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Biological activities and DNA interactions of aqueous extract of *Phlomis linearis* (Boiss. & Bal.)

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ABSTRACT

Phlomis linearis Boiss. & Bal. of the Lamiaceae family is one of the endemic species in Turkey, i.e., growing in the east, central, and southeast parts of Anatolia and used for herbal tea. This study was designed to identify the biochemical and bioactivity properties of this endemic species by DPPH scavenging activity, metal chelating activity, total phenolic content, HPLC-DAD analysis, and MTT assay. Furthermore, the plant extract was evaluated for its antimicrobial activity against bacteria and fungi by using the microdilution method. The interactions between extract and plasmid DNA and their restriction endonuclease reactions were investigated by agarose gel electrophoresis. To support our hypothesis, we performed a molecular docking analysis. The DPPH scavenging activity of the plant extract was 53.86 ± 0.50 µg/ml in terms of IC_{50} value. The IC_{50} value of the plant extract was determined as 14.71 ± 4.01 mg/ml for metal chelating assay. The phenolic content of the extract was 231.55 ± 2.11 mg/g dry weight expressed as gallic acid equivalents (GAE). HPLC-DAD results revealed that the phenolic compounds were mainly derivatives of rosmarinic acid, chlorogenic acid, luteolin, luteolin-7-glycoside, luteolin derivatives, rutin derivatives, and apigenin derivatives. Besides, the cytotoxic activity of the plant extract against L929 fibroblast, H1299 non-small-cell lung carcinoma, and Caco-2 colorectal adenocarcinoma cell lines was determined by MTT assay. Phenolic content and molecular docking results correlated with each other.

1. Introduction

Physical inactivity, smoking, use of alcohol, infectious agents, aging, and exposure to certain chemicals are contributing to the high incidence rate of cancer (Iqbal et al., 2017). One in six deaths in the world is due to cancer (Kuruppu et al., 2019). Depending upon the stage and subtype of cancer, many of these patients will receive chemotherapy, which is accompanied by certain undesirable side effects. Plant-derived products which are less toxic as compared with conventional treatment methods are significantly considered suitable candidates for anticancer drug development (Iqbal et al., 2017). Antibiotic resistance is one of the biggest threats to public health. Antibiotic resistance leads to higher medical costs and increased mortality. For this reason, scientists are in search of new compounds to treat antimicrobial infections.

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Plants have different kinds of secondary compounds as natural protection against microbial attacks and cancer. Many of these compounds have been used as whole plants or extracts for food or medical applications in humans (Wallace, 2004). One group of secondary metabolites is polyphenols in higher plants which have great antioxidant activity. Several studies have shown that plant polyphenols can be used as antioxidants against different oxidative stress-induced diseases such as cardiovascular diseases, diabetes, cancer, and neurodegenerative diseases (Zhou et al., 2019). The human body has a defense system against these oxidative stresses; however, the antioxidant supplement may be helpful for the prevention and/or treatment of these diseases afflicting people worldwide (Stagos, 2019). The flora of Turkey is comprised of approximately 11707 plant species (Davis, 2019), and only a few percent have been studied for the treatment of different diseases. Of these, some of the natural phytochemicals such as taxanes exert their anti-cancer mechanisms by binding to microtubules which are key players for cell division; camptothecin, a topoisomerase poison affects DNA replication; cephalotaxus, an inhibitor of protein synthesis; capsaicin, an activator of caspase 3 and generator of reactive oxygen species leading to apoptosis; cyanidin, an inhibitor of cell growth and division; resveratrol, a modulator of gene expression; rutin and apigenin, inducers of apoptosis and target leptin/leptin receptor activation of caspase-dependent extrinsic apoptosis pathway, inhibit signal transducer, and activate the transcription 3 (STAT3) signaling pathways (Formagio et al., 2015; Heiblig et al., 2014; Kim et al., 2010; Patel et al., 2010; Rahier et al., 2005; Seo et al., 2015; Thakore et al., 2012).

The Lamiaceae family, one of the most important herbal families, incorporates a wide variety of plants with biological and medicinal applications (Uritu et al., 2018). *Phlomis* sp. is one of the genera of Lamiaceae, consisting of 100 species mainly distributed in the Mediterranean and Iran-Turanian phytogeographic region. In Turkey, this genus is represented by 33 species of which 18 are endemics (Dadandı, 2012).

According to World Health Organisation (WHO), antibiotics are becoming increasingly ineffective as drug resistance spreads globally leading to increased difficulty in treating infections and diseases. New antibacterials are therefore urgently needed (WHO, 2015). Also with the increasing mortality rate due to cardiovascular diseases and cancer, it became essential to find alternative leads with fewer side effects for cancer and infectious diseases. Therefore, this study was designed to investigate the antimicrobial, antioxidant, cytotoxic activity, and genotoxicity of the aqueous extract of *Phlomis linearis* with the hope of discovering a new therapeutic agent. The study also determined phenolic acid and flavonoid content using HPLC-DAD and DNA cleavage activity of the extract, in combination with molecular docking simulation of the most profound compounds of the extract.

2. Materials and methods

2.1. Plant material

Plant material collected from Kahramanmaraş province was dried at 35 °C for three days. After drying, 10 g of the dried material was grounded with a homogenizer, and the sample was submerged in 100 ml of water and left to macerate for 1 day. Then, the extract was decanted, and filtered through Whatman No. 1 filter papers, and the filtrate was concentrated to dryness using a standard Buchi rotary evaporator. The resulting dry extracts were resuspended in dimethylsulphoxide (DMSO) (Kim et al., 2011).

2.2. Microorganisms and growth conditions

Gram-positive *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Enterococcus hirae* ATCC 9790, *E. faecalis* ATCC 29212, and gram-negative *Klebsiella pneumoniae* ATCC 13883, *Salmonella typhimurium* ATCC 14028, *Proteus vulgaris* RSKK 96029, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853 and yeast strains *Candida albicans* ATCC 10231 and *C. krusei* ATCC 6258 were used as test microorganisms. The bacterial and yeast cultures were grown on Mueller Hinton Broth at 37 °C and Sabouraud Dextrose Broth at 30 °C, respectively. After incubation, organisms were suspended in 10 ml of physiological saline solution and optical density readings were compared to a 0.5 McFarland standard (1.5 × 10^8 colony-forming unit/ml).

2.3. Antimicrobial activity

Minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC), and minimum fungicidal concentrations (MFC) of *P. linearis* aqueous extract were determined using sterile 96-well plates according to the Clinical & Laboratory Standards Institute (CLSI) reference methods for bacteria M7-A7 (CLSI, 2018) and yeasts M27-A3 (CLSI, 2008). Standard antimicrobial agents chloramphenicol, ampicillin, and ketoconazole were used as controls for bacteria and fungi, respectively. The analysis was carried out in triplicate.

2.4. Determination of free radical scavenging activity

The free radical scavenging potential of the extracts was determined by diphenyl-2-2-picrylhydrazyl (DPPH) assay comparing the half maximal effective concentration (EC_{50}) value of synthetic antioxidant butylated hydroxytoluene (BHT) with minor modifications according to the method described in Braca et al. (2001). A 0.5 ml extract of varying concentrations was mixed with a methanol solution of DPPH radical (0.1 mM). After 30 minutes of incubation at room temperature in the dark, the absorbance was measured spectrophotometrically at 517 nm against a blank. As a reference standard, BHT was used. The percentage of the DPPH radical scavenging effect of the extract was calculated using the following equation:

$$DPPH\ scavenging\ activity\ (\%\ inhibition) = \frac{Acontrol - Asample}{Acontrol} x\ 100$$

where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of the extract.

To determine the concentration of the extract which causes a 50% decrease in DPPH scavenging activity, an extract concentration curve versus percentage inhibition was drawn. The value calculated by linear regression analysis is referred to as EC_{50}. Higher antioxidant activity is indicated by the lower EC_{50} value. All measurements were performed in triplicate.

2.5. Determination of total phenolic content (TPC)

The total phenolic content of the extract was studied according to the method of Folin-Ciocalteu using gallic acid as standard (Afolabi & Oloyede, 2014). 0.1 ml (1 mg/ml) of the extract was mixed with 0.2 ml of diluted Folin-Ciocalteu reagent (1:1 with water). 1 ml of 2% sodium carbonate was added to the reaction mixture after incubation at room temperature for 3 minutes. The absorbance was read at 760 nm by a spectrophotometer (Shimadzu UV-1800, Japan) after 1 h of room temperature incubation in the dark. The total

phenolic content values are expressed as gallic acid equivalent (GAE) in milligrams per gram of dried extract (mg GAE/g extract). All measurements were performed in triplicate.

2.6. Determination of ferrous ion chelating capacity

The ferrous ion chelating capacity of the extract was determined according to the method mentioned by Zhou et al. (2020). 0.5 ml of the extract at varying concentrations was mixed with 1.35 ml of methanol. 0.05 ml of 2 mM $FeCl_2$ was added to the solution and incubated for 5 min. Thereafter, 0.1 ml of 5 mM ferrozine solution was mixed and stayed for 10 min. After incubation, absorbance was measured at 562 nm by spectrophotometer (Shimadzu UV-1800, Japan) against a blank. The percentage of ferrous ion chelating potential of the sample was determined using the following equation:

$$Ferrous\ ion\ chelating\ ability\ (\%) = \frac{Acontrol - Asample}{Acontrol} x\ 100$$

where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of the reaction mixture.

The EC_{50} value was determined by a linear regression curve, which is the concentration of the extracts that chelate 50% of the ferrous ion. All measurements were performed in triplicate.

2.7. Cytotoxicity assays

The cytotoxic effect of the plant extract in human L929 fibroblast, H1299 non-small-cell lung carcinoma, and Caco-2 colorectal adenocarcinoma cell lines (cell lines obtained from Kırıkkale University Culture Collection) were determined using 3-(dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay where cisplatin was used as a reference ISO 10993-5 (ISO 10993-5, 2009). Cultures were grown and seeded (10^4 cells per well in the 96-well cell culture plates) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (DMEM-10), 1% glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin. Cells were incubated at 37 °C for 24h in an atmosphere of 5% CO_2 and 100% humidity in the air. The plant extract was dissolved in DMSO (10%) and applied to cells in five different concentrations (beginning from 5 mg/ml and two-fold dilutions). The solvent DMSO (10%), cisplatin (positive), and DMEM medium (blank) were used as controls. After 24 h incubation of cells, 50 µl MTT (1 mg/ml) solution was added to each well and after 2 h of incubation at 37 °C, 100 µl of isopropanol was added to the wells, and the absorbance values of the 96-well plate were read at 570 nm in a microplate reader to determine cell viability. The cytotoxicity studies were made in triplicate and the data were given as mean ± standard deviation (SD). Based on the control groups, the percent viability was calculated by the following formula:

$$Cell\ viability\ (\%) = \frac{Extract\ OD}{Control\ OD} x\ 100$$

where OD is the optical density.

2.8. Genotoxicity

The AMES test was performed with the plate incorporation method in the presence and absence of an exogenous metabolic activation system (rat liver postmitochondrial fraction with cofactors-S9mix), by using bacterial reverse mutation test according to the OECD Guideline for testing of chemicals 471 (OECD, 1997). In these experiments, *S. typhimurium* TA97a, *S. typhimurium* TA98, *S. typhimurium* TA100, *S. typhimurium* TA102, and *S. typhimurium* TA1535 strains recommended in OECD 471 standard were used. The plant extract was dissolved in DMSO (10%) and applied in five different concentrations (beginning from non-cytotoxic concentration and serial two-fold dilutions). The strains were incubated at 37 °C for 10-15 hours until the early stationary phase of growth (approximately 10^8 cells per ml in 30 ml nutrient broth in 250 ml flasks). Solvent, negative, and positive controls were established in three replicates using each concentration. For each replicate; 2 ml of histidine-biotin supplemented semi-dissolved (at 43-48 °C) top agar, 0.1 ml of the sample (each concentration)/solvent/positive control solution (mutagen)/negative control solution (phosphate buffer) was transferred to tubes, and 0.1 ml from the bacterial suspension and 0.5 ml of S9mix (5%, v/v), and for the test in the absence of metabolic activation system, phosphate buffer was added instead of S9mix, vortexed and poured on minimal glucose agar plates. The plates were incubated at 37 °C ± 2 for 48 h. Revertant colonies were counted, and statistical analyses were made.

2.9. DNA cleavage activity

Interaction between the water extract of *P. linearis* and pBR322 plasmid DNA was carried out using gel electrophoresis following a method described by Deqnah et al. (2012).

2.10. Restriction enzyme BamHI and HindIII digestion

BamHI and HindIII are restriction endonucleases that bind at the recognition sequence 5'-G/GATCC-3', 5'-A/AGCTT-3' and cut these sequences just after the 5'-guanine and 5'-adenine on each strand, respectively (Binici et al., 2023). The plasmid DNA contains a single restriction site for both enzymes that convert the supercoiled form I and singly nicked circular form II to linear form III DNA. The compounds and the DNA were incubated for 24 and 48 h in an incubator bath at 37 °C and then subjected to restriction enzyme digestion (Okumuş et al., 2016). The mixtures were left in an incubator at 37 °C for another 1 h. The samples were run in an agarose gel. The gel was photographed using Biometra Gel Imaging Systems.

2.11. Determination of phenolic compounds in the plant extract by HPLC-DAD

Phenolic compounds from *P. linearis* were extracted and 0.0971 g dry extract was diluted to a quarter and filtered for HPLC-DAD analysis. The chromatographic analyses were performed using a Dionex (Thermo Scientific, Germering, Germany) Ultimate 3000 HPLC system equipped with an Ultimate 3000 Degasser, an Ultimate LPG-3400SD Pump, an Ultimate WPS-3000TSL Autosampler, an Ultimate 3000 DAD, and an Ultimate TCC-3000SD column compartment.

A Thermo acclaim C30 column (150 mm × 3 mm id × 3 µm pd) was used with Macherey Nagel (3 mm id) guard column. Gradient elution was used with mobile phases of A: 2% acetic acid in water and B: 70% acetonitrile-30% water. The flow rate was 0.35 ml/min and the injection volume was 10 µl. The column temperature was 25 °C. 20 phenolic standards were used for calibration and validation of the HPLC-DAD analysis method as following: gallic acid, protocatechuic acid, *p*-hydroxy benzoic acid (*p*-OH benzoic acid), catechin, chlorogenic acid, caffeic acid, syringic acid, vanillin, epicatechin, *p*-coumaric acid, ferulic acid, rutin, luteolin-7-glucoside, apigenin-7-glucoside, rosmarinic acid, resveratrol, eriodictyol, luteolin, quercetin, and apigenin. They were diluted from their stock

solution into seven different concentrations at 0.3125, 0.625, 1.25, 5, 10, 25, and 40 mg/l in a 1:1 methanol-water solution. The external calibration method (Turumtay et al., 2014) was used, and their regression coefficient (R^2) was found at least 0.999. Repeatability of the retention time and peak areas were measured as coefficient of variation (CV) which was under 0.56 for retention times and 2.55 for areas of the peaks. The limit of detection and quantification values of the peaks were under 0.11 and 0.32 mg/l for all standards. Chromatograms were processed at 254, 280, 315, and 370 nm with a diode array detector that operated 200-400 nm. Identification and quantification of peaks were accomplished by comparison of retention times and UV spectra with those of standard phenolic compounds. With different retention times from the standards, some peaks had the same or very similar UV spectra as some standards. They were identified as the derivatives of the standards with similar UV spectra and quantified as an equivalent of these standards (Turumtay et al., 2014).

Table 1. Minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC), and minimum fungicidal concentrations (MFC) of the *P. linearis* water extract and controls (mg/ml)

Microorganisms		Water extract	Positive controls		
			Ampicillin	Chloramphenicol	Ketoconazole
E. coli ATCC 35218	MIC	500	> 125	> 125	-
	MBC	> 500	> 125	> 125	-
B. subtilis ATCC 6633	MIC	> 500	62.5	3.91	-
	MBC	> 500	62.5	3.91	-
S. aureus ATCC 25923	MIC	> 500	62.5	125	-
	MBC	> 500	62.5	> 125	-
E. faecalis ATCC 29212	MIC	> 500	31.25	62.5	-
	MBC	> 500	125	> 125	-
P. aeruginosa ATCC 27853	MIC	> 500	> 125	> 125	-
	MBC	> 500	> 125	> 125	-
K. pneumoniae ATCC 13883	MIC	> 500	125	15.63	-
	MBC	> 500	125	15.63	-
S. typhimurium ATCC 14028	MIC	> 500	62.5	125	-
	MBC	> 500	62.5	125	-
P. vulgaris RSKK 96029	MIC	> 500	> 125	125	-
	MBC	> 500	> 125	> 125	-
E. hirae ATCC 9790	MIC	> 500	62.5	62.5	-
	MBC	> 500	62.5	> 125	-
C. albicans ATCC 10231	MIC	> 500	-	-	31.25
	MBC	> 500	-	-	62.5
C. krusei ATCC 6258	MIC	> 500	-	-	< 0.98
	MBC	> 500	-	-	15.63

2.12. Molecular docking studies

The docking studies of compounds were carried out using Schrödinger to investigate the binding mode and interactions between ligands and target proteins (Schrödinger Release, 2020-3a, 2020-3b, 2020-3c, 2020-3d, 2020-3e, 2020-3f, 2020-3g, 2020-3h, 2020-3i). The 2D structure of the compounds was downloaded from the ZINC15 database (Irwin & Shoichet, 2005; Irwin et al., 2012). The 3D structures of the compounds were created using MacroModel (Schrödinger Release, 2020-3d) embedded Schrödinger Platform. The LigPrep (Schrödinger Release, 2020-3c) module was used to generate all possible conformers at a pH range of 7 ± 2 and using an OPLS_2005 force field.

The crystal structures of different DNA dodecamers PDB ID: 1BNA (Drew et al., 1981), PDB ID: 2GVR (Morsy et al., 2021), and PDB ID: 2DES (Cirilli et al., 1993), as well as the crystal structures of EGFR tyrosine kinase (PDB ID: 1XKK) (Wood et al., 2004), human basic fibroblast growth factor (PDB: ID 4FGF), cytochrome P450 (PDB ID: 1OG5) (Williams et al., 2003), xanthine oxidase (PDB ID: 3NRZ) (Cao et al., 2010), pro-inflammatory gene COX-2 (PDB ID: 5IKR) (Orlando & Malkowski, 2016), and tyrosine kinase (PDB ID: 1M17) (Stamos et al., 2002) were downloaded from protein databank to further use in molecular docking studies. Structures of the proteins were prepared for docking using the Protein Preparation Wizard of Maestro (Madhavi Sastry et al., 2013; Schrödinger Release, 2020-3e). In this process, the ionization and tautomeric states were generated by Epik (Greenwood et al., 2010; Schrödinger Release, 2020-3a), and the proton states were set by PROPKA. All proteins were finally minimized by the OPLS-2005 force field.

The 3D structure of the ligands was transferred to the Receptor Grid Generation module of Maestro (Schrödinger Release, 2020-3e), and grids were produced using the default parameters: van der Waals scaling factor 1.00 and charge cutoff value 0.25 by using the force field OPLS-2005. Docking simulation was done using the extra precision (XP) scoring function of Glide (Friesner et al., 2004; Friesner et al., 2006; Halgren, 2009; Schrödinger Release, 2020-3b), and only the best scoring fit with docking score was noted for each ligand. Prime (Jacobson et al., 2002; Schrödinger Release, 2020-3f) MM/GBSA calculation was carried out to calculate relative binding free energy for 1XKK. SiteMap (Halgren, 2007; Halgren, 2009; Schrödinger Release, 2020-3i) calculation was performed for 1BNA. QikProp (Schrödinger Release, 2020-3h) module implemented the Schrödinger suite was used for evaluating the ADME properties of the ligands.

3. Results and discussion

3.1. Antimicrobial activity

The results of the antimicrobial screening revealed that the MIC value of *P. linearis* was >500 mg/ml against all bacteria and yeast strains while the MIC value of antibiotics was 3.91 - >125 mg/ml (Table 1). Unlike our high MIC value results, Göger et al. (2021) showed that the MIC value of *P. linearis* EtOAc extracts against *S. aureus* strain was 156.25 µg/ml, *E. coli* and *C. albicans* were 625 µg/ml indicating that the plant was resistant to the EtOAc extract but susceptible to the water extract of *P. linearis*. The different MIC values were attributed to the polarity of the solvent, different stages of growth, and the environment of the plant, which can significantly affect their chemical composition (Koutsoukis et al., 2019).

3.2. Antioxidant activity

Aqueous plant extract exhibited good antioxidant activity (EC_{50} = 53.86 μg/ml) relative to that of BHT as a standard antioxidant (EC_{50} = 96.47 μg/ml) according to the DPPH radical scavenging activity results (Table 2). The EC_{50} value of the *P. linearis* extract is significantly higher than that of the BHT even though both extracts were prepared using aqueous as the extraction solvent. Also, the amount of total phenolic content of the aqueous plant extract was measured and expressed in mg gallic acid equivalent per gram (mgGAE/g). The aqueous extract revealed the presence of total phenols of 231.55 ± 2.11 mgGAE/g extract (Table 2). The metal chelating activity was found at 14.71 ± 4.01 mg/ml. The total phenol content varies depending on the plant species, plant tissue, developmental stage, and environmental factors, such as temperature, water stress, and light conditions (Upadrasta et al., 2011). This paper represents the first report on the antioxidant activities of the aqueous extract of *P. linearis*.

Table 2. The antioxidant properties of *P. linearis* aqueous extract

Parameter	Result	Parameter
Total phenolic content (mg GAE/g extract)	231.55 ± 2.11	Total phenolic content (mg GAE/g extract)
Metal chelating activity (IC_{50}) (mg/ml)	14.71 ± 4.01	Metal chelating activity (IC_{50}) (mg/ml)
DPPH scavenging activity (IC_{50}) (μg/ml)	53.86 ± 0.50	DPPH scavenging activity (IC_{50}) (μg/ml)
BHT (IC_{50}) (μg/ml)	96.47 ± 0.32	BHT (IC_{50}) (μg/ml)

3.3. Cytotoxicity assays

The cytotoxicity of aqueous *P. linearis* extract was determined using MTT assay in mouse fibroblast L929 cell line, human non-small cell lung carcinoma cell line H1299, and human colorectal adenocarcinoma Caco-2 cell lines exposed to 0.16 mg/ml, 0.32 mg/ml, 0.63 mg/ml, 1.25 mg/ml, 2.50 mg/ml, and 5 mg/mL of the extracts at incubation period of 24 h.

MTT test was performed to determine the cytotoxic activity of the aqueous extract of *P. linearis*. The IC_{50} values of the extracts were determined according to cell viability data for cell lines. Figure 1 presents cell viability percentages against applied concentrations for *P. linearis* extract to cell lines. The IC_{50} values of *P. linearis* extract on L929, H1299, and CaCo-2 cells were determined as 5.99 ± 1.19 mg/ml, 2.76 ± 0.04 mg/ml, and 4.85 ± 0.68 mg/ml, respectively.

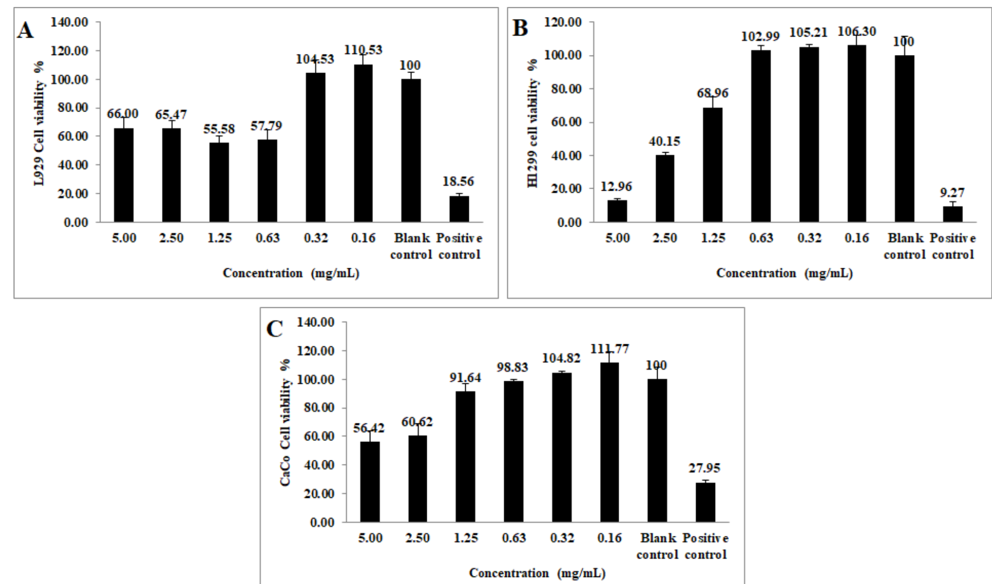

Figure 1. Cell viability percentages for applied concentrations of *P. linearis* aqueous extract on L929 mouse fibroblast cell line (A), H1299 human non-small lung carcinoma cell line (B), and human colorectal adenocarcinoma Caco-2 cell line (C)

Table 3. Genotoxicity test results of *P. linearis* water extract (0.32 mg/ml)

		Plant extract	Negative control
TA97	- S9	81.33 ± 22.5	85.00 ± 9.85
	+ S9	534 ± 38.81	456.67 ± 31.77
TA98	- S9	11.67 ± 2.08	10.67 ± 6.03
	+ S9	505.67 ± 39	431.33 ± 30.09
TA100	- S9	79.33 ± 17.67	74.33 ± 6.81
	+ S9	501.67 ± 34.50	534.67 ± 19.30
TA012	- S9	405.33 ± 28.38	393.33 ± 8.33
	+ S9	704.00 ± 42.33	744 ± 37.36
TA1535	- S9	17.67 ± 1.53	13.67 ± 3.21
	+ S9	456.33 ± 28.36	510.33 ± 47.75

It was observed that the applied doses of the *P. linearis* aqueous extract were unable to demonstrate a potentially toxic effect on L929 cells, but it was effective against H1299 human non-small lung carcinoma cells at high concentrations, as can be seen from the IC_{50} values and viability percentages. The 5 mg/ml concentration of the extract in H1299 cells had almost the same effect as cisplatin, which is widely used in cancer therapy (Ghosh, 2019).

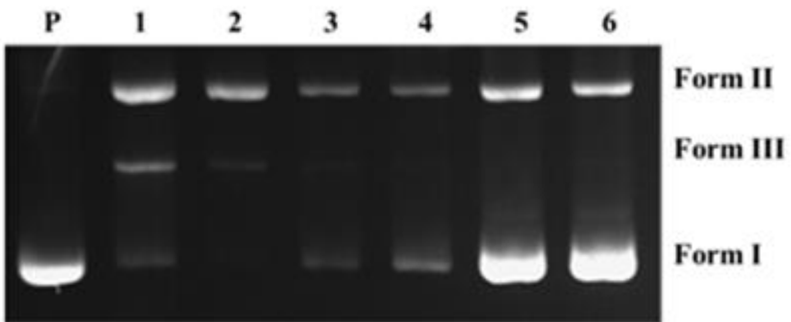

Figure 2. Results of electrophoresis after the incubation of pBR322 plasmid DNA with various concentrations of the extracts for 48 h.
The first line (P) corresponds to untreated pBR322 plasmid DNA as a control, and lines 1-6 correspond to pBR322 plasmid DNA incubated with compounds at concentrations ranging from 1000 µg/ml to 31.25 µg/ml (line 1: 1000 µg/ml; line 2: 500 µg/ml; line 3: 250 µg/ml; line 4: 125 µg/ml; line 5: 62.5 µg/ml; line 6: 31.25 µg/ml).

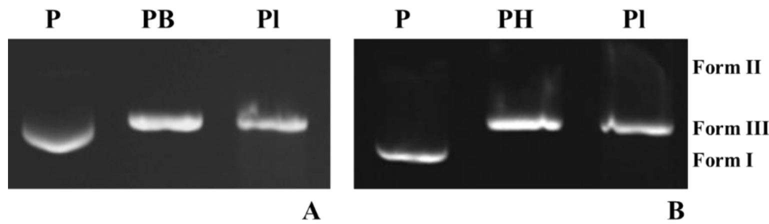

Figure 3. Electrophoretogram for the incubated mixtures of plasmid DNA followed by digestion with BamHI (A) and HindIII (B)
Lane P shows untreated DNA, lanes PB and PH depict untreated but digested plasmid DNA, lane Pl is plasmid DNA treated with the extract and enzyme-digested.

3.4. Genotoxicity

The mutagenicity of plant extract with and without metabolic activation (absence of S9 fraction) was investigated on *S. typhimurium* test strains TA97a, TA98, TA100, TA102, and TA1535. The plant extract was applied at a non-cytotoxic concentration of 0.32 mg/ml for L929 cells as required in the MTT assay results. Induction of his^{+} revertants in *S. typhimurium* strains by *P. linearis* aqueous extracts without (top line) and with (bottom line) metabolic activation (S9mix) are given in Table 3. To determine the statistical significance of the number of his^{+} revertants induced by the plant extract compared to the control, an independent sample *t*-test was done at $p \leq 0.05$.

The results were expressed in the number of revertants/plates of three independent experiments ± SD. Negative control sterile distilled water (100 µl) used as a solvent for the extract.

The use of herbs in recent years in traditional medicine has increased significantly (Sponchiado et al., 2016). However, these plants can be extremely harmful to human health if not administered properly. Studies have revealed that some herbs used even daily are potentially genotoxic (Mendonça et al., 2016). According to the AMES test results, performed both in the absence and presence of metabolic activation, the plant extract did not induce mutagenic effects on any *S. typhimurium* strains tested. There is no data in the literature regarding the genotoxic effect of *Phlomis* species (da Silva Dantas et al., 2020). Moreover, in some studies on the genotoxic effects of species belonging to the Lamiacea family, it was observed that these species also did not have genotoxic effects (Ayubi et al., 2021). It is noteworthy that the species belonging to this family are more effective in terms of anti-genotoxic effects among medicinal plants (da Silva Dantas et al., 2020).

3.5. DNA interaction with plant extract

The agarose gel electrophoresis study was carried out to gather information relating to the structural damaging interactions of the plant aqueous extract with DNA. When pBR322 plasmid DNA was exposed to the plant extract, generally two DNA bands corresponding to forms I and II were observed in both untreated and treated pBR322 plasmid DNA. As the concentration of the compounds was increased, the intensity of form II increased and form I pBR322 plasmid DNA decreased at 48 h incubation, a linear DNA form III band was observed in lane 1-2 corresponding to 1000 µg/mL and 500 µg/mL concentration (Figure 2). The mobility of form I decreased with increasing concentrations of plant extract, and also the presence of linear DNA was observed for other concentrations of the extract rather than two high concentrations indicating double-strand cleavage of the DNA. The changes in mobility and conformations of the DNA bands indicated changes in DNA conformation which may be a result of the covalent binding of some constituents of the aqueous extract with the DNA.

3.5.1. BamHI and HindIII digestion

To obtain further information on changes in DNA conformation, DNA was incubated with the plant extract which was followed by BamHI and HindIII digestion. Figure 3 gives the electrophoretogram

corresponding to the BamHI (A) and HindIII (B) digested incubated mixtures of pBR322 plasmid DNA and plant extract. BamHI and HindIII digestion of pBR322 plasmid DNA incubated with plant extract proved the binding interaction of the extract with A/A and G/G nucleotides of DNA. The compounds may also bind to other nucleotides of DNA. Analysis of phenolic compounds and molecular docking studies were performed to show the initial structural basis of DNA conformational change and DNA cleavage. The discovery of compounds that have the potential to interact with DNA macromolecule, which is the pharmacological target of many drugs currently in clinical use or advanced clinical trials, and determining their mechanism of action are important in terms of developing a new lead compound (Sirajuddin et al., 2013).

3.6. Phenolic content by HPLC-DAD analysis

As can be seen in Table 4 and Figure 4, the majority of phenolic compounds determined in the *P. linearis* aqueous extract are chlorogenic acid, derivatives of rosmarinic acid, rutin, apigenin, and *p*-coumaric acid. Luteolin-7-glycoside, luteolin, and some derivatives of luteolin, *p*-OH benzoic acid, protocatechuic acid, and caffeic acid were also determined as minor compounds.

Table 4. HPLC-DAD analysis of the extracts

No	Retention time	Compound	mg/g extract	µg/g dry plant
1	8.69	Protocatechuic acid	0.23	43.66
2	9.44	*p*-OH benzoic acid der*	0.21	41.40
3	15.54	*p*-OH benzoic acid	0.25	48.40
4	21.87	Chlorogenic acid	2.52	486.29
5	22.87	Caffeic acid	0.17	32.68
6	23.32	Rutin der	0.38	73.82
7	25.16	Apigenin der	0.94	181.00
8	25.63	Protocatechuic acid der	0.36	69.55
9	26.76	*p*-Coumaric acid	0.14	26.38
10	27.17	*p*-Coumaric acid der	0.09	17.00
11	30.22	Rosmarinic acid der	1.71	329.19
12	30.54	Rutin der	3.07	592.26
13	31.13	Rosmarinic acid der	3.38	653.14
14	31.63	Rosmarinic acid der	14.96	2886.62
15	31.85	Chlorogenic acid der	4.94	953.60
16	31.95	Rosmarinic acid der	11.29	2178.14
17	32.39	Luteolin-7-glucoside	0.48	93.04
18	32.56	Rutin der	7.34	1416.57
19	33.29	Rosmarinic acid der	1.30	250.72
20	33.84	Luteolin der	1.95	375.70
21	34.16	*p*-Coumaric acid der	0.19	37.57
22	34.54	Rosmarinic acid der	6.25	1205.75
23	35.36	Rutin der	0.61	117.56
24	35.67	Luteolin der	0.90	174.43
25	36.85	Rosmarinic acid der	0.31	60.55
26	37.636	Rosmarinic acid der	1.51	290.75
27	37.875	Rosmarinic acid der	0.96	186.01
28	38.985	Chlorogenic acid der	0.44	84.52
29	41.398	Luteolin	0.27	52.06
30	43.14	Resveratrol der	1.28	247.80
31	43.483	*p*-Coumaric acid der	0.13	25.97
32	44.53	Chlorogenic acid der	0.24	46.77
33	45.135	Resveratrol der	0.15	29.56
34	46.162	Caffeic acid der	0.41	79.00
35	46.66	Apigenin der	0.99	190.75
36	47.139	*p*-Coumaric acid der	2.84	547.99
37	50.38	*p*-Coumaric acid der	0.21	40.05

*der: derivative

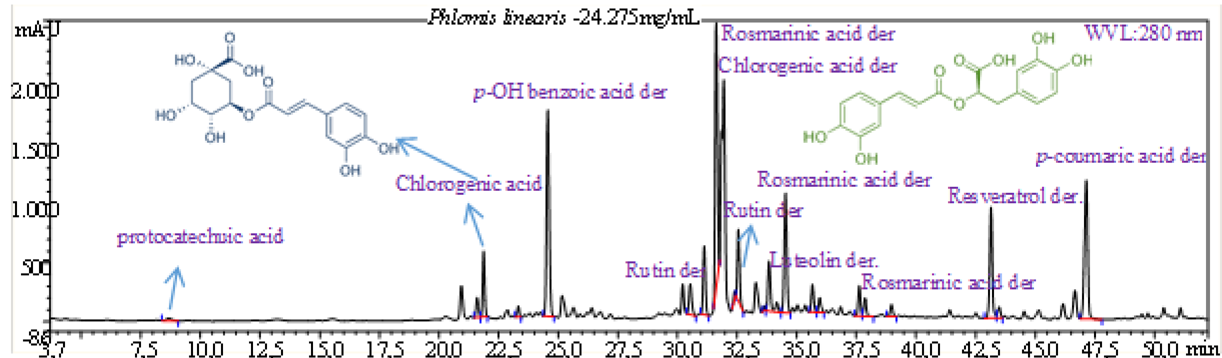

Figure 4. HPLC chromatogram of the *P. linearis* extract with the absorbance monitored at 280 nm

Demirci et al. (2003) reported that the oil of *P. linearis* was found to contain β-caryophyllene (24.2%), germacrene D (22.3%), and caryophyllene oxide (9.2%). Another study showed that the extract contained 14.9% monoterpenes with α-pinene (12.5%) as the major constituent. However, the plant sample collected in Kayseri province did not contain monoterpenes at all. In literature, the essential oil of

Phlomis species was studied. They found mainly sesquiterpene hydrocarbons as the major volatiles with caryophyllene (24.2%), germacrene D (22.3%), and *(Z)*-farnesene (6.6%) (Demirci et al., 2003). Studies regarding the chemical profiles of *P. linearis* were not well-detailed.

Table 5. Ligands in plant extract used in molecular docking*

No	Chemical structure	Chemical name	Molecular weight
1		Rutin	610.524
2		Apigenin	270.241
3		Chlorogenic acid	354.313
4		Rosmarinic acid	360.32
5		*p*-Coumaric acid	164.16

*Image from ZINC15 database (Irwin & Shoichet, 2005; Irwin et al., 2012)

Table 6. Molecular docking results of DNA with plant extract ligands

PDB IDs	XP docking score (kcal mol^{-1})				
	1	2	3	4	5
1BNA	-8.203	-1.834	-5.115	-6.569	-2.812
2GVR	-9.272	-7.245	-5.969	-7.352	-3.455
2DES	-9.999	-7.710	-9.705	-8.869	-6.021

3.7. Molecular docking studies

According to the HPLC-DAD analysis result, the top five most abundant ligands, *p*-coumaric acid, rosmarinic acid, chlorogenic acid, apigenin, and rutin were selected for molecular docking (Table 5). 2D structure of the ligands was downloaded from the ZINC15 database (Irwin & Shoichet, 2005; Schrödinger Release, 2020-3d). 3D structure, tautomers, and ionic forms of these ligands were prepared by LigPrep (Schrödinger Release, 2020-3c) module embedded in the Schrodinger Platform.

Molecular docking simulations of the plant extract ligands were performed with XP Glide (Friesner et al., 2004; Friesner et al., 2006; Halgren, 2007; Schrödinger Release, 2020-3b), docking protocol of the Schrödinger platform. Obtained XP docking scores were listed in Table 6. DNA dodecamers PDB ID: 1BNA (Drew et al., 1981), PDB ID: 2GVR (Morsy et al., 2021), and PDB ID: 2DES (Cirilli et al., 1993), as well as the other target proteins, were selected for docking studies of the major constituents of plant extract. The DNA sequence of 1BNA is that DNA (5'-D(*CP*GP*CP*GP*AP*AP*TP*TP*CP*GP*CP*G)-3') (Alizadeh et al., 2015; El-Medani et al., 2020; Morsy et al., 2021; Ozkan et al., 2020), 2GVR is that DNA (5'-D(*CP*GP*CP*GP*AP*AP*TP*TP*CP*GP*CP*G)- 3') (Morsy et al., 2021), and 2DES is that DNA (5'-D(*CP*GP*TP*AP*CP*G)-3') (Morsy et al., 2021).

The 3D models of the ligands bonded to 1BNA, and their interactions was given in Figure 5. The docked ligands' conformations were ranked according to the binding energy,

hydrogen bonding, and hydrophobic interactions between the ligands and the DNA (PDB ID: 1BNA, 2GVR, 2DES) (El-Medani et al., 2020). The docking calculations showed that docked compounds generally fit in the DNA minor groove and comprise ionic, and hydrogen bonding interactions with the DNA bases. It was also found that the optimal docking binding interactions have occurred in the G-C region. 1BNA did not have any cognate ligand, therefore, SiteMap (Schrödinger Release, 2020-3i) was used for the docking studies. Compound 1 fits in the DNA major groove as seen in Figure 6a, because it has a larger size rather than other ligands, and it fits in a wider region, i.e., the major groove in the site of 1BNA. Compound 1 has the higher binding interactions (Table 6), because of hydrogen bonding interactions between DG 16, DT 8, DA 6, DA 5, and DA 17, and the aromatic moiety and OH group. The compound 2 has hydrogen bonds between DG 16 and O group in aromatic moiety, DC 15 and OH group of 2. The binding interaction of compound 3 showed hydrogen bonds between DC 9 and OH group of 3, DC 14, and COO-, a group of 3. Also, compounds 4 and 5 shared hydrogen bonds between DG 16 and OH group, and the DT 7 and OH moiety, respectively. The order of XP docking scores is as follows:1 > 4 > 3 > 5 > 2 for 1BNA, 1 > 4 > 2 > 3 > 5 for 2GVR, and 1 > 3 > 4 > 5 > 2 for 2DES.

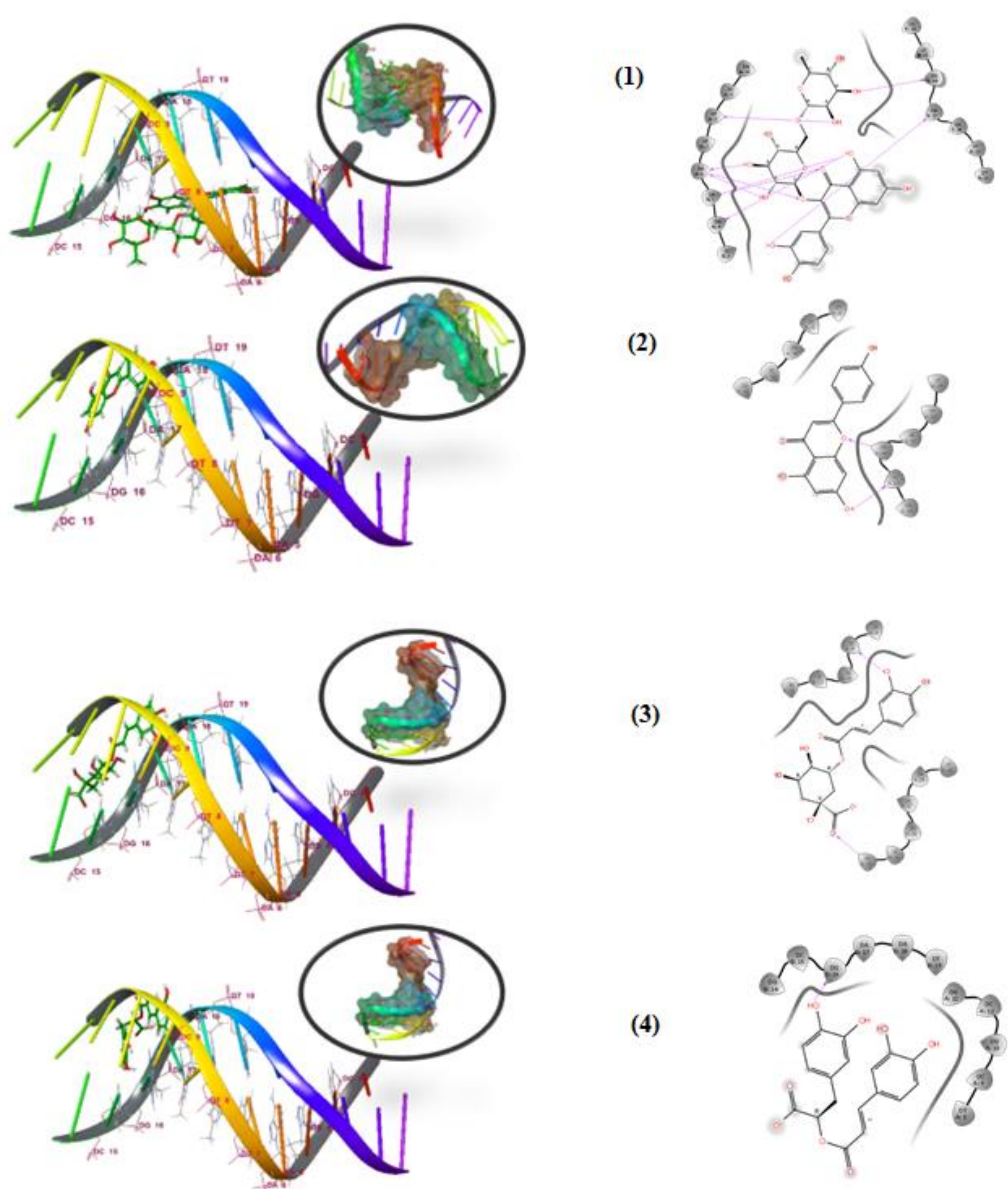

Figure 5. Binding mode of ligands 1, 2, 3, 4, and 5 with 1BNA 3D (left column) and 2D (right column) interactions

Cytochrome P450 (PDB ID: 1OG5) (Williams et al., 2003), xanthine oxidase (PDB ID: 3NRZ) (Cao et al., 2010), and pro-inflammatory gene COX-2 (PDB ID: 5IKR) (Orlando & Malkowski, 2016) were selected as target proteins for the antioxidant property. In addition, EGFR tyrosine kinase (PDB ID: 1XKK) (Wood et al., 2004), and tyrosine kinase (PDB ID: 1M17) (Stamos et al., 2002) were selected for elucidation of the structural basis of cytotoxic properties. Molecular docking studies of the ligands of plant extract and re-docking of cognate ligand was performed using XP Glide (Friesner et al., 2004; Friesner et al., 2006; Halgren, 2007; Schrödinger Release, 2020-3b) docking protocol of Schrödinger platform. Obtained XP Docking scores was listed in Table 7. A 3D model of the compound 1 bonded to the active site of the proteins, i.e., 1OG5 & 1XKK, and their 2D interactions were given in Figure 6. The Prime (Jacobson et

al., 2002; Jacobson et al., 2004; Schrödinger Release, 2020-3f) molecular mechanics/generalized born surface area (MM-GBSA) method was performed only for 1XKK to estimate the relative binding free energy of the ligands to the target protein.

Calculated binding free energy and XP docking score are well correlated. According to the docking score, generally, the antioxidant activities of the compounds were greater than cytotoxic activity, which is consistent with the experimentally obtained data.

Obtained docking score for 1OG5 shows that compounds 1 and 4 bind stronger to the active site of cytochrome P450; in other words, it can be predicted that 1 and 4 may have better antioxidant activity than cognate ligand SWF. Similarly, compound 1 in 3NRZ binds better to the active site of xanthine oxidase than cognate ligand HPA, in other words, compound 1 could have better antioxidant activity than HPA. The docking score of compound 2 for 51KR shows that compound 2 binds better to the active site of the pro-inflammatory gene COX-2, that is, compound 2 could have better antioxidant activity than cognate ligand ID8. Compound 1 was too big for the active site of 5IKR; therefore, it could not form an energetically favorable binding complex in docking calculations.

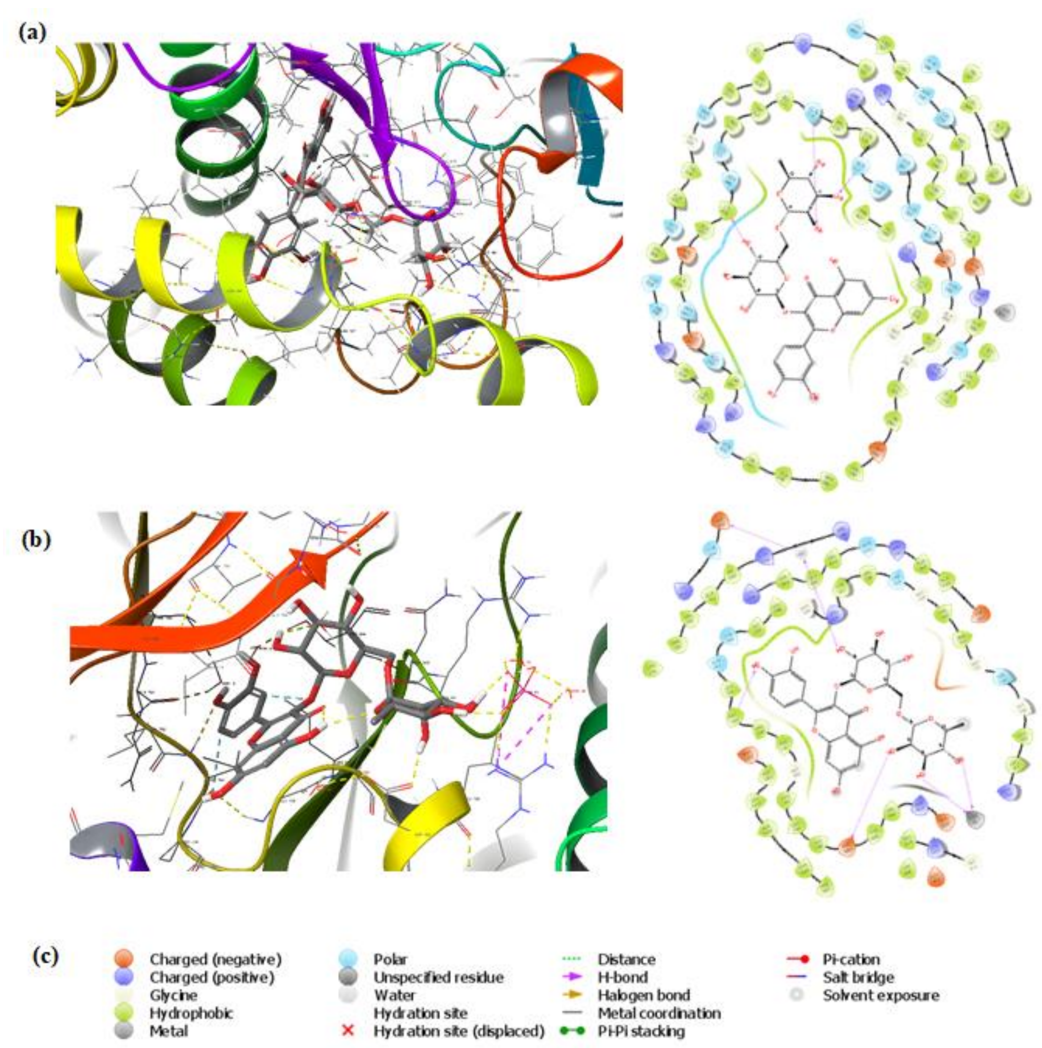

Figure 6. Binding mode of compound 1 with the active site of 1OG5 (a), 1XKK (b), and 2D interactions notations (c)

Tyrosine kinase and fibroblast growth factor were selected to evaluate the cytotoxic activity of the ligands because they are used to describe the cytotoxic activity of L929 mouse fibroblast cells, H1299 human non-small lung carcinoma cells, and human colorectal adenocarcinoma (Caco-2) cells. Tyrosine kinase is also a target enzyme related to oxidative stress. Long-term oxidative damage has been attributed to aging, cancer, neural and cardiovascular disorders, and liver diseases (Arteel, 2003; Guidi et al., 2006; Hyun et al., 2006; Kinnula & Crapo, 2004; Sas et al., 2007; Singh & Jialal, 2006). The docking score of the compounds for 1XKK is not higher than the cognate ligand FMM, that is, the usage of plant extract as an EGFR tyrosine kinase inhibitor is not suitable. The docking score of compound 1, calculated for 1M17 is a little higher than the cognate ligand AQ4, indicating that compound 1 binds stronger than the cognate ligand to the active site of tyrosine kinase. Therefore, it may be predicted that compound 1 could have better cytotoxicity than AQ4.

Compound 1 (rutin) in plant extract has antioxidant and cytotoxicity activity against cytochrome P450 and tyrosine kinase enzyme, respectively. Compound 4 (rosmarinic acid) in plant extract has higher antioxidant activity than cognate ligand SWF. Compound 2 (apigenin) in plant extract has moderate antioxidant and cytotoxic

activity. Consequently, the antioxidant activity of the plant extract is greater than the cytotoxic activity.

Compound 1 has four H-bond interactions with Ala719, Arg817, Asn818, and Asp 831; however, cognate ligand AQ4 has three with Met769, Cys773, and water molecules. Therefore, the polarity and hydrophobicity of compound 1 caused a higher number of non-covalent interactions than AQ4. Moreover, compound 1 has a bigger size compared to SWF. Similarly, compound 1 has more non-covalent interactions in the active site of 1OG5 via three H-bonds (Gln214, Asn217, and Leu208) compared to SWF with two H-bonds (Ala103 and Phe100), one π-π stacking, and one π-cation interaction as seen in Figure 5.

Table 7. Molecular docking results of cognate and plant extract ligands

PDB IDs	XP docking score (kcal mol^{-1})					
	1	2	3	4	5	Cognate
1OG5	-11.624	-7.547	-1.230	-10.555	-5.487	-6.098
3NRZ	-9.248	-7.296	-7.151	-6.440	-6.043	-6.278
5IKR	-	-10.616	-1.981	-6.658	-7.331	-10.132
1XKK	-9.145	-7.850	-6.594	-8.569	-4.901	-12.792
1M17	-12.940	-8.580	-8.122	-3.888	-3.793	-9.611
	Prime MM-GBSA ΔG binding energy (kJ mol^{-1})					
	1	2	3	4	5	
1XKK	-44.81	-39.88	-26.48	-40.60	-16.90	

Table 8. ADME results for selected ligands

No	MW	a	b	c	d	e	f	g	h	i	j
1	610.524	-2.623	9	20.55	3	15	1	-2.272	0.745	-7.436	2
2	270.241	1.635	2	3.75	0	3	3	-3.331	116.767	-3.97	0
3	354.313	-0.287	6	9.65	1	10	1	-2.563	1.652	-6.222	1
4	360.32	1.141	5	7	0	11	1	-3.344	1.512	-5.863	1
5	164.16	1.432	2	2.75	0	4	3	-1.658	62.218	-3.606	0

MW: Molecular weight, a: QPlogPo/w, b: Donor HB, c: Accpt HB, d: Rule of five, e: #rotor, f: Human oral absorption, g: QlogS, h: QPPCaco; i: QPlogKp, j: Rule of three

3.8. ADME/Tox analysis

Theoretical calculations of the ADME (absorption, distribution, metabolism, and excretion) properties of the compounds were carried out by the QikProp (Schrödinger Release, 2020-3h) module. Physically significant descriptors and pharmaceutically relevant properties of the compounds obtained with QikProp (Schrödinger Release, 2020-3h) are given in Table 8. The violation of the number five is acceptable, that is, compounds can be predicted to obey Lipinski's rule (MW < 500, QPlogPo/w < 5, donorHB ≤ 5, accptHB ≤ 10) and to have drug-like properties (Table 8). In addition, compounds are more likely to be orally available because their number of violations of Jorgensen's three rules (QPlogS > -5.7, QP PCaco > 22 nm/s, total primary metabolites < 7) is acceptable (Chen et al., 2020; Duffy & Jorgensen, 2000; Lipinski et al., 2012).

4. Conclusions

The emergence of multiple antibiotic resistance is a worldwide health issue and is motivating an increasing search for active natural and synthetic compounds that can stop antimicrobial resistance. This study showed that *P. linearis* has a more efficient antioxidant capacity compared to reference compounds. *P. linearis* could be a source of new compounds with therapeutic capacity. HPLC-DAD analysis of phenolic content revealed that the species extract is mainly rich in chlorogenic acid, derivatives of rosmarinic acid, rutin, apigenin, and *p*-coumaric acid. The extract had a strong effect on the intensity of form I supercoiled plasmid DNA and caused double-strand breaks indicating DNA cleavage activity. The molecular docking studies revealed that the docked major phytocompounds of *P. linearis* extract are oriented in DNA minor grooves via the formation of ionic, and hydrogen bonding interactions with the DNA bases. Moreover, the antioxidant activity of the plant extract was found greater than its cytotoxic activity, and the major phytocompounds demonstrated their molecular interactions (H-bonding, π-π interactions, etc.) with related target enzymes' active sites. According to ADME results, all 5 compounds showed drug-like properties and were orally available.

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None.

Conflict of interest

The authors confirm that there are no known conflicts of interest.

Statement of ethics

In this study, no method requiring the permission of the “Ethics Committee” was used.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Supplementary File

None.

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