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UPLC-MS/MS Analysis and Evaluation of the Photoprotective, Antioxidant, Anti-Inflammatory and Anti-Enzymatic Properties of Ethyl Acetate and n-Butanol Fractions from Algerian *Juniperus oxycedrus* L. Leaves

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ABSTRACT

Juniperus oxycedrus is prevalent in the arid and semi-arid regions of the Mediterranean and Near East and, is traditionally employed for both culinary and medicinal applications. This research evaluates the potential health benefits of ethyl acetate (EAJO) and n-butanol (NBJO) fractions derived from leaves. Quantitative analysis revealed enhanced concentrations of phenolic and flavonoid compounds in the EAJO fraction at 252.56 ± 3.91 mg GAE/g DW and 47.70 ± 2.21 mg QE/g DW, respectively. UPLC-ESI-MS/MS identified a rich array of twelve phenolic acids and flavonoids, with rutin and chrysin identified as the predominant compounds in both fractions. Comprehensive antioxidant assessments through seven distinct assays demonstrated strong properties, with most activities displaying an IC_{50} values under 30 µg/mL. Additionally, *in vitro* anti-inflammatory activity showed that the EAJO and NBJO fractions effectively inhibited albumin denaturation at different concentrations, indicating a moderate effect compared to the standard drug diclofenac sodium. EAJO fraction surpassed NBJO in inhibiting key enzymes associated with Alzheimer's disease, diabetes, hyperpigmentation, dermatological disorders, and select bacterial infections, evidenced by IC_{50} values for acetylcholinesterase, alpha-amylase, tyrosinase, and urease with IC_{50} values of 14.60 ± 0.98 µg/mL, 639.48 ± 7.43 µg/mL, 206.44 ± 18.48 µg/mL, and 245.77 ± 3.75 µg/mL, respectively. Both fractions also displayed promising photoprotective properties, with SPF ratings of 31.71 ± 0.31 for NBJO and 22.62 ± 1.52 for EAJO. This study highlights the significant potential of *Juniperus oxycedrus* in the development of pharmaceutical, cosmetic, and nutritional products, underlining its substantial bioactive profile with antioxidant, anti-inflammatory, anti-enzymatic, and photoprotective capabilities.

***Keywords*:** *Juniperus oxycedrus*, flavonoids, acetylcholinesterase, alpha-amylase, tyrosinase, urease.

Introduction

Historically, people across the globe have exploited the healing powers of wild plants as a practice documented in traditional recipes from ancient Greek, African, American, European, and Asian cultures.[1] As the World Health Organization outlines, traditional medicine involves a wide range of practices, knowledge, and belief systems focused on employing plant, animal, and mineral-based spiritual therapies. These techniques are exclusively applied to prevent and treat various diseases and illnesses.[2]

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In our current era, a surge of interest in the human health sector has been noticeably discernible among researchers investigating natural pharmacological agents, particularly those exhibiting minimal adverse impacts on human physiological functions.[3]

Concurrently, the demand for herbal cosmetics used in sunscreens has experienced rapid growth. This demand stems from the desire to provide enhanced protection against Ultraviolet (UV) radiation, which can potentially cause significant damage and contribute to the development of various skin diseases.[4] This rising interest arises partly from the awareness of the toxicity associated with the overuse of synthetic drugs, which have been linked to various diseases, including cancer. [5,6] This consciousness has directed research and consumer demand towards safer products devoid of artificial additives, focusing on natural antioxidants.[5] The therapeutic potential of plants has increasingly gained significant attention due to their antioxidant properties, chelate catalytic metals, and act as oxygen scavengers. Consequently, plants are viewed as a valuable resource in mitigating oxidative damage caused by free radicals.[5,7] This aspect is particularly significant given that oxidative stress plays a pivotal role in the initiation and progression of numerous diseases such as neurodegenerative disorders, inflammatory diseases, cancer, diabetes mellitus, and skin diseases,[5,8] which mainly develop from the overproduction of reactive oxygen species (ROS) within the human

body and the insufficient effectiveness of endogenous antioxidant mechanisms. This imbalance disrupts cellular homeostasis, damaging essential biological molecules such as DNA, proteins, and lipids.[8] Extensive research has focused on identifying additional antioxidants derived from natural sources, especially polyphenols and flavonoids.[9–14] These compounds identified as secondary plant metabolites, are extensively found in a diverse range of plant materials.[3,5,7] *Juniperus*, a genus in the Cupressaceae family, consists of about 75 species widespread throughout the Northern Hemisphere and especially along the Mediterranean coasts, including Algeria, Morocco, Tunisia, France, Italy and Turkey.[15,16] The Algerian flora is characterized by the presence of five distinct species of *Juniperus*; among these, *Juniperus oxycedrus* L., commonly known as "Tagga" in Algeria, which is a small shrub growing wild on semi-arid slopes and hills.[16] This plant is extensively used in traditional medicine. In Turkey, juniper tar, leaves, and fruits are famous for treating wounds, stomach and abdominal pain, gynaecological conditions, haemorrhoids, colds, coughs, bronchitis, fungal infections and kidney stones.[15,17] Additionally, *J. oxycedrus* fruit and leaf infusions are consumed internally, and its pounded fruits are used to manage diabetes.[17] Furthermore, an oil known as Cade oil, derived from the destructive distillation of the branches and wood of *Juniperus oxycedrus*, has been used for centuries to treat skin conditions in humans and animals, including psoriasis, eczema, inflamed wounds, hair issues like dandruff.[18] In various studies, *Juniperus oxycedrus* has been reported to exhibit a range of biological activities. These include essentially antioxidant,[16,19–21] antiviral,[22] anti-inflammatory and antinociceptive,[15,21] antidiabetic,[23,24] anti-cancer,[25] anti-proliferative[19] and neurodegenerative.[26]

The present study provides a comprehensive phytochemical and biological assessment of *Juniperus oxycedrus* L. leaf fractions, particularly emphasizing the use of ethyl acetate and n-butanol extracts. This marks the first time these extracts have been employed using advanced LC-ESI-MS/MS techniques for detailed analysis, and it is also the first application of these extracts for exploring antioxidant, anti-inflammatory, anti-enzymatic, and Sun protection factor (SPF) properties.

The methods chosen, including LC-ESI-MS/MS for compound identification and various bioassays for testing antioxidant and anti-inflammatory effects, are particularly suited to this study's objectives. These techniques allow for precise measurements of compound activity and interactions, essential for establishing a scientific basis for traditional uses and developing potential therapeutic applications.

This investigation explores the specific bioactive compounds of *J. oxycedrus*, uncovering novel antioxidant, anti-inflammatory, and photoprotective properties, and evaluates their *in vitro* efficacy against protein denaturation. Moreover, it examines the enzymatic inhibitory properties related to chronic diseases such as diabetes and neurodegenerative disorders, offering a new dimension to our understanding of this plant's capabilities. This approach not only confirms the known benefits of *J. oxycedrus* but also identifies potential therapeutic applications that set a precedent for future pharmacological explorations within the genus.

Materials and Methods

Chemicals

The chemical products and reagents used in our experiments encompassed various antioxidants, assay reagents, and solvents, all of analytical grade, which were procured from two main suppliers: Sigma-Aldrich (Germany) and Biochem Chemopharma. From Sigma-Aldrich, we obtained a range of key reagents including Folin-Ciocalteu's reagent, antioxidants such as butylated hydroxylanisole and butylated hydroxytoluene, compounds like galantamine, kojic acid, and thiourea, as well as biological enzymes like Acetylcholinesterase from electric eel and others including alpha amylase from *Aspergillus oryzae*, tyrosinase from mushroom, and urease from *Canavalia ensiformis*. Other significant chemicals from Sigma-Aldrich included various assay reagents like DPPH, ABTS, and compounds for chelation and reduction reactions such as neocuproine and potassium ferricyanide. From Biochem Chemopharma, we sourced essential chemicals including acetylthiocholine iodide, sodium carbonate, aluminum nitrate, several metal chlorides (iron (III) chloride, iron (II) chloride, copper (II) chloride), and other vital laboratory chemicals like potassium persulfate, potassium acetate, ammonium acetate, phosphate buffer, silver nitrate, trisodium citrate, and bovine serum albumin (BSA). The equipment used were rotavapor (BUCHI R-210), a 96-well microplate reader (Multimode Plate Reader, EnSpire, PerkinElmer, Waltham (US) United States of America and an UPLC-ESI-MS-MS Shimadzu 8040 Ultra-High sensitivity with UFMS technology was employed and equipped with binary bump Nexera XR LC-20AD.

Plant material

The aerial parts of *J. oxycedrus* subsp. *oxycedrus* were harvested in March 2019 from the Abdelaziz municipality, located in the Jijel province (36°50'45.7"N 6°01'45.4"E, Algeria). A voucher specimen was deposited at the herbarium of the faculty under the number (JNP/01/2020). The collected plant specimens were air-dried in a shaded area. Subsequently, the leaves were separated from the stems and ground into a fine powder.

Extraction

The dried aerial parts of *J. oxycedrus* (25g) were subjected to thrice maceration using 200 mL of a hydro-alcoholic solution (80 % methanol, 20 % distilled water) at ambient temperature. This process was conducted in the dark with constant shaking for 48 hours. The resulting solutions were filtered through Whatman filter paper No. 1, followed by solvent removal under pressure in a rotary evaporator. After filtration, the remaining aqueous phase underwent successive extraction with solvents of increasing polarity: 100 mL of petroleum ether (repeated twice), 100 mL of dichloromethane (twice), 100 mL of ethyl acetate (twice), and 100 mL of n-butanol (twice). These solvents were then removed using a rotary evaporator (BUCHI R-210) at (40 °C). The resulting concentrated fractions were stored dry at -20 °C in smoked glass vials until analysed. This paper focuses on two of these fractions: ethyl acetate and n-butanol.

Liquid Chromatography-Electrospray Ionization-Mass Spectrometry analysis LC-ESI-MS/MS

The analysis of various phytochemicals in the n-butanol and ethyl acetate fractions *of J. oxycedrus subsp. oxycedrus* leaves were conducted using a Shimadzu 8040 UPLC-ESI-MS-MS, featuring Ultra-high sensitivity with (UFMS) technology, equipped with a Nexera XR LC-20AD binary pump. Chromatographic separation was achieved using an Ultra-force C18 column (150 mm × 4.6 mm, 3 µm particle size; Restek). The separation process utilised a mobile phase A of water with 0.1% formic acid and methanol for mobile phase B, following a specific gradient elution schedule: 80% A for 0-1 minute, transitioning to 20% A over 1-30 minutes, then to 0% A for the next 10 minutes, maintained at 0% A for 40-45 minutes, and finally returning to 80% A for the last 15 minutes. The flow rate was maintained at 0.3 mL/min, with an injection volume of 10 µL, and the column temperature was set at 40°C. ESI conditions for the LC-MS-MS included a CID gas pressure of 230 KPs, a conversion dynode at −6.00 Kv, interface temperature at 350 °C, DL temperature at 250 °C, nebulising gas flow at 3.00 L/min, heat block at 400 °C, and a drying gas flow at 15.00 L/min. An ion trap mass spectrometer was employed in both negative and positive ion modes, utilising Multiple Reaction Monitoring (MRM).

Determination of bioactive compounds in J. oxycedrus leaves.

Determination of total phenolic content (TPC)

The total phenolic content in the ethyl acetate fraction was assessed spectrophotometrically, following the Folin-Ciocalteu method detailed by Singleton and Rossi [27] with slight modifications. In this procedure, 20 µL of the plant extract was mixed with 100 µL of Folin–Ciocalteu reagent (diluted 1:10 with distilled water) and 75 µL of sodium carbonate solution (7.5%) in a 96-well microplate. After incubation for 120 minutes at room temperature in the dark, the absorbance was measured at 765 nm using a Multimode Plate Reader, EnSpire, PerkinElmer, Waltham (US). A blank was prepared similarly,

substituting the extract with methanol. The phenolic content was determined using a standard curve generated with gallic acid (equation: y = 0.0034x + 0.1044; R^2 = 0.997). The results were expressed as micrograms of gallic acid equivalent per milligram of extract (µg GAE/mg).

Determination of total flavonoid content (TFC)
Total flavonoid content was determined using the aluminium chloride colourimetric assay described by Topçu *et al.*[28] Specifically, 50 µL of the extract (1 mg/mL) was combined with 130 µL of methanol, 10 µL of 1 M potassium acetate, and 10 µL of 10% aluminum nitrate in a 96-well microplate. After allowing the mixture to incubate at room temperature for 40 minutes. The absorbance was observed at 415nm. The flavonoid concentration was quantified by referencing a standard calibration curve of quercetin (equation: y = 0.0048x; R^2 = 0,9994), and the results were reported as micrograms of quercetin equivalent per milligram of extract (µg QE/mg).

Determination of the in vitro Antioxidant activity
DPPH free radical scavenging activity
The antioxidant potential of the fractions from *J.oxycedrus* leaves was assessed using the DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical based on the protocol established by [29]. In this assay, 40 µL of each extract, with concentrations ranging for ethyl acetate from 0.78125 to 50 µg/mL and for n-butanol from 3.125 to 200 µg/mL, combined with 160 µL of a pre-prepared DPPH solution (0.1 mM in methanol) in a 96-well microplate. The reaction mixtures were incubated in the dark at room temperature for 30 minutes. Following incubation, the absorbance was measured at 517 nm using a Multimode Plate Reader, EnSpire, PerkinElmer, Waltham (US). BHT (Butylated Hydroxytoluene) and BHA (Butylated Hydroxyanisole) were employed as antioxidant standards. The findings regarding the concentration required for 50% inhibition (IC_{50}) expressed in µg/mL were quantified.

ABTS scavenging activity
The scavenging activity against $ABTS^+$ radical was assessed using the ABTS radical cation decolourisation test, according to the method described by Re *et al.*[30] Initially, the $ABTS^{\cdot+}$ radical cation was formed by mixing 7 mM ABTS solution with 2.45 mM potassium persulfate ($K_2S_2O_8$) and allowing the reaction to occur in the dark at room temperature for 16 hours. This resulting $ABTS^{\cdot+}$ solution was then diluted with distilled water to reach an absorbance of 0.7 at 734 nm. In a 96-well microplate, 160 µL of the diluted $ABTS^{\cdot+}$ solution was combined with 40 µL of each extract prepared in methanol at varying concentrations ranging from 1.5625 to 100 µg/mL. The mixtures were incubated at room temperature for 10 minutes, and absorbance was subsequently measured at 734 nm. Standards such as BHT and BHA were used as antioxidant. The percentage of inhibition (I%) was calculated for each concentration, with results expressed as IC_{50} values, representing the concentration needed to inhibit 50% of the $ABTS^{\cdot+}$ radicals.

Reducing power assay
The reducing potential of the *Juniperus oxycedrus* fractions was assessed, employing the method described by Oyaizu *et al.*[31] In this assay, 10 µL of each extract at varying concentrations from 12.5 to 800 mg/mL, was combined with 40 µL of phosphate buffer (200 mM, pH 6.6) and 50 µL of 1% potassium ferricyanide ($K_3[Fe(CN)_6]$). The mixture was incubated at 50°C for 20 minutes. Following incubation, 50 µL of 10% trichloroacetic acid, 40 µL of distilled water, and 10 µL of 0.1% ferric chloride ($FeCl_3$) solution were added. The absorbance of the resulting solution was measured at 700 nm using a spectrophotometer. The results were expressed as $A_{0.50,}$ indicating the concentration that achieves a 0.5 absorbance. Ascorbic acid served as the reference standard.

Cupric reducing antioxidant capacity (CUPRAC)
The antioxidant capacity of the fractions in terms of cupric- reduction was measured using the CUPRAC test outlined by Apak *et al.*[32] In each well of a 96-well microplate, 40 µL of the extract at varying concentrations from 12.5 to 800 mg/mL, was combined with 50 µL of 10 mM copper (II) chloride ($CuCl_2$), 50 µL of 7.5 mM neocuproine ($C_{14}H_{12}N_2$), and 60 µL of 1 M ammonium acetate buffer (pH 7.0). The mixture was allowed to incubate at room temperature for 1 hour. After incubation, the absorbance was recorded at 450 nm using a spectrophotometer. Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) served as antioxidant standards. The $A_{0.5}$ (µg/mL) results corresponded to the concentration indicating 0.50 absorbance.

Silver nanoparticle assay (SNP)
The reduction of Ag^+ to spherical silver nanoparticles (SNPs) was according to the method developed by Özyürek *et al.*[33] The preparation of the spherical silver nanoparticles (SNP) solution, 50 mL of 1 mM silver nitrate ($AgNO_3$) was mixed with 5 mL of 1% trisodium citrate. The trisodium citrate solution was gradually introduced to the silver nitrate solution until a pale-yellow hue developed, indicating the formation of SNPs. In a 96-well microplate, 20 µL of the extracts at varying concentrations from 12.5 to 800 mg/mL were combined with 130 µL of the SNP solution and 50 µL of distilled water. The mixture was incubated at 25°C for 30 minutes. After incubation, the absorbance measurements were taken at 423 nm, and the results were expressed as $A_{0.5}$. Ascorbic acid served as the reference standard.

Galvinoxyl free radicals scavenging assay (GOR)
As proceeded by Shi et al,[34] the evaluation of the antioxidant activity utilising the galvinoxyl method was rigorously conducted. Initially, 40 µL of the extracts, at concentrations ranging from 12.5 to 800 mg/mL, were mixed with 160 µL of 0.1 mM galvinoxyl solution in methanol in a 96-well microplate. The microplate was then incubated in the dark at room temperature for 120 minutes. After the incubation period, the absorbance was measured at 428 nm. Ascorbic acid served as the standard in this study. The results were represented as EC_{50} (µg/mL), indicating the concentration at which 50% absorbance intensity was observed.

Phenanthroline assay
Based on the phenanthroline method, the antioxidant activity was evaluated following the protocol described by Szydłowska-Czerniak *et al.*[35] with slight modifications. In a microplate, 10 µL of each extract at concentrations ranging from 12.5 to 800 mg/mL was combined with 50 µL of 0.2% ferric chloride ($FeCl_3$), 30 µL of 0.5% 1,10-phenanthroline in methanol and 110 µL of methanol, making up a final volume of 200 µL. The mixture was incubated in the dark at 30°C for 20 minutes. After incubation, the absorbance was measured at 510 nm using a spectrophotometer. Data were expressed as $A_{0.50,}$ representing the concentration corresponding to a 0.50 absorbance. BHA and BHT were employed as reference antioxidant standards for comparison.

Inhibition of albumin denaturation
The *in vitro* evaluation of the anti-inflammatory properties was performed using the protein denaturation technique, according to the detailed protocol by Karthik *et al.*[36] To perform the assay, 1 mL of each extract was mixed with 1 mL of 0.2% Bovine Serum Albumin (BSA) solution prepared in a 50 mM Tris-HCl buffer (pH 6.6). The reaction mixture was incubated at 37°C for 15 minutes, followed by heating at 72°C for 5 minutes. After cooling, spectrophotometric turbidity measurements at 660 nm were carried out. Water was the blank, while a standard drug (Diclofenac) was a reference. The experiment was replicated three times. For the percentage of protein denaturation inhibition, the following equation 1 was followed:

$$\text{Inhibition denaturation } \% = \frac{[(\textbf{Absorbance control} - \textbf{Absorbance sample})]}{\textbf{Absorbance control}} \times \mathbf{100} \quad [1]$$

Anti-enzymatic activities
Evaluation of acetylcholinesterase (AchE) inhibitory activity
The fractions derived from the leaves of *Juniperus oxycedrus* were analysed to ascertain any potential inhibitory effects on AchE activity.

This quantification was done using the method that Ellman *et al.* determined.[37] Different concentrations of the sample solution, from 12.5 to 800 mg/mL (10 µL), were mixed with 150 µL of sodium phosphate buffer (100 mM, pH 8.0) and 20 µL of AChE solution in a 96-well microplate. The mixture was incubated at 25°C for 15 minutes. Following this, 10 µL of 0.5 mM dithio-bis(2-nitrobenzoic) acid (DTNB) and 10 µL of acetylthiocholine iodide were added to each well. Absorbance at 412 nm was measured using a Multimode Plate Reader, EnSpire, PerkinElmer, Waltham (US, with results given as IC_{50} values. Galantamine served as a reference standard.

Evaluation of α-amylase inhibitory activity

α-Amylase inhibitory activity was performed using the iodine/potassium iodide (IKI) method of Zengin *et al.*[38] In each well of a 96-well microplate, 25 µL of the sample at varying concentrations (12.5 to 800 mg/mL) was combined with amylase in 1 unit of sodium phosphate buffer (pH 6.9 containing 6 mM NaCl). The mixture was incubated at 37°C for 10 minutes before initiating the reaction by adding 50 µL of a 1% starch solution. Concurrently, a control was prepared, excluding the enzyme solution. After a further 20 minutes of incubation at 37°C, the reaction was stopped by adding 25 µL of 1 M hydrochloric acid and 100 µL of an iodine-potassium iodide solution. The absorbance was measured at 630 nm, and the results were given as IC_{50} value.

Evaluation of tyrosinase inhibition ability

The tyrosinase inhibition activity was measured using the method described by Deveci *et al.*[39] Samples of 10 µL of extracts at concentrations ranging from 12.5 to 800 mg/mL were placed in a 96-well microplate. To each well, 150 µL of 100 mM sodium phosphate buffer (pH 6.8) and 20 µL of tyrosinase enzyme solution (150 units/mL) were added. After a 10-minute incubation at room temperature, 20 µL of 5 mM L-DOPA was introduced to initiate the reaction. The microplate was then incubated at 37°C for another 10 minutes. Absorbance was measured at 475 nm using a Multimode Plate Reader, EnSpire, PerkinElmer, Kojic acid was used as a standard. The absorbance was measured at 475 nm, and the results were given as IC_{50} value.

Evaluation of urease inhibitory activity

The urease inhibitory capability was assessed by measuring ammonia production utilising the indophenol method described by Taha *et al.*[40] The process involved combining 25 µL of urease, 10 µL of the test sample at concentrations ranging from 12.5 to 800 mg/mL, and 50 µL of a 17 mM solution of urea in each well of a 96-well plate. This setup was incubated at 30°C for 15 minutes. Following this, 45 µL of a phenol solution (8% w/v phenol with 0.1% w/v sodium nitroprusside) and 70 µL of an alkaline mixture (2.85% NaOH and 4.7% sodium hypochlorite) were added. The reaction's absorbance was then read at 630 nm after an additional 50 minutes using a microplate reader. Thiourea was employed as a reference inhibitor, and the effectiveness of the inhibition was quantified in terms of the IC_{50} value.

Photoprotective activity (SPF)

The ethyl acetate and n-butanol fractions were solubilised in ethanol to achieve a concentration of 2 mg/mL (2000 ppm) and then spectrally analyzed across a wavelength range of 290 to 320 nm with 5 nm intervals. The sun protection efficacy was assessed through the *in vitro* determination of the Sun Protection Factor (SPF), utilizing the formula proposed by Mansur *et al.*[41] for SPF calculations. The absorbance of each sample was measured thrice, as shown in the referenced equation 2.

$$SPF_{spectrophotometry} = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda) \qquad [2]$$

Where; EE: erythemal effect spectrum, I: solar intensity spectrum, Abs: absorbance of sunscreen product, CF: correction factor (= 10). The product E(λ)x I(λ) is constant.

Statistical analysis

Results are reported as Mean ± SD of three replicates; the IC_{50} and $A_{0.50}$ values were calculated by linear regression analysis. All data were analysed using SPSS version 25. Depending on the normality of the results determined by the Shapiro-Wilk test, a one-way ANOVA with a Tukey's post hoc analysis or Kruskal-Wallis non-parametric test was carried out. Statistical difference was set at $p < 0.05$.

Results and Discussion

Exploring the bioactive contents and in vitro antioxidant activity of ethyl acetate and n-butanol fractions

Total bioactive content

Polyphenols, widely prevalent as secondary metabolites in plants, are the primary source of natural antioxidants. Due to their hydroxyl groups, they can effectively neutralise free radicals. These primary antioxidants, including phenolic acids, flavonoids, tannins, stilbenes and coumarins, among others, have been shown to offer several therapeutic properties that diminish the risk of cancer and diabetes, demonstrating antibacterial, antiviral, anti-inflammatory, and anti-allergenic properties.[42] The quantification of total phenolic and flavonoids in ethyl acetate (EAJO) and n-butanol (NBJO) fractions derived from the hydro-methanolic extract of the *J.oxycedrus* leaves is shown in Table 1. Considering the obtained findings, the EAJO fraction exhibited a superior polyphenolic concentration (252.56±3.91 µg GAE/mg dw) compared to the NBJO fraction (119.14±3.39 µg GAE/mg dw). Concurrently, the flavonoid content was notably higher in the EAJO fraction (47.70±2.20 µg QE/mg dw), followed by NBJO (31.14±2.50µg QE/mg dw).

In the literature, a range of results has been documented on the bioactive content in the aerial parts of various extracts from *J.oxycedrus*. However, studies specifically concerning ethyl acetate and n-butanol fractions were scarcely reported. According to previous studies, Chaouche *et al.*[43] reported a polyphenols content of 133.08±4.1 mg GAE/g DW in the hydro-methanolic extract of the needle from the *J.oxycedrus* collected in Tlemcen, Algeria, which was lower than the TPC content found in our EAJO fraction. Similarly, the ethanolic extract of the aerial parts of the *J.oxycedrus* from Batna, Algeria, exhibited a lower flavonoid content of 23.11±3.22 µg QE/mg,[44] which was also lower than the level we obtained. In contrast, Ben Mrid *et al.*[45] reported notably higher phenolic (292.52±11 mg GAE/g) and flavonoid contents (54.58 ± 2.98 mg QE/g) in the methanolic extract from Moroccan *J.oxycedrus* species. Similarly, Djellouli *et al.*[21] reported a high flavonoid content of 90.56±2.23 mg QE/g dw in the methanolic extract from *J.oxycedrus* leaves collected from Mascara, Algeria, which is significantly higher than our findings. The differences between these results may be attributed either to the extraction method, the geographical environments, or the plant development stage.[45]

Table 1: Total bioactive content of the ethyl acetate and n-butanol fractions of *Juniperus oxycedrus* leaves

Fractions	Total bioactive content	
	Phenolic content (µg GAE/mg)	Flavonoid content (µg QE/mg)
EAJO	252.56 ± 3.91	47.70 ±2.20
NBJO	119.14 ± 3.39	31.14±2.50

Results are expressed as means ± standard deviation of triplicate.
EAJO: ethyl acetate fraction from *Juniperus oxycedrus* leaves; NBJO: n-butanol fraction from *Juniperus oxycedrus* leaves.

Identification of the phenolic compounds by UPLC LC-MS/MS

In light of the considerable antioxidant capacities and the significant flavonoids/phenolic contents, the EAJO and NBJO fractions were selected to identify and characterize their main compounds. To our knowledge, this study is the first to employ LC-MS/MS for analyzing the secondary metabolites in these specific fractions derived from the *Juniperus oxycedrus* leaves. The UPLC-MS/MS analysis, as presented in Table 2, indicated that the EAJO and NBJO fractions from the plant leaves were almost similar. Nevertheless, there were notable differences in the presence and quantities of certain components. Overall, the analysis revealed the presence of eleven distinct compounds in the EAJO and ten compounds in the NBJO; most of

these compounds are classified as flavonoid and phenolic acid classes. Specifically, the analysis revealed the presence of two phenolic acids (salicylic acid and ferulic acid), five flavonoids (chrysin, quercetin, rutin, naringenin and hespertin), one carotenoid (beta carotene), one vitamin (folic acid), one coumarin (hydroxycoumarin) and one stilbenoid (viniferin).

The analysis demonstrated variations in the total area percentage of the identified compounds across different fractions derived from *J. oxycedrus*. Rutin was detected as the major flavonoid component in both EAJO and NBJO fractions, with 86.78 and 88.14 % area percentages, respectively. These findings were followed by the presence of chrysin, narangenin and quercetin. In the EAJO fraction, ferulic acid was the main phenolic acid, representing 1.14% of the total area. In contrast, the NBJO fraction had a minimal presence of salicylic acid, with an area percentage of 0.01%. Regarding chrysin, quercetin, rutin, naringenin, hespertin, beta carotene, folic acid and viniferin were detected in both fractions. On the other hand, ferulic acid was only found in EAJO, whereas traces of hydroxycoumarin and salicylic acid were exclusively found in the NBJO fraction (Table 2).

Only a limited number of studies have analysed the chemical composition of the *Juniperus* genus, with even fewer focusing on *J.oxycedrus*. Notably, a recent investigation by Meringolo *et al.*[19] demonstrated that (−)-epicatechin, rutin and quercetin-3-O-glucoside were the major identified constituents in the polar fractions of the aerial part of the *J. oxycedrus* collected in Italy. In a previous study, Yaglioglu *et al.*[46] demonstrated that rutin and catechin were identified as the most abundant phenolic compounds in the methanolic extract of *J. oxycedrus* collected in Turkey. Furthermore, a recent study revealed that chlorogenic acid, naringin and rutin were the most identified compounds detected in the methanolic extract of *J.oxycedrus* leaves from Algeria.[21] On the other hand, salicylic acid, hesperidin, rutin and naringenin were reported as the most abundant compounds in the methanolic extract obtained from the Moroccan *J.oxycedrus* species.[45]

The comparison of the phenolic compounds profile of EAJO and NBJO leaf fractions from *J.oxycedrus* in our study with those identified in the aerial parts of the same species in earlier research highlighted variations in the compounds detected. Indeed, rutin, naringenin and hesperitin have been previously reported in *J.oxycedrus* extracts.[21,45,47] However, catechin, caffeic acid, chlorogenic acid and gallic acid, commonly detected in the above research,[19,46] were not found in our study. In contrast, chrysin and ferulic acid detected in this study were scarcely mentioned and were first reported in *J. oxycedrus* aqueous extract from Algeria by Mehatp *et al.*[47] Concerning quercetin, beta-carotene, viniferin, and folic acid, these compounds were detected for the first time in our study, albeit in low concentrations. Our chemical composition results of *J. oxycedrus* did not completely align with the existing literature data on the same species. This difference may stem from variations in the plant species examined, the selection and the types of standard compounds used in our analysis. Further exploration using a broader range of standard compounds in future research could potentially provide a more comprehensive understanding of the chemical profile of *J. oxycedrus subsp. oxycedrus*.

Antioxidant activities

The antioxidant activity of plant extracts is commonly assessed through various *in vitro* methods. These methods primarily assess the capacity of the extracts to neutralise stable free radicals effectively. Since there is no singular method universally and precisely recommended to provide the antioxidant potential of plant extracts [5], our study employed several complementary *in vitro* tests to investigate the antioxidant activity of the EAJO and NBJO fractions, such as free-radical scavenging with DPPH, ABTS and GOR, as well as reducing abilities with CUPRAC, phenanthroline and reducing power assays, in addition to the silver nanoparticle (SNP). The findings of the antioxidant activities are presented in Table 3, expressed in terms of IC_{50} and $A_{0.5}$, compared to antioxidant standards (BHT, BHA, and ascorbic acid). In this study, the leaf fractions from *J. oxycedrus* exhibited moderate antiradical activity in the DPPH assay. The EAJO fraction was the most potent, with an IC_{50} value of 30.36±0.20 µg/mL, while the NBJO fraction followed with an IC_{50} value of 73.55±0.67 µg/mL. However, both EAJO and NBJO fractions show no significant difference compared to the standards BHT and BHA in the DPPH assay, which have IC_{50} values of 6.14±0.41µg/mL and 12.99±0.41µg/mL, respectively. This DPPH scavenging activity agreed with the findings of Meringolo *et al.*[19] and Djellouli *et al.*[21] but contrasted with those reported by Chaouche *et al.*[43]

The radical scavenging activity was also evaluated using the ABTS assays. The ABTS assay results revealed that the EAJO fraction exhibited greater antiradical activity, followed by the NBJO fraction, with an IC_{50} value of 1.57±0.09 µg/ml. This value was lower than that of the standard antioxidant BHT, which was 1.14 ±0.11 µg/ml, demonstrating the potent capacity of the EAJO fraction to neutralise ABTS free radicals. The IC_{50} value of the NBJO fraction was found to be 1.83±0.01 µg/ml, which is relatively close to the IC_{50} values of BHT and BHA 1.75±0.20 and 1.14±0.11 µg/ml, respectively. Moreover, this significant antioxidant activity is in harmony with previous research that found the methanolic extract of *J.oxycedrus* needles to have the ability to quench $ABTS^{+}$ with an IC_{50} of 0.12±0.00 mg/ml.[45] In contrast, Meringolo *et al*., [19] Jemli *et al.*[20] and Chaouche *et al*.[43] reported an ABTS activities of 9.3±1.3 µg/ml, 19.80 ± 0.55 µg/ml and 90.16 ± 4.2 µg/ml, respectively, which are less effective compared to our findings. In the reducing power assay, both EAJO and NBJO fractions demonstrated noteworthy antioxidant activity. Compared to the Ascorbic acid standard, which had an $A_{0.5}$ value of 6.73±1.15 µg/mL, the EAJO extract exhibited a considerable reducing power with an $A_{0.5}$ value of 12.67±0.5 µg/mL. Conversely, the NBJO extract exhibited a weak antioxidant capacity with an $A_{0.5}$ value of 23.44±0.57 µg/ml.

In the same context, Jemli *et al*.[20] reported that the aqueous extract of the Moroccan *J. oxycedrus* leaves also exhibited a considerable reducing power of IC_{50} of 24.23±0.07 µg/ml. In comparison to our findings, Meringolo *et al*., [19] Ben Mrid *et al*.[45] and Chaouche *et al*.[43] reported an IC_{50} of 99.5±3.7 µg/ml, 139.14±2.77 mg/ml and 290±18.3 µg/ml, respectively, this value indicated lower antioxidant activities than those observed in our study. The variation in extraction procedures could explain the differences. Specifically, using more polar solvents in the extraction process may lead to the higher antioxidant activity observed, as these solvents are known to extract a greater amount of total phenolic content (TPC), which is closely linked to antioxidant efficacy.[21,48]

Regarding the GOR assay, The EAJO was more active than the NBJO, with IC_{50} values of 7.10±0.64 µg/ml and 20.80±1.21 µg/ml, respectively. The IC_{50} value of the EAJO fraction was notably close to that of the BHA, which was 5.38±0.06 µg/ml, indicating a competitive scavenging ability. Although the activity of the NBJO fraction was higher, indicating lower potency than the Ascorbic acid, with an IC_{50} value of 6.73±0.29 µg/ml, it still demonstrated a potent free radical scavenging activity. In the CUPRAC assay, EAJO demonstrated the most potent activity with an $A_{0.5}$ value of 9.58±0.72 µg/mL, which was notably close to the $A_{0.5}$ values of the established antioxidant standards BHT and BHA, possessing an $A_{0.5}$ of 5.35±0.71 and 8.97±3.94 µg/mL, respectively. In contrast, the NBJO fraction presented an $A_{0.5}$ value of 20.80±1.21 µg/mL, indicating comparatively weaker antioxidant capacity. The assessment of metal iron reduction was carried out using the phenanthroline assay. As detailed in Table 3, the highest antioxidant activity was recorded in the NBJO fraction, with an IC_{50} of 2.09±0.57 µg/mL, followed by the EAJO fraction, with an IC_{50} of 2.77±0.28 µg/mL. Furthermore, NBJO showed a superior antioxidant capacity compared to the BHA standard, with an IC_{50} of 2.12±0.04 µg/ml. In the silver nanoparticle assay, EAJO was more efficient than the NBJO fraction, with an $A_{0.5}$ value of 16.11± 1.18 µg/ml, followed by the NBJO with an $A_{0.5}$ value of 220.00±3.54 µg/ml, demonstrating a lower concentration compared to the Ascorbic acid standard with an $A_{0.5}$ 7.14±0.05 µg/ml. The GOR, CUPRAC, phenanthroline and silver nanoparticle assay results are obtained for the first time with the Algerian *J. oxycedrus* species; no study has been carried out before.

In the aforementioned results, EAJO demonstrated superior antioxidant properties compared to the NBJO fraction. This enhanced activity may be attributed to their phenolic and flavonoid composition and quantity differences. The variety of bioactive compounds in plant

extracts, known for their antioxidant and biological benefits, play a crucial role in determining their effectiveness. These compounds contribute significantly to the overall antioxidant capacity of the fractions. These include rutin, as reported by Sharma *et al*.[49,50] In addition to chrysin, naringenin, ferulic acid and quercetin, as mentioned in recent studies.[51–54] These compounds were detected and identified through LC-ESI-MS/MS analysis, as outlined in Table 2. Additionally, the synergistic effect of these bioactive molecules, along with the sensitivity and specific mechanisms of action of the reagents used in the study, might also play a significant role in the observed differences in antioxidant efficacy between the two fractions.

Table 2: Phenolic profile determined by LC-MS-MS of EAJO and NBJO fractions of *Juniperus oxycedrus* leaves

Compound	Molecular formula	Rt Time. EAJO	Area %. EAJO	Rt Time. NBJO	Area %. NBJO
Salysilc Acid	$C_7H_6O_3$	No peak is detected	0	10.759	0.011826
Ferulic Acid	$C_{10}H_{10}O_4$	10.797	1.148471	No peak	0
Chrysin	$C_{15}H_{10}O_4$	21.687	11.6941	21.668	11.31354
Quercetin	$C_{15}H_{10}O_7$	21.661	0.01145	22.676	0.018721
Viniferin	$C_{28}H_{22}O_6$	23.042	0.006167	24.489	0.001485
Chlorogenique acid	$C_{16}H_{18}O_9$	No peak is detected.	0	No peak	0
Beta Carotene	$C_{40}H_{56}$	32.165	0.183112	32.162	0.233544
Hepertin	$C_{16}H_{14}O_6$	37.903	0.0075	39.044	0.004819
Folic Acid	$C_{19}H_{19}N_7O_6$	39.187	0.00305	37.583	0.002416
Rutin	$C_{27}H_{30}O_{16}$	40.376	86.78932	40.379	88.1493
Hydroxy coumarin	$C_9H_6O_3$	No peak is detected.	0.00	46.045	0.000176
Naringenin	$C_{15}H_{12}O_5$	47.397	0.156913	45.948	0.264179

*RT: Time retention

Table 3: Antioxidant activity of EAJO and NBJO fractions from *Juniperus oxycedrus* leaves.

	DPPH IC_{50}(µg/mL)	ABTS IC_{50}(µg/mL)	CUPRAC $A_{0,50}$ (µg/mL)	Reducing Power Assay $A_{0,50}$ (µg/mL)	SNP IC_{50} (µg/mL)	Phenanthroline IC_{50}(µg/mL)	GOR IC_{50}(µg/mL)
EAJO	30.36 ± 0.20^{b}	1.83 ± 0.01^{a}	9.58 ± 0.72^{b}	12.67 ± 0.51^{b}	16.11 ± 1.18^{b}	2.77 ± 0.28^{a}	7.10 ± 0.64^{b}
NBJO	73.55 ± 0.67^{a}	1.57 ± 0.09^{ab}	20.80 ± 1.21^{a}	23.44 ± 0.57^{a}	220.00 ± 3.54^{a}	2.09 ± 0.57^{a}	9.84 ± 1.31^{a}
BHT*	6.07 ± 0.2^{d}	1.75 ± 0.2^{b}	5.35 ± 0.71^{d}	NT	NT	0.88 ± 0.04^{a}	NT
BHA*	12.9 ± 0.24^{c}	1.14 ± 0.11^{ab}	8.97 ± 3.94^{c}	NT	NT	2.12 ± 0.05^{b}	NT
AA*	NT	NT	NT	6.73 ± 1.15^{c}	7.14 ± 0.05^{c}	NT	6.73 ± 0.29^{b}

IC_{50} and $A_{0.50}$ were calculated by linear regression analysis and expressed as Mean ± SD (n=3). The values with different superscripts (a, b, c) in the same columns differ significantly.). EAJO: ethyl acetate fraction; NBJO: n-butanol fraction; BHA: butylated hydroxyanisole; BHT: butylated hydroxytoluene; AA: ascorbic acid; NT: not tested.

In vitro anti-inflammatory test

One of the fundamental characteristics of inflammation is protein denaturation, in which proteins lose their tertiary and secondary structure by applying external stress or compounds, such as ROS. Denaturation of proteins often results in the loss of their biological functions and the induction of the inflammatory reaction dysfunctions that may manifest as rheumatic and inflammatory disorders.[55] Traditionally, plants have been utilised for treating inflammatory conditions, suggesting they could be reservoirs for novel compounds beneficial in creating medications to alleviate or cure inflammatory symptoms.[55]

In our study, the anti-inflammatory potential of *J. oxycedrus* fractions was examined by assessing their effects on protein denaturation *in vitro*. Regarding the results in Table 4, the ethyl acetate fraction (EAJO) and the n-butanol fraction (NBJO) exhibited relatively weak inhibition, with IC_{50} values of 430.27±8.22 µg/mL and 586.12±11.38 µg/mL, respectively. As shown in Fig. 1, this performance was notably less effective than the standard anti-inflammatory drug diclofenac, which exhibited a substantially lower IC_{50} value of 61.87±0.49 µg/mL and a strong anti-inflammatory effect. Furthermore, at a 500 µg/mL concentration, EAJO and NBJO showed moderate anti-inflammatory activity, inhibiting protein denaturation by 52.59% and 43.01%, respectively. These values are significantly lower than the 99.87% inhibition achieved by diclofenac under the same conditions. Corroborating our findings, a study by Djellouli *et al*.[21] reported that both methanolic and aqueous extracts of *J. oxycedrus* exhibited *in vitro* anti-inflammatory activities inferior to diclofenac. Specifically, at a 500µg/mL concentration, their activities ranged between 62.85% and 23.35%, respectively. Prior studies have suggested that the anti-inflammatory properties of plant extracts may be linked to their antioxidant capacitie.[21] In addition, compounds such as rutin, quercetin and chrysin are known for their anti-inflammatory effect.[49,52]

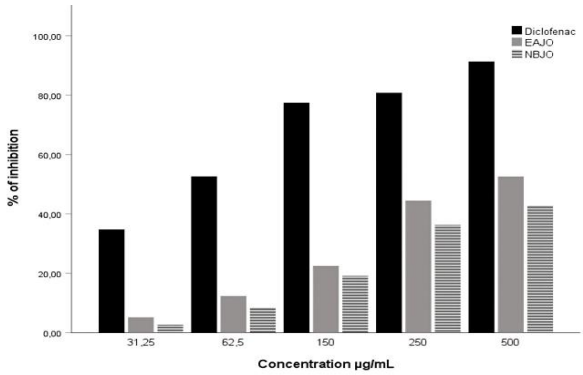

Figure 1: *In vitro* anti-inflammatory activity of ethyl acetate and n-butanol fractions derived from the *Juniperus oxycedrus* leaves. Values are mean ± SD (n = 3), compared to diclofenac.

Table 4: Effect of *Juniperus oxycedrus* leaves fractions on albumin denaturation.

Fractions/standard	BSA denaturation inhibition
	IC_{50} µg/mL
EAJO	430.27±8.22[b]
NBJO	586.12±11.38[a]
*Diclofenac	61.87±0.49[c]

IC_{50} values were calculated by linear regression analysis and expressed as Mean ± SD (n=3). The values with different superscripts (a, b, c) in the same columns differ significantly. EAJO: Ethyl acetate fraction; NBJO: N-butanol fraction; * Compounds used as standard references.

Enzymatic activity

Alzheimer's disease (AD), commonly known as dementia, represents a critical issue in global public health, affecting around 10% of the elderly population worldwide. Alzheimer's disease is associated with impairments in central cholinergic neurotransmission, primarily due to a depletion of the neurotransmitter acetylcholine, as noted by Grossberg *et al*.[56] Acetylcholinesterase (AchE) is an enzyme responsible for the hydrolysis of acetylcholine in neurons [48]. Acetylcholine is vital for the nervous system, notably in memory enhancement. By inhibiting the AChE, the acetylcholine levels in the nervous system increase, hence extenuating the risk of brain-related pathologies like Alzheimer's.[57] Previous studies suggest that oxidative stress may also contribute to the development of Alzheimer's disease (AD).[3] Furthermore, inhibiting acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) have been identified as powerful strategies for treating the symptoms of (AD).[58]

The acetylcholinesterase inhibitory activity of the *J.oxycedrus* leave fractions was evaluated, and galantamine was served for comparison, as seen in Table 5. The EAJO exhibited the most potent inhibitory effect against AChE (IC_{50}: 14.60±0.98µg/mL) followed by NBJO (IC_{50}: 201.22±1.88 µg/mL). These results were compared to the standard galantamine, which had recorded an IC_{50} of 6.27±1.15 µg/mL, where the IC_{50} measured in EAJO is closer to the standard reference. On the other hand, the available literature concerning the AchE inhibitory effect of *J.oxycedrus* leaf fractions was scarce. According to the existing data, the essential oil derived from the aerial parts of *J.oxycedrus* collected in Laghouat, Algeria, exhibited an AchE inhibitory activity of 65.88% at 200 µg/ml.[59] In the same line, Öztürk *et al*. [60] reported that the hexane extract from *J.oxycedrus* berries growing in Turkey was the most effective extract against AChE among the other six studied extracts, with an 81.40% inhibition at 200 µg/mL.[60] However, the ethanolic extract of the aerial parts of the *J. oxycedrus* badia collected in Portugal exhibited weak inhibition of 24.02 % at 200 µg/ml.[61] Similarly, Orhan *et al*. reported that the ethanolic extract derived from the Turkish *J.oxycedrus* leaves exhibited a moderate inhibition of 14.65% at 100 µg/mL, while the aqueous extract had an inhibition of 39.17% at 200 µg/mL.[26]

From the aforementioned results, we may conclude that our ethyl acetate fraction exhibited the most effective inhibitory activity against AchE, suggesting that this inhibitory capacity may be linked to the abundant phenolic compounds, especially flavonoids like chrysin, rutin, quercetin and ferulic acid are recognized for their neuroprotective properties and potential benefits in treating neurodegenerative diseases.[3,49,51,62] These compounds were present in varying quantities in both fractions, with ferulic acid exclusively found in the EAJO fraction. Ferulic acid is known for combating oxidative stress and alleviating neurodegenerative diseases.[62] This could explain the higher effectiveness of EAJO over the NBJO fraction. EAJO also exhibited a potent radical scavenging capacity, highlighting its potential as a source of compounds that can diminish oxidative stress, which is significant because oxidative stress is known to contribute to the onset and advancement of neurodegenerative diseases.[3,7,62]

Diabetes mellitus, primarily characterized by prolonged hyperglycemia, is a metabolic disorder that often results from impaired insulin production and function.[63,64] Inhibiting key enzymes involved in carbohydrate metabolism is a pivotal therapeutic strategy to manage hyperglycemia, particularly α-amylase and α-glucosidase. These enzymes in the mouth and small intestine are crucial in breaking down starch into simpler sugars.[63] Inhibiting α-amylase, which catalyses the hydrolysis of starch, can prevent the breakdown of starch and subsequent glucose absorption, leading to lower blood glucose levels.[65] This mechanism is not only beneficial in controlling diabetes but also valuable for treating obesity.[65] Consequently, significant research efforts have been directed towards developing effective α-amylase inhibitor.[63,65,66]

As seen in Table 5, the ethyl acetate and n-butanol fractions of *J. oxycedrus* exhibited significant antidiabetic activity, surpassing the acarbose standard with an IC_{50} value of 639.48±7.43µg/mL, 975.83 ±15.95 µg/mL and 3650.93±10.70 µg/mL, respectively. According to Oboh *et al*.[66] quercetin and rutin inhibit α-amylase activity in a dose-dependent manner, with rutin being more effective than quercetin. This inhibition occurs through the formation of hydrogen bonds between the hydroxyl groups in the flavonoids and the polar groups at the enzyme's allosteric site near the catalytic site. This interaction is believed to alter the enzyme's molecular structure and its hydrophilic and hydrophobic characteristics, ultimately decreasing its activity. Combinations of rutin and quercetin, especially a mix of 75% rutin and 25% quercetin, show a synergistic effect, further enhancing α-amylase inhibition.[66]

Tyrosinase plays a crucial role in the early phase of melanogenesis, the process responsible for melanin production. Melanin is the pigment that determines the colouration of skin and hair. Nevertheless, imbalances in melanin production can lead to conditions like hyperpigmentation or vitiligo.[67]

The current research exploring the effects of different fractions from *J. oxycedrus* focused on tyrosinase inhibition. As seen in Table 5, the ethyl acetate fraction of *J. oxycedrus* showed weak tyrosinase inhibitory activity, with an IC_{50} value of 206.44±18.48 µg/mL. This activity is noticeable but less potent than the standard kojic acid, which has an IC_{50} of 25.23±0.78 µg/mL. Further supporting these findings, Cheraif *et al.* [59] conducted a study that aligned with these results. Their research revealed that the essential oil (EO) from *J. oxycedrus* leaves, particularly those grown in Algeria, also exhibited limited tyrosinase inhibitory activity, measured at 36%.[59] Tyrosinase enzyme inhibition, crucial for managing hyperpigmentation, is effectively achieved by aromatic aldehydes, acids, flavonoids, and copper chelators, with rutin particularly noted for its strong anti-pigment properties.[67] However, despite its abundance in ethyl acetate (NBJO) and n-butanol (NBJO) fractions, the overall tyrosinase inhibitory activity is moderate in the EAJO and absent in the NBJO. This could suggest that in this specific context, the efficacy of rutin is influenced by factors such as the nature of its interaction within the fraction, whether synergistic or antagonistic, rather than the involvement of other bioactive molecules.[59]

Urease, a metalloenzyme found in diverse organisms like bacteria, fungi, and plants, plays a key role in converting urea into ammonia and carbamate, which subsequently decomposes into carbon dioxide and a second ammonia molecule.[40,68]

This reaction can significantly increase pH levels, impacting human health and agriculture. Urease is notably involved in serious infections in the gastric tract caused by pathogens such as *Helicobacter pylori*.[69] Its inhibition is, therefore, pivotal in developing treatments for conditions like ulcers and gastric cancer. Urease inhibitors, targeting the enzyme's active site, show promise in treating infections and mitigating urease's harmful effects on living organisms.[68,69]

With the aim of identifying urease inhibitors, as presented in Table 5, the two fractions of *J. oxycedrus* were evaluated for their urease inhibition effects in comparison to the standard compound, thiourea. The tested EAJO fraction showed a weak urease inhibitory activity with an IC_{50} of 245.77 ± 3.75µg/ml, followed by the NBJO fraction. These results are obtained for the first time with the *J. oxycedrus*

species; no study has been carried out before. A wide variety of urease inhibitors have been identified in plants, including terpenoids, polyphenols, and alkaloids. These compounds act by either competing with urea, the substrate, or by chelating nickel, an essential co-factor for the enzyme.[68] Among these inhibitors, rutin was the main compound in the fractions tested and has demonstrated the ability to inhibit urease, exhibiting an IC_{50} value of 97.8 µg/mL.[69]

Sun protection activity

Sunscreen used as a photoprotective agent for UV protection has gained widespread popularity, primarily due to its capacity to absorb, reflect, or scatter solar rays. The Sun Protection Factor (SPF) serves as a key indicator of their effectiveness against sunburn, where higher SPF values reflect superior effectiveness protection.[70]

Concurrently, prolonged exposure to UV radiation, particularly UV-B (280–320 nm), is known to escalate the risk of skin diseases like cancer and photo-allergic reactions. Recent research has turned towards natural substances as viable sunscreen options, attributable to their UV absorption and antioxidant properties. Notably, there exists a strong correlation between SPF values and the phenolic content in plant extracts, as reported by Ebrahimzadeh *et al.*[71] further endorsing the efficacy of these natural compounds in sun protection applications.

In Table 6, the sun protection factor (SPF) values of various fractions are presented. The NBJO fraction exhibited the highest SPF value at 31.71 ± 0.31, followed by the EAJO fraction, which has an SPF of 22.62 ± 1.52. The inhibitory activity observed may be attributed to the photoprotective effects of compounds such as rutin, quercetin, chrysin, ferulic acid, and beta-carotene.[72–74]

In the literature, the data about the photoprotective activity of *J.oxycedrus* was unavailable. SPF classifications are categorized into four levels: low (SPF 6-15), medium or moderate (SPF 15-30), high (SPF 30-50), and very high (SPF > 50). Based on this classification, the NBJO fraction falls into the high sunscreen activity category, whereas the EAJO fraction is categorized as having moderate sunscreen activity. Comparatively, methanolic extracts *from J. Phoenicia* leaves showed moderate sunscreen activity with an SPF of 26.13 ± 1.73, aligning closely with the findings of this study.[75]

Table 5: The enzyme inhibitory activities of EAJO and NBJO of the *Juniperus oxycedrus* leaves.

Samples	Anti –AchE IC_{50} (µg/mL)	Anti-Alpha Amylase IC_{50} (µg/mL)	Anti-Tyrosinase IC_{50} (µg/mL)	Anti-Urease IC_{50} (µg/mL)
EAJO	14.60 ± 0.98^{b}	639.48 ± 7.43	206.44 ± 18.48^{a}	245.77 ± 3.75^{a}
NBJO	201.22 ± 1.88^{a}	975.83 ± 15.95^{b}	NA	$>200^{a}$
Galantamine*	6.21 ± 0.12^{c}	NT	NT	NT
Acarabose*	NT	3650.93 ± 10.70^{a}	NT	NT
Kojic Acid*	NT	NT	25.23 ± 0.78^{b}	NT
Thiourea*	NT	NT	NT	11.35 ± 0.3^{b}

IC_{50} and $A_{0.50}$ were calculated by linear regression analysis and expressed as Mean ± SD (n=3). The values with different superscripts (a, b, c) in the same columns differ significantly. EAJO: Ethyl acetate fraction; NBJO: N-butanol fraction; NT: not tested, NA: not activated, * Compounds used as standard references.

Table 6: Sun protection factor calculation for the ethyl acetate fraction obtained from *Juniperus oxycedrus* leaves.

λ (nm)	EE x I (nm)	EAJO		NBJO		Vichy*	
		Abs	SPF	Abs	SPF	Abs	SPF
290	0.015	3.095 ± 0.12	0.464 ± 0.01	3.088 ± 0.02	0.463 ± 0.03	4.457 ± 0.02	0.668 ± 0.00
295	0.0817	2.837 ± 0.11	2.318 ± 0.09	3.156 ± 0.02	2.580 ± 0.02	4.464 ± 0.02	3.647 ± 0.01
300	0.2874	2.485 ± 0.16	7.142 ± 0.46	3.161 ± 0.04	9.084 ± 0.11	4.409 ± 0.03	12.671±0.10
305	0.3278	2.193 ± 0.16	7.190 ± 0.53	3.187 ± 0.01	10.45 ± 0.04	4.416 ± 0.03	14.47± 0.11
310	0.1864	1.990 ± 0.14	3.709 ± 0.27	3.189 ± 0.05	5.945 ± 0.09	4.428 ± 0.04	8.25 ± 0.08
315	0.0837	1.804 ± 0.13	1.510 ± 0.11	3.144 ± 0.01	2.631± 0.01	4.415 ± 0.02	3.704 ± 0.01
320	0.018	1.589 ± 0.11	0.286 ± 0.02	3.084 ± 0.06	0.555 ± 0.01	4.49 ± 0.05	0.808 ± 0.00
Total	1		22.62 ± 1.52		31.71 ± 0.31		44.23 ± 0.35

The values are expressed as Mean ± SD (n=3). * Reference compounds. EE: erythemal effect spectrum, I: solar intensity spectrum, Abs: absorbance of sunscreen product.

Conclusion

The study highlights *Juniperus oxycedrus*, collected from Jijel, Algeria, as a promising natural source for treating diabetes and skin disorders due to its α-amylase, photoprotective, and tyrosinase inhibitory activities. The ethyl acetate and n-butanol leaf fractions are rich in phenolic compounds, offering strong antioxidant and anti-inflammatory properties. These findings support its use in traditional medicine. Moreover, the study emphasises the importance of *J. oxycedrus* in developing pharmaceuticals for combating neurodegenerative disorders, owing to its potent anti-cholinesterase activity. It is concluded that the bioactive compounds isolated from the ethyl acetate fraction could offer significant therapeutic benefits, particularly in neurodegenerative and antidiabetic diseases. Additionally, the high efficacy of the n-butanol fraction as photoprotective agent suggests promising prospects for their application in cosmetic products, particularly in sunscreens. Future studies should focus on evaluating their suitability in cosmetic formulations, enhancing their value for dermatological use. Further *in vivo* investigations are required to confirm the safety and efficacy of these compounds and to fully explore their therapeutic potential. This ongoing research will be important for understanding the full spectrum of applications for *J. oxycedrus* in medical and pharmaceutical contexts, potentially leading to new treatments for chronic conditions.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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