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*Research*

# Efficacy of *Terminalia catappa* Leaves Extract As An Antimicrobial Agent Against Pathogenic Bacteria

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**ABSTRACT**

*Terminalia catappa* ("Ketapang" in Malay) is a plant that belongs to the family Combreteceae and is native to Southeast Asia. Various parts of this plant were reported to possess bioactive compounds with antimicrobial properties. However, reports on the antimicrobial activity of different ages of *T. catappa* leaf (TCL) against pathogenic bacteria are lacking. This study investigated the antibacterial activity of the different ages of TCL extracts against the pathogenic bacteria, *Staphylococcus aureus* and *Escherichia coli*. Disk diffusion assay was carried out to determine the antibacterial activity of different ages of TCL extracts. Meanwhile, the efficacy of the different leaf extracts was evaluated by performing broth microdilution (MIC & MBC determination), growth reduction, time-kill study, and membrane cell leakage assay (MCLA). The findings revealed that the extracts showed greater antibacterial activity toward the Gram-positive *S. aureus* compared to Gram-negative *E. coli*, with diameter of inhibition zone ranging from 12.33±0.53 mm to 26.33±1.4 mm and 9.23±0.32 mm to 16.21±0.42 mm, respectively. The mature leaf extract (MLE) and senescent leaf extract (SLE) exhibited higher antibacterial activity compared to young leaf extract (YLE) and shoot leaf extract (ShLE) for both Gram-positive and Gram-negative bacteria. The broth microdilution assay showed all the different ages of TCL extracts exhibited bactericidal effects towards *S. aureus*. Meanwhile, the YLE and ShLE showed a bacteriostatic effect on *E. coli*, whereas the MLE and SLE exerted bactericidal action. The time-kill study revealed that the extract activity was time- and concentration-dependent. The results of the MCLA corroborated the time-kill study, which showed that a higher concentration of extract could successfully inhibit and kill the bacterial cells, as indicated by higher protein and nucleic acid leakage. The present study suggests that all different ages of TCL extracts, particularly the MLE can act as potential antibacterial agents against both Gram-positive and Gram-negative bacteria.

**Key words:** *Terminalia catappa*, antibacterial activity, well diffusion and disk diffusion assays, MIC and MBC values, time-kill study

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## INTRODUCTION

Since the 1950s, the medical world has struggled with emerging and re-emerging infectious illnesses, with emerging pathogens now being recognized as a major public health concern. The emergence of these bacterial infections is largely caused by increased human exposure to bacterial pathogens as a result of environmental and sociodemographic changes, as well as the emergence of increasingly virulent bacterial strains and opportunistic infections, that particularly affect immunocompromised people (Vouga & Greub, 2016). These problems are exacerbated by the emergence of antimicrobial-resistant pathogens such as Penicillin-Resistant *Streptococcus pneumonia* (PRSP), Methicillin-Resistant *Staphylococcus aureus* (MRSA), and Vancomycin-Resistant Enterococci (VRE) that have retarded the success of antimicrobial drugs by becoming invulnerable to them (Adedeji, 2016). The emergence of resistant strains is due to the misuse of antibiotics in various fields, such as the widespread and prolonged use of antibiotics for therapy

in hospitals, the incorporation of antibiotics in food-producing animals for growth promotion and mass prophylaxis, inappropriate prescription and self-medication in the community and improper release of large amounts of antibiotics in wastewater (Prestinaci *et al*., 2015). These bad practices reduce the efficacy of available antibiotics, making them no longer effective against pathogenic bacteria, and have resulted in more bacterial infections that are difficult to control. Hence, the search for new effective antibacterial agents from various sources is needed to mitigate this problem.

Worldwide, humans have used plants for their medicinal qualities since the dawn of time. The pharmacological actions of plants are derived from their phytochemical components, especially from their secondary metabolites, which are amazing sources of bioactive chemicals with additional value. Plants produce secondary metabolites in response to different forms of stress to perform a range of physiological tasks. These metabolites are used in the food and beverage, cosmetics, pharmaceutical, and dietary supplement sectors (Chandran *et al*., 2020). According to Divya *et al*. (2019), T*erminalia catappa* contains various phytochemical compounds such as phenols, flavonoids, and steroidal glycosides, which are responsible for its therapeutic activity. This plant is commonly known as the sea almond, is native to Southeast Asia, and grows well in subtropical and tropical climates (Anand *et al*., 2015). The generic name came from the Latin word “terminalis”, which refers to the leaves that teem at the end of the shoot. The other local names are ketapang (Malay), Indian almond, tropical almond, and talisay (Philippines). *T. catappa* has been used as a whole plant and can be utilized as a shade, ornamental plant, and salt-tolerant street tree, while the leaves can be used as a nutrient source for Tasar silkworm and the seeds are similar to edible almonds (Chanda *et al*., 2011). Moreover, Chanda *et al*. (2011) also claimed that these trees have therapeutic potential since they have been used in many parts of Asia for alternative medicine, such as, in Java, where it is used for kidney treatment due to its cholagogue action. In India, the trees are used as a cardiac stimulant and in Southeast Asia, people have been using ketapang leaves as a folk remedy for dermatosis and hepatitis.

Furthermore, this plant has been reported to exhibit antibacterial activity against several pathogenic bacteria. For instance, methanolic *T. catappa* leaves extract exhibited antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* with a diameter of inhibition of 25.33±0.33 and 21.33±0.67 mm, respectively (Muthulakshmi & Neelanarayanan, 2021). A previous study by Tasneem and Narsegowda (2018) also revealed that this plant possesses remarkable antibacterial activity against *S. aureus* and *Escherichia coli* and antifungal activity against *Candida albicans*. This antimicrobial property is believed to be due to the presence of bioactive compounds in this plant, such as polyphenols, tannins, flavonoids, and terpenoids (Orillaneda *et al*., 2022). For instance, Anand *et al*. (2015) revealed that tannins previously isolated from *T. catappa* can disrupt the quorum sensing (QS) of pathogenic bacteria. Tannins are believed to inhibit the binding receptor of bacteria and influence their activity, and can pass through the bacterial cell wall and disrupt the cell’s metabolism (Kaczmarek, 2020). Furthermore, flavonoids which are bioactive compounds that can be extracted from *T. catappa* leaves, can inhibit the growth of bacteria by breaking down the structure of the cell membrane, preventing the synthesis of proteins, nucleic acids, or components of the cell wall, lowering energy production, and preventing the spread of resistant bacteria by blocking bacterial efflux pumps and virulence factors (Wang *et al*., 2021).

However, the available information on the antibacterial activity of different leaf maturity of *T. catappa* is scarce in the literature. Hence, this study aims to determine the antibacterial activity of different leaf maturity of *T. catappa* toward pathogenic bacteria. The findings of this study could significantly contribute to the discovery of novel antimicrobial compounds against pathogenic bacteria.

## MATERIALS AND METHODS

### Collection of samples

Four different ages of *T. catappa* leaves were collected, namely: shoot leaf (ShL), young leaf (YL), matured leaf (ML), and senescent leaf (SL). The leaves were collected at the Faculty of Applied Sciences, UiTM Shah Alam’s compound. The leaves were washed to remove any dirt and then air-dried, cut into small portions, and oven-dried (60°C), before being ground into a fine powder. The leaf powder was stored in a desiccator to maintain its dryness and prevent contamination.

### Plant extraction

The extraction process was carried out using the method described in Parthasarathy et al. (2009). To obtain the methanolic extract, 200 g of leaf powder was steeped in 99% (w/v) methanol, with a 1:90 ratio of the leaf powder to methanol volume in milliliters (mL). The combination was left to stand at room temperature for 24 hr, after which the crude methanolic fraction was extracted using a rotary evaporator. The crude extract was kept in a fume hood for 24 hr to remove any remaining solvent.

**Microorganisms and cultural maintenance**

In this investigation, two species of bacteria were employed: *E. coli*, a Gram-negative bacterium, and *S. aureus*, a Gram-positive bacterium. The obtained stock bacterial cultures were inoculated in glycerol stock and maintained in a freezer until they were ready to be sub-cultured in nutrient agar (NA) and incubated at 37°C for 24 hr.

**Inoculum preparation**

Two or three colonies of pathogenic bacteria were picked from the agar plate and were subsequently re-suspended into a universal bottle containing 5.0 mL of physiological saline solution (0.85% (w/v). The mixture was vortexed, and its turbidity was adjusted by comparing it with a 0.5 McFarland standard solution to obtain a bacterial suspension of approximately $1 \times 10^8$ CFU/mL.

**Kirby Bauer disc diffusion assay**

The disc diffusion assay was used to assess the antibacterial activity of the leaf extracts (TCLE) against the two pathogenic microorganisms using the procedures by CLLS (1999). In this study, the method described by Parthasarathy *et al*. (2009), was used to evaluate the antibacterial capability of the *T. catappa* methanolic extracts. *T. catappa* extracts (200 mg/mL) were impregnated into antibiotic discs (6 mm). The antibiotic discs were placed onto the surface of a Mueller-Hinton agar (MHA) plate that was pre-streaked with a lawn of test bacteria (inoculum size of $5 \times 10^5$ CFU/mL). For both extracts, methanol (5%), served as the negative control, and chloramphenicol (30 µg/mL) served as the positive control. The plates were incubated at 37°C for 24 hr. Any inhibitory zones that developed on the plate were measured with a ruler and the diameters were recorded.

**Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)**

A sterile 96-well microtiter plate was used for the broth microdilution experiment to determine the MIC of TCLE (Parvekar *et al*., 2020). A serial dilution procedure was used to prepare various TCLE concentrations. The microtiter well was first filled with 100 µL of sterile Muller-Hinton broth (MHB), and then with 100 µL of TCLE (20 mg/mL). After that, each well was pipetted with 50 µL of bacterial solution to produce a bacterial inoculum of $5 \times 10^5$ CFU/mL. The microtiter plate was then incubated at 37°C for 24 hr. To determine the MBC value, the wells from the microtiter plate in the MIC experiment that showed no signs of bacterial growth were streaked onto sterile MHA plates and incubated at 37°C for 24 hr. The MBC value for TCLE was determined by using the lowest concentration at which there was no discernible bacterial growth on the MHA plates.

**Bacterial growth reduction**

The methodology outlined by Jalil *et al*. (2022) was used to assess the effectiveness of the TCLE in reducing bacterial growth, with a few minor adjustments. In this investigation, all crude extracts were prepared at a 2 × MIC or MBC value. To administer the treatment, 8.9 mL of MHB and 1.0 mL of crude extract at a final concentration of 2 × MIC were added to a 50 mL Erlenmeyer flask. Next, 0.1 mL of bacterial suspension was added to the flask, resulting in a final bacterial concentration of $5 \times 10^8$ CFU/mL. The flask was then incubated for 24 hr at 37°C in a rotary shaker operating at 150 rpm. After 24 hr, 1.0 mL of the culture fluid was pipetted out and inoculated onto MHA to obtain the number of colony-forming units (CFU) using the spread plate method.

**Time-kill analysis**

A time-kill assay was conducted to determine the effectiveness of TCLE against the test bacteria at different concentrations, which were: ½ × MIC (125 mg/mL), MIC (250 mg/mL), and 2 × MIC (500 mg/mL). First, 0.1 mL of the bacterial suspension was placed into four different 50 mL Erlenmeyer flasks, labeled A, B, C, and D, each of which contained 18.9 mL of MHB, with a final bacterial suspension of about $5 \times 10^5$ CFU/mL. After that, 1.0 mL of the extracts at ½ × MIC, MIC, and 2 × MIC were added to flasks A, B, and C, respectively. As a control, 1.0 mL of 5% (v/v) methanol was added to flask D. The cultures were vortexed and then incubated for 48 hr at 37°C in an orbital shaking incubator rotating at 150 rpm. A 0.1 mL aliquot sample of the cultures was collected at every 4 hr intervals from 0 to 48 hr to count the viable cells. The aliquot samples were serially diluted, then inoculated onto new sterile MHA, and then incubated for 24 hr at 37°C. Only the plates with a colony count between 30 and 300 were used to calculate the number of CFU/mL. A time-kill curve ($\log_{10}$ CFU/mL vs. time) was constructed for

each extract concentration and control. The time required to suppress bacterial cell growth by 50%, 90%, 99%, and 99.9% was then calculated according to Taufiq and Darah (2018).

**Membrane Cell Leakage Assay (MCLA)**

The concentration of proteins and nucleic acids was used to calculate the MCLA. A bacterial pallet was obtained by centrifuging a suspension of cell biomass (18–24 hr old) at 3500 rpm for 15–20 min, after which the supernatant was discarded. The TCLE was then added to the pallet to the final concentrations of ½ × MIC, 1 × MIC, and 2 × MIC. After that, the pellet and the TCLE mixture were re-suspended in a pH 7.4 phosphate buffer solution to yield a final volume of 10 mL and then incubated at 37°C for 24 hr. After this incubation period, the mixtures were centrifuged for 15 min at 3500 rpm to obtain the supernatant, while the pellet was discarded. The nucleic acid and protein content of the supernatant was determined by utilizing a PerkinElmer UV-Vis Spectrophotometer Lambda 25 (PerkinElmer LLC, USA) at 260 and 280 nm wavelengths, respectively (Jamal *et al*., 2013). Uninoculated TCLE solutions at ½ × MIC, 1 × MIC, and 2 × MIC were employed as blanks during their respective spectrometry measurements. The occurrence of bacterial membrane cell leakage was indicated by an increase in protein and nucleic acid content in the culture medium as indicated by an increase in the absorbance at their respective wavelengths.

**Statistical analysis**

All tests were run in triplicate ($n$=3) and the data were reported as mean standard deviation, (mean ± SD). Using SPSS 15.0, One-Way ANOVA was used to evaluate the data, and the Duncan test was used to determine whether there were any mean differences. The results were considered statistically significant if $p$<0.05.

## RESULTS AND DISCUSSION

**Extraction of different ages of *Terminalia catappa* leaf**

The selection of a solvent system is crucial in extracting bioactive compounds from natural products, especially plants. Sasidharan *et al*. (2011) stated that a range of solvent solutions can be used to extract the bioactive components from natural products. When choosing a solvent, one should take selectivity, solubility, cost, and safety into account. Solvents having a polarity value close to the polarity of the solute are probably going to function better and vice versa, according to the laws of similarity and intermiscibility (like dissolves like). Alcohol solvents, specifically EtOH and MeOH, are often used in solvent extraction processes for phytochemical analysis (Zhang *et al*., 2018). In this study, the leaf was chosen instead of other plant parts to extract the bioactive compounds. This is due to a previous study suggesting that leaves generally have a higher variety and concentrations of bioactive compounds than other plant parts, such as the stem and fruit (Pateiro *et al*., 2023). Hence, the present study was done by using methanol to extract bioactive compounds from *T. catappa* leaves since several studies revealed that methanol is one of the best solvent systems for extracting bioactive compounds from plants. For instance, Sharmin *et al*. (2016) revealed that the methanol extract had a greater yield of bioactive compounds, such as total phenolic content (18.3%) compared to the water extract (14.1%) in pomegranate waste. A similar observation was reported by Papoutsis *et al*. (2016) who revealed that absolute methanol resulted in the highest extraction yields of total phenolic compounds (TPC) and total flavonoid content (TFC) from lemon (*Citrus limon* L.) pomace waste. Table 1 shows the yield of methanolic extract paste of different ages of *T. catappa* leaves obtained in the present study. The result revealed that the senescent leaf (SL) produced the highest yield of the crude extract by 12.66% followed by mature leaf (ML) and young leaf (YL) with the values of 11.03% and 7.22%, respectively. Meanwhile, the shoot leaf (ShL) showed the lowest weight of crude paste with a value of 5.57%. Vázquez-León *et al*. (2017) reported that tree age was positively correlated with the total carotenoid's contents, and inversely correlated with the ascorbic acid contents and their finding offers an understanding of variations in bioactive compounds and antiradical activity in Moringa leaves influenced by climatic factors, soil, and tree age, which may help in the estimation of the antioxidant potential present in the plants during different harvest times. Similarly, Idris *et al*. (2023) reported that the extract yield rose with the maturity stages, with old leaves exhibiting the highest percentage of extract yields. Additionally, they proposed that the extraction solvent, pH, temperature, polarity, extraction duration, extraction technique, and sample numbers employed could affect the extraction yield percentage values. In another study, Tan *et al*. (2021) also found that the maturation degree impacted the content of bioactive compounds such as vitamin C, total carotenoids, and total polyphenol in leaves.

**Table 1.** The yield of methanolic extracts paste of different ages of *T. catappa* leaf

| Extracts | Weight of TCL powder (g) | Weight of crude paste (g) | Yield (%) |
|---|---|---|---|
| Shoot | 200 | 11.14±0.6 | 5.57 |
| Young | 200 | 14.43±0.3 | 7.22 |
| Mature | 200 | 22.06±0.6 | 11.03 |
| Senescent | 200 | 25.31±0.3 | 12.66 |

**Disc diffusion assay**

The Kirby-Bauer disc diffusion susceptibility test was used to evaluate the antimicrobial drug sensitivity or resistance of facultative anaerobic and pathogenic aerobic bacteria. Figure 1 and Table 2 show the antibacterial activity of TCLEs against pathogenic bacteria in the disc diffusion assay. In terms of leaf age stages, the mature leaf extract (MLE) showed a higher inhibitory activity toward *S. aureus* with an average inhibition zone diameter of 26.33 ±1.53 mm compared to the shoot leaf extract (ShLE) (12.33 ±0.58 mm). The same observation was recorded when the Gram-negative *E. coli* was exposed to the MLE, with an average inhibition zone diameter of 16.21 ±0.42 mm. *E. coli* was the least susceptible to young leaf extract (YLE) and ShLE with average inhibition zone diameters ranging from 9.23 ±0.32 to 11.33 ±0.18 mm, respectively. The finding of the present study is in line with Shukri *et al*., (2023), who reported that the total phenolic content (TPC) and total flavonoid content (TFC), rose by 11% and 24%, respectively, from the shoot into mature leaves. Mature kenaf (*Hibiscus cannabinus*) leaves extracted in methanol and acetone solvents demonstrated high levels of antioxidant capacity and antimicrobial properties, particularly the ability to inhibit Gram-negative bacteria. Besides that, Anwar *et al*., (2017) reported that the level of phenols and flavonoids in mature leaves of *Aquilaria beccariana* was higher than in the young and old leaves and their study suggested that leaf age influences the level of secondary metabolites and their antioxidant properties. In contrast, a previous study revealed that young leaves of *Aronia melanocarpa* contain higher TPC, TFC, chlorogenic acid, and rutin (Thi & Hwang, 2014). This contradictory finding might be due to the types of plants, solvent systems, and extraction methods.

In terms of bacterial strain, the present study revealed that the Gram-positive *S. aureus* was most susceptible to all ages of the TLCE compared to the Gram-negative *E. coli*. According to Jalil & Ibrahim (2022b), the structural and morphological variations between Gram-positive and Gram-negative microorganisms may account for the varied sensitivity of the two types of bacteria to the extract. In contrast to Gram-positive bacteria, Gram-negative bacteria typically have more complicated structures like the outer membrane layer, thin peptidoglycan layer, and periplasm. The outer membrane in Gram-negative bacteria restricts the flow of molecules and acts as a selective barrier, blocking antibiotic substances while permitting the delivery of essential nutrients to the cell. Additionally, the outer membrane is attached to membrane proteins called porins that function as selective channels for the transit of hydrophilic materials of a particular size into the periplasm (Miller & Salama, 2018).

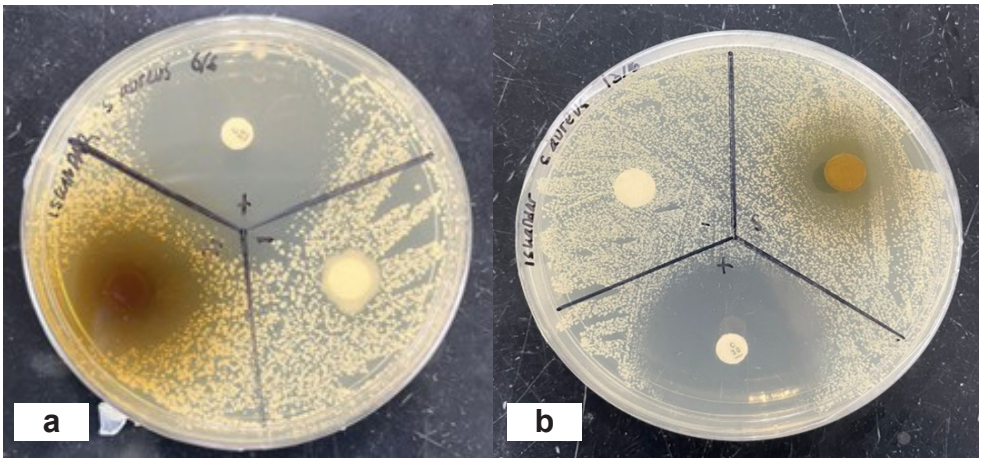


**Fig. 1.** Antimicrobial activity of methanolic extracts of *T. catappa* at different leaf ages against *S. aureus*. (a) Senescent, (b) Young.

**Table 2.** Antibacterial activity of TCLEs against pathogenic bacteria on disc diffusion assay

| Sample | Positive Control | Inhibition Zone (mm) [Mean±S.D] | |
|---|---|---|---|
| | | *Staphylococcus aureus* | *Escherichia coli* |
| Shoot | 33.00 ±1.00 | 12.33 ±0.58$^{aA}$ | 9.23 ±0.3$^{2aB}$ |
| Young | 40.67 ±1.16 | 19.33 ±1. 53$^{bA}$ | 11.33 ±0.18$^{bB}$ |
| Matured | 40.67 ±1.16 | 26.33 ±1.5$^{3cA}$ | 16.21±0.42$^{cB}$ |
| Senescent | 39.00 ±1.00 | 25.37±2.08$^{dA}$ | 15.41±0.30$^{dB}$ |

Note: Positive control is chloramphenicol, Sample is 200 mg/mL. Different superscript letters are significant at $p<0.05$, using ANOVA One-Way Tukey pairwise comparison

**Determination of MIC and MBC values**

An alternate method to assess the extract's *in vivo* antibacterial activity is the MIC determination. This technique is more accurate than the disc diffusion assay because it can estimate the level of resistance in less sensitive strains with greater precision. Additionally, combining the MIC and MBC would provide crucial information about the extract's bactericidal and bacteriostatic characteristics (Taufiq & Darah, 2022). Moreover, Moreno *et al*. (2013) reported that the good reproducibility, small sample size needed, and inexpensive cost that permits numerous replications are all benefits of broth microdilution. The procedure is simpler and more effective than the macro dilution procedure. Table 3 shows the MIC and MBC values of TCLE against test pathogenic bacteria. The present study showed that the MIC and MBC values of the TCLE against the Gram-positive *S. aureus* were 12.5 – 25.0 µg/mL and 12.5 – 50.0 µg/mL, respectively. As for Gram-negative bacterium, the MIC and MBC values of the TCLE were 25.0 µg/mL and 200 – 400 µg/mL, respectively. The finding revealed that Gram-positive bacteria are more susceptible to both extracts compared to Gram-negative bacteria. It is also noteworthy that both the Gram-positive and Gram-negative bacteria were more susceptible to the MLE and senescent leaf extract (SLE) compared to the ShLE and YLE. This may be due to the presence of a variety of bioactive compounds especially in mature and senescent leaves. However, the present study contradicts the findings of Albaa *et al*., (2024) who reported the phenolic content of *A. cordifolia* leaves decreased as the leaves aged, and young leaves appeared to have a more rapid metabolic response to thermal stress, which resulted in a greater accumulation of phenolic compounds. The present study also showed that all the TCLEs possess bactericidal effects against both Gram-positive *S. aureus* and Gram-negative *E. coli*, since their MBC/MIC ratio is less or equal to 4, though the ShLE and MLE only had a bacteriostatic effect against *E. coli*. Jalil *et al*. (2022a), stated that when the MBC/MIC ratio is 4 or below, the antimicrobial substances are regarded as bactericidal agents, while if it is larger than 4, they are regarded as bacteriostatic agents. Nevertheless, both bacteriostatic and bactericidal drugs offer benefits when used as antibiotics against pathogenic bacteria (Zulkamal *et al*., 2023). Bacteriostatic antibiotics block the growth of bacteria, while bactericidal drugs lower the number of bacteria. Unlike bactericidal drugs, bacteriostatic medications enable the immune system to fight illnesses even when the bacteria are still alive. For example, several bacteriostatic medications are effective in treating cases of clostridial and streptococcal gangrene. This is because they prevent the production of toxins, which are the primary causes of illness.

*E. coli* is less susceptible to the ShLE and YLE than *S. aureus*, possibly due to an efflux system or degrative enzymes that can flush out and degrade bioactive compounds. According to Elshimy (2023), *E. coli* can carry genes coding for enzymes, such as beta-lactamases, that can hydrolyze and inactivate beta-lactam antibiotics. *E. coli* can also express numerous efflux pumps that effectively reduce the intracellular concentration of certain antibiotics (Galindo-Méndez, 2020). Wu *et al*. (2021) reported that the predominant resistance mechanism to β-lactam antibiotics in *E. coli* is the production of plasmid-borne extended-spectrum β-lactamases (ESBLs). Furthermore, the evolution of *E. coli* resistance is significantly influenced by the mobile genetic elements transferred through horizontal gene transfer. The majority of the virulence genes of extra-intestinal pathogenic *E. coli* (ExPEC) are grouped on mobile genetic elements, typically on virulence plasmids or pathogenicity islands (PAI), displaying a distinct organization (Gregova & Kmet, 2020).

**Bacterial growth reduction**

The efficacy of the MLE at 1 × MIC was assessed against *S. aureus* and *E. coli* for 24 hr. The MLE was selected based on its prominent antibacterial activity on disc diffusion and broth microdilution assays toward test bacteria. Table 4 shows the bacterial growth reduction after 24 hr of treatment with TCLEs. After 24 hr of being treated with MLE and SLE, the growth of *S. aureus* was decreased by 99.84 and 99.88%, respectively. On the other hand, the growth of *S. aureus* was reduced by 98.39%

and 98.56% after 24 hr of treatment with ShLE and YLE, respectively. For the Gram-negative *E. coli*, the growth was suppressed by up to 99.64% and 99.79% after being treated with MLE and SLE, respectively. Whereas, 87.19% and 87.50% of bacterial growth reduction were observed after *E. coli* cells were exposed to both ShLE and YLE, respectively. The growth reduction results are in line with the MIC and MBC values (Table 4), showing the efficacy of the extracts against the test bacteria. Besides that, the present study revealed that the ShLE and YLE may exert a bacteriostatic effect against *E. coli* due to their lower growth reduction (percentage of inhibition). The lower inhibition percentage might lead to a resumption of bacterial growth if the incubation period was prolonged for more than 24 hr. Overall, the percentage of bacterial reduction depends on the leaf maturity of *T. catappa*. According to Chaliha *et al*. (2020), the developmental stage of the plants part resulted in the different concentrations of phytochemical compounds such as ascorbic acid. This is due to the time taken for the leaves to grow into a matured state involves many metabolic processes that can cause the accumulation of phytochemical compounds with antimicrobial activity such as tannins, flavonoids, and phenols. Thus, older *T. catappa* leaves have a higher potential to be an antimicrobial agent.

**Table 3.** Determination of MIC and MBC values of the crude extracts via broth microdilution assay

| Sample | Diameter of inhibition zone (mm) | | | | | |
|---|---|---|---|---|---|---|
| | *Staphylococcus aureus* | | | *Escherichia coli* | | |
| | MIC mg/mL | MBC (mg/mL) | MBC/MIC | MIC (mg/mL) | MBC (mg/mL) | MBC/MIC |
| Shoot | 25.0 | 50.0 | 2 | 25.0 | 400 | 8 |
| Young | 25.0 | 50.0 | 2 | 25.0 | 400 | 8 |
| Matured | 12.5 | 12.5 | 1 | 25.0 | 100 | 4 |
| Senescent | 12.5 | 25.0 | 2 | 25.0 | 100 | 4 |

Note: Positive control is chloramphenicol. The concentration of the sample is 400 mg/mL

**Table 4.** Bacterial growth reduction after 24 hr of treatment with the crude extracts

| Test bacteria/ Leaf extracts | *Staphylococcus aureus* | | | *Escherichia coli* | | |
|---|---|---|---|---|---|---|
| | Without extract | Extract | Bacterial reduction (R% ± SD) | Without extract | Extract | Bacterial reduction (R% ± SD) |
| Shoot | $1.93 \times 10^8$ | $3.10 \times 10^6$ | 98.39 | $2.03 \times 10^8$ | $2.60 \times 10^7$ | 87.19 |
| Young | $2.02 \times 10^8$ | $2.90 \times 10^6$ | 98.56 | $1.96 \times 10^8$ | $2.45 \times 10^7$ | 87.50 |
| Mature | $1.63 \times 10^8$ | $2.60 \times 10^5$ | 99.84 | $2.16 \times 10^8$ | $7.80 \times 10^5$ | 99.64 |
| Senescent | $1.81 \times 10^8$ | $2.25 \times 10^5$ | 99.88 | $1.51 \times 10^8$ | $3.10 \times 10^5$ | 99.79 |

## Time-kill curve

Although the MIC and MBC are the most widely used prediction techniques for antimicrobial action, they do have several drawbacks. Significantly, time-related antimicrobial effects, such as killing rate and post-antibiotic impact, are not taken into account by the MIC and MBC. In this regard, the concept of pharmacokinetic and pharmacodynamic (PK/PD) modeling has been introduced to help interpret determinations of susceptibility breakpoints (Kiem & Schentag, 2006). Time-kill curves are not commonly utilized to distinguish between bacteriostatic and bactericidal antibacterial agents; instead, they have been utilized to study the kinetics of bactericidal killing *in vitro*. They can help determine if the death of bacteria is dependent on concentration or time: Time-dependent killing occurs when increasing antibacterial concentrations to more than the MIC does not result in proportionate increases in killing (e.g., for β-lactams & oxazolidinones). Conversely, concentration-dependent killing occurs when the rate and extent of killing increases with progressively higher antibacterial concentrations (e.g., for aminoglycosides & fluoroquinolones). For time-dependent killing, the region under the serum concentration curve that is higher than the MIC is crucial (Pankey & Sabath, 2004).

In the present study, a time-kill curve was used to assess the correlation between MIC and bactericidal activity of MLE at different extract concentrations. MLE was chosen to assess the efficacy of the extract due to its excellent antibacterial activity in the disc diffusion and broth microdilution assays. Figure 2 shows the time-kill curves of *S. aureus* and *E. coli* at different concentrations of MLE (½ × MIC, 1 × MIC & 2 × MIC). In the control, the growth of *S. aureus* cells began with a log phase, followed by the lag phase and then the stationary phase. When the *S. aureus* cells were treated with MLE at a concentration of ½ × MIC, a slight growth reduction was observed after 4 hr of exposure time with $3.98 \times 10^7$ CFU/mL compared to the control ($1.0 \times 10^9$ CFU/mL). However, after 16 hr of exposure to the MLE, the number of *S. aureus* cells was slightly increased indicating the re-growth phase of the cells. A similar

observation was reported by Jalil *et al*. (2020) and they postulated that this phenomenon might be due to insufficient concentration of the extract to overcome the bacterial cells. At the MLE concentration of 1 × MIC, the *S. aureus* cells were reduced to 99.9% ($1.78 \times 10^6$ CFU/mL). Meanwhile, a reduction of 99.99% *S. aureus* cells was observed when it was treated with 2 × MIC of MLE, whereby only $1.91 \times 10^5$ CFU/mL of *S. aureus* cells was observed compared to the control ($1.38 \times 10^9$ CFU/mL) and the cells were completely killed after 24 hr of exposure time.

Meanwhile, the *E. coli* cells showed a lesser reduction in number after being treated with MLE at a concentration of ½ × MIC, whereby only 50% of bacterial cells were eliminated after 4 hr of exposure compared to control. However, the re-growth pattern was also observed for *E. coli* at a sub-MIC concentration of the extract. A sub-MIC concentration refers to the concentration of a bioactive compound that is not active on microbial growth but is still effective in changing the biochemistry and morphology of bacteria *in vitro* and *in vivo*, reducing their pathogenicity (Braga *et al*., 2000). According to Tam *et al*., (2005), two separate subpopulations with varying susceptibilities may be responsible for the regeneration phenomenon, with the resistant subpopulation taking over after the preferential killing of the susceptible subpopulation at a predetermined interaction time. Antibiotic persistence, defined as the capacity of a bacterial subpopulation to survive upon antibiotic exposure due to non-heritable phenotypic variation that is distinct from the mechanisms that generate resistance, may also induce the regrowth of bacterial cells. Although these persistent cells only make up a small portion of the total number of cells, they allow the population to survive even in high antibiotic dose exposure (Cabral *et al*., 2018). When the MLE at a concentration of 1 × MIC was used, the *E. coli* cells gradually decreased until only $6.46 \times 10^2$ CFU/mL was observed after 44 hr of exposure time compared to control ($1.95 \times 10^{12}$ CFU/mL). The present study also revealed that the MLE at a concentration of 2 × MIC completely killed all *E. coli* cells after 32 h of incubation. Overall, the time-kill curves for both *S. aureus* and *E. coli* exhibited a dose- and time-dependent effect. This trend is in line with a time-kill study by Jalil *et al*., (2021), in which they also concluded that the efficacy of the extract is dependent on dose and exposure time. A similar observation was reported by Sieberi *et al*., (2020) who revealed that the activity of the methanolic extract of *Centella asiatica* was concentration- and time-dependent against the reference strains including *Salmonella typhi* ATCC 19430*, E. coli* ATCC 25922*, Shigella sonnei* ATCC 25931*, Bacillus subtilis* ATCC 21332*,* and *S. aureus* ATCC 25923.

Table 5 shows the exposure time required to achieve 50, 90, 95, 99, and 99.9% growth reduction in the initial inoculum of *S. aureus* and *E. coli* after being exposed to MLE. The findings revealed that the growth reduction for both *S. aureus* and *E. coli* has a positive correlation with the exposure time whereby the longer exposure time led to a higher growth reduction. Regarding the MLE concentration, a positive correlation was also observed whereby the higher the concentration of extract incorporated, the shorter the time was needed to achieve a 99.9% growth reduction. This result revealed that a higher dose is needed for the MLE to show its bactericidal effect against the test bacteria and this killing activity was dependent on exposure time. However, the extract showed a bacteriostatic effect at the sub-MIC of the extract (½ × MIC). According to Zulkamal *et al*., (2023), antibiotics that are bacteriostatic or bactericidal are both effective against harmful bacteria. Bacteriostatic agents, however, only control the growth of bacteria; they do not affect the overall population of bacteria.

**Membrane Cell Leakage Assay (MCLA)**

The result for MCLA is illustrated in Figure 3 and the absorbance value was observed to have increased for both wavelengths. For the nucleic acid content (measured at 260 nm wavelength), the *S. aureus* showed a slight increase in absorption of 0.73 ± 0.06 compared to the control, 0.34 ± 0.01, when exposed to ½ × MIC concentration of MLE. Meanwhile, the absorbance value was increased when the *S. aureus* was exposed to a higher concentration of MLE at 1 × MIC and 2 × MIC, with the absorbance values of 3.01 ± 0.06 and 4.71 ± 0.17, respectively. As for the protein content (measured at 280 nm wavelength), the same trend was observed, whereby an increment of 757.14% of protein content was measured. The absorbance value was increased from 0.43 ± 0.03 (control) to 0.87 ± 0.09 (1/2 × MIC) and then showed a drastic increase at the higher concentrations of 1 × MIC and 2 × MIC, with the absorbance values of 3.62 ± 0.03 and 5.24 ± 0.30, respectively. The findings revealed that the *S. aureus* and *E. coli* cells treated with different concentrations of MLE resulted in a significant discharge of proteins and nucleic acids from the cell to the phosphate buffer medium. Higher concentrations of the MLE led to greater disruption of bacterial cell membranes, as indicated by the higher protein and nucleic acid content in the medium. The membrane cell leakage is believed to be caused by the presence of a variety of bioactive compounds in the MLE, such as terpenoids, flavonoids, alkaloids, phenols, etc. This statement is in line with Muthulakshmi and Neelanarayanan (2021) who detected the presence

of bioactive compounds, such as tannins, terpenoids, steroids, flavonoids, phenols, and alkaloids in the phytochemical screening of *T. catappa* leaf extracts using aqueous and methanol solvent systems. According to George and Brandl (2021), this membrane leakage of bacterial cells may be due to the accumulation of phenolic compounds on the bacterial surface that can lead to membrane disruption. Moreover, flavonoids that can be found in *T. catappa* extracts can also disrupt cell membrane integrity and lead to cell leakage by damaging the lipid bilayer, altering the membrane, and inhibiting protein synthesis (George & Brandl, 2020). Additionally, alkaloids, one of the bioactive compounds that can also be found in TCE, are known to damage cell membranes, leading to the release of cytoplasmic contents (Ngobeni *et al*., 2020). On the other hand, terpenoids primarily break down bacterial cell walls via their lipophilicity, and these compounds can permeate inside bacteria through their phospholipid bilayer, and exhibit antibacterial or bactericidal actions (Nazzaro *et al*., 2013). Since the integrity of the cell membrane is crucial for the normal physiological activities of bacteria, damage to the membrane caused by terpenoids will impact these activities and result in the loss of critical components, such as proteins and vital enzymes, which will ultimately lead to the antimicrobial effect (Burt & Reinders, 2003).

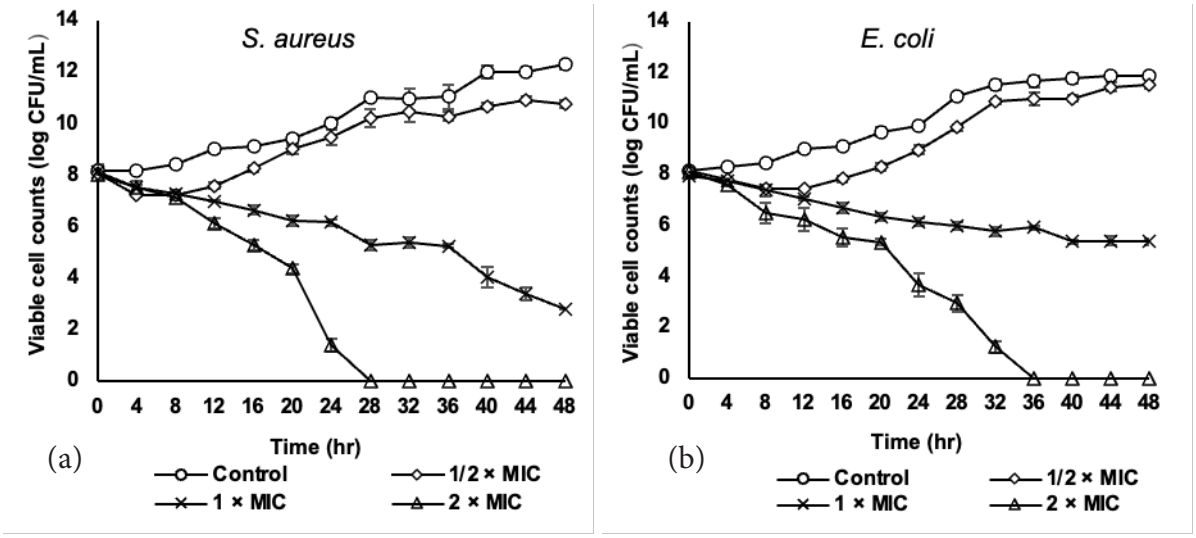


**Fig. 2.** Time-kill curve for test bacteria following exposure to methanolic *T. catappa* mature leaf extract at different extract concentrations (control, ½ × MIC, 1 × MIC, and 2 × MIC). (a) Gram-positive bacterium, *S. aureus,* and (b) Gram-negative bacterium, *E. coli*.

**Table 5.** The exposure time required to achieve 50, 90, 95, 99, and 99.9% growth reduction in initials inoculum of *S. aureus* and *E. coli*

| Percentage of reduction (%) | Time (hr) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Staphylococcus aureus* | | | | *Escherichia coli* | | | |
| | Control | ½ × MIC | 1 × MIC | 2 × MIC | Control | ½ × MIC | 1 × MIC | 2 × MIC |
| 50 | NR | 0 – 4 | 0 – 4 | 0 – 4 | NR | 0 – 4 | 0 – 4 | 0 – 4 |
| 90 | NR | 4 – 8 | 4 – 8 | 4 – 8 | NR | 4 – 8 | 4 – 8 | 4 – 8 |
| 95 | NR | 8 – 12 | 8 – 12 | 4 – 8 | NR | 16 – 20 | 8 – 12 | 4 – 8 |
| 99 | NR | NR | 8 – 12 | 8 – 12 | NR | NR | 12 – 16 | 8 – 12 |
| 99.9 | NR | NR | 16 – 20 | 12 – 16 | NR | NR | 16 – 20 | 12 – 16 |

Key: NR = not reached

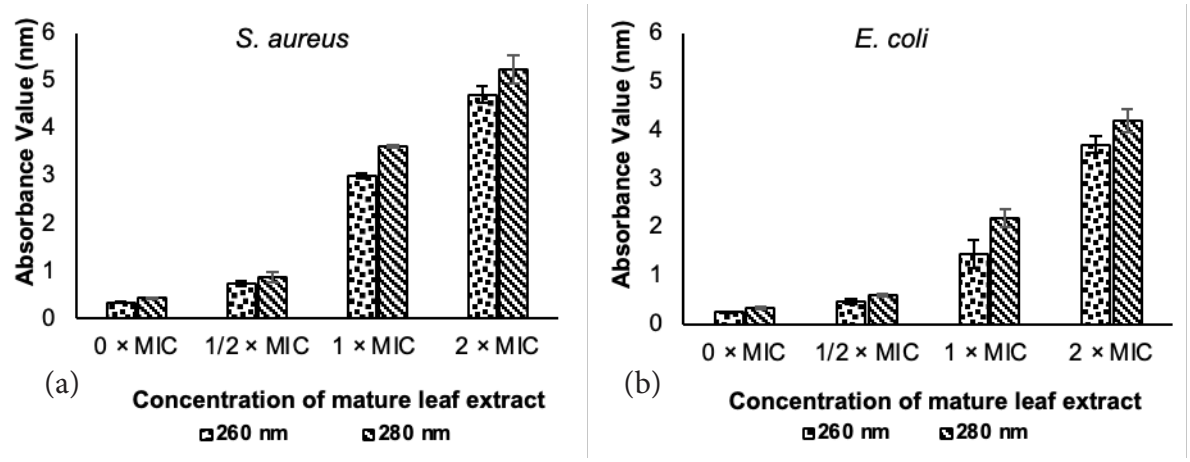


**Fig. 3.** Leakage of protein and nucleic acids from the test bacteria cell after exposure to MLE. (a) *S. aureus*, (b) *E. coli*

## CONCLUSION

The results of this study shed light on the capability of various ages of *T. catappa* leaf extracts (TCLE) as antibacterial agents against *S. aureus* and *E. coli*, with the inhibition zone diameters ranging from 9.23 ±0.32 to 26.33 ±1.53 mm in the disc diffusion assay. The MIC and MBC values for the leaf extracts against *S. aureus* and *E. coli* ranged between 12.5 to 50.0 mg/mL and 25.0 to 400.0 mg/mL, respectively. The ratio of MBC/MIC revealed that all TCLEs showed a bactericidal effect against the two tested bacteria except, for the ShLE and YLE against *E. coli* where only a bacteriostatic effect was observed. Furthermore, the bacterial growth reduction analysis showed that the MLE and SLE could inhibit the growth of Gram-positive *S. aureus* and Gram-negative *E. coli* by up to 99%. However, the ShLE and YLE can only inhibit 98% of the Gram-positive *S. aureus* and only 87% of the Gram-negative *E. coli* after 24 hr of exposure time, respectively. The time-kill curve revealed a complete kill of *S. aureus* and *E. coli* at 2 × MIC of extract within 24 hr and 36 hr of exposure time, respectively. The present study also revealed that the effect of the methanolic MLE of *T. catappa* was dose- and time-dependent. The findings from the membrane cell leakage assay supported the time-kill analysis whereby the nucleic acid and protein content in the medium for *S. aureus* and *E. coli* increased with the increment of extract concentration and thus indicated that a higher concentration of extract could effectively inhibit and kill the bacterial cells. Therefore, the *T. catappa* leaf extracts, particularly the MLE should be further tested and evaluated for potential applications in the pharmaceutical and food industries as an antibiotic and sanitizer, respectively.

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## ETHICAL STATEMENT

Not applicable.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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