

Effect of Mozart's Symphony No. 40 and Qur'anic Recitation of Surah Al-Fatihah on Bacterial Growth and Antibiotic Susceptibility of *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*

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ABSTRACT

Background: Audible sound is increasingly recognised as a physical stimulus that may influence biological systems, including microorganisms. Previous studies have reported changes in bacterial growth and antibiotic susceptibility following exposure to audible sound; however, evidence regarding Qur'anic recitation and its effects on bacteria remains limited. This study compared the effects of Mozart's Symphony No. 40 and Qur'anic recitation of Surah Al-Fatihah on bacterial growth and antibiotic susceptibility. **Methods:** *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 33591, and *Bacillus subtilis* ATCC 6633 were standardised to a 0.5 McFarland standard and investigated under three sound conditions: Mozart's Symphony No. 40, Qur'anic recitation of Surah Al-Fatihah, and a no-sound controls. Bacterial growth was monitored by measuring optical density at 600 nm (OD₆₀₀) at 30-minute intervals for 6 hours. Antibiotic susceptibility was assessed using the disk diffusion method with gentamicin, kanamycin, ampicillin, and chloramphenicol after 24 hours of incubation. Data were analysed using one-way analysis of variance (ANOVA), with statistical significance set at $p < 0.05$. **Results:** Bacterial growth responses varied according to bacterial species, sound condition and incubation time. For *E. coli*, the Qur'anic recitation group recorded the highest OD₆₀₀ of 0.786 at 4 hours, while the classical music group reached 0.766 at 5.5 hours. At 6 hours, OD₆₀₀ values were 0.517, 0.683 and 0.710 for the control, classical music and Qur'anic recitation group, respectively. For *S. aureus*, the highest OD₆₀₀ was 0.900 under Qur'anic recitation at 4.5 hours, whereas the classical music group showed higher OD₆₀₀ values during earlier period of incubation. For *B. subtilis*, Qur'anic recitation generally produced higher OD₆₀₀ values during the early and middle stages of incubation, while the classical music group recorded the highest OD₆₀₀ at 5.5 and 6 hours. One-way ANOVA demonstrated significant overall differences among sound exposure groups at the analysed growth time points ($p < 0.001$). Significant overall differences in inhibition zone measurements were also observed among exposure groups for all three bacterial species, with p values ranging from < 0.001 to 0.008. **Conclusion:** Exposure to Mozart's Symphony No. 40 and Qur'anic recitation of Surah Al-Fatihah was associated with differences in bacterial growth and inhibition zone measurements, with responses varying according to bacterial species and incubation time. These findings provide preliminary evidence that audible sound may influence bacterial responses under laboratory condition; however, the results do not establish a causal effect of sound exposure on bacterial growth or antibiotic susceptibility. Future research should incorporate controlled sound pressure levels and frequency characteristics, together with complementary measures of bacterial growth and viability, to improve reproducibility and further clarify the biological effects and potential of sound exposure.

Keywords:

audible sound; bacterial growth; antibiotic susceptibility; Quran recitation; Mozart's Symphony No. 40

INTRODUCTION

Sound is a form of mechanical energy generated by vibrations and transmitted through a medium as waves.

Although sound is primarily associated with auditory perception, increasing evidence suggests that mechanical sound waves may interact with biological systems beyond the auditory organs. Studies have demonstrated biological

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responses to sound exposure in plants, mammalian cells, and microorganisms, suggesting that sound may act as an environmental physical stimulus capable of influencing cellular processes. In microorganisms, however, the interaction between audible sound and cellular physiology remains comparatively underexplored.

Previous studies have demonstrated that audible sound can influence bacterial growth and physiological characteristics. Lee Ying et al. (2009), for example, reported that exposure to selected audible frequencies increased the growth of *Escherichia coli*, with 5 kHz producing a particularly pronounced response. Similarly, Gu et al. (2016) demonstrated that sound exposure increased biomass and specific growth rate of *E. coli* K-12 and altered intracellular macromolecular synthesis. These findings suggest that bacterial cells can respond to acoustic stimulation and that the response may depend on the characteristics of the sound stimulus.

Music represents a complex form of audible sound involving variations in frequency, amplitude, rhythm, and tempo. Research involving different musical stimuli has reported changes in microbial growth, metabolism, and antibiotic susceptibility. Sarvaiya and Kothari (2015) reported that exposure to Indian classical music increased the growth of most tested microorganisms and also increased their antibiotic susceptibility. The authors proposed that sound-induced changes in cellular physiology, including membrane-related processes, may contribute to the observed responses. More specifically, Kotwal et al. (2016) investigated several musical genres, including Mozart's Symphony No. 40, and reported differences in the growth of *E. coli* and *Staphylococcus aureus* following musical exposure. These findings indicate that microorganisms may respond differently depending on the characteristics of the acoustic stimulus.

Sound exposure has also been investigated in relation to antibiotic susceptibility. Banerjee et al. (2018) reported that different audible frequencies produced different effects on both the growth and antibiotic susceptibility of *E. coli*. Exposure to 432 Hz increased bacterial growth and antibiotic susceptibility, whereas exposure to 4 kHz produced a different response. This finding is important because it indicates that the biological effect of sound may not simply depend on the presence or absence of sound but may vary according to its acoustic characteristics.

In addition to music, Qur'anic recitation represents another form of structured audible sound characterised by specific patterns of vocalisation, rhythm, pitch, and intensity. Previous research on Qur'anic recitation has mainly investigated human psychological and physiological

responses and, to a lesser extent, cellular models. Mehrafsar and Mokhtari (2018) investigated exposure to Qur'anic recitation in a human prostate cancer cell line and reported changes in cell viability, migration, and BCL2L12 gene expression. A systematic review of speaker-based sound exposure studies also identified Qur'anic recitation as one of the less frequently investigated acoustic stimuli in biological models (Kwak et al., 2022).

Although previous studies have demonstrated that audible sound and music can influence microbial growth, metabolism and antibiotic susceptibility, research comparing different forms of audible sound under comparable experimental conditions remains limited (Sarvaiya & Kothari, 2015; Kothari & Sarvaiya, 2017; Gu et al., 2016; Banerjee et al., 2018). For example, previous studies have reported changes in bacteria growth following exposure to different sound frequencies and musical stimuli, as well as alterations in antibiotics susceptibility (Saivaiya & Kothari, 2015; Benerjee et al., 2018). However, the available literature is characterised by considerable variation in sound stimuli, experimental conditions and outcome measures, making direct comparisons between studies difficult (Kwak et al., 2022). In particular, evidence regarding the effects of Qur'anic recitation on microorganisms remains limited compared with studies investigating conventional musical or sound stimuli.

The present study addresses this gap by directly comparing two distinct forms of audible sound, namely Mozart's Symphony No. 40 as a representative of Western classical music and Al-Fatihah recitation as a form of Qur'anic recitation, under the same experimental framework. While previous studies have examined the biological effects of musical sound on microorganisms (Sarvaiya & Kothari, 2015; Kothari & Sarvaiya, 2017), and limited studies have investigated Qur'anic recitation in biological systems (Mehrafsar & Mokhtari, 2018), direct comparisons between Qur'anic recitation and a defined classical musical composition have received little attention. Importantly, the present study does not assume that either sound type would produce a superior biological effect; rather, it investigates whether these two distinct forms of audible sound are associated with similar or different microbial responses.

To provide a controlled comparison, Mozart's Symphony No. 40 and Al-Fatihah recitation were evaluated under the same experimental conditions, with a no-sound control serving as the comparator. Standardised bacterial inocula of *E. coli*, *S. aureus*, and *Bacillus subtilis* were exposed to each sound condition, and bacterial growth and antibiotic susceptibility were assessed using optical density at 600

nm (OD_{600}) and inhibition-zone measurements, respectively. This direct comparison within a common experimental framework provides a more consistent basis for evaluating whether different forms of audible sound are associated with differential responses among bacterial species. Therefore, this study aimed to determine the effects of Mozart's Symphony No. 40 and Al-Fatihah recitation on bacterial growth and antibiotic susceptibility and to compare the responses of the three selected bacterial species.

MATERIALS AND METHODS

Test Organisms

Three bacterial species were used in this study: the Gram-negative *Escherichia coli* ATCC 25922 and the Gram-positive *Staphylococcus aureus* ATCC 33591 and *Bacillus subtilis* ATCC 6633. The organisms were obtained from the Microbiology Laboratory, Centre for Medical Laboratory Technology Studies, Faculty of Health Sciences, Universiti Teknologi MARA, Puncak Alam Campus, Selangor.

Preparation and Standardisation of Bacterial Cultures

The bacterial cultures were initially grown on nutrient agar at 37°C for 18–24 hours. The identity of the selected organisms was confirmed using Gram staining and appropriate differential and biochemical tests. Fresh bacterial colonies were subsequently used to prepare suspensions. Before exposure to the experimental conditions, all bacterial suspensions were standardised to a 0.5 McFarland turbidity standard. The turbidity was verified spectrophotometrically at 625 nm, with an absorbance range of approximately 0.08–0.10.

Experimental Design and Sound Exposure

Tryptic soy broth (TSB) was used for the bacterial growth experiment. For each bacterial species and experimental condition, three independent broth cultures were prepared. Three experimental conditions were investigated: (1) Mozart's Symphony No. 40, (2) Qur'anic recitation of Surah Al-Fatihah, and (3) no-sound control. Thus, three independent cultures were prepared for each organism under each sound condition, resulting in 27 broth cultures overall.

The Mozart stimulus consisted of a recording of Symphony No. 40, while the Qur'anic recitation stimulus consisted of Surah Al-Fatihah recited by Sheikh Mishary bin Rashid

Alafasy. A speaker (AS-BS06; Malaysia) was positioned inside the incubation chamber together with the bacterial culture and the assigned sound treatment was played continuously throughout the incubation period. Control cultures were maintained in the same incubation environment without sound exposure. The experimental setup is shown in Figure 1.

The sound-pressure level, exact distance between the speaker and culture vessels, and frequency spectrum of the sound stimulus were not measured or recorded during the experiment. Therefore, the sound exposure was described based on the experimental setup and sound stimulus used rather than by quantified acoustic parameters.



Figure 1: Experimental setup for audible sound exposure of bacterial cultures.

Measurement of Bacterial Growth

Following inoculation, bacterial cultures were incubated at 37°C for 6 hours. Optical density was measured at 600 nm (OD_{600}) using a spectrophotometer at 30-minute intervals. Sterile TSB was used as the blank. Where required, samples were diluted before measurement to ensure readings remained within an appropriate measurement range.

The OD_{600} measurements represented repeated measurements obtained from the same independent broth cultures over time and did not constitute additional biological replicates. Growth profiles were evaluated by comparing OD_{600} values among the Mozart, Al-Fatihah, and no-sound conditions.

Antibiotic Susceptibility Testing

Antibiotic susceptibility was assessed using the disk diffusion method on Mueller–Hinton agar (MHA).

Following standardisation to a 0.5 McFarland suspension, bacterial cultures were evenly inoculated onto MHA using sterile swabs. Antibiotic disks containing gentamicin (CN10), kanamycin (K30), ampicillin (AMP10), and chloramphenicol (C30) were placed on the inoculated agar surface.

For each bacterial species, three independent agar plates were prepared for each experimental condition. The plates were incubated at 37°C for 18–24 hours. Following incubation, the zone of inhibition surrounding each antibiotic disk was measured in millimetres. The experimental procedure was based on the disk diffusion approach described in the Clinical and Laboratory Standards Institute guidelines (Clinical and Laboratory Standards Institute, 2020).

Statistical Analysis

All experiments were performed using three independent cultures for each organism and experimental condition. Data are presented as mean $\pm$ standard deviation (SD). Differences among the three experimental conditions were analysed using one-way analysis of variance (ANOVA) at selected measurement points. A p -value < 0.05 was considered statistically significant. Statistical analyses were conducted using Statistical Package for the Social Sciences (SPSS).

RESULTS

Effect of Audible Sound on Bacterial Growth

The bacterial growth responses varied according to bacterial species, sound condition and incubation time. The OD₆₀₀ profiles for *E. coli*, *S. aureus* and *B. subtilis* are presented in Tables 1-3 and Figures 2-4, respectively.

Escherichia coli

For *E. coli*, both sound-exposed groups generally recorded higher OD₆₀₀ values than the no-sound control during most of the incubation period. Under Mozart's Symphony No.40, the OD₆₀₀ increased from 0.288 at 0.5 hours to 0.766 at 5.5 hours. Under Al-Fatihah recitation, the OD₆₀₀ reached 0.786 at 4 hours, which was the highest recorded value among the three conditions for this organism.

At 6 hours, the OD₆₀₀ values were 0.517, 0.683 and 0.710 for the control, Mozart and Al-Fatihah conditions, respectively. One-way ANOVA demonstrated significant overall differences among the three conditions at the analysed time points of 2, 4 and 6 hours ($p < 0.001$).

Table 1: Optical density (OD) at 600nm OD₆₀₀ of *E. coli* under control, Mozart symphony No.40, and Qur'anic recitation of Surah Al-Fatihah conditions during 6 hours of incubation.

Incubation time (hour)	Optical density (OD) _{600 nm}		
	Control	Mozart	Al-Fatihah
0.5	0.225	0.288	0.307
1	0.321	0.429	0.465
1.5	0.358	0.483	0.453
2	0.410	0.500	0.562
2.5	0.452	0.637	0.579
3	0.527	0.689	0.651
3.5	0.546	0.701	0.754
4	0.621	0.756	0.786
4.5	0.639	0.693	0.735
5	0.651	0.762	0.698
5.5	0.631	0.766	0.723
6	0.517	0.683	0.710

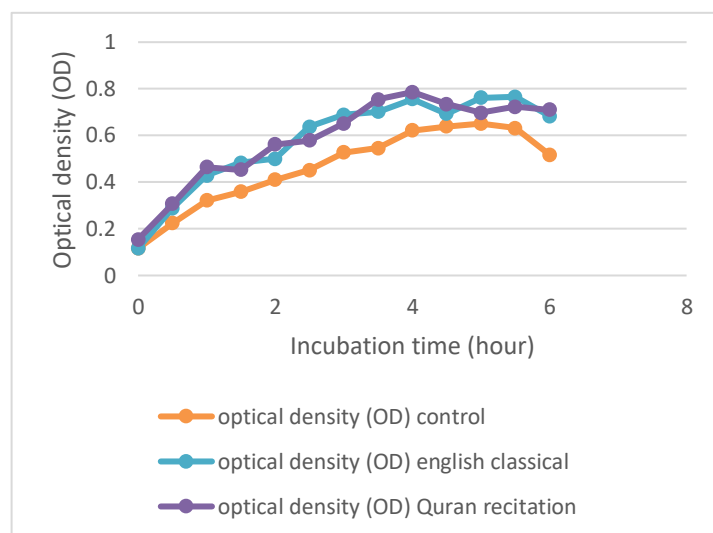


Figure 2: OD₆₀₀ of *E. coli* over incubation time under no-sound control, english classical (Mozart's Symphony No. 40) and Qur'anic recitation of Surah Al-Fatihah conditions.

Staphylococcus aureus

A distinct temporal response was observed for *S. aureus*. Exposure to Mozart's Symphony No. 40 was associated with higher OD₆₀₀ values during the earlier stages of incubation, reaching 0.652 at 2.5 hours compared with 0.304 in the control. Under Al-Fatihah exposure, the OD₆₀₀ continued to increase during the middle portion and reached its highest recorded value of 0.900 at 4.5 hours, compared with 0.481 in the control at the same time point.

At later time points, the OD₆₀₀ values fluctuated among the three conditions. At 6 hours, the values were 0.535, 0.638 and 0.659 for the control, Mozart and Al-Fatihah conditions, respectively. One-way ANOVA demonstrated

significant overall differences among the three conditions at 2, 4 and 6 hours ($p < 0.001$).

Table 2: Optical density at 600nm (OD₆₀₀) of *S. aureus* under control, Mozart symphony No.40, and Qur'anic recitation of Surah Al-Fatihah conditions during 6 hours of incubation.

Incubation time (hour)	Optical density (OD) _{600nm}		
	Control	Mozart	Al-Fatihah
0.5	0.227	0.256	0.196
1	0.255	0.311	0.252
1.5	0.287	0.418	0.347
2	0.267	0.577	0.451
2.5	0.304	0.652	0.593
3	0.349	0.514	0.636
3.5	0.381	0.518	0.751
4	0.475	0.561	0.824
4.5	0.481	0.680	0.900
5	0.708	0.684	0.771
5.5	0.637	0.723	0.837
6	0.535	0.638	0.659

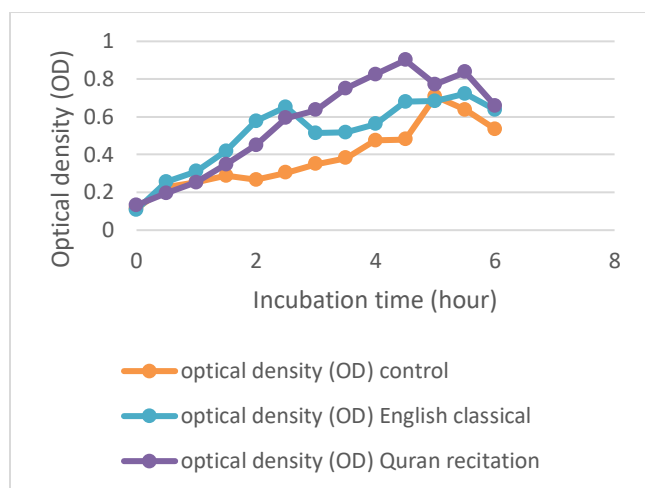


Figure 3: OD₆₀₀ of *S. aureus* over incubation time under control, english classical (Mozart's Symphony No. 40) and Qur'anic recitation of Surah Al-Fatihah conditions.

Bacillus subtilis

For *B. subtilis*, the responses to the two sound stimuli also varied throughout the incubation period. Mozart's Symphony No.40 produced a relatively high OD₆₀₀ during the early incubation period, reaching 0.282 at 0.5 hours compared with 0.127 in the control. Under Al-Fatihah exposure, higher OD₆₀₀ values were observed during

several early and middle time points, with the OD₆₀₀ reaching 0.589 at 5 hours compared with 0.433 in the control.

At 5.5 hours, the Mozart condition recorded the highest OD₆₀₀ value of 0.724, while at 6 hours the Mozart condition remained higher than both the control and Al-Fatihah conditions, with values of 0.590, 0.407 and 0.510, respectively. One-way ANOVA demonstrated significant overall differences among the three conditions at 2, 4 and 6 hours ($p < 0.001$).

Table 3: Optical density at 600nm (OD₆₀₀) of *B. subtilis* under control, Mozart symphony No.40, and Qur'anic recitation of Surah Al-Fatihah conditions during 6 hours of incubation.

Incubation time (hour)	Optical density (OD) _{600nm}		
	control	Mozart	Al-Fatihah
0.5	0.127	0.282	0.167
1	0.160	0.301	0.267
1.5	0.224	0.324	0.279
2	0.266	0.277	0.334
2.5	0.292	0.311	0.358
3	0.356	0.354	0.434
3.5	0.244	0.329	0.492
4	0.262	0.344	0.490
4.5	0.393	0.344	0.528
5	0.433	0.383	0.589
5.5	0.452	0.724	0.563
6	0.407	0.590	0.510

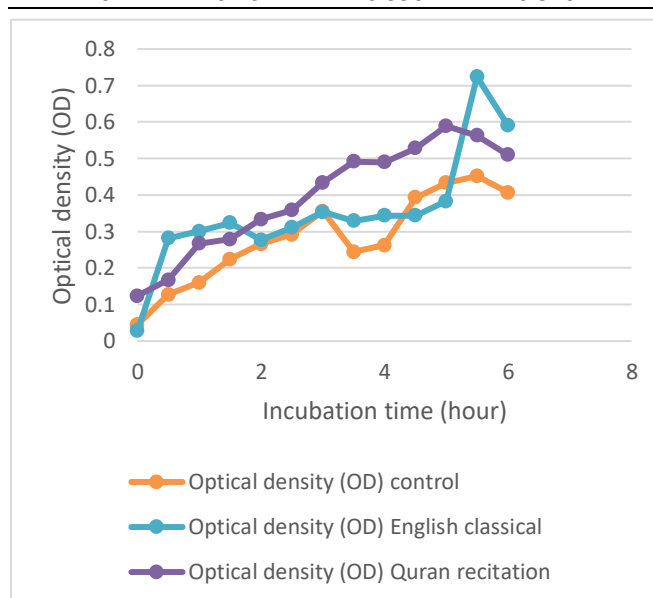


Figure 4: OD₆₀₀ of *B. subtilis* over incubation time under no-sound control, english classical (Mozart's Symphony No. 40) and Qur'anic recitation of Surah Al-Fatihah conditions.

Overall Growth Response

Overall, the OD₆₀₀ profiles demonstrated that the response to sound exposure was not uniform across bacterial species or incubation times. *E. coli* generally showed higher OD₆₀₀ values under both sound conditions than the control, whereas *S. aureus* demonstrated a pronounced middle-period response under Al-Fatihah and an earlier response under Mozart. For *B. subtilis*, Al-Fatihah was associated with higher OD₆₀₀ values at several early and middle time points, while Mozart recorded the highest values at the later 5.5- and 6-hour measurements. These findings indicate that the observed response depended on the interaction between bacterial species, sound condition and incubation time.

Effect of Audible Sound on Antibiotic Susceptibility

The inhibition-zone measurements for the four antibiotics are summarised in Figure 5-7. Differences among the three sound-exposure conditions were observed for the tested antibiotic-organism combinations.

Escherichia coli

For *E. coli*, the inhibition-zone diameters varied among the control, Mozart and Al-Fatihah conditions for all four antibiotics. Under Mozart, the largest numerical increase relative to the control was observed with ampicillin, with a mean inhibition zone of 13 mm compared with 9 mm in the control. Under Al-Fatihah, kanamycin showed the largest numerical increase relative to the control, with inhibition zones of 23 and 20 mm, respectively.

One-way ANOVA demonstrated significant overall differences among the three exposure conditions for gentamicin ($p = 0.002$), kanamycin ($p = 0.001$), ampicillin ($p < 0.001$), and chloramphenicol ($p = 0.002$).

Staphylococcus aureus

For *S. aureus*, larger inhibition zones than the control were observed under at least one sound condition for all four antibiotics. Chloramphenicol demonstrated the largest numerical difference under Al-Fatihah, with the inhibition zone increasing from 26 mm in the control to 35 mm. Under Mozart, the inhibition zone for ampicillin increased from 16 to 20 mm.

One-way ANOVA demonstrated significant overall differences among the three exposure conditions for gentamicin ($p = 0.008$), kanamycin ($p = 0.001$), ampicillin ($p = 0.001$), and chloramphenicol ($p < 0.001$).

Bacillus subtilis

For *B. subtilis*, Mozart was associated with larger inhibition zone than the control for all four antibiotics. The largest numerical difference under Mozart was observed for ampicillin, with the inhibition zone increasing from 22 to 29 mm. Under Al-Fatihah, gentamicin demonstrated the largest numerical increase relative to control, from 22 to 27 mm.

One-way ANOVA demonstrated significant overall differences among the three exposure conditions for gentamicin ($p < 0.001$), kanamycin ($p < 0.001$), ampicillin ($p = 0.002$), and chloramphenicol ($p = 0.006$).

Overall, the inhibition-zone diameters differed among exposure conditions, although the magnitude and direction of the numerical changes varied according to bacterial species, antibiotic and sound stimulus. The present analysis therefore indicates overall differences among exposure groups rather than establishing which specific pairs of groups differed statistically.

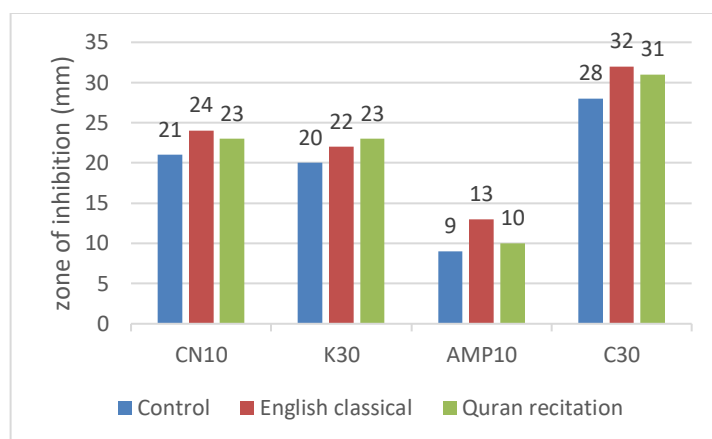


Figure 5: Antibiotic inhibition zone for *E. coli*. Values are presented as mean $\pm$ SD ($n=3$ independent biological replicates per sound-exposure group). Statistical analysis was performed using one-way ANOVA, with $p < 0.05$ considered statistically significant.

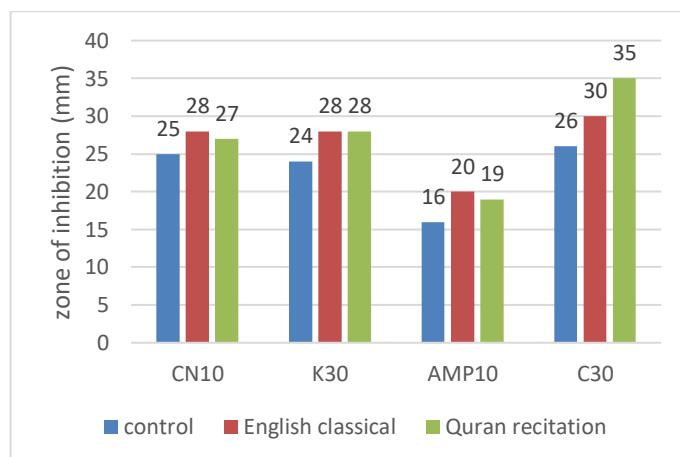


Figure 6: Antibiotic inhibition zone for *S. aureus*. Values are presented as mean $\pm$ SD (n =3 independent biological replicates per sound-exposure group). Statistical analysis was performed using one-way ANOVA, with $p < 0.05$ considered statistically significant.

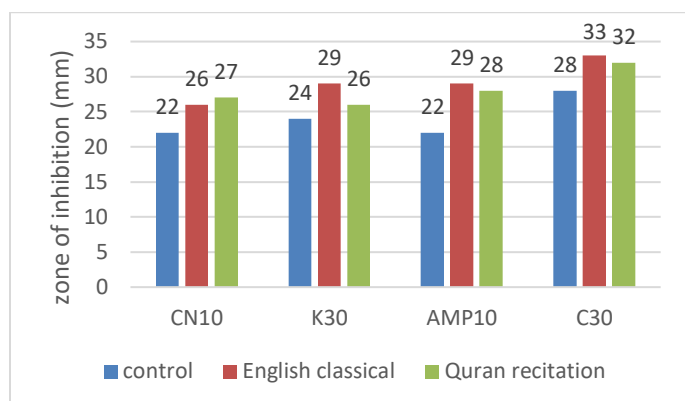


Figure 7: Antibiotic inhibition zone for *B. subtilis*. Values are presented as mean $\pm$ SD (n =3 independent biological replicates per sound-exposure group). Statistical analysis was performed using one-way ANOVA, with $p < 0.05$ considered statistically significant.

DISCUSSION

The present study demonstrated that exposure to audible sound in the form of Mozart's Symphony No. 40 and Qur'anic recitation of Surah Al-Fatihah was associated with differences in bacterial growth compared with the no-sound control. Across the three bacterial species examined, higher OD₆₀₀ values were generally observed under one or both sound-exposure conditions at several incubation time points. However, the magnitude and timing of the response varied among bacterial species and between sound stimuli. These findings are broadly consistent with previous studies reporting that audible sound can influence bacterial growth and physiological characteristics. Lee Ying et al. (2009) reported increased growth of *E. coli* following exposure to selected audible frequencies, while Gu et al. (2016) demonstrated increased biomass and specific growth rate in *E. coli* exposed to sound. These findings support the possibility that microorganisms can respond to mechanical stimulation associated with audible sound.

The response of *E. coli* in the present study was characterised by higher OD₆₀₀ values under both Mozart and Al-Fatihah exposure during most of the incubation period. The highest values were observed at 4 hours, with OD₆₀₀ values of 0.756 under Mozart and 0.786 under Al-Fatihah compared with 0.621 in the control. This pattern is consistent with previous findings that *E. coli* can exhibit altered growth characteristics following acoustic

stimulation. Gu et al. (2016), for example, reported increased biomass and specific growth rate in *E. coli* following sound exposure and identified changes in intracellular macromolecular synthesis. Nevertheless, the present study measured OD₆₀₀ as an indirect indicator of bacterial growth and did not investigate the molecular processes responsible for the observed differences.

S. aureus demonstrated a different temporal growth pattern. Mozart exposure produced a marked increase in OD₆₀₀ during the earlier incubation period, reaching 0.652 at 2.5 hours compared with 0.304 in the control. Under Al-Fatihah exposure, OD₆₀₀ continued to increase and reached 0.900 at 4.5 hours compared with 0.481 in the control. This difference in the timing of the highest OD₆₀₀ values suggests that bacterial responses to acoustic stimulation may vary according to both bacterial species and characteristics of the sound stimulus. Previous research has similarly reported differences in bacterial responses to different musical stimuli. Kotwal et al. (2016), for example, investigated Mozart's Symphony No. 40 and other musical genres and reported changes in the growth of *E. coli* and *S. aureus* following musical exposure.

For *B. subtilis*, both sound conditions were associated with higher OD₆₀₀ values than the control at several incubation points. Mozart produced a marked early increase, with an OD₆₀₀ of 0.282 at 0.5 hours compared with 0.127 in the control, whereas Al-Fatihah showed a more sustained increase during the later incubation period, reaching 0.589 at 5 hours compared with 0.433 in the control. The highest OD₆₀₀ under Mozart occurred at 5.5 hours (0.724). These findings further demonstrate that the temporal pattern of the response differed between sound stimuli and bacterial species.

The differences in bacterial growth observed following sound exposure may be related to the ability of microbial cells to respond to mechanical stimulation. Sound waves transmitted through the growth medium may produce mechanical disturbances that interact with bacterial cell surfaces and potentially influence cellular processes. Previous studies have proposed that acoustic stimulation may affect bacterial biomass, cellular morphology, protein synthesis, membrane properties, and other physiological characteristics (Sarvaiya & Kothari, 2015; Gu et al., 2016). Mechanosensitive components, including mechanosensitive ion channels, may provide one possible route through which cells respond to mechanical changes in their environment. However, these mechanisms were not directly examined in the present study. Therefore, such explanations should be regarded as possible mechanisms rather than direct conclusions from the present findings.

An important finding of the present study was the variation in inhibition-zone diameters observed following sound exposure. For most of the antibiotic-organism combinations examined, the sound-exposure groups recorded larger inhibition zones than the no-sound control for at least one of the sound conditions. For example, *E. coli* showed larger inhibition zones under Mozart for gentamicin, ampicillin, and chloramphenicol, while Al-Fatihah produced the largest numerical inhibition zone for kanamycin. Similarly, *S. aureus* showed the largest inhibition zone for chloramphenicol under Al-Fatihah, while *B. subtilis* demonstrated increased inhibition-zone diameters for several antibiotics under both sound conditions. These observations are broadly consistent with Sarvaiya and Kothari (2015), who reported changes in antimicrobial susceptibility following exposure to Indian classical music, and with Banerjee et al. (2018), who demonstrated that audible frequency could influence both bacterial growth and antibiotic susceptibility in *E. coli*.

The inhibition-zone findings require cautious interpretation. A larger inhibition zone indicates a difference in the measured response under the experimental conditions but does not, by itself, establish that the bacteria became clinically more susceptible or that sound exposure reversed antibiotic resistance. Zone diameter may be influenced by bacterial growth characteristics, physiological state, membrane properties, antibiotic diffusion, and other factors. In the present study, antibiotic susceptibility testing was performed using a standardised disk diffusion procedure based on Clinical and Laboratory Standards Institute (CLSI) guidelines. The analysis focused on comparing inhibition-zone diameters among the three sound-exposure groups rather than determining whether sound exposure altered the clinical susceptibility category of the tested bacteria. Therefore, the observed findings are interpreted as variations in inhibition-zone diameters under different sound-exposure conditions rather than definitive evidence of a change in clinical antibiotic susceptibility. Direct assessment of antibiotic uptake, membrane permeability, efflux activity, minimum inhibitory concentrations, or molecular changes would be required to further determine the mechanisms underlying the observed differences.

The findings involving Al-Fatihah are of particular interest because research investigating Qur'anic recitation using bacterial models remains limited. Previous studies involving Qur'anic recitation have predominantly focused on human psychological or physiological responses and other biological models. Mehrafsar and Mokhtari (2018), for example, investigated Qur'anic recitation in a human prostate cancer cell line and reported changes in cell viability, migration, and gene expression. The present

study therefore provides preliminary comparative data on Qur'anic recitation in a bacterial model alongside a defined classical musical stimulus. However, the findings should be interpreted within the specific experimental conditions of the present study and should not be generalised to biological effects beyond the tested bacterial models.

Although statistically significant overall differences among the three experimental conditions were observed at the selected measurement points and for the antibiotic inhibition-zone measurements, the available statistical output did not allow specific pairwise differences between individual exposure groups to be independently verified. Therefore, the present study does not interpret the data as demonstrating that Mozart or Al-Fatihah was superior to the other sound stimulus. Instead, the findings indicate that the measured bacterial responses varied among the no-sound, Mozart, and Al-Fatihah conditions. The differences observed across bacterial species, incubation times, and antibiotics further suggest that the response may depend on the interaction between the organism and the characteristics of the sound stimulus.

Several limitations should be considered when interpreting these findings. First, the sound pressure level, exact distance between the speaker and culture vessels, and frequency spectrum of the sound stimuli were not quantitatively measured or recorded during the experiment. Although the same experimental setup was used for the sound-exposure groups, the acoustic exposure therefore cannot be expressed as a defined sound pressure level or frequency-specific dose. Sound reflection within the incubation environment may also have contributed to variation in the acoustic conditions experienced by the cultures. These factors limit the reproducibility of the acoustic exposure and make direct comparison with studies using quantified acoustic parameters difficult. Kwak et al. (2022) highlighted substantial variation in acoustic parameters and experimental setups among studies investigating biological responses to sound. Future investigations should therefore incorporate calibrated measurement of sound pressure level and frequency characteristics and, where possible, use an acoustically controlled experimental environment.

Second, OD₆₀₀ was used as an indirect measure of bacterial growth. Optical density reflects turbidity or cell density but cannot distinguish between viable and non-viable cells. The observed changes in OD₆₀₀ therefore cannot independently establish whether sound exposure increased the number of viable bacteria. Complementary methods such as viable colony counts, viable-cell assays, or other direct measurements of bacterial growth and

viability would strengthen future investigations.

Third, the antimicrobial susceptibility analysis was based on inhibition-zone diameters obtained using the disk diffusion method. Although the procedure was performed using a standardised approach, inhibition-zone diameter alone does not establish the cellular mechanism responsible for differences between exposure groups. Further studies incorporating minimum inhibitory concentration determination, antibiotic uptake, membrane permeability, efflux activity, and molecular analyses could provide additional insight into how acoustic exposure may influence bacterial responses to antimicrobial agents.

Overall, the present study provides preliminary evidence that different forms of audible sound may be associated with changes in bacterial growth and inhibition-zone measurements under the experimental conditions examined. The responses varied according to bacterial species, incubation time, antibiotic, and sound stimulus. The study also provides a direct comparison of Mozart's Symphony No. 40 and Qur'anic recitation of Surah Al-Fatihah using the same experimental framework and standardised bacterial inocula. However, the findings should be regarded as evidence of an association under the specific laboratory conditions used rather than evidence of a therapeutic or clinical effect.

CONCLUSION

This study demonstrated that exposure to Mozart's Symphony No. 40 and Qur'anic recitation of Surah Al-Fatihah was associated with differences in bacterial growth, as indicated by changes in OD₆₀₀ values in *E. coli*, *S. aureus*, and *B. subtilis*. The magnitude and timing of the observed responses varied according to bacterial species and sound condition. Sound exposure was also associated with differences in antibiotic inhibition-zone diameters for the tested bacterial species and antibiotics.

Overall, the findings provide preliminary evidence that audible sound may influence bacterial growth and antibiotic response under laboratory conditions. The observed responses suggest that controlled acoustic stimulation may have potential as a non-chemical physical approach for modulating microbial behaviour, which could be of interest for future applications in industrial microbiology, biotechnology, and microbial bioprocessing. However, further investigation is required before such potential applications can be considered, particularly through experiments using controlled and quantified

acoustic parameters, larger sample sizes, and different bacterial and industrially relevant microbial systems.

The present findings should not be interpreted as evidence of a therapeutic or clinical effect, and the underlying biological mechanisms remain to be established. Future research should incorporate controlled sound pressure levels and frequency characteristics, together with complementary measures of bacterial growth and viability, to improve reproducibility and further clarify the biological effects and potential of sound exposure.

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Selective Antibacterial Activity of *Kaempferia parviflora* Root Aqueous Extract Against Selected Skin and Soft Tissue Infection Pathogens

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ABSTRACT

Skin and soft tissue infections (SSTIs) remain clinically important, while antimicrobial resistance increases the need to investigate alternative antibacterial agents. This study evaluated the antibacterial activity of *Kaempferia parviflora* root aqueous extract against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, and *Pseudomonas aeruginosa* and estimated its total phenolic content (TPC) and total flavonoid content (TFC). Aqueous extraction was selected to assess the water-soluble root fraction, which differs from the organic-solvent rhizome preparations emphasized in previous antimicrobial studies. Roots were oven-dried, powdered, extracted with distilled water, freeze-dried, and prepared as a 500 mg/mL stock. Antibacterial activity was assessed in triplicate by agar well diffusion, broth microdilution minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) assays. At 500 mg/mL, the extract produced inhibition zones of 10.00 ± 0.00 mm against *S. aureus* and 14.67 ± 0.57 mm against *S. pyogenes*, which were significantly smaller than gentamicin and ampicillin controls, respectively ($p < 0.001$). No inhibition zone was observed against *E. coli* or *P. aeruginosa*. The MIC was 500 mg/mL for all four organisms, while no bactericidal effect was detected at concentrations up to 500 mg/mL. TPC and TFC were estimated using gallic acid and quercetin colorimetric calibration curves, respectively. The aqueous root extract showed weak, selective bacteriostatic activity, mainly against the Gram-positive organisms tested. Optimization of extraction conditions and evaluation of isolated constituents or antibiotic combinations are warranted.

Keywords:

Kaempferia parviflora; antimicrobial activity; aqueous extract; skin and soft tissue infections; total phenolic content; total flavonoid content

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INTRODUCTION

Skin and soft tissue infections (SSTIs) encompass infections involving the skin, subcutaneous tissue, fascia, and muscle. *Staphylococcus aureus* and *Streptococcus pyogenes* are major Gram-positive causes of SSTIs, while Gram-negative organisms, including *Escherichia coli* and *Pseudomonas aeruginosa*, also occur in complicated infections and healthcare-associated wounds (Kaye et al., 2019; Kholifah et al., 2023). The global burden of bacterial skin diseases and the continued emergence of antimicrobial resistance create a need for additional strategies to complement conventional antibacterial therapy (Xue et al., 2022).

Medicinal plants are a potential source of antibacterial compounds because they contain chemically diverse secondary metabolites. *Kaempferia parviflora* Wall. ex Baker, commonly known as black ginger, belongs to the Zingiberaceae family and has been used traditionally in Southeast Asia. Its rhizomes are rich in methoxyflavones and other phytochemicals associated with antioxidant, anti-inflammatory, and antimicrobial activities (Chen et al.,

2018; Tan et al., 2024). Previous work has reported antimicrobial activity of *K. parviflora* extracts and individual methoxyflavones against selected microorganisms, including biofilm-forming bacteria (Mala et al., 2018; Atom et al., 2022).

The biological activity recovered from plant material depends strongly on the plant part and extraction solvent. Phenolic and flavonoid compounds differ in polarity and solubility, and aqueous extraction may recover a different chemical profile from organic solvents. Optimization studies on *K. parviflora* rhizomes have shown that extraction conditions influence total phenolic and flavonoid yields (Rahman et al., 2018). Methoxyflavones are relatively lipophilic, which may limit their recovery in water-based preparations compared with less polar solvents (Chankitisakul et al., 2025). An aqueous extract was selected in the present study to evaluate the water-soluble fraction of the root and to provide a direct contrast with the organic-solvent rhizome preparations that dominate previous antimicrobial work. This design also

tests whether antibacterial activity remains detectable when recovery of lipophilic methoxyflavones is expected to be limited.

Most published antimicrobial studies of *K. parviflora* have focused on rhizomes, organic-solvent extracts, or isolated methoxyflavones, including antibiofilm and antibiotic-combination models (Mala et al., 2018; Atom et al., 2022; Sookkhee et al., 2022). Evidence specifically addressing an aqueous root extract against SSTI-associated bacteria remains limited. This study therefore evaluated the antibacterial activity of an aqueous root extract against *S. aureus*, *S. pyogenes*, *E. coli*, and *P. aeruginosa* using agar well diffusion, MIC, and MBC assays. TPC and TFC were quantified as bulk estimates of total phenolic and flavonoid content rather than as identification of individual bioactive compounds.

MATERIALS AND METHODS

Sample Collection and Extraction

Roots of *K. parviflora* were obtained from the Green House at Universiti Teknologi MARA (UiTM) Cawangan Selangor, Puncak Alam Campus. The roots were washed, cut, rinsed with distilled water, and dried in an oven at 40°C for three days before being ground into a fine powder. The powder was soaked in distilled water at a 10:1 root-to-water ratio and mixed in an orbital shaker for 24 hours. The mixture was filtered using Whatman No. 1 filter paper, and the filtrate was freeze-dried for three days. The dried extract was stored frozen until use. A 500 mg/mL stock solution was prepared in 5% dimethyl sulfoxide (DMSO) and stored at 4°C.

Bacterial Strains and Identification

The test organisms were *S. aureus* ATCC 25923, *S. pyogenes* ATCC 12384, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 10145. Stock cultures were obtained from the Microbiology Laboratory, Centre for Medical Laboratory Technology Studies, Faculty of Health Sciences, UiTM Puncak Alam. Cultures were subcultured on blood agar and incubated at 37°C for 18-24 hours. Colony morphology and Gram staining were assessed, followed by culture on appropriate differential media and biochemical tests to confirm bacterial identity.

Total Phenolic Content

TPC was determined using the Folin-Ciocalteu colorimetric method. Diluted extract (20 µL) was mixed with 20 µL Folin-Ciocalteu reagent and allowed to stand for five minutes. Sodium carbonate solution (200 µL, 7%) and 10 µL deionized water were then added. After incubation in the dark at room temperature for 120 minutes, absorbance was measured at 750 nm using a microplate reader. Gallic acid was used to construct the calibration curve, which was described by $y = 0.4947x + 0.3661$ with

$R^2 = 0.9825$. TPC was expressed as milligrams of gallic acid equivalents per gram of extract (mg GAE/g). Samples were analyzed in triplicate.

Total Flavonoid Content

TFC was determined using the aluminium chloride colorimetric method. Plant extract or quercetin standard (50 µL) was mixed with 10 µL of 10% aluminium chloride, 150 µL distilled water, and 10 µL of 1 M sodium acetate in a 96-well microplate. The reaction mixture was incubated in the dark at room temperature for 40 minutes and absorbance was read at 410 nm. The quercetin calibration curve was described by $y = 1.6433x + 0.7643$ with $R^2 = 0.98$. TFC was expressed as milligrams of quercetin equivalents per gram of extract (mg QE/g). All measurements were performed in triplicate.

Antibiotic Susceptibility Test

Antibacterial activity was screened by agar well diffusion on Mueller-Hinton agar. Bacterial suspensions were adjusted to an optical density of 0.8-1.3 at 600 nm and lawned across the agar surface. Wells of 6 mm diameter were prepared aseptically, and 50 µL of 500 mg/mL *K. parviflora* root extract was added to the test well. Distilled water served as the negative control in the original assay. Because the extract stock was prepared in 5% DMSO, the absence of a matched 5% DMSO solvent control is acknowledged as a methodological limitation. Gentamicin (10 µg) was used as the positive control for *S. aureus*, *E. coli*, and *P. aeruginosa*, while ampicillin (10 µg) was used for *S. pyogenes*. Plates were incubated at 37°C for 18-24 hours, and inhibition zones were measured in millimetres. Experiments were performed in triplicate.

Minimum Inhibitory Concentration

MIC was determined using a broth microdilution assay in 96-well microtiter plates. The root extract was serially diluted twofold in Mueller-Hinton broth from 500 to 0.98 mg/mL. Bacterial suspension was added to the test wells, with separate growth and sterility controls. Plates were incubated at 37°C for 16-24 hours. Bacterial growth was assessed visually from turbidity and confirmed by absorbance at 600 nm. MIC was defined as the lowest extract concentration with no detectable bacterial growth. The assay was performed in triplicate.

Minimum Bactericidal Concentration

MBC was assessed by subculturing samples from MIC wells onto Mueller-Hinton agar. Plates were incubated at 37°C for 18-24 hours and examined for colony growth. The lowest concentration producing no bacterial growth was defined as the MBC. The assay was performed in triplicate.

Statistical Analysis

Data were summarized as mean $\pm$ standard deviation

where applicable. Mean inhibition zones for the extract and respective positive-control antibiotics were compared using an independent t-test for *S. aureus* and *S. pyogenes*, the two organisms with measurable inhibition by the extract. A 95% confidence interval and $p < 0.05$ were used as the significance threshold. TPC and TFC assay outputs were summarized descriptively because values expressed as mg GAE/g or mg QE/g extract require normalization to the amount of extract analyzed and should not be interpreted as independent concentration-dependent outcomes across sample dilutions.

RESULTS

Confirmation of Test Organisms

The four bacterial strains were confirmed from their colony characteristics, Gram-stain morphology, selective or differential media, and biochemical profiles. *S. aureus* and *S. pyogenes* were Gram-positive cocci, while *E. coli* and *P. aeruginosa* were Gram-negative bacilli. The confirmed cultures were used for subsequent antimicrobial testing.

Antibiotic Susceptibility Test

At 500 mg/mL, the *K. parviflora* root aqueous extract inhibited the two Gram-positive organisms but produced no measurable inhibition against the Gram-negative organisms. The mean inhibition zone was 10.00 ± 0.00 mm for *S. aureus* and 14.67 ± 0.57 mm for *S. pyogenes*. Gentamicin and ampicillin produced larger inhibition zones than the extract. For *S. aureus*, the mean difference between the extract and gentamicin was -14.33 mm (95% CI: -15.26 to -13.41 ; $t = -43.00$, $df = 4$; $p < 0.001$). For *S. pyogenes*, the mean difference between the extract and ampicillin was -25.33 mm (95% CI: -26.26 to -24.41 ; $t = -76.00$, $df = 4$; $p < 0.001$).

Table 1: Inhibition zones of *K. parviflora* root extract and positive controls.

Organism	Extract (mm $\pm$ SD)	Positive control (mm $\pm$ SD)
<i>S. aureus</i>	10.00 ± 0.00	Gentamicin 24.33 ± 0.58
<i>S. pyogenes</i>	14.67 ± 0.57	Ampicillin 40.00 ± 0.00
<i>E. coli</i>	0.00 ± 0.00	Gentamicin 24.33 ± 0.57
<i>P. aeruginosa</i>	0.00 ± 0.00	Gentamicin 27.00 ± 0.00

Minimum Inhibitory and Bactericidal Concentrations

The MIC was 500 mg/mL for all four bacterial strains. At 250 mg/mL and lower concentrations, visible growth and increased OD600 values were detected. Subculture of the MIC assay demonstrated bacterial growth for every organism at all tested concentrations. Thus, no MBC was identified up to 500 mg/mL.

Table 2: MIC and MBC of *K. parviflora* root aqueous extract.

Organism	MIC (mg/mL)	MBC result
<i>S. aureus</i>	500	No MBC detected up to 500 mg/mL
<i>S. pyogenes</i>	500	No MBC detected up to 500 mg/mL
<i>E. coli</i>	500	No MBC detected up to 500 mg/mL
<i>P. aeruginosa</i>	500	No MBC detected up to 500 mg/mL

Total Phenolic Content

The gallic acid calibration curve showed good linearity ($R^2 = 0.9825$) with the regression equation $y = 0.4947x + 0.3661$. Across the tested extract dilutions, the original analysis produced apparent values ranging from 0.38 ± 0.002 to 8.65 ± 0.004 mg GAE/g. Because TPC expressed as mg GAE/g extract should be normalized to the amount of extract represented in the assay, these dilution-dependent values are not interpreted as separate concentration-dependent TPC outcomes. A single normalized TPC estimate should be derived from the raw assay data using the appropriate dilution and mass corrections.

Table 3: Assay-derived apparent TPC values of *K. parviflora* root aqueous extract across tested dilutions before final mass normalization.

Concentration (mg/mL)	Apparent TPC (reported mg GAE/g)
20	0.38
40	1.48
60	2.96
80	5.32
100	8.65

Total Flavonoid Content

The quercetin calibration curve showed strong linearity ($R^2 = 0.98$) with the regression equation $y = 1.6433x + 0.7643$. Across the tested extract dilutions, the original analysis produced apparent values ranging from 0.36 ± 0.015 to 1.48 ± 0.004 mg QE/g. Because TFC expressed as mg QE/g extract should be normalized to the amount of extract represented in the assay, these dilution-dependent values are not interpreted as separate concentration-dependent TFC outcomes. A single normalized TFC estimate should be derived from the raw assay data using the appropriate dilution and mass corrections.

Table 4: Assay-derived apparent TFC values of *K. parviflora* root aqueous extract across tested dilutions before final mass normalization.

Concentration (mg/mL)	Apparent TFC (reported mg QE/g)
2	0.36
4	0.60
6	1.06
8	1.22
10	1.48

DISCUSSION

The present findings show selective antibacterial activity of *K. parviflora* root aqueous extract against the Gram-positive SSTI-associated organisms tested. At 500 mg/mL, measurable zones were obtained for *S. aureus* and *S. pyogenes*, while *E. coli* and *P. aeruginosa* showed no inhibition in the agar well diffusion assay. The activity against both Gram-positive organisms was significantly weaker than the corresponding conventional antibiotics. This pattern is consistent with reports that plant extracts often display organism-dependent antimicrobial activity according to their phytochemical composition and bacterial susceptibility (Adjeroh et al., 2020; Rahim et al., 2023).

Differences in cell-envelope architecture offer a plausible explanation for the lack of zones against the two Gram-negative species. Gram-negative bacteria possess an outer membrane that restricts entry of many antibacterial molecules and contributes to intrinsic resistance. *P. aeruginosa* has additional resistance mechanisms, including efflux systems and biofilm formation, which further reduce susceptibility to antimicrobial compounds (Hassena et al., 2025). These features may have limited the action or diffusion of water-extracted constituents in the present study.

MIC testing showed that all four organisms required the highest tested concentration, 500 mg/mL, for growth inhibition. The subsequent MBC assay demonstrated viable bacterial growth after subculture from all tested concentrations, including 500 mg/mL. These findings indicate weak bacteriostatic potency under the study conditions rather than bactericidal activity. For *E. coli* and *P. aeruginosa*, the MIC of 500 mg/mL despite the absence of inhibition zones in agar may reflect methodological differences between diffusion and broth assays. Agar well diffusion depends on the ability of extract constituents to migrate through the agar matrix, whereas broth microdilution provides direct and sustained contact between the bacterial cells and the extract. Poor diffusion of water-extracted constituents could therefore produce no visible zone while direct broth exposure at a very high

concentration suppresses growth. Because 500 mg/mL was also the highest concentration tested, the MIC should be interpreted as inhibition only at the upper assay limit rather than evidence of strong antibacterial activity. Comparisons with stronger activity reported for *K. parviflora* rhizome fractions or purified methoxyflavones should remain qualitative because those studies used different plant parts, extraction solvents, and chemically enriched constituents (Sookkhee et al., 2022).

TPC and TFC provide bulk estimates of phenolic and flavonoid content, respectively, but they do not identify individual compounds. Accordingly, these assays do not confirm the presence or amount of specific *K. parviflora* methoxyflavones. Phenolics and flavonoids as broad chemical classes have been associated with antimicrobial mechanisms such as membrane disruption and interference with cellular processes, but the present data do not establish which constituents produced the observed antibacterial effect. The link between the measured TPC/TFC responses and antibacterial activity should therefore be interpreted cautiously until the extract is characterized using compound-specific analytical methods such as chromatographic or spectrometric profiling (Chen et al., 2018; Atom et al., 2022).

Direct quantitative comparison of the current TPC and TFC values with published organic-solvent preparations is premature until the assay outputs are recalculated as mass-normalized representative values. Qualitatively, extraction solvent and plant part are expected to influence chemical recovery. Sitthichai et al. (2022), for example, evaluated organic-solvent extracts of *K. parviflora* rhizomes, whereas the present study examined an aqueous root preparation. Relatively lipophilic methoxyflavones have limited aqueous solubility, and differences between roots and rhizomes may also alter phytochemical composition (Chankitisakul et al., 2025). Therefore, differences in both extraction solvent and plant part should be considered when comparing antibacterial potency across studies.

This study was limited to four reference strains, one aqueous extraction approach, and in vitro endpoints. Agar diffusion was performed at a single extract concentration, while the study did not isolate or identify individual active constituents. In addition, distilled water rather than a matched 5% DMSO solvent control was reported for the agar diffusion assay, which limits attribution of any solvent-related effect. These factors limit conclusions regarding spectrum, mechanism, chemical drivers, and clinical relevance. Future studies should compare water with ethanol, methanol, or mixed-solvent extraction, quantify and identify active compounds, use an appropriately matched solvent control, and evaluate interactions between *K. parviflora* constituents and

standard antibiotics. Solvent optimization and combination testing may clarify whether the weak activity observed in the aqueous root preparation can be improved (Kekeçoğlu & Sorucu, 2022; Sookkhee et al., 2022).

CONCLUSION

K. parviflora root aqueous extract showed limited and selective antibacterial activity against the tested SSTI-associated bacteria. Agar diffusion demonstrated inhibition of *S. aureus* and *S. pyogenes* at 500 mg/mL, while *E. coli* and *P. aeruginosa* were not inhibited. The MIC was 500 mg/mL for all four organisms and no bactericidal effect was detected at concentrations up to 500 mg/mL, indicating weak antibacterial potency under the tested conditions. TPC and TFC assays provide bulk estimates of phenolic and flavonoid content but do not identify specific methoxyflavones or establish which compounds are responsible for antibacterial activity. The findings support further evaluation of optimized extraction methods, compound-specific phytochemical characterization, purified constituents, matched solvent controls, and combinations with conventional antibiotics.

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In vitro Anti-Inflammatory Effects of Bromelain on Interleukin-6 And Thymic Stromal Lymphopoietin Regulation in Lipopolysaccharide-Stimulated Human Keratinocytes

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ABSTRACT

Background: Atopic dermatitis (AD) is a chronic inflammatory skin condition characterized by immune dysregulation and keratinocyte-derived cytokines, such as Interleukin-6 (IL-6) and Thymic Stromal Lymphopoietin (TSLP). This study investigated the anti-inflammatory potential of bromelain, a proteolytic enzyme from pineapple (*Ananas comosus*) using a lipopolysaccharide (LPS)-stimulated human keratinocyte cell model. **Methods:** The enzyme was extracted and partially purified using ammonium sulphate precipitation, followed by further purification through ultrafiltration to obtain a refined enzyme fraction, ultrafiltrated bromelain (UFB). Cell viability, proliferation, and the expression of inflammation-related genes were examined following bromelain treatment. **Results:** The findings demonstrated that bromelain exhibited no cytotoxic effects, maintaining normal cellular morphology and nuclear integrity. At a concentration of 250 µg/mL, bromelain significantly enhanced cell survival to 91% under LPS-stimulated stress, remaining well below the half-maximal inhibitory concentration (IC₅₀) threshold. Furthermore, this specific concentration effectively suppressed the mRNA expression of IL-6 and TSLP. **Conclusion:** These findings demonstrated that bromelain can successfully modulate key pro-inflammatory mediators in keratinocytes. Consequently, bromelain represents a promising natural therapeutic agent for the management of atopic dermatitis and other inflammatory skin disorders.

Keywords:

Inflammation; cytokines; Atopic dermatitis; *Ananas comosus*; proteolytic enzyme.

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INTRODUCTION

Atopic Dermatitis (AD) is a chronic inflammatory disease characterized by immune dysregulation and defective skin barrier function, with inflammation with recurrent eczematous lesions and pruritus. AD affects up to 20% of children and 1–3% of adults worldwide and the prevalence continues to rise steadily (Laughter et al., 2021). The global numbers are high, but statistics from developing countries are showing a significant burden in the region. For example, In Malaysia, 20% of Malaysian children have AD and more than 55% of affected children have 'atopic march' (Mehta & Fulmali, 2022). Researchers have pointed out the major cytokines highly expressed in AD patients to find the causes of this common disease which are Interleukin-6 (IL-6) and Thymic Stromal Lymphopoietin (TSLP). These keratinocyte derived cytokines induce an inflammatory response by triggering an overactive Th2

immune response which leads to chronic irritation and further impairing the skin barrier (Makowska et al., 2023). These molecules cause inflammation by stimulating an overactive Th2 immune response, leading to persistent irritation of the skin and a compromised skin barrier.

Currently, the prescription for AD treatment still includes immunosuppressive drug (Ross, 2023). However, data from previous studies indicate that immunosuppressants used for AD may increase the risk of certain health complications. For example, Dupilumab therapy has been associated with adverse effects such as injection site reactions, conjunctivitis, headache, and nasopharyngitis (Albader et al., 2019). Additionally, the side effects of steroid treatment have led to the emergence of steroid phobia, with an increasing number of patients unwilling to undergo such treatment (Contento et al., 2021). Therefore, the study of natural plant-based substitutes

may provide safer and more effective treatment for AD (Ghadigaonkar, 2024).

Proteolytic enzymes are promising candidates since they can modulate complex immune responses (Venetikiidou et al., 2025). Its significance lies in its therapeutic potential in the management of inflammatory conditions including AD via modulation of pro-inflammatory cytokines, inhibition of immune cell activation and reduction of oxidative stress (Pavan et al., 2012). Bromelain is a natural proteolytic enzyme that is found mainly in the pineapple fruit (*Ananas comosus*). Earlier studies showed that bromelain is able to downregulate IL-6 and tumour necrosis factor- α (TNF- α) which are important key cytokines in chronic inflammation (Rathnavelu et al., 2016). Bromelain has been investigated for the treatment of a variety of inflammatory disorders, such as osteoarthritis, inflammatory bowel disease and cancer related inflammation, either as a substitute or complement, due to its wide spectrum anti-inflammatory effects (Bottega et al., 2021). In addition, bromelain has cytoprotective potential that may protect cells from damage caused by inflammation and is a promising natural therapeutic agent for the management of inflammation-related diseases such as AD (Kansakar et al., 2024).

While bromelain has potential therapeutic benefits, the lack of evidence for its effects on the regulation of IL-6 and TSLP genes require further investigation to elucidate its immunomodulatory role and therapeutic relevance in the management of inflammatory skin disorders. So, the aim of this study was to investigate the possible role of bromelain in attenuation of IL-6 and TSLP gene expression level in LPS-stimulated HaCaT cell model. The study offers significant information regarding the potential of bromelain as a natural anti-inflammatory product, by demonstrating its effects on cell behavior and cytokine expression and providing preliminary evidence to support its therapeutic value in the treatment of inflammatory skin disorders such as atopic dermatitis.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The HaCaT cells (ATCC, USA) a human keratinocyte cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma, USA) and 1% penicillin-streptomycin (Gibco, USA). Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂.

Ultrafiltrated Bromelain (UFB) Extraction and Purification

The extraction and purification method used in this study was prepared according to our previously established method (Tarmizi et al., 2023). Briefly, MD2 Hybrid pineapple cores were provided by SMART KJ Agro Asia Plt (Kedah, Malaysia) were homogenized in distilled water and centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was subjected to ammonium sulphate (50% saturation) and precipitated overnight. Ultrafiltration was then performed using a membrane with a molecular weight cut-off (MWCO) of 10 kDa to concentrate and purify the bromelain enzyme. The purified bromelain fractions were collected and filtered with 0.22 μ m syringe filter and stored at -80°C until further use.

Alamar Blue Assay

The cytotoxic effects of UFB on HaCaT cell viability were evaluated using the Alamar Blue (Biorad, USA), following the protocol provided by the manufacturer. HaCaT cells with a density of 2×10^4 HaCaT cells that were pre-seeded in 96-well plate were introduced with the various UFB concentrations of 15.63 μ g/mL, 31.25, 62.50, 125, and 250 μ g/mL respectively. The negative control group was HaCaT cells cultured in DMEM medium only. Untreated cells serve as the positive control for inflammation model. After 24 hours of exposure, 10 μ L of Alamar blue reagent was added to each well, and the plates were incubated at 37°C for 4 hours. Absorbance at $\lambda = 570$ nm was then measured using a microplate reader (Tecan, Switzerland). The cell viability results were expressed as the percentage of viability of the control cells whose viability was defined as 100%.

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of Treated cells (OD)}}{\text{Absorbance of Untreated Cells (OD)}} \times 100$$

Cell Morphological Analysis

Microscopic observation using inverted phase-contrast microscopy was performed to evaluate the morphology and nuclear integrity of HaCaT cells. A total of 1×10^6 HaCaT cells were seeded on a coverslip in a 6-well plate and treated with 250 μ g/mL UFB. The negative control group was HaCaT cells were seeded in DMEM medium only. Then, cells were washed with PBS, permeabilized with 0.1% Triton X-100 and stained with NucSpot-488 (Biotium, USA) for 20 min. The images of stained cells were taken under a confocal microscope (LSM 900, Zeiss). Changes in cell structure and nuclear morphology were observed in both untreated and treated HaCaT cells.

Cell Proliferation Assay

A total of 1.5×10^4 HaCaT cells were seeded in each well of a 96-well plate and incubated for 24 hours. Then, they were stimulated with $1 \mu\text{g/mL}$ of LPS (Thermo Fisher, USA) for 1 hour and further treated with or without UFB for further 24 hours. Meanwhile the control group was maintained with DMEM medium only without exposure to both LPS and UFB. All groups were incubated in a humidified incubator at 37°C , $5\% \text{CO}_2$ and for 24 hours. Cell viability was assessed using the Alamar Blue assay at 24 hours upon treatment with UFB and a graph plotting cell concentration against cell viability percentage was generated.

Gene Expression Analysis

A total of 1×10^6 HaCaT cells were seeded into a 6-well plate. The LPS-stimulated HaCaT cells were treated with or without $250 \mu\text{g/mL}$ UFB for 24 hours. The RNA extraction was performed by using InnuPREP RNA Mini (Jena, Germany) according to the manufacturer's recommendations. Extracted RNAs were quantified by a Nanodrop 2000 spectrophotometer, and the absorbance ratio of 260/280 was used to determine the purity of the RNAs. For quantitative reverse transcription PCR (RT-qPCR), 100 ng template RNA was mixed with specific primers, IL-6 (IDT, USA) (forward - 5'-ACA GCC ACT CAC CTC TTC AG-3', reverse - 5'-CCA TCT TTT TCA GCC ATC TTT-3') and TSLP (IDT, USA) (forward - 5'-TCC TCT GAA GAC CTG ACC-3', reverse - 5'-TCT CCT TTC TCC CTA ATC CTC-3') and SensiFAST One Step Mix (Bioline, Australia). The RNA amplification was conducted using RT-qPCR thermocycler (CFX96 Bio-Rad iCycler). The expression level of IL-6 and TSLP was normalized to GAPDH (forward - 5'-AAA CAG ATG AAG GTG CTC CTT -3', reverse - 5'-TGG AGA ACA CCA CTT GTT GC-3'). The RT-qPCR amplification included 40 cycles of the following: reverse transcription at 55°C for 10 min, initial denaturation at 95°C for 1 min, denaturation at 95°C for 10 sec and extension at 60°C for 30 sec.

Statistical Analysis

All tests were carried out in triplicate with three independent experiments. Statistical differences among multiple experimental groups were initially assessed using one-way analysis of variance (ANOVA), followed by Bonferroni's multiple-comparison post hoc test to determine the differences between individual groups. A p-value of < 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism version 10 (GraphPad Software, San Diego, CA, USA).

RESULTS

Cytotoxicity of UFB on HaCat Cells

The cytotoxicity effect of UFB was assessed by treating HaCaT cells with a series of different concentrations of UFB (15.62 , 31.25 , 62.5 , 125 and $250 \mu\text{g/mL}$) for 24 hours using the Alamar Blue assay. Incubation of HaCaT cells with different concentrations of UFB for 24 hours demonstrated no significant cytotoxic effects on cellular viability (Figure 1). The loss of viability did not exceed the IC_{50} threshold, confirming that UFB is a relatively safe compound. Therefore, $250 \mu\text{g/mL}$ UFB was chosen as the optimal concentration for further testing.

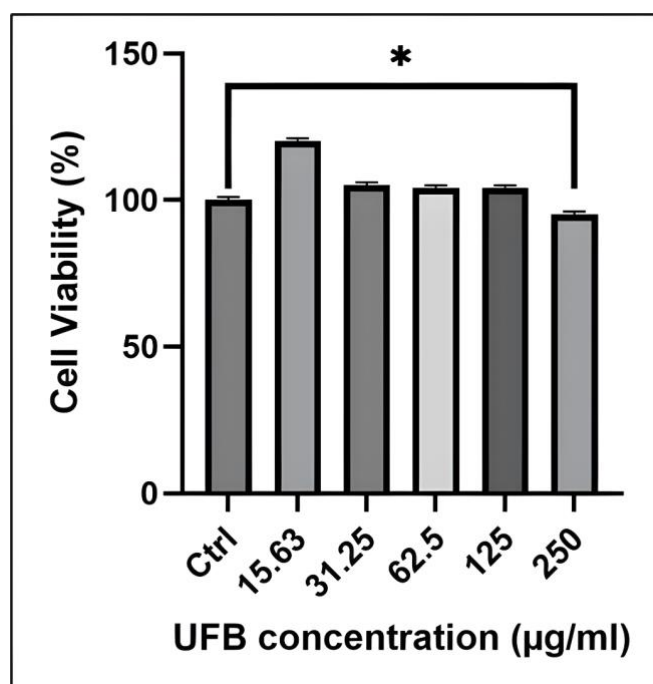


Figure 1: Viability analysis of HaCaT cells treated with UFB. HaCaT cells were exposed to various concentrations of UFB in serum-free medium, and cell viability was assessed after 24 hours using the Alamar Blue assay. Data are presented as mean $\pm$ SD from three independent experiments. Statistical significance is indicated by single asterisks (*), representing $p < 0.05$.

Effect of UFB on Morphological Changes and Nuclear Integrity in HaCat Cells

The non-toxic effect was subsequently examined on HaCaT cells when the optimum UFB dose of $250 \mu\text{g/mL}$ was introduced. Morphological changes and nuclear integrity of HaCaT cells were evaluated using phase-contrast and confocal microscopy. As shown in Figure 2, untreated HaCaT cells maintained their normal spindle-shaped morphology. Following treatment with $250 \mu\text{g/mL}$ UFB, cells exhibited slight morphological changes, including

minor shrinkage, but remained viable. To further confirm these observations, nuclear staining with NucSpot-488 revealed that nuclei in both untreated and treated cells remained compact and intact, with no evidence of chromatin damage. These results suggest that UFB exerts minimal cytotoxic effects, supporting its biocompatibility and ability to preserve HaCaT cell morphology and nuclear integrity.

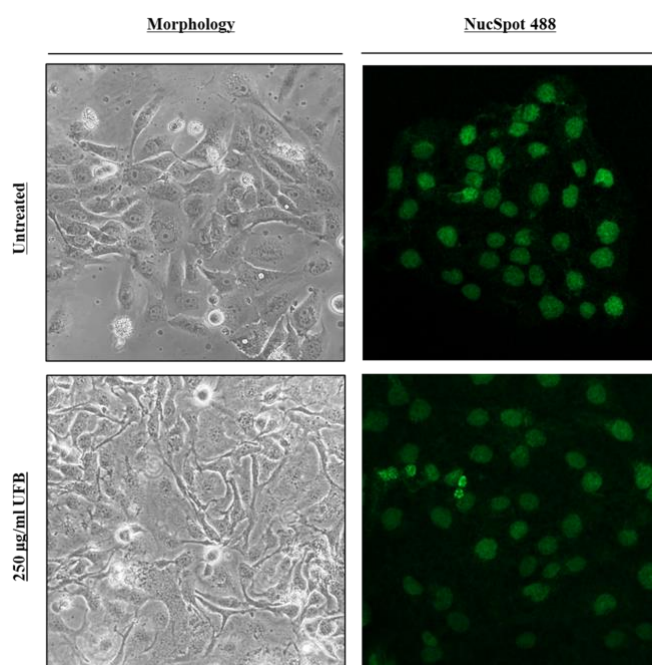


Figure 2: Morphological and immunofluorescence analysis of HaCaT cells treated with or without 250 µg/mL UFB. Phase-contrast microscopy (left) shows the overall morphology of HaCaT cells following UFB treatment, while immunofluorescence imaging (right) with NucSpot-488 highlights nuclear integrity.

Anti-inflammation Activity of UFB on LPS-Stimulated HaCaT Cells

To evaluate the cytoprotective potential of UFB against endotoxin-induced cell damage, HaCaT cells were exposed to 1 µg/mL of lipopolysaccharide (LPS) in the presence or absence of 250 µg/mL UFB. As shown in Figure 3, treatment with LPS alone significantly reduced cell viability to approximately 87% compared to the untreated control group ($p < 0.01$). However, co-treatment with 250 µg/mL UFB significantly attenuated LPS-induced cytotoxicity, increasing cell viability to approximately 91% group ($p < 0.01$). These findings demonstrate that UFB exerts a significant protective effect against LPS-induced cytotoxicity in HaCaT keratinocytes.

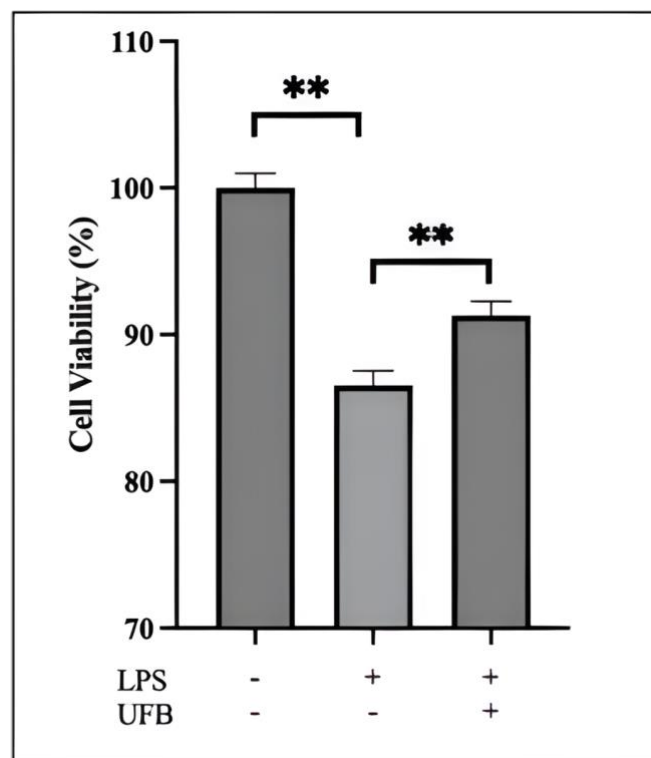


Figure 3: Protective effect of UFB on LPS-stimulated cytotoxicity in HaCaT cells. HaCaT cells were stimulated with 1 µg/mL of lipopolysaccharide (LPS) in the presence or absence of 250 µg/mL UFB. Cell viability was expressed as a percentage relative to the untreated control group. Data are presented as mean $\pm$ SD. Statistical significance is indicated by double asterisks (**) ($p < 0.01$).

Effect of UFB on Pro-Inflammatory Cytokines in LPS-Stimulated HaCaT Cells

To further evaluate the anti-inflammatory activity of UFB, the expression of interleukin-6 (IL-6) and thymic stromal lymphopoietin (TSLP) was examined in LPS-stimulated HaCaT cells using RT-qPCR (Figure 4). LPS stimulation markedly increased the expression of both inflammatory markers compared with the untreated control. IL-6 expression increased substantially following LPS stimulation, reaching approximately 35-fold relative to the control group ($p < 0.0001$). Treatment with 250 µg/mL UFB significantly reduced IL-6 expression to approximately 8-fold compared with the untreated LPS-stimulated cells ($p < 0.0001$).

Similarly, LPS stimulation increased TSLP expression relative to the control ($p < 0.01$). Treatment with 250 µg/mL UFB significantly decreased TSLP expression to approximately 1.1-fold compared with the LPS-stimulated group ($p < 0.01$). Overall, these findings demonstrate that UFB treatment significantly suppressed the LPS-induced expression of IL-6 and TSLP in HaCaT cells, indicating its potential to attenuate inflammatory responses at the gene expression level.

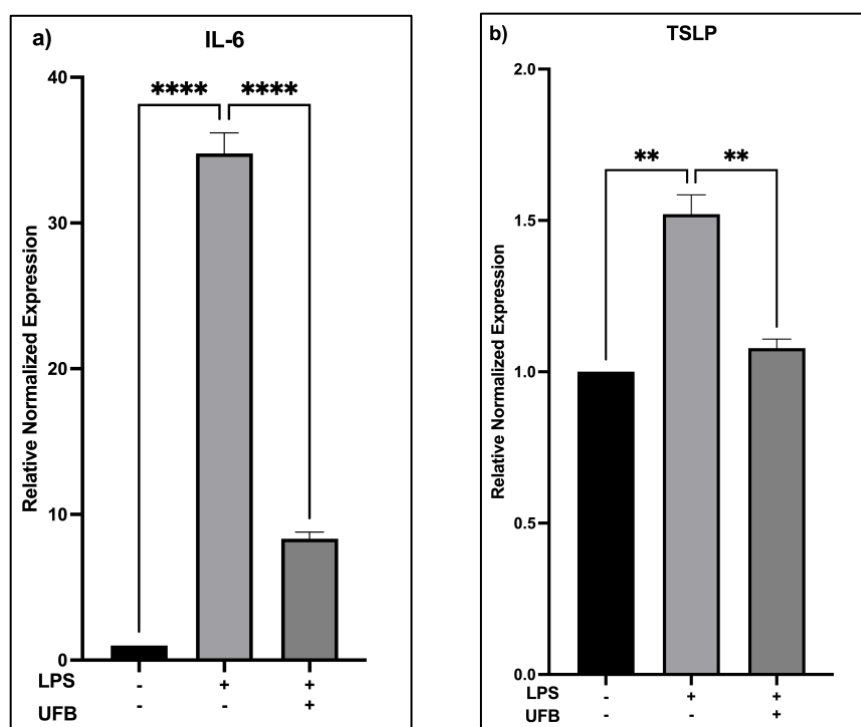


Figure 4: Gene expression level of (a) IL-6 and (b) TSLP in LPS-stimulated HaCaT cells treated with 250 µg/mL UFB was measured using RT-qPCR. Treatment with 250 µg/mL UFB resulted in a significant reduction in IL-6 and TSLP expression compared to the untreated LPS-stimulated group (**p < 0.01, ****p < 0.0001).

DISCUSSION

The cytotoxicity assessment confirmed that bromelain exhibited no significant cytotoxic effects at concentrations up to 250 µg/mL, as HaCaT cell viability remained above the IC₅₀ threshold. This aligns with previous studies highlighting the biocompatibility of bromelain, supporting its potential as a safe bioactive compound for therapeutic applications (Tarmizi et al., 2023). The non-toxic effect of bromelain was further supported by a study by Esen et al. (2023) which showed that bromelain was non-toxic to mouse fibroblast NIH/3T3 cells and promoted their growth, suggesting its potential for wound healing and tissue repair. Morphological and nuclear assessments using phase-contrast and confocal microscopy confirmed bromelain's safety. Untreated HaCaT cells had a normal spindle-shaped morphology, but treatment with 250 µg/mL UFB caused minor shrinkage without compromising cell viability (Figure 2). Nuclear staining with NucSpot-488 revealed that nuclei in both treated and untreated cells were compact and intact, with no signs of chromatin damage. These findings show that bromelain preserves cellular morphology and nuclear integrity, confirming its biocompatibility and ability to maintain HaCaT cell

morphology and nuclear integrity. Therefore, 250 µg/mL UFB is selected as optimal concentration in this study as it maintained cell viability above a critical threshold while potentially exerting biological effects on cytokine regulation, providing an appropriate balance between safety and efficacy for further investigation.

HaCaT cells, an immortalized human keratinocyte cell line, are widely used in dermatological research for their stable differentiation, ease of culture, and high proliferative capacity (Colombo et al., 2017). To investigate the anti-inflammatory effects of bromelain in an *in vitro* model, its activity on LPS-induced HaCaT cell proliferation was examined to mimic the inflammatory environment associated with AD. HaCaT cells were stimulated with 1 µg/mL LPS and subsequently treated with or without 250 µg/mL UFB for 24 hours.

LPS stimulation was applied for 24 hours to establish an inflammatory response, as previously described by (Kim et al., 2023). A significant decrease in cell viability of about 13% was observed in LPS-stimulated groups compared with the control group, indicating that inflammatory stimulation compromised keratinocyte survival (Figure 3).

Interestingly, LPS-stimulated cells that were treated with UFB show a higher viability of 91%. This indicates that bromelain mitigated the cytotoxic effects of LPS, suggesting a protective role under inflammatory stress.

LPS is known to induce inflammation in HaCaT cells by activating inflammation signalling pathways such as NF- κ B and MAPK. Previous studies on HaCaT and RAW 264.7 cells have similarly reported reduced cell viability following LPS stimulation due to oxidative and inflammatory stress (M. J. Kim et al., 2023). The decline in viability is primarily attributed to the generation of reactive oxygen species (ROS) and pro-apoptotic cytokines triggered by TLR4-mediated signalling. These events promote mitochondrial dysfunction, DNA fragmentation, and caspase activation, leading to programmed cell death (Baek et al., 2020).

The ability of UFB to improve cell viability under these conditions suggests its cytoprotective effect against LPS-induced stress. Similar protective effects were reported for Schizandrin A, which alleviated LPS-induced apoptosis and oxidative stress in HaCaT cells by modulating NF- κ B and Nrf2/HO-1 pathways (Xu et al., 2020). Although the precise molecular mechanism by which bromelain exerts its cytoprotective in HaCaT cells remains unclear, it is plausible that bromelain interferes with upstream receptor interactions during LPS recognition or attenuates downstream inflammatory signalling cascades.

Therefore, bromelain's proteolytic nature might contribute to the degradation or modulation of cell-surface receptors, thereby reducing LPS binding affinity or TLR4 signalling. Alternatively, bromelain may enhance antioxidant defences or stabilize mitochondrial integrity, leading to improved cellular resilience under inflammatory stress. These results demonstrate that bromelain enhances cell survival under inflammatory conditions, reinforcing its potential as a therapeutic agent for AD and other inflammation-mediated skin disorders.

To further investigate the anti-inflammatory potential of bromelain using an *in vitro* inflammation model, the efficacy of UFB was evaluated for its regulatory effects on inflammatory cytokines in LPS-induced HaCaT cells. The mRNA expression levels of IL-6 and TSLP were quantified using RT-qPCR analysis (Figure 4). LPS stimulation markedly increased the expression of both IL-6 and TSLP after 24 hours, indicating successful induction of an inflammatory response in HaCaT keratinocytes. However, LPS-Stimulated HaCaT cells that were treated with UFB significantly demonstrated downregulation in the expression of both cytokines. A comparable inhibitory pattern of IL-6 and TSLP gene expression was observed when TNF/IFN-induced HaCaT cells were treated with

Punicalagin (Huang et al., 2024). These findings suggest that UFB effectively suppresses proinflammatory cytokine expression, supporting its potential as a natural anti-inflammatory agent. Signalling pathways such as MAPK and NF- κ B are commonly activated upon stimulation by LPS and other microbial components (Zhang et al., 2025). Activation of Toll-like receptor 4 (TLR4) by LPS triggers a cascade of protein phosphorylation events, including the activation of IKK, p38, ERK, and JNK kinases, which ultimately lead to the translocation of NF- κ B into the nucleus and transcription of proinflammatory cytokines. IL-6 and TSLP are among the cytokines transcriptionally regulated through these pathways and are key mediators of keratinocyte-driven inflammatory responses. Elevated IL-6 levels promote immune cell recruitment, cell stress, and proliferation, while TSLP plays a critical role in causing epithelial inflammation, particularly in atopic dermatitis (Smolinska et al., 2023). Thus, the observed reduction in both cytokines following bromelain treatment suggests that bromelain may modulate deactivation of upstream signalling events within these canonical inflammatory cascades.

Previous studies have reported that bromelain exerts its anti-inflammatory effects through multiple mechanisms, including inhibition of NF- κ B activation, suppression of MAPK phosphorylation, and degradation of cell-surface receptors involved in leukocyte adhesion (Narayanan, 2025). The ability of bromelain anti-inflammatory activity has been linked to inhibit key signalling pathways involved in inflammation. Bromelain treatment has been shown to reduce phosphorylation of NF- κ B (p65) and MAPKs such as ERK, JNK, and p38, thereby attenuating downstream cytokine production in LPS-stimulated cells (Insuan et al., 2021). Additionally, recent experimental evidence indicates that bromelain suppresses NF- κ B-mediated pro-inflammatory cytokine secretion and MAPK-associated signalling in inflammatory models (Sezen et al., 2025). In HaCaT keratinocytes specifically, LPS-induced stimulation activates both MAPK and NF- κ B signalling cascades, leading to increased expression of inflammatory mediators, which can be modulated by anti-inflammatory agents (Plata-Babula et al., 2025). In addition, bromelain's proteolytic and antioxidant properties contribute to reduced oxidative stress and cytokine production in inflamed tissues (Bahar et al., 2024). The findings from this study are consistent with these reports, highlighting that bromelain attenuates inflammation at both transcriptional and signalling levels.

Furthermore, given the central role of IL-6 and TSLP in AD pathogenesis, the ability of bromelain to suppress their expression highlights its potential as a therapeutic candidate for AD management, as depicted in the

proposed mechanism in Figure 5. Future studies should explore the detailed molecular interactions between bromelain and intracellular signalling pathways such as MAPK and NF- κ B. Collectively, these findings demonstrate that bromelain possesses strong anti-inflammatory activity and may serve as a promising adjunct or alternative therapy for inflammation-related skin disorders.

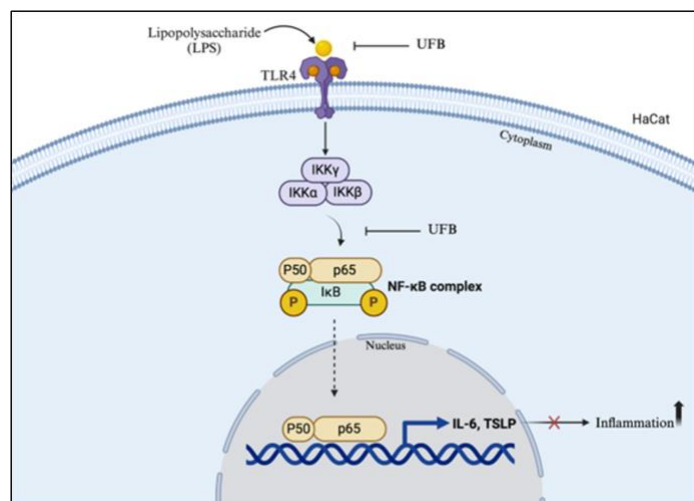


Figure 5: Proposed mechanism illustrating the potential inhibitory sites of UFB in the NF- κ B signalling pathway. UFB is suggested to interfere with multiple points along the pathway, including the inhibition of upstream signalling events, suppression of I κ B degradation, and prevention of NF- κ B translocation into the nucleus, ultimately reducing the transcription of pro-inflammatory genes.

CONCLUSION

This study confirms the hypothesis that bromelain serves as a potent anti-inflammatory agent capable of modulating key mediators associated with keratinocyte-driven skin inflammation. Current AD therapies, including topical corticosteroids and calcineurin inhibitors, are associated with adverse effects following long-term use. Therefore, natural bioactive compounds such as bromelain may represent a promising adjunct or alternative strategy with potentially fewer side effects. By effectively regulating pro-inflammatory signalling without inducing cytotoxicity, bromelain proves to be a viable natural candidate for the management of atopic dermatitis. These findings validate its potential as a therapeutic alternative in dermatology, although further investigation into its clinical translational relevance remains necessary.

In addition to its anti-inflammatory properties, bromelain may contribute to the preservation of epidermal barrier integrity by protecting keratinocytes from oxidative and apoptotic damage, thereby supporting overall skin

homeostasis. Given that barrier dysfunction and chronic inflammation are central features in AD pathogenesis, the dual cytoprotective and immunomodulatory actions of bromelain position it as a multifunctional therapeutic candidate. Furthermore, its proteolytic activity and reported antioxidant potential may synergistically reduce inflammatory amplification within the cutaneous microenvironment.

However, the present findings are limited to an *in vitro* HaCaT model and may not fully recapitulate the complex immune interactions observed *in vivo*. Future studies should investigate the effects of bromelain on cytokine secretion profiles, oxidative stress markers, and barrier-related proteins such as filaggrin and loricrin, as well as evaluate its efficacy in animal models of AD. Clinical trials assessing optimal dosage, formulation, delivery systems, and long-term safety will be essential to determine its therapeutic applicability. Collectively, this study provides foundational evidence supporting bromelain as a promising natural anti-inflammatory compound for the development of safer and more targeted dermatological interventions.

ACKNOWLEDGEMENT

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Effects of Chemotherapeutic Agents on *TET2* and *DNMT3A* Gene Expression in TF-1 Erythroleukemia Cells

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ABSTRACT

Background: Epigenetic dysregulation involving DNA methylation regulators such as *TET2* and *DNMT3A* plays a central role in the pathogenesis of myeloid malignancies. Cytarabine and daunorubicin remain the backbone agents of standard induction chemotherapy for acute myeloid leukaemia, yet their direct effects on the transcriptional expression of *TET2* and *DNMT3A* in erythroid leukemic cells remain poorly characterized. This study aimed to evaluate and compare the effects of cytarabine, daunorubicin, and their combination on *TET2* and *DNMT3A* gene expression in the TF-1 erythroleukemia cell line. **Methods:** TF-1 cells were seeded in 96-well plates and treated with the IC₅₀ concentrations of cytarabine (43 nM) and daunorubicin (35 nM), individually and in combination, for 48 hours, alongside an untreated control. The experiment consisted of one independent cell-culture experiment, with 3 wells per treatment group. Total RNA was extracted, and its concentration, purity, and integrity were assessed prior to reverse transcription. Relative expression of *TET2* and *DNMT3A* was quantified by real-time PCR using the comparative Ct ($2^{-\Delta\Delta Ct}$) method, with GAPDH as the reference gene and untreated cells as the calibrator. **Results:** All primer pairs demonstrated high specificity and acceptable amplification efficiency (101.65–111.40%; $R^2 \geq 0.9958$). Cytarabine treatment was associated with *TET2* and *DNMT3A* expression levels of 0.78-fold and 0.95-fold, respectively, relative to the untreated control. Daunorubicin was associated with expression levels of 0.71-fold for *TET2* and 0.87-fold for *DNMT3A*. Following combined cytarabine and daunorubicin treatment, *TET2* expression was 0.91-fold, while *DNMT3A* expression was 1.03-fold relative to the untreated control. **Conclusion:** The present preliminary findings indicate observable differences in *TET2* and *DNMT3A* gene expression following chemotherapy exposure in TF-1 cells. However, the absence of biological replication prevents confirmation of treatment-associated effects. Independent biological replicates and further validation are required to determine the reproducibility and biological significance of these observations.

Keywords:

Eg: *TET2*; *DNMT3A*; cytarabine; daunorubicin; TF-1 erythroleukemia cells

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INTRODUCTION

Epigenetic dysregulation is now recognized as a central mechanism in the pathogenesis of haematological malignancies, alongside classical genetic lesions. Aberrant changes in the epigenome are now recognized to be important in driving the development of multiple human cancers, including acute myeloid leukaemia (AML), with recurrent mutations identified in genes that regulate DNA methylation, including *DNMT3A*, *TET2*, and *IDH1/2*, promoting self-renewal and blocking differentiation of haematopoietic stem and progenitor cells (Schrezenmeier & Huntly, 2025). These epigenetic alterations are, in principle, reversible, that have attracted particular interest as both prognostic markers and potential therapeutic targets in myeloid neoplasms.

Among the epigenetic modifiers implicated in haematopoietic transformation, *DNMT3A* and *TET2* occupy antagonistic but interlinked positions in the regulation of DNA methylation (Zhang et al., 2016).

DNMT3A catalyses the addition of 5-methylcytosine, whereas *TET2* oxidizes 5-methylcytosine as a first step in active DNA demethylation, yet mutations in both genes recur across a similar spectrum of human hematopoietic malignancies and frequently co-occur with oncogenic driver mutations. Mutations in *DNMT3A* and *TET2* arise across a comparable range of lymphoid and myeloid disorders and are also common in clonal haematopoiesis associated with aging, likely reflecting a selective competitive advantage conferred on the mutant hematopoietic stem cell clone (Zhang et al., 2016). These genes have been shown to jointly repress lineage-specific transcription factors, including the erythroid master regulator KLF1, in hematopoietic stem cells, linking their epigenetic activity directly to erythroid commitment.

Cytarabine and daunorubicin are the two backbone agents of standard cytotoxic induction therapy for AML, typically delivered together as the "7+3" regimen, and each interacts with the genome through a distinct mechanism that could plausibly extend to the regulators of DNA

methylation. Cytarabine is a cytidine nucleoside analogue that is incorporated into replicating DNA, and have been demonstrated to cause changes in DNA methylation patterning and downstream gene expression in leukemic cells, and combination with hypomethylating agent decitabine have shown that cytarabine can modulate the demethylating activity of decitabine when the two are administered together (Qin et al., 2007). Daunorubicin, by contrast, is an anthracycline that intercalates into DNA and stabilizes the DNA–topoisomerase II cleavable complex, producing single- and double-strand breaks. Daunorubicin has also been reported to alter regulation of gene expression as part of its broader mechanism of action (Boulanger et al., 2023). Since both *TET2* and *DNMT3A* expression have separately been shown to shift in response to cytotoxic and hypomethylating drug exposure in other malignant contexts, it is plausible that cytarabine and daunorubicin, despite acting through different primary mechanisms, could each perturb *TET2/DNMT3A* transcription in leukaemic cells, though whether these two agents act concordantly on this axis has not been established.

The TF-1 cell line provides a well-established model for investigating these questions in an erythroid malignant context. TF-1 erythroblasts were isolated from the bone marrow of a pancytopenia patient and provide a system for investigating the proliferation and differentiation of myeloid progenitor cells. The line is of immature erythroid origin, requires GM-CSF, IL-3, or erythropoietin for growth, and retains the capacity to differentiate along either the erythroid or macrophage lineage depending on the inducing stimulus, while showing constitutive expression of globin genes consistent with commitment to the erythroid lineage. These properties make TF-1 a suitable platform for probing how the two principal agents of AML induction chemotherapy perturb the epigenetic machinery governing erythroid differentiation and malignant proliferation, independent of the confounding genetic heterogeneity present in primary patient samples.

Despite the established roles of cytarabine and daunorubicin in AML treatment and the accumulating evidence that *TET2* and *DNMT3A* are dysregulated and pharmacologically responsive across a range of myeloid malignancies, direct data on how these two specific agents affect the transcriptional expression of *TET2* and *DNMT3A* within an erythroleukemic cellular background are lacking. Clarifying this relationship is important for understanding the mechanistic basis of chemotherapy-induced epigenetic remodelling during induction therapy, and for anticipating how such changes in *TET2/DNMT3A* expression might influence differentiation status or contribute to the emergence of drug resistance. The

present study therefore aims to evaluate and compare the effects of cytarabine and daunorubicin on *TET2* and *DNMT3A* gene expression in the TF-1 erythroleukemia cell line, contributing mechanistic insight into how the two agents of the standard AML induction backbone act on the epigenetic regulatory machinery of erythroid leukemic cells.

MATERIALS AND METHODS

Cell Seeding and Drug Treatment

TF-1 cells were seeded in 96-well plates at a density of 5×10^4 cells per well in 100 μ L RPMI-1640 complete growth medium and incubated for 24 hours (37°C, 5% CO₂) prior to chemotherapy treatment, according to the treatment groups summarized in Table 1. The experiment consisted of one independent cell-culture experiment, with 3 wells per treatment group. Cells were subsequently treated with 43 nM cytarabine and 35 nM daunorubicin, corresponding to the IC₅₀ concentrations previously determined in our laboratory using a dose-response MTT assay in TF-1 cells. For the combination treatment, cytarabine and daunorubicin were applied simultaneously at their respective IC₅₀ concentrations (43 nM cytarabine + 35 nM daunorubicin). An untreated group was included as the control. The cells were then incubated for a further 48 hours. Following the 48-hour treatment period, the cell suspension from each well was transferred to a microcentrifuge tube and centrifuged at 300 $\times$ g for 5 minutes. The supernatant was discarded, and cell pellets were washed twice with phosphate-buffered saline (PBS) before the final supernatant was removed. Pellets were stored at –80°C until RNA extraction.

Table 1: Treatment groups for TF-1 cells

Treatment Group	Description	Concentration
Control	Untreated	-
Monotherapy	Cytarabine	43 nM
	Daunorubicin	35 nM
Combination Therapy	Cytarabine + Daunorubicin	43 nM + 35 nM

RNA Extraction and Assessment

Total RNA was extracted from cell pellets using Cells & Tissue RNA Extraction Kit (Infusion Tech, Korea) according to the manufacturer's protocol. RNA concentration and purity were determined using a Thermo Scientific NanoDrop 2000 spectrophotometer, based on UV absorbance at 260 nm and the A260/A280 ratio and RNA

integrity was assessed by agarose gel electrophoresis.

Primer Design

Primers for *TET2*, *DNMT3A* and *GAPDH* (endogenous control) were designed using Primer-BLAST (NCBI). Primer specificity was further verified *in silico* using UCSC In-Silico PCR to confirm unique binding to the intended target sequence. Primers were reconstituted to 100 μ M in Tris-EDTA buffer upon receipt and stored at -20°C . Working stocks (10 μ M) were prepared in nuclease-free water prior to use. Primer sequences and characteristics are shown in Table 2.

Table 2: Primers used for qPCR analysis

Gene	Primer Sequence (5'–3')	Tm ($^{\circ}\text{C}$)	Size (bp)
<i>GAPDH</i>	F: TTGCAATGAGATACCCACA	59.92	200
	R: TGCAAACCAACAAAGATGGA	60.09	
<i>TET2</i>	F: CTTTTCGTGGAGTGTGTGG	59.97	160
	R: AGCGAAGAACATCTGGAGCC	60.11	
<i>DNMT3A</i>	F: GGAGCGAGATCCCTCCAAAAT	59.86	197
	R: GGCTGTTGTCATACTTCTCATGG	59.38	

Real-Time PCR (qPCR)

Total RNA was reverse-transcribed to cDNA using NZY RT SuperMix 5 $\times$ according to the manufacturer's protocol. qPCR reactions were prepared using NEXpro™ R3Charge 2 $\times$ Green qPCR Master Mix in a total reaction volume of 20 μ L according to the manufacturer's protocol. qPCR was performed on a Bio-Rad CFX96 Real-Time PCR Detection System. Melting curve analysis was performed at the end of each run to confirm amplicon specificity. Each cDNA sample was analysed in 3 technical qPCR replicates for each *TET2*, *DNMT3A* and *GAPDH*. Technical qPCR replicates were used to assess assay repeatability and were not considered independent biological replicates.

Target gene expression was normalized to *GAPDH*, and relative expression (fold change) was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method, with untreated TF-1 cells as the calibrator. Mean Ct values were used to calculate:

$$\Delta\text{Ct} = \text{Ct (target gene)} - \text{Ct (reference gene)}$$

$$\Delta\Delta\text{Ct} = \Delta\text{Ct (treated)} - \Delta\text{Ct (control)}$$

$$\text{Fold change} = 2^{(-\Delta\Delta\text{Ct})}$$

The gene expression changes were presented descriptively and were interpreted as preliminary observations rather than statistically confirmed treatment effects since the present study was based on a single independent cell-culture experiment without biological replication

RESULTS

Primer Validation and qPCR Performance

Primer Specificity

Primer specificity for *TET2*, *DNMT3A*, and the reference gene *GAPDH* was confirmed by melting curve analysis following qPCR (Figure 1). Each primer pair produced a single, distinct melting peak with no secondary peaks, indicating specific target amplification without primer-dimer formation, and confirming suitability for subsequent gene expression analysis.

Amplification Analysis

Amplification curves for *GAPDH*, *TET2*, and *DNMT3A* showed a consistent, progressive increase in fluorescence across cycles in all samples (Figure 2), confirming successful and reliable target amplification.

PCR Efficiency

Amplification efficiency for each primer pair was determined from standard curves generated using serially diluted cDNA (Figure 3), based on the linear relationship between Ct value and log dilution factor. The amplification efficiencies were 101.65% for *GAPDH*, 106.52% for *TET2*, and 111.40% for *DNMT3A*, with R^2 values of 0.9998, 0.9958, and 0.9971, respectively (Table 3). The *GAPDH* and *TET2* primer pairs were within the predefined efficiency range of 90–110%, whereas the *DNMT3A* primer pair showed a slightly elevated efficiency of 111.40%. Although the R^2 value indicated a strong linear relationship, the slightly elevated *DNMT3A* efficiency represents a methodological limitation and warrants further primer optimization. Therefore, the *DNMT3A* expression findings should be interpreted cautiously.

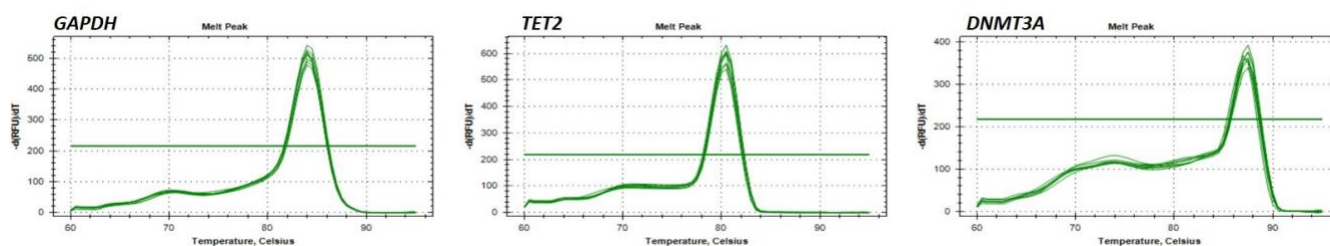


Figure 1: Melting curve analysis of qPCR products for *GAPDH*, *TET2* and *DNMT3A*.

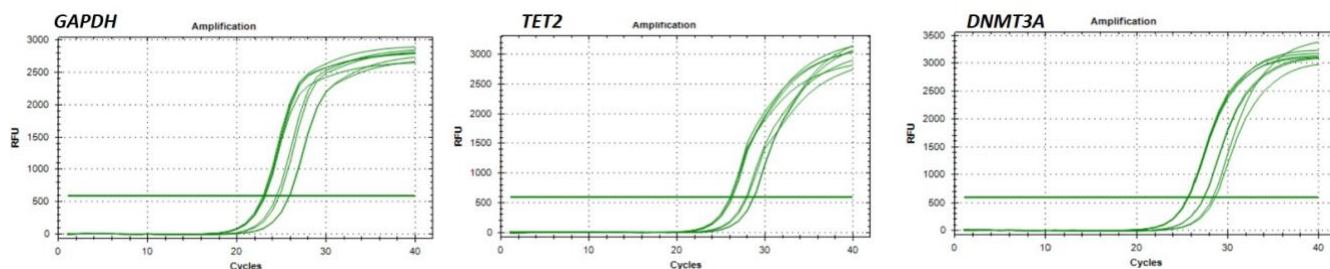


Figure 2: qPCR amplification curves for *GAPDH*, *TET2* and *DNMT3A*

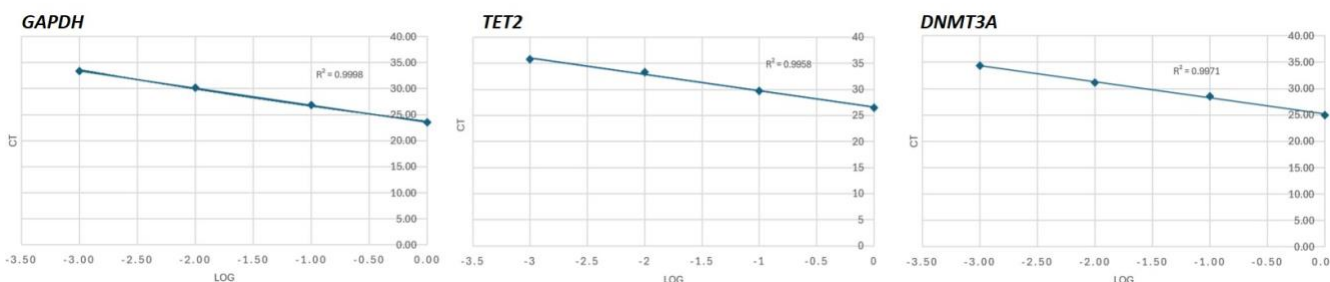


Figure 3: Standard curves of Ct values from serially diluted cDNA for *GAPDH* and *DNMT3A*

Table 3: PCR amplification efficiency of primer pairs

Primer	Slope	PCR Efficiency (%)	R ² Value
<i>GAPDH</i>	-3.283	101.65	0.9998
<i>TET2</i>	-3.175	106,52	0.9958
<i>DNMT3A</i>	-3.076	111.40	0.9971

Effect of Chemotherapeutic Agents on Gene Expression in TF-1 Cells

TET2 Gene Expression

Relative *TET2* expression following cytarabine, daunorubicin, and combination treatment was determined by the comparative Ct ($2^{-\Delta\Delta Ct}$) method, with *GAPDH* as the reference gene and untreated cells as the calibrator (Table 4, Figure 4). *TET2* expression was 0.78-fold relative to the untreated control following cytarabine treatment and 0.71-fold following daunorubicin treatment. The combined treatment resulted in a *TET2* expression level of 0.91-fold relative to the untreated control. Thus, the observed differences

represented modest reductions in *TET2* expression relative to the untreated control, with the largest observed difference occurring following daunorubicin treatment.

DNMT3A Gene Expression

Relative *DNMT3A* expression was similarly determined by the $2^{-\Delta\Delta Ct}$ method (Table 5, Figure 5). *DNMT3A* expression was 0.95-fold relative to the untreated control following cytarabine treatment and 0.87-fold following daunorubicin treatment. The combined treatment resulted in a *DNMT3A* expression level of 1.03-fold relative to the untreated control. These values indicate relatively small differences from the untreated control, with the combined treatment showing an expression level close to

the reference value of 1.0-fold.

Table 4: Relative *TET2* expression by treatment group

Treatment	Mean Ct ± SD	ΔCt	ΔΔCt	Fold Change (2 ^{ΔΔCt})
Untreated	28.72 ± 0.042	2.83	0	1
Cytarabine	26.245 ± 0.021	3.19	0.36	0.78
Daunorubicin	27.92 ± 0.127	3.33	0.50	0.71
Cytarabine-Daunorubicin	26.06 ± 0.000	3.00	0.13	0.91

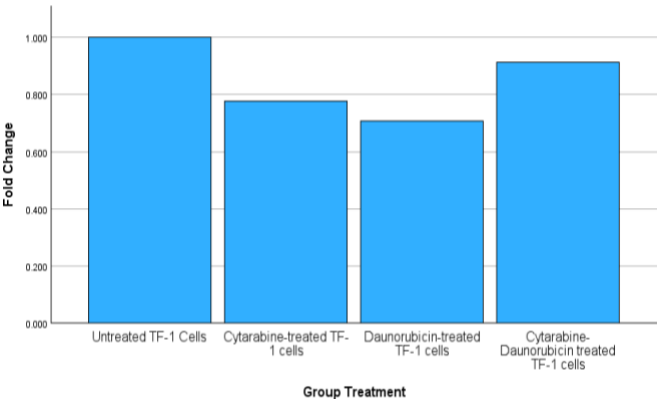


Figure 4: Relative *TET2* expression across treatment groups

Table 5: Relative *DNMT3A* expression by treatment group

Treatment	Mean Ct ± SD	ΔCt	ΔΔCt	Fold Change (2 ^{ΔΔCt})
Untreated	28.44 ± 0.177	2.54	0	1
Cytarabine	25.67 ± 0.000	2.62	0.08	0.95
Daunorubicin	27.33 ± 0.028	2.74	0.20	0.87
Cytarabine-Daunorubicin	25.61 ± 0.035	2.50	-0.04	1.03

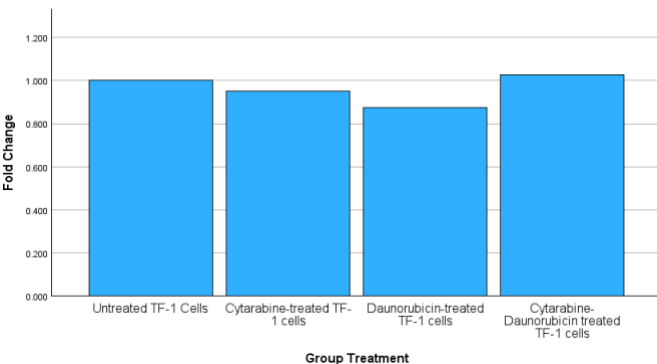


Figure 5: Relative *DNMT3A* expression across treatment groups

DISCUSSION

This study examined the effect of cytarabine, daunorubicin, and their combination on transcription of the epigenetic regulator *TET2* in TF-1 cells. Both agents, administered as monotherapy, downregulated *TET2*

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expression relative to untreated controls, with daunorubicin producing the greater reduction. The combination treatment, by contrast, produced only a slight reduction in *TET2* expression and remained higher than either monotherapy alone.

The downregulation observed after cytarabine treatment may relate to its mechanism of action. As a pyrimidine nucleoside analogue, cytarabine is incorporated into newly synthesized DNA during S phase, inhibiting DNA polymerase activity and arresting replication. Although cytarabine primary target is DNA synthesis rather than the epigenetic machinery, the cellular stress generated by this disruption may secondarily affect transcription of genes involved in DNA methylation and demethylation, including *TET2*, consistent with the broader role TET enzymes play in stress-responsive transcriptional programs (Lio & Rao, 2019).

Daunorubicin produced the largest reduction in *TET2* expression among all treatment groups. As an anthracycline, daunorubicin acts through DNA intercalation and topoisomerase II inhibition, generating extensive DNA damage and triggering apoptotic signalling. The greater suppression of *TET2* expression relative to cytarabine may therefore reflect a more severe cellular stress response, consistent with the more extensive genotoxic mechanism of anthracyclines relative to nucleoside analogues (More et al., 2023).

The combination treatment produced a distinct pattern, although *TET2* expression remained below untreated levels, it was higher than under either single agent. This suggests that simultaneous exposure to both drugs elicits a transcriptional response distinct from either monotherapy, potentially reflecting the concurrent activation of DNA damage repair, stress adaptation, and survival signalling pathways that partially offset the suppressive effect of either drug alone. This pattern is consistent with a compensatory response aimed at preserving epigenetic homeostasis under combined cytotoxic pressure, though this interpretation remains speculative in the absence of functional or pathway-level data. These findings contrast with a previous study that reported reduced *TET2* expression in AML patients is associated with disease pathogenesis and increased promoter methylation, and suggested that restoring *TET2* expression could support normal demethylation and hematopoietic differentiation (Ghasemi et al., 2024). In the present study, however, conventional chemotherapy did not restore or increase *TET2* expression under any treatment condition; all groups remained downregulated relative to untreated controls. This discrepancy likely reflects differences in study context of which the previous

study examined *TET2* dysregulation as a driver of AML pathogenesis in patient samples, while the present study assessed the transcriptional response of a non-*TET2*-mutated erythroleukemia cell line to short-term cytotoxic exposure. Similarly, a previous study has largely focused on *TET2*-mutated AML or epigenetic-targeted agents rather than the transcriptional effects of conventional chemotherapy on wild-type *TET2* (Kim, Wu, Zhang, Yang, & Chang, 2020). It should also be noted that mRNA-level changes measured here do not necessarily reflect corresponding changes in *TET2* protein abundance or enzymatic activity.

Effect on *DNMT3A* Gene Expression

DNMT3A maintains DNA methylation patterns by catalysing methyl group addition at CpG dinucleotides, a process essential for normal hematopoietic development and differentiation. In AML, *DNMT3A* mutations are among the most frequently identified genetic alterations and are associated with aberrant methylation patterns and altered treatment response (Chan & Majeti, 2013). Characterizing how chemotherapy affects *DNMT3A* expression may therefore help clarify how cytotoxic treatment intersects with epigenetic regulation in leukemic cells.

Similar with *TET2*, both cytarabine and daunorubicin monotherapy downregulated *DNMT3A* expression relative to untreated cells, with daunorubicin again producing the greater reduction. However, the combination treatment produced a slight upregulation relative to untreated cells, showing a pattern distinct from either monotherapy. The downregulation observed under monotherapy may reflect chemotherapy-induced cellular stress since DNA methylation is mechanistically linked to DNA replication and chromatin regulation, disruption of these processes by either agent could secondarily suppress *DNMT3A* transcription. The larger reduction seen with daunorubicin is consistent with the more extensive DNA damage response associated with anthracycline exposure relative to nucleoside analogue treatment.

The slight upregulation observed under combination treatment suggests that concurrent exposure to both agents elicits a different transcriptional response than either drug alone, potentially reflecting a compensatory mechanism that helps preserve epigenetic stability under compounded cytotoxic stress. While gene expression changes alone cannot establish pharmacological synergism, this discrepancy indicates that combined treatment exerts a qualitatively different influence on *DNMT3A* regulation than single-agent exposure. This could possibly reflect transcriptional adaptations in surviving

cells aimed at maintaining core cellular functions, including methylation-pathway regulation. This interpretation remains speculative, however, as the present study measured only *DNMT3A* mRNA expression and did not assess methyltransferase activity or protein-level changes.

Previous investigations of *DNMT3A* in AML have largely focused on mutation status and prognostic significance rather than chemotherapy-induced changes in expression, limiting direct comparison with the present findings (Blau et al., 2015; Chan & Majeti, 2013). Similarly, it was also demonstrated that cytarabine–daunorubicin combination therapy improves therapeutic efficacy in AML (More et al., 2023); however, this study did not examine *DNMT3A* expression that constrain direct comparison.

Several technical and biological factors may also contribute to the differences between these findings and previous reports, including variability introduced during cDNA synthesis, cell passage number, and differences in treatment response across AML/erythroleukemia cell models. A key limitation of the present study is the absence of biological replicates; the reported changes in gene expression should therefore be interpreted as preliminary trends derived from technical replicates rather than confirmed biological effects. In addition, gene expression was assessed at a single time point (48 hours post-treatment), and given the dynamic nature of transcriptional responses, effects occurring earlier or later in the treatment course may not have been captured.

Overall, these results suggest that cytarabine and daunorubicin exposure influences *TET2* and *DNMT3A* transcription in TF-1 cells, with all treatment groups showing downregulation relative to untreated controls. While the precise mechanism underlying this response remains unclear, the findings support the broader premise that conventional chemotherapy affects not only pathways involved in cell survival but also the expression of genes governing epigenetic regulation. Future studies incorporating biological replicates, protein-level analysis, DNA methylation profiling, and multiple post-treatment time points (e.g., 24, 48, and 72 hours) are needed to clarify the functional significance and temporal dynamics of chemotherapy-induced changes in *TET2* expression.

CONCLUSION

In conclusion, treatment of TF-1 cells with cytarabine, daunorubicin, or their combination was associated with relatively small differences in *TET2* and *DNMT3A* expression compared with untreated cells. *TET2* expression ranged from 0.71-fold to 0.91-fold, while

DNMT3A expression ranged from 0.87-fold to 1.03-fold. The *DNMT3A* expression level following combined treatment was close to the untreated reference level. These findings are descriptive and should not be interpreted as definitive evidence of gene upregulation, downregulation, or a chemotherapy-mediated regulatory mechanism. Further studies incorporating independent biological replicates, multiple treatment time points, and complementary analyses at the protein and DNA methylation levels are required to determine whether these observed expression differences represent reproducible biological effects.

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Staphylococcus aureus in Raw Retail Chicken Meat from Puncak Alam, Selangor: Antimicrobial Susceptibility and Detection of *nuc* and *mecA*

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ABSTRACT

Background: Raw poultry is a recognised vehicle for *Staphylococcus aureus*, and methicillin-resistant *S. aureus* (MRSA) in the food chain has made retail meat an important surveillance target. Malaysia has among the highest per-capita poultry consumption worldwide, yet local data on *S. aureus* in raw chicken at the point of retail sale remain sparse. This study determined the occurrence of *S. aureus* in raw chicken meat sold in Bandar Puncak Alam, Selangor, characterised the antimicrobial susceptibility of the isolates, and confirmed identification and methicillin resistance by real-time PCR (qPCR) targeting the *nuc* and *mecA* genes. **Methods:** Thirty raw chicken samples were collected in three batches from six retail premises comprising two supermarkets and four retail shops, selected by convenience sampling on the basis of accessibility. Samples were enriched in tryptic soy broth, cultured on mannitol salt agar, and identified by colonial morphology, Gram staining, catalase and coagulase testing. Confirmed isolates underwent Kirby–Bauer disc diffusion against six agents and SYBR Green qPCR. **Results:** Nineteen of 30 samples (63.3%) yielded mannitol-fermenting growth, from which 24 pure isolates were recovered. Eight isolates (33.3%), from six samples (20.0%), satisfied all phenotypic criteria for *S. aureus*. All eight were *nuc*-positive by qPCR, amplifying between cycles 16 and 19, and *mecA*-negative, with normal internal control amplification. All eight isolates were susceptible to amoxicillin, ampicillin, penicillin, oxacillin and streptomycin; two isolates (25.0%) showed no inhibition zone around the vancomycin disc. Uniform oxacillin susceptibility was concordant with the absence of *mecA*, indicating that no MRSA was detected. All six positive samples arose from a single sampling batch, so premises type, individual outlet and collection date were completely confounded; the concentration of positives in the retail shops cannot be attributed to premises type, and no comparison between outlet types is possible. **Conclusion:** Raw chicken meat in this locality carried *S. aureus* at a moderate rate but yielded no evidence of methicillin resistance. The vancomycin observation cannot be interpreted as resistance, because disc diffusion is not a valid method for vancomycin in staphylococci and requires confirmation by minimum inhibitory concentration testing.

Keywords:

poultry meat; food safety; antimicrobial resistance; methicillin resistance; real-time PCR

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INTRODUCTION

Staphylococcus aureus is a Gram-positive coccus that colonises the skin and anterior nares of a substantial proportion of healthy people and, at the same time, ranks among the leading causes of foodborne intoxication worldwide. Ingestion of preformed staphylococcal enterotoxins produces a short, self-limiting but sometimes severe gastroenteritis, and the organism is also responsible for a wide spectrum of invasive disease (Kadariya et al., 2014). Because enterotoxins are heat-stable and survive cooking temperatures that kill the organism itself, contamination of raw ingredients has consequences that persist into the finished dish.

The public-health importance of *S. aureus* in food has been amplified by antimicrobial resistance. Methicillin-resistant *S. aureus* (MRSA), defined by carriage of the *mecA* gene encoding the low-affinity penicillin-binding protein PBP2a, was historically a healthcare-associated pathogen but is now established in livestock and in the food chain. The World Health Organization retains MRSA on its bacterial priority pathogens list (World Health Organization, 2024). A review of 259 studies found MRSA reported in 84.3% of investigations of meat from retail outlets, although sample-level prevalence was typically below 20%, with the highest rates in pork and chicken (González-Machado et al., 2024). Whether food acts as a genuine transmission route or mainly as a passive vehicle remains debated

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(Sergelidis & Angelidis, 2017), but the presence of resistant strains on retail meat is a recognised One Health concern.

Poultry occupies a particular position in this picture. Chicken is the most widely consumed meat in Malaysia, where per-capita poultry consumption is among the highest in the world, and it is economically accessible and acceptable across the country's religious and ethnic groups. Antimicrobials are used in poultry production for therapy, prophylaxis and, in some settings, growth promotion, and this use has been linked to the selection of resistant staphylococci in birds and in the people who handle them (Ali et al., 2017; Chai et al., 2022). Contamination of carcasses can occur at many points — during scalding and defeathering, on conveyors and cutting equipment, and through handling at the point of sale — so that the organism recovered from retail meat may originate from the bird, the abattoir environment or the food handler.

Published Malaysian data on *S. aureus* in raw retail chicken remain limited. An earlier study in Kota Bharu, Kelantan, recovered *S. aureus* from 24% of chicken meat samples and reported the associated resistance profiles (Aklilu et al., 2016), and Malaysian reviews have repeatedly identified hygiene along the food chain as a recurring weakness (New et al., 2017). Comparable data for the Klang Valley conurbation, and for the distinction between supermarket and small retail outlets within a single locality, are scarce.

Identification of *S. aureus* by culture and biochemical testing is inexpensive but slow and imperfectly specific, since other mannitol-fermenting, catalase-positive staphylococci grow indistinguishably on selective media (Paramithiotis, 2023). Real-time PCR shortens the turnaround time and provides species-level confirmation through the thermonuclease (*nuc*) gene, while *mecA* detection gives a genotypic determination of methicillin resistance that is independent of the phenotypic assay (Hadi et al., 2023). This study therefore set out to determine the occurrence of *S. aureus* in raw chicken meat sold at retail premises in Bandar Puncak Alam, Selangor; to characterise the antimicrobial susceptibility of the isolates recovered; and to confirm species identity and methicillin resistance by SYBR Green qPCR targeting *nuc* and *mecA*.

MATERIALS AND METHODS

Study Design and Sample Collection

A cross-sectional study was conducted at the Microbiology, Pathogen and Molecular Laboratories, Faculty of Health Sciences, Universiti Teknologi MARA,

Puncak Alam Campus, Selangor. Thirty samples of raw chicken meat, comprising breast, thigh and keel portions, were purchased from six retail premises in Bandar Puncak Alam, Selangor. The six premises were selected by convenience sampling on the basis of accessibility within the township; neither randomisation nor stratification was applied, so the premises are not a statistically representative sample of retail outlets in the area. Breast, thigh and keel portions were taken as available at each premises rather than allocated by design, and portion type was not recorded for individual samples; the distribution of anatomical sites across the 30 samples therefore cannot be reported. Sampling was carried out during March 2026 in three batches of ten samples collected on separate occasions: batch 1 from two supermarkets, batch 2 from two retail shops in Fasa 3, and batch 3 from retail shops in Fasa 1 and Fasa 2 (Table 2). Samples were placed in an insulated box immediately after purchase and transported to the laboratory for processing on the same day.

Sample Preparation and Enrichment

Each sample was cut into approximately 25 cm² portions using a sterile knife and board, then immersed in 15 mL of 0.85% saline for 15 min at room temperature with gentle agitation. Two millilitres of the resulting suspension were transferred into 10 mL of tryptic soy broth (Merck, Germany) and incubated at 37 °C for 19–24 h.

Isolation and Phenotypic Identification

A loopful of the enriched broth was streaked onto mannitol salt agar (Oxoid Ltd, England) by the quadrant method and incubated at 37 °C for 24 h. Colonies showing morphology consistent with *S. aureus*, together with the surrounding change of the phenol red indicator from red to yellow, were subcultured onto nutrient agar (Oxoid Ltd, England) and incubated for a further 24 h to obtain pure growth. Where a sample yielded two distinct colony types consistent with *S. aureus*, both were carried forward as separate isolates.

Pure isolates were characterised by Gram staining and examined at ×1000 under oil immersion; by the slide catalase test using freshly prepared 3% (v/v) hydrogen peroxide; and by the tube coagulase test, in which two drops of thawed plasma were added to a broth suspension of the isolate and incubated at 37 °C for up to 4 h, with clot formation read as positive. An isolate was regarded as *S. aureus* when it showed compatible colonial morphology on both media, Gram-positive cocci in clusters, a positive catalase reaction and a positive tube coagulase reaction. *S. aureus* ATCC 25923 served as the positive control throughout.

Antimicrobial Susceptibility Testing

Confirmed isolates were tested by the Kirby–Bauer disc diffusion method on Mueller–Hinton II agar (Becton Dickinson, USA). Inocula were prepared in sterile saline, adjusted to the 0.5 McFarland standard and used within 15 min. Six discs (Oxoid Ltd, England) were applied: amoxicillin 10 µg, ampicillin 10 µg, penicillin 10 units, oxacillin 1 µg, streptomycin 25 µg and vancomycin 30 µg. Plates were incubated at 37 °C for 24 h and zones of inhibition were interpreted against Clinical and Laboratory Standards Institute breakpoints (Clinical and Laboratory Standards Institute, 2024). Isolates were recorded as susceptible or as showing no measurable zone; minimum inhibitory concentration testing was not performed.

DNA Extraction

Template DNA was prepared by boiling lysis (Dashti et al., 2009). Four to five colonies from nutrient agar were suspended in 500 µL of sterile distilled water, vortexed for 10 s and centrifuged at 10,000 rpm for 10 min at 27 °C. The supernatant was discarded, the pellet resuspended in 50 µL of sterile distilled water, and the suspension heated at 90–95 °C for 10 min on a heating block before immediate transfer to ice for 2–5 min. After a final centrifugation at 10,000 rpm for 1 min, the supernatant was collected and stored at –20 °C until amplification.

Real-Time PCR

Primers targeting a 181-bp fragment of the *nuc* gene and a 163-bp fragment of the *mecA* gene were adopted from the assay described by Aklilu et al. (2016) and synthesised commercially (AITbiotech, Singapore, and Integrated DNA Technologies, Singapore, respectively). Primer sequences and amplicon sizes are given in Table 1. Amplification was performed on a C1000 Touch thermal cycler with a CFX96 optical module (Bio-Rad, USA) and analysed using CFX Manager 3.0. Cycling comprised pre-incubation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 10 s and combined annealing and extension at 57 °C for 30 s; the same annealing temperature was used for both targets. Each 25 µL reaction contained 12.5 µL of QuantiFast SYBR Green PCR master mix (Qiagen, UK), 0.5 µL of each primer, 1.5 µL of RNase-free water and 10 µL of template. Reactions were assembled inside a class II biological safety cabinet. *S. aureus* ATCC 25923 served as the positive control for *nuc* and RNase-free water as the no-template control. Because a characterised MRSA strain could not be handled in the teaching laboratory, an internal amplification control was used in place of a positive control for *mecA*; this monitors reaction chemistry and instrument performance but does not demonstrate that the *mecA* assay itself can detect a true *mecA*-positive strain. Melting-curve analysis was performed from 65 to 95 °C after the final cycle.

Table 1: Primers used for SYBR Green real-time PCR detection of the *nuc* and *mecA* genes, adopted from Aklilu et al. (2016). The amplicon size is given once for each primer pair. nt, nucleotides; bp, base pairs

Gene	Primer	Sequence (5'→3')	Length	Amplicon
<i>nuc</i>	Forward	ATGGACGTGGCTTAGCGTAT	20 nt	181 bp
<i>nuc</i>	Reverse	GCGTTGTCTTCGCTCCAAAT	20 nt	
<i>mecA</i>	Forward	ACTGCTATCCACCCTCAAAC	20 nt	163 bp
<i>mecA</i>	Reverse	CTGGTGAAGTTGTAATCTGG	20 nt	

Isolation and Identification

Data Analysis

Findings are reported descriptively as counts and percentages. No inferential statistical testing was performed; the number of positive samples was too small to support hypothesis testing, and the batch structure of the sampling confounds premises with collection date.

Ethical Considerations

The study used commercially purchased food samples only and involved no human participants or animals; institutional ethical approval was therefore not required.

RESULTS

Nineteen of the 30 samples (63.3%) produced growth on mannitol salt agar with colonial morphology and mannitol fermentation consistent with *S. aureus*, evident as a change of the medium from red to yellow around the growth (Figure 1A). Colonies varied in pigmentation from creamy white to golden yellow. Five of these samples yielded two distinct colony types, so that 24 pure isolates in total were subcultured onto nutrient agar (Figure 1B).

On microscopy, 22 of the 24 isolates (91.7%) were Gram-positive cocci in irregular clusters (Figure 1C); the remaining two were a Gram-positive bacillus and a Gram-positive diplococcus. Twenty isolates (83.3%) were catalase-positive, with vigorous bubbling within 10 s (Figure 1D), and 13 (54.2%) produced a visible clot in the

tube coagulase test within 4 h. Eight isolates satisfied all phenotypic criteria simultaneously and were accepted as *S. aureus*, representing 33.3% of the 24 isolates. These eight isolates originated from six of the 30 samples, giving a sample-level occurrence of 20.0%. Four of the six positive samples yielded a single confirmed isolate each, while two samples each yielded two confirmed isolates of differing pigmentation, accounting for the difference between the sample and isolate counts in Table 2.

All six positive samples belonged to batch 3 and were purchased from the two retail shops in Fasa 1 and Fasa 2; no *S. aureus* was recovered from either supermarket or from the Fasa 3 retail shops (Table 2). Because batch and premises were collected on different occasions, this distribution reflects both location and sampling date and cannot be attributed to premises type alone.

Table 2: Distribution of raw chicken meat samples and confirmed *S. aureus* isolation by batch and premises. “Positive samples” counts meat samples from which at least one isolate was confirmed as *S. aureus*; “Isolates” counts the individual cultures confirmed as *S. aureus*. The two totals differ because two samples each yielded two distinct colony types that were both confirmed. The percentage is calculated on the total of 30 samples

Batch	Premises	Samples (n)	Positive samples (n)	Isolates (n)
1	Supermarket A	5	0	0
1	Supermarket B	5	0	0
2	Retail shop A, Fasa 3	5	0	0
2	Retail shop B, Fasa 3	5	0	0
3	Retail shop, Fasa 1	5	4	4
3	Retail shop, Fasa 2	5	2	4
Total		30	6 (20.0%)	8

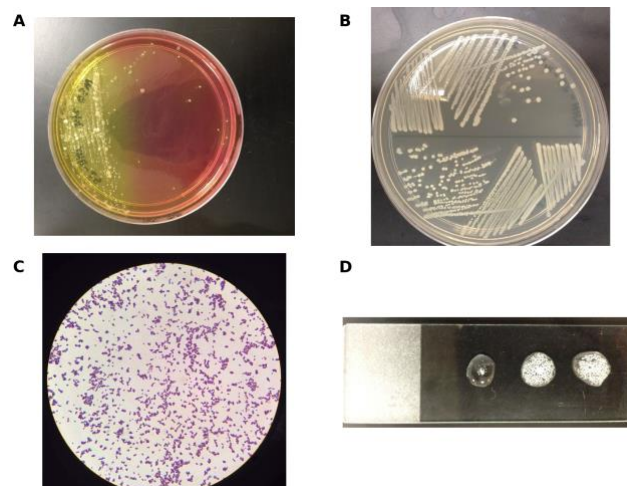


Figure 1: Culture and phenotypic identification of isolates from raw chicken meat. (A) Mannitol salt agar showing mannitol-fermenting growth, with the phenol red indicator changed from red to yellow around the colonies; (B) pure subculture on nutrient agar, showing two colony morphologies recovered from a single sample; (C) Gram stain of *S. aureus* ATCC 25923 showing Gram-positive cocci in irregular clusters, oil immersion, ×1000; (D) slide catalase test, with the left-hand drop showing no reaction (negative) and the two right-hand drops showing vigorous bubbling (positive). Images are representative

Antimicrobial Susceptibility

All eight confirmed isolates were susceptible to amoxicillin, ampicillin, penicillin, oxacillin and streptomycin (Table 3, Figure 2). Two isolates (25.0%) produced no measurable zone of inhibition around the 30 µg vancomycin disc, while the remaining six produced clear zones. No isolate was non-susceptible to more than one agent, and no multidrug-resistant phenotype was observed.

Table 3: Disc diffusion results for the eight confirmed *S. aureus* isolates. Disc diffusion is not an acceptable method for determining vancomycin susceptibility in staphylococci; the vancomycin column therefore records the presence or absence of an inhibition zone as observed, and is not an interpretation of resistance

Antimicrobial agent	Disc potency	Susceptible n (%)	No zone n (%)
Amoxicillin (AML)	10 µg	8 (100)	0 (0)
Ampicillin (AMP)	10 µg	8 (100)	0 (0)
Penicillin (P)	10 units	8 (100)	0 (0)
Oxacillin (OX)	1 µg	8 (100)	0 (0)
Streptomycin (S)	25 µg	8 (100)	0 (0)
Vancomycin (VA)	30 µg	6 (75.0)	2 (25.0)

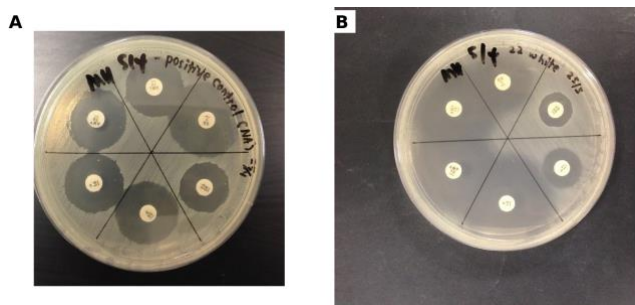


Figure 2: Kirby-Bauer disc diffusion on Mueller-Hinton II agar. (A) *S. aureus* ATCC 25923 control; (B) a representative test isolate. Discs are amoxicillin (AML 10 µg), ampicillin (AMP 10 µg), penicillin (P 10 units), oxacillin (OX 1 µg), streptomycin (S 25 µg) and vancomycin (VA 30 µg), arranged in six sectors

Molecular Confirmation

All eight isolates were positive for the *nuc* gene, crossing the fluorescence threshold between cycles 16 and 19, together with the ATCC 25923 positive control (Figure 3A). Melting-peak analysis produced a single peak of closely comparable temperature for every isolate and for the control, with no peak in the no-template control (Figure 3B), indicating amplification of a single product and confirming all eight isolates as *S. aureus*.

None of the eight isolates amplified with the *mecA* primers. Only the internal amplification control crossed the threshold, at approximately cycle 15, while all test isolates and the no-template control remained flat throughout the 40 cycles (Figure 3C), and only the internal control produced a melting peak (Figure 3D). No MRSA was therefore detected among the isolates recovered in this study. This genotypic result was concordant with the phenotypic finding of uniform oxacillin susceptibility.

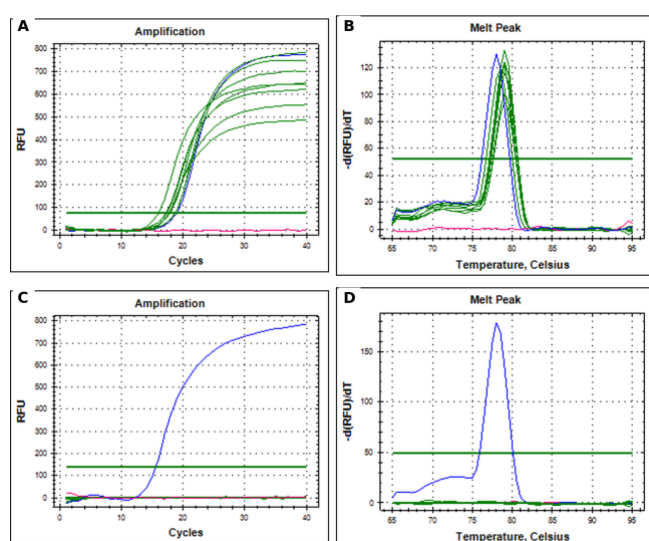


Figure 3: SYBR Green real-time PCR detection of the *nuc* and *mecA* genes. (A) Amplification plot for *nuc*: all eight test isolates and the positive control crossed the threshold between cycles 16 and 19, with no signal in the no-template control; (B) melting-peak analysis for *nuc*, showing a single peak of comparable

temperature for all isolates and the control; (C) amplification plot for *mecA*: only the internal amplification control amplified, at approximately cycle 15, with no signal from any test isolate; (D) melting-peak analysis for *mecA*, in which only the internal control produced a peak

DISCUSSION

Three findings require interpretation: an occurrence of 20.0% that is unremarkable by regional standards, a complete absence of *mecA* that is more informative than it first appears, and two antimicrobial results — for vancomycin and for the β -lactams — that are unlikely to be biologically real. Each is considered in turn, together with what the confounded sampling structure does and does not permit.

The sample-level occurrence of 20.0% sits within the range reported elsewhere. A study of chicken meat from a wet market in Kota Bharu, Kelantan, recovered *S. aureus* from 24% of samples (Aklilu et al., 2016), and the international review by González-Machado et al. (2024) found that sample-level prevalence in retail meat is usually below 20%, with chicken among the more frequently contaminated commodities. The closeness of the present figure to the Kelantan estimate is more informative than either value alone. The two studies sampled different states, different retail formats and different years, yet converged on a similar rate, which argues that *S. aureus* contamination is a general property of the Malaysian retail poultry chain rather than a feature of particular outlets or regions. This comparison should nevertheless be made cautiously: enrichment protocols, selective media and confirmation criteria differ between studies, and a single-colony picking strategy such as the one used here will under-detect samples carrying *S. aureus* at low density alongside competing staphylococci. Reported prevalence in this field is therefore as much a function of method as of true contamination, and cross-study comparison sets a context rather than a benchmark.

The absence of *mecA* in all eight isolates is the most clear-cut finding, and it is strengthened by its concordance with the phenotypic data, since every isolate was also susceptible to oxacillin. Aklilu et al. (2016) likewise found no MRSA in Malaysian chicken meat, and MRSA has been reported at low frequency in Malaysian animal handlers (Chai et al., 2022). The contrast with the international picture is instructive: González-Machado et al. (2024) found MRSA reported in the large majority of published investigations of retail meat, yet usually at low sample-level prevalence. Two consecutive Malaysian studies returning no MRSA is therefore consistent with genuinely low circulation in this supply chain rather than with its

absence, and the more defensible reading is that any MRSA present is too infrequent to be captured by studies of this size. This should nevertheless be interpreted with care. Eight isolates provide very limited power to detect a phenotype that is typically present at low prevalence: with a true prevalence of 5%, the probability of observing no positive isolate in a sample of eight exceeds 65%. The result is best read as the absence of evidence for MRSA in a small set of isolates from one locality, not as evidence that MRSA is absent from the Malaysian poultry chain. A further constraint is that no characterised *mecA*-positive strain was available as a positive control; the internal amplification control confirms that the reaction chemistry and instrument were functioning, but it does not establish the analytical sensitivity of the *mecA* assay itself.

Two isolates produced no inhibition zone around the vancomycin disc. This observation must not be reported as vancomycin resistance. Disc diffusion does not reliably separate vancomycin-susceptible from vancomycin-intermediate staphylococci, and current CLSI guidance requires that vancomycin susceptibility in *S. aureus* be determined by a minimum inhibitory concentration method (Clinical and Laboratory Standards Institute, 2024). Vancomycin-intermediate and vancomycin-resistant *S. aureus* remain rare, and the prior probability of encountering two such isolates among eight from retail chicken is very low. A technical explanation is considerably more likely: an inoculum denser than the 0.5 McFarland standard, uneven agar depth, or disc handling and storage are all recognised causes of falsely small or absent zones. Until MIC testing is performed, these two isolates should be described only as having shown no zone, and the finding treated as a prompt for confirmatory testing.

The uniform susceptibility of all eight isolates to penicillin, ampicillin and amoxicillin also deserves comment, because it runs counter to most published data. Penicillin resistance mediated by the β -lactamase gene *blaZ* is the commonest resistance phenotype in *S. aureus* globally, and studies of food isolates typically report it in the great majority of strains (Sergelidis & Angelidis, 2017; González-Machado et al., 2024). Complete penicillin susceptibility in eight consecutive isolates is therefore an unusual result. It may be genuine and reflect limited antimicrobial pressure in the supply chain serving these premises, but it may equally reflect difficulty in reading penicillin zone edges, which requires specific attention to the appearance of the zone margin rather than its diameter alone. Confirmation by a penicillin zone-edge test or by *blaZ* detection would settle the question and would materially strengthen the paper.

That all six positive samples came from the two retail

shops sampled in batch 3, with none from the supermarkets or the Fasa 3 shops, is a striking pattern, and it is tempting to attribute it to differences in cold-chain management and handling hygiene between larger and smaller outlets. The study design does not permit that inference. Each batch was collected on a different occasion, so premises type, individual shop and sampling date are completely confounded; the pattern is equally consistent with a single contaminated delivery, a warmer sampling day, or a change in laboratory handling between batches. The observation is reported because it may help design a future study, not as a finding about supermarkets and retail shops.

Several further limitations should be acknowledged. The sample size of 30 was the practical minimum and permits only descriptive reporting. The eight isolates are also not eight independent observations, since two pairs were recovered from the same meat sample and may represent the same strain; without genotyping this cannot be resolved, and the molecular findings should be read at the level of six samples rather than eight independent isolates. No enumeration was performed, so the results cannot be compared with microbiological criteria expressed as counts per gram. Identification relied on a conventional phenotypic panel, and although *nuc* qPCR provided species confirmation, the isolates were not characterised further by enterotoxin gene profiling, *spa* typing or sequencing, so their potential to cause staphylococcal food poisoning is unknown. Susceptibility testing covered six agents, omitting classes such as tetracyclines, macrolides and fluoroquinolones that are relevant to poultry production, and zone diameters were not retained in the analysable record, so results are reported as categorical outcomes. Minimum inhibitory concentrations were not determined for any agent. Because portion type was not recorded for individual samples, contamination could not be examined by anatomical site. Any variation in *S. aureus* carriage between breast, thigh and keel portions is therefore unmeasured here, and would in any case have been confounded with premises and batch in the same way as the outlet-type comparison; recording portion type per sample is a straightforward improvement for future work. Finally, sampling was confined to one township over a short period and cannot be generalised.

Future work in this setting should enrol a larger sample across multiple townships and seasons, decouple premises type from sampling occasion by collecting from all premises on each visit, and report zone diameters alongside MIC determination for vancomycin. Screening confirmed isolates for enterotoxin genes and for *blaZ*, and extending the panel of antimicrobial agents, would allow the public-health significance of the isolates to be assessed

rather than inferred. Given that raw chicken is handled in almost every Malaysian kitchen, such data would support targeted messaging on cross-contamination and cooking practice.

CONCLUSION

Staphylococcus aureus was recovered from 20.0% of raw chicken meat samples obtained from retail premises in Bandar Puncak Alam, Selangor, and all eight isolates were confirmed by *nuc*-targeted real-time PCR. No isolate carried the *mecA* gene, and all were susceptible to oxacillin, so no MRSA was detected. Two isolates produced no vancomycin inhibition zone, but this requires confirmation by minimum inhibitory concentration testing before it can be interpreted, and complete β -lactam susceptibility across all isolates is unusual enough to warrant verification. All six positive samples came from a single sampling batch, so premises type, individual outlet and collection date are completely confounded; no conclusion can be drawn about whether supermarkets and small retail shops differ, and this should be resolved by design in any future study. Within the limits of a small, single-locality study, these findings indicate that raw chicken sold in this township carries *S. aureus* at a rate comparable with international reports while showing no evidence of methicillin resistance, and they support continued periodic surveillance of retail poultry rather than any conclusion that the food chain is free of resistant staphylococci.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Evaluation of Cytonucleomorphometric Parameters for Differentiating Normal, Atrophy, and Low-Grade Squamous Intraepithelial Lesion in Cervical Cytology

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ABSTRACT

Background: Conventional cervical cytology relies on morphological interpretation, which may be challenging when atrophic and low-grade squamous intraepithelial lesion (LSIL) cells show overlapping features. Cytonucleomorphometric may provide objective measurements to support differentiation. This study aimed to establish baseline morphometric data for normal intermediate, atrophic, and LSIL cells for future computer-assisted cervical cytology applications. **Methods:** A quantitative cross-sectional comparative study analysed 300 archived conventional Pap smear cells comprising 100 normal intermediate, 100 atrophic, and 100 LSIL cells. Images were captured at 40× objective magnification and analysed using ImageJ for four biologically distinct parameters: nuclear area, nuclear perimeter, maximum nuclear diameter, and nuclear-to-cytoplasmic (N/C) ratio. Kruskal–Wallis testing followed by post-hoc Bonferroni-adjusted pairwise comparisons were performed. **Results:** There were significant overall differences among the three groups for all four primary morphometric parameters ($p < 0.001$). LSIL cells exhibited significantly larger mean nuclear area ($91.16 \pm 20.92 \mu\text{m}^2$), perimeter ($34.05 \pm 4.03 \mu\text{m}$), and maximum diameter ($11.71 \pm 1.62 \mu\text{m}$) compared to both normal intermediate and atrophic cells. The mean N/C ratio was lowest in normal intermediate cells (0.0274 ± 0.0093) and significantly higher in atrophic (0.0977 ± 0.0379) and LSIL cells (0.1331 ± 0.1161); however, post-hoc pairwise comparisons revealed no significant difference in N/C ratio between atrophy and LSIL ($p = 0.831$). **Conclusion:** Cytonucleomorphometric analysis provides objective quantitative features that successfully differentiate LSIL from its benign morphological mimic, atrophy, using nuclear size parameters (area and perimeter). The N/C ratio, while useful for distinguishing normal cells, fails to differentiate atrophy from LSIL due to overlapping morphometric profiles.

Keywords:

cervical cytology;
Cytonucleomorphometric; normal
intermediate cells; atrophy; LSIL

INTRODUCTION

Cervical cancer remains an important preventable cause of morbidity and mortality worldwide, with screening enabling detection of cervical abnormalities before progression to invasive disease (Arbyn et al., 2022). Conventional Papanicolaou (Pap) smear cytology remains an important cytological method, but interpretation depends substantially on visual assessment. Subtle abnormalities and benign changes may share morphological features, creating diagnostic challenges and inter-observer variability (Zhang et al., 2022; Pulkkinen et al., 2023).

Atrophic cells are particularly relevant because reduced cellular maturation may produce relatively prominent, hyperchromatic nuclei and scant cytoplasm, potentially mimicking squamous intraepithelial abnormalities. LSIL cells, in contrast, characteristically demonstrate nuclear

enlargement and other HPV-associated cytological changes (Alrajjal et al., 2021). Quantitative assessment of nuclear and cellular morphology may therefore provide additional objective information when conventional morphological interpretation is equivocal.

Cytonucleomorphometric uses digital image measurements such as nuclear area, perimeter, diameter, and N/C ratio to quantify cellular morphology. Mishra et al. (2023) demonstrated significant differences in several morphometric characteristics across categories of squamous epithelial abnormality in conventional cervico-vaginal Pap smears. However, focused comparisons involving normal intermediate, atrophic, and LSIL cells remain limited. The present study addressed this gap by measuring four practical morphometric parameters and comparing their distributions across the three cell groups. The study aimed to establish baseline data that could support standardised quantitative assessment and future

computer-assisted cervical cytology applications.

MATERIALS AND METHODS

Study Design and Setting

A quantitative, cross-sectional comparative study was conducted in the Cytology Laboratory, Faculty of Health Sciences, Universiti Teknologi MARA, Puncak Alam, Selangor, Malaysia. Archived conventional Pap smear slides were examined for normal intermediate, atrophic, and LSIL cells.

Sample Selection

A total 60 slides were included, consisting of 20 Normal, 20 Atrophy, and 20 LSIL slides. Selecting 5 cells per slide, 100 cells per diagnostic category were selected. Slides were included when cellularity and staining were adequate and the target cells had intact morphology, distinct nuclear borders, and clearly visible cytoplasmic margins. Cells were selected using predefined morphological criteria to minimise observer selection bias. For each slide, the observer systematically examined the available cellular fields and selected the first suitable cell encountered that met the inclusion criteria, rather than selecting cells based on the perceived severity of morphological features. Selected cells had clearly defined nuclear and cytoplasmic boundaries, adequate staining and fixation, and sufficient cellular integrity for accurate measurement. Cells showing poor fixation or staining, overlap, folding, degeneration, obscured borders, excessive blood or inflammatory exudate, or other artefacts were excluded from measurement. The same selection criteria and measurement procedure were applied consistently across all three cell groups.

Image Acquisition and Morphometric Analysis

Image acquisition was performed using a LEICA DM750 light microscope equipped with a LEICA ICC50 digital

NIH, USA). For each cell, the nuclear boundary was manually traced using the ImageJ 'Freehand Selections' tool to obtain nuclear area (μm^2), nuclear perimeter (μm), and maximum nuclear diameter (μm). The N/C ratio was calculated by dividing the nuclear area by the total cellular area.

Statistical Analysis

Data were recorded in Microsoft Excel and analysed using SPSS version 30.0. Descriptive statistics were calculated for each parameter. Shapiro–Wilk and Levene's tests were used to assess distribution and variance assumptions. Because the data did not consistently satisfy parametric assumptions, Kruskal–Wallis tests were applied to compare the three groups. Significant overall differences were followed by all-pairwise comparisons with Bonferroni adjustment. Statistical significance was set at $p < 0.05$.

RESULTS

A total of 300 cells were successfully analysed with no missing observations. LSIL cells generally demonstrated larger nuclear dimensions and a higher N/C ratio than normal intermediate and atrophic cells. The descriptive findings are summarised in Table 1.

Table 1: Descriptive statistics of Cytonucleomorphometric parameters

Parameter	Normal (n=100)	Atrophy (n=100)	LSIL (n=100)
Nuclear area (μm^2)	48.55 $\pm$ 8.99	45.62 $\pm$ 9.70	91.16 $\pm$ 20.92
Nuclear perimeter (μm)	24.84 $\pm$ 2.52	23.94 $\pm$ 2.64	34.05 $\pm$ 4.03
Maximum nuclear diameter (μm)	8.49 $\pm$ 0.93	8.17 $\pm$ 1.00	11.71 $\pm$ 1.62
N/C ratio	0.0274 $\pm$ 0.0093	0.0977 $\pm$ 0.0379	0.1331 $\pm$ 0.1161

Kruskal–Wallis testing demonstrated significant differences among the three cell groups for nuclear area ($H = 195.080$), nuclear perimeter ($H = 194.610$), maximum nuclear diameter ($H = 185.741$), and N/C ratio ($H = 195.330$); all $p < 0.001$. Bonferroni pairwise results are presented in Table 2.

The four nuclear size-related parameters consistently distinguished LSIL from both normal intermediate and atrophic cells, while normal intermediate and atrophic cells did not differ significantly for these parameters. In

Table 2: Bonferroni pairwise comparisons

Parameter	Normal vs Atrophy	Normal vs LSIL	Atrophy vs LSIL
Nuclear area	0.586	<0.001	<0.001
Nuclear perimeter	0.387	<0.001	<0.001
Maximum nuclear diameter	0.356	<0.001	<0.001
N/C ratio	<0.001	<0.001	0.831

camera. Calibration was executed under a 40 $\times$ objective magnification using a physical stage micrometer (1 mm divided into 100 parts, yielding 10 μm per division) to establish a standard pixel-to-micrometer conversion factor of 3.12 pixels/ μm within ImageJ software (Version 1.54g,

contrast, N/C ratio significantly differentiated normal intermediate cells from both atrophic and LSIL cells but did not significantly differentiate atrophic from LSIL cells.

DISCUSSION

The principal finding was the consistent enlargement of LSIL nuclei across three related measurements. Nuclear area, perimeter, and maximum diameter were all significantly greater in LSIL cells than in normal intermediate and atrophic cells. This convergence suggests that nuclear enlargement was a robust measurable feature of LSIL within the present dataset rather than an effect limited to a single measurement.

The nuclear area finding is broadly consistent with Mishra et al. (2023), who reported a mean LSIL nuclear area of approximately $104.32 \mu\text{m}^2$ compared with $91.16 \mu\text{m}^2$ in the present study. The lower value may reflect differences in study population, cell-selection criteria, slide preparation, staining, calibration, and image acquisition. Similarly, the present mean LSIL nuclear perimeter of $34.05 \mu\text{m}$ was close to the approximately $36.74 \mu\text{m}$ reported by Mishra et al. Differences in boundary tracing and segmentation can influence perimeter measurements.

Maximum nuclear diameter was $11.71 \mu\text{m}$ in the present LSIL group, compared with approximately $11.00 \mu\text{m}$ reported by Mishra et al. Direct numerical comparison should be made cautiously because the present study measured maximum nuclear diameter directly, whereas Mishra et al. derived diameter from nuclear area.

The lack of a statistically significant difference in N/C ratio between the atrophic and LSIL groups ($p = 0.831$) has a profound biological basis that highlights a major diagnostic trap in exfoliative cervical cytology. Under low-oestrogen states, immature parabasal cells may predominate because of impaired maturation into intermediate and superficial cells, resulting in characteristic atrophic cytological features (Ali et al., 2024). Morphologically, these atrophic parabasal-like squamous cells suffer from cytoplasmic shrinkage (extreme scantiness of cytoplasm) while maintaining a normal, non-dysplastic nucleus (mean nuclear area: $45.62 \pm 9.70 \mu\text{m}^2$). This physical shrinkage naturally produces a highly elevated N/C ratio (0.0977 ± 0.0379). Conversely, LSIL represents a true neoplastic process driven by human papillomavirus (HPV) infection, which causes true nuclear enlargement (mean nuclear area: $91.16 \pm 20.92 \mu\text{m}^2$) but retains a moderate, mature cytoplasmic volume. Consequently, despite their entirely different pathophysiological origins (hormonal withdrawal vs. viral neoplasia), atrophic and LSIL cells exhibit a highly

overlapping N/C ratio (0.0977 vs. 0.1331 , respectively). This statistical and morphologic convergence explains why the N/C ratio fails to resolve the atrophy-LSIL diagnostic pitfall, and confirms that clinical pathologists must rely on absolute nuclear size dimensions (such as nuclear area and perimeter) to make a definitive diagnosis

The absence of significant differences between normal intermediate and atrophic cells for the four nuclear size parameters, together with the significant N/C-ratio difference, suggests that nuclear size alone may not capture the morphological changes associated with atrophy. Conversely, the significant differences across all four nuclear parameters between atrophic and LSIL cells suggest that a combination of nuclear measurements may be more informative for this diagnostically challenging distinction.

These findings have relevance for computer-assisted cytology because quantitative models require consistently defined and labelled features. The present study provides preliminary morphometric data that could contribute to future reference or ground-truth datasets. Furthermore, a crucial limitation of this baseline study is the inherent selection bias associated with purposive sampling. By intentionally excluding folded, overlapping, degenerated, or dirty cells to obtain clean boundaries for ImageJ tracing, we created an "idealised" cellular cohort. While this is necessary to establish precise baseline quantitative data, it does not represent the messy, highly variable environment of routine clinical Pap smears. When training computer-assisted screening or artificial intelligence (AI) deep-learning models, algorithms trained on such idealised datasets often suffer from severe performance degradation when applied to uncrated, real-world clinical slides. Future studies must therefore incorporate "noisy" and imperfect cytological images to ensure clinical generalisability.

The study also has several limitations. The dataset contained only 300 cells and was selected purposively, creating potential selection bias. Difficult, overlapping, folded, degenerated, or artefactual cells were excluded. Only five morphometric parameters were evaluated, while additional nuclear shape, cellular, chromatin, and texture features may provide further discriminatory information. Furthermore, cytological group classification was not independently validated against histopathology. Future studies should incorporate larger independent datasets and evaluate diagnostic performance using sensitivity, specificity, predictive values, receiver operating characteristic analysis, and area under the curve.

CONCLUSION

In conclusion, cytonucleomorphometric analysis demonstrates significant quantitative differences that can be exploited to show significant differences among normal, atrophic, and LSIL squamous cells in cervical cytology. Nuclear area, perimeter, and maximum diameter serve as the most robust, statistically significant parameters to distinguish LSIL from benign atrophic mimics. In contrast, due to hormonal-induced cytoplasmic shrinkage, the N/C ratio exhibits overlapping profiles between atrophic and LSIL cells, rendering it unsuitable as a standalone diagnostic parameter. These findings establish a baseline quantitative framework for the future development of computer-assisted cervical cancer screening.

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Characterisation of *Zingiber officinale*: Phytochemical composition, Antioxidant and Antidiabetic Activities

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ABSTRACT

Background: *Zingiber officinale* (ginger) contains various bioactive compounds associated with antioxidant and antidiabetic properties. This study aimed to characterise the phytochemical composition and evaluate the antioxidant and antidiabetic activities of aqueous and 70% ethanol extracts of *Zingiber officinale*. **Methods:** The extracts were evaluated for total phenolic content (TPC), total flavonoid content (TFC), and gallic acid concentration using Folin–Ciocalteu, aluminium chloride, and HPLC methods, respectively. Antioxidant activity was assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and ferric-reducing antioxidant power (FRAP) assays. The antidiabetic potential was evaluated based on α -amylase and α -glucosidase inhibitory activities. **Results:** The ethanol extract showed higher TPC and TFC than the aqueous extract, with values of 203.67 ± 3.38 μ g GAE/ml and 122.80 ± 13.83 μ g QE/ml, respectively, at 1000 μ g/ml. HPLC analysis confirmed the presence of gallic acid in both extracts, with a significantly higher concentration in the ethanol extract (78.93 ± 1.03 μ g/ml) than in the aqueous extract (11.54 ± 1.59 μ g/ml). The ethanol extract also demonstrated stronger DPPH radical-scavenging activity, with a lower IC₅₀ value (115.88 ± 0.51 μ g/ml) compared with the aqueous extract (1176.76 ± 12.69 μ g/ml). In the FRAP assay, the ethanol extract also showed a lower IC₅₀ value (11.55 ± 0.16 μ g/ml) than the aqueous extract (321.62 ± 2.05 μ g/ml). Both extracts exhibited α -amylase and α -glucosidase inhibitory activities, although their activities were significantly lower than acarbose. **Conclusion:** Overall, the findings indicate that 70% ethanol was more effective than water in extracting phenolic and flavonoid compounds and enhancing antioxidant activity, while both extracts demonstrated moderate potential to inhibit carbohydrate-digesting enzymes. These findings support the potential of *Zingiber officinale* as a source of bioactive compounds with antioxidant and antidiabetic properties.

Keywords:

Zingiber officinale; α -amylase; α -glucosidase; antioxidant; phytochemical

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INTRODUCTION

Zingiber officinale, commonly known as ginger, belongs to the family Zingiberaceae, which comprises more than 50 genera and 1,200 species, mainly distributed in tropical Asia (Kumari et al., 2020). Originally from Southeast Asia, *Zingiber officinale* is now cultivated worldwide, with Asia contributing approximately 81% of global production (Poudel et al., 2022). In Malaysia, Bentong ginger (*Zingiber officinale* Roscoe var. Bentong) is one of the commonly

cultivated varieties. It is preferred for its strong aroma, lower fibre content, and spicy flavour (Suhaimi et al., 2014; Again et al., 2022). Other varieties reported in Malaysia include halia betai, halia bara, and halia indang.

The health-promoting properties of *Zingiber officinale* are associated with its diverse bioactive compounds, including phenolic compounds such as gallic acid, zingerone, gingerenone-A, quercetin, and 6-dehydrogingerdione, as well as terpenes and gingerols. Phenolic and polyphenolic

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compounds are particularly important contributors to the antioxidant activity of ginger (Mao et al., 2019). Antioxidants can neutralise free radicals and reduce oxidative damage caused by excessive reactive oxygen species (ROS), which have been associated with various chronic diseases (Poprac et al., 2017).

In addition to antioxidant activity, the bioactive compounds in *Zingiber officinale* may possess antidiabetic properties. α -Amylase and α -glucosidase are key carbohydrate-digesting enzymes that contribute to the release of absorbable sugars. Inhibition of these enzymes can delay carbohydrate digestion and reduce the subsequent increase in blood glucose levels (Aleixandre et al., 2022; Li et al., 2022). Therefore, plant-derived compounds with inhibitory activity against these enzymes may have potential as complementary sources of antidiabetic agents.

The choice of extraction solvent is an important factor in recovering bioactive compounds from plant materials. Differences in solvent polarity can influence the type and amount of compounds extracted. Water is a polar solvent, whereas ethanol has intermediate polarity and can extract a broader range of phytochemicals (Abubakar & Haque, 2020). Therefore, comparison of different extraction solvents is important for determining the extract with the most favourable phytochemical composition and biological activity.

MATERIALS AND METHODS

Zingiber officinale Extraction

The powder of *Zingiber officinale* were purchased from Mr Bentong World Trading, Malaysia. The crude extracts of *Zingiber officinale* were prepared using 70% ethanol and distilled water. For ethanol extraction, fine rhizome powder was mixed with 70% ethanol at a ratio of 1:3 (w/v) and kept in a foil-wrapped Schott bottle for four days on an orbital shaker, with daily shaking. The mixture was filtered using muslin cloth and Whatman No. 1 filter paper, and the filtrate was concentrated using a rotary evaporator under reduced pressure, followed by drying with a centrifugal vacuum concentrator (Mustafa et al., 2021). For aqueous extraction, fine rhizome powder was mixed with distilled water at a ratio of 1:4 (w/v) and extracted by decoction at 100°C for 20 minutes. The mixture was then centrifuged at 1000 rpm for 10 minutes at 4°C. The supernatant was filtered and freeze-dried to obtain the aqueous extract (Princy et al., 2023).

Phytochemical Analysis

Total phenolic content (TPC)

The total phenolic content of *Zingiber officinale* extract was determined using the Folin–Ciocalteu colorimetric assay according to Azizan et al. (2020). Briefly, 20 μ l of extract at concentrations ranging from 31.25 to 1000 μ g/ml was mixed with 100 μ l of Folin–Ciocalteu reagent. After 5 minutes, 80 μ l of 0.75% sodium carbonate was added, and the mixture was incubated in the dark for 20 minutes at room temperature. A blank was prepared using distilled water instead of the extract. Absorbance was measured at 765 nm using a microplate reader. Gallic acid (0–100 μ g/ml) was used as the standard to construct a calibration curve, and the total phenolic content was expressed as μ g gallic acid equivalents (GAE)/ml of extract.

Total flavonoid content (TFC)

The total flavonoid content of *Zingiber officinale* extract was determined using the aluminium chloride colorimetric assay according to Manan et al. (2019). Briefly, 100 μ l of extract at concentrations ranging from 31.25 to 1000 μ g/ml was mixed with 2% aluminium chloride solution and incubated at room temperature for 15 minutes. The absorbance was measured at 415 nm using a microplate reader. Quercetin (0–100 μ g/ml) was used as the standard to construct a calibration curve, and the total flavonoid content was expressed as μ g quercetin equivalents (QE)/ml of extract.

Gallic acid quantification by High-Performance Liquid Chromatography (HPLC) analysis

The HPLC analysis was performed according to Faqer et al. (2022) with slight modifications. An Eclipse Plus C18 column (4.6 $\times$ 250 mm, 5 μ m) was used at 40°C with a maximum pressure of 4000 psi. The mobile phase consisted of solvent A (12% acetonitrile) and solvent B (distilled water), with isocratic elution using 88% distilled water. The sample (1000 μ g/ml) was injected at a volume of 20 μ l, with a flow rate of 1.2 ml/min and detection at 280 nm. The analysis was conducted for 6 minutes, and the peaks obtained from the *Zingiber officinale* extract were compared with the gallic acid standard. Gallic acid standards at five concentrations (20–100 μ g/ml) were analysed in triplicate to construct the calibration curve. The gallic acid concentration in the extract was determined using the regression equation obtained from the calibration curve.

Antioxidant Activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH radical scavenging assay was performed according to Shu et al. (2023) with slight modifications. A 60 µg/ml DPPH solution was prepared by dissolving 1200 µg of DPPH in 20 ml of methanol: water (4:1). In a 96-well microplate, 180 µl of DPPH solution was mixed with 20 µl of the *Zingiber officinale* extract. Ascorbic acid was used as the positive control. The plate was incubated in the dark for 30 minutes, and absorbance was measured at 517 nm using a microplate reader. All measurements were performed in triplicate. The DPPH radical scavenging activity was calculated as follows:

$$\text{Inhibition (\%)} = [(Ac - As)/Ac] \times 100$$

where As is the absorbance of the sample and Ac is the absorbance of the control.

Ferric-reducing antioxidant power (FRAP) activity

The FRAP assay was performed according to Athilah et al. (2020) with slight modifications. The FRAP reagent was prepared by mixing acetate buffer (pH 3.6), 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM hydrochloric acid, and ferric chloride in a 10:1:1 ratio. The reagent was incubated at 37°C for 30 minutes before use. The ethanol and aqueous *Zingiber officinale* extracts were serially diluted to concentrations of 31.25–1000 µg/ml. A total of 150 µl of FRAP reagent was mixed with 50 µl of extract in a 96-well microplate. Absorbance was measured at 593 nm using a microplate reader. All measurements were performed in triplicate. The inhibition percentage was calculated using the following equation:

$$\text{Inhibition (\%)} = [(As - Ac)/As] \times 100$$

where As is the absorbance of the sample and Ac is the absorbance of the control.

Antidiabetic Activity

Evaluation of α-glucosidase activity

The *in vitro* α-glucosidase inhibition assay was performed in a 96-well microplate according to Lankatillake et al. (2021) with slight modifications. Assay buffer I consisted of 100 mM potassium phosphate buffer (pH 6.8) containing 2% dimethyl sulfoxide (DMSO), while buffer II contained potassium phosphate buffer without DMSO. Test extracts and acarbose (positive control) were prepared at 1000 µg/ml using buffer I. The *Saccharomyces cerevisiae* α-

glucosidase enzyme (10 µg/ml) and *p*-nitrophenyl-α-D-glucopyranoside (*p*-NPG; 5 mM) were prepared using buffer II. For the assay, 50 µl of buffer I and 50 µl of test extract or acarbose were added to the respective wells to obtain a final concentration of 500 µg/ml. A negative control and sample, acarbose, and control blanks were also prepared. Subsequently, 30 µl of α-glucosidase enzyme was added to all wells except the blank wells. The plate was incubated in the dark at 37°C for 10 minutes. Then, 20 µl of *p*-NPG substrate was added to all wells except the blank wells, followed by incubation at 37°C in the dark for 20 minutes. Absorbance was measured at 405 nm using a microplate reader maintained at 37°C. All measurements were performed in triplicate. The percentage of α-glucosidase inhibition was then calculated.

Evaluation of α-amylase activity

The *in vitro* α-amylase inhibition assay was performed according to Karakaya et al. (2018) with slight modifications. *Zingiber officinale* extracts were diluted to 100 µg/ml using 5% DMSO. A 1% starch solution was prepared in 0.2 M acetate buffer (pH 5.0). Briefly, 20 µL of α-amylase from *Aspergillus oryzae* (500 µg/mL) was mixed with 20 µL of the extract in a 96-well microplate and incubated at 50°C for 10 minutes. The reaction was initiated by adding 20 µl of 1% starch solution, followed by incubation at 50°C for another 10 minutes. The reaction was stopped by adding 40 µl of 3,5-dinitrosalicylic acid (DNS) reagent (96 mM), and the plate was heated in a boiling water bath for 5 minutes. The plate was then cooled to room temperature, followed by the addition of 80 µl of distilled water. Absorbance was measured at 540 nm using a microplate reader. Acarbose (100 µg/ml) was used as the positive control, and all measurements were performed in triplicate. The percentage of α-amylase inhibitory activity was then calculated.

Statistical Analysis

Data were presented as mean ± standard error of the mean (SEM). Differences among the extracts were analysed using one-way analysis of variance (ANOVA), followed by Duncan's or Tukey's post hoc test, as appropriate. A *p*-value < 0.05 was considered statistically significant.

RESULTS

Total Phenolic Content of *Zingiber officinale*

The total phenolic content (TPC) of *Zingiber officinale* increased with increasing extract concentration in both aqueous and ethanol extracts (Table 1). The aqueous extract showed the lowest TPC at 31.25 µg/ml (0.65 ± 0.53 µg GAE/ml) and the highest at 1000 µg/ml (24.36 ± 3.81 µg GAE/ml). Similarly, the ethanol extract showed an increase from 9.20 ± 3.04 µg GAE/ml at 31.25 µg/ml to 203.67 ± 3.38 µg GAE/ml at 1000 µg/ml. Overall, the ethanol extract demonstrated a higher TPC than the aqueous extract across the tested concentrations.

Table 1: TPC of aqueous and ethanolic extract of *Zingiber officinale*

Concentration (µg/ml)	Total Phenolic Content (µg GAE/ml ± SEM)	
	Aqueous	Ethanol
31.25	0.65 ± 0.53	9.20 ± 3.04
62.5	0.91 ± 0.70	11.32 ± 0.58
125	2.36 ± 0.58	24.79 ± 0.58
250	5.52 ± 0.53	48.21 ± 1.13
500	11.14 ± 2.12	100.73 ± 3.38
1000	24.36 ± 3.81	203.67 ± 3.38

Total Flavonoid Content of *Zingiber officinale*

The total flavonoid content (TFC) of *Zingiber officinale* increased with increasing extract concentration in both aqueous and ethanol extracts (Table 2). The aqueous extract showed the lowest TFC at 31.25 µg/ml (0.46 ± 0.64 µg QE/ml) and the highest at 1000 µg/ml (22.49 ± 8.62 µg QE/ml). Similarly, the ethanol extract showed an increase from 4.67 ± 0.42 µg QE/ml at 31.25 µg/ml to 122.80 ± 13.83 µg QE/ml at 1000 µg/ml. Overall, the ethanol extract demonstrated a higher TFC than the aqueous extract across the tested concentrations.

Table 2: TFC of aqueous and ethanolic extract of *Zingiber officinale*

Concentration (µg/ml)	Total Flavonoid Content (µg QE/ml ± SEM)	
	Aqueous	Ethanol
31.25	0.46 ± 0.64	4.67 ± 0.42
62.5	0.93 ± 0.72	10.49 ± 1.02
125	2.28 ± 1.15	21.90 ± 2.03
250	5.28 ± 2.25	41.33 ± 4.41
500	10.93 ± 4.36	74.12 ± 7.72
1000	22.49 ± 8.62	122.80 ± 13.83

Gallic Acid in *Zingiber officinale*

The HPLC chromatograms of the gallic acid standard and *Zingiber officinale* extracts are shown in Figure 1. The gallic acid standard showed a retention time of 1.996 ± 0.013 min. The aqueous and ethanol extracts showed retention times of 1.950 ± 0.039 and 1.919 ± 0.022 min, respectively. These retention times were close to that of the gallic acid standard, indicating the presence of gallic acid in both extracts. As shown in Table 3, the aqueous extract had an area under the curve (AUC) of 34.8395 and a gallic acid concentration of 11.54 ± 1.59 µg/ml. In comparison, the ethanol extract showed a higher AUC of 192.6320 and a significantly higher gallic acid concentration of 78.93 ± 1.03 µg/ml ($p < 0.05$). Therefore, the ethanol extract contained a higher amount of gallic acid than the aqueous extract.

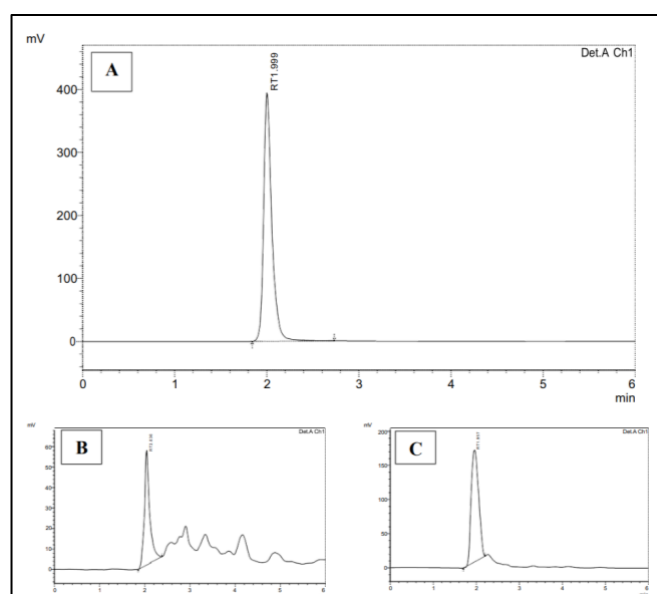


Figure 1: Chromatogram of references standard for gallic acid (A), aqueous (B) and ethanol (C) extracts of *Zingiber officinale* at 280 nm

Table 3: The area under the curve (AUC) and concentration of gallic acid for aqueous and ethanol extracts of *Zingiber officinale*

Sample	Area Under Curve (AUC)	Concentration of Gallic Acid (µg/ml ± SEM)
Aqueous	34.8395	11.54 ± 1.59^a
Ethanol	192.6320	78.93 ± 1.03^b

*Different letters indicate significant differences using Duncan multiple tests, $p < 0.05$

DPPH Radical Scavenging Activity of *Zingiber officinale*

Table 4 shows that the DPPH radical scavenging activity of *Zingiber officinale* extracts generally increased with increasing concentration. The activity of the aqueous and ethanol extracts was compared with ascorbic acid. Ascorbic acid showed the highest scavenging activity of $94.92 \pm 0.12\%$ at $100 \mu\text{g/ml}$. The aqueous extract showed a maximum activity of $69.65 \pm 0.14\%$ at $5000 \mu\text{g/ml}$, while the ethanol extract showed $91.89 \pm 0.20\%$ at $1000 \mu\text{g/ml}$. The IC_{50} values were determined using linear interpolation and represent the concentration required to achieve 50% DPPH radical scavenging. As shown in Table 4, the IC_{50} values were $47.86 \pm 0.81 \mu\text{g/ml}$ for ascorbic acid, $115.88 \pm 0.51 \mu\text{g/ml}$ for the ethanol extract, and $1176.76 \pm 12.69 \mu\text{g/ml}$ for the aqueous extract. The ethanol extract showed a significantly lower IC_{50} value than the aqueous extract ($p < 0.05$), indicating stronger DPPH radical scavenging activity.

Table 4: Scavenging activity of DPPH of ascorbic acid and *Zingiber officinale* extracts

Sample	Concentration ($\mu\text{g/ml}$)	% Inhibition (Mean $\pm$ SEM)	IC_{50} Value ($\mu\text{g/ml} \pm \text{SEM}$)
Ascorbic Acid	3.125	10.51 ± 0.79	47.86 ± 0.81^a
	6.25	13.02 ± 0.45	
	12.5	19.84 ± 0.33	
	25	31.57 ± 0.49	
	50	51.73 ± 0.68	
	100	94.92 ± 0.12	
Aqueous Extract	156.25	14.30 ± 0.24	1176.76 ± 12.69^b
	312.5	21.44 ± 0.79	
	625	34.78 ± 0.42	
	1250	52.02 ± 0.46	
	2500	67.77 ± 0.34	
	5000	69.65 ± 0.14	
Ethanol Extract	31.25	21.56 ± 0.20	115.88 ± 0.51^c
	62.5	34.53 ± 0.36	
	125	52.64 ± 0.18	
	250	67.77 ± 0.34	
	500	85.74 ± 0.42	
	1000	91.89 ± 0.20	

*Different superscript letters indicate significant differences according to Tukey's post hoc test ($p < 0.05$)

FRAP Activity of *Zingiber officinale*

Table 5 shows that the ferric ion-reducing activity of *Zingiber officinale* extracts increased with increasing concentration. Ascorbic acid showed the highest activity, increasing from $38.10 \pm 0.79\%$ at $3.125 \mu\text{g/ml}$ to $95.03 \pm 0.07\%$ at $100 \mu\text{g/ml}$. The aqueous extract increased from $9.26 \pm 0.51\%$ at $31.25 \mu\text{g/ml}$ to $73.66 \pm 0.30\%$ at $1000 \mu\text{g/ml}$, while the ethanol extract increased from $21.61 \pm 0.23\%$ at $3.125 \mu\text{g/ml}$ to $88.37 \pm 0.10\%$ at $100 \mu\text{g/ml}$. The IC_{50} values were $5.15 \pm 0.11 \mu\text{g/ml}$ for ascorbic acid, $11.55 \pm 0.16 \mu\text{g/ml}$ for the ethanol extract, and $321.62 \pm 2.05 \mu\text{g/ml}$ for the aqueous extract. The ethanol extract showed a lower IC_{50} value than the aqueous extract, indicating stronger ferric ion-reducing activity. The difference between the extracts was statistically significant ($p < 0.05$).

Table 5: Ferric ion reduction by ascorbic acid and *Zingiber officinale* extracts

Sample	Concentration ($\mu\text{g/ml}$)	% Inhibition (Mean $\pm$ SEM)	IC_{50} Value ($\mu\text{g/ml} \pm \text{SEM}$)
Ascorbic Acid	3.125	38.10 ± 0.79	5.15 ± 0.11^a
	6.25	56.49 ± 0.54	
	12.5	72.17 ± 0.35	
	25	84.08 ± 0.25	
	100	95.03 ± 0.07	
Aqueous Extract	31.25	9.26 ± 0.51	321.62 ± 2.05^b
	62.5	18.35 ± 0.20	
	125	28.87 ± 0.36	
	250	45.12 ± 0.28	
	1000	73.66 ± 0.30	
Ethanol Extract	3.125	21.61 ± 0.23	11.55 ± 0.16^a
	6.25	35.07 ± 0.30	
	12.5	52.69 ± 0.54	
	25	67.52 ± 0.43	
	100	88.37 ± 0.10	

*Different superscript letters indicate significant differences according to Tukey's post hoc test ($p < 0.05$)

α -Glucosidase Inhibitory Activity of *Zingiber officinale*

Figure 2 shows the α -glucosidase inhibition activity of acarbose and *Zingiber officinale* extracts at a concentration of 500 μ g/ml. Acarbose showed the highest inhibition activity at $17.26 \pm 0.73\%$, followed by the ethanol extract at $12.51 \pm 0.56\%$ and the aqueous extract at $10.25 \pm 1.02\%$. Acarbose showed significantly higher α -glucosidase inhibition than both aqueous and ethanol extracts ($p < 0.05$). However, no significant difference was observed between the aqueous and ethanol extracts. These findings indicate that both *Zingiber officinale* extracts exhibited lower α -glucosidase inhibitory activity than acarbose at 500 μ g/ml.

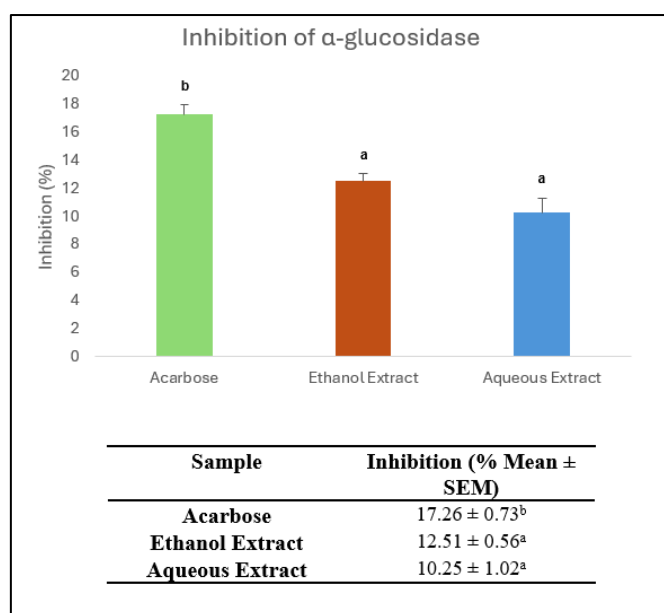


Figure 2: Percentage of α -glucosidase inhibition by acarbose and *Zingiber officinale* extracts at 500 μ g/ml. Different letters indicate significant differences according to Tukey's post hoc test ($p < 0.05$)

α -Amylase Inhibitory Activity of *Zingiber officinale*

Figure 3 shows the α -amylase inhibition activity of acarbose and *Zingiber officinale* extracts at 100 μ g/ml. Acarbose, used as the positive control, showed the highest inhibition activity at $53.20 \pm 0.10\%$. The ethanol extract showed an inhibition activity of $26.78 \pm 0.23\%$, while the aqueous extract showed $22.34 \pm 2.34\%$. Acarbose showed significantly higher α -amylase inhibition than both *Zingiber officinale* extracts ($p < 0.05$). However, no significant difference was observed between the ethanol and aqueous extracts. These findings indicate that both *Zingiber officinale* extracts exhibited lower α -amylase inhibitory activity than acarbose at 100 μ g/ml.

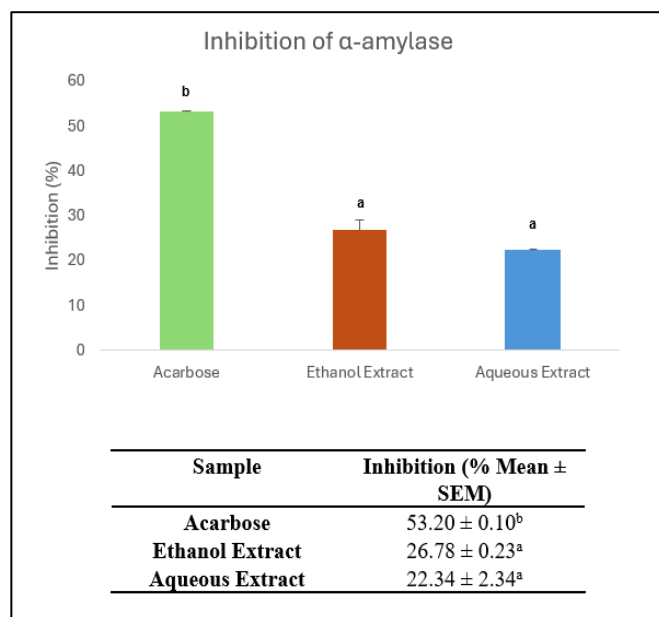


Figure 3: Percentage of α -amylase inhibition by acarbose and *Zingiber officinale* extracts at 100 μ g/ml. Different letters indicate significant differences according to Tukey's post hoc test ($p < 0.05$)

DISCUSSION

Phytochemical Analysis of *Zingiber officinale*

The type of extraction solvent significantly affected the total phenolic content (TPC) of *Zingiber officinale*. Ethanol extract showed a significantly higher TPC ($203.67 \pm 3.38 \mu$ g GAE/ml) than the aqueous extract ($24.36 \pm 3.81 \mu$ g GAE/ml). This finding is consistent with previous studies reporting higher phenolic contents in ethanol extracts of ginger compared with water and other solvents. Kaur and Kapoor (2002) reported a TPC of 221.3 ± 9.4 mg GAE/100 g in ethanol extract of ginger rhizome. Similarly, Cai et al. (2004) and Hinneburg et al. (2006) reported that ethanol was more effective in extracting phenolic compounds from ginger than methanol and water. Prakash (2010) also reported the highest TPC in ethanol extract, followed by methanol and acetone extracts. These findings suggest that ethanol is an effective solvent for extracting phenolic compounds from *Zingiber officinale*.

A similar trend was observed for total flavonoid content (TFC). The ethanol extract contained significantly higher flavonoid levels ($122.80 \pm 13.83 \mu$ g QE/ml) than the aqueous extract ($22.49 \pm 8.62 \mu$ g QE/ml). This difference may be related to the polarity of the extraction solvents, which can influence the recovery of flavonoid compounds. Previous studies have also reported higher flavonoid concentrations in ethanol extracts compared with methanol and acetone extracts (Ghasemzadeh et al., 2011; Tanweer et al., 2020; Nouioura et al., 2024). Therefore, the present findings further support the suitability of ethanol

for extracting flavonoid compounds from *Zingiber officinale*.

Gallic acid, a phenolic compound with antioxidant and antimicrobial properties, was also detected in both extracts. The ethanol extract contained a significantly higher concentration of gallic acid ($78.93 \pm 1.03 \mu\text{g/ml}$) compared with the aqueous extract ($11.54 \pm 1.59 \mu\text{g/ml}$). This finding is consistent with Das et al. (2019), who reported a higher gallic acid content in ethanol extract than in aqueous extract using HPLC analysis. The higher recovery of gallic acid in the ethanol extract may be attributed to differences in solvent polarity and the greater affinity of ethanol-based solvents for extracting phenolic compounds from plant matrices (Riaz et al., 2023).

Overall, the present findings indicate that ethanol was more effective than water for extracting phenolic compounds, flavonoids, and gallic acid from *Zingiber officinale*. The higher levels of these phytochemicals in the ethanol extract may contribute to its stronger antioxidant activity observed in the present study.

Antioxidant Activity of *Zingiber officinale*

DPPH and FRAP assays were used to evaluate the antioxidant activity of *Zingiber officinale* extracts. In the present study, the ethanol extract demonstrated stronger antioxidant activity than the aqueous extract at lower concentrations. In the DPPH assay, the ethanol extract showed $21.56 \pm 0.20\%$ inhibition at $31.25 \mu\text{g/ml}$, whereas the aqueous extract showed $14.30 \pm 0.24\%$ inhibition at $156.25 \mu\text{g/ml}$. Similarly, in the FRAP assay, the ethanol extract showed $21.61 \pm 0.23\%$ inhibition at $3.125 \mu\text{g/ml}$, compared with $9.26 \pm 0.51\%$ for the aqueous extract at $31.25 \mu\text{g/ml}$. These findings indicate that the ethanol extract exhibited greater antioxidant activity at lower concentrations. The IC_{50} results further supported this finding. For the DPPH assay, the ethanol extract had a lower IC_{50} value ($115.88 \pm 0.51 \mu\text{g/ml}$) than the aqueous extract ($1176.76 \pm 12.69 \mu\text{g/ml}$). Similarly, in the FRAP assay, the ethanol extract showed a lower IC_{50} value ($11.55 \pm 0.16 \mu\text{g/ml}$) than the aqueous extract ($321.62 \pm 2.05 \mu\text{g/ml}$).

The higher antioxidant activity observed in the ethanol extract may be associated with the greater extraction of antioxidant compounds. Ethanol can solubilise a wider range of compounds with different polarities compared with water (Singh et al., 2021). The higher TPC, TFC, and gallic acid content observed in the ethanol extract in the present study may also contribute to its stronger DPPH radical-scavenging and ferric reducing activity. Phenolic and flavonoid compounds have been reported to

contribute to the antioxidant properties of ginger extracts (Jelled et al., 2015). In addition, compounds such as gallic acid and gingerol may neutralise free radicals and terminate oxidative chain reactions (Sanjeevkumar et al., 2016).

Antidiabetic Activity of *Zingiber officinale*

α -Amylase and α -glucosidase are important enzymes involved in carbohydrate metabolism, as they catalyse the breakdown of polysaccharides into smaller sugars, including disaccharides and monosaccharides (Li et al., 2022). Inhibition of these enzymes can therefore reduce carbohydrate digestion and subsequent glucose release, making them important targets in the management of postprandial blood glucose levels.

In the present study, acarbose demonstrated significantly higher α -amylase inhibitory activity ($53.20 \pm 0.10\%$) at $100 \mu\text{g/ml}$ compared with both ethanol and aqueous extracts of *Zingiber officinale*. Similarly, acarbose showed significantly higher α -glucosidase inhibitory activity ($17.26 \pm 0.73\%$) at $500 \mu\text{g/ml}$ than both *Zingiber officinale* extracts. These findings are consistent with Ahmed and Kumar (2023). The stronger inhibitory activity of acarbose may be attributed to its high affinity for the active sites of carbohydrate-digesting enzymes, thereby interfering with their catalytic activity (Nelson, 2015). In contrast, the lower inhibition observed with the ethanol and aqueous extracts suggests that their constituent compounds may have weaker or less direct interactions with these enzymes.

Despite the lower inhibitory activity compared with acarbose, both ethanol and aqueous extracts demonstrated similar inhibitory effects. This similarity may be associated with the decoction method used in the present study, particularly the thermal transformation of 6-gingerol into 6-shogaol. High temperatures during extraction can promote this transformation, potentially increasing the presence of 6-shogaol in the extracts. Yi et al. (2019) reported that 6-shogaol exhibited antidiabetic potential by reducing blood glucose levels in diabetic mice. Furthermore, Jiao et al. (2023) demonstrated that 6-shogaol contributed to α -glucosidase inhibition and promoted glucose uptake. Li et al. (2021) also reported that 6-shogaol, 6-gingerol, and 6-paradol contributed to α -amylase inhibitory activity. Therefore, the presence of these bioactive compounds may partly explain the α -amylase and α -glucosidase inhibitory activities observed in the *Zingiber officinale* extracts.

The comparatively moderate enzyme inhibition observed in the *Zingiber officinale* extracts may also be relevant

when considering the limitations associated with strong α -amylase and α -glucosidase inhibition. Mohammed et al. (2017) reported that excessive inhibition of these enzymes, particularly by acarbose, may contribute to gastrointestinal side effects such as bloating, flatulence, and abdominal discomfort. However, the lower inhibitory activity of *Zingiber officinale* extracts should not be interpreted as evidence that they necessarily cause fewer side effects, as this would require specific safety and clinical investigations.

Overall, although both ethanol and aqueous *Zingiber officinale* extracts exhibited lower α -amylase and α -glucosidase inhibitory activities than acarbose, their similar inhibitory effects indicate the presence of bioactive compounds with potential carbohydrate-digesting enzyme inhibitory properties. The contribution of compounds such as 6-gingerol, 6-shogaol, and 6-paradol may explain these activities. Further studies are required to identify the specific compounds responsible, clarify their mechanisms of action, and evaluate the efficacy and safety of *Zingiber officinale* as a potential complementary approach for glycaemic control.

CONCLUSION

Zingiber officinale contains various phytochemicals, including gallic acid. The phytochemical analysis and antioxidant assays demonstrated that the 70% ethanol extract was superior to the aqueous extract in terms of phytochemical content and antioxidant activity. However, both ethanol and aqueous extracts exhibited comparable antidiabetic potential, as demonstrated by their inhibition of α -amylase at 100 $\mu\text{g/ml}$ and α -glucosidase at 500 $\mu\text{g/ml}$. These findings suggest that *Zingiber officinale* possesses potential antioxidant and antidiabetic activities, which may be attributed to its diverse phytochemical constituents. Further studies are recommended to identify the active compounds and confirm their mechanisms, efficacy, and safety.

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HPLC Profiling and *In Vitro* Cytocompatibility Assessment of Ultrafiltration-Purified Bromelain from Pineapple (*Ananas comosus*) Core Waste

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ABSTRACT

Background: Bromelain, a proteolytic enzyme complex derived from pineapple (*Ananas comosus*), is highly regarded for its therapeutic properties and potential for sustainable recovery from agro-industrial waste. This study characterized ultrafiltration-purified bromelain (UFB) obtained from pineapple core waste through chromatographic profiling and *in vitro* cytocompatibility assessment.

Methods: Purification was achieved through crude extraction, 50% ammonium sulfate precipitation, and membrane ultrafiltration. Characterization was conducted using high-performance liquid chromatography (HPLC) profiling alongside *in vitro* cytotoxicity evaluations via the Alamar Blue assay on human gingival fibroblasts (HGF) and mouse fibroblasts (L929). **Results:** HPLC profiling demonstrated a major chromatographic peak in UFB with a retention time comparable to that of the commercial bromelain reference. *In vitro* cytotoxicity evaluations demonstrated significant cytocompatibility (>80% cell viability) in both HGF and L929 at UFB concentrations ≤125 µg/mL. However, higher concentrations induced dose-dependent reductions in cell viability, with L929 fibroblasts displaying greater susceptibility (64% viability) at 250 µg/mL. **Conclusion:** Overall, the findings support ultrafiltration as an effective approach for obtaining an enzymatically active bromelain-rich preparation with favorable cytocompatibility at concentrations up to 125 µg/mL, providing a basis for further investigation of its potential therapeutic or biomaterial applications.

Keywords:

Bromelain; *Ananas comosus*; ultrafiltration; HPLC profiling; cytotoxicity

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INTRODUCTION

Bromelain is a heterogeneous mixture of thiol endopeptidases, phosphatases, glucosidases, peroxidases, cellulases and glycoproteins, and its biological activities are thought to be due to the synergistic action of these components (Colletti et al., 2021). Bromelain is a mixture of proteolytic enzymes extracted from the pineapple plant (*Ananas comosus*) and has been the subject of much scientific interest due to its wide-ranging biological activities including antibacterial, anti-inflammatory, and anticancer effects (Mamo, 2019). Bromelain has attracted much interest in biomedical applications such as wound healing, oral health care products, anti-inflammatory formulations, and drug delivery systems (Hong et al., 2021). Furthermore, the bromelain composition is dependent on the pineapple cultivar, source tissue, extraction methods and purification strategies, which in turn influence the enzymatic activity and biological efficacy (Varilla et al., 2021). The industrial demand of this enzyme system has opened up the exploration of agricultural residues as sustainable sources for recovery (Seguí & Fito Maupoey, 2018). Notably, the pineapple core

is an abundant source of bromelain in comparison with other processing wastes, providing an interesting opportunity for green waste valorization (Nath et al., 2023).

However, the biological efficacy of the recovered enzyme is highly dependent on downstream extraction and purification parameters that can drastically alter its protein profile and functional stability (Colletti et al., 2021). Our previous study established an efficient downstream purification protocol for bromelain, resulting in enhanced purification and specific protease activity. Furthermore, the purified UFB matrix demonstrated significant anti-biofilm activity against *Streptococcus mutans* biofilm formation *in vitro* (Johari et al., 2023). Although our previous studies established the purification efficiency and bioactivity activities of UFB, the chromatographic profile and cellular compatibility of the resulting preparation have not been systematically evaluated. The present study therefore focuses specifically on chromatographic comparison with a commercial bromelain reference and preliminary cytocompatibility assessment in human gingival and murine fibroblasts. This

dual evaluation provides a fundamental baseline for the relationship between the chromatographic purity of bromelain from waste and its further biological compatibility.

MATERIALS AND METHODS

Extraction and Purification of UFB

Bromelain was recovered from MD2 pineapple core using the standardized protocol described by Johari et al. (2023). Briefly, the pineapple core was homogenized with distilled water and centrifuged at 13,000 rpm for 40 min at 4 °C. The supernatant was subjected to protein fractionation with 50% (w/v) ammonium sulfate at 4 °C overnight. The resulting precipitate was resuspended in an equivalent volume of 0.2 M phosphate buffer (pH 7.0), filtered through a 0.22 µm membrane, and subjected to ultrafiltration using Amicon centrifugal filter units (Millipore) with a 10 kDa molecular weight cut-off. The samples were centrifuged at 13,000 rpm for 30 min at 4 °C. The resulting ultrafiltration-purified bromelain (UFB) was collected for subsequent HPLC profiling and *in vitro* cytocompatibility assessment.

SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine the molecular weight of the UFB fraction. A commercial bromelain standard (1,000 µg/mL; molecular weight 23–25 kDa) was used as the reference, while serially diluted UFB samples (31.25, 62.5, 125, 250, 500, and 1,000 µg/mL) were analysed for protein band detection and visualization. The UFB was mixed with the sample buffer and denatured by heating at 95 °C for 5 min. Electrophoresis was performed at a constant voltage of 100 V using a gel composed of 4% stacking and 12% resolving layers. After electrophoresis, the gel was stained with Coomassie Brilliant Blue for 15 min and destained overnight in 10% acetic acid. Protein bands were subsequently visualized and documented using an ImageQuant™ LAS 500 gel documentation system.

Bradford's Assay

Protein concentration in the UFB sample was determined using the Pierce™ Bradford Protein Assay Kit in a 96-well microplate. Bovine serum albumin (BSA) at 2 mg/mL was used to generate the standard curve. Briefly, 5 µL of UFB sample was mixed with 250 µL of Bradford reagent and incubated for 10 min at room temperature. Absorbance was measured at 595 nm using a microplate reader. Protein concentration was calculated from the BSA

standard curve.

Enzymatic Assay

The assay used 1.1 mM L-tyrosine as the standard and 0.65% casein as the substrate. Absorbance of the L-tyrosine standards at different concentrations was measured at 660 nm using a spectrophotometer, and a standard curve was generated. For proteolytic activity, 0.65% casein was prepared in 0.05 M phosphate buffer (pH 7.0) and mixed with 500 µL of UFB. The mixture was incubated at 37 °C for 10 min, followed by the addition of 0.11 M trichloroacetic acid (TCA). The mixture was then filtered, and 1 mL of the filtrate was mixed with 2.5 mL sodium carbonate solution. Subsequently, 500 µL of Folin's reagent was added, mixed thoroughly, and incubated at 37 °C for 30 min. Absorbance was then measured at 660 nm. Proteolytic activity was calculated using the following formula:

$$\text{Activity (U/ml)} = \frac{\text{Tyrosine equivalent released} \times \text{total volume of reaction}}{\text{Volume of enzyme sample} \times \text{reaction time} \times \text{cuvette volume}} \quad (1)$$

HPLC

High-performance liquid chromatography (HPLC) analysis was carried out using a Shimadzu Prominence system, which included an LC-20AT pump, DGU-20A5 inline degasser, SPD-20A UV-VIS detector, SIL-20A autosampler, CTO-10AS VP column oven, and was operated via LC Solution software version 11 to validate the identity and integrity of the bromelain complex following the methodology of Yantih et al. (2019) with minor modifications. Both UFB samples and bromelain standards were prepared at 1000 µg/mL for analytical comparison. Chromatographic separation was achieved using a C18 reversed-phase column (150 mm × 4.6 mm) as the stationary phase. The analysis utilized a methanol–water mobile phase (7:3, v/v) at a flow rate of 1 mL/min and a column temperature of 25 °C. Detection was carried out using a photodiode array detector at 270 nm. The injection volume was 20 µL for each run. The results were presented in a form of chromatogram that shows peaks of each compound.

Cell cultures

Human gingival fibroblasts (HGF; CRL-2014) and mouse fibroblasts (L929, CCL-1) were purchased from American Type Culture Collection (ATCC, USA). Both cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma, USA) and 1% penicillin-streptomycin (Gibco, USA). Cells were grown at 37 °C in a humidified

atmosphere of 5 % CO₂.

Alamar Blue Assay

The cytocompatibility of UFB sample against HGF and L929 cell lines was evaluated using Alamar Blue assay (Bio-Rad, USA). 1×10^4 cells were seeded into 96-well plates until 80 % confluency. The treatment groups were treated with two-fold serial dilutions of UFB ranging from 15.62 µg/mL to 250 µg/mL and the control groups were maintained in serum-free media. After a 24 h incubation period the medium was aspirated and the cells washed with sterile DPBS. Each well was subsequently replenished with 10 µL of Alamar Blue reagent and 90 µL of fresh medium, followed by a 4-hour incubation at 37 °C in a darkened, humidified 5 % CO₂ atmosphere. Fluorescence intensity was measured at 570 nm using a microplate reader.

RESULTS

Protein Visualization

SDS-PAGE analysis was performed to determine the molecular weight and protein profile of the UFB. As shown in Figure 1, the commercial bromelain standard produced a distinct protein band at approximately 25 kDa. A corresponding band was observed in all UFB samples at a similar molecular weight. The predominant UFB protein band migrated at approximately 25 kDa, consistent with the reported molecular mass of bromelain. No prominent high molecular weight protein bands were detected in the UFB samples.

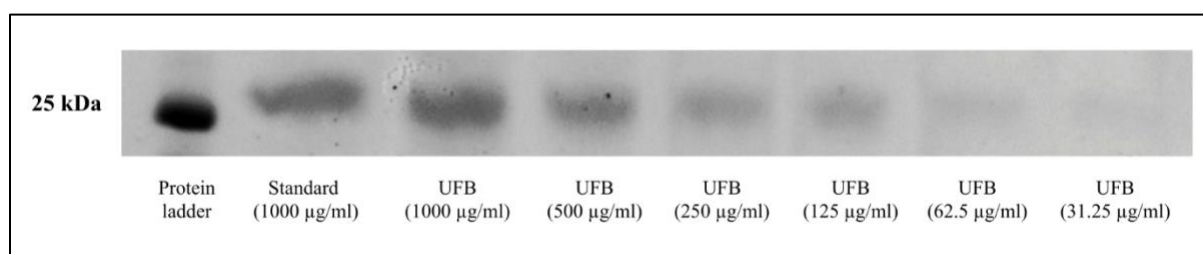


Figure 1: The detection of bromelain enzyme by using SDS-PAGE analysis

Protein Quantification and Enzymatic Activity of UFB

The protein concentration and proteolytic activity of the UFB were determined using the Bradford protein assay and specific enzymatic activity assay. The UFB exhibited a protein concentration of 1321 µg/mL, proteolytic activity of 79.68 CDU/mL, and a specific activity of 71.16 CDU/mg, as represented in Table 1.

Table 1: Protein concentration, proteolytic activity and specific activity of UFB

Sample	Protein Content (ug/mL)	Proteolytic Activity (CDU/mL)	Specific Activity (CDU/mL)
UFB	1321	79.6771	71.1693

HPLC Profiling

HPLC analysis was performed to compare the chromatographic profile of UFB with a commercial bromelain standard. The UFB sample showed a major chromatographic peak at a retention time of 3.8–4.1 min which was comparable to that of commercial bromelain standard (Figure 2). The major peak represented 71.5% of the total chromatographic area in UFB, compared with 75.9% in the commercial bromelain standard (Table 2). Both samples showed their major peaks at a similar retention time when monitored at 270 nm.

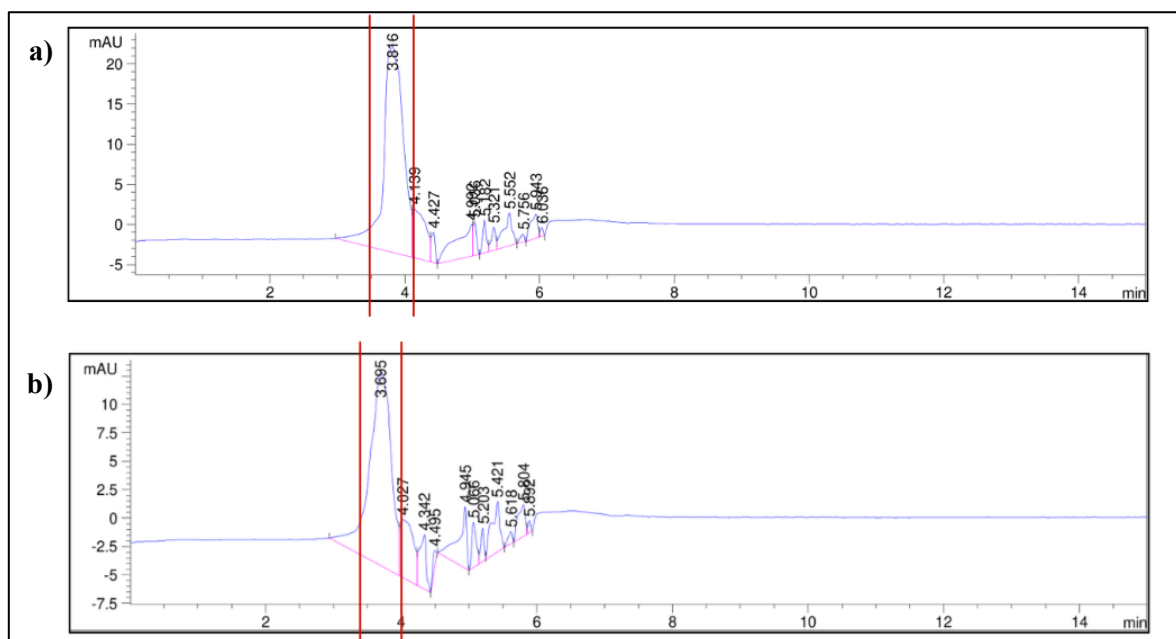


Figure 2: Chromatogram of bromelain in standard (a) and UFB sample (b) at 270 nm

Table 2. The retention time and peak area of bromelain in (a) standard and (b) UFB sample.

Wavelength (nm)	Retention Time (min)	Area (%)	
		Standard	UFB
270	3.8 – 4.1	75.9	71.5

Cytocompatibility of UFB

The cytocompatibility of UFB was evaluated using the Alamar Blue assay following 24 hours exposure to concentrations ranging from 15.62 to 250 $\mu\text{g/mL}$ in human gingival fibroblasts (HGF) and L929 mouse fibroblasts

(Figure 3). Cell viability was expressed relative to untreated controls. UFB maintained more than 80% cell viability in both HGF and L929 cells at concentrations of $\leq 125 \mu\text{g/mL}$. At 250 $\mu\text{g/mL}$, cell viability decreased to approximately 86% in HGF cells and 64% in L929 cells.

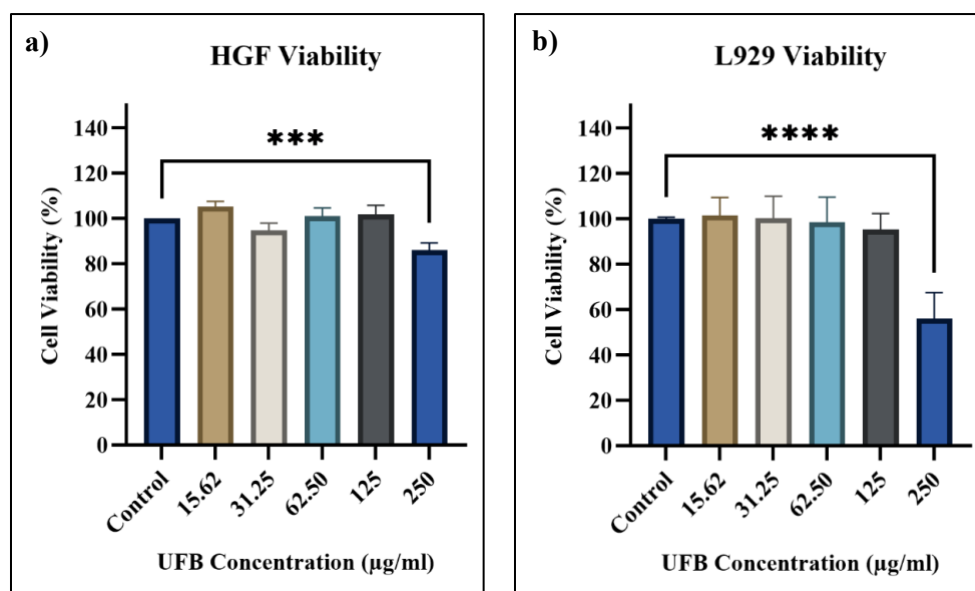


Figure 3: Cytotoxic effects of UFB on mammalian cell lines. (a) HGF viability and (b) L929 viability following treatment with various concentrations of UFB in serum-free medium. Data are presented as mean $\pm$ standard deviation from three independent experiments. Statistical significance was determined by one-way ANOVA, where *** p < 0.001 and **** p < 0.0001 compared to the control group.

DISCUSSION

The present study evaluated the physicochemical characteristics, enzymatic activity, chromatographic profile, and preliminary cytocompatibility of UFB recovered from pineapple (*Ananas comosus*) core. The UFB exhibited an apparent molecular weight of 23–25 kDa, consistent with established values for stem bromelain, with minor variations that may arise from differences in cultivar, tissue origin, or glycosylation extent (Fissore et al., 2023). Combining ammonium sulfate precipitation and membrane ultrafiltration provided an efficient purification process by concentrating the protein and removing residual salts, sugars, pigments, and low-molecular-weight peptides (Porfírio et al., 2024; Suherman et al., 2024). This downstream strategy successfully preserved functional catalytic behavior, yielding high total proteolytic and specific activity (Johari et al., 2023; Halim et al., 2025).

High-performance liquid chromatography (HPLC) further verified the structural preservation and identity of UFB. The major chromatographic peak of the UFB preparation accounted for 71.5% of the total area, closely matching the 75.9% observed for the commercial standard. Furthermore, both UFB and the standard exhibited major peaks within a narrow retention time window of 3.8–4.1 min. Because substantial shifts in retention time typically signify protein denaturation, degradation, or structural modification during processing, this close alignment

indicates that the primary bromelain constituents remained structurally intact throughout extraction, precipitation, and ultrafiltration (Wätzig et al., 2021). Detection at 270 nm provided sensitive monitoring of aromatic residues (tyrosine and tryptophan) necessary for accurate protein profiling (Eid et al., 2022). Although HPLC does not resolve individual isoenzymes or tertiary protein folding directly, it serves as a reliable metric for verifying chromatographic identity, evaluating sample purity, and confirming process consistency for MD2 pineapple core-derived enzymes (Porfírio et al., 2024). Cellular metabolic activity was evaluated using the Alamar Blue assay, where the reduction of resazurin to fluorescent resorufin measures mitochondrial function rather than direct cell necrosis (Vieira-da-Silva & Castanho, 2023). Following exposure across a broad concentration spectrum, UFB exhibited favorable cytocompatibility and maintained high cell viability in both HGF and mouse L929 fibroblasts up to moderate dosage levels. These findings align with recent reports demonstrating that bromelain maintains acceptable biocompatibility within defined therapeutic windows, whereas excessive doses induce concentration-dependent cellular stress, pro-apoptotic signaling, or cytotoxicity (Bernasińska-Słomczewska et al., 2026).

At elevated dosage levels, a cell-line-specific response emerged: primary HGF cells retained markedly higher viability than immortalized L929 fibroblasts. This differential sensitivity suggests that human oral fibroblasts

are more resilient to bromelain exposure, likely owing to variations in species origin, membrane composition, proliferation dynamics, and extracellular matrix (ECM) production (Petiti et al., 2024). While L929 cells serve as a standard reference for biomaterial testing, HGF cells represent a clinically relevant model for oral mucosal applications (Bürgers et al., 2022). Mechanistically, the reduced viability in L929 cells at higher doses reflects elevated proteolytic stress rather than intrinsic chemical toxicity; excessive protease exposure accelerates ECM and cell-surface receptor degradation, disrupting cell adhesion and metabolic activity (Hong et al., 2021).

CONCLUSION

In conclusion, this study demonstrates that ammonium sulfate precipitation followed by membrane ultrafiltration is an effective approach for recovering and concentrating bromelain from pineapple (*Ananas comosus*) core waste. The HPLC profile of UFB was comparable to that of a commercial bromelain standard, providing additional chromatographic information for the characterization and quality assessment of pineapple core-derived bromelain. UFB also retained proteolytic activity and showed favorable cytocompatibility in HGF and L929 fibroblasts at appropriate concentrations. Overall, this study contributes to the characterization and sustainable valorisation of pineapple processing waste and provides a basis for further standardization and evaluation of UFB for potential biomedical and oral healthcare applications.

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Inter-observer and intra-observer reliability and diagnostic accuracy of medical laboratory technology students in screening pleural fluid cytology following virtual cytology education: a single-centre descriptive study

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ABSTRACT

Background: Cytology education requires students to develop competency in recognizing and interpreting cellular features through microscopic examination. With the use of Online Distance Learning method (ODL) virtual method, students may rely primarily on image-based materials to learn cytology. Therefore, evaluating their inter- and intra-observer reliability and diagnostic accuracy following Virtual cytology education (VCE)-based learning is important. This study aimed to assess inter- and intra-observer reliability and determine the diagnostic accuracy of students in screening pleural fluid cytology cases following VCE-based learning. **Methods:** This was a single-arm, descriptive study without a comparator group. The study was conducted at the cytology laboratory, Universiti Teknologi MARA Selangor, Puncak Alam Campus. Five slide observers were selected among students who learn cytology through VCE to screen 20 pleural fluid cases in two screening sessions with a month gap. Inter- and intra-reliability tests were measured using Fleiss' kappa and Cohen's kappa values, respectively. Meanwhile, the diagnostic accuracy was measured using the operating parameters. **Results:** For inter-observer reliability, the Fleiss' kappa value recorded a 'Fair' agreement with 0.258 in the first screening session (A) and a 'Moderate' agreement with 0.490 in the second screening session (B). Analysis using Cohen's kappa for intra-observer reliability showed agreement significantly better than chance for two of five observers (SO1, $\kappa = 0.560$; SO5, $\kappa = 0.821$), which was not demonstrated for the remaining three (SO2, SO3, SO4). Meanwhile, the overall diagnostic accuracy was recorded at 85%. **Conclusion:** In conclusion, the reliability test showed fair to moderate inter-observer agreement between the two sessions, indicating some improvement but still limited consistency between observers. However, the average diagnostic accuracy was recorded at 85%, which suggests that although the observers only learned through VCE with only help by images, majority of them still can screen the slides correctly with their knowledge.

Keywords:

virtual cytology education; diagnostic accuracy; pleural fluid; inter-observer reliability; intra-observer reliability

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INTRODUCTION

Online Distance Learning (ODL) virtual method has become increasingly adopted approach in higher education, providing students with opportunities to access learning materials through online platforms (Schueler & West, 2023). However, the implementation of ODL in Virtual Cytology Education (VCE) may present several challenges, including limited access to appropriate technology, unreliable internet connectivity, and potential limitations in the effectiveness of online learning for developing practical cytology and diagnostic skills (Deraman et al., 2021). These limitations may affect

cytology students, who may be restricted in their ability to analyse cytology slides when learning cellular morphology primarily through images. Routine examination of cytology slides is necessary to ensure the accuracy of observers when assessing patient samples. During the learning sessions, instructors and laboratory staff provided slide images for students to study.

Due to their dependence on the slide image presented, students may have limited experience in manipulating slides under a microscope to locate abnormal cells, a process known as screening. Lack of proficiency in examining slides under a microscope may result in students missing abnormal or malignant cells. To perform effective slide screening, cytology observers must have adequate experience. Adequate training is important for effective screening and interpretation of cytology slides (Cubillos et al., 2024).

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This study focuses on pleural fluid, the fluid surrounding the lungs. Pleural fluid examination can be used to evaluate for the presence of malignant cells (D'Agostino & Edens, 2020). Inter-observer reliability refers to the degree of agreement among different observers, whereas intra-observer reliability refers to the consistency of observations made by the same observer across repeated screening sessions (Mohamad et al., 2020; Alrehily, 2024). Diagnostic accuracy is an important measure of observer performance. Low diagnostic accuracy may result in incorrect interpretation by the observers.

This study aimed to determine the inter- and intra-observer agreement and the observers' diagnostic accuracy following VCE-based learning, as the observers blindly screened pleural fluid slides under a microscope.

MATERIALS AND METHODS

Experimental pedagogical method

This study was conducted following ethical clearance granted by the Faculty Research Ethics Committee (Approval No.: FERC/FSK/MR/2022/0089). A two-hours practical session on respiratory cytology was conducted via Google Meet, where a live microscopic view was displayed on a 52-inch LCD monitor through a LEICA ICC50 digital camera-equipped LEICA DM750 compound microscope. Additionally, the high definition (HD) web camera connected to a laptop allowed remote students to participate in real time, as shown in Figure 1.



Figure 1: The teaching and learning process of hybrid virtual cytology education using Google Meet and face-to-face in the Cytology Laboratory at UiTM

Selection of cases

Twenty slide cases were randomly selected based on slide selection criteria of satisfactory staining quality, no broken slides, appropriate sample collection, no obscured blood, and no contaminants or artefacts. The slides were renamed cases 1 through 20 for later analysis.

Selection of observers

The inclusion and exclusion criteria were used to select five observers randomly. The slide observers were Medical Laboratory Technology students at UiTM Puncak Alam who had participated in VCE sessions to learn cytology-related topics. Observers could determine cell morphological characteristics and operate light microscopes.

Screening session

Each observer independently screened all 20 cases, presented in two batches to limit fatigue. Slide observers were given a worksheet to record their diagnosis. They observed the slides and completed the diagnosis worksheet. This study aimed to classify results as benign, atypical, or malignant. The observers had a one-month interval between the first and second screenings to prevent recall. This gap was for the intra-observer reliability test. Intra-observer reliability measures agreement between the same slide observers. In contrast, inter-observer reliability measures agreement between separate slide observers. The second purpose was to assess the diagnostic accuracy of post-VCE slide observers in diagnosing pleural slide cases. The actual hospital diagnosis was compared to the observers' results as a control. Data was tabulated and tested using formulas and software to show diagnostic accuracy and reliability for all slide observers.

Data analysis

The data were analysed with SPSS (Statistical Package for Social Science) for Windows version 28.0, IBM Corp, Armonk, New York, United States. Inter- and intra-observer reliability were assessed using kappa statistics. Fleiss' kappa was used to assess agreement among the five observers, while Cohen's kappa was used to assess agreement between ratings made by the same observer on different occasions (Fleiss, 1971; Cohen, 1960). The reliability test was analysed using specified kappa values. Inter-observer reliability was assessed by comparing the agreement between all observers using Fleiss' kappa value, while intra-observer was assessed by comparing the same observer's diagnoses on different occasions using Cohen's kappa value. For diagnostic accuracy, data was compared to hospital outcomes. Data were categorised as true positives (TP), true negatives (TN), false positives (FP), and false negatives (FN). Sensitivity, specificity, PPV, NPV, positive likelihood ratio ($LR+ = \text{sensitivity} \div [1 - \text{specificity}]$), and diagnostic accuracy were determined from the data, with $LR+$ calculated from pooled counts across the five observers because it is undefined for

individual observers with zero false positives. Receiver Operating Characteristics (ROC) curves and Area Under the Curve (AUC) were also determined to assess the diagnostic performance of the observers (Laerd Statistics, 2019). The test is more accurate if the curve were closer to the left and top of the ROC area.

RESULTS

The inter-observer agreement is the agreement between different observers. Fleiss' kappa value was used for inter-observer reliability as the observers were more than two. In screening session A, the observers show 'Fair' agreement with 0.258 kappa value, and for screening session B, the observers show 'Moderate' agreement with 0.490, as shown in Table 1.

Table 1: Overall Fleiss' kappa for screening sessions A and B

Screen session	Kappa	Agreement	ASE	Z	P-value	95% CI	
						Lower	Upper
A	0.258	Fair	0.052	4.921	<0.001	0.155	0.361
B	0.490	Moderate	0.052	9.401	<0.001	0.388	0.593

ASE = asymptotic standard error; CI = confidence interval

Kappa interpretation: <0.20 Poor, 0.21-0.40 Fair, 0.41-0.60 Moderate, 0.61-0.80 Good, 0.81-1.00 Very good (Landis & Koch, 1977; Altman, 1999).

Category-specific agreement for benign, atypical and malignant cases is given in Table 2.

Table 2: Category-specific Fleiss' kappa for screening sessions A and B

Ses	Cate	Agree	κ	ASE	Z	P-value	95% CI	95% CI
sio	gory	ment					Lower	Upper
n								
A	Beni	Fair	0.371	0.071	5.242	<0.001	0.232	0.509
A	Atyp	Poor	0.085	0.071	1.207	0.227	-0.053	0.224
A	Mali	Fair	0.255	0.071	3.603	<0.001	0.116	0.393
B	Beni	Good	0.625	0.071	8.839	<0.001	0.486	0.764
B	Atyp	Poor	0.153	0.071	2.165	0.030	0.015	0.292
B	Mali	Mode	0.592	0.071	8.373	<0.001	0.453	0.731
	gnant	rate						

ASE = asymptotic standard error; CI = confidence interval

Kappa interpretation: <0.20 Poor, 0.21-0.40 Fair, 0.41-0.60 Moderate, 0.61-0.80 Good, 0.81-1.00 Very good (Landis & Koch, 1977; Altman, 1999).

Intra-observer reliability measured the agreement between each observer in the first and second screening. Cohen's kappa was used because intra-observer reliability assessed agreement between two ratings made by the same observer on different occasions. Intra-observer agreement varied between the two sessions, as tabulated in Table 3. Two observers showed agreement significantly

better than chance (SO1, $\kappa = 0.560$; SO5, $\kappa = 0.821$). For SO2 ($\kappa = 0.170$, $p = 0.268$), SO3 ($\kappa = 0.318$, $p = 0.051$) and SO4 ($\kappa = 0.227$, $p = 0.168$), this was not demonstrated. As only 20 cases were screened, these values should be interpreted with caution and are reported individually rather than as a single average.

Table 3: Cohen's kappa value of intra-observer reliability test

Slide observer (SO)	Cohen's kappa value	Strength of agreement	P-value	Approx. 95% CI*
SO1 (A vs B)	0.560	Weak	0.002	0.205 – 0.915
SO2 (A vs B)	0.170	None	0.268	-0.131 – 0.471
SO3 (A vs B)	0.318	Minimal	0.051	-0.001 – 0.637
SO4 (A vs B)	0.227	Minimal	0.168	-0.096 – 0.550
SO5 (A vs B)	0.821	Strong	<0.001	0.332 – 1.000†

†Upper bound truncated at 1.000, the maximum possible value of kappa. Strength-of-agreement categories per McHugh (2012).

The distribution of true positive, true negative, false positive and false negative results for each observer is given in Table 4 (session A) and Table 5 (session B).

Table 4: Distribution of TP, TN, FP, and FN of slide observer in screening session A

SO	TP (%)	TN (%)	FP (%)	FN (%)
SO1	6 (30)	13 (65)	0	1 (5)
SO2	6 (30)	9 (45)	4 (20)	1 (5)
SO3	4 (20)	12 (60)	1 (5)	3 (15)
SO4	4 (20)	10 (50)	3 (15)	3 (15)
SO5	6 (30)	11 (55)	2 (10)	1 (5)

Percentages are of the 20 cases screened by that observer. Likelihood-ratio calculations in Table 6 use counts pooled across all five observers rather than these individual rows, because several observers have zero false positives (FP = 0), for whom specificity is 100% and the positive likelihood ratio is undefined

Table 5: Distribution of TP, TN, FP, and FN of slide observer in screening session B

SO	TP (%)	TN (%)	FP (%)	FN (%)
SO1	6 (30)	13 (65)	0	1 (5)
SO2	7 (35)	7 (35)	6 (30)	0
SO3	6 (30)	13 (65)	0	1 (5)
SO4	6 (30)	12 (60)	1 (5)	1 (5)
SO5	7 (35)	12 (60)	1 (5)	0

Percentages are of the 20 cases screened by that observer. Likelihood-ratio calculations in Table 6 use counts pooled across all five observers rather than these individual rows, because several observers have zero false positives (FP = 0), for whom specificity is 100% and the positive likelihood ratio is undefined

The operating parameters for diagnostic accuracy were recorded in Table 6. Although sensitivity and specificity can trade off against each other in general, in this study both increased together from session A to session B (sensitivity 74.26% to 91.42%; specificity 84.62% to 87.68%). Meanwhile, the positive likelihood ratio (LR+) means how

much more likely a positive result is in a true malignant case than in a true non-malignant case, calculated as sensitivity divided by (1 – specificity). Table 7 recorded the results of AUC of ROC for both screening sessions and the total average.

Table 6: Average value of each operating parameter in screening sessions A and B

Operating parameter	Average value		Total average
	A	B	
Sensitivity	74.26% (54.86–93.71)	91.42% (81.71–100)	82.84% (72.30–93.41)
Specificity	84.62% (69.52–99.71)	87.68% (63.72–100)	86.15% (75.21–97.10)
Positive Predictive Value (PPV)	74.42% (53.00–95.86)	85.40% (61.97–100)	79.91% (67.04–92.81)
Negative Predictive Value (NPV)	86.30% (77.23–95.35)	95.62% (90.62–100)	90.96% (85.64–96.25)
Positive Likelihood Ratio (LR+)	4.83	7.43	5.98
Diagnostic Accuracy	81.00% (69.06–92.94)	89.00% (75.54–100)	85.00% (77.46–92.54)

Values are means (with 95% CI) of the five observers for that session. Total average is the mean of sessions A and B, except for Positive Likelihood Ratio (LR+), which is calculated from counts pooled across both sessions rather than as the mean of the A and B columns.

Table 7: Area under the curve (AUC) of slide observer in screening sessions A and B

Slide observer (SO)	Area under curve (AUC)		Total average
	A	B	
SO1	0.929	0.929	0.929
SO2	0.775	0.769	0.772
SO3	0.747	0.929	0.838
SO4	0.670	0.890	0.780
SO5	0.852	0.962	0.907
Average	0.795	0.896	0.846

AUC = area under the receiver operating characteristic curve. Total average (right column) is the mean of sessions A and B for each observer; Average (bottom row) is the mean across the five observers.

The overall performance also can be seen in Figure 2 and Figure 3, where the value was plotted for sensitivity and 1-specificity. The diagnostic accuracy can be seen as an area under the line.

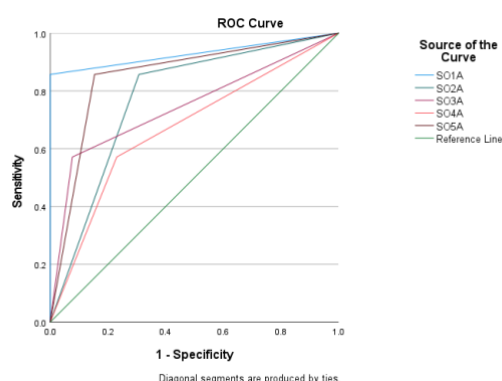


Figure 2: ROC curve showing the relationship of sensitivity and 1-specificity of slide observers in screening session A

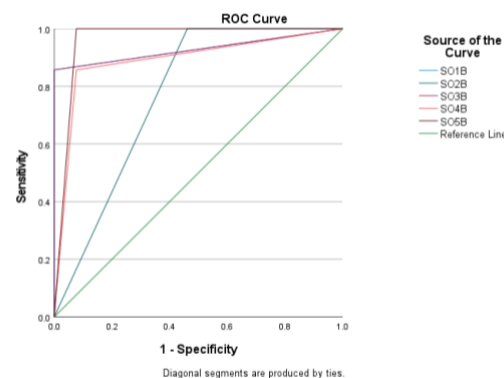


Figure 3: ROC curve showing the relationship of sensitivity and 1-specificity of slide observers in screening session B

DISCUSSION

Inter-observer reliability

Fleiss' kappa was used to represent the result of inter-observer as there were more than two observers. The data were analysed for overall agreement and based on benign, atypical, and malignant categories. Twenty cases were screened by five randomly chosen observers based on inclusion and exclusion criteria. The level of agreement was adapted from Landis and Koch (1977), Altman (1999), which stated that k value < 0.20 indicated "Poor", 0.21-0.40 as "Fair", 0.41-0.60 as "Moderate", 0.61-0.80 as "Good" and 0.81-1.00 as "Very good".

The overall agreement of the observers showed fair agreement in the first screening session (A) with 0.258 kappa value. In contrast, in the second screening session (B), the observers showed moderate agreement of 0.490. The 95% confidence interval for the kappa estimate in screening session A was 0.155–0.361, while that for screening session B was 0.388–0.593.

As for the individual categories, the observers reported mixed results (Table 2). In screening session A, the observers showed 'Fair' agreement for benign and malignant cases, but for atypical cases, the observers showed a 'Poor' agreement. This may be because the observers had difficulty diagnosing the atypical cases and misinterpreted them as benign or malignant. However, in screening session B, the observers recorded a 'Good' agreement in benign cases 'Poor' agreement for atypical, and 'Moderate' agreement for malignant cases. The second screening session showed an increase in agreement compared to the first as the observers were making revisions and gaining more confidence in diagnosing pleural fluid cases for the second screening.

Intra-observer reliability

Intra-observers' reliability measured the agreement of each observer between the first and second screening sessions (Nayar et al., 2018). Intra-observer reliability is crucial to evaluate the consistency of each observer in screening the same slide cases. Therefore, a one-month interval was given between the two screening sessions to minimise recall of the first screening results. Cohen's kappa value was used to measure the agreement for the intra-observer reliability test. The value was categorised as 0-0.2 interpreted as 'None' agreement, 0.21-0.39 as 'Minimal' agreement, 0.40-0.59 as 'Weak' agreement, 0.60-0.79 as 'Moderate' agreement, 0.80-0.90 as 'Strong' agreement and above 0.90 as 'Almost perfect' agreement.

The agreement varied among the observers, with the highest agreement recorded by SO5 with a 'Strong' agreement, followed by SO1 with a 'Weak' agreement. SO3 and SO4 recorded 'Minimal' agreement, while SO2 recorded 'None' agreement. The result shows that some of the observers are inconsistent with their diagnosis. The inconsistency may be due to the observers being confused when observing for the first screening. They study for the second screening, leading them to give different diagnoses during the second screening.

Diagnostic accuracy

This study's second objective is to measure each slide observer's diagnostic accuracy. The cases were divided into non-malignant and malignant cases. The results were classified as true positive, true negative, false positive, and false negative. The results were then compared with actual results obtained from the donated hospital. The benign cases were categorised as non-malignant, while atypical and malignant were categorised under malignant. In screening session A, the observers recorded 81% diagnostic accuracy, while in session B, the observers recorded 89% diagnostic accuracy. The increasing diagnostic accuracy suggests that the observers still can screen the slide cases, although only learning through VCE with only the images provided by the lecturers. Piletska (2023) reported that the use of cytology cases as an educational tool improved trainees' exposure to cytology. The increase in diagnostic accuracy between the first and second screening session also shows that the observers can adjust very well to using microscope and revise cytology studies after the first screening session. The average diagnostic accuracy of the first and second screening sessions (A and B) was 85%. This exceeds the 65% baseline accuracy that would be obtained by classifying every case as benign (13 of the 20 cases were non-malignant); however, 12 of the 70 malignant readings

(17.1%) were missed across both sessions, which would have clinical implications if used diagnostically. Although the diagnostic accuracy in this study was lower compared to a study that was made by Ahmad, (2020) using serous fluid cases, which are 92.25%, it can be accepted as the previous research focused on the absence of demographic data and the previous observers were experienced in screening session during the learning session (Ahmad, 2020). Thus, the findings in this study revealed that most of the observers could accurately diagnose the pleural fluid cases although only learning through VCE.

Most of the results are correctly classified into true positive and true negative, as shown in Table 4 and Table 5. The results proved that most observers could diagnose non-malignant and malignant cases accurately. In addition, the false positive and false negative results, particularly the 17.1% false-negative rate for malignant cases, would have clinical implications and should not be regarded as having little effect on performance. These results may be due to the observers misclassifying their diagnosis as non-malignant or vice-versa. The total average overall performance from screening sessions A and B recorded 82.84% for sensitivity, 86.15% for specificity, 79.91% for PPV, 90.96% for NPV, 5.98 for the positive likelihood ratio, and 0.846 for AUC of ROC.

The primary malignant case of pleural fluid was mesothelioma, but mesothelioma was not common cancer. It was caused by asbestos which is not widely used nowadays (Ahmad, 2020). Usually, the malignant cells found in the pleural fluid were metastasized cancer cells such as adenocarcinomas, squamous cell carcinoma, and other metastasized carcinomas. The pleural fluid cases distributed to the observers consist of 13 non-malignant and seven malignant cases.

The normal mesothelial cell shows eccentric, located nucleus, normal nuclear to cytoplasmic ratio, and presence of windows or clear space between cells. Meanwhile, the cells can be seen in tight clusters arrangement with 3D appearances, high N:C ratio, and hyperchromatic nucleus for malignant cases. Most observers misdiagnosed the atypical cases as non-malignant, while the actual result is atypical, which is in the malignant category. The atypical cell shows a slightly high N:C ratio, smooth nuclear membrane with the presence of macrophages, erythrocytes, and lymphocytes in the background. According to Roslan et al. (2021), without clinical history, the observers may misdiagnose the cases as they rely only on the morphological. This may be one of the reasons most of the observers misdiagnosed the cases, as they were not provided with clinical history.

Limitation

Slides were selected based on inclusion criteria consisting of good staining quality, no broken slide, adequate sample collected, no obscured blood, and absence of contaminants and artifacts. Due to the requirements, some of the slides cannot be used. Only seven out of 20 cases were malignant, which caused the slide selection to be imbalanced between malignant and non-malignant. Other than that, the selection of slide observers also has limitations. This study only focused on medical laboratory technology students who were studying through VCE and also were doing their final year projects throughout the time. Due to this, the time for the observers to screen slides was not specific as need to consider their time for their own project. Besides, in the reliability test, the observers' results were compared within observers for the inter-reliability test and within themselves for the intra-reliability test. However, inter-, and intra-observer reliability assess agreement rather than validity against the reference diagnosis. Therefore, agreement results cannot be interpreted as confirmation of diagnostic validity.

CONCLUSION

In conclusion, inter-observer agreement was fair in the first session and moderate in the second, showing some improvement but still limited consistency between observers. Meanwhile, the intra-reliability reported various results from 'none' to 'strong' agreement. This indicates that the observers were inconsistent in diagnosing the pleural fluid cases. However, the diagnostic accuracy of the observers was 85% on average, above the 65% baseline obtainable by classifying every case as benign. This diagnostic accuracy suggests that although the observers are only learning through VCE with the only help of images, most of them can still screen the slides correctly with their knowledge.

For future study, observers should be properly prepared with their knowledge and use of a microscope before screening for the first time, and further research could examine whether this improves their ability to make a diagnosis based on cell characteristics.

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Comparative Antibacterial Activity of Methanol, Ethyl Acetate and Hexane Extracts of *Parkia speciosa* Hassk. Pericarp Against Selected Bacterial Pathogens

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ABSTRACT

Background: The persistent emergence of multidrug-resistant bacterial strains presents an urgent global public health threat, driving the need to discover novel antimicrobial agents from natural plant sources. *Parkia speciosa* Hassk, commonly known as petai, is widely consumed in Southeast Asia for its nutritional and medicinal properties. While the green seeds are traditionally eaten, the outer pericarp is discarded as agricultural waste. This study evaluated the antibacterial potential of crude methanol, ethyl acetate, and hexane extracts of *P. speciosa* pericarp against twenty one selected bacterial strains. **Methods:** Dried, ground pericarp was subjected to exhaustive extraction using methanol, ethyl acetate, and hexane. Antimicrobial activity was evaluated using agar disk diffusion, while Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) were determined via broth microdilution assays across a concentration range of 0.90–1000 mg/mL. **Results:** Methanol and ethyl acetate extracts inhibited *S. aureus* and *B. cereus* in a concentration-dependent manner, whereas hexane showed limited activity only at 500 and 1000 mg/mL. The largest inhibition zone was observed with ethyl acetate against *S. aureus* at 1000 mg/mL (18.0 ± 0.0 mm), while no inhibition was observed against *E. coli* or *S. typhi*. MIC and MBC testing against 21 bacterial strains showed that *B. cereus* was the most susceptible to both methanol and ethyl acetate extracts. Ethyl acetate generally showed greater potency, with lower MIC and MBC values than methanol against most organisms. MBC values were identical to the corresponding MIC values for all tested strains. **Conclusion:** These findings demonstrate that *P. speciosa* pericarp possesses promising antibacterial activity, particularly in its ethyl acetate and methanol extracts, and may represent a potential source of bioactive compounds for the development of natural antimicrobial agents.

Keywords:

Parkia speciosa; pericarp, antibacterial activity; ethyl acetate; methanol, hexane

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INTRODUCTION

Bacterial resistance to conventional antibiotics has emerged as one of the most pressing global public health challenges, compromising the effective treatment of infectious diseases and increasing the risk of therapeutic failure, prolonged illness, healthcare burden, and mortality (Muteeb et al., 2023). The World Health Organization reported in 2026 that approximately one in six laboratory-confirmed bacterial infections worldwide in 2023 was resistant to antibiotics, highlighting the continuing need for new antibacterial strategies and sources of bioactive compounds (World Health Organization, 2026). As such, plant-derived products became of interest because their chemically diverse secondary metabolites may provide lead compounds for antimicrobial development.

Parkia speciosa Hassk., commonly known as petai or stink bean, is widely consumed in Southeast Asia. Although the seeds are the principal edible portion, the pod or pericarp represents a substantial discarded plant material. Reviews of *P. speciosa* describe a broad range of phytochemicals and biological activities, including antioxidant, hypoglycaemic and antimicrobial effects. From previous studies, it has also been noted that different plant parts and extraction solvents can yield markedly different bioactivities (Kamisah et al., 2013; Saleh et al., 2021). Hence, valorisation of its pericarp is therefore attractive, both as a phytochemical resource and as an approach to reduce agricultural or food waste.

The antibacterial activity of *P. speciosa* has been attributed to the presence of various bioactive plant metabolites. However, the magnitude of antibacterial activity may vary depending on factors such as the geographical origin of the plant material and the extraction conditions employed (Ghasemzadeh et al., 2018). A previous study by Cheong et al. (2023) has

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demonstrated the efficacy of an ethyl acetate pericarp extract against *S. aureus* and *B. cereus*. More recently, Goh et al. (2025) reported that mid-polar fractions of *P. speciosa* pods, particularly ethyl acetate-derived fractions, exhibited notable antibacterial activity against clinically relevant bacterial species. However, these studies primarily focused on ethyl acetate extracts or fractions and therefore provide limited insight into how solvent polarity influences the antibacterial activity of *P. speciosa* pericarp extracts. In particular, a comparison of extracts obtained using solvents representing different polarities, such as methanol, ethyl acetate, and hexane, against a broader range of bacterial species has not been adequately established. Therefore, the present study advances previous work by systematically comparing the antibacterial activity of methanol, ethyl acetate, and hexane extracts of *P. speciosa* pericarp, looking into the influence of solvent polarity on the spectrum and magnitude of antibacterial activity. Disc diffusion was initially performed as a preliminary screening assay using four selected bacterial species, followed by broth microdilution assays to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) across a broader panel of twenty-one bacterial strains. This comparative approach provides a more comprehensive assessment of the antibacterial potential of *P. speciosa* pericarp and facilitates identification of the extract with the greatest antibacterial activity for further investigation.

MATERIALS AND METHODS

Plant Material and Extraction

P. speciosa Hassk. was purchased from local markets in Puncak Alam, Selangor, Malaysia. Botanical identity was verified by an authorized officer at the Forest Research Institute Malaysia (FRIM), Kepong, and the specimen was recorded as PID 090316-09. The pericarp was cut into small pieces, washed with distilled water, sun-dried and ground to powder using a mechanical blender. The powdered material was weighed and stored at 4°C until extraction. The powdered pericarp was then separately soaked in methanol, ethyl acetate and hexane at a powder-to-solvent ratio of 1:3. For exhaustive extraction, the mixture was filtered through Whatman No. 1 filter paper every three days and fresh solvent was added to the residual plant material until the filtrate remained colorless. Subsequently, the combined filtrates were concentrated using a rotary evaporator. Crude extracts were stored in foil-wrapped containers at 4 °C and prior to analyses, each extract was dissolved in dimethyl sulfoxide (DMSO) to achieve a stock concentration of 1000 mg/mL.

Bacterial Strains and Culture Preparation

The antibacterial activity of the extracts was evaluated against a panel of twenty-one bacterial strains, comprising nine Gram-positive and twelve Gram-negative bacteria. All bacterial stock cultures were obtained from the Microbiology Laboratory, Faculty of Health Sciences, Universiti Teknologi MARA, Puncak Alam. The gram-positive panel comprised of *S. aureus* ATCC 33591, *S. epidermidis* ATCC 12228, *S. saprophyticus* ATCC 15305, *S. agalactiae* ATCC 13813, *S. pyogenes* Group A ATCC 19615, *S. mutans* ATCC 25175, *B. cereus* ATCC 11778, *L. monocytogenes* ATCC 19111 and *E. faecalis* ATCC 29212.

As for the gram-negative bacteria, *V. parahaemolyticus* ATCC 17802, *P. mirabilis* ATCC 35659, *P. vulgaris* ATCC 8427, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883, *S. enterica* serovar Paratyphi A ATCC 11511, *S. enterica* serovar Typhimurium ATCC 14028, *E. coli* ATCC 25922, *K. oxytoca* ATCC 700324, *S. boydii* serovar 1 Group C ATCC 9207, *S. sonnei* ATCC 25931 and *S. flexneri* serovar 26 Group B were used. Each stock culture was then streaked onto nutrient agar and incubated at 37°C for 18-24 h. Single colonies were subjected to Gram staining, biochemical identification and growth on appropriate differential media for confirmation.

Disc Diffusion Assay

Antibacterial susceptibility screening was performed on Mueller-Hinton agar by disc diffusion. Bacterial suspensions of *S. aureus*, *B. cereus*, *E. coli* and *S. typhi* prepared in Mueller-Hinton broth were spread evenly over the agar surface using sterile cotton swabs. Filter-paper discs were loaded with 10 µL of extract at 1000, 500, 250, 125, 62.5 or 31.25 mg/mL and placed on the inoculated agar. Plates were incubated at 37°C for 24 h and inhibition-zone diameters were measured in millimeters. Ampicillin (10 µg) served as positive control and DMSO was used as negative control.

MIC and MBC Determination

MIC was determined using a broth microdilution approach in 96-well round-bottom microtiter plates. Extracts were serially diluted in Mueller-Hinton broth to obtain final test concentrations spanning approximately 0.9-1000 mg/mL (1000, 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.9, 1.95 and 0.97 mg/mL). Each well received 50 µL of diluted extract and 50 µL of bacterial suspension. Antibiotic control, bacterial growth control and DMSO control wells were included. Plates were incubated at 37°C for 24 h. The MIC was defined as the lowest tested concentration showing no visible bacterial growth. For

MBC determination, aliquots from test dilutions without visible growth were subcultured onto Mueller-Hinton agar and incubated at 37°C for 24 h. The MBC was defined as the lowest extract concentration yielding no bacterial growth after subculture.

Data Analysis

All experiments were performed using three independent tests for each organism and experimental condition. Data are presented as mean $\pm$ standard deviation (SD) where applicable. Mean inhibition-zone diameters were analysed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons. A p -value < 0.05 was considered statistically significant. Statistical analyses were performed using IBM SPSS Statistics.

RESULTS

Disc Diffusion Activity

Methanol and ethyl acetate extracts inhibited both *S. aureus* and *B. cereus* across the full concentration range tested, whereas the hexane extract produced measurable inhibition only at 500 and 1000 mg/mL (Table 1). Significant differences in inhibition-zone diameters were observed among the groups for all extract–organism combinations (one-way ANOVA, $p < 0.001$). Result for *S. aureus* which demonstrated the largest inhibition zone was produced by the ethyl acetate extract at 1000 mg/mL (18.0 ± 0.0 mm), followed by methanol (16.5 ± 0.0 mm) and hexane (8.0 ± 0.1 mm).

However, all extract concentrations produced significantly smaller inhibition zones than the respective ampicillin controls ($p < 0.001$). As for *B. cereus*, the ethyl

acetate extract produced the largest zone at 500 mg/mL (14.0 ± 0.1 mm), while the methanol extract produced a maximum inhibition zone of 12.5 ± 0.5 mm at 1000 mg/mL. Interestingly, methanol at 250–1000 mg/mL and ethyl acetate at 125–1000 mg/mL produced significantly larger inhibition zones than their respective ampicillin controls ($p < 0.001$). No significant difference was observed between methanol at 125 mg/mL ($p = 0.056$), or between ethyl acetate at 62.5 mg/mL ($p = 0.934$) when compared to ampicillin. The hexane extract showed weaker activity, producing inhibition zones of 8.0 ± 0.5 mm and 6.5 ± 0.1 mm at 1000 and 500 mg/mL, respectively. Both values were significantly lower than those of the ampicillin control. No inhibition zones were observed against *E. coli* or *S. typhi* for any of the extracts.

MIC and MBC

As the hexane extract exhibited negligible antibacterial activity in this preliminary screening, subsequent broth microdilution assays were conducted only with the methanol and ethyl acetate extracts. Their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined against a broader panel of twenty-one bacterial strains. It was observed that the MIC and MBC values were generally comparable across the tested bacterial strains.

The methanol and ethyl acetate extracts inhibited all organisms within the tested concentration range (Table 2) indicating that the extracts showed antibacterial activities at variable degrees.

Table 1: Mean inhibition-zone diameters of *P. speciosa* pericarp extracts against *S. aureus* and *B. cereus*

Concentration (mg/mL)	Methanol <i>S. aureus</i> $\pm$ SD	Methanol <i>B. cereus</i> $\pm$ SD	Ethyl acetate <i>S. aureus</i> $\pm$ SD	Ethyl acetate <i>B. cereus</i> $\pm$ SD	Hexane <i>S. aureus</i> $\pm$ SD	Hexane <i>B. cereus</i> $\pm$ SD
1000	16.5 ± 0.0	12.5 ± 0.5	18.0 ± 0.0	12.5 ± 0.1	8.0 ± 0.1	8.0 ± 0.5
500	15.5 ± 0.1	12.0 ± 0.0	17.0 ± 0.1	14.0 ± 0.1	7.0 ± 0.3	6.5 ± 0.1
250	14.0 ± 0.0	10.5 ± 0.0	16.0 ± 0.0	12.5 ± 0.0	0 ± 0.0	0 ± 0.0
125	11.5 ± 0.0	8.5 ± 0.1	14.5 ± 0.3	12.0 ± 0.0	0 ± 0.0	0 ± 0.0
62.5	9.5 ± 0.3	7.5 ± 0.0	12.5 ± 0.2	10.0 ± 0.5	0 ± 0.0	0 ± 0.0
31.25	7.5 ± 0.0	7.0 ± 0.0	9.5 ± 0.0	8.5 ± 0.0	0 ± 0.0	0 ± 0.0
Ampicillin 10 μ g	37.5 ± 0.2	9.0 ± 0.0	35.0 ± 0.0	9.8 ± 0.3	25.0 ± 0.1	9.0 ± 0.0
DMSO	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0

Values are expressed as mean inhibition-zone diameter $\pm$ standard deviation (SD) from triplicate measurements ($n = 3$).

Table 2: MIC and MBC values of methanol and ethyl acetate extracts of *P. speciosa* pericarp against 21 bacterial strains

Organism	Methanol MIC (mg/mL)	Methanol MBC (mg/mL)	Ethyl acetate MIC (mg/mL)	Ethyl acetate MBC (mg/mL)
<i>S. aureus</i>	7.81	7.81	7.81	7.81
<i>B. cereus</i>	1.95	1.95	1.95	1.95
<i>S. epidermidis</i>	3.9	3.9	3.9	3.9
<i>E. coli</i>	500	500	31.25	31.25
<i>S. enterica</i>	500	500	31.25	31.25
<i>P. aeruginosa</i>	500	500	62.5	62.5
<i>P. mirabilis</i>	62.5	62.5	62.5	62.5
<i>S. saprophyticus</i>	31.25	31.25	3.9	3.9
<i>S. agalactiae</i>	31.25	31.25	3.9	3.9
<i>S. pyogenes</i>	31.25	31.25	3.9	3.9
<i>S. mutans</i>	31.25	31.25	3.9	3.9
<i>L. monocytogenes</i>	125	125	3.9	3.9
<i>E. faecalis</i>	125	125	62.5	62.5
<i>V. parahaemolyticus</i>	125	125	3.9	3.9
<i>P. vulgaris</i>	500	500	7.81	7.81
<i>K. pneumoniae</i>	500	500	7.81	7.81
<i>S. paratyphi A</i>	500	500	31.25	31.25
<i>K. oxytoca</i>	500	500	31.25	31.25
<i>S. boydii</i>	250	250	3.9	3.9
<i>S. sonnei</i>	250	250	3.9	3.9
<i>S. flexneri</i>	1000	1000	62.5	62.5

The lowest MIC observed for both extracts was 1.95 mg/mL against *B. cereus*. As for the methanol extract, the next lowest MIC values were 3.9 mg/mL against *S. epidermidis* and 7.81 mg/mL against *S. aureus*. However, several gram-negative organisms required substantially higher methanol concentrations (500 mg/mL), including *E. coli*, *P. aeruginosa*, *P. vulgaris* and *K. pneumoniae*, while *S. flexneri* required 1000 mg/mL.

For the ethyl acetate extract, the MIC results indicate generally strong activity against several organisms, with the lowest MIC observed was for *B. cereus* at 1.95 mg/mL, as stated earlier. MICs of 3.9 mg/mL were recorded for *S. epidermidis*, *S. saprophyticus*, *S. agalactiae*, *S. pyogenes*, *S. mutans*, *L. monocytogenes*, *V. parahaemolyticus*, *S. boydii*, *S. sonnei*, and *S. Flexneri* while *S. aureus*, *P. vulgaris*, and *K. pneumoniae* showed MICs of 7.81 mg/mL. Higher MIC values were observed for *E. coli*, *S. enterica*, *S. paratyphi A*, and *K. oxytoca* at 31.25 mg/mL, while *P. aeruginosa*, *P. mirabilis*, and *E. faecalis* required 62.5 mg/mL. Overall, the ethyl acetate extract exhibited lower MIC values than the methanol extract against most of the tested organisms, indicating greater antibacterial activity.

Corresponding with the MIC findings, both methanol and ethyl acetate extracts exhibited bactericidal activity against all tested bacterial strains, with MBC/MIC ratios equal to 1. This indicates that the bactericidal concentrations of both extracts were equivalent to their respective inhibitory concentrations.

DISCUSSION

The present comparative analysis indicates that the antibacterial activity of *P. speciosa* pericarp is strongly influenced by the extraction solvent. Methanol and ethyl acetate exhibited greater activity than hexane in the disc diffusion assay, while ethyl acetate generally produced lower MIC and MBC values against a broader range of bacterial species. This solvent-dependent pattern suggests that the principal antibacterial constituents of the pericarp are more efficiently recovered using polar to mid-polar solvents rather than with a non-polar solvent such as hexane. This may be related to the differences in solvent polarity and extraction selectivity between the tested solvents. Solvent polarity influences the type and relative abundance of secondary metabolites recovered from plant material, as compounds vary in their solubility

(Wakeel et al., 2019). Methanol can extract a relatively broad range of polar and moderately polar constituents, whereas ethyl acetate may selectively concentrate compounds of intermediate polarity. In contrast, hexane predominantly extracts non-polar constituents, which may explain its comparatively weak antibacterial activity in the present study.

The solvent-dependent pattern observed in the present study is comparable with previous findings for *P. speciosa*. Hasim et al. (2015) reported substantially lower antibacterial activity of n-hexane peel extract than ethyl acetate extract against *S. aureus* and *E. coli*, further suggesting that the major antibacterial constituents may be preferentially recovered in solvents of greater polarity. Likewise, Goh et al. (2025) reported greater antibacterial activity in mid-polar fractions of *P. speciosa* pods, supporting the possibility that compounds of intermediate polarity contribute substantially to the observed activity. The present findings also agree with Cheong et al. (2023), who reported susceptibility of *S. aureus* and *B. cereus* to ethyl acetate extract of *P. speciosa* pericarp in their disc diffusion assay. Nevertheless, solvent polarity alone does not determine antibacterial potency, as extraction time, temperature, plant-to-solvent ratio, compound stability and interactions among constituents may also influence the composition and biological activity of crude extracts. In addition, disc diffusion assay should be interpreted primarily as a screening method rather than as a direct measure of intrinsic antibacterial potency, particularly for crude plant extracts, because the observed inhibition zone is influenced not only by antimicrobial activity but also by the ability of individual constituents to diffuse through the agar matrix. Consequently, compounds with antibacterial activity may demonstrate limited inhibition zones if their diffusion from the paper disc is poor, thus emphasising the importance of interpreting disc diffusion findings together with MIC and MBC measurements.

The MIC and MBC findings further demonstrate the solvent-dependent antibacterial activity of *P. speciosa* pericarp observed in the disc diffusion assay. Both methanol and ethyl acetate extracts exhibited activity against all bacterial strains tested, although their effectiveness varied considerably among organisms. *B. cereus* was the most susceptible organism to both extracts, followed by *S. epidermidis* and *S. aureus*. In contrast, the methanol extract required considerably higher concentrations to inhibit several organisms, particularly *E. coli*, *S. enterica*, *P. aeruginosa*, *P. vulgaris*, *K. pneumoniae*, *S. paratyphi* A and *K. oxytoca*, while the MIC against *S. flexneri* reached 1000 mg/mL. These

findings indicate marked variation in bacterial susceptibility to the crude pericarp extracts. Ethyl acetate generally demonstrated greater antibacterial potency than methanol, particularly against organisms that were relatively less susceptible to the methanol extract. This broader activity supports the possibility that ethyl acetate preferentially concentrates antibacterial constituents of intermediate polarity that are present at lower relative abundance in the methanol extract. A similar pattern was reported by Goh et al. (2025), in which the ethyl acetate fraction of *P. speciosa* pods exhibited the greatest antibacterial activity among the fractions evaluated.

Although several gram-negative species required relatively high concentrations of the extracts, the susceptibility pattern was not uniformly associated with gram-negative status. The comparatively high MIC values recorded for organisms such as *E. coli*, *P. aeruginosa*, *K. pneumoniae* and *Salmonella* spp., particularly with the methanol extract, may partly reflect the intrinsic permeability barrier provided by the lipopolysaccharide-containing outer membrane of gram-negative bacteria (Nikaido, 2003; Delcour, 2009). The additional membrane can restrict the penetration of many antimicrobial compounds, while efflux systems may further reduce their intracellular accumulation. Nevertheless, some gram-negative organisms, including *V. parahaemolyticus*, *S. boydii* and *S. sonnei*, were inhibited by relatively low concentrations of the ethyl acetate extract. Thus, susceptibility appears to depend not only on bacterial cell-envelope structure but may also be linked to the chemical composition of the extract and the specific susceptibility characteristics of individual bacterial species.

At present, the mechanisms underlying the antibacterial activity of *Parkia* species have not been fully elucidated. Terpenoids present in these plants have been proposed to disrupt bacterial membrane function by altering membrane lipid dynamics, thereby increasing permeability and promoting cellular damage (Behuria and Sahu, 2020). Other potential mechanisms may involve interactions with essential bacterial proteins and interference with nucleic acid metabolism. Plant-derived flavonoids, for example, have been reported to inhibit bacterial DNA gyrase and DNA synthesis, among other cellular targets (Mori et al., 1987; Plaper et al., 2003; Donadio et al., 2021). Such mechanisms may partly explain the variation in susceptibility observed among the tested organisms; however, they have not yet been specifically confirmed for *P. speciosa* pericarp extracts. Further mechanistic studies are therefore required to identify the active constituents and determine their specific antibacterial targets.

In the present study, the MBC findings closely followed the MIC pattern, with the MBC values of both methanol and ethyl acetate extracts being identical to their corresponding MIC values. Consequently, the calculated MBC/MIC ratio was 1 in all cases. An MBC/MIC ratio of ≤ 4 is commonly interpreted as indicative of bactericidal activity, whereas higher ratios are generally associated with predominantly bacteriostatic effects. Accordingly, the present findings suggest that both methanol and ethyl acetate extracts exerted bactericidal effects against the bacterial strains tested, with bacterial killing occurring within the same two-fold dilution interval as growth inhibition. The ethyl acetate extract was particularly notable because bactericidal activity was achieved at substantially lower concentrations than the methanol extract for many of the organisms. Nevertheless, the identical MIC and MBC values should be interpreted with some caution. As MIC and MBC were determined using a two-fold serial dilution series, identical MIC and MBC values were obtained when growth inhibition and bacterial killing occurred within the same tested concentration, although the exact effective concentrations may not necessarily be identical. Furthermore, susceptibility testing of crude plant extracts may be influenced by factors such as solubility, precipitation, extract colour and turbidity, which can complicate the determination of broth microdilution endpoints. Further confirmation using narrower concentration intervals, viable bacterial counts or time-kill assays would therefore provide a more precise assessment of the bactericidal activity and concentration-dependent killing kinetics of *P. speciosa* pericarp extracts.

CONCLUSION

P. speciosa pericarp demonstrated clear solvent-dependent antibacterial activity. Methanol and ethyl acetate extracts exhibited substantially greater activity than the hexane extract in the preliminary disc diffusion assay. The ethyl acetate extract produced lower MIC and MBC values across the broader bacterial panel. Among the tested organisms, *B. cereus*, *S. epidermidis*, and *S. aureus* were among the most susceptible. Further studies employing standardised extraction procedures, quantitative phytochemical profiling, bioassay-guided fractionation, mechanistic investigations, and toxicity assessments are warranted to identify and characterise the active compounds and to evaluate the potential of *P. speciosa* pericarp as a potential source of antibacterial compounds.

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In Vitro Antibacterial Activity of Commercial Lemongrass (*Cymbopogon citratus*) Essential Oil Against *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* Typhimurium

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ABSTRACT

Background: Foodborne bacterial pathogens remain a major public-health burden, and rising antimicrobial resistance has renewed interest in plant-derived antimicrobials as adjuncts to conventional food-preservation strategies. Lemongrass (*Cymbopogon citratus*) essential oil is rich in citral and has documented antibacterial properties, but commercial preparations vary and few studies report both diffusion-based and broth-based endpoints for the same oil batch. This study evaluated the in vitro antibacterial activity of a single commercial lemongrass essential oil batch against three foodborne reference strains. **Methods:** *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922 and *Salmonella enterica* serovar Typhimurium ATCC 14028 were tested by agar disc diffusion at 5, 10, 20, 50 and 100% (v/v) and by broth microdilution over 0.078–10.0% (v/v), each in triplicate, with vehicle, growth and sterility controls. **Results:** On disc diffusion, *S. aureus* showed the largest zones at 100% (25.00 ± 8.00 mm, including the 6 mm disc) and *E. coli* smaller but consistent zones (11.67 ± 2.08 mm); *S. Typhimurium* produced no inhibition beyond the disc at any concentration. Two-way analysis of variance indicated significant effects of organism, concentration and their interaction (all $p < 0.001$). In broth, however, all three organisms were inhibited within the tested range: replicate minimum inhibitory concentrations (MICs) were ≤0.078, ≤0.078 and 0.156% for *S. aureus*; 0.313, 0.313 and 0.156% for *E. coli*; and 0.625, 0.156 and 0.156% for *S. Typhimurium*. Operational no-growth-on-subculture endpoints were 0.156% for *S. aureus* and 0.313% for *E. coli*; the *S. Typhimurium* assay was inconclusive and is not reported. **Conclusion:** The marked discordance between the two methods, most striking for *S. Typhimurium*, indicates that agar diffusion substantially underestimates the activity of a hydrophobic essential oil and should not be used alone to conclude that an organism is unaffected. Lemongrass essential oil inhibited all three pathogens at low concentrations in liquid medium, supporting further evaluation in food matrices.

Keywords:

essential oil; citral; foodborne pathogens; disc diffusion; natural antimicrobials

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INTRODUCTION

Foodborne illness remains a substantial and largely preventable contributor to the global disease burden. *Staphylococcus aureus*, *Escherichia coli* and *Salmonella enterica* are among the organisms most frequently implicated, causing disease through preformed enterotoxins, invasive infection or both (Kadariya et al., 2014). In Malaysia, reviews of the food chain have repeatedly identified hygiene and temperature control as recurring weaknesses, and foodborne outbreaks continue to be reported despite established control programmes (New et al., 2017).

The problem is compounded by antimicrobial resistance. Resistant foodborne organisms circulate between livestock, food products and people, and the World Health Organization continues to list several such pathogens as priorities for research and development (World Health Organization, 2024). Consumer resistance to synthetic preservatives has grown in parallel, and both pressures have renewed interest in plant-derived antimicrobials that might supplement rather than replace conventional preservation.

Lemongrass (*Cymbopogon citratus*) essential oil (LGEO) is among the most studied of these. Its principal constituent

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is citral, an isomeric mixture of geranial and neral, accompanied by myrcene, geraniol, linalool and limonene; recent GC-MS characterisation of a Thai lemongrass oil, for example, reported citral at 79.24% (geranial 44.52%, neral 34.72%) with β -myrcene at 5.56% (Luang-In et al., 2024). Terpenoids of this class are lipophilic and partition into the bacterial cytoplasmic membrane, increasing permeability and dissipating the proton-motive force, which is the mechanism generally invoked for their antibacterial effect (Guimarães et al., 2019). Because Gram-negative bacteria possess an outer membrane whose lipopolysaccharide layer restricts the entry of hydrophobic molecules (Nikaido, 2003), essential oils are commonly reported to be more active against Gram-positive organisms, although the magnitude of this difference varies considerably between studies (Valková et al., 2022).

Two limitations recur in this literature. First, essential oil composition varies with plant chemotype, harvest and extraction, so results obtained with laboratory-extracted oils are difficult to transfer to the commercial preparations that a food producer would actually buy. Second, many studies report only agar diffusion endpoints. Where both formats have been applied to lemongrass oil, broth dilution has yielded inhibition at appreciably lower concentrations than agar diffusion (Naik et al., 2010), yet the implications of that discrepancy are rarely pursued. Agar diffusion is convenient but depends on the test substance migrating through a hydrophilic matrix, and a poorly diffusing lipophilic oil may therefore appear inactive against an organism that is in fact susceptible in liquid medium. Where both methods are applied to the same oil and the same strains, the comparison itself becomes informative.

This study therefore evaluated a single, GC-MS-characterised commercial batch of LGEO against three foodborne reference strains using both agar disc diffusion and broth microdilution, with the aims of establishing concentration-dependent inhibition on solid medium, determining minimum inhibitory and operational bactericidal endpoints in liquid medium, and comparing what the two methods indicate about the same organism–oil combinations.

MATERIALS AND METHODS

Essential Oil

A commercially available *C. citratus* essential oil (NHR Organic Oils, United Kingdom; batch 171221-2) was purchased and used throughout. A commercial preparation was chosen in preference to laboratory extraction because it offers a defined, batch-consistent

composition supported by a supplier gas chromatography–mass spectrometry (GC-MS) certificate, and because it represents the material a food producer would use in practice. The oil was stored in amber glass at 4 °C and protected from light. The composition stated on the supplier certificate of analysis for this batch is given in Table 1; total citral (neral plus geranial) was 69.82%, within the certificate specification of 68–75%, with geraniol at 6.03% as the next most abundant constituent. Physical parameters were specific gravity 0.8882 at 20 °C, refractive index 1.4795 at 20 °C and optical rotation -2.5° . The composition is taken from the supplier certificate; no independent GC-MS analysis was performed in this laboratory.

Table 1: Composition of the tested lemongrass essential oil, transcribed from the supplier certificate of analysis for batch 171221-2 (NHR Organic Oils, United Kingdom; origin Sri Lanka; steam distilled; CAS 91844-92-7). Values are as reported by the supplier and were not independently verified

Component	GC value (%)
Camphene	1.63
Methyl heptenone	1.76
Limonene	0.35
Neral	30.33
Geranial	39.49
<i>Total citral (neral + geranial)</i>	<i>69.82</i>
Geraniol	6.03
Geranyl acetate	2.90
β -Caryophyllene	1.43
α -Pinene	0.29

Bacterial Strains and Inoculum Preparation

Three reference strains were used for the antibacterial assays: *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922 and *Salmonella enterica* serovar Typhimurium ATCC 14028. *S. aureus* ATCC 29213 served as the experimental strain for both the disc diffusion and broth microdilution assays. *S. aureus* ATCC 25923 was used separately as the quality-control strain for antibiotic disc diffusion, in line with Clinical and Laboratory Standards Institute recommendations, and was not exposed to LGEO; its zone diameters were not pooled with the experimental data. Identity and purity were confirmed before testing by colonial morphology on blood agar and on selective media (mannitol salt agar, MacConkey and eosin methylene blue agar, and *Salmonella–Shigella* agar respectively), Gram staining, and biochemical testing — catalase and tube coagulase for *S. aureus*, and triple sugar iron, urease, citrate, motility, indole, methyl red and Voges–Proskauer for the Gram-negative strains. Cultures were subcultured

every 14 days and maintained on nutrient agar at 4 °C.

Inocula were prepared by direct colony suspension from 18–24 h nutrient agar cultures into sterile tryptic soy broth and adjusted to 0.5 McFarland turbidity (approximately 1.5×10^8 CFU/mL) using a densitometer, or spectrophotometrically at 625 nm to an optical density of 0.08–0.13 where tube diameter precluded densitometry. Suspensions were used within 15 min of preparation.

Disc Diffusion Assay

Disc diffusion followed the approach of Valková et al. (2022) with modifications. LGEO was prepared in sterile distilled water containing 0.5% (v/v) Tween-80 as emulsifier and diluted to 5, 10, 20, 50 and 100% (v/v). Standardised inoculum was swabbed evenly onto Mueller–Hinton agar (90 mm plates, 4 mm depth). Sterile 6 mm filter paper discs were impregnated with 10 µL of each dilution and dried for 30 min at 25 ± 2 °C in a laminar flow cabinet. Plates were held for a 15 min pre-diffusion period and incubated at 37 °C for 18–24 h. Ampicillin/sulbactam (SAM, 20 µg) served as the antibiotic control for *S. aureus* and *E. coli* and gentamicin (CN, 30 µg) for *S. Typhimurium*; 0.5% Tween-80 served as the vehicle control. Zone diameters, inclusive of the 6 mm disc, were measured to the nearest whole millimetre. Each organism–concentration combination was tested in triplicate on independent plates ($n = 3$).

Antibiotic discs were included to confirm organism viability and expected susceptibility, not as potency comparators for the oil. Direct comparison of zone diameters between a defined-mass antibiotic disc and a volumetrically applied essential oil is not methodologically valid, as the two differ in molecular mass, polarity and diffusion behaviour in agar. Assay performance was verified against Clinical and Laboratory Standards Institute quality-control ranges (Clinical and Laboratory Standards Institute, 2024).

Broth Microdilution: MIC and MBC

MIC was determined in 96-well microtitre plates. Mueller–Hinton broth containing 1% (v/v) Tween-80 was dispensed into columns 2–8, and 200 µL of 20% (v/v) LGEO stock was placed in column 1. Two-fold serial dilution was carried out from column 1 to column 8, discarding the final 100 µL, after which standardised inoculum was added at a 1:1 ratio. This gave final LGEO concentrations of 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156 and 0.078% (v/v) and a final Tween-80 concentration of 0.5% (v/v), matching the disc diffusion assay. Four controls were included: a growth control (broth plus inoculum), a sterility control (broth only), a vehicle control (1% Tween-80 broth plus inoculum)

and an oil-turbidity blank (LGEO stock plus broth, without inoculum). Plates were sealed and incubated at 37 °C for 18–24 h, and the MIC was read as the lowest concentration showing no visible turbidity or pellet. All assays were performed in triplicate.

Following the approach of Guimarães et al. (2019), 10 µL from each well was subcultured onto Mueller–Hinton agar for MBC determination and incubated at 37 °C for 18–24 h. All wells were subcultured because essential oil emulsions at higher concentrations produce turbidity that can obscure visual MIC reading. No chemical neutraliser or wash step was used, so residual oil carry-over onto the subculture plate cannot be excluded. Because viable counts of the starting inoculum were not performed, the conventional $\geq 99.9\%$ reduction criterion could not be verified; endpoints are therefore reported as operational “no growth on subculture” values rather than as proven bactericidal concentrations.

Statistical Analysis

Zone diameters are summarised as mean $\pm$ standard deviation of three replicates. The effects of organism, LGEO concentration and their interaction were examined by two-way analysis of variance (ANOVA) on the replicate-level data, with partial eta squared reported as the effect size. Concentration–response monotonicity within each organism was assessed by Spearman rank correlation. MIC and MBC values derive from two-fold dilutions and are ordinal and interval-censored; they are therefore reported descriptively as replicate-level endpoints together with the modal value and the lowest concentration inhibiting all three replicates, rather than analysed parametrically. Analyses were performed in Python 3.12 (SciPy 1.17, statsmodels), with significance set at $p < 0.05$.

Two features of the design constrain the inference. Cells in which every replicate equalled 6 mm have zero variance, and the 6 mm disc imposes a floor below which inhibition cannot be recorded; ANOVA assumptions of normality and variance homogeneity are therefore not fully satisfied. The analysis is presented for the structure it reveals, with effect sizes and replicate spread given equal weight to the p values.

Biosafety and Ethical Considerations

All work was performed in a Biosafety Level 2 laboratory using a certified Class II biosafety cabinet, and contaminated material was autoclaved at 121 °C and 15 psi for 20 min before disposal. The study used commercial reference strains only and involved no human participants, clinical specimens or animals; institutional bioethics

approval was therefore not required.

RESULTS

Disc Diffusion

Zone diameters are given in Table 2 and the concentration–response relationships in Figure 1. The vehicle control produced no inhibition for any organism, confirming that 0.5% Tween-80 did not contribute to the observed zones, and all antibiotic controls, including the *S. aureus* ATCC 25923 quality-control run, fell within expected ranges.

S. aureus showed no inhibition at 5 and 20% and only marginal inhibition at 10% (6.67 ± 1.15 mm), but zones increased sharply thereafter to 12.33 ± 6.66 mm at 50% and 25.00 ± 8.00 mm at 100%, exceeding the ampicillin/sulbactam control (23.33 ± 0.58 mm). Replicate spread was substantial at these two concentrations, with individual values of 8–20 mm at 50% and 17–33 mm at 100%. *E. coli* showed smaller but more consistent and monotonically increasing zones, from 6.00 ± 0.00 mm at 5% to 11.67 ± 2.08 mm at 100%. Concentration–response was monotonic for both organisms (Spearman $\rho = 0.83$ for *S. aureus* and $\rho = 0.95$ for *E. coli*, both $p < 0.001$).

S. Typhimurium produced no measurable inhibition beyond the 6 mm disc at any concentration tested, including undiluted oil, while its gentamicin control produced a 20.00 ± 0.00 mm zone, confirming organism viability and assay validity (Figure 2).

Table 2: Zone of inhibition (mm, mean $\pm$ SD, $n = 3$) for lemongrass essential oil and controls. Values include the 6 mm paper disc, so 6.00 mm denotes no inhibition beyond the disc. Antibiotic control was ampicillin/sulbactam (20 μ g) for *S. aureus* and *E. coli* and gentamicin (30 μ g) for *S. Typhimurium*; vehicle control was 0.5% (v/v) Tween-80

Treatment	<i>S. aureus</i>	<i>E. coli</i>	<i>S. Typhimurium</i>
LGEO 5%	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
LGEO 10%	6.67 ± 1.15	7.33 ± 1.15	6.00 ± 0.00
LGEO 20%	6.00 ± 0.00	8.33 ± 0.58	6.00 ± 0.00
LGEO 50%	12.33 ± 6.66	9.67 ± 0.58	6.00 ± 0.00
LGEO 100%	25.00 ± 8.00	11.67 ± 2.08	6.00 ± 0.00
Antibiotic control	23.33 ± 0.58	20.00 ± 0.00	20.00 ± 0.00
Vehicle control	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00

Two-way ANOVA on the replicate-level data showed significant effects of organism, concentration and their

interaction (Table 3). The interaction term carried the largest effect size, reflecting that the three organisms responded to increasing concentration in qualitatively different ways rather than differing by a constant amount.

Table 3: Two-way analysis of variance of zone diameter by organism and LGEO concentration. η^2_p , partial eta squared. Assumption limitations are described in Materials and Methods

Source of variation	df	F	p	η^2_p
Organism	2, 30	13.11	< 0.001	0.47
Concentration	4, 30	13.37	< 0.001	0.64
Organism $\times$ concentration	8, 30	7.15	< 0.001	0.66

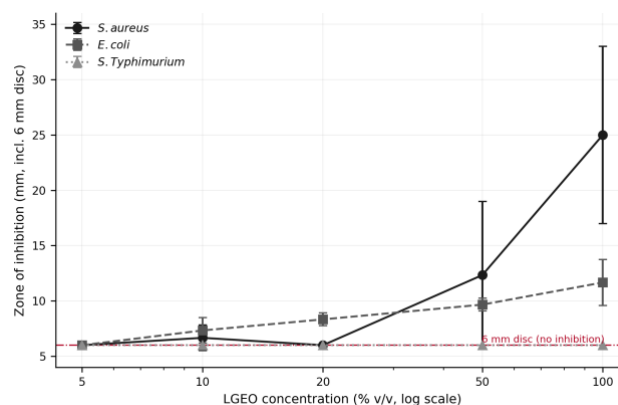


Figure 1: Concentration–response relationship for lemongrass essential oil against the three test organisms by agar disc diffusion. Points are means of three replicates and error bars are standard deviations. The dash-dotted line marks the 6 mm disc diameter, below which no inhibition can be recorded. *S. Typhimurium* remained at this floor at every concentration tested

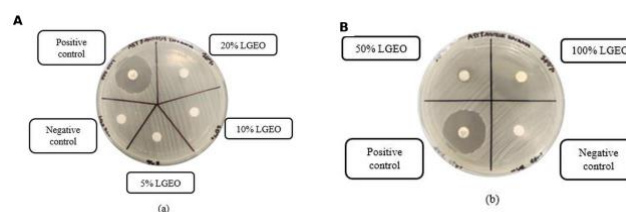


Figure 2: Representative disc diffusion plates for *S. aureus* ATCC 29213 on Mueller–Hinton agar. (A) LGEO at 5, 10 and 20% (v/v) with antibiotic and vehicle controls; (B) LGEO at 50 and 100% (v/v) with controls. Labels are as annotated on the original plates

MIC and MBC

In broth microdilution, all three organisms were inhibited within the tested range, including the organism that had shown no activity on agar (Table 4, Figure 3). Growth controls were turbid, sterility controls remained clear, vehicle controls were turbid in every replicate, and oil-turbidity blanks remained clear, confirming that the readings reflected bacterial growth rather than emulsion turbidity.

For *S. aureus*, two replicates showed no visible growth down to the lowest concentration tested ($\leq 0.078\%$) and the third was turbid at 0.078% with an endpoint of 0.156%. For *E. coli*, replicate MICs were 0.313, 0.313 and 0.156%, giving a modal value of 0.313%. For *S. Typhimurium*, replicate MICs were 0.625, 0.156 and 0.156%; the modal value was 0.156% but the lowest concentration inhibiting all three replicates was 0.625%, and the inter-replicate spread was correspondingly the widest of the three organisms.

Operational no-growth-on-subculture endpoints were 0.156% for *S. aureus* and 0.313% for *E. coli*, the latter

Table 4: MIC and operational MBC endpoints for lemongrass essential oil, expressed as % (v/v). Replicate MICs are listed individually because the summary rule affects the reported value. "All-replicate MIC" is the lowest concentration inhibiting all three replicates. $\leq 0.078\%$ denotes inhibition at the lowest concentration tested (left-censored). MBC values are operational no-growth-on-subculture endpoints, not verified $\geq 99.9\%$ reductions

Organism	Replicate MICs	Modal MIC	All-replicate MIC	MBC
<i>S. aureus</i>	≤ 0.078 , ≤ 0.078 , 0.156	≤ 0.078	0.156	0.156
<i>E. coli</i>	0.313, 0.313, 0.156	0.313	0.313	0.313
<i>S. Typhimurium</i>	0.625, 0.156, 0.156	0.156	0.625	Not reportable

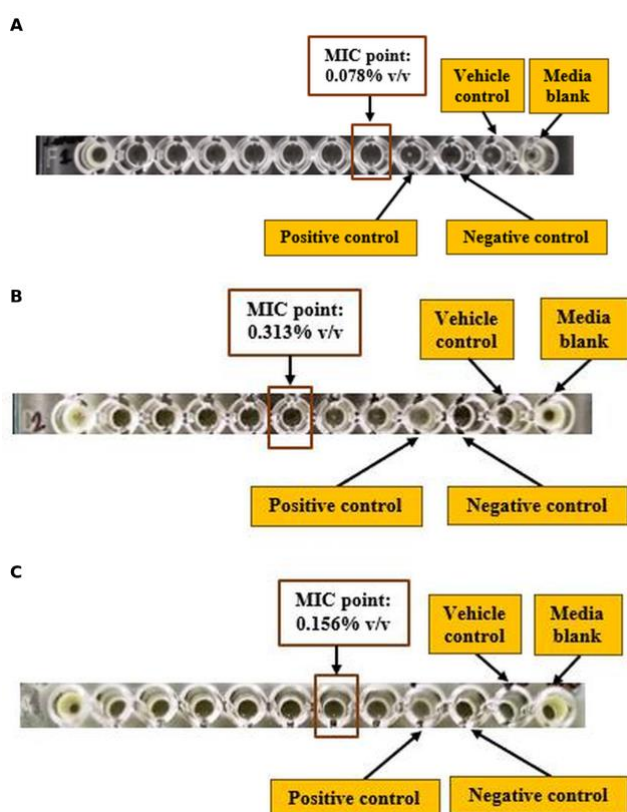


Figure 3: Representative broth microdilution rows after 18–24 h at 37 °C, showing the MIC endpoint and the control wells. (A) *S. aureus* ATCC 29213, endpoint at 0.078% (v/v) in this replicate; (B) *E. coli* ATCC 25922, endpoint at 0.313% (v/v); (C) *S. Typhimurium* ATCC 14028, endpoint at 0.156% (v/v). Each panel shows a single representative replicate; replicate-level endpoints are given in Table 4

DISCUSSION

matching its MIC in all three replicates. One anomaly requires disclosure: in *S. aureus* replicate 3, the well at 0.078% was turbid at MIC reading yet yielded no growth on subculture, which taken literally places the bactericidal endpoint below the inhibitory one. This is not biologically interpretable and most likely reflects either misreading of a faintly turbid well or oil carry-over onto the subculture plate; no bactericidal ratio has been calculated from this replicate. The *S. Typhimurium* subculture assay gave internally inconsistent results and was judged uninterpretable; no MBC is reported for that organism and the assay requires repetition.

Three findings emerge from this study, and the most consequential is not the potency of the oil but the disagreement between the two methods used to measure it. On agar, *S. Typhimurium* appeared entirely unaffected by LGEO at every concentration up to undiluted oil; in broth, the same strain was inhibited at 0.156–0.625% (v/v). Any study relying on disc diffusion alone would have concluded that this organism was resistant, and would have been wrong.

The explanation is methodological rather than biological. Agar diffusion requires the test substance to migrate through a hydrophilic gel, and citral and the accompanying monoterpenes are lipophilic and poorly water-soluble. An emulsified oil applied to a paper disc therefore establishes only a shallow concentration gradient, and a measurable zone forms only where the organism is susceptible to the very high local concentration immediately around the disc. Broth microdilution removes this constraint by dispersing the oil throughout the medium with an emulsifier. This interpretation is supported by the only comparable study to apply both formats to lemongrass oil: Naik et al. (2010) reported that all their test organisms were inhibited at lower concentrations by broth dilution than by agar diffusion. The present data extend that observation to its limiting case, in which an organism scored as wholly inactive on agar proved susceptible in broth at well under 1% (v/v). The practical implication is that disc diffusion is acceptable for ranking activity but should not be used on its own to declare an organism unaffected by an essential oil — a conclusion that applies well beyond the present

three strains.

The second finding concerns the Gram-positive selectivity commonly reported for essential oils. On agar, the expected pattern appeared clearly: *S. aureus* produced the largest zones at high concentration, exceeding its antibiotic control, and this is consistent with the conventional explanation that the outer membrane of Gram-negative bacteria restricts the entry of hydrophobic terpenoids (Nikaido, 2003; Valková et al., 2022). Naik et al. (2010) reached the same conclusion for lemongrass oil, as did Valková et al. (2022) for structurally related citrus oils. In broth, however, the MIC values for the three organisms fell within a two- to four-fold range of one another, which is narrow for a dilution assay and considerably narrower than the agar data would suggest. The apparent selectivity was therefore substantially an artefact of the assay format. Luang-In et al. (2024) similarly found only a narrow separation between *E. coli* and *S. aureus* when a citral-rich lemongrass oil was assessed quantitatively, which is difficult to reconcile with the large differences routinely reported from zone diameters alone. This does not overturn the outer-membrane explanation, but it does indicate that its magnitude has probably been overstated in studies that rest on diffusion endpoints alone.

Third, the concentrations required in broth were low. Inhibition at 0.078–0.625% (v/v) is at the more active end of the published range for lemongrass oil and is plausible for a citral-rich commercial preparation. Citral is the constituent to which most of this activity is attributed: it partitions into the cytoplasmic membrane, increases permeability and dissipates the proton-motive force (Guimarães et al., 2019), and isolated citral reproduces much of the activity of the whole oil against *S. aureus* (Gao et al., 2020). Direct numerical comparison across studies is nevertheless difficult, because potency is variously expressed as % (v/v), µg/mL or µL/disc and because oils differ in citral content; Luang-In et al. (2024), for instance, reported inhibition of *E. coli* and *S. aureus* at 8–10 µg/mL for an oil containing 79.24% citral, somewhat richer than the 69.82% of the batch tested here, a value that cannot be placed on the same axis as a volumetric percentage without knowing the density and citral fraction of both preparations. This is itself an argument for reporting the GC-MS composition of the tested oil alongside its MIC, which comparatively few studies do. Whether such concentrations translate into a food matrix is a separate question: essential oils partition into lipid and protein, are inactivated by high fat content, and impose a strong sensory penalty at concentrations far below those tested here. These results should therefore be read as establishing in vitro potential rather than as supporting any immediate application in food preservation.

Several limitations should be acknowledged. A single commercial batch was tested, so batch-to-batch variation was not assessed, and the composition of the oil is taken from a supplier certificate rather than from independent GC-MS analysis in this laboratory, which limits comparison with studies that report their own citral quantification (Luang-In et al., 2024). Only three replicates were performed per condition, and the replicate spread for *S. aureus* at 50 and 100% was wide enough (8–20 mm and 17–33 mm respectively) that the mean values at those concentrations should be treated as indicative; a repeat using calliper measurement would be needed before those two means could carry any weight. Zone diameters were measured with a ruler to the nearest whole millimetre, which is coarse relative to that spread. The two-way ANOVA is reported for the structure it reveals, but the 6 mm floor produced zero-variance cells and its assumptions are not fully met. No neutraliser was used in the MBC subculture, so oil carry-over cannot be excluded, and viable counts were not performed, so the endpoints are operational rather than verified bactericidal concentrations. The *S. Typhimurium* MBC is missing entirely, and one *S. aureus* replicate produced an internally inconsistent MIC–MBC pair. Finally, only three reference strains were examined; food isolates may differ from laboratory-adapted ATCC strains in susceptibility.

Future work should test multiple batches with independent GC-MS confirmation, increase replication, and add time-kill kinetics, which would give a far better measure of bactericidal activity than the subculture endpoint used here. Repeating the *S. Typhimurium* MBC with a validated neutralisation step, and re-testing the single *S. aureus* replicate that gave an internally inconsistent MIC–MBC pair, would close the two remaining gaps in the present dataset; both assays are in progress. Evaluation in a defined food matrix, with sensory assessment alongside microbiological endpoints, would be the logical next stage.

CONCLUSION

Commercial lemongrass essential oil inhibited *Staphylococcus aureus*, *Escherichia coli* and *Salmonella Typhimurium* in broth microdilution at 0.078–0.625% (v/v), with operational no-growth endpoints of 0.156% for *S. aureus* and 0.313% for *E. coli*. On agar, activity was concentration-dependent for *S. aureus* and *E. coli* but entirely absent for *S. Typhimurium* at every concentration tested, including undiluted oil. This discordance is the study's principal message: agar disc diffusion substantially underestimates the activity of a lipophilic essential oil and should not be used alone to conclude that an organism is unaffected. Within the limits of a small in vitro study using

a single oil batch and three reference strains, these findings support the antibacterial potential of lemongrass oil against common foodborne pathogens and indicate that broth-based endpoints should be reported whenever essential oils are screened.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Beyond Fibre: Exploring *Hibiscus cannabinus* L. Root Extract for *Aedes albopictus* Larval Control

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ABSTRACT

Background: Escalating insecticide resistance among mosquito vectors necessitates the development of plant-derived alternatives for larval control. While *Hibiscus cannabinus* L. is heavily cultivated for its stem fibre, its root system remains largely underexplored. This study evaluated the larvicidal potential of an aqueous root extract of *H. cannabinus* L. against *Aedes albopictus* to establish quantitative baseline mortality thresholds. **Methods:** Dried, pulverized *H. cannabinus* L. roots were extracted via aqueous maceration (1:20 w/v) for 72 h, filtered, and lyophilized. Field-collected larvae were morphologically identified, and third- to fourth-instar *Ae. albopictus* specimens were exposed to the extract at concentrations ranging from 200 to 1,000 ppm. Mortality was recorded at 24 and 48 h post-exposure, analysed via one-way ANOVA, and subjected to log-probit analysis to estimate LC₅₀ and LC₉₀ values. **Results:** The extract induced significant, concentration-dependent larval mortality across both exposure intervals ($p < 0.001$). At 600 ppm, mortality reached 97.33%, while complete mortality (100%) was achieved at concentrations ≥ 800 ppm. Probit analysis indicated an exposure-time component, with lethal concentration thresholds decreasing from 24 to 48 h. **Conclusion:** Aqueous *H. cannabinus* L. root extract demonstrates effective larvicidal activity against wild *Ae. albopictus*. Repurposing this agricultural biomass offers an eco-friendly source for alternative vector control tools. However, phytochemical profiling, non-target ecotoxicological screening, and semi-field trials are required to determine its practical field feasibility.

Keywords:

Aedes albopictus; botanical larvicide; *Hibiscus cannabinus*; larvicidal activity

Article history:

INTRODUCTION

Dengue remains a critical mosquito-borne viral disease, imposing a substantial healthcare burden worldwide. In Malaysia, specific climatic and environmental factors actively favour the proliferation and year-round persistence of *Aedes* vectors (Hii et al., 2016). The severity of localized transmission is underscored by epidemiological data from 2024, which recorded 122,423 dengue cases and 117 deaths across the country (Ministry of Health Malaysia [MOH], 2025), reflecting a sustained historical trend documented over the past decade (Khaw et al., 2024). *Aedes albopictus* acts as a primary secondary vector driving this transmission cycle, alongside *Aedes aegypti* (Torto & Tchouassi, 2024).

Because commercial dengue vaccines face deployment limitations, disease mitigation depends heavily on suppressing vector populations (Low et al., 2025). Larval control operations are particularly pivotal as they target the immobile, aquatic stages of development, successfully preventing the emergence of adult mosquitoes (Hong et al., 2025). Historically, synthetic chemical larvicides, particularly the organophosphate temephos, have formed the cornerstone of Malaysian vector-control programs (Wan-Norafikah et al., 2023). Continuous, intensive applications of these synthetic compounds, however, exert high selective pressure on wild populations. Recent surveillance confirms that repetitive chemical exposure has severely compromised vector susceptibility, giving rise to widespread multi-class insecticide resistance across various domestic field strains (Rasli et al., 2021).

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Insecticide resistance, coupled with the environmental consequences of repeated chemical applications, necessitates the development of plant-derived larvicides. Botanical extracts serve as viable alternatives to conventional insecticides due to their diverse, naturally occurring bioactive compounds (Mohd Salleh et al., 2022; Noorazlan et al., 2024). Previous studies have demonstrated that plant-derived compounds can induce larval mortality and disrupt physiological and biochemical processes in mosquitoes (Rohmah et al., 2020; Rodrigues et al., 2021; Sofi et al., 2022; Boulkenafet et al., 2023).

Hibiscus cannabinus L. is primarily cultivated for its stem fibre. While its nutritional profile, general phytochemistry, and diverse pharmacological attributes including antioxidant, antimicrobial, and anti-inflammatory properties, have been documented (Sim & Nyam, 2021), research has focused predominantly on the leaves, seeds, flowers, and stems (Norhisham et al., 2023). Consequently, other plant fractions remain underexplored, with the root system being substantially less examined. Although Kujoana et al. (2025) reported the presence of phenolic compounds within *H. cannabinus* L. roots, the larvicidal potential of this specific aqueous fraction against *Ae. albopictus* has not been characterized. This study therefore aimed to evaluate the larvicidal efficacy of an aqueous *H. cannabinus* L. root extract against third- and fourth-instar *Ae. albopictus* larvae.

MATERIALS AND METHODS

Plant Material and Aqueous Extraction

Roots of *H. cannabinus* L. were collected from Lembaga Kenaf dan Tembakau Negara, Felda Panching Timur, Kuantan, Pahang, Malaysia (3°49'07" N, 103°11'58" E). The material was washed, dried at 40°C for 9 days, and pulverized. The resulting powder was macerated in distilled water (1:20 w/v) for 72 h under orbital agitation (180–190 rpm) at room temperature. Following centrifugation at 3,000 rpm for 5 min, the supernatant was filtered, frozen at –80°C and lyophilized. The dried aqueous extract was stored at –20°C until further use.

Mosquito Collection and Identification

A total of 200 ovitraps were deployed across three selected locations within the Universiti Teknologi MARA (UiTM) Puncak Alam Campus, Kuala Selangor, Malaysia (3°12'03" N, 101°26'51" E). The traps were retained in the field for 3–4 days, after which the collected eggs and larvae were transferred to the Vector Control Research Laboratory, placed in decanted water and fed *ad libitum*. Third- and fourth-instar larvae were examined

morphologically, and only *Ae. albopictus* specimens were selected for the study.

Preparation of Test Concentration

A stock solution of the freeze-dried *H. cannabinus* L. extract (10 mg/mL, 1% w/v) was prepared via sonication for 30 min at 30–38°C. The stock was subsequently diluted to yield final working concentrations of 200, 400, 600, 800, and 1,000 ppm.

Larvicidal Bioassay

The larvicidal bioassay was conducted in accordance with World Health Organization guidelines (WHO, 2005), with modifications. Groups of 25 healthy third- to fourth-instar larvae were exposed to 100 mL of test medium in triplicate. Positive (1 mg/L temephos) and negative (1% ethanol–water) controls were included. Although the extract was prepared by aqueous maceration, ethanol was subsequently added as a co-solvent to facilitate dissolution; hence, 1% ethanol–water was included as the negative control to account for potential solvent effects (Chan et al., 2022). Controlled conditions were maintained at 27 ± 2°C under a 12:12 h light:dark cycle without food provision. Unresponsive or moribund larvae were scored as dead after 24 and 48 h.

Statistical Analysis

Statistical analyses were performed using SPSS. Data from the triplicate assays were pooled and evaluated via one-way analysis of variance (ANOVA), with statistical significance defined at $p < 0.05$. Log-probit analysis was used to estimate the LC₅₀ and LC₉₀ values, along with their 95% confidence intervals.

RESULTS

Morphological Identification

Morphological examination confirmed the test larvae as *Ae. albopictus* based on characteristic diagnostic keys, specifically the alignment and structure of the comb scales and ventral brush.

Larvicidal Bioassay and Statistical Evaluation

The larvicidal activity of *H. cannabinus* L. root extract against *Ae. albopictus* demonstrated a clear concentration-dependent mortality profile at both 24 and 48 h of exposure (Figure 1).

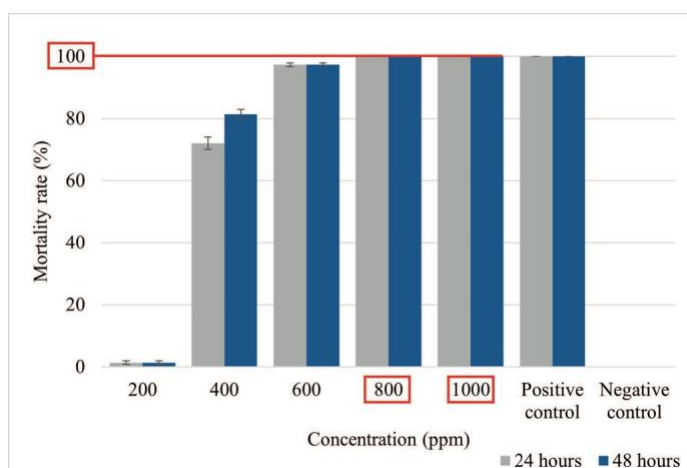


Figure 1: Larval mortality (%) of *Ae. albopictus* following 24 and 48 h of exposure to varying concentrations of *H. cannabinus* L. aqueous root extract. Data points represent the mean of triplicate bioassays

The lowest concentration (200 ppm) exhibited negligible larvicidal efficacy, causing only 1.33% larval mortality at both time points, which was not statistically distinct from the negative control (0%; $p > 0.05$). In contrast, the positive control (1 mg/L temephos) yielded 100% mortality uniformly across all tested timepoints. A sharp increase in efficacy was observed at 400 ppm, with mortality rising from 72.00% at 24 h to 81.33% at 48 h. Strong larvicidal activity was achieved at 600 ppm (97.33%), while complete larval mortality was successfully induced at concentrations of 800 ppm and 1,000 ppm across both exposure windows. One-way ANOVA confirmed highly significant variations in larval mortality across the tested groups at both 24 h ($F(6, 14) = 609.98$, $p < 0.001$) and 48 h ($F(6, 14) = 960.78$, $p < 0.001$).

Probit Analysis

Log-probit analysis indicated that the larvicidal efficacy of the extract enhanced with longer exposure periods, as demonstrated by the decrease in lethal concentration thresholds from 24 h to 48 h (Table 1). The LC_{50} value declined from 347.89 ppm (95% CI: 321.48–372.12 ppm) at 24 h to 330.83 ppm (95% CI: 305.44–354.38 ppm) at 48 h. Similarly, the LC_{90} values dropped from 487.38 ppm (95% CI: 452.18–538.21 ppm) to 458.88 ppm (95% CI: 425.88–504.75 ppm) over the same period.

Table 1: Lethal concentration (LC_{50} and LC_{90}) values of *H. cannabinus* L. aqueous root extract against *Ae. albopictus* larvae

Exposure Period (h)	LC_{50} (ppm)	95% Confidence Interval	LC_{90} (ppm)	95% Confidence Interval
24	347.89	321.48–372.12	487.38	452.18–538.21
48	330.83	305.44–354.38	458.88	425.88–504.75

DISCUSSION

The larvicidal efficacy observed in this study represents the first report on the larvicidal properties of *H. cannabinus* L. root extract against field-derived *Ae. albopictus*. The clear concentration-dependent mortality profile, coupled with the temporal reduction in lethal concentration values (LC_{50} and LC_{90}) from 24 to 48 hours, demonstrates that larvicidal activity is a function of both phytochemical density and exposure duration. While lower concentrations proved insufficient, the rapid achievement of complete mortality at elevated doses suggests the presence of highly active bioactive constituents within the root biomass.

The concentration-dependent response observed is consistent with findings reported for other botanical larvicides. Rohmah et al. (2020), for example, reported concentration-dependent mortality of *Ae. aegypti* exposed to *Averrhoa bilimbi* fruit extract, with an LC_{50} of 977 ppm. This value was higher than the LC_{50} recorded for *H. cannabinus* root extract in the present study. Differences in lethal concentrations between botanical studies may be influenced by factors such as the mosquito species, plant material, and extraction method (Rodrigues et al., 2021).

The larvicidal activity of the root extract is also evident when compared with other recent botanical assays. For example, *Delphinium speciosum* extracts required much higher toxicity thresholds, exceeding LC_{50} and LC_{90} values of 500 ppm and 1,000 ppm, respectively, at 24 h post-treatment (Sanei-Dehkordi et al., 2024). Given that complete mortality was achieved at 800 ppm, the crude aqueous *H. cannabinus* L. root extract represents a highly promising biopesticide candidate. Nevertheless, further evaluation is required before its suitability for practical vector control can be established.

Previous phytochemical profiling of *H. cannabinus* roots identified a range of phenolic acids, flavonoids and other compounds, with kelampayoside A, oxyisocyclointegrin, suberic acid and sibiricose A3 reported at relatively high levels in the roots (Kujoana et al., 2025). Although their

larvicidal properties have not been established, plant-derived phenolic compounds have been associated with several mechanisms of mosquito toxicity. Phenylpropanoids isolated from *Piper sarmentosum*, for instance, demonstrated larvicidal activity against *Ae. albopictus* together with acetylcholinesterase inhibition (Hematpoor et al., 2016), while flavonoids have been shown to inhibit Noppera-bo, a glutathione S-transferase involved in ecdysteroid biosynthesis and larval development in *Ae. aegypti* (Inaba et al., 2022). Other botanical extracts have also been associated with larval midgut damage (Boulkenafet et al., 2023; Rohmah et al., 2020) and inhibition of detoxifying enzymes (Rodrigues et al., 2021). Collectively, these findings illustrate the diverse mechanisms underlying botanical larvicidal activity, including neurotoxic, growth-regulatory, midgut-damaging and detoxification-related effects. However, the mechanism underlying the larvicidal activity of *H. cannabinus* root extract could not be elucidated in the present study. As phytochemical screening was not performed and the extraction solvent differed from that used by Kujoana et al. (2025), the specific constituents responsible for the observed larvicidal activity remain unknown.

The use of field-derived *Ae. albopictus* is relevant because wild mosquito populations may differ from laboratory strains in their susceptibility to larvicides. However, wild populations possess inherent genetic heterogeneity and potential insecticide resistance from localized vector control operations, as documented by Rasli et al. (2021). However, this study did not evaluate the toxicity to non-target aquatic organisms. Further studies should assess its effects on non-target organisms, and evaluate the extract under semi-field conditions.

CONCLUSION

Aqueous *H. cannabinus* L. root extract exhibits effective, concentration-dependent larvicidal activity against field-derived *Ae. albopictus*. These findings provide a valuable baseline for developing alternative, eco-friendly biopesticides and highlight a potential use for the underutilized root biomass of *H. cannabinus*, a crop primarily cultivated for fibre. However, the crude extract remains a candidate raw material rather than a finished formulation. Advancing toward field application requires subsequent bioassay-guided fractionation to isolate primary active compounds, mechanism-of-action characterization, and ecotoxicological safety evaluations against non-target aquatic species.

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Knowledge, HPV Vaccination Willingness, Awareness and Concerns Regarding HPV Self-Sampling Among Business Students at UiTM Puncak Alam

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ABSTRACT

Background: Human papillomavirus (HPV) is a sexually transmitted infection associated with cervical cancer and other diseases. Knowledge of HPV prevention is important among university students, particularly non-health university students. This study assessed knowledge of HPV infection and vaccination, vaccination status and willingness, and awareness and concerns regarding HPV self-sampling among Bachelor of Business in Event Management (Honours) students at Universiti Teknologi MARA (UiTM) Puncak Alam. **Methods:** A cross-sectional study involved 121 students using a bilingual, self-administered online questionnaire. Data were analysed using IBM SPSS Statistics version 29.0. **Results:** The overall HPV knowledge score was 7.98/21 (38.02%), with 76.0% classified as having poor knowledge. Mean infection and vaccination knowledge scores were 3.76/10 (37.60%) and 4.22/11 (38.39%), respectively. HPV infection knowledge was moderately and positively correlated with HPV vaccination knowledge (Spearman's $r_s = .573$, $p < .001$). Overall, 56 (46.3%) respondents reported HPV vaccination. Among the 65 unvaccinated respondents, 20 (30.8%) were willing, 26 (40.0%) were unwilling, and 19 (29.2%) were unsure. Lack of information was the main reason for unwillingness (41.2%). Only 41 respondents (33.9%) had previously heard of HPV self-sampling. Among the 19 respondents who identified specific concerns, accuracy and sample quality and pain, discomfort, or hymen-related concerns were the most frequently reported (13/19, 68.4% each). In reduced binary logistic regression involving 64 unvaccinated respondents, previous awareness of HPV self-sampling was associated with higher odds of vaccination willingness (odds ratio = 5.325, 95% confidence interval 1.293–21.927, $p = .021$), although the model was not statistically significant ($\chi^2(3) = 7.515$, $p = .057$). **Conclusion:** These findings indicate substantial gaps in HPV knowledge and self-sampling awareness, supporting targeted, evidence-based, gender-inclusive HPV education.

Keywords:

human papillomavirus; HPV vaccination; HPV self-sampling; knowledge; awareness

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INTRODUCTION

Human papillomavirus (HPV) infection is a major global public health concern and the necessary cause of cervical cancer. Persistent infection with high-risk HPV types is responsible for the vast majority of cervical cancer cases, making HPV prevention and early detection central to cervical cancer control. Among more than 200 identified HPV types, HPV-16 and HPV-18 account for approximately 70% of cervical cancers worldwide and contribute substantially to other anogenital cancers, including anal, vulvar, vaginal, and penile cancers. In contrast, low-risk HPV-6 and HPV-11 are predominantly associated with anogenital warts (de Martel et al., 2017; Regasa et al., 2023). Although cervical cancer is largely preventable

through HPV vaccination, screening, and appropriate treatment, it remains an important global health burden. The World Health Organization (WHO) has therefore established a global cervical cancer elimination strategy based on the 90–70–90 targets: 90% of girls fully vaccinated against HPV by age 15, 70% of women screened using a high-performance test by ages 35 and 45, and 90% of women with cervical disease receiving appropriate treatment (WHO, 2024).

HPV vaccination represents an important primary prevention strategy, while cervical cancer screening provides an essential opportunity for secondary prevention through the early detection of high-risk HPV infection and cervical precancerous lesions. Conventional

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cytology-based screening, including the Pap smear, has increasingly been complemented by primary HPV DNA testing. Compared with cytology, HPV-based screening provides greater sensitivity for detecting high-risk HPV infection and may support earlier identification of women at increased risk of cervical precancer and cancer, as well as longer screening intervals (Verma et al., 2025; Shao et al., 2024). Current WHO guidance supports HPV DNA testing as a high-performance screening approach and allows either healthcare-provider-collected or self-collected samples where HPV testing is available (WHO, 2021). In Malaysia, national cervical cancer screening guidance has incorporated HPV testing and self-sampling as screening approaches, reflecting the growing role of HPV-based strategies in cervical cancer prevention (Ministry of Health Malaysia, 2023).

HPV self-sampling may further improve access to cervical cancer screening by providing greater privacy, convenience, and flexibility for individuals who may face barriers to facility-based screening. WHO recommends HPV self-sampling as an additional approach to cervical cancer screening and notes its potential to increase screening participation and coverage (WHO, 2023). Evidence among university students also indicates that awareness and knowledge of HPV, vaccination, and self-sampling remain variable, suggesting that the availability of preventive technologies alone may not ensure their effective uptake (Shao et al., 2024; Tiiti et al., 2025). In particular, Tiiti et al. (2025) demonstrated the relevance of HPV knowledge and awareness of self-sampling among female university students, highlighting the importance of accessible and appropriate HPV education within young adult populations.

Despite advances in HPV vaccination and screening, knowledge and awareness remain important determinants of preventive health behaviour. University students represent an important population for HPV prevention because they are at an age when health knowledge, attitudes, and behaviours may influence future decisions regarding vaccination, sexual health, and participation in cervical cancer screening. However, students enrolled in non-health-related programmes may have fewer opportunities for formal exposure to HPV, sexual and reproductive health, and cervical cancer prevention information. Consequently, gaps in knowledge may affect their understanding of HPV transmission, associated diseases, vaccination, screening, and emerging approaches such as HPV self-sampling. In Malaysia, limited knowledge and awareness of HPV testing have been reported within university settings, further highlighting the need to strengthen HPV-related health education among young adults (Yun-Vern et al., 2024).

Assessing HPV-related knowledge, vaccination practices and willingness, and awareness of HPV self-sampling among non-health students is therefore important for identifying specific educational needs and informing targeted health promotion. Although previous studies have examined HPV knowledge, vaccination, and self-sampling among university populations, evidence remains limited regarding students from non-health disciplines in the Malaysian context. Furthermore, there is a need to understand not only what students know about HPV, but also their vaccination practices, willingness to be vaccinated, and awareness of newer screening approaches. Addressing these areas may help universities develop more relevant and targeted health education interventions that promote HPV prevention and informed participation in cervical cancer screening.

Therefore, this study aimed to assess HPV-related knowledge, vaccination practices and willingness, and awareness of HPV self-sampling among Bachelor of Business in Event Management students at Universiti Teknologi MARA (UiTM) Puncak Alam, Malaysia. The findings are expected to identify important gaps in HPV-related knowledge and preventive awareness and provide evidence to support targeted, campus-based HPV health education and prevention strategies.

MATERIALS AND METHODS

This study employed a quantitative cross-sectional design to assess HPV knowledge, vaccination practices and willingness, and awareness and perceptions of HPV self-sampling among BA266 students at UiTM Puncak Alam. Data were collected at a single point using a self-administered online questionnaire via Google Forms. This approach enabled efficient, objective, and confidential data collection and statistical analysis.

Sample Design and Setting

The study population comprised undergraduate Business in Event Management students at UiTM Puncak Alam, Selangor. This population was selected because these students are from a non-health-related programme and may have limited exposure to reproductive health education, including HPV infection, vaccination, and self-sampling. Therefore, they represent an appropriate group for assessing HPV-related knowledge and awareness.

Sample Selection

The study population comprises all undergraduate Business in Event Management Students enrolled at UiTM Puncak Alam, Selangor. These students were chosen

because they represent a non-health science group whose awareness of HPV self-sampling testing is expected to be lower compared to students in health-related programs.

Sample Size

The total population of BA266 Business in Event Management students at UiTM Puncak Alam is 177. The required sample size was determined using the Raosoft sample size formula, considering population size, margin of error, confidence level, and response distribution. This approach ensures an appropriate and statistically reliable sample for the quantitative study (Alanazi et al., 2024).

Accordingly, this study used the same proportion (18.4%) to estimate the population parameter for sample size calculation. The sample size was calculated using Eq. (1).

$$n = \frac{Z^2[P(1-p)]}{e^2} \quad (1)$$

where, n = sample size; Z = the z-score, a measure of how confident the study wants to be in its results (1.96 for a 95% confidence level); P = the estimated proportion of the population; and e = the amount of error the study will tolerate in its results (0.06 for a 6% margin of error).

For the BA266 programme, the minimum required sample size was 121 respondents, based on a 95% confidence level and a $\pm 5\%$ margin of error. This sample size was considered appropriate and feasible for the descriptive cross-sectional study using an online survey method.

Questionnaire Development

The main instrument used in this study will be a self-administered online questionnaire created using Google Form. The questionnaire aims to assess the students' knowledge of human papillomavirus (HPV), awareness and understanding of HPV vaccination, and perceptions toward HPV self-sampling as an alternative cervical cancer screening method. This instrument is adapted directly from (Tiiti et al., 2025), entitled "Knowledge of Human Papillomavirus (HPV) and Self-Sampling Including Vaccination Practices Among Female University Students in the Free State, South Africa." The questionnaire that will be used in this study will be in both English and Bahasa Melayu and has been validated for reliability and internal consistency. It has been adopted from previous research with minor changes to fit the Malaysian context and university setting. The questionnaire consists of five sections as described below:

Part A: Demographic information

The first section collected participants' demographic and

health-related information, including age, marital status, education level, sexual activity, contraceptive use, recent healthcare visits, history of sexually transmitted infections (STIs), and family history of cervical cancer. These characteristics were collected to describe the respondents and examine their potential associations with HPV knowledge and preventive behaviours.

Part B: Knowledge of HPV infection

The second section assessed participants' knowledge of HPV infection using 10 statements with "True," "False," and "Don't Know" response options. The items covered HPV transmission, symptoms, natural clearance, and its association with cervical cancer. This section aimed to identify respondents' level of understanding and common misconceptions regarding HPV infection.

Part C: Knowledge of HPV vaccination

The third section assessed participants' knowledge of HPV vaccination using 11 True/False/Don't Know statements. The items covered vaccine availability, purpose, effectiveness, eligibility, vaccination schedules, and its role in preventing cervical cancer and genital warts. This section aimed to determine students' understanding of HPV vaccination and identify misconceptions that may affect vaccine uptake.

Part D: HPV vaccination practices

This section assessed participants' HPV vaccination practices, including vaccination status, willingness to receive the vaccine, and reasons for hesitancy or refusal. Respondents could select multiple barriers, such as limited information, safety concerns, fear of injections, religious considerations, or perceived lack of necessity. The findings help identify factors influencing HPV vaccine acceptance among university students.

Part E: HPV self-sampling awareness and perceptions

The final section assessed participants' awareness and perceptions of HPV self-sampling for cervical cancer screening. Respondents were asked about their prior awareness and concerns, including accuracy, discomfort, effects on virginity, lack of information, cost, and accessibility. Before data collection, the questionnaire was pilot-tested among students excluded from the main sample to assess clarity, consistency, and reliability. Feedback was used to refine the questionnaire and ensure that the items were clear, culturally appropriate, and relevant to the target population.

Ethics Approval from the Committee

Ethical approval was obtained from the Research Ethics Committee of Universiti Teknologi MARA (UiTM) under Reference No. FERC/FSK/MR/2026/00075 before data collection commenced. Participation was entirely voluntary, with no incentives or coercion involved. All ethical principles were maintained throughout the research process, including respect for participants' autonomy, confidentiality, and privacy.

Statistical Analysis

After data collection, responses were analysed using IBM SPSS Statistics version 29.0. Data were checked for completeness and accuracy before analysis. Descriptive statistics, including frequency, percentage, mean, standard deviation, and median, were used to summarise respondents' characteristics and HPV knowledge scores. Cronbach's alpha assessed questionnaire reliability, while the Shapiro–Wilk test evaluated data normality. As the data were not normally distributed, appropriate non-parametric tests were applied.

RESULTS

A total of 121 respondents participated in the study. Most were aged 23–25 years (58.7%), female (76.0%), single (92.6%), and pursuing a bachelor's degree (100.0%). Respondents were distributed across all four years of study, with Year 2 comprising the largest proportion (28.1%). Most were not sexually active (89.3%) and did not use contraception (92.6%). Slightly more than half had visited a clinic or health centre within the previous six months (51.2%). None reported a history of sexually transmitted infections, while 2.5% reported a personal or family history of cervical cancer (Table 1).

Table 1: Demographic and background characteristics of respondents

Characteristic	Category	n	%
Age	Under 20 years	1	0.8
	20–22 years	21	17.4
	23–25 years	71	58.7
	Over 25 years	28	23.1
Gender	Male	28	23.1
	Female	92	76.0
	Others	1	0.8
Marital status	Single	112	92.6
	Married	9	7.4
Education	Bachelor's degree	121	100.0
Year of study	Year 1	28	23.1
	Year 2	34	28.1
	Year 3	31	25.6
	Year 4	28	23.1
Currently in a sexual relationship	Yes	13	10.7
	No	108	89.3
Currently using contraception	Yes	9	7.4
	No	112	92.6
Visited clinic in past 6 months	Yes	62	51.2
	No	59	48.8
History of STI in past 6 months	No	121	100.0
Personal/family history of cervical cancer	Yes	3	2.5
	No	118	97.5

Note. N = 121. Percentages may not total exactly 100 due to rounding.

Overall HPV Knowledge Scores and Levels

The scores were calculated separately for HPV infection knowledge, HPV vaccination knowledge, and overall HPV knowledge. The mean, standard deviation, and median were used to describe the scores, while the respondents were also grouped into poor, moderate, and good knowledge levels.

Table 2 shows that the mean HPV infection knowledge score was 3.76 out of 10, while the mean vaccination knowledge score was 4.22 out of 11. For overall HPV knowledge, the respondents obtained a mean score of 7.98 out of 21. After conversion into percentages, the mean overall score was 38.02%. The scores for infection and vaccination knowledge were also low, at 37.60% and 38.39%, respectively. This indicates a generally low level of HPV knowledge among the respondents.

Table 2: Descriptive statistics for HPV knowledge scores

Measure	Possible range	M	SD	Median
HPV infection knowledge	0–10	3.76	1.92	4.00
HPV vaccination knowledge	0–11	4.22	2.58	4.00
Overall HPV knowledge	0–21	7.98	4.08	8.00
HPV infection knowledge (%)	0–100	37.60	19.19	40.00
HPV vaccination knowledge (%)	0–100	38.39	23.46	36.36
Overall HPV knowledge (%)	0–100	38.02	19.45	38.10

Table 3 shows that most respondents were classified as having poor knowledge in all three knowledge areas. For HPV infection knowledge, 66.9% had poor knowledge, while only 4.1% achieved a good level. For vaccination knowledge, 74.4% were classified as poor, and only 5.0% were classified as good. Overall, 76.0% of respondents had poor HPV knowledge, 19.0% had moderate knowledge, and only 5.0% had good knowledge. This finding highlights a clear knowledge gap among the respondents

Table 3: Levels of HPV knowledge

Knowledge domain	Poor n (%)	Moderate n (%)	Good n (%)
HPV infection	81 (66.9)	35 (28.9)	5 (4.1)
HPV vaccination	90 (74.4)	25 (20.7)	6 (5.0)
Overall HPV knowledge	92 (76.0)	23 (19.0)	6 (5.0)

Correlation Between HPV Infection and Vaccination Knowledge

A statistically significant, moderate positive relationship was found between HPV infection knowledge and HPV vaccination knowledge, $r = .573$, $p < .001$. Respondents with higher knowledge of HPV infection tended to have higher knowledge of HPV vaccination.

HPV Vaccination Practice and Willingness Among Respondents

Among the 121 respondents, 56 respondents or 46.3% had received the HPV vaccine. In comparison (Table 4), 65 respondents or 53.7% had not received it. Among the unvaccinated respondents, only 30.8% were willing to receive the vaccine. Meanwhile, 40.0% were unwilling and 29.2% remained unsure. This indicates that HPV vaccination uptake and willingness were still low within the study population.

Table 4: HPV vaccination practice and willingness

Variable	Category	n	%
Received HPV vaccine	Yes	56	46.3
	No	65	53.7
Willingness among unvaccinated respondents	Yes	20	30.8
	No	26	40.0
	Not sure	19	29.2

Note. Willingness percentages in the final analysis were calculated among the 65 unvaccinated respondents.

HPV Self-Sampling Awareness and Concern

Only 33.9% of respondents had previously heard about HPV self-sampling, while 66.1% had never heard about this screening method. This demonstrates a low level of awareness among the respondents. Regarding concerns, 35.5% stated that they had concerns about HPV self-sampling, whereas 64.5% did not report any concerns. However, only 19 respondents selected at least one specific concern. (Table 5)

Table 5: Awareness and concerns regarding HPV self-sampling

Variable	Category	n	%
Previously heard of HPV self-sampling	Yes	41	33.9
	No	80	66.1
Has concerns about HPV self-sampling	Yes	43	35.5
	No	78	64.5

Reasons for the Concerns Regarding HPV Self-Sampling

Accuracy and sample quality were among the main concerns, reported by 68.4% of respondents who selected a concern (Table 6). Pain, discomfort or hymen-related issues were reported by the same percentage. Lack of information and possible effects on health were each selected by 57.9%, while cost and availability were reported by 47.4%. These findings indicate that uncertainty about the reliability, safety and procedure of self-sampling may affect respondents' views towards the method.

Table 6: Reasons for the concerns regarding HPV self-sampling

Concern	Responses, n	Percent of cases
Accuracy and sample quality	13	68.4
Pain, discomfort, or hymen-related concerns	13	68.4
Lack of information or education	11	57.9
Cost and availability	9	47.4
Effects on health	11	57.9
Other concerns	2	10.5

Note. Multiple responses were allowed. Percentages are based on 19 respondents who selected at least one concern; therefore, percentages exceed 100%.

Differences in Overall HPV Knowledge

Mann–Whitney U and Kruskal–Wallis tests were conducted to determine whether statistically significant differences existed in overall HPV knowledge across selected demographic and health-related groups.

There were no statistically significant differences in overall HPV knowledge were found by gender, vaccination status, clinic attendance, or prior awareness of HPV self-sampling. Vaccinated respondents had a higher mean rank than unvaccinated respondents, but the difference did not reach statistical significance ($p = .069$).

Correlation with Overall HPV Knowledge Level

The chi-square analysis found no significant association between overall HPV knowledge level and gender, year of study, vaccination status or previous awareness of HPV self-sampling. All recorded p -values were above .05. Year of study produced the largest Cramer’s V value at .185. However, the strength of the association was still small and not statistically significant. The results should also be interpreted carefully because several expected cell counts were below five. (Table 7)

Table 7: Chi-square tests of associations with overall knowledge level

Variable	χ^2	df	p	Cramér's V
Gender	1.150	4	.886	.069
Year of study	8.277	6	.219	.185
HPV vaccination status	3.626	2	.163	.173
Previous awareness of HPV self-sampling	1.353	2	.508	.106

Note. Several contingency tables contained expected cell counts below five; therefore, chi-square results should be interpreted cautiously.

DISCUSSION

This study examined HPV-related knowledge, vaccination practices and willingness, and awareness and concerns regarding HPV self-sampling among students enrolled in a non-health academic programme at UiTM Puncak Alam. The findings demonstrate substantial gaps in HPV-related health literacy and engagement with preventive strategies in this population. Overall HPV knowledge was low, with a mean score of 7.98 out of 21 (38.02%), and 76.0% of respondents were classified as having poor knowledge. Although 46.3% of respondents reported having received HPV vaccination, more than half remained unvaccinated, and only 30.8% of unvaccinated respondents indicated willingness to receive the vaccine. Awareness of HPV self-sampling was also limited, with only 33.9% reporting that they had heard of the method previously. These findings suggest that being enrolled in higher education does not necessarily translate into adequate knowledge or awareness of HPV prevention, particularly among students outside health-related programmes.

HPV-Related Knowledge

The most prominent finding was the low level of HPV-related knowledge among the respondents. The mean overall knowledge score was only 38.02%, with 76.0% of students classified as having poor knowledge. Knowledge was similarly limited for both HPV infection and HPV vaccination, with mean percentage scores of 37.60% and 38.39%, respectively. This finding is consistent with evidence from university student populations showing that HPV knowledge remains inadequate despite relatively high levels of general educational attainment. A recent systematic review and meta-analysis by Li et al. (2025) reported considerable gaps in HPV knowledge among university students, particularly regarding HPV transmission, prevention and vaccination. Similarly, Tiiti et al. (2025) reported inadequate HPV-related knowledge among university students, highlighting the need for targeted educational interventions among young adults.

The present findings extend these observations to students from a non-health academic programme. Unlike students who receive formal education related to health, medicine or public health, students in programmes such as event management may have fewer opportunities to encounter structured information about HPV and cervical cancer prevention. Therefore, the low knowledge observed in this study may reflect an educational gap rather than a lack of general educational attainment. This is important because HPV prevention requires knowledge of several interconnected components, including HPV infection, vaccination and screening. The World Health

Organization (WHO, 2021, 2023) emphasises vaccination and HPV-based screening as key components of cervical cancer elimination, meaning that inadequate knowledge at the student level may reduce awareness of available preventive opportunities.

A moderate positive correlation was also observed between knowledge of HPV infection and knowledge of HPV vaccination (Spearman's $r_s = 0.573$, $p < 0.001$). This suggests that respondents who demonstrated greater understanding of HPV infection also tended to have better knowledge of HPV vaccination. The finding supports the importance of integrated HPV education rather than addressing vaccination as an isolated health behaviour. Understanding how HPV is transmitted and its relationship with cervical cancer may provide a basis for understanding why vaccination is recommended. However, because this study was cross-sectional, the observed correlation should not be interpreted as evidence that improving infection knowledge would directly increase vaccination knowledge or uptake.

Interestingly, overall HPV knowledge did not differ significantly according to gender, vaccination status, previous healthcare attendance or previous awareness of HPV self-sampling. Similarly, no significant association was observed between knowledge category and gender, year of study, vaccination status or previous awareness of HPV self-sampling. Although vaccinated respondents showed a relatively higher mean knowledge rank, the difference did not reach statistical significance ($p = 0.069$). These findings suggest that the knowledge gap may be relatively widespread across the study population rather than concentrated within a single demographic subgroup. Nevertheless, the absence of statistically significant associations should be interpreted cautiously because the sample size was modest and several categorical analyses contained small cell counts, which may have limited the statistical power to detect meaningful differences.

HPV Vaccination Uptake and Willingness

Almost half of the respondents (46.3%) reported having received HPV vaccination, while 53.7% remained unvaccinated. Although the vaccination proportion indicates that a substantial number of students had already accessed HPV vaccination, the proportion of unvaccinated students remains important from a prevention perspective. Among the unvaccinated respondents, only 30.8% were willing to receive the vaccine, whereas 40.0% were unwilling and 29.2% were unsure. Thus, the findings demonstrate not only an uptake gap but also a considerable willingness gap among those who had not yet been vaccinated.

The coexistence of relatively poor HPV knowledge and incomplete vaccination uptake is consistent with findings reported among university students in previous studies. Li et al. (2025) highlighted that knowledge and awareness can influence HPV vaccination-related attitudes and intentions, although willingness to vaccinate does not necessarily translate into actual vaccination. The present study similarly demonstrates a distinction between vaccination status and future willingness. Importantly, the 29.2% of unvaccinated respondents who were unsure represent a potentially important group for intervention because uncertainty may be more amenable to education and counselling than firm refusal.

However, the present findings do not demonstrate a statistically significant relationship between overall HPV knowledge and vaccination status. Therefore, it would be inappropriate to conclude that poor knowledge alone explains why students were unvaccinated. HPV vaccination behaviour is likely to be influenced by multiple factors, including perceived susceptibility, perceived benefits and risks, accessibility, cost, healthcare provider recommendation, previous exposure to vaccination information and social or cultural influences. Previous research among young adults and university students has similarly indicated that HPV vaccination decisions are multifactorial rather than determined by knowledge alone (Li et al., 2025; Tiiti et al., 2025). Consequently, interventions should combine accurate HPV education with practical information on vaccine availability, eligibility, safety and access.

The findings should also be interpreted in the context of Malaysia's broader cervical cancer prevention strategy. The Ministry of Health Malaysia (MOH, 2023) and WHO (2024) emphasise prevention through HPV vaccination and screening as essential components of cervical cancer elimination. Improving vaccination uptake among university students may therefore represent an opportunity to strengthen primary prevention before students enter later adulthood. University-based health promotion could provide accessible information and facilitate referral or linkage to appropriate vaccination services.

Awareness of HPV Self-Sampling

Awareness of HPV self-sampling was another important gap identified in this study. Only 33.9% of respondents had previously heard of HPV self-sampling, while 66.1% had not. This finding is broadly consistent with previous research showing that awareness of HPV self-sampling remains limited among populations who could potentially benefit from this screening approach. Tiiti et al. (2025), for

example, identified gaps in awareness and understanding of HPV-related prevention among university students, while studies by Shao et al. (2024) and Yun-Vern et al. (2024) similarly highlight the importance of increasing awareness and understanding of HPV self-sampling and HPV screening among relevant populations.

The low awareness observed in the present study is particularly relevant because HPV self-sampling has been promoted as a strategy that may improve accessibility and acceptability of HPV-based screening. WHO (2021, 2023) recognises HPV testing, including approaches involving self-collected samples where appropriately implemented, as an important component of cervical cancer screening strategies. Evidence from Malaysia, including the ROSE programme, has also demonstrated the potential of HPV self-sampling to support screening participation by addressing practical and personal barriers associated with conventional screening approaches (Khoo et al., 2025; Yun-Vern et al., 2024). Therefore, the finding that two-thirds of respondents had never heard of HPV self-sampling indicates a communication gap that should be addressed alongside broader HPV education.

The present study also found that 35.5% of respondents reported having concerns about HPV self-sampling. However, the results indicate that only 19 respondents selected at least one specific concern. Therefore, the finding should be interpreted as evidence that a proportion of respondents had concerns, rather than as evidence that particular concerns were prevalent across the entire sample. Previous studies have identified issues such as uncertainty regarding sample accuracy, discomfort, embarrassment, procedure-related concerns and insufficient information as potential influences on perceptions of self-sampling (Duan et al., 2025; Ji et al., 2025; Mishra et al., 2025). These findings reinforce the importance of providing clear explanations regarding how self-sampling is performed, what the test detects, the accuracy of the method and the importance of appropriate follow-up after an abnormal result.

Importantly, this study assessed awareness and concerns rather than actual acceptance or use of HPV self-sampling. Therefore, the present findings cannot establish whether increasing awareness would result in greater willingness to use self-sampling. Future studies should directly assess intention to use self-sampling, perceived acceptability and factors influencing uptake.

Factors Associated with Vaccination Willingness

The multivariable analysis provided an additional finding of interest. Previous awareness of HPV self-sampling was

identified as a significant predictor of vaccination willingness within the regression model, although the overall regression model was marginally non-significant. This finding should therefore be interpreted as an exploratory association rather than evidence of a definitive predictive relationship.

The association may indicate that students who have previously encountered information about HPV self-sampling are also more engaged with HPV-related health information generally. In other words, awareness of one HPV prevention strategy may reflect broader health awareness or previous exposure to sexual and reproductive health information. However, the cross-sectional design prevents determination of whether awareness of self-sampling preceded or influenced vaccination willingness. It is also possible that students with greater pre-existing health awareness were more likely both to encounter information about self-sampling and to express willingness towards vaccination.

The marginally non-significant overall regression model further suggests that the identified association should not be overinterpreted. The relatively small sample size and limited number of respondents within some outcome categories may have reduced the precision and statistical power of the multivariable analysis. Larger studies should therefore examine whether awareness of self-sampling remains independently associated with vaccination willingness after adjustment for relevant factors such as age, gender, year of study, healthcare utilisation, previous HPV information exposure and socioeconomic characteristics.

Implications for University-Based HPV Prevention

Taken together, the findings indicate that HPV prevention among university students should move beyond general awareness campaigns towards targeted and integrated health education. The particularly low knowledge scores suggest that students require basic, accurate information about HPV infection, transmission, health consequences, vaccination and screening. Education about vaccination should additionally address practical issues such as vaccine benefits, safety, availability and where students can obtain further advice.

The findings also support incorporating information about HPV self-sampling into university health promotion. Because most respondents had never heard of the method, introducing self-sampling without first establishing basic understanding may be insufficient. Educational materials should explain the purpose of HPV testing, the self-collection procedure, test accuracy,

privacy and the importance of follow-up. Evidence from previous studies suggests that appropriate education and clear communication can improve understanding and acceptability of HPV self-sampling (Duan et al., 2025; Ji et al., 2025; Mishra et al., 2025).

Although the study population consisted predominantly of female students, HPV prevention should remain gender-inclusive. HPV infection affects both women and men, and vaccination contributes to prevention of HPV-related diseases beyond cervical cancer. Therefore, university-based interventions should avoid presenting HPV solely as a women's health issue. A gender-inclusive approach may also help reduce misconceptions and improve awareness of HPV vaccination among the wider student population.

Limitations

Several limitations should be considered when interpreting these findings. First, convenience sampling may have introduced selection bias because students who were more interested in HPV, vaccination or health-related issues may have been more likely to participate. Consequently, the findings may not represent students who were less interested in or less knowledgeable about HPV.

Second, the study was conducted within a single academic programme at one university campus. The participants were all BA266 Event Management students at UiTM Puncak Alam, and therefore the findings cannot be assumed to represent students from other academic disciplines, universities or geographical settings. In particular, health-related students may have substantially different exposure to HPV information. The single-programme design therefore limits the generalisability of the findings to the wider Malaysian university population.

Third, the online questionnaire was voluntary and self-administered, creating the possibility of non-response and self-selection bias. Students who chose not to participate may have differed systematically from participants in their HPV knowledge, vaccination experience or health-seeking behaviour. The direction and magnitude of this potential bias cannot be determined from the present study.

Fourth, several variables, including vaccination status and sensitive behavioural information, were self-reported. Self-reported vaccination status may be affected by recall error, particularly if vaccination occurred several years earlier or outside the university setting. Sensitive questions concerning sexual relationships and related health behaviours may also be affected by social desirability bias, whereby respondents may provide

answers they perceive as more socially acceptable. These issues may have influenced the accuracy of the reported estimates.

Fifth, the cross-sectional design means that exposure and outcome variables were measured at the same point in time. Consequently, causal relationships cannot be established. This limitation is particularly relevant to the finding that previous awareness of HPV self-sampling was associated with vaccination willingness, as the study cannot determine whether awareness influenced willingness or whether both were related to another underlying factor.

Sixth, the sample had an imbalance in gender distribution, with 76.0% female respondents and 23.1% male respondents, while only one respondent was categorised as another gender. This imbalance limits the ability to make robust comparisons between gender groups, particularly for the smallest category. The gender distribution may also reduce the representativeness of the findings for a more balanced university population.

Finally, the relatively small sample size and small cell sizes in several categorical analyses limited statistical power. Several chi-square analyses contained expected cell counts below five, which reduces the reliability of conventional chi-square inference. The limited number of observations also reduced the precision of the multivariable logistic regression and may have increased the risk of Type II error, whereby meaningful associations were not detected. Therefore, statistically non-significant findings should not necessarily be interpreted as evidence that no association exists. Larger, multicentre studies with more balanced samples are needed to confirm the observed patterns and identify independent determinants of HPV vaccination and screening-related behaviours.

Overall Interpretation

Overall, this study demonstrates that substantial gaps in HPV prevention knowledge and awareness remain among students in a non-health academic programme. The low knowledge scores, incomplete vaccination uptake, limited willingness among unvaccinated respondents and low awareness of HPV self-sampling indicate that university students may remain insufficiently informed about available HPV prevention strategies. At the same time, the absence of significant associations across several demographic and behavioural variables suggests that the observed knowledge gap may be broadly distributed within this population rather than limited to a single subgroup.

The findings support the need for integrated, accessible and gender-inclusive HPV health education within university settings. Such programmes should address HPV infection, vaccination and HPV-based screening together, while providing practical information that enables students to make informed preventive health decisions. However, because of the methodological limitations and the cross-sectional nature of the study, these findings should be regarded as evidence of an important educational and public health gap rather than evidence of causal relationships. Future research involving larger and more diverse student populations, probability-based sampling and longitudinal or intervention designs would help determine whether improvements in HPV knowledge and awareness translate into greater vaccination uptake and engagement with HPV screening.

CONCLUSION

Overall, this study identified substantial gaps in HPV knowledge, HPV vaccination knowledge, and awareness of HPV self-sampling among non-health university students. Although nearly half of the respondents reported having received HPV vaccination, willingness to vaccinate among unvaccinated students remained limited, with lack of information being the most frequently reported barrier. Awareness of HPV self-sampling was also low, and among respondents who identified specific concerns, concerns regarding sample accuracy and sample quality and pain, discomfort, or hymen-related issues were frequently reported. These findings highlight the need for targeted, evidence-based and gender-inclusive HPV education within university settings to improve knowledge, address information gaps, and increase awareness of HPV vaccination and HPV-based screening options, including self-sampling.

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Volcanic-Derived Soils and Lycopene Accumulation in Tomato: A Narrative Review of Mineral, Agronomic, and Mechanistic Evidence

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ABSTRACT

Background: Lycopene is a health-promoting carotenoid with high concentrations in tomatoes. Commercial and popular narratives often assert that tomatoes grown in the volcanic soils of Mount Vesuvius and Mount Etna contain particularly high lycopene concentrations, an assertion that has largely gone untested in controlled comparative studies. This narrative review summarises the current available evidence for the association between mineral origin of soil and lycopene accumulation in tomato, and distinguishes direct from indirect evidence throughout. **Methods:** A structured literature review was performed across Web of Science, PubMed, Scopus, and Google Scholar, with the final query conducted in August 2026. Restricted to English-language, peer-reviewed publications published between 2010–2026, the search criteria included tomato agriculture, volcanic soil science (Andisol/andic characteristics), soil mineralogy, mineral bioavailability, carotenoid/lycopene biosynthesis, and fruit-quality results. Following removal of duplicates, review of titles/abstracts and then full text, the initial 340 articles were narrowed down to 45 included studies. The remaining categories of evidence, such as volcanic soil mineralogy, nutrient uptake/bioavailability, and carotenoid-pathway biochemistry, were kept as indirect, plausibility-supporting evidence. Only research carried out in volcanic soil systems approached direct evidence on tomato lycopene. **Results:** Volcanic soils, especially Andisols, had high phosphorus retention due to allophane and imogolite clay minerals and were rich in iron, magnesium, calcium, potassium and phosphorus, while the mineral profile of conventionally farmed soils was mainly determined by fertilisation regime, not parent material. Magnesium, iron and zinc have been proposed as cofactors for some enzymes in the carotenoid biosynthesis pathway. Elevated lycopene content in Vesuvian volcanic soils was associated with biostimulant application rather than with volcanic soil origin itself, because no non-volcanic comparator was included; methodological heterogeneity concerning cultivar, fertilisation intensity and extraction method prevented pooled comparison. **Conclusion:** There is evidence that volcanic and conventional soils have different mineral profiles and that there is a possible biochemical mechanism that connects the minerals in the soil to the production of lycopene. Since the impact of matching volcanic and conventional soils on tomato lycopene accumulation under controlled settings has not been explicitly compared in any published study, the mineral-lycopene relationship should be viewed as a testable hypothesis rather than an established effect. To test this theory, research should use uniform cultivar selection, fertilization intensity, and extraction techniques.

Keywords:

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INTRODUCTION

A naturally occurring lipophilic red carotenoid, lycopene is found in high concentrations in tomatoes (8.8–42.0 µg/g fresh weight), watermelon, pink grapefruit, papaya, apricots, and guava (Carvalho et al., 2020; Sigurdson et al., 2017). Lycopene must be received through diet because humans are unable to synthesise it from scratch (Puah et al., 2021). Epidemiological evidence links dietary lycopene to a reduced risk of several chronic diseases, including some cancers and metabolic disorders (Crupi et al., 2023). Given this nutritional and therapeutic value, maximizing lycopene accumulation can be done through soil management practices, particularly fertilization regime, irrigation scheduling and cultivar selection.

Crop quality is significantly influenced by environmental and edaphic (soil-related) factors, according to a large body of research (Blasa et al., 2010). Typically, synthetic fertilisers mixed with organic inputs like vermicompost and enhanced with micronutrients are used in arable agricultural systems to supply minerals. According to field data, these techniques can improve soil fertility in cropping sequences by increasing soil organic carbon and making nitrogen-phosphorus-potassium (NPK) and other micronutrients more accessible (Deepchandra et al., 2025). Essential elements for plant biological processes are found in volcanic ash, and when present in sufficient quantities, these materials can promote plant growth; however, too much can become toxic and have a

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detrimental effect on physiological systems (Carrera-Beltrán et al., 2024). While commercial and popular narratives frequently assert that the volcanic soils of Mount Vesuvius and Mount Etna yield tomatoes with exceptionally high lycopene concentrations, this claim remains largely untested in controlled comparative design (Parisi et al., 2021).

For clarity, this review defines volcanic-derived soil as soil formed from significant amounts of pyroclastic material, which act as parent materials for soil formation or the rejuvenation of existing soils, are frequently produced by volcanic eruptions. The most prevalent types of volcanic ash soils are called andisols or andosols. Approximately 5.395 million hectares, or 2.9% of Indonesia’s total land area are covered by these soils (Kartawisastra & Dariah, 2014). As the original tropical rainforest land region, andisols are widely used for productive agricultural soil, especially for horticulture crops, tea plantations, and coffee plantations (Anda & Dahlgren, 2020).

This review synthesizes the existing evidence bearing on this specific comparison. It first describes the mineral profiles of conventional and volcanic soils independently. Second, it looks at what is known about the bioavailability and uptake of certain minerals. Third, it takes into account how metal cofactors function mechanistically in the process of lycopene biosynthesis. The paper then summarises the findings from fruit studies, including the widely held beliefs that spur this investigation. Lastly, it describes the research approach needed to bridge this gap and discusses the methodological obstacles that have hindered a direct comparison.

MATERIALS AND METHODS

This is a narrative review with a structured, but not systematic, literature search; no protocol was registered and no PRISMA flow diagram, formal risk-of-bias assessment or meta-analysis was undertaken, consistent with the scope of a narrative review for IJAHS. This study endeavoured to gather a comprehensive selection of 340 articles from various databases, including Web of Science (WoS), PubMed, Scopus and Google Scholar. Search terms spanned tomato cultivation, soil mineralogy, carotenoid and lycopene biosynthesis, and volcanic soil science. Each record was screened against a set of inclusion and exclusion criteria.

Records were included if they addressed one of five evidence categories: (1) volcanic soil mineralogy, including Andisol and Andosol classification and andic soil

properties; (2) conventional soil mineralogy under managed N-P-K and micronutrient fertilization regimes; (3) nutrient uptake and bioavailability, including rhizosphere effects and mineral amendment trials; (4) the biochemical pathways of lycopene biosynthesis, including MEP-pathway enzymes and their metal cofactors; and (5) indirect fruit-quality studies conducted in volcanic soil systems, retained only to support mineral-uptake plausibility rather than as direct lycopene evidence.

Only category 5 approaches direct evidence, and even these studies confuse soil origin with farming system, biostimulant treatment, or cultivar because no matched conventional-soil comparator was tested alongside a volcanic one. Categories 1–4 do not themselves report a tomato lycopene outcome and are treated throughout this review as indirect, plausibility-supporting evidence rather than direct evidence for the central claim. This fixes the apparent discrepancy in Table 1, where some included publications do not disclose a lycopene outcome despite the specified exclusion condition. These articles were purposefully kept as indirect supporting evidence and are marked as such in Tables 1–3.

Records were excluded if they lacked lycopene or carotenoid outcome data, if fertilizer intensity confounded any comparison of soil type, if methodology was incompatible with cross-study synthesis (for example, differing extraction or quantification methods, mismatched cultivars, or unpaired micronutrient and carotenoid data), or if the only available evidence was anecdotal or industry-sourced rather than drawn from a controlled trial. This exclusion criterion was applied only to category-5 fruit studies, for which a lycopene outcome was expected; it was not applied to the mechanistic and soil-mineralogy categories retained as indirect evidence.

Table 1: Inclusion and exclusion criteria for study selection

Inclusion criteria	Exclusion criteria
Volcanic soil mineralogy (Andisols, andic properties)	No lycopene/carotenoid outcome data
Conventional soil mineralogy (NPK + micronutrients)	Fertilizer intensity confounds soil comparison
Nutrient uptake and bioavailability (rhizosphere, amendments)	Incompatible methods (cultivar mismatch, unpaired data)
Lycopene biosynthesis (MEP pathway, metal cofactors)	Anecdotal or industry-sourced evidence
Volcanic soil fruit studies (plausibility only)	

RESULTS

The comprehensive literature search initially identified 340 articles as potentially relevant to the study. Following screening, 295 records were excluded, leaving 45 articles for full-text assessment. The selected articles that fulfilled the criteria are shown in Table 2.

Table 2: Articles included in the review following full-text assessment

Authors	Year	Title	Category
da Silva Martins, J V et al.	2024	A Combined Soil and Water Management Strategy to Improve the Nutrition and Marketability of Tomato Variety 'Heinz 9553'	Lycopene + Fertilization (Indirect)
Kalore, S et al.	2025	A comparative study on tomato (<i>Solanum lycopersicum</i>) quality under different soil and nutrient management	Lycopene + Fertilization (Indirect)
Bonanomi, G et al.	2025	Agronomic impacts of chemically and microbiologically characterized compost tea in Mediterranean volcanic soils	Volcanic Soil (Indirect)
Fusco, G M et al.	2026	Agronomic, Biochemical and Metabolic Responses of Tomato to Vermicompost and Mineral Fertilization in Loam and Clay Soils	Lycopene + Fertilization (Indirect)
Khan, M Y et al.	2017	Antioxidant compounds and minerals in tomatoes by Trichoderma-enriched biofertilizer	Lycopene + Fertilization (Indirect)
Tóth, E et al.	2024	Are the Nutritional Properties of Organic Tomatoes Altered by Single and Combined Microbial Soil Inoculants?	Lycopene + Fertilization (Indirect)
Mehlferber, E et al.	2025	Azomite, a volcanic ash-based fertilizer modulates gene expression during photomorphogenesis	Volcanic Soil (Indirect)
Oo, A Z et al.	2018	Aerated Irrigation and Pruning Residue Biochar on N ₂ O Emission, Yield and Ion Uptake of Komatsuna	Volcanic Soil (Andosol) (Indirect)
Iacomino, G et al.	2022	Biochar and Compost Application either Alone or in Combination Affects Vegetable Yield in a Volcanic Mediterranean Soil	Volcanic Soil (Indirect)
Flores, I R et al.	2021	Bioactive compounds in tomato and their relationship with soil mineral content	Lycopene + Fertilization (Indirect)
Carillo, P et al.	2020	Biostimulatory Action of Arbuscular Mycorrhizal Fungi Enhances Productivity, Functional and Sensory Quality in Cherry Tomato	Lycopene + Fertilization (Indirect)
Fortis-Hernández, M et al.	2018	Changes in nutraceutical quality of tomato under different organic substrates	Lycopene + Fertilization (Indirect)
Wu, Y et al.	2020	Combined application of soluble organic and chemical fertilizers in drip fertigation improves tomato yield and quality	Lycopene + Fertilization (Indirect)
Dermawan, R et al.	2024	Comparison of Five Phosphorus (P) and Potassium (K) Extraction Methods in Tomato Cultivation in Andisols and Inceptisols	Volcanic Soil (Indirect)
Dermawan, R et al.	2024	Evaluation of five soil nutrient extraction methods for tomato fertilization in Andisols-Garut, Indonesia	Volcanic Soil (Indirect)
Carricondo-Martínez, I et al.	2022	Different Organic Fertilisation Systems Modify Tomato Quality	Lycopene + Fertilization (Indirect)
Daoud, B et al.	2020	Different potassium fertilization levels influence water-use efficiency, yield, and fruit quality of cocktail tomato	Lycopene + Fertilization (Indirect)
Brezeanu, P M et al.	2022	Effects of Organic Inputs Application on Yield and Qualitative Parameters of Tomatoes and Peppers	Lycopene + Fertilization (Indirect)
Kong, L et al.	2020	Effects of arbuscular mycorrhizal fungi on photosynthesis, ion balance of tomato under saline-alkali soil	Lycopene + Fertilization (Indirect)
Fan, H et al.	2023	Effects of Organic Fertilizer Supply on Soil Properties, Tomato Yield, and Fruit Quality: A Global Meta-Analysis	Lycopene + Fertilization (Indirect)
Mehlferber, E C et al.	2022	Temporally Selective Modification of the Tomato Rhizosphere and Root Microbiome by Volcanic Ash Fertilizer	Volcanic Soil (Indirect)
Sabatino, L et al.	2019	Interactive Effects of Genotype and Molybdenum Supply on Yield and Overall Fruit Quality of Tomato	Lycopene + Fertilization (Indirect)
He, Y R et al.	2025	Modulation of Potassium-to-Calcium Ratio in Nutrient Solution Improves Quality Attributes and Mineral Composition	Lycopene + Fertilization (Indirect)

Authors	Year	Title	Category
Moriyama, Y et al.	2018	Soil biological and physiochemical factors limiting the growth potential of tomato planted in waterlogged volcanic soil	Volcanic Soil (Indirect)
Agbede, T M et al.	2018	Soil properties, growth, fruit yield, mineral, lycopene and vitamin C of tomato with green manures and NPK	Lycopene + Fertilization (Indirect)
Verma, S B et al.	2023	Sustaining soil health and tomato production through organic inputs and bacterial inoculations in alluvial soils	Lycopene + Fertilization (Indirect)
Caruso, G et al.	2019	Yield and Nutritional Quality of Vesuvian Piennolo Tomato PDO as Affected by Farming System and Biostimulant	Volcanic Soil (Vesuvius) (Indirect)
Stoleru, V et al.	2020	Yield and nutritional response of greenhouse grown tomato cultivars to sustainable fertilization and irrigation	Lycopene + Fertilization (Indirect)
Perreau, F et al.	2020	ABSCISIC ACID-DEFICIENT4 Has an Essential Function in Both cis-Violaxanthin and cis-Neoxanthin Synthesis	Biochemistry (Pathway) (Indirect)
Changan, S S et al.	2018	Abscisic acid biosynthesis under water stress: NCED1 gene in rice	Biochemistry (Fe cofactor) (Indirect)
Yan, F et al.	2020	BEL1-LIKE HOMEODOMAIN4 regulates chlorophyll accumulation, chloroplast development in tomato	Biochemistry (Indirect)
Sun, T et al.	2023	Co-chaperoning of chlorophyll and carotenoid biosynthesis by ORANGE family proteins in plants	Biochemistry (Indirect)
Li, X D et al.	2022	Glycine-rich RNA-binding cofactor RZ1AL is associated with tomato ripening and development	Biochemistry (Lycopene) (Indirect)
Li, W et al.	2024	Mutation of YFT3, an isomerase in the isoprenoid pathway, impairs carotenoid accumulation in tomato	Biochemistry (Indirect)
Zhao, X D et al.	2018	Role of the tomato TAGL1 gene in regulating fruit metabolites	Biochemistry (Carotenoid) (Indirect)
Leiva-Ampuero, A et al.	2020	Salinity impairs photosynthetic capacity and enhances carotenoid-related gene expression in tomato	Biochemistry (Gene Expression) (Indirect)
Dyachenko, E A et al.	2020	Structural and functional features of phytoene synthase isoforms PSY1 and PSY2 in pepper	Biochemistry (Mg binding) (Indirect)
Wang, J et al.	2024	The transcription factor CaBBX10 promotes chlorophyll and carotenoid pigment accumulation in pepper	Biochemistry (Indirect)
Alam, M Z et al.	2023	Arbuscular Mycorrhizal Fungi Enhance Biomass Growth, Mineral Content, and Antioxidant Activity in Tomato under Drought	Mineral Uptake (Indirect)
Irina, Z et al.	2024	Assessment of vitamin- and mineral-content stability of tomato fruits for functional food	Mineral Uptake (Indirect)
Vergara, C et al.	2017	Dark Septate Endophytic Fungi Help Tomato to Acquire Nutrients from Ground Plant Material	Mineral Uptake (Indirect)
Pérez, C M et al.	2022	Leaf area and its impact in yield and quality of greenhouse tomato	Volcanic Soil (tezontle) (Indirect)
Magallanes-López, A M et al.	2020	Post-Harvest Quality of 40 Collections of Tomato Native of Mexico	Volcanic Soil (sand substrate) (Indirect)
Ligot, N et al.	2023	Grain size modulates volcanic ash retention on crop foliage and potential yield loss	Volcanic Soil (Indirect)
Aina, O E et al.	2019	Effect of organic and inorganic sources of nutrients on bioactive compounds and antioxidant activity of tomato	Lycopene + Fertilization (Indirect)

Volcanic-Derived Soil Mineral Profile (Indirect Evidence) are typically rich in primary minerals containing essential plant nutrients (Oo et al., 2018).

The mineral profile of volcanic soils has been extensively studied in systems such as the Andisols of Garut, Indonesia (Dermawan et al., 2024a, 2024b), volcanic soils of the Mediterranean (Bonanomi et al., 2025; Iacomino et al., 2022), and the Vesuvian soils of Italy (Caruso et al., 2019). These soils derive from the weathering of volcanic ash and In addition to trace minerals like zinc, boron, and molybdenum, the mineral profile of volcanic soils is noticeably rich in elements like iron, magnesium, calcium, potassium, and phosphorus (Dermawan et al., 2024a, 2024b). Weathering processes eventually liberate these minerals, which are derived from the original volcanic

material. Volcanic soils from Mediterranean regions have unique chemical and microbiological properties that affect plant growth and nutrient availability, according to research by Bonanomi et al. (2025).

Andisols, in particular, are known for their high phosphorus retention capacity due to the presence of allophane and imogolite clay minerals (Dermawan et al., 2024a, 2024b). This characteristic has a major impact on crops' access to phosphorus and calls for particular fertilisation techniques. The extraction effectiveness varies significantly depending on the kind of soil and the particular approach used, according to a comparison of five phosphorus and potassium extraction methods in tomato growing in Andisols and Inceptisols (Dermawan et al., 2024a). The significance of method selection for precise nutrient assessment was also demonstrated by the evaluation of five soil nutrient extraction techniques for tomato fertilisation in Andisols in Garut, Indonesia (Dermawan et al., 2024b).

It has been demonstrated that Azomite, a fertiliser based on volcanic ash, modulates gene expression during photomorphogenesis, indicating that volcanic mineral sources can affect plant developmental processes in ways other than just providing nutrients (Mehlferber et al., 2025). Furthermore, volcanic soil systems have shown the ability to maintain microbial communities that support plant health and nutrient cycling, as evidenced by the temporally selective alteration of the tomato rhizosphere and root microbiome after the application of volcanic ash fertiliser (Mehlferber et al., 2022).

Studies conducted on Vesuvian soils have indicated that the unique mineral composition contributes to the distinctive nutritional quality of tomatoes grown in this region (Caruso et al., 2019). The grain size of volcanic ash has been shown to modulate retention on crop foliage, with potential implications for nutrient delivery and yield (Ligot et al., 2023). Furthermore, soil biological and physiochemical factors limiting the growth potential of tomato planted in waterlogged volcanic soil have been investigated (Moriyama et al., 2018). Research on tezontle, a volcanic soil substrate used in Mexico, has examined its impact on greenhouse tomato quality (Pérez et al., 2022). Volcanic sand substrates have also been evaluated for their effects on post-harvest quality of native Mexican tomato collections (Magallanes-López et al., 2020); none of these studies included a matched conventional-soil comparator.

Conventional Farming Soil Mineral Profile

Fertilisation techniques, not parent material, play a major role in determining the mineral profile of conventionally

treated soils. Studies have looked at how different fertilisation schedules affect crop yields and soil characteristics. Research has examined how organic inputs affect soil nutrient availability and how they interact with synthetic fertilisers (Brezeanu et al., 2022; Carillo et al., 2020; Carricondo-Martínez et al., 2022; Fan et al., 2023; Fusco et al., 2026; Iacomino et al., 2022; Tóth et al., 2024; Verma et al., 2023). Tomato output and quality have been demonstrated to increase when soluble organic and chemical fertilisers are applied together in drip fertigation (Wu et al., 2020).

Both synthetic nutrient solutions and organic amendments are usually applied in conventional soil management. According to field data, these activities can improve soil fertility in cropping sequences by increasing soil organic carbon and the availability of nitrogen, phosphorus, potassium, and other micronutrients (Agbede et al., 2018; Kalore et al., 2025). Nutrient release patterns and general soil health have been found to be influenced by the ratio of organic to inorganic inputs (Tóth et al., 2024; Verma et al., 2023).

One important aspect that can complicate comparisons of soil types is fertilisation intensity (Tóth et al., 2024). Organic inputs and bacterial inoculations help maintain soil health and tomato production, according to research on alluvial soils (Verma et al., 2023). Similar effects on soil characteristics, growth, fruit output, mineral content, lycopene, and vitamin C in tomatoes have been demonstrated by green manure and NPK applications (Agbede et al., 2018).

Conventional agricultural soils' chemical composition is usually controlled by predetermined fertilisation regimens. The impacts of several organic fertilisation systems on tomato quality have been studied (Carricondo-Martínez et al., 2022). It has been demonstrated that adjusting the potassium-to-calcium ratio in nutritional solutions enhances mineral composition and qualitative features (He et al., 2025). Additionally, it has been shown that varying potassium fertilisation levels affect cocktail tomato productivity, fruit quality, and water-use efficiency (Daoud et al., 2020).

Bioactive compounds in tomatoes have been shown to relate to soil mineral content (Flores et al., 2021). Trichoderma-enriched biofertilizer has been found to affect antioxidant compounds and minerals in tomatoes (Khan et al., 2017). Arbuscular mycorrhizal fungi have been demonstrated to enhance productivity, functional and sensory quality in cherry tomatoes (Carillo et al., 2020), while also affecting photosynthesis and ion balance in tomatoes under saline-alkali soil conditions (Kong et al.,

2020). Different organic fertilisation systems have been shown to modify tomato quality (Carricondo-Martínez et al., 2022).

Comparative Mineral Bioavailability and Uptake Dynamics (Indirect Evidence)

Nutrient availability and plant acquisition mechanisms differ significantly between volcanic and ordinary soils, according to comparative studies on mineral bioavailability and absorption kinetics. Nutrient release patterns and plant uptake efficiency are influenced by the distinct mineralogical characteristics of volcanic soils, such as their high phosphorus retention capacity and allophane clay content (Dermawan et al., 2024a, 2024b; Moriyama et al., 2018).

The accumulation of phytochemicals in fruit tissues is directly influenced by the availability of minerals in the soil matrix, according to research on the link between bioactive compounds in tomatoes and soil mineral concentration (Flores et al., 2021). Soil physicochemical characteristics, such as pH, organic matter content, and cation exchange capacity, seem to influence the bioavailability of minerals from volcanic soils (Iacomino et al., 2022; Moriyama et al., 2018).

Mineral bioavailability is significantly influenced by rhizosphere effects. In drought-stricken tomatoes, arbuscular mycorrhizal fungi have been demonstrated to increase biomass growth, mineral content, and antioxidant activity (Alam et al., 2023). According to Vergara et al. (2017), dark septate endophytic fungi aid tomato plants in obtaining nutrients from ground plant debris. Numerous studies have established the involvement of plant-growth-promoting microbes in nutrient uptake (Alam et al., 2023; Carillo et al., 2020; Khan et al., 2017; Kong et al., 2020; Vergara et al., 2017).

The impact of *Trichoderma*-enriched biofertilizer on antioxidant compounds and minerals in tomatoes has been studied (Khan et al., 2017). Agronomic effects that are chemically and microbiologically characterised have been shown when compost tea is applied to Mediterranean volcanic soils (Bonanomi et al., 2025). Vegetable yield in volcanic Mediterranean soils has been demonstrated to be impacted by the use of compost and biochar, either separately or in combination (Iacomino et al., 2022).

Water availability and mineral uptake are closely linked. Research on aerated irrigation and pruning residue biochar has demonstrated effects on N₂O emission, yield, and ion uptake in crops (Oo et al., 2018). The interaction between soil mineral composition and plant physiological processes

has been investigated through studies on the effects of organic fertilizer supply on soil properties, tomato yield, and fruit quality (Fan et al., 2023; Verma et al., 2023). The assessment of vitamin and mineral content stability in tomato fruits for functional food applications has provided insights into post-harvest dynamics (Irina et al., 2024).

Studies have looked at how applying organic inputs affects tomato and pepper yield and qualitative characteristics, emphasising how soil management affects nutrient bioavailability (Brezeanu et al., 2022). It has been studied how genotype and molybdenum availability combine to affect tomato yield and overall fruit quality (Sabatino et al., 2019). Greenhouse-grown tomato cultivars' yield and nutritional response to sustained irrigation and fertilisation have been reported (Stoleru et al., 2020).

The comparative mineral bioavailability and uptake dynamics between volcanic and conventional soils remain an area requiring further investigation. While individual studies have examined nutrient availability in each soil type, direct comparative studies using controlled experimental designs are limited.

Mechanistic Link: Minerals as Cofactors in Carotenoid Biosynthesis (Indirect Evidence)

Biologically, lycopene can be produced by condensing two precursors, dimethylallyl diphosphate (DMAPP) and isopentenyl pyrophosphate (IPP), followed by chain elongation events (Cheng et al., 2019). The condensation of two geranylgeranyl diphosphate molecules is catalysed by phytoene synthase (PSY), the first enzyme involved in carotenoid production. The characterisation of the structural and functional characteristics of the pepper phytoene synthase isoforms PSY1 and PSY2 has consequences for comprehending cofactor requirements and enzyme control (Dyachenko et al., 2020).

As a cofactor in several stages of the carotenoid biosynthesis process, magnesium is essential. Magnesium ions are necessary for the activity of the enzyme geranylgeranyl diphosphate synthase (GGPPS), which catalyses the production of geranylgeranyl diphosphate. Magnesium-binding requirements have been established by research on the structural and functional characteristics of phytoene synthase isoforms PSY1 and PSY2 (Dyachenko et al., 2020). Understanding the molecular processes behind carotenoid accumulation has been made possible by studies on the function of the tomato TAGL1 gene in controlling fruit metabolites (Zhao et al., 2018).

Minerals also play a cofactor role in the control of gene expression. Studies on the BEL1-LIKE HOMEODOMAIN4 gene have shown how it controls tomato chlorophyll

accumulation and chloroplast formation (Yan et al., 2020). It has been demonstrated that carotenoid accumulation is hampered by mutations in YFT3, an isomerase in the isoprenoid pathway, underscoring the significance of metal-dependent enzymatic steps (Li, W. et al., 2024). Tomato ripening and development have been linked to the glycine-rich RNA-binding cofactor RZ1AL (Li, X. D. et al., 2022). These gene-regulation investigations, which establish the biological plausibility of a mineral-gene expression link rather than the link itself, were not carried out under a volcanic vs normal soil comparison.

The mechanistic understanding of mineral cofactors in carotenoid biosynthesis provides a biochemical framework for explaining how soil mineral composition influences lycopene accumulation. However, direct evidence linking specific volcanic soil minerals to enhanced cofactor availability and subsequent lycopene biosynthesis remains limited.

Synthesis of Fruit-Level Evidence (Direct and Indirect Evidence)

Two studies, Fusco et al. (2026), which studied loam and clay soils supplemented with vermicompost and minerals, and Caruso et al. (2019), which concentrated on Vesuvian Piennolo tomatoes, use a direct fruit-level technique to assess tomato lycopene in volcanic soils. However, because neither study included a matching conventional-soil control grown under the same conditions, it is impossible to separate the effects of treatment from the effects of soil origin. The combination of this fruit-level data indicates a complicated connection between the origin of soil minerals and tomato lycopene accumulation. More generally, research on growing tomatoes in volcanic soils, especially those from Mount Vesuvius and Mount Etna, has repeatedly shown increased antioxidant content and nutritional quality (Caruso et al., 2019; Magallanes-López et al., 2020; Pérez et al., 2022).

Further studies on volcanic soil include the effects of farming systems and biostimulants on Vesuvian Piennolo (Caruso et al., 2019), the effects of tezontle substrate on greenhouse tomato yield and quality (Pérez et al., 2022), the effects of volcanic sand on Mexican tomato post-harvest quality (Magallanes-López et al., 2020), and the effects of volcanic ash grain size on foliage retention and yield loss (Ligot et al., 2023).

Soil type and fertilisation have an impact on bioactive accumulation: mineral availability influences antioxidant/lycopene synthesis (Flores et al., 2021); vermicompost/mineral fertilisation drives agronomic/biochemical responses in loam/clay (Fusco et al., 2026); combined soil-water management enhances

‘Heinz 9553’ nutrition/marketability (da Silva Martins et al., 2024); comparative nutrient-management studies are available (Kalore et al., 2025). Organic systems alter quality (Carricondo-Martínez et al., 2022), organic inputs impact tomato/pepper parameters (Brezeanu et al., 2022), combined soluble organic/chemical drip fertigation improves yield/quality (Wu et al., 2020), and organic substrate composition affects nutraceutical quality (Fortis-Hernández et al., 2018), while combined soluble organic/chemical drip fertigation enhances yield/quality (Wu et al., 2020), organic systems modify quality (Carricondo-Martínez et al., 2022), and organic inputs affect tomato/pepper parameters (Brezeanu et al., 2022). Agronomic factors and genotype interact: fruit composition is influenced by molybdenum supply (Sabatino et al., 2019); post-harvest vitamin/mineral stability is evaluated (Irina et al., 2024); water use, yield, and cocktail tomato quality are influenced by potassium levels (Daoud et al., 2020).

Bioactive chemicals and antioxidant activity are influenced by the source of nutrients (Agbede et al., 2018; Aina et al., 2019). A global meta-analysis has confirmed the impact of organic fertiliser on soil characteristics, yield, and quality (Fan et al., 2023). Antioxidants and minerals are impacted by Trichoderma-enriched biofertilizer (Khan et al., 2017), and organic tomato nutrition is changed by single or mixed microbial inoculants (Tóth et al., 2024).

Mycorrhizal fungi impact photosynthesis and ion balance in saline-alkali conditions (Kong et al., 2020); sustainable fertigation and irrigation impact greenhouse cultivar yield and nutrition (Stoleru et al., 2020); adjusting the K/Ca ratio enhances quality and mineral composition (He et al., 2025); green manures and NPK impact soil properties, growth, yield, minerals, lycopene, and vitamin C (Agbede et al., 2018); and organic inputs and bacterial inoculations maintain alluvial-soil health and tomato production (Verma et al., 2023).

Controlled comparison studies that specifically examine the effects of volcanic versus standard soil minerals on tomato lycopene are still scarce, despite a wealth of data on individual parameters. There is a clear possibility to determine the precise contributions of volcanic soil profiles to improved lycopene accumulation, as most research isolates individual soil/fertilization variables instead of comparing mineral provenance.

DISCUSSION

The current review summarises the information that is currently available about the connection between tomato lycopene accumulation and soil mineral origin. The results

show that normal farming soils and volcanic soils have different mineral profiles. Conventional farming systems supply minerals through managed N-P-K programs supplemented with micronutrients (Agbede et al., 2018; Flores et al., 2021; Sabatino et al., 2019), whereas volcanic soils, especially Andisols, have a high capacity to retain phosphorus because of allophane and imogolite clay minerals (Dermawan et al., 2024a, 2024b). These should not be interpreted as showing that one type of soil produces more lycopene than another because they represent variations in soil chemistry rather than variations in a matched experimental comparison.

Biochemical evidence that metal cofactors are crucial to the carotenoid biosynthesis pathway supports the mechanistic relationship between soil minerals and lycopene accumulation. Important enzymatic processes, such as phytoene synthase activity, depend on magnesium, iron, and zinc (Dyachenko et al., 2020; Li, W. et al., 2024). These metal cofactors are necessary for the MEP pathway, which generates precursors for carotenoid synthesis (Perreau et al., 2020; Sun et al., 2023; Wang et al., 2024).

Evidence at the fruit level suggests that soil type and fertilisation technique have an impact on lycopene accumulation. According to research on Vesuvian soils, tomatoes grown on volcanic substrates have higher nutritional quality (Caruso et al., 2019). Applications of biostimulants have been demonstrated to increase antioxidant activity, total phenols, and lycopene content (Caruso et al., 2019; Carillo et al., 2020; Colla et al., 2017). In particular, biostimulant-treated plants showed a greater lycopene content (196.3 mg 100g⁻¹ fw with protein hydrolysate) than untreated controls (150.2 mg 100g⁻¹ fw), according to Caruso et al. (2019).

Nevertheless, meta-analytic pooling is not possible due to the methodological heterogeneity of the evidence base across cultivar type, fertilisation intensity, and extraction techniques (Caruso et al., 2019; Tóth et al., 2024). Additionally, there are very few controlled comparison studies that explicitly compare the effects of conventional versus volcanic soil minerals on lycopene accumulation, which is a major gap in the literature (Parisi et al., 2021). This gap, rather than any demonstrated soil-origin effect, is the review's principal finding.

CONCLUSION

This narrative review shows that volcanic-derived and conventionally managed soils differ in mineral profile, and that a biologically plausible mechanism could link soil minerals to lycopene production through metal-

dependent steps in the MEP and carotenoid pathways. The higher lycopene values reported in Vesuvian tomatoes, however, reflect biostimulant or farming-system treatments rather than volcanic soil origin per se; no study found in this search directly compared matched volcanic and conventional soils for their effect on tomato lycopene accumulation under controlled conditions. The title and wording of the review have been changed to reflect the fact that the mineral–lycopene link discussed here should be viewed as a tested hypothesis rather than an established effect.

Nevertheless, there are currently no direct comparative studies comparing the impact of volcanic vs normal soil minerals on the accumulation of lycopene under controlled conditions. Future research should employ a controlled comparative design with standardized cultivar selection, fertilization intensity, and extraction methodology to validate the claim that volcanic soils yield tomatoes with exceptionally high lycopene concentrations.

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Cytotoxicity Evaluation of *Gynura procumbens* Leaf Extract Containing Polyphenol on Macrophages via XTT assay

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ABSTRACT

Background: *Gynura procumbens* (GP) is a traditional medicinal plant widely used in Southeast Asia, with reported anti-inflammatory and antioxidant activity attributed largely to its polyphenol content. Before evaluating the anti-inflammatory potential of GP leaf extract (GPLE) containing polyphenol, establishing a non-cytotoxic concentration range in a relevant immune cell model is essential. This study evaluated the cytotoxicity of GPLE on RAW264.7 macrophages using the XTT assay. **Methods:** RAW264.7 macrophages were treated with GPLE (7.82 to 125 µg/mL) for 24 hours. Viability was determined using the XTT colorimetric assay. Cell-free GPLE blanks at matching concentrations were used to correct for the extract's intrinsic colour, and viability values were calculated relative to untreated control (NC). Data are presented as mean ± SD (n = 3). **Results:** GPLE showed a concentration-dependent effect on RAW264.7 viability. Viability remained close to or above control levels from 7.82 to 62.5 µg/mL (103.95% to 145.49%), while viability decreased markedly to 37.22 ± 5.03% at 125 µg/mL. The half-maximal inhibitory concentration (IC₅₀) of GPLE against RAW264.7 macrophages was estimated at 84.25 µg/mL. **Conclusion:** GPLE is non-cytotoxic to RAW264.7 macrophages at concentrations up to 62.5 µg/mL, providing a suitable concentration range for the subsequent evaluation of its anti-inflammatory potential in this model.

Keywords:

Gynura procumbens; RAW264.7 macrophages; XTT assay; cell viability; colorimetric interference

INTRODUCTION

Gynura procumbens (GP), a herbaceous plant traditionally consumed as a vegetable and folk remedy across Southeast Asia, has been reported to possess antihypertensive, antihyperglycaemic, antioxidant, and anti-inflammatory properties, largely attributed to its flavonoid and polyphenol content (Bhowmik et al., 2025; Mohd Nor et al., 2025; Wan Yusoff et al., 2016). The plant, commonly known as “Sambung Nyawa” in Malaysia and Indonesia, has been widely used in traditional medicine for the treatment of various inflammatory conditions (Mohamed et al., 2023; Manogaran et al., 2019). Ethanolic extracts and extracts containing polyphenol from GP leaves have been shown to suppress nitric oxide production and pro-inflammatory cytokine expression in lipopolysaccharide (LPS) stimulated RAW264.7 macrophages via modulation of the PI3K/Akt and MAPK

signalling pathways (Cao et al., 2022).

The RAW264.7 murine macrophage cell line is widely used as an in vitro model for studying inflammatory responses. Prior to evaluating the anti-inflammatory activity of a candidate extract in this model, it is standard practice to first establish the concentration range over which the extract does not itself compromise cell viability, so that any subsequent reduction in inflammatory markers can be attributed to a genuine anti-inflammatory effect rather than to cytotoxicity (Kamiloglu et al., 2020).

While the cytotoxicity of crude GP ethanol extracts and their solvent fractions on RAW264.7 macrophages has been previously reported (Manogaran et al., 2019), and GP extracts containing polyphenol prepared by a related liquid-liquid partitioning method have been screened for antioxidant and COX-2 binding activity (Kim et al., 2021),

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the cytotoxicity of this specific ethyl acetate fraction toward RAW264.7 macrophages evaluated using inferential statistics has not been established. Determining this non-cytotoxic concentration range is a necessary prerequisite before the extract's anti-inflammatory potential in this model can be meaningfully evaluated. Therefore, this study aimed to evaluate the cytotoxic effect of GPLE on RAW264.7 macrophages using the XTT assay to establish a statistically supported non-cytotoxic concentration range suitable for subsequent anti-inflammatory investigation.

MATERIALS AND METHODS

Plant Material and Sample Preparation

Fresh GP leaves were thoroughly rinsed with distilled water to remove adhering impurities and subsequently cut into smaller pieces. The leaves were initially air-dried under continuous ventilation, followed by sun-drying to facilitate preliminary dehydration. Final drying was performed using an oven until the plant material was completely dried. The dried leaves were then ground into a fine powder using a laboratory-grade grinder prior to extraction.

Extraction and Enrichment of Polyphenols

The extraction and enrichment of polyphenols from GP leaves were performed based on the method of Kim et al. (2021), with modifications. Briefly, the dried leaf powder was macerated in aqueous methanol, filtered, and concentrated under reduced pressure. The concentrated extract then underwent liquid-liquid partitioning using hexane and ethyl acetate to remove non-polar constituents and enrich the polyphenol fraction. The resulting ethyl acetate fraction was dried, concentrated, and stored at -70 °C until further use.

Cell Culture

RAW264.7 murine macrophage cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin streptomycin and maintained at 37 °C in a humidified atmosphere of 5% CO₂. Cells were subcultured upon reaching 80 to 90% confluence.

XTT Cell Viability Assay

The effect of GPLE on RAW264.7 macrophage viability was determined using the XTT Cell Viability Assay Kit (Biotium, Fremont, CA, USA), following the manufacturer's protocol (Biotium, 2012). Cells were seeded into 96-well plates at a

density of 10,000 cells per well and allowed to adhere prior to treatment. Cells were then treated with GPLE at five concentrations (125, 62.5, 31.25, 15.53, and 7.82 µg/mL) prepared by two-fold serial dilution, for 24 hours. Untreated cells served as the negative control (NC), 0.2% (v/v) DMSO served as the vehicle control (VC), and 10% (v/v) DMSO served as the positive control (PC) for cytotoxicity. Following treatment, activated XTT solution was added to each well and plates were incubated for 4 hours at 37 °C. Absorbance was measured at a test wavelength of 450 nm with a reference wavelength of 650 nm using a microplate reader.

As GP leaf extract possesses intrinsic colour, cell-free blank wells containing GPLE at each corresponding concentration were included to correct for extract-derived background absorbance. The corrected absorbance for each GPLE treated well was calculated by subtracting the corresponding cell-free GPLE blank absorbance from the treated well absorbance. Percentage cell viability was calculated relative to the mean corrected absorbance of the NC group using Eq. (1):

$$\text{Cell Viability \%} = \frac{\text{Corrected absorbance of treated well}}{\text{Mean corrected absorbances of NC}} \times 100 \quad (1)$$

Statistical Analysis

Data are expressed as mean ± standard deviation (SD) of three replicate wells. The half-maximal inhibitory concentration (IC₅₀) was determined by nonlinear regression (log(inhibitor) vs. response, variable slope) of all replicate viability values using GraphPad Prism 10.6.0.

RESULTS

Effect of GPLE on RAW264.7 Macrophage Viability

Treatment of RAW264.7 macrophages with GPLE for 24 hours produced a concentration dependent effect on cell viability (Table 1, Figure 1). Viability remained close to or above the untreated control at the lower concentrations tested, reaching 145.49 ± 6.93% at 7.82 µg/mL, 135.21 ± 8.59% at 15.53 µg/mL, 105.87 ± 18.54% at 31.25 µg/mL, and 103.95 ± 7.90% at 62.5 µg/mL. At the highest concentration tested, 125 µg/mL, viability decreased markedly to 37.22 ± 5.03%.

Table 1: Effect of GPLE on cell viability (%) determined by XTT assay.

Concentration ($\mu\text{g/mL}$)	Cell Viability (% Mean $\pm$ SD)
125	37.22 $\pm$ 5.03
62.5	103.95 $\pm$ 7.90
31.25	105.87 $\pm$ 18.54
15.53	135.21 $\pm$ 8.59
7.82	145.49 $\pm$ 6.93

Values are expressed as mean $\pm$ SD ($n = 3$). Viability (%) was calculated relative to NC, corrected for extract background absorbance using GPLE (no cell).

The vehicle control (0.2% DMSO) maintained viability at $103.16 \pm 24.78\%$, confirming that the solvent used to prepare GPLE stock solutions was not independently cytotoxic at the concentration used. The positive control (10% DMSO) reduced viability to $-9.05 \pm 11.76\%$, confirming assay sensitivity to a known cytotoxic stimulus (Table 2).

Table 2: Cell viability (%) of assay controls.

Control	Cell Viability (% Mean $\pm$ SD)
Vehicle Control (VC)	103.16 ± 24.78
Negative Control (NC)	100.00 ± 24.85
Positive Control (PC)	-9.05 ± 11.76

Values are expressed as mean $\pm$ SD ($n = 3$). VC = vehicle control; NC = negative control (defined as 100% viability); PC = positive control (10% DMSO). The $\pm 24.85\%$ SD on NC reflects well-to-well variability in raw absorbance readings used to define the 100% baseline, not variability in a "result."

Nonlinear regression (log(inhibitor) vs. response, variable slope; GraphPad Prism 10.6.0) estimated the IC_{50} of GPLE against RAW264.7 macrophages at $84.25 \mu\text{g/mL}$.

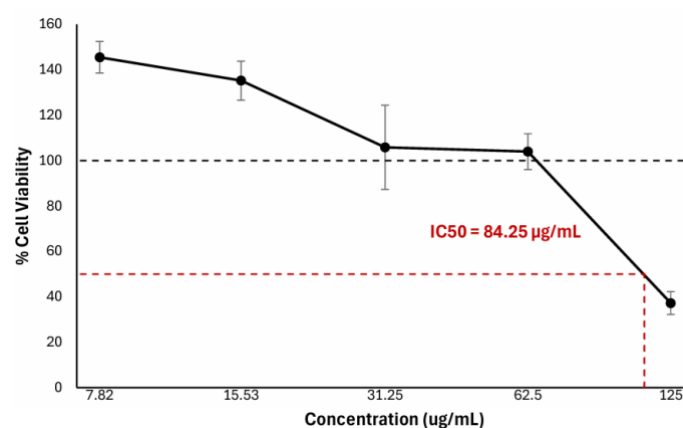


Figure 1: Dose response effect of GPLE on the viability of

RAW264.7 macrophages at 24 h. Cells were seeded at 1×10^4 cells/well and treated at the indicated concentrations for 24 hours; viability was determined by XTT assay. Results are expressed as mean $\pm$ SD ($n = 3$) relative to untreated negative control (NC, dashed line = 100%). The IC_{50} of GPLE against RAW264.7 macrophages was estimated at $84.25 \mu\text{g/mL}$ by nonlinear regression (GraphPad Prism 10.6.0).

DISCUSSION

This study evaluated the cytotoxicity of GPLE on RAW264.7 macrophages using the XTT assay, with correction for the extract's intrinsic colour interference. The results show that GPLE exerts a concentration dependent effect on cell viability, with viability maintained near or above control levels at low concentrations (7.82 to $62.5 \mu\text{g/mL}$) and a marked reduction in viability at the highest concentration tested ($125 \mu\text{g/mL}$).

The apparent increase in viability at the lowest GPLE concentrations following background subtraction is noteworthy. This could reflect a genuine proliferative or metabolic stimulatory effect of GPLE at low concentrations, a phenomenon commonly reported for polyphenolic compounds exhibiting hormetic properties (Perrone & D'Angelo, 2025). Polyphenols are well recognised to exhibit hormetic, concentration dependent relationships with cell viability: protective at low concentrations, but pro oxidant and cytotoxic at higher concentrations, as reactive oxygen species generation from polyphenol autoxidation increasingly outweighs their radical scavenging capacity (Vrankova et al., 2026). The relatively high apparent viability values observed here may also reflect the inherent difficulty of perfectly matching background absorbance in coloured plant extracts, even with careful cell-free blank correction (Gavanji et al., 2023).

The cytotoxicity observed at $125 \mu\text{g/mL}$ contrasts with the largely proliferative profile reported for crude GP ethanol extract and its solvent fractions on RAW264.7 macrophages at comparable concentrations (Manogaran et al., 2019). This comparison should be interpreted with some caution, as the two extracts were prepared using different primary solvents (methanol in the present study versus ethanol in Manogaran et al., 2019); differences in extraction efficiency and selectivity for phenolic compounds between these solvents may themselves contribute to the divergent phytochemical yield, independent of the partitioning step described below. A plausible explanation is that the liquid-liquid partitioning and polyphenol enrichment steps used to prepare GPLE concentrate polyphenolic constituents relative to a crude

extract, potentially enhancing both the bioactive and cytotoxic properties of the final preparation. activity in RAW264.7 macrophages in future studies.

Using the $\geq 70\%$ viability threshold described in ISO 10993-5 as a reference (ISO, 2009), concentrations up to 62.5 $\mu\text{g/mL}$ were considered non-cytotoxic, establishing the working range for subsequent anti-inflammatory evaluation.

The RAW264.7 murine macrophage cell line employed in this study is a widely recognised and reproducible in vitro model for evaluating the cytotoxicity and immunomodulatory activity of natural products (Riastri et al., 2023). Its uniform genetic background minimises biological variability, supporting reliable dose response comparisons and the use of this model for initial screening of plant extracts prior to more complex in vivo investigations.

A key methodological consideration in this study was the intrinsic colour of GPLE, a recognised source of interference in colorimetric viability assays such as XTT when applied to pigmented plant extracts (Gavanji et al., 2023). Without correction, extract colour can be misread as formazan signal, producing falsely elevated viability estimates. The use of GPLE cell-free blanks at matching concentrations, combined with plate-matched controls, helps minimise the contribution of assay interference to the reported viability values.

This study has several limitations. Data were obtained from a single experimental batch (three technical replicate wells), and independent biological replicates across separate culture passages and days would strengthen confidence in the reported viability estimates. The upper limit of GPLE's cytotoxic potential beyond 125 $\mu\text{g/mL}$ remains to be characterized in future work. Additionally, distinguishing between true metabolic stimulation and residual background correction artefacts at the lowest concentrations would benefit from orthogonal validation using a non-colorimetric method, such as cell counting or DNA quantification.

CONCLUSION

This study established a non-cytotoxic concentration range of GPLE on RAW264.7 macrophages using the XTT assay, fulfilling the objective of this study. GPLE was found to be non-cytotoxic at the lower to moderate concentrations tested, while cytotoxicity was evident at the highest concentration. These findings indicate that GPLE has potential for further investigation, providing a suitable non-cytotoxic basis for evaluating its anti-inflammatory

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