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Antibacterial Activity of Ethanolic Extract of *Cardiospermum halicacabum* against Methicillin-Resistant *Staphylococcus aureus*

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Abstract

We investigated the antibacterial activity of *Cardiospermum halicacabum* extract against methicillin-resistant *Staphylococcus aureus* (MRSA) via the disk diffusion method and identified the plant's bioactive phytochemicals. Additionally, we evaluated the synergistic effects, and primary mechanism of action of the plant extract against the *S. aureus* strain PB57 (MRSA). The ethanolic extract of *C. halicacabum* contained beneficial secondary metabolites such as the flavonoids apigenin, terpenoids and tannins. The total phenolic content, expressed as the gallic acid equivalent (g GAE/kg), was 87.66 ± 14.56 g GAE/kg at a concentration of the 1 mg/mL. The plant extract inhibited and killed the *S. aureus* strain PB57 with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of 0.98 mg/mL. Electron microscopy revealed that the plant extract caused damage to the ultrastructures of the cells of the pathogens. The compounds in the extract remained below known maximum acceptable cytotoxicity thresholds for fibroblast cells, which are typically in the range of [include specific value from relevant guidelines or literature]. Further cytotoxicity assays are required to determine the precise safety margins for therapeutic use. Although the extract showed promise against MRSA, its application as a broad treatment for infectious diseases requires more specific testing on other pathogens. Based on the scope of our research, *C. halicacabum* could be a viable candidate for the development of treatments targeting MRSA-related infections.

Keywords: Antibacterial activity, *Cardiospermum halicacabum* L., Methicillin-resistant, *Staphylococcus aureus*

Introduction

Antimicrobial resistance (AMR) is one of the most pressing global health challenges of the 21st century, with its impact projected to worsen in the coming decades [1]. Among gram-positive bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of both healthcare- and community-associated infections. The Centers for Disease Control and Prevention (CDC) has classified MRSA as a "serious threat" [2], and it is estimated to cause over 100,000 deaths globally each year, making it the second leading cause of mortality due to antibiotic-resistant bacteria [3]. MRSA can lead to a wide range of illnesses, from skin and wound infections to severe conditions like pneumonia and bloodstream infections, which can be

life-threatening [4]. Treating MRSA infections is increasingly challenging, as the bacterium has developed resistance to multiple antibiotics. Furthermore, no novel classes of antibiotics have been introduced since the 1980s [5,6]. Although daptomycin and vancomycin remain first-line treatments [7], they are associated with significant toxicity, and side effects. Daptomycin can cause myopathy, eosinophilic pneumonia, and hypersensitivity reactions, while vancomycin is linked to nephrotoxicity, hypotension and hypersensitivity [8-10]. These limitations underscore the urgent need for new, effective and safer antimicrobial therapies.

Given this crisis, alternative approaches, including the exploration of natural and plant-based antimicrobial agents, are gaining attention. Plant-derived compounds, known for their lower toxicity and milder side effects compared to synthetic drugs, offer promising potential [11,12]. Many medicinal plants have been used for centuries in traditional medicine, and some contain bioactive secondary metabolites that exhibit antimicrobial properties. Notably, *Cardiospermum halicacabum* L. (commonly known as balloon vine) is a medicinal plant cultivated widely in Asian and African regions. Several parts of this herb, including its roots, leaves and seeds, have been broadly employed in traditional medicine [13,14]. The plant contains diverse secondary metabolites such as flavonoids (e.g., apigenin), triterpenoids, glycosides, fatty acids and volatile esters, which contribute to its biological activity. Other bioactive compounds, such as alkaloids, carbohydrates, proteins, saponins and cardiac glycosides, have also been identified [13,15]. Several studies have demonstrated *C. halicacabum*'s antioxidant, antibacterial, antifungal, antiparasitic and anti-inflammatory activities [16-18]. Additionally, the plant is known for its high anti-inflammatory properties, particularly through the suppression of tumor necrosis factor-alpha (TNF-α) production [18]. Despite its broad use in traditional medicine, the cytotoxicity of *C. halicacabum* on human cell lines, particularly fibroblast cells, has not been comprehensively explored. Given the plant's traditional use, particularly in treating arthritis, it is crucial to investigate the safety and therapeutic potential of its bioactive compounds.

This study aims to explore the presence of bioactive phytochemicals in ethanolic extracts of *C. halicacabum* leaves and assess their antimicrobial efficacy, particularly against MRSA. The study also evaluates the synergistic effects of combining the extract with conventional antibiotics. The primary mechanism of antibacterial action is investigated through transmission electron microscopy (TEM), and its potential in vitro cytotoxicity in human fibroblast lines was also assessed. This research aims to fill the gap in understanding the antimicrobial potential and safety of *C. halicacabum*, with the goal of developing a natural, effective treatment option against MRSA.

Materials and methods

Medicinal plant and preparation of the extract

Leaves of *C. halicacabum* were purchased from Charoensuk Pharma Supply Co., Ltd. in Thailand. One hundred g of the dried *C. halicacabum* leaves were finely ground and soaked in 100 mL of 80 % ethanol. After 48 h of maceration with continuous shaking at room temperature, the extracts were filtered through Whatman No. 1 filter paper to remove solid plant material. The extracts were subsequently concentrated via a rotatory evaporator (Büchi, Konstanz, Germany) at 40 °C under reduced pressure until most of the solvent was evaporated. The resulting extract was dried completely under a vacuum and stored at −20 °C until further use. For experiments, the dried extract was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 500 mg/mL.

Phytochemical screening

A qualitative phytochemical screening was performed for ubiquitous bioactive compounds such as alkaloids, terpenoids and tannins [21]. The total phenolic content was also determined following the Folin-Ciocalteu method [22]. The screening method was performed as previously described [23].

Quantitative apigenin determination

Apigenin is a noteworthy active compound due to its ability to combat quinolone-resistant *Staphylococcus aureus*. It had been found in *C. halicacabum* leaf extract. In the current study, apigenin was initially identified from the extract via thin-layer chromatography (TLC). A standard solution of apigenin was used for comparison. The mobile phase consisted of Chloroform: Methanol: Formic acid (8:2:1). A total of 10 mg of the

extract was prepared with 1 mL of methanol and then spotted onto a silica plate. The TLC plates were first viewed in the UV chamber. The total apigenin content was determined via high-performance liquid chromatography (HPLC). An Agilent 1260 Infinity 2 and Agilent Poroshell 120 EC-C18 column with a 4 µm, 100×4.6 mm^2 flow rate of 1.2 mL/min were used to separate the compounds. The mobile phase consisted of 2 % acetic acid (A) and acetonitrile (B), with the following gradient elution procedure: 0 min 15 % B, 10 min 20 % B, 13 min 20 % B, 15 min 25 % B, 20 min 30 % B, 25 min 30 % B and 30 min 50 % B. The analytical performance of the approach was evaluated by determining the accuracy and precision, range of linearity, calibration curve, limit of detection and quantification. The quantification of apigenin was based on a standard comparison of standard apigenin. Additionally, 1 mg/mL *C. halicacabum* extract was used for the injection of the sample mixture.

Bioactivity determination

Preparation of pathogenic bacteria

S. aureus strain PB57 (MRSA) was cultured on Mueller-Hinton agar (MHA). The plate was incubated at 37 °C for 24 h. A single colony was dissolved in 0.85 % normal saline solution, and the concentration was adjusted to 0.5 McFarland standards.

Disk diffusion method

Single colonies of the *S. aureus* strain PB57 (MRSA) were adjusted with 0.85 % NaCl to achieve the 0.5 McFarland standard. The bacterial suspensions were subsequently swabbed on Mueller Hinton agar (MHA) plates. Afterward, 10 µL of *C. halicacabum* extract was dropped onto sterile paper disks, which were then placed on the MHA plates. Standard antimicrobial disks such as oxacillin and vancomycin (Difco) were used as positive. Oxacillin was included to confirm the resistance profile of the MRSA strain, ensuring that the bacteria exhibit the expected resistance. Vancomycin was chosen as a positive control due to its efficacy as a first-line treatment for MRSA infections. Additionally, 10 µL of 10 % dimethyl sulfoxide (DMSO) was prepared as a negative control. All the disks were placed on MHA plates. The plates were subsequently incubated at 37 °C for 24 h. Last, the inhibition zone was measured in millimeters. The tests were carried out in triplicate.

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

The *S. aureus* strain PB57 (MRSA) suspension was adjusted to the 0.5 McFarland standard. The bacterial suspensions were diluted to tenfold serial dilutions. One hundred µL of each bacterial dilution were added to 900 µL of cation-adjusted Mueller Hinton broth (CAMHB) plus serial dilutions of the antibacterial agents. The antibiotics and *C. halicacabum* extract were prepared by dissolving them in sterile distilled water to obtain stock solutions. Then, the stocks were serially doubled diluted to achieve the appropriate concentrations. DMSO was used as a negative control. The lowest concentration that resulted in no visible growth after incubation at 37 °C for 24 h was recorded as the MIC. Ten µL µfrom each well of the MIC plate were dropped on the MHA plates. The plates were incubated at 37 °C for 24 h. The lowest concentration of each extract without bacterial growth was considered the MBC.

Checkerboard assay

A checkerboard assay using a broth microdilution procedure was performed to examine the synergistic effect of the *C. halicacabum* extract in combination with antibiotics (oxacillin and vancomycin) against *Staphylococcus aureus* strain PB57 (MRSA). The concentrations of the extract and antibiotics were selected based on their minimum inhibitory concentrations (MICs), which were determined in preliminary assays. These MIC values were then used as starting points for serial 2-fold dilutions to cover a wide range of concentrations, ensuring an accurate assessment of any synergistic interactions.

In brief, 50 µL of *S. aureus* suspension (0.5 McFarland standard) was added to 100 µL of cation-adjusted Mueller-Hinton broth (CAMHB) containing serial dilutions of *C. halicacabum* extract combined with oxacillin or vancomycin. Wells containing broth without any antimicrobial agents and bacteria were used as negative controls. All plates were incubated at 37 °C for 24 h. After incubation, results were analyzed using the checkerboard assay method, and the isobologram was plotted. The fractional inhibitory concentration (FIC) index of the antibacterial combinations was calculated via the following formula:

1) FIC of *C. halicacabum* extract = MIC of *C. halicacabum* extract in combination/MIC of *C. halicacabum* extract alone

2) FIC of antibiotics = MIC of antibiotics in combination/MIC of antibiotics alone

3) Therefore, the FIC index = FIC of *C. halicacabum* extract + FIC of antibiotics.

The results of the FIC index (FICI) was interpreted as follows: FICI ≤ 0.5, synergistic; 0.5 < FICI < 1, partially synergistic; FICI = 1, additive; 1 < FICI ≤ 4, indifferent; FICI > 4, antagonistic [24]. The selection of concentrations for the assay allowed for the identification of both optimal and suboptimal dosages in combination, facilitating the identification of any potential synergistic or antagonistic effects.

Time-kill assay

A time-kill assay was performed to determine the antibacterial activity of the extract either alone or in combination with antibiotics and confirm the results of the checkerboard assay. A total of 500 µL of the *S. aureus* strain PB57 (MRSA) at 107 CFU/mL in 0.85 % NaCl was added to each 4.5 mL CAMHB containing 5 mL of the *C. halicacabum* extract alone, antibiotics alone, or the combination of the antibacterial agents. All test mixtures and the control were incubated at 37 °C. A sample of 1 mL from each mixture was taken every 1 h interval, ranging from 0 - 8 h, and at 24 h for viable plate counts. A 0.1 mL aliquot from each 1 mL sample was dropped aseptically and spread evenly on MHA. All the plates were subsequently incubated at 37 °C for 24 h, after which the colony-forming units (CFUs) of the MRSA were determined. A graph of the viable count was plotted against time.

Transmission electron microscopy

To analyze the impact of *C. halicacabum* extract on bacterial cell structure, *S. aureus* strain PB57 (MRSA) was exposed to the extract and antibiotics, then prepared for transmission electron microscopy (TEM) (JEOL, Tokyo, Japan). After treatment, bacterial cells were fixed in 2.5 % glutaraldehyde, post-fixed in 1 % osmium tetroxide and embedded in Spurr's resin (EMS). Thin sections (60 - 80 nm) were cut using an ultramicrotome and stained with uranyl acetate and lead citrate. TEM images were captured at various magnifications. Quantitative analysis involved measuring the extent of cell wall damage, membrane disruption and intracellular changes using image analysis software. The severity of these alterations was compared between treated and untreated cells to assess the impact of the treatments. The extent of damage was categorized based on predefined criteria, such as the degree of membrane disruption or loss of cell wall integrity.

Cytotoxicity test (MTT assays)

The fibroblasts (ATCC PCS-201-010) were seeded in a 96-well plate containing high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FBS and 1 % penicillin/streptomycin at a density of 1×10^4 cells/well. After incubation overnight, the cells were treated with different concentrations of *C. halicacabum* extract as follows: 2,000, 1,500, 1,000, 500, 250, 125, 25 and 0 µg/mL. These concentrations were chosen to cover the below and above concentrations of MIC and MBC values. Then, the cells were incubated for an additional 24 h. Afterward, the culture medium was removed, and PBS was added to each well to wash the residue of the extract. Then, DMEM was added to each well. Subsequently, 20 µL of 5 mg/mL MTT in PBS was added to each well. The cells were further incubated for 2 h. Afterward, the culture medium was removed again. The formazan crystals generated by viable cells were dissolved in 100 µL of DMSO, and the absorbance was measured at 540 nm with a microplate spectrophotometer (Synergy H1, BioTek, USA). The percentage of cell viability and the IC50 (50 % inhibitory concentration) were calculated with the GraphPad Prism program.

Statistical analysis

The MTT assay was performed in 3 independent experiments with triplicate wells for each condition. One-way analysis of variance (ANOVA) with Tukey's comparison test was performed to assess the statistically significant differences between the experimental groups.

Results and discussion

Phytochemical screening and total phenolic content

The crude extract of *C. halicacabum* contained favorable plant secondary metabolites such as terpenoids and tannins. Dragendorff's test demonstrated that alkaloids were absent in the extract. The total phenolic content was measured using the Folin-Ciocalteu technique, with gallic acid as the reference biomarker. The total phenolic content of the extract was calculated from a gallic acid calibration curve. The content of the extract at 1 mg/mL, gallic acid equivalent (GAE), was 87.66 ± 14.56 g GAE/kg.

Quantitative determination of apigenin in the extract

The results indicated that the crude extract of *C. halicacabum* contained apigenin. To confirm this finding, HPLC was performed. The HPLC chromatogram of the standard apigenin appeared at a retention time of 22.47 min (**Figure 1 (A)**), whereas the chromatogram of the *C. halicacabum* extract also displayed peaks that corresponded to apigenin, suggesting that this extract contained apigenin as one of its bioactive compounds (**Figure 1(B)**). A calibration curve was constructed to quantify the total amount of apigenin present in the *C. halicacabum* extract (**Figure 2**). With respect to the chromatographic method validation, the linearity of the HPLC method was defined as having an R2 equal to 1.00 at concentrations ranging from 7.81 to 1,000.00 µg/mL. The limit of detection (LOD) value of apigenin was 0.08 pg/mL, while the limit of quantification (LOQ) was 0.52 pg/mL. This method was precise with relative standard deviations (% RSD) of intra- and interday less than 3.48 and 5.00 %, respectively. The accuracy of the method is presented as the percentage recovery, which ranges from 90 to 110 %. Therefore, this HPLC method was accurate and reliable for determining of the amount of apigenin in the *C. halicacabum* extract. The results demonstrated that 1 mg of the extract contained 0.154 ± 0.005 mg of apigenin, thus constituting 15.40 % of the extract.

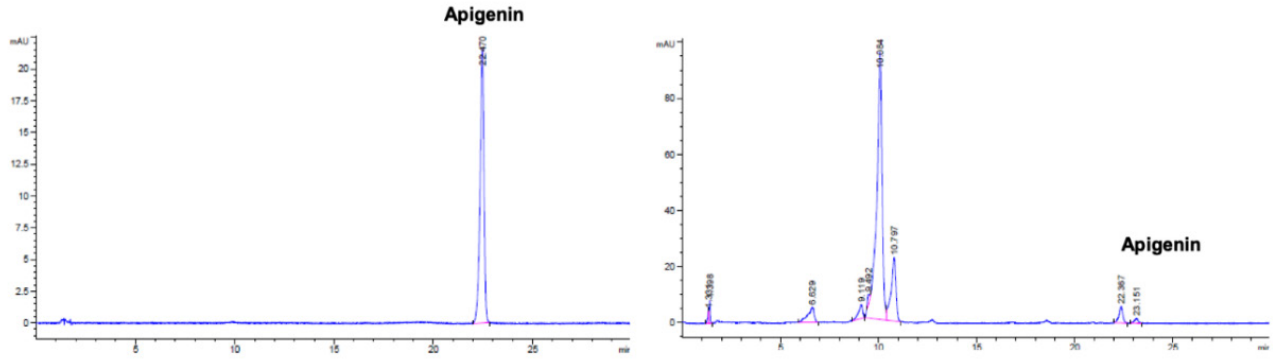

Figure 1 High-performance liquid chromatography chromatograms of a standard apigenin solution (A) and the *C. halicacabum* extract (B).

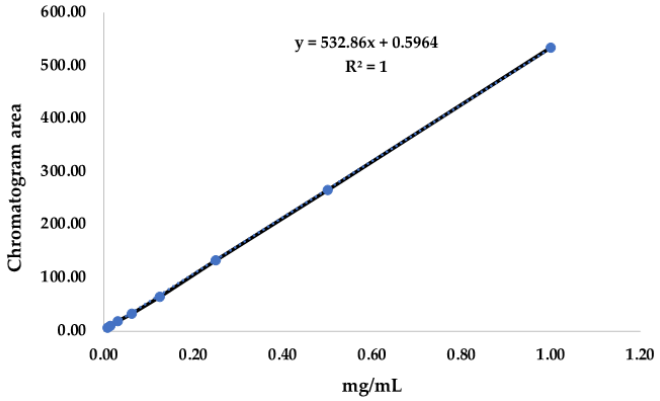

Figure 2 Calibration curve of a standard apigenin solution.

Bioactivity determination

Disk diffusion method

A disk diffusion screening test demonstrated that the potential antimicrobial activity of *C. halicacabum* has an inhibitory effect on the growth of the *S. aureus* strain PB57 (MRSA), which has a 15 mm inhibition zone. The positive control exhibited an inhibition zone of 19 mm.

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

The MIC and MBC of the *C. halicacabum* extract, oxacillin and vancomycin were tested against the *S. aureus* strain PB57 (MRSA). The MIC and MBC of the *C. halicacabum* extract against MRSA were 0.98 mg/mL, while those of oxacillin were 1 mg/mL and for vancomycin, 0.004 mg/mL.

Checkerboard assay

The FIC indices for the combinations of *C. halicacabum* extract with oxacillin and *C. halicacabum* extract with vancomycin against *S. aureus* strain PB57 (MRSA) were calculated as 1 and 1.5, respectively. According to these results, the combination of *C. halicacabum* extract with oxacillin showed an additive effect (FIC index = 1), indicating that while the 2 agents work together, their combined effect is equivalent to the sum of their individual effects, without demonstrating enhanced synergy. In contrast, the combination with vancomycin (FIC index = 1.5) was categorized as indifferent, suggesting that the extract and vancomycin do not interact in a way that significantly improves or reduces each other's antibacterial activity (**Table 1**).

Table 1 MICs and FICs index of oxacillin and vancomycin when used either alone or in combination with the *C. halicacabum* extract against *S. aureus* strain PB57 (MRSA).

Combination of agents	MIC in combination (A + B)	FIC index	Type of interaction
C. halicacabum extract	0.5	1	Additive
+ Oxacillin	0.5		
C. halicacabum extract	0.5	1.5	Indifferent
+ Vancomycin	1		

Time-kill assay

The results of the time-kill assay of *S. aureus* strain PB57 (MRSA) after treatment with *C. halicacabum* extract are shown in **Figure 3**. The untreated *S. aureus* strain PB57 (MRSA) was found to have no reduction in viable cell count and grew normally over 24 h. Bacterial cells treated with the *C. halicacabum* extract (500 mg/mL) demonstrated a gradual decrease in viability after 3 h and a further reduction to 103 CFU/mL within 6 h of treatment. In

bacterial cells treated with oxacillin and vancomycin, the viability decreased after 2 h of treatment and continued to decrease until no viability was observed at 24 h of exposure.

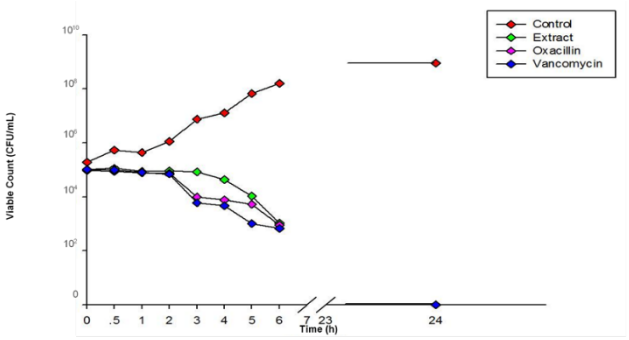

Figure 3 Time kill curves of *S. aureus* strain PB57 (MRSA) after exposure to *C. halicacabum* extract, oxacillin and vancomycin. *S. aureus* strain PB57 (MRSA) cultured alone was used as the control.

Transmission electron microscopy (TEM)

The descriptive results from TEM displayed that the cytoplasmic membrane and cell wall of untreated *S. aureus* strain PB57 (MRSA) cells were intact and well-distinguished (**Figures 4(A)** and **4(B)**). In *S. aureus* strain PB57 (MRSA) treated with *C. halicacabum* extract, significant damage and abnormal morphology were observed (**Figures 4(C)** and **4(D)**). The *C. halicacabum* extract resulted in unwell-defined cell membrane and cell wall with cell distortion. Furthermore, the density of the cytoplasm significantly changed and differed from that of the untreated control. Leakage of intracellular material was also detected in *C. halicacabum* extract treated cells (**Figures 4(C)** and **4(D)**).

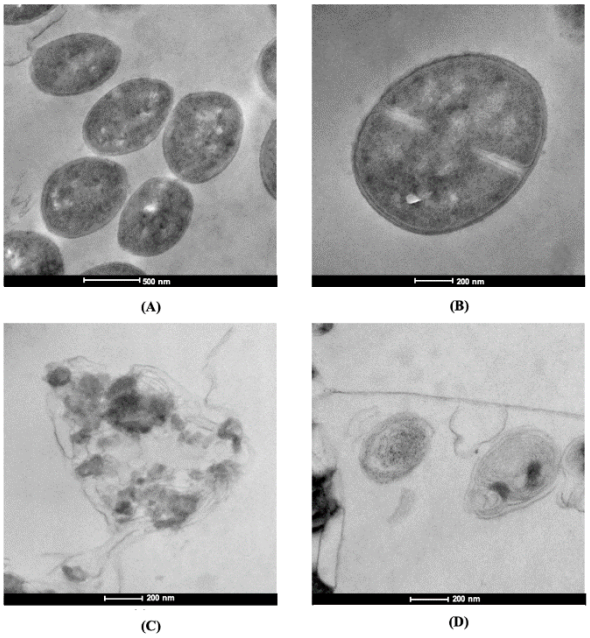

Figure 4 Ultrathin sections of log-phase *S. aureus* strain PB57 (MRSA) grown in CAMHB containing control (drug-free) (A,B) or *C. halicacabum* extract (C,D).

Cytotoxicity test

The cytotoxic effect of *C. halicacabum* extract on fibroblasts was tested via an MTT assay. As shown in **Figure 5**, the number of viable fibroblasts was inhibited by the *C. halicacabum* extract in a concentration-dependent manner. A fibroblast viability of less than 75

% was initially observed after treatment with the *C. halicacabum* extract at a concentration of 1,000 µg/mL, which is less than the MIC of the *C. halicacabum* extract against the *S. aureus* strain PB57 (MRSA) (**Figure 5(A)**). The concentration of the herbal extract that reduced the number of viable cells to 50 % (IC50) was 813.5 µg/mL (**Figure 5(B)**). These results showed that the extract had a cytotoxic effect on the fibroblasts after 24 h of exposure.

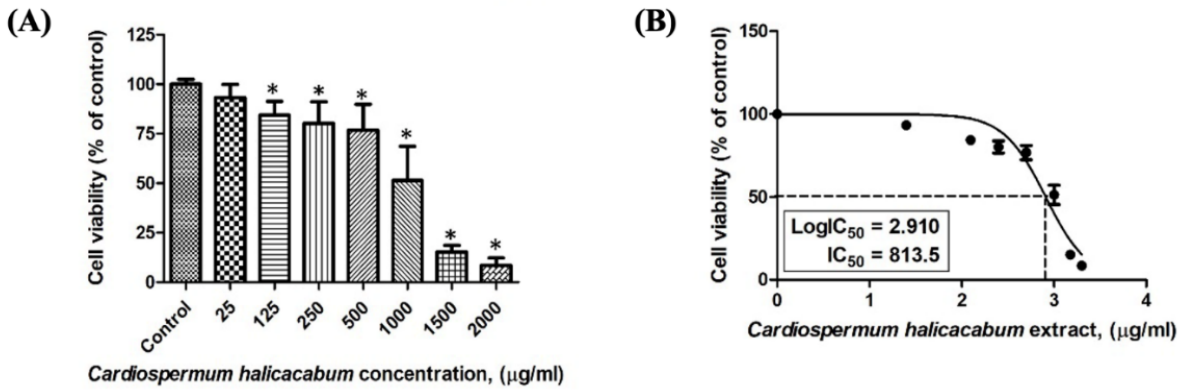

Figure 5 Effects of different concentrations of *C. halicacabum* extract (A) and the IC50 values of the *C. halicacabum* extract on fibroblasts (B). Data from 3 independent experiments, each of which was performed in triplicate, are shown as the mean ± standard deviation (SD), n = 3, $*p < 0.05$.

Discussion

Phytochemical studies of *C. halicacabum* in this work revealed that the herbal extract contained terpenoids, tannins, flavonoids and polyphenols, especially apigenin, constituting 15 % of the extract's total compound composition. The phenolic compound group contained 87.66 ± 14.65 g GAE/kg. Other studies have demonstrated that the main classes of secondary metabolites for this plant include flavonoids, triterpenoids, glycosides, fatty acids and volatile esters [15,25,26]. In this study, the antibacterial effect of *C. halicacabum* extract was evaluated via a disk diffusion assay, MIC and MBC tests. While the qualitative link between phytochemical content and antibacterial efficacy is well-supported, a detailed quantitative analysis is needed to understand the relative contribution of each compound, particularly apigenin, flavonoids, and phenolic compounds, to the overall antibacterial activity. Given that flavonoids, phenolic compounds as well as apigenin are known for their broad-spectrum antimicrobial properties [27-31].

Several studies have reported that apigenin possesses antibacterial properties against bacteria, including *S. aureus*, *Streptococcus suis*, *Bacillus subtilis*, *Escherichia coli* and some antibiotic-resistant strains. It is believed to function by disrupting bacterial cell membranes and inhibiting specific bacterial enzymes [29-31]. The present study revealed that *C. halicacabum* contains apigenin, suggesting that its antibacterial activity may be attributed to the presence of apigenin/ However, it would be valuable to investigate their individual and combined effects through fractionation or bioassay-guided isolation in future studies. Such an approach would provide a more precise understanding of how these compounds interact to inhibit MRSA growth.

Antimicrobial susceptibility results via the disk diffusion method demonstrated that the *C. halicacabum* extract potentially inhibited the *S. aureus* strain PB57 (MRSA) with an inhibition zone of 15 mm. The MIC and MBC results confirmed the antibacterial activity as the extract inhibited and killed the *S. aureus* strain PB57 (MRSA) at an MIC and MBC of 0.98 mg/mL. The MIC and MBC results revealed that the *S. aureus* strain PB57 (MRSA) was highly resistant to oxacillin and still susceptible to vancomycin as well as the herbal extract, which seems consistent with previous findings [32,33]. A comparison with standard antibiotics further highlights vancomycin's continued efficacy, while the resistance to oxacillin underscores the growing need for alternative treatment options. Additionally, the results also indicated that the herbal extract was bactericidal

against the MRSA strain as the MIC and MBC of the crude extract against *S. aureus* strain PB57 were equal at 0.98 mg/mL. The results align with the findings of Gaziano *et al.* [16] regarding the ability of the *C. halicacabum* extract to inhibit various types of pathogenic bacteria depending on different solvents or extraction techniques. Moreover, the ethanol fraction was shown to be more effective than the aqueous extract against gram-positive bacterial strains, especially *Staphylococcus epidermidis*, *Streptococcus faecalis*, *S. aureus*, *Bacillus subtilis* and *Bacillus cereus* [1,34,35]. In line with previous studies, the ethanolic extracts of *C. halicacabum* showed intense activity against *S. aureus* [28,36]. This result may be explained by the fact that the antimicrobial activities of phenol- and flavonoid-rich plant extracts are likely due to their ability to directly interfere with heat shock protein 90 (Hsp90). In this context, Hsp90 is involved in several processes and has emerged as a potential target for antimicrobial therapy. They play key cellular roles by eliciting molecular responses to environmental changes, morphogenesis, drug resistance and pathogenicity [16].

On another note, medications available to treat MRSA are becoming increasingly limited. Additionally, previous *in silico* findings on fungi suggested that *C. halicacabum* extract, alone or in combination with lower doses of traditional antimycotic medications, could represent a novel promising therapeutic strategy for the treatment of fungal infections [32,37]. Therefore, we aimed to determine the synergistic activity of the combination of current resistant antibiotics and the herbal extract against the MRSA strain. However, in this study, checkerboard assays shown that the *C. halicacabum* extract does not enhance the efficacy of oxacillin on *S. aureus* strain PB57 (MRSA) with the FIC index of 1. The failure to observe synergistic interactions could be due to the mechanisms of action of oxacillin and the herbal extract operating independently, which warrants further exploration. For this reason, it was crucial to first investigate the extract's effects on *S. aureus* strain PB57 (MRSA), leading to more in-depth experiments. The time-killing curve showed a reduction in the viable cell count of the *S. aureus* strain PB57 (MRSA) from 10^5 to 10^3 CFU/mL within 6 h. In contrast, the untreated cells exhibited continued to increase 24 h. Transmission electron microscopy (TEM) analysis further revealed significant structural damages in the extract-treated cells, including disruption of the cell wall and cytoplasmic membrane, compared to the intact structure of untreated cells. These observations suggested that *C. halicacabum* extract may exert its antibacterial effect by compromising the integrity of the bacterial cell envelope, leading to cell lysis and reduced viability. However, further studies are needed to elucidate the exact molecular pathways involved.

Lastly, the cytotoxic effect of the crude extract of *C. halicacabum* on human fibroblast cells was assessed to gain its safety profile for further utilization in treating *S. aureus*-mediated human diseases such as abscess, folliculitis, and impetigo [38]. *C. halicacabum* had the IC50 value of 813.5 µg/mL on primary dermal fibroblast cells, whereas MIC and MBC values against *S. aureus* PB57 (MRSA) was 980 µg/mL. The results indicated that the value of IC50 is less than the MIC and MBC of the extract against the *S. aureus* PB57 (MRSA), implying that a high concentration of the *C. halicacabum* extract had a cytotoxic effect on fibroblasts after 24 h of exposure. Indeed, the *in vitro* cytotoxicity testing is recommended to conduct when the crude extract shows the IC_{50} value less than 30 mg/mL based on US NCI plant screening program [39]. In line with previous report, plant extracts containing high phenolics, hydrocyanic acids and triterpenoids exhibit a cytotoxic effect [38]. However, the use of this plant and its individual constituents is not considered for a specific health concern in the EU. Earlier investigations on a dried powdered form of *C. halicacabum* revealed no oral toxicity in rats at doses up to 40 g/kg of body weight. Further, methanol leaf extract of *C. halicacabum* on an oral toxicity study in mice showed that the extract was safe at acute toxicity doses (2,000 mg/kg/day). Regarding to sub-chronic toxicity test, mice treated with a dose of 400 mg/kg did not show any signs of toxicity and the death rate decreased over a period of 45 days [20]. The ethanolic extract of *C. halicacabum* at 200 µg/mL has been reported to inhibit the growth of Ehrlich ascites carcinoma cell lines by about 60 % [40]. Moreover, *C. halicacabum* at 50 µg/mL showed strong anti-inflammatory effect on LPS-induced murine macrophages (In press). According to the high MIC and MBC values (980 µg/mL) in this study, it may limit the use of *C. halicacabum* extract on antibacterial activity. However, various evidence has

suggested the alternative application of *C. halicacabum* for anti-cancer, anti-inflammation actions.

Conclusions

This study highlighted the potential of the crude extract of *C. halicacabum* as a promising candidate for novel antimicrobial agents, particularly for treating hospital-acquired or associated infections caused by MRSA. The extract demonstrated significant antibacterial activity, with MIC and MBC values indicating its efficacy against the *S. aureus* strain PB57 (MRSA). Phytochemical analysis revealed the presence of biologically active compounds, such as terpenoids, flavonoids and polyphenols, which likely contribute to the observed antibacterial effects. However, while the extract showed promising antibacterial properties, it also exhibited cytotoxicity toward human fibroblast cells at certain concentrations, raising concerns about its therapeutic application. The extract was reportedly non-toxic in previous oral toxicity studies, but further clarification is needed regarding the safety of specific doses and modes of administration. Given these findings, more in-depth investigations are required to isolate the pharmacologically active components from *C. halicacabum* and assess their specific contributions to antimicrobial activity. Additionally, the cytotoxic effects observed *in vitro* must be further explored to ensure that concentrations effective against bacteria do not pose a risk to human cells. Future research should focus on developing a more refined extract, with targeted studies on its safety profile, potential clinical applications and mechanisms of action. This will help bridge the gap between preliminary *in vitro* results and therapeutic use.

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