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ORIGINAL ARTICLE / ÖZGÜN MAKALE

IN VITRO AND *IN SILICO* STUDIES ON LIGNAN SECOISOLARICIRESINOL DIGLUCOSIDE

LİGNAN SEKOİZOLARİSİRESİNOL DİGLUKOSİT ÜZERİNE İN VİTRO VE İN SİLİKO ÇALIŞMALAR

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ABSTRACT

Objective: *Lignans are important biologically active compounds in diphenolic structure. Secoisolariciresinol diglucoside (SDG) is a significant type of lignan known to have anti-cancer properties. This study aimed to investigate the antiproliferative activity properties of SDG on hepatocellular carcinoma cells (HepG2), colorectal cancer cells (DLD-1), lung carcinoma (A549), and prostate cancer (PC3) cell lines.*
Material and Method: *Cell viability of cancer cells was determined by the MTT method after treatment with various concentrations of SDG at 48 or 72 hours. The DFT (Density Functional Theory) analysis of the SDG was performed using Spartan'10 and visualized. Drug-likeness and absorption, distribution, metabolism, excretion, and toxicity (ADME-Tox) properties of this compound were examined. Molecular docking was carried out to research the biological activity of SDG.*
Result and Discussion: *Our results showed that SDG exhibited significant cytotoxicity only against DLD-1 cells with IC_{50} value of 37.45 µM, but inactive against other cancer cell lines as in vitro. 4UYA, which biomarker for colon cancer, is the crystal structure of the MLK4 kinase domain. The binding energy value for the SDG-MLK4 kinase domain was calculated as -6.1 kcal/mol. Anticancer potential was verified by in vitro assay and in silico molecular docking study. In conclusion, this study revealed the protective aspect of SDG against colon cancer and showed that it has promising anticancer activity.*
Keywords: *Cancer, cytotoxicity, DFT, molecular docking, SDG*

ÖZ

Amaç: *Lignanlar, difenolik yapıda biyolojik olarak aktif önemli bileşiklerdir. Sekoizolarisiresinol diglukosit. (SDG), kanser önleyici özelliklere sahip olduğu bilinen önemli bir lignan türüdür. Bu çalışmada SDG'nin hepatoselüler karsinom hücreleri (HepG2), kolorektal kanser hücreleri (DLD-1), akciğer karsinomu (A549) ve prostat kanseri (PC3) hücre hatları üzerindeki antiproliferatif*

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aktivite özelliklerinin araştırılması amaçlanmıştır.
Gereç ve Yöntem: *Kanser hücrelerinin hücre canlılığı, 48 veya 72 saatte çeşitli SDG konsantrasyonları ile muamele edildikten sonra MTT yöntemiyle belirlendi. SDG'nin DFT (Yoğunluk Fonksiyonel Teorisi) analizi Spartan'10 kullanılarak yapıldı ve görselleştirildi. Bu bileşiğin ilaca benzerliği ve emilim, dağılım, metabolizma, atılım ve toksisite (ADME-Tox) özellikleri incelendi. SDG'nin biyolojik aktivitesini araştırmak için moleküler yerleştirme gerçekleştirildi.*
Sonuç ve Tartışma: *Sonuçlarımız SDG'nin yalnızca IC_{50} değeri 37,45 µM olan DLD-1 hücrelerine karşı anlamlı sitotoksisite sergilediğini, diğer kanser hücre hatlarına karşı ise in vitro olarak inaktif olduğunu gösterdi. Kolon kanseri biyobelirteci olan 4UYA, MLK4 kinaz bölgesinin kristal yapısıdır. SDG-MLK4 kinaz alanına ait bağlanma enerjisi değeri -6,1 kcal/mol olarak hesaplandı. Antikanser potansiyeli in vitro analiz ve in silico moleküler yerleştirme çalışmasıyla doğrulandı. Sonuç olarak bu çalışma SDG'nin kolon kanserine karşı koruyucu yönünü ortaya koyarak umut verici antikanser etkinliğe sahip olduğunu göstermiştir.*
Anahtar Kelimeler: *DFT, kanser, moleküler yerleştirme, SDG, sitotoksisite*

INTRODUCTION

One of the main ways to deal with degenerative diseases such as cancer has long been seen as a focus on diet, and as a result of increasing awareness in people, the demand and orientation for functional foods is increasing. In this context, particular emphasis is placed on foods rich in lignans. Lignans, belonging to the phytoestrogen class, are natural compounds in diphenolic structure and have different biological activities [1,2]. Secoisolariciresinol diglucoside (SDG) is an essential bioactive lignan species that is present in small amounts in a variety of foods and plants but is particularly high ratio in flaxseed (Linum usitatissimum) [3,4]. After ingestion of SDG, it is metabolized by colon bacteria to the mammalian lignans enterodiol and enterolactone [5,6]. It has been observed that studies involving lignans especially focus on the estrogenic activities of these structures and their potential for effect on cancer types such as hormone-related breast cancer [7-9]. The chemical structure of SDG is shown in Figure 1.

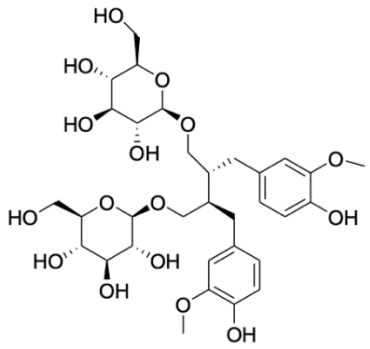


Figure 1. Chemical structure of SDG

After consumption of plant lignan SDG, it is metabolized to mammalian lignans enterodiol and enterolactone by demethylation and dehydroxylation processes by some bacteria in the human colon, such as *Peptostreptococcus Eubacterium* and *Eggerthella* [5,10]. The biological activity of SDG is generally attributed to this metabolic conversion [2]. It has been reported that lignans from flaxseed have the ability to interfere with the cellular properties of malignant tumors, affect molecular signaling junctions, and regulate related signaling pathways [11]. It has been demonstrated by various studies that SDG exhibits protective effects against various cancers such as colon, prostate, and breast, with its antiproliferative, antioxidant, and anti-estrogenic properties and/or by inhibiting metabolic-related enzymes [2,10,12-14]. There are more studies showing the anti-cancer treatment potential of the SDG metabolites enterodiol and enterolactone alone or in combination [7,8,15,16]. There are relatively few studies in the literature on the effects of SDG alone on cancer cells.

In this study, the cytotoxic activity of SDG on DLD-1 human colon cancer, A549 lung cancer, PC3 prostate cancer, and HepG2 liver cancer cell lines was evaluated. In addition, docking study was performed to analyze the binding conformation of the SDG molecule at the 4UYA active site, and *in silico* ADME-Tox profile studies were carried out. This study aimed to observe the inhibitory effectiveness of SDG, which is considered a potential anticancer candidate, in preventing cancer cell development, through molecular structure compatibility and cytotoxicity.

MATERIAL AND METHOD

Reagents, Solvents, and Materials

Human liver carcinoma (HepG2) (ATCC® HB-8065™) cell line, human lung cancer (A549) (ATCC® CCL-185™) cell line, human colon cancer (DLD-1) (ATCC® CCL-221™) cell line, and human prostate cancer (PC3) (ATCC® CRL-1435™) cell line was purchased from American Type Culture Collection. SDG was purchased commercially from Cayman chemical company and used without further purification. SDG was dissolved in 0.5% DMSO for cell culture studies.

In vitro Cytotoxic Activity Studies

The cells were seeded into 96-well plates at 5 x 10^3 cells/well densities for cytotoxic activity studies [17,18]. Cells were exposed to the SDG for different concentrations, varying from 300 to 0.5 µM (for A549), 300 to 37.5 µM (for HepG2), 300 to 9.375 µM (for DLD-1 and PC3), after 24 h. MTT stock solution was prepared at 5 mg/ml and 50 µl was added to each well after 48 h (for HepG2 and DLD-1) and 72 h (for A549 and PC3) and incubated for a further 2 h. The absorbance values were measured with an Epoch 2 Elisa plate reader at 590 nm.

Computational Methods

Frontier molecular orbitals (FMOs) and molecular electrostatic potential (MEP) map calculations were performed utilizing the Spartan software program (Spartan'10, version 1.1.0. Wavefunction) [19] with DFT: B3LYP/6–31G* method [20]. Drug-likeness and ADMET analysis of the SDG compound was performed using SwissADME [21], Pro Tox-II [22], and SwissTargetPrediction [23] prediction tools. The docking studies were performed utilizing UCSF Chimera and AutoDock Vina software [24]. From the PubChem database (https://pubchem.ncbi.nlm.nih.gov), the available structures of SDG were retrieved.

RESULT AND DISCUSSION

Cytotoxic Activity Studies

The cytotoxic activities of SDG were tested in the hepatocellular carcinoma cells (HepG2), colorectal cancer cells (DLD-1), lung carcinoma (A549), and prostate cancer (PC3) cell lines at concentrations of 300, 150, 75, 37.5, 18.75, and 9.375 µM as *in vitro* for 48 or 72 h. The IC_{50} values calculated with the GraphPad Prism 5 program are given in Table 1.

Table 1. The calculated IC_{50} results for SDG

Compound	**IC_{50} (µM)**			
	HepG2	**A549***	**DLD-1**	**PC3**
SDG	>300	>300	37.45	>300

Considering that SDG is metabolized in the colon, it is possible to say that lignan may exert its inhibitory effect on colon tumor cells through mechanisms other than estrogenic activity [2]. Özgöçmen *et al*. determined the IC_{50} values as 100 µM for 24 h and 150 µM for 48 and 72 h in SW480 cells in which they applied 40-200 µM SDG and found that cell proliferation was inhibited by almost half at all-

time intervals [25]. In another study, lower doses (0-40 µM) of SDG application showed a dose- and time-dependent decrease in cell numbers in SW480 human colon cancer cells, but cell viability was recorded above 80%. It has been claimed that SDG may be mediated by a cytotoxic mechanism associated with cyclin A expression in colon cancer cells [12]. Chen *et al*. found that SDG treatment was able to significantly inhibit cell viability over time (0-24 h) in a different colon cancer cell line HCT116. The IC_{50} value for HCT116 cells was determined as 24.5 µmol/l [26]. The results we obtained support the literature, and it was found that among the cancer cell lines studied, colon cancer cells (DLD-1) were the cells most affected by SDG cytotoxicity with an IC_{50} value of 37.45 µM. It was observed that SDG did not have an antiproliferative effect (IC_{50}>300 µM) against A549 and HepG2 cancer cells in the studied conditions. Only at a high concentration of SDG (300 µM), HepG2 cell viability was determined as 37%. PC3 cells were also cytotoxictly affected by 75 µM and above SDG concentration, and cell viability decreased depending on the concentration (87.1%, 67.2%, and 47.8% for 75, 150, and 300 µM, respectively). Considering the estrogenic properties of lignan, inhibition of the proliferation of hormone-dependent cancer types may be possible through a mechanism based on this.

It has been reported that SDG can show higher stability than its metabolite enterolactone, and this high stability is due to the ability of bulky glucose groups in its chemical structure to resist possible electrophile attacks [12]. However, in a study examining the antiproliferative activity of SDG, END, and ENL on acute myeloid leukemia cell lines (KG-1 and Monomac-1), ENL showed promising cytotoxicity activity in both cell lines, whereas SDG was found to have a minimal anti-proliferative effect in KG-1 cells after 24 hours of application. However, it was found that SDG had a proliferative effect for 48 hours against KG-1 cells. Contrary to expectations, it was determined that cell proliferation increased in both time periods (24 and 48 hours) due to the increase in SDG and END concentrations in Monomac-1 cells [16]. From these results and the data we obtained from the study, it is possible to assume that the relevant lignans may exhibit different metabolic behaviors depending on the cell type.

Computational Structural Analysis

The highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) are referred FMOs [27]. The reactivity and stability of compounds are estimated using molecular orbital energies. The energy difference (HOMO-LUMO gap) is a critical factor in the chemical kinetic stability and reactivity of the compound. The small energy difference between the HOMO-LUMO molecular orbitals indicates that the molecule is soft, on the contrary, it is hard when it is large [28-30]. The optimization of the SDG compound was carried out using DFT with a functional B3LYP/6–31G* basis set in the gaseous phase. In the gaseous environment, the ΔE value was found to be 3.8531 eV for SDG. The HOMO, LUMO energies, ΔE energy ranges, and the visuals of them were given in Table 2 and Figure 2.

The MEP method is a very useful and practical method for determining the electrophilic and nucleophilic fields of molecules. The MEP map is a coded by colours map of the electron density surfaces of molecules. In this map, the red colour symbolizes electron-rich regions (partially negative charge) and the blue colour symbolizes electron-poor regions (partially positive charge). The yellow colour indicates regions with fewer electrons than the other regions and the green colour represents neutral regions with zero potential [31,32]. The molecular potential surfaces of the SDG were easily obtained using the Spartan program. As shown in Figure 3, analysis of the MEP map reveals that negative regions marked in red are located on O atoms in the rings. As can be understood from the MEP map, it is predicted that the compound will exhibit nucleophilic behaviour from the red region where the oxygens are present.

Table 2. The HOMO, LUMO energies, and ΔE energy ranges of SDG

SDG				
Medium	**$E_{HOMO(a.u.)}$**	**$E_{LUMO(a.u.)}$**	**$\Delta E_{(a.u.)}$**	**$\Delta E_{(eV)}$**
Gaseous	-0.2912	-0.1496	0.1416	3.8531

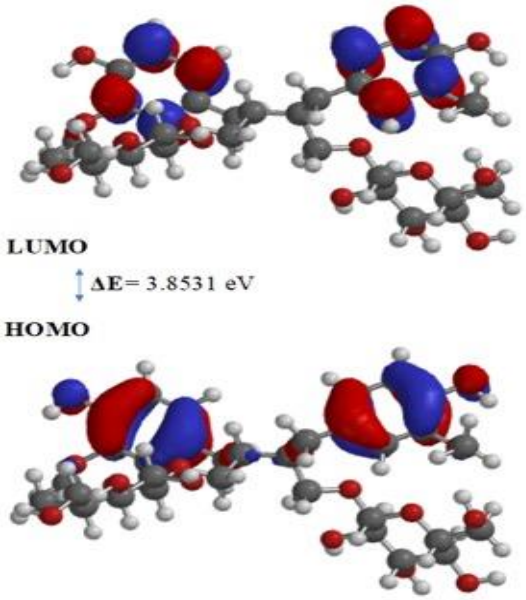


Figure 2. HOMO and LUMO energy plots of SDG in the gaseous media

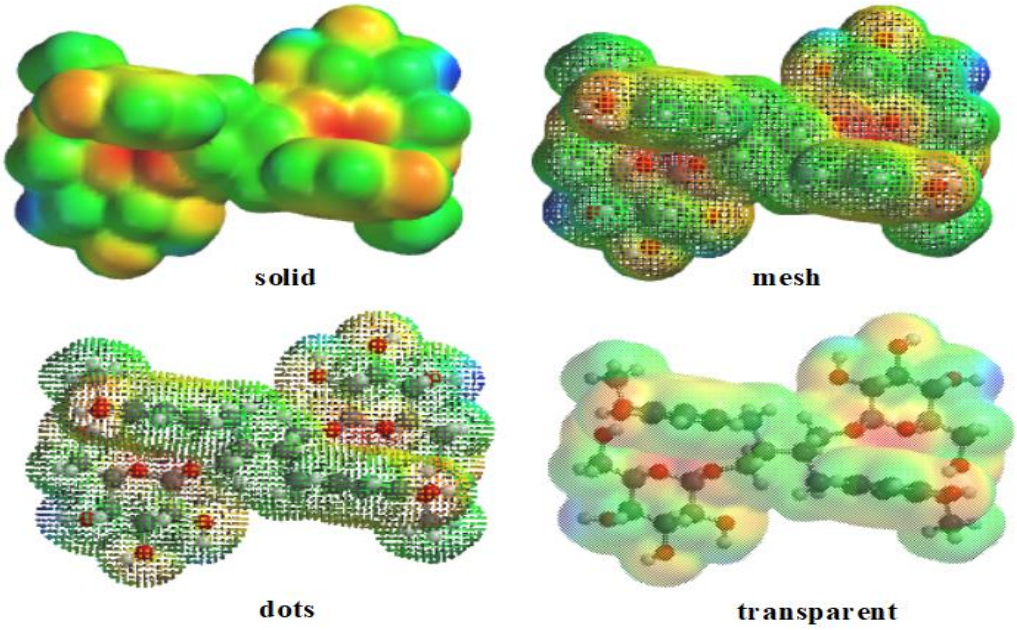
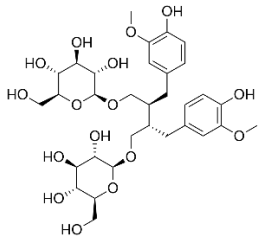


Figure 3. Showing the molecular electrostatic potential maps of the SDG

Molecular Docking

Molecular docking analyses aim at predicting the possible molecular interactions between proteins and ligands. The interaction between the SDG molecule and the MLK4 kinase was determined using docking analysis. The good energy value of the docking result (-6.1 kcal/mol) for the SDG molecule is shown in Table 3. Ligand-receptor 3D interactions were visualized owing to the Biovia Discovery Studio Visualizer program [33]. The 3D structure of the MLK4 kinase domain (PDB ID: 4UYA) at 2.80 Å resolution was acquired via the RSCB PDB website (https://www.rcsb.org/). SDG molecule formed secondary interactions with the MLK4 kinase. These interactions are shown in Figure 4.

Table 3. Docking analysis results of SDG compound

Compound Name	Chemical Structure Depiction	Docking Score (kcal/mol)	Amino Acid Residues
SDG		-6.1	ILE16, VAL21, LYS34, LEU71, LEU73, THR145, ASP146

The best interaction of the SDG compound was determined as conventional hydrogen bond, π-donor hydrogen bond, van der Waals interaction, alkyl interaction, and π-alkyl interaction including ILE16, VAL21, LYS34, LEU71, LEU73, THR145, ASP146 residues.

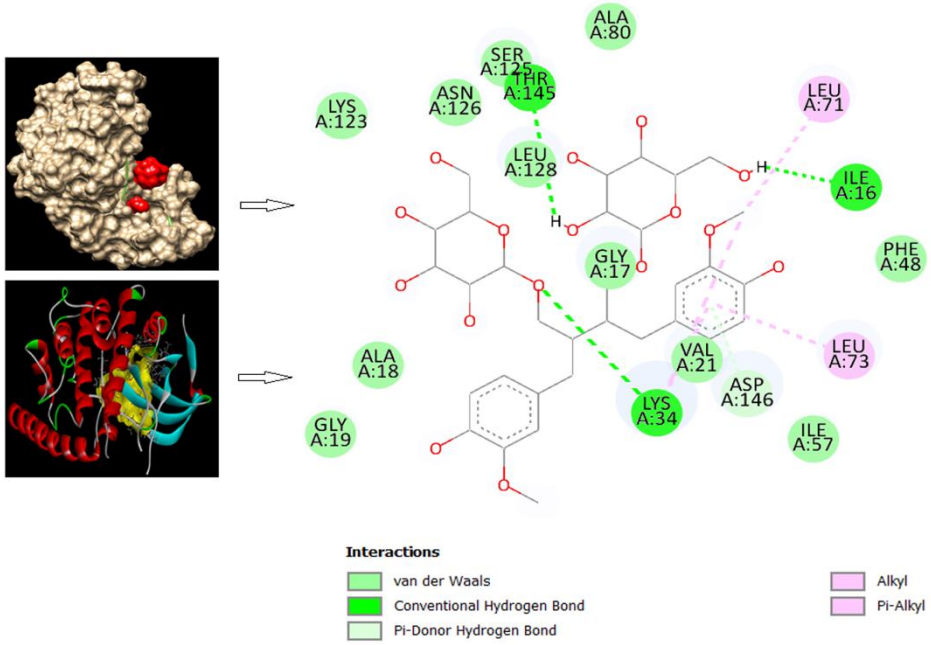


Figure 4. Images of the protein-ligand interaction by Discovery Studio Visualizer

Druggability and ADMET Properties

Druggability features refer to the physicochemical parameters and ADME-Tox properties of the compound. *In silico* methods have been widely used to estimate the ADME properties of molecules because *in vivo* and *in vitro* analyses are costly and time-consuming [34]. To determine the ADME-Tox properties of the SDG, several web-based *in silico* tools were used, including SwissADME, SwissTargetPrediction, and ProTox-II.

Prediction of pharmacokinetic and toxicokinetic properties greatly increases the success of reaching the target in the discovery of potential drug candidate compounds. Lipinski's rule of five has become standard for the prediction of the drug-likeness of the compounds [35]. The SDG does not comply with Lipinski's four rules. It complies with a rule only because its lipophilicity coefficient is LogP≤5. Due to the bulky nature of SDG, their gastrointestinal absorption is low, it cannot cross the blood-brain barrier (BBB) and cannot be used as substrates of P-glycoprotein (P-gp). The solubility of SDG in octanol/water is also low. SDG molecule does not interact with or inhibit CYP2C19, CYP1A2,

CYP2D6, CYP2C9 and CYP3A4. The results obtained by the SwissADME web tool are shown in Table 4.

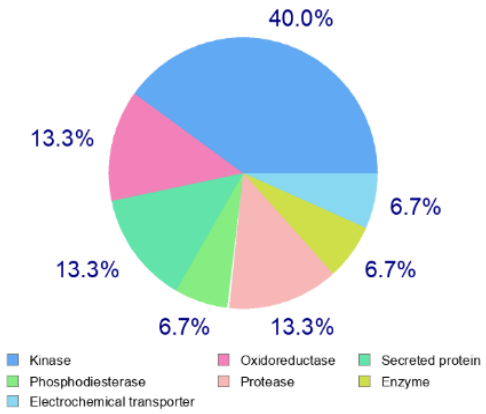
Table 4. ADME features estimated by SwissADME of the SDG

	SwissADME
	Physicochemical Properties
Formula	$C_{32}H_{46}O16$
Molecular weight	686.70 g/mol
Number heavy atoms	48
Number aromatic heavy atoms	12
Fraction Csp^3	0.62
Number rotatable bonds	15
Number hydrogen bond acceptors	16
Number hydrogen bond donors	10
Molar refractivity	164.05
TPSA	257.68 $Å^2$
Lipophilicity	
$LogP_{o/w}$	1.07
Water Solubility	
LogS	-2.87
Solubility	9.25e-01 mg/ml
Absorption	
GI absorption	Low
Distribution	
BBB permeation	No
P-gp substrate	No
Metabolism	
CYP1A2 inhibitor	No
CYP2C19 inhibitor	No
CYP2C9 inhibitor	No
CYP2D6 inhibitor	No
CYP3A4 inhibitor	No
$LogK_p$ (skin permeation)	-10.96 cm/s
Drug-likeness	
Lipinski	No
Ghose	No
Veber	No
Medicinal Chemistry	
PAINS	0 alert
Brenk	0 alert
Leadlikeness	No; 2 violation: MW >350
Synthetic Accessibility	6.71
Bioavailability Score	0.17

With regard to Table 5 of the SDG could be an inhibitor of 40% probability for kinase, 13.3% for oxidoreductase, 13.3% for secreted protein, 13.3% for protease, 6.7% for phosphodiesterase, 6.7% for enzyme and 6.7% for electrochemical transporter. In line with this data obtained using SwissTargetPrediction, kinase proteins were preferred for molecular docking analysis.

The toxicity prediction was performed owing to the Pro-Tox II web tool. The predicted data are given in Table 6. In terms of toxicity results, the SDG molecule was not hepatotoxic, carcinogenic, mutagenic, or cytotoxic but had immunotoxic effects. The predicted toxicity class of SDG was categorized as 5.

Table 5. The predicted biological target list and percentage distribution



Target	Common name	Uniprot ID	ChEMBL ID	Target Class
Protein kinase C delta	PRKCD	Q05655	CHEMBL2996	Kinase
Tyrosinase	TYR	P14679	CHEMBL1973	Oxidoreductase
Testis-specific androgen-binding protein	SHBG	P04278	CHEMBL3305	Secreted protein
Phosphodiesterase 5A	PDE5A	O76074	CHEMBL1827	Phosphodiesterase
Protein kinase C gamma	PRKCG	P05129	CHEMBL2938	Kinase
Protein kinase C beta	PRKCB	P05771	CHEMBL3045	Kinase
Protein kinase C epsilon	PRKCE	Q02156	CHEMBL3582	Kinase
Protein kinase C eta	PRKCH	P24723	CHEMBL3616	Kinase
Protein kinase C theta	PRKCQ	Q04759	CHEMBL3920	Kinase
Thrombin	F2	P00734	CHEMBL204	Protease
Coagulation factor VII	F7	P08709	CHEMBL3991	Protease
Tyrosyl-DNA phosphodiesterase 1	TDP1	Q9NUW8	CHEMBL1075138	Enzyme
Interleukin-2	IL2	P60568	CHEMBL5880	Secreted protein
Steroid 5-alpha-reductase 1	SRD5A1	P18405	CHEMBL1787	Oxidoreductase
Sodium/glucose cotransporter 2	SLC5A2	P31639	CHEMBL3884	Electrochemical transporter

Table 6. The toxicity computation of SDG molecule by Pro-Tox II web tool

Toxicity Model Report (Predicted Toxicity Class:5)			
Classification	**Target**	**Shorthand**	**Prediction**
Organ toxicity	Hepatotoxicity	dili	Inactive
Toxicity end points	Carcinogenicity	carcino	Inactive
Toxicity end points	Immunotoxicity	immuno	Active
Toxicity end points	Mutagenicity	mutagen	Inactive
Toxicity end points	Cytotoxicity	cyto	Inactive

In this study, the SDG molecule, which is commercially purchased, was tested *in vitro* against different cancer cell lines. The results showed that SDG, which is an important lignan species, had antiproliferative activity against the colon cancer cell line (DLD-1) while it was found to be inactive against other cell lines such as prostate, lung, and liver. The molecular docking behaviour of commercially purchased SDG against MLK4 kinase was further investigated. The predicted binding energy was found to be -6.1 kcal/mol. The docking results showed that SDG was inhibited through secondary interactions.

In general, it can be said that SDG may exhibit a greater therapeutic potential for the prevention of colon cancer based on the cytotoxic analyses obtained. As seen in the literature, the therapeutic efficacy of SDG for oncological cases is related to the type of malignant tumor and cancer cell

characteristics. However, further studies are needed to clearly demonstrate the validity of SDG supplementation/treatment.

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AUTHOR CONTRIBUTIONS

Concept: İ.B., S.Ç.Y., S.A.; Design: İ.B., S.Ç.Y.; Control: S.A.; Kaynaklar: İ.B., S.Ç.Y.; Sources: İ.B., S.Ç.Y., S.A.; Data Collection and/or Processing: İ.B., S.Ç.Y., S.A.; Analysis and/or Interpretation: İ.B., S.Ç.Y., S.A.; Literature Review: İ.B., S.Ç.Y., S.A.; Manuscript Writing: İ.B., S.Ç.Y., S.A.; Critical Review: İ.B., S.Ç.Y., S.A.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that ethics committee approval is not required for this study.

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