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Contrasting alkaloid and polyphenol-rich plants: LC-MS/MS based characterization and bioactivity assessment of *Papaver somniferum* and *Celtis australis*

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Abstract: This study involves a comparative evaluation of *Papaver somniferum* L. and *Celtis australis* L., two phytochemically different medicinal plants from Türkiye, by focusing on their phenolic content, antioxidant capacity, and enzyme inhibition properties. Ethanolic and aqueous extracts of both plants were analyzed by LC–MS/MS, and the results revealed different secondary metabolites. *P. somniferum* was determined to be rich in terms of flavonoids, including luteolin, apigenin, and naringenin. Meanwhile, *C. australis* was found to be rich in terms of phenolic acids, including rosmarinic, syringic, vanillic, and chlorogenic acids. Antioxidant evaluation through ABTS radical scavenging, CUPRAC, FRAP, and Fe^{3+}-reducing assays showed that the aqueous extract of *C. australis* (WECA) exhibited significant radical scavenging capacity; on the other hand, the ethanolic extract of *P. somniferum* (EEPS) possessed stronger reducing power, which was evaluated as consistent with their respective phenolic compositions determined by LC-MS/MS analysis. Enzyme inhibition assays of both extracts demonstrated potent α-amylase inhibition by EEPS with an IC_{50}: 5.02±0.73 µg/mL, suggesting potential antidiabetic effects, and remarkable human carbonic anhydrase II isoenzyme (hCA II) inhibition by *P. somniferum* water extract with IC_{50}: 3.11±1.98 µg/mL, outperforming the standard inhibitor. Both plants also showed moderate anticholinergic effect, indicating a possible neuroprotective property. In conclusion, while the flavonoid-rich *P. somniferum* showed excellent reducing ability and potent inhibition of key metabolic enzymes, including α-amylase, acetylcholinesterase (AChE), and hCA II, the phenolic acid–dominant *C. australis* exhibited a stronger radical scavenging efficiency, reflecting distinct yet complementary antioxidant and therapeutic potentials that may be harnessed for functional or pharmacological applications.

Keywords: *Papaver somniferum; Celtis australis;* LC-MS/MS; antioxidant activity; enzyme inhibition.

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1. Introduction

Since antiquity, plants have been employed as a remedy for diverse ailments [1-3]. Although systematic and scientific approaches are largely absent, traditional medical practices across cultures rely extensively on herbal preparations [4-6]. Current research increasingly confirms that these long-standing applications are merely empirical, as many traditional uses closely related to the pharmacological abilities now identified in the respective plants [7-9].

Celtis australis and *Papaver somniferum* are well-known members of traditional medicine and nutrition plants [10,11]. Although they belong to distinct botanical families, they are characterized by different chemical classes of phytochemicals. Both plants have a shared common usage in folk practices for managing pain, inflammation, and gastrointestinal ailments [12-14]. *C. australis* fruits are traditionally consumed as food and are known to be rich in flavonoids and phenolics, whereas *P. somniferum* is primarily recognized as alkaloid-rich latex but is also used in the form of seeds and seed oil, and nutraceutical applications [11]. Despite the emphasis in the literature on opium alkaloids, the phenolic profile of *P. somniferum* remains comparatively underexplored [15].

To express the phytochemical diversity of both species into a functional relationship, both antioxidant and enzyme inhibition studies were performed in the present study. Oxidative stress is the major factor in the development of metabolic, neurodegenerative, and cardiovascular diseases, and therefore, determining the antioxidant potential of plant extracts provides a first indication of their possible health-promoting properties [16,17]. In this study, multiple complementary methods, including radical scavenging assays were used. Radical scavenging assays measure a compound's ability to directly neutralize free radicals by donating hydrogen atoms or electrons, thereby interrupting chain reactions that damage biomolecules such as lipids, proteins, and DNA [18]. This activity is particularly relevant for mimicking intermediate, direct antioxidant effects that would occur in biological systems under oxidative stress [19,20]. In contrast, reducing power-based methods assess the electron-donating capacity of an antioxidant against oxidized intermediates or transition metals. Such reducing activity is associated with the regeneration of endogenous antioxidant systems and prevention of metal-catalyzed ROS formation via Fenton-type reactions. This mechanism represents a more sustained protective effect, complementing radical scavenging by strengthening redox homeostasis [21-25].

Applying this dual antioxidant evaluation to *C. australis* and *P. somniferum* is particularly relevant because the two species are dominated by two distinct classes of secondary metabolites [11,12]. *C. australis*, rich in flavonoids and phenolics, is expected to show pronounced reducing capacity due to the multiple hydroxyl groups that enable electron transfer and metal chelation [26]. In contrast, *P. somniferum* contains isoquinoline alkaloids alongside some phenolics, and alkaloids are generally weaker reducing agents, but may still exert radical scavenging effects through specific functional groups [12]. Therefore, a comparative analysis helps researchers to determine how these divergent phytochemical profiles translate into different modes of antioxidant defense, offering insight into their relative value as dietary or therapeutic sources of bioactive compounds. Beyond their antioxidant role, the extracts were tested against several clinically relevant enzymes, such as α-amylase, which is directly associated with the modulation of carbohydrate metabolism and the control of postprandial hyperglycemia, making it a key therapeutic target in type II diabetes mellitus (T2DM) [27-31]. AChE and butyrylcholinesterase (BChE) are enzymes responsible for the breakdown of acetylcholine, and their inhibition has been recognized as a central strategy in the treatment of Alzheimer's disease (AD) and related cognitive disorders [32-37]. hCA play essential roles in pH regulation, respiration, and electrolyte balance [38-40]. Therefore, their inhibitions have pharmaceutical implications for glaucoma, epilepsy, and osteoporosis.

By combining LC-MS/MS-based metabolite profiling with these functional assays, this study aims to provide a comparative perspective on how the distinct phytochemical classes of *C. australi*s and *P. somniferum* contribute to antioxidant and enzyme inhibitory activities. Such an approach provides not only chemotaxonomic insights but also highlights how different phytochemical repertoires, characterized by polyphenol dominance in *C. australis* and alkaloid association with phenolic content in *P. somniferum*, contribute to their bioactivity. Beyond their pharmacological relevance, this approach highlights the dual significance of these plants as both medicinal and nutritional sources, establishing a baseline of data that may inform future pharmacognostic, nutraceutical, and functional food research.

2. Experimental

2.1. Materials and Methods

DPPH, ABTS, standard antioxidants ascorbic acid, butylated hydroxytoluene (BHT), and Trolox were purchased from Sigma Aldrich GmbH (Steinheim, Germany). Standards used in LC-MS/MS have ≥ 95% purity and are purchased from Sigma. AChE from electric, Type VI-S lyophilized powder 200–1000 U/mg protein), BChE from equine serum, and α-glycosidase enzymes type I from Baker's Yeast 100UN were purchased from Sigma Aldrich. Acetylthiocholine iodide 99.0%, butyrylthiocholine iodide ≥ 98%, 5,5′-dithiobis (2-nitrobenzoic acid), 4-nitrophenyl β-D-glucopyranoside (p-NPG) ≥ 98%(TLC), and 4-nitrophenyl acetate (p-NFA) ≥ 99.0% (GC) were purchased from Sigma Aldrich.

2.2. Preparation of Plant Extract

C. australis fruit and *P. somniferum* seeds were dried and ground up to a size of 0.5-1.0 µm. Briefly, 10 g of each extract was weighed and prepared separately for the water and ethanol extraction. Ethanol extracts were prepared by mixing dried powder with 100 mL of ethanol for 5 hours. Then, the extracts were filtered through Whatman paper (No.1), and the organic phase was then collected and evaporated (Heidolph Hei-Vap PrecisionG3 Rotary Evaporator, Schwabach, Germany). Water extracts were prepared by mixing dried powder with 100 mL of distilled water overnight. Then, the extracts were filtered through Whatman paper (No.1), and the remaining turbid filtration was centrifuged at 1000 rpm for 10 min until a transparent solution was obtained. Lastly, dry extracts were obtained by lyophilization (Christ Alpha1-4 LD Plus, Harz, Germany) [41,42]. The extraction yields were calculated the amount of remaining extracts from the initial amount of dried extract.

2.3. Phytochemical Analysis

2.3.1. Determination of Total Phenol and Flavonoid Content

The samples' total flavonoid contents were determined through the $Al(NO_3)_3$ colorimetric method [43,44]. For this purpose, 0.5 mL of the sample in ethanol was mixed with 1.5 mL of 95% methanol. Then, 0.5 mL of 1.0 M potassium acetate, 2.3 mL of distilled water, and 1.5 mL aluminum nitrate (10%) were added to the reaction mixture, and the samples were incubated at 25°C for 40 min after vigorous shaking. After incubation, absorbances were recorded at 415 nm of each reaction, and the results were expressed as µg quercetin equivalents (QE)/mg of dried plant extract. A standard curve of quercetin was obtained within a concentration range of 1-500 µg/mL. The total phenolic contents in extracts were determined by the Folin-Ciocalteu method as previously applied [45]. The results were expressed as µg gallic acid equivalents (GAE)/mg dried plant extract, with concentrations ranging from 0 to 200 µg/mL.

2.3.2. LC-MS/MS Determination of Secondary Metabolites

A Shimadzu-Nexera ultrahigh performance liquid chromatography (UHPLC) with a tandem mass spectrometer was used for evaluation of standard phytochemicals quantitatively. The reversed-phase UHPLC was coupled with an autosampler (SIL-30AC model), a column oven (CTO-10ASvp model), binary pumps (LC-30AD model), and a degasser (DGU-20A3R model). The chromatographic conditions were optimized to achieve better separation for 53 phytochemicals. Different columns such as Agilent Poroshell 120 EC-C18 model (150 mm×2.1 mm, 2.7 µm) and RP-C18 Inertsil ODS-4 (100 mm×2.1 mm, 2 µm), different mobile phases (B) such as acetonitrile and methanol, different mobile phase additives such as ammonium format, formic acid, ammonium acetate, and acetic acid, different column temperatures such as 25, 30, 35 and 40°C were tried and applied until the optimum conditions were achieved. Consequently, the chromatographic separation was achieved on a reversed-phase Agilent Poroshell 120 EC-C18 model (150 mm×2.1 mm, 2.7 µm) analytical column. The column temperature was set to 40°C. The elution gradient comprised eluent A (distilled water+5 mM ammonium formate+0.1% formic acid) and eluent B (Methanol+5 mM ammonium formate+0.1% formic acid). The

following gradient elution profile was used: 20-100% B (0-25 min), 100% B (25-35 min), 20% B (35-45 min). Furthermore, the solvent flow rate and injection volume were set as 0.5 mL/min and 5 µL, respectively [46].

Mass spectrometry was detected using a Shimadzu LCMS-8040 model tandem mass spectrometer with an electrospray ionization source operating in negative and positive ionization modes. The data acquisition and processing were made using the advanced LabSolutions software (Shimadzu). The multiple reaction monitoring (MRM) mode was used to quantify the phytochemicals. The MRM method was optimized to selectively detect and quantify phytochemical compounds based on screening specified precursor phytochemical-to-fragment ion transitions. The collision energies (CE) were optimized to generate optimal phytochemical fragmentation and maximal transmission of the desired product ions. The MS operating conditions were set as follows: drying gas (N2) flow, 15 L/min; nebulizing gas (N2) flow, 3 L/min; DL temperature, 250°C; heat block temperature, 400°C; and interface temperature, 350°C [47]. Because the method was based on quantitative multiple reaction monitoring (MRM), the acquisition was restricted to predefined precursor–product ion transitions, and full MS/MS spectral data were not collected. Calibration standards exhibiting excessive variability were excluded according to the predefined acceptance criterion (CV% > 15%)

A detailed method validation study and data have been determined by our study group and published [46]. Therefore, a new validation was not performed. The method demonstrated excellent linearity (r^2: 0.957–0.999), high repeatability with intra-day precision (RSD%) below 2.13% and inter-day precision below 2.51%, and high accuracy with recoveries ranging from 99.2% to 100.8%. Limits of detection (LOD) and limit of quantification (LOQ) were established for all 53 analytes. Measurement uncertainty (U%, k:2) values were also reported and found to be within acceptable limits for routine quantitative analysis. Since the same chromatographic system, mass spectrometric settings, internal standards, and calibration levels were used in this study, the method performance characteristics apply directly to the present study.

2.3. Antioxidant Capacity

2.3.1. Radical Scavenging Assays

DPPH radical scavenging activity determination assay was performed as previously applied [48]. An ethanolic solution of DPPH (0.1 mM) was prepared and incubated in the dark by mixing 16 hours for pre-radicalization. Then, 0.5 mL DPPH and 0.5 mL plant samples in ethanol (15-45 µg/mL) were mixed and incubated at 30°C for 30 min. The sample only contains DPPH and ethanol used as a control reaction. The absorbances of each sample were recorded at 517 nm. Scavenging was performed based on the bleaching ability of different concentrations of the plant extracts. The bleaching ability of the samples was measured by the decrease in absorbance ABTS radicals at 734 nm [49,50]. The sample contains ABTS and buffer solution used as a control reaction. DPPH and ABTS assays were also performed on reference antioxidants, including ascorbic acid, BHT, and Trolox. Each test was performed in triplicate. Percentages of the inhibition were calculated by using the formula;

DPPH Scavenging (%) = (Sample absorbances/Control absorbances) x 100

2.3.2. Reducing Capacity Assays

The ability of plant extracts was determined with three different methods conducted in triplicate for each sample and compared with the positive controls, BHT, and Trolox [51,52].

Fe^{3+} Reducing Assay: Briefly, 0.75 mL of different concentrations between 15-45 µg/mL of plant samples in distilled water was mixed with 1.25 mL of 0.20 M phosphate buffer (pH 6.6) and 1% (w/w) potassium ferrocyanide. Then, the mixture was incubated at 50°C for 30 min before adding 1.25 mL of trichloroacetic acid (10%, w/w). Lastly, 0.25 mL of 0.1 % $FeCl_3$ was added to the mixture, and absorbance

was recorded at 700 nm. Reaction mixtures were analyzed against a blank sample that contained distilled water instead of the extract solution via a Shimadzu UV-1800 UV Spectrophotometer [53].

CUPRAC Reducing Assay: 10 mM of $CuCl_2$, 7.5 mM neocuprine, and 1.0 M ammonium acetate buffer were mixed in equal amounts in a test tube, and then, 0.5 mL of different concentrations (15-45 µg/mL in ethanol) of the samples were added. The total reaction volume was adjusted to 2 mL with distilled water, yielding Cu^{2+}-neocuprine complex from Cu^{+}-neocuprine complex by electron donor after 30 min incubation a 25ºC was measured spectrophotometrically using the blue-colored final reaction mixture at 450 nm. The blank sample contained ethanol instead of the extract solution [54].

Fe^{3+}-TPTZ Reducing Assay: FRAP reagent containing 10 mM TPTZ (0.4 mM, HCl): 20 mM $FeCl_3$, sodium acetate buffer (0.3 M, pH: 3.6) in a ratio of 1:1:10 was prepared before use. 0.5 mL of plant extracts in acetate buffer was mixed with an equal volume of 20 mM $FeCl_3$ and FRAP reagent, resulting in a 5 mL final volume. The sample containing buffer instead of the samples was used as a blank, and each reaction's absorbance was measured at a wavelength of 593 nm after 30 min incubation at 37ºC [55].

2.4. Enzyme Inhibition Analysis

2.4.1. α-Amylase Inhibition

The inhibition effect of plant extracts over α-amylase was investigated by simple incorporation of certain concentrations of extracts into the enzyme activity assay. α-Amylase activity was determined through the starch-iodine method, and a decrease in the delta absorbance of the reaction mixture compared to a control reaction that does not consist of the enzyme was employed to evaluate activity inhibition [56-58]. The assay reaction consisted of 0.5 mL of plant extracts in phosphate buffer (0.1 M, pH 6.9), 1 mL of starch (4%), and 0.5 mL of enzyme solutions 1 mg/mL, 5 U per reaction. After 30 min of the reaction at 30°C, 0.5 mL of 1 M HCl was added to stop enzyme activity, followed by the addition of 1 mL of iodine reagent (2.5 mM iodine in 5 mM KI). A sample that does not contain starch was also set up as a blank, and the absorbances of all the samples were measured at 593 nm [59,60].

2.4.1. Cholinergic Enzyme Inhibitions

The extracts at varying concentrations to complete inhibition were added to a mixture containing an equal 10 mM dTNB and 10 mM substrate in 1 mL of the reaction solution. The substrate was acetylcholine iodide for AChE and butyrylcholine iodide for BChE. Right after enzyme addition (10 µL), the absorbance at 412 nm of the mixture was measured for three minutes at minute intervals. Control and blank were set up without inhibitors and enzymes. Donepezil was used as positive control for the AChE and BChE inhibition [61].

2.4.2. Carbonic Anhydrase I and II Isoenzymes (hCA I and hCA II) Inhibition

Human carbonic anhydrase I and II isoenzymes were purified by Sepharose 4B-L-Tyrosine sulfanilamide affinity column and characterized by SDS-PAGE as previously applied [62]. Various concentrations of the plant samples in Tris-SO_4 (0.05 M, pH 7.4), 0.07 mM of p-nitrophenylacetate (in 1:25 acetone: water), and 20 µL of the enzyme were gently vortexed, and as soon as the addition of the enzyme, the absorbance change was monitored at 348 nm by measuring at minute intervals for three minutes. Control reactions and blank reactions were set up without inhibitors and enzymes. These steps were repeated until more than half of the enzyme activity was inhibited [63, 64].

3. Results and Discussion

3.1. Phenolic Composition Analysis

The qualitative and quantitative secondary metabolite profiling of *C. australis* and *P. somniferum* extracts was performed using LC–MS/MS and spectrophotometric analysis, revealing substantial variation in both phenolics' diversity and abundance among species and different solvent types. These differences provide a chemical rationale for the different antioxidant capacities determined in the biochemical assays. The LC–MS/MS analysis was supported by spectrophotometric quantification of total phenolic content (TPC) and total flavonoid content (TFC) to further evaluate the compositional

differences between *C. australis* and *P. somniferum* extracts. The TPC of the ethanolic and aqueous extracts was expressed as gallic acid equivalents (µg/mg extract), while the TFC was calculated as quercetin equivalents (µg/mg extract).

Among all samples, *P. somniferum* exhibited markedly higher total phenol and flavonoid levels when compared with *C. australis*. The ethanolic extract of *P. somniferum* (EEPS) contained 428.27 µg GAE/mg of phenolics and 426.91 µg/mg of flavonoids, while its aqueous extract (WEPS) also showed substantial phenols of 209.69 µg GAE/mg extract and total flavonoid content of 185.07 µg QE/mg extract. In contrast, *C. australis* displayed significantly lower TPC and TFC values, particularly in the aqueous extract (9.18 µg GAE/mg extract and 78.38 µg QE/mg extract, respectively). These differences confirm that *P. somniferum* is quantitatively richer in polyphenolic constituents, although *C. australis* contains phenolic acids of strong individual antioxidant potency, as evidenced by its LC–MS/MS composition. These data highlight that both qualitative and quantitative parameters jointly determine the antioxidant potential of plant extracts. While *P. somniferum* stands out for its overall abundance of polyphenols and flavonoids, *C. australis* demonstrates that even extracts with moderate phenolic levels can exhibit potent antioxidant responses when enriched in structurally active phenolic acids. This interplay between phenolic concentration and composition aligns with previous findings on phenolic–antioxidant relationships in plant systems [65,66]. In *C. australis*, phenolic acids and simple polyphenols predominated, particularly in the WECA. Detected compounds included quinic acid (0.0178 µg/mg extract), vanillic acid (0.0341 µg/mg extract), syringic acid (0.019 µg/mg extract), chlorogenic acid (0.0104 µg/mg extract), and Rosmarinic acid (0.1157 µg/mg extract). The presence of these highly hydroxylated phenolic acids indicates that *C. australis* is a rich source of hydrophilic antioxidants capable of donating hydrogen atoms or electrons to neutralize ROS. These results agree with earlier reports that identified vanillic, chlorogenic, and rosmarinic acids as major antioxidant components of *C. australis* leaves and fruits. Previous studies have also noted that aqueous and hydro-alcoholic extracts of *C. australis* exhibit strong free radical scavenging and metal-chelating capacities, largely attributed to their high phenolic acid content [26. 67,68]. In contrast, EECA contained only trace levels of phenolic compounds, consistent with its relatively weak performance in the DPPH and reducing assays. The DPPH radical scavenging assay measures antioxidant capacity by monitoring the reduction of the stable DPPH radical. Antioxidants donate electrons or hydrogen atoms, causing a color change quantifiable spectrophotometrically [68].

The EEPS displayed a broader spectrum of flavonoids and low-molecular-weight phenolics compared to WEPS (Table 1). The principal compounds identified were protocatechuic acid (0.037 µg/mg extract), protocatechuic aldehyde (0.028 µg/mg extract), naringenin (0.009 µg/mg extract), luteolin (0.008 µg/mg extract), apigenin (0.010 µg/mg extract), and acacetin (0.007 µg/mg extract). Flavonoids such as luteolin and apigenin possess conjugated ring systems and catechol-type hydroxyl substitutions that enable efficient electron transfer and metal ion reduction, properties consistent with the strong reducing capacity of EEPS observed in CUPRAC and FRAP assays [69,70]. The aqueous extract (WEPS) also contained notable amounts of quinic acid (0.292 µg/mg extract) and protocatechuic acid (0.228 µg/mg extract), confirming that some polar antioxidants are extractable in water, though generally in lower concentration. Detailed LC-MS/MS chromatogram of the extracts were shown in Figure S1-S4. Method validation summary table can be found in Table S1. These findings extend previous work on *Papaver* species, where several flavonoids and phenolic acids were reported to contribute to antioxidant and anti-inflammatory activities [10].

Collectively, the LC–MS/MS analysis demonstrate that solvent polarity plays a decisive role in the extraction of bioactive phenolics. Water preferentially extracted hydrophilic phenolic acids such as Rosmarinic and chlorogenic acids, while ethanol was more effective for moderately lipophilic flavonoids, including luteolin, apigenin, and naringenin. This compositional trend is consistent with previous observations that solvent polarity governs phenolic yield and antioxidant potency in many plant systems [65]. These findings suggest the hypothesis that species-specific secondary metabolites are responsible for the antioxidant behavior and indicate the potential of plants as promising natural sources of multifunctional phenolic antioxidants with potential nutraceutical and pharmacological importance [71].

Table 1. Quantitative LC-MS/MS results: EECA; Ethanol extract of *C. australis*, EEPS; Ethanol extract of *P. somniferum*, WECA; Water extract of *C. australis*, WEPS; Water extract of *P. somniferum.* Results were expressed as µg in the mg of samples, < LOD; under the limit of detection

Analyte	EECA	EEPS	WECA	WEPS
Quinic acid	<LOD	<LOD	0.0178	0.292
Protocatechuic acid	<LOD	0.037	<LOD	0.228
Chlorogenic acid	<LOD	<LOD	0.0104	<LOD
Protocatechuic aldehyde	<LOD	0.028	<LOD	<LOD
Vanillic acid	<LOD	<LOD	0.0341	<LOD
Caffeic acid	<LOD	<LOD	<LOD	<LOD
Syringic acid	<LOD	<LOD	0.019	<LOD
Rosmarinic acid	<LOD	<LOD	0.1157	<LOD
Naringenin	0.012	0.009	<LOD	<LOD
Luteolin	0.009	0.008	<LOD	0.007
Apigenin	0.007	0.01	0.0073	0.005
Acacetin	<LOD	0.007	<LOD	0.006

3.2. Antioxidant Capacity Testing Results

The antioxidant capacities of *C. australis* and *P. somniferum* extracts were systematically evaluated through multiple complementary assays to explore their radical scavenging and reducing properties. These assays collectively provide insight into two distinct antioxidant mechanisms, including hydrogen atom transfer and single electron donation, which are influenced by the phytochemical composition in each extract [72].

In the DPPH radical scavenging assay, all tested extracts exhibited concentration-dependent activity, although their effects were weaker than those of standard antioxidants such as Trolox and ascorbic acid. Among the plant samples, the WECA demonstrated the strongest DPPH scavenging activity with an IC_{50} value of 47.01±1.82 µg/mL, indicating a high capacity to neutralize free radicals. The EEPS followed with moderate activity with a IC_{50} value of 67.10±1.88 µg/mL, while WEPS and EECA showed comparatively lower scavenging properties. These results indicate that WECA contains efficient hydrogen or electron-donating constituents capable of stabilizing reactive radicals.

In the CUPRAC assay, as can see in Table 2, the reducing power followed the order EEPS > WEPS > WECA > EECA, with absorbance values ranging from 0.17 to 1.14 at 450 nm. EEPS exhibited the highest reducing ability (A_{450}: 1.14±0.07), approaching the performance of reference antioxidants such as BHT (A_{450}: 1.66±0.16) and Trolox (A_{450}: 1.70±0.03). These findings suggest that the ethanolic extract of *P. somniferum* contains phenolic compounds with high electron-donating potential, possibly linked to its phenolic composition. Similarly, the FRAP and Fe^{3+} reducing assays provided consistent results, showing that EEPS and WECA had comparatively stronger ferric ions reducing abilities than the other extracts. In the FRAP assay, absorbance values ranged from 0.18 to 0.79 at 595 nm, with EEPS again exhibiting the highest reducing activity. The Fe^{3+} reducing assay also indicated higher absorbance for EEPS (A_{700}: 0.60±0.01), suggesting effective reduction of Fe^{3+} ions.

Table 2. Antioxidant capacity assay result of *C. australis* and *P. somniferum* Extracts: EECA; Ethanol extract of *C. australis*, EEPS; Ethanol extract of *P. somniferum*, WECA; Water extract of *C. australis*, WEPS; Water extract of *P. somniferum*

Antioxidants	DPPH· Scavenging		Cu^{2+} reducing		FRAP reducing		Fe^{3+} reducing	
	(IC_{50})	r^2	λ (450 nm)	r^2	λ (595 nm)	r^2	λ (700 nm)	r^2
BHT	12.45±2.68	0.9957	1.66±0.16	0.9878	0.64±0.01	0.9793	1.70±0.08	0.9941
Trolox	9.42±2.43	0.9958	1.70±0.03	0.9960	1.08±0.01	0.9993	1.69±0.01	0.9972
Ascorbic acid	10.44±1.59	0.9808	-	-	-	-	-	-
EECA	76.07±1.06	0.9948	0.17±0.02	0.9972	0.25±0.04	0.9715	0.25±0.04	0.9715
WECA	47.01±1.82	0.9918	0.36±0.03	0.9447	0.18±0.03	0.9880	0.14±0.01	0.9882
EEPS	67.10±1.88	0.9854	1.14±0.07	0.9423	0.79±0.01	0.9497	0.60±0.01	0.9168
WEPS	71.76±1.48	0.9881	0.54±0.03	0.9912	0.26±0.03	0.9087	0.27±0.01	0.9698

The reducing power assays including CUPRAC, FRAP, and Fe^{3+} reduction, provided a complementary perspective by measuring the electron transfer capacity of the extracts. In all three

methods, the ethanolic extract of *P. somniferum,* EEPS, consistently exhibited the highest reducing power (A_{450}: 1.14 ± 0.07; A_{595}: 0.79±0.01; A_{700}: 0.60±0.01, Figure 1and Table 2). This strong reducing performance suggests that EEPS possesses compounds capable of donating electrons to reduce oxidized intermediates or transition metal ions, thereby interrupting oxidative chain reactions [65,67,71]. The WECA also exhibited moderate reducing activity, while EECA and WEPS were less potent in this regard. A detailed graphical representation of the antioxidant assay results is shown in Figure 1.

The role of radical-scavenging activities is highly significant due to the damaging impact that free radicals can have on biological systems [72]. The antioxidant performance of our *P. somniferum* extract shows a pattern highly consistent with the findings of Sharopov *et al*. [73] although the relative strength of each assay varies between studies. In Sharopov's work, different tissues of *P. somniferum* demonstrated notable antioxidant activity, with DPPH IC_{50} values ranging from 35.1 to 157.6 µg/mL and exceptionally strong activity in alkaloid-rich fractions (IC_{50}, 7–8 µg/mL). Similarly, our ethanolic *P. somniferum* extract exhibited meaningful DPPH radical scavenging effects (IC_{50}: 67.10 µg/mL), placing it within the same bioactive range reported for several poppy tissues. In both studies, flavonoid and alkaloid-containing fractions were responsible for the strongest electron-transfer activity, which is reflected in our results as well *P. somniferum* showed the highest reducing power among all extracts tested. This alignment indicates that antioxidant capacity in *P. somniferum* is largely determined by secondary-metabolite richness, particularly flavonoids, phenolic acids, and alkaloids. When evaluated together, both datasets demonstrate a shared phytochemical-driven antioxidant mechanism in poppy species, with radical scavenging and reducing power strongly shaped by extract composition and plant tissue chemistry [73]. The antioxidant results obtained for *C. australis* in our study show a coherent yet assay-dependent relationship with those reported [74]. In our work, the aqueous extract of *C. australis* (WECA) exhibited the highest radical scavenging ability among the tested *C. australis* extracts, as indicated by its strong DPPH activity with a 47.01 µg/mL IC_{50} value and moderate reducing capacity. Also, it was reported that the ethanolic leaf extract displayed the strongest radical scavenging property determined by DPPH and ABTS radical scavenging assays (254.66 and 522.11 mg Trolox equivalent (TE)/g, respectively). Both datasets therefore agree that Celtis leaves represent the most bioactive organ, but differ in the solvent yielding the highest potency: in our analysis, water extracted the dominant phenolic acids responsible for scavenging activity, including rosmarinic, syringic, and vanillic acids, whereas, ethanol extraction preferentially enriched antioxidant phenolics, leading to stronger TE-based radical scavenging values [74].

Overall, the antioxidant assay results reveal two distinct functional profiles: *C. australis*, particularly its aqueous extract, is more effective in direct radical scavenging, whereas *P. somniferum*, especially its ethanolic extract, shows stronger electron-donating and metal-reducing capacity. These contrasting patterns align well with their expected phytochemical differences. LC-MS/MS screening confirmed that *C. australis* extracts are rich in phenolic acids such as rosmarinic, vanillic, and syringic acids, compounds known for their hydrogen atom transfer ability, while *P. somniferum* extracts contained flavonoids such as luteolin, apigenin, and naringenin, which contribute more strongly to electron transfer–based antioxidant mechanisms.

Taken together, these findings support the hypothesis that the distinct phytochemical compositions of *C. australis* and *P. somniferum* dictate their antioxidant modes of action. While *C. australis* primarily acts through radical scavenging, *P. somniferum* exerts its antioxidant effect predominantly via reducing processes. This duality highlights how differences in secondary metabolite profiles translate into complementary antioxidant strategies, emphasizing the potential of both species as valuable sources of natural antioxidants. secondary metabolites are biologically active compounds produced by plants that are not directly involved in primary metabolic processes such as growth or reproduction. Many of these metabolites, including flavonoids, phenolic acids, alkaloids, and terpenoids, exhibit strong antioxidant properties. Their antioxidant activity arises from their ability to neutralize ROS binds metal ions and protects cellular structures from oxidative stress. This protective function helps plants survive environmental challenges such as UV radiation, pathogens, and drought. In humans, secondary metabolites with antioxidant capacity contribute to reduced oxidative damage, potentially lowering the risk of chronic diseases and supporting overall health [75].

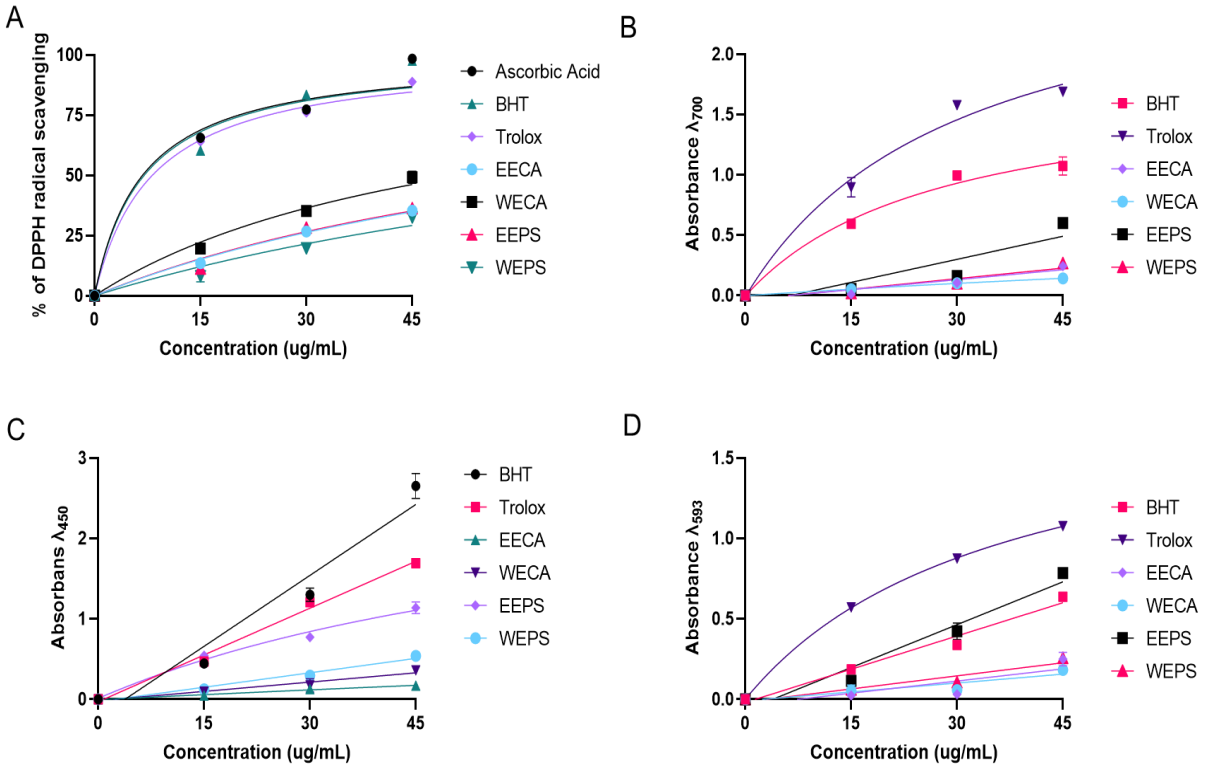

Figure 1. Graphical representation of antioxidant assays: A) DPPH radical scavenging assay, B) Fe^{3+} reducing assay, C) CUPRAC assay, D) FRAP assay EECA; Ethanol extract of *C. australis*, EEPS; Ethanol extract of *P. somniferum*, WECA; Water extract of *C. australis*, WEPS; Water extract of *P. somniferum.*

3.2. Enzyme Inhibition Profiles of C. australis and P. somniferum

The inhibitory activities of *C. australis* and *P. somniferum* extracts were evaluated against α-amylase, AChE, BChE, and hCA II (Table 3). Distinct differences were observed depending on both the plant source and the extraction solvent. Among all samples, the EEPS (IC_{50}: 5.02±0.73 µg/mL) exhibited the most potent α-amylase inhibition, with IC_{50}: 5.02±0.73 µg/mL, comparable to the standard drug Acarbose, which inhibited the α-amylase with IC_{50} value of 9.43±0.35 µg/mL. In contrast, *C. australis* extracts showed weaker inhibition (IC_{50}:73.60±6.06 µg/mL for EECA, IC_{50}:42.22±12.01 µg/mL for WECA). The remarkable potency of EEPS may be attributed to the presence of flavonoids such as apigenin, luteolin, and naringenin, which were detected at quantifiable levels in LC-MS/MS analysis. These compounds are known as α-amylase inhibitors through competitive inhibition at the catalytic center, therefore, reducing postprandial glucose levels [69-71]. Plant extracts contain diverse bioactive compounds, such as polyphenols, flavonoids, alkaloids, and terpenoids, which can interact with metabolic enzymes and alter their activity. Many of these compounds act as enzyme inhibitors by binding to active sites or modifying enzyme structure, thereby reducing catalytic function. Enzyme inhibition by plant extracts is important in pharmacology, as it can regulate biochemical pathways linked to diseases such as diabetes, AD, and inflammation. For example, some extracts inhibit enzymes like α-amylase, AChE, or tyrosinase. Through these interactions, plant extracts demonstrate therapeutic potential and contribute to the development of natural drug candidates [76,77].

All extracts demonstrated noteworthy cholinesterase inhibitory ability. EECA, WECA, EEPS, and WEPS exhibited IC_{50} values in the range of 18–26 µg/mL against AChE, comparable to or slightly weaker than the donepezil (8.82±0.20 µg/mL). Interestingly, BChE inhibition was generally stronger in *C. australis* (IC_{50}: 23.61±2.16 for EECA, IC_{50}: 26.06±2.61 µg/mL for WECA) than in *P. somniferum* ethanolic extract (IC_{50}: 39.53±5.38 µg/mL for EEPS), while WEPS was relatively more active (IC_{50}: 22.98±1.17 µg/mL). The LC-MS/MS profiles support this trend: *P. somniferum* extracts contained apigenin, luteolin, and acacetin, flavones known to interact with the peripheral anionic site of

cholinesterase, enhancing inhibitory potential [7,69-71]. In *C. australis*, the presence of rosmarinic acid, syringic acid, and vanillic acid may also contribute to cholinesterase inhibition through hydrogen bonding with key active-site residues [7].

All extracts showed measurable inhibition of hCA II isoenzyme, an enzyme linked to glaucoma and edema control [78-90]. The strongest IC_{50} value was recorded for WEPS with a 3.11±1.98 µg/mL, outperforming the standard acetazolamide inhibition with an IC_{50} value of 9.96±0.21 µg/mL. This exceptional inhibition could be related to the synergistic effect of phenolic acids (quinic, protocatechuic) and flavonoids (luteolin, apigenin), which can chelate the Zn^{2+} ion at the enzyme's catalytic center [81,82]. Conversely, *C. australis* extracts displayed moderate inhibition effect (EECA: 49.03±8.65 µg/mL), consistent with their lower phenolic diversity. In general, the EEPS showed the most balanced enzyme inhibition profile, while the WEPS excelled specifically against hCA II isoenzyme. The *C. australis* extracts, particularly WECA, showed moderate but consistent inhibition across all enzymes, reflecting the contribution of hydroxycinnamic acids and rosmarinic acid derivatives. The integration of LC-MS/MS and bioassay results suggests that enzyme inhibition is primarily driven by flavonoid aglycones (apigenin, luteolin, naringenin) and simple phenolic acids (protocatechuic, vanillic, rosmarinic acids), which act synergistically. The solvent polarity clearly influenced both phenolic composition and inhibitory potency, with ethanol favoring flavonoid extraction and water enhancing the recovery of phenolic acids [83-89].

α-Amylase plays a crucial role in digestion by initiating the breakdown of dietary starch into maltose and glucose. Produced mainly in saliva and the pancreas, it helps convert complex carbohydrates into absorbable sugars, supporting efficient nutrient uptake and overall digestive function throughout the gastrointestinal tract [90,91]. Table 3 provides a summary of the α-amylase enzyme inhibition effect of both plant extracts. The most effective plant extract was found as EEPS, which demonstrated an IC_{50} value of 5.02±0.73 µM (r^2 = 0.9572) towards α-amylase. For comparison, the standard α-amylase inhibitor, Donepezil, demonstrated weaker inhibition with an IC_{50} value of 9.43±0.35 µM (r^2 = 0.9995) when compared to EEPS.

Table 3. Enzyme inhibition results of *C. australis* and *P. somniferum* extracts: EECA; Ethanol extract of *C. australis*, EEPS; Ethanol extract of *P. somniferum*, WECA; Water extract of *C. australis*, WEPS; Water extract of *P. somniferum*. *Acarbose was employed as a positive control for α-amylase. Donepezil for was used as clinical inhibitor for AChE and BChE [92-94]. Acetazolamide was used as an inhibitor of carbonic anhydrase II isoenzyme [95-99]

Samples	α-Amylase		AChE		BChE		hCA II	
	IC_{50}	r^2	IC_{50}	r^2	IC_{50}	r^2	IC_{50}	r^2
EECA	73.60±6.06	0.9138	18.89±1.15	0.9991	23.61±2.16	0.9985	49.03±8.65	0.9561
WECA	42.22±12.01	0.9763	19.96±1.52	0.9987	26.06±2.61	0.9968	-	-
EEPS	5.02±0.73	0.9572	18.78±3.38	0.9927	39.53±5.38	0.9868	8.82±0.94	0.9334
WEPS	47.10±8.89	0.9308	19.85±1.02	0.9995	22.98±1.17	0.9996	3.11±1.98	0.9971
Standards*	9.43±0.35	0.9995	8.82±0.20	0.9836	15.51±0.40	0.9836	9.96±0.21	0.9930

4. Conclusions

This study comparatively evaluated the phytochemical composition and bioactivity of *P. somniferum* and *C. australis* collected from the western region of Türkiye. LC–MS/MS profiling revealed that *P. somniferum* is characterized by flavonoid-rich ethanolic extracts, notably containing luteolin, apigenin, and naringenin, while *C. australis* predominantly harbors phenolic acids such as rosmarinic, syringic, and vanillic acids, especially in its aqueous extract. These compositional differences were reflected in their biological activities: *C. australis* showed stronger radical scavenging potential, whereas *P. somniferum* demonstrated superior reducing and enzyme inhibitory capacities. EEPS exhibited remarkable α-amylase inhibition, suggesting potential for antidiabetic applications, while WEPS

displayed exceptional hCA II isoenzyme inhibition, indicative of possible antiglaucoma activity. Moderate cholinesterase inhibition by both plants further supports their neuroprotective potential. Collectively, these findings highlight how the divergent phytochemical profiles of alkaloid and polyphenol-dominant plants translate into complementary antioxidant and enzyme inhibitory mechanisms. Both species can thus be regarded as promising sources of multifunctional natural compounds with prospective nutraceutical and pharmaceutical relevance, warranting further in-depth isolation, mechanism, and *in vivo* studies. In addition, the comparative antioxidant patterns observed between the two species underscore the critical influence of extraction solvent and organ specificity on the detection methods. The strong radical scavenging capacity of aqueous *C. australis* extracts, contrasted with the robust reducing power of ethanolic *P. somniferum* extracts, highlights how solvent polarity can selectively enrich phytochemical classes. Furthermore, the enzyme inhibition profiles obtained here provide the first integrated evaluation of α-amylase, both cholinesterase, and carbonic anhydrase inhibition abilities for these species from this geographical region, expanding their documented pharmacological spectrum. These results not only confirm the therapeutic potential of both taxes but also position them as valuable candidates for future formulation studies, including anticholinergic, antidiabetic, and ocular health-related applications. Overall, this work offers a comprehensive chemical–biological framework that can guide the targeted use of *P. somniferum* and *C. australis* in functional product development. Future investigations should integrate fractionation, structure–activity relationship analysis, cell-based assays, and *in vivo* models to validate the bioefficacy and safety of the active constituents identified herein.

Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/ journal-of-chemical-metrology

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