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Exploring the Pharmacological Potential of the Chemically Characterized Essential Oil from *Clinopodium nepeta* subsp. *ascendens*: A Combined *In Vitro* and *In Silico* Analysis

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Abstract: This extensive investigation investigates the many biological properties found in the essential oil extracted from *Clinopodium nepeta subsp*. ascendens (CNEO), a previously unknown subspecies native to eastern Morocco. The study begins with a detailed examination of the phytochemical makeup of CNEO using gas chromatography-mass spectrometry, which reveals a complex spectrum of 24 terpene chemicals. Key components include linalyl acetate (23.28%), trifluoroacetyl-α-terpineol (13.66%), camphor (13.28%), and menthol (9.22%). Beyond compositional clarification, the study carefully explores CNEO's different biological functions. The essential oil has a high total antioxidant capacity (TAC) of 315.07 µg AA/mg and effectively inhibits DPPH free radicals (IC50 = 112.97 ± 2.67 µg/ml). CNEO has shown remarkable antibacterial activity against a variety of pathogens, occasionally surpassing gentamicin, indicating its promise as an antibacterial agent. Equally substantial antifungal activity, surpassing that of cycloheximide, is found with low minimum inhibitory concentrations (MIC), demonstrating its powerful antifungal characteristics. CNEO inhibits xanthine oxidase (IC50 = 30.82 ± 0.78 µg/ml), α-amylase (IC50 = 40.13 ± 1.60 µg/ml), and α-glucosidase (IC50 = 45.30 ± 0.69 µg/ml) activities, indicating potential for glycemic management therapy. Additionally, the essential oil has strong anti-tyrosinase activity (IC50 = 29.78 ± 1.01 µg/ml), suggesting possible dermatoprotective applications for melanin control. Trifluoroacetyl-α-terpineol, a key component of CNEO, inhibits proteins having antibacterial, antifungal, and dermatoprotective activities, according to molecular docking analysis. The implications of these findings lay a solid framework for future research into the entire therapeutic potential of CNEO in medical, cosmetic, and industrial settings.

Keywords: *Clinopodium nepeta subsp. Ascendens; Essential oil; GC-MS; Biological activities; Molecular docking.*

1. Introduction

Herbal medicine persists as a prevalent practice globally, rooted in both biomedical advantages and cultural beliefs (Leonti and Casu 2013). In Morocco, traditional herbal therapy boasts a rich history, delivering substantial benefits in treating various disorders, despite limited scientific exploration of the therapeutic and chemical qualities of the plants involved (Bourhia *et al.,* 2019; El-Mernissi *et al.,* 2023). Numerous traditional Moroccan medicinal herbs are reported to possess remarkable bioactivities, potentially enhancing community health (Ait-Sidi-Brahim, Markouk and Larhsini 2019). Throughout history, people have relied on the healing properties of medicinal aromatic herbs, with medicinal plants offering a wide array of bioactive compounds, including antimicrobial, anti-inflammatory, and antioxidant potentials (Bencheikh *et al.,* 2021; Giannenas *et al.,* 2020; Ramawat, Dass and Mathur 2009). Derived from crude extracts, plant-based medicines utilize a complex combination of phytochemicals to address both chronic and infectious diseases (Adegboye *et al.,* 2021; Pandey *et al.,* 2011; Thomas *et al.,* 2021). While various plant species harbor a vast pool of secondary metabolites, only a few have been extensively studied and proven as significant sources of bioactive compounds (Bencheikh *et al.,* 2023; Cragg and Newman 2001). Essential oils, secondary metabolites responsible for protecting plants against various threats, find applications in antimicrobial, sedative, food additive, and cosmetic products (Diniz do Nascimento *et al.,* 2020; Shanthi and Diwan 2023). Numerous research teams are dedicated to exploring the basic components and properties of essential oils, including their antioxidant, anticorrosive, anthelmintic, antibacterial and antifungal aspects. (Diniz do Nascimento *et al., 2020;* Lazrak *et al.,* 2021*;* Taibi *et al.,* 2023b; Tarfaoui *et al.,* 2021; Tariq *et al.,* 2019; Upadhyay 2010)

The escalating resistance to antibiotics and anotifungals constitutes a substantial threat to global health, stemming from widespread and often inappropriate usage. This resistance poses a critical menace to essential medical treatments, emphasizing the urgent need for responsible antimicrobial practices, the development of new therapeutic strategies, and innovative interventions on a global scale (McEwen and Collignon 2018; Talebi Bezmin Abadi *et al.,* 2019) Diabetes, characterized by the dysregulation of glucose metabolism, emerges as a burgeoning global concern intricately linked to contemporary lifestyles. The rise of Type 2 diabetes, primarily driven by unhealthy behaviors, underscores the imperative for implementing preventive measures and awareness programs to curb its increasing incidence worldwide (Tan *et al.,* 2020; Tinajero and Malik 2021). In dermatology, melanin-related skin disorders, encompassing hyperpigmentation and hypopigmentation, present intricate challenges that impact both aesthetics and psychological well-being (Bolognia and Pawelek 1988; Green *et al.,* 1999; Marks *et al.,* 1986; St Tan, Tang and Peng 2013). The multifaceted nature of these health issues necessitates a comprehensive and interdisciplinary approach to address the diverse dimensions of these global health challenges within the academic discourse.

In the ongoing research focused on the volatile compounds from various endemic plant species, this study delves into the essential oil composition of *Clinopodium nepeta* subspecies in the Eastern region of Morocco. The studied subspecies, *C. nepeta subsp. ascendens*, is distinguished by its purple or pale pinkish-white flowers, measuring 8-10 mm in length. The calyx slightly swells at the base when mature (Quézel and Santa 1962). While subspecies such as *Clinopodium nepeta subsp. nepeta* and *Clinopodium nepeta subsp. glandulosum*, have received significant scientific attention, the focus of this study is on the relatively unexplored *C. nepeta*

subsp. Ascendens. This subspecies presents a unique opportunity for scientific discovery, as it has not been extensively studied before.

Molecular docking is a crucial method in medicinal research for exploring the therapeutic potential of natural substances, including those derived from traditional herbal medicine (Elbouzidi *et al.,* 2024; Ouahabi *et al.,* 2023). Molecular docking utilizes computational simulations to forecast the binding relationships between bioactive compounds and target proteins, providing an efficient method for selecting potential candidates for drug development (Khaldan 2020, 2021). Efficient screening procedures are necessary in the setting of herbal medicine due to the wide range of bioactive chemicals found in plant extracts.

This investigation explores the antioxidant, antimicrobial properties, and impact on glycemic regulation of *C. nepeta subsp. Ascendens*, which has not been extensively studied. Notably, this study combines in vitro and in silico methods. Many studies have highlighted the lack of research on essential oils from endemic plant species, especially regarding their pharmacological potential. These studies have shown the effectiveness of using combined in vitro and in silico approaches to understand the biological benefits of these compounds (Helaly, El-Bindary and Elsayed 2023; Rezk *et al.,* 2023). The in silico analysis complements the in vitro assays, providing a more comprehensive understanding of the essential oil's pharmacological potential. By employing this multidimensional approach, this work aims to uncover the full range of biological activities associated with the essential oil from *C. nepeta subsp. ascendens* (CNEO), thereby contributing novel insights into its pharmacological applications.

2. Methodology

2.1. Plant material

The research centered on *Clinopodium nepeta subsp. ascendens*, indigenous to Ahfir province in northeastern Morocco. The plant's aerial parts, including leaves and flowers, were methodically acquired from a local market in the spring of 2022 and subsequently transported to the Faculty of Sciences at Mohammed 1er University in Oujda. The primary objective of this botanical inquiry was the meticulous taxonomic identification of the specimens, an essential process for the precise classification of the plant species in question. It is noteworthy that the collection deliberately included both vegetative and reproductive structures to ensure a holistic taxonomic assessment, considering the full morphological spectrum and floral attributes of *Clinopodium nepeta subsp. ascendens*. The identification was conducted by the esteemed botanist, Professor Mohammed ADDI, affiliated with the Faculty of Science in Oujda.

2.2. Essential oil extraction

Essential oil extraction was carried out from the *Clinopodium nepeta* sp. *ascendens* aerial part using the hydrodistillation method with a modified Clevenger configuration. This technique was chosen to preserve the volatile components of the essential oil, in line with the methodology described by Taibi *et al.,* (Taibi *et al.,* 2023a, 2023b).

2.3. Phytochemical composition by GC-MS

In order to elucidate the chemical composition of *Clinopodium nepeta subsp. ascendens* essential oil (CNEO. The analytical setup included a Shimadzu GC system and an MS QP2010, both procured from Kyoto, Japan. These instruments were equipped with a BPX25 capillary column featuring a 5% diphenyl and 95% dimethylpolysiloxane phase, measuring 30 m in length,

0.25 mm in inner diameter, and 0.25 µm in film thickness. Pure helium gas (99.99%) served as the carrier gas at a constant flow rate of 3 mL/min. The operational temperatures for injection, ion source, and interface were uniformly maintained at 250°C. The column oven temperature was initially set at 50°C for 1 minute, followed by a ramp to 250°C at a rate of 10°C/min, also held for 1 minute. Ionization of sample components occurred in electron impact (EI) mode at 70 eV, scanning a mass range of 40-300 m/z. In a splitless injection mode with a split ratio of 90:1, 1 µL of each diluted extract was introduced into the system. Duplicate analyses were performed for all samples. Constituent identification was based on retention time alignment and mass spectral fragmentation pattern matching with verified standards and databases, including the National Institute of Standards and Technology (NIST) library. Data acquisition and processing were facilitated by Laboratory Solutions software version 2.5 (Elbouzidi *et al.,* 2024; Loukili *et al.,* 2023; Ouahabi *et al.,* 2023). This robust analytical approach facilitates a comprehensive exploration of the phytochemical composition inherent in this essential oil. By establishing a detailed understanding of the chemical constituents present, this analysis significantly contributes to unraveling the nuanced properties and potential applications of CNEO within the academic realm of botanical and pharmaceutical research.

2.4. Non-Enzymatic Antioxidant Assays: 2,2-Diphenyl-1-Picrylhydrazyl (DPPH), and Total Antioxidant Capacity (TAC)

The essential oil's capacity to scavenge 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) radicals was evaluated according to the method described in (Al-Mijalli *et al.*, 2023; Haddou *et al.*, 2023; Zrouri *et al.,* 2021), with slight modifications. Each concentration was tested three times. A 0.1 mM DPPH (2,2-diphenyl-1-picrylhydrazyl) solution was prepared, and various extract concentrations (0.2 to 1 mg/mL) were used. Ascorbic acid was employed as a positive control (Ouahabi *et al.,* 2023). Total antioxidant activity measures how well CNEO can protect the body from damage caused by harmful substances called free radicals (Chaudhary *et al.,* 2015). The total antioxidant activity, assessing the ability of CNEO to protect the body from damage caused by free radicals, was determined using the phosphorus-molybdenum technique, similar to the method described by (Elbouzidi *et al.*, 2023). A standard curve was constructed based on ascorbic acid, and the findings were subsequently presented in terms of ascorbic acid equivalents (Prieto, Pineda and Aguilar 1999).

2.5. Antimicrobial activity

2.5.1. Disk Diffusion Method

The bacterial strains, comprising *S. aureus (ATCC 6538), M. luteus (LB 14110)* and*, P. aeruginosa*, (ATCC 15442), *E. coli (ATCC 10536),* as well as the fungal strains, encompassing, *P. digitatum* (clinical isolate)*, A. niger* (clinical isolate), *C. glabrata* (clinical isolate)*,* and *R. glutinis* (ON 209167), were introduced onto Mueller-Hinton agar plates and PDA plates, respectively. Sterile paper discs were impregnated with 15 µL of *Clinopodium nepeta subsp. ascendens* essential oil (CNEO) for bacteria, and fungi. Gentamicin (1 mg/mL) functioned as the positive control for bacteria, while cycloheximide (1 mg/mL) served as the positive control for fungi. Following specific incubation durations (24 hours at 37°C for bacteria and 48 hours at 25°C for fungi), the resulting inhibition zones were measured around the discs for each concentration of CNEO and the positive controls (gentamicin and cycloheximide) (Elbouzidi *et al.,* 2023; Haddou *et al.,* 2024; Hayani *et al.,* 2022). This methodological standardization facilitated a comprehensive assessment

of CNEO's antimicrobial efficacy against a diverse array of bacterial and fungal strains, contributing substantively to the understanding of its potential as a valuable antimicrobial agent within scientific discourse.

2.5.2. Determination of MIC

The experimental protocol used in this study to determine the minimum inhibitory concentration (MIC) of CNEO followed the 96-well microplate method. Mueller-Hinton culture medium containing 0.15% agar was chosen because of the insolubility of essential oils in water. MIC was assessed over a concentration range from 8 to 0.0015%. Microplates were incubated at specific temperatures (37°C for 24 hours for bacteria, 25°C for 48 hours for fungi) (Al-Mijalli *et al.,* 2023; Mrabti *et al.,* 2023; Taibi *et al.,* 2024). Resazurin was used to monitor growth, with visual observations of color change. Gentamicin and cycloheximide were used as positive controls for bacteria and fungi, respectively. Results were obtained by conducting the test in triplicate to ensure the reproducibility of observations, thereby enabling a reliable assessment of the antimicrobial efficacy of CNEO against various bacterial and fungal strains.

2.5.3. Determination of MBC and MFC

To determine the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC), 3 µL samples from the wells showing no observable microbial growth, were transferred to MHA growth medium and YEG culture medium suitable for bacteria and fungi, respectively. Samples were then incubated at specific temperatures: 37°C for 24 hours for MBC, 25°C for 48 hours for *Rhodotorula glutinis* and *C. glabrata*, and 72 hours for *P. digitatum* and *A. niger* for MFC. After the incubation periods, minimum concentrations were established as those of CNEO causing no observable bacterial growth and those preventing fungal growth.

2.5.4. Antidiabetic activity/ α-Amylase Inhibition, α-Glucosidase Inhibition, and Xanthine Oxidase Assays

For the exploration of the potential inherent antidiabetic properties in *Clinopodium nepeta* subsp. *ascendens* essential oil (CNEO), this study embarks on a comprehensive assessment integrating three pivotal assays. The α-amylase and α-glucosidase inhibition assays serve as foundational components, thoroughly delving into CNEO's inhibitory effects on key enzymes central to glucose metabolism (Ouahabi *et al.,* 2023; Bouslamti *et al.,* 2023), For the α-amylase assay, isolated compounds from CNEO undergo pre-incubation with α-amylase solution at 1 U/mL for 10 minutes at 37 °C. The reaction initiates with the addition of 30 µL of soluble starch (0.5% in deionized water), followed by a 6-minute incubation at 37 °C. The reaction is then terminated with 20 µL of hydrochloric acid (1 M) and 120 µL of a 0.25 mM iodine solution. Absorbance at 565 nm is measured using a MultiskanTM Microplate Spectrophotometer. Simultaneously, the α-glucosidase assay utilizes a methanolic stock solution mixed with 0.1 M potassium phosphate buffer and α-glucosidase enzyme solution (0.5 U/mL) for a 6-minute incubation at 25 °C. The reaction includes a subsequent addition of 20 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside substrate, followed by an 8-minute incubation. Termination is achieved with 100 µL of 0.2 M Na_2CO_3, and absorbance is recorded at 405 nm (Yin *et al.,* 2014). The XO test, commonly used for its relevance in antidiabetic assays and conducted under aerobic conditions, serves as a diagnostic tool for diabetes, critically explores CNEO's inhibitory potential on xanthine oxidase. The assay mixture comprises 50 ml of the test solution, 35 ml of 70 mM phosphate buffer (pH

7.5), and 30 ml of enzyme solution (0.01 units/ml) for a 15-minute preincubation at 25 °C. The reaction begins with the addition of 60 ml of substrate solution (150 mM xanthine in the same buffer) and incubation at 25 °C for 30 minutes (Umamaheswari *et al.,* 2007). The reaction is halted with 25 ml of 1 N HCl, and absorbance at 290 nm is measured using a Perkin-Elmer HTS-7000 Bio Assay Reader. The amalgamation of insights derived from these three methodologically distinct assays collectively enriches our understanding of the multifaceted antidiabetic potential within CNEO. The intricate details gleaned from the α-amylase and α-glucosidase assays, coupled with the nuanced insights provided by the XO assay, collectively position CNEO as a promising candidate for further exploration in the domain of diabetes management. This study underscores the pivotal role of CNEO within the realm of diabetes research and its potential therapeutic applications.

2.5.5. Dermatoprotective Activity: Tyrosinase Inhibition Assay

To assess the dermatoprotective potential of CNEO, we employed a modified version of the method described by Bouyahya *et al.,* (Bouyahya *et al.,* 2019, 2021) to evaluate tyrosinase inhibitory activity. In brief, 25 µL of the CNEO sample was combined with 100 µL of tyrosinase solution (333 U/mL, 50 mM phosphate buffer, pH 6.5) and incubated at 37 ◦C for 10 minutes. Following this, 300 µL of L-DOPA (5 mM) was added, and the mixture underwent a 30-minute incubation at 37 ◦C. Absorbance readings were then taken at 510 nm using a spectrophotometer. Tyrosinase inhibition levels were computed at CNEO concentrations of 40, 60, 120, and 160 µg/mL, and the IC50 values were determined. Quercetin was employed as the positive control in this study.

2.6. Molecular Docking Protocol

2.6.1. Ligand and Protein Preparation

Table 1 shows the structures of the 24 volatile compounds that have been found in CNEO. Their PubChem CIDs are also reported [source: https://pubchem.ncbi.nlm.nih.gov/; accessed on September 26, 2023]. It is important to emphasize that, for the docking study, we specifically selected 14 primary compounds out of the 24 identified in CNEO. These 14 compounds were chosen because they collectively account for over 90% of the overall composition of the essential oil. A Collection of standard reference drugs, encompassing the antioxidant BHT (PubChem CID: 31404), the antibacterial and antifungal agents' trimethoprim (PubChem CID: 5578), and itraconazole (PubChem CID: 55283), were retrieved in SDF format. Subsequent to the acquisition of these compounds, they were incorporated into Discovery Studio version 4.5, and a comprehensive ligand library in PDB format was generated, utilizing the PubChem CIDs as identifiers. To refine the accuracy of molecular interactions for all identified phytoconstituents and standard ligands, the semi-empirical Pm6 method was applied (Bikadi and Hazai 2009). Employing a computational paradigm, we sought to prognosticate the potential antioxidant, antibacterial, antifungal, anti-inflammatory, cytotoxic, and dermatoprotective properties latent within the identified compounds. Specific proteins were thoroughly chosen based on precedent studies for interaction: bacterial dihydrofolate reductase from *Staphylococcus aureus* (DHFR) enzyme (PDB ID: 2W9G) for its antibacterial effects (Khatun *et al.,* 2021), lanosterol alpha-sterol demethylase from *Saccharomyces cerevisiae* (CYP51, PDB ID: 5EQB), tyrosinase (PDB ID: 5I3B) (di Giacomo *et al.,* 2021) for dermatoprotective activity (El Hachlafi *et al.,* 2024; Heaslet *et*

al., 2009; Loukili *et al.*, 2023; Monk *et al.*, 2014; Taibi *et al.*, 2023a; Wood *et al.*, 2004). The three-dimensional crystal structures of these carefully selected proteins were retrieved from the RCSB Protein Data Bank (source: https://www.rcsb.org/; accessed on September 26, 2022) in PDB format. Following this retrieval, a processing phase ensued, employing the PyMOL 2.3 tool to eliminate extraneous water molecules and superfluous protein residues. Nonpolar hydrogen atoms were introduced to ensure structural completeness. Subsequently, the energy levels of these proteins were methodically optimized to their lowest state using the Swiss PDB viewer, an energy minimization tool (Guex and Peitsch 1997). After this cleaning and optimization process, the macromolecules were saved in PDB format, setting the stage for a more comprehensive and in-depth analysis.

2.6.2. Ligand-Protein Interaction, and Docking Validation

The exploration of potential binding patterns and affinities between isolated plant metabolites and specific target biomolecules was undertaken through the implementation of a molecular docking approach (Elbouzidi *et al.*, 2022). This computational method involved utilizing a semi-flexible modeling approach and was executed with the widely recognized molecular docking software, PyRx AutoDock Vina. The preparation and formatting of target proteins as macromolecules in PyRx served as a crucial preliminary step, facilitating the subsequent stages of the computational interaction process (Pawar and Rohane 2021). In our study, we employed the ligand minimization tool provided by the Schrödinger Suite software. Ligand structures were minimized using the OPLS3 force field to optimize their geometries and energetics prior to molecular docking analysis (Harder *et al.*, 2016). The selection of the most optimal hit was based on this energetically optimized state (O'Boyle *et al.*, 2011). Grid boxes were then strategically established, centering and mapping active binding sites for proteins. To provide a specific example, grid mapping for proteins was defined at table coordinates (refer to **Table S1** in the supplementary material). Throughout this process, all other docking parameters were maintained at their default settings, and AutoDock Vina 1.1.2 was employed for the docking process. Upon completion of the docking process, the analysis results were projected. The outcomes, together with the anchored macromolecules and ligands, were exported in pdbqt format as output files. Subsequently, these files for ligands and the macromolecule were merged and saved in PDB format. This prepared data set the stage for a more in-depth examination, and the PyMol software was utilized for this purpose. Lastly, two-dimensional (2D) visualizations were generated through the application of Discovery Studio Visualizer (version 4.6), providing comprehensive insights into the molecular interactions and potential binding modes.

The validation of the docking procedure involved the measure of the quality of the reproduction of a known binding pose with the docking protocol used for the experiments. This was achieved by calculating the root-mean-square deviation (RMSD) between the co-crystallized ligand and the best docking pose, by using 2.5 Å as threshold (Dickerhoff *et al.*, 2021). The validation process was conducted using PyMOL version 2.3, and RMSD values were then calculated (**Table S1**). 3D images of the superimposed ligands were given in the supplementary file **Figure S1**. The native co-crystallized orientation of the redocked ligands were found to have RMSD values ranging from 0.03 to 2.04 Å, which suggests a successful docking procedure (Dickerhoff *et al.*, 2021).

3. Results and Discussion

3.1. Phytochemical composition

Gas chromatography-mass spectrometry (GC-MS) analysis of CNEO reveals a diverse compound composition, primarily comprising 24 terpene compounds, including notable percentages of linalyl acetate (23.28%), Trifluoroacetyl-α-terpineol (13.66%), camphor (13.28%), and menthol (9.22%) (**Figures 1 & 2**; **Table 1**). These findings underscore the richness of terpene compounds, renowned for their antioxidant, anti-inflammatory, antibacterial, and anti-fungal properties. Linalyl acetate, the predominant compound, imparts calming properties, while Linalyl acetate enhances antimicrobial effects (Alqahtani *et al.,* 2023; Khayyat 2020; Peana *et al.,* 2002). Camphor and menthol contribute to soothing effects with their anti-inflammatory properties. Additionally, terpene compounds like eucalyptol and terpinen-4-ol offer respiratory benefits (Galan *et al.,* 2020).

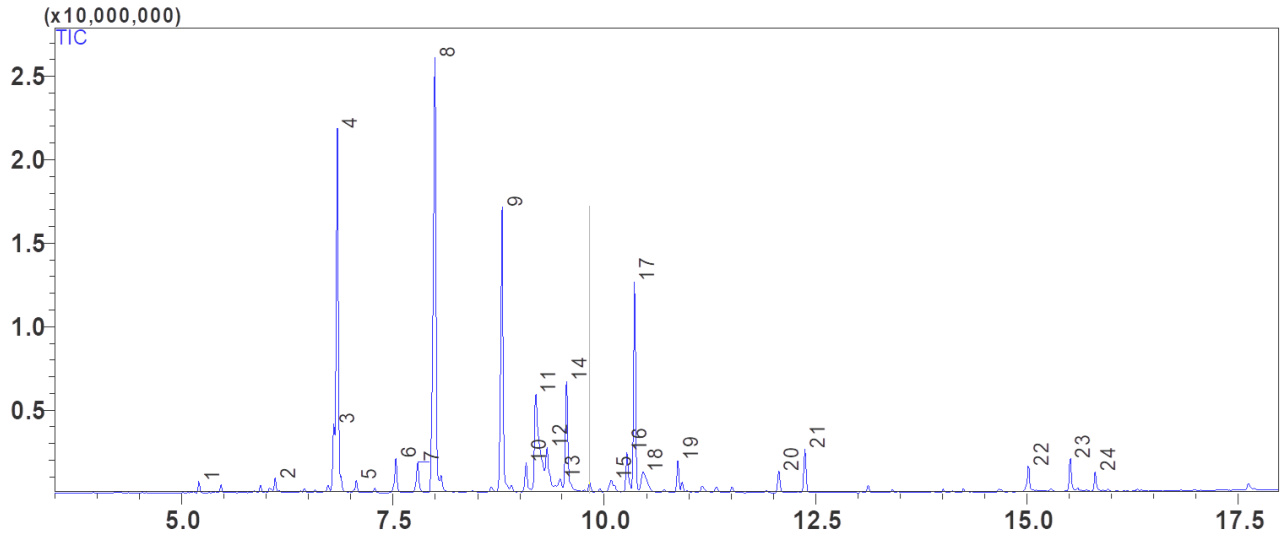

Figure 1. GC-MS CNEO chromatogram. The peaks in the chromatogram represent the volatile compounds identified and quantified in **Table 1**.

The abundance of terpene compounds in CNEO suggests a potential synergy of health benefits, particularly in relaxation, anti-inflammation, and pathogen control (Beddiar *et al.,* 2021). These bioactive molecules may play a pivotal role in the diverse biological activities of this aromatic and medicinal plant. The intricate interplay of these compounds could underlie the plant's pharmacological effects, spanning antimicrobial, anti-inflammatory, and even anticancer properties. The identification of the phytochemical composition opens avenues for further exploration into the biological properties and therapeutic implications of these specific compounds. Several studies have been carried out on essential oil extracted from *C. nepeta* (Beddiar *et al.,* 2021; Benkhaira *et al.,* 2023; Debbabi *et al.,* 2020; ÖZTÜRK *et al.,* 2021; Rodenak-Kladniew *et al.,* 2023). Still, the present study marks the first investigation into essential oil extracted from the Moroccan subspecies *C. nepeta subsp. ascendens*. GC-MS analysis revealed an abundance of terpene compounds in *C. nepeta subsp. ascendens* essential oil, including linalyl acetate, trifluoroacetyl-α-terpineol, camphor, and menthol stand out as major components. These constituents are recognized for their biological and pharmacological properties, and even minority compounds can interact synergistically to influence these biological activities. The results obtained differ from those reported by Rodenak-Kladniew *et al.,* (2023), who found that the essential oil of

another *C. nepeta* (L.) subspecies, Kuntze *subsp. spruneri*, is dominated by the presence of other major terpene compounds such as pulegone (37.2%), menthone (26.6%), and isomenthone (11.7%) (Rodenak-Kladniew *et al.,* 2023). They also differ from the results found by Öztürk *et al.,* (2021) for oil extracted from *C. nepeta* subsp. *glandulosum* (Req.), who identified piperitone oxide (47.8%), limonene (18.6%) and piperitone oxide II (13.6%) as major components (ÖZTÜRK *et al.,* 2021). These variations in chemical composition can be attributed to a number of factors, including subspecies, environmental factors, the vegetative cycle, plant age, and even genetic factors (Massad *et al.,* 2011).

Table 1. Phytochemical constituents of CNEO. Rt: retention time.

N°	Compounds	Rt (min)	% Relative Area	Identification
1	α-pinene	5.202	0.38	MS, RT
2	β-myrcene	6.104	0.55	MS, RT
3	Eucalyptol	6.800	3.17	MS, RT
4	**Trifluoroacetyl-α-terpineol**	6.840	**13.66**	MS, RT
5	p-Cymene	7.065	0.49	MS, RT
6	Linalool oxide	7.533	1.56	MS, RT
7	Linalool	7.792	1.46	MS, RT
8	**Linalyl acetate**	7.995	**23.28**	MS, RT
9	**Camphor**	8.790	**13.28**	MS, RT
10	Borneol	9.074	1.56	MS, RT
11	**Menthol**	9.189	**9.22**	MS, RT
12	Terpinen-4-ol	9.322	3.07	MS, RT
13	4,6-Octadienoic acid, 2-acetyl-2-methyl-, ethyl ester	9.476	0.96	MS, RT
14	**α-terpineol**	9.553	**6.43**	MS, RT
15	Ethanol, 2-(3,3-dimethylcyclohexylidene)-, (Z)-	10.081	0.96	MS, RT
16	Pulegone	10.268	2.02	MS, RT
17	**Linalyl anthranilate**	10.359	**7.74**	MS, RT
18	Geranyl vinyl ether	10.460	2.42	MS, RT
19	Lavandulyl acetate	10.870	1.28	MS, RT
20	Neryl acetate	12.066	0.97	MS, RT
21	Geranyl acetate	12.374	1.86	MS, RT
22	Caryophyllene oxide	15.019	1.39	MS, RT
23	Bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-methyl-9-methylene-	15.514	1.43	MS, RT
24	α-Bisabolol	15.809	0.86	MS, RT
	Monoterpene hydrocarbons		9	16.65
	Oxygenated monoterpenes		12	80.86
	Sesquiterpenes hydrocarbons		1	1.43
	Oxygenated sesquiterpenes		-	-
	Others		2	1.92
	Total identified (%)		24	99.43

3.2. Antioxidant Activity

The outcomes of the assessment of CNEO's antioxidant activity were derived from two distinct methodologies, encompassing the measurement of total antioxidant capacity (TAC) and

the evaluation of antioxidant activity against the DPPH free radical (**Table 2**). Determining the total antioxidant capacity (TAC) of CNEO yielded a noteworthy outcome of 315.07 ± 4.55 µg AA/mg CNEO, providing insight into the comprehensive antioxidant potential of the essential oil. Regarding specific antioxidant activity against the DPPH free radical, CNEO exhibited a value of 112.97 ± 2.67 µg/mL. Although marginally lower than that of ascorbic acid (125.23 ± 3.89 µg/mL), employed as a positive control in this study, these findings authenticate the antioxidant effectiveness of CNEO. This remarkable antioxidant activity can be attributed to bioactive terpene compounds, such as menthol, the major compound in this essential oil

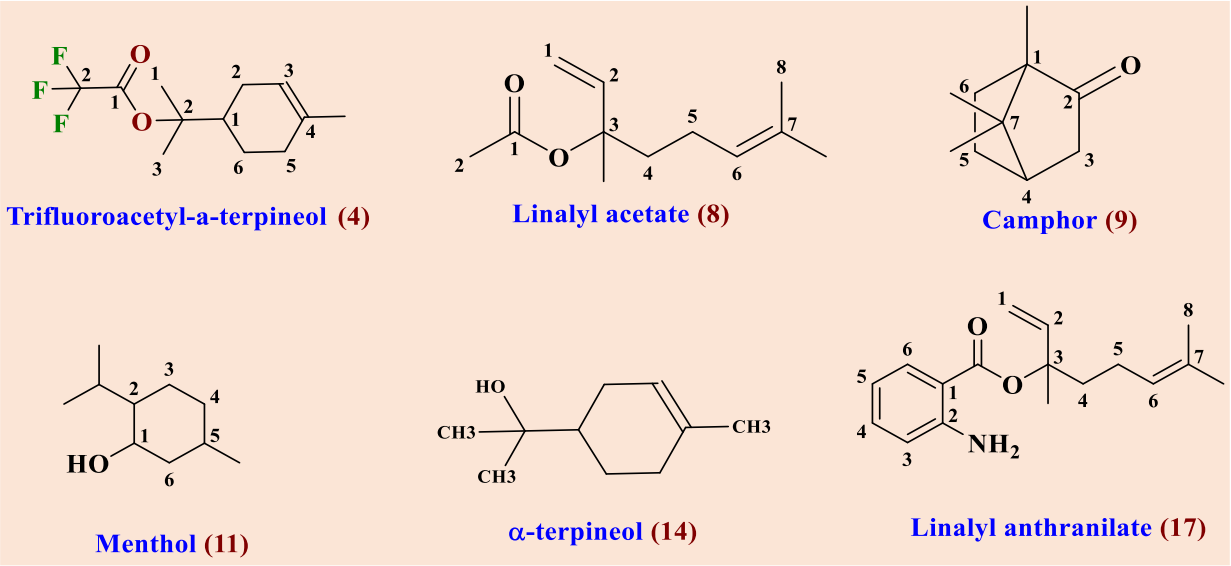

Figure 2. Chemical structures of the major compounds identified in CNEO.

Rozza *et al.,* demonstrated that menthol possesses remarkable antioxidant activity, reinforcing the understanding of CNEO as a promising antioxidant agent (Rozza *et al.,* 2014). These findings suggest exciting avenues for future applications in health and nutrition. These results align with those found by Benkhaira, *et al.,* and Beddiar, *et al.,* who found that essential oil extracted from other subspecies of this plant showed remarkable antioxidant results. All these results conclusively confirm CNEO's remarkable antioxidant capacities, highlighting its strong potential as a natural antioxidant agent (Beddiar *et al.,* 2021; Benkhaira *et al.,* 2023). This evidence suggests that CNEO is a promising alternative against oxidative stress, heralding potential applications as a natural antioxidant agent.

Table 2. Evaluation of the antioxidant activity of CNEO

EO/Reference	Antioxidant Activity	
	TAC (µg AA/mg)	DPPH (µg/mL)
CNEO	315.07 ± 4.55	112.97 ± 2.67
Ascorbic acid	-	125.23 ± 3.89

3.3. Antimicrobial activity

3.3.1 Antibacterial activity

The detailed results of evaluating the antibacterial activity of the essential oil extracted from *Clinopodium nepeta* subsp. *ascendens* (CNEO), presented in **Table 3**, reveal promising properties against various bacterial strains. These include *Staphylococcus aureus* and *Micrococcus luteus* as

Gram-positive bacteria, and *Escherichia coli* and *Pseudomonas aeruginosa* as Gram-negative bacteria. offering a significant prospect compared with gentamicin, a commonly used antibiotic. Inhibition zone diameters, such as 32.5 mm for *S. aureus*, 22 mm for *M. luteus*, 24 mm for *E. coli*, and 16 mm for *P. aeruginosa*, demonstrate significant activity of the essential oil. These results even surpassed those of gentamicin in some cases. The results of the Minimum Inhibitory Concentrations (MIC) and Bactericidal Concentrations (MBC) present crucial aspects of evaluating the antibacterial activity of CNEO. These parameters offer valuable insights into the efficacy and potency of the essential oil in its interaction with the bacterial strains tested. The MIC values, ranging from 0.125% to 2% (*v/v*), testify to the remarkable efficacy of the essential oil at relatively low concentrations. This observation is particularly encouraging, underlining the ability of the essential oil to inhibit bacterial growth even at moderate doses. A low MIC indicates that low quantities of essential oil are required to inhibit bacterial proliferation, suggesting strong inhibitory activity. Minimum Bactericidal Concentrations (MBC), ranging from 0.25% to 4% (*v/v*), underlining the ability of the essential oil to inhibit bacterial growth and actively eliminate the bacteria tested. A low MBC indicates that essential oil can prevent bacterial growth and eradicate existing bacteria. This notable bactericidal property reinforces the essential oil's position as a powerful antimicrobial agent. The remarkable efficacy of essential oil at low concentrations has major practical implications, improving its tolerance while preserving its effectiveness and opening up opportunities for application in medicine and industry. Its remarkable bactericidal property reinforces its status as a potent antimicrobial agent, suggesting medical applications ranging from local disinfection to infection prevention. MIC and MBC results underline the versatility and efficacy of this essential oil, warranting further research to explore its molecular mechanisms and clinical and industrial applications.

Table 3. Antibacterial Efficacy Evaluation

Bacterial Strain	***S. aureus***	***M. luteus***	***E. coli***	***P. aeruginosa***
Essential oil, IZ [1,2]	32.5	22	24	16
Gentamicin, IZ [1,2] (1 mg/mL)	19.5	21.5	22.5	20.5
DMSO	0.0	0.0	0.0	0.0
MIC (% *v/v*)	0.125	1	1	2
MBC (% *v/v*)	0.25	4	2	4

All values in this table represent mean ± SD (n = 3).
[1]: 15µL was the volume used for the disc diffusion method.
[2]: diameter of inhibition zone (mm).

3.3.2 Antifungal activity

As delineated in the **Table** below, the outcomes of assessing the antifungal potential of the essential oil extracted from *Clinopodium nepeta subsp. ascendens* unveil its notable efficacy against a diverse array of fungal strains. The comparisons made with cycloheximide, a widely employed antifungal agent, provide valuable insights into the essential oil's performance. The diameters of the Inhibition Zone (IZ) prominently showcase the remarkable antifungal capabilities of the essential oil, surpassing cycloheximide in several instances. Specifically, the IZ measurements of 43.5 mm for *Aspergillus* spp. and 35.5 mm for *Candida glabrata* underscore the potent inhibitory effect on fungal growth. The Minimum Inhibitory Concentration (MIC) values

shed light on the essential oil's effectiveness at concentrations ranging from 0.125% to 2% (*v/v*) (**Table 4**). This indicates a substantial capacity to impede fungal growth, even at relatively modest concentrations, emphasizing its potential utility at lower doses. Furthermore, the Minimum fungicidal Concentrations (MFC), spanning from 0.5% to 4% *v/v*, emphasize the inhibitory and active eradication capabilities of the essential oil against the tested fungal strains.

These findings strongly imply that the essential oil harbors noteworthy antifungal activity, expanding its application horizons into promising realms such as medicine, agronomy, and industry. The multifaceted efficacy showcased against fungal strains positions this essential oil as a versatile and potent agent with broad potential applications in various fields.

Table 4. Antifungal Potency Assessment

Fungal Strain	*P. digitatum*	*A. niger*	*C. glabrata*	*R. glutinis*
Essential oil, IZ [1,2]	19.5	43.5	35.5	31.5
Cycloheximide, IZ [1,2] (1 mg/mL)	19.5	21.5	22.5	20.5
DMSO	0.0	0.0	0.0	0.0
MIC (% *v/v*)	1	0.125	0.5	2
MFC (% *v/v*)	2	0.5	2	4

All values in this table represent mean ± SD (n = 3). [1]: 15µL was the volume was used for the disc diffusion method. [2]: diameter of inhibition zone (mm).

This notable antimicrobial efficacy can be ascribed to the intricate chemical composition of the essential oil, particularly the presence of bioactive molecules such as terpenes, exemplified by linalyl acetate. Antecedent investigations have substantiated the inhibitory prowess of linalyl acetate against a spectrum of bacterial and fungal strains, denoting a wide-ranging scope of action (Khayyat 2020; Mishra *et al.,* 2012). Parallel research has underscored the antimicrobial potential of camphor, the primary organic constituent of this essential oil. Findings signify its inhibitory capabilities vis-à-vis the growth of diverse bacterial strains, predominantly those of Gram-positive nature, through the disruption of cellular membranes. Additionally, camphor has exhibited antifungal properties by impeding the growth of select fungi (Kong *et al.,* 2022; Shokova, Kim and Kovalev 2016). These outcomes align with prior investigations, including those conducted by Debbabi et al, who observed antifungal attributes in the essential oils of *Clinopodium nepeta subsp. nepeta* and *subsp. glandulosum* (Debbabi *et al.,* 2020). They also lend support to the conclusions posited by Benkhaira *et al.,*, revealing the efficacy of *C. nepeta* essential oil against a spectrum of pathogenic microorganisms, encompassing both bacterial and fungal entities (Benkhaira *et al.,* 2023).

3.4. In Vitro Inhibition of Xanthine Oxidase, α-Amylase and α-Glucosidase

The results of CNEO's antidiabetic activity (**Table 5**) reveal valuable information on its potential as a therapeutic agent, considering the molecular mechanisms involved in blood glucose regulation. Concerning inhibition of xanthine oxidase, an enzyme involved in purine metabolism, CNEO demonstrated significant activity with an IC50 of 30.82 ± 0.78 µg/mL. Xanthine oxidase is a key enzyme in the production of uric acid from xanthine and hypoxanthine. Although allopurinol, the positive control, showed slightly higher activity with an IC50 of 23.34 ± 0.09 µg/mL, CNEO remains promising in this capacity, suggesting potential for managing hyperuricemia-related complications in diabetic patients. About the inhibition of α-amylase, a

digestive enzyme crucial in breaking starch into glucose units, CNEO exhibited an IC50 of 40.13 ± 1.60 µg/mL. α-Amylase plays a key role in digesting complex carbohydrates into glucose, and its inhibition may help regulate postprandial glucose release. Although this value is slightly higher than that of acarbose, the positive control, which showed an IC50 of 35.48 ± 0.69 µg/mL, CNEO maintains significant activity in modulating this process. The most remarkable aspect is the inhibition of α-glucosidase, an enzyme in the intestinal mucosa that catalyzes the breakdown of complex carbohydrates into glucose. CNEO showed an IC50 of 45.30 ± 0.69 µg/mL, surpassing the efficacy of acarbose (IC50: 65.41 ± 2.10 µg/mL). Inhibition of α-glucosidase is crucial for attenuating glucose absorption in the intestinal tract, thus contributing to blood glucose regulation. This remarkable anti-diabetic activity could be attributed to the richness of its composition in bioactive terpene molecules, especially linalyl acetate, recognized for its proven anti-diabetic properties according to previous studies (Alqahtani *et al.,* 2023). The work of El Omari *et al.,* also highlighted camphor as a major compound in this essential oil, also endowed with proven anti-diabetic activity (Benkhaira *et al.,* 2023). These two compounds, linalyl acetate and camphor, thus emerge as key players that could be responsible for the exceptional anti-diabetic activity observed in the essential oil in question (El Omari *et al.,* 2023). This convergence of results suggests a possible synergy between linalyl acetate and camphor, acting in tandem to exert a beneficial influence on glucose metabolism enzymes. These findings align with those presented by Benkhaira *et al.,* who evaluated the activity of *C. nepeta* essential oil from another region (Middle Atlas of Morocco) and another subspecies (Benkhaira *et al.,* 2023). All these overall results suggest that CNEO could be a promising candidate for developing antidiabetic therapies.

Table 5. xanthine oxidase, α-amylase, and α-glucosidase inhibitory activities of *C. nepeta subsp. ascendens* essential oil (CNEO) in terms of IC_{50} values.

Essential oil/Positive control	IC50 (µg/mL), ± SD		
	Xanthine Oxidase Inhibition	α-Amylase Inhibitory Activity	α-Glucosidase Inhibitory Activity
CNEO	30.82 ± 0.78 [a]	40.13 ± 1.60 [a]	45.30 ± 0.69 [a]
Allopurinol	23.34 ± 0.09 [b]	-	-
Acarbose *	-	35.48 ± 0.69 [b]	65.41 ± 2.10 [b]

* Positive controls. Values are means ± SD (standard deviation); a,b, indicate significant differences in each column at $p < 0.05$.

3.5. Tyrosinase inhibition assay

The evaluation of anti-tyrosinase activity holds paramount importance in dermatological research due to the enzyme's pivotal role in melanogenesis—the biosynthetic pathway for melanin. Melanin is the primary pigment responsible for the coloration of skin, hair, and eyes. It is synthesized through the enzymatic conversion of tyrosine to DOPA, a reaction catalyzed by tyrosinase (El Hachlafi *et al.,* 2024). This process is not only crucial for melanosome formation but also for the subsequent production of melanin, thereby significantly influencing pigmentation and the overall appearance of skin and its texture. Understanding and modulating tyrosinase activity is therefore essential for developing dermatoprotective strategies that can manage pigmentation disorders.

In the present study, the anti-tyrosinase activity of CNEO was assessed using the tyrosinase inhibitor assay (**Table 6**). The results revealed a value of 29.78 ± 1.01 µg/mL, for the anti-tyrosinase activity of CNEO, compared with that of the control, quercetin, which showed a value

of 22.15 ± 0.12 µg/mL. These data highlight the ability of CNEO essential oil to modulate tyrosinase activity, suggesting a dermatoprotective potential in the regulation of melanin synthesis. These findings imply the potential of CNEO in dermatoprotection and the regulation of melanin synthesis, thereby presenting promising prospects for the development of dermatological products targeting hyperpigmentation concerns and promoting balanced skin pigmentation. The observed activity is tentatively attributed to camphor, with existing studies indicating its inhibitory impact on tyrosinase, thereby impeding the conversion of tyrosine to DOPA and mitigating melanin production (Shokova, Kim and Kovalev 2016). These observations underline the importance of pursuing investigations into the possible applications of CNEO in the development of dermatological products aimed at alleviating hyperpigmentation problems and maintaining balanced skin pigmentation, thus offering appreciable aesthetic benefits.

Table 6. *In vitro* dermatoprotective activity using Tyrosinase inhibition assay.

Assay	CNEO	Control (Quercetin)
	IC50 (µg/mL)	
Tyrosinase	29.78 ± 1.01	22.15 ± 0.12

Values are mean ± SEM (n = 3)

3.6. Molecular Docking Predictions of the Possible Mechanisms of Action of CNEO Compounds

To comprehend how *C. nepeta subsp. ascendens* Essential Oil (CNEO) impacts pharmacological activities, we conducted molecular docking of its bioactive components with corresponding molecular receptors using various computational methods. The binding strength is inversely proportional to the numerical value of the binding affinity (kcal/mol). The prediction of the top docking demonstrated an expected binding affinity with a root mean square deviation of zero (Ouahabi *et al.*, 2023). In this study, we employed a method to assess the binding affinities of 14 compounds present in CNEO (that represent more than 90% of the total chemical composition of the essential oil). These compounds were tested against five proteins associated with diverse biological functions: Dihydrofolate reductase from *S. aureus* (DHFR, PDB ID: 2W9G) for antibacterial activity, lanosterol 14-alpha demethylase (CYP51, PDB ID: 5EQB) for antifungal activity (El Hachlafi *et al.*, 2024; Heaslet *et al.*, 2009; Loukili *et al.*, 2023; Monk *et al.*, 2014; Taibi *et al.*, 2023a; Wood *et al.*, 2004).

The results of the molecular docking studies are presented in a heatmap table utilizing a color gradient ranging from red to green, with a transition to yellow (at the 50th percentile) to emphasize the docking score energies. The lowest energy scores, often corresponding to the native ligand or a highly effective inhibitor, are depicted in red, indicating the most optimal matches. Green or other shades of green denote higher energy values, suggesting a weaker affinity with the target. This approach facilitates the identification of chemical compounds likely to inhibit specific targets.

3.6.1. Interactions with dihydrofolate reductase (PDB: 2W9G): Antibacterial activity

Dihydrofolate reductase (DHFR) plays a crucial role in the synthesis of tetrahydrofolate, essential for the production of purines, pyrimidines, and certain amino acids (Andrews et Dyer, 2022). Inhibiting DHFR is a major target for the development of antibacterial, antifungal, antitubercular, and anticancer drugs, as disrupting this pathway hinders the cells' ability to replicate and grow (Bhagat *et al.*, 2022; Chawla *et al.*, 2021). The *Staphylococcus aureus* DHFR protein

structure, identified by the PDB code 2W9G, was retrieved from the RCSB Protein Data Bank (Heaslet *et al.*, 2009). This structure is a promising target for the development of compound with antibacterial activity, given that *S. aureus* is a virulent pathogenic bacterium responsible for various infections, including those of the skin, soft tissues, and bones (Bhabha *et al.*, 2013). Targeting DHFR proves strategically important, as inhibiting this enzyme disrupts tetrahydrofolate biosynthesis, leading to the depletion of essential folate coenzymes necessary for the synthesis of nucleic acids (DNA and RNA) and specific amino acids (Wróbel *et al.*, 2020). Thus, DHFR represents an attractive focal point for the advancement of antibacterial drugs, with the potential to effectively impede bacterial growth and proliferation (Bayazeed *et al.*, 2022).

In this study, only one compound, trifluoroacetyl-α-terpineol, achieved a docking score similar to that of the native ligand, trimethoprim, a broad-spectrum antibiotic known for its inhibitory potential against dihydrofolate reductase, with a docking score of -6 kcal/mol. Trimethoprim establishes one hydrogen bond with amino acid residue located in the protein's binding pocket, Asn29, and one van der Waals interaction with a key amino acid residue from the binding site, Asp27 (Heaslet *et al.*, 2009). The binding site of this protein (DHFR, PDB: 2W9G), was found to be surrounded with five important amino acidic residues, namely Leu5Ile, Asp27, Phe92, Phe98Tyr, and Thr111 (Heaslet *et al.*, 2009). In contrast, trifluoroacetyl-α-terpineol did exhibit two hydrogen bonds with amino acid residues from the active site, namely Lys29, and Lys33 (**Figure 3**)**.** This finding underscores the significance of Trifluoroacetyl-α-terpineol as a potential therapeutic compound with a docking profile similar to that of ciprofloxacin, known for its antibacterial efficacy. The notable proximity of the docking scores suggests a potential mechanism of action that merits further investigation.

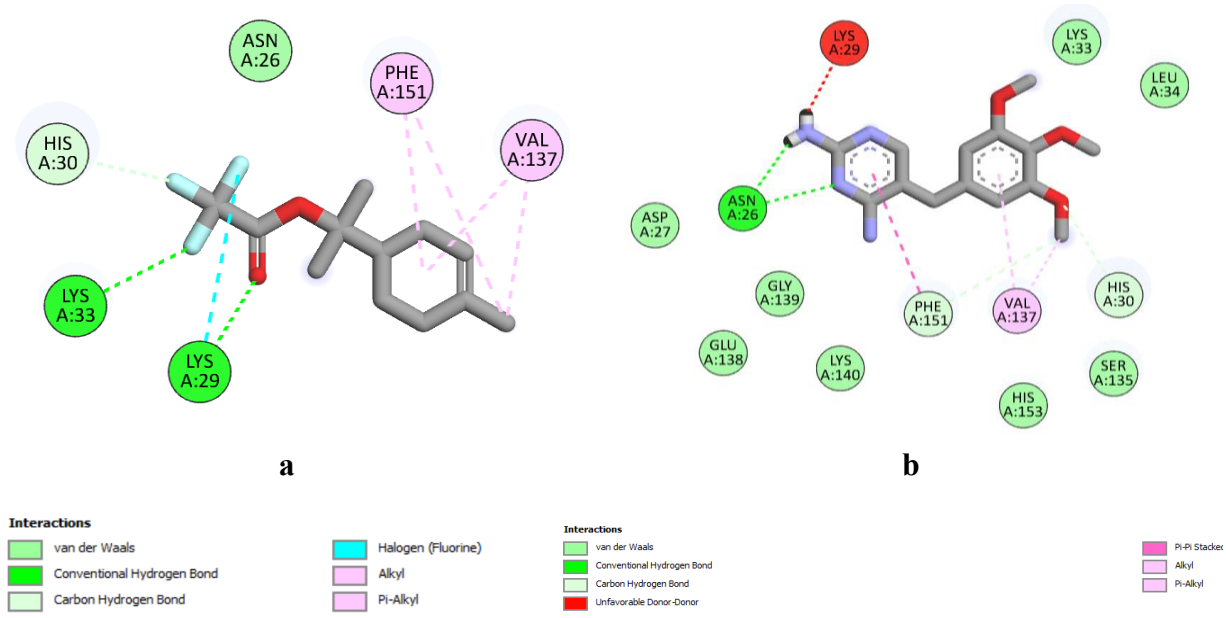

Figure 3. 2D Molecular Docking Interactions of trifluoroacetyl-α-terpinene (A), and trimethoprim (B), with Dihydrofolate Reductase from S. aureus (PDB: 2W9G).

3.6.2. Interactions with lanosterol 14-α demethylase (CYP51, PDB: 5EQB): Antifungal activity

The CYP51 enzyme, also known as lanosterol 14-alpha demethylase, plays a pivotal role in the ergosterol biosynthesis pathway, exclusive to fungal cells. Ergosterol is a crucial component of the fungal cell membrane, akin to cholesterol in human cells (Shanmugavani *et al.*, 2022). By

inhibiting CYP51, the ergosterol synthesis process is disrupted, leading to a reduction in ergosterol levels and the accumulation of harmful sterol intermediates (Jordá and Puig 2020). This disturbance compromises the integrity and fluidity of the fungal cell membrane, making it more permeable and less functional. CYP51 inhibition affects a broad spectrum of pathogenic fungi. As ergosterol is a fundamental component of fungal cell membranes, targeting CYP51 can be effective against various fungal species, including yeasts and molds (Parker *et al.*, 2014; Zhang *et al.*, 2019). This broad-spectrum activity is crucial for the development of antifungal agents capable of combating different types of fungal infections. One of the advantages of targeting CYP51 is that ergosterol is not produced in human cells, which primarily utilize cholesterol for membrane structure (Lepesheva *et al.*, 2008; Suárez *et al.*, 2002). This key difference in sterol biosynthesis between humans and fungi allows for the development of antifungal agents that selectively target fungal cells with minimal toxicity to the host.

The binding site of this protein, CYP51 (PDB: 5EQB), was found to be surrounded with acidic residues, namely, Thr322, Met313, Ile139, Tyr140, His381, Ser382, Phe241, Pro238, among others, as depicted by (Monk *et al.*, 2014). In this investigation, 2 out of the 14 compounds derived from CNEO exhibited docking scores comparable to or lower than the antifungal agent itraconazole (-7.1 kcal/mol), with 1,6-Octadien-3-ol, 3,7-dimethyl-, 2-aminobenzoate having a similar docking score with itraconazole, and trifluoroacetyl-α-terpineol displaying the lowest binding free energy (-8 kcal/mol). This suggests a potent inhibitory capacity against the CYP51 enzyme. Trifluoroacetyl-α-terpineol was observed to establish one conventional hydrogen bond, with residue Gln316, and 6 hydrophobic interactions with essential amino acid residues, one with the amino acid residue Met313, from the binding pocket **(Figure 4).**

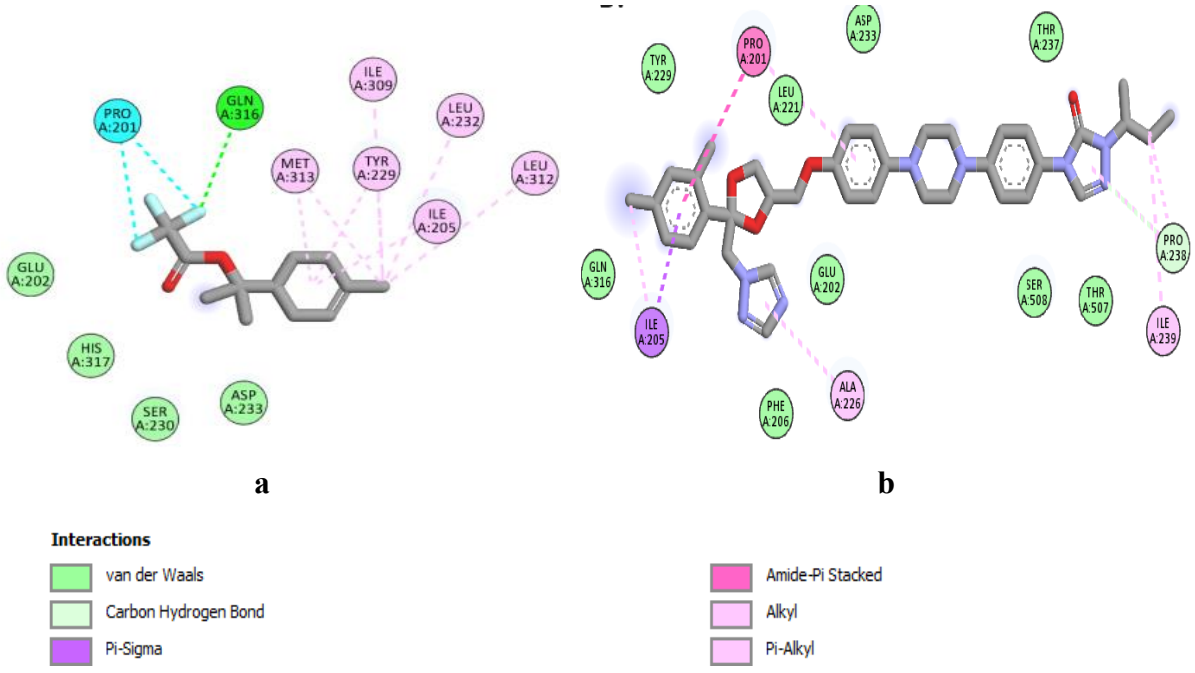

Figure 4. 2D Molecular docking interactions of trifluoroacetyl-α-terpinene (A), and Itraconazole (B) with lanosterol alpha-demethylase from *Saccharomyces cerivisiae* (PDB: 5EQB).

These results underscore the potential of trifluoroacetyl-α-terpineol as a promising candidate for further exploration as an antifungal agent, highlighting its favorable docking characteristics and interaction patterns with the CYP51 enzyme.

3.6.3. Interactions with tyrosinase (PDB ID: 5I3B): Dermatoprotective activity

Tyrosinase holds a central position as the primary regulatory enzyme in the melanin biosynthesis pathway, exerting significant influence, especially during the initial two stages of the process. These crucial stages involve the transformation of tyrosine into 3,4-dihydroxyphenylalanine (DOPA) and the subsequent oxidation of DOPA to dopaquinone (Abdanipour *et al.*, 2016). In the intricate cascade of melanin production, tyrosinase's key role unfolds during these fundamental steps, underscoring its pivotal contribution to the synthesis of melanin, the pigment responsible for skin, hair, and eye coloration. The enzymatic activities of converting tyrosine to DOPA and then oxidizing DOPA to dopaquinone are integral components of this intricate biological pathway, emphasizing the indispensable role played by tyrosinase in the intricate and finely regulated process of melanin biosynthesis.

In our current study, we have made a significant discovery regarding the binding interactions of 1,6-Octadien-3-ol, 3,7-dimethyl-, 2-aminobenzoate (Linalyl anthranilate), Trifluoroacetyl-α-terpineol, Pulegone, Acetic acid geraniol ester, Cyclohexanone, 5-methyl-2-(1-methylethyl)-, (2S-trans)-, 3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)- with the studied protein, indicating their strong inhibitory potential, with scores of -6 kcal/mol, -5.9 kcal/mol, -5.9 kcal/mol, -5.9 kcal/mol, -5.9 kcal/mol, -5.8 kcal/mol, respectively (**Table 8**).

Table 8. The molecular free binding affinity (measured in kcal/mol) obtained from computational simulations of the identified compounds in CNEO.

N°	Compounds	2W9G (Antibacterial)	5EQB (Antifungal)	5I3B (Dermatoprotective)
		Free Binding energy (Kcal/mol) *		
-	**Native Ligand**	**-6**	**-7.1**	**-5.5**
1	Linalyl acetate	-5.1	-6.7	-4.5
2	Trifluoroacetyl-α-terpineol	**-6.2**	**-8**	**-6.0**
3	Bicyclo[2,2,1]heptan-2-one, 1,7,7-trimethyl-, (1S)-	-4.8	-5.4	-5.0
4	Menthol	-5	-7	-5.0
5	1,6-Octadien-3-ol, 3,7-dimethyl-, 2-aminobenzoate (Linalyl anthranilate)	-5.5	**-7.1**	**-5.9**
6	α-terpineol	-5.3	-7	-5.3
7	Eucalyptol	-4.9	-5.9	-4.8
8	Terpinen-4-ol	-5	-7	**-5.8**
9	Geranyl vinyl ether	-4.6	-6.7	-4.5
10	Pulegone	-5.5	-5.8	**-5.9**
11	Geranyl acetate	-5.2	-6.9	**-5.9**
12	Linalool	-5	-6.6	-4.3
13	Borneol	-4.9	-5.4	**-5.9**
14	Linalool oxide	-5.3	-6.7	-4.7

To contextualize these results, we compared them to the binding affinity of the native ligand, hydroquinone. The interaction of Trifluoroacetyl-α-terpineol revealed the formation of 9 hydrophobic interactions with the amino acid residues in the binding site, while It is noteworthy

that when comparing these results to the native ligand, we observed that the native ligand established two hydrogen bonds, as illustrated in **Figure 5**. These findings reveal the potential of these molecules as inhibitors of the studied protein. Our *in silico* analysis revealed that Trifluoroacetyl-α-terpineol, which contains three fluorine atoms, and an α-terpineol unit, has a promising results regarding its binding affinity with major protein targets implicated in the investigated biological activities. In conclusion, the manifold biological and pharmacological properties observed in CNEO warrant further in-depth exploration. These auspicious findings not only contribute to elucidating the potential applications of CNEO but also advocate for sustained research efforts to harness its therapeutic potential across diverse realms of health. Nevertheless, it is strongly advised to conduct additional toxicological and clinical investigations to validate the safety and effectiveness of the essential oil derived from this plant

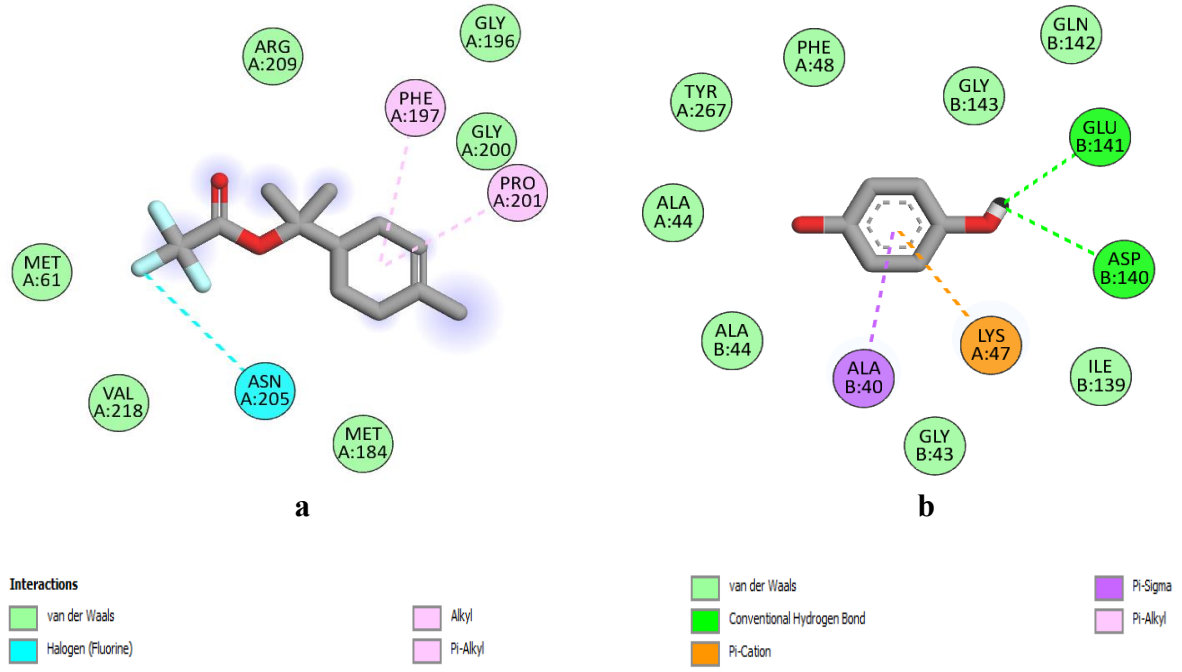

Figure 5. 2D Molecular docking interactions of trifluoroacetyl-α-terpineol (A), and hydroquinone (native ligand of Tyr), with tyrosinase (PDB: 5I3B).

Conclusion

This comprehensive investigation delved into the analysis of the essential oil derived from the Moroccan subspecies *C. nepeta subsp. ascendens*, elucidating a composition abundant in terpene compounds, notably linalyl acetate, Trifluoroacetyl-α-terpineol, camphor, and menthol. The antioxidant analysis of CNEO demonstrated a significant total antioxidant capacity (TAC) of 315.07 µg AA/mg and an effective DPPH free radical inhibition with an IC50 value of 112.97 ± 2.67 µg/ml, corroborating existing research and solidifying its standing as a promising natural antioxidant entity. The assessment of antimicrobial efficacy demonstrated robust inhibitory activity against diverse bacterial and fungal strains, underscoring its potential as an efficacious antibacterial and antifungal agent. Encouraging outcomes in anti-diabetic activity hinted at CNEO's capacity to modulate enzymes involved in glucose metabolism, with linalyl acetate and camphor emerging as pivotal contributors (El Omari *et al.,* 2023; Peana *et al.,* 2002; Shokova *et al.,* 2016; Singh *et al.,* 2023). The anti-gout and antidiabetic potential of CNEO manifests through

significant inhibition of xanthine oxidase (IC50 = 30.82 ± 0.78 µg/ml), α-amylase (IC50 = 40.13 ± 1.60 µg/ml), and α-glucosidase (IC50 = 45.30 ± 0.69 µg/ml) activities, suggesting therapeutic prospects in glycemic regulation. Furthermore, the essential oil showcases compelling anti-tyrosinase activity (IC50 = 29.78 ± 1.01 µg/ml), indicating potential dermatoprotective applications in melanin regulation. The implications of these findings provide a robust foundation for future investigations, unraveling the full therapeutic potential of CNEO within medical, and cosmetic contexts.

Conflict of interest: The authors declare no conflict of interest.

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