B. Akçeşme et al. / Hacettepe J. Biol. & Chem., 2026, 54 (1), 9-19

Research Article

Hacettepe Journal of Biology and Chemistry

journal homepage: https://hjbc.hacettepe.edu.tr/
Dergipark Akademik: https://dergipark.org.tr/en/pub/hjbc

Investigation of Phytochemicals Derived from *Gentiana Dinarica* As Potential Drug Candidates For Colorectal Cancer by Computational Approaches

Gentiana Dinarica'dan Türetilen Fitokimyasalların Kolorektal Kanser için Potansiyel İlaç Adayları Olarak Hesaplamalı Yaklaşımlarla İncelenmesi

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ABSTRACT

Colorectal cancer is one of the deadliest cancers worldwide, necessitating early intervention for the most promising outcomes. Current treatments include chemotherapeutic drugs, which, although effective in reducing the risk of metastasis, often harm healthy cells and cause significant patient distress due to adverse side effects. Phytochemicals, which tend to have fewer negative effects, may serve as viable alternative treatment options. This study investigates the potential of phytochemicals derived from *Gentiana dinarica* for colorectal cancer treatment by targeting proteins involved in the Wnt signaling pathway using computational biology approaches. Our results identify gentioside as a promising candidate, showing the highest binding affinity toward β-catenin, DVL1, and WNT5a proteins, while isoorientin demonstrates the greatest potential against the COX2 protein. However, due to violations of drug-likeness rules by gentioside and isoorientin, norswertianin and gentiopicroside were further analyzed. These compounds exhibit high binding affinity and favorable drug-likeness profiles, making them strong candidates for inhibiting Wnt pathway receptors. Comprehensive in vitro and in vivo studies are recommended to validate the effectiveness of these phytochemicals in the treatment of colorectal cancer.

Key Words
Colorectal cancer, *Gentiana dinarica*, phytochemicals, computational biology.

ÖZ

Kolorektal kanser, dünya çapında en ölümcül kanserlerden biridir ve en umut verici sonuçlar için erken müdahale gerektirir. Mevcut tedaviler arasında, metastaz riskini azaltmada etkili olmalarına rağmen, genellikle sağlıklı hücrelere zarar veren ve olumsuz yan etkiler nedeniyle hastada önemli sıkıntıya neden olan kemoterapötik ilaçlar bulunur. Daha az olumsuz etkiye sahip olma eğiliminde olan fitokimyasallar, uygulanabilir alternatif tedavi seçenekleri arasındadır. Bu çalışma, hesaplamalı biyoloji yaklaşımlarını kullanarak Wnt sinyal yolunda yer alan proteinleri hedefleyerek Gentiana dinarica'da bulunan fitokimyasalların kolorektal kanser tedavisi için potansiyelini araştırmaktadır. Sonuçlarımıza göre, β-catenin, DVL1 ve WNT5a proteinlerine karşı en yüksek bağlanma afinitesini gösteren umut verici bir aday olarak gentioside'i belirlerken, isoorientin COX2 proteinine karşı en büyük potansiyeli göstermektedir. Ancak, gentioside ve isoorientin tarafından ilaç benzerliği kurallarının ihlal edilmesi nedeniyle norswertianin ve gentiopikrozid için ileri analizler yapıldı. Bu bileşikler yüksek bağlanma afinitesi ve uygun ilaç benzerliği profilleri sergiler ve bu da onları Wnt yolağı reseptörlerini inhibe etmek için güçlü adaylar yapar. Bu fitokimyasalların kolorektal kanser tedavisindeki etkinliğini doğrulamak için kapsamlı in vitro ve in vivo çalışmalar önerilmektedir.

Anahtar Kelimeler
Kolorektal kanser, *Gentiana dinarica*, fitokimyasallar, hesaplamalı biyoloji.

Article History: Received: Jun 3, 2025; Accepted: Aug 20, 2025; Available Online: Dec 30, 2025.
DOI: https://doi.org/10.15671/hjbc.1712451
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INTRODUCTION

Colorectal cancer (CRC) is one of the most common and deadliest malignant diseases, caused by the uncontrollable growth and spread of abnormal colon cells (colon cancer) or rectal cells (rectal cancer) [1]. Development of CRC significantly depends on genetic predisposition, the adopted lifestyle of an individual, and environmental conditions [2]. CRC impacts both sexes, with a worldwide report from 2020 indicating that males are generally more affected, having 20% more fatal outcomes than females [3]. In 2020 close to 2 million new cases of CRC were recorded worldwide, making it the third most common form of cancer, and with almost a million deaths in the same year, it represents a major health concern, particularly for groups which are considered high-risk for CRC development, which includes the elderly, overweight individuals, as well as people with chronic inflammatory bowel disease [1,3].

While early detection efforts, adequate screening measures, and advancements in treatment have made the disease more manageable and decreased the death rate, it is estimated that by the year 2040, over 3 million cases of CRC will be diagnosed annually [3]. This estimate, alongside an alarming increase in the prevalence of the disease in younger generations, compounded with the fact that in over 50% of all CRC cases the cancer metastasizes, most commonly to the liver, has increased the urgency of finding new measures to combat the disease [2,4]. An increase in unhealthy living practices, lack of exercise, and increased favoritism of Western eating habits have led to an increase in the prevalence of CRC on a global scale, with this trend being expected to continue for the foreseeable future [4]. This phenomenon has not escaped Bosnia and Herzegovina either, with 1865 cases being recorded in 2020, making it the second most common cancer type in the country [5].

Treatments for CRC are varied, often including abdominal surgery, radiation therapy, and chemotherapy, depending on the disease development stage [6]. The side effects of chemotherapy and radiation therapy are known to be stressful to the patient, causing harm to other areas of the body and not just cancerous regions. Some of the side effects include nausea, hair loss, fatigue, vomiting, radiation dermatitis, oral and gastrointestinal mucositis, etc., with some effects even having life-long implications for the patients [7,8]. A consistent effort is being made to lessen the negative side effects of these treatments; alongside new research being conducted in attempting to develop new treatments with milder side effects.

The need for more accessible and effective drugs for the prevention and treatment of CRC has led to the consideration of phytochemicals as a potential solution to this problem [9]. Phytochemicals, also called secondary metabolites, can be defined as any form of biologically active compound produced by plants via several chemical pathways [10]. Phytochemicals have biological activity in the plant host, and they function in plant growth and defense against pathogens. Although all the benefits and functions of secondary metabolites have not yet been discovered, it has been confirmed that phytochemicals have health-promoting and disease-preventing effects in humans [11]. They are also considered modulators of key cellular signaling pathways; thus, secondary metabolites affect cancer cell behavior such as proliferation, apoptosis, and invasion, at different levels, including intercellular messaging, transcriptional or post-transcriptional regulation, and protein activation [12].

The Wnt/β-catenin signaling pathway is a conserved cellular signaling pathway that has an important role in regulating embryonic development and maintaining tissue homeostasis in adults, as well as processes like cell proliferation, differentiation, survival, and migration [13]. The functioning of this pathway can be divided into 4 key parts: the extracellular segment, the membrane segment, the cytoplasmic segment, and the nuclear segment. The extracellular segment consists of the secreted glycoproteins called Wnt proteins (e.g., Wnt1, Wnt3a, Wnt5a), which initiate the signaling cascade by acting as primary ligands. Following this, in the membrane segment of the pathway, frizzled (FZD) receptors and low-density lipoprotein receptor-related proteins (LRP5/6) work together to propagate the signal into the cell. In the absence of Wnt signals, in the cytoplasm, proteins of the so-called destruction complex, Adenomateous Polyposis Coli (APC), AXIN, Casein Kinase 1 (CK1), and Glycogen Synthase Kinase 3 Beta (GSK-3β), have the function of phosphorylating β-catenin, marking it for degradation by proteasomes, preventing it from accumulating in the cell. [14]. However, when a signal is present, dishevelled (DVL) protein is recruited to the membrane, where it acts by disrupting the destruction complex, thus allowing β-catenin to accumulate in the cell. The stabilized β-catenin then translocates to the

nucleus, marking the nuclear segment of the signaling pathway, where it interacts with transcription factors such as T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) to activate target gene expression [15]. These target genes are involved in various cellular functions, including cell proliferation and survival. Shortly, in the absence of Wnt ligands, β-catenin is continually degraded by the destruction complex. TCF/LEF factors in the nucleus are bound by inhibitors, repressing the transcription of Wnt target genes. Conversely, when Wnt ligands bind to FZD and LRP5/6 receptors, the destruction complex is inhibited, leading to the accumulation of β-catenin in the cytoplasm. β-catenin then translocates to the nucleus, displaces inhibitors, and works with TCF/LEF to activate transcription of target genes that drive cell proliferation and other processes. Due to its importance in the development of various diseases, this pathway is an attractive target for therapeutic intervention [16]. One example of such implementation in cancer treatment is the use of inhibitors for the destruction complex, which restores normal β-catenin degradation in cancer cells, thereby preventing the activation of oncogenic pathways [16].

In recent years, research efforts have included Gentiana dinarica, a perennial plant species endemic to the regions of the southwest Balkan, including the high mountains of Bosnia and Herzegovina, where the carbonate soils of the sub-alpine regions present a favourable environment for its growth [17]. Plants of the Gentiana genus have a long history of use for medicinal purposes, particularly for digestive issues. Analysis of G. dinarica has revealed it to be rich in various phytochemicals which have the potential to be used in the treatment of various cancers and other diseases [18, 19, 20].

In this study, four essential proteins were selected in the Wnt signaling pathway, including beta-catenin (β-catenin), Cyclooxygenase 2 (COX2), Wnt5a, and DLV, as targets for inhibiting their function. The phytochemicals amarogentin, gentiopicroside, gentioside, silibinin, isoorientin, sweroside, norswertianin and swertiamarin from *G. dinarica* were selected to investigate their inhibitory role in the Wnt signaling pathway as promising candidates against colorectal cancer. Potential ligands were analyzed for their drug likeness and molecular properties as well as their bioactivities. The interactions between the proteins and ligands were studied using molecular docking, and the efficacy of these interactions was studied as described in the methodology.

MATERIALS and METHODS

Selection of target proteins and phytochemicals

The proteins used in this study, β-catenin, COX2, Wnt5a, and DVL1 were chosen after an extensive literature review. It was shown that these proteins are crucial for signal transduction in the Wnt signaling pathway, in which a malfunction can lead to the development of CRC. The β-catenin (PDB ID: 1JDH; UniProt ID: P35222) and DLV1 (PDB ID:6LCB; UniPort ID: O14640) protein models used in the study were chosen from the PDB structures with the highest identification quality and longest amino acid sequence representation in Uniprot. Selected PDB structures were used in further analysis [21, 22]. The FASTA sequence of COX2 (UniProt ID: P35354) was uploaded to the Swiss model server, and the PDB structure with the highest resulting global model quality estimate (GMQE) was used as the template of the model [23]. For Wnt5a (UniProt ID: P41221, no experimental structure has been determined, therefore the model used in further analysis was predicted by AlphaFold [24]. For PDB structures that contain the target protein bound to another ligand, the ligand was deleted from the structure using AutoDockTools 1.5.7, prior to continuing the preparation for molecular docking of the protein and phytochemicals [25]. Two different ligand binding sites were identified for the Wnt5a protein. Two separate molecular docking analyses were performed and named as Wnt5a_1 and Wnt5a_2.

Swertiamarin, norswertianin, gentiopicroside, gentioside, sweroside, amarogentin, and isoorientin, from *G. dinarica* were selected as the potential phytochemicals with high prospects for successful results following a literature search to explore their potential role as inhibitors in the Wnt signaling pathway (PubChem ID: 442435, 5281658, 88708, 15559486, 161036, 115149, and 114776, respectively). Irinotecan [26], capecitabine [27], and silibinin [28] are selected as control ligands which are known to be effective in this pathway (PubChem ID: 60838, 60953, and 31553, respectively) (Figure 1). The structures of the phytochemicals were derived from the PubChem database, first downloaded in the structure data format (SDF) before being converted into the PDB format.

OpenBabelGUI was used to convert the SDF format of the phytochemicals into the PDBQT format needed for the molecular docking, described below [29]. The canonical SMILES for all the phytochemicals were extracted

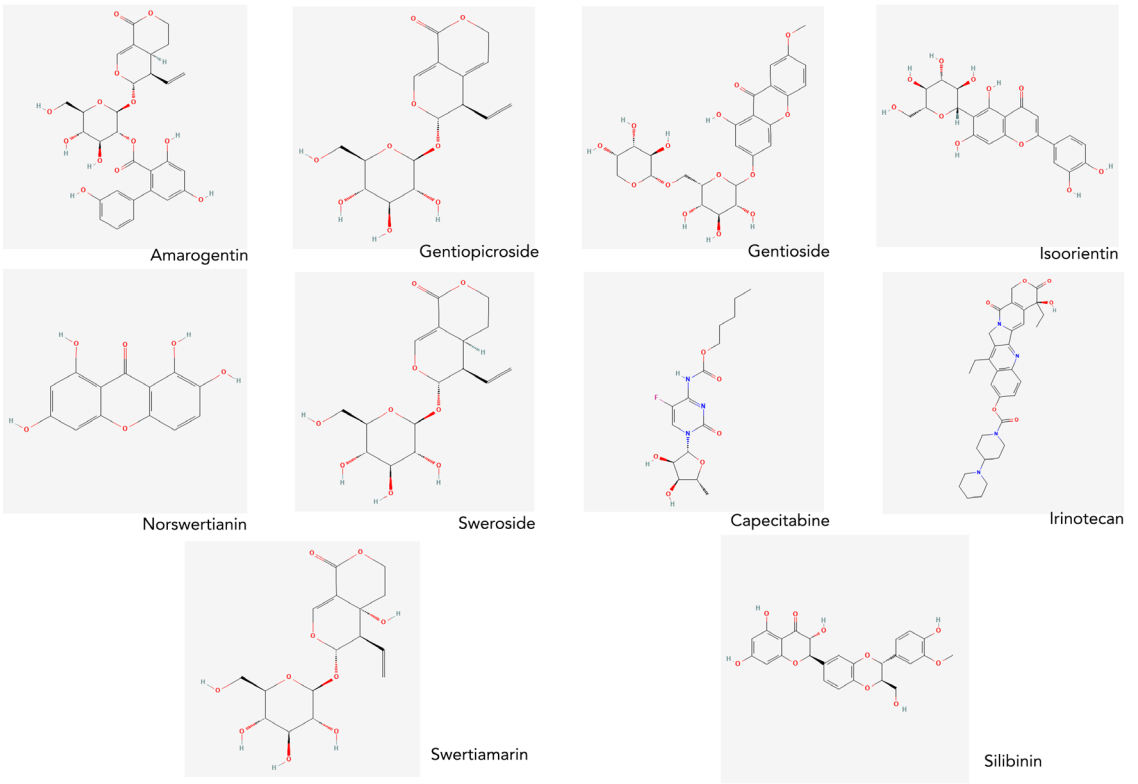

Figure 1. Structures of phytochemicals (ligands) derived from PubChem database.

from their respective PubChem database websites and were then used for further analysis of the drug-likeness of these phytochemicals as described below.

Predicting the drug-likeness and toxicity of selected phytochemicals

Initially, two different servers were used to predict the drug-likeness of the selected phytochemicals, Molinspiration Cheminformatics Software (https://www.molinspiration.com/) and SwissADME (http://www.swissadme.ch/) [30,31]. The drug-likeness was calculated based on the Lipinski rule of 5 (includes molecular weight (MW<500 Da), calculated lipophilicity (ALogP), Polar surface area (PSA), number of hydrogen bond acceptors (HBA), and number of hydrogen bond donors (HBD), with the oral bioavailability being calculated using Veber's rule. SwissADME was used to determine whether the phytochemicals violated the Lipinski or Veber rule.

Additionally, the admetSAR server (http://lmmd.ecust.edu.cn/admetsar2/) was used to predict ADMET properties, which stand for absorption, distribution, metabolism, excretion, and toxicity, important aspects to consider during the drug design process [32]. For this purpose, the following properties were considered: human intestinal absorption (HIA), human oral bioavailability, Caco-2 permeability, plasma protein binding, blood-brain barrier penetration (BBB), acute toxicity, carcinogenicity (binary), and mutagenicity.

Preparation of protein models and phytochemicals for docking

To predict the bound protein-ligand complex structure, molecular docking principles were utilized. This allows for visualizing the conformational space of the ligands within the binding site of the protein. Since a comparison method is required, a scoring function was performed by the utilized software which output the free energy of binding between the protein and the ligand in each docking pose or conformation.

The protein models obtained using the procedures explained above, were prepared for docking by using AutoDockTools 1.5.7 [25]. The results were that the models were converted from the PDB format to the PDBQT format. This was done, firstly by deleting any ligands bound to the present protein structures, after which

all water molecules were removed. The structure was checked for any missing atoms, which were promptly fixed. Afterwards, polar hydrogens and Kollman charges were added, with the charge deficit being spread over all atoms in the residue. The dimensions of the grid box necessary for determining the area to which the ligands should bind (active sites) were determined by observing other structures derived from PDB that contain the target protein bound to a ligand, which gave additional information about binding sites on the protein. The phytochemicals were used in their PDBQT format, and all phytochemicals were simultaneously analyzed for only one protein at a time. The configuration file containing the grid box dimensions for the protein and the number that specifies how many different binding options to consider (as a default it was set to 10 for all proteins) was written as a text file and used by AutoDock Vina while performing the protein-ligand docking. The results of the docking were visualized in BIOVIA Discovery Studio 2021 [33].

The whole methodological design of the study is shown in Figure 2.

RESULTS and DISCUSSION

Drug-likeness and molecular physicochemical properties of phytochemicals

Molinspiration Cheminformatics server was used to calculate molecular physicochemical properties and to predict bioactivity of the target ligands. The results obtained from Molinspiration Cheminformatics for ligand properties which are used in Lipinski Rule and Veber Rule (mentioned in method) including Method for logP prediction developed at molinspiration (miLogP), topological polar surface areas (TPSA), number of atoms (N atoms), molecular weight (MW), number of hydrogen bond acceptor and donor, number of rotatable bonds (Nrotb), molecular volume (vol) are shown in Table 1. Lipinski Rule or Rule of Five (ROF) states that a potential drug should not violate more than one rule. However, these compounds should not be completely removed from the research considering that many drugs do not fulfill ROF criteria [34]. miLogP is an important parameter for the measurement of the lipophilicity or hydrophobicity of the ligand, which affects the absorption, transportation and distribution of the ligand in the body as well as its formulation and dosage. LogP

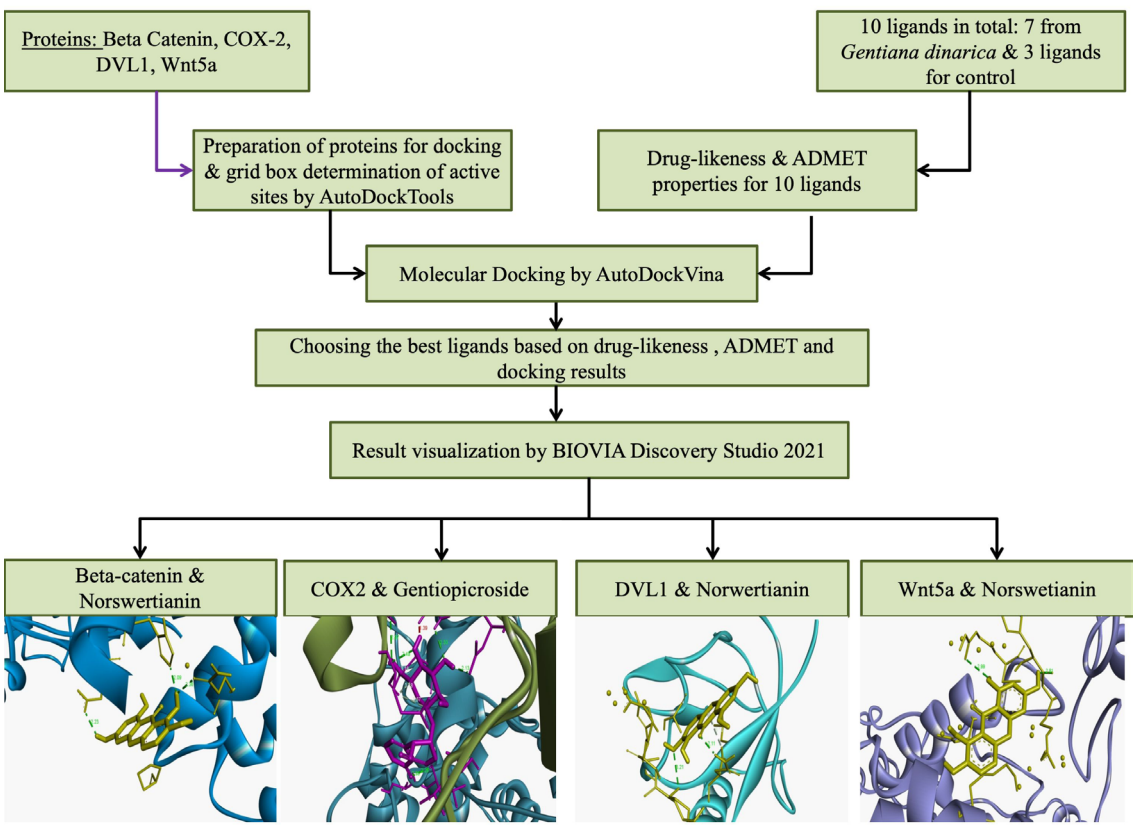

Figure 2. The workflow of the study.

Table 1. Molecular physicochemical properties of selected phytochemicals obtained by using Molinspiration Chemoinformatics Server.

Chemical	PubChem ID	miLogP	TPSA	N atoms	MW	H-acceptor	H- donor	Nrotb	Vol
Swertiamarin	442435	-1.66	155.15	26	374.34	10	5	4	314.97
Norswertianin	5281658	2.01	111.12	19	260.2	6	4	0	204.65
Gentiopicroside	88708	-0.9	134.92	25	356.33	9	4	4	301.06
Gentioside	15559486	-0.26	217.98	39	522.49	14	7	6	453.56
Sweroside	161036	-0.71	134.92	25	358.34	9	4	4	307.28
Amarogentin	115149	2.04	201.68	42	586.55	13	6	8	494.1
Isoorientin	114776	0.03	201.27	32	448.38	11	8	3	363.22
Irinotecan	60838	4.1	114.21	43	586.69	10	1	5	530.67
Capecitabine	60953	1.88	122.92	25	359.35	9	3	7	311.36
Silibinin	31553	1.47	155.15	35	482.44	10	5	4	400.86

value <5, better if it is between 1.35-1.8 for good oral and intestinal absorption. The values represented in the leftmost column show that all candidates pass this condition (<5). According to Veber's Rule, polar surface area must be smaller than 140 Å2. Norswertianin, gentiopicroside, and sweroside are the three ligands among the potential seven ligands that fulfill this criterion. Molecules must contain between 20 and 70 atoms (50 on average) according to Veber's Rule. All ligands except norswertianin (N atoms: 19) pass this parameter. Molecular mass must be less than 500 Daltons for both the Lipinski and Veber rules. Except gentioside (522.49) and amarogentin (586.55), all other ligands have a MW under 500 Daltons.

The molecule must have no more than 5 hydrogen bond donor sites and that of 10 hydrogen bond acceptor sites according to both Lipinski and Veber rule. Gentiopicroside and sweroside are the only ligands contain less than 10 H-acceptor sites and less than 5 H-donor sites. Nrotb is a topological parameter that measures molecular flexibility, and it can be considered as a good descriptor of oral bioavailability of drugs. Lipinski's rule of five limits the number of rotatable bonds to less than 10 whereas Veber's rule states that a compound should have 10 or fewer rotatable bonds (average 4.9). All ligands contain Nrotb below the number 10.

Predicted bioactivity properties of the potential ligands considered in calculating the effectiveness of a compound during the drug design process are shown in Table 2. Some parameters for drug-likeness properties predicted by Molinspiration Chemoinformatics Server include G protein-coupled receptors (GPCR) ligand, ion channel modulator, kinase inhibitor, nuclear receptor ligand, protease inhibitor, and enzyme inhibitor. Compounds that have a bioactivity score equal to or more than 0.00 are most likely to exhibit considerable biological activity, while values between -0.50 and 0.00 are expected to be moderately active. Bioactivity scores that are less than -0.50 are presumed to be inactive [35]. Our seven candidate ligands have shown either biologically active or moderately active scores.

The far right two columns of Table 2 give the results obtained from the SwissADME server, showing that 3 phytochemicals failed both the Lipinski and Veber's rules, including gentioside, amarogentin, and isoorientin. Norswertianin, gentiopicorside, and sweroside are three phytochemicals that passed both rules whereas swertiamarin passed only the Lipinski rule but failed Veber's rule with one violation.

AdmetSAR is a web server for predicting around 50 drug properties related to absorption, distribution, metabolism, excretion, and toxicity. Among many ADMET properties analyzed by the admeSAR server, commonly used properties are listed in Table 3. The score derived from admetSAR is as follows: scores are adjusted between 0 and 1 according to the scores of the oral drugs in DrugBank, in which "1" indicates the best and "0" means the worst [32].

Human intestinal absorption (HIA), human oral bioavailability (HOB), and Caco-2 permeability are considered as absorption. Norswertianin has shown the highest score (0.8801) for HIA, whereas swertiamarin and amarogentin have shown the highest score (0.8286) for HOB.

Table 2. Bioactivity properties and drug likeness of selected phytochemicals obtained by using Molinspiration Chemoinformatics and SwissADME servers.

Chemical	GPCR Ligand	Ion channel modulator	Kinase inhibitor	Nuclear receptor ligand	Protease inhibitor	Enzyme inhibitor	Lipinski Rule	Veber's rule	Vol
Swertiamarin	0.17	0.26	-0.23*	0.04	0.26	0.43	Yes (0)	No (1)	314.97
Norswertianin	-0.22*	-0.22*	-0.01*	0.21	-0.46*	0.27	Yes (0)	Yes (0)	204.65
Gentiopicroside	0	0.13	-0.45*	-0.06*	-0.02*	0.38	Yes (0)	Yes (0)	301.06
Gentioside	0	-0.25*	-0.08*	-0.06*	-0.08*	0.31	No (3)	No (1)	453.56
Sweroside	0.17	0.26	-0.28*	0.06	0.14	0.45	Yes (0)	Yes (0)	307.28
Amarogentin	0.06	-0.26*	-0.3*	-0.04*	0.03	0.13	No (3)	No (1)	494.1
Isoorientin	0.11	0.01	0.16	0.2	0.01	0.46	No (2)	No (1)	363.22
Irinotecan	0.33	-0.45*	-0.1*	-0.15*	0.02	0.54	Yes (1)	Yes (0)	530.67
Capecitabine	0.67	0.01	0.26	-0.58**	0.37	0.91	Yes (0)	Yes (0)	311.36
Silibinin	0.07	-0.05*	0.01	0.16	0.02	0.23	Yes (0)	No (1)	400.86

* Moderately active, ** inactive, unlabeled are active.

Table 3. Estimation of ADMET analysis for selected phytochemicals by admetSAR.

Chemical	Human Intestinal Absorption	Human oral bioavailability	Caco-2 permeability	Plasma protein binding	Blood-brain barrier penetration (BBB)	Acute toxicity	Carcinogenicity (binary)	Mutagenicity
Swertiamarin	0.55	0.8286	0.8615	0.376	0.55	1.532	1	0.64
Norswertianin	0.8801	0.5857	0.5865	1.098	0.775	1.32	1	0.66
Gentiopicroside	0.5678	0.8143	0.8386	0.398	0.6	1.669	1	0.59
Gentioside	0.5545	0.6857	0.8951	0.794	0.65	1.817	0.99	0.77
Sweroside	0.619	0.8143	0.8586	0.245	0.625	0.4512	1	0.627
Amarogentin	0.4645	0.8286	0.9123	0.716	0.775	0.4552	0.98	0.61
Isoorientin	0.6665	0.6143	0.9224	0.893	0.7	2.833	1	0.6129
Irinotecan	0.9766	0.9286	0.8305	0.796	0.7	0.56	0.89	0,55
Capecitabine	0.9761	0.7571	0.7551	0.706	0.5	0.685	0.7338	0.6908
Silibinin	0.9045	0.7571	0.8574	0.888	0.8	0.7236	0.99	0.57

Isoorientin is the ligand with the highest Caco-2 permeability score (0,9123). In silico analysis of the distribution of the studied ligands throughout the body, the volume of distribution parameter, the ability to bind to blood plasma proteins, plasma protein binding (PPB) and cross the blood–brain barrier penetration (BBB) was considered. Norswertianin has the highest PPB and BBB scores of 1.098 and 0.775, respectively. Mutagenicity score is the lowest for gentiopicroside (0.59), whereas the acute toxicity score is lowest for sweroside (0.4512).

Analysis of protein-ligand molecular docking

Table 4 shows the molecular docking results obtained for all four proteins and selected ligands. Norswertianin, one of the phytochemicals which passed the drug-likeness test, was found to have the highest binding affinity with β-catenin, DVL1, and both models of WNT5a, with -6.7 kcal/mol, -6.6 kcal/mol, -6.6 kcal/mol, -5.9 kcal/mol, respectively. The non-violating phytochemical which had the highest binding affinity with COX2 was gentiopicroside, with a binding affinity of -8.4 kcal/mol. It is interesting to note that the control molecule capecitabine showed a lower binding affinity than the chosen phytochemicals for every protein model tested.

Figure 3 shows a 2D and 3D representation of the bonds between the proteins and the phytochemicals with the highest bonding affinity with no violation of RO5. In the molecular docking of norswertianin and β-catenin, norswertianin formed 3 conventional hydrogen bonds,

Table 4. Binding affinities (kcal/mol) of ligands to the corresponding proteins.

Proteins	β -catenin	COX2	DVL1	Wnt5a_1	Wnt5a_2
Ligands					
Swertiamarin	-6.2	-8.5	-6.7	-5.9	-5.3
Norswertianin	-6.7	-7.9	-6.6	-6.6	-5.9
Gentiopicroside	-6.1	-8.4	0	-6.3	-5.6
Gentioside	-7.9	-9.3	-8	-8.1	-6.6
Sweroside	-6.4	-8.2	-6.4	-6.3	-5.4
Amarogentin	-7.1	-9.1	-7.4	-6.9	-6.2
Isoorientin	-6.6	-10.9	-6.8	-6.9	-6.3
Irinotecan	-8.1	-12.7	-8.6	-8	-7.8
Capecitabine	-5.7	NA	-6.3	-6.2	-4.8
Silibinin	-7.2	-10.4	-8.2	-8.2	-6.7

NA: No data available

with Glu567, His544, and Gln601. In the case of COX2 and gentiopicroside, gentiopicroside forms 5 conventional hydrogen bonds, with Leu145, Ser146, Asp229, Gln241, and Arg333. Norswertianin and DLV1 are shown to have 2 conventional hydrogen bonds, with Asn255 and Glu257. Additionally, Pi-donor hydrogen bonds are also present. Norswertianin also formed 2 conventional hydrogen bonds withWnt5a, with Arg109, and His110 (Wnt5a_1 was visualized due to its higher binding affinity than Wnt5a_2). Potential ligands that show the highest binding affinity regardless of their violation of Lipinski and Veber rules are gentioside with β-catenin, DVL1 and Wnt5a proteins and isoorientin with COX2 protein interactions are shown in Supplementary Figure 1S. Even though these phytochemicals violate Lipinski and Veber rules, gentioside and isoorientin indicated strong binding power to the target proteins, which can be considered in further chemical studies to improve their drug-like properties [34].

DISCUSSION

Aberrant functioning (hyperactivation) of the WNT signaling pathway has proven to be present in most of CRC types [36]. This is to be expected since this pathway plays a crucial role in tissue homeostasis, as well as regulation of endogenous stem cells (including cancer stem cells), therefore malfunctioning in the cascades of the pathway could lead to the development of CRC starting from the colorectal epithelium [37].

β-catenin, DVL1, Wnt5a and COX2 proteins are analyzed in this study since they have essential roles in WNT signaling pathway. Research has already been conducted with ligands either targeting β-catenin directly or some of its complexes, during various cancer treatments, such as for hepatocellular carcinoma, breast cancer, and even for colorectal cancer [38, 39, 40]. DVL1 can activate alternative Wnt pathways and interacts with various proteins to regulate cellular processes like proliferation and polarity. Irregular activity of Dishevelled proteins can lead to uncontrolled cell proliferation, contributing to cancer development, and indeed, overexpression of DVL1 is often encountered in cancerous cells [41, 42]. Irregular activity of Wnt5a is connected to various diseases and conditions such as inflammation, fibrosis, asthma, vascular calcification, and tumor development [43]. Wnt5a can act as both a tumor suppressor and an oncoprotein, depending on the type of neoplasm [44]. Therefore, the treatments targeting Wnt5a vary depending on the type of role Wnt5a is playing in the disease development. While it is not inherently part of the Wnt signaling pathway, COX2 produces prostaglandin E2 (PGE2), which binds to various receptors that can activate numerous signaling pathways, including that of the Wnt/β catenin pathway. Targeting COX2 would subsequently lead to the inhibition of the Wnt signaling pathway. This protein is already being targeted by cyclooxygenase inhibitors and non-steroidal inflammatory drugs in various cancer treatments, since the inhibition of this protein affects many other pathways which are also related to cancer occurrence and growth [45, 46].

The results of this study showed that norswertianin and gentiopicroside are the phytochemicals with the

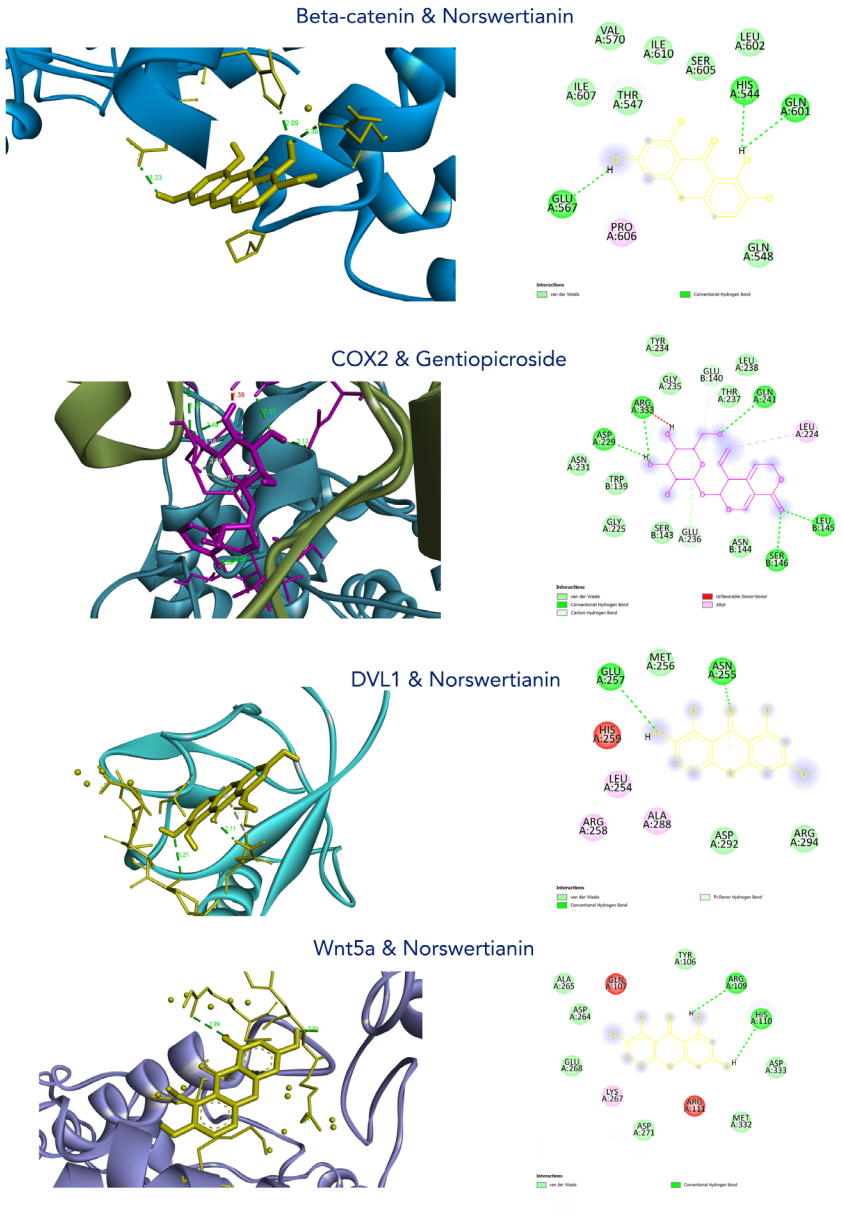

Figure 3. 3D (left) and 2D (right) representation of the interactions formed between the proteins and ligands norswertianin and gentiopicroside (PubChem ID: 5281658 and 88708, respectively). Only the ligands with highest affinity docking results with no violation of rule of five are shown.

greatest potential to be used as therapeutic agents during colorectal cancer treatments, when targeting the four proteins investigated. Norswertianin, one of the phytochemicals which passed the drug-likeness test, showed to have the highest binding affinity with β-catenin, DVL1, and both models of WNT5a, with -6.7 kcal/mol, -6.6 kcal/mol, -6.6 kcal/mol, -5.9 kcal/mol, respectively. The non-violating phytochemical which had the highest binding affinity with COX2 was gentiopicroside, with a binding affinity of -8.4 kcal/mol. Gentiopicroside is a common phytochemical found in many Gentiana species and is classified as a secoiridoid glycoside [47]. Various beneficial properties have been attributed to these glycosides, including antifungal, antibacterial, anti-inflammatory, garstroprotective, hepatoprotective, smooth-muscle relaxing, antinociceptive, and even antitumor characteristics [48, 49]. Due to its anti-inflammatory and oxidative stress-reducing properties, various studies have demonstrated successful results of using gentiopicroside in treating diseases such as

pulmonary fibrosis, alleviating chemotherapy drug-induced heart failure, and even for the treatments of various cancers such as ovarian cancer, cervical cancer, and gastric cancer [49, 50, 51]. Norswertianin is classified as a xanthone which has been found to have glucose-regulating properties, exhibit antioxidant characteristics, and some xanthones are also reported to have anti-inflammatory effects [52]. It should be noted that the characteristics were observed for xanthones derived from different plant species and not norswertianin specifically. Norswertianin, however, has been reported to cause the induction of autophagy and reactive oxygen species (ROS) -dependent differentiation of human glioblastoma cell lines [53]. This discovery could lead to better treatments for cancer patients, since such induction would mean that the cancer cells are less resilient as they are more susceptible to programmed cell death, and that the cells are more likely to differentiate into less aggressive phenotypes. While our findings provide valuable computational insights into the potential of *Gentiana dinarica* phytochemicals as drug candidates, it is important to note that no experimental validation has been performed in this study. Therefore, the proposed interactions and predicted activities should be considered preliminary and require further in vitro and in vivo experimental studies to confirm their biological relevance.

CONCLUSION

Overall, the proteins and phytochemicals investigated in this study have an established history of being researched to discover new cancer treatments. While norswertianin and gentiopicroside showed promising results, their effects and safety cannot be accurately assessed until validated under physiological conditions in cell lines and model organisms. This study highlights the power of computational drug design in identifying promising phytochemicals from *Gentiana dinarica* as potential drug candidates. However, as no experimental validation was performed, further in vitro and in vivo studies are essential to confirm these computational predictions and assess their therapeutic potential.

Acknowledgments

The authors gratefully acknowledge Muhammed Yasir Goz of the Visual Arts and Communication Design program at the International University of Sarajevo for his assistance in editing the figures presented in this article.

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