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Research Article

Targeting Nosocomial *Stenotrophomonas maltophilia* With Plant Extracts: A Combined Antimicrobial, Antibiofilm and Molecular Docking Study

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Abstract

Background and Objective: Currently, there is a need for alternative antimicrobial and anti-biofilm strategies owing to the combined challenges of multidrug resistance and biofilm formation by *Stenotrophomonas maltophilia*. Thus, this study aimed to investigate the antibacterial and antibiofilm effects of extracts from *Thymus serpyllum*, *Mentha piperita*, *Rosmarinus officinalis*, *Tilia cordata*, *Salvia officinalis*, and *Thymbra spicata* against *S. maltophilia*, as well as the interactions of carnosic acid, luteolin, and carnosol compounds in these extracts with potential target molecules. **Materials and Methods**: Plant extracts were obtained using a Soxhlet device. Antimicrobial activity against 16 clinical *S. maltophilia* isolates was evaluated using the disk diffusion method, and the antibiofilm effect was assessed using the microtiter plate method. Carnosic acid, luteolin, and carnosol compounds in the extracts were selected as ligands, and a binding analysis was performed with proteins. **Results**: The *T. serpyllum* extract showed the highest inhibition zone (20.5 $\pm$ 2.8 mm; $p < 0.005$), with dose-dependent antimicrobial activity (1024 µg/mL $>$ 512 µg/mL; $p < 0.05$). Among the assessed 15 biofilm-producing strains, *T. serpyllum* inhibited 10, *S. officinalis* inhibited six, and *R. officinalis* inhibited five strains. Molecular docking indicated strong binding energies (carnosic acid: –8.51 kcal/mol, luteolin: –7.62 kcal/mol, carnosol: –9.23 kcal/mol) and multiple interactions with the MlaC protein. **Conclusion**: These findings suggest that extracts from *T. serpyllum*, *S. officinalis*, and *R. officinalis* may target the Mla pathway and exhibit promising antimicrobial and antibiofilm effects against multidrug-resistant *S. maltophilia*, likely through the associated active compounds. The molecular docking analyses further supported the potential of these extracts to disrupt membrane integrity by interfering with the Mla system, thereby enhancing bacterial susceptibility to antimicrobial agents. However, additional studies are required to validate these mechanisms and investigate their broader biological implications.

Keywords: *Stenotrophomonas maltophilia*; *Thymus serpyllum*; luteolin; biofilms/drug effects; molecular docking simulation

1. Introduction

S. maltophilia is an emerging nosocomial pathogen with intrinsic antibiotic resistance that primarily affects immunocompromised patients. *S. maltophilia* usually causes respiratory tract infections with a mortality rate of 20–60% [1]. These bacteria cause respiratory and urinary tract infections by colonizing the surfaces of medical devices and treatment equipment such as urinary catheters, endoscopes, and ventilators [2]. *S. maltophilia* has many structural components that contribute to its virulence and pathogenicity. Pili/flagella/fimbrial/adhesins enable colonization on living and non-living surfaces; outer membrane lipopolysaccharide causes antibiotic resistance and biofilm formation as well as cell death; diffusible signaling factor causes motility, extracellular enzyme production, lipopolysaccharide synthesis, microcolony formation, and tolerance to antibiotics and heavy metal ions [3,4]. *S. maltophilia* has the ability to form biofilms on abiotic surfaces and host (biotic) tissues. This is an important virulence factor for bacteria that plays an important role in nosocomial and multi-bacterial infections, significantly reducing the therapeutic efficacy of important antibiotics, including aminoglycosides, fluoroquinolones, and tetracyclines [5].

Trimethoprim/sulfamethoxazole (TMP-SMX) is the first-choice therapeutic option for treating infections due to its *in vitro* efficacy and favorable clinical outcomes. Meanwhile, alternative antibiotics such as levofloxacin, tigecycline, ceftazidime, colistin, and ticarcillin-clavulanic acid are also used in cases of resistance to TMP-SMX [6]. Due to the recent increase in antimicrobial resistance in *S. maltophilia* and because of the biofilm component that contributes to this resistance, there is a need to develop new antimicrobial and antibiofilm agents against *S. maltophilia*.

Studies have shown that *T. serpyllum* (wild thyme) plant extracts are effective against both Gram-positive and Gram-negative bacteria, including *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis* [7,8]. *M. piperita* (peppermint), *R. officinalis* (rosemary), *T. cordata* (*T.* linden), *S. officinalis* (sage), and *T. spicata* (spiked thyme) plants have also been reported to promote antimicrobial effects [9–12]. Although some studies have examined the antimicrobial effects of these plant species, no comprehensive research has evaluated the activity of these extracts against multidrug-resistant *S. maltophilia*, except for *T. serpyllum*. Unlike previous studies, this study compares six plant extracts and further integrates a molecular docking analysis to explore potential mechanisms of action, thereby offering a more mechanistic and comparative insight into the antimicrobial and antibiofilm potential of these extracts.

In silico molecular docking is used to describe the reactions of molecules and predict the potential associated macroscopic properties. Data obtained from experiments can provide novel information; however, these data do not reveal the mechanism of action in the biological system. Therefore, these mechanisms should be further studied in computer modeling analyses using the structures of biological systems [13].

This study aimed to investigate the antimicrobial and antibiofilm activities of *T. serpyllum*, *M. piperita*, *R. officinalis*, *T. cordata*, *S. officinalis*, and *T. spicata* extracts against *S. maltophilia* strains and to investigate the possible interactions of the compounds in the most active extracts with the targets in terms of antibacterial activity through the molecular docking method.

2. Materials and Methods

2.1 Plants and Extraction

T. serpyllum, *M. piperita*, *R. officinalis*, *T.cordata*, *S. officinalis*, and *T. spicata* plants were purchased in dried form from an herbalist in Gaziantep in 2023. The purchased material was washed with distilled water and left to dry in a sunless environment for one week. The plant material was then ground into powder, and methanol extracts (50 grams of plant material in 250 mL of methanol) were obtained using a Soxhlet extraction apparatus (Thermo Fisher Scientific, Waltham, MA, USA). The methanol extracts were concentrated using a rotary evaporator (Heidolph, Schwabach, Germany) at 40 °C and then allowed to dry completely at ambient temperature in a light-protected environment. The dried extracts were stored in a cool, dark place until further use.

2.2 Bacterial Strains

The bacterial strains used in this study consisted of eight TMP-SMX-resistant and eight TMP-SMX-susceptible *S. maltophilia* strains, which were obtained as part of the routine microbiological diagnostic workflow at the hospital. The samples sent to the microbiology laboratory were inoculated on 5% sheep blood agar, MacConkey agar, and chocolate agar media. The medium plates were incubated at 35 $\pm$ 2 °C for 24–48 hours under aerobic conditions. Bacterial colonies in the plates in which growth was detected were identified at the species level using the VITEK®2 COMPACT (VITEK 2 bioMérieux, France) automated system. Antibiotic susceptibilities were evaluated according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations. This study was approved by the Istanbul Health Sciences University Umraniye Training and Research Hospital Clinical Research Ethics Committee (approval number: 228473746, date: 02 November 2023). The study was carried out in accordance with the guidelines of the Declaration of Helsinki. Written consent was obtained from the patients or their families/legal guardians.

2.3 Determination of Antibacterial Activity

Plant extracts were dissolved in 10% dimethyl sulfoxide (DMSO), and concentrations of 1024 µg/mL and 512 µg/mL were prepared. A total of 20 µL of the prepared solution was pipetted and impregnated onto sterile 6 cm diameter disks. As a control, 20 µL of 10% DMSO alone was impregnated onto a sterile disk. The prepared disks were left to dry at room temperature for 24 hours. Inoculum with a density of 0.5 McFarland (BioMerieux, Marcyl'Étoile, France) was prepared (1×10^8 CFU/mL) from overnight cultures of bacterial strains. A total of 100 µL was added to the surface of the Mueller–Hinton agar (MHA) (Merck, Darmstadt, Germany), and the mixture was spread with a sterile swab. Extract disks were placed on the agar with sterile forceps. Petri dishes were incubated at 35 °C for 24 hours. The zone diameters formed at the end of the incubation were measured with a ruler and recorded. The antibiofilm activity of three plant extracts, among those showing the largest zone diameters, was studied.

2.4 Biofilm Quantification

Biofilm production by bacterial isolates was investigated using the method described by Christensen *et al*. [14]. With this method, the absorbance of the crystal violet dye formed in the wells of the microplates was measured in the spectrometer (Multiskan Go/Thermo Scientific, ABD) at 570 nm. At the end of the measurement, the mean optical density (OD) of the control group was recorded as the OD cut-off (ODc) value, and the mean OD of the isolates (ODisolate) was calculated. Results were evaluated according to the criteria described by Stepanović *et al*. [15]: ODisolate $\leq$ ODc as no biofilm formation; ODc $<$ ODisolate $\leq$ (2ODc) as weak biofilm; (2ODc) $<$ ODisolate $\leq$ (4ODc) as moderate biofilm; ODisolate $>$ (4ODc) as strong biofilm.

2.5 Determination of Antimicrobial Activity Against Mature Biofilms

The method used by Pompilio *et al.* [16] was modified and applied. Fresh inoculum adjusted to 0.5 McFarland turbidity (1×10^8 CFU/mL) was diluted 1/10 (1×10^7 CFU/mL). A total of 200 µL of this inoculum was added to each well of a 96-well polystyrene flat-bottom microplate and incubated at 37 °C for 24 h. Planktonic bacteria were removed by washing the wells twice with phosphate-buffered saline (PBS) (200 µL). A total of 200 µL of 4 mg/mL TMP-SMX, 8 mg/mL rosemary, 8 mg/mL thyme, and 8 mg/mL sage extracts, prepared in cationic Mueller–Hinton broth (CAMHB), were added to the remaining biofilm-formed wells in the microplate, separately against each strain. The CAMHB without any antibiotic or extract was used as a positive control. The wells to which 200 µL of DMSO was added were considered as negative controls. Measurements were recorded at an optical density of 600 nm (OD_{600}) before and after incubation. The reduction (inhibition) rates of the live bacteria in the biofilm were evaluated by comparing the OD values of the positive and negative controls.

2.6 Computer-Aided In Silico Molecular Docking Analysis

This study analyzed the compounds carnosol, carnosic acid, and luteolin, which had been previously identified in the literature via high-performance liquid chromatography (HPLC) as bioactive components in *R. officinalis*, *T. serpyllum*, and *S. officinalis* [17–19]. Carnosic acid, luteolin, and carnosol compounds were evaluated within the scope of Lipinski's Rule of Five, which is traditionally used for small-molecule therapeutics. The three-dimensional (3D) structures of these molecules were downloaded in SDF format from the PubChem database (https://pubchem.ncbi.nlm.nih.gov).

Subsequently, the structure of each molecule was converted into the PDB format using the BIOVIA Discovery Studio Visualizer program (Dassault Systèmes, VélizyVillacoublay, France). The *E. coli* phospholipid-binding protein MlaC (PDB ID: 5UWA) was selected as the receptor molecule and was downloaded in PDB format from the Protein Data Bank (https://www.rcsb.org) database.

Molecular docking analyses were performed using AutoDock 4.0 (The Scripps Research Institute, La Jolla, CA, USA) [20] to predict the possible binding sites of carnosic acid and carnosol, which are abundant in *R. officinalis* and *S. officinalis*, and luteolin ligands, which are abundant in *T. serpyllum*, on the crystal structure of the MlaC receptor. The MlaC crystal structure, with a resolution of 1.50 Å, was selected as the target (receptor) molecule. AutoDock Tools (ADT) software was used to prepare the parameters of the receptor and ligand molecules before initiating the docking analysis. Polar hydrogen atoms in the receptor and ligand molecules were retained while nonpolar hydrogens were incorporated. Gasteiger charges were calculated using ADT as previously described by Ricci and Netz [21] and Nasab *et al.* [22]. During the molecular docking experiment, all rotatable bonds in the ligands were allowed to rotate. The prepared receptor and ligand structures were then saved in the PDBQT format. A grid box size of $60 \times 60 \times 60$ Å was set with a grid spacing of 0.375 Å. The dockings were generated from 25 GA (Genetic Algorithm) runs, 5×10 [5]. An energy evaluation was conducted, utilizing a maximum of 27,000 generations and an initial population of up to 150 individuals. Values of 0.02 and 0.8 were selected as the mutation and crossover rates, respectively, for the population. After conducting 100 independent docking runs with the MlaC protein for each ligand, the program clustered all possible binding modes and ranked the selected poses based on their binding free energy. The ranking focused on the conformation with the lowest binding free energy, measured in kcal/mol, which represents the best docking pose for each ligand. The best docking pose obtained between the ligand and the receptor using AutoDock 4.0 was analyzed using BIOVIA Discovery Studio Visualizer 2016 [23].

2.7 Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 27 (IBM Corp., Armonk, NY, USA). Since the data did not exhibit a normal distribution (as verified by the Shapiro–Wilk test), non-parametric tests were employed. The Kruskal–Wallis H-test was applied to compare inhibition zone diameters and biofilm inhibition rates among different plant extract groups. When the Kruskal–Wallis test indicated statistically significant differences ($p < 0.05$), pairwise comparisons were conducted using the Mann–Whitney U-test to determine which specific groups differed from each other. Strains exhibiting inhibition were coded as "1" in the biofilm inhibition analysis, while those showing no inhibition were coded as "0". All results were evaluated at a 95% confidence level ($p < 0.05$).

3. Results

3.1 Antimicrobial Activity

The inhibition zone diameters for the *S. maltophilia* strain formed using the plant extracts are presented in Table 1. The zone diameter for *T. serpyllum* was found to be the highest ($p < 0.005$). While there was no significant difference between the zone diameters for *M. piperita*, *R. officinalis*, and *S. officinalis*, the activity of these extracts was higher than that of *T. cordota* and *T. spicata* ($p < 0.005$). The antimicrobial activity of *R. officinalis* and *T. cordata* extracts was found to be more effective against TMP-SMX-susceptible strains than against resistant ones ($p < 0.05$). However, no statistically significant difference was found between susceptible and resistant strains in other extracts ($p > 0.05$). Antimicrobial activity increased with the dose of the extracts (Fig. 1). The activities of the extracts at the 1024 µg/mL dose were greater than those at the 512 µg/mL dose ($p < 0.05$).

Table 1. Inhibition zone diameters formed by plant extracts for *S. maltophilia* strains.

		Zone diameter (mm)											
		T. serpyllum		*M. piperita*		*R. officinalis*		*T. cordata*		*S. officinalis*		*T. spicata*	
Strain number	TMP-SMX susceptibility	1024 μg/mL	512 μg/mL	1024 μg/mL	512 μg/mL	1024 μg/mL	512 μg/mL	1024 μg/mL	512 μg/mL	1024 μg/mL	512 μg/mL	1024 μg/mL	512 μg/mL
1	S	20	12	20	16	15	10	17	12	19	12	12	0
2	S	19	15	17	13	20	13	0	0	17	0	13	12
3	S	19	11	18	15	20	16	0	0	15	7	13	12
4	S	20	12	19	13	20	11	0	0	15	0	10	0
5	S	18	10	14	7	13	7	0	0	16	0	10	0
6	S	28	16	24	16	21	16	14	6	21	0	15	10
7	S	19	11	16	7	16	10	14	10	16	11	18	13
8	S	26	19	20	16	21	19	21	19	19	14	14	11
9	R	18	11	15	11	15	9	15	13	16	0	14	0
10	R	19	9	14	6	17	12	0	0	15	0	10	6
11	R	20	16	17	17	18	15	0	0	15	0	13	8
12	R	21	15	20	17	17	14	0	0	17	14	11	7
13	R	24	16	20	15	17	14	0	0	20	14	17	12
14	R	20	15	17	15	16	12	0	0	18	0	12	8
15	R	19	12	16	15	16	12	0	0	16	0	12	6
16	R	18	9	14	8	14	7	0	0	14	0	8	0

R, resistance; S, susceptible; TMP-SMX, Trimethoprim/Sulfamethoxazole. The susceptibility of *S. maltophilia* strains to TMP/SMX was determined using the VITEK®2 COMPACT device. The inhibition rates of plant extracts against these strains at concentrations of 1024 μg/mL and 512 μg/mL were determined using the disk diffusion test. Zone diameters were measured with a ruler. The length was recorded in millimeters.

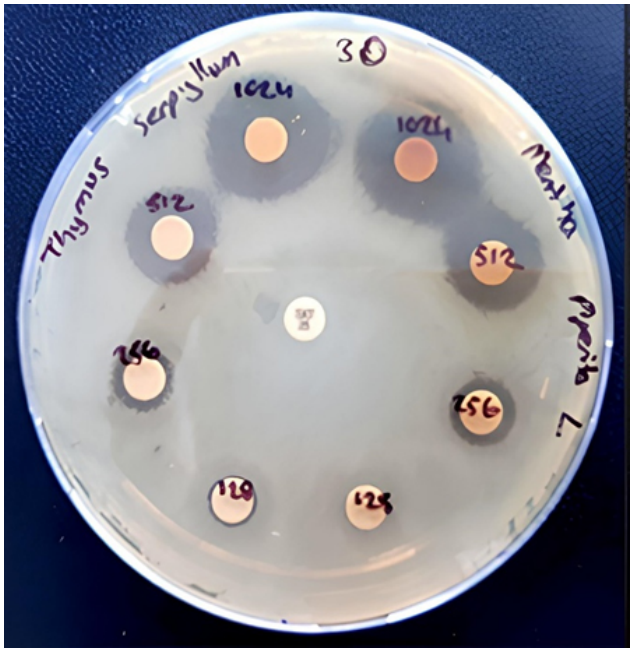

Fig. 1. Inhibition zones for *T. serpyllum* and *M. piperita* extracts against *S. maltophilia* strain (number 12) at concentrations of 1024 µg/mL, 512 µg/mL, 256 µg/mL, and 128 µg/mL.

3.2 Biofilm Formation Ability of S. maltophilia

In total, 15 of the *S. maltophilia* strains isolated from 16 different clinical samples produced a biofilm, whereas one strain did not. When the biofilm production capacities of the strains were examined, 11 were observed as strong, 2 were medium, and 2 were weak (Table 2).

3.3 Antibiofilm Activity

Among the 15 biofilm-producing strains, while TMP-SMX inhibited bacterial growth in all strains to varying degrees, rosemary inhibited bacterial growth in five strains, sage in six strains, and thyme in 10 strains. Among the extracts, the highest activity against the strain was observed for the *T. serpyllum* (thyme) extract ($p < 0.05$). The absorbance values in the biofilm wells to which the extract or antibiotic was added were measured at OD_{600} and compared with those in the control wells containing pure medium with biofilm. Table 2 shows that there was less growth than the control, expressed as a percentage decrease based on absorbance. The wells in which a decrease was not observed are denoted as "0". Since strain number one did not form a biofilm, the absorbance values are not provided (Table 2).

3.4 Docking Analysis

The amino acid interactions of the ligands with the target protein MlaC are shown in Figs. 2,3,4. The lowest Gibbs free binding energies (ΔG) for carnosic acid, luteolin, and carnosol were determined to be –8.51, –7.62, and –9.23 kcal/mol, respectively. Carnosic acid and carnosol each formed interactions with 18 amino acids (via five and six different bond types, respectively), while luteolin interacted with 14 amino acids via five different bond types. All binding energies were considered significant, as the recorded energies were lower (more negative) than the threshold of –6.0 kcal/mol.

4. Discussion

S. maltophilia is naturally resistant to many antibiotics, including beta-lactams, aminoglycosides, macrolides, tetracyclines, and carbapenems. This poses serious challenges in the treatment of *S. maltophilia* infections. Currently, the most effective antibiotic is TMP-SMX; however, resistance to TMP-SMX has increased over recent years [24,25]. Indeed, a systematic review reported that the resistance rate of *S. maltophilia* to TMP/SMX was 9.2% worldwide between 2000 and 2022 [26]. These data highlight that reliance on TMP-SMX alone may not be sustainable in the long term, and alternative therapeutic strategies are urgently required.

Moreover, in a study by Pompilio *et al*. [16], which analyzed 109 *S. maltophilia* isolates from various clinical samples, the authors noted that the majority of the assessed strains (91.7%) were capable of producing biofilms. Additionally, Bilgin *et al*. [27] determined that a significant portion (87.2%) of the 78 evaluated *S. maltophilia* isolates from pulmonary and extrapulmonary samples formed biofilms, and Sun *et al*. [28] reported that 42 (82%) of 51 hospital-acquired *S. maltophilia* isolates also formed biofilms.

In the present study, 15 of the 16 assessed clinical *S. maltophilia* strains (93%) were found to produce biofilms; furthermore, 11 were determined to have a strong biofilm production capacity, two a moderate capacity, and two a weak capacity. The results of our study are consistent with those of other studies, indicating that *S. maltophilia* strains exhibit a high capacity for biofilm production. This strong biofilm-forming ability may partly explain the persistence and multidrug resistance of *S. maltophilia* in clinical environments. Meanwhile, no significant difference was found in our study between the biofilm production capacities of TMP-SMX-resistant and susceptible strains ($p > 0.05$), indicating that biofilm formation is a common adaptive trait independent of TMP-SMX resistance.

To our knowledge, no previous studies have investigated the antibacterial activity of *T. serpyllum*, *R. officinalis*, *T. cordata*, and *S. officinalis* extracts against *S. maltophilia*. However, our study demonstrates significant antibacterial activity by the *T. serpyllum* methanolic extract. Balkan *et al*. [29] reported that essential oils from *T. serpyllum* have shown inhibition zones >20 mm with 20 µL applications, while Galovičová *et al*. [30] reported a 15.67 mm inhibition zone using 10 µL of essential oil. In our study, the methanolic extract at a concentration of 1024 µg/mL produced a larger inhibition zone of 20.5 mm. These results not only demonstrate a dose–response relationship for the essential oil but also suggest that the methanolic extract may

Table 2. Antibiofilm activity against a preformed *S. maltophilia* biofilm.

Strain no.	R/S	Biofilm degree	TMP-SMX	*T. serpyllum*	*S. officinalis*	*R. officinalis*
1	S	-	N	N	N	N
2	S	+++	90	56	68	77
3	S	+	100	68	55	0
4	S	++	99	32	0	0
5	S	+++	90	7	23	27
6	S	+++	90	0	0	0
7	S	+++	99	0	0	0
8	S	+++	90	8	0	0
9	R	+++	99	0	0	0
10	R	+++	100	0	0	0
11	R	+	100	82	56	0
12	R	++	90	57	0	83
13	R	+++	90	73	54	57
14	R	+++	94	30	0	0
15	R	+++	91	76	58	56
16	R	+++	99	0	0	0

R, resistance; S, susceptible; N, none; +++, strong biofilm; ++, medium biofilm; +, weak biofilm; -, no biofilm.

After biofilm formation was confirmed in the microplates, planktonic bacteria were washed with TMP-SMX (4 mg/mL), and extracts of rosemary, thyme, and sage (8 mg/mL) were added to the remaining biofilms. CAMHB was used as a positive control, and 100% DMSO was used as a negative control. Biofilm inhibition rates were calculated using OD_{600} measurements and are presented as percentages compared to the controls.

be more effective than essential oils. This enhanced activity may be attributed to the synergistic effects of polar compounds (such as phenolic acids and flavonoids) extracted by methanol, emphasizing the importance of the extraction technique in maximizing antimicrobial potential.

A study on methanol and water extracts from *T. spicata* showed the formation of inhibition zones of 23 mm and 16 mm, respectively [31]. In this study, the inhibition zone of the methanol extract from *T. spicata* was found to be 12.63 $\pm$ 2.55 mm. This difference may be due to the dose used. Joma *et al*. [31] used an extract concentration of 50 mg/mL, whereas our study employed an extract concentration of 1 mg/mL. The results show that the diameter of the inhibition zone increases with the dose of the antibiotic. In this study, a dose-dependent increase in the inhibition zone was also demonstrated in measurements performed with concentrations of 512 µg/mL and 1000 µg/mL ($p < 0.05$). These results confirm a dose–response effect, but also suggest the need for standardized extract concentrations in antimicrobial studies to allow comparability across research.

While the minimum inhibitory concentration (MIC) for the ethanol extract from *M. piperita* against *S. maltophilia* is reported in the literature as >80 mg/mL [32], our study observed inhibition of 17.56 $\pm$ 2.67 mm at a concentration of 1 mg/mL, demonstrating that this extract may be effective even at low concentrations.

Although, to our knowledge, no previous antimicrobial studies have been conducted on *T. cordata* extracts against *S. maltophilia*, Ali *et al*. [11] tested alkaloid, flavonoid, and glycoside extracts on *S. aureus* and *E. coli*. Notably, while no antimicrobial activity was observed against *E. coli* in this study [11], the flavonoid extract showed activity against *S. aureus*, producing an 18 mm inhibition zone at a high concentration of 3 mg/mL. This low level of antimicrobial activity is consistent with the weak antibacterial effect of *T. cordata* extracts observed in our study. Furthermore, the significantly reduced impact of *T. cordata* extracts on resistant *S. maltophilia* strains in our findings ($p < 0.05$) provides additional evidence of the limited antimicrobial potential of this extract. Therefore, *T. cordata* extracts may not represent a promising candidate for therapeutic development against *S. maltophilia* infections.

Zhang *et al*. [33] reported that a disc impregnated with 50% *R. officinalis* essential oil produced a 13.83 mm inhibition zone against *S. maltophilia*. In our study, the methanol extract (primarily containing polar compounds) demonstrated a larger inhibition zone (17.25 mm). This observation suggests that the bioactive compounds in the polar fraction of *R. officinalis* could exhibit stronger antimicrobial effects compared to the essential oil. Additionally, partial evaporation of volatile components from the essential oil during testing might have contributed to this difference. These findings could be interpreted to indicate that the polar extract of *R. officinalis* may potentially offer therapeutic advantages over the essential oil against resistant *S. maltophilia* strains. Moreover, this finding implies that

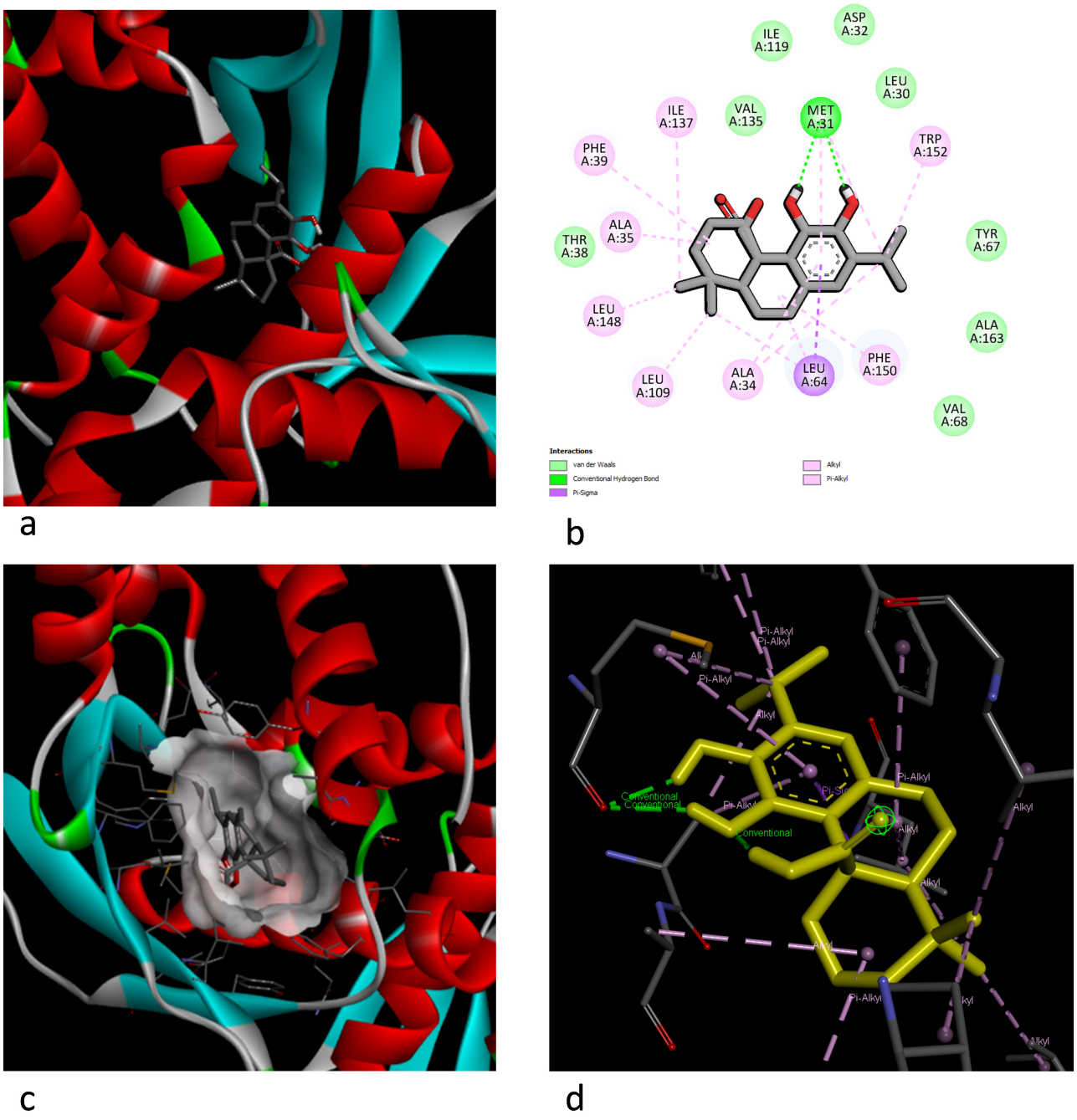

Fig. 2. Representation of the docking interaction between carnosic acid and MlaC. (a) Best 3D docking pose. (b) The two-dimensional (2D) aa interaction and chemical bond types. (c) The 3D electric field interaction. (d) Ligand interaction pose.

polar extracts may be advantageous for developing stable formulations compared to volatile essential oils.

In our study, we observed an inhibition zone of 16.81 mm against *S. maltophilia* using a total extract of *S. officinalis*. Interestingly, this finding is comparable to results reported by Kačániová *et al.* [34], who demonstrated a 16.47 $\pm$ 0.58 mm inhibition zone using *S. officinalis* essential oils. Although essential oils generally contain a higher concentration of volatile bioactive compounds, the similar levels of antibacterial activity observed suggest that well-prepared total extracts—despite having a different composition—may achieve comparable efficacy. This supports our earlier observations that the synergistic interaction of both polar and nonpolar compounds in total extracts can significantly contribute to antimicrobial activity. Therefore, total extracts may offer not only a broader spectrum of bioactive components but also advantages in terms of stability and formulation potential. This indicates that both the volatile and non-volatile fractions of *S. officinalis* contribute toward the observed antimicrobial properties, potentially offering formulation flexibility for therapeutic use.

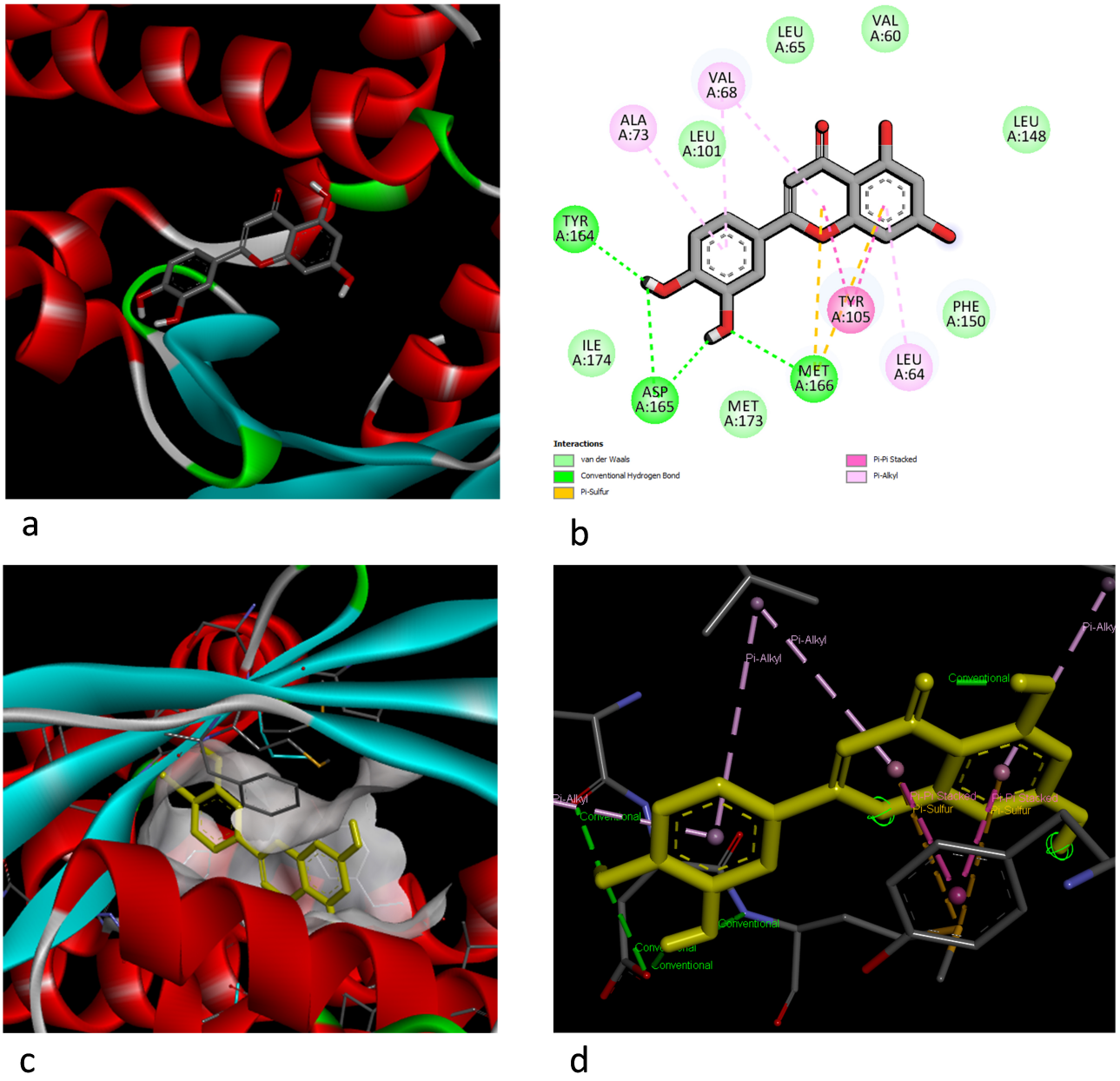

Fig. 3. Illustration of the docking interaction between luteolin and MlaC. (a) Best 3D docking pose. (b) The 2D aa interaction and chemical bond types. (c) The 3D electric field interaction. (d) Ligand interaction pose.

Pietruczuk-Padzik *et al.* [35] reported that among all the tested plant extracts against *Staphylococcus* species, *T. serpyllum* and *Taraxacum officinale* leaf extracts exhibited the most promising antibacterial activity, both against planktonic cultures and biofilm forms. Kačániová *et al.* [34] found that *T. serpyllum* essential oils inhibited *Pseudomonas aeruginosa* biofilm at a concentration of 0.236 mg/mL, while Čabarkapa *et al.* [36] reported that this essential oil prevented biofilm formation by *Salmonella enteritidis* strains. In our study, the *T. serpyllum* extract demonstrated biofilm inhibition activity in 66.7% of the *S. maltophilia* strains. These findings support the potential of *T. serpyllum* to suppress biofilm formation in various bacterial species.

Wijesundara and Rupasinghe [37] reported that the essential oils from *S. officinalis* significantly inhibited biofilm formation of *Streptococcus pyogenes* strains at 0.25 mg/mL and were able to eradicate existing biofilms at 0.5 mg/mL. Ünlü *et al.* [38] also reported that this essential oil inhibited biofilm formation in 41.2% of the tested methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *Staphylococcus aureus* (MSSA) isolates, with varying degrees of inhibition. In the present study, *S. officinalis* extract exhibited biofilm inhibition activity in 40% of *S. maltophilia* strains. These findings indicate that *S. officinalis* can exert biofilm-suppressing effects on various bacterial species.

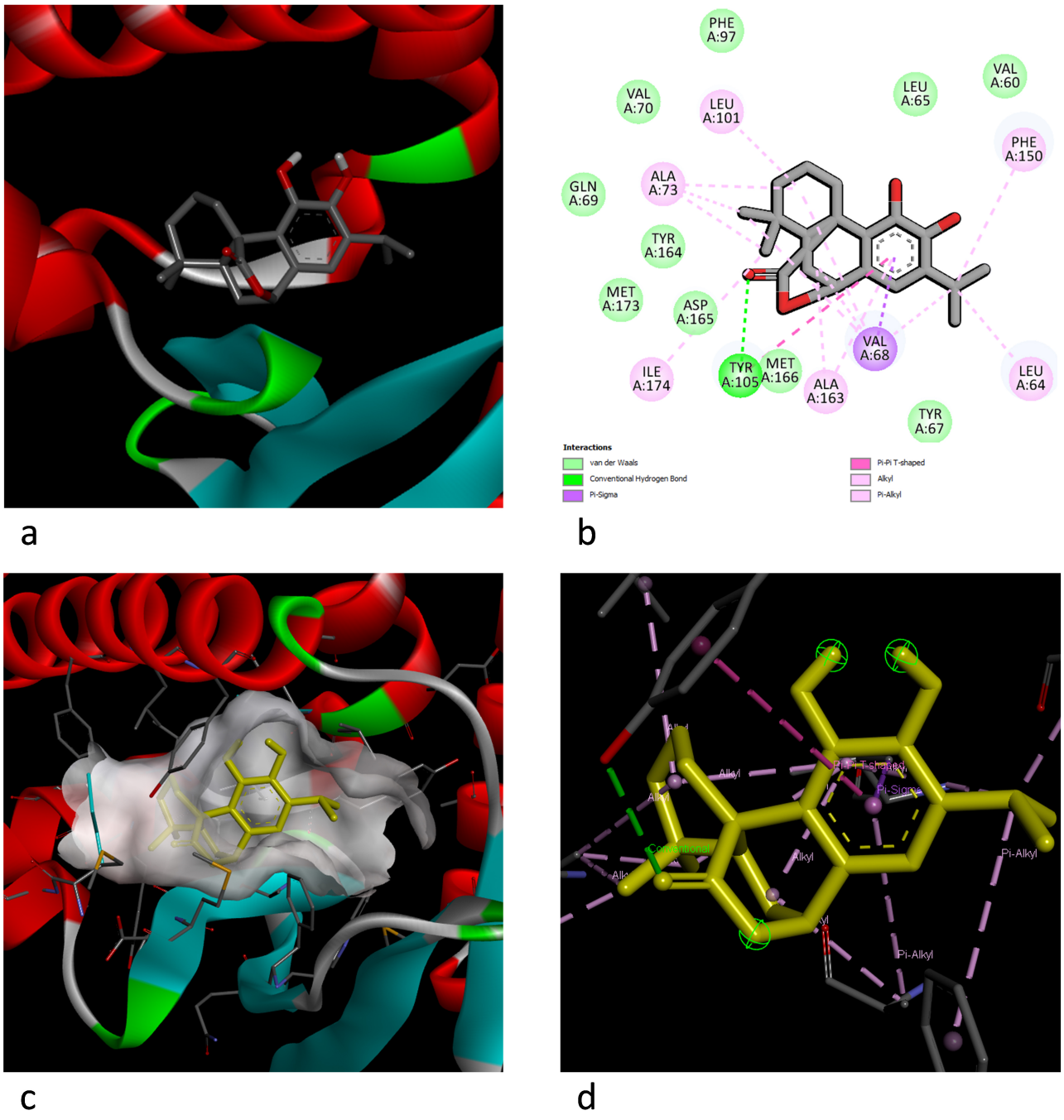

Fig. 4. Illustration of the docking interaction between carnosol and MlaC. (a) Best 3D docking pose. (b) The 2D aa interaction and chemical bond types. (c) The 3D electric field interaction. (d) Ligand interaction pose.

Ben Abdallah *et al.* [39] reported the potent antibiofilm activity of *R. officinalis* (rosemary) essential oils against MRSA, and Jardak *et al.* [40] demonstrated the effectiveness of the same essential oils against *Staphylococcus epidermidis* biofilms. Galovičová *et al.* [41] also reported that these essential oils inhibited *S. maltophilia* biofilms. In our study, the methanolic extract of *R. officinalis* showed lower inhibition (33.3%) against *S. maltophilia* biofilms. This difference in efficacy may be due to differences in extract type and concentration ratio.

Su *et al.* [42] reported that the minimum biofilm eradication concentration was above 1 mg/mL for all the studied strains. Similarly, Río-Chacón *et al.* [43] reported that the minimum biofilm eradication concentration value was above 5 mg/mL for the majority of strains. In our study, a TMP-SMX concentration of 4 mg/mL inhibited the formation of mature biofilm structures in all tested strains. Our results, when evaluated in conjunction with the literature, indicate that high doses of TMP-SMX are necessary for inhibiting *S. maltophilia* biofilms. However, systemic application of this concentration in humans is not possible.

Therefore, the obtained data may simply guide the evaluation of alternative treatment strategies, such as topical applications, implant coatings, or the development of new drug delivery systems.

According to our results, the *T. serpyllum* extract exhibited the highest antibacterial and antibiofilm activity with statistical significance ($p < 0.05$). This suggests that *T. serpyllum* may be a potential antimicrobial agent against *S. maltophilia*. While *M. piperita*, *R. officinalis*, and *S. officinalis* extracts showed similar levels of antibacterial activity ($p > 0.05$), *T. cordata* and *T. spicata* extracts demonstrated lower activity ($p < 0.05$). The fact that *T. serpyllum* was the most effective extract in terms of both antibacterial and antibiofilm activities suggests a possible relationship between these effects. Therefore, these findings indicate that *T. serpyllum* has the potential to be evaluated as a natural antimicrobial and antibiofilm agent.

In the analyses conducted by Sonmezdag *et al*. [44] using the gas chromatography–mass spectrometry–olfactometry (GC–MS–olfactometry) and liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS) techniques, 24 volatile compounds were identified in the aroma profile of *T. serpyllum*, most of which were classified as terpenes. Phenolic compound analysis revealed 18 different phenolics, with luteolin-7-O-glucoside, luteolin, and rosmarinic acid being the most prominent.

Recent metabolomic studies using ultra-high-performance liquid chromatography–electrospray ionization–quadrupole-time-of-flight mass spectrometry (UHPLC–ESI–QTOF-MS) have elucidated the distinct yet overlapping phytochemical profiles of *R. officinalis* and *S. officinalis* [45,46]. These analyses revealed that rosemary is particularly rich in bioactive diterpenes, with Soxhlet extraction yielding high concentrations of carnosol (22,000.67 $\pm$ 77.39 µg/g) and carnosic acid (2915.40 $\pm$ 33.23 µg/g), along with notable levels of luteolin-7-O-glucoside (209.95 $\pm$ 8.78 µg/g). In contrast, sage demonstrated higher accumulations of rosmarinic acid (17,678.7 $\pm$ 673.4 µg/g) and 12-methoxy carnosic acid (21,918.3 $\pm$ 715.4 µg/g), while still containing detectable quantities of luteolin derivatives. Previous studies have reported that carnosol, carnosic acid, and luteolin compounds, which are biocomponents of *R. officinalis*, *T. serpyllmus*, and *S. officinalis* species, exhibited strong antimicrobial and antibiofilm activities, and that the mechanism of action of these compounds occurred on the cell membrane [47–49]. In line with these findings, our study identified an active transport mechanism located in the cell membrane, which maintains membrane integrity through lipid transport, as a potential target for improving the understanding of the possible antimicrobial mechanism of action. In this context, a molecular docking analysis was performed to evaluate the interaction of the selected compounds through this mechanism.

The Mla ABC transport system, which is known to transport phospholipids across the periplasm in Gram-negative bacteria and plays a role in maintaining outer membrane homeostasis, is also found in *S. maltophilia*. The Mla system plays a role in basic resistance to antimicrobial and antibiofilm agents. The periplasmic substrate-binding protein MlaC is an important component in this system and has been shown to bind to phospholipids, thereby contributing to basic resistance to various antimicrobial agents [50]. In this study, the *E. coli* phospholipid-binding protein MlaC (PDB ID: 5UWA) was selected as a model receptor in the docking analysis. When we examined the molecular docking results, strong hydrogen bond interactions were observed with MET31 in the amino acid residues of the carnossic acid molecule and the MlaC receptor-binding motif (RBM), while van der Waals interactions were observed with the THR38, VAL135, ILE119, ASP32, LEU30, TYR67, ALA163, and VAL68 residues. In addition, a Pi-Sigma bond interaction was observed with LEU64, while alkyl and Pi–alkyl bond interactions were determined with ALA34, LEU109, LEU148, ALA35, PHE39, ILE137, TRP152, and PHE150. When the luteolin and MlaC docking interaction was examined, hydrogen bonds were observed for the TYR164, ASP165, and MET166 residues, and van der Waals interactions were observed for the ILE174, LEU101, LEU65, VAL60, PHE150, and MET173 residues. The MET166 residue also formed a second interaction: a Pi–sulfur bond. While the TYR105 residue showed a Pi–Pi interaction, the LEU65, ALA73, and VAL68 residues exhibited Pi–alkyl interactions.

In the carnosol–MlaC docking interaction, van der Waals interactions were observed for the MET166, ASP165, MET173, TYR164, GLN69, VAL70, PHE97, LEU65, VAL60, and TYR67 residues, and both hydrogen bonding and Pi–Pi–T interactions were observed for TYR105. Additionally, carnosol exhibited Pi–Sigma interactions for the VAL68 and ALA163 residues. Moreover, the ILE174, ALA73, LEU101, PHE150, and LEU64 residues showed alkyl and Pi–alkyl interactions.

The binding energy threshold is accepted as –6.0 kcal/mol. In our study, the results were accepted as significant because all ligand–receptor Gibbs free binding energies were more negative than –6.0 kcal/mol. When designing molecular docking analyses, the antimicrobial activity was predicted to occur via the Mla pathway. The Mla system consists of 6 proteins, MlaA, MlaB, MlaC, MlaD, MlaE, and MlaF, which transport phospholipids from the outer leaflet of the outer membrane to the inner membrane. MlaA is a lipoprotein located in the outer membrane and transfers phospholipids to MlaC, which is a periplasmic protein [51]. MlaC transfers phospholipids to the Mla–FEDB complex in the inner membrane, and Mla–FEDB then inserts phospholipids into the inner membrane [52]. In this study, the MlaC protein within the Mla system was specifically targeted owing to the central and unique role MlaC plays in the lipid transport function in the Mla sys-

tem. MlaC is the sole periplasmic, soluble protein that transports phospholipids between MlaA in the outer membrane and the Mla–FEDB complex in the inner membrane. Since most other Mla components are membrane proteins, these components are structurally less accessible; in contrast, MlaC offers a directly inhibitory target [53]. Previous studies have demonstrated that the functional loss of *mlaC* increases bacterial susceptibility to several classes of antibiotics. Bernier *et al*. [54] showed that *mlaC* mutants in *Burkholderia cepacia* complex strains exhibited increased sensitivity to tetracyclines, chloramphenicol, macrolides, fluoroquinolones, and rifampin compared to wild-type strains. This highlights the functional relevance of MlaC as a potential target in antimicrobial resistance mechanisms. Moreover, inactivating the Mla system eliminates the lipid asymmetry of the outer membrane and increases the sensitivity to various antimicrobial agents in many Gram-negative bacteria [53]. When these results are evaluated in conjunction with *in vitro* antimicrobial activity studies, carnosic acid, carnosol, and luteolin compounds, which are abundantly found in *T. serpyllum, R. officinalis*, and *S. officinalis* extracts, are highlighted in terms of antimicrobial activity and may be potential active antibacterial substances.

This study has certain limitations, including the relatively small number of clinical isolates and the exclusive use of *in vitro* assays, which may not fully reflect the complexity of *in vivo* conditions. Moreover, variations in extract composition related to plant origin, harvest time, and extraction method could affect reproducibility. Despite these limitations, these findings provide preliminary evidence supporting the antibacterial and antibiofilm activity of selected plant extracts against *S. maltophilia*. Importantly, this work addresses a gap in the literature by, to our knowledge, being the first to evaluate the activity of *T. serpyllum*, *R. officinalis*, *T. cordata*, and *S. officinalis* extracts against *S. maltophilia* and by proposing the Mla system as a possible molecular target. Nonetheless, further investigations focusing on the standardization of extract preparation, the exploration of potential synergistic effects with existing antibiotics, and the experimental validation of molecular docking predictions would strengthen the clinical relevance of these results.

5. Conclusion

This study demonstrated that extracts of *T. serpyllum*, *S. officinalis*, and *R. officinalis* possess measurable antimicrobial and antibiofilm activities against multidrug-resistant *S. maltophilia*. The most active extract was from *T. serpyllum*, producing an inhibition zone of 20.5 $\pm$ 2.8 mm at a concentration of 1024 µg/mL and inhibiting biofilm formation in 66.7% of the tested strains. *S. officinalis* and *R. officinalis* were also effective, with inhibition zones of 16.8 $\pm$ 2.0 mm and 17.3 $\pm$ 2.4 mm, and observed biofilm inhibition in 40% and 33.3% of the evaluated strains, respectively. Notably, all three extracts retained activity against TMP-SMX-resistant isolates. Meanwhile, molecular docking analyses revealed strong binding of carnosol (–9.23 kcal/mol), carnosic acid (–8.51 kcal/mol), and luteolin (–7.62 kcal/mol) to the MlaC protein, suggesting a possible mechanism via the disruption of the Mla system. These findings provide the first evidence that these plant extracts and their key phytochemicals could serve as promising alternative therapeutic agents or potent adjuvants against challenging *S. maltophilia* infections.

Availability of Data and Materials

The datasets used in this study are available from the corresponding author upon reasonable request.

Author Contributions

YA and ZAT designed the research study. YA, HA, and IHK performed the research and conducted experiments. YA, HA, and IHK analyzed the data. BNE and FFS contributed to literature review, methodology development, and writing – review & editing. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Approval for this study was obtained from the Istanbul Health Sciences University Umraniye Training and Research Hospital Clinical Research Ethics Committee (approval number: 228473746, date: 02 November 2023). The study was carried out in accordance with the guidelines of the Declaration of Helsinki. Written consent was obtained from the patients or their families/legal guardians.

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Conflict of Interest

The authors declare no conflict of interest.

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