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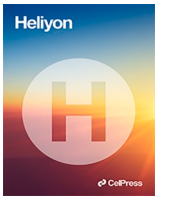

Research article

Antibacterial effect of ethanolic *Gnetum gnemon* L. leaf extract on food-borne pathogens and its application as a natural preservative on raw quail eggs

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A R T I C L E I N F O

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A B S T R A C T

Gnetum gnemon L. is an evergreen tree that belongs to the Gnetaceae family and is commonly used as a vegetable and medicinal plant among indigenous people. The key goal of this study was to assess the antibacterial efficacy of ethanolic *G. gnemon* leaf extract (EGLE) against six food-borne pathogens. The antimicrobial activity of EGLE was evaluated using multiple methods, including the well diffusion assay (WDA), minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and time-kill assay. Gas Chromatography-Mass Spectrometry (GC-MS) analysis was used to identify active volatile compounds responsible for EGLE's antibacterial activities. Total plate count (TPC) was conducted to measure microbial populations and evaluate the efficacy of EGLE as a natural preservative in raw quail eggs. 100 g of dried and powdered sample yielded an average of 11.58 ± 0.38 % post-extraction. The inhibition zone in WDA ranged from 11.00 ± 0.57–13.50 ± 0.58 mm, MIC ranged from 6.25 to 50.00 mg/mL, and MBC values were between 12.5 and >50 mg/mL. Results from the time-kill study showed that at 4 × MIC *Bacillus pumilus* and *B. megaterium* were completely killed in 1 h incubation time and other bacteria were killed within 2–4 h. Findings from TPC demonstrated that at the highest tested concentration of EGLE, there was no significant bacterial growth for a 30-day observation period. Thereby, suggesting that it had the potential to function as a natural preservative for raw quail eggs. EGLE may be a viable alternative to synthetic preservatives in combating food-borne pathogens.

1. Introduction

Foodborne illnesses caused by pathogenic microorganisms in contaminated food have profound public health implications and impose considerable economic and human burdens on society [1]. Improving food safety is crucial for sustainable development and public health. The interconnectedness of food safety and health is evident, as the consumption of unsafe food promotes an unhealthy pattern of sickness and lack of nutrition. Regulating harmful bacteria in food is essential for quality standards, as nearly 10 % of the

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List of uncommon abbreviations

EGLE	Ethanolic *Gnetum gnemon* leaf extract
WDA	- Well diffusion assay
TPC	Total plate count
PCA	Plate count agar
MIC	- Minimum inhibitory concentration
MBC	- Minimum bactericidal concentration
GC-MS	- Gass Chromatography-Mass Spectrometry

global population suffers from foodborne illnesses, resulting in 420,000 deaths annually [2]. The utilization of natural and eco-friendly products has become a prominent subject of discussion. Industries ranging from paint to food prioritize the utilization of natural methods for production, preservation, and degradation [3]. Researchers are now prioritizing the exploration of potent antimicrobial compounds derived from plant sources, to solve the issue of food safety and antibiotic resistance [1,4]. These natural substances can effectively regulate bacterial infection, prolong shelf life, and eradicate hazardous diseases. They have been utilized for the treatment of different infections, as well as for the preservation of food and as natural agents for controlling pests in ecological agriculture [5].

Scientists are currently focusing on plant extracts, essential oils, and antioxidants found in plants as potential substitutes for food preservatives to prevent spoilage. Research has been conducted to determine the efficacy of essential oils and antioxidant properties in various plants for their potential application as antibacterial agents in the food industry. The many applications highlight their potential as valuable resources in the advancement of natural and sustainable antibacterial agents [2,5–7].

Medicinal and edible plants are valuable resources for discovering novel antimicrobial agents [1]. Previous studies have been conducted on plants such as *Teucrium polium* L. and Areca nut due to their conventional medicinal use in the treatment of bacterial infections, gastrointestinal discomfort, constipation, and disorders related to the reproductive system. Various components of these plants, such as leaves, roots, flowers, and seeds, have been utilized by people for ages without any notable adverse reactions [4,6]. *Gnetum gnemon* L. is an example of the above kind of plant that has been used for medicinal and culinary purposes by indigenous populations for centuries [8,9].

Gnetum gnemon L. is an evergreen tree found in Southeast Asia and the western Pacific Ocean islands. It thrives in tropical and lower montane forests. It is a significant source of nutrition, especially for underprivileged regions with limited access to protein-rich foods [8,9]. The plant is also abundant in various beneficial compounds, including antioxidants, tyrosinase inhibitors, antimicrobial agents, anti-ageing substances, and other bioactive compounds [8]. In Malaysia and Indonesia, the common name for *G. gnemon* is *Melinjo*. The *Gnetum* genera, particularly *G. gnemo*n, have been historically employed in traditional Chinese ethnomedicine for treating conditions such as arthritis, bronchitis, and asthma [9].

The extraction method of biologically active compounds from plant materials relies on the type of solvent used. Organic solvents like ethanol, acetone, and methanol are commonly used, with ethanol being the most common due to its safety for consumers [1,10]. Studies on different parts of *G. gnemon* plant showed they have antibacterial properties against *Escherichia coli* and *Staphylococcus aureus* [8]**.** Hexane extract suppressed *S. aureus* proliferation, while dichloromethane was effective against *E. coli* [11]. Dry *G. gnemon* leaf extract with a 96 % ethanol solution showed significant anti-acne properties, effectively preventing the spread of *Propionibacterium acnes* [12].

Edible plants contain bioactive elements with various physiological effects, including anticancer, antioxidant, anti-inflammatory, and antibacterial actions [13]. Previously phytochemical compounds were analyzed in *G. gnemon* using various methods, including column chromatography, and high-performance liquid chromatography [14,15]. These compounds include stilbenoids, resveratrol, saponins, flavonoids, tannins, gnemonoside C, gnetin C, gnemonoside A, C, and D [14,16], vitamin C, beta-carotene, lutein, polyphenols, and vitamin E [15]. The current study used GC-MS to identify and characterize volatile compounds that might contribute to the observed antibacterial activity in EGLE. This method offers a more comprehensive understanding of the chemical constituents responsible for the antimicrobial properties of this plant. Our choice of GC-MS aimed to address a critical gap in the current literature. Specifically, we sought to identify and characterize volatile compounds that might contribute to the observed antibacterial activity in EGLE. GC-MS offers the advantage of detecting compounds that may not have been thoroughly explored in previous studies, allowing for a more comprehensive understanding of the chemical constituents responsible for the antimicrobial properties of this plant.

Quails play a vital role in supplying meat and eggs in many Asian countries. Quail eggs have a higher nutritional value compared to chicken eggs since they include increased levels of antioxidants, minerals, and vitamins [17,18]. Tasić (2022) conducted a study that found that microbial contamination is common in quail eggs [19]. This contamination is mostly caused by inadequate storage conditions, improper handling techniques during storage, and the use of contaminated feed for the birds. The study conducted by Tasić (2022) examined the occurrence of *Salmonella* spp. on the surfaces of quail eggshells [19]. To mitigate the risk of microbial contamination, it is a common practice to refrain from washing farm-fresh eggs since this action could potentially compromise the quality of the outer cuticle that effectively covers the pores of the eggshell [20]. Quail eggs stored at room temperature for 15 days, instead of the recommended 5 days, can decrease albumen quality. However, refrigeration techniques can prolong the longevity of quail eggs by preventing natural degradation during storage [20,21]. Egg shelf-life preservation has been a topic of interest, with synthetic preservatives being a potential solution. However, concerns about safety and toxicity have led to a growing demand for natural ingredients and food preservatives. Plants, with their abundant bioactive compounds, are a promising choice as they are widely

available and can serve as natural preservatives in various food products [22].

The main objective of this research was to evaluate the antibacterial properties of ethanolic *G. gnemon* leaf extract (EGLE). In vitro susceptibility assays were conducted to determine its effectiveness against several food-borne bacteria including Gram-negative and Gram-positive ones. Additionally, total plate count (TPC) analysis on raw quail eggs treated with EGLE assessed its potential as a natural preservative by measuring microbial load reduction. These findings are notable due to the limited studies on using EGLE as an antibacterial agent and natural preservative in food products.

2. Materials and methods

2.1. Sample preparation

The leaf sample of *Gnetum gnemon* was sourced from the Botanical Garden of Universiti Putra Malaysia (UPM), located in Selangor, Malaysia. The leaves were washed and dried at 45 °C for 48 h using an oven dryer (SMA-113, Smoke Master, Japan), before being crushed and pulverized with a food processor (MX-G1012, Panasonic, Japan). Then 100 g of the dried *G. gnemon* leaf powder was mixed with 400 mL of 95 % ethanol (Systerm, ChemAR, Kielce, Poland) and left to stand at room temperature with occasional shaking for 7 days, as described in Rukayadi et al. (2009) [23]. The solution was then filtered using Whatman No. 1 filter paper (Whatman International Ltd., Middlesex, England) and concentrated using a rotary vacuum evaporator (BUCHI Rotavapor R-200, Switzerland) at 50 °C and 150 rpm for 2–3 h. The crude concentrated extract was further dissolved in 100 % dimethyl sulfoxide (DMSO) (Fisher Scientific, Leicestershire, United Kingdom). The resulting concentration was 1000 mg/mL. To achieve the final concentration of 100 mg/mL, the extract was diluted with distilled water at a volumetric ratio of 1:10.

2.2. Yield of extraction

The determination of the percentage of yields for crude plant extract was performed by considering the dry weight of the extract, employing the subsequent formula:

The percentage yield of extract (%) = [(Weight of the resulting dried crude extract, g)/The mass of the dried leaves applied to the extraction process, g] × 100 %

2.3. Antibacterial susceptibility test

2.3.1. Bacterial strains

The antibacterial activity of the EGLE was tested against *Bacillus pumilus* ATCC 14884, *Bacillus megaterium* ATCC 14581, *Staphylococcus aureus* ATCC 29737, *Enterobacter aerogenes* ATCC 13048, *Escherichia coli* ATCC 43895, and *Salmonella* Typhimurium ATCC 14028. The bacterial strains selected for this study were sourced from the American Type Culture Collection (ATCC, Rockville, Maryland, United States).

2.3.2. Preparation of bacterial inoculum

The bacterial strains were introduced onto Muller Hinton Agar (MHA; Oxoid, Hampshire, England) culture plates and subjected to incubation at a temperature of 37 °C for 24 h. The bacteria were produced in compliance with the guidelines provided by the Clinical and Laboratory Standards Institute [24]. Bacterial colonies were introduced into a 1 mL volume of Muller Hinton broth (MHB; Oxoid, Hampshire, England) and incubated for 24 h. Afterwards, the bacterial suspensions were introduced into a solution of 10 mL of MHB. The turbidity of the inoculum was controlled to a range of 10^6–10^8 CFU/mL using both the conventional broth microdilution method and the quantification method. The enumeration of the inoculum was conducted after incubation at a temperature of 37 °C for a duration of 24 h, as stated by the Clinical and Laboratory Standards Institute [24] and Rukayadi et al. (2013) [25].

2.3.3. Well diffusion assay

The Well Diffusion assay was carried out according to the Clinical and Laboratory Standards Institute [24] guidance and tested 100 mg/mL concentration of EGLE against selected bacterial strains. A sterile cotton swab was used to disperse the bacterial colony onto freshly prepared Mueller-Hinton agar (MHA) media and uniform wells with a diameter of 6 mm were produced. Into each well, 50 μL of EGLE was placed along with 50 μL of 0.1 % chlorhexidine (CHX; Sigma Aldrich, USA) as a positive control, and 50 μL of 10 % DMSO as a negative control. The plates were appropriately labelled and incubated at 37 °C for 24 h. The results were measured by observing cleared circles indicating the inhibition zone surrounding the wells and recorded in millimeters (mm).

2.3.4. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The study applied the broth microdilution method according to the Clinical and Laboratory Standards Institute [24] to calculate EGLE's minimum inhibitory concentration (MIC) against selected bacterial strains. The experiment was conducted using a sterile 96-well microtiter plate, where the initial concentration of the inoculum was around 10^6 CFU/mL. A broth medium containing no inoculum or antimicrobial agent was considered a negative control. In contrast, the positive growth control was made up of an entire microbial culture absent of an antimicrobial agent. The microtiter plates were subjected to aerobic incubation at 37 °C for 24 h in MHB medium. MIC was determined for the concentration of extract at which no observable growth was detected. Additionally, to determine the minimum bactericidal concentration (MBC), 10 μL of suspension was taken from each well of the sample in the MIC test and placed

on MHA media. The MHA media were then incubated at 37 °C for 24 h and MBC was recorded. The MBC values were determined by the lowest concentration of EGLE at which no growth on MH agar after 24 h incubation [1].

2.3.5. Time kill assay

The time-kill assay was performed using the methodology outlined by the Clinical and Laboratory Standards Institute [24]. The inoculum suspension for each bacterial strain was prepared separately in Mueller-Hinton broth (MHB) with an approximate concentration of 10^6 CFU/mL. The MHB medium, which included the inoculum, was utilized to dilute the EGLE to attain final concentrations of 0 × MIC, 0.5 × MIC, 1 × MIC, 2 × MIC, and 4 × MIC for every bacterial strain. The mixtures were incubated at a temperature of 30 °C while being agitated at 200 rpm. Samples with a volume of 100 μL were collected at predetermined time points of 0 h, 0.5 h, 1 h, 2 h, and 4 h. Subsequently, a series of dilutions was performed on the samples using a 1 % sterile phosphate buffer saline (PBS; Oxoid, Hampshire, England). The diluted samples were plated onto MHA. The plates were incubated at a temperature of 37 °C for duration of 24 h, after which the colonies were counted. A graph was constructed to display the relationship between log10 CFU/mL and incubation duration.

2.4. Determination of phytochemical compounds in EGLE using gas chromatography-mass spectrometry (GC-MS) analysis

An analysis using gas chromatography-mass spectrometry (GC-MS) was performed to identify the volatile compounds present in *G. gnemon* leaf extract. *G. gnemon* leaf extract was dissolved in high-performance liquid chromatography (HPLC) grade methanol (MEOH; Sigma Aldrich, USA) at a concentration of 50 mg/mL. The analysis was performed utilizing the QP2010 Ultra gas chromatograph-mass spectrometer (Shimadzu Corporation, Kyoto, Japan), which was coupled with an electron multiplier detector. The analyzer utilized a chromatographic column, namely the Rxi-5ms, which had precise measurements of 30.0 m in length, 0.25 mm in internal diameter, and a film thickness of 0.25 μm. The carrier gas used was helium, with a flow rate of 0.80 mL/min for the column. The functional specifications of the analyzer include a column oven with a starting temperature of 50 °C. The temperature was thereafter raised at a rate of 3 °C per minute until it reached 300 °C and remained unchanged for 10 min. The injection temperature and ion-source temperature were both set at 250 °C and 200 °C, respectively. The peaks in the extract of *G. gnemon* were identified by assessing their similarity index, retention index (RI), and mass fragment patterns, comparing them to the reference spectra in the Shimadzu GC-MS NIST/Wiley library. The data were additionally compared to earlier published findings on the volatile compounds found in *G. gnemon* leaf extract.

2.5. Soaking treatment of quail egg sample with EGLE

Samples of raw quail eggs were collected from a wet market in Selangor, Malaysia. The fresh specimens were carefully packed inside the ice box to maintain their freshness. Immediately, all samples were sent to the laboratory for analysis. Various concentrations (0.00 %, 0.05 %, 0.50 %, and 5.00 %) of EGLE were prepared and administered to soak the egg samples. The immersed samples were stored in two conditions: at controlled room temperature (25.0 ± 2.0 °C) and refrigerated environment (4.0 ± 2.0 °C) for 30 days. Then, the microbiological analysis was conducted at multiple time intervals (0 d, 1 d, 3 d, 6 d, 9 d, 14 d, and 30 d). During 0 d, the cracked quail eggs were introduced into a 25 mL solution of sterile saline water (0.1 %) and subsequently agitated (eight-stroke blending per second) using a stomacher machine (BagMixer 400P, France). Then, serial dilution was performed on EGLE-treated quail egg samples using phosphate-buffered saline (0.1 %) as diluent. The mixture was homogenized using a vortex and this process resulted in the dilutions of 10^{-3} to 10^{-6}. The plate count agar (PCA: Sigma Aldrich, United States) was introduced with 0.1 mL diluent from each dilution to perform spread plate and incubate at 37 °C for 24 h. The results were expressed as the logarithmic values of colony forming unit (Log_{10} CFU/g).

2.6. Statistical analysis

The experiments were carried out in triplicate, with each replication being repeated three times. The means were computed utilizing Microsoft Excel 2022. The data analysis for the analysis of variance (ANOVA) was performed using Minitab® Version 21.4.0 for Windows (Minitab Inc.) software. The statistical relevance of the observed differences between the treatments was evaluated by applying Tukey's test, with a significance level set at $P < 0.05$. The results were interpreted as the analysis's means ± standard deviation (SD).

Table 1
Extraction yield of *G. gnemon*.

Ethanolic extraction	Sample weight (g)	Yield of extraction (g)	Percentage of yield (%)
1	100	11.25	11.25
2	100	12.00	12.00
3	100	11.50	11.50
Mean ± SD	100.00 ± 0.00	11.58 ± 0.38	11.58 ± 0.38

Footnote: Results were expressed as means ± standard deviation (SD); n = 3 × 3.

3. Results and discussion

3.1. Yield of G. gnemon leaf extract

The investigation showed that 100 g of dried and powdered samples yielded an average of 11.58 ± 0.38 % after the completion of the extraction process. Table 1 refers to the extraction yield of *G. gnemon* leaf powder.

In food-related studies, organic solvents, such as ethanol, and hexane are often used to extract leaves. While methanol is more polar, ethanol is preferred due to its safety profile, as methanol is toxic to the human body [26–28]. Ethanol, on the other hand, is effective in extracting the desired constituents and generally considered safe for consumption [29]. Therefore, ethanol was chosen as the solvent for extraction in this study for its efficacy and safety profile. Maceration was the extraction method employed in the current study, and the sample was subjected to maceration for seven days to increase the likelihood of obtaining a good yield of the desired compounds. Previous research on the extraction of tannins from plant leaves found that a longer maceration duration led to a better yield, with a six-day maceration period being the most effective [30]. Similarly, the seven-day maceration period in this study allowed sufficient time for ethanol to penetrate EGLE powder, solubilizing desired bioactive compounds within the cell walls through molecular diffusion.

3.2. Antibacterial activity of EGLE

3.2.1. Well diffusion assay

The study utilized the agar well diffusion method to assess the antibacterial effectiveness of EGLE at a concentration of 100 mg/mL. Six strains of both Gram-positive and Gram-negative bacteria were tested, including *Bacillus megaterium, Bacillus pumilus, Staphylococcus aureus*, *Enterobacter aerogenes*, *Escherichia coli*, *Salmonella* Typhimurium. Chlorhexidine (CHX) at 1 mg/mL served as the positive control, while a 10 % concentration of dimethyl sulfoxide (DMSO) served as the negative control. The inhibition zones (Fig. 1) observed ranged from 15.00 ± 0.29 to 18.00 ± 0.29 mm across the bacterial strains tested. The observed inhibition zones in well diffusion assay are displayed in Fig. 1.

The results, documented in Table 2, illustrate the antibacterial activity of the *G. gnemon* L. extract against both Gram-positive and Gram-negative bacteria.

The ethanolic extract of *G. gnemon* had been found to possess antibacterial properties against all six strains of tested foodborne bacteria. However, upon examination of the diameter of the inhibition zone of each bacterial strain, it was evident that Gram-positive

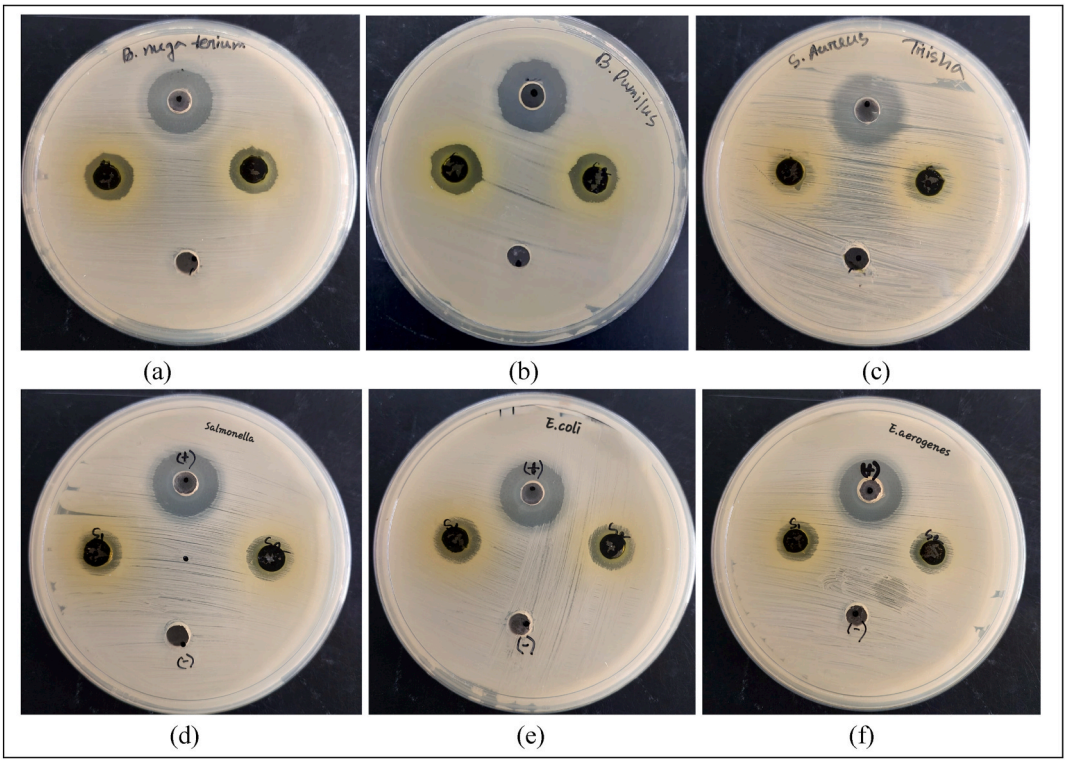

Fig. 1. Inhibition zone of (a) *Bacillus megaterium* (b) *Bacillus pumilus* (c) *Staphylococcus aureus* (d) *Salmonella enterica* serovar Typhimurium (e) *Escherichia coli* (f) *Enterococcus aerogenes* after being treated with 10 % *G. gnemon* leaf extract.

Table 2
Inhibition zone of *G. gnemon* leaf extract against foodborne bacteria.

Bacterial Strains	Diameter of inhibition zone (mm) ± SD		
	G. gnemon leaf extract [10 % (w/v)]	CHX [0.1 % (w/v)]	DMSO [10 % (v/v)]
B. megaterium **ATCC 14581**	13.00 ± 0.29^{ab}	17.00 ± 0.29	n.a
B. pumilus **ATCC 14884**	13.50 ± 0.58^{a}	18.00 ± 0.29	n.a
S.aureus **ATCC 29737**	12.50 ± 0.29^{cd}	18.00 ± 0.29	n.a
E.aerogenes **ATCC 13048**	11.50 ± 0.29^{cd}	17.00 ± 0.29	n.a
E. coli **ATCC 43895**	12.00 ± 0.50^{bc}	15.00 ± 0.29	n.a
S. **Typhimurium ATCC 14028**	11.00 ± 0.57^{d}	15.50 ± 0.50	n.a

Footnote: n.a: not active (no inhibition).
The diameter of the inhibition zone is in (mm) including well diameter = 6.00 mm.
Positive control (Chlorhexidine: CHX; 0.1 %); Negative control (DMSO; 100 %).
Results were expressed as means ± standard deviation (SD); n = 3 × 3. One Way ANOVA analysis showed significant differences ($p < 0.05$) between tested bacterial strains and inhibition zones from *Melinjo* (*G.gnemon* L.) leaf extract. The data indicated by different lowercase superscripts (a–f) in the same column are significantly different, whereas similar superscripts imply that there are no significant differences.

bacterial strains display a greater inhibition zone compared to their Gram-negative counterparts. Numerous studies have demonstrated that antibacterial agents produced from plants exhibit greater efficacy against Gram-positive bacteria. This phenomenon can be related to the intrinsic resistance of Gram-negative bacteria, as well as the specific composition and features of plants [31,32]. Research indicated that Gram-positive bacteria have cell walls that are more susceptible to a variety of antimicrobial drugs and herbal remedies than Gram-negative bacteria [33–35]. Gram-negative bacteria's periplasmic space and lipopolysaccharide layer contributed to their greater resistance [31]. The bacterial strains utilized in this research displayed varying levels of susceptibility to EGLE at a 100 mg/mL concentration. Gram-positive bacterial strains such as *B. pumilus, B. megaterium,* and *S. aureus* exhibited promising effects with inhibition zones of 13.50 ± 0.58, 13.00 ± 0.29, and 12.50 ± 0.29 mm, respectively. Meanwhile, Gram-negative bacterial strains like *E. coli, S.* Typhimurium, and *E. aerogenes* displayed inhibition zones of 12.00 ± 0.50, 11.00 ± 0.57, and 11.50 ± 0.29 mm, respectively. Notably, the Gram-positive bacterial strains *B. pumilus* and *B. megaterium* displayed a greater inhibition zone in the presence of the ethanol extract. This observation confirmed the heightened antibacterial activity of the ethanol extract as the size of the inhibition zone is positively correlated with its effectiveness against bacterial growth.

Ghavam et al. (2022) [5] conducted a study on four different plant Lamiaceae family that are native to Iran. The study was conducted on different Gram-negative and Gram-positive bacterial strains. Upon all the bacterial strains the highest antimicrobial activities were obtained with the essential oil from *Thymus daenensis* against Gram-positive strains such as *Staphylococcus aureus* with inhibition zone of 39 ± 10 mm. Another study [6] was done on *Teucrium polium* L, an Iranian medicinal plant that showed the highest antibacterial activity against *S. aureus* with the largest zone of inhibition (~14.29 mm). And for Gram-negative bacteria, *Escherichia coli* showed the highest inhibition zone (~9.00 mm).

Another study by Parhusip et al. (2011) [36] evaluated the antibacterial activity of *G. gnemon* seed and peel extract against pathogenic bacteria, using various solvents such as ethanol, ethyl acetate, and hexane. The extracts were found to be more effective against Gram-positive bacteria *(B. cereus* and *S. aureus)* than Gram-negative bacteria *(P. aeruginosa)* for all solvents. In this study, *E. coli* showed the highest inhibition zone (12.00 ± 0.50 mm) among all the Gram-negative bacteria. Given that *E. coli* is naturally resistant to

Table 3
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *G. gnemon* leaf extract against bacterial strains.

Bacterial Strains	*G. gnemon* leaf extract (mg/mL)		CHX (mg/mL)	
	MIC	MBC	MIC	MBC
B.megaterium **ATCC 14581**	12.5	25.0	0.98	0.98
B.pumilus **ATCC 14884**	6.25	12.5	0.98	0.98
E.aerogenes **ATCC 13048**	12.5	25.0	0.78	1.56
E. coli **ATCC 43895**	25.0	>50.0	0.39	0.78
S.aureus **ATCC 29737**	12.5	>50.0	0.98	0.98
S. **Typhimurium ATCC 14028**	12.5	25.0	0.98	0.98

Footnote: Positive control (chlorhexidine: CHX; 0.1 %).

most clinically relevant antimicrobial drugs [37], EGLE may have the potential to serve as an antimicrobial agent against *E. coli*.

The agar well diffusion assay is a simple and cost-effective method commonly used in studies on plants to assess antibacterial activity [38,39]. Nevertheless, the study's results primarily consisted of qualitative data, which proved to be inadequate for a precise assessment of antibacterial effectiveness. To acquire accurate quantitative data, it was imperative to conduct MIC and MBC assays during the assessment of the antibacterial efficacy of the ethanolic *G. gnemon* leaf extract against foodborne pathogens.

3.2.2. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC range of all six bacterial strains was presented in Table 3.

The MIC range for the bacterial strains was between 6.25 mg/mL and 25.0 mg/mL, while the MBC range was between 12.5 mg/mL and >50.0 mg/mL. All the bacterial strains showed bacteriostatic effect against EGLE. Among the Gram-positive bacterial strains, *B. pumilus*, *B. megaterium*, and *S. aureus* had MIC concentrations of 6.25 mg/mL, 12.5 mg/mL, and 12.5 mg/mL, respectively, with MBC concentrations of 12.5 mg/mL, 25.0 mg/mL, and 25.0 mg/mL. Meanwhile, *E. coli*, *S.* Typhimurium, and *E. aerogenes* had MIC values of 25.0 mg/mL, 12.5 mg/mL, and 12.5 mg/mL with MBC values of >50.0 mg/mL, 25.0 mg/mL, and >50.0 mg/mL, respectively.

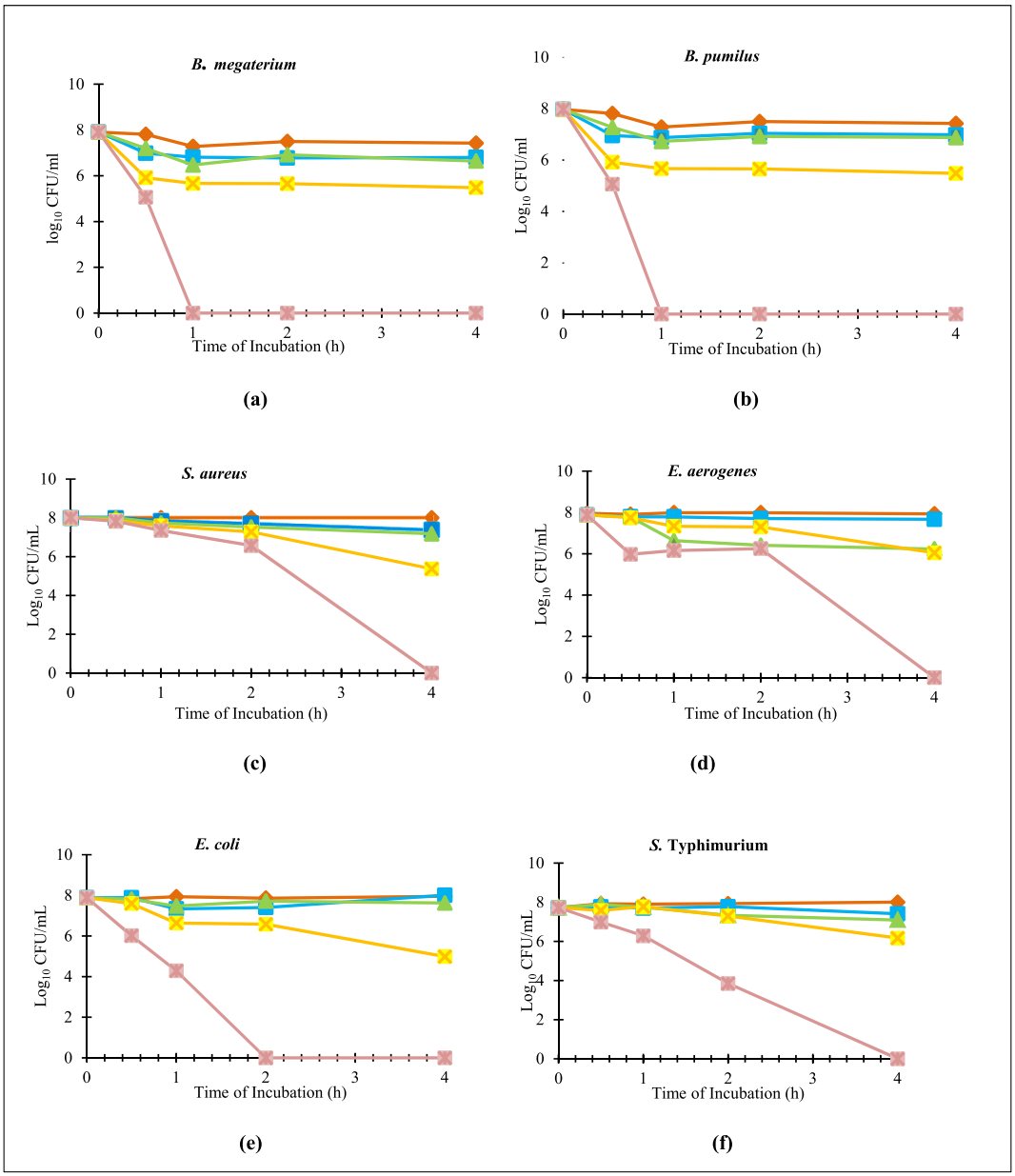

Fig. 2. Time-kill curve of six bacterial strains (a) *B. megaterium* (b) *B. pumilus* (c) *S. aureus* (d) *E. aerogenes* (e) *E. coli* (f) *S.* Typhimurium at different concentrations; 0 × MIC-◆, 0.5 × MIC-■, 1.0 × MIC-▲, 2.0 × MIC-× and 4.0 × MIC-■.

A recent study conducted by Dayoh et al. (2021) [40] examined the effects of EGLE on *S. aureus* ATCC25923 and found that the MIC value for *S. aureus* was 640 mg/mL. Another study [36] investigated the efficacy of ethanolic *G. gnemon* peel extract against *B. cereus*, *P. aeruginosa,* and *S. aureus*. Of these three bacteria, only *B. cereus* and *S. aureus* had MIC values of 1.40 μg/mL and 0.90 μg/mL, respectively and *G. gnemon* peel extract showed no activity for the Gram-negative bacteria. While the MIC concentration obtained for *G. gnemon* peel was comparatively lower than that of our current leaf extract, both of them showed antibacterial susceptibility towards *S. aureus*. In both studies, *S. aureus* required a lower concentration of extract than other bacteria, indicating that the *G. gnemon* plant has higher antibacterial activity against *S. aureus*. It should be noted, however, that the MIC and MBC assay have certain limitations in providing comprehensive insights into the kinetics of antibacterial action. Therefore, a time-kill experiment was conducted to establish the relationship between the rate of bactericidal activity, the incubation period in hours, and the concentration of the extract.

3.2.3. Determination of time-kill assay curve

The monitoring of bacterial growth and mortality for all six bacterial strains throughout a 4 h incubation period with five different concentrations, including positive control (no extract), to assess the antibacterial efficacy of the extract, is depicted in Fig. 2(a–f).

According to data from Fig. 2(a) (*B. pumilus*), the initial count was 8 Log_{10} CFU/mL, and a plateau trend was observed across 0 × MIC, 0.5 × MIC, 1.0 × MIC, and 2.0 × MIC, indicating no significant difference in incubation time. However, at 4.0 × MIC (25.0 mg/mL), there was a notable decrease in Log_{10} CFU/mL count after only 1 h of incubation. Previous research by Aiyegoro et al. (2008) [41] found that methanolic *Helichrysum pedunculatum* leaf extract demonstrated bactericidal activity on *B. pumilus* at concentrations of 5 mg/mL (1 × MIC) and 10 mg/mL (2 × MIC) after 12 h of incubation. Fig. 2(b). (*B. megaterium*) demonstrated that at a concentration of 4 × MIC (50.0 mg/mL) of EGLE, all bacteria were eradicated within only 1 h. In a previous study by Mahbub et al. (2011) [42] a concentration of 4.50 mg/mL of *Crescentia cujete* extract and 2.50 mg/mL of *Moringa oleifera* extract displayed a bacteriostatic effect on *B. megaterium* after 24 h of incubation. In contrast, the current research indicates that *G. gnemon* extract can eliminate *B. megaterium* at a higher concentration within only 1 h of incubation time.

Based on the results presented in Fig. 2(c), it was found that EGLE at 4 × MIC (50.0 mg/mL) completely eradicated all bacterial growth of *S. aureus* within 4 h. Another study conducted by Kyahar et al. (2021) [43] focused on the medicinal plant *Adenodolichos paniculatus*, which is known for its traditional use in treating sore throat infections. The research revealed that a concentration of 6.25 mg/mL with an incubation time of 12 h produced complete eradication of *S. aureus*. Interestingly, the study found that a higher concentration of 25.0 mg/mL was required for complete elimination of *S. aureus* within a 5 h incubation period. This aligns with our current findings, which suggest that a higher concentration of *G. gnemon* leaf extract *S. aureus* requires a shorter incubation period to achieve bactericidal effects. In Fig. 2(d) (*E. aerogenes*), it was demonstrated that bacterial growth was eliminated within a 4 h incubation period at a concentration of 4.0 × MIC (50.0 mg/mL). A study by Ogunsina (2020) [44] found that administering the methanolic extract from *Lannea acida* at a concentration of 50 mg/mL for 7 h exhibited bacteriostatic properties against *E. aerogenes*. However, our recent research has shown that EGLE was significantly more effective as a bactericidal agent against *E. aerogenes* than the previously mentioned study.

According to the findings in Fig. 2(e), which pertains to *E. coli*, a concentration of 100.0 mg/mL (4.0 × MIC) resulted in a significant reduction of bacterial count. In fact, within a mere 2 h of incubation time, *E. coli* was entirely eradicated. Murray et al. (2020) [45] also reported on the bactericidal activity of *Agrimonia pilosa* extract against 4.0 × MIC of *E. coli*. In that study, *E. coli* was eliminated at a concentration of 0.03 mg/mL within 4 h. It's worth noting that the concentration of *G. gnemon* extract used in the current study was higher than that of *A. pilosa*. This discrepancy in concentration could be due to the presence of bioactive compounds in different plants.

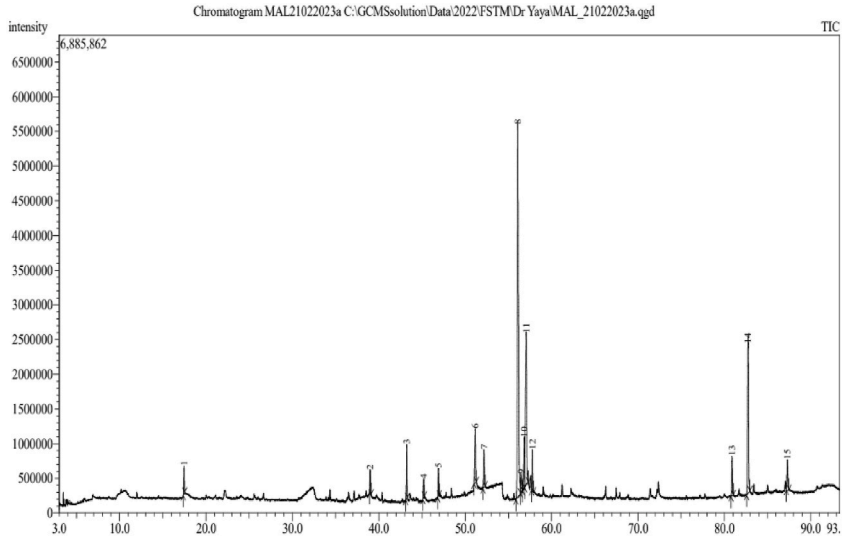

Fig. 3. Chromatogram of GC-MS of *G. gnemon* L. leaf extract.

According to the findings in Fig. 2(f)–*S*. Typhimurium was successfully eliminated at a concentration of 4.0 × MIC and 50.0 mg/mL. A recent study conducted by Loo et al. (2018) [46] explored the effects of silver nanoparticles (AgNPs) obtained from *pu-erh* tea leaves on *S*. Typhimurium. The results of the time-kill assay showed that *S*. Typhimurium was eradicated after only 1 h of incubation at concentrations of 15.6 μg/mL and 31.2 μg/mL, corresponding to 4 × MIC and 8 × MIC, respectively.

The results derived from the time-kill assay bolster the idea that eradicating bacterial populations depends on both the amount of extract used and the duration of exposure. This is demonstrated by the fact that higher concentrations of the extract caused a more rapid reduction in population size, culminating in complete elimination.

3.3. Gas chromatography-mass spectrometry (GC-MS) profile of EGLE

In the GC-MS chromatogram of the crude EGLE, a total of 15 peaks were detected, as shown in Fig. 3.

To identify the compounds, we computed the similarity index and retention indexes (RI) and compared the mass fragment patterns with the standard spectra available in the Shimadzu GC-MS NIST/Wiley library. The most abundant compound was phytol (38.76 %; Peak no. 8), followed by cis, cis,*cis*-7-10-13-hexadecatrienal (18.67 %; Peak no. 11), and vitamin E (12.81 %) (Peak no.14). The remaining twelve compounds were present in amounts of less than 5 % Table 4.

Phytol is a naturally occurring diterpene alcohol found in plants that is produced during the breakdown of chlorophyll, a crucial process in photosynthesis. A study [53] conducted a time-kill assay to assess the impact of phytol on *S. aureus* growth, revealing that the presence of 0.00015 mg/mL of the diterpene inhibited growth due to its adverse effect on the cell membrane. Lee et al. (2016) [57] examined phytol's antibacterial activity in *P. aeruginosa*, revealing that phytol induces oxidative stress in the tested bacterium. *Cis*-7, 10,13-hexadecatrienal is a fatty aldehyde that occurs naturally in selected plant-based and essential oils. According to another study [58] *Cis*-7,10,13-hexadecatrienal was obtained from GC-MS analysis of *Litsea cubeba* oil, exhibited antibacterial properties against *E. coli* ATCC8739. Another abundant compound of EGLE is vitamin E, a fat-soluble vitamin with antioxidant properties, which has been shown to have antibacterial activities. It includes lipophilic antioxidants called tocopherols and has been shown to enhance the bactericidal effects of antibiotics by interfering with lipocalin binding. Vitamin E has been found to be most potent against *Candida albicans*, *S. aureus* and *S. epidermidis*. Studies have shown that vitamin E concentrations ranging from 50 to 400 IU/mL can inhibit various bacterial pathogens, including *E. coli*, and *P. aeruginosa* [59–61].

In our current research, we have discovered the presence of 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-, mega-stigmatrienone, neophytadiene, ethyl linoleate, 9,12,15-octadecatrienoic acid, ethyl ester, (Z,Z,Z)-, and 2,3-Bis(1-methylallyl)pyrrolidine in EGLE. While these specific compounds have not been previously identified in the *G. gnemon*, it is important to note that they have been found in other plant extracts. GC-MS analysis also detected other volatile compounds such as Omega-3 fatty acid, tocopherol, diterpene, flavonoid, methoxyphenols, and ascorbic acid. Notably, most of the compounds examined in our study have been reported to exhibit antibacterial or antimicrobial properties, further supporting *G. gnemon* leaf extract as a potential antimicrobial agent.

3.4. Effect of G. gnemon leaf extract on microbial population in raw quail egg

Over the years, there has been a growing demand for natural food preservatives, prompting extensive research into their effectiveness in preserving perishable food items [62]. This trend is largely fueled by concerns over food-borne illnesses, which have far-reaching implications for not only consumers but also the food industry and safety regulators. Natural antimicrobial compounds have emerged as a promising solution, effectively combating bacterial and fungal growth and ultimately enhancing the quality and longevity of food products [63].

The current study analyzed the effects of using EGLE as a natural preservative. Different concentrations of EGLE (0.00 %, 0.05 %, 0.50 %, and 5.00 %) were tested at various time intervals (0 d, 1 d, 3 d, 6 d, 9 d, 14 d, 30 d) under different temperature conditions, including room temperature and chiller temperature. To determine food hygiene levels and bacterial presence in food, water, and other sanitary materials, plate count agar (PCA) was used as a non-selective medium. This medium is commonly used to cultivate bacteria that can thrive at temperatures ranging from 20 to 45 °C [64].

From the information presented in Fig. 4, it is apparent that the microbial count fluctuates across various concentrations of EGLE over a range of storage times (0 d, 1 d, 3 d, 6 d, 9 d, 14 d, and 30 d) at a temperature of 25.0 ± 2.0 °C. Log_{10} CFU/g was employed as a method of measuring microbial count. At concentrations of 0.00 % and 0.05 % of EGLE, there was a consistent increase in bacterial growth. Due to a significant increase in bacterial population from 2.95 Log_{10} CFU/g to 7.77 Log_{10} CFU/g and 7.50 Log_{10} CFU/g, respectively, bacteria analysis at concentrations of 0.00 % and 0.05 % was terminated on the sixth day. As a result, EGLE concentrations of 0.50 % and 5.00 % were used to assess the period between day 9 and day 30. At a concentration of 0.50 %, bacterial growth increased on the fourteenth day, whereas there was no significant increase in the bacterial population at a concentration of 5.00 %. The findings indicate that EGLE is more effective as an antibacterial agent at a concentration of 5.00 % than at lower concentrations such as 0.00 %, 0.05 %, and 0.50 %. As a result, the use of EGLE concentration has a significant impact on bacterial growth in raw quail eggs. A study conducted by Mukhlisah et al. (2020) [65] provided evidence that the augmentation of *G. gnemon* leaf extract concentrations plays a role in the preservation of egg quality.

Fig. 5 shows a significant difference in the result between the chiller and room temperature. When the samples were exposed to lower temperatures in the chiller, bacterial growth declined. Some of the samples even did not exhibit any bacterial growth when subjected to chiller conditions. The findings indicated that varying doses of EGLE, ranging from 0.05 % to 5.00 %, did not lead to noteworthy bacterial proliferation in the sample. This has occurred because the temperature in the chiller can effectively maintain the

Table 4
List of volatile compounds detected in GC-MS.

Peak No.	Constituent	Molecular formula	Molecular mass	$[M]^+$	t_R (min)	Mass fragmentation (m/z)	Area (%)	RI	SI (%)	Classification	Reported Activity	References
1	4H-Pyran-4-one,2,3 dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144.1253	372	17.462	43,72,101,144	1.54	1269	92	Flavonoid	Antimicrobial, Anti-inflammatory, and Antioxidant	[47]
2	Megastigmatrienone	$C_{13}H_{18}O$	190.2814	385	39.009	41,91,133,175	1.43	1454	87	Carotenoid	Aroma	[48]
3	Coniferyl alcohol	$C_{10}H_{12}O_3$	80.0786	377	43.232	77,91,124,137	3.70	1667	91	Methoxyphenols	Antibacterial	[49]
4	2,3-Bis(1-methylallyl) pyrrolidine	$C_{12}H_{21}N$	179.3018	332	45.201	41,69,95,124	1.10	1328	78	Terpene	**Sweetener**	[50]
5	Neophytadiene	$C_{20}H_{38}$	278.2973	358	46.911	41,68,82,123	1.63	1774	94	Diterpene	Antimicrobial	[51]
6	l-(+)-Ascorbic acid 2,6-dihexadecanoate	$C_{38}H_{68}O_8$	652.4914	289	51.167	43,73,85,129	4.12	4765	90	Ascorbic acid	Anticancer, Antioxidant, Antibacterial	[52]
7	cis-Sinapyl alcohol	$C_{11}H_{14}O_4$	210.0892	290	52.191	77,167,182,210	2.71	1842	74	Alcohol	No report	**This study**
8	Phytol	$C_{20}H_{40}O$	296.5310	369	56.104	43,57,71,81	38.76	2106	93	Acyclic diterpenoids alcohol	Antimicrobial, Antioxidant	[53]
9	Linoleic acid	$C_{18}H_{32}O_2$	280.4472	305	56.483	41,55,81,95	1.52	2183	83	Omega-3 fatty acid	Antimicrobial, antioxidant	[11]
10	Ethyl linoleate	$C_{20}H_{36}O_2$	308.4986	363	56.835	41,55,67,95	3.91	2193	85	Fatty acid	Antimicrobial	[54]
11	Cis, cis,*cis*-7-10-13-Hexadecatrienal	$C_{16}H_{26}O$	234.3770	341	57.060	41,55,67,70	18.67	1824	88	Omega-3 fatty acid	Antibacterial	[55]
12	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-	$C_{20}H_{34}O_2$	306.4828	356	57.780	67,79,95,108	2.63	2201	93	Acid Ethyl Ester	Anti-inflammatory	[56]
13	Gamma-Tocopherol	$C_{28}H_{48}O_2$	416.6795	375	80.891	151,191,205,416	2.69	3036	91	Tocopherol	Antibacterial,	[16]
14	Vitamin E	$C_{29}H_{50}O_2$	430.7061	322	82.744	41,43,165,430	12.81	3149	90	Tocopherol	Antibacterial	[15]
15	Gamma-Sitosterol	$C_{29}H_{50}O$	414.7067	389	87.291	43.107,145,414	2.77	2731	84	Stigmastans and derivatives	Antimicrobial,	[11]

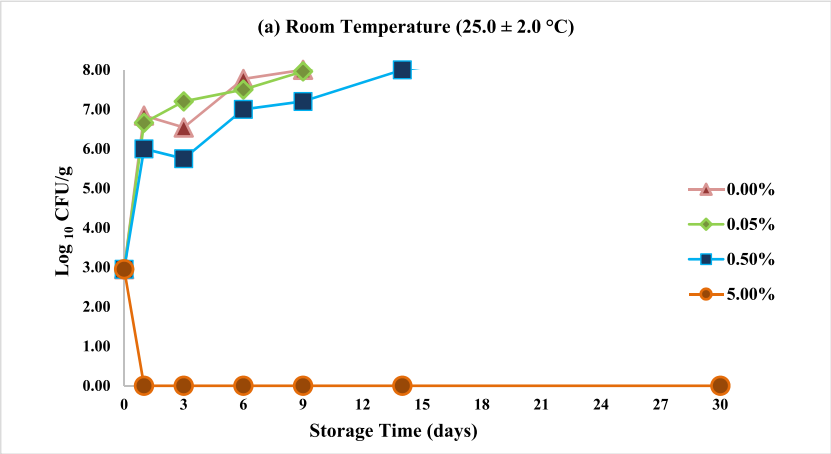

Fig. 4. Microbial counts were measured after storing raw quail eggs for 30 days at room temperature under different extract concentrations (▲-0.00 %;♦-0.05 %,■-0.50 %;●- 5.00 %).

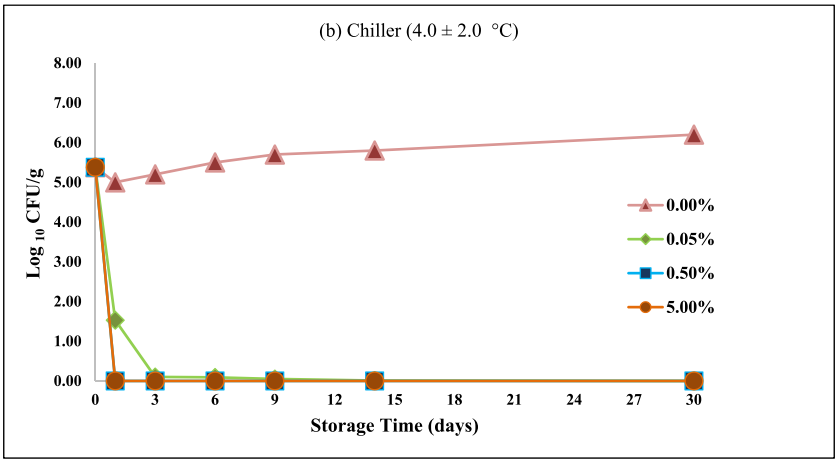

Fig. 5. Microbial counts were evaluated after 30 days of storage with varying extract concentrations (▲-0.00 %;♦-0.05 %,■-0.50 %;●-5.00 %) under different chiller temperatures.

quality and longevity of products.

A study published by Wong et al. (2021) [66] suggested that raw, half-boiled, or minimally cooked eggs have higher chances of microbial contamination compared to hard-boiled eggs. Eggs can become the vehicle of microbial transmission if they are raw, improperly cooked, or stored without refrigeration [67,68]. Egg quality, shelf life, and subsequently consumer health is impacted by storage conditions and time [20]. Based on storage conditions, it has been established that eggs stored at room temperature can experience decreased quality and that refrigeration can prolong the freshness and shelf life of eggs [21]. This study indicated that ethanolic *G. gnemon* leaf extract held promise as a natural preservative for raw quail eggs. Generally, it is not recommended to maintain room temperature as it is less preferable compared to the chiller. However, both temperatures showed a decline in the microbial population found in uncooked quail eggs.

4. Conclusion

Ethanolic *Gnetum gnemon* L. leaf extract showed antimicrobial activity against six food-borne pathogens *Bacillus pumilus* ATCC 14884, *Bacillus megaterium* ATCC 14581, *Staphylococcus aureus* ATCC 29737, *Enterobacter aerogenes* ATCC 13048, *Escherichia coli* ATCC 43895, and *Salmonella* Typhimurium ATCC 14028. GC-MS analyzed 15 volatile compounds, most of which displayed antibacterial properties. This supports the observed antibacterial activity and highlights EGLE's potential as a natural source of antimicrobial agents. Moreover, it had the potential to function as a natural preservative in raw quail eggs at room temperature and chilling, making it a viable alternative to synthetic preservatives in combating food-borne pathogens. Hence, future studies can be done on the application of ethanolic *G. gnemon* leaf extract as a natural preservative on other perishable foods such as fresh-cut fruits, vegetables,

and meat.

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CRediT authorship contribution statement

Mansura Rahman Trisha: Writing – review & editing, Writing – original draft, Visualization, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Vyenna Deavyndra Gunawan:** Investigation. **Jun Xian Wong:** Formal analysis. **Mohd Sabri Pak Dek:** Validation, Supervision, Resources. **Yaya Rukayadi:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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