



Using a combination of in vitro and in silico approaches to explore the biological potential of *Arceuthobium oxycedri* extracts: innovative insights from the lab to functional applications

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Abstract

In the present study, the antioxidant capacity, enzyme inhibitory activities, and chemical composition of various extracts from *Arceuthobium oxycedri* were assessed by LC-MS/MS. Extracts were further evaluated for their cytotoxicity by the resazurin reduction assay and for their antioxidant activity by qRT-PCR in liver carcinoma HepG2 cells. The LC-MS/MS indicated that in both ethanolic and ethanol/water (70%) extracts, there were highly identified phenolic compounds, and they exhibited greater antioxidant effects. These extracts exhibited potential inhibitory activities against acetylcholinesterase and butyrylcholinesterase with the values of 4.72 and 4.41 mg GALAE/g, respectively. The ethanol extract showed the highest α -glucosidase inhibitory activity, 2.19 mmol ACAE/g, probably due to its high content of proanthocyanidins and hydroxycinnamic acids. Furthermore, results demonstrate that some of the extracts significantly reduce the expression of the antioxidant gene *HO-1*, while γ -GCS was slightly increased. To further investigate the molecular mechanisms, network pharmacology approach was used to identify potential targets related to HO-1 and NRF2. The analysis revealed key proteins associated with oxidative stress and metabolic pathways. Subsequently, molecular docking was performed on these identified targets, along with standard enzymes such as AChE, BChE, α -amylase, α glucosidase, and tyrosinase. The best-performing protein-ligand interactions were then selected for molecular dynamics simulations. These findings enable us to infer that ethanol and ethanol/water (70%) extracts represent the most promising candidates based on their chemical composition for their medicinal importance, represented by their rich contents of phenolic and flavonoid compounds.

Keywords *A. oxycedri* · Chemical profiling · Oxidative stress · Antioxidants · Enzyme inhibition · Network pharmacology · In silico

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Introduction

Phytochemicals, also called phytonutrients, are crucial in pharmaceutical development for chronic diseases, utilizing their bioactive properties to prevent and treat diseases [1, 2]. Recent studies show that about 80% of the world's population expects medication benefits from such chemical sources, which have influenced the pharmaceutical industry [3]. Roughly 25% of the prescription drugs are derived from phytochemicals in the USA [4]. About 80,000 plant species are used in different phytochemical-based medications since WHO reported their effectiveness against chronic and acute diseases with less harmful side effects than other chemical-based drugs [5]. A total of 121 medicinal medicines originating from phytochemicals have been developed

throughout the last century around the world; many of these emanate from ancient knowledge systems dating as far back as 2800 BCE [5].

Plants are rich in natural bioactive chemicals, including secondary metabolites and antioxidants [6–8]. The most abundant of these are phenolics, which play a crucial role in the colouring of plants, their development, reproduction and their protection against pathogens and herbivores. This is mainly due to their phytoalexin properties and strong astringency [9, 10]. Phenolic compounds exhibit numerous bioactivities, such as anti-allergic, anti-inflammatory, antioxidant, hepatoprotective, antiviral, and anticarcinogenic activities [11, 12]. Their antioxidant activity, however, has been the most interesting, especially regarding their potential to reduce the formation and scavenge free radicals in vivo [13].

The genus *Arceuthobium*, commonly known as dwarf mistletoe, is particularly biologically important because it comprises one of the evolutionarily most specialized groups of the Loranthaceae, now reclassified to Santalaceae [14]. This genus's 8–15 species are distributed between central Spain/southern Europe, North Africa, the Near East, the Himalayas, and western China. *Arceuthobium* species are semi-parasitic plants that mostly parasitize *Juniperus* species and other members of the Cupressaceae family [15].

Regardless of their biological importance, there is a scant phytochemical study on these plants. Crawford and Hawksworth (1979) studied the flavonoid composition of 36 taxa of *Arceuthobium*, including *Arceuthobium oxycedri*, and found that all species contained flavonol 3-*O*-glycosides, mainly derivatives of quercetin and myricetin [16]. Tocher et al. (1982) studied triacyl glyceride fatty acids of three *Arceuthobium* species [17]. However, until now, no research on the biological studies of this genus has been conducted.

Arceuthobium oxycedri (DC) M. Bieb., the most common species in Turkey, generally parasitizes on branches of *Juniperus oxycedrus*. In Anatolian folk medicine, the plant was traditionally used for viral and inflammatory affections of upper respiratory ways, gastrointestinal disorders, and as a hypotensive agent as tea [18, 19]. Various *Arceuthobium* species have been used to treat lung and oral hemorrhages, tuberculosis, cachexia, abdominal pain, cough, colds, rheumatism, and diabetes [16].

The current study assessed the antioxidant activities, cytotoxicity, qRT-PCR of important oxidative stress genes, and enzyme inhibitory potential of five different extracts from the aerial parts of *A. oxycedri* using various in vitro methods. In addition to biological activities, HPLCMS was used for chemical profiling. In order to provide further elucidation regarding the molecular mechanisms underlying the observed bioactivities and to explore the interactions between the phytochemical constituents and potential target

proteins, a multifaceted approach was employed, integrating network pharmacology analyses along with molecular docking and molecular dynamics simulation studies. This comprehensive strategy offers novel insights into the therapeutic potential of *A. oxycedri* and contributes to a more profound comprehension of its intricate biological activities.

Materials and methods

Plant collection

In 2020, botanical specimens were collected from the city forest of Konya, Türkiye. Dr. Evren Yildiztugay conducted the taxonomic identification, and a voucher specimen was preserved in the herbarium of the Selcuk University (Voucher number: EY-3021). The plant samples were dried in the shade at ambient temperature, powdered, and thereafter stored away from light.

Plant extract preparation

The extraction procedure included four solvents: *n*-hexane, ethyl acetate, ethanol, a 70% ethanol/water mixture, and water. Each 10 g sample was macerated with 200 mL of *n*-hexane, ethyl acetate, ethanol, and a mixture of ethanol/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.

Spectrophotometric assay for total phenolic and flavonoid contents

Total phenolics and flavonoids were quantified in our previous paper [20]. Gallic acid and rutin served as reference standards in the experiments, with results reported as gallic acid equivalents (GAE) and rutin equivalents (RE).

LC-MS/MS metabolomic analysis

Liquid chromatography coupled with mass spectrometry (UHPLC/MS/MS) was used to analyze the extracts. The equipment used for this analysis was the Dionex Ultimate 3000RS UHPLC system, which was fitted with a mass spectrometer (Q-Exactive Orbitrap, Thermo, USA). Our previous publication included all analytical details (Zengin et al. 2018).

Assays for in vitro antioxidant capacity

As previously described [21], various antioxidant tests were performed. DPPH, ABTS radical scavenging, CUPRAC, and FRAP results were milligrams of Trolox equivalents (TE) per gram. In millimoles of TE per gram of extract, the phosphomolybdenum (PBD) test measured antioxidant potential, and in EDTAE, metal chelating activity (MCA) was measured.

Inhibitory effects against some key enzymes

Enzyme inhibition studies were performed on samples according to methods [21], Acarbose equivalents (ACAE) per gram of extract inhibited amylase and glucosidase, while milligrams of galanthamine equivalents (GALAE) inhibited acetylcholinesterase (AChE) and butyrylcholinesterase. To evaluate tyrosinase inhibition per gram of extract, milligrams of kojic acid equivalents were used.

Cell cultivation

Human hepatoblastoma cells (HepG2: Deutsche Sammlung von Mikroorganismen und Zellkulturen DSMZ, Braunschweig, Germany) were cultivated in T75 cell culture flask (Sarstedt, Nümbrecht, Germany) supplied with 15 mL of Dulbecco's Modified Eagle Medium (DMEM) (Pan-Biotech, Aidenbach, Germany), 10% fetal bovine serum (Pan-Biotech) and 5% 10.000 U mL⁻¹ penicillin; 10 mg mL⁻¹ streptomycin. Cells were incubated at 37 °C in an CO₂ incubator (Heracell™ VIOS160i, ThermoFisher Scientific, Darmstadt, Germany) at 5% CO₂. Twice a week, cells were detached from the cell culture flask with 2 mL trypsin EDTA (Pan-Biotech, Aidenbach, Germany) and an incubation at 37 °C for 8 min. After the incubation, cells were centrifuged at 5000 x g for 5 min and the supernatant was removed. Cells were resuspended in 2 mL DMEM and 200 µL were transferred into a new T75 cell culture flask for further cultivation.

Cytotoxicity assay

To ensure that test samples were not cytotoxic to HepG2 cells, a cytotoxicity assay with resazurin was performed. At first, *A. oxycedri* extracts were solved in methanol at stock concentrations of 10 mg mL⁻¹. Thus, methanol was used as solvent control. For the cytotoxicity assay, HepG2 cells were seeded into a 96 well plate (Sarstedt) at a density of 5,000 cells in 200 µL medium per well in 60 wells. Cells were grown for 24 h prior to treatment. After 24 h of attachment, cells were treated with the tested extracts for 24 h at a concentration of 100 µg mL⁻¹. In case of hexane and ethyl

acetate extracts concentrations of 50 µg mL⁻¹ and 75 µg mL⁻¹ were additional included. Triton X at a concentration of 1% was used as negative control. After 24 h of incubation, the treatment solutions were removed and 100 µL of a 10% resazurin in medium solution was added to the wells. The plate was incubated at 37 °C for 4 h in an CO₂ incubator. After the incubation period, the fluorescence signal (excitation wavelength of 560 nm, emission wavelength of 690 nm) was measured in a multiplate reader (Infinite M200, Tecan, Männedorf, Switzerland).

Cell culture experiments for subsequent qPCR analysis

For the analysis of gene expression, HepG2 cells were seeded at a density of 1×10^6 cells per well in 6-well plates (Sarstedt). Cells were grown for 24 h before treatment. Afterwards, cells were treated with the plant extracts at 100 µg mL⁻¹. Cells were treated for 6 h, and afterwards washed with 500 µL phosphate buffered saline (PBS) (ChemSolute, Renningen, Germany). Cells were detached from the wells using 250 µL trypsin EDTA (Pan-Biotech, Aidenbach, Germany) and incubation at 37 °C in an CO₂ incubator (ThermoFisher Scientific) for 6 min. The cell suspension was mixed with medium and then transferred into a 2 mL tube and centrifuged at 1000 xg for 5 min. The supernatant was removed and the pellets were frozen at -80 °C until the extraction of RNA.

RNA extraction

Total RNA was extracted using the phenol chloroform extraction method (Hornbacher et al., 2022). To extract RNA, cell pellets were homogenized in 1 mL of a mixture containing 48% water-saturated phenol (Applichem, Darmstadt, Germany), 33% deionized water, 5% glycerol (VWR, Darmstadt, Germany), 3.5% 3 M sodium acetate (NaAc) buffer (pH=5.0) (Merck, Darmstadt, Germany), 0.4 M ammonium thiocyanate (Riedel-de Haën, Seelze, Germany) and 0.8 M guanidium thiocyanate (Roth, Karlsruhe, Germany). Cell pellets were homogenized for 30 min at room temperature on a shaker (CellMedia, Zeitz, Germany).

Subsequently, 200 µL chloroform (Sigma-Aldrich, Taufkirchen, Germany) was added to the mixture and inverted by hand for 15 s and incubated for 3 min at room temperature. Tubes were then centrifuged at 12,000 x g at 4 °C for 15 min. After centrifugation, 400 µL of the upper phase were transferred into a new 1.5 mL tube and mixed with 500 µL of isopropanol (Walter-CMP, Kiel, Germany). The mixture was inverted 10–15 times, incubated at room temperature for 10 min and then centrifuged for 10 min at 4 °C at 12,000 x g. The supernatant was removed and the

pellet washed with 1 mL 70% ethanol (Walter, Kiel, Germany), shaken for 10 min on a shaker and then centrifuged at 7,500 $\times$ g at 4 °C for 5 min. Afterwards, ethanol was completely removed with a pipette and the pellet was allowed to dry. To resuspend the RNA, the pellet was resolved in 25 μ L of nuclease free water (Life Technologies, Darmstadt, Germany). Until further analysis, RNA samples were stored at –20 °C.

Synthesis of cDNA

RNA samples were diluted 1:10 with nuclease free water. Additionally, randomly selected samples were diluted 1:4 and 1:16 (v/v) afterwards to ensure linearity of cDNA synthesis. For the removal of genomic DNA, 2 μ L of diluted RNA was added to 1.2 μ L DNase (ThermoFisher Scientific, Darmstadt, Germany), 1.2 μ L DNase buffer (ThermoFisher Scientific) and 7.6 μ L nuclease free water in a 96-well plate (Sarstedt, Nümbrecht, Germany). After sealing the plate with an adhesive cover (Sarstedt,), the plate was incubated at 37 °C for 30 min on a thermal shaker. To degrade the DNase, the plate was incubated at 70 °C for 15 min subsequently. After cooling on ice, the plate was briefly centrifuged and 1 μ L of random nonamer primer (50 pmol μ L^{–1}) was added. Secondary nucleic acid structures were resolved by incubating the plate at 70 °C for 5 min and the primer was annealed by cooling the plate immediately on ice. After brief centrifugation, 0.5 μ L reverse transcriptase (Promega, Walldorf, Germany), 4 μ L reverse transcriptase buffer (Promega, Walldorf, Germany), 2 μ L of 10 mM dNTPs (Thermo Scientific) and 0.5 μ L nuclease free water were added. Random additional samples were assigned to ensure full removal genomic DNA in which reverse transcriptase was exchanged with nuclease free water. After brief centrifugation, reverse transcription was performed at 37 °C for 1 h and enzymes were inactivated at 70 °C for 15 min. Until further analysis, samples were stored at –20 °C.

Quantitative polymerase chain reaction (qPCR)

To enable relative quantification of RNA, 2 μ L of each sample was pooled and a serial dilution with the dilution factor 1:4 was prepared to prepare a standard curve with the obtained transcription data. To perform qPCR analyses, 2 μ L cDNA, 2.2 μ L nuclease free water, 5 μ L SYBR Green Mix (Applied Biosystems, Darmstadt, Germany) and 0.4 μ L forward and reverse Primer, respectively (Eurofins, Hamburg, Germany) were added in a white nuclease free 96 well plate (Sarstedt). qPCR was performed on a Thermocycler (QuantStudio3 Real-Time PCR System; ThermoFisher Scientific, Darmstadt, Germany). The PCR setup consisted of 40 cycles, each cycle underwent the following steps: 95 °C

for 10 min, 60 °C for 60 s. After 40 cycles, a dissociation curve was recorded by using the following regimen: 95 °C for 15 s, 60 °C for 60 s and heating to 95 °C at 0.15 °C s^{–1}.

Disease targets and acquitting intersection targets

The analysis was conducted on the plant composition, with the compounds employed to suggest potential gene targets. The target data of these compounds was obtained from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) and The Comparative Toxicogenomics Database CTD (<https://ctdbase.org/>). Subsequently, all the data files were consolidated and refined to remove redundancy, as necessary for the subsequent screening. Transcription factors associated with the *NFE2L2* and *HMOX1* gene were identified using QIAGEN Ingenuity Pathway Analysis (IPA) and prioritized based on a significance threshold of p -value ≤ 0.05 . Among the transcription factors selected were transporter, transmembrane receptor, translation regulator, phosphatase, peptidase, ligand-dependent nuclear receptor, ion channel, growth factor, G-protein coupled receptor, cytokine, enzymes, kinases, and complexes. Finally, the genes and compounds targets were obtained from the online tool Venny 2.1.0 and used to make a venn diagram, which helped in finding common genes. Subsequent examination of these common genes was undertaken to facilitate further downstream analysis [22, 23].

Protein–protein interaction network construction

The protein–protein interactions (both direct and indirect) of target proteins were predicted using the STRING database (<https://string-db.org/>). In this case, the focus was on the human species ‘‘*Homo sapiens*’’ with a confidence score of 0.7. The subsequent generation and storage of the resulting interaction diagram network of the target was facilitated by the generation and storage of the diagrams in TSV format. Subsequently, the data was exported to the Cytoscape 3.7.2 software for its visualization. Subsequently, the STRING plugin was implemented to identify the *NFE2L2* and *HMOX1* first node [24].

Functional annotation of common genes

Common targets were incorporated into DAVID, v6.8 (<https://david.ncifcrf.gov/home.jsp>), for functional annotation enrichment analysis, including biological process (BP), cellular component (CC), molecular function (MF), and KEGG pathway enrichment analysis [23]. In addition, the KEGG pathway database revealed the presence of potential regulated pathways. To identify statistically relevant KEGG pathways and GO keywords, a threshold of p value = 0.05

was established [24]. The graphs of KEGG Pathway and GO were plotted based on the counts of the top pathways and top GO terms of 20 and 10, respectively [25]. All graphical analyses, including KEGG pathway and GO enrichment visualizations, were performed using R v4.3.3 software.

Molecular docking

Molecular docking simulations were performed to explore the interactions of major phytochemicals from *A. oxycedri* extracts with selected protein targets. The 3D crystal structures of all proteins were retrieved from the Protein Data Bank (PDB) using their respective PDB IDs (Supplementary Table S7). The selected targets included key enzymes such as AChE [26], BChE [27], and amylase [28], glucosidase [25], tyrosinase [22] as well as *NFE2L2* and *HMOX1* related protein *TNF- α* , *SIRT1*, *PPAR γ* [29], *Akt1*, *Akt2*, *MAPK8* [25], *c-Myc*, *c-Fos* [30], *AP-1*, *IL6*, *eNOS*, *TLR2*, *TLR4*, and *BRCA1*. Ligand structures were obtained from the PubChem database and optimized using Avogadro v1.2.0 [31]. Protein and ligand preparations were performed using AutoDock v4.2.6 [32]. Docking grid coordinates and box dimensions were defined based on literature data or predicted using POCASA v1.1 [33, 34]. Detailed grid information is provided in Table S7. Docking simulations were carried out using AutoDock Vina v1.1.2 with exhaustiveness set to 32. Validation of the docking protocol was performed by re-docking the co-crystallized ligands, and Root Mean Square Deviation (RMSD) values were calculated [35]. Protein-ligand interaction profiles were analyzed using the Protein-Ligand Interaction Profiler (PLIP) [36, 37], and catalytic residues were identified using HOTSPOT WIZARD v3.1 (Table S8).

Molecular dynamics simulation and MM/PBSA free energy calculation

Molecular dynamics (MD) simulations were performed using GROMACS 2023.1. The initial system setup was conducted using CHARMM-GUI Solution Builder (<http://charmm-gui.org>) following standard protocols. The CHARMM36m force field was applied for protein parameterization [30]. Protein-ligand complexes were solvated in a TIP3P water model within a periodic boundary box, maintaining a 10 Å buffer distance. Counterions were added to neutralize the system and to achieve a physiological salt concentration of 0.15 M NaCl [36]. Energy minimization was performed using the steepest descent algorithm, followed by equilibration under NVT and NPT conditions at 310 K [38]. Production MD simulations were conducted for 100 ns. The LINCS algorithm was used to constrain bond lengths, and long-range electrostatic interactions were

computed using the particle mesh Ewald (PME) method [39]. Binding free energy calculations were performed using the gmx_MMPBSA tool to estimate the ligand-binding affinity and assess the stability of the protein-ligand complexes. MM/PBSA calculations were applied to the following complexes based on their docking scores and interaction profiles: AKT1_prodelphinidin B1, eNOS_prodelphinidin B1, PPAR γ _3-O-feruloylquinic acid, PPAR γ _prodelphinidin B1, PPAR γ _prunin, SIRT1_3-O-p-coumaroylquinic acid, PPAR γ _3-O-caffeoylquinic acid, and TLR2_prunin [40, 41].

Statistical analysis

The mean was calculated using three independent biological replicates. Statistical analysis was performed using Graphpad Prism 10 (Graphpad Software Inc, Boston, USA). Statistically significant differences were determined by performing ANOVA using the Tukey's test. Significance thresholds were set as follows: $p \leq 0.05 = *$, $p \leq 0.01 = **$, $p \leq 0.001 = ***$.

Result and discussion

Total phenolic (TPC) and flavonoid content (TFC)

The presence of polyphenols in plant extracts plays a vital role in determining their pharmacological ability for specialized metabolic products. TPC is an essential indicator of total antioxidant capacity and is widely used in evaluating plant antioxidant properties [42, 43]. The diversity of phenolics and antioxidants in plants, which exhibit different structures, molecular sizes, and polarity, makes the type of extraction solvents a determinant factor in analyses [44].

According to the results, the ethanol extract exhibited the highest TPC (109.38 mg GAE/g) and highest TFC (24.09 mg RE/g), which indicated that this extract is rich in phenolic compounds responsible for antioxidant activity. The ethanol/water (70%) extract showed a comparable TPC with 108.58 mg GAE/g but a much lower TFC of 11.53 mg RE/g, indicating a preferential extraction of non-flavonoid phenolics. On the other hand, the water extract exhibited a relatively high TPC (94.40 mg GAE/g) but the lowest TFC (7.90 mg RE/g). *n*-Hexane and ethyl acetate extracts had lower values of TPC and TFC, reflecting their poor ability to solubilize polyphenols and flavonoids (Table 1).

Chemical composition

Chemical characterization of the different extracts of *A. oxycedri* was analyzed by the latest HPLC-MS, and the list of

Table 1 Extraction yields (%), total phenolic (TPC) and flavonoid contents (TFC) in the tested extracts*

Extracts	Extraction yields (%)	TPC (mg GAE/g)	TFC (mg RE/g)
<i>n</i> -Hexane	1.03	18.97±0.09 ^d	17.88±0.42 ^b
Ethyl acetate	1.52	26.10±0.59 ^c	11.75±0.13 ^c
Ethanol	5.38	109.38±0.89 ^a	24.09±0.14 ^a
Ethanol/water (70%)	22.58	108.58±0.66 ^a	11.53±0.01 ^c
Water (infused)	26.59	94.40±1.01 ^b	7.90±0.03 ^d

GAE gallic acid equivalents, RE rutin equivalents

Different letters indicate significant differences between the extracts ($p < 0.05$)

*Values are reported as mean±SD of three parallel measurements

all identified compounds is presented in Table 2. The chemical profiles of the extracts showed a wide range of bioactive compounds, which have been grouped into phenolic acids, flavonoids, proanthocyanidins, hydroxycinnamic acids, organic acids, and fatty acids, these varied significantly with the different extraction methods and thus impacted the biological activities of the extracts, such as antioxidant potential and enzyme inhibition (Table S2–S6).

It emphasized phenolic acids like gallic acid, protocatechuic acid, and chlorogenic acid in the ethanol and ethanol/water (70%) extracts. Gallic acid, a widely recognized compound showing strong antioxidant and tyrosinase-inhibitory activities [45, 46], was found to be present in ethanol, ethanol/water, and water but absent in the *n*-hexane and ethyl acetate extracts. Both ethanol and ethanol/water (70%) extracts demonstrated a high amount of protocatechuic acid with a proven ability to contribute to free radical scavenging and reducing power [47, 48], while its contribution was rather low in extracts prepared with *n*-hexane, ethyl acetate, and water. Chlorogenic acid, identified as a known compound with noteworthy antioxidant and anti-diabetic activities [49, 50], was included in ethanol, ethanol/water (70%), and ethyl acetate extracts but lacked the presence of *n*-hexane and water extracts. The absence of these key phenolic acids in *n*-hexane and water extracts underlines their poor solubilization of polar compounds, impacting negatively on their overall biological performance.

Flavonoids, such as rutin, catechin, and epigallocatechin, were highly represented in ethanol, ethanol/water, and ethyl acetate extracts and greatly contributed to the antioxidant and enzyme-inhibitory activities of these extracts. Rutin, a potent metal chelator and free-radical scavenger [51, 52], was not detected in hexane and water extracts, which diminished the antioxidant activity of these extracts. Catechin and epigallocatechin, though important for DPPH and ABTS radical scavenging activities [53], were not detected in *n*-hexane and water extracts. The non-detection of these two compounds in both the non-polar and aqueous extracts

is directly linked with the poor performance of the latter in antioxidant assays.

Ethanol and ethanol/water (70%) extracts were highly enriched with proanthocyanidins, such as prodelphinidins and procyanidins. The compounds are of utmost importance when it comes to antioxidant mechanisms like reducing power and copper ion reduction [54, 55]. The absence of these compounds in extracts of *n*-hexane and water explains the poor antioxidant capacity in these extracts. Ethyl acetate extract revealed a content of these compounds in moderate amounts but without the diversity observed for ethanol-based extracts, which underlines the selective efficiency of ethanol extraction.

Hydroxycinnamic acids like caffeic acid and its ester 3-*O*-feruloylquinic acid were present in ethanol, ethanol/water, and ethyl acetate extracts. These are important compounds with their dual antioxidant and enzyme-inhibitory properties [56], especially as tyrosinase inhibitors [57]. Absence of caffeic acid in *n*-hexane and water extracts depicts a diminished capacity against oxidative stress and enzymatic activity for these extracts.

Quinic acid and citric acid were the organic acids present in all extracts, except water and *n*-hexane, respectively. Quinic acid is a precursor for many metabolic pathways [58] and was determined in ethanol, ethanol/water (70%), and ethyl acetate extracts. It was detected in ethyl acetate, ethanol, and water extracts but was not present in *n*-hexane. Such acids are expected to contribute to bioactivity because they improve solubility and stability.

Fatty acids, including α -linolenic acid, linoleic acid, and long-chain saturated fatty acids represented by cerotic acid and montanic acid, were present exclusively in *n*-hexane extracts. These non-polar phytochemicals are well known for their anti-inflammatory and lipid-modulating activities [59] but are not major contributors to antioxidant and enzyme-inhibitory activities, which are highly contributed by polar phenolic compounds [60, 61]. The high content of fatty acids explained the observed selective bioactivity of the *n*-hexane extract.

The bioactivity of these extracts is sometimes limited by compounds not present within the specific extracts. For example, hexane extract, due to the absence of flavonoids and phenolic acids such as rutin, chlorogenic acid, and catechin, thus accounts for low antioxidant activity; water extract lacks proanthocyanidin and hydroxycinnamic acids and expressed moderate-grade antioxidant activity and an equally moderate enzyme inhibition activity. Ethyl acetate extract, though containing a moderate diversity of phenolics, lacks the richness of ethanol-based extracts, thus limiting its efficacy in certain bioactivities.

Table 2 Chemical characterization of the tested extracts

Compounds	<i>n</i> -Hexane	Ethyl acetate	EtoH	EtoH /water	Water
Quinic acid	+	+	+	+	–
Shikimic acid	–	+	+	–	–
Citric acid	–	+	+	–	+
Gallic acid (3,4,5-trihydroxybenzoic acid)	–	–	+	+	+
Prodelphinidin B isomer 1	–	–	+	+	+
Protocatechuic acid (3,4-dihydroxybenzoic acid)	–	–	+	+	–
Gallocatechin	–	–	+	+	–
Protocatechuic acid- <i>O</i> -glucoside	–	–	+	+	+
Prodelphinidin C isomer 1	–	–	+	+	+
Prodelphinidin C isomer 2	–	–	+	+	+
Prodelphinidin B isomer 2	–	–	+	+	+
Procyanidin B <i>O</i> -hexoside	–	–	+	+	+
2-hydroxy-3-(2-hydroxyphenyl)propanoic acid	–	–	+	+	+
Neochlorogenic acid (5- <i>O</i> -caffeoylquinic acid)	–	+	+	+	–
Prodelphinidin C isomer 3	–	–	+	+	+
Procyanidin B isomer 1	–	+	+	+	+
Procyanidin B isomer 2	–	–	+	+	+
Prodelphinidin B isomer 3	–	–	+	–	–
3- <i>O</i> -(<i>p</i> -coumaroyl)quinic acid cis isomer	–	–	+	–	+
3- <i>O</i> -(<i>p</i> -coumaroyl)quinic acid	–	–	+	–	+
Catechin	–	+	+	+	+
Epigallocatechin	–	–	+	+	+
6- <i>O</i> -methyлаucubin or isomer	–	+	+	+	+
Procyanidin B isomer 3	–	–	+	+	+
Procyanidin B isomer 4	–	–	+	+	+
Prodelphinidin C isomer 4	–	–	+	+	–
Chlorogenic acid (3- <i>O</i> -caffeoylquinic acid)	–	+	+	+	–
3- <i>O</i> -feruloylquinic acid	–	–	+	+	+
Caffeic acid	–	+	+	+	–
Ampelopsin (dihydromyricetin)	–	+	+	+	+
Kynurenic acid	–	–	+	+	–
Dihydrokaempferol- <i>O</i> -hexoside	–	+	+	+	+
Epicatechin	–	–	+	+	+
5- <i>O</i> -(<i>p</i> -coumaroyl)quinic acid	–	–	+	+	+
<i>p</i> -Coumaric acid	–	+	+	+	–
5- <i>O</i> -(<i>p</i> -coumaroyl)quinic acid cis isomer	–	–	+	+	+
Cinchonain I isomer 1	–	+	+	+	+
Myricetin-3- <i>O</i> -glucoside (Isomyricitrin)	–	+	+	+	+
Ethyl chlorogenate	–	–	+	–	–
Cinchonain I isomer 2	–	+	+	+	+
Matairesinol	–	+	+	+	–
Prunin (Naringenin-7- <i>O</i> -glucoside)	–	+	+	+	+
Myricetin- <i>O</i> -pentoside	–	+	+	+	+
Dihydrokaempferol (3,4',5,7-tetrahydroxyflavanone)	–	+	+	+	+
Unidentified myricetin- <i>O</i> -hexoside derivative	–	–	+	+	+
Quercetin- <i>O</i> -pentosylhexoside	–	+	+	+	+
Naringenin- <i>O</i> -hexoside	–	+	+	+	+
Isoquercitrin (quercetin-3- <i>O</i> -glucoside)	–	+	+	+	+
Methoxy-pentahydroxy(iso)flavone- <i>O</i> -pentosylhexoside	–	+	+	+	+
Rutin (quercetin-3- <i>O</i> -rutinoside)	–	+	+	+	+
Methoxy-pentahydroxy(iso)flavone- <i>O</i> -hexoside	–	+	+	+	+
Azelaic acid (Nonanedioic acid)	+	+	–	–	–
Myricetin (3,3',4',5,5',7-hexahydroxyflavone)	–	+	+	+	–
Eriodictyol (3',4',5,7-tetrahydroxyflavanone)	–	+	+	+	+
Dimethoxy-tetrahydroxy(iso)flavone- <i>O</i> -hexoside	–	+	+	+	+

Table 2 (continued)

Compounds	<i>n</i> -Hexane	Ethyl acetate	EtoH	EtoH /water	Water
Naringenin (4',5,7-trihydroxyflavanone)	–	+	+	+	+
Quercetin (3,3',4',5,7-pentahydroxyflavone)	–	+	+	+	–
Methoxy-pentahydroxy(iso)flavone	–	+	+	+	–
Dimethoxy-tetrahydroxy(iso)flavone	–	+	+	+	–
Bisapigenin isomer 1	–	+	+	+	–
Bisapigenin isomer 2	–	+	+	+	–
Phytosphingosine	–	–	+	+	–
13-Hydroxyoctadecatrienoic acid	+	+	–	–	–
Hexadecanedioic acid	+	+	–	–	–
9-hydroxyoctadecadienoic acid	+	+	–	–	–
Juniperic acid (16-hydroxyhexadecanoic acid)	+	+	–	–	–
α -linolenic acid	+	+	–	–	–
Linoleic acid	+	+	–	–	–
Oleic acid	+	+	–	–	–
Gondoic acid or isomer	+	–	–	–	–
Behenic acid (docosanoic acid)	+	–	–	–	–
Lignoceric acid (tetracosanoic acid)	+	–	–	–	–
Hyenic acid (pentacosanoic acid)	+	–	–	–	–
Cerotic acid (hexacosanoic acid)	+	–	–	–	–
Carboceric acid (Heptacosanoic acid)	+	–	–	–	–
Montanic acid (Octacosanoic acid)	+	–	–	–	–

+ present, – absent

Table 3 Antioxidant properties of the tested extracts*

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)
Hexan	19.98±0.38 ^c	36.12±1.19 ^c	52.27±2.57 ^c	27.50±0.56 ^c	9.33±0.75 ^c	1.50±0.13 ^b
Ethyl acetate	26.78±0.16 ^d	55.78±0.42 ^d	83.16±7.18 ^d	40.61±2.37 ^d	21.72±0.91 ^b	1.84±0.11 ^b
Ethanol	315.12±3.01 ^b	599.91±6.35 ^b	464.33±12.76 ^b	344.78±13.80 ^b	11.83±1.33 ^d	2.53±0.23 ^a
Ethanol/water (70%)	345.02±3.88 ^a	654.61±5.90 ^a	507.53±16.29 ^a	388.09±15.73 ^a	15.26±0.48 ^c	2.49±0.11 ^a
Water (infused)	233.90±1.16 ^c	422.15±6.54 ^c	388.47±10.64 ^c	293.47±0.83 ^c	29.07±0.07 ^a	1.64±0.05 ^b

PBD phosphomolybdenum, MCA metal chelating activity, TE trolox equivalent, EDTAE EDTA equivalent

Different letters indicate significant differences between the extracts ($p < 0.05$)

*Values are reported as mean±SD of three parallel measurements

Antioxidant activities

To maintain a healthy life, it is crucial to maintain a balance between oxidants and free radicals. Free radicals cause disturbance by destroying essential biomolecules. Maintaining a balance between oxidants and free radicals is essential for optimal health [62, 63].

Excess free radicals must be neutralized, and antioxidants must be ingested to maintain redox equilibrium in the body.

To evaluate the antioxidant potential of *A. oxycedri*, the plant extracts were submitted to different in vitro assays, including radical scavenging, reducing power, chelating power, and total antioxidant capacity for their antioxidant activities. Significant differences observed among the analyzed extracts (Table 3) are primarily related to chemical composition and mode of extraction.

Different assays have been carried out for in vitro antioxidant activity so that quick screening of the compounds can be done because, generally, such compounds that have poor in vitro antioxidant activity will not be able to show significant action in vivo [64]. Free radicals play a significant role in most of the clinical conditions. Antioxidants scavenge free radicals and thereby protect us from several diseases. They act either by scavenging the reactive oxygen species or by protecting antioxidant defence systems [65].

The free radical-scavenging capacity of the extracts was assessed using DPPH and ABTS assays [66]. Indeed, the 70% ethanol/water solution of ethanol/water exhibited the highest radical-scavenging activities in both DPPH and ABTS assays with values of 345.02 mg TE/g and 654.61 mg TE/g, respectively, due to high contents of phenolic acids like gallic and chlorogenic acids and flavonoids such as

rutin and catechin. The ethanol extract showed values of 315.12 mg TE/g and 599.91 mg TE/g for DPPH and ABTS, respectively. The moderate activities of the ethyl acetate extract could be explained by their relatively lower contents of phenolics and flavonoids compared with ethanol-based extracts; hence, these extracts provided 26.78 mg TE/g for DPPH and 55.78 mg TE/g for ABTS. The aqueous and hexane extracts were the least active radical scavengers due to their insufficient phenolic content; hexane showed the lowest DPPH value of 19.98 mg TE/g.

The FRAP and CUPRAC measurements showed the reductive capability of the extracts that behaved similarly. Thus, 70% ethanol/water extract had the maximum reductive capacity among all prepared extracts with a FRAP and CUPRAC value of 388.09 mg TE/g and 507.53 mg TE/g, respectively. The corresponding values of FRAP and CUPRAC of ethanol extract that were 344.78 mg TE/g and 464.33 mg TE/g, respectively, were equal to that from ethanol/water extract. These results arise from the powerful reductants in those extracts, such as gallic acid, protocatechuic acid, and prodelpinidins. The ethyl acetate extract exhibited moderate activity: FRAP, 40.61 mg TE/g; CUPRAC, 83.16 mg TE/g, while hexane and water extracts showed poor reducing power. The CUPRAC value of hexane was only higher than that determined by FRAP, 52.27 and 27.50 mg TE/g, respectively, indicating the possibility of lipophilic reductants at lower levels.

The water extract exhibited the highest metal chelation activity, a measure of the ability of the extracts to form complexes with and sequester transition metal ions, with a chelating value of 29.07 mg EDTAE/g. Among them, the ethanol/water (70%) and ethanol extracts exhibited chelating activity of 15.26 and 11.83 mg EDTAE/g, respectively, whereas the lowest was from the hexane extract with 9.33 mg EDTAE/g. The higher chelating capacity of the aqueous extract is attributed to hydrophilic molecules, including organic acids such as citric acid, which are known for exhibiting strong metal-complexing properties.

The phosphomolybdenum assay determined the overall antioxidant activity, and the results showed that the extracts prepared with ethanol and 70% ethanol/water acted more efficiently. The maximum value of PBD was for ethanol extract with 2.53 mmol TE/g, followed by ethanol/water extract with 2.49 mmol TE/g. While the total antioxidant capacity of the ethyl acetate extract was moderate, 1.84 mmol TE/g, that of the hexane and water extracts were lower, 1.50 and 1.64 mmol TE/g, respectively. High PBD values of the ethanol-based extracts indicated their rich chemical composition of reductive and electron-donating chemicals such as proanthocyanidins and flavonoids.

In the previous investigation [67] the antioxidant activities of *A. oxycedri* extracts were tested using in vitro assays

such as DPPH, metal-chelating, and FRAP assays; it was disclosed that the ethyl acetate extract had the highest radical quenching activity against DPPH at $89.83 \pm 0.98\%$, followed by the butanol extract with $79.87 \pm 0.89\%$.

Enzyme inhibitory effects

The enzyme inhibitory potential of the different extracts of *A. oxycedri* was tested against important enzymes related to diabetes and neurodegenerative problems, including α -amylase, α -glucosidase, acetylcholinesterase, and butyrylcholinesterase. Furthermore, we also evaluated tyrosinase inhibition, as it is a significant enzyme for the management of melanin production and hyperpigmentation [68, 69].

AChE and BChE are essential enzymes in neurotransmission [70]. Their hyperactivities have been implicated in neurodegenerative diseases such as Alzheimer's disease [71, 72]. Inhibition prevents the hydrolysis of acetylcholine and hence enhances cognitive function [70, 73]. Our results indicated the ethanolic extract of *A. oxycedri* resulted in quite strong inhibition for BChE, with the best value at 4.72 mg GALAE/g. Correspondingly highly inhibiting effects are observed towards AChE-2.37 mg GALAE/g of inhibition. Ethanol/water extract showed 4.41 and 2.28 mg GALAE/g in the case of inhibition in BChE and AChE activity. The finding accordance with the plentiful occurrence of phenolics and, especially, gallic acid, protocatechuic acid, rutin, and catechin-known as typical neuroprotectors within those extracts [74, 75]. The ethyl acetate extract exhibited moderate inhibition of 3.96 mg GALAE/g for BChE and 2.16 mg GALAE/g for AChE, probably because of its selective extraction of flavonoids and phenolic acids. The *n*-hexane extract, although containing a small amount of phenolics, showed AChE inhibition (2.37 mg GALAE/g) comparable to that of the ethanol extract, probably due to lipophilic substances such as fatty acids [76]. However, it exerted weaker inhibition against BChE with an activity of 3.04 mg GALAE/g, which indicates relatively lower bioactivity of this extract. The hydrophilic extract indeed exhibited the poorest inhibition: 1.45 mg GALAE/g for AChE and 3.20 mg GALAE/g for BChE, because of the limited diversity of flavonoids and the absence of key proanthocyanidins.

In a previous study, the extracts from *A. oxycedri* demonstrated high inhibitions on BChE; the highest was the ethyl acetate extract, $94.74 \pm 2.43\%$, which is comparable to galantamine. AChE inhibitions were only observed in the ethyl acetate extract, $45.37 \pm 1.23\%$. The standard compound catechin did not show any inhibitory activity [67]. Our analysis indicated that the ethanol extract showed 4.72 mg GALAE/g for remarkable in vitro BChE inhibition activity; for AChE, this was 2.37 mg GALAE/g. A similar performance was obtained for the ethanol/water extract.

These results confirm the earlier studies related to the high cholinesterase inhibitory activities of extracts from *A. oxycedri* and showed our evidence in compliance with the support of bioactivity reported previously.

For several years, plant extracts have received much attention in modern medicine and cosmetic formulation. Many researchers supported the usage of natural medicinal products from plants [77]. Phytochemicals have been evaluated as agents of cosmetics and pharmaceuticals that decrease excessive melanin production and whiten the skin. Skin whitening can be caused by numerous factors. The action of antioxidants was believed to reduce melanin production [78]. Tyrosinase also has been implicated in the neurotoxicity and neurodegeneration of dopamine-containing neurons associated with Parkinson's disease [79]. The extracts were analyzed for their tyrosinase activity, and the results are presented in Table 4. Among the tested extracts, the ethanol extract provided the maximum inhibition with a value of 80.40 mg/KAE/g, followed by ethanol/water (70%). While the water extract displayed minimal activity, Ethyl acetate and hexane displayed moderate activity in inhibiting this critical enzyme, indicating their low total phenolic and flavonoid contents (Table 4).

Diabetes Mellitus is a chronic disorder characterized by a disturbance in the normal metabolic processes of carbohydrates, lipids, and proteins, inducing various biochemical changes throughout the body, eventually leading to characteristic hyperglycemia, a hallmark of the disease [80]. Alpha-amylase is among the most vital enzymes in the process of digestion and plays an essential role in the hydrolysis of polysaccharides; it exists in large amounts in saliva and pancreatic juice [81]. The inhibition of the enzyme is therefore considered a useful strategy for decreasing abnormally high postprandial blood glucose levels [82]. The current findings indicated that the ethyl acetate extract possesses the maximum inhibition with the highest activity, 1.06 mmol ACAE/g, followed by the hexane extract, which has an inhibition activity equal to 0.92 mmol ACAE/g. The ethanol and ethanol/water at 70% exhibited a moderate range of inhibition. These were followed by ethanol and ethanol/water at 70%, which exhibited 0.75 and 0.64 ACAE/g inhibition,

respectively. Contrarily, the water extract provides minimum inhibition with an average of 0.48 ACAE/g among all the tested extracts.

The α -glucosidase enzyme is among the most important enzymes lining the mucosal brush border of the small intestine. It is responsible for the digestion and breakdown of complex carbohydrates into simpler, smaller, and absorbable forms. Inhibition of α -glucosidase effectively delays glucose absorption and prevents elevation of postprandial blood sugar levels, which could prevent the development of diabetes [83, 84]. For the inhibitory activity of α -glucosidase, ethanol extract displayed the highest activity at 2.19 mmol ACAE/g. In comparison, hexane and ethyl acetate provided a modest range of activities, with inhibition of 1.96 and 1.9 mmol ACAE/g, respectively. In contrast, water extract did not show any inhibition (Table 4).

Cytotoxicity of extracts

Prior to the antioxidant cell culture studies, all extracts were tested for cytotoxicity. Even if there was a dose-dependent decrease in cell viability by $50 \mu\text{g mL}^{-1}$ and $75 \mu\text{g mL}^{-1}$ for *n*-hexane and ethyl acetate extracts, the results were non-significant compared to the medium control (Fig. 1). Concentrations of $100 \mu\text{g mL}^{-1}$ led to cell viability ranging from 78 to 100% with no significant reduction.

Effects on the expression of HO-1 (heme oxygenase-1), NRF2 (nuclear factor erythroid 2-related factor 2), and γ -GCS (gamma-glutamylcysteine synthetase) in HepG2 cells

The treatment of HepG2 cells with various extracts of *A. oxycedri* resulted in differential expression of key genes responsible for antioxidant function, such as HO-1, NRF2, and γ -GCS. Figure 2a shows the expression of HO-1 was significantly reduced by the solvent control methanol compared to medium control. However, the extracts generated by using ethanol and infusion further significantly reduced the HO-1 mRNA level. This critical enzyme enhances the antioxidant potential of the cell by breaking down heme to

Table 4 Enzyme inhibitory properties of the tested extracts*

Extracts	AChE ^a (mg GALAE/g)	BChE (mg GALAE/g)	Tyrosinase (mg KAE/g)	Amylase (mmol ACAE/g)	Glucosidase (mmol ACAE/g)
Hexan	2.37±0.03 ^a	3.04±0.99 ^b	53.06±0.58 ^c	0.92±0.01 ^b	1.96±0.04 ^b
Ethyl acetate	2.16±0.24 ^a	3.96±0.11 ^{ab}	58.66±1.10 ^b	1.06±0.03 ^a	1.92±0.06 ^b
Ethanol	2.37±0.08 ^a	4.72±0.05 ^a	82.40±0.24 ^a	0.75±0.03 ^c	2.19±0.01 ^a
Ethanol/water (70%)	2.28±0.03 ^a	4.41±0.06 ^a	80.23±0.42 ^a	0.64±0.01 ^d	na
Water (infused)	1.45±0.03 ^b	3.20±0.12 ^b	24.50±1.63 ^d	0.48±0.01 ^e	na

GALAE galantamine equivalent, KAE kojic acid equivalent, ACAE acarbose equivalent, na not active

Different letters indicate significant differences between the extracts ($p < 0.05$)

*Values are reported as mean±SD of three parallel measurements

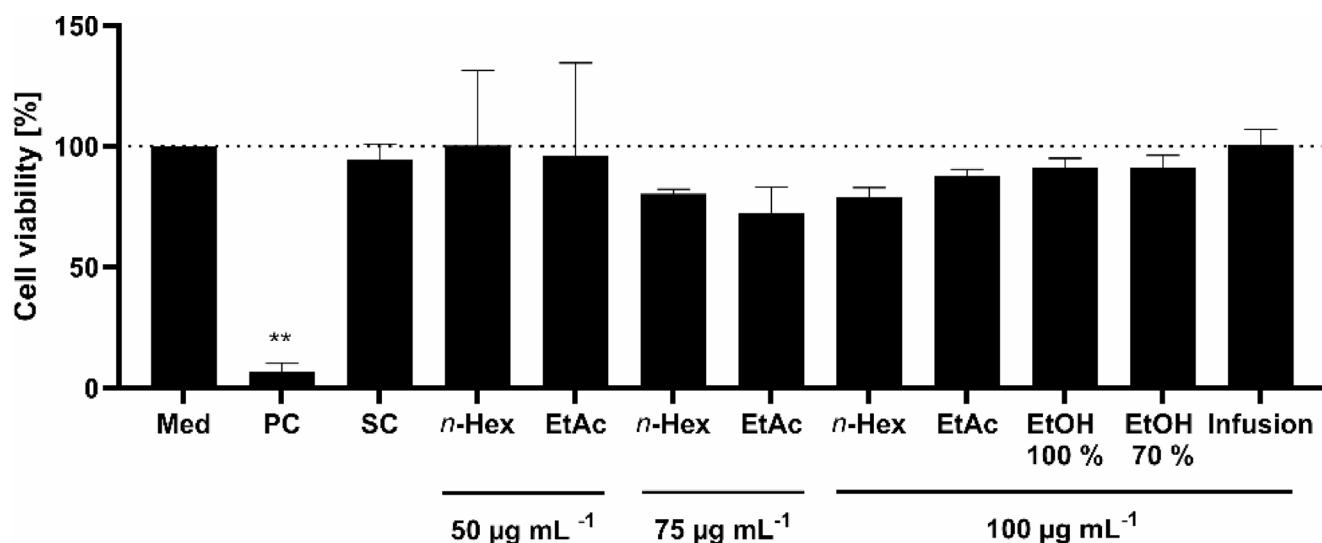


Fig. 1 Non-significant cytotoxicity after 24 h of treatment with different extracts of *Arceuthobium oxycedri*. All extracts were solved in methanol, which was used as solvent control (SC). Triton X with a

concentration of 1% was used as positive control (PC) and medium (Med) as negative control. *n-Hex* n-hexane, *EtAc* ethyl acetate, *EtOH* ethanol (** $p < 0.01$ compared to Med)

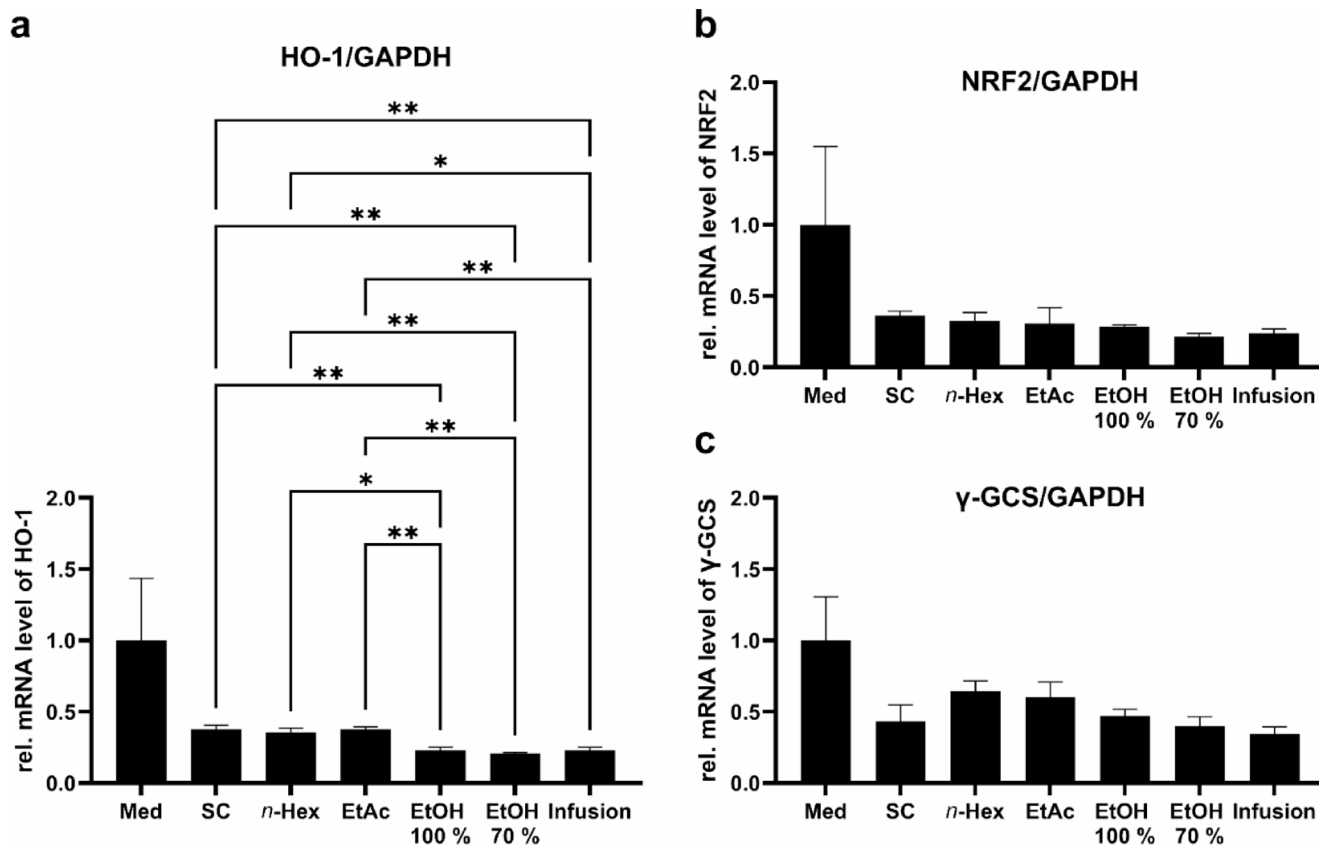


Fig. 2 HO-1, NRF-2 and γ -GCS were expressed after 6 h of treatment with the different extracts of *Arceuthobium oxycedri* in relation to GAPDH. **a** HO-1 expression was reduced in cells treated with water, 100% ethanol and 70% ethanol extracts; **b** NRF2 expression was non-significantly affected by the extracts; **c** γ -GCS expression was slightly

increased with *n*-Hexane and ethyl acetate extract treatments. Methanol was used as solvent control (SC); *n-Hex* n-hexane, *EtAc* ethyl acetate, *EtOH* ethanol. Significant differences are indicated as (* $p < 0.05$; ** $p < 0.01$)

form biliverdin, carbon monoxide, and iron, which all contribute to reducing oxidative stress [85, 86]. The reduced levels of HO-1 could be an indicator for the high antioxidant potential of these extracts. While in each cell ROS is generated for instance by mitochondrial activity, the extracts may reduce the cellular ROS level and the constant expression of HO-1 is reduced likewise. Interestingly, the reduction of HO-1 by ethanol and infusion extracts correlated with the antioxidant potential demonstrated in the DPPH assay as well as the high TPC. The results of NRF2 expression were similar to HO-1. NRF2 expression was highest in untreated cells, while the solvent control and the extracts reduced the mRNA level after 6 h of treatment (Fig. 2b). NRF2 is a key transcription factor that controls antioxidative response genes like HO-1 and plays a crucial role in the upkeep of cellular redox homeostasis through its involvement in enhancing phase II detoxification and antioxidant enzyme activity [87, 88]. Slightly reduced NRF2 expression following ethanol and infusion extract treatment indicates that such extracts might more markedly reduce the NRF2 pathway and the antioxidant defense mechanism in cells due to less cellular ROS [89]. Lastly, Fig. 2c suggested that the *n*-hexane and ethyl acetate-extract treatment caused a light but non-significant upregulation of γ -GCS, the rate-limiting enzyme for glutathione synthesis. The significance of γ -GCS in glutathione biosynthesis, a potent cellular antioxidant, suggests that the extracts can induce glutathione biosynthesis and thus increase the protection of the cell against reactive oxygen species (ROS) [90, 91].

The relationship between total phenolic content (TPC) values, DPPH antioxidant activity, and gene expression levels provides insight into these findings. The extracts that showed high TPC values, including water (infusion) and 70% ethanol, contained more significant antioxidant activity, as measured by the DPPH assay. This enhanced antioxidant capacity is associated with reduced NRF2, HO-1, and γ -GCS expression levels. The relationship between these parameters indicates that high antioxidant activity, especially from phenolic compounds, would decrease intracellular ROS levels. As NRF2 is induced by reactive oxygen species, lower levels of ROS would, therefore, reduce the demand for NRF2 activation and the induction of its target genes, including HO-1 and γ -GCS [92, 93]. This accounts for reduced levels of these gene mRNAs in more efficacious antioxidant-treated cells since diminished HO-1 and γ -GCS expression versus cells exposed to other extracts also implies. The correlation of NRF2 expression with target genes reveals more insight into what its mechanism entails [92].

Since NRF2 activation is crucial for the transcription of antioxidative genes such as HO-1 and γ -GCS, low levels of NRF2 would consequently lower the transcription of these

genes [92, 94]. This corresponds with the hypothesis that the antioxidant potential of the extracts influences ROS levels, hence minimizing the requirement for NRF2-dependent gene activation. Literature has proved this concept, and it was established that phenolic compounds could modulate NRF2 activation and its impact [95], demonstrating the promise of these extracts to affect cell responses to oxidative stress. The results show that *A. oxycedri* extracts, water, 100% ethanol, and 70% ethanol have high antioxidative activities.

Disease targets and acquitting intersection targets

Pubchem and CTD were used to predict potential gene targets for each hub phytochemical which is gallic acid, prodelphinidin B1, galocatechin, 3-*O*-(*p*-coumaroyl)quinic acid, prodelphinidin C1, caffeic acid, prunin, 5-*p*-coumaroylquinic acid, epicatechin, epigallocatechin, catechin, ampelopsin, *p*-coumaric acid, 5-*O*-caffeoylquinic acid, 3-*O*-caffeoylquinic acid, 3-*O*-feruloylquinic acid. After combining all data and removing duplicates, 4,078 unique targets were identified. Subsequently, IPA software predicted 645 upregulated genes (p -value ≤ 0.05) (Fig. 3A). Duplicate genes were further filtered using Cytoscape, and gene mapping was performed via the STRING plugin. Comparison of these genes with plant-derived targets using Venny 2.1.0 revealed 228 common intersection targets (Fig. 3B).

Protein–protein interaction (PPI) network analysis

Protein–protein interaction (PPI) network analysis was conducted using the STRING and Cytoscape platforms based on the 228 identified target genes. The resulting network comprised 228 nodes and 1,918 edges, representing proteins and their interactions, respectively. Nodes with higher degrees were considered central to the network. In particular, NFE2L2 and HMOX1 emerged as key hub proteins, showing extensive interactions with multiple targets, including inflammatory mediators (*IL1B*, *IL6*, *TNF*), transcription factors (*JUN*, *FOS*, *MYC*), antioxidant-related proteins (*SOD1*, *TXN*, *GCLC*, *GCLM*), and key regulators such as *AKT1*, *SIRT1*, and *PPARG*. Additionally, a subset of 19 proteins were found to be commonly interacting with both *NFE2L2* and *HMOX1* (Fig. 3C).

GO enrichment analysis

GO enrichment analysis of the 228 target genes using STRING revealed a total of 430 GO terms, including 762 biological processes (BP), 183 molecular functions (MF), and 72 cellular components (CC), with a significance threshold of $p \leq 0.05$. The distribution of enriched GO terms

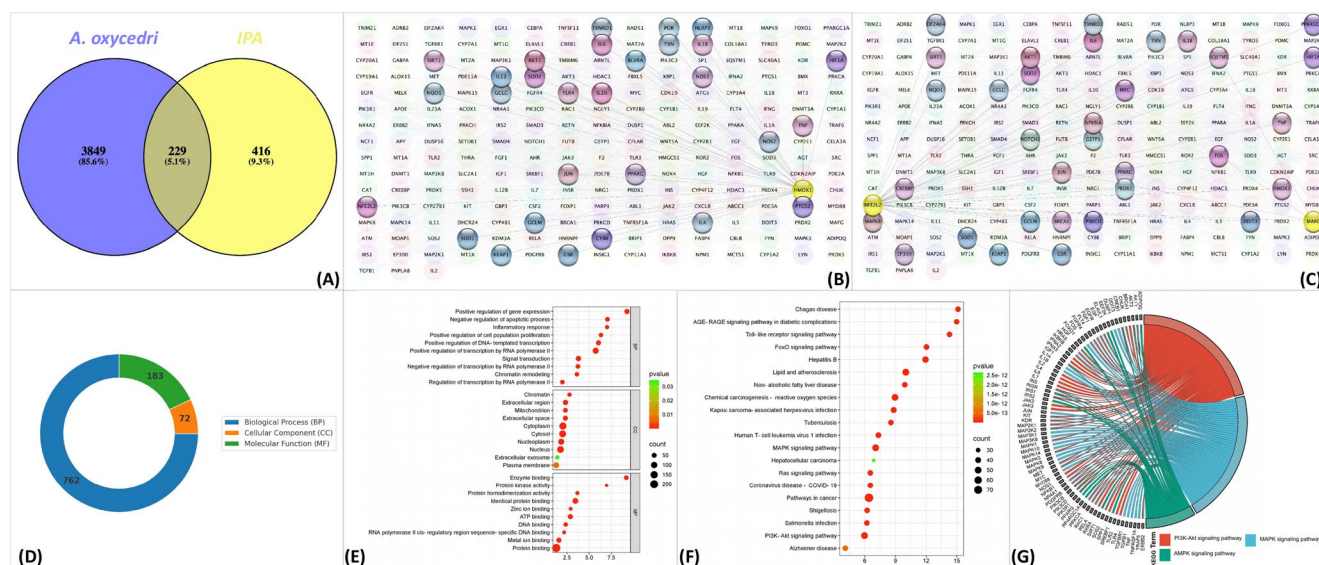


Fig. 3 Network pharmacology workflow chart. **A** Venn diagram showing overlapping genes related to *HMOX1*, *NFE2L2* and *A. oxycedri*. **B** *HMOX1* gene-based first node hub targets. **C** *NFE2L2* gene-based first node hub targets. **D** Pie chart representing all Gene Ontology

indicated that BP terms represented the largest proportion (74.9%), followed by MF (18.0%) and CC (7.1%) (Fig. 3D). Among the BP terms, key enriched processes were positive regulation of transcription by RNA polymerase II (84 genes), signal transduction (59 genes), positive regulation of gene expression (55 genes), and regulation of apoptosis (42 genes). These processes predominantly involved pivotal regulatory genes such as *MYC*, *BRCA1*, *CREB1*, *AKT1*, and *TNF*, which are associated with transcriptional control, intracellular signaling, and cell survival. In the CC category, highly enriched compartments included the cytosol (133 genes), cytoplasm (124 genes), nucleoplasm (120 genes), nucleus (82 genes), and plasma membrane (79 genes), reflecting the localization of the identified target proteins. MF analysis highlighted protein binding (210 genes) as the most abundant term, followed by identical protein binding (73 genes), metal ion binding (54 genes), ATP binding (53 genes), and enzyme binding (41 genes) [96–98]. These results emphasize the involvement of *A. oxycedri*-derived compounds in critical cellular processes related to transcriptional regulation, signaling pathways, apoptosis, and chromatin remodeling (Fig. 3E), providing potential molecular targets for therapeutic intervention in cancer.

KEGG pathway enrichment analysis

KEGG pathway enrichment analysis of the 229 selected target genes using DAVID identified 177 pathways. The top 20 enriched pathways were ranked based on gene counts (Fig. 3F). Among them, “Pathways in cancer” exhibited

(GO) terms. **E** Top 10 GO terms ranked by enrichment analysis. **F** Top 20 KEGG pathways ranked by gene enrichment analysis. **G** Chord diagram depicting the top 3 KEGG pathways and their corresponding gene targets

the highest gene enrichment (79 genes), followed by “Lipid and atherosclerosis” (50 genes), “PI3K-Akt signaling pathway” (50 genes), “MAPK signaling pathway” (49 genes), and “Chemical carcinogenesis - reactive oxygen species” (47 genes). Figure 3G visualizes the distribution of target genes within four major pathways: PI3K-Akt, MAPK, AMPK, and PPAR. These pathways are closely associated with cancer development, inflammation, metabolic regulation, and stress response mechanisms. The PI3K-Akt pathway showed enrichments of key regulators including *AKT1*, *AKT2*, *BRCA1*, *IGF1*, *IL6*, *MYC*, and *PIK3* genes, indicating their central role in cell survival and proliferation. Similarly, the MAPK pathway involved *MAPK8*, *JUN*, *FOS*, *AP-1*, and related genes critical for transducing mitogenic and stress signals. In the AMPK and PPAR pathways, genes such as *SIRT1*, *PPARγ*, *FOXO1*, and *AKT1* were prominently enriched, indicating their importance in energy metabolism and inflammation control. Furthermore, inflammatory mediators and immune response genes including *TNF-α*, *IL6*, *TLR2*, *TLR4*, and *eNOS* were frequently involved across these pathways (Fig. 3G). To further investigate the regulatory roles of *NFE2L2* and *HMOX1*, pathway mapping was performed using the Pathview tool in R. Figure 4A displays their involvement in the AMPK pathway (via *PGC-1α*, *SIRT1*, *PPARγ*, and *AKT*), Fig. 4B shows their role in the MAPK pathway (via *TNF-α*, *IL1*, *AKT*, *c-MYC*, *c-Fos*, and *AP-1*), and Fig. 4C highlights their association within the PI3K-Akt pathway (*TLR2/4*, *Cytokines*, *eNOS*, *AKT*, *BRCA1*, and *MYC*). These findings support the pivotal roles of *NFE2L2* and *HMOX1* in regulating multiple

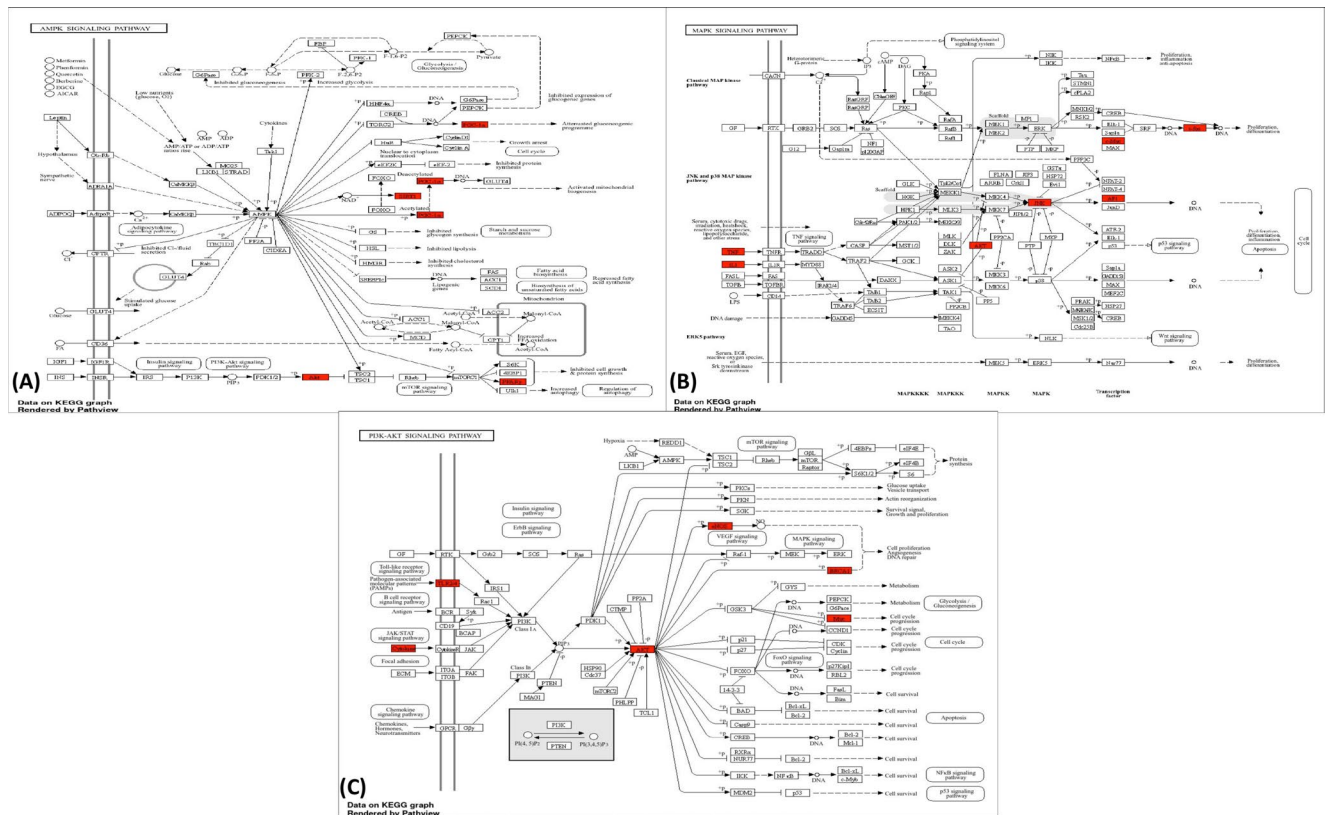


Fig. 4 Functional pathways and key protein interrelationships. **A** AMPK signaling pathway. **B** MAPK signaling pathway. **C** PI3K-Akt signaling pathway

signaling pathways related to cancer, oxidative stress, and inflammation.

Molecular docking results

This study employed an in silico evaluation to investigate both the standard metabolic enzymes and colon cancer-associated properties of compounds identified in *A. oxycedri*. The chemical profiling revealed a significant number of bioactive compounds, including gallic acid, prodelphinidin B1, gallicocatechin, 3-*O*-(*p*-coumaroyl)quinic acid, prodelphinidin C1, caffeic acid, prunin, 5-*p*-coumaroylquinic acid, epicatechin, epigallocatechin, catechin, ampelopsin, *p*-coumaric acid, 5-*O*-caffeoylquinic acid, 3-*O*-caffeoylquinic acid, 3-*O*-feruloylquinic acid.

These compounds were evaluated against standard metabolic enzymes (AChE, Amylase, BChE, Glucosidase, and TYR) and colon cancer-associated proteins (TNF- α , SIRT1, PPAR γ , Akt1, Akt2, MAPK8, c-MYC, c-Fos, AP-1, IL-6, eNOS, TLR2, TLR4, and BRCA1). Binding modes, free energies, RMSD values, and hydrogen bond interactions were assessed (Fig. 4), while Table 5 summarizes the compounds exhibiting binding energies equal to or lower than -9 kcal/mol. Binding energies ranged from -11.7 to

-3.9 kcal/mol, with RMSD values between 0.1 and 11.3 Å. HOTSPOT WIZARD analysis revealed that most compounds formed hydrogen bonds within catalytic or conserved residues, except for Glucosidase, eNOS-Ampelopsin, and SIRT1-3-*O*-Feruloylquinic acid (Table 5 and Table S8). Regarding standard metabolic enzymes, prodelphinidin C1 exhibited the strongest binding affinity towards BChE (-11.7 kcal/mol), while prodelphinidin B1 showed high binding against AChE (-10.5 kcal/mol) and amylase (-9.7 kcal/mol). Other compounds, such as catechin, epicatechin, epigallocatechin, gallicocatechin, ampelopsin, and prunin, also displayed favorable binding affinities, interacting predominantly with catalytic residues like *GLU202* and *TYR124* in AChE, *ASP70* and *TYR332* in BChE, and *GLN63* and *ARG195* in Amylase. However, in the case of glucosidase, although favorable binding affinities were recorded, the interactions were mainly localized outside the catalytic region, suggesting an alternative binding mode.

Among cancer-associated protein targets, AKT1 was identified as a key target, with prodelphinidin C1 (-10.2 kcal/mol) (Fig. 5D) and prodelphinidin B1 (-9.4 kcal/mol) forming multiple hydrogen bonds with essential residues. Similarly, prunin (-10.5 kcal/mol) showed superior binding affinity against eNOS, while favorable interactions were

Table 5 The Docking score (kcal/mol) and interacting residues of the enzyme and protein

Compound	Target	PDB ID	Bind- ing energy	RMSD	Interaction		Binding site	Catalytic site
					Type	Number		
Catechin	BChE	3DJY	−9.2	0.8	Hbond	8	ASP A:70;ASP A:70;TRP A:82;ASN A:83;GLU A:197;TYR A:332;TYR A:332;HIS A:438	ASP A:70; TRP A:82; GLU A:197; TYR A:332; HIS A:438
Epicatechin	BChE	3DJY	−9.2	0.45	Hbond	6	ASP A:70;ASP A:70;ASN A:83;GLU A:197;TYR A:332;TYR A:332	ASP A:70; GLU A:197; TYR A:332
Epigallocatechin	BChE	3DJY	−9.1	nd	Hbond	5	ASN A:68;ASP A:70;ASP A:70;GLU A:197;TYR A:332	ASP A:70; GLU A:197; TYR A:332
Gallocatechin	BChE	3DJY	−9.1	1.06	Hbond	5	ASP A:70;ASP A:70;ASN A:83;GLU A:197;TYR A:332	ASP A:70; GLU A:197; TYR A:332
Prunin	BChE	3DJY	−9.6	1.06	Hbond	5	ASN A:68;ASN A:68;ASP A:70;THR A:284;SER A:287	ASP A:70; SER A:287
Prodelphinidin C1	BChE	3DJY	−11.7	0.56	Hbond	14	ASN A:68;ASN A:68;ASP A:70;TRP A:82;TRP A:82;GLU A:276;GLY A:283;GLY A:283;SER A:287;TYR A:332;TYR A:332;TRP A:430;TYR A:440;TYR A:440	ASP A:70; TRP A:82; SER A:287; TYR A:332
Prodelphinidin B1	BChE	3DJY	−11.0	0.61	Hbond	6	THR A:120;TYR A:128;TYR A:128;TYR A:332;TYR A:332;HIS A:438	THR A:120; TYR A:128; TYR A:332; HIS A:438
Ampelopsin	BChE	3DJY	−9.4	1.06	Hbond	4	ASP A:70;ASP A:70;ASN A:83;TYR A:332	ASP A:70; TYR A:332
Prodelphinidin C1	Glucosidase	3W37	−9.1	0.61	Hbond	13	ASP A:232;ALA A:234;ASN A:237;SER A:474;SER A:474;SER A:497;SER A:497;SER A:505;SER A:505;LYS A:506;ASP A:568;ASP A:568	nd
Prodelphinidin B1	Glucosidase	3W37	−9.3	0.42	Hbond	6	ASP A:232;ALA A:234;ASP A:469;ASP A:469;ARG A:552;ASP A:568	nd
3-O-Feruloylquinic acid	AChE	2Y2V	−9.2	1.11	Hbond	6	GLY A:121, GLY A:122, TYR A:124, ALA A:204, ARG A:296, HIS A:447	GLY A:121; GLY A:122; TYR A:124; ALA A:204; HIS A:447
Catechin	AChE	2Y2V	−9.6	1	Hbond	4	TYR A:72, TYR A:72, ASP A:74, GLU A:202	GLU A:202
Epicatechin	AChE	2Y2V	−9.6	0.13	Hbond	4	GLN A:71, ASP A:74, TYR A:133, HIS A:447	GLN A:71; TYR A:133; HIS A:447
Epigallocatechin	AChE	2Y2V	−9.8	1.05	Hbond	6	TYR A:72, TYR A:72, ASP A:74, GLY A:120, TYR A:124, GLU A:202	GLY A:120; TYR A:124; GLU A:202
Gallocatechin	AChE	2Y2V	−9.5	0.3	Hbond	5	GLN A:71, TRP A:86, ASN A:87, TYR A:124, TYR A:133	GLN A:71; TRP A:86; TYR A:124; TYR A:133
3-O-caffeoylquinic acid	AChE	2Y2V	−9.3	0.82	Hbond	11	ASP A:74, GLY A:121, GLY A:122, ALA A:204, ALA A:204, SER A:293, PHE A:295, ARG A:296, ARG A:296, HIS A:447	GLY A:121; GLY A:122; ALA A:204; PHE A:295; HIS A:447
Prunin	AChE	2Y2V	−10.3	1.04	Hbond	4	ASP A:74, TRP A:86, PHE A:295, ARG A:296	TRP A:86; PHE A:295
5-O-caffeoylquinic acid	AChE	2Y2V	−9.4	1	Hbond	5	GLY A:121, GLY A:122, TYR A:124, PHE A:295, TYR A:337	GLY A:121; GLY A:122; TYR A:124; PHE A:295; TYR A:337

Table 5 (continued)

Compound	Target	PDB ID	Binding energy	RMSD	Interaction		Binding site	Catalytic site
					Type	Number		
5-p-coumaroylquinic acid	AChE	2Y2V	−9.5	1.01	Hbond	3	GLY A:120, PHE A:295, TYR A:341	GLY A:120; PHE A:295
Prodelphinidin B1	AChE	2Y2V	−10.5	0.09	Hbond	12	TYR A:72, TYR A:72, THR A:75, THR A:75, LEU A:76, TYR A:124, GLU A:292, GLU A:292, SER A:293, SER A:293, SER A:293, PHE A:295	TYR A:124; PHE A:295
Ampelopsin	AChE	2Y2V	−10.2	0.23	Hbond	6	GLN A:71, ASP A:74, ASN A:87, SER A:125, TYR A:133, HIS A:447	GLN A:71; SER A:125; TYR A:133; HIS A:447
Epicatechin	Amylase	2QV4	−9.0	1.07	Hbond	7	GLN A:63; ARG A:195; ARG A:195; ASP A:300; ASP A:300; ASP A:300; HIS A:305	GLN A:63; ARG A:195; ASP A:300; HIS A:305
Epigallocatechin	Amylase	2QV4	−9.0	0.18	Hbond	6	TYR A:62; GLN A:63; ASP A:197; ASP A:197; HIS A:299; ASP A:300	TYR A:62; GLN A:63; ASP A:197; ASP A:300; HIS A:299
Gallocatechin	Amylase	2QV4	−9.0	0.67	Hbond	8	GLN A:63; GLN A:63; HIS A:101; ARG A:195; ALA A:198; GLU A:233; HIS A:299; ASP A:300	GLN A:63; HIS A:101; ARG A:195; ALA A:198; GLU A:233; HIS A:299; ASP A:300
Prunin	Amylase	2QV4	−9.6	0.28	Hbond	1	ALA A:198	ALA A:198
Prodelphinidin C1	Amylase	2QV4	−10.0	0.5	Hbond	8	TRP A:59; TYR A:62; GLN A:63; ASN A:105; ASN A:105; ALA A:106; ASP A:300; HIS A:305	TRP A:59; TYR A:62; GLN A:63; ASP A:300; HIS A:305
Prodelphinidin B1	Amylase	2QV4	−9.7	1.08	Hbond	10	ASN A:53; ASN A:53; TYR A:62; GLN A:63; HIS A:101; ASN A:105; ASN A:105; ALA A:106; THR A:163; THR A:163	TYR A:62; GLN A:63; HIS A:101; THR A:163
Prunin	TLR2	2Z7X	−9.2	0.29	Hbond	4	PHE A:322; TYR A:326; LEU A:334; SER A:346	PHE A:322; SER A:346; LEU A:334
3-O-p-coumaroylquinic acid	eNOS	4D1O	−9.1	1.17	Hbond	6	VAL A:185; GLY A:355; TRP A:356; TRP A:356; TRP A:356; MET A:358	VAL A:185; GLY A:355; TRP A:356; MET A:358
Prunin	eNOS	4D1O	−10.5	1.07	Hbond	4	VAL A:185; GLY A:186; TRP A:356; TRP A:356	VAL A:185; GLY A:186; TRP A:356
Prodelphinidin C1	eNOS	4D1O	−10.0	11.3	Hbond	7	GLN A:247; ARG A:250; ARG A:250; ASN A:338; TRP A:356; ARG A:365; ARG A:372	TRP A:356; ARG A:365
Prodelphinidin B1	eNOS	4D1O	−10.4	1.08	Hbond	8	VAL A:185; GLN A:247; ARG A:250; ARG A:250; TRP A:356; TRP A:356; GLU A:361; ASN A:366	VAL A:185; TRP A:356; GLU A:361; ASN A:366
Ampelopsin	eNOS	4D1O	−9.4	1.05	Hbond	8	SER A:78; THR A:80; GLU A:432; GLN A:435; CYS A:441; CYS A:441; GLN A:462; VAL A:465	nd
Prunin	AKT1	4GV1	−9.0	0.38	Hbond	6	LYS A:158; GLU A:228; ALA A:230; LYS A:276; ASN A:279; ASN A:279	LYS A:158; LYS A:276; ASN A:279
Prodelphinidin C1	AKT1	4GV1	−10.2	10.1	Hbond	8	LYS A:158; LYS A:179; GLU A:198; GLU A:234; GLU A:234; LYS A:276; ASN A:279; ASP A:439	LYS A:158; LYS A:179; GLU A:198; GLU A:234; LYS A:276; ASN A:279

Table 5 (continued)

Compound	Target	PDB ID	Bind- ing energy	RMSD	Interaction		Binding site	Catalytic site
					Type	Number		
Prodelphinidin B1	AKT1	4GV1	−9.4	0.64	Hbond	11	LYS A:158;THR A:160;GLY A:162;LYS A:179;GLU A:191;HIS A:194;THR A:195;LYS A:276;ASN A:279;ASN A:279;GLY A:294	LYS A:158; THR A:160; GLY A:162; LYS A:179; GLU A:191; HIS A:194; THR A:195; LYS A:276; ASN A:279; GLY A:294
3-O-feruloylquinic acid	PPAR γ	7E0A	−9.7	0.21	Hbond	5	GLY A:284;GLY A:284;ARG A:288;ARG A:288;GLU A:291	GLY A:284; ARG A:288; GLU A:291
Catechin	PPAR γ	7E0A	−9.8	0.7	Hbond	3	GLY A:284;GLU A:291;LEU A:340	GLY A:284; GLU A:291; LEU A:340
3-O-p-coumaroylquinic acid	PPAR γ	7E0A	−9.9	9.9	Hbond	6	ARG A:288;ARG A:288;ARG A:288;ARG A:288;GLU A:291;GLU A:343	ARG A:288; GLU A:291; GLU A:343
Epicatechin	PPAR γ	7E0A	−9.8	0.59	Hbond	1	GLU A:291	GLU A:291
Epigallocatechin	PPAR γ	7E0A	−9.3	0.86	Hbond	2	ARG A:288;GLU A:343	ARG A:288; GLU A:343
Gallocatechin	PPAR γ	7E0A	−9.4	0.93	Hbond	2	GLU A:291;GLU A:343	GLU A:291; GLU A:343
3-O-caffeoylquinic acid	PPAR γ	7E0A	−9.5	0.01	Hbond	10	LEU A:228;LEU A:228;GLY A:284;ARG A:288;ARG A:288;ARG A:288;ARG A:288;LEU A:330;GLU A:343;GLU A:343	LEU A:228; GLY A:284; ARG A:288; LEU A:330; GLU A:343
Prunin	PPAR γ	7E0A	−9.8	0.3	Hbond	5	ARG A:288;ARG A:288;ARG A:288;ARG A:288;TYR A:327	ARG A:288; TYR A:327
5-O-caffeoylquinic acid	PPAR γ	7E0A	−9.4	0.3	Hbond	2	ARG A:288;GLU A:343	ARG A:288; GLU A:343
5-p-coumaroylquinic acid	PPAR γ	7E0A	−9.4	2.24	Hbond	0		
Prodelphinidin B1	PPAR γ	7E0A	−9.1	0.41	Hbond	6	GLU A:291;GLN A:294;GLN A:294;GLU A:298;SER A:342;GLU A:343	GLU A:291; GLN A:294; SER A:342; GLU A:343
Ampelopsin	PPAR γ	7E0A	−9.8	0.28	Hbond	3	PHE A:287;ARG A:288;GLU A:343	PHE A:287; ARG A:288; GLU A:343
Catechin	SIRT1	4I5I	−9.3	nd	Hbond	11	ARG A:274;ARG A:274;TYR A:280;TYR A:280;HIS A:363;VAL A:412;GLY A:440;SER A:441;SER A:442;LEU A:443;LYS A:444	ARG A:274; TYR A:280; HIS A:363; VAL A:412; GLY A:440; SER A:441; SER A:442; LYS A:444
3-O-p-coumaroylquinic acid	SIRT1	4I5I	−9.2	0.82	Hbond	6	TYR A:280;PHE A:297;PHE A:297;VAL A:412;VAL A:412;PHE A:414	TYR A:280; PHE A:297; VAL A:412; PHE A:414
Epigallocatechin	SIRT1	4I5I	−9.4	0.91	Hbond	12	ALA A:262;ARG A:274;PHE A:297;GLN A:345;HIS A:363;VAL A:412;PHE A:414;SER A:441;SER A:442;LEU A:443;LYS A:444;VAL A:445	ALA A:262; ARG A:274; PHE A:297; GLN A:345; HIS A:363; VAL A:412; PHE A:414; SER A:441; SER A:442; LYS A:444; VAL A:445
Prunin	SIRT1	4I5I	−9.4	nd	Hbond	8	ALA A:262;PHE A:297;PHE A:414;GLY A:415;GLU A:416;SER A:441;ARG A:446;ARG A:446	ALA A:262; PHE A:297; PHE A:414; GLY A:415; GLU A:416; SER A:441; ARG A:446

Table 5 (continued)

Compound	Target	PDB ID	Bind- ing energy	RMSD	Interaction		Binding site	Catalytic site
					Type	Number		
5-O-Caffeoylquinic acid	SIRT1	4I5I	−9.3	1.05	Hbond	9	ALA A:262;GLY A:263;PHE A:273;ARG A:274;GLN A:345;GLY A:440;SER A:442;LEU A:443;LYS A:444	ALA A:262; GLY A:263; PHE A:273; ARG A:274; GLN A:345; GLY A:440; SER A:442; LYS A:444
3-O-Feruloylquinic acid	SIRT1	4I5I	−9.7	11.9	Hbond	4	SER A:365, LYS A:408, GLU A:410, ASN A:417	nd

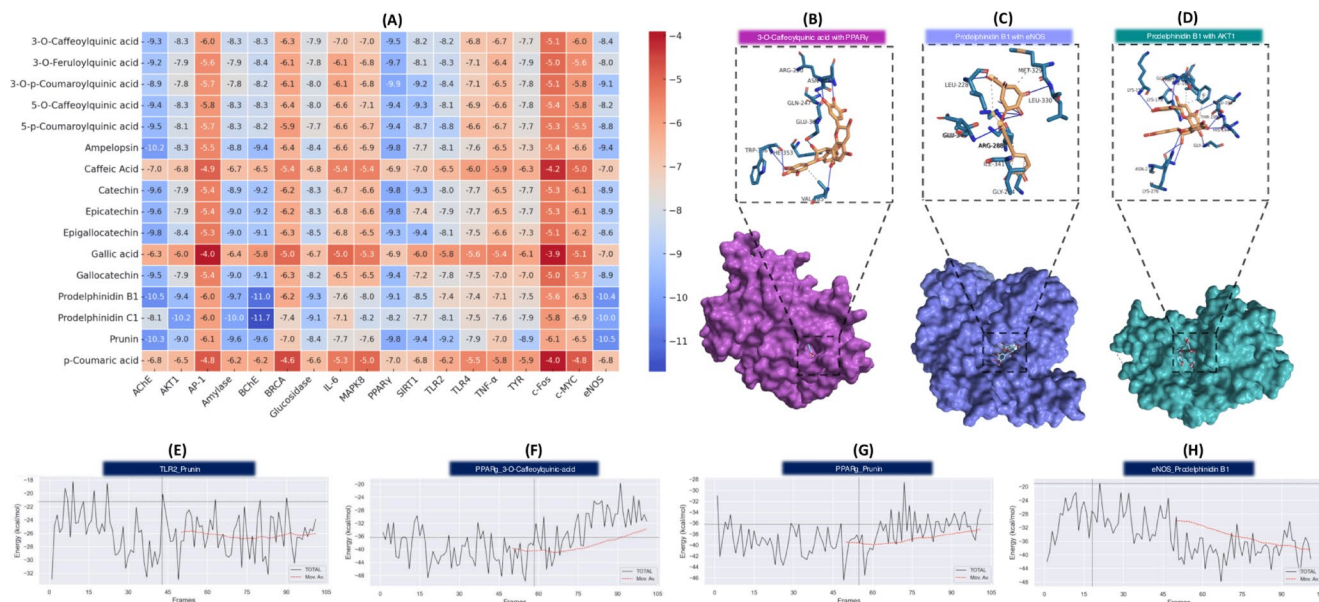


Fig. 5 A comprehensive analysis of the binding interactions between enzymes/proteins and the selected compounds, along with MM/PBSA binding free energy calculations: **A** A graphical representation of docking scores for relevant proteins and enzymes. **B** Molecular interaction analysis of 3-O-caffeoylquinic acid with PPAR γ . **C** Molecular interaction analysis of prodelphinidin B1 with eNOS. **D** Molecular interac-

tion analysis of prodelphinidin B1 with AKT1. **E** MM/PBSA binding free energy calculation of the TLR2_Prunin complex. **F** MM/PBSA binding free energy calculation of the PPAR γ _3-O-caffeoylquinic acid complex. **G** MM/PBSA binding free energy calculations of the PPAR γ _Prunin. **H** MM/PBSA binding free energy calculation of the eNOS_Prodelphinidin B1

also observed for TLR2, PPAR γ , and SIRT1 with various compounds, particularly involving residues *GLY284*, *ARG288*, *GLU291*, and *PHE297*. HOTSPOT WIZARD analysis confirmed that most active compounds bound within the catalytic regions of these proteins, except for eNOS-Ampelopsin and SIRT1-3-O-Feruloylquinic acid, which engaged residues outside these sites. Additionally, the docking poses of the best-performing complexes further illustrated the appropriate orientation and favorable accommodation of the ligands within the active sites of their corresponding targets, as visualized in Fig. 5B–D. Overall, these results demonstrate that *A. oxycedri*-derived compounds, particularly prodelphinidin C1, prodelphinidin B1, and prunin, exhibit strong multi-target inhibitory potential against both metabolic enzymes and colon cancer-related

proteins, suggesting their potential application in the development of novel phytochemical-based therapeutics.

Binding free energy analysis: MM/PBSA results and implications for ligand efficacy

Binding free energy analysis using MM/PBSA calculations was conducted to evaluate the binding stability of eight selected protein-ligand complexes (Table S9). Among these, PPAR γ _Prunin (−38.27 kcal/mol), PPAR γ _3-O-caffeoylquinic acid (−36.82 kcal/mol), eNOS_Prodelphinidin B1 (−34.29 kcal/mol), and TLR2_Prunin (−25.98 kcal/mol) demonstrated the most favorable binding free energy values, suggesting their strong and stable interactions with their respective protein targets. The detailed energy profiles and component contributions of these top-ranked complexes

are illustrated in Fig. 5E–H: TLR2_Prurin (Fig. 5E), PPAR γ 3-O-caffeoylquinic acid (Fig. 5F), PPAR γ _Prurin (Fig. 5G), and eNOS_Prodelphinidin B1 (Fig. 5H). Overall, van der Waals and electrostatic interactions were the major stabilizing forces across these complexes, while polar solvation energy contributed to reduced binding affinity in some cases. These findings highlight PPAR γ _Prurin, PPAR γ 3-O-caffeoylquinic acid, eNOS_Prodelphinidin B1, and TLR2_Prurin as promising ligand candidates for further experimental validation.

Molecular dynamics simulation

Molecular dynamics simulations were conducted to evaluate the structural stability, flexibility, binding dynamics, and hydrogen bonding behavior of four selected protein-ligand complexes: PPAR γ _Prurin, PPAR γ 3-O-caffeoylquinic acid, eNOS_Prodelphinidin B1, and TLR2_Prurin.

RMSD analysis revealed that all complexes achieved equilibrium throughout the simulation period (Fig. 6A). PPAR γ 3-O-caffeoylquinic acid and PPAR γ _Prurin

exhibited slightly higher fluctuations, indicating conformational changes, whereas eNOS_Prodelphinidin B1 and TLR2_Prurin displayed relatively stable RMSD profiles. Root mean square fluctuation (RMSF) analysis was performed to evaluate the flexibility of residues within each complex (Fig. 6B). The results indicated that PPAR γ _Prurin and PPAR γ 3-O-caffeoylquinic acid exhibited relatively higher regional fluctuations compared to eNOS_Prodelphinidin B1 and TLR2_Prurin. Particularly, PPAR γ complexes showed increased flexibility at specific residues, while eNOS and TLR2 complexes demonstrated lower fluctuation patterns, indicating more stable conformations. SASA analysis was performed to monitor the changes in surface exposure of the complexes during the simulation (Fig. 6C). Among the studied complexes, PPAR γ _Prurin exhibited a gradual increase in SASA values over time, suggesting a possible conformational rearrangement that may lead to increased ligand exposure. In contrast, PPAR γ 3-O-caffeoylquinic acid, eNOS_Prodelphinidin B1, and TLR2_Prurin maintained relatively stable SASA profiles throughout the simulation period, indicating more compact and stable binding

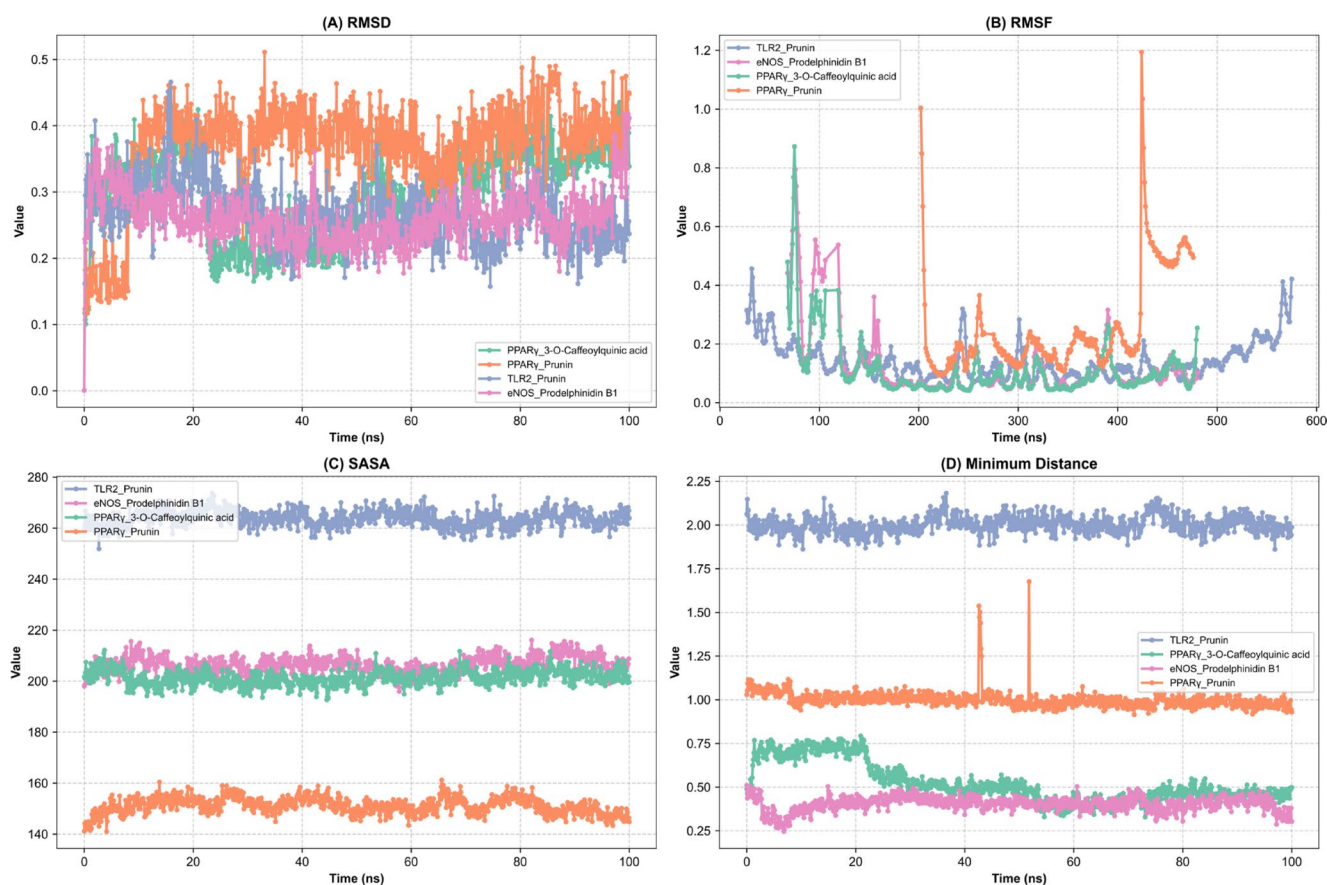


Fig. 6 Presentation of molecular dynamics simulations in graphical form; **A** RMSD of the eNOS_Prodelphinidin B1, PPAR γ _Prurin, PPAR γ 3-O-Caffeoylquinic acid, and TLR2_Prurin complexes. **B** RMSF of the eNOS_Prodelphinidin B1, PPAR γ _Prurin, PPAR γ 3-O-Caffeoylquinic acid, and TLR2_Prurin complexes. **C** Solvent Acces-

sibility of the eNOS_Prodelphinidin B1, PPAR γ _Prurin, PPAR γ 3-O-Caffeoylquinic acid, and TLR2_Prurin complexes. **D** Minimum Distance of the eNOS_Prodelphinidin B1, PPAR γ _Prurin, PPAR γ 3-O-Caffeoylquinic acid, and TLR2_Prurin complexes

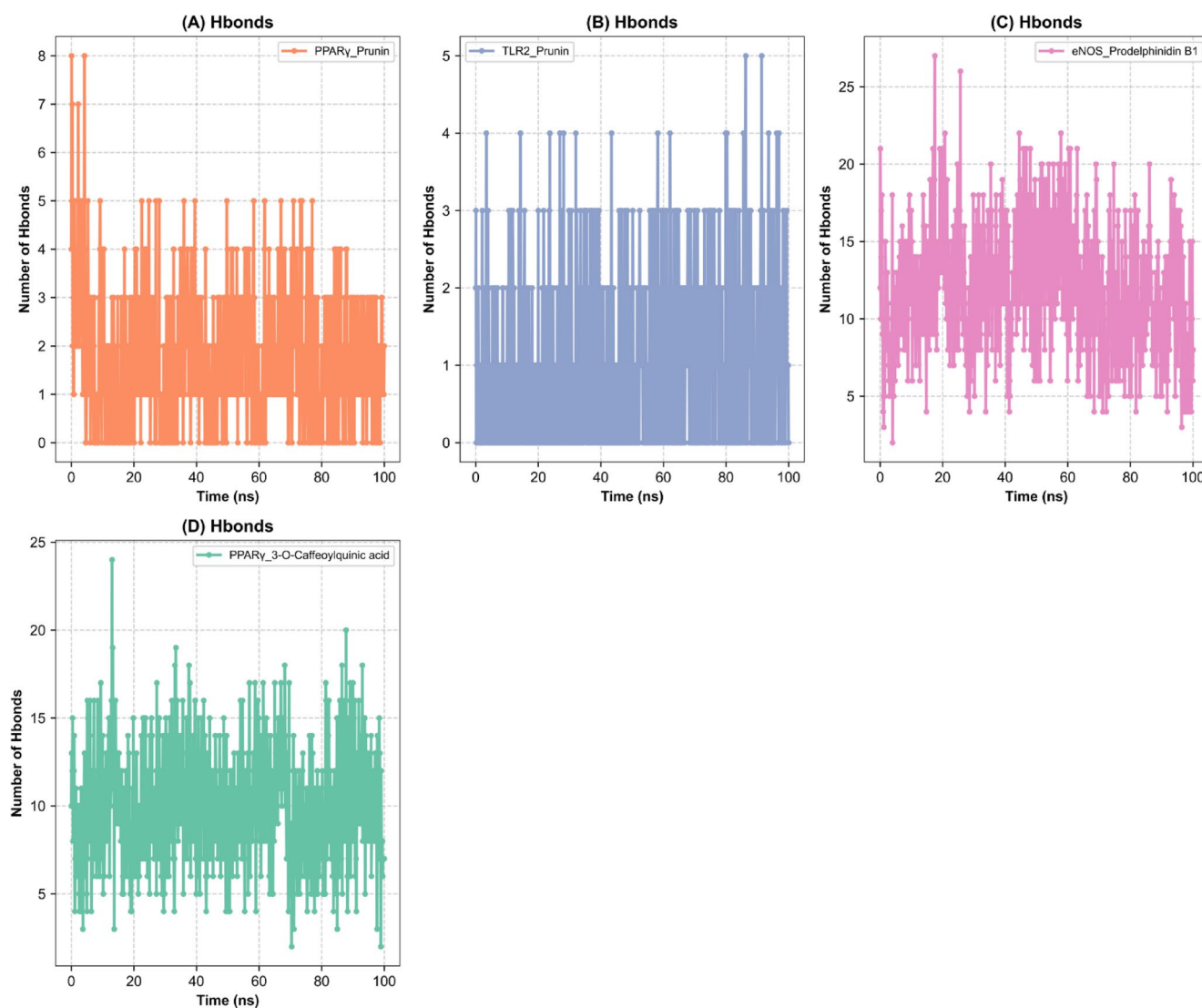


Fig. 7 Hydrogen bonds analysis. **A** Hydrogen bonds of the PPAR γ _Prunin complex. **B** Hydrogen bonds of the TLR2_Prunin complex. **C** Hydrogen bonds of the eNOS_Prodelphinidin B1 complex. **D** Hydrogen bonds of the PPAR γ _3

conformations. Hydrogen bond (Hbond) analysis was performed using the gmx hbond tool in GROMACS, applying a donor-acceptor distance cutoff of 3.5 Å and a hydrogen bond angle cutoff of 120°, which are widely accepted geometric criteria for identifying specific Hbond interactions. In this context, the total number of hydrogen bonds formed between protein and ligand was calculated at each simulation frame, providing insights into the dynamic nature of binding interactions. It should be noted that this approach captures both transient and stable hydrogen bonds, resulting in higher overall counts compared to persistent hydrogen bonds maintained throughout the simulation (Fig. 7A–D). The analysis revealed that the eNOS-Prodelphinidin B1 complex exhibited dynamic but generally stable Hbond patterns, fluctuating between 9 and 12 during the simulation (Fig. 7C). Similarly, the PPAR γ -3-O-caffeoylquinic acid

complex maintained a relatively constant Hbond number of around 10, indicating stable binding behavior (Fig. 7D).

In contrast, the PPAR γ -Prunin complex showed a gradual reduction in Hbond numbers from 3 to 2 over time, suggesting decreased binding stability (Fig. 6A). The TLR2_Prunin complex demonstrated the lowest and most unstable Hbond profile, fluctuating around 1, indicating a weak and highly dynamic interaction (Fig. 7B).

Conclusion

The antioxidant and enzyme inhibitory properties of *A. oxycedri* extracts obtained with different solvents were investigated. Ethanol and ethanol/water (70%) showed the best and most stable antioxidant activity in all the tests, and high

inhibition against AChE, BChE, and tyrosinase turned these extracts into candidates for neurodegenerative disorders and hyperpigmentation. It showed selective action, inhibiting only α -amylase, which might be helpful in the control of postprandial glucose levels. Hexane extract demonstrated moderate inhibitions against α -glucosidase and α -amylase, while an aqueous extract had the least activity for overall results. HPLC-MS QTOF chemical profiling identified high-value bioactive chemicals such as flavonoids and phenolic acids supporting the observed bioactivity. Molecular docking and molecular dynamics simulations suggest that prodelphinidin B1 may act as a potential eNOS inhibitor, while 3-O-caffeoylquinic acid exhibits strong interactions with PPAR γ , indicating its potential inhibitory effect. Overall, the presence of these bioactive compounds suggests that *A. oxycedri* extracts could be valuable for targeting metabolic and oxidative stress-related disorders and hepatocellular carcinoma. However, further in vivo studies are necessary to validate these findings and to explore their pharmacological potential.

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Data availability Data will be made available on request.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval Not applicable.

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Consent for publication Not applicable.

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