Ekstrak *Moringa oleifera* Sebagai Antiseptik Terhadap *Escherichia coli* dan *Streptococcus pyogenes* Menggunakan Metode *Percentage Kill*

Moringa oleifera Extract as An Antiseptic Towards Escherichia coli and Streptococcus pyogenes by Using Percentage Kill Method

Conny Riana Tjampakasari[1], Ariyani Kiranasari[1], Alfia Chairunnisa[2], Fatindhiya Mahira Hilmy[2]

[1]Department of Microbiology, Faculty of Medicine, Universitas Indonesia
[2]Medical Education, Faculty of Medicine, Universitas Indonesia

*Korespodensi Penulis :
Conny Riana Tjampakasari
Email : connyrianat@yahoo.com

Abstrak

Latar belakang: Kandungan kimia daun kelor (*M.oleifera*) menunjukan sifat antibakteri terhadap bakteri Gram positif dan Gram negatif dengan menghambat sintesis dan metabolisme DNA serta menghancurkan dinding sel. Penelitian ini bertujuan untuk mengetahui signifikansi kemampuan ekstrak daun *M.oleifera* sebagai antiseptik terhadap *E. coli* dan *S. pyogenes.* **Metode:** Uji *percentage kill* dilakukan untuk mengetahui persentase kematian bakteri setelah kontak dengan *M. oleifera* pada menit ke 1, 2 dan 5. Variabel yang digunakan yaitu kontrol dan perlakuan yang dilakukan secara bersamaan. Uji dinyatakan memenuhi standar bila memberikan hasil ≥90% untuk setiap waktu kontak. **Hasil:** Uji terhadap *E. coli* pada menit ke 1, 2 dan 5 menunjukkan hasil masing-masing 93.41%, 94.14%, dan 96.87%, sedangkan terhadap *S. pyogenes* masing-masing 73.27%, 83.15% dan 94.19%. Terhadap *S. pyogenes* pada menit ke-5 memenuhi standar. **Kesimpulan:** *M. oleifera* efektif mengeliminasi *E. coli* karena nilai *percentage kill* untuk semua waktu kontak ≥90% dengan nilai tertinggi pada menit ke-5 (96.87%), sedangkan terhadap *S. pyogenes* ekstrak daun *M. oleifera* menunjukkan aktifitas mikroba yang baik pada waktu kontak 5 menit (94.19%).

Kata kunci: *M. oleifera*, ekstrak *E. coli*, *S. pyogenes*, *percentage kill*

Abstract

Background: Chemical constituents of *Moringa oleifera* (*M.oleifera*) leaves show antibacterial properties against Gram-positive and Gram-negative bacteria by inhibiting the DNA synthesis and metabolism extract as an antiseptic for *E.coli and S. pyogenes.* **Methods:** Using the Percentage Kill test to determine the percentage of bacterial death after contact with *M. oleifera* extract at 1, 2 and 5 minutes. There are two variables that will be used, namely control and treatment that are carried out at the same time. The Percentage Kill test is considered meet the standards if the result is ≥90% for each contact time. **Results:** The discovered that the Percentage Kill in minute 1, 2, and 5 to *E. coli* are 93.41%, 94.14%, and 96.87% respectively. While to *S. pyogenes* are 73.27%, 83.15% and 94.19%. The result found in the fifth minute is categorized as good as it fulfilled the minimum criteria of ≥ 90% for *S. pyogenes.* **Conclusions:** *M. oleifera* was effective to eliminate *E. coli* because the percentage kill value for all contact times was above 90% with 5th minute as the highest kill percentage (96.87%). While to *S. pyogenes* the *M. oleifera* leaves extract presented a good antimicrobial activity in a contact time of 5 minute (94.19%).

Keywords: *M. oleifera*, extract, *E. coli*, *S. pyogenes*, percentage kill

Background

Escherichia coli which is a predominant facultative member of the intestinal microbiota in human can also be a major causative agent of infections in extraintestinal organs such as sepsis (SEPEC or sepsis-associated *E. coli*), bacteremia and pyelonephritis. This occurs when the pathogenic bacteria adhere, invade and be followed by colonization of tissues. There has been an increased number of *Escherichia coli* sepsis and bacteremia events in recent years from the contamination of urinary infections and skin infections.[1]

Streptococcus pyogenes a Group A streptococci (GAS) is one of the major Gram-positive bacterial pathogen exclusive to humans. The interaction of GAS and humans ranges extensively. In normal healthy individuals, manifestation of GAS is usually limited to mild purulent infections of the skin and mucosal membranes. While in vulnerable individuals with pre-existing conditions GAS may present severe systemic manifestations. A prime example of these manifestations include streptococcal toxic shock syndrome and necrotizing fasciitis which produce high morbidity and mortality rate. A high burden caused by GAS is particularly apparent in developing countries with minimal income and facilities.[2,3]

Antiseptic is defined by the WHO as a disinfectant which can damage or prevent the development of microorganisms present in the tissue without producing any harmful effect on the body. Antiseptics are used on the skin or mucous membranes with unbroken skin or open wounds in order to prevent sepsis through the killing of microorganisms within the area.[1,2]

Moringa oleifera (*M. oleifera*) is one of the most common species of the family *Moringaceae* and can be easily found around Africa and Asia. *M. oleifera* contains a wide range of bioactive components. These active components include saponins, phenolic acids, flavonoids, tannins, alkaloids, isothiocyanates and triterpenoids. *M. oleifera* are recognized for having pharmacological and medicinal benefits as well as antibacterial purposes that are beneficial. The WHO states that in developing countries, 80% of the populations opt more towards the use of herbals as the main therapy in favor of modern day drugs. Making *M. oleifera* a suitable option for

antibacterial or antiseptic use, which is also supported by the extensive safety margin of *M. oleifera* for humans and animals. *M. oleifera* specifically the leaves have very high active contents are proven to provide great advantages in chronic conditions, such as diabetes, high blood pressure, cancer, hypercholesterolemia, insulin resistance, and high blood pressure.[4,5]

Based on the risk of developing infection due to sepsis, *E. coli* and *Streptococcus pyogenes,* the usage of antiseptic becomes crucial in order to prevent severe manifestation due to the infection. Along with the high burden that this bacteria produced in developing countries and the preferred usage of herbals as therapy, *M. oleifera* which is abundant in those countries becomes an appropriate option. In order to prove the efficacy of *M. oleifera* as an antiseptic against *E. coli and S. pyogenes*, it will be evaluated using the percentage kill method.[4,5,6]

Methods

The simplicia leaves of *M. oleifera* Lamk were obtained from the Tropical Biopharmaceutical Study Center of IPB University which was then used as extract in the Department of Medical Pharmacy, Faculty of Medicine, Universitas Indonesia.

Extract weight 120.8495 g which also consists of 15.30% water content. By subtracting 100% out of the water content, it will results in 84.70% and it accounts for the purity of the extract, later it will be divided with 100 ml to obtain the concentration of the extract, resulting in 847 mg/ml. This number further defines that in each 1 mL volume taken from the sample consists of 847 mg concentrated *M. oleifera* extract. This research will target of using an 800 mg concentration and 50 mL volume before dilution process using Carboxymethyl cellulose (CMC) Na 1%. CMC is prepared firstly by mixing CMC into the disc and combine it with 50 ml warm sterile water until the color is clear and then add on another 50 ml of warm water.[7] After the CMC is ready, a simple dilution chemistry formula is used to obtain the concentration volume of extract from the sample should be, as shown below $V_1M_1 = V_2M_2$.

Bacterial strain culture of *E. coli* and *S. pyogenes* were obtained from the Department of Microbiology,

Faculty of Medicine Universitas Indonesia.

Bacteria optimization

Bacteria is then incubated for 18-24 hour and the turbidity is equalized to 0.5 McFarland. One inoculating loop of bacteria is added to 10 ml of Tryptic Soy Broth (TSB) (Oxoid). Afterward, a ten-fold serial dilution is made. Each were poured to petri dish, added with another 15 mL of of molten Plate Count Agar (PCA) (Ikapharmindo) and mix gently. Once solid, it is incubated at 35ºC for 24-48 hours in inverted position. Electronic colony counter is used to identify the statistically viable plates containing between 100 to 200 colonies. This concentration of bacteria is used for percentage kill test.[7]

Percentage Kill test

Mix 0.5 mL of bacteria (concentration 10^5 in TSB) in 4.5 mL *M. oleifera* extract (X) and 0.5 mL bacteria (concentration 10^5 in TSB) in 4.5 mL sterile water (C). Pour to petri dish after 1, 3, and 5 minutes (in triplicate). Mix it gently with 15 mL of of molten PCA and incubate at 35ºC for 24-48 hours once its solidify. Both are held at the same time. The growing colonies counted using an electronic colony counter. Percentage killed is measure by the following equation :[8,9,10]

Percentage Kill = (C–X)/C x 100%

C : Total colony of control

X :Total colony of *M.oleifera* mixture

Significant percentage kill is achieved with > 90% .

Results

Based on the preliminary result, we find the optimal concentration of bacteria was obtained in 10^5 both *E.coli* and *S. pyogenes*. Diluting the colony of *E. coli* and *M. oleifera* with 1, 2 and 5 minutes contact time using the Percentage Kill methods will produce the result as shown in the Table 1.

The results showed the number of colonies of *E. coli* in control with three times repetition with a contact time of 1 minute, respectively 183, 166 and 152 colonies. For a contact time of 2 minutes, the number of colonies is 147, 141 and 139. While for a contact time of 5 minutes, the number of colonies was 112, 109 and 99. On the contrary, the number of colonies of *E.coli* in treatment which is given *M. oleifera* extract with three times repetition of a 1 minute contact time is 12, 11 and 10. While on the second minutes, the data shows 12, 7 and 6 respectively. Then, on the fifth minutes, it shows

5, 3 and 2 colony growth. The bar chart represents the average *E.coli* colony growth within the first, second and fifth minutes in both control and treatment. A significant difference can be observed within each minutes, thus, we could state that *M. oleifera* leaves extract hinder the growth of *E.coli* even from the first minute, since it results in more than 90% (Figure 1).

Results of the percentage kill procedure with contact time of 1, 2, and 5 minutes and a three time repetition for each contact time is presented in Table 2. Based on the data, the average growth of *S. pyogenes* colony which is given *M. olefera* and control in 1, 2, and 5 minutes are acquired. For the control, the average colony growth in the first minute is 173.33, in the second minute the obtained mean is 152.33, while the average growth of colony in the fifth minute is 143.33.

Table 1. Average Amount of *E. coli* colony growth in control and *M. oleifera*

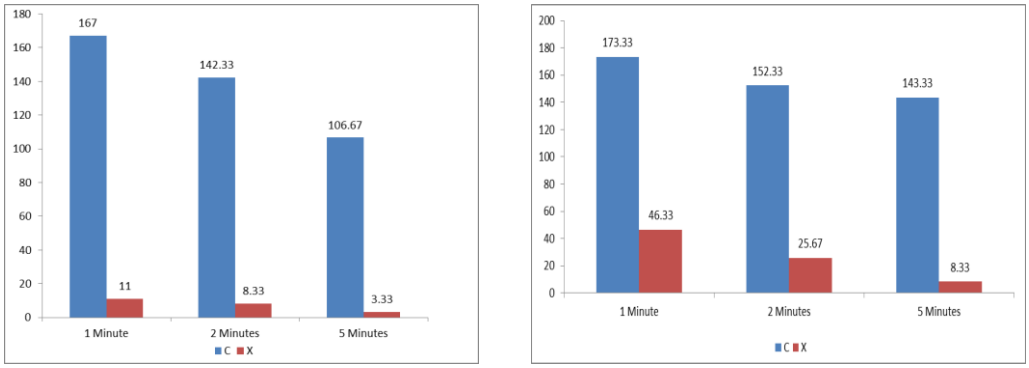
Time	Colony Count (C)			Average Colony Growth (C)	Colony Count (X)			Average Colony Growth (X)
	I	II	III		I	II	III	
1 Minute	183	166	152	167	12	11	10	11
2 Minutes	147	141	139	142.33	12	7	6	8.33
5 Minutes	112	109	99	106.67	5	3	2	3.33

I : First Repetition, II : Second Repetition, III : Third Repetition
C : Total colony of control
X :Total colony of *M. oleifera* mixture

Table 2. Average amount of *S. pyogenes* colony growth in control and *M. oleifera*

Time	Colony Count (C)			Average Colony Growth (C)	Colony Count (X)			Average Colony Growth (X)
	I	II	III		I	II	III	
1 Minute	160	170	190	173.33	60	48	31	46.33
2 Minutes	140	155	162	152.33	30	26	21	25.67
5 Minutes	127	145	158	143.33	11	9	5	8.33

I : First Repetition, II : Second Repetition, III : Third Repetition I
C : Total colony of control
X :Total colony of *M. oleifera* mixture



(A) **(B)**

Figure 1. (A) Average *E. coli* bacterial colony growth. (B) Average *S. pyogenes* bacterial colony growth. C : Total colony of control, X :Total colony of *M. oleifera* mixture

In order to obtain the final outcome of the Percentage Kill method, the results is then applied to the Percentage Kill formula. Based on the calculations, the final results of the Percentage Kill method during minutes 1, 2, and 5 respectively are 93.41%, 94.14% and 96.87% for *E. coli* and 73.27%, 83.15%, 94.188% respectively for *S. pyogenes.* According to these results, It can be stated that the higher the contact time, the higher the percentage kill results, thus showing the eradication level of *E. coli* is bigger. The Percentage Kill results can be interpreted as good because it exceeded ≥ 90%. From the first minute, it has already shown a higher percentage level (above 90%), hence we can state that it has already given benefit in terms of hinder the growth of *E. coli.* Meanwhile for *S. pyogenes,* findings in the first and second minute does not fulfill the minimum requirement of ≥90%. Therefore, only results of the fifth minute is able to meet the specified standard.

Discussion

This experiment proved the presence of antimicrobial efficacy *of M. oleifera* to suppress the growth of *E.coli* and *S. pyogenes*. Supported by the smooth growth of colony which is given treatment in comparison to the higher growth of colony found in control.

From the average colony number of these repetitions, it can be stated that the extract of *M. oleifera* can eradicate *E. coli*. The hypothesis of the above mentioned notion is due to the antiseptic properties of *M. oleifera* extract that can slow the growth rate of *E. coli.* It can be explained by several possible mechanisms. The first one is that the phytoconstituents from the *M. oleifera* itself such as Isothiocyanate and it derivates, for instance Allyl isothiocyanate (AITC) as it will provide

effects towards the bacterial membrane and influence the metabolism of bacteria by inhibiting the activity of thioredoxin which is a key role enzyme for its metabolism.[11]

Moreover, aqueous extract shows MIC results that are effective towards Gram negative bacteria such as *E. coli* due to the active components of Tannins, Phenol and Alkaloids. Polyphenols that existed accumulate on the surface and favored by the bacteria's increased hydrophobicity and eventually an increase in bacterial growth inhibition percentage. On the other hand, Tannins' antibacterial efficacy is explained by their capacity to penetrate through the bacterial cell wall up to the interior membrane, causing interference with the cell's metabolism and, as a result, its death. Tannic acid prevents germs from adhering to surfaces since it acts as the inhibitor of NorA efflux pump which is considered as the main mechanism for its antibacterial activity. Thus, bacterial cell death comes from a loss of adherence to the surface. Tannic acid also inhibits the absorption of sugar and amino acids, limiting the bacteria's development. Tannins are multidentate ligands that may bind to proteins primarily through hydrophobic interactions and hydrogen bonding. As a result, the metabolism of bacteria is inhibited.[12,13]

Tannin also provides antiviral activity, its action is dependent on viral cell membrane adsorption, which results in the virus's activity and capacity to assault human cells being inhibited. All these possible mechanisms lead to the decrease of *E. coli* growth in the colony thus contributing to the probability of *M. oleifera* effectivity towards the elimination of *Escherichia coli* and even may acts as an antiviral, indicated by the significant difference on the colony results based on time contact and repetition in comparison to control.[14,15]

Previous research by Dima LR et al, in Sam Ratulangi University, Manado, in 2016 stated that different concentration of *M. oleifera* in 5%, 10%, 20%, 40% and 80% have shown a potent antibacterial result, shown when at the highest concentration percentage which is 80% can results the highest MIC zone diameter of 24.00 mm in *E. coli*, and the smallest diameter can be found in the lowest concentration which is 12mm from 5% concentration. The smallest concentration has even shown a significant result as antibacterial. Combining the highest percentage and number of contact time might eradicate a higher number of *E. coli* growth.[16,17] Based on their research we continued

with antiseptic potency test using the percentage kill method and the result showed promising.

On the comparison of contact time towards the growth of *E. coli*, there is an average number of colony growth on minute 1, 2 and 5 minutes which are 11, 8.33 and 3.33 respectively. The extract of *M. oleifera* has antibacterial properties from the Percentage Kill results on minute 1, 2 and 5 with 93.41%, 94.14% and 96.87% respectively. It indicates that one minute contact time using extract of *M. oleifera* has already given a prominent and effective results and five minutes of contact time shown the highest percentage kill results, hence the highest eradication number of *E. coli.*

Meanwhile the result derived from the Percentage Kill method of *S. pyogenes* showed that the influence of *M. oleifera* extract can already be identified within the first 1 minute. Although the outcome of 73.27% and 83.15% found in the first and second minute respectively still does not fulfill the minimum requirement of an effective antimicrobial which is $\geq$90%. However, a notable improvement of the plant's antimicrobial action is identified in the fifth minute demonstrated by a result of 94.188%. This information signify that the increase of *M. oleifera* antimicrobial efficacy is associated with longer duration of contact.

Furthermore, this outcome is affected by the presence of active substances or phytochemicals contained in *M. oleifera* which is extracted by certain solvents including aquadest and ethanol. The extraction of *M. oleifera* leaves using ethanol generate polyphenol and flavonoid, while the involvement of aquadest in extraction produce tannin, saponin, triterpenoid, flavonoid, and steroid. Flavonoid takes part in generating antimicrobial activity in *M. oleifera* through the disruption of bacterial cell wall and cytoplasm which inhibit the cell division process. Supported by the ability of flavonoid to form a complex with extracellular proteins and bacterial cell wall.[18,19] Another phytochemical in *M. oleifera* is alkaloid which possess bacterial DNA disturbing characteristics by suppressing enzyme activation functioning in directing nucleotide to single strand DNA. This interruption of bacterial DNA results in other interference of bacterial cell division and consequently the normal structure of the bacteria along with suppression of protein synthesis which takes part in the metabolism and construction of bacterial cell wall. In addition to the prior antimicrobial components, a polyphenol substance called Tannin is

also involved in the inhibition of extracellular enzyme inducing membrane cell disturbance.[20,21]

Another study conducted by Esimone *et al* in 2006 also suggest that antimicrobial effects of the leaf extract is further enhanced by the activity of Beta-lactams located on the transpeptidation of the cell wall. The interaction between antimicrobial peptides and cell membrane is comprised of two phases. In the first phase, phospholipid head groups present on the surface exhibit negative charges which attracts cationic amino acids.[22] Next, hydrophobic peptide patches interact with aliphatic fatty acids while positively charged peptides interact with the anionic components. Resulting in membrane disruption along with changes in membrane potential, changes in membrane permeability, disrupted lipid distribution, leakage of cytoplasmic materials, blockage of anionic cell elements, and stimulation of autolytic enzymes which contributes to the death of bacteria. Therefore it can be acknowledge that the proteins or peptides pose a crucial role in the antimicrobial defense system of *M. oleifera*.[23]

The outcome of this research is consistent with the report of Oluduro in 2012 which noted that the aqueous extract derived from the leaf of *M.oleifera* manifested antimicrobial action towards Gram positive bacteria. Furthermore, this research finding is also congruent with another study performed by Floorentina in 2015 which reported the efficacy of *M.oleifera* in inhibiting the growth *of S. pyogenes* by evaluating the minimum inhibitory zone created by the bacteria. This particular research also noted the association between a higher concentration of extract and a lower growth of bacteria as there was also higher level of antimicrobial properties.[23,24]

Conclusion

We conclude that *M. oleifera* leaf extract has potential as an antiseptic against *E. coli* and it is necessary to do a concentration increase examination of *M. oleifera* leaf extract against *S. pyogenes.*

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