ORIGINAL ARTICLE

Non-Toxic Flavonoids of Artemisia annua can be used as Anti-Cancer Compounds: A Computational Analysis

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ABSTRACT

Objective: To identify potential flavonoids of *Artemisia annua* effective against cancer using computational approaches.

Study Design: Computational approaches were used to predict the anticancer activity of flavonoids through CDRUG, comparing it with the standard anticancer drug, followed by determining physiochemical properties and toxicity prediction of the selected flavonoids.

Place and Duration of Study: The study was carried out at Department of Bioinformatics and Biosciences of Capital University of Science and Technology (CUST) Islamabad, from December 2017 to July 2018.

Materials and Methods: The flavonoids of *Artemisia annua L* were downloaded and computational techniques such as similarity search, toxicity prediction, targets identification etc. were applied to investigate their anti-cancer activities.

Results: *Luteoline, Cirsilineol, Cirsiliol, Eupatorin, Crisimaritin* and *Artemetin* showed positive results among all the tested flavonoids. These compounds have the potential to replace anti-cancer drugs because of anti-cancer activity, toxicity against cancer cells and similarity with approved anti-cancer agents.

Conclusion: The screened compounds are good candidate for future drugs to be used against cancer. However, this is an in-silico study requiring further laboratory and enzymatic assays confirmation, which can be done in-vitro in future.

Keywords: *Artemisia annua, Cancer, Drug Discovery, Flavonoids, Medicinal Plants.*

How to cite this: *Dilshad E, Idrees M, Sajid B, Munir A, Fazal S. Non-Toxic Flavonoids from Artemisia annua can be used as Anti-Cancer Compounds: A Computational Analysis. Life and Science. 2022; 3(4): 151-162. doi: http://doi.org/10.37185/LnS.1.1.195*

Introduction

Cancer is a group of diseases in which cells grow abnormally, uncontrollably and migrate to attack different tissues of the body.[1] According to current prevalent cancer theories cancer is an uncontrolled somatic cell proliferation caused by the progressive accumulation of random mutations in critical genes that control cell growth and differentiation.[2] In 2020 about 1,806,590 new cases of cancer occurred globally (not including skin cancer other than melanoma). It caused about 606,520 deaths.[3] By 2030, it is predicted that there will be 26 million new cancer cases and 17 million cancer deaths per year. Common treatment measures include surgery, combination therapy, and radiation therapies, accounting for the large number of side effects. Several cancer therapies show resistance against disease due to improper mechanisms and wrong targets.[4] Today, despite considerable efforts, cancer remains an aggressive killer worldwide. Current treatments include chemotherapy, radiotherapy, and chemically derived drugs. Treatments such as chemotherapy put patients under a lot of strain and damage their health. Therefore, there is a need to focus on using alternative treatments and therapies against cancer.[4]

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Funding Source: NIL; Conflict of Interest: NIL
Received: Mar 21, 2021; Revised: May 31, 2021
Accepted: Dec 08, 2021

Medicinal plants are nature's gift to human beings that can help them pursue a disease-free healthy life and thus can play an important role in preserving health. A survey conducted by the World Health Organization (WHO) regarding medicinal plant uses reported that more than 1/3rd of the world's population relies on herbs for their primary health care.[5] Medicinal plants are considered local heritage with global significance.[6] As there is an increasing trend towards improving the "quality of life", there is consequently increased demand for medicinal plants.[7] These plants are either used directly as folk medicines or indirectly as pharmaceutical preparations of modern medicine,[8] being a source of a variety of compounds with different therapeutic significance.[9]

Many plants derived anti-cancer drugs have been used in western medicine. These include vinblastine, vincristine, paclitaxel, camptothecin, epipodophyllotoxin and many more.[10]

Generally, traditional drugs against cancer are used as they are non-lethal.[11] The anti-cancer properties of plants have been recognized for decades. Isolation of podophyllotoxin and a few different elements (known as lignans) from the regular mayapple (*Podophyllum peltatum*) led to the development of drugs used to treat testicular and small cell lung cancer.[12] The National Cancer Institute (NCI) has screened nearly 35,000 plant species for potential anti-cancer activities.[13]

Artemisia annua, also known as sweet wormwood and annual wormwood, is a common type of plant that is native to temperate Asia but naturalized throughout the world. It belongs to the family of Asteraceae and has bright yellow flowers, fern-like leaves, and a camphor-like scent. Glandular structures produce a broad variety of bioactive compounds (generally terpenoids), found on the surface of leaves, stems and flowers.[14] Artemisinin found in *A. annua* and its semi-synthetic artemisinin derivatives (including artesunate, dihydroartemisinin and artemether) are used for the production of combination therapies for the treatment of malaria.[15] Artemisinin extract from this herb is being used as an ingredient in anti-malarial medicines to help prevent anti-malaria drug resistance. Furthermore, it is used for treatment against bacteria, viruses and other microorganisms.[16]

Flavonoids consist of a huge collection of polyphenolic compounds having a benzo-pyrone structure and are universally present in plants. Secondary metabolites of phenolic nature including flavonoids are responsible for a variety of pharmacological activities. Flavonoids can prevent DNA mutations that occur in critical genes, such as oncogenes or tumour-suppressing genes, thus preventing cancer initiation or progression.[17] *A annua L* contains hydroxylated and polymethoxylated flavonoids such as chrysosplenol D, eupatin, cirsilineol, casticin, chrysoplenetin, that have beneficial effects and inverse relationships with cardiovascular disease and also with parasitic diseases such as malaria.[18] *A. annua* preparations have received a lot of interest in recent years because of their anti-cancer effects. In breast cancer cells, *Artemisia annua* extracts cause cell cycle arrest. *A. annua* extract's primary components, arteannuin B, casticin, chrysosplenol D, arteannuic acid, and 6,7-dimethoxycoumarin, are also used to treat a variety of malignancies.[18]

Despite efforts the treatment of several cancers is still inadequate. The use of synthetic chemotherapeutic drugs to treat cancer has not been successful in fulfilling expectations. Therefore, there is a need to develop more effective anti-cancer drugs with fewer adverse effects.[19] Natural products are considered novel and potential sources of chemopreventive and therapeutic agents.[20] Thus, in the quest of exploring anticancer drugs from natural resources, this research aimed to identify anti-cancer flavonoids from *A. annua* by using the bioinformatics techniques.

Materials and Methods

I. Flavonoids* of *A. annua Retrieval

In order to identify anti-cancer flavonoids from *A. annua* using computational approaches, we downloaded flavonoids of *A. annua* from the Zinc database in SMILES string format, which contains a library of 727,842 molecules[21] for virtual screening.[22] In total 32 flavonoids were downloaded for the current study. Detailed information on the downloaded compounds is given in Table 1.

ii. Prediction of Anti-cancer Activity of Flavonoids

The anti-cancer activity of downloaded flavonoids was predicted by using CDRUG, which uses the molecular descriptive method and a hybrid score to measure the similarity between the query and the active compound. CDRUG is effective to predict the anti-cancer activity of the chemical compounds.[23] The confidence interval (*p-value*) was calculated to predict whether a compound has anti-cancer activity. We used the default ($P < 0.05$) cutoff in CDRUG to screen 32 flavonoids.

iii. ***Comparison of Similarity between Flavonoids and Anti-cancer Drugs***

After the prediction of the anti-cancer activity of the 32 selected flavonoids, the similarity of predicted anti-cancer flavonoids with anti-cancer drugs in different developmental stages was performed according to the reported procedure.[24] The information concerning anti-cancer drugs in pre-clinical, clinical and approved stages was retrieved from SIB (Swiss Institute of Bioinformatics). SIB is a database that integrates biology, chemistry and pharmacology data to provide researchers with reliable, detailed, and current information to support successful drug discovery.[25]

iv. ***Identification of Physiochemical Properties***

The molecular properties of predicted anti-cancer flavonoids and anti-cancer drugs were calculated using Pipeline Pilot v8.5. Pipeline Pilot database v8.5 calculates molecular weight, and the number of rotatable bonds, rings, aromatic rings, H-bond acceptors and H-bonds donors.[26] The most common fragments and their frequency were calculated using the protocol 'Most Frequent Fragment' Pipeline Pilot v8.5.[27]

v. ***Identification of Structural Similarity among Flavonoids and Anti-cancer Drugs***

The structural similarity was measured using the Tanimoto coefficient (Tc) defined as Tc = C(i, j)/U(i, j), where C(i, j) is the number of common features in the fingerprints of molecules i and j and where U(i, j) is the number of all features in the union of the fingerprints of molecules i and j. The fingerprint MACCS implemented in the Pybel57 were generated for each structure and used to calculate TC. Two compounds are considered structurally similar if their fingerprints have a Tc of 0.70 or greater.[28]

vi. ***Toxicity Analysis of Flavonoids***

All the predicted anti-cancer compounds were tested for toxicity and drug likelihood by applying Lipinski's 'rule of five. Orally administered drugs are more likely in areas of chemical space defined by a limited range of molecular properties, which were encapsulated in Lipinski's 'rule of five'. This states that, historically, 90% of orally absorbed drugs had fewer than 5 H-bond donors, less than 10 H-bond acceptors, the molecular weight of less than 500 daltons and A logP values of less than 5.[29]

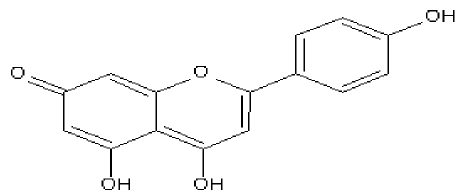
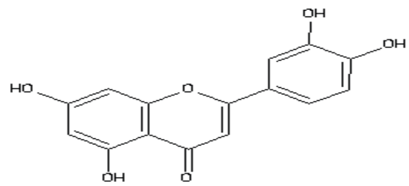
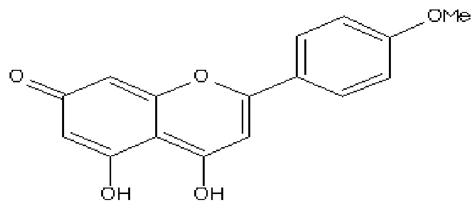
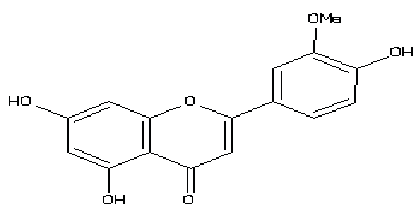
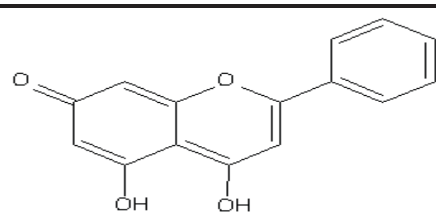
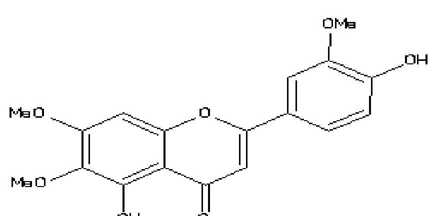
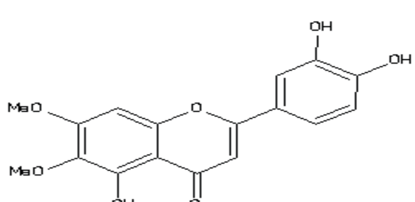
Results and Discussion

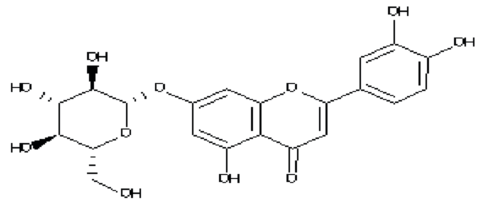
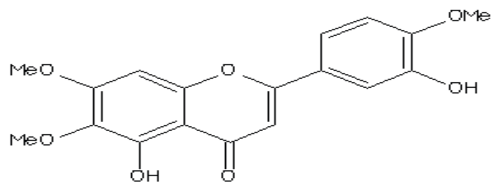
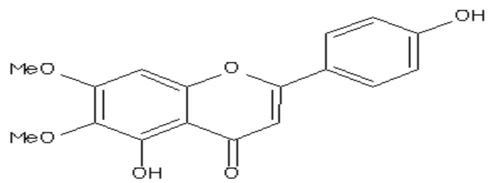
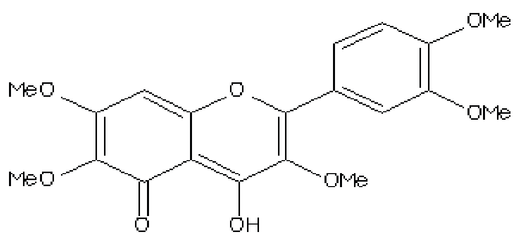
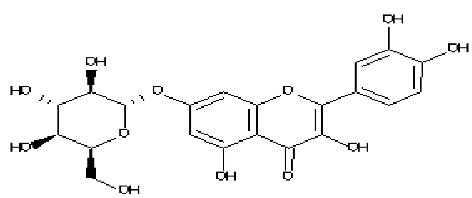
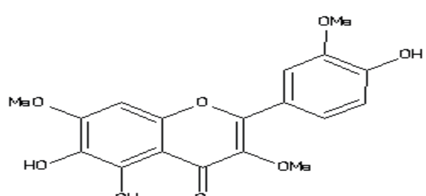
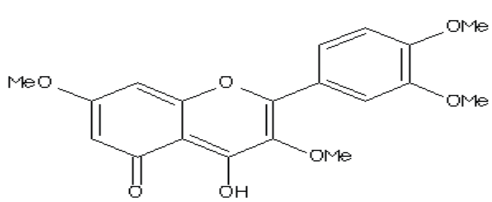
The mole2 format and SMILES string of 32 flavonoids of *A. annua* were downloaded from Zinc database. Flavonoids of *A. annua* are: apigenin, luteolin, acacetin, chrysoeriol, chrysin, cirsilineol, cirsiliol, cynaroside, eupatorin, cirsimaritin, artemetin, quercimeritrin, chrysosplenol C, retusin, chrysosplenol D, rhamnetin, mikanin, isorhamnetin, astragalin, rutin, axillarin, casticin, eupatin, chrysosplenetin, kaempferol, tamarixetin, syringetin, myricetin, isokaempferide, laricitrin, mearnsetin, quercetin shown in Table 1.

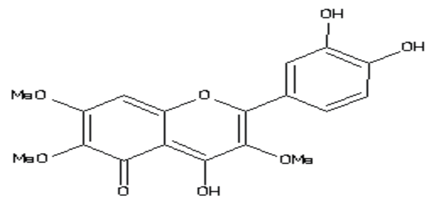
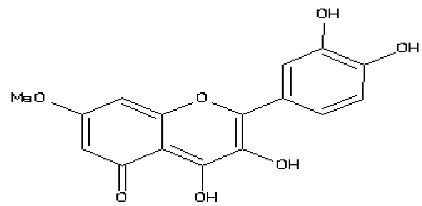
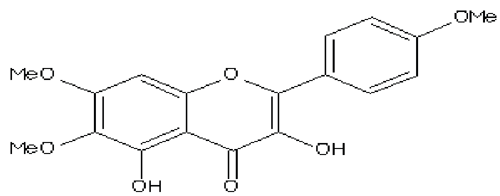
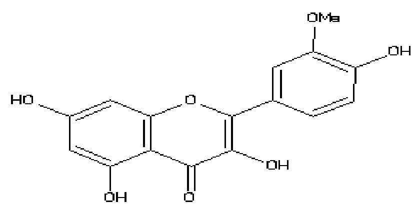
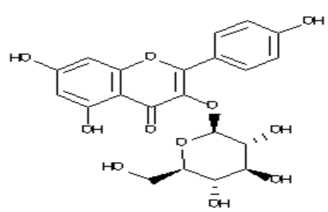
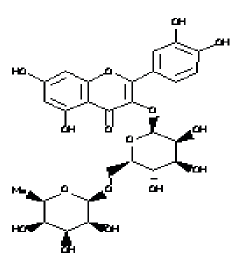
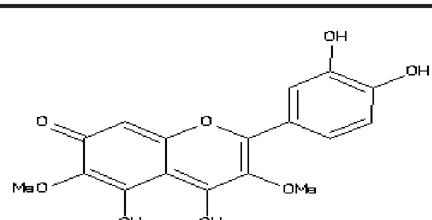
Prediction of anti-cancer activity of flavonoids was done by using CDRUG, which uses molecular descriptive method (Mean_logG150) and hybrid score (HSCORE) to measure the similarity between the query and the active. *P-value* was calculated to predict the anti-cancer activity of flavonoid. *P-value* is the probability of whether flavonoid has anti-cancer ability or not. In this study, a default *p*-value ($P<0.05$) was used in CDRUG for screening. Only 7 flavonoids named Apigenin, luteolin, Cirsilineol, Cirsiliol, Eupatorin, Crisimaritin and Artemetin have shown their ability to replace anti-cancer drugs because they have anti-cancer activity.

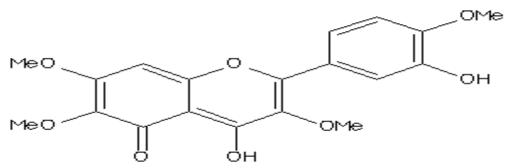
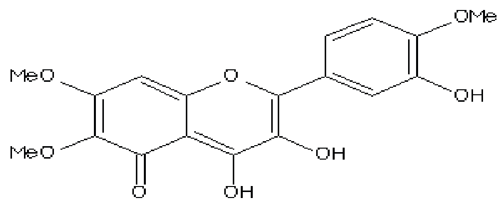
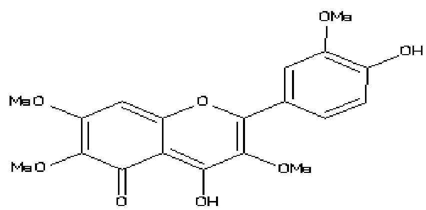
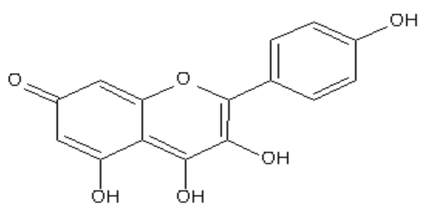
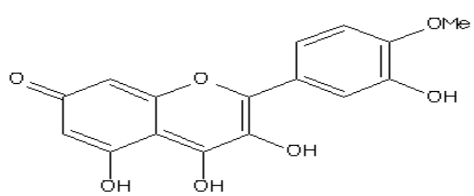
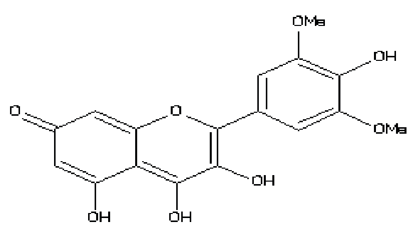
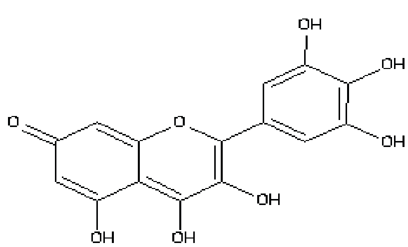
Apigenin is a naturally occurring plant flavone, mostly present in fruits and vegetables and is recognized as a bioactive flavonoid shown to have anti-inflammatory and anti-cancer properties. One of the common sources of apigenin consumed as an ingredient in herbal tea is chamomile, prepared from dried flowers of *Matricaria chamomilla.*[30] Luteolin, 3', 4', 5, 7-tetrahydroxyflavone, is a common flavonoid, which exists in many types of plants including fruits, vegetables and medicinal plants. Plants rich in luteolin have been used in Chinese

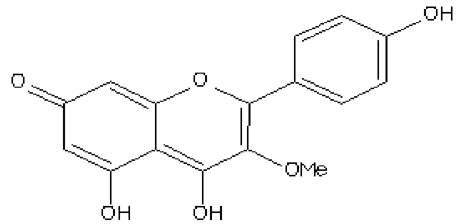
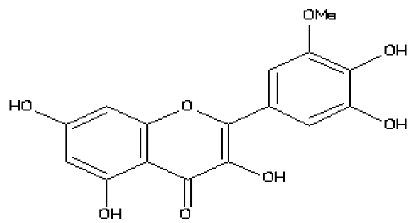
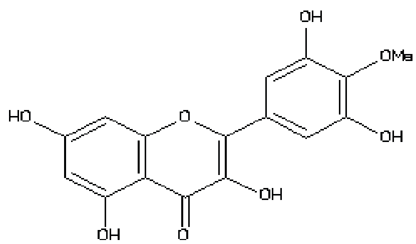
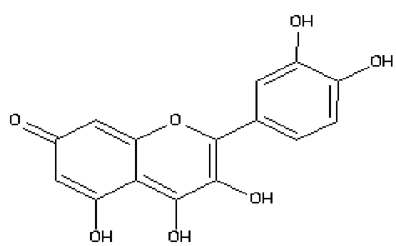
Table 1: The flavonoids of *A. annua* along with their molecular structures and SMILES format

Flavones	**Molecular Structure**	**SMILE string**
Apigenin		c1cc(ccc1c2cc(=O)c3c(cc(cc3o2)O)O)O
Luteolin		c1cc(c(cc1c2cc(=O)c3c(cc(cc3o2)O)O)O)O
Acacetin		COc1ccc(cc1)c2cc(=O)c3c(cc(cc3o2)O)O
Chrysoeriol		COc1cc(ccc1O)c2cc(=O)c3c(cc(cc3o2)O)O
Chrysin		c1ccc(cc1)c2cc(=O)c3c(cc(cc3o2)O)O
Cirsilineol		COc1cc(ccc1O)c2cc(=O)c3c(o2)cc(c(c3O)OC)OC
Cirsiliol		COc1cc2c(c(=O)cc(o2)c3ccc(c(c3)O)O)c(c1O C)O

Cynaroside		c1cc(c(cc1c2cc(=O)c3c(cc(cc3o2)O[C@H]4[C@@H]([C@H]([C@@H]([C@H](O4)CO)O)O)O)O)O)O
Eupatorin		COc1ccc(cc1O)c2cc(=O)c3c(o2)cc(c(c3O)OC)OC
Cirsimaritin		COc1cc2c(c(=O)cc(o2)c3ccc(cc3)O)c(c1OC)O
Artemetin		COc1ccc(cc1OC)c2c(c(=O)c3c(o2)cc(c(c3O)OC)OC)OC
Quercimeritrin		c1cc(c(cc1c2c(c(=O)c3c(cc(cc3o2)O[C@H]4[C@@H]([C@H]([C@@H]([C@@H](O4)CO)O)O)O)O)O)O)O
Chrysosplenol C		COc1cc(ccc1O)c2c(c(=O)c3c(o2)cc(c(c3O)O)OC)OC
Retusin		COc1ccc(cc1OC)c2c(c(=O)c3c(cc(cc3o2)OC)O)OC

Chrysosplenol D		COc1cc2c(c(c1OC)O)c(=O)c(c(o2)c3ccc(c(c3)O)O)OC
Rhamnetin		COc1cc(c2c(c1)oc(c(c2=O)O)c3ccc(c(c3)O)O)O
Mikanin		COc1ccc(cc1)c2c(c(=O)c3c(o2)cc(c(c3O)OC)OC)O
Isorhamnetin		COc1cc(ccc1O)c2c(c(=O)c3c(cc(cc3o2)O)O)O
Astragalin		c1cc(ccc1c2c(c(=O)c3c(cc(cc3o2)O)O)O[C@H]4[C@@H]([C@H]([C@@H]([C@H](O4)CO)O)O)O)O
Rutin		C[C@@H]1[C@@H]([C@@H]([C@@H]([C@@H](O1)OC[C@@H]2[C@H]([C@@H]([C@@H]([C@@H](O2)Oc3c(=O)c4c(cc(cc4oc3c5ccc(c(c5)O)O)O)O)O)O)O)O)O)O
Axillarin		COc1c(cc2c(c1O)c(=O)c(c(o2)c3ccc(c(c3)O)O)OC)O

Casticin		COc1ccc(cc1O)c2c(c(=O)c3c(o2)cc(c(c3O)OC)OC)OC
Eupatin		COc1ccc(cc1O)c2c(c(=O)c3c(o2)cc(c(c3O)OC)OC)O
Chrysosplenetin		COc1cc(ccc1O)c2c(c(=O)c3c(o2)cc(c(c3O)OC)OC)OC
Kaempferol		c1cc(ccc1c2c(c(=O)c3c(cc(cc3o2)O)O)O)O
Tamarixetin		COc1ccc(cc1O)c2c(c(=O)c3c(cc(cc3o2)O)O)O
Syringetin		COc1cc(cc(c1O)OC)c2c(c(=O)c3c(cc(cc3o2)O)O)O
Myricetin		c1c(cc(c(c1O)O)O)c2c(c(=O)c3c(cc(cc3o2)O)O)O

Isokaempferide		COc1c(=O)c2c(cc(cc2oc1c3ccc(cc3)O)O)O
Laricitrin		COc1cc(cc(c1O)O)c2c(c(=O)c3c(cc(cc3o2)O)O)O
Mearnsetin		COc1c(cc(cc1O)c2c(c(=O)c3c(cc(cc3o2)O)O)O)O
Quercetin		c1cc(c(cc1c2c(c(=O)c3c(cc(cc3o2)O)O)O)O)O

traditional medicine for treating various diseases such as hypertension, inflammatory disorders, and cancer.[30] Cirsilineol is a bioactive flavone isolated from *Artemisia* and *Teucrium gnaphalodes*.[31] Cirsimaritin is a flavonoid also known as 4',5-dihydroxy-6,7-dimethoxyflavone used in treating cancer and also has anti-inflammatory properties. For all the anti-cancer compounds predicted through CDrug, the H-score and *P-values* were lying in the ratio of 0.4 – 0.7 and 0.003 – 0.2, respectively.

Similarly, a comparison of predicted anti-cancer compounds with approved and investigational drugs was done by using the tool SwissSimilarity which comes under the SIB. Similarity comparison was performed based on two methods i.e. FP2 fingerprinting and the combined method. Both of these methods use SMILE's string format as a query. FP2 is a path-based fingerprint which indexes small molecule fragments based on linear segments of up to 7 atoms.[32] A molecule's structure is examined to isolate linear fragments of length from 1-7 atoms. Single-atom fragments were ignored. The fragment was dismissed if the atoms formed a ring. For each of the fragment the atoms, bonding or whether they create a complete ring were recorded and saved in a set so that there is only one of each fragment type. The results of fingerprinting are shown in Table 2.

Genistein is an iso-flavonoid that is derived from soy products. It inhibits protein-tyrosine kinase and topoisomerase-II (DNA topoisomerases, type II) activity and is used as an antineoplastic and antitumor agent.[33] Experimentally, it has been shown

Table 2: The results of FP2-fingerprinting and combined methods of the Swiss Similarity search tool

Flavonoid	Similar Approved Compound	Similar Investigational Compound	Method
Apigenin	No	Genistein	FP2- Fingerprints
Luteolin	No	Genistein	FP2- Fingerprints
Acacetin	No	Genistein Flavopiridol	Combined Method FP2- Fingerprints
Casticin	Sunitinib	No	Combined Method
Chrysin	No	Genistein Flavopiridol	Combined Method FP2- Fingerprints
Cirsilineol	Crizotinib Imatinib Sunitinib	No	Combined Method
Cirsiliol	Vandetanib		Combined Method
Cynaroside	Epirubicin	No	Combined Method
Eupatorin	Crizotinib Imatinib	No	Combined Method
Cirsimaritin	Sunitinib Vandetanib	No	Combined Method
Artemetin	Crizotinib Imatinib	No	Combined Method
Quercimeritrin, Chrysosplenol C, Retusin, Chrysosplenol D, Rhamnetin, Mikanin, Isorhamnetin, Astragalin, Rutin, Axillarin, Eupatin, Chrysosplenetin, Kaempferol, Tamarixetin, Syringetin, Myricetin, Isokaempferide, Laricitrin, Mearnsetin, Quercetin, Chrysoeriol	No	No	Combined Method FP-2 fingerprinting

to induce G2 phase arrest in human and murine cell lines. It is a synthetic flavonoid founded in an extract from an Indian plant for the potential cure of cancer. It works by inhibiting cyclin-dependent kinase, arresting cell division and causing apoptosis in non-small lung cancer cells.[34] Crizotinib is used for the treatment of advanced non-small-cell lung cancer (NSCLC), which is anaplastic-lymphoma kinase (ALK)-positive which is detected by an FDA-approved test.[35] Imatinib is a small molecule kinase inhibitor used to treat certain types of cancer. More importantly, it is used for curing chronic myelogenous leukaemia (CML), gastrointestinal stromal tumours (GISTs) and many other malignancies.[36] Sunitinib is an oral small-molecule or multi-targeted receptor tyrosine kinase (RTK) inhibitor that inhibits multiple RTKs, some of which are implicated in tumour growth, pathologic angiogenesis, and metastatic development of cancer.[37] Vandetanib is an oral kinase inhibitor of cancer angiogenesis and cancer cell propagation with the potential for use in a broad range of cancer types. It is used to treat advanced or metastatic medullary thyroid cancer in adult patients.[38] Epirubicin is used as a component of adjuvant therapy in patients with an indication or evidence of axillary node tumour involving resection of primary breast cancer.[39]

Target identification of downloaded flavonoids was done using Swiss Target Prediction which comes under the SIB. Swiss Target Prediction is a web server for the target prediction or identification of bioactive small molecules. It is based on the observation that similar bioactive molecules are more likely to share similar targets.[31] The targets of a molecule were predicted by identifying proteins with known ligands that were highly similar to the query molecule. Target prediction can be done by combining different methods or measures of chemical similarity based on both chemical structure and molecular shape.[33] These results indicate that the combined approach is especially efficient when no ligand has the same scaffold. A large number of proteins or enzymes targets were predicted for all the 32 query flavonoids, the predicted targets include enzymes, ser-thr kinases, membrane proteins, transcription factors, metalloproteases, transporter proteins, serine proteases, and several unclassified proteins.(shown in Supplementary material)

The toxicity test for the downloaded flavonoids was done using the SBIO tool, based on the Lipinski's rule of five. As Lipinski's rule states that, in general, an orally active drug has no more than one violation of the following criteria: i.e there should be no more than 5 hydrogen bond donors (the total number of nitrogen–hydrogen and oxygen-hydrogen bonds, no more than 10, hydrogen bond acceptors (all nitrogen or oxygen atoms), Molecular mass should be less than 500 Daltons and LOGP not greater than 5 (40). Properties of the compounds are given in Table 3.

Table 3: Details of Lipinski rule of five for all 32 Flavonoids

Flavonoid	Mass	HBDs	HBAs	LogP	Molar refractivity
Apigenin	270	3	5	2.42	70.81
Leutolin	286	4	6	2.13	72.48
Acacetin	284	2	5	2.72	75.70
Chrysoeriol	300	3	6	2.43	77.37
Chrysin	254	2	4	2.71	69.15
Crisilineol	344	2	7	2.73	88.81
Cirsiliol	330	3	7	2.44	83.92
Cymaroside	448	7	11	-0.402	105.21
Eupatorin	344	2	7	2.74	88.81
Cirsimaritin	314	2	6	2.73	82.25
Artemetin	388	1	6	3.02	99.64
Quercimeritrin	464	8	12	-0.52	106.78
Crysosplenol	360	3	8	2.41	89.87
Retusin	358	1	7	3.01	93.09
Chrysosplenol D	360	3	8	2.41	89.87
Rhamnetin	316	4	7	2.31	78.94
Isohmnetin	316	4	7	2.31	78.94
Asragalin	448	7	11	-0.4362	103.61
Rutin	610	10	16	-1.88	137.49
Azilarin	346	4	8	2.11	84.98
Casticin	374	2	8	2.71	94.76
Eupatin	360	3	8	2.63	90.38
Chrysosplentin	374	2	8	2.71	94.76
Kaempferol	286	4	6	2.30	72.39
Tamarixetin	316	4	7	2.31	78.94
Syringetin	346	4	8	2.32	85.49
Myricetin	318	6	8	1.72	75.72
Isolaempferide	300	3	6	2.39	76.77
Laricitrin	332	5	8	2.02	80.60
Mearnsetin	332	5	8	2.02	74.05
Quercetin	302	5	7	2.01	74.05

According to the results, most of the flavonoids show the best results except a few i.e. cymaroside, quercimeritrin, astragalin and rutin. The cymaroside has hydrogen bond acceptors of more than 10 i.e. 11, quercimeritrin also have hydrogen bond acceptors more than 10 i.e 12. Other than these flavonoids apigenin, acacetin, chrysoeriol, chrysin, artimetin, cirsiliol, retusin, isolaempferide, azillarin, myricetin, mearnsetin showed that these flavonoids are best according to the rules defined for toxicity prediction using Lipinski's rule of five as they have mass less than 500 Daltons and hydrogen bond donors less than 5, hydrogen bond acceptors less than 10, their logP value is less than 5 and their molecular refractivity is between 40-130.[40]

Conclusion

In this study, 32 flavonoids of *Artemisia Annua* were downloaded and different tests were applied to them to confirm their anti-cancer activities i.e. prediction of anti-cancer activity of flavonoids by using CDRUG tool, similarity comparison with approved drugs and drugs that are in investigational stage by using SWISS similarity tool and toxicity testing by using Lipinski rule of five. Among all the downloaded flavonoids, only luteoline, cirsilineol, cirsiliol, eupatorin, crisimaritin and artemetin have the ability to replace anti-cancer drugs because of anti-cancer activity, anti-cancer toxicity and approved drugs similarity. Also, they show positive results among all tests applied to them. However, this is an in-silico study requiring further laboratory and enzymatic assays confirmation which can be done in-vitro in future.

Acknowledgement

The authors are grateful to the Capital University of Science and Technology Islamabad, Pakistan for providing a platform to conduct research.

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