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RESEARCH ARTICLE

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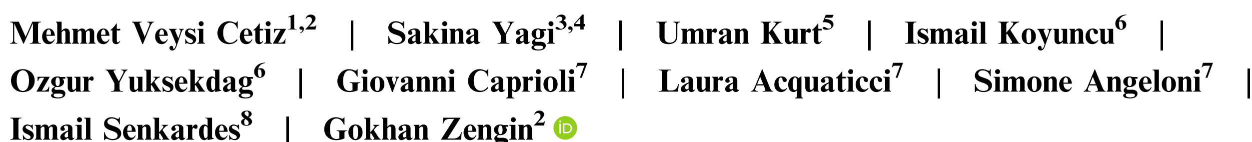
Bridging HPLC-ESI-MS/MS analysis and in vitro biological activity assay through molecular docking and network pharmacology: The example of European nettle tree (*Celtis australis* L.)



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Abstract

Celtis australis L. (Family: Cannabaceae) is commonly used to treat many diseases like gastrointestinal problems, menstrual bleeding and amenorrhea. The present study was designed to investigate the chemical constituents, antioxidant, enzyme inhibitory and cytotoxic properties of different extracts from twigs, fruits and leaves of *C. australis*. EtOAc, EtOH, 70% EtOH and aqueous extracts were prepared by maceration. Results showed that the EtOH extract of the leaves had the highest total phenolic content and possessed remarkable antiradical, ion reducing and total antioxidant activities. Additionally, the leaves (EtOH or EtOAc extracts) exerted the best enzyme inhibition properties. The polar extracts of the leaves had significant cytotoxic effect against the human colorectal adenocarcinoma (HT-29) and human prostate cancer (DU-145) cell lines while the EtOAc of the twigs was effective against the former cell line. Phytochemically, the twigs and fruits accumulated high content of vanillic acid, 4-hydroxy benzoic acid and syringic acid. Through a combination of in vitro and in silico approaches, we identified key phytochemicals exhibited significant inhibitory effects on several cancer-related proteins, through in vitro and in silico approaches that show significant inhibition of cancer-related proteins. In conclusion, these findings indicated that *C. australis* could be a promising source of bioactive molecules for food, pharmaceutical and cosmetic industries.

KEYWORDS

antioxidant, bioactive agents, *Celtis australis*, enzyme inhibition, network pharmacology, solvent extracts

1 | INTRODUCTION

The genus *Celtis* comprises about 60–70 species and is widely distributed in Asia, Mediterranean region, North Australia and North and South America (Liu et al., 2021). It was previously belonging to the family Ulmaceae and then reclassified under the family Cannabaceae. Morphologically, the genus has characteristic leaf structure, which is deciduous, alternate, and distichous with three veins. Flowers are small, greenish, and either unisexual or bisexual. Fruits are fleshy and one-seeded (Sattarian, 2006). It is used in traditional medicine to treat a wide range of ailments like diabetics, gastrointestinal, amenorrhea, pain, headache, and fever (Samadd et al., 2024). Generally, few

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studies were performed to evaluate the chemical constituents and biological activities of *Celtis* species, nevertheless, studied species possess anticancer, anti-inflammatory, antimicrobial and antioxidant activities and they are rich in phenolic acids, anthocyanins, lignans, steroids, terpenes, ceramides and amides (Ayanlowo et al., 2020; Kagho et al., 2020; Samadd et al., 2024).

Celtis australis is deciduous tree grown widely in Mediterranean region and is commonly known as the European nettle tree or lote tree (Perović et al., 2023). Traditionally, the plant is used to cure bone fracture and to treat many diseases like gastrointestinal problems, menstrual bleeding and amenorrhea among others (Anuj et al., 2023). Phytochemical studies demonstrated that *C. australis* contains phenolic acids, flavonoids, tannins, coumarins, (Safari et al., 2023). The flavone C-glycosides isovitexin and cytisoside were isolated from the leaves (Spitaler et al., 2009). Other C-glycosides namely, 4‴-α-rhamnopyranosyl-2″-O-β-D-galactopyranosylvitexin, 2″-O-α-L-rhamnopyranosylvitexin, isovitexin, 2″-O-α-L-rhamnopyranosyl-7-O-methylvitexin, cytisoside 18, and acacetin 7-O-glucoside were also isolated (El-Alfy et al., 2011; Sommavilla et al., 2012). Five terpenes, a steroid and an anthraquinone were isolated from the bark and fruits (Badoni et al., 2011). Previous studies have showed that *C. australis* possess antibacterial, antifungal (Badoni et al., 2010), anti-inflammatory (BSemwal & Semwal, 2012), antioxidant (Safari et al., 2023) and antitumor (Erden et al., 2024) activities. Indeed, *C. australis* has long-standing traditional uses and more studies on their phytoconstituents and biological activities are warranted to explore its potential role towards human health.

The recent advancements in bioinformatics and molecular biology have considerably expanded the potential for investigating plant-derived compounds. Network pharmacology, an emerging field, integrates a variety of in silico techniques, including molecular docking, protein-protein interaction (PPI) network analysis via search tool for the retrieval of interacting genes/proteins (STRING), and pathway enrichment analyses using Kyoto Encyclopedia of Genes and Genomes (KEGG) and GO, to provide a comprehensive understanding of how compounds can affect multiple targets simultaneously. This holistic approach is crucial for elucidating the molecular mechanisms underlying therapeutic effects and has proven to be particularly valuable in identifying multi-target strategies for complex diseases like cancer. Additionally, disease ontology semantic enrichment (DOSE) analysis serves as a powerful tool for linking gene sets to specific diseases, offering further insights into their roles in disease pathology (Bultinck et al., 2012; Kanehisa et al., 2010; Yu et al., 2015).

Based on lights of the above-mentioned informations, the present study aimed to investigate the chemical constituents of different extracts from twigs, fruits and leaves of *C. australis* and to evaluate their antioxidant activity based on their capacity to scavenge free radicals, chelate and reduce metal ions as well as their ability to inhibit enzymes implicated in diabetes, skin hyperpigmentation and Alzheimer's diseases. Also, their cytotoxicity against a panel of cancer cells was examined. We employed a range of network pharmacology tools, including DOSE, KEGG, GO, STRING analyses, and molecular docking, with the objective of identifying key phytochemicals. Our findings, initially highlighted through STRING and pathway enrichment analyses and further confirmed by molecular docking, reinforce the therapeutic potential of these phytochemicals. This comprehensive approach provides a robust foundation for subsequent in vivo studies, which may ultimately lead to the development of novel multi-target cancer therapies.

2 | MATERIALS AND METHODS

2.1 | Plant collection

In 2021, botanical specimens were collected from the Maltepe, Başıbüyük area in Istanbul, Turkey. Dr. Ismail Senkardes conducted the taxonomic identification, and a voucher specimen was preserved in the herbarium of the Pharmacy Faculty at Marmara University (Voucher number: MARE-23028). The fruits, twigs, and leaves were segregated, dried in the shade at ambient temperature, pulverized, and thereafter stored away from light.

2.2 | Plant extract preparation

The extraction procedure included four solvents: ethyl acetate, ethanol, a 70% ethanol (in water), and water. Each 10 g sample was macerated with 200 mL of ethyl acetate, ethanol, and a mixture of ethanol and water for 24 h at ambient temperature. The aqueous extract was prepared by infusing 10 g of plant material in boiling water for 15 min. Organic solvents were removed via evaporation under low pressure, and the aqueous extract was subjected to freeze-drying.

2.3 | Assay for total phenolic and flavonoid contents

Total phenolics and flavonoids were quantified according to the procedures outlined by (Slinkard & Singleton, 1977). Gallic acid (GA) and rutin (RE) were used as reference standards in the studies, with results expressed as gallic acid equivalents (GAE) and rutin equivalents (RE).

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2.4 | Analysis by using HPLC-ESI-MS/MS triple quadrupole

HPLC-MS/MS studies were performed using an Agilent 1290 Infinity series and a Triple Quadrupole 6420 from Agilent Technology (Santa Clara, CA) equipped with an electrospray ionization (ESI) source operating in negative and positive ionization modes. All separation and identification details are given in the supplemental materials (Supporting Information S1: Table S1).

2.5 | Assays for in vitro antioxidant capacity

In accordance with the methodologies detailed in our prior publication (Grochowski et al., 2017), multiple antioxidant assays were conducted. The results from the DPPH, ABTS radical scavenging, CUPRAC, and FRAP assays were expressed in milligrams of Trolox equivalents (TE) per gram of extract. The phosphomolybdenum (PBD) assay quantified antioxidant potential in millimoles of TE per gram of extract, whilst the metal chelating activity was measured in milligrams of disodium edetate equivalents (EDTAE) per gram of extract.

2.6 | Inhibitory effects against some key enzymes

In accordance with the established protocols (Grochowski et al., 2017), experiments on enzyme inhibition were performed on the samples. The activities that inhibit amylase and glucosidase were quantified in acarbose equivalents (ACAE) per gram of extract, while the inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) was assessed in milligrams of galanthamine equivalents (GALAE) per gram of extract. Tyrosinase (Tyr) inhibition was quantified in milligrams of kojic acid equivalents (KAE) per gram of extract.

2.7 | Cytotoxic evaluation

2.7.1 | Cell culture

The study utilized cancer and normal cell lines acquired from ATCC and were preserved in liquid nitrogen. DU-145 (Prostate Carcinoma), MDA-MB-231 (Breast Adenocarcinoma), HELA (Cervix Adenocarcinoma), HT-29 (Colon Adenocarcinoma), HCT-116 (Colorectal Carcinoma), A549 (Lung Adenocarcinoma), HGC-27 (Gastric Carcinoma), and HEK-293 (Embryonic Kidney Epithelial) cells were cultivated in DMEM-F12/RPMI-1640 media enriched with 10% Fetal Bovine Serum (FBS) and 100 μg/mL of streptomycin/100 IU/mL of penicillin in incubators at 37°C under humidified conditions with 5% CO_2.

2.7.2 | Cell viability assay

The cytotoxic effects of the extracts were evaluated utilizing the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) test. The cells (DU-145, MDA-MB-231, HELA, HT-29, HCT-116, A549, HGC-27, and HEK-293) were cultured in a sterile 96-well plate for 24 h at a density of 1 × 10^4 cells per well. The medium were eliminated, and the extracts were incubated at concentrations of 0, 2.5, 5, 10, 25, 50, 100, and 200 μg/mL for a duration of 24 h. 10 μL of MTT (0.5 mg/mL) was applied to each well as the reactive agent. Following a 4-h incubation, the material was discarded and replaced with 100 μL of DMSO, following which optical density measurements were conducted at OD570-OD690 nm utilizing a plate reader (Thermo Multiskan GO, Thermo). Subsequent to these measurements, plots were generated and the IC50 value was determined.

2.7.3 | Disease ontology enrichment analysis

A comprehensive Disease Ontology (DOSE) enrichment analysis was performed using the DOSE package V3.30.1 in R. The methodology employed is in accordance with the principles delineated by Yu et al., 2015 and is further supported by an array of literature in bioinformatics and systems biology. The genes associated with the molecules were obtained from databases including CTD, PubChem, and SwissTarget.

2.7.4 | Screening of potential targets

The identification of therapeutic targets represents a pivotal stage in the development of novel pharmaceutical agents within the context of medical research. In this study, gene targets for specific cancer types, including breast cancer, prostate cancer, colon, gastric, and lung cancer, were identified using the Comparative Toxicogenomics Database (CTD) and GeneCards. The molecular targets of the compounds were determined through databases such as the CTD, PubChem, and SwissTarget Prediction. The data obtained were then analyzed using the Venny V2.1.0 tool to identify common genes for each cancer type. These common genes were subsequently utilized for STRING, KEGG, and GO analyses.

2.7.5 | Protein-protein interaction (PPI) network analysis

To investigate the properties associated with breast cancer, colon cancer, and prostate cancer, a PPI

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network was constructed using the STRING V12.0 database. The primary objective of this analysis was to identify functional interactions between proteins. A confidence score threshold of ≥0.4 was applied to select potential targets, with "*Homo sapiens*" designated as the species of interest. The analysis was conducted using Cytoscape software, V3.10.2 (Yagi et al., 2024).

2.7.6 | KEGG and GO enrichment analysis

A KEGG pathway enrichment analysis was conducted to explore the biological processes and signaling pathways associated with key targets that may be influenced by *C. australis*. The analysis was conducted using the Database for Annotation, Visualization, and Integrated Discovery (DAVID, V6.8, https://david.ncifcrf.gov/home.jsp). The parameters were configured as follows: the official gene symbol was utilized as the identifier, *Homo sapiens* was selected as the species, and the gene list was designated as the list type. A *p* value of less than 0.05 was deemed to be the appropriate threshold for statistical significance. The figures and tables related to this analysis were generated using R V4.3.3 software.

2.7.7 | Molecular docking

The proteins and enzymes utilized in this study were obtained from the Protein Data Bank (PDB), with detailed information provided in Supporting Information S1: Table S1 for reference. The co-crystallized ligands, cofactors, and water molecules were removed using BIOVIA Discovery Studio Visualizer V4.5. Ligands were obtained from PubChem, and subsequently optimized with OpenBabel V3.1.1. The preparation of protein and enzyme structures was conducted using MGL Tools, version 1.5.6. The active sites within the proteins and enzymes were identified using POCASA V1.1, inhibitors, or methods corroborated by studies in the literature (Supporting Information S1: Table S1) (Duran et al., 2024; Yu et al., 2010). Molecular docking was conducted with AutoDock Vina V1.1.2, and grid boxes were delineated according to the methodology described by Trott and Olson (2010).

2.7.8 | Statistical analysis

Experiments were performed in triplicate, and differences between the extracts were compared using one-way ANOVA with post-hoc Tukey test. Graph Pad Prism (version 9.2) was used for the analysis. A *p* value of <0.05 was considered significant.

3 | RESULTS AND DISCUSSION

3.1 | Total phenolic (TPC) and flavonoids (TFC) contents

The TPC and TFC of extracts from twigs, leaves and fruits of *C. australis* were determined and results are presented in Table 1. TPC was in the range of 7.99–75.99 mg GAE/g with highest values recorded from the EtOH and aqueous extracts of the leaves. In fact, leaves and twigs extracts had the higher TPC than their respective fruits extracts, except the aqueous extract. On the other hand, the EtOAc and EtOH extracts from the fruits recorded the highest TFC (22.94 and 20.99 mg RE/g respectively). This followed by the leaves aqueous extract (19.13 mg RE/g) and twigs EtOAc and EtOH extracts (18.22 and 16.4 mg RE/g respectively). All other extracts from the three organs displayed low TFC (0.56–9.98 mg RE/g). Previous studies on the phenolic content were mainly performed for the fruits and leaves. Safari et al., 2023 found that the TPC in 85% methanolic extract of fruits of 20 genotypes of *C. australis* grown in Iran was in the range of 1176.73–398.27 mg GAE/100 g and the TFC was in the range of 313.19–197.41 mg catechin (C)E/100 g. Filali-Ansari et al. (Filali-Ansari et al., 2015) reported values of TPC and TFC for the leaves hydromethanolic extract as 387.35 mg GAE/100 g and 152.14 mg CE/g respectively. Nasirifar et al. (Nasirifar et al., 2013) reported values of TPC as 8.9 mg GAE/100 g and TFC as 3.34 mg quercetin/g. Globally, the results in the present study revealed that the TPC in the

TABLE 1 Total phenolic and flavonoid contents in *Celtis australis* extracts.

Parts	Extracts	TPC (mg GAE/g)	TFC (mg RE/g)
Twigs	EtOAc	29.99 ± 0.73^{c}	18.22 ± 0.03^{d}
	EtOH	28.58 ± 0.61^{cd}	16.40 ± 0.20^{e}
	70% EtOH	29.09 ± 0.82^{c}	4.50 ± 0.03^{g}
	Water	17.99 ± 0.23^{g}	1.41 ± 0.22^{i}
Fruits	EtOAc	18.51 ± 0.18^{g}	22.94 ± 0.28^{a}
	EtOH	18.67 ± 0.08^{g}	20.99 ± 0.15^{b}
	70% EtOH	7.99 ± 0.15^{h}	1.18 ± 0.07^{ij}
	Water	24.07 ± 0.26^{f}	0.56 ± 0.06^{j}
Leaves	EtOAc	25.49 ± 0.47^{ef}	3.08 ± 0.29^{h}
	EtOH	75.99 ± 1.15^{a}	4.89 ± 0.54^{g}
	70% EtOH	26.90 ± 0.54^{de}	19.13 ± 0.42^{c}
	Water	33.72 ± 0.83^{b}	9.98 ± 0.27^{f}

Note: *Values are reported as mean ± SD of three parallel measurements. Different letters indicate significant differences between the tested extracts ($p < 0.05$).

Abbreviations: GAE, gallic acid equivalents; RE, rutin equivalents.

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leaves and twigs were superior to that of the fruits and were in line to the findings of Ota et al. (2017) who indicated that the EtOH extract of the leaves had the highest TPC. Furthermore, Ota et al. (2017) noted that the TPC of the leaves and fruits varied according to growth stage and season. Indeed, other factors like solvent and method of extraction affected the recovery of phenolic content.

3.2 | Chemical profile

The chemical profile of different extracts from *C. australis* were determined using HPLC-ESI-MS/MS triple quadrupole and results are presented in Table 2. Chromatograms are given in supplemental materials (Supporting Information S1: Figure S1–S12) Results showed that the chemical profile of the plant varied according to the plant's organ and type of extract. The number of compounds detected in the EtOAc, EtOH, 70%EtOH and aqueous extracts from the twigs was 6, 15, 15 and 5 respectively and in the fruits as follows: EtOAc = 5, EtOH = 9, 70% EtOH = 11, H_2O = 2 while in the leaves as follows: EtOAc = 4, EtOH = 15, 70% EtOH = 17, H_2O = 6. The highest concentrations of total individual compounds were observed in 70% EtOH (3653.34 mg/kg) and EtOH (2401.67 mg/kg) extracts of the leaves. The four extracts of the twigs were characterized by high accumulation of vanillic acid (268.00–211.93 mg/kg) with highest content recorded from the EtOH and 70% EtOH extracts respectively. Also, 4-hydroxy benzoic acid was found in high content in the four extracts (118.76–197.90 mg/kg) while syringic acid (148.62–225.65 mg/kg) were only detected in the three organic solvent extracts. A considerable quantity of hesperidin (206.67 mg/kg) was only identified in the 70% EtOH. Neochlorogenic acid, chlorogenic acid, ferulic acid, rutin, isoquercitrin, delphindin 3,5 diglucoside and kaempferol-3-glucoside were only detected in the EtOH and 70% EtOH extracts. On the other hand, the EtOAc and EtOH extracts of the fruits were dominated by the presence of 4-hydroxy benzoic acid (101.77 and 104.99 mg/kg respectively) while the 70% EtOH extract with syringic acid (141.00 mg/kg) followed by neochlorogenic acid (134.85 mg/kg) and 4-hydroxy benzoic acid (104.00 mg/kg) and low content of neochlorogenic acid (16.81 mg/kg) and gallic acid (5.66 mg/kg) was detected in the aqueous extract. The EtOH and 70% EtOH extracts of the leaves were characterized by a remarkable abundance of neochlorogenic acid (646.44 and 1426.74 mg/kg respectively), catechin (954.78 and 333.84 mg/kg respectively) and rutin (278.19 and 371.41 mg/kg respectively). These compounds were also detected in the aqueous extract but in low amount. Furthermore the 70% EtOH extract accumulated also high content of chlorogenic acid (643.45 mg/kg), 4-hydroxy benzoic acid (110.04 mg/kg), syringic acid (138.71 mg/kg) and procyanidin A2 (119.12). Other compounds like gallic acid, p-coumaric acid, ferulic acid, caffeic acid, hyperoside, isoquercitrin, delphindin 3,5 diglucoside and kaempferol were detected in lower concentrations in some extracts. However, the majority of these compounds were previously identified, with varied concentrations, in the fruit and leaves extracts (Ota et al., 2017; Safari et al., 2023).

3.3 | Antioxidant activity

The antioxidant activity of extracts from twigs, leaves and fruits of *C. australis* were evaluated by measuring their free radical scavenging potential, through the DPPH and ABTS assays, chelating power, reducing capacity through the CUPRAC and FRAP assays and their total antioxidant activity via the PBD assay. Results are presented in Table 3. The EtOH extract of the leaves exerted potent free radical scavenging activity in both the DPPH (254.66 mg TE/g) and ABTS (522.11 mg TE/g) assays. Also, the aqueous and 70% EtOH extracts of the leaves exhibited considerable anti-ABTS (121.36 and 104.09 mg TE/g respectively) and anti-DPPH (45.00 and 44.96 mg TE/g respectively) activities. Although less effective than the leaves, extracts of the twigs had also considerable antiradical activity with highest effect recorded from the ABTS assay (41.93–109.46 mg TE/g). Additionally, the best antiradical activity of the fruits extracts was obtained from the aqueous extract (ABTS = 109.14 mg TE/g; DPPH = 30.45 mg TE/g). Similarly, the EtOH, 70% EtOH and aqueous extracts of the leaves displayed the best reducing capacity (CUPRAC = 115.75–425.67 mg TE/g; FRAP = 50.80–207.26 mg TE/g) with highest values recorded from the EtOH extract. The twigs and fruits extracts showed also significant Cu^{++} reducing capacity with highest values recorded from the 70% EtOH and EtOH extracts of the twigs and aqueous and EtOH extracts of the fruits respectively. The two extracts of the twigs also displayed considerable Fe^{+++} reducing activity. On the other hand, the aqueous extract of the fruits exerted the best chelating power (25.30 mg EDTAE/g) followed by that of the leaves (20.00 mg EDTAE/g). The EtOH extract of the leaves (2.30 mmol TE/g) followed by that of the twigs (1.70 mmol TE/g) revealed the highest total antioxidant activity while the EtOH extract of the fruit had the same effect as that exerted by the 70% EtOH extract of the twigs (1.55 mmol TE/g). Overall, the leaves (specifically the EtOH extract) recorded the highest antioxidant activity (5/6 assays), however, other organs showed also antioxidant activity but were less effective than the leaves. A previous studied on *C. australis* showed that 85% methanolic extract from the fruits of

TABLE 2 Chemical profile of *Celtis australis* extracts expressed in (mg/kg, dry weight).

	Twigs				Fruits				Leaves			
Compounds	**EtOAc**	**EtOH**	**EtOH (70%)**	**Water**	**EtOAc**	**EtOH**	**EtOH (70%)**	**Water**	**EtOAc**	**EtOH**	**EtOH (70%)**	**Water**
Total Anthocyanins												
Delphindin3-galactoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cyanidin-3-glucoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Petunidin-3-glucoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Pelargonidin-3-glucoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Pelargonidin-3-rutinoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Malvidin-3-galactoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Delphindin 3,5 diglucoside	n.d.	43.40	19.28	n.d.	n.d.	6.50	n.d.	n.d.	n.d.	75.66	88.18	n.d.
Total Flavonols												
Rutin	n.d.	76.76	31.22	n.d.	n.d.	23.74	16.79	n.d.	5.20	278.19	371.41	14.40
Isoquercitrin	n.d.	35.54	9.69	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	61.52	70.00	n.d.
Quercitrin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Hyperoside	1.11	42.26	17.45	n.d.	n.d.	2.89	1.13	n.d.	n.d.	71.77	87.30	0.97
Isorhamnetin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Myricetin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Kaempferol-3-glucoside	n.d.	4.00	1.30	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.92	3.54	n.d.
Quercetin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Kaempferol	n.d.	65.93	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	16.09	n.d.
Total Flavan-3-ols												
Catechin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	80.79	n.d.	n.d.	954.78	333.84	81.54
Epicatechin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	21.24	n.d.	n.d.
procyanidin B2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Procyanidin A2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	119.12	n.d.
Total Dihydrochalcones												
Phloretin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Phloridzin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.48	2.36	n.d.
Total Flavanones												
Hesperidin	n.d.	n.d.	206.67	n.d.	21.26	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Naringin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total Phenolic acids												
Gallic acid	7.12	32.24	36.93	13.78	8.47	8.95	32.93	5.66	n.d.	11.78	62.14	n.d.
Neochlorogenic acid	n.d.	74.56	73.56	15.12	n.d.	35.55	134.85	16.81	17.24	646.44	1426.74	66.83
Chlorogenic acid	n.d.	11.43	15.01	n.d.	1.10	3.87	18.52	n.d.	1.91	82.67	643.45	24.49
4-Hydroxy benzoic acid	118.76	197.90	171.68	150.19	101.77	104.99	104.00	n.d.	n.d.	76.06	110.04	n.d.
3-Hydroxy benzoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Caffeic acid	n.d.	55.33	59.17	n.d.	n.d.	n.d.	51.85	n.d.	n.d.	41.14	65.15	n.d.
Vanillic acid	211.93	268.00	264.41	225.51	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Syringic acid	148.62	214.36	225.65	n.d.	n.d.	n.d.	141.00	n.d.	n.d.	n.d.	138.71	n.d.

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TABLE 2 (Continued)

Compounds	Twigs EtOAc	Twigs EtOH	Twigs EtOH (70%)	Twigs Water	Fruits EtOAc	Fruits EtOH	Fruits EtOH (70%)	Fruits Water	Leaves EtOAc	Leaves EtOH	Leaves EtOH (70%)	Leaves Water
p-Coumaric acid	12.09	22.79	25.94	29.02	10.93	13.12	14.95	n.d.	11.35	18.16	32.90	12.83
Ferulic acid	n.d.	87.40	101.66	n.d.	n.d.	73.20	73.74	n.d.	n.d.	56.84	82.36	n.d.
Ellagic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total phenolic compounds	**499.64**	**1231.89**	**1259.61**	**433.62**	**143.53**	**272.80**	**670.54**	**22.47**	**35.70**	**2401.67**	**3653.34**	**201.05**

Abbreviation: n.d., not detected.

TABLE 3 Antioxidant activity of *Celtis australis* extracts.

Parts	Extracts	DPPH (mg TE/g)	ABTS (mg TE/g)	CUPRAC (mg TE/g)	FRAP (mg TE/g)	Chelating (mg EDTAE/g)	PBD (mmol TE/g)
Twigs	EtOAc	13.97 ± 0.38^{e}	41.93 ± 1.96^{g}	64.54 ± 1.99^{g}	28.54 ± 1.45^{f}	na	1.41 ± 0.08^{de}
	EtOH	25.96 ± 0.56^{d}	90.96 ± 0.77^{e}	101.56 ± 1.09^{d}	45.01 ± 0.13^{d}	2.37 ± 0.09^{f}	1.70 ± 0.04^{b}
	70% EtOH	31.61 ± 0.17^{c}	109.46 ± 0.31^{c}	106.77 ± 0.24^{d}	49.49 ± 0.97^{c}	13.05 ± 1.16^{d}	1.55 ± 0.01^{cd}
	Water	11.07 ± 0.45^{f}	71.63 ± 0.82^{f}	45.31 ± 0.82^{i}	19.85 ± 0.23^{g}	16.55 ± 0.60^{c}	0.96 ± 0.01^{g}
Fruits	EtOAc	2.68 ± 0.54^{h}	7.65 ± 0.69^{k}	53.07 ± 1.17^{h}	10.35 ± 0.21^{i}	10.87 ± 0.99^{e}	1.37 ± 0.07^{e}
	EtOH	2.61 ± 0.33^{h}	13.20 ± 0.96^{j}	73.60 ± 0.27^{f}	20.81 ± 0.14^{g}	16.44 ± 1.14^{c}	1.55 ± 0.08^{bcd}
	70% EtOH	10.22 ± 0.08^{f}	27.78 ± 1.20^{h}	27.63 ± 0.30^{j}	14.37 ± 0.18^{h}	15.12 ± 0.81^{cd}	0.47 ± 0.01^{i}
	Water	30.45 ± 0.17^{c}	109.14 ± 0.17^{c}	82.92 ± 0.85^{e}	33.91 ± 0.35^{e}	25.30 ± 0.46^{a}	0.70 ± 0.01^{h}
Leaves	EtOAc	5.17 ± 0.11^{g}	24.89 ± 0.84^{h}	68.54 ± 1.44^{fg}	20.99 ± 0.72^{g}	na	1.68 ± 0.08^{bc}
	EtOH	254.66 ± 0.93^{a}	522.11 ± 1.61^{a}	425.67 ± 7.64^{a}	207.26 ± 2.41^{a}	2.56 ± 0.28^{f}	2.30 ± 0.01^{a}
	70% EtOH	44.96 ± 0.88^{b}	104.09 ± 0.12^{d}	115.75 ± 2.14^{c}	50.80 ± 0.85^{c}	10.17 ± 0.06^{e}	1.16 ± 0.08^{f}
	Water	45.00 ± 0.88^{b}	121.36 ± 1.03^{b}	123.19 ± 0.26^{b}	54.87 ± 0.83^{b}	20.00 ± 1.13^{b}	1.12 ± 0.01^{f}

Note: *Values are reported as mean ± SD of three parallel measurements. Different letters indicate significant differences between the tested extracts ($p < 0.05$).
Abbreviations: EDTAE, EDTA equivalent; MCA, metal chelating activity; na, not active; PBD, phosphomolybdenum; TE, trolox equivalent.

20 genotypes of *C. australis* showed antiradical activity in the range of 14.12%–88.24% from the DPPH and reducing capacity with values between 44.35 and 117.87 mg Fe^{2+}/100 g from the FRAP assay (Safari et al., 2023). They also demonstrated a positive correlation with the TPC and antiradical activity. Nasirifar et al. (2013) recorded an anti-DPPH value of 63.45% while Ota et al. (2017) found that EtOH extract of leaves collected at the end of October exerted anti-DPPH 50-fold higher than those collected at end of June and this high antioxidant activity is associated to an increase of epicatechin, cyanidin-3,5-di-O-glucoside and vanillic acid levels. They also found that the aqueous extract and EtOH extract of the leaves exerted the highest anti-DPPH activity in agree with the results of the present study. As mentioned before, the best antioxidant activity in the present study was recorded from the EtOH extract, however, this extract was characterized by the high accumulation of catechin and neochlorogenic acid which were proven for their potent antioxidant activity. The number and location of hydroxyl groups in catechin molecule and the presence of one vicinal dihydroxyl group on the B ring and a galloyl group at the 3-position are suggested to be responsible of its free radical property (Munteanu & Apetrei, 2022; Nanjo et al., 1999). Neochlorogenic acid was identified as a major antioxidant compound in *Polygonum Cuspidatum* leaves (Kurita et al., 2016). Vanillic acid was previously isolated from hydromethanolic of the *C. australis* leaves and was found to inhibit the free DPPH radicle by an IC_{50} 8.2 µg/mL (Filali-Ansari et al., 2016).

3.4 | Enzyme inhibition activity

The twigs, leaves and fruits of *C. australis* were evaluated for their enzyme inhibition property against AChE, BChE, Tyr, α-amylase and α-glucosidase enzymes. Results are presented in Table 4. The anti-AChE activity

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TABLE 4 Enzyme inhibitory activity of *Celtis australis* extracts.

Parts	Extracts	AChE (mg GALAE/g)	BChE (mg GALAE/g)	Tyrosinase (mg KAE/g)	Amylase (mmol ACAE/g)	Glucosidase (mmol ACAE/g)
Twigs	EtOAc	2.82 ± 0.12[ab]	2.05 ± 0.13[cd]	52.47 ± 0.41[bc]	0.94 ± 0.07[b]	0.10 ± 0.02[f]
	EtOH	2.84 ± 0.02[ab]	2.39 ± 0.18[bc]	50.09 ± 1.67[c]	0.68 ± 0.03[c]	1.18 ± 0.05[c]
	70% EtOH	2.40 ± 0.07[d]	1.83 ± 0.04[cd]	54.68 ± 0.78[bc]	0.61 ± 0.01[cd]	1.22 ± 0.01[c]
	Water	0.84 ± 0.19[f]	0.95 ± 0.09[g]	12.51 ± 0.54[f]	0.09 ± 0.01[e]	na
Fruits	EtOAc	2.41 ± 0.12[d]	1.56 ± 0.23[def]	37.84 ± 1.26[d]	0.95 ± 0.06[b]	na
	EtOH	2.83 ± 0.01[ab]	2.74 ± 0.37[ab]	51.30 ± 0.34[bc]	0.87 ± 0.02[b]	0.96 ± 0.04[d]
	70% EtOH	2.56 ± 0.01[bcd]	2.09 ± 0.17[cd]	52.89 ± 0.31[bc]	0.56 ± 0.02[d]	0.97 ± 0.05[d]
	Water	1.34 ± 0.10[e]	0.99 ± 0.04[fg]	17.84 ± 0.14[e]	0.14 ± 0.02[e]	1.14 ± 0.08[c]
Leaves	EtOAc	2.76 ± 0.19[abc]	3.27 ± 0.24[ab]	56.36 ± 4.83[b]	1.06 ± 0.03[a]	na
	EtOH	2.91 ± 0.08[a]	2.82 ± 0.27[efg]	70.40 ± 2.91[a]	0.91 ± 0.03[b]	1.57 ± 0.01[a]
	70% EtOH	2.49 ± 0.06[cd]	1.24 ± 0.19[efg]	51.53 ± 0.83[bc]	0.56 ± 0.01[d]	1.42 ± 0.01[b]
	Water	0.76 ± 0.06[f]	1.61 ± 0.01[de]	10.28 ± 0.88[f]	0.08 ± 0.01[e]	0.73 ± 0.01[e]

Note: **Values are reported as mean ± SD of three parallel measurements. Different letters indicate significant differences between the tested extracts ($p < 0.05$).
Abbreviations: ACAE, acarbose equivalent; GALAE, galantamine equivalent; KAE, kojic acid equivalent; na, not active.

of different extracts was in the range of 0.76–2.91 mg GALAE/g with highest effect recorded from the EtOH extract of the leaves followed by the EtOH extract of the twigs and fruits in addition to the EtOAc extract of the former (2.82–2.84 mg GALAE/g, $p \geq 0.05$). Generally, the aqueous extracts of the three organs exhibited the least enzyme inhibitory properties in most assays. The anti-BChE activity was in the range of 0.99–3.27 mg GALAE/g with highest effect obtained from the EtOAc extract of the leaves. This followed by its EtOH extract (2.82 mg GALAE/g), and that of the fruits (2.74 mg GALAE/g) and twigs (2.39 mg GALAE/g) respectively. The Tyr inhibitory activity of different extracts was in the range of 10.28–70.40 mg KAE/g with the EtOH (70.40 mg KAE/g) and EtOAc (56.36 mg KAE/g) extracts of the leaves revealed respectively the highest activity. This followed by the EtOAc and 70% EtOH extracts of the twigs, EtOH and 70% EtOH extracts of the fruits and 70% EtOH extract of the leaves which exerted comparable effect (51.30–54.68 mg KAE/g, $p \geq 0.05$). The α-amylase inhibitory activity was in the range of 0.08–1.06 mmol ACAE/g with the highest inhibition effect observed in the EtOAc extract of the leaves followed by the EtOAc extract of the twigs and fruits and the EtOH extract of the latter and that of the leaves (0.87–0.95 mmol ACAE/g, $p \geq 0.05$). Concerning the inhibitory property against the α-glucosidase enzyme, the best effect was obtained respectively from the EtOAc (1.57 mmol ACAE/g) and EtOH (1.42 mmol ACAE/g) extracts of the leaves while the EtOH and 70% EtOH extracts of the twigs and aqueous extract of the fruit exerted comparable inhibitory effect (1.14–1.22 mmol ACAE/g, $p \geq 0.05$). Overall, it was clear that the leaves displayed the best enzyme inhibitory properties.

Previously the enzyme inhibitory property of *Celtis* species was determined for *C. tournefortii* where the leaf methanolic extract exhibited an anti-AChE activity with IC_{50} 13.58 μg/mL (Baran & Keskin, 2023). However, pure compounds isolated from *Celtis* species were tested for their cholinesterase inhibitory properties. For example three amides were isolated from *C. africana* were shown to exert moderate anti-AChE activity (IC_{50} 84.3–98.3 μM) (Al-Taweel et al., 2012). 18 compounds isolated from *C. adolphi-friderici* exhibited also moderate activity with IC_{50} values ranged from 45.2 to 100.0 μM (Jumeta et al., 2021). N-p-Coumaryltyramine isolated from *C. chinensis* showed weak AChE inhibitory activity with IC_{50} value of 122 Mm (Suganthy et al., 2009). Results of the present study suggested that beside the possibility of synergistic action, the chemical profile of different extracts showed the presence of some compounds that were proven to possess enzyme inhibitory activity. For example, 4-hydroxy benzoic acid, vanillic acid and syringic acid was found to display an AChE inhibitory activity with IC_{50} values of 6.36, 6.79 and 6.96 μmol/μmol AChE respectively (Budryn et al., 2022). Catechin was found to exert anti-Tyr activity with IC_{50} 30.2 μg/mL (Ko et al., 2011) and, hence, could be a major contributor in the anti-Tyr effect of the leaves and fruits extracts. Also chlorogenic acid was also suggested to exert significant anti-Tyr property (Oh et al., 2019).

Although there are no reports on the inhibitory properties of *Celtis* species towards the α-glucosidase and α-amylase, 70% ethanolic extract of *C. iguanaea* leaf was found to reduce the serum glucose level in rats and reduces the maltase and sucrase level (Zanchet et al., 2018). Furthermore, it was suggested that 4-hydroxy benzoic acid and vanillic acid were the major contributor for the

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α-glucosidase and α-amylase inhibitory effect of *Elaeagnus angustifolia* leaves extracts (Saltan et al., 2017). Vanillic acid (IC_{50} = 27.89 mM; 0.35 mg/mL), syringic acid (IC_{50} = 44.81 mM, 0.39 mg/mL) and gallic acid (IC_{50} = 1.25 mM; 0.75 mg/mL) exerted considerable α-amylase inhibitory effect (Aleixandre et al., 2022; Tan et al., 2017; Zaharudin et al., 2018). Overall, it could be stated that *C. australis* could be considered as a promising source of enzyme inhibitors.

3.5 | Cytotoxicity

The cytotoxicity of different extracts from the twigs, leaves and fruits of *C. australis* were evaluated against the breast cancer (MDA-MB-231), colorectal adenocarcinoma (HT-29), cervical carcinoma (HELA), prostate cancer (DU-145), colorectal cancer (HCT-116), lung cancer (A549), gastric cancer (HGC-27) and human embryonic kidney (HEK-293) cell lines and results are given in Table 5. Anticancer activity extract with IC_{50} < 20 µg/mL is considered significant according to the National Cancer Institute (NCI) (Geran et al., 1972). The NCI considered IC_{50} upper limit criteria of ≤30 µg/mL is a promising crude extract for further purification (Campoccia et al., 2021; Suffness et al., 1990). Other reports consider an IC_{50} ≤ 100 µg/mL is effective as cytotoxic extract (Canga et al., 2022). According to these criteria, the majority of extracts displayed cytotoxicity with IC_{50} ≤ 100 µg/mL against all the tested cancer cells and no extract had IC_{50} < 20 µg/mL. However, four extracts namely; EtOAc of the twigs and EtOH, 70% EtOH and aqueous extracts of the leaves exerted cytotoxic effect with IC_{50} ≤ 30 µg/mL and they were effective against the HT-29 with IC_{50} of 23.90, 23.86, 26.31 and 28.13 µg/mL respectively. Also, the EtOH and 70% EtOH extracts of the leaves were cytotoxic against the DU-145 cell line with IC_{50} of 29.78 and 29.47 µg/mL respectively. Thus, these four extracts could be considered as promising candidates for isolation of anticancer molecules. The ripe fruits of *C. australis* was previously shown to possess cytotoxicity against human ovarian cancer cell line A2780 (IC_{50} 251.43 µg/mL) (Erden et al., 2024). The ethanolic and aqueous extracts of the leaves revealed considerable toxicity against human hepatocellular carcinoma (HEP-G2) (EC_{50} 26.10–26.90 µg/mL), colon adenocarcinoma (COLO 205) (EC_{50} 25.65–63.45 µg/mL) and gastric carcinoma (NCI-N87) (EC_{50} 45.15–35.10 µg/mL) (El-Alfy et al., 2011). Previous studies also demonstrated the anticancer activity of *Celtis* species like *C. aetnensis* against colon cancer (Caco2) cell (Acquaviva et al., 2016) and *C. tournefortii* against human liver cancer (HepG2) cells (Kavitha Krishna Nadiger et al., 2024). Kim et al. (2005) showed some isolated steroids and terpenoids to be responsible for the antitumor property of *C. sinensis* while a new glucosphingolipid isolated from *C. africana* was found to exert potent cytotoxic activity against mouse lymphoma cell line L5178Y (EC_{50} 7.8 µg/mL) (Perveen et al., 2015). Okafor et al. (2021) suggested that the inhibition of colon cancer by *Vigna subterraenea* extracts could be partly due to the synergistic or additive effect of phenolic acids and flavonoids present in the extract. In addition, neochlorogenic acid has been found to suppress the growth of estrogen-independent MDA-Mb-435 breast cancer cells (Noratto et al., 2009). Rutin was found to have toxicity against HTC hepatic cells at concentration 810 µM (Cristina Marcarini et al., 2011). Catechin effectively inhibited the MCF7 cell in a concentration dependent manner (Endang et al., 2014). Thus, it could be postulated that the cytotoxic effect of *C. australis* in the present study could be partly to the presence of phenolic acids and flavonoids.

3.6 | *C. australis* of Active Compounds: Targeting Cancer

The present study focused on the phytochemicals of *C. australis*, including catechin, isoquercitrin, vanillic acid, 4′-hydroxybenzoic acid, neochlorogenic acid,

TABLE 5 Cytotoxic effects of *Celtis australis*-extracts on cancer and normal cell lines (IC_{50} [µg/mL]).

	Twigs				Fruits				Leaves			
Cells	EtOAc	EtOH	70% EtOH	Water	EtOAc	EtOH	70% EtOH	Water	EtOAc	EtOH	70% EtOH	Water
MDA-MB-231	49.49	56.71	100.10	112.20	109.60	58.86	116.30	101.20	33.90	30.89	78.99	86.05
HT-29	23.90	44.45	36.50	38.15	86.07	63.53	62.04	31.13	45.29	23.86	26.31	28.13
HELA	62.03	80.73	90.47	73.63	97.42	97.82	92.37	73.74	72.78	79.71	75.05	81.02
DU-145	37.26	33.55	115.5	62.02	77.57	70.09	77.75	39.97	66.41	29.78	29.47	72
HCT-116	31.25	38.51	45.14	51.1	112.65	86.32	99.65	65.52	55.02	69.24	82.56	95.63
A549	52.2	74.12	69.9	77.71	96.35	79.68	106.54	45.68	45.3	35.63	69.53	76.35
HGC-27	69.54	79.65	85.6	74.12	79.65	100.9	89.65	58.51	35.03	74.12	56.98	83.36
HEK-293	65.25	71.67	81.24	78.61	105.23	98.65	120.36	86.65	70.25	55.21	61.65	71.84

chlorogenic acid, hesperidin, hyperoside, caffeic acid, delphinidin 3-5-diglucoside, and rutin. The gene targets of these compounds were identified through an analysis of the CTD, PubChem, and SwissTargetPrediction databases. The DOSE analysis yielded significant associations between a set of genes of interest and a range of diseases. These findings provide crucial insights into the potential biological functions of the investigated genes and their implications for disease. The enrichment analysis identified significant associations with multiple cancer types, including musculoskeletal system cancer, stomach cancer, connective tissue cancer, bone cancer, head and neck cancer, head and neck carcinoma, and bone sarcoma (Supporting Information S1: Figure S13). These findings underscore the importance of the genes under investigation in oncogenic processes and highlight their relevance in cancer biology. Moreover, the identification of diseases such as ischemia, hepatitis, and liver cirrhosis indicate that these genes may play a potential role in the development of these conditions. The results of the cell line study indicated that the HGC-27 cell line was associated with gastric cancer, as evidenced by the R plot results. Nevertheless, the observed efficacy in this cell line was not robust. Following the identification of genes associated with *C. australis* phytochemicals, a Venn diagram was employed to analyze the intersections between these genes and those specific to breast, colon, prostate, gastric, and lung cancers. The analysis involved a comparison of 245 breast cancer-related genes, 99 colon cancer-related genes, 39 prostate, 240 gastric, and 630 lung cancer-related genes obtained from the CTD and GeneCards databases with the 4002 genes associated with *C. australis* compounds. The Venn diagram provided a visual representation of the overlap between the gene sets, indicating which genes are shared between the *C. australis* phytochemicals and the selected cancer types (Figure 1).

This analysis yielded valuable insights into the potential biological functions of the overlapping genes and their relevance as therapeutic targets in cancer treatment. These findings contribute to a more profound comprehension of the molecular mechanisms underlying cancer biology and provide a foundation for future research in this domain. The STRING database analysis for breast cancer revealed a gene interaction network comprising 236 nodes and 11,042 edges. By applying the maximal clique centrality (MCC) method through the CytoHubba plugin, key hub genes such as TP53, AKT1, HIF1A, STAT3, and CASP3 were identified (Supporting Information S1: Figure S14). The analysis of colon cancer targets within the STRING database produced a network of 97 nodes and 2,340 edges. The MCC method identified critical hub genes including BCL2, TP53, CTNNB1, PTEN, and ERBB2 (Supporting Information S1: Figure S15). Prostate cancer, the STRING database analysis led to the construction of a network consisting of 38 nodes and 776 edges, with TP53, AKT1, PTEN, CDKN2A, and PIK3CA emerging as the key hub genes (Supporting Information S1: Figure S16). In the case of gastric cancer, the STRING database analysis led to the construction of a network consisting of 225 nodes and 8430 edges, with TP53, PTEN, STAT3, CTNNB1 and MYC emerging as the key hub genes (Supporting Information S1: Figure S17). Similarly, lung cancer the STRING database analysis led to the construction of a network consisting of 585 nodes and 21654 edges, with TP53, BCL-2, ACTB, CTNNB1 and MYC emerging as the key hub genes.

The findings of this study indicate that the phytochemicals of *C. australis*, particularly those including hesperidin, and rutin, possess significant potential as valuable agents for cancer treatment. The comprehensive interaction network, defined by the STRING database, demonstrates that these compounds may affect cancer pathways through various mechanisms and may target multiple genes involved in different types of cancer. The identification of different cancer types' critical hub genes, such as TP53, AKT1, and PTEN, further emphasizes the central role of these genes in oncogenic processes. TP53, which is known as the "guardian of the genome" plays a well-established and widely accepted role in regulating the cell cycle and apoptosis, and is a well-known tumor suppressor gene. The continuous identification of TP53 as a hub gene in breast, colon, prostate, gastric, and lung cancer pathways substantiates its pivotal role in cancer biology and its potential as a therapeutic target (Lindström et al., 2022). However, the variable activity observed in the HGC-27 cell line suggests that these phytochemicals may be affected by factors such as cellular environment, gene expression profiles, and specific mutations. This situation clearly indicates the necessity for further research to determine the optimal conditions for the maximum therapeutic efficacy of these compounds. Additionally, the relationships between these non-cancer conditions *and C. australis* phytochemicals suggest that these substances may have therapeutic potential beyond cancer treatment. The identification of these relationships has opened up new avenues for research into the therapeutic applications of these compounds in a wider range of diseases, which is a significant development in this field of study.

3.7 | KEGG and GO analysis

A target-pathway enrichment analysis was conducted using the DAVID database to investigate the potential biological effects of *C. australis* components on breast, colon, prostate, gastric, and lung cancers. The analysis identified 119 statistically significant pathways for breast cancer, 134 for colon cancer, and 102 for prostate cancer ($p < 0.05$). The components of *C. australis* were found to enrich several key biological processes for cancer. Such as, colon cancer these components

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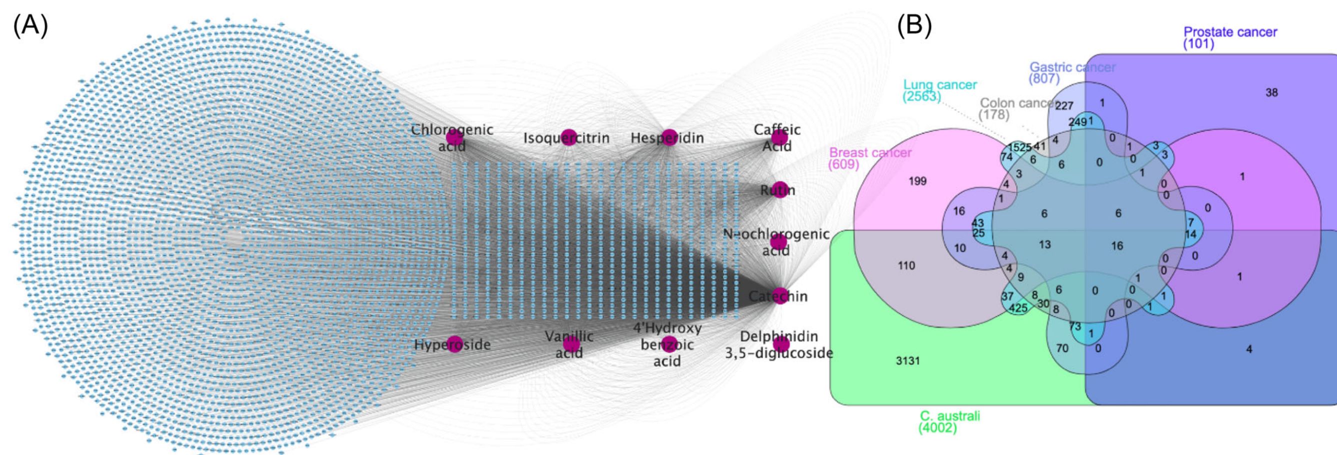

FIGURE 1 Target analysis of *C. australis* and cancer. (A) Target analysis of *C. australis*. (B) Venn diagram showing the overlap between *C. australis*-related breast, colon, prostate, gastric, and lung cancer-associated genes.

were found to enrich pathways including Pathways in cancer; Gastric cancer; Colorectal cancer; Endometrial cancer; Hepatocellular carcinoma; Breast cancer; Prostate cancer; Proteoglycans in cancer; and MicroRNAs in cancer. In the context of prostate cancer, the enriched pathways included Pathways in cancer; Gastric cancer; Colorectal cancer; Hepatocellular carcinoma; Breast cancer; Prostate cancer; MicroRNAs in cancer, and the PI3K-Akt signaling pathway. These findings indicate that the components of *C. australis* may possess broad therapeutic potential, capable of influencing multiple biological processes across various cancer types (Figure 2B).

GO analysis revealed significant enrichment in various biological processes, molecular functions, and cellular components associated with breast, colon, prostate, gastric, and lung cancer. For breast cancer, key enriched processes include positive regulation of transcription by RNA polymerase II, phosphorylation, negative regulation of cell population proliferation, and positive regulation of DNA-templated transcription. These processes are critical for cell growth, gene expression, and regulation of apoptosis (programmed cell death) and play a central role in cancer development and progression. In the context of colon cancer, significant enrichment has been observed in processes such as cell cycle regulation, negative regulation of transcription by RNA polymerase II, and negative regulation of apoptotic processes (Hunter, 1995; Orphanides et al., 1996). These processes are directly related to the uncontrolled proliferation of cancer cells and their ability to evade programmed cell death (Sherr, 1996), which are fundamental features of colon cancer pathology. In prostate and breast cancer, the enriched processes include both positive and negative regulation of gene expression as well as metal ion binding. These findings suggest that the components of *C. australis* may influence transcriptional regulation and metal ion homeostasis, both of which play important roles in the development of prostate cancer (Orphanides et al., 1996) (Figure 2A). From a molecular function perspective, the analysis revealed significant enrichment in functions such as DNA binding, ATP binding, protein binding, protein homodimerization activity, identical protein binding, ubiquitin protein ligase binding, and enzyme binding. These functions are essential for various cellular processes, including DNA replication, repair, and ubiquitin-mediated protein degradation, which are often dysregulated in cancer cells. Overall, the GO enrichment results suggest that the components of *C. australis* may exert therapeutic effects by modulating key biological processes and molecular functions that are critical for the development and progression of breast, colon, prostate, gastric and lung cancer.

3.8 | Molecular docking

As part of the study, molecular docking was conducted for enzymes and proteins. The requisite coordinates and grid sizes for these analyses are provided in Supporting Information S1: Table S2. From the multitude of compounds identified in *C. australis*, catechin, isoquercitrin, vanillic acid, 4′-hydroxybenzoic acid, neochlorogenic acid, chlorogenic acid, hesperidin, hyperoside, caffeic acid, delphinidin 3-5-diglucoside, and rutin, were selected for comprehensive analysis due to their pervasive distribution. The study selected four proteins for analysis in breast cancer: EGFR, ARO, Eg5, and PI3K delta, based on previous research (Alossaimi et al., 2024; Badawi et al., 2023). For colon cancer (HT-29), the proteins identified were E2F1, c-Fos, IFN-γ, IL-2, IRS-1, TGF-β1, CDK4, Cyclin D1, CDKN1A, TP53, and NF-κB p65 (Ahmad et al., 2022; Ikwu, Isyaku, et al., 2020; Li et al., 2019). In HeLa cells, BCL-2, BCL-W, MCL-1, AKT1, and BRAF were the proteins selected for further investigation (Çankaya et al., 2021). Regarding prostate

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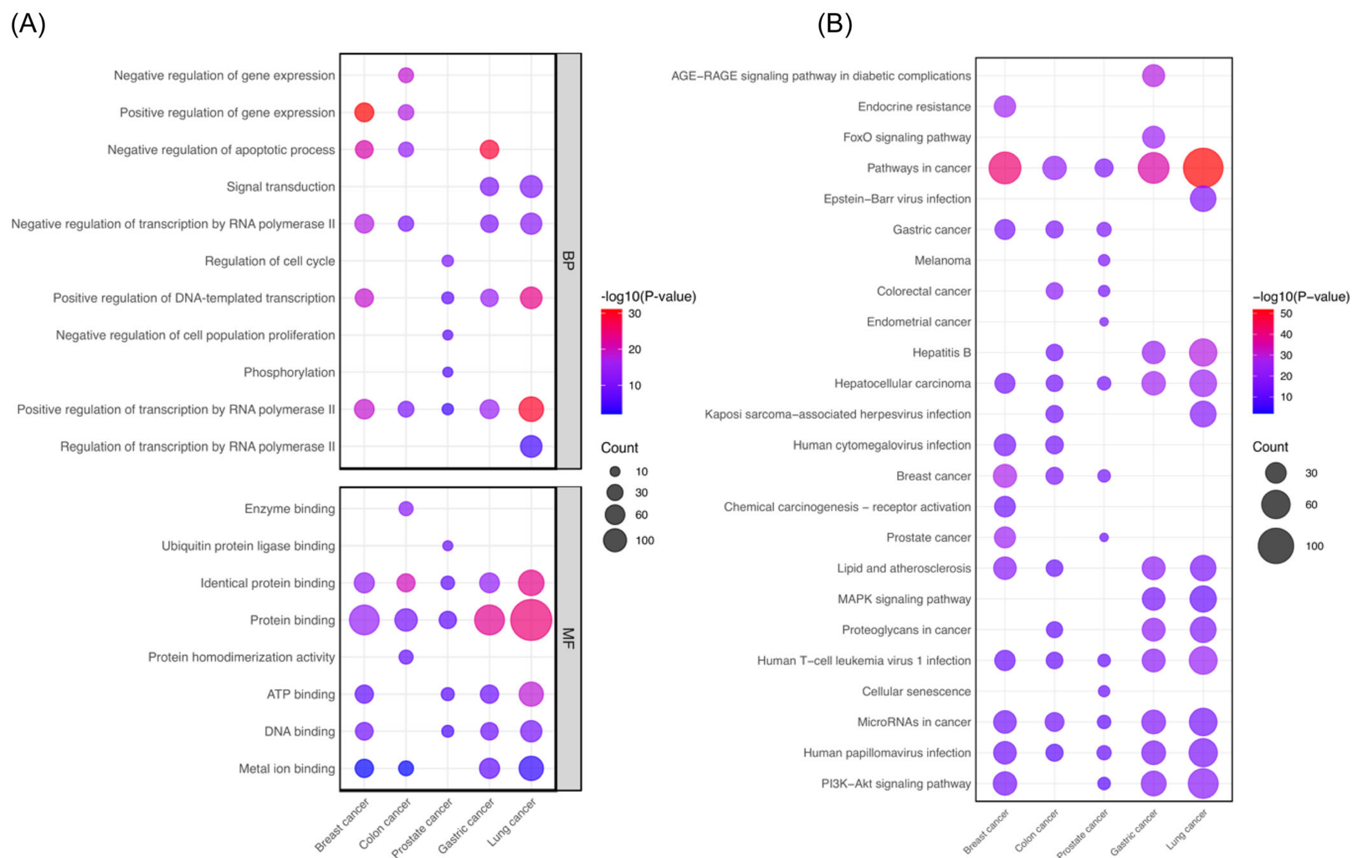

FIGURE 2 Cancer-related term enrichment analysis using KEGG pathways and GO biological processes and molecular functions. (A) shows the GO term enrichment analysis for biological processes (BP) and molecular functions (MF) across different types of cancer. (B) shows the KEGG pathway enrichment analysis for the 12 most related pathways to cancer types.

cancer (Du-145 and HTC-116), the analysis focused on AR, TOP2B, CDK2, and PTEN (Ikwu, Shallangwa, et al., 2020; Jamaspishvili et al., 2018; Rangaswamy et al., 2023). For lung cancer (A549), the chosen proteins included cIAP1-BIR3, EGFR, and MDM2. Finally, gastric cancer (HGC-27), AKT-1, BCL-2, TRAF2, CDK2, Caspase-3 (Casp-3), and TUBA1B was specifically selected (Hu et al., 2022; Ma et al., 2022; Ning et al., 2021; Ren et al., 2020; Xu et al., 2021). In addition to the enzymes AChE, BChE, Tyr, amylase, and glucosidase that were selected for molecular docking, the chosen compounds demonstrated binding energies within a range of −11.5 to −3.6 kcal/mol when interacting with both enzymatic targets and cancer-related proteins (Supporting Information S1: Tables S3 and S4). Subsequently, compounds that met the minimum docking score threshold of −8.0 kcal/mol and formed at least one hydrogen bond were subjected to further analysis. A hydrogen bond distance limit of 4 Å was employed to facilitate the evaluation process. Figure 3 illustrates this, and Supporting Information S1: Table S4 lists the compounds that surpassed the −8.0 kcal/mol docking score threshold. The results of the molecular docking study show that out of eleven molecules, hesperidin (31), rutin (25), delphinidin 3-5-diglucoside (21), isoquercitrin (16), neochlorogenic acid (16), hyperoside (16), catechin (15) and chlorogenic acid (14), have remarkable binding energies with proteins. Analysis of the binding energies for enzyme residues revealed higher interactions between AChE and isoquercitrin, AChE and hesperidin, AChE and hyperoside, ARO and hesperidin, AChE and delphinidin 3-5-diglucoside, TRAF2 and hesperidin, respectively. In these interactions, it was observed that bonds such as pi-pi, pi-sigma, pi-sulfur, pi-alkyl, and conventional hydrogen bonds were more prevalent than hydrogen bonds (Figure 3) (Supporting Information S1: Table S4).

Molecular docking analyses were performed to evaluate the inhibitory effects of *C. australis* hub molecules on the enzymes α-amylase, AChE, BChE, α-glucosidase and Tyr. Hesperidin proved to be a potent inhibitor of all enzymes, exhibiting the lowest binding energies and the highest number of hydrogen bonds: α-amylase (−10.0 kcal/mol, five hydrogen bonds), AChE (−11.5 kcal/mol, five hydrogen bonds), BChE (−10.4 kcal/mol, six hydrogen bonds), α-glucosidase (−9.7 kcal/mol, five hydrogen bonds), and Tyr (−8.9 kcal/mol, seven hydrogen bonds). In addition, molecules such as hesperidin, hyperoside, rutin, and isoquercitrin showed remarkable inhibitory activities with high binding energies and high numbers of hydrogen bonds with several

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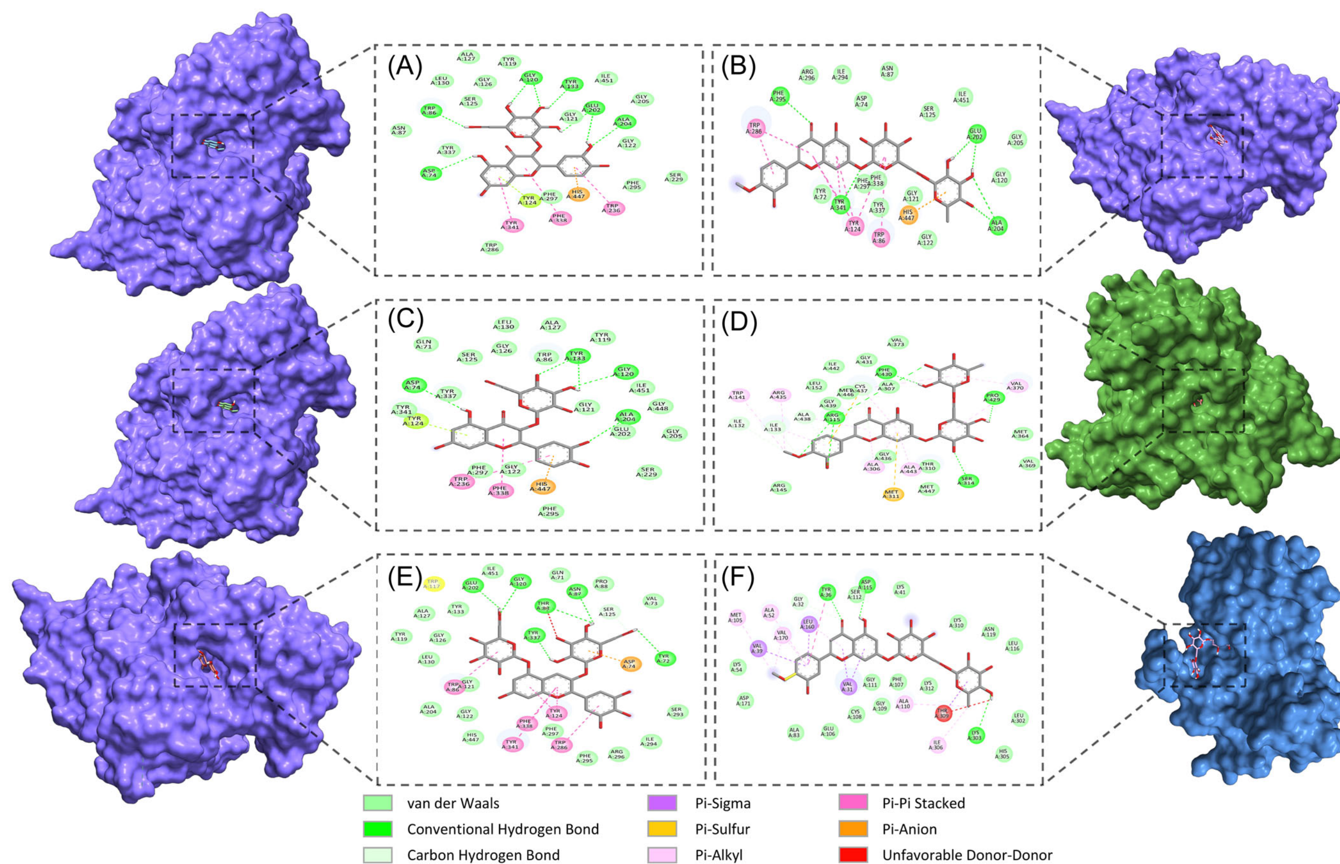

FIGURE 3 Enzymes and proteins' active sites with compounds showing the best binding energy; (A) Interaction between AChE and isoquercitrin. (B) Interaction between AChE and hesperidin. (C) Interaction between AChE and hyperoside. (D) Interaction between ARO and hesperidin. (E) Interaction between AChE and delphinidin 3-5-diglucoside. (F) Interaction between TRAF2 and hesperidin.

enzymes. For example, rutin interacted strongly with α-amylase (−9.2 kcal/mol, seven hydrogen bonds), AChE (−10.0 kcal/mol, three hydrogen bonds), BChE (−10.6 kcal/mol, five hydrogen bonds), glucosidase (−9.1 kcal/mol, four hydrogen bonds), and Tyr (−8.8 kcal/mol, five hydrogen bonds). Isoquercitrin showed high affinities with α-amylase (−8.9 kcal/mol, two hydrogen bonds), AChE (−11.0 kcal/mol, eight hydrogen bonds), and BChE (−10.0 kcal/mol, eight hydrogen bonds), whereas hyperoside was effective against BChE (−10.0 kcal/mol, nine hydrogen bonds) and AChE (−10.8 kcal/mol, five hydrogen bonds). In addition, other molecules such as delphinidine 3-5-diglucoside and neochlorogenic acid also showed good results against several enzymes. Delphinidin 3-5-diglucoside showed significant interactions with α-amylase (−8.8 kcal/mol, five hydrogen bonds) and Tyr (−8.3 kcal/mol, six hydrogen bonds). Analysis of the interactions of these inhibitors with α-amylase revealed that residues Asp A:197 and Glu A:233 interacted with five different compounds, while Gln A:63 interacted with four different compounds. For AChE, residues Glu A:202 and Ala A:204 interacted with four compounds and Gly A:120 with three compounds. For BChE, residues Leu A:286 and His A:438 interacted with five compounds and residues Asp A:70, Ala A:199 and Glu A:197 interacted with four compounds. In addition, residue Asp A:568 of glucosidase was found to interact with three different compounds. These results suggest that these molecules have high inhibitory potential and can be considered as potential therapeutic agents for the treatment of various diseases such as diabetes, and Alzheimer's disease. The low binding energies and high number of hydrogen bonds further emphasize their potential efficacy as enzyme inhibitors (Yagi et al., 2024) (Table S3).

C. australis exhibits potent inhibitory effects on several key proteins associated with breast, colon, prostate, gastric, and lung cancers. Hesperidin emerged as the most potent inhibitor with the highest binding affinities for many of the proteins tested in this study. For example, hesperidin demonstrated potent inhibitory effects on proteins such as TP53 (−8.6 kcal/mol, colon cancer), PTEN (−10.2 kcal/mol, prostate cancer), EGFR (−10.4 kcal/mol, breast and lung cancer), ARO (−10.9 kcal/mol, breast cancer), Eg5 (−8.8 kcal/mol, breast cancer), and PI3K delta (−10.3 kcal/mol, breast cancer). These results highlight the potential of hesperidin as a versatile inhibitor in cancer therapy. In addition to hesperidin, other molecules such as rutin showed high binding affinities with TRAF2 (−10.1 kcal/mol, gastric cancer), caspase-3 (−9.0 kcal/mol, gastric cancer), EGFR (−9.9 kcal/mol, breast and lung cancer), ARO (−10.6 kcal/mol, breast cancer), and

PI3K delta (−9.6 kcal/mol, breast cancer). Delphinidine 3-5-diglucoside was also effective against CDK2 (−9.0 kcal/mol, gastric cancer), Cyclin D1 (−8.1 kcal/mol, colon cancer), and EGFR (−9.7 kcal/mol, breast and lung cancer). Isoquercitrin showed high binding energies with CDK4 (−9.6 kcal/mol, colon cancer), ARO (−9.3 kcal/mol, breast cancer), and Eg5 (−8.8 kcal/mol, breast cancer). Chlorogenic acid showed the highest binding energies with TUBA1B (−9.0 kcal/mol, gastric cancer) and AKT (−8.4 kcal/mol, gastric cancer). Hyperoside was effective against BCL-2 (−8.8 kcal/mol, gastric cancer) and ARO (−9.3 kcal/mol, breast cancer), whereas neochlorogenic acid showed high affinities for TRAF2 (−8.5 kcal/mol, gastric cancer) and TUBA1B (−8.4 kcal/mol, gastric cancer). These results suggest that in addition to hesperidin, other molecules such as rutin, delphinidin 3-5-diglucoside, isoquercitrin, chlorogenic acid, hyperoside, and neochlorogenic acid are effective inhibitors against multiple targets (See Supporting Information S1: Table S4). In particular, hesperidin has demonstrated potent inhibitory effects on proteins associated with several types of cancer, making it a promising candidate for the treatment of breast, prostate, colon, gastric, and lung cancers. Molecules such as rutin and delphinidin 3-5-diglucoside have shown high potency against specific proteins, suggesting their potential as components in combination therapies. The effects of chlorogenic acid on TUBA1B and AKT are particularly noteworthy for the inhibition of gastric cancer related genes. In conclusion, the results of this study indicate that the hub molecules of *C. australis*, particularly hesperidin, rutin, delphinidin 3-5-diglucoside, isoquercitrin, chlorogenic acid, hyperoside and neochlorogenic acid, have the potential to develop treatments that target multiple cancer types by inhibiting key proteins such as TP53, PTEN, EGFR, ARO, Eg5, PI3K delta, IFN-γ, IL-2, IRS-1, TGF-β1, CDK4, Cyclin D1, BCL-2, BCL-W, MCL-1, AKT-1, AR, TOP2B, cIAP1-BIR3, MDM2, TUBA1B CDK2, TRAF2, and casp-3. Future research should focus on validating these molecules through in vivo studies and exploring their full potential in cancer therapy.

4 | CONCLUSION

The present study represents the first detailed investigation of the antioxidant, enzyme inhibitory and cytotoxic properties of *C. australis*. Extracts of all organisms were rich in phenolics. The twigs and fruits were characterized by high accumulation of vanillic acid, 4-hydroxy benzoic acid and syringic acid, in addition to neochlorogenic acid in the fruits. The leaves were characterized by a remarkable abundance of neochlorogenic acid, catechin and rutin. Accordingly, the tested biological activities of the three organs varied. Indeed, the three organs displayed significant biological activities, but the leaf exerted the best antioxidant activity in most assays (5/6). Also, it exerted the best inhibitory property against AChE, BChE, Tyr, α-amylase and α-glucosidase enzymes. The leaves had considerable cytotoxicity against the HT-29 and DU-145 cell lines. The twigs were also effective against the HT-29 cell line while the fruit recorded the best chelating power. The identified compounds in the extracts demonstrated robust inhibitory effects on a range of cancer-related proteins, such as including ARO, PI3K delta, AKT-1, PTEN, and TRAF2, as well as all the enzymes under investigation. In conclusion, this study showed that *C. australis* is a rich source of bioactive compounds with potential for future development of phytopharmaceuticals targeting specific oxidative stress-linked diseases and for variable food and cosmetic applications. These findings indicate that these phytochemicals have the potential to be developed into multi-target cancer therapies. Further in vivo and in vitro validation is required to confirm these results.

AUTHOR CONTRIBUTIONS

Mehmet Veysi Cetiz: Data curation; methodology; visualization; Writing—original draft. **Sakina Yagi**: Investigation; methodology; supervision; Writing—original draft; Writing—review and editing. **Umran Kurt**: Investigation; methodology; visualization; Writing—original draft. **Ismail Koyuncu**: Data curation; investigation; methodology; Writing—original draft; Writing—review and editing. **Ozgur Yuksekdag**: Data curation; investigation; methodology; Writing—original draft; Writing—review and editing. **Giovanni Caprioli**: Data curation; investigation; methodology; Writing—original draft; Writing—review and editing. **Laura Acquaticci**: Data curation; investigation; methodology; visualization; Wwriting—original draft; Writing—review and editing. **Simone Angeloni**: Investigation; methodology; supervision; writing—original draft; Writing—review and editing. **Ismail Senkardes**: Investigation; software; Writing—original draft. **Gokhan Zengin**: Conceptualization; data curation; methodology; Writing—original draft; Writing—review and editing.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

ETHICS STATEMENT

None declared.

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