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Antibacterial and Phytochemical Properties of *Stachys schtschegleevii* Oil Extract: Investigating Interaction and Antimicrobial Activity against Urinary Tract Infection Bacteria Through in Silico and in Vitro

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Abstract
Urinary tract infections represent a global health challenge increasingly complicated by antimicrobial resistance. This study explores the therapeutic potential of *Stachys schtschegleevii* essential oil against common uropathogens. Gas chromatographic-Mass Spectrometry analysis revealed four principal bioactive compounds (α-Pinene, β-Pinene, Linalool, and Hexadecanoic Acid) exhibiting remarkable synergistic antimicrobial activity. Molecular docking simulations demonstrated exceptional binding affinities between these phytocompounds and bacterial dihydrofolate reductase enzymes, with α-Pinene and β-Pinene forming strongest complexes with *Enterococcus faecalis* DHFR (-6.1 kcal/mol) and Hexadecanoic Acid with *Staphylococcus aureus* DHFR (-6.1 kcal/mol). In vitro evaluation confirmed significant antimicrobial efficacy, with substantial inhibition zones (*E. faecalis* 20.16±0.2mm, *E. coli* 17.7±0.45mm, *S. aureus* 20.53±1.47mm) and impressive minimum inhibitory concentrations *(E. faecalis* 6.25mg/ml, *E. coli* 1.56mg/ml, *S. aureus* 3.12mg/ml). The multi-component nature of these extracts creates a complementary mechanism of action whereby multiple compounds simultaneously target different bacterial pathways, significantly reducing resistance development probability compared to single-compound therapeutics. This synergistic interaction, coupled with the plant's documented anti-inflammatory properties, presents schtschegleevii as an exceptional candidate for developing novel phytotherapeutic approaches against increasingly resistant uropathogens, offering an optimal balance of therapeutic efficacy, economic viability, and patient safety in urinary tract infection management.

Keywords
Antibacterial; In silico; In vitro; *Stachys schtschegleevii*; Urinary tract infection.

1. INTRODUCTION

Urinary tract infections (UTIs) are among the most prevalent bacterial illnesses worldwide, affecting ~150 million people annually. Although UTIs can affect both sexes, they disproportionately impact women, with a 50% lifetime risk. The recurrence pattern is particularly concerning, as 25% of women initially diagnosed with cystitis experience recurrent UTIs within six months, with some experiencing six or more episodes within a year following the initial infection [1]. Among children, UTIs account for approximately 1.5 million outpatient visits annually in the United States alone, with 8% of children aged 1 month-11 years experiencing at least one UTI episode, and 30% of these cases developing recurrent infections within a year [2]. In elderly populations, UTIs present unique diagnostic challenges, requiring specific criteria including local genital symptoms, evidence of urinary tract inflammation through pyuria, and confirmatory urine culture, though no universally accepted definition exists despite numerous consensus standards for surveillance purposes [3].

The etiological landscape of UTIs is dominated by uropathogenic *Escherichia coli* (UPEC), responsible for more than 80% of community-acquired bacterial infections. Secondary pathogens include *Staphylococcus*, *Proteus*, *Enterobacter*, *Klebsiella*, and *Enterococcus* species, which play particularly significant roles in catheter-associated and hospital-

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acquired infections [4]. Concerningly, global surveillance data indicate a progressive increase in multidrug-resistant uropathogens, rendering existing antibiotic therapies increasingly ineffective for both acute infection management and recurrence prevention. This escalating antimicrobial resistance compromises quality of life and poses serious health implications across all affected demographic groups [5].

The limitations of conventional antibiotics have renewed scientific interest in traditional herbal remedies and their natural constituents. Aromatic plants and herbal medicines are frequently employed in phytotherapy due to their diverse biological activities, including antibacterial, anti-carcinogenic, carminative, hepatoprotective, antiviral, and antioxidant properties [6, 7]. *Stachys schtschegleevii*, belonging to the Lamiaceae family, has historically been utilized in traditional medicine, with its branches and leaves frequently employed to treat rheumatism, reduce inflammation, and disinfect the respiratory system [8, 9]. A notable advantage of medicinal plants like *Stachys schtschegleevii* is the synergistic interaction between their constituent compounds, where multiple bioactive components work through different mechanisms simultaneously, potentially enhancing antimicrobial efficacy while reducing the likelihood of resistance development compared to single-compound treatments. Natural products and their derivatives have long been recognized as sources of bioactive substances with antimicrobial efficacy, low toxicity, and cost-effectiveness, presenting advantages over synthetic alternatives that are expensive to produce and rarely demonstrate both preclinical and clinical efficacy against bacterial or fungal infections.

Despite extensive research on antimicrobial resistance, significant gaps remain in our understanding of natural alternatives to conventional antibiotics, particularly regarding their mechanisms of action and specific bioactive components. While monoterpenes such as pinenes, found in turpentine, rosemary, lavender, and coniferous tree essential oils [10, 11], and compounds like linalool ($C_{10}H_{18}O$) and hexadecanoic acid (palmitic acid) have demonstrated promising antimicrobial properties [12], their potential against uropathogens and their synergistic interactions remain insufficiently explored. Furthermore, while dihydrofolate reductase (DHFR) a key enzyme in folate metabolism crucial for DNA precursor production and de novo synthesis of glycine and purines [13] represents a potential antimicrobial target, few studies have investigated the interaction between plant-derived compounds and this enzyme in the context of UTIs. This research gap is particularly significant given the urgent need for alternative approaches to combat increasingly resistant uropathogens.

This study aims to explore the antibacterial potential of *Stachys schtschegleevii* extracts against three common bacteria responsible for UTIs, with emphasis on maximizing effectiveness while minimizing cost and ensuring patient safety. This work combines in silico analyses with laboratory experiments to identify and evaluate novel bioactive components, their synergistic interactions, and their mechanisms of action against uropathogenic bacteria. Specifically, we investigate the interaction between key phytochemical constituents of *Stachys schtschegleevii* and bacterial dihydrofolate reductase, to explore a plausible mechanistic basis for the observed antimicrobial effects. These findings enhance our understanding of natural antimicrobial agents and hold significant implications for developing alternative UTI treatments to address the growing challenge of antibiotic resistance. By elucidating both the composition and efficacy of this traditional medicinal plant, we bridge ethnopharmacological knowledge with modern scientific methodologies to advance the development of nature-based therapeutic strategies against UTIs.

2. EXPERIMENTAL

2.1. Materials and methods

The herbal *Stachys schtschegleevii* (Angiosperms →Lamiaceae →*Stachys* →*Stachys schtschegleevii*) was collected from a virgin mountain in the West Azerbaijan region of Iran called Shahindezh (36.685959N, 46.562307E). Voucher specimens (Herbarium No. 83580 TARI) have been placed at the Research Institute of Forest and Rangelands, Tehran, Iran's Central Herbarium [9]. For the purpose of this investigation, every microbe utilized came from the IROST in Tehran, Iran. We looked at *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922), and *E. faecalis* (ATCC 29212). The investigation's microbiological growth conditions (for transfer, cultivation, and differentiation) were all purchased from Merck, and all reagent materials were obtained from Sigma-Aldrich Chemicals.

2.2. Preparation of urinary bacteria

Within an actual span for six months, a cohort of 411 patients spanning from

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neonates to individuals of 80 years of age, encompassing both male and female genders, and presenting with initial symptomatic manifestations indicative of urinary tract infections, availed themselves at the medical center of the Tabriz University of Medical Sciences, thus being meticulously selected for the purpose of this comprehensive investigation. Subsequent to the provision of standardized training pertaining to the meticulousness of urine sampling, wherein sterile urine collection was duly allocated, the process of urinary sample procurement was meticulously executed [14]. The collected specimens were forthwith subjected to discerning analytical scrutiny (Urine Analysis) conducted by adept laboratory specialists (*E. coli*: Citrate: Negative, VP test: Negative, Methyl red: Positive, Indole test: Positive, and Gram staining: Gram negative, [15]; *S. aureus*: DNase: Positive, Coagulase: Positive, Pigment: Positive, Catalase: Positive, and Gram staining: Cocci gram positive, [16]; *E. faecalis*: Pyruvate fermentation test: Positive, Growth in sodium chloride 6.5%, Bile Esculin: Positive, Catalase: Negative, and Gram staining: cocci Gram-positive ,[17]), immediately followed by the essential microbiological cultivation procedures (Urine Culture) pivotal for the seamless progression of the investigative endeavor. Differential tests were conducted, and essential data were collected. Five series of the three studied bacteria (*E. faecalis*, *E. coli*, and *S. aureus*) were isolated and purified through repeated culturing (up to five times) to ensure purity. Furthermore, supplementary differential examinations proved systematically reiterated to corroborate the heightened veracity for the investigation's findings in a meticulous manner. Ultimately, the purified bacterial strains derived from the urinary samples of the patients were obtained. A simplified illustration of the investigation's methodology is shown in Figure 1 , depicting the activity cycle of the DHFR enzyme and its inhibition, emphasizing the significance of interfering with the enzyme's activity through drugs or chemical compounds.

2.3. Essential oil extraction

After being gathered, the plant *Stachys schtschegleevii* had been thoroughly dried in the shade and then ground into a fine powder via a mixer. Everything was prepared to begin the procedure of extraction. Shredded 70 g of leaf stems and 90 g of leaves were added to an SDE with 35 mL of pentane for 120–150 minutes in order to extract the essential oils. They need to be able to absorb the likely water; 2 g of sodium

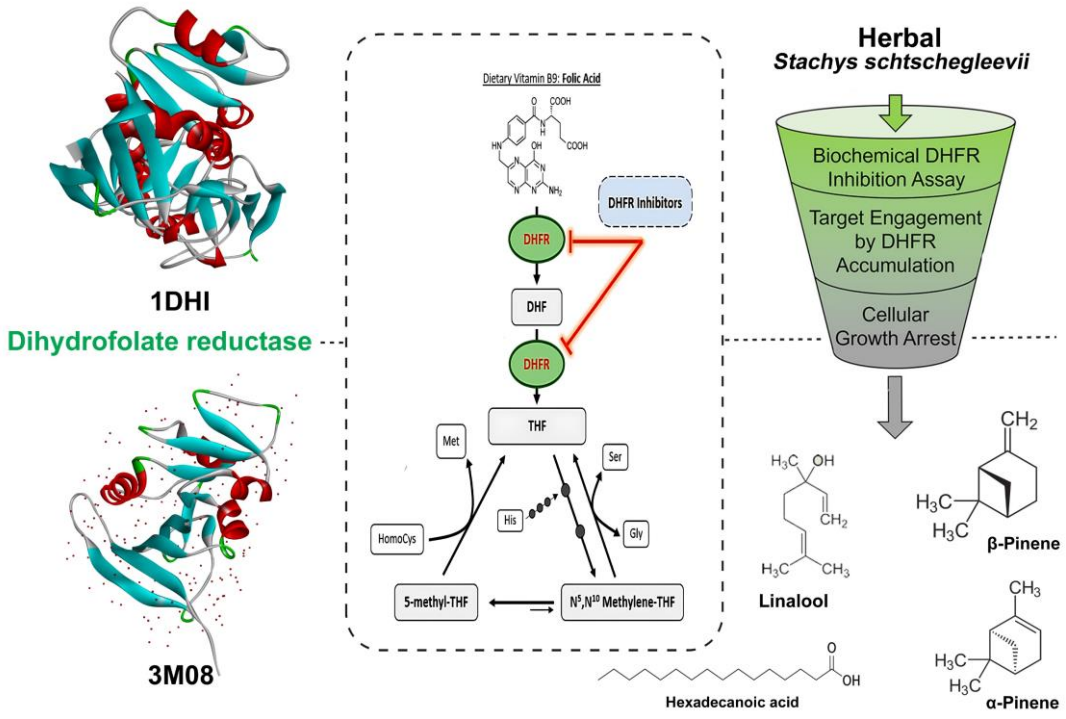

Fig. 1. The schematic illustrates the activity cycle of the enzyme DHFR and its inhibition, alongside the 3D structure of the proteins and the chemical structures of synthesized compounds derived from the herbal plant *Stachys schtschegleevii* as presented in the study.

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sulfate were added at the conclusion of the extraction process. The refrigerator was used to store the essential oil for 24 hours. The residual pentane was carefully transferred into a vial and allowed to evaporate at room temperature, after which the concentrated extract was stored at 4 °C until subsequent gas chromatographic analysis [18].

2.4. Pharmaceutical analysis

A Restek HP-5MS 5% phenylmethyl siloxane capillary column with (0.25mm i.d., 30m, 0.25m film layer of thickness), an Agilent HP-5973 Mass Selective Detector (MSD) equipped with a gas chromatograph Agilent HP-6890 were used to perform the GC-MS analysis under the same conditions as previously mentioned. A similar sequence of n-alkanes was injected under circumstances identical to those of the sample, and retention indices were computed for each component. The components of the oil's essential were identified utilizing retention indices (RI) in respect to n-alkanes, computerized corresponding via the Wiley7n.L and Wiley275.L, to be effectively analyses of the dispersion structure of the mass spectrum with data from the research [19].

2.5. Pharmacological effects in silico

Chemical components with pharmaceutical properties from *Stachys schtschegleevii* were identified through gas chromatography (GC-MS) analysis. After rigorous screening of all GC-MS detected compounds, three key bioactive components were specifically selected due to their significant pharmacological potential, and their chemical structures were retrieved from the PubChem repository. Subsequently, the 2D configuration of these compounds was delineated using the IUPAC nomenclature. This 2D structure was then translated into 3D form through the utilization of the ChemBio3D software, where energy minimization and molecular dynamics were executed to attain the most enduring natural conformation [20].

The protein structure of dihydrofolate reductase, across three bacterial species under scrutiny, underwent assessment through the Uniports database and was subsequently extracted from the Protein Data Bank (PDB) [21, 22]. Primary preparation procedures, encompassing the elimination of prior ligands and extraneous water molecules external to the structure, addition of hydrogen atoms, excision of redundant residues, introduction of charges to amino acids, and reduction of the protein's structure energy, were performed using the Chimera software. This preparatory phase led to the creation of a molecular docking-ready state.

Molecular docking was orchestrated via the AutoDock Vina software in conjunction with PyRX. Notably, the selection of ligands capable of binding effectively to the target protein was accomplished with heightened precision. Moreover, the pharmacokinetic attributes of the selected compounds were scrutinized using online repositories like the Protein Ligand Interaction Profiler (PLIP)[23] and AdmetSAR [24]. The effectiveness of each component individually or in combination demonstrates the significant impact of this plant material, and the higher the potency and concentration of these compounds in the plant, the greater the efficacy of the herbal product.

2.6. Pharmacological effects in vitro

Three series of bacteria extracted and purified from the urine of the patient (all three bacterial strains under study) and one series of purchased standard bacteria for control purposes were cultivated in the Mueller-Hinton agar environment to evaluate the effects for discs prepared from the extracted oil essence of the plant. After the incubation period of 24 hours, the findings were recorded. In addition, the essence was diluted in accordance with prescribed concentrations using the broth dilution procedure. The next step was the injection of bacteria. The tube under investigation, which displays no discernible development after 24 hours of incubation, was classified as a MIC [25].

2.7. Statistical Analyses

The R programming language was used to carry out the analysis after the experiments were run in triplicate [26]. The findings were expressed as mean values ± standard deviations, and Tukey's test in the programming language R was used to get the modified p-values. When the corrected p-value in the treatment comparison was equal to or less than 0.05, it was considered significant. Then, using R's ggplot2 program, visual representations were produced [27].

3. RESULTS

3.1 Microbiology and antibiogram of the urine

In a six-month study involving 411 patients presenting with symptoms of urinary tract infection, bacterial cultures were isolated and identified following urinalysis (UA) and urine culture (UC) evaluations. As depicted in Supplement Table 1, *Escherichia coli* (31%), *Enterococcus faecalis* (23%), and Klebsiella pneumoniae (10%) emerged as the predominant urinary pathogens. For subsequent investigations, three key bacterial species were selected: *E. coli* (Gram-negative), *E. faecalis* (Gram-positive), and *Staphylococcus aureus* (Gram-positive).

Supplement Table 2 presents the efficacy of nine conventional antibiotics (Ampicillin, Chloramphenicol, Co-trimoxazole, Erythromycin, Gentamicin, Oxacillin, Streptomycin, Tetracycline, and Vancomycin) against these bacterial isolates, assessed via diffusion disk antibiotic susceptibility testing and minimum

inhibitory concentration (MIC) determinations. Results indicate that co-trimoxazole currently demonstrates sensitivity in more than 50% of cases across all three bacterial species (*E. faecalis* = 55.3%, *E. coli* = 71.93%, and *S. aureus* = 80%). Conversely, streptomycin exhibits sensitivity in less than 50% of cases for all three bacteria (*E. faecalis* = 0%, *S. aureus* = 15.38%, and *E. coli* = 45.61%), suggesting significant bacterial resistance to this antibiotic. Moreover, tetracycline demonstrated notable resistance patterns in *S. aureus* (72.8%), *E. faecalis* (64%), and *E. coli* (55%).

Of the nine antibiotics investigated, *E. coli* and *E. faecalis* exhibited resistance to five antibiotics each (*E. coli*: Ampicillin, Gentamicin, Oxacillin, Tetracycline, Vancomycin; *E. faecalis*: Chloramphenicol, Erythromycin, Gentamicin, Oxacillin, Streptomycin), while *S. aureus* demonstrated resistance to three antibiotics (Ampicillin, Streptomycin, Tetracycline).

3.2. Pharmaceutical analysis: Gas Chromatography -Mass Spectrometry

Gas chromatography–mass spectrometry (GC-MS) profiling of the essential oil extracted from *Stachys schtschegleevii* leaves revealed a chemically diverse composition, comprising terpenes, sesquiterpenes, and fatty acids with potential bioactivity. A total of 32 distinct phytochemical constituents were identified, accounting for the complex phytochemical profile of the extract (Table 1). Among these, four major compounds—α-pinene (5.7%), β-pinene (1.91%), linalool (0.76%), and hexadecanoic acid (6.95%)—were selected for further investigation due to their reported antimicrobial and pharmacological relevance.

The chromatographic analysis demonstrated that sesquiterpenes such as Germacren-D (25.68%) and Valencene (8.28%) were also present in considerable amounts, contributing to the extract's chemical diversity and potentially influencing its biological activity. Retention indices (RI) were calculated against a homologous series of n-alkanes, confirming accurate compound identification with high analytical confidence. The comprehensive compositional profile outlined in Table 1 provided the basis for selecting the key bioactive molecules that were subsequently evaluated through molecular docking and in vitro antimicrobial assays.

Table 1. The chemical constituents of the essential oil extracted from *Stachys schtschegleevii* leaves.

Row	Retaining time	Component	Composition (%)	RI[b]	RI[c]
1	6.39	α-Pinene	5.7	896	896
2	7.46	β-Pinene	1.91	933	932
3	8.32	δ-Carene	0.29	961	849
4	8.86	DL-Limonene	0.78	979	873
5	9.06	cis-Ocimene	0.64	986	982
6	11.09	Linalool	0.76	1042	898
7	13.65	3-Cyclohexen-1-ol,4-methyl-	0.40	1109	1056
8	19.94	α-Copaene	1.03	1259	1078
9	20.47	β-Elemene	2.46	1272	1092
10	21.32	Hexadecane	0.84	1292	1099
11	21.63	β-Cubebene	0.51	1299	1110
12	22.52	Vulgarol B	1.34	1321	1116
13	23.47	Germacren-D	25.68	1344	1123
14	23.76	Valencene	8.28	1351	1141
15	24.28	α-Amorphene	0.74	1364	1173
16	24.55	β-Eudesmol	3.96	1370	1179
17	26.36	Spathunelol	4.74	1415	1201
18	26.51	Vulgarol-B	1.60	1418	1233
19	26.74	Salvial-4-(14)-en-1-one	1.24	1424	1244
20	27.06	Valencene	1.26	1432	1254
21	27.31	Armodendrene	1.35	1439	1268
22	28.15	α-Cadinol	3.97	1460	1276
23	28.56	t-Muurolol	6.34	1470	1289
24	29.47	Vulgarol-B	2.65	1493	1309
25	30.72	Minsulfide	0.97	1526	1317
26	33.29	2-Pentadecanone,6,10,14-trimethyl	2.79	1593	1336
27	34.43	Ethyl linoleate	1.91	1612	1343
28	34.65	11,14,17-Eicosatrienoic acid	3.81	1616	1365
29	36.81	Hexadecanoic acid	6.95	1646	1375
30	37.69	2,6-Octadien-1-ol,3,7-dimethyl	1.63	1658	1385
31	40.57	9,12-Octadecanoic acid	1.42	1699	1392
32	40.77	Ethyl linoleolate	1.85	1905	1399

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3.3. Pharmacological effects in silico simulation

3.3.1. Molecular Dynamics and Docking Simulation

The bacterial dihydrofolate reductase pathway, a common target for numerous antibiotics, was selected for investigation. The specifications of the bacterial proteins evaluated are presented in Table 2, along with the outcomes of molecular docking between four ligands (α-Pinene, β-Pinene, Linalool, and Hexadecanoic Acid) and the bacterial proteins (1DHI, 3M08, and 4M7V).

Our investigation into molecular interactions revealed distinct binding profiles across bacterial species. The Gram-negative *E. coli* exhibited energetically favorable interactions with two terpene compounds, demonstrating binding energies of -5.0 kcal/mol for α-pinene and -5.6 kcal/mol for linalool. In contrast, the Gram-positive *S. aureus* displayed significant binding affinity with three compounds, where hexadecanoic acid showed the strongest interaction (-6.1 kcal/mol), followed by linalool (-5.6 kcal/mol) and α-pinene (-5.5 kcal/mol). Additionally, *E. faecalis*, another Gram-positive bacterium, formed stable complexes with α-pinene and β-pinene (both at -6.1 kcal/mol) as well as hexadecanoic acid (-5.8 kcal/mol). The three-dimensional conformational arrangements of the most energetically favorable complexes, specifically 4M7V with α-pinene, 4M7V with β-pinene, and 3M08 with hexadecanoic acid (all exhibiting -6.1 kcal/mol binding affinity), are visualized in Figure 2, highlighting the structural basis for these molecular interactions.

3.3.2. Prediction of medicinal properties (Admet)

Figure 3 and Table 3 provide comprehensive analyses of the physicochemical, toxicological, and pharmacokinetic properties of α-pinene, β-pinene, linalool, and hexadecanoic acid, integrating structural visualization with bioinformatics data. Figure 3 presents the three-dimensional molecular structures alongside radar charts illustrating key molecular descriptors, including molecular weight (MW), quantitative estimate of drug-likeness (QED), partition coefficient (SlogP), topological polar surface area (TPSA), hydrogen bond acceptors (HBA), hydrogen bond donors (HBD), number of rotatable bonds (nRot), and ring systems (nRing). These parameters provide valuable insights into molecular complexity, solubility, and drug-likeness. Additionally, toxicity profiles highlight potential eye and skin irritation, phototoxicity, and acute dermal toxicity—factors crucial for assessing their pharmaceutical and industrial safety profiles.

Table 3, derived from the AdmetSAR bioinformatics database, complements these findings with detailed pharmacokinetic and toxicological data. The analysis reveals that β-pinene, α-pinene, and linalool localize primarily in lysosomes, whereas hexadecanoic acid predominantly accumulates in mitochondria. All four compounds, due to their low molecular weight and size, demonstrate the ability to cross the blood-brain barrier (BBB), suggesting potential neurological activity. Notably, all compounds exhibit negative AMES mutagenicity, indicating minimal risk of genetic mutations. α-Pinene and β-pinene demonstrate positive human oral bioavailability, indicating efficient gastrointestinal absorption, hepatic metabolism, and maintenance of therapeutic plasma concentrations prior to renal clearance. While α-pinene, β-pinene, and hexadecanoic acid showed no nephrotoxicity, they exhibited hepatotoxic effects, necessitating careful consideration in therapeutic applications.

Table 2. The results of Auto Dock Vina (binding affinity), active site, PDB and UniProt code.

Organism	Protein			Binding Affinity (kcal/mol)			
	UniProt	PDB	Active Site	α-Pinene	β-Pinene	Linalool	Hexadecanoic acid
Escherichia coli	P0ABQ4	1DHI	ILE5 / LEU183	-5	-4.6 *	-5.6	-4.7 *
Staphylococcus aureus	P0A017	3M08	THR46 / PHE92	-5.5	-4.9 *	-5.6	-6.1
Enterococcus faecalis	Q834R2	4M7V	ALA7 / LEU20	-6.1	-6.1	-4.8 *	-5.8

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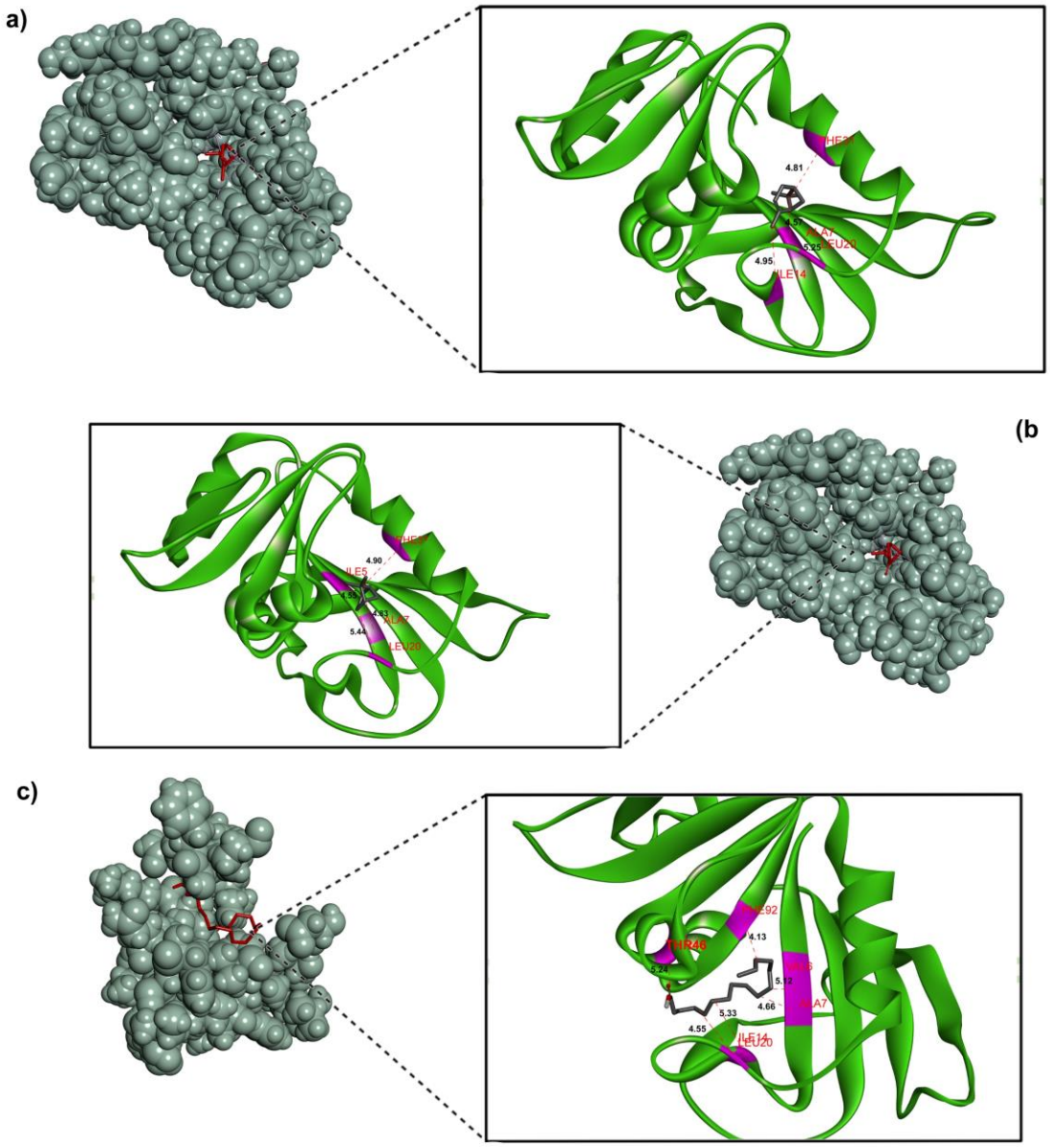

Fig. 2. 3D structure resulting from the docking a) 4M7V - α-Pinene; b)4M7V - β-Pinene; c) 3M08-Hexadecanoic acid.

Table 3. The results of AdmetSAR database.

Name of the drug	Admet SAR						
	Subcellular localization	Molecular Weight	Blood Brain Barrier	Human Oral Bioavailability	Nephrotoxicity	Hepatotoxicity	Ames Mutagenesis
α-Pinene	Lysosomes	136.24	+	0.8286 (+)	0.7361 (-)	0.7125 (+)	0.9800 (-)
β-Pinene	Lysosomes	136.24	+	0.7857 (+)	0.6858 (-)	0.7875 (+)	0.9800(-)
Linalool	Lysosomes	154.25	+	0.5429 (-)	0.5972 (+)	0.5267 (-)	0.9700 (-)
Hexadecanoic acid	Mitochondria	256.43	+	0.6714 (-)	0.5720 (-)	0.7625 (+)	1.0000 (-)

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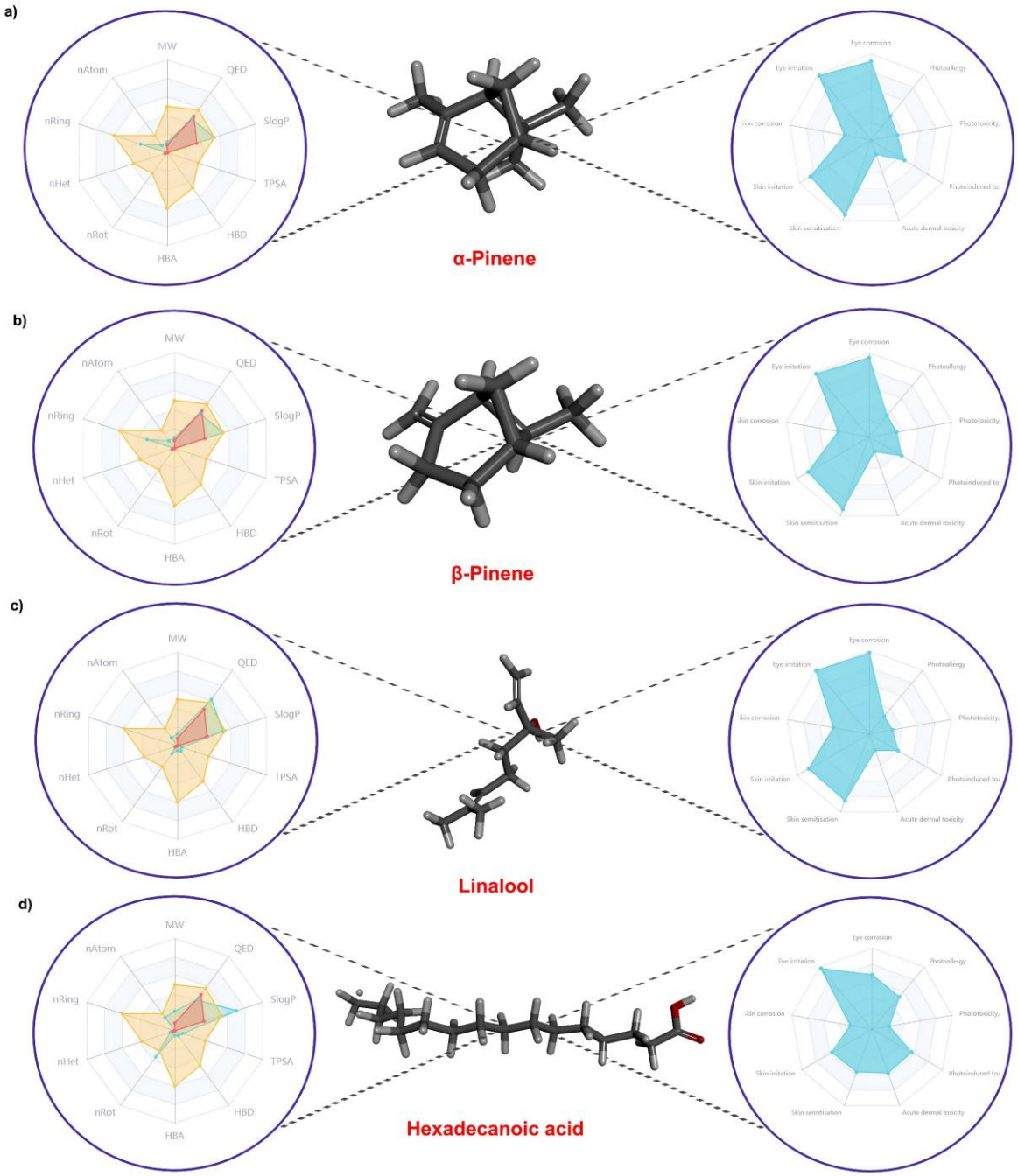

Fig. 3. 3D molecular structures alongside radar charts depicting molecular and toxicological properties. The left radar charts represent key molecular properties, while the right radar charts illustrate the toxicological characteristics of the drug molecules.

3.4. Pharmacological effects in vitro

The antimicrobial efficacy of *Stachys schtschegleevii* extract was evaluated against bacterial isolates in vitro, examining the extract as a whole rather than isolated components. Table 4 presents the results of antibiogram disk diffusion and MIC assays. In the inhibition zone (IZ) analysis, the median diameters of inhibition halos were recorded as: *E. faecalis* = 20.16 ± 0.2 mm, *E. coli* = 17.7 ± 0.45 mm, and *S. aureus* = 20.53 ± 1.47 mm. The minimum inhibitory concentrations (MIC) were determined as: *E. faecalis* = 6.25 mg/ml, *E. coli* = 1.56 mg/ml, and *S. aureus* = 3.12 mg/ml. Control data corroborated the primary findings, confirming the reliability and validity of *Stachys schtschegleevii*'s antimicrobial activity against the tested organisms. Figure 4 illustrates

the three phases of the in vitro experimental outcomes. Section "a" depicts the pharmaceutical preparation process, with references provided for validation methodologies employed in pharmaceutical production. Section "b" illustrates key steps in the isolation and preparation of bacterial isolates from patient urine samples. The average bacterial colony-forming units (CFU) were determined in paragraph "b5" and expressed as CFU/ml for both UTI and non-UTI conditions, with p-values for *S. aureus*, *E. faecalis*, and *E. coli* reported as p=0.06, p=0.09, and p=0.06, respectively. Section "c" demonstrates the effects of plant-derived pharmaceuticals on bacterial growth using MIC tests and disk diffusion methods. The disk diffusion test results are reported in Section "c2" (p-values for *S. aureus*, *E. faecalis*, and *E. coli* were p=0.06, 0.05, and 0.07, respectively), while minimum inhibitory concentration outcomes are visually presented in Section "c4" based on microbial inhibition proportions. Precise numerical data are provided in accordance with the referenced figures, with additional details available in Table 4.

Table 4. Antibacterial activity of *Stachys schtschegleevii* extracts.

Ligand	Inhibition Zone (mm)			MIC Test (mg/ml)		
	E-Coli	*E. faecalis*	*S. aureus*	*E-Coli*	*E. faecalis*	*S. aureus*
Stachys schtschegleevii + UTI subcultures	17.7 ± 0.45	20.16 ± 0.2	20.53 ± 1.47	1.56	6.25	3.12
Stachys schtschegleevii + control bacteria	18	19.5	20.1	1.56	6.25	3.12

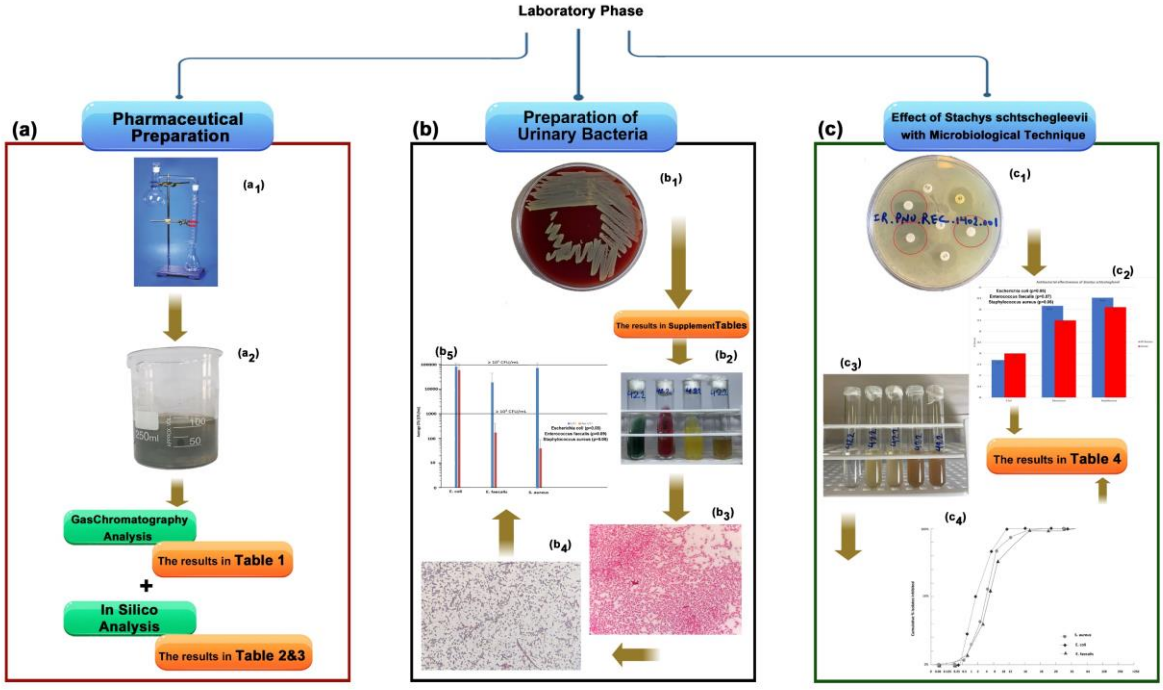

Fig. 4. (a) Pharmaceutical Preparation; (a1) Distillation and extraction process; (a2) B Beaker containing an extract of *Stachys schtschegleevii*; The verified findings from Pharmaceutical production have been published in Table 1,2, and 3, as referenced in section a; (b) How to Prepare Urinary Bacteria; (b1) Cultured *E. Coli* in blood agar; (b2) Illustrates tests for differentiating bacteria; (b3) Gram staining of *E. coli* under a microscope; (b4) Gram staining of *S. aureus* under a microscope; (b5) Both under UTI and non-UTI settings, the average bacterial colony-forming unit (CFU) count was determined and reported in CFU/ml. The following are the p-values for *S. aureus*, *E. faecalis*, and *E. coli*: p=0.06, p=0.09, and p=0.06, respectively; (c) Examine *Stachys schtschegleevii*'s effects using microbial techniques; (c1) displays the disk diffusion (prepared disks from *Stachys schtschegleevii*); (c2) The disk diffusion test findings are visually displayed and measured in millimeters (mm) for both control strains and UTI bacteria. The following are the p-values for *Staphylococcus aureus*, *Enterococcus faecalis*, and *Escherichia coli*: p=0.06, p=0.05, and p=0.07, respectively; (c3) shows the use of the MIC test *Stachys schtschegleevii*; (c4) The percentage of inhibition is used to graphically represent the MIC test findings; Table 4 provides more specific information.

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4. DISCUSSION

This investigation demonstrates significant antimicrobial efficacy of both conventional antibiotics and *Stachys schtschegleevii* essential oil against predominant urinary tract infection (UTI) pathogens. The observed high resistance to antibiotics such as streptomycin and tetracycline across the tested bacterial isolates (*E. coli*, *E. faecalis*, *S. aureus*) corroborates findings by Yisiak Oumer et al. (2021) on bacterial isolates from Southern Ethiopia, which similarly reported tetracycline resistance in *E. coli* and *E. faecalis* with relative sensitivity in *S. aureus*. The minor variations in resistance percentages between our findings and previous reports may be attributed to regional differences in microbial mutations, methodological approaches, and sample sizes [28]. The global emergence of multidrug-resistant bacterial strains represents a significant challenge to public health [29]. Excessive antibiotic usage leads to numerous health complications, primarily bacterial resistance, which diminishes therapeutic efficacy, complicates disease management, and presents substantial challenges for researchers and healthcare practitioners. Consequently, natural alternatives including essential oils and their constituent phytocompounds have been extensively investigated for their antimicrobial properties.

Phytochemical analysis through gas chromatography identified key components with potential antimicrobial activities. Pinenes demonstrated a favorable composition profile and established antimicrobial associations, enhancing their relevance to this investigation [30]. (-)-α-Pinene functions through multiple mechanisms, including metabolic interference, microbial flow inhibition, and membrane integrity reduction, effectively modulating antibiotic resistance [31]. Our findings suggest that pinene compounds possess considerable potential for antimicrobial therapy to inhibit bacterial proliferation, either independently or as antibiotic adjuvants. Linalool, identified as the most prevalent monoterpene in food products, has been detected in 23 different food sources at varying concentrations. Its significance stems from diverse biological properties, including anti-inflammatory, antioxidant, anticancer, cardioprotective, and antibacterial activities [32]. Hexadecanoic acid, a fatty acid compound, is recognized for its antibacterial properties and represents a viable candidate for next-generation antibacterial drug development against various bacterial infections. Fatty acids play crucial roles in host defense mechanisms against numerous pathogens, including multidrug-resistant bacteria [33]. The exceptional ability of hexadecanoic acid to enhance the antibacterial effects of other compounds when used in combination represents a crucial characteristic that is uncommon among other naturally occurring substances

Molecular docking simulations revealed favorable binding affinities between compounds such as α-pinene, β-pinene, and hexadecanoic acid with bacterial dihydrofolate reductase (DHFR) proteins, suggesting a potential mechanistic basis for their antibacterial effects. DHFR is essential in the de novo pathway of thymidine and purine synthesis, and small molecules targeting this specific enzyme have demonstrated promise as antibiotic candidates [34]. Furthermore, *E. coli* DHFR presents a potentially effective genetic approach and valuable biological tool for therapeutic applications in molecular biology [35]. While it might be valuable to address small molecules targeting both plasmid and chromosomal encoded DHFR from an antibiotic development perspective, the current investigation employed only chromosomal DHFR as a model structure due to the abundance of structural and mechanical data available for this target and its essentiality for bacterial survival, making it an excellent candidate for drug development.

Daniela Bomfim de Barros et al. (2022) investigated alpha-pinene's antifungal activity against C. albicans, confirming a minimum inhibitory concentration (MIC) ranging from 128–512 µg/ml. In the present study, we corroborated these findings regarding the antibacterial activity of α-pinene (identified in *Stachys schtschegleevii* along with other components), demonstrating MICs of 6.25 mg/ml for *E. faecalis*, 1.56 mg/ml for *E. coli*, and 3.12 mg/ml for *S. aureus*. Furthermore, Priscilla et al.'s 2021 investigation demonstrated α-pinene's synergistic activity with conventional antibiotics, enhancing the efficacy of tetracycline and erythromycin against antibiotic-resistant *Staphylococcus aureus* infections [36]. This finding, similar to our observations, underscores the significance of α-pinene against *S. aureus*.

An intraperitoneal injection of *Stachys schtschegleevii* Sosn extract was used in 2008 research by Maleki-Dizaji et al. to assess the anti-inflammatory characteristics of the extract, and the results showed a decrease in the inflammatory response [37]. Given that inflammation is one of the most significant indicators of urinary tract infections and the promising findings from Maleki-Dizaji's study, this plant becomes a highly suitable candidate for further investigation in the current research. In a different investigation, Sonboli et al. (2005) found that the oil of *Stachys schtschegleevii* had a moderately negative impact on gram-positive bacteria. It was *S. aureus* that was most delicate. Gram-negative microbes, with a few exceptions of *E. coli*, were resistant to the oil concentrations utilized (15 ml/disc) [38]. Similarly, methanol

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extracts have been found to be more efficient against gram-positive bacteria in a study on the antibacterial properties of four Stachys creatures from Iran [39]. The authors of the current study validated the findings of Sonboli et al. regarding the impact of the plant *Stachys schtschegleevii* on three bacteria, namely *S. aureus*, *E. faecalis*, and *E. coli*, as an herbal antibiotic. *Stachys schtschegleevii* methanol extracts show strong bacteriostatic and bactericidal effects. The extract of *Stachys schtschegleevii* had considerable bacteriostatic properties against gram-negative bacteria. While the current study, similar to the study by Azimi, confirms the antibacterial effects of *Stachys schtschegleevii* [40].

The global proliferation of antibiotic-resistant pathogens necessitates innovative therapeutic approaches. This investigation identified four potent antibacterial components (α-pinene, β-pinene, linalool, and hexadecanoic acid) within *Stachys schtschegleevii*, each demonstrating distinct efficacy against various uropathogens. The synergistic interactions among these phytocompounds enhance their collective antimicrobial potential, with molecular docking analyses confirming differential binding affinities to bacterial DHFR targets. This natural multi-component synergy offers a significant advantage over single-compound therapeutics by addressing the multifaceted nature of antibiotic resistance through concurrent mechanistic pathways. Combined with the plant's documented anti-inflammatory properties, these bacteriostatic and bactericidal effects against both Gram-positive and Gram-negative pathogens position this phytotherapeutic approach as particularly promising for urinary tract infection treatment. These findings provide a foundation for further phytopharmaceutical development and potential industrial application against escalating antimicrobial resistance challenges.

5. CONCLUSIONS

Stachys schtschegleevii demonstrated significant antimicrobial properties against UTI-associated pathogens (*E. faecalis*, *S. aureus*, and *E. coli*), likely targeting dihydrofolate reductase (DHFR). Gas chromatographic analysis of the essential oil extract identified four bioactive compounds (α-Pinene, β-Pinene, Linalool, and Hexadecanoic Acid) with differential binding affinities to bacterial DHFR proteins, as revealed by in-silico analysis. In vitro experiments confirmed substantial antimicrobial activity through significant inhibition zones and low MIC values. Notably, the synergistic interactions among these phytocompounds enhance their collective efficacy, providing a multi-targeted approach against bacterial resistance that offers advantages over single-compound therapeutics. This natural multi-component synergy, combined with the plant's documented anti-inflammatory properties, positions *Stachys schtschegleevii* as a promising candidate for urinary tract infection management. Future research should explore extraction optimization and potential synergies with conventional antibiotics to address emerging antimicrobial resistance challenges.

Declaration of interest

There are no conflicts to declare. The authors report no conflicts of interest. Also, the authors are responsible for the writing and content of this article.

Research Involving Humans and Animals Statement

This study has obtained ethical approval from the PNU university Ethics Committee with the approval number IR.PNU.REC.1402.001. The research adheres to ethical standards, ensuring the rights and well-being of human participants. All participants in this study received adequate explanation and training, and their consent for data review and publication has been obtained.; Animals Statement: not applicable.

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خواص ضد باکتریایی و فیتوشیمیایی عصاره روغنی گیاه *Stachys schtschegleevii*: بررسی برهمکنش و فعالیت ضد میکروبی علیه باکتری‌های عامل عفونت ادراری از طریق in silico و in vitro

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چکیده

عفونت‌های دستگاه ادراری یکی از چالش‌های مهم سلامت جهانی هستند که به‌طور فزاینده‌ای با مشکل مقاومت میکروبی روبه‌رو شده‌اند. این مطالعه، پتانسیل درمانی روغن اسانسی گیاه ***Stachys schtschegleevii*** را در برابر باکتری‌های شایع ایجادکننده این عفونت‌ها بررسی می‌کند. آنالیز کروماتوگرافی گازی چهار ترکیب زیست‌فعال اصلی را شناسایی کرد (آلفا–پینن، بتا–پینن، لینالول و اسید هگزادکانوئیک) که فعالیت ضد میکروبی هم‌افزا و قابل توجهی نشان دادند. شبیه‌سازی‌های داکینگ مولکولی نشان داد که این ترکیبات گیاهی تمایل اتصال بالایی به آنزیم دی‌هیدروفولات ردوکتاز باکتریایی دارند؛ به‌ویژه آلفا–پینن و بتا–پینن بیشترین پیوند را با DHFR باکتری ***Enterococcus faecalis*** (با انرژی ۶٫۱ –کیلوکالری بر مول) و اسید هگزادکانوئیک با آنزیم ***Staphylococcus aureus*** (نیز با انرژی ۶٫۱– کیلوکالری بر مول) برقرار کردند. آزمایش‌های در شرایط آزمایشگاهی (in vitro) نیز اثربخشی بالای ضد میکروبی این روغن را تأیید کرد؛ به‌طوری که قطر ناحیه بازدارندگی قابل توجهی ثبت شد (برای ***E. faecalis*** معادل ۰٫۲± ۲۰٫۱۶ میلی‌متر، ***E. coli*** برابر ۰٫۴۵ ±۱۷٫۷میلی‌متر، و ***S. aureus*** برابر ۱٫۴۷± ۲۰٫۵۳ میلی‌متر) و مقادیر حداقل غلظت مهارکننده (MIC) چشمگیری نیز به دست آمد (برای ***E. faecalis*** معادل ۶٫۲۵ میلی‌گرم بر میلی‌لیتر، ***E. coli*** برابر ۱٫۵۶ میلی‌گرم بر میلی‌لیتر، و ***S. aureus*** برابر ۳٫۱۲ میلی‌گرم بر میلی‌لیتر). ماهیت چندجزئی این عصاره‌ها، مکانیسم عمل مکملی را ایجاد می‌کند که طی آن چندین ترکیب به‌طور هم‌زمان مسیرهای متفاوتی از باکتری‌ها را هدف قرار می‌دهند، و در نتیجه، احتمال بروز مقاومت دارویی نسبت به درمان‌های تک‌جزئی را به‌شدت کاهش می‌دهد. این هم‌افزایی، همراه با خواص ضدالتهابی گزارش‌شده‌ی این گیاه، آن را به گزینه‌ای برجسته برای توسعه درمان‌های گیاهی نوین در برابر پاتوژن‌های مقاوم در عفونت‌های ادراری تبدیل کرده است؛ درمانی که تعادل مناسبی بین اثربخشی، صرفه اقتصادی، و ایمنی برای بیمار فراهم می‌آورد.

کلید واژه ها

ضدباکتری؛ شبیه‌سازی مولکولی؛ بررسی آزمایشگاهی؛ ***Stachys schtschegleevii***؛ عفونت دستگاه ادراری.