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ORIGINAL ARTICLE

Essential oil composition and biological activities of three *Hypericum* species: *In silico* and *in vitro* studies

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Abstract: *Hypericum* species, which have been used in folk medicine for a long time due to their wound healing, antidepressant, and antihypertensive effects, have an important place among medicinal plants. In this study, the chemical compositions of the essential oils from three *Hypericum* species (*H. empetrifolium* subsp. *empetrifolium*, *H. hyssopifolium* var. *elongatum*, *H. pseudolaeve*) grown in Anatolia were determined by GC-MS. Cytotoxic, antimicrobial and antioxidant activities as well as anticholinesterase, antiurease, tyrosinase, antielastase, and collagenase enzyme-inhibitory activities of the essential oils obtained were also determined. As major components, α-pinene (16.68%) was determined for *H. empetrifolium* subsp. *empetrifolium*, 2-methyloctane (20.79%) was determined for *H. hyssopifolium* var. *elongatum*, cis-β-ocimene (21.64%) was determined for *H. pseudolaeve*. Of these three *Hypericum* species, 2-methyloctane, α-pinene, cis-β-ocimene, caryophyllene, germacrene D, and caryophyllene oxide compounds were prominent constituents. The especially noteworthy aspects of each species is the acetyl- (inhibition%: 72.65 ± 0.13), butyryl-cholinesterase (inhibition%: 70.73 ± 1.69), tyrosinase (inhibition%: 59.62 ± 0.82), elastase (inhibition%: 38.12 ± 0.18), and collagenase (inhibition%: 22.09 ± 0.10) enzyme activities for *H. pseudolaeve* sample, the cytotoxic properties of the essential oils in *H. empetrifolium* subsp. *empetrifolium* species and the high potential for antimicrobial activity in *H. hyssopifolium* var. *elongatum* species were determined. Additionally, examinations of the major constituents of the species have been conducted on the investigated enzymes both *in vitro* and *in silico*. These findings on biological activity suggested that three essential oils from the *Hypericum* species warrant further investigation in validated biological models.

Keywords: *Hypericum*, β-ocimene, essential oils, biological activities, *in silico* and *in vitro*, GC-MS

1 Introduction

It is known that the genus *Hypericum* L. has approximately 500 species in the world. Türkiye is an important center of *Hypericum* species and 48 of the 97 species reported in Türkiye are endemic (Babacan et al., 2017). In Turkey, St. John's Wort is known by the names sarı kantaron, binbirdelikotu, koyunkıran and kılıç otu (Baytop, 1984). *Hypericum* species contain many bioactive compounds. The main ones are flavonoids, xanthones, procyanidin compounds, hypericin and pseudohypericin compounds, known as naphthodianthrone group compounds, and hyperforin and adhyperforin compounds, known as phloroglucinol derivatives (Patocka, 2003; Seyrekoglu et al., 2022). Extracts from *Hypericum* species are used in the treatment of depression, in stomach ailments, as an appetizer, in jaundice, as an anti-inflammatory in external wounds, in athlete's foot, in gingivitis, as an expectorant in sinusitis, in bowel inflammation, in hemorrhoids, as an antipyretic in febrile diseases and traditionally in the treatment of many diseases with its blood-forming properties (Volz, 1997). Because of their wound healing and anti-aging characteristics, *Hypericum* species are used especially in the treatment of burns (Baytop, 1984). Especially *H. perforatum* L. and *H. triquetrifolium*

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Turra. are used more widely (Baytop, 1984). In Anatolia, the use of these species, especially nonpolar extracts (St. John's Wort oil), is quite common. St. John's wort oil is traditionally prepared by macerating 100 g of flowers in 250 g of olive oil, then keeping the mixture in the sun for 15 days with occasional shaking. It is then used by filtering through cotton (Baytop, 1984). There are many studies in the literature on the essential oil content of *Hypericum* species. Components such as 2-methyloctane, nonane, undecane, α-pinene, β-pinene, limonene, β-myrcene, *cis*-β-ocimene, germacrene-D, caryophyllene and caryophyllene oxide have come into prominence in essential oil contents of *Hypericum* species (Bagci & Bekci, 2010; Bagci & Yuce, 2010; Akdeniz et al., 2020; Seyis et al., 2020). There are more than 3,000 studies in the literature on species belonging to the genus *Hypericum* (Silva et al., 2021). Although many studies have examined the chemical composition of the essential oils of *Hypericum* species, few provide detailed accounts of their biological activities.

In this study, essential oil components of *H. empetrifolium* subsp. *empetrifolium* Willd., *H. hyssopifolium* var. *elongatum* Ledeb. Woronow, and *H. pseudolaeve* Robson species obtained by the hydrodistillation method were determined with the GC-MS/FID device. Their anticholinesterase, antiurease, antityrosinase, antielastase, and anticollagenase capacities were also examined, as were their activities (DPPH free radical and ABTS cation radical scavenging activities, and CUPRAC techniques). Also, the toxic effects of these essential oils on a non-malignant cell culture (PDF) and their cytotoxic effects on cancerous MCF-7 (breast cancer) and HT-29 (colon cancer) cell lines were determined by the MTT method. In addition, the broth microdilution method and minimum inhibitory concentration were used to define their antimicrobial activity. Although the literature contains studies on the chemical composition and certain biological activities of the essential oils of these species, no study evaluates all of these parameters together.

2 Materials and Methods

2.1 Plant Material

H. empetrifolium subsp. *empetrifolium* Willd (S1) was collected by Dr. Abdulselam Ertaş from Okurcalar/Antalya in July 2016 and was identified by Mehmet Fırat. *H. hyssopifolium* var. *elongatum* Ledeb (S2) from Kelkit/Gümüşhane and *H. pseudolaeve* Robson (S3) from Bahçesaray/Van were collected and diagnosed/identified by Mehmet Fırat (from the Department of Biology, Faculty of Education, Van YüzüncüYıl University) in July 2016. Herbarium numbers of S1-S2-S3 samples are M. Fırat 32888 (VANF), M. Fırat 32910 (VANF), and M. Fırat 32920 (VANF), respectively. In order to obtain the essential oils, the shade-dried plant material from each sample was finely ground and subjected to hydrodistillation for 3 h using approximately 100 g of the aerial parts of the species in 300 mL of distilled water. The yield of the essential oil was calculated as S1 (0.25%), S2 (0.20%) and S3 (0.23%) (v/w) based on dry weight.

2.2 GC-MS Analysis

At the Dicle University Faculty of Pharmacy, the components of the essential oil samples (shade-dried aerial parts) obtained by hydrodistillation using the Clevenger apparatus were analyzed on an Agilent 7890A gas chromatograph equipped with an FID and an Agilent 5977B mass spectrometer (MS). GC-MS/FID analyses were performed on a nonpolar Agilent HP-5MS UI column (30 m x 0.25 mM x 0.25 µM film thickness) and helium (1 mL/dk) was used as carrier gas. The oven temperature was initially held at 60°C for 5 min, increased to 240°C at a rate of 3°C/min, and maintained at this final temperature for another 5 min. The injector temperature was maintained at 250°C, the split ratio was 1:25, with an injection volume of 1.0 µL. The MS transfer line and ion source temperatures were set at 230°C and 300°C, respectively. Mass spectra were recorded in electron ionization (EI) mode at 70 eV across an *m/z* range of 40–400 (Bakir et al., 2020; Ertaş and Yener, 2020; Saruhan-Fidan et al., 2021; Demirel et al., 2026; Sicak et al., 2026). By comparing the retention times and mass spectra of the constituent parts, essential oils were identified. The compounds were identified by comparing their mass spectra with entries in the NIST and Wiley libraries (Yigitkan et al., 2026).

2.3 Antioxidant Activities

The antioxidant properties of the samples were evaluated using DPPH, ABTS, and CUPRAC assays. In all three methods, α-tocopherol (α-TOC) and butylated hydroxytoluene (BHT) served as reference standards, and sample concentrations of 1, 10, 25, 50, and 100 µg/mL were utilized for IC_{50} calculations (Yigitkan et al., 2021). Values exceeding the upper limit of the established linear calibration range (1–100 µg/mL) were reported as ≥100, which were determined by mathematical extrapolation of the calibration curve. For the DPPH assay, 40 µL of the sample solutions in ethanol at different concentrations were mixed with 160 µL of a 0.1 mM DPPH solution in ethanol; after 30 min, the absorbance values were recorded at 517 nM (Blois, 1958). For the ABTS assay, the ABTS•+ radical cation was generated by mixing 7 mM ABTS in water with 2.45 mM potassium persulfate and storing the solution in the dark at 25°C for 12 h. This solution was then diluted with ethanol to adjust the absorbance to 0.700 ± 0.025 at 734 nM. Aliquots of 160 µL of the ABTS•+ solution were added to each sample concentration, and after 30 min, the percentage inhibition at 734 nM was calculated relative to an ethanol blank (Re et al., 1999). Lastly, for the CUPRAC assay, 61 mL of 1.0×10^{-2} M copper (II) chloride, 61 µL of ammonium acetate buffer (1 M, pH 7.0), and 61 µL of 7.5×10^{-3} M neocuproine solution was combined. Various volumes of the sample solution (x µL: 2.5, 6.25, 12.5, and 25 µL) and distilled water (67 − x µL) were added to achieve a final volume of 250 µL. After 1 h of incubation, the absorbance at 450 nM was measured against a reagent blank (Apak et al., 2004).

2.4 Cytotoxic Activities

In order to determine the cytotoxic effects of the samples, the MTT method developed by Mojarraba et al. (Mojarraba

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et al., 2013), with minor modifications was used. A primary dermal fibroblast cell line (PDF) was used to test the toxic effects of the samples, while colon cancer cell line (HT-29) and breast cancer cell line (MCF-7) were used to test the cytotoxic effects of the samples (Yener et al., 2018). The MTT assay was conducted 48 hours post-treatment. Each well received 10 µL of a 5 mg/mL MTT solution, and cells were then incubated for 3 hours at 37°C with 5% CO_2, 95% air, and 100% humidity. The medium was taken out and replaced with 100 µL of DMSO after three hours. After 15 minutes of room temperature plate shaking, the optical density (OD) of each well was measured using a plate reader set at 540 nM (Multi Scan Go, Thermo). The samples with concentrations of 200, 100, 50, 25, 10, and 1 µg/mL were used to perform IC_{50} calculations.

2.5 Anticholinesterase Inhibitory Activity

The acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities were determined using a modified Ellman's method (Ellman et al., 1961). Briefly, aliquots of 150 µL of sodium phosphate buffer (100 mM, pH 8.0) were mixed with 10 µL of the sample solution and 20 µL of either BChE or AChE solution. After stirring, the mixture was incubated at 25°C for 15 min, followed by the addition of 10 µL of DTNB. The enzymatic reaction was subsequently initiated by adding 10 µL of either acetylthiocholine iodide or butyrylthiocholine iodide, maintaining a final sample concentration of 100 µg/mL. The hydrolysis of the substrates was continuously monitored at 412 nM using a BioTek PowerWave XS microplate reader. In this test procedure, galantamine served as the standard reference material. Sample inhibition percentages were computed at a concentration of 100 µg/mL for all enzyme inhibition methods. In addition, the enzyme inhibition techniques employed an identical volume of ethanol.

2.6 Urease Inhibitory Activity

Urease inhibitory activity of the essential oils was determined according to the reported protocol (Hina et al., 2015). Final volume of reaction is 200 µL at pH 8.2. Twenty-five microliters of urease (Jack bean) solution were mixed with 10 µL of each sample (1000 µg/mL) and incubated at 30°C for 15 min. Aliquots were taken and immediately transferred to assay mixtures containing urea (100 mM) in buffer (50 µL) and reincubated for 30 min in 96-well plates. Forty-five microliters from each of phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) and 70 µL of alkali reagent (0.5% w/v sodium hydroxide and 0.1% sodium hypochlorite) were added to wells. Increase in absorbance was measured after 50 min at 630 nM against blank. All reactions were performed in triplicates. Thiourea was used as a positive control.

2.7 Inhibition of Enzymes Associated with Skin Aging and Pigmentation

The samples' anti-aging capability was assessed using the inhibitory activities of tyrosinase, elastase, and collagenase.

Tyrosinase inhibition assays were performed according to the method Hearing's protocol (Hearing & Jimenez, 1987). Briefly, the samples were screened for the *o*-diphenolase inhibitory activity of tyrosinase using L-DOPA as substrate. All samples were dissolved in ethanol to reach to a concentration of 1000 µg/mL. One hundred fifty microliters of phosphate buffer (pH = 6.8), 10 µL of the samples and 20 µL of the enzyme solution were added to the wells in the microplate, and the initial absorbance at 475 nM was read after stirring for 3 min. This solution was then incubated for 10 minutes at 37°C, after 10 minutes 20 µL of L-DOPA was added and incubated again at 37°C, after 10 minutes the final absorbance at 475 nM was read in the Microplate ELISA reader, with kojic acid serving as the standard reference.

Elastase inhibitory activity was measured using a slightly modified version of the Kraunsoe et al. (Kraunsoe et al., 1996) technique. In summary, 10 µL of sample ethanol solution and 20 µL of elastase enzyme solution were added to 40 µL of buffer solution (0.1 M Tris-Cl, pH = 8) and incubated at 37°C for 10 min. Subsequently, 30 µL of 1.015 mM substrate (*N*-Succinyl-Ala-Ala-p-nitroanilide) were added and incubated for 20 min at 37 degrees Celsius in buffer solution (0.1 M Tris-Cl, pH = 8). Finally, absorbance at 410 nM was measured using oleanolic acid as the standard reference.

Collagenase inhibitory activity was measured using a slightly modified version of the Thring et al. (Thring et al., 2009) method. The sample solution was prepared in 20 µL of DMSO, and 10 µL of collagenase enzyme solution (0.8 U/mL) was added to 50 µL of phosphate buffer (pH: 7.5) and incubated at 25°C for 15 min. Afterwards, 20 µL of substrate solution (*N*-(3-[2-furyl]acryloyl)-Leu-Gly-Pro-Ala) was added, incubated at 25°C for 20 min, and absorbance was measured at 340 nM. Epicatechin gallate was used as standard reference.

The urease, tyrosinase, elastase, collagenase, acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and urease inhibitions were computed using the following equation. AChE, BChE, urease, tyrosinase, elastase and collagenase inhibition (%) = 100 − (OD test well/OD control) × 100.

2.8 Antimicrobial Activity

By using the broth dilution method, the antimicrobial activity was determined against the following bacteria and yeast (*Candida albicans* ATCC10231), Gram-negative (*Escherichia coli* ATCC25922, *Pseudomonas aeroginosa* ATCC27853) and Gram-positive (*Staphylococcus aureus* 25923, *Streptococcus pyogenes* ATCC19615) (CLSI (Clinical and Laboratory Standards Institute) M7-A7, 2006; Tintino et al., 2014). 96-well plates containing 0.1 mL of Mueller-Hinton broth and 0.1 mL of different concentrations of EOs were inoculated with 0.005 mL of a culture containing 10^5 CFU/mL. MIC values were ascertained following incubation at the proper temperature and length of time. For the bacteria and yeast, positive controls were ampicillin and fluconazole, respectively. Every test was conducted three times.

2.9 Molecular Docking

Computer-aided drug design, particularly molecular docking, has become an indispensable computational tool in

structural biology for predicting the non-covalent interactions between small- molecule ligands and macromolecular receptors. This *in silico* approach provides atomistic insights into the binding mechanisms by identifying crucial hydrogen bonds, hydrophobic interactions, and electrostatic forces that stabilize the ligand-receptor complex. In this study, DOCK 6.5 (Kuntz Lab programs are available free of charge for academic institutions) was used to predict the binding poses of 2-methyloctane, α-pinene, *cis*-β-ocimene, and caryophyllene oxide to determine the locations of the enzyme's active sites for anticholinesterase, antityrosinase, antielastase, and anticollagenase. The docking analyses were performed using the Protein Data Bank (PDB) templates 2x8b.pdb, 4bbz.pdb, 5i38.pdb, 1bru.pdb, and 2d1n.pdb which represent AChE, BChE, tyrosinase, elastase, and collagenase, respectively. Crystallographic water molecules were removed from all the structures and the missing coordinates of the atoms were modelled using xLeAP and an ff99SB force field. Atoms on proteins were assigned the PARM99 charges, and all ionizable residues were set at their default protonation states at neutral pH. All structures were further processed by the xLeAP module of AMBER. The molecular systems were neutralized by the addition of counterions. The selected target proteins were minimized with Amber force field (Case et al., 2025; Duan et al., 2003; Bayly et al., 1993; Pettersen et al., 2004). The Discovery Studio 4.1 Client a visualizing tool was used to generate the hydrophobicity graphs and graphical depiction of target proteins. The protein Ramachandran graph was accessed through PDB. The Discovery Studio 2.1 Client (Accelrys Software Inc, 2009) was used to view 3D structure of target proteins. The calculations were performed according to the reports (Lang et al., 2007; Carletti et al., 2013; Deri et al., 2016; Nakanishi et al., 2020). Because the essential oils showed no detectable urease inhibition under the tested conditions, a molecular modeling study wasn't needed and was not performed. The *in-silico* analyses other than the one described above were performed according to Yener et al. (Yener et al., 2020). Dock 6.5 (Case et al., 2025) module allows all stages of a docking process to be performed with the generation of ligand conformations, ligand docking, and the scoring of the binding modes. As in this case, where a rigid receptor approximation was used, it is expected that 130 the different receptors considered will lead to different ligand binding modes depending on the initial size of the enzyme-binding cavity. Thus, fourteen designed molecules were docked onto available receptors following a multistep procedure. In order to describe receptor-binding properties, a grid of potential energy was calculated for atoms taking part in the binding pocket. These atoms were obtained from the analysis of each protein-ligand complex. In this step, default parameters were used. The ligand was then docked using the calculated grid to place it into the cavity and score the proposed binding mode.

2.10 Statistical Analysis

The activity assay findings were displayed as means ± standard meaning error (S.E.M.). An unpaired *t*-test and a one-way analysis of variance (ANOVA) were used to assess the findings. It was determined that the differences were statistically significant at $p < 0.05$.

3 Results and Discussion

3.1 Essential Oil Composition

The essential oil contents of the three studied *Hypericum* species were determined by GC-MS and GC-MS/FID, and 67 constituents were identified, accounting for 91.59%, 93.88%, and 91.79% (Table 1 and Figure S1). The results shown that the monoterpene and sesquiterpene hydrocarbon contents differed among the three samples studied, but the S3 sample is richer, in terms of oxygenated sesquiterpenes. The major components in the studied samples were determined as follows: α-pinene (16.68%), 2-methyloctane (11.52%), and thymol (4.88%) in S1; 2-methyloctane (20.79%), α-pinene (10.81%), and germacrene D (8.71%) in S2; and cis-β-ocimene (21.64%), caryophyllene (16.91%), and α-pinene (14.18%) in S3 (Table 1).

In the previous studies, the major components of the essential oils from the studied samples have been reported as follows: *H. empetrifolium* subsp. *empetrifolium* has 19.0% α-pinene, 12.5% germacrene D, and 8.7% β-pinene; (Grafakou et al., 2020) *H. hyssopifolium* var. *elongatum* has 57.3% α-pinene, 9.0% β-pinene, and 6.2% D-limonene; (Cakir et al., 2004) *H. pseudolaeve* has 23.92% caryophyllene, 11.19% D-limonene, 10.67% α-cadinol, 7.96% caryophyllene oxide and 5.76% α-pinene (Bagci & Yuce, 2010). Overall, 2-methyloctane, α-pinene, *cis*-β-ocimene, caryophyllene, germacrene D, and caryophyllene oxide compounds are prominent, from a chemotaxonomic standpoint, in the samples investigated. Furthermorethe results presented in this study are in agreement with previous reports, and differences in the major and other constituents may depend on variables such as soil structure and climatic conditions.

3.2 Antioxidant and Cytotoxic Activities

The results shown that all samples studied exhibit low DPPH free radical scavenging activity (Table 2). Although all investigated essential oil samples showed moderate activity, the S1 was determined to be the most active sample (IC_{50}: 11.27 ± 0.26 µg/mL), in the ABTS cation radical scavenging activity method. Consistent with previous results, the antioxidant activity of the samples in the CUPRAC method indicated that S1 showed moderate to high activity and remained the most active sample ($A_{0.5}$: 40.70 ± 1.21 µg/mL). The cytotoxicity of the used samples used was evaluated by the MTT assay using the PDF healthy cell line, the MCF-7 estrogen-dependent breast cancer cell line, and the HT-29 colon cancer cell line. The results show that all samples exhibit moderate to high toxicity against both normal and cancer cells (Table 2). The high toxicity of these species on healthy cell lines has shown that caution is needed when using them. To evaluate the preference of the samples toward malignant cells over healthy counterparts, the selectivity indices (SI) were calculated using the formula: SI = IC_{50} of PDF normal cells/IC_{50} of cancer cells. An SI value greater than 1.0 indicates selective cytotoxicity toward cancer cells.

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Table 1. Essential oil composition of aerial parts of *Hypericum* species

No	RI[a]	Constituents[b]	S1 Ess[c]	S2 Ess	S3 Ess	No	RI[a]	Constituents[b]	S1 Ess	S2 Ess	S3 Ess	Identification Methods
1	848	2-Hexenal	0.10	0.23	0.14	37	1434	Himachala-2,4-diene	–	–	0.20	Co-GC, MS, RI
2	859	2-Methyloctane	11.52	20.79	0.12	38	1436	β-Cubebene	0.50	–	0.24	Co-GC, MS, RI
3	899	Nonane	7.87	4.84	1.27	39	1446	Aromadendrene	0.14	0.17	–	Co-GC, MS, RI
4	927	3-Thujene	–	0.95	–	40	1457	α-Himachalene	0.99	–	–	Co-GC, MS, RI
5	935	α-Pinene	16.68	10.81	14.18	41	1458	*trans*-β-Farnesene	–	–	0.21	Co-GC, MS, RI
6	950	Camphene	0.45	0.11	0.11	42	1460	*cis*-β-Famesene	–	0.65	–	Co-GC, MS, RI
7	967	3-Methylnonane	6.57	2.89	0.18	43	1462	Humulene	0.70	0.62	1.09	Co-GC, MS, RI
8	975	Sabinen	–	0.73	–	44	1468	Alloaromadendrene	0.21	0.36	–	Co-GC, MS, RI
9	981	β-Pinene	2.36	5.09	0.39	45	1473	Cyclodecane	–	4.23	–	Co-GC, MS, RI
10	991	β-Myrcene	0.80	1.02	0.86	46	1483	γ-Muurolene	1.61	1.4	1.04	Co-GC, MS, RI
11	999	Decane	0.26	0.15	–	47	1490	Germacrene D	7.66	8.71	4.26	Co-GC, MS, RI
12	1018	α-Terpinen	0.37	0.29	–	48	1495	β-Selinene	–	–	0.13	Co-GC, MS, RI
13	1026	*p*-Cymene	0.66	0.38	0.12	49	1501	Valencene	2.25	0.46	–	Co-GC, MS, RI
14	1030	Limonene	2.07	0.69	0.39	50	1505	Bicyclogermacrene	1.54	1.74	–	Co-GC, MS, RI
15	1036	trans-β-Ocimene	0.15	0.82	1.23	51	1506	α-Muurolene	–	–	0.66	Co-GC, MS, RI
16	1047	*cis*-β-Ocimene	0.12	2.55	21.64	52	1509	α-Farnesene	–	0.54	–	Co-GC, MS, RI
17	1059	γ-Terpinene	0.97	0.70	0.11	53	1521	γ-Cadinene	0.96	0.72	0.61	Co-GC, MS, RI
18	1062	2-Methyldecane	3.27	3.08	–	54	1530	δ-Cadinene	1.78	1.39	1.78	Co-GC, MS, RI
19	1091	Terpinolen	0.55	0.17	0.12	55	1540	Cubenene	0.13	–	–	Co-GC, MS, RI
20	1099	Undecane	2.83	2.05	5.50	56	1545	α-Cadinene	0.20	0.18	–	Co-GC, MS, RI
21	1104	Nonanal	–	0.16	0.31	57	1551	α-Calacorene	0.15	–	0.13	Co-GC, MS, RI
22	1180	Terpinen-4-ol	0.11	0.39	–	58	1566	Nerolidol	0.11	0.44	–	Co-GC, MS, RI
23	1193	α-Terpineol	0.20	–	0.59	59	1587	Spathulenol	1.65	1.07	0.75	Co-GC, MS, RI
24	1200	Myrtenol	0.15	–	–	60	1594	Caryophyllene oxide	1.26	1.76	14.01	Co-GC, MS, RI
25	1263	2-Methyldodecane	–	0.37	–	61	1602	Viridiflorol	–	0.41	–	Co-GC, MS, RI
26	1299	Tridecane	–	0.13	0.15	62	1613	Globulol	0.66	–	–	Co-GC, MS, RI
27	1301	Thymol	4.88	–	–	63	1620	Bisabolene epoxide	–	0.19	–	Co-GC, MS, RI
28	1354	α-Cubebene	–	0.13	–	64	1649	*tau*-Cadinol	0.67	0.4	0.77	Co-GC, MS, RI
29	1358	α-Longipinene	0.57	0.24	–	65	1663	α-Cadinol	1.28	0.43	0.99	Co-GC, MS, RI
30	1376	α-Ylangene	0.10	–	–	66	1676	1-Tetradecene	–	0.86	–	Co-GC, MS, RI
31	1382	α-Copaene	0.27	0.34	0.32	67	1690	α-Bisabolol	1.03	–	–	Co-GC, MS, RI
32	1391	β-Bourbonene	0.12	0.34	0.17			Total identified (%)	91.59	93.88	91.79	
33	1396	β-Elemene	–	–	0.11			Monoterpene hydrocarbons	32.01	30.63	39.33	
34	1421	β-Funebrene	–	0.51	–			Oxygenated monoterpenes	5.34	0.39	0.59	
35	1422	α-Cedrene	0.33	–	–			Sesquiterpene hydrocarbons	21.99	24.70	27.86	
36	1427	Caryophyllene	1.78	6.20	16.91			Oxygenated sesquiterpenes	6.66	4.70	16.52	
								Others	25.59	33.46	7.49	

Note: [a]Kovats index on HP 5MS fused silica column; [b]A nonpolar Agilent HP-5MS fused silica column; [c]Percentage composition; Co-GCCo-injection with authentic compounds; S1-Ess: *H. empetrifolium* subsp. *empetrifolium* collected in Antalya, S2-Ess: *H. hyssopifolium* var. *elongatum* collected in Gümüşhane, S3-Ess: *H. pseudolaeve* collected in Van; [RI]Retention Index literature comparison.

For HT-29, the calculated SI values were found to be 0.59, 0.36, and 0.19 for Samples S1, S2, and S3, respectively. Similarly, the SI values for MCF-7 were determined as 0.69, ≤ 0.19, and 0.21. All obtained SI values were significantly lower than 1.0, explicitly demonstrating that the studied samples exhibit general cytotoxicity rather than preferential anticancer activity. Therefore, the high toxicity of these species on healthy cell lines has shown that caution is needed when using them in potential biomedical applications.

There are a few previous studies that investigate the chemical content of the used *Hypericum* samples and reported similar results to those we obtained in the literature. On the other hand, there are some studies on antioxidant and cytotoxic activities of different *Hypericum* species extracts and their essential oils (Akdeniz et al., 2020; Ozdemir et al., 2020; Balikci et al., 2020). However, the cytotoxic effects of essential oils of the samples extracted from, *H. hyssopifolium* var. *elongatum* and *H. pseudolaeve* have been investigated in detail for the first time in this study, according to our knowledge.

3.3 Enzyme Inhibitory Activities

The anticholinesterase activities of the essential oils from the studied *Hypericum* species were determined using acetylcholinesterase and butyrylcholinesterase inhibition assays (Table 2). The results showed that all three samples exhibited moderate to high cholinesterase activity. It was found that all three samples showed moderate to high cholinesterase enzyme activity. It was determined that the S3 sample showed

Table 2. Biological activities of essential oil of aerial parts of *Hypericum* species

	Antioxidant activity[a]			Cytotoxic activity[a]					Enzyme activity (100 µg/mL)[a]					
	IC_{50} (µg/mL)		$A_{0.5}$ (µg/mL)	IC_{50} (µg/mL)					inhibition (%)					
Samples	**DPPH**	**ABTS**	**CUPRAC**	**HT-29**	**SI(HT-29)**	**MCF-7**	**SI(MCF-7)**	**PDF**	**AChE**	**BChE**	**Urease**	**Tyrosinase**	**Elastase**	**Collagenase**
S1	≥100	11.27 ± 0.26[a]	40.70 ± 1.21[a]	93.78 ± 0.18[a]	0.59	79.90 ± 0.09[a]	0.69	54.89 ± 1.08[a]	22.65 ± 0.52[a]	59.27 ± 1.34[a]	*na*	1.32 ± 1.06[a]	10.25 ± 0.11[a]	6.45 ± 0.09[a]
S2	≥100	≥100	≥100	105.23 ± 1.33[b]	0.36	≥200	≤0.19	37.48 ± 0.85[b]	38.29 ± 1.36[b]	52.16 ± 2.39[b]	*na*	1.78 ± 2.50[a]	12.74 ± 0.12[b]	7.17 ± 0.02[b]
S3	≥100	≥100	≥100	123.30 ± 1.14[c]	0.19	114.15 ± 1.02[b]	0.21	23.68 ± 0.28[c]	72.65 ± 0.13[c]	70.73 ± 1.69[c]	*na*	59.62 ± 0.82[b]	38.12 ± 0.18[c]	22.09 ± 0.10[c]
2-Methyloctane	≥100	≥100	98.02 ± 0.92[b]	–	–	–	–	–	58.09 ± 1.12[d]	25.83 ± 0.16[d]	42.41 ± 0.87[a]	12.03 ± 0.53[c]	22.58 ± 0.72[d]	15.79 ± 0.67[d]
α-Pinene	≥100	≥100	56.23 ± 1.02[c]	–	–	–	–	–	48.64 ± 0.87[e]	11.93 ± 0.04[e]	37.66 ± 0.65[b]	*na*	16.52 ± 0.13[e]	12.54 ± 0.11[e]
cis-*β*-Ocimene	≥100	≥100	41.65 ± 0.56[a]	–	–	–	–	–	63.03 ± 0.78[f]	08.61 ± 0.06[f]	*na*	34.84 ± 0.46[d]	55.04 ± 0.78[f]	28.04 ± 0.23[f]
Caryophyllene oxide	≥100	≥100	38.46 ± 0.78[a]	–	–	–	–	–	50.01 ± 0.13[e]	65.78 ± 0.59[c]	*na*	*na*	29.98 ± 0.26[g]	*na*
BHT[b]	54.68 ± 0.47[a]	15.24 ± 0.63[b]	8.42 ± 0.25[d]	–	–	–	–	–	–	–	–	–	–	–
α-TOC[b]	14.55 ± 0.26[b]	9.52 ± 0.36[c]	19.53 ± 0.34[e]	–	–	–	–	–	–	–	–	–	–	–
Galanthamine[b]	–	–	–	–	–	–	–	–	91.45 ± 0.84[g]	78.92 ± 0.65[g]		–	–	–
Thiourea[b]	–	–	–	–	–	–	–	–	–	–	93.47 ± 1.48[c]	–	–	–
Kojic acid[b]	–	–	–	–	–	–	–	–	–	–	–	96.75 ± 1.42[e]	–	–
Oleanolic acid[b]	–	–	–	–	–	–	–	–	–	–	–	–	42.38 ± 0.48[h]	–
Epicatechin gallate[b]	–	–	–	–	–	–	–	–	–	–	–	–	–	83.84 ± 1.78[g]

Notes: [a]Values expressed are means ± S.E.M. of three parallel measurements and values were calculated according to the negative control. Values with different letters in the same column were significantly different ($p < 0.05$). SI: Selectivity indices. **S1:** *H. empetrifolium* subsp. *empetrifolium* collected in Antalya, **S2:** *H. hyssopifolium* var. *elongatum* collected in Gümüşhane, **S3:** *H. pseudolaeve* collected in Van. [b]Standard compound. *na*: Not active.

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Table 3. Antimicrobial activities of *Hypericum* species essential oils (MIC values as µg/mL)

Samples	Microorganisms				
	Gram-negative		Gram-positive		Yeast
	E. coli	*P. aeruginosa*	*S. aureus*	*S. pyogenes*	*C. albicans*
S1	200 ± 0.5[a]	200 ± 0.2[a]	200 ± 0.3[a]	200 ± 0.7[a]	200 ± 0.7[a]
S2	50 ± 0.6[b]	200 ± 0.1[a]	60 ± 0.1[b]	50 ± 0.4[b]	50 ± 0.3[b]
S3	80 ± 0.6[c]	200 ± 0.4[a]	60 ± 0.3[b]	50 ± 0.8[b]	50 ± 0.8[b]
PC	7.82 ± 0.40[d]	*na*[b]	95 ± 0.37[c]	7.82 ± 0.10[c]	3.13 ± 0.20[c]

Note: Values with different letters in the same column were significantly different ($p < 0.05$). **S1:** *H. empetrifolium* subsp. *empetrifolium* collected in Antalya, **S2:** *H. hyssopifolium* var. *elongatum* collected in Gümüşhane, **S3:** *H. pseudolaeve* collected in Van, *PC:* positive controls that are ampicillin for bacteria and fluconazole for Yeast, *na*: not active.

Table 4. Docking scores and scoring components for the investigated ligands

Compounds	AChE			BChE			Tyrosinase			Elastase			Collagenase		
	VdW	es	DockS	VdW	es	DockS	VdW	es	DockS	VdW	es	DockS	VdW	es	DockS
2-Methyloctane	−29.04	−0.03	−29.07	−28.13	−0.01	−28.14	−28.58	−0.06	−28.64	−25.25	−0.13	−25.40	−28.55	−0.08	−28.62
α-Pinen	−16.77	−0.09	−16.86	−23.99	−0.18	−24.18	−21.85	−0.03	−21.88	−16.95	−0.12	−17.08	−22.16	−0.182	−22.34
cis-β–Ocimene	−28.44	−0.91	−29.35	−29.76	−27.33	−0.44	−27.77	−0.41	−30.16	−26.24	−0.01	−26.25	−30.03	−0.26	−30.29
Caryophylene oxide	−20.96	-0,65	−21.69	−31.63	−0.05	−31.63	−20.29	-0,46	−20.75	−22.33	−0.071	−22.40	−25.36	−0.46	−25.82
Galantamine[a]	−66.53	−11.68	−78.21	−63.05	−7.14	−71.19	–	–	–	–	–	–	–	–	–
Kojic acid[a]	–	–	–	–	–	–	−69.19	−10.97	−80.16	–	–	–	–	–	–
Olenaionic acid[a]	–	–	–	–	–	–	–	–	–	−31.39	-0,86	−32.25	–	–	–
Epicatechin gallate[a]	–	–	–	–	–	–	–	–	–	–	–	–	−7.20	−53.90	−61.11

Note: [a]Standard compound, VdW: Van der Waals energy, es: electrostatic energy, DockS: Dock score energy, all energy parameters in kcal/mol.

the highest inhibitory activity in both acetyl- (inhibition %: 72.65 ± 0.13) and butyryl-cholinesterase (inhibition%: 70.73 ± 1.69) inhibition. However, the urease enzyme activity could not be detectable in any of the three samples investigated (Table 2). While the essential oil samples did not exhibit urease activity, the moderate activity observed in some of their major components suggests this is related to antagonistic interactions. Sample S3 was identified as the most effective for inhibiting tyrosinase activity, compared to the other two samples (inhibition%: 59.62 ± 0.82). In concordance, it was determined that sample S3-dependent inhibition of the elastase (inhibition%: 38.12 ± 0.18) and collagenase (inhibition%: 22.09 ± 0.10) activities was also higher than in the other two samples (Table 2).

Akdeniz et al. (Akdeniz et al., 2020) reported that the essential oils of *H. lysimachioides* var. *spathulatum* have low cholinesterase inhibitory activity. Furthermore, Sur et al. (Sur et al., 2020) showed the neuropharmacological potential of essential oils of *H. scabrum*, in another study. On the other hand, previous studies have demonstrated the wound-healing effects of extracts derived from various *Hypericum* species, as well as their inhibitory activities against the enzymes tyrosinase, elastase, and collagenase (Silva et al., 2021). However, while studies on the inhibitory activities of the essential oils of *H. empetrifolium* subsp. *empetrifolium* against these enzymes are available in the literature (Akdeniz et al., 2023), no studies have been found that examine the inhibitory effects of the essential oils of *H. hyssopifolium* var. *elongatum* and *H. pseudolaeve*. To the best of our knowledge, the present study is the first to demonstrate the inhibitory activities of the essential oils of these two species against the aforementioned enzymes.

3.4 Antimicrobial Activity

Although many studies have reported antimicrobial activities of *Hypericum* species (Bertoli et al., 2011), the antimicrobial activities of the species used in this study have not been reported. The results showed that all used *Hypericum* samples have high antimicrobial activities although they have different activity rates. Indeed, they could be arranged as S2 > S3 > S1 according to their antimicrobial activities, with MIC values ranging from 50 to 200 µg/mL (Table 3). Our results are in concordance with the previous studies which showed that *α*-pinene, *β*-pinene, and caryophyllene have antimicrobial potential (Guedes et al., 2011).

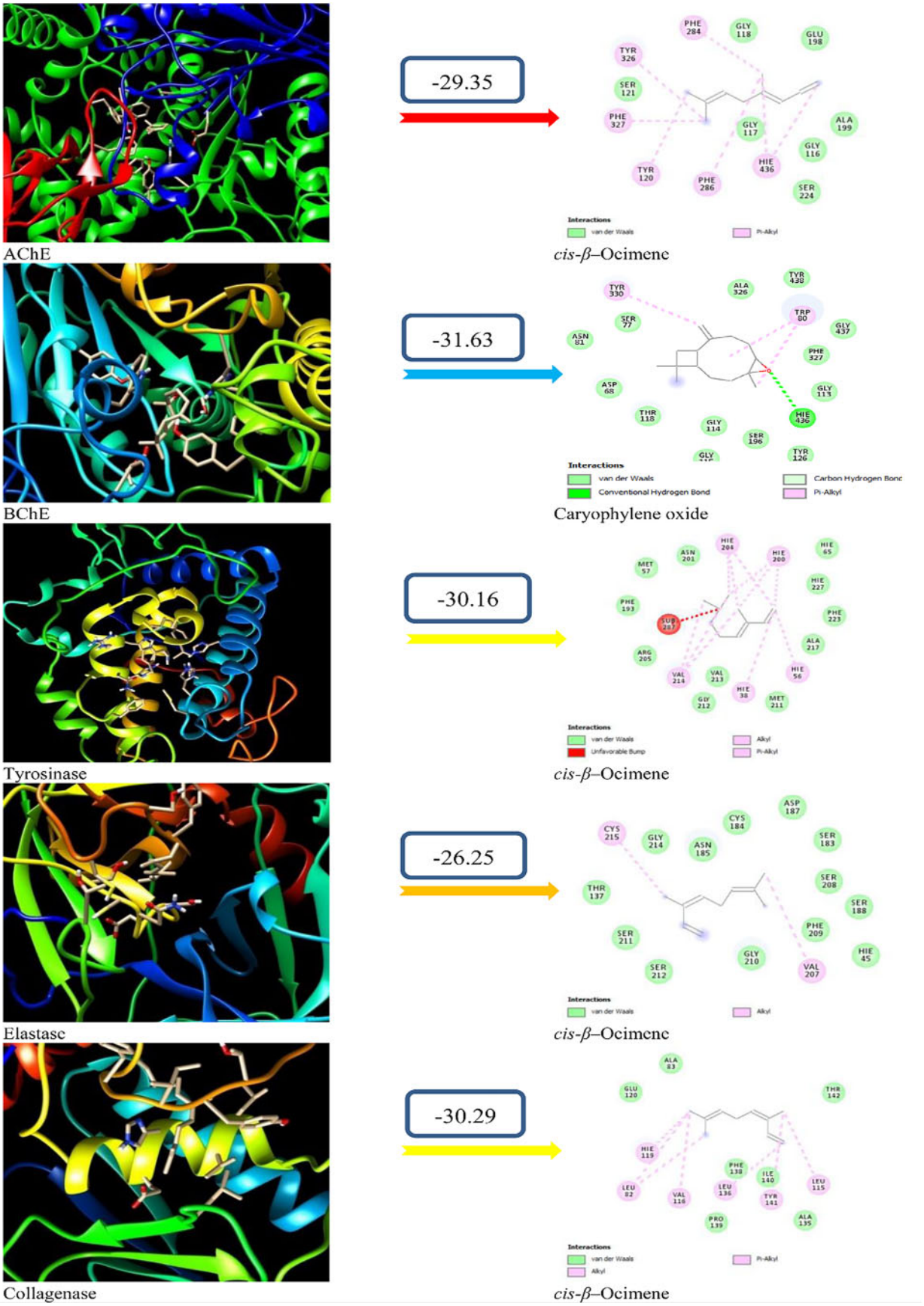

Figure 1. Predicted binding poses and two-dimensional interaction diagrams of selected ligands in the target enzymes

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3.5 In vitro and in silico studies of major components

The enzyme-inhibitor interactions were assessed using docking calculations, in which binding free energy was recorded for each possible position. The docking scores obtained for the investigated compounds were as follows: for AChE is from −16.86 kcal/mol to −29.35 kcal/mol, for BChE is from −0.44 kcal/mol to −31.63 kcal/mol, for tyrosinase is from −20.75 kcal/mol to −30.16 kcal/mol, for elastase is from −17.08 kcal/mol to −26.25 kcal/mol, and for collagenase is from −22.34 kcal/mol to −30.29 kcal/mol (Table 4). It was shown that the main constituents exhibited activity with comparable interactions in all the enzymes examined because their chemical structures are similar, with van der Waalls and π-alkyl interactions being predominant (Figure 1).

In vitro enzyme activities of 2-methyloctane, α-pinene, *cis*-β-ocimene, and caryophyllene oxide at 100 µg/mL were investigated and showed variable inhibitory activity against the corresponding enzymes. Indeed, we have seen that the essential oil sample S3 has the highest activity in the inhibition of AChE (inhibition%: 72.65 ± 0.13) compared to other samples, as described above. In concordance, *cis*-β-*ocimene*, which is a major component of S3, has the highest inhibition ratio in the AChE activity (inhibition%: 63.03 ± 0.78). Consistent with the activity of S3 (inhibition %: 59.62 ± 0.82, 38.12 ± 0.18, and 22.09 ± 0.10, respectively), *cis*-β-*ocimene* (inhibition %: 34.84 ± 0.46, 55.04 ± 0.78, and 28.04 ± 0.23, respectively) was also found to be the most effective compound in inhibiting tyrosinase, elastase, and collagenase activities. Similarly, another major component of S3 (inhibition%: 70.73 ± 1.69), which has the highest inhibitory activity against BChE, is caryophyllene oxide (inhibition%: 65.78 ± 0.59).

4 Conclusion

The essential oil compositions of *H. empetrifolium* subsp. *empetrifolium, H. hyssopifolium* var. *elongatum*, and *H. pseudolaeve* species were determined by GC-MS/FID, and the biological activities of these samples were investigated in detail.

It was determined that the essential oil samples and their major components investigated in this study exhibit similar enzyme-inhibitory activities *in vitro* and *in silico*. It has been determined that the S1 sample exhibits prominent antioxidant activity, whereas the S3 sample shows the highest inhibition of the tested enzymes among the three oils.

In conclusion, while the essential oils of the *Hypericum* species examined reveal promising findings regarding various biological activities, the current results are preliminary in nature; further research is needed to elucidate the mechanisms of action of these compounds, comprehensively evaluate their safety and toxicity profiles, and validate their biological effects using appropriate *in vivo* models.

The main limitations of this study are that the research was restricted to three *Hypericum* species, biological activities were evaluated only using *in vitro* and *in silico* methods, and the mechanisms of action have not been fully elucidated. Furthermore, given the cytotoxic effects observed in healthy cells, the safety profiles and biological activities of the essential oils must be validated through further mechanistic studies and appropriate *in vivo* models.

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MA, IY and AE: Writing, Editing, Data curation, Visualization, Investigation, LV, MF and AE: Writing, Editing, Investigation, Data curation, EA and EVO: Conceptualization, Supervision, Writing, Editing, Methodology, Investigation, MF and MY: Investigation, Data curation, Writing, AE and MA: Methodology, Data curation, AE, IY and LV: Writing, Data curation, MA and AE: Methodology, Data curation. MA, IY and AE wrote and revised the manuscript, LV: revised the manuscript. All authors read and approved the final manuscript.

Availability of Data and Materials

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request. Source data are provided with this paper.

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The authors affirm that no Artificial Intelligence (AI) tools were employed in the preparation of this manuscript, which includes language editing, figure generation, or image editing while Claude AI was employed to aid in the creation of the graphical abstract.

Conflicts of Interest

The authors declare that they do not have any conflict of interest.

Supporting Information

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