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Essential oils of indigenous citrus varieties of Northeast India as potential antibiofilm agents against foodborne pathogens: An *in vitro* and *in silico* study

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Abstract: The unique structural and biological diversity found in plants renders them a distinctive and sustainable source for discovering new antibacterial, antifungal and antiparasitic compounds. In the present study, antimicrobial and antibiofilm properties of essential oils of citrus varieties of Northeast India were studied against selected foodborne pathogens using both *in vitro* and *in silico* approaches. These essential oils showed significant antimicrobial and antibiofilm activities against foodborne pathogens. i.e. *Bacillus cereus* MTCC430 and *Yersinia enterocolitica* MTCC859. It was observed that the treatment with essential oil disturbed the membrane integrity of the pathogens, thereby causing the release of nucleic acids. This study also postulated that active compounds of the essential oils interact with different target proteins of the pathogens and provide an explanation for the mechanisms of antimicrobial and antibiofilm action of the essential oils of citrus varieties against foodborne pathogens.

Keywords: antimicrobial activity; hydro-distillation; membrane integrity; minimum inhibitory concentration; molecular docking

Diseases caused by the intake of any food contaminated with foodborne microorganisms are a major concern for global public health (Bajpai et al. 2013). Food industries are looking for natural products to design preservatives that can control the growth of foodborne pathogens without affecting the nutri-

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tional and physicochemical properties of food products (Bajpai et al. 2013; Picone et al. 2023). Nowadays, plant-derived essential oils are applied in food processing industries because they are safe and effective in controlling the risk of foodborne disease outbreak (Diao et al. 2013). Plant-derived essential oils are mainly mixtures of monoterpenes and sesquiterpenes which are used in food processing industries for their scents and flavours. Lemon essential oils contain different antimicrobial compounds that provide protection against infections (Gültepe 2020; Denkova-Kostova et al. 2021). The north-eastern part of India has a very rich biodiversity and some varieties of citrus fruits are protected by geographical indication (GI) tags (Rajan 2023). There are limited research works available for the determination of antimicrobial activities of essential oils extracted from these citrus varieties.

The essential oils mainly inhibit microbial growth by disrupting the cell membrane and cause metabolic damage and leakage of the intracellular constituents (Das et al. 2022; Mahmud et al. 2023; Sateriale et al. 2023). *Bacillus cereus* and *Yersinia*, as foodborne pathogens, pose a significant threat to the food industry due to their capacity to form biofilms. The spores produced by *B. cereus*, being hydrophobic in nature, have the property to adhere to surfaces, contributing to their role in foodborne illnesses. However, *B. cereus*-related foodborne illness is mild in nature, as a result of which it often remains unreported and often mistaken for common gastrointestinal ailments (Granum et al. 2012). Moreover, in foods with prolonged shelf-life, *Bacilli* and their spores prevail as prominent pathogens, posing challenges to their detection and management. *Yersinia enterocolitica,* a foodborne pathogen known for its resistance to cold, has the ability to contaminate food in post-processing steps, subsequently leading to an illness in consumers (Bhaduri et al. 2013). The major barriers created by biofilm are the exopolymer matrices that make the bacteria tolerant to antimicrobial agents by reducing the penetration of the antimicrobial agents (Mazzantini et al. 2016; Kerekes et al. 2019; Sivasankar et al. 2020; El-Tarabily et al. 2021). In recent times, many researches on essential oils focused on their antimicrobial and antibiofilm activities because different compounds present in essential oils interfere with biofilm formation mechanisms of bacteria (Rossi et al. 2022; Gado et al. 2023). Molecular docking is used for calculating the binding energy of protein-ligand interactions which may suggest affinity of the examined compounds towards the target ligand (Noshad et al. 2022). This study aimed to explore the antimicrobial and antibiofilm properties of essential oils extracted from native citrus varieties of Northeast India. Additionally, *in silico* studies were conducted to observe interactions between the major compounds of the oils with the target proteins, aiming to elucidate potential mechanisms underlying the inhibition of specific foodborne pathogens.

MATERIAL AND METHODS

Collection of samples and extraction of essential oils. Four indigenous citrus varieties of Northeast India *Citrus jambhiri* (PL1), *Citrus medica* (PL2), *Citrus limon* (elongated lemon; PL3), and *Citrus aurantifolia* (rough lemon; PL4) were collected from different parts of Northeast India. The fruits were surface sterilised with 70% ethanol followed by washing and peeling. The extraction of essential oils from the peels was performed using the Clevenger apparatus and kept at 20 °C until needed. Characterisation of the extracted essential oils was reported in another publication (Loying et al. 2023).

Bacterial strains and their growth conditions. The selected bacteria were obtained from MTCC (microbial type culture collection) and Gene Bank (IMTECH Chandigarh, India). The bacterial strains *B. cereus* MTCC430 and *Y. enterocolitica* MTCC859 were used in the studies and grown in lysogeny broth (LB) medium at 37 °C.

Paper disk diffusion assay. The antimicrobial activities of the essential oils were determined by paper disk diffusion assay. The solidified LB medium was spread with 100 µL of bacterial broth culture of 10^5 to 10^7 CFU·mL^{-1} (CFU – colony forming unit) and allowed to dry for a few minutes. And then autoclaved paper disks were loaded with 15 µL of essential oils and placed on the LB medium spread with bacterial broth culture and they were incubated at 37 °C for 24 h. After 24 h of incubation, culture plates were observed for the zone of inhibition (ZOI).

Minimum inhibitory concentration (*MIC*) of the essential oils. To determine the *MIC* of the EOs against the bacterial strains the guidelines of the National Committee for Clinical Laboratory Standards, USA were used (NCCLS, Methods for dilution antimicrobial susceptibilty tests for bacteria that grow aerobically, 2006). The EOs were double diluted in LB and incubated overnight with 1:100 diluted bacterial culture in 96-well plates and incubated at 37 °C for 24 h.

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After incubation, the growth inhibition of the bacterial strains was observed. The minimum concentration which completely inhibited the visible growth of the bacterial strains was considered the minimum inhibitory concentrations (*MIC*s) of the EOs. The Tween 80 of 0.1% (*v*/*v*) concentration was used to mix the EOs with the medium completely and only concentrations lower than *MIC* were used for the experiments on biofilm inhibition as described by Kavanaugh et al. (2012).

Biofilm inhibition activity. For the screening of biofilm inhibition, the tissue culture plate method was used as described by Gupta et al. (2014). The bacterial culture of 100 µL with 10^6 to 10^7 CFU·mL^{-1} was added in LB medium. To the bacterial culture, two different concentrations of EOs were added: 1/2 *MIC* and 1/4 *MIC* and incubated at 37 °C for 48 h. After the incubation period, the wells were washed twice with phosphate buffered saline (PBS) of pH 7.4 to remove free-floating planktonic bacteria. The adhered cells were stained with 0.1% crystal violet and incubated at 37 °C for 30 min. After incubation, the wells were washed with PBS to remove excess stain and air dried. Then methanol was added to each well to solubilise the bound crystal violet and untreated wells were used as control. The percentage of biofilm inhibition was checked by using a microplate reader at optical densities at 570 nm and inhibition was calculated using the formula:

$$Biofilm\ inhibition\ (\%) = \frac{\left(OD_{control} - OD_{test}\right)}{OD_{control}} \times 100 \quad (1)$$

where: $OD_{control}$ – the absorbance of the untreated control; OD_{test} – the absorbance of the treated well.

Crystal violet staining assay. The biofilm inhibition of EOs by crystal violet staining was performed using a method described by Gupta et al. (2014). Briefly, the biofilms of the specific bacteria were allowed to form on coverslips and incubated at 37 °C for 24 h in media containing the exact concentrations of EOs that led to the highest inhibition of biofilm in the previous step. Control plates were also maintained alongside the test plates. After incubation, the coverslips were stained with 0.1% crystal violet for 20 min and then washed with PBS three times. The air-dried coverslips were observed under a light microscope (Magnus CH20i LED; Magnus Opto Systems India Pvt. Ltd., India) at 1 000× magnification.

Effects of essential oils on cell membrane integrity. The destruction of the cell membrane integrity by EOs was determined by the method described by Mitra et al. (2022) with minor modifications. Briefly, the bacterial samples were centrifuged at 4 500 revolutions per minute (rpm) for 6–10 min. The pellets obtained were washed with PBS with pH 7.2 and 0.9% saline was added, followed by treatment with *MIC*s of the EOs and incubation at 37 °C. Controls were also maintained for each bacterium which were not treated with EOs. The bacterial suspensions were removed at 2-hour intervals and were centrifuged at 6 000 rpm for 5 min. The supernatant was taken and the absorbance was measured at 260 nm for the detection of nucleic acids in the suspension.

Molecular docking. Using Autodock (version 4.2.1), docking studies were carried out between Quorum-Sensing Transcriptional Activator from *Yersinia enterocolitica* (yenR; PDB ID: 5L07) and non-haemolytic enterotoxin from *Bacillus cereus* (PDB ID: 4K1P) with the most abundant compounds in the extracted oils, i.e. citronellol and limonene (Loying et al. 2023). The docking studies were carried out in triplicates and the number of computed solutions was 50 in each case. The number of evaluations was 2 500 000 with maximum generation number 27 000 and other default values were allowed. The root mean square deviation (RMSD) clustering maps were produced to find the best cluster having the lowest energy and a great number of populations as mentioned by Forli et al. (2016).

Statistical analysis. All experiments were performed in triplicates and one-way analysis of variance (ANOVA) was performed followed by Tukey's post-hoc tests at a significance level of $P = 0.05$ (95% confidence intervals) to compare different groups.

RESULTS AND DISCUSSION

Antimicrobial activities of essential oils. EOs are the secondary metabolites of the plants that have well-documented history of serving as natural remedies. Antimicrobial activities of the EOs extracted from indigenous citrus varieties against selected foodborne pathogens are represented as zones of inhibitions as shown in Table 1. It was found that PL4 and PL1 has the highest activity against *B. cereus* MTCC430 and *Y. enterocolitica* MTTCC859, respectively (MTCC – microbial type culture collection).

Although no statistical significance was observed for zones of inhibition among the four EOs studied, it was documented that PL1 showed maximum antimicro-

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Table 1. Antimicrobial activity of the essential oils against foodborne pathogens in citrus varieties

Bacteria	Zone of inhibition (mm)			
	PL1	PL2	PL3	PL4
Bacillus cereus MTCC430	10.50 ± 0.50[a]	10.50 ± 0.87[a]	10.33 ± 0.58[a]	11.33 ± 0.58[a]
Yersinia enterocolitica MTTCC859	13.67 ± 0.50[a]	10.17 ± 0.77[b]	10.33 ± 0.58[b]	12.67 ± 1.04[b]

[a,b] Different letters represent significant differences at $P < 0.05$; MTTC – microbial type culture collection; PL1 – *Citrus jambhiri*; PL2 – *Citrus medica*; PL3 – *Citrus limon* (elongated lemon); PL4 – *Citrus aurantifolia* (rough lemon)

bial activity against both the studied test organisms. As per the previously published report, D-limonene was the major compound in all citrus essential oils (Loying et al. 2023). But in this study we did not find any consistency in the zones of inhibition. This suggests that the antimicrobial activity might result from a 'matrix effect' generated by the interaction between the primary compound D-limonene and the secondary compounds found in various essential oils at different concentrations (Fatima et al. 2021).

Determination of minimum inhibitory concentration. A dependable evaluation of *MIC*s profoundly influences the selection of a therapeutic approach, thereby impacting on the effectiveness of infection treatment (Kowalska-Krochmal et al. 2021). The minimum inhibitory concentrations (*MIC*s) of the essential oils against selected foodborne pathogens are shown in Table 2. For the oil PL1, *MIC* against *B. cereus* MTCC430 and *Y. enterocolitica* MTTCC859 were 0.42 ± 0.19% and 0.84 ± 0.35% (*v*/*v*), respectively. For PL2 and PL3, the respective *MIC* were found to be 0.53 ± 0.190% and 0.26 ± 0.086% (*v*/*v*) against *B. cereus* MTCC430. Against *Y. enterocolitica* MTCC859, PL2, and PL3 exhibited *MIC* of 0.26 ± 0.087% and 0.31 ± 0% (*v*/*v*), respectively. The *MIC* value for the PL4 against both the bacteria was found to be similar, i.e. 0.31 ± 0% (*v*/*v*). The results of *MIC*s when compared among the four EOs clearly indicated that PL3 and PL4 are associated with promising results compared to PL1. On the other hand, PL2 exhibited moderate *MIC* values. The results for PL2, PL3, and PL4 are in similar line to previously published data on essential oils (Afonso et al. 2018; Song et al. 2019).

Biofilm inhibition assay using microtitre plate. The percentages of different biofilm inhibition concentrations (1/2 *MIC* and 1/4 *MIC*) of the essential oils are shown in Figure 1. At 1/2 *MIC,* the percentage of biofilm inhibition was found to be significant for all the essential oils against the selected foodborne pathogens compared to the other concentration, i.e. 1/4 *MIC*. At 1/2 *MIC*, the percentage of biofilm inhibition against *B. cereus* MTCC430 was found to be the highest for PL1 (78.97 ± 1.76%), followed by PL2, PL4, and PL3 (77.03 ± 1.06, 68.37 ± 0.72, and 61.2 ± 0.87%, respectively). The percentage of biofilm inhibition against *Y. enterocolitica* MTTCC859 at 1/2 *MIC* of the essential oils was found to be 80.33 ± 0.96% for PL1, 79 ± 1.34% for PL2, 77.47 ± 1.30% for PL3, and 72.13 ± 1.6% for PL4. From this experiment it is observed that the sub-*MIC* concentration of EO is good enough to inhibit biofilm formed by a monoculture of bacteria. This result corroborates a previously reported finding indicating that the biofilm inhibition is not solely dependent on lethal concentration. Instead, sublethal damage can also prevent the bacterial attachment to a surface, which represents the initial stage of biofilm formation (Kerekes et al. 2019) The antibiofilm property observed in all the four studied EOs may be due to the abundance of monoterpenoids as described by previous studies (Li et al. 2014; Loying et al. 2023). These findings are corroborated by the results of the crystal violet staining test (Figure 2). It was observed that due to the application of 1/2 *MIC* concentrations of EOs, the development of biofilm was reduced.

Effects of EOs on membrane integrity assay. The inhibitions of pathogens found in the present

Table 2. Minimum inhibitory concentration (*MIC*) of the essential oils against foodborne pathogens in citrus varieties

Bacteria	*MIC* (% *v*/*v*)			
	PL1	PL2	PL3	PL4
Bacillus cereus MTCC430	0.42 ± 0.19[a]	0.53 ± 0.190[a]	0.26 ± 0.086[a]	0.31 ± 0.00[a]
Yersinia enterocolitica MTTCC859	0.84 ± 0.35[a]	0.26 ± 0.087[b]	0.31 ± 0.000[b]	0.31 ± 0.00[b]

[a,b] Different letters represent significant differences at $P < 0.05$; MTTC – microbial type culture collection; PL1 – *Citrus jambhiri*; PL2 – *Citrus medica*; PL3 – *Citrus limon* (elongated lemon); PL4 – *Citrus aurantifolia* (rough lemon)

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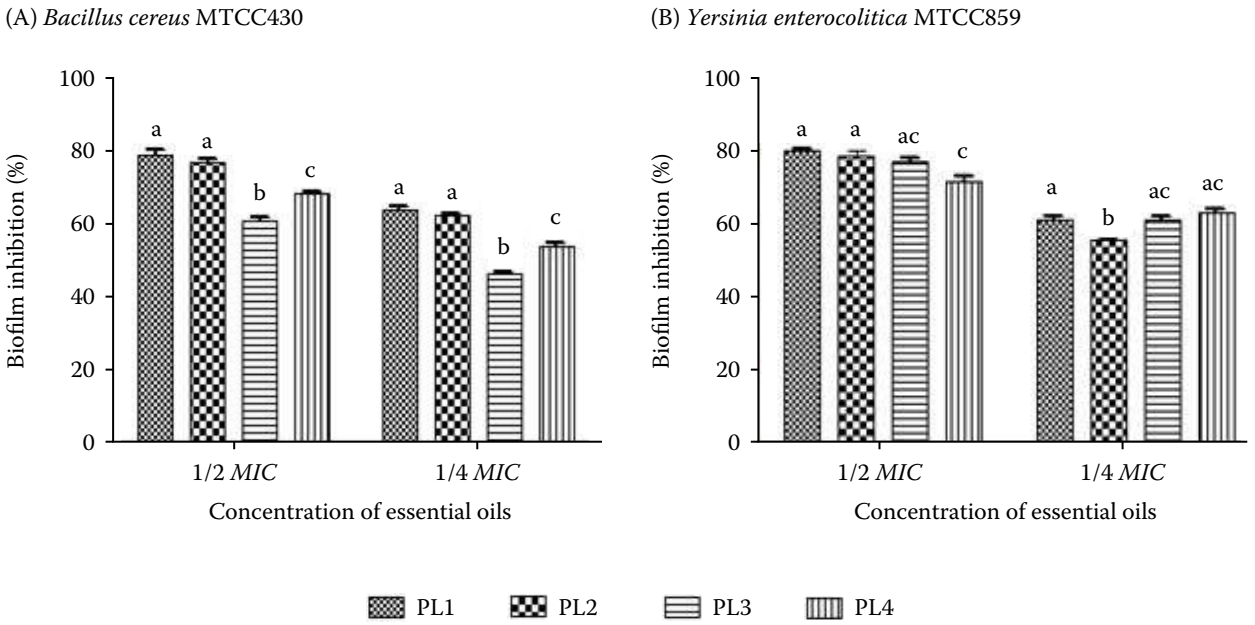

Figure 1. The percentage of biofilm inhibition of essential oils against (A) *Bacillus cereus* MTCC430 and (B) *Yersinia enterocolitica* MTTCC859

a–c – different letters represent significant differences at $P < 0.05$; *MIC* – minimum inhibitory concentration; MTCC – microbial type culture collection; PL1 – *Citrus jambhiri*; PL2 – *Citrus medica*; PL3 – *Citrus limon* (elongated lemon); PL4 – *Citrus aurantifolia* (rough lemon)

study may be due to the impairment of the cell membrane by EOs causing the cell membrane rupture, blocking the enzyme system and disrupting the ion exchange phenomenon (Lang et al. 2012). Since EOs are hydrophobic in nature, they might move across the cell membranes of bacteria, thereby disrupting the cell wall due to a change in the permeability of the membrane. The membrane damage can be detected by measuring the concentration of nucleic acids leaked from bacterial cells. The impairment of the membrane integrity is represented in Figure 3. The highest increase in absorbance was found in case of PL4 and PL3 treatment against *B. cereus* MTCC430 and *Y. enterocolitica* MTTCC859, respectively. Throughout

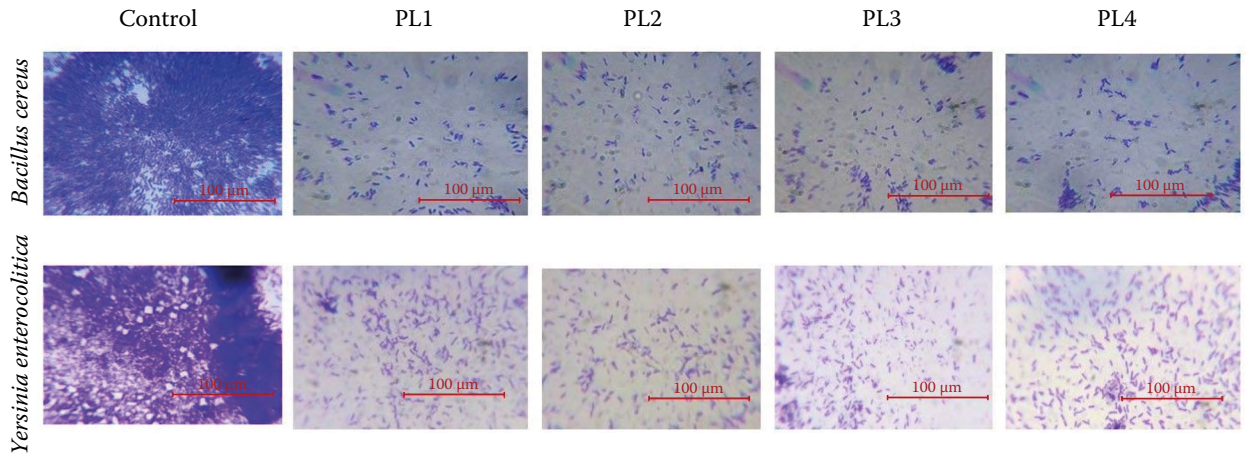

Figure 2. Crystal violet staining of *Bacillus cereus* and *Yersinia enterocolitica* biofilms

Untreated biofilms are represented as control groups while other biofilms were treated with half the minimum inhibitory concentration concentrations of PL1 (*Citrus jambhiri)*, PL2 (*Citrus medica*), PL3 (*Citrus limon*), and PL4 (*Citrus aurantifolia*)

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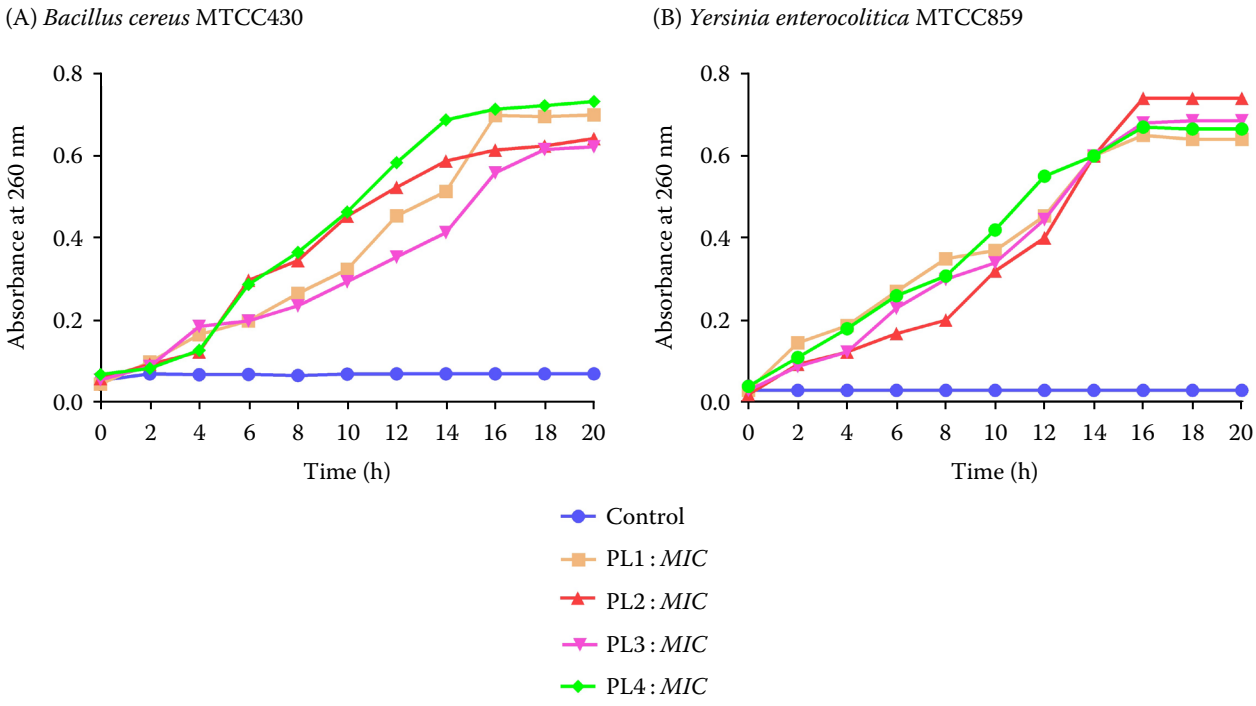

Figure 3. The effects of essential oils on membrane integrity for (A) *Bacillus cereus* MTCC430 and (B) *Yersinia enterocolitica* MTTCC859

MIC – minimum inhibitory concentration; MTCC – microbial type culture collection; PL1 – *Citrus jambhiri*; PL2 – *Citrus medica*; PL3 – *Citrus limon* (elongated lemon); PL4 – *Citrus aurantifolia* (rough lemon)

the experiment, the controls exhibited no increase in absorbance and remained consistently near zero. The finding of the present study is in accordance with the previous findings of Bouyahya et al. (2019), who reported that the absorbance of nucleic acids in bacterial suspension of *E. coli* treated with the essential oil of *Origanum compactum* increased up to 0.67 ± 0.02 at 2× *MIC* level.

Molecular docking assay. From a docking analysis, it was found that ligands (citronellol and limonene) were interacting stably with the target proteins, i.e. 4K1P (Figure 4) and 5L07 (Figure 5). The ligand citronellol has many interactions with different amino acid residues of the binding cavity of the target protein 4K1P. In Figure 4A, there were van der Waals interactions with the residues GLU200 (GLU – glutamic acid), ASP242 (ASP – aspartic acid), THR245 (THR – threonine), ILE201 (ILE – isoleucine), ILE202, and ARG197 (ARG – arginine). Alkyl type interactions were found with ILE194, ILE197, and ALA240 (ALA – alanine) residues. The hydrogen bonding was observed with the LYS203 (LYS – lysine) residue. Similarly, in Figure 4B van der Waals interactions of limonene with 4K1P were observed with the following residues: GLU200, ASP242, THR245, ILE237, GLU236, ILE194, PRO198 (PRO – proline), and GLN199 (GLN – glutamine). In addition, alkyl interactions were observed with ALA241 and ALA240. The interactions of citronellol with 5L07 are shown in Figure 5A, the interactions are mainly van der Waals, alkyl, Pi-alkyl, and hydrogen bond type. These interactions were with the following residues: ALA101, ILE106, ILE70, PHE98 (PHE – phenylalanine), VAL69 (VAL – valine), ASP67, ASN125 (ASN – asparagine), SER32 (SER – serine), TYR50 (TYR – tyrosine), TRP82 (TRP – tryptophan), TYR58, TRP54, LEU34 (LEU – leucine), and LYS91. The interactions of limonene with 5L07 are visualised in Figure 5B. The van der Waals interactions were observed with the ALA101, ILE106, ILE70, PHE98, VAL69, ASP67, ASN125, and SER32 residues. The binding affinities of citronellol and limonene with 4K1P were found to be –4.7 and –5 ΔG, kcal·mol^{-1} (ΔG – standard Gibbs free energy change). The binding energies –6.1 and –7 ΔG, kcal·mol^{-1} were observed for citronellol and limonene with 5L07, respectively. Considering the facts, the ligands showed the highest binding energy with the target protein 5L07. Similar to the present studies different types of hydrophobic interactions between ligands and amino acid residues of the binding sites of the receptors were reported by Santana et al. (2020).

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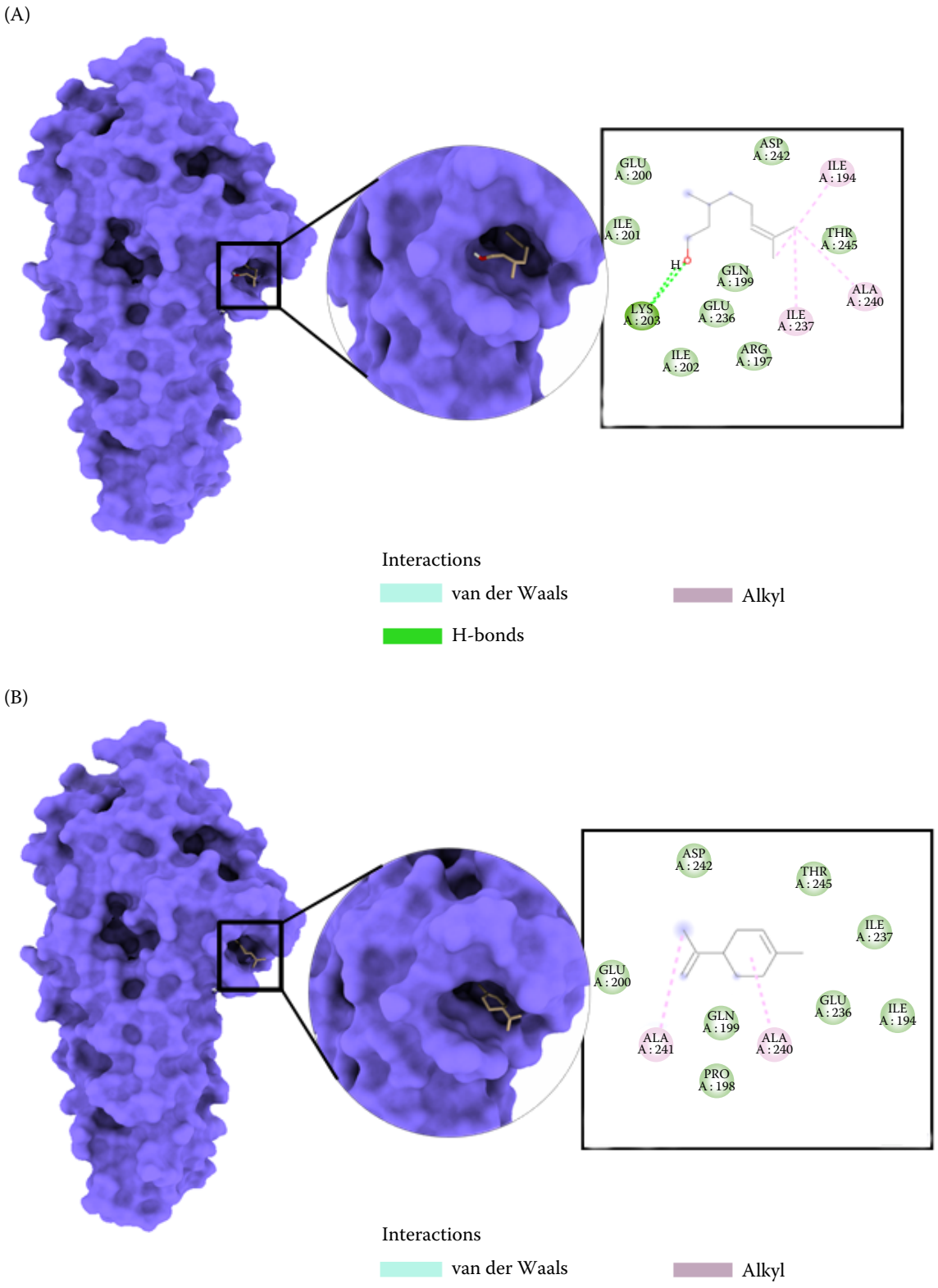

Figure 4. Dock poses of the ligands (A) citronellol and (B) limonene with 4K1P (non-haemolytic enterotoxin from *Bacillus cereus*)

The left panel shows a surface view of 4K1P having a deep core accommodating the ligands; the right panel exhibits 2D interaction of binding cavity residues of 4K1P with the ligands; ALA – alanine; ARG – arginine; ASP – aspartic acid; GLN – glutamine; GLU – glutamic acid; ILE – isoleucine; LYS – lysine; PRO – proline; THR – threonine

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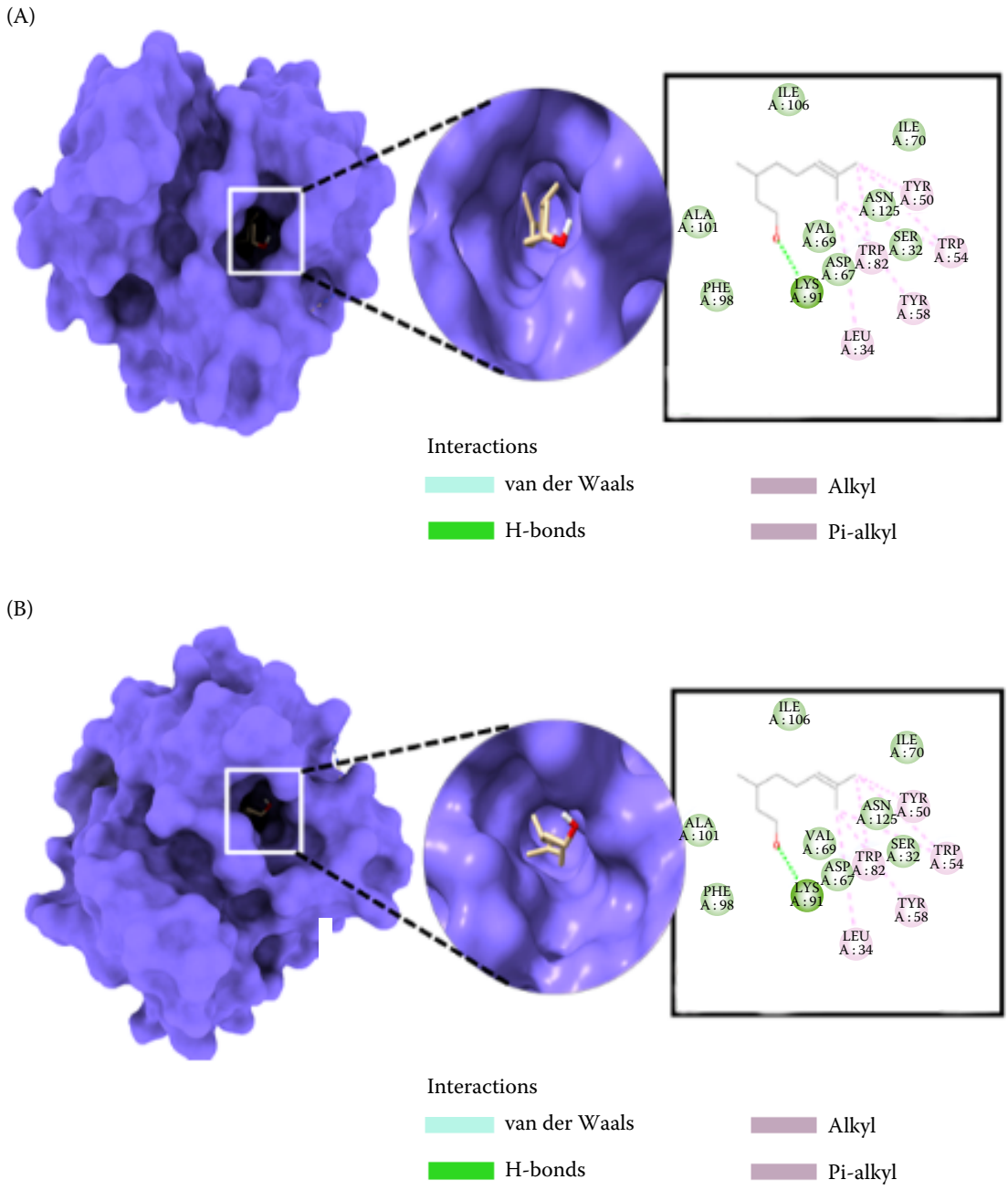

Figure 5. Dock poses of the ligands (A) citronellol and (B) limonene with 5L07 (Quorum-Sensing Transcriptional Activator from *Yersinia enterocolitica*)

The left panel shows a surface view of 5L07 having a deep core accommodating the ligands; the right panel exhibits 2D interaction of binding cavity residues of 5L07 with the ligands; ALA – alanine; ASN – asparagine; ASP – aspartic acid; ILE – isoleucine; LEU – leucine; LYS – lysine; PHE – phenylalanine; SER – serine; TRP – tryptophan; TYR – tyrosine; VAL – valine

CONCLUSION

In the present study the essential oils of four different citrus varieties of Northeast India showed both antimicrobial and antibiofilm activities against food-borne pathogens. The antimicrobial activities of the essential oils were significant against the pathogens. The citrus essential oils also showed the damage to the bacterial membrane and caused a release of nucleic acids in surrounding medium, thereby causing an increase in absorbance with time. Molecular docking showed different types of interactions between abun-

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dant compounds of the oils and the target sites of the target proteins and also unveiled good confluence and stable conformation between active compounds of the essential oils and the target proteins of the foodborne bacterial pathogens. So the findings of the present study showed that the essential oils from citrus varieties are potential antibacterial agents, which was confirmed by both *in vitro* and *in silico* studies.

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