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Original Article

Evaluation of the Antibacterial and Anti-biofilm Activity of *Satureja khuzestanica* Essential Oil and Coumarin Against *Stenotrophomonas maltophilia*

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A B S T R A C T

Introduction: Antimicrobial resistance has become a serious global health issue; hospital-acquired infections are now much harder to treat effectively. *Stenotrophomonas maltophilia* emerges as an opportunistic Gram-negative bacterium with built-in resistance to numerous antimicrobial agents. The present work examined the antibacterial and anti-biofilm effects of *Satureja khuzestanica* essential oil and coumarin against a standard strain of *Stenotrophomonas maltophilia*.

Materials and Methods: Essential oil was extracted by hydrodistillation from the aerial parts of *Satureja khuzestanica*. Agar well diffusion tested basic susceptibility. Broth microdilution measured minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values. Crystal violet assay assessed biofilm inhibition at sub-inhibitory concentrations (sub-MIC) concentrations of the essential oil.

Results: Inhibition zones measured 40.0 mm for the essential oil and 30.0 mm for coumarin. The oil gave an MIC of 31.25 µg/mL and an MBC of 62.50 µg/mL. A ratio of 2.0 indicated bactericidal activity. Coumarin displayed poor solubility with precipitation in broth medium; reliable MIC and MBC values remained undetermined. Biofilm formation fell by 62.1% at half the MIC of the essential oil ($p < 0.001$).

Conclusion: *Satureja khuzestanica* essential oil effectively killed planktonic cells of *Stenotrophomonas maltophilia* and substantially reduced biofilm development. Data support the potential application of this oil in plant-derived therapies for multidrug-resistant strains and chronic biofilms in clinical settings. Improved formulations may yield viable options in the near term. Addition to conventional antibiotics could prolong their utility.

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Introduction

Antimicrobial resistance (AMR) is acknowledged as a paramount global health challenge of the 21st century, linked to elevated mortality rates and significant economic impacts on healthcare infrastructures worldwide [1]. Among the various resistant pathogens, opportunistic Gram-negative bacteria pose unique therapeutic challenges due to their complex cell wall structure and multiple innate and acquired resistance mechanisms [2]. These pathogens, responsible for a significant proportion of nosocomial infections, have severely limited the available therapeutic options. *Stenotrophomonas maltophilia* (*S. maltophilia*) is a prominent example of such a resistant pathogen and constitutes the primary focus of the present study.

S. maltophilia is a multidrug-resistant, opportunistic Gram-negative pathogen classified as an emerging pathogen of high clinical significance. This classification stems from its intrinsic resistance to a broad spectrum of antimicrobial agents, including carbapenems, aminoglycosides, and most beta-lactam antibiotics [3]. This microorganism is particularly capable of causing severe infections such as pneumonia, bacteremia, and medical device-related infections in susceptible populations, including immunocompromised patients, individuals with cystic fibrosis, and those on mechanical ventilation [4]. A fundamental strategy underpinning its survival and persistence is its remarkable capacity to develop biofilms. A biofilm is a structured community of bacterial cells encapsulated in a self-produced extracellular polymeric matrix, which significantly contributes to pathogen survival in hostile environments by limiting antibiotic penetration [5]. Given the intrinsic resistance and high biofilm-forming capacity of *S. maltophilia*, the identification and development of novel therapeutic compounds, particularly those from natural origins, are an undeniable necessity.

In the face of the AMR crisis, natural compounds derived from plants have garnered increasing attention as valuable sources for the discovery and development of novel antimicrobial drugs [6]. Essential oils and phenolic compounds, owing to their multi-target mechanisms of action, demonstrate considerable potential to overcome bacterial resistance. Compared to synthetic antibiotics, these secondary metabolites often present advantages such as biodegradability, lower cytotoxicity to host cells, and the ability to directly target the biofilm structure or inhibit its development [7]. Iran's rich biodiversity makes it a significant source of medicinal plants with high therapeutic potential, which necessitates rigorous scientific validation. In this context, the endemic plant *Satureja khuzestanica* (*S. khuzestanica*) and the natural compound coumarin were selected as candidates for this study.

S. khuzestanica, a plant endemic to Iran, is the source of an essential oil rich in phenolic compounds, particularly carvacrol (>90%), which has been shown to exhibit prominent biological activities, including antimicrobial, antioxidant, and anti-inflammatory activities

[8, 9]. Previous studies have established the potent inhibitory effects of this essential oil against a wide range of pathogenic bacteria [10, 11]. On the other hand, coumarin, a naturally occurring benzopyrone compound, is recognized for its wide array of biological activities. Specifically, coumarin and its derivatives have been reported to inhibit the biofilm formation process in important pathogens like *Pseudomonas aeruginosa* by interfering with bacterial cell-to-cell communication systems, known as quorum sensing, which refers to the cell-to-cell communication mechanism employed by microorganisms to coordinate collective behaviors in response to population density [12, 13]. Although coumarin possesses significant antimicrobial potential, its low aqueous solubility remains a persistent technical challenge in laboratory assays and clinical applications. Therefore, evaluating the effects of these two compounds on *S. maltophilia* has the potential to enhance the current understanding of strategies for managing resistant infections.

Notwithstanding the current evidence regarding the antimicrobial properties of *S. khuzestanica* essential oil and coumarin, a distinct research gap exists regarding the comprehensive evaluation of these compounds' potential against *S. maltophilia*. Specifically, the anti-biofilm potential of these two compounds, individually, against this challenging nosocomial pathogen has not yet been systematically investigated. Accordingly, the present study was designed to quantitatively evaluate and compare the antibacterial effects determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) and to investigate the anti-biofilm efficacy of *S. khuzestanica* essential oil and coumarin was evaluated against a reference strain of *S. maltophilia*. It is anticipated that the findings of this research will contribute to the development of alternative, natural-product-based therapeutic strategies for managing infections caused by resistant *S. maltophilia* and help reduce the reliance on conventional antibiotics.

Materials and Methods

Plant material and test compounds

The essential oil of *S. khuzestanica* was obtained through extraction from the aerial components, specifically the leaves and stems, of the plant. The plant material was collected during the flowering stage from the native regions of Lorestan Province, Iran. Following collection, the samples were shade-dried. The essential oil was then extracted by hydro-distillation for 3 hours using a Clevenger-type apparatus. The resulting essential oil was stored in dark glass vials at 4 °C until further analysis. To ensure methodological reproducibility and quality verification, the chemical profile of the essential oil was cross-referenced with the standardized gas chromatography mass spectrometry analysis reported by Saidi for *S. khuzestanica* populations in the neighboring Ilam Province (western Iran). According to this study, carvacrol constitutes the predominant bioactive component of the species in this

geographical zone, exceeding 90% of the total oil composition [9].

Coumarin (purity ≥99%) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). To prepare the coumarin stock solution (50 mg/mL), 500 mg of coumarin powder was accurately weighed using an analytical balance and transferred to a 10 mL volumetric flask. The powder was subsequently dissolved in approximately 8 mL of DMSO, after which the final volume was precisely adjusted to the calibration mark with additional DMSO. The antibiotic gentamicin and ceftazidime/ clavulanic acid (CZA; 30/10 µg) antimicrobial susceptibility disks were procured from reputable commercial suppliers.

Bacterial strain and culture conditions

Although prior research has demonstrated the antimicrobial properties of *S. maltophilia*, this study specifically employed the standard strain ATCC 13637. The strain was procured from the Iranian National Center for Genetic and Biological Resources. For activation purposes, the bacterium was subcultured onto Mueller-Hinton Agar (MHA) and subsequently incubated at 37 °C for a duration of 24 hours. For all subsequent assays, a bacterial inoculum was prepared by suspending colonies in either Mueller-Hinton Broth (MHB) or a sterile saline solution. A cell density of roughly 1.5×10^8 colony-forming units (CFU) per milliliter was achieved by adjusting the suspension's turbidity to match the 0.5 McFarland standard.

Agar Diffusion Susceptibility Testing

The antibacterial properties of the compounds were assessed employing the agar well diffusion technique. A lawn culture was established by uniformly swabbing a standardized bacterial inoculum (0.5 McFarland standard) onto the surface of MHA plates [14]. Subsequently, aseptic techniques were used to create 6 mm diameter wells in the agar medium. The test agents were then added to the individual wells: 100 µl of coumarin dissolved in DMSO, 150 µl of pure *S. khuzestanica* essential oil, and 100 µl of DMSO to serve as a negative control. Simultaneously, a standard Kirby-Bauer disk containing ceftazidime/clavulanic acid (CZA) was applied to the agar medium to evaluate antibiotic susceptibility. The diameters of the zones of inhibition (ZOI) were measured in millimeters after a 24-hour incubation period at 37 °C.

Determination of MIC and MBC

MIC and MBC values of *S. khuzestanica* essential oil and gentamicin (used as a positive control antibiotic) were determined using the broth microdilution method in 96-well microplates, following the guidelines of the Clinical and Laboratory Standards Institute. Serial two-fold dilutions of the essential oil and gentamicin were made in MHB.

Thereafter, each well was inoculated with the bacterial suspension to attain a final bacterial concentration of approximately 1.5×10^8 (CFU/mL). Wells inoculated with the bacterial suspension in the absence of any test compound functioned as the positive control (growth control), whereas wells containing only sterile broth without bacterial inoculation served as the negative control (sterility control). The microplates were then incubated

for 24 hours at 37 °C .The MIC was defined as the lowest concentration of the test agent that resulted in no visible bacterial growth (i.e., no turbidity). To determine the MBC, an aliquot (typically 10 µl) from each well showing no visible growth was subcultured onto fresh MHA plates. Following a 24-hour incubation period, the MBC was recorded as the lowest concentration that yielded no colony growth on the agar surface The characterization of the antimicrobial activity as either bactericidal or bacteriostatic was established by evaluating the ratio of the MBC to the MIC [15]. All experiments were performed in at least three independent biological replicates on different days, and the data were expressed as mean ± standard deviation.

Biofilm formation inhibition assay

The ability of the essential oil to inhibit biofilm formation was evaluated using the crystal violet colorimetric assay. This assay was conducted in 96-well flat-bottomed microplates. Each test well was inoculated with 100 µl of the bacterial suspension in MHB supplemented with 1% glucose and 100 µl of *S. khuzestanica* essential oil at sub-inhibitory concentrations (specifically, 1/2 MIC and 1/4 MIC). Negetive control wells, containing only the bacteria and culture medium, were also included. The plates were then incubated statically at 37 °C for 48 hours. Following the incubation period, the liquid contents were removed, and the wells were washed three times with phosphate-buffered saline to remove non-adherent, planktonic cells. Subsequently, the remaining biofilms were stained for a duration of 15 minutes using a 0.1% (w/v) crystal violet solution. After a subsequent washing step and air-drying, the stain bound to the biofilm was solubilized by adding 70% ethanol to each well. The adherent biomass was quantified by measuring the optical density at a wavelength of 595 nm using a microplate reader. The percentage of biofilm inhibition was calculated employing the following formula [16]:

$$\text{Biofilm Inhibition}\,(\%) = \left(\frac{OD_{Control} - OD_{Test}}{OD_{Control}}\right) \times 100$$

Statistical analysis

All experiments were performed in at least three independent replicates, and the data are expressed as the mean ± standard deviation. Differences between the control and treatment groups were analyzed for statistical significance using an independent samples t-test in GraphPad Prism (version 9.0). A p-value <0.05 was considered statistically significant. Different levels of significance are indicated in figures and tables as follows: $p < 0.05$, $p < 0.01$, and $p < 0.001$.

Results

S. khuzestanica essential oil and coumarin exhibit potent antibacterial activity against *S. maltophilia*

The antibacterial activities of *S. khuzestanica* essential oil and coumarin were evaluated using the agar diffusion method. The essential oil exhibited exceptionally potent activity, as evidenced by a mean ZOI of 40.0±1.73 mm. This ZOI was significantly larger than the 30.0±1.0 mm zone produced by coumarin ($p<0.001$). Notably, the *S. maltophilia* strain was completely resistant to CZA antibiotic,

while the negative control (DMSO) exhibited no inhibitory activity (Fig. 1). These results are summarized in Table 1.

S. khuzestanica essential oil exhibits a bactericidal effect on _S. maltophilia_

To quantitatively evaluate the antimicrobial activity, the MIC and MBC values were determined. *S. khuzestanica* essential oil exhibited an MIC of 31.25 µg/mL and an MBC of 62.50 µg/mL. The determined MBC/MIC ratio of 2.0 substantiates the bactericidal properties of the essential oil against *S. maltophilia* [17]. The control antibiotic, gentamicin, also confirmed its bactericidal action with an MIC of 0.976 µg/mL, an MBC of 1.953 µg/mL, and a corresponding MBC/MIC ratio of 2.0. However, due to coumarin's low solubility and subsequent precipitation in the broth culture medium, determining its MIC and MBC values wasn't feasible. These findings are summarized in Table 2.

S. khuzestanica essential oil effectively inhibits _S. maltophilia_ biofilm formation

The anti-biofilm activity of *S. khuzestanica* essential oil was investigated at a sub-inhibitory concentration (1/2 MIC; 15.625 µg/mL). Treatment with the essential oil resulted in a 62.1% reduction in biofilm formation, as evidenced by a significant decrease in mean optical density (OD_{595}) from 0.29 ± 0.02 in the control group to 0.11 ± 0.01 in the treated group ($p < 0.001$) (Fig. 2A). These quantitative data were visually corroborated; the dense purple staining characteristic of the control group's robust biofilm was markedly diminished in the essential oil -treated wells (Fig. 2B). Detailed results are summarized in Table 3.

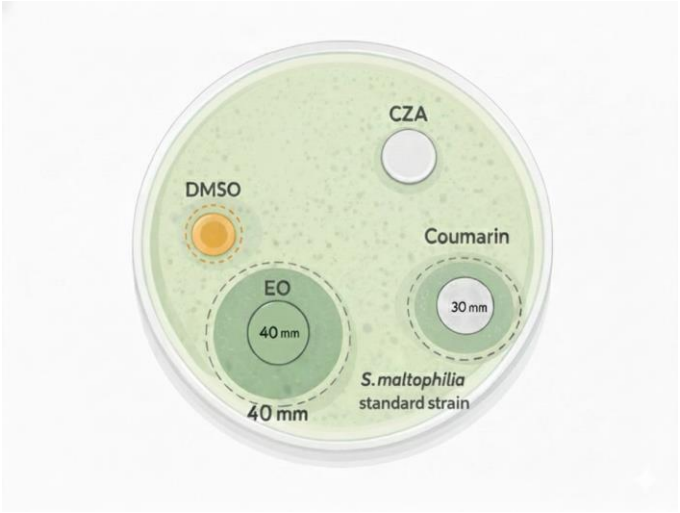

Fig. 1. Antibacterial susceptibility of *Stenotrophomonas maltophilia* to *Satureja khuzestanica* essential oil and coumarin. The representative agar well diffusion plate shows a potent zone of inhibition for the essential oil and coumarin. In contrast, the negative control (DMSO) and the Ceftazidime/ clavulanic acid (CZA) disk exhibit no inhibitory zones, confirming the strain's resistance to CZA.

Table 1. Antibacterial activity of *S. khuzestanica* essential oil and coumarin against *S. maltophilia* ATCC 13637

Test agent	Inhibition zone (mm) ± SD	Interpretation
***S. khuzestanica* essential oil**	40.0 ± 1.73	Highly susceptible
Coumarin	30.0 ± 1.00	Susceptible
Dimethyl sulfoxide (control)	0.0 ± 0.00	–
Ceftazidime/ Clavulanic acid	0.0 ± 0.00	Resistant

Values are mean ± SD of three independent experiments.

Table 2. Antimicrobial activity parameters of *S. khuzestanica* essential oil and gentamicin against *S. maltophilia*

Test compound	MIC (µg/ml)	MBC (µg/ml)	MBC/MIC ratio	Type of effect
***Satureja khuzestanica* essential oil**	31.25 ± 0.00	62.50 ± 0.00	2.0	Bactericidal
Gentamicin	0.976 ± 0.00	1.953 ± 0.00	2.0	Bactericidal
Coumarin	—	—	—	Not determined

MIC= Minimum inhibitory concentration; MBC= Minimum bactericidal concentration

Data are presented as Mean ± SD.

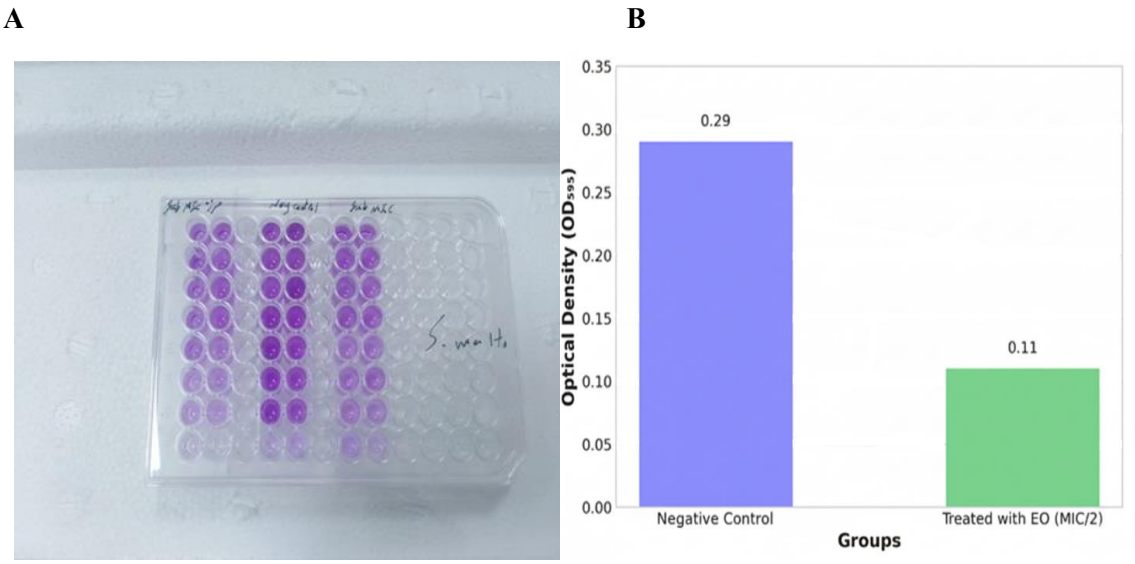

Fig. 2. Inhibition of *Stenotrophomonas maltophilia* biofilm formation by of *Satureja khuzestanica* essential oil. **A:** A representative image from the crystal violet assay visually confirms this inhibition. The dense purple staining in the control wells indicates robust biofilm, which is markedly diminished in the essential oil -treated wells.
B: Quantitative analysis shows a significant reduction in biofilm biomass, measured by optical density (OD_{595}), following treatment with the essential oil. Data are presented as mean ± SD ($p < 0.001$ vs. control).

Table 3. Quantitative analysis of the anti-biofilm activity of *S. khuzestanica* essential oil against *S. maltophilia*

Group	Mean normalized optical density (Mean ± SD)	Biofilm Inhibition (%)
Negetive control	0.29 ± 0.02	0
Treatment with essential oil (MIC/2)	0.11 ± 0.01 *	62.1

*Significant difference compared to negetive control ($p < 0.001$)

Discussion

The global crisis of AMR, particularly the emergence of multidrug-resistant opportunistic pathogens like *S. maltophilia*, has compelled the scientific community to search for novel therapeutic strategies. This Gram-negative bacterium has become a formidable clinical challenge, especially for immunocompromised patients, owing to its intrinsic resistance and high capacity for biofilm formation [5]. In response to this need, the present study evaluated the potential of plant-derived compounds. A pivotal finding of this research was the demonstration of the potent dual bactericidal and anti-biofilm activity of *S. khuzestanica* essential oil against *S. maltophilia*. The significance of this achievement is further highlighted by the complete resistance of the *S. maltophilia* strain to the standard combination of ceftazidime/clavulanic acid.

The bactericidal activity of *S. khuzestanica* essential oil against *S. maltophilia* was quantitatively confirmed, with MIC and MBC values determined to be 31.25 µg/mL and 62.50 µg/mL, respectively. The resulting MBC/MIC ratio of 2.0 offers conclusive evidence of the oil's bactericidal properties [18]. Although direct chemical analysis of the specific essential oil batch used in this study was not performed, the remarkable biological activity observed is highly consistent with the well-documented chemical profile of this endemic Iranian plant. Extensive scientific literature identifies the phenolic compound carvacrol as the principal and dominant component (often >90%) of *S. khuzestanica* essential oil [9, 19, 20]. Therefore, the potent antibacterial effect is likely attributable to this compound. The primary antibacterial mechanism of carvacrol involves the disruption of the bacterial membrane, leading to the leakage of intracellular contents and ultimately cell death [21]. Our results are in full agreement with prior studies; for example, Resende et al. confirmed the antibacterial activity of carvacrol against a wide range of pathogens [22], and a study by Asadi et al. reported its potent effects on *Escherichia coli* [23]. Although the susceptibility of the standard strain used in this research may differ from that of clinical isolates in other studies, the consistent efficacy of this compound across various studies highlights its potential as a broad-spectrum antibacterial agent [24].

The evaluation of coumarin, the second natural compound studied, yielded noteworthy results. While the compound demonstrated significant antibacterial activity in the agar diffusion assay, consistent with previous reports such as Qais et al., a fundamental methodological challenge precluded its quantitative evaluation [25]. The limited aqueous solubility of coumarin made the determination of its MIC unfeasible. This limitation is considered an important finding in itself, as it highlights the necessity for future studies on improving coumarin's formulation, possibly through nanotechnology, to fully exploit its potential as an inhibitor of quorum sensing mechanisms [26]. From a clinical perspective, the most

prominent achievement of this research was the ability of the *S. khuzestanica* essential oil to inhibit biofilm formation by 62.1% at a sub-inhibitory concentration (sub-MIC). This phenomenon, occurring at a concentration devoid of direct killing effects, suggests that the agent interferes with key virulence pathways, including the quorum sensing system and initial adhesion processes. The importance of this finding is undeniable, given the pivotal role of biofilms in the persistence of chronic infections and the phenotypic resistance of *S. maltophilia*. These results are consistent with the findings of Cáceres et al. and Walczak et al., who proved that carvacrol impedes biofilm development by inhibiting bacterial quorum sensing mechanisms [27, 28]. Furthermore, a study by Ghaderi et al., which reported the anti-biofilm effect of the same essential oil on *Pseudomonas aeruginosa*, provides further confirmation of this natural agent's broad potential in combating bacterial biofilms [20].

Synthesizing these findings introduces *S. khuzestanica* essential oil as a therapeutic agent with a dual mode of action; it not only targets planktonic cells but also prevents the formation of the resilient biofilm structure. This multifaceted attribute opens new horizons for clinical applications, such as its use as an adjuvant to conventional antibiotics, the development of antimicrobial coatings for medical devices, and the formulation of safe and efficacious natural disinfectants.

Despite the promising results, the interpretation of these findings requires consideration of the study's inherent limitations. As noted, a primary limitation was the lack of direct chemical analysis of the specific essential oil batch used. Furthermore, this research was conducted under *in vitro* conditions and focused on a single standard ATCC strain; therefore, the generalizability of the results to diverse clinical isolates of *S. maltophilia* warrants further investigation. These limitations, along with the methodological challenges encountered in the quantitative evaluation of coumarin, delineate clear pathways for future studies to validate these findings in *in vivo* models and to establish a definitive link between the chemical profile and the observed biological activity.

Conclusion

The results of this study provide compelling evidence that the essential oil of *S. khuzestanica* possesses considerable potential as a therapeutic agent against the opportunistic and resistant pathogen, *S. maltophilia*. Its dual activity both bactericidal against planktonic cells and highly inhibitory against biofilm formation positions this natural agent as an outstanding candidate for the development of novel therapeutic strategies. The present findings establish the scientific groundwork for further investigations into pharmaceutical formulations and preclinical evaluations, representing a step forward in combating the global crisis of antibiotic resistance.

Ethical Considerations

This study was conducted using a standard, commercially available bacterial strain (ATCC 13637) and did not involve the use of human subjects or live animals. Therefore, formal ethical

approval was not required for this research. All laboratory procedures were performed in accordance with standard microbiological safety guidelines.

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Conflict of Interest
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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

Authors' Contributions
K. K-Ch. and F.G. were responsible for designing the review protocol, conducting the literature review, providing feedback on the manuscripts, writing the manuscript and improving the interpretation of the results. S. S-K. was responsible for writing the manuscript, assembling data, analyzing data, and interpreting analyses.

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