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# The Potential Antioxidant Mechanism of Essential Oils extracted from *Cupressus sempervirens* grown in Saudi Arabia

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**Abstract**

This study examined the chemical composition and antioxidant activity of essential oils (EOs) obtained from *Cupressus sempervirens var. horizontalis* grown in Abha region of Saudi Arabia. Essential oils (EOs) were isolated by hydro-distillation and characterized by GC-MS and GC-FID, revealing 88 compounds, among which α-pinene (35.1%) and δ-3-carene (25.5%) were the major components. The EO was assessed for antioxidant capacity in vitro by means of radical scavenging activity against DPPH and ABTS, which showed a significant scavenging activity with $IC_{50}$ values as low as 0.352 mg/mL and TEAC values greater than that of BHT, a synthetic antioxidant. To better describe the mechanism behind antioxidant activity, an in silico molecular docking study was carried out using NADPH oxidase (2CDU) as the target protein. Among these EO components, Germacrene-D, Cedrol, and Terpinen-4-ol were identified as having the best ΔG values, with hydrogen bonds and hydrophobic interactions all contributing to their strong binding affinities. These results highlight the potential for *C. sempervirens* EO to act as a natural antioxidant and a promising alternative to synthetic compounds in the pharmaceutical, food, and cosmetic industries.

Keywords: *Cupressus sempervirens*; Essential oil; Composition; Antioxidative activity; molecular docking.

## 1. Introduction

Essential oils (EOs) are invaluable extracts derived from plants through intricate extraction or distillation processes, capturing the volatile essence of these botanical wonders. Owing to their multifaceted biological and medicinal attributes [1], EOs have demonstrated impressive antibacterial and antifungal properties, making them pivotal in herbal medicine [2]. Their effectiveness spans the spectrum of antiseptic prowess against bacterial infections [3], including the prevention and treatment of oral diseases [4,5] and against fungal infections caused by dermatophyte fungi [6]. Additionally, the therapeutic utility extends to genital infections in pregnant women [7].

The antioxidant properties of essential oils (EOs) have garnered significant interest because of their ability to protect foods from the harmful effects of oxidants [8] and their potential role in preventing adverse health effects including cardiovascular, neurologic, immunologic, and carcinogenic effects by neutralizing free radicals [9,10]. With growing evidence linking these diseases to cellular damage caused by free radicals, the demand for safe, natural antioxidants has increased. This has driven extensive research into the antioxidant potential of EOs, especially as concerns grow over the potential health risks associated with synthetic antioxidants like butylated hydroxyanisole and butylhydroxytoluene [11,12].

Cupressus sempervirens is a tree originating from the Mediterranean basin but has a substantial presence in North America and Asia, including Saudi Arabia [13].

The Saudi Green Initiative, a commendable effort to combat climate change and enhance environmental sustainability, involves the ambitious planting of ten billion trees. Valued at a staggering 2.094 billion riyals, this initiative aims to contribute to a healthier environment and elevate overall quality of life [14]. In this context, *C. sempervirens* emerged as a particularly intriguing botanical entity because of its notable resistance to water and heat stresses [15,16], a trait invaluable in arid climates. Moreover, it has been shown that *C. sempervirens* is not influenced by physiographic factors or physicochemical properties of the soil [17]. However, given that the chemotype of plants is variable and may be influenced by the region and seasonality [18-20], and that environmental conditions, including pollution and edaphic factors, influence the composition of the secondary metabolites of plants [21,22], we aimed in the present study to determine the chemical composition of EOs of *C. sempervirens* var. *horizontalis* grown in the Abha region of Saudi Arabia. Scientific investigations have consistently demonstrated that *C. sempervirens* exhibits a range of biological activities to varying degrees, including antioxidant, antimicrobial, and antiplatelet effects. These diverse biological activities are closely linked to the plant's phytochemical composition [23,24].

The primary objectives of the present study were to determine the chemical composition and perform a comprehensive analysis of the antioxidant activity of the EOs extracted from *C. sempervirens* grown in Saudi Arabia. Antioxidant activity was studied using the DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) methods.

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These assays are sensitive and can assess the antioxidant activity of complex matrices, making them suitable for studying the potential health benefits of essential oils. The potential mechanism of action will also be investigated using in-silico molecular docking to determine the possible interactions between the principal essential oil components and NADPH oxidase, thereby contributing to the growing body of knowledge on natural antioxidants and supporting their potential use in food preservation, therapeutic applications, and disease prevention strategies, particularly in combating oxidative stress-related conditions.

## 2. Results and Discussion

### 2.1 Gas Chromatograph/Mass Spectrum (CC—MS) Analysis

Chromatographic analysis of the essential oils extracted from the leaves of *C. sempervirens var. horizontalis*, using GC/MS and GC-FID, with Linear Retention Index system (LRI), revealed a complex chemical composition comprising 88 compounds, including those present in trace amounts. Table 1 shows the chemical composition of the essential oils, with their corresponding LRI values calculated using polar and non-polar capillary columns. The most predominant compounds in these essential oils include α-pinene, δ-3-carene, α-terpinolene, cedrol, sabinene, 4-terpinene-nol, and germacrene-D.

**Table 1: Chemical composition of essential oils extracted from *Cupressus sempervirens* var. *horizontalis***

| No | Compound | LRI[1] | | Concentration (%) | Identification[4] |
|---|---|---|---|---|---|
| | | NPC[2] | PC[3] | | |
| 1 | Tricyclene | 926 | 1014 | 0.9 | LRI, GC-MS |
| 2 | α-thujene | 931 | 1030 | 0.3 | LRI, GC-MS |
| 3 | α-pinene | 939 | 1033 | 35.1 | LRI, GC-MS |
| 4 | α-fenchene | 950 | 1059 | 0.9 | LRI, GC-MS |
| 5 | camphene | 950 | 1068 | <0.1 | LRI, Co-inj[5], GC-MS |
| 6 | thuja-2,4(10)-diene | 957 | 1186 | <0.1 | LRI, GC-MS |
| 7 | verbenene | 967 | 1049 | <0.1 | LRI, GC-MS, Co-inj[5] |
| 8 | sabinene | 968 | 1125 | 2.8 | LRI, Co-inj[5], GC-MS |
| 9 | β-pinene | 976 | 1115 | 1.0 | LRI, Co-inj[5], GC-MS |
| 10 | myrcene | 991 | 1152 | 3.1 | LRI, Co-inj[5], GC-MS |
| 11 | δ-2-carene | 1001 | 1126 | <0.1 | LRI, GC-MS |
| 12 | α-phellandrene | 1007 | 1160 | - | LRI, GC-MS |
| 13 | δ-3-carene | 1011 | 1148 | 25.5 | LRI, GC-MS, Co-inj[5] |
| 14 | α-terpinene | 1016 | 1183 | 0.7 | LRI, GC-MS, Co-inj[5] |
| 15 | p-cymene | 1026 | 1258 | 0.3 | LRI, GC-MS, Co-inj[5] |
| 16 | limonene | 1033 | 1218 | 2.9 | LRI, GC-MS, Co-inj[5] |
| 17 | (Z)-β-ocimene | 1040 | 1230 | - | LRI, GC-MS |
| 18 | (E)-β-ocimene | 1050 | 1251 | - | LRI, GC-MS |
| 19 | γ-terpinene | 1062 | 1236 | 1.8 | LRI, Co-inj[5], GC-MS |
| 20 | p,α-dimethyl styrene | 1071 | 1459 | - | LRI, GC-MS |
| 21 | α-terpinolene | 1088 | 1280 | 4.4 | LRI, Co-inj[5], GC-MS |
| 22 | linalool | 1098 | 1547 | 0.1 | LRI, Co-inj[5], GC-MS |
| 23 | α-thujone | 1115 | 1430 | <0.1 | LRI, GC-MS |
| 24 | *cis*-p-menth-2-en-1-ol | 1117 | 1642 | <0.1 | LRI, GC-MS |
| 25 | *trans*-p-menth-2-en-1-ol | 1122 | 1650 | 0.1 | LRI, GC-MS |
| 26 | α-campholenal | 1126 | 1508 | - | LRI, GC-MS |
| 27 | *trans*-pinocarveol | 1127 | 1645 | 0.1 | LRI, GC-MS |
| 28 | *cis*-p-mentha-2,8-dien-1-ol | 1129 | 1609 | <0.1 | LRI, GC-MS |
| 29 | *cis*-limonene oxide | 1132 | 1449 | <0.1 | LRI, GC-MS |
| 30 | pinocarvone | 1134 | 1574 | <0.1 | LRI, GC-MS |
| 31 | camphor | 1142 | 1473 | <0.1 | LRI, GC-MS |
| 32 | isoborneol | 1145 | 1642 | - | LRI, GC-MS |
| 33 | borneol | 1147 | 1680 | <0.1 | LRI, GC-MS |
| 34 | camphene hydrate | 1148 | 1442 | <0.1 | LRI, GC-MS |
| 35 | sabina ketone | 1156 | 1622 | <0.1 | LRI, GC-MS |

| | | | | | |
|---|---|---|---|---|---|
| 36 | p-mentha-1,5-dien-8-ol | 1159 | 1665 | - | LRI, GC-MS |
| 37 | δ-terpinol | 1163 | 1662 | - | LRI, GC-MS |
| 38 | umbellulone | 1166 | 1643 | <0.1 | LRI, GC-MS |
| 39 | myrtenal | 1168 | 1548 | <0.1 | LRI, GC-MS |
| 40 | myrtenol | 1176 | 1586 | <0.1 | LRI, GC-MS |
| 41 | terpinen-4-ol | 1179 | 1571 | 2.0 | LRI, Co-inj[5], GC-MS |
| 42 | p-cymen-8-ol | 1184 | 1820 | <0.1 | LRI, GC-MS |
| 43 | α-terpineol | 1196 | 1673 | 0.3 | LRI, GC-MS |
| 44 | cis-dihydrocarvone | 1198 | 1636 | - | LRI, GC-MS |
| 45 | verbenone | 1204 | 1730 | 0.1 | LRI, GC-MS |
| 46 | *trans*-piperitol | 1205 | 1675 | <0.1 | LRI, GC-MS |
| 47 | *trans*-dihydrocarvone | 1206 | 1656 | - | LRI, GC-MS |
| 48 | *cis*-sabinene hydrate acetate | 1219 | 1564 | 0.1 | LRI, GC-MS |
| 49 | fenchyl acetate | 1234 | 1640 | <0.1 | LRI, GC-MS |
| 50 | methyl thymol | 1238 | 1595 | - | LRI, GC-MS |
| 51 | methyl carvacrol | 1244 | 1970 | <0.1 | LRI, GC-MS |
| 52 | linalyl acetate | 1260 | 1550 | <0.1 | LRI, Co-inj[5], GC-MS |
| 53 | bornyl acetate | 1278 | 1562 | 0.1 | LRI, GC-MS |
| 54 | thymol | 1280 | 2180 | 0.1 | LRI, GC-MS |
| 55 | carvacrol | 1286 | 2230 | <0.1 | LRI, GC-MS |
| 56 | terpenyl-4-acetate | 1330 | 1666 | <0.1 | LRI, GC-MS |
| 57 | α-terpinyl acetate | 1337 | 1667 | 1.7 | LRI, GC-MS |
| 58 | *trans*-piperitol acetate | 1346 | 1678 | 0.5 | LRI, GC-MS |
| 59 | α-cubebene | 1354 | 1458 | <0.1 | LRI, GC-MS |
| 60 | α-ylangene | 1372 | 1485 | - | LRI, GC-MS |
| 61 | α- copaene | 1373 | 1519 | 0.1 | LRI, Co-inj[5], GC-MS |
| 62 | β-bourbonene | 1384 | 1520 | - | LRI, GC-MS |
| 63 | β-elemene | 1387 | 1595 | - | LRI, GC-MS |
| 64 | longifolene | 1398 | 1635 | - | LRI, GC-MS |
| 65 | β-cubebene | 1409 | 1564 | 0.1 | LRI, GC-MS |
| 66 | β-caryophyllene | 1420 | 1598 | 0.2 | LRI, GC-MS |
| 67 | β-gurjunene | 1423 | 1537 | - | LRI, GC-MS |
| 68 | α-cedrene | 1432 | 1755 | - | LRI, GC-MS |
| 69 | aromandendrene | 1435 | 1593 | 0.2 | LRI, GC-MS |
| 70 | α-humulene | 1448 | 1672 | 0.2 | LRI, Co-inj[5], GC-MS |
| 71 | allo-aromadendrene | 1460 | 1637 | - | LRI, GC-MS |
| 72 | γ-muurolene | 1477 | 1695 | - | LRI, GC-MS |
| 73 | germacrene D | 1478 | 1721 | 1.9 | LRI, Co-inj[5], GC-MS |
| 74 | β-selinene | 1486 | 1705 | 0.1 | LRI, GC-MS |
| 75 | *epi*-cubebol | 1493 | 1996 | 0.2 | LRI, GC-MS |
| 76 | α-muurolene | 1499 | 1738 | - | LRI, GC-MS |
| 77 | β-bisabolene | 1502 | 1725 | 0.1 | LRI, GC-MS |
| 78 | γ-cadinene | 1508 | 1772 | 0.4 | LRI, GC-MS |
| 79 | cis-calamenene | 1510 | 1851 | - | LRI, GC-MS |
| 80 | δ-cadinene | 1515 | 1720 | <0.1 | LRI, GC-MS |
| 81 | cadina-1,4-diene | 1517 | 1785 | <0.1 | LRI, GC-MS |
| 82 | α-cadinene | 1521 | 1758 | 0.1 | LRI, GC-MS |
| 83 | elemol | 1526 | 2087 | - | LRI, GC-MS |
| 84 | α-calacorene | 1542 | 1907 | - | LRI, GC-MS |
| 85 | germacrene B | 1552 | 1845 | - | LRI, GC-MS |
| 86 | β-calacorene | 1560 | 1921 | - | LRI, GC-MS |

| | | | | | |
|---|---|---|---|---|---|
| 87 | β-caryophyllene oxide | 1576 | 2008 | 0.1 | LRI, GC-MS |
| 88 | cedrol | 1592 | 2093 | 4.1 | LRI, GC-MS |
| 89 | humulene epoxide II | 1606 | 2002 | 0.1 | LRI, GC-MS |
| 90 | t-cadinol | 1616 | 2190 | <0.1 | LRI, GC-MS |
| 91 | 1-*epi*-cubenol | 1626 | 2081 | 0.1 | LRI, GC-MS |
| 92 | t -muurolol | 1627 | 2208 | 0.1 | LRI, GC-MS |
| 93 | α-acorenol | 1630 | 2163 | 0.6 | LRI, GC-MS |
| 94 | β-acorenol | 1637 | 2212 | 0.8 | LRI, GC-MS |
| 95 | *epi*-α-cadinol | 1640 | 2170 | 0.1 | LRI, GC-MS |
| 96 | α-muurolol | 1645 | 2192 | 0.2 | LRI, GC-MS, Co-inj[5] |
| 97 | β-eudesmol | 1649 | 2242 | 0.1 | LRI, GC-MS |
| 98 | α-eudesmol | 1652 | 2188 | 0.1 | LRI, GC-MS |
| 99 | α-cadinol | 1653 | 2221 | 0.1 | LRI, GC-MS |
| 100 | cadalene | 1674 | 2200 | 0.2 | LRI, GC-MS |
| 101 | kaur-15-ene | 1966 | 2337 | 0.1 | LRI, GC-MS |
| 102 | sandaracopimaradiene | 1967 | 2250 | 0.8 | LRI, GC-MS |
| 103 | manoyl oxide | 1993 | 2350 | 0.3 | LRI, GC-MS |
| 104 | isopimaradiene | 2013 | 2170 | <0.1 | LRI, GC-MS |
| 105 | 8,13-diepimanoyl oxide | 2016 | 2355 | 0.8 | LRI, GC-MS |
| 106 | abietatriene | 2044 | 2530 | 0.9 | LRI, GC-MS |
| 107 | manool | 2055 | 2180 | - | LRI, GC-MS |
| 108 | kaurene | 2060 | 2425 | - | LRI, GC-MS |
| 110 | abietadiene | 2080 | 2502 | <0.1 | LRI, GC-MS |
| 111 | 13(16),14-labdien-8-ol | 2087 | 2255 | - | LRI, GC-MS |
| 112 | (11E,13Z)-labdadien-8-ol | 2095 | 2266 | - | LRI, GC-MS |
| 113 | phyllocladanol | 2210 | 2147 | <0.1 | LRI, GC-MS |
| 114 | sempervirol | 2223 | 2275 | 0.1 | LRI, GC-MS |
| 115 | 4-epi-abietal | 2238 | 2265 | <0.1 | LRI, GC-MS |
| 116 | totarol | 2253 | 2280 | 0.1 | LRI, GC-MS |
| 117 | ferruginol | 2320 | 2295 | 0.1 | LRI, GC-MS |
| | | Total Identified (%) | | 98.4 | |
| | | Yield % (m/m) | | 1.1 | |

[1]Linear Retention Index.
[2] Linear Retention Index with an HP-5 non-polar column.
[3] Linear Retention Index with an HP-INNOWax polar column.
[4]Identification method.
[5]Co-inj: Co-injection.

**2.2. Study of the chemical Composition of Essential Oils from *C. sempervirens var. horizontalis***

The results presented in Table 2 reveal that essential oils derived from *C. sempervirens var. horizontalis* are predominantly composed of hydrocarbon monoterpenes (79.7%), followed by sesquiterpenes (10.3%) and diterpenes in lower concentrations (3.2%).

**Table 2: Metabolic classes of the studied essential oils**

| Metabolic class | *C.S. Horizontalis* (%) |
|---|---|
| Hydrocarbon monoterpene | 79.4 |
| Aromatic hydrocarbon | 0.3 |
| Monoterpene alcohol | 2.7 |
| Monoterpene ketone | 0.1 |
| Monoterpene ester | 2.4 |
| Sesquiterpene | 3.6 |
| Sesquiterpene alcohol | 6.5 |
| Sesquiterpene ether | 0.2 |
| Diterpene | 0.9 |
| Diterpene ether | 1.1 |
| Aromatic diterpene | 0.9 |
| Diterpene alcohol | 0.3 |

Among hydrocarbon monoterpenes, α-pinene was the most prevalent, constituting 35.1% of the EOs. This concentration was higher in the EO extracted from *C. sempervirens var. horizontalis* than those found in the same variant in France (13.07%) [25], and lower than those registered in Turkish plants (47.3%) [26]. Zhang et al. [27] reported a synergistic interaction of this monoterpene with paclitaxel in combating non-small-cell lung carcinoma. The second most abundant compound was δ-3-carene, accounting for 25.5% of the EOs. This compound has been shown to have an inhibitory effect on the growth of cancer cells, including lung and prostate human cancer cells [28]. Fahed *et al.* reported a higher α-pinene concentration (57.5%) and did not detect δ-3-carene in EOs extracted from plants grown in Lebanon [29]. In contrast, EOs extracted from plants in Turkey exhibited δ-3-carene concentrations ranging from 22.9 to 25.5% [26]. α-Terpinolene reached 4.4%, limonene 2.9 %, and sabinene 2.8%, following α-pinene (35.1%) and δ-3-carene (25.5%) in terms of prevalence. Slightly lower limonene concentration (2.8%) has been reported in Iranian *C. sempervirens var. horizontalis* EOs [30]. Sesquiterpene hydrocarbons were present at lower concentrations in essential oils (3.7%). Emami *et al.* (2006) [30] reported a higher concentration of sesquiterpene hydrocarbons (6.3%) in the essential oil extracted from *C. sempervirens* var. *horizontalis* plants grown in Iran [30]. In addition, the authors observed a lower concentration of sesquiterpene alcohols (4.6%) in the essential oils extracted from the leaves of *C. sempervirens* var. *horizontalis* compared to those found in the present study (6.5%).

### 2.3. Antioxidant Assays

#### 2.3.1. DPPH˙ Method

The antioxidant activity of various solutions was determined using EOs, specifically the concentration of the product required to reduce the DPPH radical by 50%. The $IC_{50}$ values for all the solutions were determined after 30 min.

Figure 1 shows the variation in the percentage of inhibition (PI) of the essential oils extracted from the twig organs of the studied C. sempervirens specimens, compared to the reference antioxidant BHT.

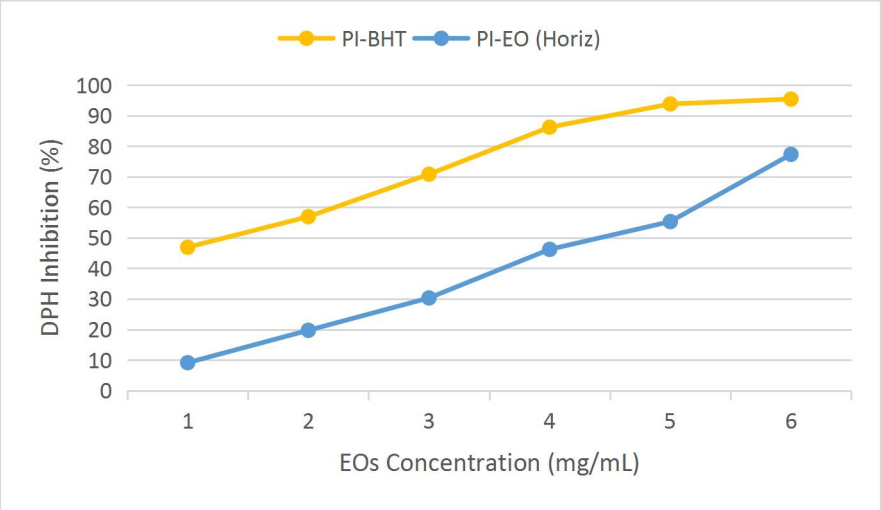



**Fig. 1. Curve of the PI variation as a function of the concentration of EOs from *C. sempervirens* var. *horizontalis*.**

Table 3 represents the IC50 value obtained for the essential oil extracted from the leaves of the plant ($IC_{50}$ = 0.352 mg/mL), showing high antioxidant activity. These results support that the EOs possess strong antioxidative potential and would be beneficial to utilize in the health, food, and cosmetics industries. Further research is warranted to harness and optimize the benefits of these highly active essential oils.

**Table 3: $IC_{50}$ value**

| Cupressus essential oil solution | $IC_{50}$ (mg/ml) |
|---|---|
| *C. sempervirens var. horizontalis* | 0.352 |

#### 2.3.2. ABTS Method (TEAC)

To validate the results obtained using the DPPH method, we tested the same essential oils using ABTS assay.

The ABTS method allows the direct measurement of the reactivity of an antioxidant compound without the need to examine the degradation products. In the ABTS assay, the $ABTS^{\bullet+}$ radical cation is reduced by the reference Trolox antioxidant solution and results in a decrease of the absorbance at 734 nm. The percentage of reduction is calculated based on concentration, using the calibration curve equation generated with Trolox (Y = -0.1245 + 0.5365). Antioxidant activity is expressed as Trolox Equivalent Antioxidant Capacity (TEAC). These TEAC values were compared with those of BHT, used as a standard antioxidant control. The results are presented in Table 4.

**Table 4: TEAC value**

| Essential oil | TEAC (mmol/g) |
|---|---|
| *C. sempervirens var. horizontalis* | 0.650 |

The findings from our study clearly indicate that *Cupressus sempervirens* var. *horizontalis* display significantly higher antiradical activity than that achieved using BHT, a common reference antioxidant. The considerable difference in antiradical efficacy indicated that *Cupressus* EOs could potentially be promising as effective antioxidants. Furthermore, it's noteworthy to highlight that these results are consistent with the data derived from the DPPH method, reinforcing the credibility and reliability of our findings. Consistency across these distinct methodologies underscores the robustness of the evidence regarding the remarkable antiradical potential of *Cupressus* EOs. These results highlight the importance of exploring and utilizing the antioxidant properties of *Cupressus* essential oils, emphasizing their potential applications in diverse fields such as food preservation, pharmaceuticals, and other industries. Further research in this field may reveal the full potential of these natural antioxidants.

**2.4. Molecular Docking**

Several studies have explored the antibacterial or antiviral activity of the essential oil components of *Cupressus* species using molecular docking [31-35], but to our knowledge, only a few studies have evaluated the antioxidant activity using this technique [36]. In the present study, we investigated the molecular docking interactions of the main EOs' components with NADPH oxidase (2CDU). The analysis included α-pinene (35.1%), δ-3-carene (25.5%), α-terpinolene (4.4%), cedrol (4.1%), myrcene (3.1%), limonene (2.9%), sabinene (2.8%), terpinene-4-ol (2.0%), Germacrene-D (1.9%), γ-terpinene (1.8%), α-terpinyl acetate (1.7%), β-pinene (1.0%). The free energy binding (ΔG) for the docked compounds is shown in Figure 2. The results demonstrated notable differences in binding affinities, providing important insights into the potential antioxidant mechanisms of these compounds. The docking protocol was validated through flexible re-docking of the co-crystallized ligands using the MMFF94 force field.

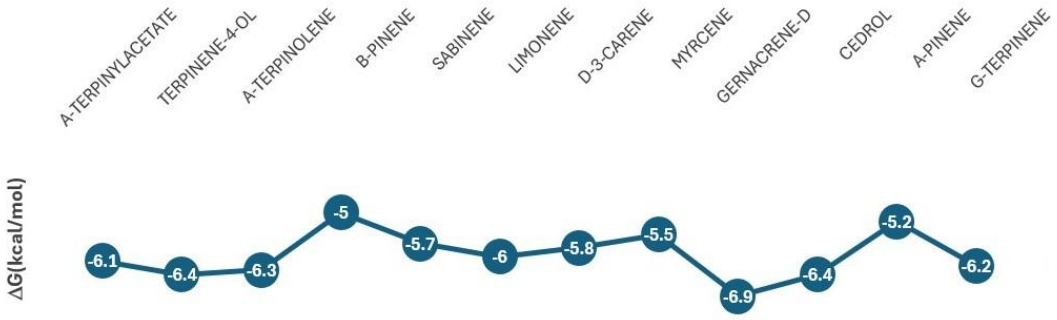
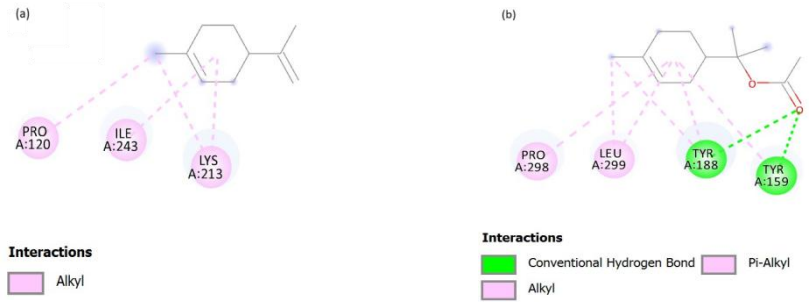



**Fig. 2. The free binding energies for the main components of *C. sempervirens* var. *horizontalis* EOs.**

Binding free energy (ΔG) analysis of the major essential oil (EO) components with the 2CDU receptor revealed significant variations in their interaction strengths, shedding light on their potential antioxidant mechanisms. Components with the lowest ΔG values exhibited the strongest binding to the receptor, indicating a high probability of effective interaction with 2CDU to inhibit oxidative stress pathways, such as reactive oxygen species (ROS) production. In contrast, components with higher ΔG values exhibited weaker binding, suggesting less activity. These results highlight specific EO components as promising candidates for natural antioxidants. Compared with known inhibitors or co-crystallized ligands, the strong binding affinities of some EO components highlight their potential as viable alternatives to synthetic antioxidants. Overall, this analysis highlights the relevance of molecular docking to elucidate the antioxidant potential of essential oil components through their interactions with target receptors.



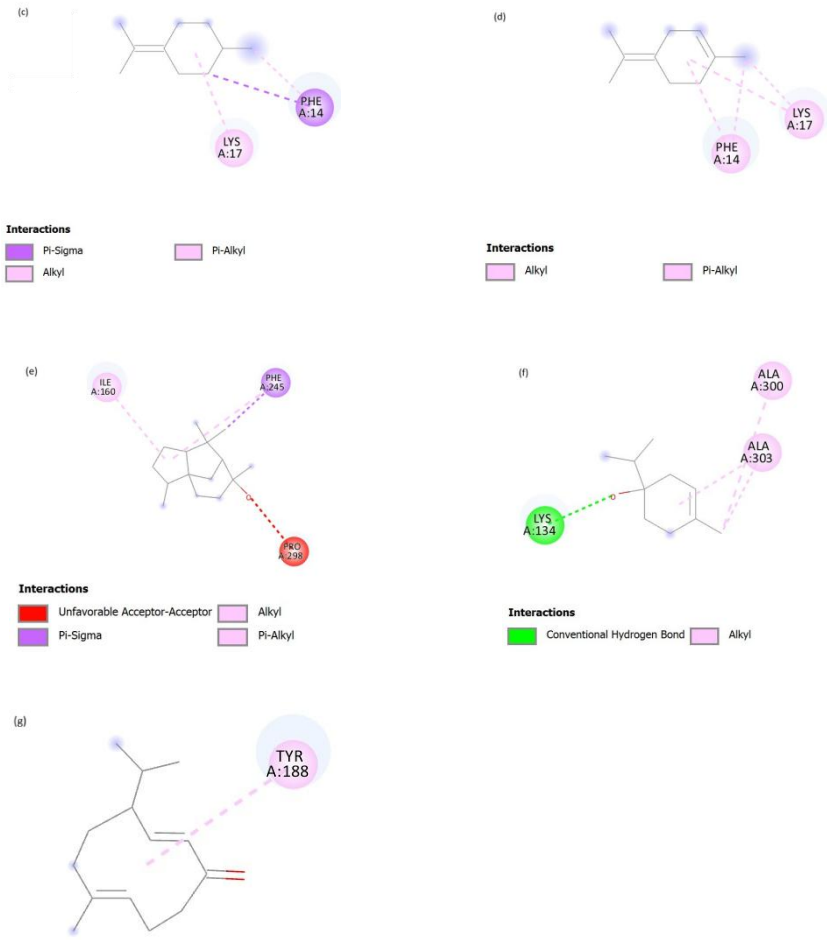



**Fig. 3. Interactions of the major EOs components with 2CDU, (a) Limonene (ΔG = -6.0 Kcal/mol), (b) α-terpinyl acetate (ΔG = -6.1 Kcal/mol), (c) γ-terpinene (ΔG = -6.2 Kcal/mol), (d) α-terpinolene (ΔG = -6.3 Kcal/mol), (e) Cedrol (ΔG = -6.4 Kcal/mol), (f) Terpinene-4-ol (ΔG = -6.4 Kcal/mol), and (g) Germacrene-D (ΔG = -6.9 Kcal/mol).**

Significant variation in the interaction strength was observed, with Germacrene-D showing the lowest ΔG value (-6.9 Kcal/mol), followed by Cedrol and Terpinene-4-ol (-6.4 Kcal/mol). Lower but significant binding energy was also observed for α-terpinolene (-6.3 Kcal/mol), γ-terpinene (-6.2 Kcal/mol), α-terpinyl acetate (-6.1 Kcal/mol), and Limonene (-6.0 Kcal/mol), indicating a high probability of effective interaction with 2CDU to inhibit oxidative stress pathways, such as reactive oxygen species (ROS) production.

These compounds with the lowest binding free energy could serve as potential natural antioxidants and offer an alternative to synthetic antioxidants.

The results of the docking molecular study of the interactions of these compounds with 2CDU are shown in Figure 3.

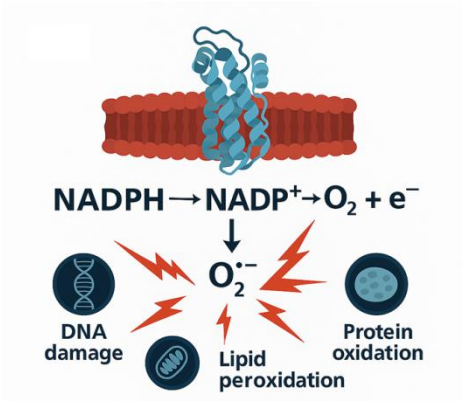
The interaction diagram between Limonene and the 2CDU protein (Figure 3 – a) highlights hydrophobic interactions involving alkyl groups. The residues involved included Proline (PRO120), Isoleucine (ILE243), and Lysine (LYS213). Proline and Isoleucine, which are nonpolar residues, form hydrophobic interactions with the alkyl groups of Limonene via van der Waals forces. Lysine, although typically polar, contributes to the interaction through its aliphatic chain, which participates in hydrophobic stabilization. These interactions indicated that Limonene binds within a nonpolar pocket of 2CDU, where the hydrophobic residues create a favorable environment for binding. The absence of hydrogen bonds or polar interactions suggests that the binding is primarily driven by hydrophobic forces, which may result in weaker binding affinity compared to ligands with polar or charged interactions. If Limonene binds near or within the active site of 2CDU, it could potentially act as an inhibitor by obstructing substrate access or altering conformational dynamics of the protein. Further docking studies or molecular dynamics simulations could provide more detailed insights into the binding strength and the biological significance of this interaction. Figure 3-b shows the interactions between α-terpinyl acetate and 2CDU. α-Terpinyl acetate creates standard hydrogen bonds with TYR188 and TYR159, which seem to be important residues in the active site of the 2CDU receptor. These hydrogen bonds have intensive interactions that may increase the binding affinity and are essential for the

stabilization of the ligand within the binding pocket. There are also hydrophobic pi-alkyl and alkyl interactions with PRO298 and LEU299. These non-covalent interactions help to further stabilize the ligand into the receptor's hydrophobic regions, thus strengthening the favorable interaction. Strategic hydrogen bonds with key tyrosine residues, alongside hydrophobic contacts with proline and leucine, suggests that the binding mode for the α-terpinyl acetate is quite stable and energetically favorable. The described interactions indicate that the compound might play a major role in the antioxidant activity of *C. sempervirens* essential oil through its potent interaction with the enzyme NADPH oxidase (2CDU), which catalyzes the formation of reactive oxygen species (ROS). Hydrophobic interactions are the predominant type of binding that stabilizes the interaction of γ-terpinene with the 2CDU receptor based on docking analysis, as evidenced by pi-sigma and pi-alkyl interactions with PHE14 and alkyl interaction with LYS17 (Figure 3-c). These non-covalent interactions may imply that the aromatic ring of γ-terpinene can make favorable contacts with the receptor's hydrophobic pocket, even in the total absence of conventional hydrogen bonds. This supports a stable intermolecular binding conformation such that γ-terpinene could successfully bind to NADPH oxidase and consequently modulate activity. This also supports the role of γ-terpinene as a potential natural antioxidant. α-terpinolene have a similar structure to that of γ-terpinene and interacts with the same amino acids (PHE14 and LYS17). Docking analysis showed that its binding was promoted by hydrophobic interactions in the guise of pi-alkyl and alkyl interactions, suggesting that the hydrophobic regions α-terpinolene are well accommodated in the binding pocket of the receptor. Although no hydrogen bonds were formed, the presence of a few hydrophobic contacts contributed positively to the overall stability of the ligand-receptor complex. This indicates that α-terpinolene may bind well to the 2CDU receptor and thus could affect its activity, supporting its position as a natural antioxidant constituent of *Cupressus sempervirens* essential oil. Considering the molecular docking analysis of cedrol with the 2CDU receptor, it may be noted that its interaction is predominantly governed by hydrophobic contacts, which include pi-alkyl contacts with ILE160 and PHE245 that help bind the molecule within the pocket. These interactions represent favorable binding with the hydrophobic regions of the receptor, but a poor acceptor-acceptor interaction was observed with PRO298, represented in red. This disfavored interaction may destabilize the binding conformation to a very small extent or reduce the overall binding affinity of cedrol compared with the other essential oil constituents. However, the presence of stabilizing hydrophobic interactions shows that cedrol is still able to bind to the 2CDU receptor, possibly contributing to the antioxidant activity of *Cupressus sempervirens* essential oil, but less so than constituents that do not have any disfavored interactions. In the molecular docking interaction between terpinen-4-ol and the 2CDU receptor (NADPH oxidase), a combination of hydrophilic and hydrophobic interactions contributed to binding stability. There was one key interaction, which was a conventional hydrogen bond defined based on the hydroxyl group of terpinen-4-ol and LYS134, likely playing a critical role in anchoring the molecule in the active site. In addition, alkyl interactions with ALA300 and ALA303 stabilized the ligand in the hydrophobic pocket of the receptor. This combined interaction mode (hydrogen bonding and hydrophobic contacts) suggests that terpinen-4-ol may demonstrate high binding affinity and play a role in modifying or regulating the oxidative activity of NADPH oxidase; thereby justifying its potential antioxidant role in *Cupressus sempervirens* essential oil. The results of the molecular docking of germacrene D with the 2CDU receptor displayed a single hydrophobic π-alkyl interaction with TYR188, indicating that germacrene D interacts with the receptor mainly via non-polar interactions and hydrophobic contribution (both make a smaller contribution to binding stability than polar interactions). The absence of hydrogen bonding or multiple interaction points to strong polarity indicates that there is less specific and weaker binding than for the other essential oil components. The interaction with the TYR188 residue (shown to be significant in other strong-binding ligands) indicates that, while germacrene D may have a lower affinity as it engages TYR188, it may still engage the receptor with a modulating function. This suggests a secondary role for germacrene D, supporting the antioxidant potential of *Cupressus sempervirens* essential oil.

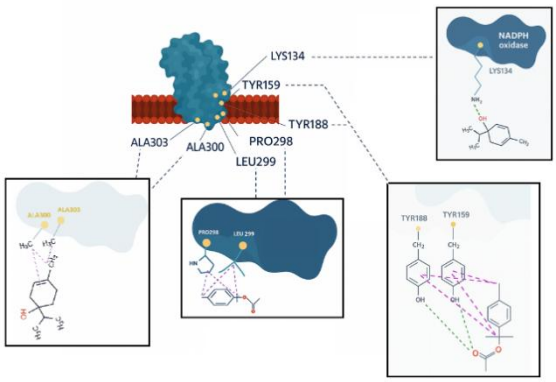
The overall molecular docking results of the principal components of *Cupressus sempervirens* essential oil with the NADPH oxidase receptor (2CDU) highlight a range of binding affinities and interactions that support their potential antioxidant activities. Certain compounds, such as α-terpinyl acetate and terpinen-4-ol, exhibit strong binding patterns characterized by classic hydrogen bonds and hydrophobic interactions, reflecting more stable and specific interactions with the receptor. These findings show that these compounds can effectively inhibit NADPH oxidase activity and reduce the production of reactive oxygen species (ROS), justifying their use as effective natural antioxidants. The remaining major components, including γ-terpinene, α-terpinolene, limonene, and germacrene D, primarily involved the receptor with alkyl and pi-alkyl interactions.

Even though such interactions are less specific and weaker than hydrogen bonding, they still contribute to stabilizing the ligand-receptor complex and indicate moderate antioxidant activity. Cedrol, even though it involves multiple hydrophobic contacts, exposes an unfavorable acceptor-acceptor interaction, which would reduce its binding stability and overall antioxidant activity. Taken together, the results show that the antioxidant activity of C. sempervirens essential oil can be attributed to a synergistic effect of multiple components, with α-terpinyl acetate and terpinen-4-ol being the most likely candidates for further biological evaluation.

(a)




(b)




(c)

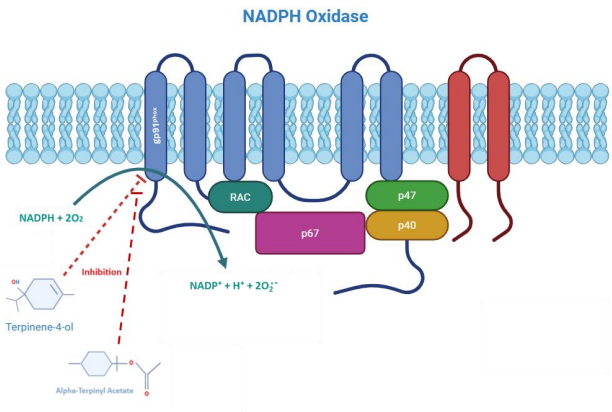



**Fig. 4. Proposed antioxidant mechanism of Cupressus sempervirens essential oil components via NADPH oxidase inhibition, (a) Normal production of ROS species by NADPH oxidase, (b) Molecular docking interactions of terpinen-4-ol and α-terpinyl acetate, (c) Proposed inhibitory effect of terpinen-4-ol and α-terpinyl acetate.**

In order to explain the inhibitory process more clearly, we constructed a summarized figure (Fig. 4) showing the interaction between the main components of essential oils and NADPH oxidase. Under physiological conditions, the NADPH oxidase complex converts electrons from NADPH to molecular oxygen, producing superoxide anions ($O_2^{\cdot -}$), as shown in Fig. 4a, which are then transformed into other reactive oxygen species (ROS) like hydrogen peroxide ($H_2O_2$) and hydroxyl radicals (OH·). These species harm proteins, lipids, and DNA, among other cellular constituents, which leads to oxidative stress. The molecular docking interactions of terpinen-4-ol and α-terpinyl acetate with NADPH oxidase are depicted in Figure 4b. Important interactions are highlighted, including hydrophobic contacts with residues like ALA300, ALA303, PRO298,

and LEU299 and hydrogen bonding (e.g., between terpinen-4-ol and LYS134 or α-terpinyl acetate with TYR188/TYR159). These interactions imply a possible disruption of the enzyme's catalytic activity, which could result in a suggested decrease in ROS production, as shown in Fig. 4c. This mechanistic basis supports the antioxidant activity of Cupressus sempervirens essential oil and bioactive compounds.

This study underscores the importance of molecular docking in predicting bioactivity and highlights the use of C. sempervirens EO as a natural source of antioxidants, which can offer an alternative to synthetic compounds for food and pharmaceutical applications.

## 3. Experimental

### 3.1 Plant Materials and Reagents

Leaves of *C. sempervirens* were collected in March and September 2023 from the Abha region of Saudi Arabia. Two plant specimens, CH 01 and CP01, were identified by botanists from the Department of Agriculture at King Abdulaziz University, Saudi Arabia, and deposited in the herbarium of the department, with voucher specimen No CSH05862. The identification was based on their morphological characteristics (type of ramification, shape of branches and leaves, appearance of the rhytidome, or characteristics of the cones). Before hydro-distillation, the leaves were dried in ambient air for a week until the weight stabilized.

2,2-diphenyl-1-picrylhydrazyl (DPPH) was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

### 3.2 Essential oil extraction

To extract oil from the leaves, we employed a hydro-distillation technique. Specifically, we subjected 100 g of dry leaves to a Clevenger separator for a duration of six hours. The resulting essential oil, characterized by its pale-yellow color, was dried using anhydrous $Na_2SO_4$ and subsequently stored at 40°C. The yield of this extraction, calculated in relation to the dry matter, was 0.84%. The refractive index of the oil was 1.4875.

### 3.3 Gas Chromatograph/Mass Spectrometry (GC—MS) Analysis

The chemical constituents of the essential oil of *C. sempervirens*, diluted to 1% in hexane, were identified using a gas chromatograph (HP 5890 — SERIES II) coupled with a quadrupole mass spectrometer (HP-MSD 5972 A) in electronic impact mode. Two types of columns were employed: a non-polar HP-5MS capillary column (30 m length, 0.25 mm internal diameter, 0.52µm film thickness) with a stationary phase of 5% diphenyl and 95% dimethylpolysiloxane, and an HP-INNOWAX polar column (30 m length, 0.25 mm internal diameter, 0.25µm film thickness) with a polyethylene glycol stationary phase. The injector, source, and interface temperatures were set to 250, 175, and 280°C, respectively. Helium served as the carrier gas at 1.2 ml/min, with a working pressure of 9 Psi. Temperature programming for the non-polar column ranged from 50°C to 280°C at 50°C/min with two hold periods, and the polar column temperature ranged from 50°C to 250°C at 50°C/min with two hold periods. One microliter of the sample (diluted to 1% in hexane) was injected, and spectral data were acquired from 50 to 550 atomic mass units (amu) in scanning mode with an electronic impact source at 70 eV. Compounds were identified using NBS75K.L and WILEY275.L spectral libraries, using concordance notes (NIC) to confirm the proposed structures.

### 3.4 Gas chromatography GC-FID/KI analysis

The chemical constituents were identified and quantified using a gas chromatograph (GC) equipped with a flame ionization detector (FID) and two types of columns: a non-polar HP-5 capillary column (30m length, 0.25 mm internal diameter, 0.52µm film thickness) consisting of 5% diphenyl and 95% dimethylpolysiloxane as the stationary phase, and a polar HP-INNOWAX column (30m length, 0.25 mm internal diameter, 0.25µm film thickness) with a polyethylene glycol stationary phase. The injector and detector temperatures were maintained at 250 and 280°C, respectively. Nitrogen was used as carrier gas at a flow rate of 1.2 mL/min, and a working pressure of 9 psi. The temperature program for the non-polar column ranged from 50°C to 280°C at a rate of 50°C/min with two hold periods, whereas the temperature program for the polar column ranged from 50°C to 250°C at the same rate with two hold periods. One microliter of sample (diluted to 1% in hexane) was injected. The Linear Retention Indices (LRI) for all identified constituents were calculated using the Van den Dool and Kratz equation, which is appropriate for temperature-programmed gas chromatography conditions. The resulting LRI values were compared with those of reference C9–C28 alkanes [37]. Quantitative analysis was performed using HP ChemStation software, assuming that all response coefficients were similar, equating the percentages of constituents to the percentages of peak areas in the chromatogram.

The Linear Retention Index (LRI) was calculated using the Van den Dool and Kratz formula, which is appropriate for retention indexing under non-isothermal (temperature-programmed) GC conditions [38]:

$$\mathrm{LRI_A} = \frac{100\,\mathrm{N} + 100\,\mathrm{X}\,(\log \mathrm{Tr(A)} - \log \mathrm{Tr(N)}}{\log \mathrm{Tr(N+1)} - \log \mathrm{Tr(N)}}, \quad (1)$$

Where A is the compound to be identified, N is the number of carbon atoms of the hydrocarbon which comes out just before compound A, Tr(A) is the retention time of compound A, Tr(N) is the retention time of the hydrocarbon that leaves just before compound A, and Tr(N+1) is the retention time of the hydrocarbon that comes out just after the compound.

These techniques have facilitated the comprehensive identification of nearly all essential oil constituents.

### 3.5. Antioxidant Assays

#### 3.5.1. DPPH• Method

One milliliter of the sample solution (essential oil in ethanol) was combined with 3 mL of a 100 mmol/L ethanolic solution of DPPH• (1,1-diphenyl-2-picrylhydrazyl) with vigorous stirring. The resulting mixture was incubated for 30 min at room temperature, and the absorbance was measured at 517 nm. The difference in absorbance between the sample and methanol (used as an analytical blank) was considered an indicator of antioxidant activity, and a comparison was made with butylated hydroxytoluene (BHT) and vitamin E as reference standards. The anti-radical activity was quantified using the PI value, which represents the percentage of inhibition, where the absorbance of the sample alone was zero at 517 nm.

$$\mathrm{PI} = 1 - \frac{\text{Absorbance of the sample}}{\text{Absorbance of the blank}} \mathrm{X100}, \quad (2)$$

A PI curve was generated by plotting PI values as a function of essential oil solution concentration (mg/L). To determine the $IC_{50}$ (mg/mL), the point corresponding to 50% PI on the curve was projected onto the x-axis [39].

#### 3.5.2. $ABTS^{+\bullet}$ (TEAC) Method

The protocol involved dissolving ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) in water to achieve a concentration of 7 mM. $ABTS^{+\bullet}$ radicals were generated by the reaction of the stock solution with potassium persulfate (2.45 mM). The solution was shielded from light and left to stand for 12-16 hours. The ABTS stock solution was further diluted in ethanol to achieve an absorbance of 0.70 (±0.02) at 734 nm.

The same procedure was applied to three different concentrations of methanolic oil solutions (0.01, 0.5, and 1 mg/mL). The reduction in color absorbance was calculated as a percentage relative to concentration and was expressed as Trolox equivalents. Antioxidant activity was assessed at three concentrations aligned with the doses on the Trolox curve. The antioxidant capacity was quantified in Trolox(R) equivalent (TEAC), which represents the concentration (mmol/L or mg/L) of Trolox(R) that exhibits an activity equivalent to a unit concentration of the substance being tested.

### 3.6 Molecular Docking

To acquire the 3D structure of the EO's components of interest, based on their binding energy (ΔG), we used the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Thereafter, the obtained files were minimized and converted to ligand for the analysis of the interactions with NADPH oxidase, referenced as 2CDU in the protein data bank using AutoDock. The protein was used as a receptor after removing ligands (ADP and FAD) and water molecules and introducing polar hydrogen atoms. Subsequently, docking analysis was performed using Discovery Studio Visualizer.

## 4. Conclusions

A comprehensive investigation of *Cupressus sempervirens var. horizontalis* essential oil highlighted its biochemically rich and heterogeneous content with hydrocarbon monoterpenes such as α-pinene and δ-3-carene being the predominant constituents. In the DPPH and ABTS tests, the essential oil showed strong antioxidant activity, demonstrating its efficiency in scavenging free radicals. In silico molecular docking also confirmed such findings by showing stable interaction with significant constituents of EO and NADPH oxidase, an important enzyme for the generation of reactive oxygen species (ROS). Compounds like terpinen-4-ol and α-terpinyl acetate demonstrated strong and specific binding interactions, indicating a significant role in modulating oxidative stress. The overall antioxidant potential seems to come from the synergistic effects of several constituents, even though some, like cedrol, showed less favorable binding because of destabilizing interactions. These outcomes support the potential application of *C. sempervirens* essential oil as a natural, prosperous, and safer alternative to synthetic antioxidants in therapeutics, food preservation, and cosmetic formulations.

## 5. Conflicts of interest

There are no conflicts to declare.

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