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Antibiotic Resistance Modulatory Activities of *Mentha cordifolia* Opiz and *Mentha arvensis* L. buffered Leaves Crude Extracts against Methicillin-resistant *Staphylococcus aureus* Phenotypes

Aprilyn F. Francisco[1,2], Lean Kristin F. Ugdang[3], Keziah Amor S. Catulong[1] and Alfredo A. Hinay, Jr[1,2*]

[1] *College of Medical and Biological Sciences, University of the Immaculate Conception, Davao City, Philippines*
[2] *Graduate School Department, University of the Immaculate Conception, Davao City, Philippines*
[3] *Clinical Laboratory and Training Center, University of the Immaculate Conception, Davao City, Philippines*

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***Corresponding author**
ahinay@uic.edu.ph

ABSTRACT

Methicillin-resistant strains of *Staphylococcus aureus* strains pose a critical challenge to healthcare, necessitating the development of novel therapeutic approaches. This study investigated the potential of secondary metabolites from *Mentha cordifolia* Opiz and *Mentha arvensis* L. to modulate antibiotic resistance in methicillin-resistant *S. aureu*s clinical isolates. A rapid *p*-iodonitrotetrazolium chloride colorimetric assay was employed to evaluate the antibacterial activity, antibiotic resistance-modulating activities, and effects of *M. cordifolia* and *M. arvensis* extracts. This study focused on the interaction between these extracts and oxacillin, an antibiotic that typically exhibits high minimum inhibitory concentrations (MICs) against methicillin-resistant *S. aureus*. Remarkably, the addition of the buffered crude extracts of *M. cordifolia* and *M. arvensis* to oxacillin resulted in significant modulatory activity with a modulatory factor of 2 to 413.3 at a concentration of 125 µg/ml. This modulation was observed as a decrease in oxacillin MIC against the tested methicillin-resistant *S. aureus* strains. Moreover, both *M. cordifolia* and *M. arvensis* demonstrated potent modulatory effects, accounting for 71% of all tested methicillin-resistant phenotypes. The results of this study open new avenues for combating the growing threat of antibiotic-resistant bacterial infections in the healthcare setting. By potentially enhancing the efficacy of existing antibiotics, such as oxacillin, this approach could lead to improved treatment outcomes for patients with methicillin-resistant *S. aureus* infections.

Keywords: Philippine mint, Modulatory activity, Modulatory effect, Methicillin-resistant *Staphylococcus aureus*

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INTRODUCTION

Antimicrobial resistance (AMR) has emerged as a critical global health emergency over the past decade, posing a significant threat to modern medicine and public health [1]. The widespread and indiscriminate use of antibiotics has led to a dramatic increase in multidrug-resistant (MDR) bacteria, pushing society closer to a potentially catastrophic post-antibiotic era [2, 3]. The most concerning MDR pathogens are members of the ESKAPE family, including Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp.

Staphylococcus aureus stands out within the ESKAPE group as a notorious bacterium that constantly acquires antibiotic-resistance genes, leading to severe nosocomial infections. This pathogen has emerged as a superbug that significantly contributes to global morbidity (60%) and mortality (64%) [4, 5]. A critical resistance mechanism in *Staphylococcus aureus* is the multidrug efflux pump, which extrudes several classes of antibiotics, specifically beta-lactam drugs. This mechanism, along with other resistance factors, contributes significantly to the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA strains have developed the ability to resist multiple antibiotics, including methicillin and other beta-lactams, largely because of the acquisition of mecA and the expression of efflux pumps. This resistance mechanism is of particular concern because of its widespread distribution, with Asian countries (63.8%) leading, followed by Europe (16%), the United States (13.8%) and Africa (5.2%) [5, 6].

The increasing prevalence of methicillin-resistant *S. aureus* underscores the urgent need for innovative therapeutic strategies. Efflux pump inhibitors (EPIs), a class of antibiotic resistance modulators, have long been recognized as effective tools to combat multidrug-resistant bacteria, reverse drug resistance, and rejuvenate conventional antibiotics. These molecules interfere with bacterial efflux pumps and protein complexes that actively expel antibiotics from the bacterial cells. As resistance

modulators, EPIs increase the intracellular concentration of antibiotics, restore the efficacy of drugs that have become less effective owing to efflux-mediated resistance, and potentially reverse acquired drug resistance in bacteria overexpressing efflux pumps. This modulation can significantly enhance the potency of existing antibiotics and expand their spectrum of activity, often reducing the minimum inhibitory concentrations required to combat resistant bacterial strains[6–8]. Currently, plant-derived alkaloids (reserpine), flavonoids, polyphenols, and phenolic diterpenes have been found to possess promising action to reverse efflux pump-related resistance in *Staphylococcus aureus* [2]. In addition, carbonyl cyanide m-chlorophenyl hydrazine (CCCP) and other synthetic efflux pump inhibitors (EPIs) have been found to be very effective, making them very close to ideal EPI. However, these EPIs are currently not licensed for clinical use or clinical trials, owing to their potency, spectrum of activity, and pharmacokinetics [2, 9, 10].

To address this gap in combating multidrug resistance, the continuous search for potential EPIs from edible sources with low toxicity is a promising strategy. Edible botanical extracts containing tannins, steroids, triterpenes, and polyphenols have been shown to possess antibiotic-resistance modulatory activity against methicillin-resistant *Staphylococcus aureus* phenotypes and are believed to act as potential EPIs [11]. Notably, some of these phytochemicals are present in the essential oils of mint and are extensively used in the pharmacological and non-pharmacological industries [8, 12, 13]. In this study, we revealed the significant antibiotic resistance modulatory activity of crude extracts buffered with *Mentha arvensis* and *Mentha cordifolia* leaves on the efflux pump of methicillin-resistant *S. aureus* phenotypes. These results suggest that mint extract can effectively modulate antibiotic-resistance mechanisms. This study provides valuable insights for further research on plant-derived sources that may offer potential solutions for reversing antimicrobial resistance and enhancing the efficacy of conventional antibiotics.

MATERIALS AND METHODS

Plant Collection

Intact mint leaves, stems, and roots, regardless of plant age, were purchased from local vendors in Davao City, Philippines. Prior to extraction, the intact plants were identified and authenticated as *M. arvensis* L. and *M. cordifolia* Opiz. Environmental conditions such as soil quality, humidity, and other environmental factors were not considered during procurement.

Buffered Extraction and lyophilization

The leaves (*M. cordifolia* and *M. arvensis*) were thoroughly washed with distilled water and air-dried. In a 500 ml conical flask, a buffered extract was prepared by electrically blending 25 g of plant material with 100 ml of 0.1 M, pH 7.0 phosphate buffer (Sigma Aldrich). To remove any remaining cell debris in the preparation, the homogenate was filtered through gauze and the filtrate was centrifuged at 3,400 RPM for 30 min. The clear supernatant obtained represented the crude extract, which was subjected to lyophilization [14].

Test Microorganism

Ten clinical isolates of *S. aureus* were obtained from a private hospital in Davao City, the Philippines. The isolates were initially cultured on Mannitol Salt Agar (HiMedia, India) to purify and avoid a mixed culture. Subsequently, the isolates were cultured and maintained on Tryptic Soy Agar plates (HiMedia) at 4 °C. Colony morphology examination, Gram staining, catalase test, and coagulase test were performed for the phenotypic identification of the clinical isolates, and Vitek 2 was used to identify oxacillin resistance.

Evaluation of the Antibacterial Activity

The antibacterial activity of the extracts was determined using a rapid *p*-iodonitrotetrazolium chloride (INT) colorimetric assay[15]. Two-fold serial dilutions of the extract (dissolved in dimethyl sulfoxide (DMSO)/MHB) were prepared in 96-well microplates. Then, 100 µl of inoculums (1.5×10^8 bacteria/ml) prepared in MHB was added. The plates were covered with a sterile plate sealer, agitated with a shaker to mix the contents of the wells, and incubated at 37 °C for 18 h. Wells containing MHB, 100 µl of inoculum, and DMSO at a final concentration of 1% served as negative controls. The minimum inhibitory concentration (MIC), defined as the lowest sample concentration that prevented bacterial growth, was determined after adding 40 µL of INT (0.2 mg/ml) to each well of the plates and incubated at 37 °C for 30 min.

Evaluation of the Role of Efflux Pumps in the Resistance of Selected Bacteria

To evaluate the contribution of efflux pumps to 10 clinical isolates of methicillin-resistant *Staphylococcus aureus* strains, the proton motive force uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as an efflux pump inhibitor[11]. The Minimum Inhibitory Concentration (MIC) of oxacillin, with and without CCCP, was determined by computing the ratio to identify its modulatory activity. Significant modulatory activity (a ratio equal to or greater than 2). A significant reduction in MICs upon CCCP treatment indicated the presence of active efflux pumps, suggesting their role in the observed antibiotic resistance mechanism of methicillin-resistant *S. aureus*. All assays were performed in duplicate.

The modulating activity of the *M. cordifolia* and *M. arvensis* buffered crude extracts

Antibiotic resistance modulation activity of the leaf-buffered crude extracts was determined by computing the modulatory activities based on the MIC of Oxacillin in the absence and presence of plant extracts and the control

(CCCP). Crude-buffered samples were tested at a concentration of 125 µg/ml against MDR *Staphylococcus aureus*. Briefly, after serial dilutions of antibiotics, the extract was added to each well (125 µg/ml) in duplicate. The 10 MDR *Staphylococcus* bacterial concentrations were prepared in 1.5×10^6 colony-forming units (CFU)/ml using spectrophotometry (absorbance ranges: 0.08-0.10 OD). Wells 1 and 12 served as the positive and negative controls, respectively. The MIC was determined after incubation and addition of INT. Wells that received antibiotic dilutions without extracts and wells with a combined solution (antibiotic and extract) were used as MICs for antibiotics and buffered lead crude extracts, respectively. Modulation activity was calculated as MIC Oxacillin/MIC Oxacillin in *Mentha* sp. buffered crude extract combination.

Modulating effect of *M. cordifolia* and *M. arvensis* buffered crude Extracts

The modulation factor was defined as the ratio of the MIC of the antibiotic alone to that of the antibiotics in the presence of *Mentha sp.* extract. A modulation factor ≥ 2 was set as the cutoff for biologically significant antibiotic resistance-modulating effects [16].

RESULTS

Antibacterial Activity of *M. cordifolia* and *M. arvensis* buffered Crude Extracts

The extracts tested showed no activity against *S. aureus* ATCC 25923 or clinical multidrug-resistant (MDR) *S. aureus* isolates, with minimal inhibitory concentration (MIC) values recorded at 496 µg/ml for three-fold dilutions of 1000 µg/ml, 500 µg/ml, and 125 µg/ml.

Phytochemical Screening of Buffered Crude Extracts of *M. cordifolia* and *M. arvensis*

Phytochemical screening of lyophilized buffered crude extracts of *M. cordifolia* Opiz and *M. arvensis* L. showed that both species tested positive for phenolic compounds, tannins, and saponins, indicating the presence of secondary metabolites. However, both Mentha extracts tested negative for triterpenoids and flavonoids.

Evaluation of Resistance Mechanism of Methicillin-resistant *S. aureus*

The mechanism of drug resistance was evaluated using EPI-CCCP control. Methicillin-resistant *Staphylococcus aureus* with modulatory activities of > 2 and <2 was classified as efflux-pump-related and non-efflux-pump-related drug resistance, respectively. Table 1 presents the classification of the methicillin-*resistant S. aureus* isolates based on their drug-resistance mechanisms. Among the 10 clinical isolates, seven were classified as efflux pump-related drug resistance.

The Modulatory Activity of *Mentha* species in different concentrations against Efflux-pump related drug-resistant *S. aureus*

To screen for the ability of the extracts to potentiate the action of antibiotics, the modulating activity of *Mentha* species was evaluated. This was done by computing the MIC of the Oxacillin with and without the presence of the extract at two different concentrations (1000, 500, and 125 µg/ml) at which the extracts did not exhibit inhibitory or antibacterial activity. The results, as shown in Table 2, revealed that both *M. cordifolia* Opiz and *M. arvensis* L. buffered crude extracts significantly potentiated the action of oxacillin, even at the lowest concentration tested (125 µg/ml), which represents a four-fold dilution from the highest concentration.

Table 1 Efflux-pump-related resistance mechanism of clinically Isolated *Staphylococcus aureus*

Bacteria used	Tested samples, MIC (ug/mL)		
	Oxacillin	Oxacillin + CCCP	Efflux-pump-related drug resistance
Clinical isolate 01	496	372 (1.3)	Non-efflux-pump
Clinical isolate 02	186	77.5 (2.4)	Efflux-pump
Clinical isolate 03	372	496 (0.8)	Non-efflux-pump
Clinical isolate 04	186	62.45 (3.0)	Efflux-pump
Clinical isolate 05	186	310 (0.6)	Non-efflux-pump
Clinical isolate 06	248	0.9 (275.6)	Efflux-pump
Clinical isolate 07	248	0.9 (275.6)	Efflux-pump
Clinical isolate 08	496	0.9 (551.1)	Efflux-pump
Clinical isolate 09	496	0.9 (551.1)	Efflux-pump
Clinical isolate 10	496	0.9 (551.1)	Efflux-pump

MIC Minimal Inhibitory Concentration; *CCCP* efflux pump inhibitor, Carbonyl Cyanide m-chlorophenylhydrazone; (), *Modulating factor*; values in bold represent modulating factor ≥ 2.

Table 2 The modulatory activity and modulatory effect of *Mentha* species against clinically isolated efflux-pump-related multidrug-resistant *Staphylococcus aureus* phenotypes

Bacteria used	Tested samples, MIC (µg/ml)					
	Oxacillin + *M. cordifolia*			Oxacillin + *M. arvensis*		
	1,000 µg/ml	500 µg/ml	125 µg/ml	1,000 µg/ml	500 µg/ml	125 µg/ml
Clinical isolate 02	0.9 (206.7)	15.5 (20)	15.5 (20)	3.87 (48)	3.87 (48)	19.37 (16)
Clinical isolate 04	0.9 (413.3)	0.9 (413.3)	0.9 (413.3)	0.9 (413.3)	0.9 (413.3)	0.9 (413.3)

Modulatory effect; percentage modulation of the extract against efflux-pump-related MDR *Staphylococcus aureus* phenotypes*; MIC;* Minimal Inhibitory Concentration; (): *Modulating factor*; values in bold represent the modulating factor ≥ 2.

Table 3 The modulatory activity and modulatory effect of *Mentha* species against clinically isolated efflux-pump-related multidrug-resistant *Staphylococcus aureus* phenotypes

Bacteria used	Tested samples, MIC (µg/mL)	
	Oxacillin + *M. cordifolia* Opiz	Oxacillin + *M. arvensis* L.
Clinical isolate 02	15.5 (20)	19.37 (16)
Clinical isolate 04	0.9 (413.3)	0.9 (413.3)
Clinical isolate 06	23.25 (8)	46.5 (4)
Clinical isolate 07	93 (2.7)	23.25 (10.7)
Clinical isolate 08	62 (6)	186 (2)
Clinical isolate 09	372 (1.3)	496 (1)
Clinical isolate 10	496 (1.0)	496 (1)
Modulatory effect	71%	71%

Modulatory effect; percentage modulation of the extract against efflux-pump-related MDR *Staphylococcus aureus* phenotypes*; MIC;* Minimal Inhibitory Concentration; (): *Modulating factor*; values in bold represent the modulating factor ≥ 2.

Modulatory activity and effects of Mentha species against clinically multidrug-resistant *S. aureus* phenotypes

The modulatory activity of *M. cordifolia* and *M. arvensis* on oxacillin against seven clinically isolated *S. aureus* strains is shown in Table 3. The results revealed that *Mentha cordifolia* -buffered crude extract at 125 µg/ml modulated the antibacterial activity of oxacillin against five clinical isolates of methicillin-resistant *S. aureus* phenotypes, with a modulatory effect of 71%.

DISCUSSION

Mentha cordifolia plant extract has been previously documented for its antimicrobial activity against some pathogenic bacteria, including methicillin-resistant *Staphylococcus aureus* [17]. Several studies have indicated that antibacterial activity is due to different chemical agents in the extract, specifically flavonoids, and triterpenoids[17, 18]. In this study, these compounds were qualitatively negative after buffered extraction. This can explain the lack of antibacterial activity of the buffered crude extract against the ATCC *S. aureus* and methicillin-resistant *S. aureus* phenotypes. Remarkably, when the buffered crude extract was added to oxacillin, which has a relatively high MIC against methicillin-resistant *S. aureus*, a significant modulatory activity (decrease in MIC) at 125 µg/ml was observed. This could serve as a pharmacological advantage since there is a direct relationship between the concentration of the plant extract and possible toxicity. Moreover, these findings suggest that secondary metabolites, specifically tannins, saponins, and phenolic compounds, isolated from buffered crude extracts of *M. cordifolia* and *M. arvensis*, can be used as potential antibiotic resistance modulators. Several *in vitro* studies have demonstrated the modulatory activity of secondary metabolites from plant extracts against antibiotics in different methicillin-resistant phenotypes. Moreover, several reports have suggested that phenolic compounds, tannins, and flavonoids reverse efflux pump-related drug resistance in *S. aureus.*

One of the primary mechanisms is the inhibition of efflux pumps and protein complexes that actively expel antibiotics from the bacterial cells. Secondary metabolites, particularly phenolic compounds, tannins, and flavonoids, can directly block these pump channels or interfere with their energy sources, thereby preventing antibiotic expulsion [19–21]. Certain compounds in the Mentha extract can inhibit specific bacterial enzymes that contribute to antibiotic resistance, such as β-lactamases and topoisomerases. Furthermore, these secondary metabolites can interfere with biofilm formation and maintenance, making bacteria more susceptible to antibiotics. Many of these compounds work synergistically with conventional antibiotics to enhance their penetration into bacterial cells or to interfere with resistance mechanisms. Some secondary metabolites can also alter the expression of genes involved in antibiotic resistance, either by downregulating genes encoding

resistance mechanisms or upregulating genes that increase bacterial susceptibility to antibiotics [12, 13, 20, 21].

It has been previously reported that plant extracts with >70% modulatory effects in combination with oxacillin can serve as potential antibiotic resistance modulators[11]. In this study, we report the modulatory effects of *M. cordifolia* and *M. arvensis*, which account for 71% of all methicillin-resistant phenotypes. This indicates that the phytoconstituents of *Mentha* sp. can act as potential efflux pump inhibitors or modulating agents. Several studies have demonstrated the potential of EPI and its modulatory action on secondary metabolites including tannins and phenolic compounds in some plants[9, 11, 22, 23]. Interestingly, these plant-derived phytochemicals were qualitatively detected in the *Mentha* sp. used in this study. Research on *Mentha* sp. extracts as antibiotic-resistance modulators presents a promising approach to combating methicillin-resistant *S. aureus*, demonstrating the modulating effects of conventional antibiotics. The incomplete characterization of the active compounds and the absence of mechanistic insights are notable weaknesses; however, this study's alignment with previous research on plant-derived efflux pump inhibitors strengthens these findings. Despite the need for more comprehensive quantitative analyses and toxicity assessments, this study provides a valuable foundation for future investigations of plant-derived compounds as potential solutions to antibiotic resistance, balancing limitations with significant potential in addressing critical healthcare challenges.

CONCLUSIONS

Research on natural products such as *Mentha* spp. is crucial due to the escalating global issue of antibiotic resistance. *S. aureus*, particularly multidrug-resistant strains, poses significant challenges in healthcare settings. Finding ways to enhance the effectiveness of existing antibiotics, such as oxacillin, using natural compounds could offer promising solutions. This study demonstrates the potential of secondary metabolites of Mentha *spp*. to modulate antibiotic resistance, thereby suggesting a pathway for the development of new treatments for drug-resistant bacterial infections. This highlights their potential as antibiotic resistance modulators and offers a promising avenue for the development of novel therapeutic strategies against resilient bacterial infections.

Ethical Approval

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of The University of The Immaculate Conception Protocol Code GS-84-02-23.

Data Availability

All data generated or analyzed during this study are included in this published article.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this article.

Authors' Contribution

All authors have made significant contributions to this work. All authors were equally involved in drafting, writing, and reviewing the manuscript. They agreed to the journal to which the manuscript was submitted and approved its final version for publication.

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