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ORIGINAL PAPER

Assessment of chemical composition and in vitro and in silico anticarcinogenic activity of bee bread samples from Eastern Anatolia (Kars)

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Abstract
In this study, aqueous extracts of bee bread samples collected from five different regions in Eastern Anatolia (Kars, Turkey) were obtained by ultrasonic wave-assisted extraction method. Moisture, ash, acidity and pH values of bee bread samples were measured. Phenolic components of aqueous extracts were determined by LC-HRMS method. The cytotoxicity of these extracts on colon (DLD-1), breast (MCF-7), cervix (HeLa) and prostate cancer (PC-3) cells was determined by the MTT method. The interactions of genistein, naringenin and vitamin C, which are major components of the extracts, with important cancer targets such as human thymidylate synthase, c-MYC transcription factor, cyclin-dependent kinase and androgen receptor prostate cancer targets were investigated by Molecular Docking method. According to the findings, all extracts caused higher cytotoxicity on MCF-7 cancer cells compared to other cell lines, while they caused low cytotoxicity on PC-3 cells. Molecular Docking results show that the naringenin compound, whose anticarcinogenic effects have been supported by previous studies, may be a successful agent in inhibiting the active sites of selected cancer cell targets. When all these results are evaluated, it is recommended that the antitumoral effect of bee bread for the treatment of cancer disease be supported by further studies.

Keywords Bee bread · Anticarcinogenic activity · Chemical composition · Molecular Docking

Introduction

In recent years, there has been an increasing interest in natural products for the purpose of balanced and healthy nutrition due to some undesirable effects of synthetic foods or food additives [1]. In this context, there is increasing interest in research on bee products, which are natural functional products with high nutritional value and positive effects on health [2]. Honeybee products such as honey, pollen, bee bread, royal jelly, propolis, bee venom, and beeswax have been used as traditional medicine since ancient times [3]. The use of honeybee products in areas such as alternative medicine, healthy nutrition, and apitherapy is increasing day by day. Bee products are rich in proteins, carbohydrates, lipids and fatty acids, vitamins, micro and macro elements, and phenolic compounds, and the biologically active properties of bee products are attributed to these rich contents [4, 5]. Bee bread is defined as a honeybee product obtained from pollen collected by bees, processed, fermented, and stored in the hive [3]. There are promising results regarding the antioxidant [6, 7], antimicrobial [6, 8], and antitumoral activity of bee bread. The chemical composition of bee products is affected by many natural factors such as geographical region, type of source plant, and season of collection, and therefore their bioactivity varies according to these factors [9]. In addition to these natural

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factors, the extraction method and extraction solvent used also cause different chemical components to be obtained from the product [9–12]. Bee bread is known for its health benefits due to the rich nutrients and bioactive compounds it contains. Similar to other bee products, it contains various nutrients such as protein, carbohydrates, fat, vitamins, minerals, and enzymes. Additionally, it contains bioactive compounds such as flavonoids, polyphenols, phytosterols, enzymes, and amino acids [13]. Some in vitro (cell-based) and in vivo studies on the anticarcinogenic activity of bee bread have shown promising results. Bee bread samples collected from northeastern Portugal showed moderate antitumor activity in MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma) cells; however, these samples were reported to not cause toxicity on PLP2 liver normal cells [14]. The cytotoxicity of ethanolic extracts of bee bread samples collected from Poland on astrocytoma (DASC), and human glioblastoma multiforme (U87MG) cells was examined, and the researchers associated the cytotoxic effect with the specific composition of that natural bee bread product [15]. It has also been reported that bee bread inhibits the growth of cancer cells and initiates the process of apoptosis (programmed cell death) of tumor cells [13]. In general, bee bread is a new bee product and today it is recommended to be collected and consumed as a food supplement called "bee bread" [15]. It is known that the biological activity of natural compounds varies depending on the conditions of the region where they are taken. Studies on the biological activity of bee bread samples are also limited in this context. To contribute to the literature, this study used an ultrasonic wave-assisted extraction method to obtain aqueous extracts of bee bread samples taken from five distinct regions in Eastern Anatolia, Turkey. Samples of bee bread were examined for moisture content, ash content, acidity, and pH. The LC-HRMS method was used to identify the phenolic components of the aqueous extracts. The MTT technique was used to assess the cytotoxicity of these extracts on cells from the colon (DLD-1), breast (MCF-7), cervix (HeLa), and prostate cancer (PC-3) cells. Using the Molecular Docking method, the interactions between the extracts' main components, genistein, naringenin, and vitamin C, and significant cancer targets, such as human thymidylate synthase, the transcription factor c-MYC, cyclin-dependent kinase, and androgen receptor prostate cancer targets, were evaluated.



Fig. 1 Bee bread on honeycomb

Material method

Chemicals and instruments

Sodium hydroxide, phenolphthalein, phosphate buffered saline solution, Dulbecco's Modified Eagle Medium (DMEM, for DLD-1, HeLa and MCF-7 cell line), dimethylsulfoxide and fetal bovine serum (FBS) (Sigma-Aldrich, Germany), RPMI 1640 (NutriCulture, Turkey for PC-3 cell line), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and penicillin/streptomycin (Thermo Fisher Scientific) used for the determination of anticancer properties were purchased commercially. MCF-7 cells (ATCC-HTB22), DLD-1 (ATCC-CCL-221), and HeLa Cells (ATCC-CCL-2) were cultured in DMEM-high glucose supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin (ThermoFisher) at 37 °C with 5% CO_2. PC-3 cells (ATCC CRL-1435) were cultured in RPMI-1640 supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin (ThermoFisher) at 37 °C with 5% CO_2. 5-Fluorouracil are reference drug that was purchased from Lilly and Kocak Farma, respectively. Hed Lab X BIO MSC CLASS II Biosafety cabinet, Thermo Fisher EVOS FL Inverted Microscope, Panasonic MCO-170AICUVH-PE CO_2 Incubator, and BioTek Epoch UV-Vis Spectrophotometer were used in anticancer studies.

Collection and preparation of samples

10.0 g of bee bread samples (Fig. 1) collected from Kars, Eastern Anatolia, Turkey (Fig. 2) were taken and 50 mL of distilled water was added to it. To prepare extracts from this mixture, the mixture was kept in an ultrasonic bath at 35 °C for 1 h. Then, water was removed from the mixture in a rotary evaporator and the samples to be used for chemical composition and anticarcinogenic activity experiments were stored at + 4 °C. Samples 1–5 were collected in August 2023 from coordinates 40°00'37.1"N 42°39'50.0"E (Sarikamis, Kars), 40°27'29.9"N 42°54'55.2"E (Selim, Kars), 40°34'30.0"N 43°02'17.5"E (Pasacayiri location, Kars),

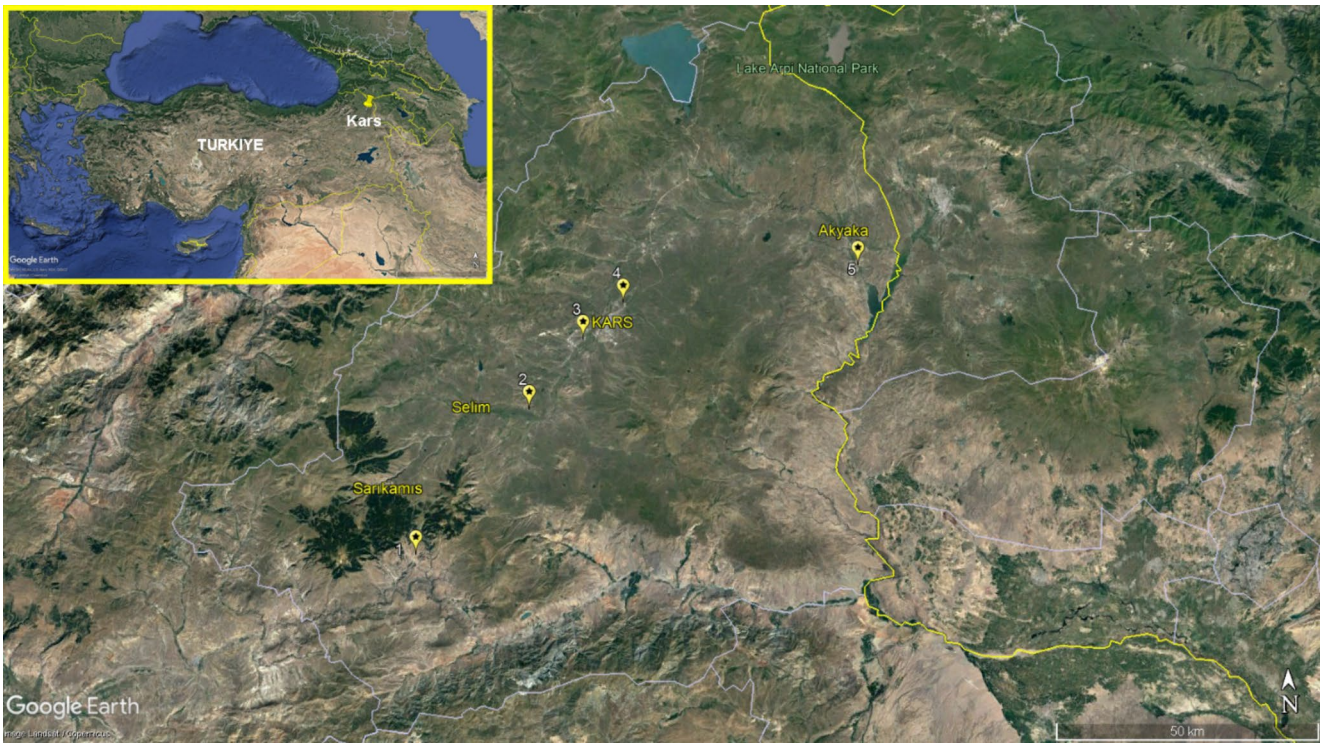


Fig. 2 Representation on the map of the regions where bee bread samples were collected (It was created by entering coordinates into Google Earth)

40°38’04.9"N 43°07’44.4"E (Karadag location, Kars) and 40°41’19.6"N 43°39’12.6"E (Akyaka, Kars), respectively.

Moisture, Ash, pH, and acidity measurements

Shimadzu MOC63U device was used to determine the moisture content of the samples. To determine the pH value of bee bread samples, 20 g of the samples were weighed, 50 mL of pure water was added and homogenized, and the pH of the prepared solutions was measured with a digital Thermo Scientific Orion Star A211 model pH-meter. For the ash determination analysis, the crucibles used in the analysis were kept in the muffle furnace at 550–600 °C for 1 h, cooled in the desiccator, and then weighed on a precision scale with a sensitivity of 0.0001 g, to bring them to a constant weight before the analysis. 3 g of each bee bread sample was placed in the crucibles, kept at 200 °C for the first hour, then burned in the muffle furnace at 550 °C for 5–6 h until the standard ash color was obtained and left to cool in the desiccator. Then, by weighing again, the weight differences before and after burning were determined and the amount of ash was calculated as a percentage by weight. For acidity determination, 1 g of the samples was taken and suspended in 5 mL of distilled water. It was titrated with 0.05 M NaOH solution until the pH reached 8.5 ± 0.1. The volume of NaOH consumed in the titration was read from the burette and the acidity of the samples was calculated in terms of lactic acid.

Liquid Chromatography–High Resolution Mass Spectrometry (LC–HRMS) device

LC-HRMS analyses were performed using an Exactive Plus Orbitrap (Thermo Fisher Scientific) high-resolution MS composite with a heated electrospray ionization interface and LC system containing a DIONEX UltiMate 3000 RS pump, DIONEX UltiMate 3000 RS autosampler, and DIONEX UltiMate 3000 RS column oven. The Orbitrap-MS instrument was calibrated with positive (Pierce™ LTQ Velos ESI Positive Ion Calibration Solution) and negative calibration (Pierce™ Negative Ion Calibration Solution) solutions using an auto syringe injector (Thermo Fisher Scientific, USA).

In the LC-HRMS analyses, the LC and MS parts were run simultaneously with the TraceFinder 3.2 (Thermo Scientific) program installed on the system computer, and the data were collected and recorded with the Xcalibur software version 2.1.0.1140 (Thermo Fisher Scientific).

Chromatography and high-resolution MS conditions

Analyzes performed used a Phenomenex® Gemini® 3 µm NX-C18 110 Å (100 mm × 2 mm) column. The column

oven temperature was operated at 30 °C. In the elution gradient, 2% (v/v) glacial acetic acid prepared in ultrapure water obtained with the Ultrapure water system (GFL 2004/ Human power 1) in the mobile A phase, and 99.9% pure LC-MS grade methanol (Sigma) in the mobile B phase used.

Separation was performed with a sample injection volume of 20.0 µL and a flow rate of 0.3 mL/min. Analysis time was set to 20 min in total.

The Orbitrap HRMS equipped with a heated electrospray ionization interface was operated in both positive (Full MS/ AIF) and negative (Full MS/AIF) modes. Ionization interface sheath gas flow rate 35; auxiliary gas flow rate 7; spray voltage 3.5 kV; capillary temperature 350 °C; auxiliary gas temperature 350 °C; S-lens RF level was set to 50. MS scan range 60–800 m/z; resolution 17,500; ACG target 3.106; maximum IT 2 ms; CE (collision energy)/ step CE was carried out under 25 V conditions.

For the phytochemical compounds belonging to the LC–Orbitrap HRMS analysis method, benzoic acid, 4-hydroxybenzoic acid, 3-hydroxybenzoic acid, 3-hydroxyphenyl acetic acid (3-HPA), syringic acid, gallic acid (3,4,5-trihydroxybenzoic acid), protocatechuic acid (3,4-dihydroxybenzoic acid), protocatechuic acid ethyl ester (ethyl 3,4-dihydroxybenzoate), 3,4-dihydroxybenzaldehyde (protocatechuic aldehyde), 2,4-dihydroxybenzoic acid (beta-resorcylic acid), vanillic acid, homovanillic acid ((4-hydroxy-3-methoxyphenylacetic acid), vanillin, gentisic acid, 3,4-dihydroxyphenylacetic acid (DOPAC, homoprotocatechuic acid), trans cinnamic acid, coumaric acid (trans-3-hydroxycinnamic acid), caffeic acid, caffeic acid phenhyl ester (CAPE), ferulic acid, sinapic acid, chlorogenic acid, quinic acid, 3-(4-hydroxyphenyl) propionic acid, vitamin C (L-ascorbic acid), α-cyano-4-hydroxycinnamic acid, catechin (cianidanol)-p, epigallocatechin, epigallocatechin gallate, chrysin (5,7-dihydroxy-2-phenyl-4H-chromen-4-one), apigenin (5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one), acacetin (5,7-dihydroxy-2-(4-methoxyphenyl)-4H-chromen-4-one), rhoifolin (apigenin 7-O- neohesperidoside), vicenin 2, apigenin 7-glucuronide, apigenin 7-glucoside, genkwanin (4',5-dihydroxy-7-methoxyflavone, apigenin 7-O-methyl ether), apiin (apigenin-7-(2-O-apiosylglucoside), vitexin (apigenin 8-C-glucoside), schaftoside, rutin hydrate M-OH2, luteolin, luteolin-7-O-glucuronide (luteolin-7-O-β-D-glucuronide), diosmetin (luteolin 4′-methyl ether), orientin, isoorientin, luteoloside (luteolin 7-glucoside), luteolin 7-rutinoside, galangin (3,5,7-trihydroxy-2-phenyl-4 H-chromen-4-one), quercetin, isoquercitrin (quercetin 3-glucoside), narcissin (narcissoside, isorhamnetin 3-rutinoside), quercetin 3-rutinoside 7-glucoside, isorhamnetin (quercetin 3′-methyl ether), hyperoside (quercetin 3-D-galactoside), kaempferol, afzelin (kaempferol 3-rhamnoside), kaempferide, kaempferitrin, nicotiflorin (kaempferol 3-rutinoside, kaempferol 3-O-β–rutinoside), astragalin (kaempferol 3-glucoside), tiliroside, leucoside (kaempferol 3-sambubioside), myricetin, fisetin hydrate, naringin, naringenin, sakuranetin (naringenin 7-O-methyl ether), narirutin (narirutinsa, naringenin rutinoside), taxifolin, taxifolin M+3 H, hesperidin, neohesperidin, liquiritigenin, liquiritin (4′,7-dihydroxyflavanone 4′-glucoside), genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4 h-chromen-4-one), daidzin, formononetin (neochanin), kuromanine (cyanidin 3-glucoside chloride), ellagic acid hydrate, phloridzin, rosmarinic acid, glabridin, arbutin, emodin, etoposide, procyanidin B2, doxorubicin hydrochloride, and ethylgallate were used a standard. A 10-point calibration curve was applied in the range of 10–500 ppb and injected three times each.

10 mg of each bee bread sample was weighed and dissolved in 10 mL of 50% methanol-purified water mixture. Then, it was filtered through a 25 mm diameter PTFE syringe filter with a pore size of 0.22 µm, and it was taken into a 1.5 mL vial and injected into the device [16].

MTT Assay

In this study, the anticarcinogenic activities of bee bread extracts were investigated on colon cancer (DLD-1), breast cancer (MCF-7), cervical cancer (HeLa), and prostate cancer (PC-3) cell lines. To prepare the medium that will enable the cells to live and multiply, media appropriate to the cell type (DMEM or RPMI 1640) were prepared. Cells were counted using a Thoma slide. After cell counting was completed, anticarcinogenic activity was determined using the MTT test method in the biosafety cabinet. After adding 100 µL of the prepared medium to 96-well microplates, 100 µL (5×10^5 cells/well) of cell suspension was added to each well. The microplates were kept in an incubator at 37 °C and 5% CO_2 for 24 h to allow the cells to proliferate and adhere to the surface. Stock solution was prepared by weighing 10 mg of each bee bread sample and dissolving it in 10 mL of 50% methanol-purified water mixture. Application concentrations of 7.81 µg/mL, 15.62 µg/mL, 31.25 µg/mL, 62.50 µg/mL, 125 µg/mL, 250 µg/mL, 500 µg/mL, and 1000 µg/mL were prepared from this stock solution. When the incubation period was completed, 100 µL of solutions of different concentrations of bee bread extracts (7.81 µg/mL, 15.62 µg/mL, 31.25 µg/mL, 62.50 µg/mL, 125 µg/mL, 250 µg/mL, 500 µg/mL, 1000 µg/mL), whose anticarcinogenic effects were investigated, were added to the culture medium. Similarly, solutions of the same concentrations of 5-fluorouracil (given as standard in the Figs. 3, 4, 5 and 6) used as the reference drug were prepared. At this stage, 100 µL of medium was added to the wells designated as the

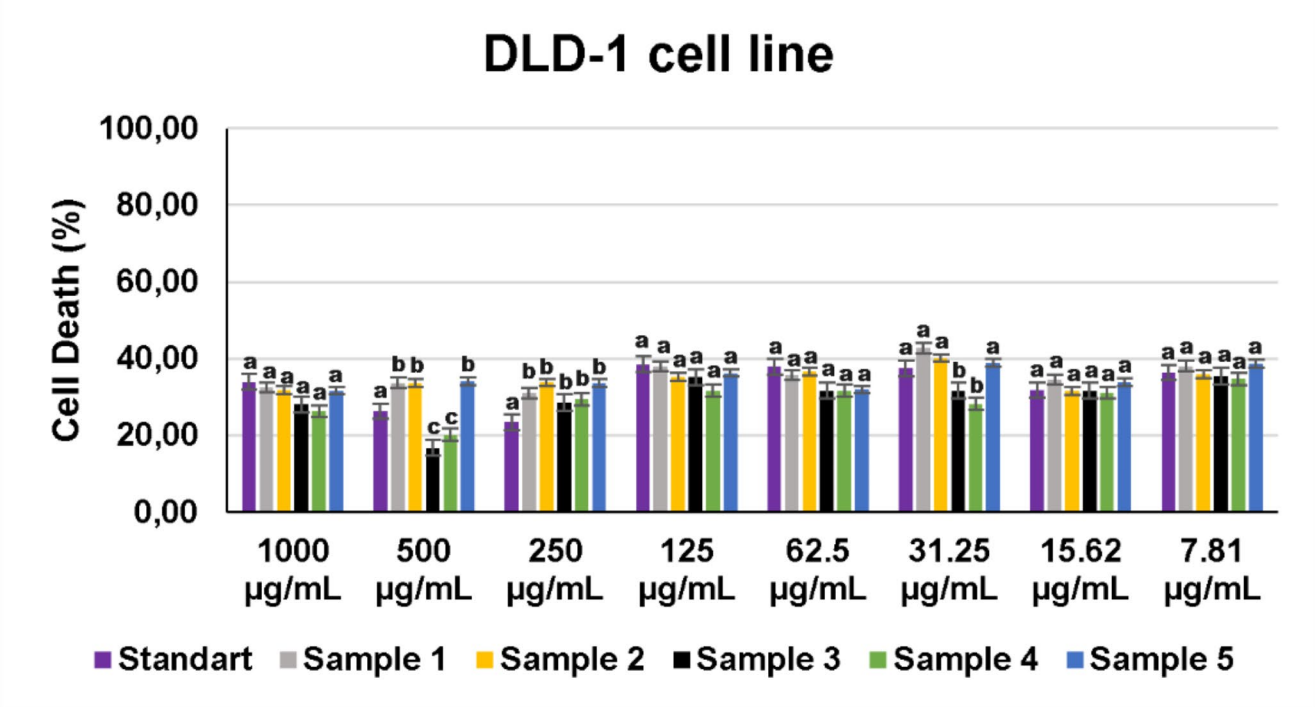


Fig. 3 Cell death rates of standard drug and bee bread extract on DLD-1 cancer cells. Statistically, the groups were evaluated among themselves

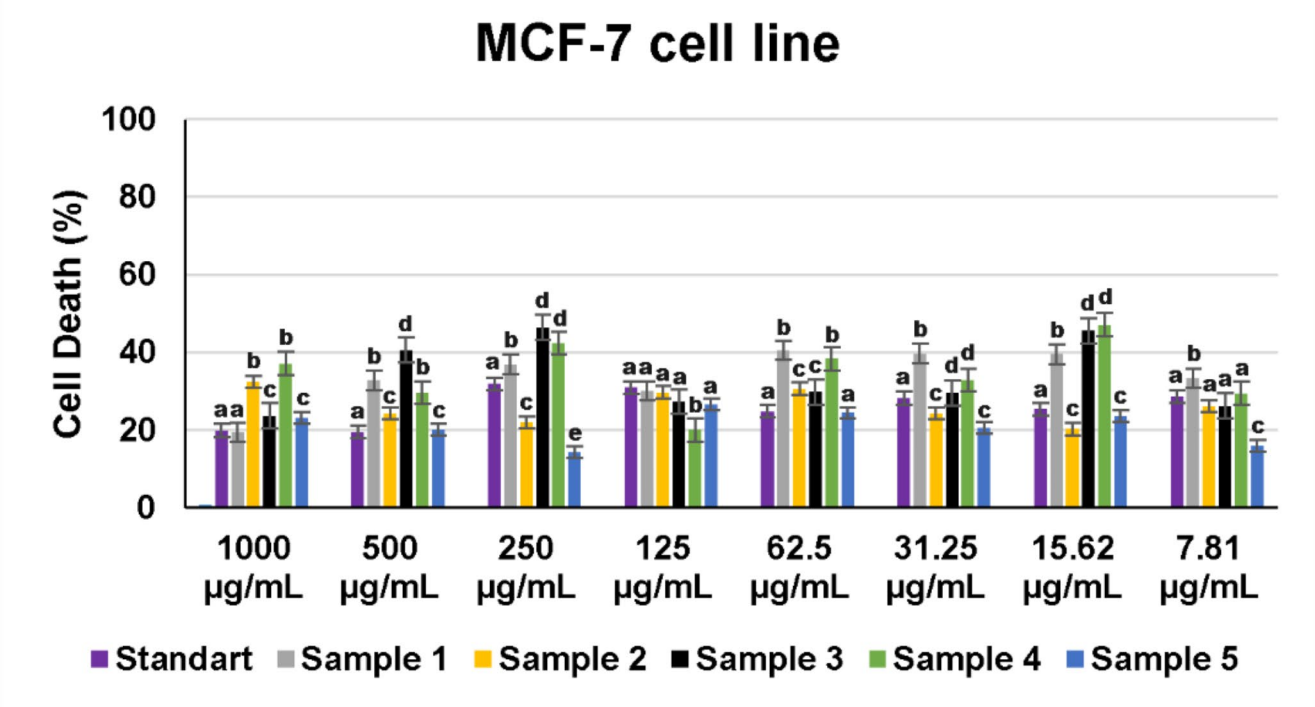


Fig. 4 Cell death rates of standard drug and bee bread extract on MCF-7 cancer cells. Statistically, the groups were evaluated among themselves

control group. Cells were incubated at 37 °C for 24 h in a 5% CO_2 incubator. When the incubation period was completed, 20 µL of the previously prepared MTT reagent was added to each well. The 96-well plate was gently mixed on a shaker and incubated for 3–4 h at 37 °C in a 5% CO_2 incubator. After the medium in the wells was completely withdrawn, 200 µL DMSO was added to each well. After keeping the microplates in an incubator with 5% CO_2 at

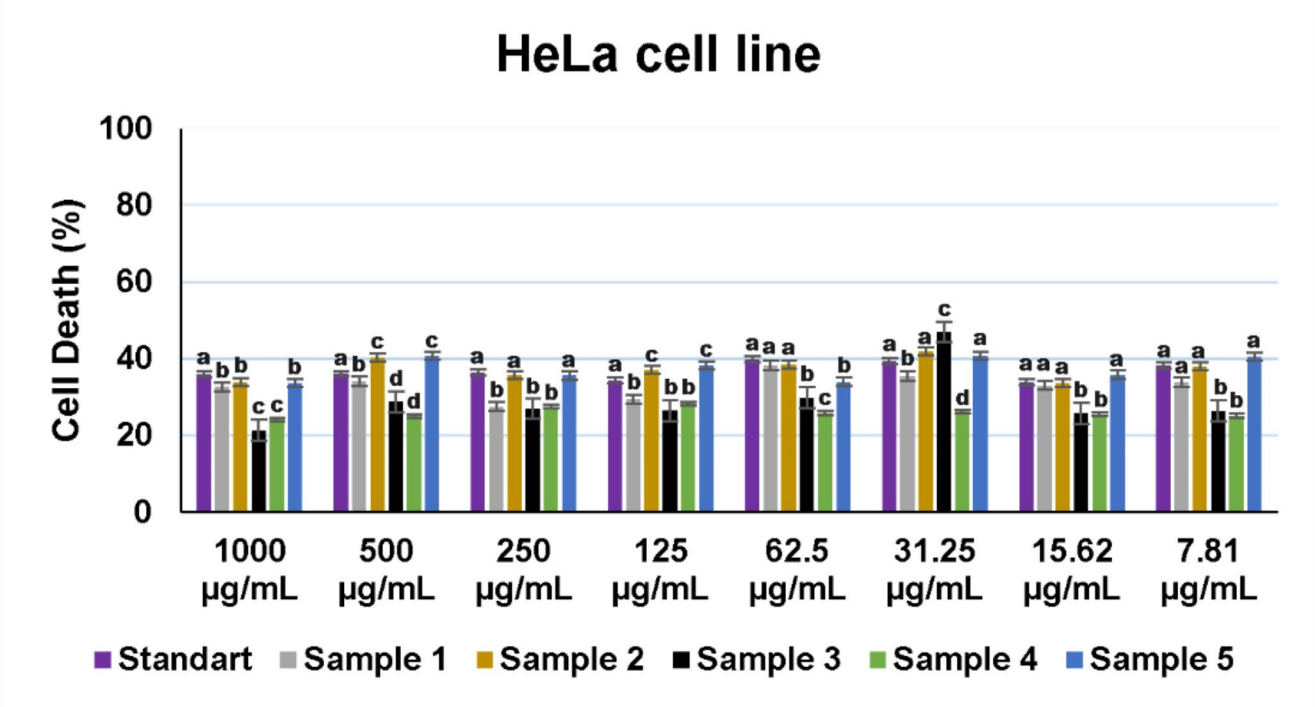


Fig. 5 Cell death rates of standard drug and bee bread extract on HeLa cancer cells. Statistically, the groups were evaluated among themselves

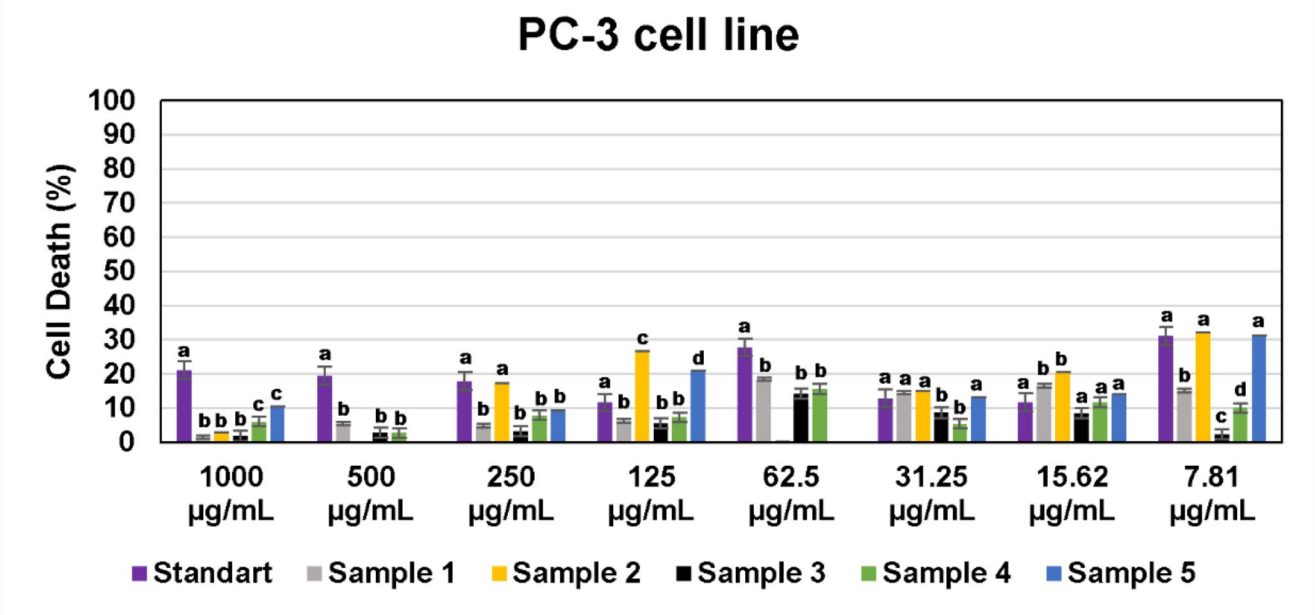


Fig. 6 Cell death rates of standard drug and bee bread extract on PC-3 cancer cells. Statistically, the groups were evaluated among themselves

37 °C for 24 h to dissolve the formed formazan crystals, the absorbance values of the microplates were measured with a spectrophotometer at 570 nm. The experiments were performed in triplicate. Cell viabilities were calculated using the formula below.

Cell Viability = (Absorbance of test well / Absorbance of control well) x 100.

Statistical analysis

Two-way ANOVA (Tukey) test included in the IBM SPSS Statistics for Windows (version 22.0, IBM Corp., Armonk, NY, USA) package program was used for the statistical calculations of the absorbance values obtained. Statistically

significance level is accepted at 95% ($p<0.05$). Graphs were created in Microsoft Excel program.

In silico Assays

Human thymidylate synthase (TS), the c-MYC transcription factor, cyclin-dependent kinases, and the androgen receptor are important targets in cancer drug development due to their critical roles in DNA synthesis, cell proliferation, and signaling pathways that drive cancer progression. Targeting these molecules, either individually or in combination, can lead to the development of more effective cancer therapies. The crystal structure of human thymidylate synthase (PDB ID: **1HVY)** [17], c-MYC transcription factor (PDB ID: **6G6K)** [18], cyclin-dependent kinase (PDB ID: **2WXV)** [19] and androgen receptor prostate cancer targets (PDB ID: **2Q7K**) [20] and were downloaded from the protein data bank. Pre-processing of ligands (genistein, naringenin, and vitamin C) and targets was done with the help of AutoDockTools-1.5.7 software [21]. To prepare the target file, polar hydrogens, and Kollman charges were added, and the target file was saved in pdbqt format. Molecular docking was carried out using AutoDockTools-1.5.7, and the docking results and visualization studies were used by Discovery Studio 2021 software [22].

Results

As seen in Table 1, the moisture content of the samples varies between 6.37 and 12.78%. The mineral matter of the samples is in the range of 2.40–3.03%. It was determined that the pH values of the samples were very close to each other. Acidity varies between 488.57 mEq/kg and 541.78 mEq/kg (Table 1).

Table 1 Details regarding moisture (%), ash (%), acidity (mEq/Kg), and pH of the samples

Sample Name	Coordinates	Moisture (%)	Ash (%)	Acidity (mEq/Kg)	pH
Sample 1	40.0086 N 42.6575 E	10.82	2.40	531.26	4.82
Sample 2	40.4583 N 42.9153 E	6.37	2.43	506.67	5.01
Sample 3	40.5750 N 43.0382 E	10.47	2.67	488.57	4.76
Sample 4	40.6347 N 43.1290 E	12.78	3.03	531.26	5.24
Sample 5	40.6887 N 43.6535 E	10.01	2.73	541.78	4.69

LC-HRMS results

According to the findings, the phenolic components of bee bread samples vary depending on the region where they were collected. Benzoic acid, 4-hydroxybenzoic acid, vitamin C, routine hydrate, isorhamnetin, naringenin, and genistein were found to be present in all samples. The amount of isorhamnetin (1114.51 µg/g), the most abundant phenolic compound in Sample 1, in Samples 2–5 is 243.91 µg/g, 691.58 µg/g, 226.85 µg /g, and 127.90 µg/g, respectively. The amount of genistein in the samples was determined as 1013.33 µg/g, 1014.76 µg/g, 1014.70 µg/g, 1014.78 µg/g, and 1005.57 µg/g for Samples 1–5, respectively. Genistein is the most abundant phenolic compound in Samples 2–5. Phloridzin, the third most abundant compound in Sample 1 (999.15 µg/g) and the most abundant compound in Sample 2 (999.36 µg/g), was not found in the other three samples. The amount of naringenin, one of the most abundant compounds in the samples, varies between 816.69 µg/g - and 820.36 µg/g. Vitamin C amounts in Samples 1–5 were determined as 520.56 µg/g, 627.19 µg/g, 709.57 µg/g, 560.29 µg/g and 516.31 µg/g, respectively. Leucoside, taxifolin and taxifolin M+3 H are only found in Sample 1. Afzelin is only found in Sample 2, isoquercitrin is only found in Sample 3, gallic acid is only found in Sample 4, and luteolin is only found in Sample 5. Data on all detected phenolic compounds are given in Table 2.

***ND: Not detected.**

MTT Assay Results

In this study, it was determined that bee bread's water extracts on DLD-1, MCF-7, HeLa, and PC-3 cell lines. The results were compared with the cell control group and the reference drug 5-fluorouracil (standard). When the effects of bee bread extracts on DLD-1 cancer cells were examined, it was seen that it caused cell death close to the standard drug 5-fluorouracil at all concentrations. When Fig. 3 is examined, it is seen that all bee bread extracts at 250 µg/mL concentration show superior anticarcinogenic activity than the standard drug. There is no difference in the anticarcinogenic effect exhibited by the samples at 250 µg/mL concentration on the DLD-1 cancer cell. However, there are differences between samples and standard drug. It was determined that Sample 1 and Sample 2, especially at concentrations of 31.25 µg/mL, 15.62 µg/mL, and 7.81 µg/mL, were more effective than the standard drug and caused more cell death. Sample 3 and Sample 4 showed lower anticarcinogenic effects than the standard drug and other extracts at a concentration of 500 µg/mL (Fig. 3). It was determined that the cytotoxicity exhibited by the bee samples and the standard

Table 2 Chemical compositions of the bee bread samples from Eastern Anatolia (Kars)

Target Compounds	SAMPLE 1 (µg/g)	SAMPLE 2 (µg/g)	SAMPLE 3 (µg/g)	SAMPLE 4 (µg/g)	SAMPLE 5 (µg/g)
Benzoic acid	46.87	4.85	5.19	30.13	40.08
4-Hydroxybenzoic acid	90.00	17.36	20.70	23.97	19.07
Vanillic acid	12.36	29.89	28.93	10.00	ND*
Gentisic acid	9.08	261.74	ND*	ND*	ND*
Homoprotocatechuic acid	128.80	ND*	95.36	95.13	156.72
Coumaric acid	33.37	ND*	7.08	14.22	23.11
Vitamin C	520.56	627.19	709.57	560.29	516.31
Rutin hydrate	19.98	32.89	153.91	1.19	68.41
Quercetin	5.37	112.42	ND*	37.60	105.01
Isorhamnetin	1114.51	243.91	691.58	226.85	127.90
Kaempferol	6.66	ND*	51.96	20.99	221.14
Leucoside	625.25	ND*	ND*	ND*	ND*
Naringenin	818.10	818.81	820.36	816.69	819.36
Taxifolin	423.49	ND*	ND*	ND*	ND*
Taxifolin M+3H	7.07	ND*	ND*	ND*	ND*
Genistein	1013.33	1014.76	1014.70	1014.78	1005.57
Phloridzin	999.15	999.36	ND*	ND*	ND*
Arbutin	21.17	20.13	11.30	13.67	ND*
Ferulic acid	ND*	38.81	53.22	ND*	ND*
Narcissin	ND*	142.86	ND*	8.82	ND*
Afzelin	ND*	24.42	ND*	ND*	ND*
Astragalin	ND*	21.63	ND*	ND*	63.01
Kuromanine	ND*	21.24	ND*	ND*	63.24
Protocatechuic acid	ND*	ND*	6.45	1.46	ND*
Isoquercitrin	ND*	ND*	585.78	ND*	ND*
Gallic acid	ND*	ND*	ND*	2.86	ND*
3-(4-hydroxyphenyl) propionic acid	ND*	ND*	ND*	20.14	20.46
Luteolin	ND*	ND*	ND*	ND*	3.83

at concentrations of 1000 µg/mL, 62.50 µg/mL, 15.62 µg/mL, and 7.81 µg/mL was not statistically different.

As seen in Fig. 4, it was determined that the extracts, except Sample 1, at a concentration of 1000 µg/mL had a higher anticarcinogenic effect on MCF-7 cancer cells than the standard drug. Statistically, the anticarcinogenic effect of Samples 2 and 4 on MCF-7 cells is different from the standard and other samples. This difference is also seen between Samples 3 and 5 and the standard drug. Sample 1 appears to cause more cell death than the standard drug at all concentrations except 1000 µg/mL and 125 µg/mL. Sample 2 showed better anticarcinogenic performance than the standard drug at only three concentrations (1000 µg/mL, 500 µg/mL, and 62.5 µg/mL). Sample 3 and Sample 4 exhibited higher antitumoral effects than the standard drug at all concentrations except 125 µg/mL and 7.8 µg/mL. Remarkably, Sample 3 caused more than twice as much cell death on MCF-7 cancer cells than the standard drug at a concentration of 500 µg/mL. The percentages of cell death caused by Sample 3 and the standard drug on MCF-7 cancer cells are 40.64% and 19.56%, respectively. When the antitumoral activity of Sample 5 is evaluated in general, it can be seen in Fig. 4 that it performs lower than other extracts. Although Sample 5 has low anticarcinogenic activity, it has been determined that it causes cell death close to that of the standard drug at some concentrations (Fig. 4).

Figure 5 shows that the extracts showed lower anticarcinogenic effects than the standard drug at concentrations of 1000 µg/mL, 250 µg/mL, and 62.5 µg/mL. It was determined that Sample 2 and Sample 5 showed higher antitumoral effects than the standard drug at concentrations of 500 µg/mL, 125 µg/mL, and 31.5 µg/mL. When the death

rates caused by all extracts and the standard drug on HeLa cancer cells were examined, it was determined that Sample 3 had the highest anticarcinogenic effect at a concentration of 31.25 µg/mL (Fig. 5).

It was determined that the extracts and the standard drug showed very low anticarcinogenic activity on PC-3 cancer cells in the concentration range of 1000–125 µg/mL. The standard drug and Sample 2 caused cell death at very similar values (~17.50%) at a concentration of 250 µg/mL. At a concentration of 125 µg/mL, Sample 2 caused more cell death than the standard drug. As seen in Fig. 6, the highest anticarcinogenic activity was detected at the lowest application concentration of 7.81 µg/mL. At this concentration, Sample 2, Sample 5, and standard drug caused cell death at the rate of 32.17%, 31.18%, and 31.09%, respectively (Fig. 6). Samples 2 and Sample 5 did not cause cell death at a concentration of 500 µg/mL and 62.5 µg/mL concentrations.

Molecular docking results

Genistein bonds conventional hydrogen bond interactions with amino acids arg50, his256, glu87, and ser16, pi-donor hydrogen bonding with the amino acid residues cys195 and asn226, pi-sulfur with cys195, pi-pi stacking interactions with the amino acid trp109 and pi-alkyl interacitons with amino acid residues leu192 and cys195 in the active site of the human thymidylate synthase (Fig. 7a and b). Naringenin makes conventional hydrogen bond interactions with amino acids gln214 and asp218, pi-donor hydrogen bonding with the amino acid cys195, and pi-pi stacking interactions with the amino acid trp109 in the active site of the human thymidylate synthase (Fig. 7c and d). Vitamin C interacts conventional hydrogen bond interactions with arg163 (2.94 Å), thr167 (2.23 Å), ile178 (2.07 Å), trp182 (2.66 Å), arg176 (2.21 Å) amino acid residues in the active site of the human thymidylate synthase (Fig. 7e and f). The binding scores between the colon cancer target and genistein, naringenin, and vitamin C are −8.2 kcal/mol, -8.7 kcal/mol, and −6.0 kcal/mol, respectively. Naringenin exhibits a stronger binding affinity to this target than genistein and vitamin C.

Genistein contacts via conventional hydrogen bonds with asp86 (3.08 Å) and his84 (2.65 Å), pi-sigma interactions with leu194, pi-pi stacked with phe80 amino acid, and pi-alkyl interactions with ile10 and val18 in the active site of cyclin-dependent kinase (Fig. 8a and b). Naringenin interacts through conventional hydrogen bonds with asp145 (3.02 Å), and leu83 (2.18 Å), pi-donor hydrogen bond with tyr15, pi-sigma interactions with ile10, pi-pi stacked interactions with phe82, pi-alkyl interactions with val18, ala31, and leu134 amino acid residues in the active site of the cyclin-dependent kinase. The conventional hydrogen bond lengths between naringenin asp145 and leu83 were found as 3.02 Å, and 2.18 Å, respectively (Fig. 8c and d). Vitamin C connects via conventional hydrogen bonds with ser247 (d=2.03 Å) and leu243 (d=2.27 Å) and carbon-hydrogen

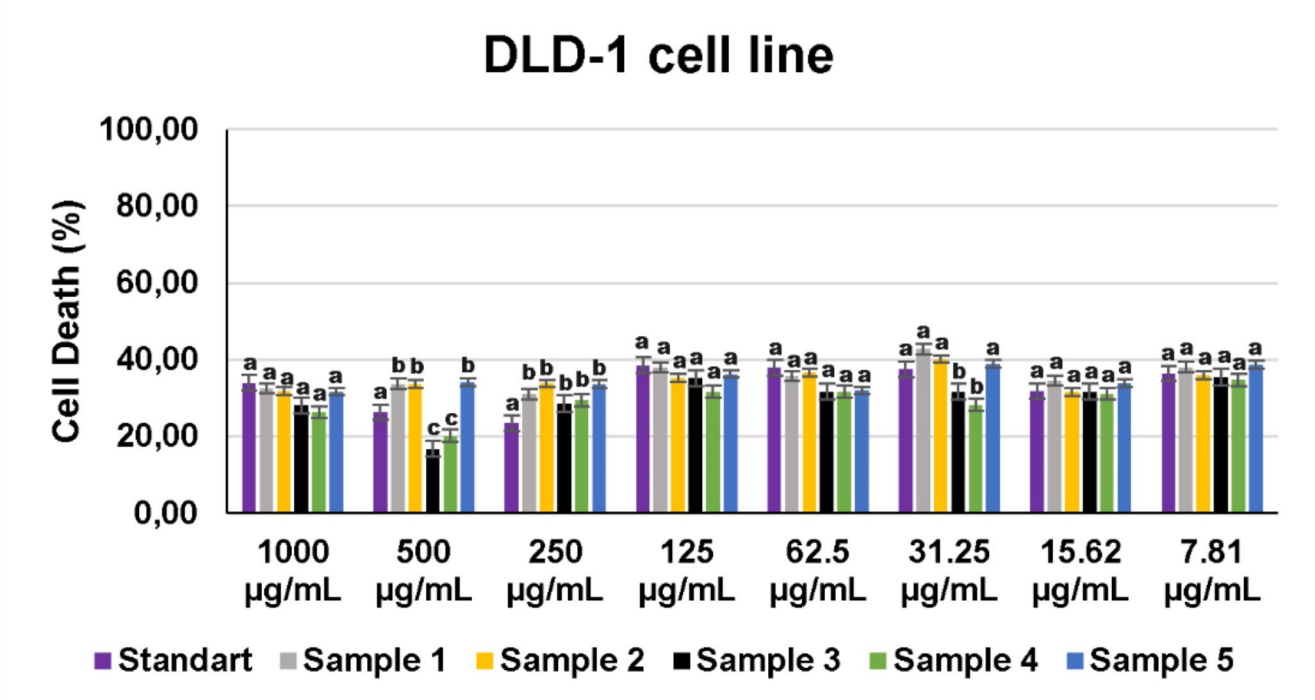


Fig. 7 Molecular docking of genistein (**a**), naringenin (**c**), and Vitamin C (**e**) into the active site of human thymidylate synthase. 2D view of the interactions of genistein (**b**), naringenin (**d**), and vitamin C (**f**) with the active site of human thymidylate synthase

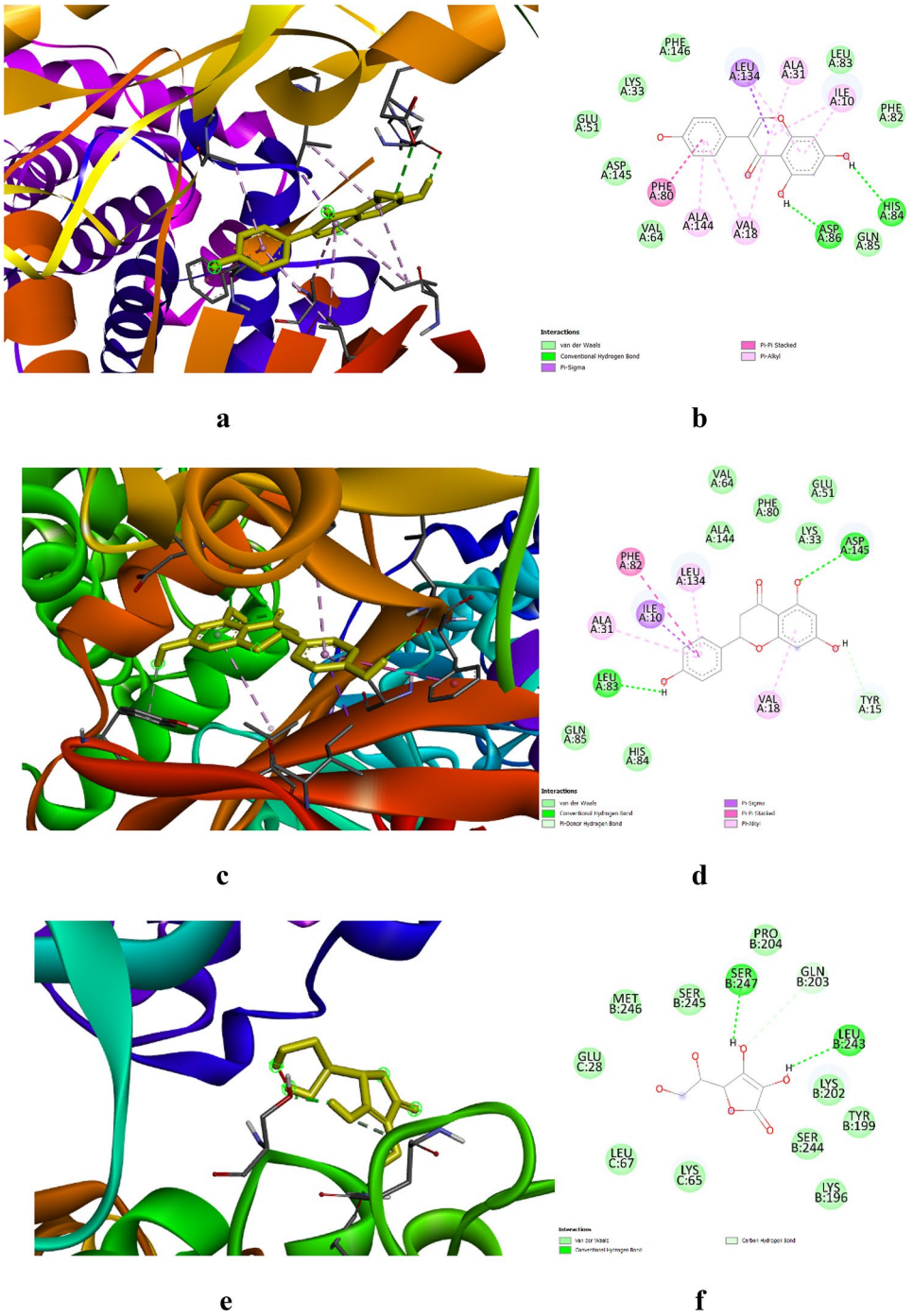


Fig. 8 Molecular docking of genistein (**a**), naringenin (**c**), and Vitamin C (**e**) into the active site of cyclin-dependent kinase. 2D view of the interactions of genistein (**b**), naringenin (**d**), and vitamin C (**f**) with the active site of cyclin-dependent kinase

bond with gln293 amino acid residue in the active site of the cyclin-dependent kinase (Fig. 8e and f). Naringenin and vitamin C interact with cyclin-dependent kinase only through hydrogen bond interactions. No electrostatic or hydrophobic interaction was found between the compounds and this target. The binding energies between genistein, naringenin, and vitamin C with the cyclin-dependent kinase were found as -8.5 kcal/mol, -8.5 kcal/mol, and −5.6 kcal/mol, respectively.

The binding energy between genistein and the androgen receptor was found to be -7.8 kcal/mol. Genistein interacts through a conventional hydrogen bond with glu681, carbon-hydrogen bond with lys808 amino acid, pi-cation with arg752, and lys808, pi-sigma with val715, pi-pi stacked with trp718, pi-alkyl interacts with ala748, arg752, pro682, and amino acid residues in the active site of the androgen receptor prostate cancer (Fig. 9a and b). Naringenin binds through a conventional hydrogen bond with gly683, pi-pi

T-shaped interactions with trp718, pi-alkyl interacts with ala748, arg752, pro682, val715, and lys808, and pi-cation with arg752 and lys808 amino acid residues in the active site of the androgen receptor prostate cancer (Fig. 9c and d). The binding energy between narigenin and the prostate cancer target was determined to be -8.1 kcal/mol. Vitamin C interacts with the active site of the prostate cancer target only by van der Waals interactions (Fig. 9e and f). The binding energy between vitamin C and the prostate cancer target was determined to be -6.0 kcal/mol.

Genistein binds through a conventional hydrogen bond with ser224, val940, arg214, and his217, pi-cation with lys944 and arg214, pi-sigma with val940 and leu210, pi-pi stacked with his217, pi-alkyl interacts with lys944, arg214, lys213 amino acid residues in the active site of c-MYC transcription factor (Fig. 10a and b). Naringenin interacts through conventional hydrogen bonds with thr947 (2.61 Å)

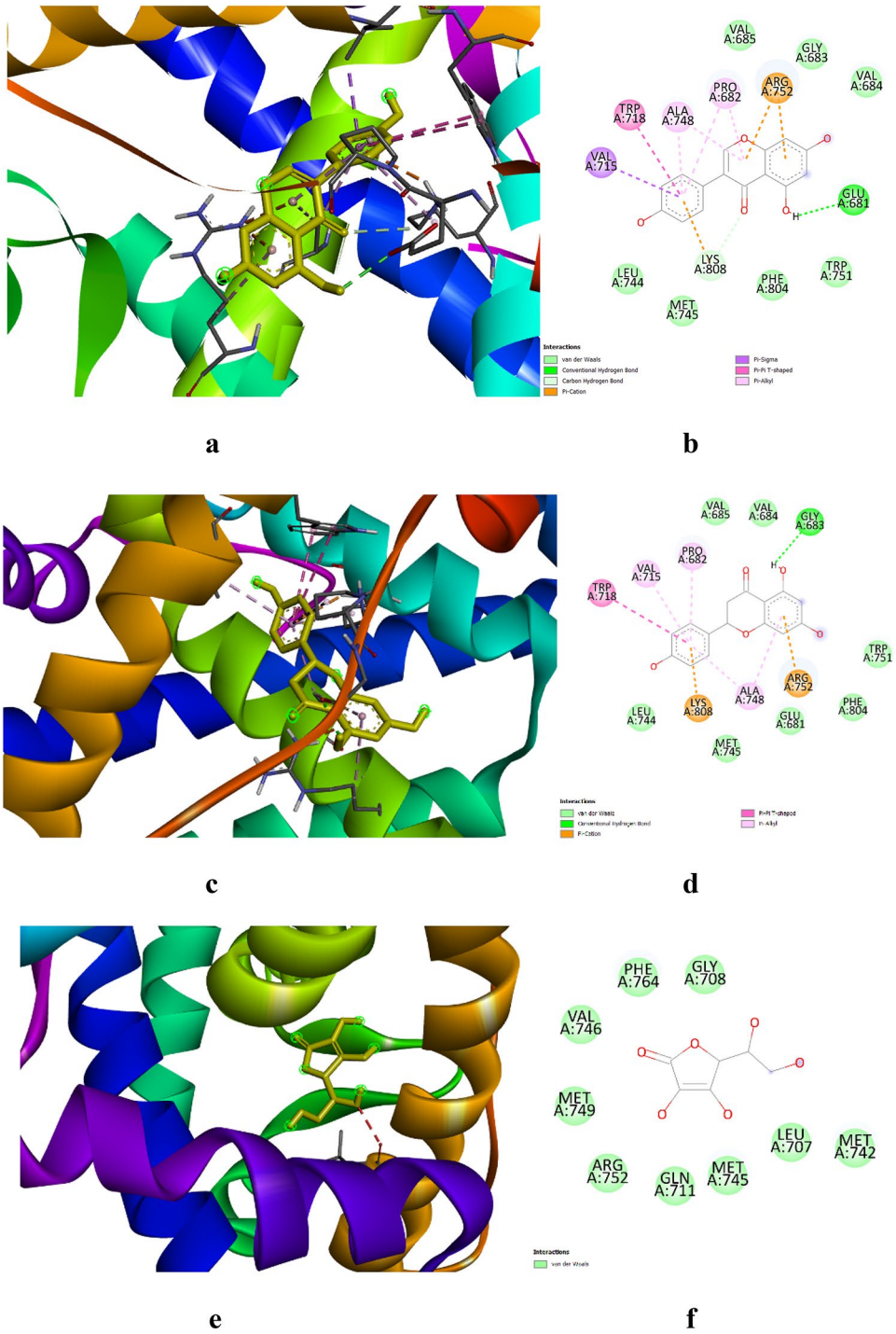


Fig. 9 Molecular docking of genistein (**a**), naringenin (**c**), and Vitamin C (**e**) into the active site of androgen receptor prostate cancer. 2D view of the interactions of genistein (**b**), naringenin (**d**), and vitamin C (**f**) with the active site of androgen receptor prostate cancer

and arg214 (2.25 Å), carbon-hydrogen bonds with ser221 and ser224 amino acid residues in the active site of the c-MYC transcription factor (Fig. 10c and d). Vitamin C binds to the active site of the target protein only through van der Waals bonds (Fig. 10e and f). The binding energies of genistein, naringenin, and vitamin C to the breast cancer target were determined as -8.0 kcal/mol, -8.1 kcal/mol, and 5.3 kcal/mol, respectively. Data on the Molecular Docking of genistein, naringenin, and vitamin C with human thymidylate synthase, c-MYC transcription factor, cyclin-dependent kinase, and androgen receptor prostate cancer targets are given in Table 3.

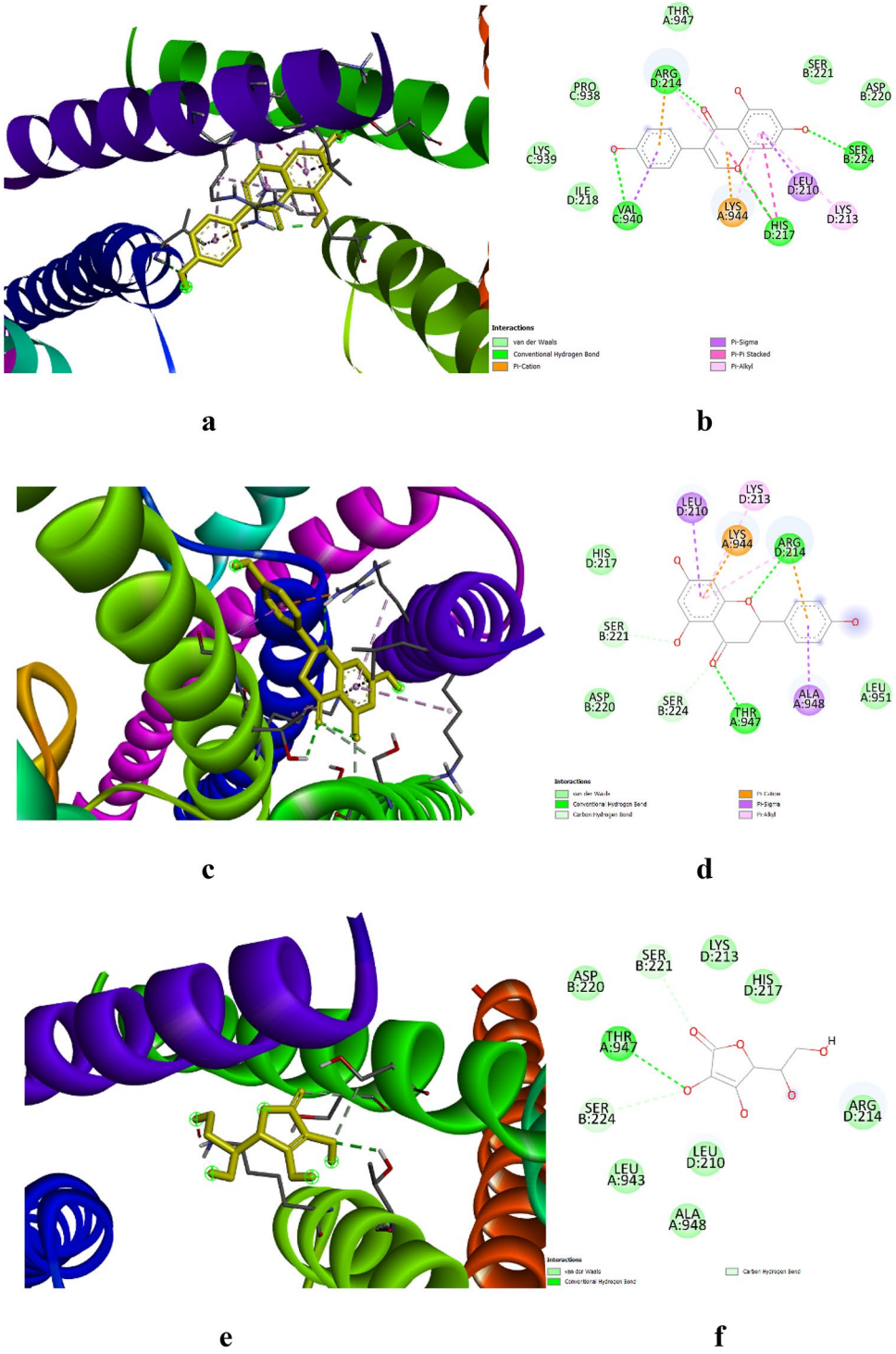


Fig. 10 Molecular docking of genistein (**a**), naringenin (**c**), and Vitamin C (**e**) into the active site of c-MYC transcription factor. 2D view of the interactions of genistein (**b**), naringenin (**d**), and vitamin C (**f**) with the active site of the c-MYC transcription factor

Discussion

Bee bread attracts attention with its high nutritional value and biological benefits. It is an important food source for health with its antibacterial, antifungal, antioxidant, and immune-supporting properties. However, depending on regional differences, pollen diversity, climate conditions, and vegetation, changes may occur in the biological properties of bee bread. Therefore, bee bread produced in different regions may differ in terms of content and biological activity.

Tavdidishvili et al. extracted bee bread samples from the Imereti region of western Georgia with 70% ethanol. The flavonoid content of the samples obtained was determined by HPLC. It was determined that the samples contained naringin, rutin, and quercetin [23]. In our samples, quercetin was found to be 5.37 µg/g, 112.42 µg/g, 37.60 µg/g and 105.00 µg/g in Samples 1, 2, 4, and 5 respectively, in the samples of Tavdidishvili et al., the amount of quercetin was detected in the range of 3.86–19.32 µg/g. Considering that Tavdidishvili et al. collected bee bread samples from Western Georgia and ours from Northeastern Türkiye, it is thought that altitude and climatic conditions cause variability not only in the diversity of phenolic compounds but also in their content.

Phenolic components of bee bread samples collected from northeastern Portugal were determined by HPLC-DAD-ESI/MS. While the components and their amounts varied in the samples, kaempferol-3-O-rutinoside, quercetin-3-O-glucoside, and quercetin 3-O-rutinoside were found to be present in all samples. Although the location where bee bread samples were collected varies, the samples are rich in the flavonoids isorhamnetin-O-hexosyl-O-rutinoside, quercetin-O-hexosyl-O-hexoside, kaempferol-O-hexosyl-rutinoside, isorhamnetin-O-pentosyl-hexoside, kaempferol-3-O-rutinoside, and quercetin-3-O-rhamnoside [14]. Sobral et al. detected 32 different compounds in six bee bread samples. In our study, 28 compounds were determined in 5 different samples. Sobral et al. reported that isorhamnetin-O-hexosyl-O-rutinoside, isorhamnetin-O-pentosyl-hexoside, and quercetin-3-O-rhamnoside were the most abundant compounds in methanolic extracts of bee bread. These compounds were not detected in the aqueous extracts of the bee bread samples we collected. This shows that in addition to regional differences, the extraction solvent and the method of obtaining the extract play an important role in the diversity of phenolic compounds to be obtained.

Suleiman et al. determined, that alcohol, water, and hot water extracts of bee bread samples collected from the east coast of Malaysia in different seasons contained gallic acid, caffeic acid, mangiferin, trans-ferulic acid, trans-3-hydroxycinnamic acid, 2-hydroxycinnamic acid, quercetin, kaempferol, and apigenin [24].

Elsayed et al. detected the presence of gallic acid (1.60 µg/g), p-hydroxybenzoic acid (253.64 µg/g), catechin (11.59 µg/g), vanillic acid (20.32 µg/g), caffeic acid (4.198 µg/g), syringic acid (0.948 µg/g), benzoic acid (852.20 µg/g), ferulic acid (207.40 µg/g), rutin (535.00 µg/g), ellagic acid (16.34 µg/g), o-coumaric acid (10.90 µg/g), resveratrol (53.20 µg/g), cinnamic acid (313.80 µg/g), quercetin (717.40 µg/g), myricetin (24.50 µg/g), kaempferol (34.02 µg/g) in bee bread samples they collected from Egypt [25].

Among these components, gallic acid, p-hydroxybenzoic acid, vanillic acid, benzoic acid, ferulic acid, coumaric acid, quercetin, and kaempferol are also included in our samples. 4-Hydroxybenzoic, ferulic acid, caffeic acid, and p-coumaric were reported as major components of these samples. In our study, both the components and the amount of components vary according to the samples and are not found as major components.

Some studies are showing that natural bioactive compounds can be considered drugs that synergistically inhibit the growth and proliferation of some bacteria and cancer cells at relatively low concentrations [26, 27]. Some basic phytochemicals such as epigallocatechin gallate, naringenin, lycopene, genistein, and resveratrol contained in bee products have been used in cancer treatment [27–31]. Not only flavonoids, polyphenols, phytosterols, enzymes, and amino acids, but also minerals and vitamins in bee products, especially vitamin C, are among the bioactive components in bee products. Vitamin C is known for its numerous health benefits. It is undoubtedly a compound that gives bee products their unique biological active potential [32–34]. When all these data are evaluated, it is thought that the phenolic species obtained from bee bread samples and the change in their amount may vary depending on climate, altitude, extraction solvent and method, and the time of sample collection.

Sobral et al. investigated the anticarcinogenic effects of bee bread samples collected from the northeast of Portugal on MCF-7 (breast adenocarcinoma) and HeLa (cervical carcinoma) cancer cell lines using the sulforhodamine B method. According to the results obtained, it was reported that six different bee bread extracts [methanol: water (80:20, v/v)] caused 25% cell death above 400 µg/mL concentration. However, even at the lowest concentration used in our study (7.81 µg/mL), cell death rates were found to be in the range of 15–40%. It was even determined that this rate increased up to 47.13% depending on the increasing concentration. Although methanol, a more effective solvent in revealing biologically active species, was used, the water

Table 3 Molecular Docking results of the interactions of genistein, naringenin and vitamin C with human thymidylate synthase (PDB ID: **1HVY)** [17], c-MYC transcription factor (PDB ID: **6G6K)** [18], cyclin-dependent kinase (PDB ID: **2WXV)** [19] and androgen receptor prostate cancer targets (PDB ID: **2Q7K**) [20]

Compound-PDB ID	Docking Score (Kcal/mol)	Hydrogen bond	Hydrophobic	Electrostatic	Other
Genistein- **1HVY**	-8.2	**Conventional Hydrogen Bond**: Arg50 (2.05 Å), His256 (3.07 Å), Glu87 (2.49 Å), Ser216 (2.99 Å) **Pi-Donor Hydrogen Bond**: Cys195 (3.91 Å), Asn226 (2.79 Å)	**Pi-Pi T-shaped**: Trp109 (5.13 Å) **Pi-Alkyl**: Leu192 (5.43 Å), Cys195 (5.05 Å)		**Pi-Sulfur**: Cys195 (5.4 Å)
Genistein-**6G6K**	-8.0	**Conventional Hydrogen Bond**: Ser224 (2.08 Å), Val940 (2.75 Å), Arg214 (2.37 Å), His217 (2.82 Å)	**Pi-Sigma**: Val940 (3.48 Å), Leu210 (3.98 Å) **Pi-pi Stacked**: His217 (5.02 Å) **Pi-Alkyl**: Lys944 (4.40 Å), Arg214 (4.12 Å), Lys944 (4.00 Å), Lys213 (5.04 Å)	**Pi-Cation**: Lys944 (3.31 Å), Arg214 (4.31 Å)	
Genistein-**2WXV**	-8.5	**Conventional Hydrogen Bond**: Asp86 (3.08 Å), His84 (2.64 Å)	**Pi-Sigma**: Leu134 (3.66 Å), Pi-Pi Stacked: Phe80 (4.45 Å) **Pi-Alkyl**: Ile10 (5.11 Å), Val18 (5.41 Å), Ala31 (4.85 Å), Leu134 (4.88 Å).	Genistein-**2WXV**	
Genistein- **2Q7K**	-7.8	**Conventional Hydrogen Bond**: Glu681 (2.60 Å) **Carbon-Hydrogen Bond**: Lys808 (3.54 Å)	**Pi-Sigma**: Val715 (3.96 Å) **Pi-Pi T-Shaped**: Trp718 (5.54 Å) **Pi-Alkyl**: Pro682 (4.65 Å), Ala748 (4.30 Å), Lys808 (5.10 Å)	**Pi-Cation**: Arg752 (3.40 Å), Lys808 (3.25 Å)	
Naringenin- **1HVY**	-8.7	**Conventional Hydrogen Bond**: Gln214 (2.87 Å), asp218 (2.05 Å) **Pi-Donor Hydrogen Bond** cys195 (4.18 Å)	**Pi-Pi Stacked**: Trp109 (5.08 Å)		
Naringenin-**6G6K**	-8.0	**Conventional Hydrogen Bond**: Thr947 (2.61 Å), Arg214(2.25 Å), **Carbon-Hydrogen Bond**: Ser221 (3.10 Å), Ser224 (3.71 Å)	**Pi-Sigma**: Leu210 (3.96 Å), **Pi-Alkyl**: Ala748 (5.22 Å), Lys944 (4.07 Å), Lys213 (5.19 Å), Arg214 (5.40 Å)	**Pi-Cation**: Lys944 (4.26 Å), Arg214 (3.78 Å)	
Naringenin- **2WXV**	-8.5	**Conventional Hydrogen Bond**: Asp145 (3.02 Å), Leu83 (2.18 Å), **Pi-Donor Hydrogen Bond**: Tyr15 (2.96 Å)	**Pi-Sigma**: Ile10 (3.68 Å) Pi-Pi Stacked Phe82 (5.99 Å) **Pi-Alkyl**: Val18 (5.25 Å), Ala31 (5.29 Å), Leu134 (4.70 Å)		
Naringenin- **2Q7K**	-8.1	**Conventional Hydrogen Bond** Gly683 (2.21 Å)	**Pi-Pi T-shaped**: Trp718 (5.37 Å) **Pi-Alkyl**: Ala748 (5.22 Å), Arg752 (4.73 Å), Pro682 (4.67 Å), Val715 (4.86 Å), Lys808 (5.03 Å)	**Pi-Cation**: Arg752 (3.96 Å), Lys808 (3.09 Å)	
Vitamin C- **1HVY**	-6.0	**Conventional Hydrogen Bond**: Arg163 (2.94 Å), thr167 (2.23 Å), Ile178 (2.07 Å), Trp182 (2.66 Å), Arg176 (2.21 Å)			
Vitamin C-**6G6K**	-5.3	**Conventional Hydrogen Bond**: Thr947 (2.49 Å), **Carbon Hydrogen Bond**: Ser221 (2.98 Å) Ser224 (3.43 Å)			
Vitamin C- **2WXV**	-5.6	**Conventional Hydrogen Bond**: Ser247 (2.03 Å), Leu243 (2.27 Å), **Carbon Hydrogen Bond**: Gln293 (3.75 Å)			
Vitamin C- **2Q7K**	-6.0	Van der Waals interactions			

extracts in our study showed a higher cytotoxic effect on MCF-7 and HeLa cancer cells [14].

In a study conducted by Elsayed et al., the anticarcinogenic effects of Egyptian citrus bee bread ethanolic extracts on PC-3 cancer cells were investigated using the MTT method. According to the research results, the IC_{50} value was determined as 314 µg/mL. In the same study, it was reported that bee bread extracts at a concentration of 1000 µg/mL reduced the viability of PC-3 cancer cells to below 20% [25]. When compared to the data obtained from our study, there is a significant difference between the results of Elsayed et al. It is thought that this difference arises because the bee bread samples used were collected from different geographical regions and the extraction method was different. It is normal for the content of natural products such as bee bread to change (such as chemical composition, mineral content, fatty acid content, total phenolic and flavonoid contents) depending on many variables such as soil structure, vegetation, and climatic conditions of different geographical regions. Therefore, these changes may cause the biologically active properties of natural products to differ.

Conclusions

In this study, aqueous extracts of bee bread samples collected from five different regions in eastern Turkey (Kars) were taken using ultrasonic wave-assisted extraction. Moisture, ash, acidity, and pH values of these extracts were investigated. Eastern Anatolian bee bread is rich in phenolic components. Naringenin, genistein, and vitamin C, which are identified as major components, are known to have antitumor effects. According to Molecular Docking results, the binding energy of naringenin to all selected cancer targets was found to be higher than the binding energy of genistein and vitamin C to the identified cancer targets. That is, naringenin can inhibit the active sites of these targets. Furthermore, compared to vitamin C and genistein, naringenin interacts with selected targets through hydrogen bonding, and electrostatic and hydrophobic interactions. When the antitumor effects of bee bread extracts obtained from bee bread collected from five different regions are evaluated, it is seen that the extracts cause more cancer cell death than the standard drug, especially on MCF-7 cancer cells. It is also observed that the extracts have a moderate anticarcinogenic effect on HeLa and DLD-1 cancer cells, respectively. Minimal antitumoral activity was detected on PC-3 cancer cells. As a result, the findings obtained from this study may be guiding and promising for further research to investigate the anticarcinogenic effects of bee bread extracts, taken from different locations, with different concentrations and methods, as alternative treatments or food supplements.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no conflict of interest.

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