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Medicinal value of *Lippia multiflora* Mondenke flowers in the fight of oral and dental infections

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Biofilm

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ABSTRACT

Oral infections pose a significant global health issue. This study assessed the antibacterial properties of methanol and dichloromethane extracts from *Lippia multiflora* flowers against *Staphylococcus aureus* ATCC 43300 and *Streptococcus mutans* ATCC 2517, two bacteria known to cause oral infections. The study measured the ability of these flower extracts to inhibit the growth and biofilm formation of *S. aureus* and *S. mutans* using micro-dilution and crystal violet methods, respectively. Additionally, we analyzed the presence of secondary metabolites in the extracts both qualitatively and quantitatively. The antioxidant properties of the extracts were evaluated using DPPH, ABTS, and FRAP methods. The results indicated that the dichloromethane extract demonstrated a more substantial bactericidal effect than the methanolic extract against *S. mutans* and *S. aureus*, with minimal bactericidal concentrations of 0.25 ± 0.02 mg/mL and 3.13 ± 0.30 mg/mL, respectively. Furthermore, the dichloromethane extract at a 100 µg/mL concentration exhibited the highest anti-biofilm activity against both *S. aureus* and *S. mutans*. Phytochemical screening revealed the presence of alkaloids, flavonoids, quinones, and tannins in both extracts. The total phenolic content was higher in the methanolic extract (49.57 ± 2.74 mg EAG/100 mg) compared to the dichloromethane extract (25.71 ± 0.39 mg EAG/100 mg). Similarly, the total flavonoid content was more significant in the methanolic extract (2.87 ± 0.049 mg EQ/100 mg) than in the dichloromethane extract (2.24 ± 0.02 mg EQ/100 mg). The methanolic extract also exhibited superior anti-DPPH and anti-ABTS activities, as well as a higher Fe (III) reduction potential than the

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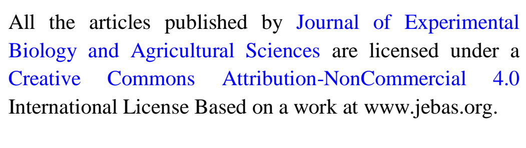

dichloromethane extract (P < 0.05). These findings suggest that *L. multiflora* flowers could serve as a potential source of antimicrobial agents for combating oral infections.

1 Introduction

Oral health is vital in maintaining individual and population health (Hung et al. 2019). Oral infections affect people across all social strata, with varying degrees of severity (Kilinc et al. 2024). Furthermore, these infections can hinder chewing and speaking, negatively impact physical appearance, and alter an individual's social life (Furuta and Yamashita 2013). A significant portion of the global population experiences oral infections at some point, with school children particularly susceptible to dental caries (Youssefi and Afroughi 2020). The primary oral infections affecting individuals are dental caries and periodontal disease. Dental caries remains the third most prevalent global health issue in terms of morbidity. In Burkina Faso, approximately 60% of the population suffers from oral infections due to poor oral hygiene and dietary habits (Clauss et al. 2021).

Antibiotics and specific oral hygiene practices, such as brushing teeth and mouthwash, are recommended to combat oral infections. However, the accessibility of certain antibiotics poses a challenge for populations in low-income countries. Additionally, the ability of bacteria to form biofilms is a significant factor in developing bacterial resistance, which can lead to the failure of conventional antibiotic treatments (Nadar et al. 2022). Bacteria within biofilms are up to 1,000 times more virulent than their planktonic counterparts (Kalia et al. 2023). This biofilm-induced resistance limits the penetration of antibiotics into the biofilm matrix, thereby enhancing the production of various virulence factors, facilitating the exchange of virulent genes among bacteria, and coordinating bacterial behaviour (Sharma et al. 2023). Consequently, discovering effective and bioavailable antibacterial drugs from medicinal plants with potent antibiofilm properties is a promising alternative to combat oral infections, particularly in developing nations.

Lippia multiflora, commonly known as the Gambian tea bush in English and Kwilg-wisaoré in Moore, is a perennial plant characterized by its erect woody stem and aromatic, camphoraceous odour. It is found in many African countries and typically grows in savannahs, reaching heights of 2.7 to 4 meters. This plant is frequently used in various forms of traditional medicine in Burkina Faso to treat several microbial infections, including oral and anal candidiasis (Bangou et al. 2012). Research by Rouamba et al. (2024a) demonstrated that extracts from *L.*

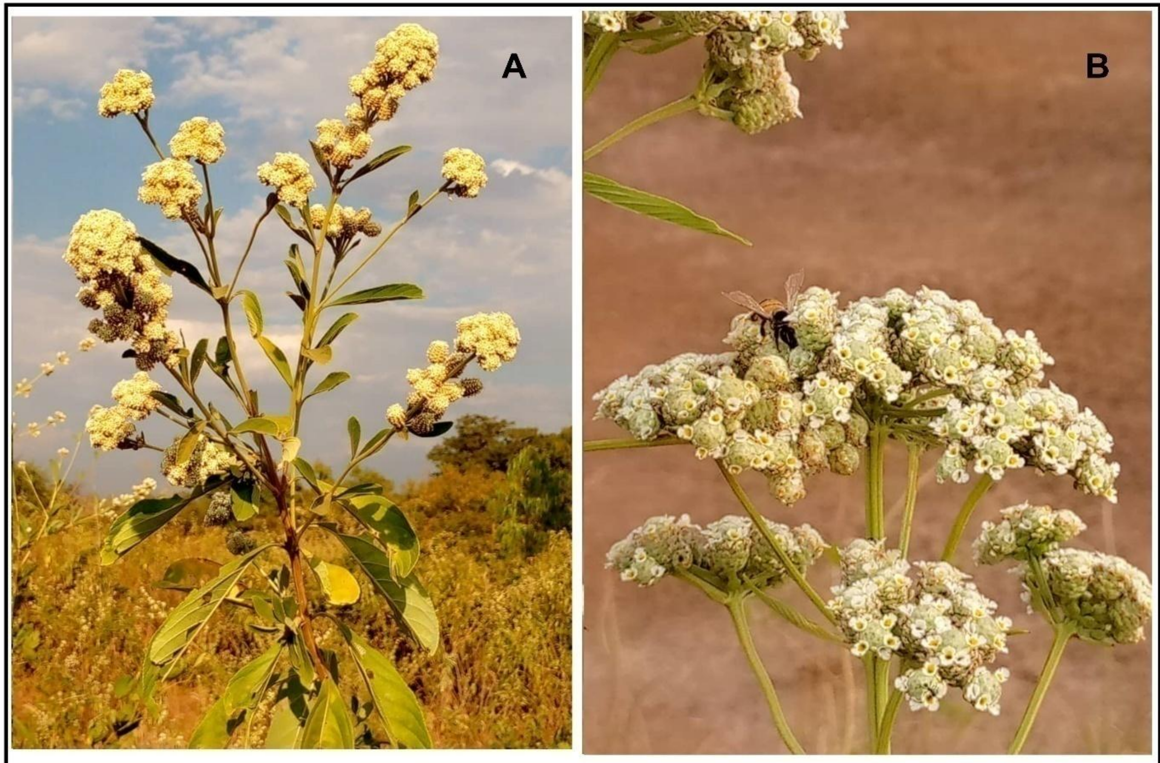

Figure 1 *Lippia multiflora* (A) whole plant, and (B) inflorescence

multiflora leaves significantly enhanced the bactericidal effects of cefotaxime against methicillin-resistant *Streptococcus aureus* (ATCC 43300). Furthermore, Rouamba et al. (2024b) found that the essential oils from *L. multiflora* flowers strongly inhibited biofilm formation and enhanced the motility of *Pseudomonas aeruginosa* (PAO1). While many studies have focused on the bactericidal properties of *L. multiflora* leaves or flower extracts against various Gram-positive and Gram-negative bacterial strains, scientific information regarding the antibiofilm potential of *L. multiflora* flower extracts against bacteria responsible for oral and dental infections, such as *S. mutans* and *S. aureus*, is limited. This study evaluated the antibacterial properties of methanol and dichloromethane extracts of *L. multiflora* flowers against *S. aureus* (ATCC 43300) and *S. mutans* (ATCC 2517), which are associated with oral infections.

2 Materials and Methods

2.1 Plant collection

The flowers of *L. multiflora* were collected in September 2022 from Loumbila, Burkina Faso (coordinates: 12°31'5.39"N; 1°22'8.39"W). An expert taxonomist at the Plant Ecology Laboratory, UFR/SVT, identified the collected plant samples at Université Joseph KI-ZERBO in Burkina Faso. Herbarium sheets, prepared with various plant parts and flowering tops, have been deposited at the herbarium of Université Joseph KI-ZERBO under the ID number CI-922.

2.2 Extraction

The flowers of *L. multiflora* were first ground into a powder. This powder was then subjected to maceration in dichloromethane at 10 g per 100 mL, with mechanical stirring for 24 hrs at 37 °C. Afterwards, the mixture was centrifuged for 20 minutes at 800 g, and the supernatant was collected. This supernatant was concentrated using a rotary evaporator and evaporated to dryness to yield the dichloromethane crude extract. The remaining residue was dried and then subjected to maceration in methanol under the same conditions to produce the methanol crude extract.

2.3 Determination of minimal inhibitory and bactericidal concentrations

The extracts' minimum inhibitory concentration (MIC) was determined using the microdilution method (Roy and Gupta 2022). Different concentrations of the extracts were added to 96-well plates containing 10 µL of a bacterial inoculum (at a specified concentration) and incubated for 24 hours at 37 °C. After the incubation, iodonitrotetrazolium was added. The lowest concentration of the extract in which no colour change occurred, indicating the absence of bacterial growth, was recorded as the MIC. The minimum bactericidal concentration (MBC) was assessed using a solid LB-agar medium (Septya et al. 2023). Samples were taken from the wells that showed no bacterial growth during the MIC determination and transferred to Petri dishes containing LB-agar medium. After 24 hours of incubation at 37 °C, the lowest concentration of the extract in the petri dish that displayed any visible bacterial colonies was considered the MBC.

2.4 Antibiofilm assay

Using the crystal violet method, a non-bacteriostatic concentration of the extracts was utilized to evaluate the antibiofilm activity (Kamimura et al. 2022). In this experiment, 10 µL of the inoculum of each bacterium (10^6 CFU/mL) was incubated with each extract at a final concentration of 100 µg/mL for 24 hours at 37°C in 96-well plates. After incubation, the supernatant and any planktonic bacteria were removed from the wells. The remaining bacterial biofilm was washed with distilled water and fixed with methanol for 15 minutes. Following the removal of methanol, crystal violet was added to the wells. After incubating for 30 minutes at 37°C, excess crystal violet was removed, and the fixed crystal violet in the biofilm membrane was solubilized using an acetic acid solution. The intensity of the dissolved crystal violet colour is proportional to the quantity of biofilm formed. Optical densities were measured at 590 nm, and the results were expressed as the percentage inhibition of biofilm formation compared to the control without extracts, with salicylic acid used as a reference compound.

2.5 Antioxidant assay

The antioxidant potential of the extracts was evaluated by assessing their ability to neutralize DPPH and ABTS radicals and reduce iron(III) using the DPPH, ABTS, and FRAP methods, respectively (Compaoré et al. 2016). Ascorbic acid was utilized to generate standard curves for the DPPH radical scavenging assay ($Y = 0.058X + 0.130$; $R^2 = 0.994$) and the iron(III) reduction tests ($Y = 0.013X - 0.018$; $R^2 = 0.999$). Trolox was used to create the standard curve for the ABTS radical scavenging activity ($Y = 0.016X + 0.096$; $R^2 = 0.997$). The results were expressed as mg of EAA/10 g of extract for the DPPH radical trapping test, mmol of EAA/10 g of extract for the iron(III) reduction test, and mg of ET/10 g of extract for the ABTS radical quenching test.

2.6 Phytochemical screening

The total flavonoid content was measured using iron chloride, as described by Hilma et al. (2018). Quercetin was utilized to create the standard curve, represented as $Y = 0.031X + 0.019$ ($R^2 = 0.999$). The results are reported as mg of EQ per 100 mg of extract. Total phenolics were quantified with the Folin-Ciocalteu reagent following the method outlined by Lucas et al. (2022). Gallic acid served as the standard for generating the curve, expressed as $Y = 39.543X + 0.039$ ($R^2 = 0.999$), and the results are presented as mg

of EAG per 100 mg of extract. Qualitative phytochemical analysis was conducted using standard analytical tests to screen for primary and secondary metabolites following the methodology described by Yamin et al. (2021).

2.7 Statistical analysis

The results are presented as the mean value from multiple independent experiments (n > 3) with the standard deviation included. A one-way ANOVA, followed by the Tukey post-test, was conducted to assess the significance of the results. A statistical difference was noted when $P < 0.05$.

3 Results

3.1 Minimal inhibitory and bactericidal concentrations

The results of the minimal inhibitory and minimal bactericidal concentrations indicated that the dichloromethane and methanolic extracts of *L. multiflora* strongly inhibited the growth of *S. mutans* compared to *S. aureus* ($P < 0.05$). These findings suggest that *S. mutans* is more sensitive to the extracts of *L. multiflora* than *S. aureus*. Additionally, the dichloromethane extract exhibited a higher bactericidal effect than the methanolic extract against both *S. mutans* and *S. aureus*, with minimal bactericidal concentrations of 0.25 ± 0.02 mg/mL and 3.13 ± 0.30 mg/mL, respectively (Table 1). These results are promising in the fight against oral infections, mainly because *S. mutans* is the primary bacterium associated with oral and dental infections, such as dental caries, while *S. aureus* is an opportunistic pathogen.

3.2 Anti-biofilm activity

After evaluating the extracts' capacity to kill bacteria (bactericidal effect) or inhibit bacterial growth (bacteriostatic effect), we assessed their ability to prevent bacterial biofilm formation, with the results displayed in Figure 2. Both extracts effectively inhibited

Table 1 Minimal inhibitory and bactericidal concentrations of extracts

Extract	Bacterial strain	MIC (mg/mL)	MBC (mg/mL)	MBC/MBC	Sensibility
MeOH	*S. mutans*	0.78 ± 0.07^{b}	3.13 ± 0.30^{b}	4.00 ± 0.40	Bacteriostatic
	S. aureus	3.13 ± 0.30^{a}	$> 12.5 \pm 1.20^{a}$	> 3.99	Nd
DCM	*S. mutans*	0.13 ± 0.01^{d}	0.25 ± 0.02^{d}	8.00 ± 0.80	Bacteriostatic
	S. aureus	0.25 ± 0.02^{c}	$> 0.50^{c}$	> 2.00	Nd

MIC: minimal inhibitory concentration, MBC: minimal bactericidal concentration, MeOH: methanol, DCM: dichloromethane, Nd: Not determined, values with different superscript letters in each column differ statistically ($P < 0.05$)

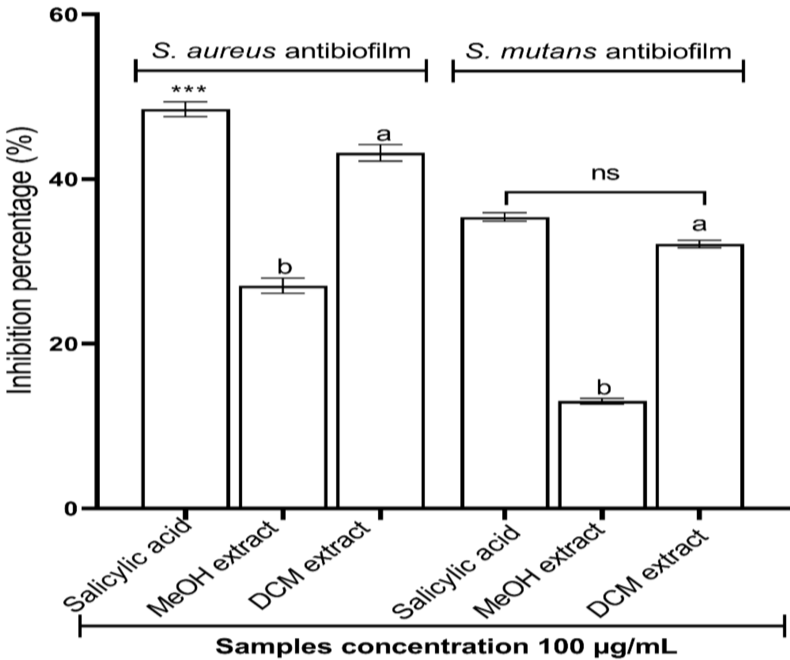

Figure 2 Antibiofilm activity of *L. multiflora* extract [*** *p<0.001* versus the corresponding salicylic acid, $^{a,b}P<0.05$ compared between extracts (ANOVA followed by Tukey test), ns: nonsignificant, MeOH: Methanol, DCM: dichloromethane]

biofilm formation in *S. aureus* and *S. mutans*. The dichloromethane extract demonstrated superior inhibitory activity against biofilm formation compared to the methanol extract for both *S. aureus* and *S. mutans* ($P < 0.05$). Notably, the dichloromethane extract and salicylic acid showed similar levels of inhibitory activity against biofilm formation in *S. mutans*. However, the dichloromethane extract was less effective than salicylic acid in inhibiting biofilm formation in *S. aureus*. These findings suggest that the dichloromethane extract of *L. multiflora* flowers is a promising source of antibiofilm compounds for combating *S. mutans*.

3.3 Antioxidant activities of tested extracts

Bacteria can cause oxidative stress during their infection processes. The capacity of the extract to quench free radicals (antioxidant effect) was measured, and the data were presented in Table 2. All the extracts showed higher DPPH and ABTS radical scavenging activities and higher Fe(III) reducing power. The methanolic extract showed more anti-DPPH activity (11.69 ± 0.24mg AAE/10g), more anti-ABTS activity (943.38 ±3.41mg TE/10g) and more Ferric reducing power (41.04± 0.89 mmol AAE /10g) than dichloromethane extract ($P < 0.05$) which exhibited the anti-DPPH activity of 9.20 ± 0.12mg AAE/10g, the anti-ABTS activity of 938.44 ±2.91mg TE/10 g and the Ferric reducing power of 13.96± 0.28mmol AAE /10g.

3.4 Phytochemical screening

To evaluate the antibacterial potential of the extract, we conducted a quantitative and qualitative screening of its bioactive phytomolecules. The results of the quantitative phytochemical screening are presented in Table 3. The methanol extract demonstrated higher total phenolic (49.57 ± 2.74 mg GAE/100 mg) and flavonoid (2.87 ± 0.05 mg QE/100 mg) contents compared to the dichloromethane extract, which showed total phenolic and flavonoid contents of 25.71 ± 0.39 mg GAE/100 mg and 2.24 ± 0.02 mg QE/100 mg, respectively ($P < 0.05$). The extraction yields indicated that the compounds in *L. multiflora* flowers were more polar and more soluble in methanol (7.78%) than dichloromethane (2.07%). For the qualitative phytochemical screening, we documented the results by photographing the test tubes, and the images are displayed in Figure 3.

Table 2 Antioxidant activities of tested *L. multiflora* extracts

Extract	DPPH (mg AAE/10g)	FRAP (mmol AAE /10 g)	ABTS (mg TE/10 g)
Dichloromethane	9.20 ± 0.12^{b}	13.96 ± 0.28^{b}	938.44 ± 2.91^{b}
Methanol	11.69 ± 0.24^{a}	41.04 ± 0.89^{a}	943.38 ± 3.41^{a}

AAE: Ascorbic acid equivalent, TE: Trolox equivalent, values with different superscript letters in each column differ statistically ($P < 0.05$)

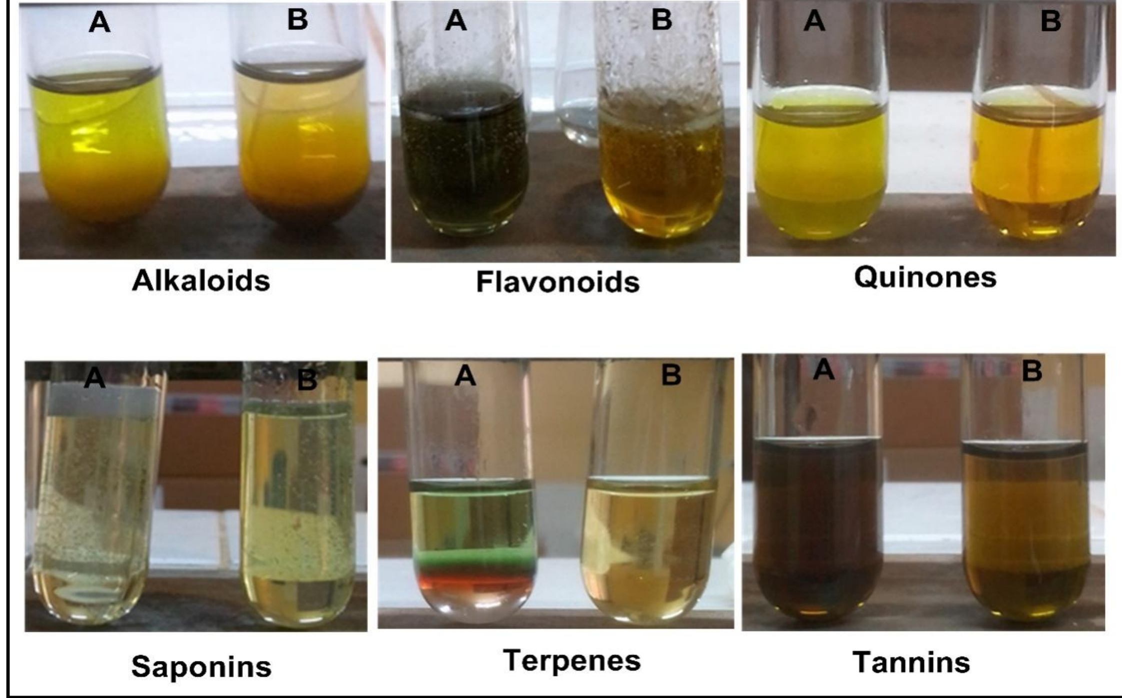

Figure 3 Photo of the different tubes for the secondary metabolites screening, (A) methanol extract, (B) dichloromethane extract

Table 3 Phenolic and flavonoid contents in the tested *L. multiflora* flower extracts

Extract	R (%)	Total phenolic (mg GAE/100 mg)	Total flavonoid (mg QE/100 mg)
Dichloromethane	2.07^{b}	25.71 ± 0.39^{b}	2.24 ± 0.02^{b}
Methanol	7.78^{a}	49.57 ± 2.74^{a}	2.87 ± 0.05^{a}

R: Rendement of extraction, GAE: Gallica cid equivalent, QE: Quercetin equivalent, values with different superscript letters in each column differ statistically ($P < 0.05$)

Table 4 Presence of the Secondary metabolites in prepared extracts of *L. multiflora*

Extract	Alkaloids	Flavonoids	Quinones	Saponins	terpenes	Tannins
MeOH	+	+	+	+	+	+
DCM	+	+	+	-	-	+

MeOH: methanol, DCM: dichloromethane, (+) presence, (-): absence

The analysis of Figure 3, which displays the colour of the tubes and the development of micelles, allowed us to identify various classes of secondary metabolites present in each extract. In tube A, the yellow precipitate, pink/orange colouration, yellow colouration, purple colour ring, brown colouration, and persistent moss indicated the presence of alkaloids, flavonoids, quinones, terpenes, tannins, and saponins, respectively, in the methanol extract. Conversely, tube B's absence of persistent moss and the purple colour signified that the dichloromethane extract does not contain terpenes or saponins. Table 4 summarizes the presence or absence of secondary metabolites in methanol and dichloromethane extracts. Alkaloids, flavonoids, quinones, and tannins were detected in both extracts, while saponins and terpenes were not found in the dichloromethane extract.

4 Discussion

Methanol and dichloromethane extracts of *L. multiflora* demonstrated significant bactericidal and antibiofilm activity against two bacteria, *S. aureus* and *S. mutans*, which are associated with dental and oral infections. Furthermore, these extracts exhibited strong antioxidant activities against DPPH and ABTS radicals and notable ferric-reducing power. The antibacterial and antioxidant properties of the extracts may be attributed to the presence of alkaloids, flavonoids, tannins, quinones, terpenes, and saponins. *S. mutans* is a commensal organism found in humans' and animals' oral cavities and respiratory tracts (Abranches et al. 2018). Under certain conditions, it can act as an opportunistic pathogen, leading to infections such as septicemia and endocarditis (Nomura et al. 2020). *S. mutans* produces lactic acid, dissolving hard tissues and extracellular polysaccharides, enhancing adhesion to dental surfaces and accelerating biofilm formation (Krzysciak et al. 2014). Similarly, *S. aureus* is an opportunistic pathogen in oral diseases, primarily causing suppurative cutaneous infections like whitlow and boils (Del-Giudice 2020). Both bacteria are significant contributors to oral infections, including dental caries. In biofilms, they exhibit increased resistance to conventional antibiotic treatments. The oral biofilm comprises cariogenic bacteria, particularly *S. mutans* and *S. sobrinus*, which initiate caries disease. Other pathogens, such as *Lactobacillus*, *Actinomyces*, *S. aureus*, and *P. aeruginosa*, further contribute to biofilm progression (Zhu et al. 2023). The process of biofilm formation occurs in several stages: the establishment of an exogenous film, bacterial adhesion to surfaces, bacterial co-aggregation, maturation of the biofilm, and eventual detachment (Zubair et al. 2017).

In this study, the flowers of *L. multiflora*, particularly the dichloromethane extract, revealed very low minimal inhibitory and minimal bactericidal concentrations, indicating a robust bactericidal potential against *S. mutans* and *S. aureus*. This bactericidal potential of the extract could be advantageous for treating oral infections by effectively eliminating planktonic bacteria and preventing their growth and pathogenicity. The bactericidal effect of the extract may be attributed to various mechanisms, including disruption of the bacterial cell wall, inhibition of bacterial enzymes, blocking of bacterial DNA synthesis, or interference with bacterial efflux pumps. The presence of multiple phytochemical groups in the extract, especially tannins, alkaloids, quinones, and flavonoids, could play a role in these antibacterial mechanisms. Tannins and quinones are known for their bactericidal properties, as they inhibit the synthesis of peptidoglycans, a vital component of the bacterial cell wall. Additionally, alkaloids inhibit DNA and protein synthesis and ATP synthetase activity (Vaou et al. 2021). Flavonoids are bacterial enzyme inhibitors and efflux pump blockers (Waditzer and Bucar 2021).

Traditional anti-infective therapies (antibiotics) target bacteria during their planktonic phase; however, these methods face a significant limitation due to potential resistance development. Consequently, strategies aimed at different stages of biofilm formation present a promising alternative to combat antibiotic resistance. The dichloromethane extract exhibited the highest antibiofilm potential against *S. mutans* and *S. aureus*. The extract's

antibiofilm effect may be explained by inhibiting the synthesis of the biofilm's polysaccharide coating, disrupting biofilm adhesion by altering medium composition, or quelling biofilm maturation and detachment. Phenolic compounds, including flavonoids and phenolic acids, are recognized for inhibiting the synthesis of the protective biofilm envelope, which may elucidate the antibiofilm effects observed in this study (Nassima et al. 2019).

Additionally, *S. mutans* and *S. aureus* influence the cellular oxidative stress response during their pathogenic processes, which may exacerbate oral diseases (Wen et al. 2017). The malonyl dialdehyde (MDA) produced during oxidative stress is found in the superficial layers of cartilage and is associated with dental caries (de Sousa Né et al. 2023). In this study, methanolic and dichloromethane extracts exhibited vital antioxidant activities by inhibiting DPPH and ABTS radicals and demonstrating ferric (III) reduction capacity. These antioxidant properties of the extracts may help mitigate oxidative stress induced by bacteria involved in oral infections, thereby limiting the onset and progression of these infections.

Conclusion

The dichloromethane extract of *L. multiflora* flowers demonstrated significant inhibition of bacterial growth in both *S. mutans* and *S. aureus*. It also exhibited the most vigorous antibiofilm activity against these two bacteria, which are known to be responsible for oral infections. While previous studies have screened the extracts of *L. multiflora* leaves and flowers for their bactericidal properties, this is the first time that the antibiofilm potential of the flower extract has been evaluated specifically for *S. aureus* and *S. mutans*. This study indicates that *L. multiflora* flowers can potentially be a valuable source of therapeutic phytomolecules for addressing planktonic and biofilm-forming bacteria associated with oral infections.

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Conflict of interest

All authors declared that no competing interest exists

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