:
Positive control: Amoxicillin antibiotic disc (30 µg).
Negative control: DMSO.

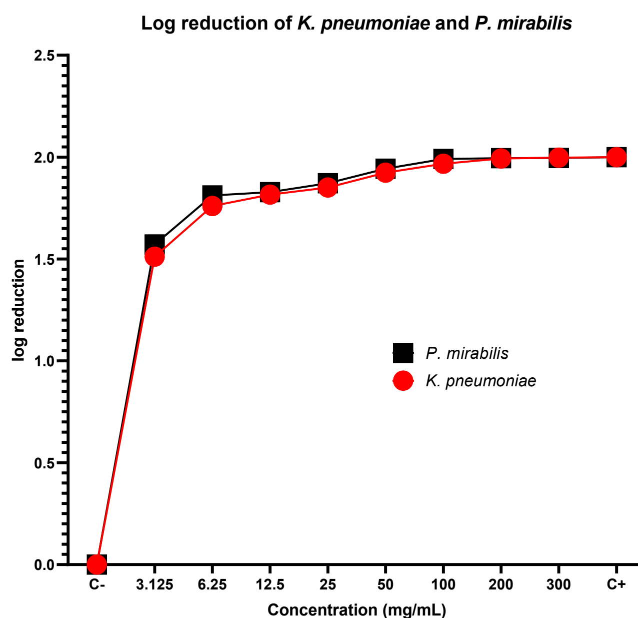


Figure 3 Graph of log reduction against *K. pneumoniae* and *P. mirabilis*.

Membrane leakage cell (DNA and Protein)

Cell leakage is the phenomenon of the release of nucleic acids and protein nitrogen from bacterial cells to the outside of the cells [44]. The treatment in this test showed that there was a release of nucleic acid material at the absorption wavelength of 260 nm (DNA) and 280 nm (protein). The measurement results can be seen in **Figure 4** (*K. pneumoniae*) and **Figure 5** (*P. mirabilis*). The mechanism of membrane leakage cell can be caused by the mechanism of action of secondary metabolites as antibacterial agents. The content of flavonoids as antibacterial by forming complex compounds against extracellular proteins that are able to interfere with the permeability of bacterial cell walls and membranes [45]. Apart from flavonoid compounds, ethanolic extract of avocado leaves also contains tannin compounds where the mechanism of action of tannin as an antibacterial is by inhibiting the synthesis of chitin in the formation of cell walls and also damaging cell membranes, resulting in bacterial cell lysis. This is also related to the mechanism of saponins as antibacterial agents that can damage porins, which are the entrance and exit of antibacterial agent compounds [46]. The value of the ratio of 260/280 nm is provided in **Figure 6**. The value of the ratio of 260/280 nm indicates the purity of the absorption between DNA and protein contained. In the tested cell leakage, results showed the absorbance ratio value of 260/280 nm did not exceed the value of 1.000. This shows that the purity of the absorption that occurs in cell leakage is not pure, because the purity of DNA is characterized by a value between 1.8 to 2 in the isolation of DNA from a tissue [47], while in this test it does not isolate DNA, it only measures the absorption at a wavelength of 260 and 280 nm [48]. Previous studies have reported the mechanism of cell leakage of ethanol extract of Butterfly pea flower against *Streptococcus mutans*, showing absorption at wavelengths of 260 and 280 nm [49]. This is also consistent with the findings of Sun *et al*. [50], who investigated the antibacterial impact and mechanism of anthocyanin-rich Chinese wild blueberry extract.

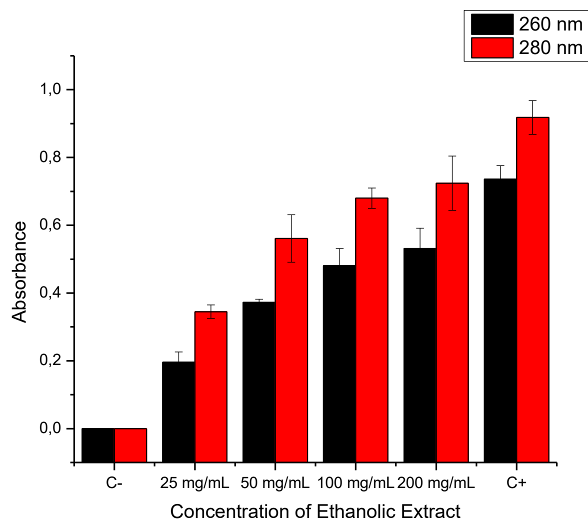


Figure 4 Cell leakage absorbance at 260 and 280 nm against *K. pneumoniae*.

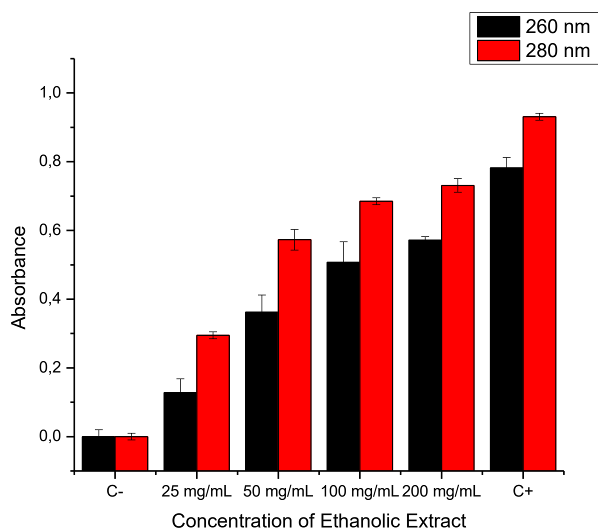


Figure 5 Cell leakage absorbance at 260 and 280 nm against *P. mirabilis*.

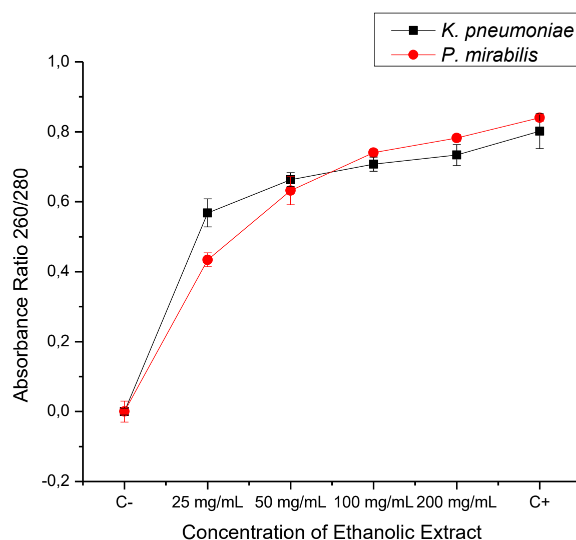


Figure 6 Ratio of 260/280 nm against *K. pneumoniae* and *P. mirabilis*.

Conclusions

Avocado leaves ethanol extract has a potency as an antibacterial which is characterized by its inhibitory zones at a concentration of 300 mg/mL of 11.77 ± 0.15 mm (*K. pneumonia*), 11.10 ± 0.20 (*P. mirabilis*) with a strong category and a MIC of 3.125 mg/mL of 6.43 ± 0.06 mm (*K. pneumonia*) and 6.73 ± 0.15 mm (*P. mirabilis*) in the moderate category. In addition, it was also characterized by the presence of cell leakage on measurements with UV-VIS spectrophotometry (260 nm for DNA and 280 nm for protein). The scope of work in the future can be prefer in the search for active compounds that have great potential in antibacterial properties against clinical isolates of bacteria.

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