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FULL PAPER

LC-MS/MS and GC-MS profiling, antioxidant, enzyme inhibition, and antiproliferative activities of *Thymus leucostomus* HAUSSKN. & VELEN. extracts

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Abstract

The chemical composition as well as antioxidant, antiproliferative, and enzyme inhibition activities of extracts from aerial parts of *Thymus leucostomus* HAUSSKN. & VELEN. obtained with hexane, methanol, and water were evaluated. Results showed that the methanol extract had significantly ($p < 0.05$) the highest total phenolic content (TPC; 107.80 mg GAE/g) and total flavonoids content (TFC; 25.21 mg RE/g) followed by the aqueous extract (102.72 mg GAE/g and 20.88 mg RE/g, respectively). LC-MS/MS-guided profiling of the three extracts revealed that rosmarinic acid (34.8%), hesperetin (42.9%), and linoleic acid (18%) were the dominant compounds in the methanol, aqueous and hexane extracts, respectively. GC-MS analysis of the hexane extract showed that ɣ-sitosterol (29.9%) was the major constituent. The methanol extract displayed significantly ($p < 0.05$) the highest Cu^{++}, Fe^{+++}, and Mo(VI) ions scavenging and reducing properties while the aqueous extract exerted significantly ($p < 0.05$) the highest metal chelating power (42.51 mg EDTAE/g). Both the hexane and methanol extracts effectively inhibited the acetylcholinesterase enzyme (2.63 and 2.65 mg GALAE/g, respectively) while the former extract exerted significantly ($p < 0.05$) the highest butyrylcholinesterase (2.32 mg GALAE/g), tyrosinase (19.73 mg KAE/g), and amylase (1.16 mmol ACAE/g) inhibition capacity. The aqueous extract exhibited the best glucosidase inhibition property (0.49 mmol ACAE/g). The methanol and hexane extracts exerted a higher cytotoxic effect on HT-29 (IC_{50}: 8.12 µg/mL) and HeLa (IC_{50} = 8.08 µg/mL) cells, respectively. In conclusion, these results provide valuable insight into the potential use of *T. leucostomus* bioactive extracts in different pharmaceutical applications.

KEYWORDS

antioxidant, antiproliferative, chemical profile, enzyme inhibition, *Thymus leucostomus*

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1 | INTRODUCTION

Aromatic plants have been used since ancient times as food additives for their aroma and flavor and to preserve food against pathogenic microorganisms.[1–6] They are also widely used to treat several human, animal, and plant diseases due to their variable biological activities like antimicrobial, insecticidal, and antioxidant properties.[7] Volatile and nonvolatile metabolites from different organs of aromatic plants include terpenes, phenols, sterols, and hydrocarbons among others.[7,8]

Plants belonging to the genus *Thymus* (family Lamiaceae) are aromatic herbs with versatile culinary and medicinal applications.[9] The genus *Thymus* comprises about 215 species and is found mainly in the Mediterranean region as herbaceous perennials or small shrubs.[10,11] They are used in different traditional systems to cure diseases associated with the respiratory, urinary, and digestive systems, hypertension, and diabetes.[12] The aerial parts of *Thymus serpyllum* L. (wild thyme) have been reported in the ethnomedicine for the treatment of respiratory and gastrointestinal disorders [13] while *Thymus daenensis* Celak herb is known for its antioxidant, antimicrobial, insecticidal, and cytotoxic properties.[14] The North African *Thymus* species have been known to treat diabetes as well as several circulatory, digestive, respiratory, and urinary disorders[9,15] whereas garden thyme (*Thymus vulgaris* L.) has chiefly been reported for wound healing due to its antiseptic properties.[16] About 39 *Thymus* species and 64 taxa are recorded in the flora of Turkey with 47% being endemic.[17] They are known locally as kekik and many of them are consumed as herbal tea and condiment as well as for medicinal purposes.[18] Many of them were examined for their essential oils composition, which was found to be mainly dominated by thymol and/or carvacrol.[18–22] Besides, they were mainly evaluated for their antimicrobial and antioxidant activities. For example, essential oils extracted from *Thymus leucostomus* growing in Turkey are rich in monoterpenes with carvacrol, cymene, thymol, α-terpinyl acetate, borneol, and linalool as major compounds.[23,24] Additionally, the essential oil from the leaves is reported to possess high antibacterial, antifungal, and antioxidant activities.[23]

Recently, solvent extracts of *Thymus* species have become an appealing target for identifying promising bioactive extracts/molecules with potential applications.[25] For example, aqueous extracts of *Thymus longicaulis* subsp. *chaubardii* and subsp. *Longicaulis* possess significant antioxidant activity.[18] Hydromethanolic and methanolic extracts of *Thymus algeriensis* have high antioxidant, antimicrobial, and anti-acetylcholinesterase activity.[26,27] Hydroethanolic and aqueous extracts of *Thymus citriodorus* and *T. vulgaris* are found to display high antiproliferative activity.[28] Generally, investigations on the chemical composition and biological activities of organic and aqueous extracts from Turkish *Thymus* species are limited and few studies have been published.[29] Hence, the present study aimed to investigate the chemical constituents and biological activities of extracts from aerial parts of *T. leucostomus* Hausskn. & Velen. Chemical composition was examined using spectrophotometric, GC/MS, and HPLC-MS/MS techniques. The antioxidant activity of extracts was determined by evaluating their capacity to scavenge free radicals and to chelate/reduce metal ions. Their ability to inhibit enzymes implicated in diabetes, skin hyperpigmentation, and Alzheimer's diseases was also evaluated and their cytotoxic effect was investigated against prostate cancer, human breast, HeLa cervical carcinoma, human colorectal adenocarcinoma, and human gastric carcinoma cell lines.

2 | RESULTS AND DISCUSSION

2.1 | Chemical composition

2.1.1 | Total phenolic (TPC) and total flavonoid contents (TFC) of extracts

The TPC and TFC on hexane, methanol, and aqueous extracts from aerial parts of *T. leucostomus* were determined and results are presented in Table 1. The methanol extract showed significantly ($p < 0.05$) the highest TPC (107.80 mg GAE/g) and TFC (25.21 mg RE/g) followed by the aqueous extract (102.72 mg GAE/g and 20.88 mg RE/g, respectively) while the hexane extract revealed the least contents (29.93 mg GAE/g and 13.14 mg RE/g, respectively). Comparing these results with published data, it was noted that the TPC value obtained in the present study was comparable to that obtained from methanolic extract (104.47 mg GAE/g) of the aerial part of Turkish *Thymus zygioides* but was higher than its aqueous extract (67.25 mg GAE/g). Additionally, both extracts recorded higher TFC (57.57–91.86 mg QE/g) than those obtained in the present study.[29] In turn, these values were generally superior to published data of TPC and TFC of methanolic extract of Tunisian *T. algeriensis* leaf, which were in the range of 14.8–34 mg GAE/g and 3–11 mg RE/g, respectively.[26] In fact, the distribution of phenolic contents varies according to the genotype of plants and depends on many exogenous and endogenous factors.[30]

2.1.2 | LC-MS/MS-guided profiling of extracts

Metabolites were identified based on their MS^2 fragments retrieved from literature and online resources (Mass Bank) and results are summarized in Table 2. A total of 15 compounds were identified

TABLE 1 Total phenolic and flavonoid contents of *Thymus leucostomus* aerial part extracts.

Extracts	Total phenolic content (mg GAE/g)	Total flavonoid content (mg RE/g)
Hexane	29.93 ± 0.42^c	13.14 ± 0.74^c
Methanol	107.80 ± 0.42^a	25.21 ± 0.57^a
Water	102.72 ± 0.68^b	20.88 ± 0.42^b

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

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

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TABLE 2 LC-ESI-MS/MS analysis of the *n*-hexane (Hex), aqueous, and methanol (MeOH) extracts of *Thymus leucostomus*.

Peak no.	t_R	$[M-H]^-$	MS^2	Identification	Chemical class	Relative area (%) Hex	MeOH	Water	Ref.
1	0.76	377	**191**, 93	Quinic acid derivative	Quinic acid derivatives	–	4.1	–	[31, 32]
2	0.82	191	**191**	Quinic acid		–	–	5.1	[33]
3	6.60	595	287, **151**, 135	Hydroxylated naringin	Flavonoids	–	28.1	–	[34]
4	7.13	579	**271**, 151, 119	Naringin		–	3.6	–	[35]
5	7.42	359	197, 179, **161**, 135, 73	Rosmarinic acid	Caffeic acid derivatives	–	34.8	33.3	[36, 37]
6	8.41	287	**135**, 107, 83, 65	Eriodictyol (tetrahydroxy flavanone)	Flavonoids	–	–	5.7	[38]
7	8.54	593	309, **285**, 241, 164	Kaempferol rutinoside		–	10.1	–	[39–41]
8	9.29	271	177, 165, **151**, 119, 107, 93	Naringenin		–	1.6	4.2	[42]
9	9.60	301	**301**, 286, 242, 199, 174, 164	Hesperetin		–	2.8	42.9	[43]
10	11.7	285	**285**, 270, 243, 164, 151	Tetrahydroxy flavone or methoxylated naringenin		4.0	–	8.5	[44, 45]
11	14.7	293	**275**, 195, 179	Hydroxylinolenic acid		8.1	–	–	[46]
12	21.8	279	**180**	Linoleic acid (C18:2)	Fatty acids	13.8	5.9	–	[47, 48]
13	24.2	255	247, 219, 208, 170, 143, **120**, 119	Palmitic acid (C16:0)		7.6	3.9	–	[47]
14	24.7	281	235, 203, 96, **36**	Oleic acid (C18:1)		6.6	–	–	[47]
15	27.9	283	**283**, 268, 209, 125	Stearic acid (C18:0)		1.5	–	–	[47]

Note: MS^2 in bold refers to the major (base peak) fragment.
Abbreviation: t_R, retention time in minutes.

belonging to the flavonoids, quinic and caffeic acid derivatives, and fatty acids (Figure 1). Quinates (eluted early in the chromatogram) were only detected in methanol and/or water extracts, whereas fatty acids (eluted late in the chromatogram) were solely observed in hexane and methanol extracts. Flavonoid glycosides were detected primarily in the methanol and aqueous fractions yet in different ratios. The assessment of quinic acid and its derivative (peaks 1 and 2) was based on the observation of the ion at *m/z* 191 corresponding to quinic acid. Peak 5 with $[M-H]^-$ molecular ion peak at *m/z* 359 produced fragment at *m/z* 197 (hydroxylated hydrocaffeic acid), 179 (caffeoyl ion), and 161 (dehydrated caffeoyl ion) supporting the structure of rosmarinic acid. Naringin (peak 4) showed an $M-H^-$ peak at *m/z* 579 and a fragment ion at 271 corresponding to the loss of rutinose moiety and another fragment at *m/z* 151 due to retro Diels–Alder of the fragment ion at 271. Hydroxy naringin (peak 3) showed 16 mass units over naringin yet with similar fragments corresponding to the neutral loss of rhamnose and glucose (*m/z* 287). Peak 7 with a deprotonated molecular ion peak at *m/z* 593 corresponding to a rutinoside derivative of a tetrahydroxy flavone like kaempferol with a fragment ion at *m/z* 285 corresponding to the loss of rhamnose (146 amu) and glucose (162 amu). Peak 6 showed an $[M-H]^-$ peak at *m/z* 287 with a fragment at *m/z* 135 characteristic of eriodictyol.

Few data have been reported on the chemical composition of *T. leucostomus*. A study conducted on the 70% methanol extract reported the identification of phenolics like rosmarinic acid, luteolin, naringenin, and apigenin glucuronide.[49] In the present study, rosmarinic acid, naringenin, and naringin were similarly detected in the polar fraction. Phenolic acids such as quinic acid, caffeic acid, and their derivatives have previously been described in thymus species like *T. vulgaris*, *T. serpyllum*, *Thymus comosus*, *Thymus zygis*, *Thymus pulegioides*, and *Thymus fragrantissimus*.[50–55] Naringenin and eridioctyol flavanones and their glycosides have previously been detected in *T. vulgaris*, *Thymus herba-barona*, and *Thymus webbianus*.[54,56–58] Luteolin has been reported in several *Thymus* species, however, kaempferol and its glycosides were generally minor.[54] Previous work on *T. zygis*, *T. pulegioides*, and *T. fragrantissimus* showed that the ratio of polyunsaturated to saturated fatty acids (PUFA/SFA) was significantly high ranging from 1.62 to 1.83. These data are in agreement with our results, where the PUFA/SFA ratio is approximately 1.52 in the hexane extract of *T. leucostomus*.[59] It is worth noting that the consumption of food and spice high in PUFA/SFA ratio is strongly recommended by the World Health Organization to minimize the risk of cardiovascular disorders.[60]

2.1.3 | GC-MS-guided profiling of the hexane extract

GC-MS investigations on the hexane extract revealed the presence of saturated long-chain alkanes (from C_{23} to C_{33}) as major components

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FIGURE 1 Diversity of metabolites in *Thymus leucostomus* hexane (in green), methanol (in pink), and water (in purple) extracts. Those compounds detected in more than one extract are presented in red.

TABLE 3 GC-MS investigations on the *n*-hexane extract of *Thymus leucostomus*.

Peak no.	t_R	Annotated compounds	$KI_{exp.}$	KI_{rep}	Relative area (%)	Formula	Chemical class	Identification
1	40.2	Tricosane	2289	2298	1.31	$C_{23}H_{48}$	Saturated alkanes	MS/KI
2	41.4	Oleamide	2358	n.d.	7.20	$C_{18}H_{35}NO$	Fatty acid amides	MS
3	41.9	Tetracosane	2389	2400	3.19	$C_{24}H_{50}$	Saturated alkanes	MS/KI
4	43.5	Pentacosane	2488	2498	4.90	$C_{25}H_{52}$		MS/KI
5	45.1	Hexacosane	2588	2598	5.96	$C_{26}H_{54}$		MS/KI
6	46.6	Heptacosane	2687	2699	7.20	$C_{27}H_{56}$		MS/KI
7	48.1	Octacosane	2787	2798	6.51	$C_{28}H_{58}$		MS/KI
8	49.5	Nonacosane	2886	2899	7.36	$C_{29}H_{60}$		MS/KI
9	50.4	2-Methyloctacosane	2949	n.d.	–	$C_{29}H_{60}$		MS
10	50.9	Triacontane	2986	2998	4.55	$C_{30}H_{62}$		MS/KI
11	52.2	Hentriacontane	3086	3099	8.32	$C_{31}H_{64}$		MS/KI
12	53.1	3-methyl hentriacontane	3160	3169	–	$C_{32}H_{66}$		MS/KI
13	53.4	Dotriacontane	3185	3200	3.60	$C_{32}H_{66}$		MS/KI
14	54.3	Campesterol	3247	n.d.	3.57	$C_{28}H_{48}O$	Sterols	MS
15	54.9	Tritriacontane	3285	3300	6.33	$C_{33}H_{68}$	Saturated alkanes	MS/KI
16	55.7	γ-Sitosterol	3338	3351	29.98	$C_{29}H_{50}O$	Sterols	MS/KI
		Total identified compounds (%)			99.98			
		Fatty acid amide (%)			7.20			
		Saturated alkane (%)			59.23			
		Sterols (%)			33.55			
		Triterpene (%)			–			

Note: Identification was done based on comparing the mass spectrum and the experimental Kovat index with the reported data on the NIST-17 database (National Institute of Standards and Technology) and with previously published data.

Abbreviations: KI_{exp}, Kovat index experimental; KI_{rep}, Kovat index reported; MS, mass data; n.d., not detected; t_R, retention time.

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representing about 59.2% of the total metabolites with hentriacontane being the dominating alkane (8.3%) (Table 3). Sterols are the second predominant class reaching ca. 33.5% of the total peak areas with ɣ-sitosterol (29.9%) being the major one. Oleamide (7.2%) was the only fatty amide detected in the hexane extract. The present study is the first report on the chemical composition of the hexane extract of *T. leucostomus*. Previous work was performed on essential oil showing the presence of thymol and borneol as major constituents[61]

2.2 | Antioxidant properties

Antioxidants contribute to the prevention of a wide range of diseases by scavenging or neutralizing reactive oxygen species that accumulate in different tissues.[62] The antioxidant properties of hexane, methanol, and aqueous extracts from aerial parts of *T. leucostomus* were evaluated through their capacity to scavenge free radicals and to reduce/chelate metals (Table 4). The methanol extract exerted significantly ($p < 0.05$) the highest antioxidant activity in all tested assays except in the metal chelating one where the highest activity was recorded from the aqueous extract. This was followed by the aqueous extract, which displayed significantly ($p < 0.05$) better antioxidant activity (5/6 assays) with higher remarkable values than the hexane extract. The methanol extract had a 1.6-fold higher ability to scavenge the DPPH radical than the ABTS one and a 1.7-fold higher power to reduce the Cu^{++} ions than the Fe^{+++} ones. On the other hand, the aqueous extract was 2.5 and 3.3 times more effective to chelate iron than the methanol and hexane extracts, respectively. The antioxidant activity of *T. leucostomus* was only reported for the essential oil extracted from the leaf by Elkiran and Avsar using the DPPH assay and their results revealed that it possessed significant antioxidants.[23] In another study, it was suggested that the antioxidant activity of aqueous and methanolic extracts of *Thymus longicaulis*,[18,63] *Thymus capitatus*,[64] and *Thymus mastichina*[65] was attributed to the richness of these extracts in rosmarinic acid, which was also detected in the present study in considerable amount (ca. 34% in both methanol and water extracts as indicated from the relative area under each peak). Also, naringin and naringenin, which were identified in the methanolic extract in the present study, were reported to contribute to the antioxidant of *Thymus* species.[65] Also, the present results were in accordance with previous studies reporting that polar solvent extracts revealed higher antioxidant activity than non-polar solvent ones.[66]

2.3 | Enzyme inhibition

Enzyme inhibitors remain a key target for the development of drugs for treating many health disorders including, skin pigmentation, diabetes, and Alzheimer's disease.[67] To the best of our knowledge, only a few studies reported the enzyme inhibition properties of *Thymus* species. In the present study, extracts from the aerial parts of *T. leucostomus* were examined against acetylcholinesterase (AChE), butyrylcholinesterase (BChE), tyrosinase (Try), amylase, and glucosidase enzymes. Results are presented in Table 5. The hexane and methanol extracts exerted comparable capacity ($p > 0.05$) to inhibit the AChE (2.63 and 2.65 mg GALAE/g, respectively). Besides, the hexane extract revealed significantly ($p < 0.05$) the highest anti-BChE (2.32 mg GALAE/g), anti-Try (19.73 mg KAE/g), and anti-α-amylase (1.16 mmol ACAE/g) activities. Although the aqueous extract did not inhibit the two cholinesterase enzymes and tyrosinase one, it showed significantly ($p < 0.05$) the highest activity against the α-glucosidase enzyme

TABLE 4 Antioxidant properties of *Thymus leucostomus* aerial part extracts.

Methods	DPPH (mg TE/g)	ABTS (mg TE/g)	CUPRAC (mg TE/g)	FRAP (mg TE/g)	PBD (mmol TE/g)	MCA (mg EDTAE/g)
Hexane	28.45 ± 0.11^c	33.82 ± 0.62^c	85.63 ± 9.78^c	43.87 ± 0.57^c	2.22 ± 0.08^b	13.70 ± 2.32^b
Methanol	433.91 ± 4.02^a	269.50 ± 5.70^a	569.72 ± 10.59^a	336.84 ± 7.61^a	2.93 ± 0.22^a	16.78 ± 0.81^b
Water	292.65 ± 3.06^b	224.50 ± 4.68^b	402.19 ± 6.90^b	252.46 ± 1.44^b	1.96 ± 0.03^c	42.51 ± 0.33^a

Note: Values are reported as mean ± SD of three parallel measurements. Different letters indicate significant differences in the tested extracts ($p < 0.05$).
Abbreviations: EDTAE, EDTA equivalent; MCA, metal chelating activity; PBD, phosphomolybdenum; TE, Trolox equivalent.

TABLE 5 Enzyme inhibitory effects of *Thymus leucostomus* aerial part extracts.

Methods	AChE (mg GALAE/g)	BChE (mg GALAE/g)	Tyrosinase (mg KAE/g)	Amylase (mmol ACAE/g)	Glucosidase (mmol ACAE/g)
Hexane	2.63 ± 0.02^a	2.32 ± 0.03	19.73 ± 0.95^a	1.16 ± 0.06^a	0.49 ± 0.02^b
Methanol	2.65 ± 0.13^a	na	4.22 ± 0.52^b	0.72 ± 0.01^b	Na
Water	na	na	na	0.21 ± 0.02^c	0.88 ± 0.01^a

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

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(0.88 mmol ACAE/g). The presence of ɣ-sitosterol as a major compound in hexane extract could be implicated in the cholinesterase, butyrylcholinesterase, and amylase inhibition activity as a recent study revealed that β-sitosterol was effective against Alzheimer's[68] and diabetes.[69] Furthermore, it was found that linoleic acid, which was accumulated in the hexane extract, contributed to the enhancement of digestive enzyme ability.[70]

2.4 | Antiproliferative activity

Cancer accounted for nearly 10 million deaths in 2020 alone, making it an important health concern that requires effective prevention and treatment actions (WHO 2021). Plant extracts are considered an important source of combatting cancer molecules. In the present study, hexane, methanol, and water extracts of *T. leucostomus* aerial parts were evaluated for their antiproliferative effect against five cancer cells (DU-145 prostate cancer cells, MDA-MB-231 human breast, hela cells cervical carcinoma, HT-29 human colorectal adenocarcinoma, and human gastric carcinoma HGC-27 cells) in addition to normal HEK-293 cell line (Table 6). The highest antiproliferative activity with high selectivity on cancer cells was obtained, respectively, from the methanol extract against HT-29 (IC_{50} = 8.12 µg/mL; SI = 6.6) (Figure 2) and hexane one against HeLa (IC_{50} = 8.08 µg/mL; SI = 5.3) cells. Both extracts respectively revealed also considerable antiproliferative activity against DU-145 (IC_{50} = 12.16 µg/mL; SI = 4.4) and HGC-27 (IC_{50} = 12.72 µg/mL; SI = 3.3) cells. Aqueous extract was less toxic against all tested cell lines. A previous study on the antiproliferative activity of *Thymus* species was mainly associated with the composition of their essential oils. Relatively few studies were performed on solvent extracts, for example, the methanolic extract of *T. vulgaris* was shown to possess significant antitumor activity against MCF-7 and MDA-MB-231 breast cancer.[71] Also, the ethanolic extract from *T. cilicicus* inhibited the proliferation of the human alveolar basal epithelial adenocarcinoma (A549).[72] However, rosmarinic acid, which was the dominant constituent of the methanol extract in the present study, was previously isolated from *T. praecox* subsp. *grossheimii* and found to inhibit significantly the HT-29 cells.[73]

TABLE 6 Cytotoxic effects of *Thymus leucostomus* (hexane, methanol, and water) extracts on cancer and normal cell lines (IC_{50}).

Cell lines	Hexane IC_{50} (µg/mL)	Hexane SI	Methanol IC_{50} (µg/mL)	Methanol SI	Water IC_{50} (µg/mL)	Water SI
DU-145	113.7	0.4	12.16	4.4	46.47	0.7
MDA-MB-231	80.62	0.5	117.0	0.5	41.71	0.8
HeLa	8.077	5.3	33.73	1.6	21.55	1.5
HT-29	13.04	3.3	8.12	6.6	27.25	1.2
HGC-27	12.72	3.3	32.11	1.7	31.02	1.1
HEK-293	42.57		53.19		32.94	

Note: SI is calculated as the average of the IC_{50} value in the HEK-293 normal cell line divided by the IC_{50} value in the cancer cell lines obtained in each independent experiment.

Abbreviation: SI, selectivity index.

2.5 | Molecular modeling

In this study, the binding affinity of compounds in *T. leucostomus* extracts against different target enzymes was predicted and the results are shown in Figure 3. The resulting protein-ligand interaction in some selected complexes was examined. Naringin is strongly bound to AChE (Figure 4a) and BChE (Figure 4b) in opposite orientations via multiple H-bonds and van der Waals interactions, with a couple of π-π stacked interactions in the AChE complex. Rosmarinic acid was found to demonstrate strong binding to amylase by forming H-bonds near the entrance to, and deep inside, the active site (Figure 4c). Rosmarinic acid was also buried in the narrow active site of tyrosinase mainly via H-bonds, hydrophobic contacts, and van der Waals interactions (Figure 4d). Interestingly, rosmarinic acid showed an interesting binding mode in the active site of glucosidase through a combination of the interactions described above (Figure 4f).

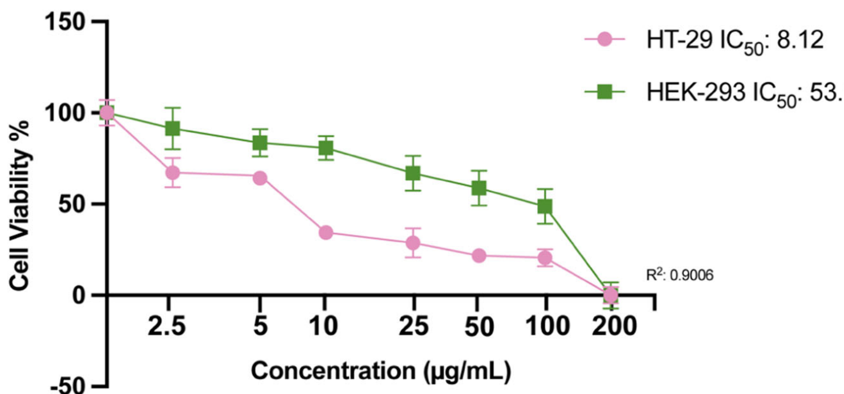

FIGURE 2 Evaluation of cytotoxic effects of *Thymus leucostomus* (methanol) on HT-29 and HEK-293 cell lines by MTT assay. All data are expressed as the mean ± SD from three independent experiments. The IC_{50} value was calculated using the GraphPad Prism 9 program.

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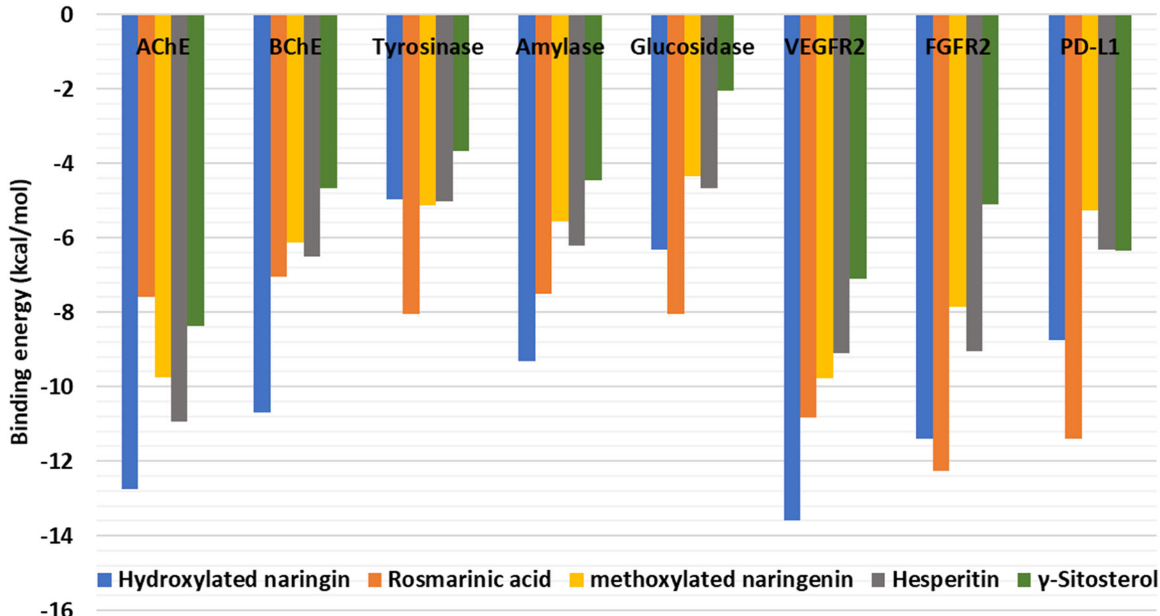

FIGURE 3 Docking score of the dominant compounds in three extracts from *Thymus leucostomus*.

Furthermore, to check their anticancer potential, these compounds were docked into the active site of potential cancer target proteins: FGFR2 [10], VEGFR2 [11], and PD-L1 [12]. Hesperitin, naringin, and rosmarinic acid display potential inhibitory activities against these proteins. Different interactions comprising H-bonds, π-π stacked interaction, and hydrophobic contact as well as multiple van der Waals interactions all over the binding site were formed. The specific interacting residues are shown for the interaction of VEGFR-2 with hesperitin (Figure 5a), FGFR2 with naringin (Figure 5b), and PD-L1 with rosmarinic acid (Figure 5c). Together, these interactions may explain the observed enzyme inhibitory activity of the different extracts from *Thymus leucostomus*.

3 | CONCLUSION

Results obtained from the present study highlighted the chemical composition and potential efficiency of *T. leucostomus* aerial part extracts as antioxidants, inhibiting the proliferation of cancer cells, as well as enzymes associated with hyperpigmentation, neurodegenerative, and endocrine diseases. Rosmarinic acid, hesperitin, and γ-sitosterol are the dominant compounds of the methanol, aqueous, and hexane extracts, respectively, and their presence is suggested to contribute significantly to the biological potential of the extracts. Generally, the methanolic extracts displayed the highest antioxidant in most of the tested assays while the hexane extract revealed the best enzyme inhibition. The two extracts also displayed significant antiproliferative activity. Hence, *T. leucostomus* extracts have many health benefits and properties and can be a promising source for various pharmaceutical applications. Isolation of metabolites responsible for biological activities as well as their mechanism of action is warranted.

4 | EXPERIMENTAL

4.1 | Plant materials and extraction

In 2022, plant materials were obtained from a field investigation (Turkey, B3 Eskişehir: Seyitgazi, Kırka village, roadsides, 39°16′29″N; 30°32′26″E, 960 m, Voucher No: SV3602). Taxonomical identification was carried out by Prof. Selami Selvi. The aerial parts were carefully separated and dried in the shade at room temperature, then ground into powder using a laboratory mill and stored in the dark.

The extracts were prepared using three solvents including *n*-hexane, methanol, and water. Overnight, the plant material (10 g) was macerated with *n*-hexane and methanol (200 mL each)at room temperature. For the preparation of the water extract, the plant material (10 g) was mixed in boiled water for 15 min (as infusion method). The organic solvents were evaporated to remove solvents, whereas the water extract was dried using a freeze-dryer.

4.2 | Assay for TPC and TFC

The quantification of bioactive compounds involved measuring the TPC and TFC, following the procedures outlined in the paper.[74]

4.3 | High-performance liquid chromatography coupled to electrospray mass ionizer (HPLC-ESI-TQ-MS/MS) analysis of *T. leucostomus n*-hexane, methanol, and aqueous extracts

HPLC-ESI-TQ-MS/MS was used to assign the chemical composition of *T. leucostomus*. Extracts were injected into a Shimadzu® 8045 HPLC

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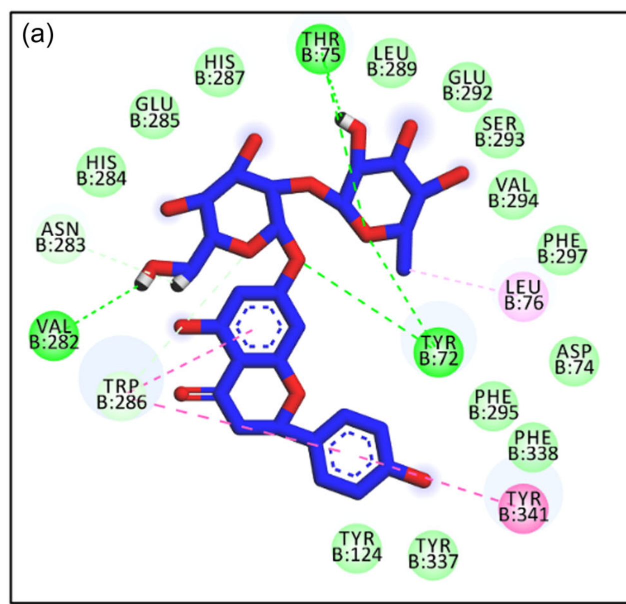

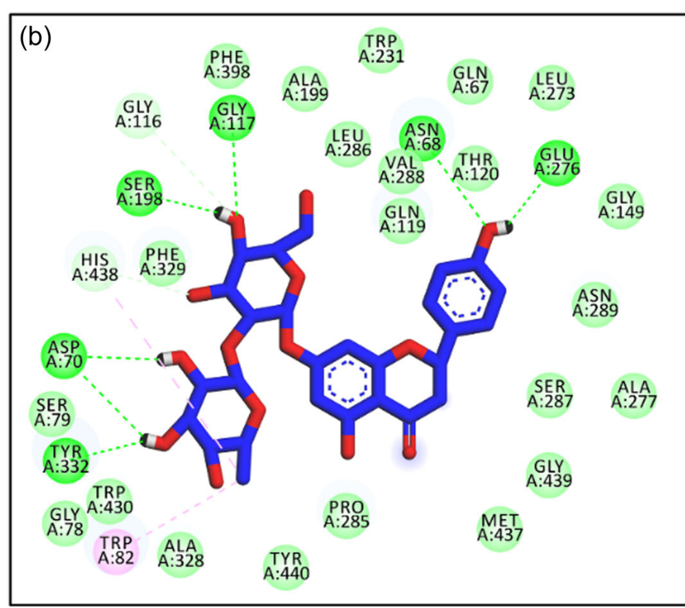

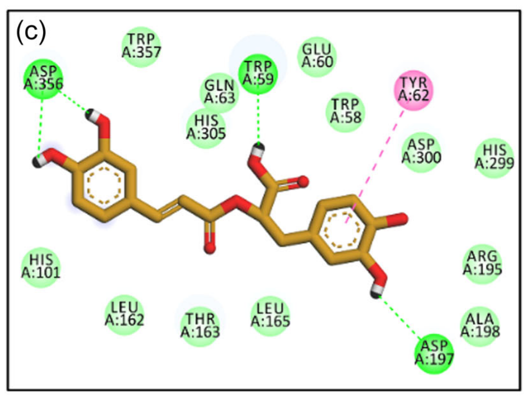

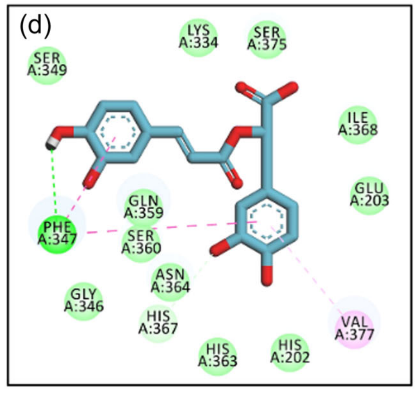

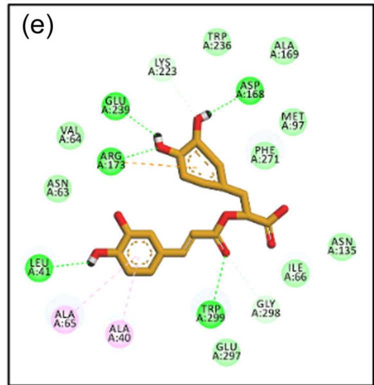

Interactions

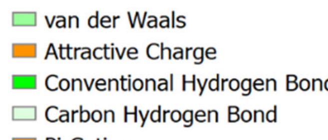

FIGURE 4 Interaction between target enzymes and the dominant compounds in different extracts from *Thymus leucostomus*: (a) AChE and naringin, (b) BChE and naringin, (c) tyrosinase and rosmarinic acid, (d) amylase and rosmarinic acid, (e) glucosidase and rosmarinic acid.

system coupled to a triple quadrupole mass analyzer (Shimadzu® Corporation). The extracts were dissolved in HPLC-grade methanol and filtered using a 0.2 µm PTFE filter. Chromatographic separation was achieved using a Shim-pack Velox C18 reversed-phase column (2.7 µm, 2.1 × 150 mm); the injection volume was set to 10 µL. A mobile phase consisting of water (A) and acetonitrile (B) was applied with the optimized gradient program as follows: 0–5 min, 10% B; 5–15 min, 10%–30% B; 15–22 min, 30%–70% B; 22–30 min, 70%–80% B; 30–35 min, 80%–10% B. Mass detection was performed in negative ESI ionization mode, interface temperature was set at 300°C, desolvation temperature at 526°C, cone gas flow was set to 50 L/h, and the nebulizing gas flow was adjusted to 3 L/min. For collision-induced dissociation (CID) MS/MS measurements, collision energy was adjusted for each peak in a range from 20 to 50 eV and

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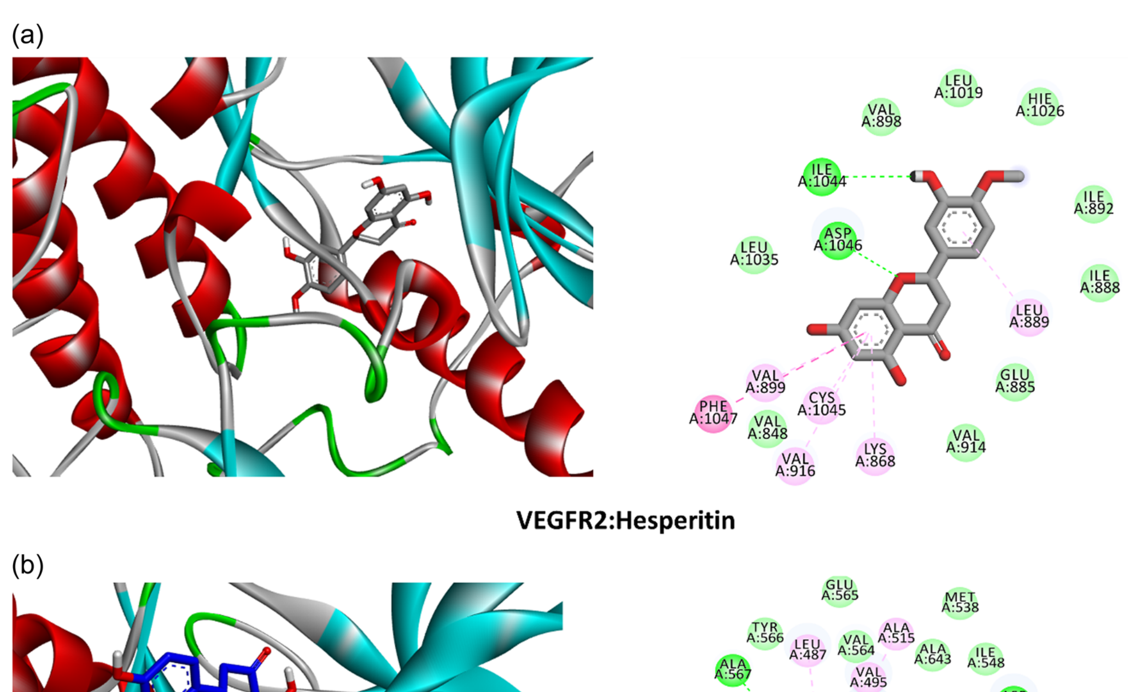

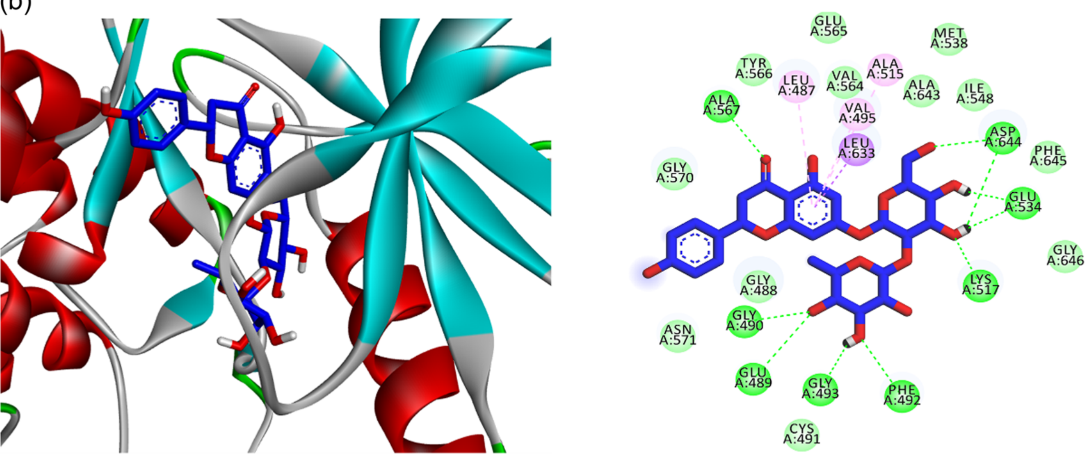

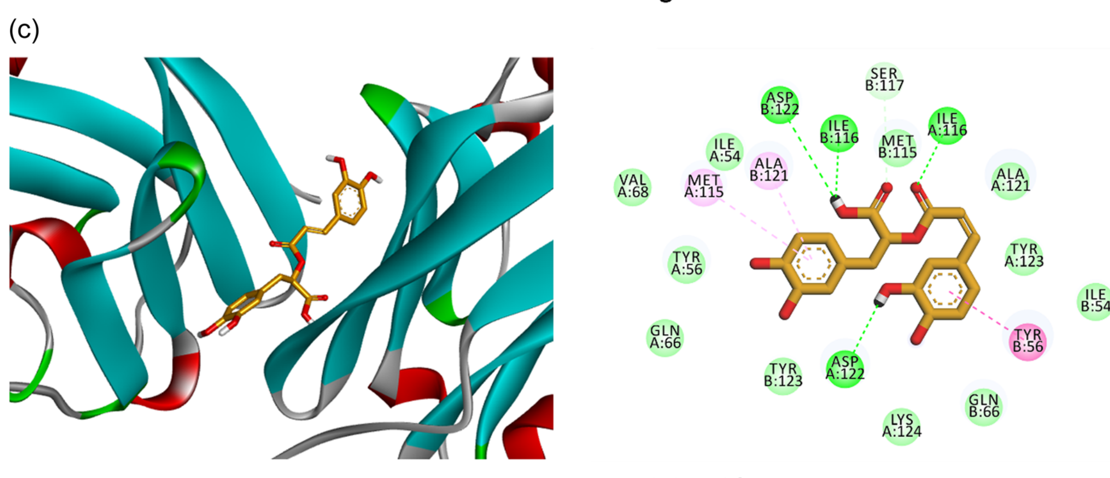

FIGURE 5 Binding mode and interaction of the dominant compounds in different extracts from *Thymus leucostomus* with cancer target proteins: (a) VEGFR-2 and hesperitin, (b) FGFR2 and naringin, and (c) PDL1 and rosmarinic acid.

mass detection was performed in a mass range *m/z* 100–1200. Lab solutions software was used for data processing.

4.4 | GC-MS analysis of *T. leucostomus n*-hexane extract

The hexane extract of *T. leucostomus* was injected into a gas chromatography coupled to mass spectrometry (Shimadzu GCMS-QP 2010) operating in EI mode at 70 eV and mass spectrum acquisition performed in the mass range of 35–500 *amu*. The instrument was equipped with an Rtx-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness: Restek). One microliter sample was injected in a split injection mode with a split ratio of 10:1. Separation was achieved using an initial oven temperature at 45°C for 2 min (isothermal), then gradually increased to 300°C at a rate of 5°C/min (ramp) and kept constant at 300°C for another 5 min (isothermal). Helium was used as a carrier gas with a flow rate set at 1.4 mL/min. The injector temperature was maintained at 250°C. The mass unit interface temperature was set at 280°C and the ion source temperature was adjusted to 200°C. Retention indices (RI) were calculated relative to a homologous series of *n*-alkanes (C_8-C_{30}) injected under the same GC conditions. Identification of the compounds was done by comparing their mass spectra and retention indices with data reported in NIST-17 and Wiley library databases as well as published data in the literature.

4.5 | Antioxidant tests

To assess the antioxidant potential of the extracts, a set of six complementary in vitro spectrophotometric tests were performed. These included the ABTS and DPPH assays, which examine the antioxidants' ability to neutralize free radicals. FRAP and CUPRAC assay, which evaluates the extract's reduction capabilities, as well as metal chelating ability (MCA) and phosphomolybdenum (PBD) assays. Each of these assays, except for MCA, was evaluated using the Trolox standard. The comparison for MCA was made in terms of equivalent EDTA equivalent per gram of extract. All used procedures are given in our previous work.[75]

4.6 | Enzyme inhibitory tests

To assess the inhibitory effects of the tested extracts on various enzymes, we employed acetylcholinesterase (AChE), butyrylcholinesterase (BChE), tyrosinase, amylase, and glucosidase. Details of the experimental procedures can be found in our prior publication.[75] We quantified AChE and BChE inhibition as milligrams of galanthamine equivalents (GALAE) per gram of extract, tyrosinase inhibition as milligrams of kojic acid equivalents (KAE) per gram of extract, and α-amylase and α-glucosidase inhibition as millimoles of acarbose equivalents (ACAE) per gram of extract.

4.7 | Cell assays

4.7.1 | Materials

The following materials were used in this study: The cell culture medium (DMEM-F12/RPMI 1640; Sigma-Aldrich Cat No: D0697/R8758), fetal bovine serum (FBS; Sigma-Aldrich Cat No: F7524), %1 penicillin/streptomycin (Sigma-Aldrich Cat No: P4333), L-glutamine (Sigma-Aldrich Cat No: 59202C), trypsin-EDTA solution (Sigma-Aldrich Cat No: 59417C), dimethyl sulfoxide (DMSO) (Sigma-Aldrich Cat No: PHR1309), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich Cat No: M2128). The culture plates (96-well) were purchased from Nunc (Brand Products).

4.7.2 | Cell culture

The following cancer and normal cell lines obtained from ATCC and stored in liquid nitrogen were used for the study: DU-145 (Prostate Carcinoma), MDA-MB-231 (Breast Adenocarcinoma), HeLa (Cervix Adenocarcinoma), HT-29 (Colon Adenocarcinoma), HGC-27 (Gastric Cancer), and HEK-293 (Normal Human Embryonic Kidney) cells were cultured in DMEM-F12/RPMI-1640 media supplemented with 10% FBS, 100 µg/mL of streptomycin/100 IU/mL of penicillin in incubators at 37°C under humid conditions containing 5% CO_2.

4.7.3 | Cell viability assay

The cytotoxic effects of *T. leucostomus* (hexane, methanol, and water) were assessed using the MTT assay. The cells (DU-145, MDA-MB-231, HELA, HT-29, HGC-27, and HEK-293) were incubated in a 96-well sterile plate for 24 h with 1×10^4 cells per well. The media were removed and the extracts were incubated at doses of 0, 2.5, 5, 10, 25, 50, 100, and 200 µg/mL for 24 h; 10 µL of MTT (0.5 mg/mL) was added into each well as the reactive agent. After 4 h of incubation, the media was removed and substituted with 100 µL of DMSO, after which measurements were performed at OD_{570}-OD_{690} nm using a plate reader (Thermo Multiskan GO, Thermo). Following these measurements, plots were formed and the IC_{50} value was calculated.

4.8 | Molecular modeling

The prepared crystal protein structures of AChE (PDB ID: 6O52),[76] α-amylase (PDB ID: 1B2Y),[77] and BChE (PDB ID: 6EQP)[78] were retrieved.[79] The homology-modeled structures of tyrosinase and α-glucosidase built using the crystal structures of tyrosinase from *Priestia megaterium* (PDB ID: 6QXD)[80] and α-glucosidase from *Mus musculus* (PDB ID: 7KBJ),[81] respectively, were also retrieved.[82] Furthermore, the following crystal structures of cancer target proteins were downloaded from the Protein Data Bank (https://

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www.rcsb.org/)[83]: FGFR2 kinase catalytic domain complexed with ARQ 069 (PDB ID: 3RI1),[84] VEGFR2 complexed with tivozanib (PDB ID: 4ASE),[85] and PD-L1 complexed with small-molecule inhibitor (PDB ID: 5N2F).[86] All protein preparation was done on the PlayMolecule Proteinprepare server.[87] The ChEMBL database (https://www.ebi.ac.uk/chembl/) was used to obtain all ligand 3D structures, which were then optimized using UCSF Chimera.[88] All non-polar hydrogen atoms were merged, and gasteiger charges were added to all atoms using MGLTools 1.5.6 software. Docking simulation was carried out using AutoDock 4.2.6 (https://autodock.scripts.edu)[89] and an adopted docking protocol was applied.[90] Biovia DS Visualizer v4.5 (BIOVIA) examined protein-ligand interaction.

4.9 | Statistical analysis

The experiments were performed in triplicate, and differences between the extracts were compared using an ANOVA and Tukey's test. GraphPad Prism (version 9.2) was used for the analysis.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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