

Antibacterial And Antibiofilm Activities Of Supercritical Fluid Extract And Post-Extraction Waste Of *Salvia officinalis*

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ABSTRACT: The increasing prevalence of biofilm-associated infections necessitates the discovery of alternative antivirulence strategies in the management of resistant and chronic infections caused by opportunistic pathogens. In this study, the antibacterial and antibiofilm activities of the extract by supercritical fluid extraction (SFE) of *Salvia officinalis* L. and the waste fractions after extraction were investigated in vitro. Antibiofilm activity was determined using the crystal violet method on biofilm-producing strains *Acinetobacter baumannii* ATCC 19606, *Pseudomonas aeruginosa* ATCC 15692, and *Staphylococcus epidermidis* ATCC 35984, while antibacterial activity was assessed as minimum inhibitory concentration (MIC) by the broth microdilution method in accordance with EUCAST standards. The results revealed that all fractions exhibited limited activity against Gram-negative strains (*A. baumannii* and *P. aeruginosa*) with an MIC value of 2500 µg/mL, whereas the SO and SO-EA fractions showed antibacterial effect against the Gram-positive and high-biofilm-capacity *S. epidermidis* with an MIC of 312.5 µg/mL, and the SO-E fraction with an MIC of 625 µg/mL. The most striking antibiofilm activity results were observed with *S. epidermidis*, where SO achieved 94.3%, SO-E 90.1%, and SO-EA 87.1% inhibition rates, demonstrating very strong effects; in *P. aeruginosa*, SO-E exhibited the highest inhibition at 68.1%; and in *A. baumannii*, only the SO-E and SO-EA fractions displayed significant inhibition around 68–69%. These findings highlight the potential of *S. officinalis* SFE extracts – and particularly the extraction waste that are typically discarded – as natural, effective, and sustainable antimicrobial/antibiofilm agents.

KEYWORDS: *Salvia officinalis*; supercritical fluid extraction; antibiofilm; antibacterial; post-extraction waste.

1. INTRODUCTION

The rapid global increase in antibiotic resistance has become a major public health concern, particularly due to the rising prevalence of opportunistic pathogens capable of biofilm formation [1, 2]. Biofilm-associated infections pose critical challenges in chronic, device-related, and nosocomial infections because bacteria in biofilms exhibit 10- to 1000-fold higher tolerance to antibiotics, disinfectants, and host immune responses compared to their planktonic forms [3, 4]. This issue is particularly pronounced among ESKAPE pathogens such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis*; *A. baumannii* is classified as a "critical" priority pathogen in hospital-acquired infections, *P. aeruginosa* enhances biofilm resistance through quorum sensing (QS) mechanisms, and *S. epidermidis* develops tolerance to glycopeptides via its biofilm matrix in catheter/prosthesis-related infections [5-7].

In recent years, plant-derived bioactive compounds have attracted attention as alternative or complementary antimicrobial agents due to their multimodal mechanisms of action and chemical diversity. *Salvia officinalis* L. (sage) is rich in phenolic diterpenoids (carnosic acid and carnosol), rosmarinic acid, and terpenoids (1,8-cineole, thujone, and camphor) and has been extensively studied for its antimicrobial and antibiofilm potential [8-10]. These compounds act against Gram-positive and Gram-negative bacteria by disrupting membrane integrity, inhibiting adhesion, and affecting oxidative balance, while diterpenoids such as carnosic acid and carnosol have been reported to provide biofilm inhibition particularly in methicillin-resistant *Staphylococcus aureus* (MRSA) and other pathogens [11, 12]. Furthermore, *S. officinalis* extracts have

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been shown to reduce biofilm development in *P. aeruginosa* and *Candida* species by targeting QS and extracellular polymeric substance production [13, 14].

The extraction method is a critical factor determining the chemical profile and biological efficacy of plant material. While traditional solvent-based methods can degrade thermolabile compounds, supercritical fluid extraction (SFE) is a green technology that uses carbon dioxide as a supercritical solvent and isolates heat-sensitive compounds like carnosic acid and carnosol with high yield and without solvent residues [10, 15, 16].

SFE has become a popular green extraction method in recent years. This is because it uses non-toxic, eco-friendly solvents, works at relatively low temperatures (which helps protect sensitive bioactive compounds from thermal degradation), and is quick and selective. Notably, while the primary focus of SFE has been on the target compounds, the residual plant material, often regarded as waste, may still contain substantial amounts of valuable phytochemicals [17]. Unfortunately, these valuable compounds are frequently disregarded and discarded as waste within numerous experimental setups. However, studies exploring the bioactive potential of residual plant material following SFE are limited [18, 19].

In context, this study aims to evaluate the antibacterial and antibiofilm activities of the extract (SO) obtained from *S. officinalis* via SFE extraction, and the post-extraction waste fractions (SO-E and SO-EA) against standard reference biofilm-forming strains: *A. baumannii* ATCC 19606, *P. aeruginosa* ATCC 15692, and *S. epidermidis* ATCC 35984. In comparison with the reference antibiotic ciprofloxacin, this investigation seeks to elucidate the superior potential of SO, SO-E, and SO-EA fractions particularly against Gram-positive and biofilm-associated pathogens, and to highlight the value of extraction process residual plant material as sustainable sources of bioactive agents [14, 20].

2. RESULTS

2.1. Antibacterial activity

MIC values determined for the SFE extract (SO) of *S. officinalis* and the post-extraction waste fractions (SO-E and SO-EA) varied among the tested strains. For *A. baumannii* ATCC 19606 and *P. aeruginosa* ATCC 15692, the MIC values of all *Salvia* fractions were determined 2500 µg/mL, revealing that Gram-negative bacteria exhibit lower susceptibility to plant extracts. In contrast, against the high biofilm-forming capacity strain *S. epidermidis* ATCC 35984, the SO and SO-EA fractions displayed antibacterial activity with a MIC of 312.5 µg/mL, while the SO-E fraction exhibited a MIC of 625 µg/mL (Table 1). Overall, the obtained findings indicate that *Salvia officinalis*-derived extracts by SFE, possess higher antibacterial potential particularly against Gram-positive and biofilm-associated pathogens, and suggest that the waste remaining after extraction may also be biologically valuable.

Table 1. Minimum inhibition concentrations (MIC) of the fractions

Strain	SO MIC (µg/mL)	SO-E MIC (µg/mL)	SO-EA MIC (µg/mL)	Ciprofloxacin MIC (µg/mL)
<i>A. baumannii</i> ATCC 19606	2500	2500	2500	0.5
<i>P. aeruginosa</i> ATCC 15692	2500	2500	2500	0.25
<i>S. epidermidis</i> ATCC 35984	312.5	625	312.5	0.25

2.2. Antibiofilm activity

In the study, the biofilm production inhibitory capacities of the three different fractions (SO, SO-E, and SO-EA) were evaluated on standard reference strains according to the method of Adeyemo et al. (2022) (Table 2) [21]. In *A. baumannii* ATCC 19606, although the control biofilm optical density (OD) value was relatively low (0.495 ± 0.06), the inhibitory effect of SO remained quite limited (10.6%), whereas the SO-E and SO-EA derivatives exhibited similar and significant inhibition (69.6% and 68.6%, respectively). In *P. aeruginosa* ATCC 15692, the control biofilm production was found to be higher (1.845 ± 0.04); SO provided moderate inhibition (43.3%), SO-E demonstrated the highest effect (68.1%), and SO-EA exhibited moderate activity (54.7%). The

most striking results were observed with *S. epidermidis* ATCC 35984; while the control OD value was 1.197 ± 0.21 , all tested substances yielded very high biofilm inhibition rates (SO: 94.3%; SO-E: 90.1%; SO-EA: 87.1%).

These findings demonstrate that the tested substances (particularly SO) possess very strong antibiofilm activity against *S. epidermidis*, while in *A. baumannii* only SO-E and SO-EA showed similar efficacy. In *P. aeruginosa*, SO-E's clear superiority over the others is remarkable. These results suggest that the biofilm prevention potentials of the substances against Gram-positive and Gram-negative bacteria exhibit species-dependent differences.

Table 2. Biofilm productions and inhibitions

Strain	Biofilm production $\pm$ S.D. ^a	Inhibition $\pm$ S.D.*			Inhibition %		
		SO	SO-E	SO-EA	SO	SO-E	SO-EA
<i>A. baumannii</i> ATCC 19606	0.495 ± 0.06	0.375 ± 0.14	0.127 ± 0.04	0.131 ± 0.02	10.61	69.65	68.61
<i>P. aeruginosa</i> ATCC 15692	1.845 ± 0.04	1.040 ± 0.24	0.589 ± 0.08	0.835 ± 0.09	43.27	68.06	54.73
<i>S. epidermidis</i> ATCC 35984	1.197 ± 0.21	0.683 ± 0.02	0.354 ± 0.18	0.154 ± 0.10	94.29	90.13	87.14

*Standard deviation (n=3)

3. DISCUSSION

In this study, the antibacterial and antibiofilm activities of the main extract (SO) obtained from *Salvia officinalis* via SFE extraction, and the post-extraction waste fractions (SO-E and SO-EA) were evaluated against Gram-negative (*Acinetobacter baumannii* ATCC 19606, *Pseudomonas aeruginosa* ATCC 15692) and Gram-positive (*Staphylococcus epidermidis* ATCC 35984) reference strains. The obtained findings reveal that these fractions exhibit pronounced efficacy particularly against Gram-positive bacteria and biofilm formation, emphasizing that even the post-extraction waste fractions (SO-E and SO-EA) carry significant antibacterial/antivirulence potential. These results support the role of natural products in alternative or adjuvant strategies against antibiotic resistance [14].

In terms of antibacterial efficacy, all fractions showed a MIC value of 2500 µg/mL against Gram-negative strains, whereas markedly lower values – 312.5 µg/mL for SO and SO-EA, and 625 µg/mL for SO-E – were determined against the Gram-positive *S. epidermidis* ATCC 35984 strain. This difference suggests that, since present study was conducted only with sage extract and pulp, the structural components of sage may be ineffective against these two Gram-negative bacteria. Furthermore, other studies have found that various herbal extracts are less effective against Gram-negative bacteria than Gram-positive bacteria; this can be explained by the natural resistance of bacteria to terpenoid and phenolic compounds due to their outer membrane lipopolysaccharide layer, efflux pumps, and limited permeability. In the literature, it is frequently reported that essential oils and extracts of *S. officinalis* exhibit stronger inhibition against Gram-positive bacteria (particularly *Staphylococcus* and *Bacillus* species), while higher MIC values are generally detected against Gram-negatives (including *P. aeruginosa*). For instance, Delamare et al. (2007) reported that the essential oil of *S. officinalis* cultivated in South Brazil was more effective against Gram-positives, with increased resistance observed in Gram-negatives [22]. Similarly, although Cui et al. (2017) reported equal efficacy (MIC 0.05–0.1%) of *S. sclarea* essential oil against both Gram-positive and Gram-negative bacteria, studies on *S. officinalis* commonly show superiority against Gram-positives; various reports indicate MIC values against *P. aeruginosa* ranging from 0.05% to 5%, while much lower concentrations are effective against Gram-positives [23].

However, in supercritical extraction, the lipophilic volatiles, including terpenoids such as -thujone, 1,8-cineole, camphor and borneol, alongside the diterpenes carnosic acid and carnosol are selectively solubilized and removed in the first phase of extraction [10, 24, 15]. The residual solid matrix of *S. officinalis* is enriched in highly polar/hydrophilic polyphenols, mainly rosmarinic acid [26, 27]. On the other hand, the lipophilic abietane skeleton of the carnosic acid and carnosol diterpenes is also soluble in solvents of moderate polarity (acetone, ethyl acetate, methanol) [10, 28–30].

Independent quantitative studies on *S. officinalis* have shown varying distributions of key bioactive components. The lighter monoterpenes of the essential oil occur in wide ranges depending on the cultivar (α -thujone 5.45–19.23%, β -thujone 5.45–6.17%, camphor 5.08–29.2%, and 1,8-cineole 8.85–39.18), the highest values reported for hydrodistilled oils, particularly North African and Saudi Arabian [25, 31, 32]. The heavier sesquiterpenes (viridiflorol 9.94–22.1%, borneol $\approx$ 8.4%, bornyl acetate $\approx$ 2.2%, α -humulene 1.93–8.94%) were preferentially partitioned into supercritical CO₂ fractions than hydrodistilled oils [25, 31]. Diterpenes, on the other hand, partition into intermediate- and low-polarity solvents rather than into aqueous residues: carnosic acid 1.25–32.42 mg/g in commercial dried sage, 0.29–120.0 μ g/mg in SFE extracts and 23.62 mg/g in acetone Soxhlet extracts; carnosol 0–9.06 mg/g in commercial sage and 0.46–65.5 μ g/mg under SFE [10, 28, 33]. The polar hydroxycinnamic acids and flavonoid glycosides: rosmarinic acid (3.2–20.6 mg/g in commercial sage, 7.45 mg/g in 70% ethanolic macerates, 8.90 mg/g in aqueous Soxhlet and 17678.67 ± 673.37 μ g/g by UHPLC-MS profiling), luteolin-7-O-glucoside, apigenin-7-O-glucoside, luteolin-7-O-glucuronide, apigenin-7-O-glucuronide and the salvianolic acids, are accumulated in the aqueous, hydroethanolic and decoction phases [28, 30, 33–36].

Evidence from the literature indicates that the antimicrobial activity of *S. officinalis* extracts is largely dependent on the polarity and relative abundance of their phytochemical constituents. The strongest inhibitory activity against four pathogenic bacterial strains, in particular *Bacillus subtilis*, was reported by Pavić et al. (2019) for SFE extract enriched with carnosic acid and carnosol [10]. Carnosic acid was also found to inhibit biofilm formation MRSA at 0.05 mg/mL with significant downregulation of the expression of QS regulatory genes [37]. Moreover, carnosic acid has a broad-spectrum antimicrobial activity with a dual cell wall-membrane inhibitory mechanism by disrupting bacterial Lipid II cycle and promoting membrane permeabilization and depolarization [38]. Similarly, carnosol has been reported to induce metabolic dysregulation and inhibit biofilm formation in *S. aureus* (MRSA and MSSA) planktonic and biofilm cultures [39]. Jordán et al. (2012) tested 27 different methanolic extracts of *S. officinalis* and further showed that the relative distribution of carnosic acid and carnosol is a key determinant of antibacterial activity [40]. Extracts with a higher proportion of carnosol showed significantly higher antibacterial efficacy. The relevance of diterpenes was also evidenced by Moreno et al. (2006) in a comparative study on *Salvia rosmarinus* (*Rosmarinus officinalis*). In that study the highest antimicrobial spectrum was observed for a methanolic extract with MIC values ranging from 2–15 μ g/mL against Gram-positive bacteria, 2–60 μ g/mL against Gram-negative bacteria and 4 μ g/mL against yeast strains, containing about 30% carnosic acid, 16% carnosol and 5% rosmarinic acid [29]. On the contrary, the aqueous extract, where the major component is rosmarinic acid ($\sim$ 15%), showed much lower antimicrobial activity, with MIC values comparable to those of pure rosmarinic acid. In accordance with these results, pure rosmarinic acid has been reported to have antibacterial MIC values from 0.002 to >0.8 mg/mL [26]. Its antimicrobial activity against *Escherichia coli* (MIC 0.8 mg/mL; MBC 0.9 mg/mL), *S. aureus*, *Salmonella* spp., and *B. subtilis* has been ascribed to increased membrane permeability, disruption of intracellular proteins, and inhibition of Na⁺/K⁺-ATPase activity [41].

The high Gram-negative MIC values obtained in present study (2500 μ g/mL) are consistent with the selective compound profile of supercritical extraction (remaining diterpenoids, carnosic acid, rosmarinic acid) and reflect Gram-negative resistance in planktonic cells. The comparable efficacy of the post-extraction waste fractions (SO-E, SO-EA) to SO highlights that the retained active compounds preserve their biological value and underscores the reuse potential of industrial waste.

In antibiofilm activities, inhibition rates measured according to the method of Adeyemo et al. (2022) showed marked strain-dependent differences [21]. In *A. baumannii*, the limited inhibition by SO (10.6%) detected, SO-E and SO-EA displayed high rates (69.6% and 68.6%, respectively), suggesting that hydrophilic compounds remaining in the by-products penetrate the biofilm matrix more effectively. In *P. aeruginosa*, SO-E's superior performance at 68.1% points to a higher concentration of compounds targeting QS and matrix disruption. The highest efficacy was observed in *S. epidermidis*; inhibition rates of 94.3% for SO, 90.1% for SO-E, and 87.1% for SO-EA demonstrate that the fractions possess strong antibiofilm activity against Gram-positive biofilm pathogens. These differences can be explained by the difficulty of inhibiting QS systems and complex polysaccharide matrices in Gram-negatives, versus easier cell wall targeting in Gram-positives. In the literature, the antibiofilm effects of *S. officinalis* derivatives are associated with QS inhibition, matrix disruption, and increased membrane permeability; for example, Wijesundara and Rupasinghe (2018) reported that *S. officinalis* essential oil inhibited *Streptococcus pyogenes* biofilm formation by more than 50% [42]. Additionally, diterpenoids such as carnosic acid have been shown to exhibit specific efficacy against *Staphylococcus* biofilms (e.g., inhibition in MRSA) with more limited effects on *P. aeruginosa* biofilms [11]. The

high inhibition observed in this study against *S. epidermidis* aligns with this strain's simpler biofilm matrix structure, highlighting the fractions' advantage in targeting adhesion and QS [2,7,10].

In summary, the data suggest that the accumulation of polar phenolic compounds (e.g. rosmarinic acid) in the *S. officinalis* SFE residue fractions and the enrichment of more lipophilic diterpenes (e.g. carnosic acid and carnosol), and terpenoids (e.g. -thujone, 1,8-cineole, camphor) in the *S. officinalis* SFE fractions may act together to influence the antibacterial and antibiofilm activity profiles of the extracts via a synergistic multi-target mechanism.

Overall, the antibiofilm effects of the fractions are more pronounced than their antibacterial effects; this is explained by the easier effectiveness of the extract compounds in targeting biofilm formation and dispersion outside the cell wall barrier, rather than on planktonic cells, thus demonstrating the superiority of natural agents in targeting biofilm structures. Mechanistically, compounds (thujone, camphor, 1,8-cineole, carnosic acid, rosmarinic acid) play roles in disrupting cell membranes, affecting protein synthesis, and inhibiting QS signals [9,11]. However, the results are limited to *in vitro* conditions; *in vivo* efficacy, cytotoxicity, pharmacokinetics, and validation with clinical isolates need to be confirmed. Future studies are recommended to include GC-MS and HPLC profiling, synergistic antibiotic combinations, and molecular docking.

4. CONCLUSION

This study demonstrates the antimicrobial and antibiofilm potential of extracts (SO) and their post-extraction waste fractions (SO-E and SO-EA) obtained from *S. officinalis* by supercritical fluid extraction (SFE). Although their antibacterial activity against Gram-negative planktonic bacteria was limited, the low MIC values (312.5–625 µg/mL) observed against *S. epidermidis* and the high levels of biofilm inhibition (68.0–94.3%) across the tested strains highlight the potential of these fractions for combating biofilm-associated infections. A key finding of this study is that the post-extraction waste fractions (SO-E and SO-EA) retained biological activity comparable to that of the main extract, demonstrating that valuable bioactive properties are preserved following the SFE process. These findings also suggest that the fractions may interfere with biofilm development, although the underlying mechanisms require further investigation. To the best of our knowledge, this is the first study to evaluate the antibacterial and antibiofilm activities of extracts prepared from *S. officinalis* SFE residues. Overall, these findings support the potential of SFE-derived waste fractions as sustainable and value-added natural resources for the development of complementary strategies to combat biofilm-associated infections and antimicrobial resistance.

5. MATERIALS AND METHODS

5.1. Extraction material

The main SFE extract (SO) of *Salvia officinalis* leaf and the residual plant material obtained from LUK Botanik (Gebze, Türkiye).

5.2. Preparation of extracts

For SO-E and SO-EA, the residual plant material obtained from SFE of *S. officinalis* was extracted by maceration. Dry powder post-extraction waste (10 g per extract) was macerated at room temperature with ethyl acetate (3 × 100 mL) and ethanol (3 × 100 mL) for extraction. Then the extracts concentrated to dryness under decreased pressure and lyophilized in vacuo [43]. The extraction yields of the ethyl acetate and ethanol extracts were 7.62% and 17.43%, respectively. Extract codes and types are given in Table 3.

Table 3. Codes and types of *S. officinalis* leaf extracts

Codes of extracts	Extract types
SO	The main SFE extract
SO-E	SFE residual ethanolic extract
SO-EA	SFE residual ethyl acetate extract

The main extract (SO: 41 mg) obtained from *S. officinalis* by SFE and the post-extraction waste fractions (SO-E: 40 mg, SO-EA: 41.3 mg) were used. Each fraction was dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions reaching a concentration of 10000 µg/mL in the first well. Stock solutions were subjected to serial twofold dilutions to obtain the test concentration ranges. As solvent control, the final concentration of DMSO was always kept below 1%.

5.3. Bacterial strains

Antibacterial and antibiofilm activities were evaluated against three reference biofilm-producing bacterial strains: *Acinetobacter baumannii* ATCC 19606, *Staphylococcus epidermidis* ATCC 35984, and *Pseudomonas aeruginosa* ATCC 15692.

5.4. Antibacterial activity assay

The antibacterial activity of *S. officinalis* SFE extract and the residual plant extracts was determined as minimum inhibitory concentration (MIC) using the broth microdilution method in accordance with European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards [44]. Twofold serial dilutions of the test substances were prepared in cation-adjusted Mueller-Hinton Broth (MHB). Fresh bacterial cultures were adjusted to a 0.5 McFarland standard and diluted 1:100. Then, 100 µL of inoculum was added to each well to achieve a final inoculum density of approximately 5×10^5 CFU/mL. Plates were incubated at 35 ± 1 °C for 18–20 hours. All experiments were performed in triplicate.

5.5. Biofilm formation assay

Biofilm formation was determined using a modified Christensen et al.'s (1982) method, using the crystal violet [45]. Bacterial suspensions were adjusted to a 0.5 McFarland standard, diluted 1:100, and 100 µL was added to wells containing tryptic soy broth (TSB) supplemented with 2% sucrose. Plates were incubated at 35 ± 1 °C for 24 hours to allow biofilm development. After incubation, non-adherent cells were removed, wells were washed three times with distilled water, and stained with 0.1% crystal violet for 15 minutes. Excess dye was removed by washing, and bound dye was solubilized with 33% glacial acetic acid. Biofilm biomass was quantified by measuring optical density (OD) at 620 nm wavelength. All experiments were performed in triplicate.

5.6. Evaluation of antibiofilm activity

The antibiofilm activity of the test substances was evaluated at 1/2 sub-inhibitory concentrations according to modified Christensen et al.'s (1982) method [45]. These concentrations were prepared based on previously determined MIC values and added to the incubation medium. After incubation (35 ± 1 °C, 24 hours), planktonic cells were removed, wells were washed three times with distilled water, and the remaining adherent biofilm cells were stained with 0.1% crystal violet for 10 minutes. Excess dye was removed by washing, and bound dye was solubilized with 33% glacial acetic acid. Biofilm biomass was determined by measuring OD at 620 nm wavelength. Antibiofilm activity was calculated as percentage inhibition [21]. All experiments were performed in triplicate.

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