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Project work on

'Understanding the Mechanism of Shilajit by Using Network Pharmacology Approach'

Submitted by

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FACULTY OF ENGINEERING & TECHNOLOGY

DEPARTMENT OF BIOTECHNOLOGY

PROGRAM B Tech

CERTIFICATE

This is to certify that the Project Report entitled

'Understanding the Mechanism of Shilajit by Using Network Pharmacology Approach'

is a bonafide work carried out by

Rhiya Sharma (01FB15EBT038)

In partial fulfillment for the completion of 8th semester course work in the Program of Study B.Tech.- Biotechnology under rules and regulations of PES University, Bengaluru during the period Jan. 2019 – Apr. 2019. It is certified that all corrections/suggestions indicated for internal assessment have been incorporated in the report. The project report has been approved as it satisfies the 8th semester academic requirements in respect of project work.

Signature with date & Seal *Internal Guide*	*Signature with date & Seal* *Chairperson*	*Signature with date & Seal* *Dean of Faculty*

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Name of Examiner: ***Signature with date***

FACULTY OF ENGINEERING & TECHNOLOGY

DEPARTMENT OF BIOTECHNOLOGY

PROGRAM B Tech

DECLARATION

I, **Rhiya Sharma,** hereby declare that the Project Work entitled, **'Understanding the Mechanism of Shilajit by Using Network Pharmacology Approach',** is an original work done by us under the guidance of **Dr. Jhinuk Chatterjee**, Associate Professor, PES University, and is being submitted in partial fulfillment of the requirements for completion of 8th Semester course work in the Program of Study B.Tech in Biotechnology.

PLACE: BANGALORE
DATE:

Rhiya Sharma (01FB15EBT038)

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Abbreviations:

Abbreviation	Full Form
WHO	World Health Organisation
Fas	Fulvic acids
Has	Humic acids
HMs	Humins
SMILES	Simplified molecular-input line-entry system
STITCH	Search Tool for Chemicals Interacting
TTD	Therapeutic Target Database
GDA	Gene Disease Associations
IC50	Half Maximal Inhibitory Concentration
TP53	Tumor Protein p53
PTGS2	Prostaglandin G/H synthase 2
PTGS2	Prostaglandin G/H synthase 2
EGFR	Epidermal growth factor receptor
EDFR	Epidermal growth factor
MET	MET proto-oncogene, receptor tyrosine kinase
ESR1	Estrogen receptor 1
VEGFA	Vascular endothelial growth factor A
TNF	Tumor necrosis factor
DNMT1	DNA methyltransferase 1
KDR	Kinase insert domain receptor
MAPK3	Mitogen-activated protein kinase 3
PLAU	Plasminogen activator, urokinase
NOS2	Nitric oxide synthase 2
SRC	Proto-oncogene tyrosine-protein kinase SRC
MMP1	Matrix metallopeptidase 1
IGF1R	Insulin like growth factor 1 receptor
CA1	Carbonic anhydrase 1

CA2	Carbonic anhydrase 2
CA9	Carbonic anhydrase 9
CA12	Carbonic anhydrase 12
CNR1	Cannabinoid receptor 1
CNR2	Cannabinoid receptor 1
PRKCB	Protein kinase C beta
MAPK8	Mitogen-activated protein kinase 8
MAPK14	Mitogen-activated protein kinase 14
SERPINE1	Serpin family E member 1
F3	Coagulation factor III, tissue factor
CSNK2A1	Casein kinase 1 alpha 1
SYK	Spleen associated tyrosine kinase
HSF1	Heat shock transcription factor 1
AKR1B1	Aldo-keto reductase family 1 member B
PRTN3	Proteinase 3

ABSTRACT

Natural products from the Indian system of traditional medicine, like Ayurveda, are considered as appealing options for new drug discovery. Ayurveda uses intelligent formulations which contain several bioactives and are capable of modulating several disease targets; however, their scientific reasoning and methods remain largely unexplored. It is now possible to study the complex interactions between the bioactives, targets, diseases and genes with the help of the up-and-coming technique called Network Pharmacology. This technique combines systems biology and computational biology to study multi-component and multi-targeted formulations. This report presents the potential of network pharmacology to understand the foundation of an Ayurveda formulation known as Shilajit. Pharmacology networks of Shilajit have been developed based on the information gathered from different databases and using the software Cytoscape. The networks depict the interaction of bioactives with molecular targets and their relation with cancer. This pioneering effort might open new possibilities to know the pharmacodynamics of Ayurvedic drugs like Shilajit and also help in the discovery of new leads and targets for cancer.

HYPOTHESIS

Pharmacological network of Shilajit will depict the interaction of bio-actives with molecular targets of cancer.

OBJECTIVES

1. To find the bio-actives of Shilajit.
2. To predict the targets of the components of Shilajit.
3. To construct a pharmacology network of the mechanism of the formulation of Shilajit.
4. To validate the findings.

INTRODUCTION

Drug research has undergone many changes over the past few years. Although high-throughput technologies are available, the number of new drug candidates entering into the market has reduced. In addition, the number of drugs withdrawn after market launch is increasing. There seems to be a need to boost the discovery of new drugs [1]. Natural products and traditional medicine can play a vital role to overcome the current impasse in drug discovery [2].

Drug discovery usually follows the one-gene / one-target / one drug approach, but the smarter approach may be a multi-target, multi-component formulation form. Many single-target drugs can not completely correct a complex disease condition such as cancer [3]. Due to the presence of polygenic syndromes and not just isolated diseases, multi-target approaches are needed. Therefore, the strategy must be shifted from one that focuses on a single target-new chemical entity as a drug to one of a multi-target, synergistic approach to formulation discovery [4].

Network pharmacology is an emerging technique that is helpful in formulation discovery, and integrates the recent advances in omics technology, system and computational biology [5]. It is also known as poly-pharmacology and attempts to understand drug action [6]. At present, efforts are being made to design ligands with maximum selectivity to act on specific targets for drugs. Like enzyme action, the drug-receptor relationship is also considered to be highly specific – similar to a “lock and key”. However, just as one master key can open many locks, many drugs can modulate multiple target proteins rather than acting on single targets.

Several drugs used to treat diseases in specialties such as cardiology, oncology, and psychiatry, have effects on multiple targets. To better understand the underlying complex biological and pharmacological processes for these complex diseases, it is important to know the systems network of various pathways where the drugs are likely to act [7]. Cancer, for example, is a complex disease that depends on multiple genes. A multi-drug formulation that binds to multiple disease related targets will be far more effective and advantageous than a single drug that binds to a single target. This allows the multi-target formulation to regulate pathways that function in the diseased state. A network of interactions between natural products, in formulations from traditional systems like Ayurveda, and cancer target proteins helps identify potential drug leads and new interactions [8].

Ayurveda, a traditional Indian medicinal system, offers several sophisticated formulations which have been used for thousands of years. The Traditional Knowledge Digital Library (TKDL, http://www.tkdl.res.in) contains more than 36,000 Ayurveda formulations, about 100 of which are very popular and are even used as “over-the-counter products” at the community level. Some of these drugs continue to be used for preventive and primary health care as a home remedy in India. Shilajit is one of the broadly used Ayurveda formulations [2].

Shilajit, also known as Salajit, Shilajatu, Mumie or Mummiyo is a pale-brown to blackish-brown exudate of variable consistency from rock layers in many of the world's high mountain ranges, particularly the Indian subcontinent's Himalayan ranges [9]. It is also found in Russia, Tibet, Norway and other countries, where it is collected at altitudes between 1000 and 5000 m in small quantities from steep rock faces.

Shilajit is known to be a rejuvenator (‘Rasayana’) of traditional Hindu Ayurvedic origin, which has attracted considerable interest in India. Rasayana medicines, an Ayurvedic pharmacology classification, improve the quality of 'Rasa' (plasma), thereby strengthening or promoting the health of all body tissues [10]. Shilajit has been associated with a number of pharmacological activities and has been used as a rejuvenator for ages and in the treatment of a variety of diseases. Modern scientific studies have systematically validated a number of its properties and showed that Shilajit is a genuine panacea in Oriental medicine [11]. Since there are a number of such remedies described in our ancient texts, it is essential that research be carried out to validate their claims and uses.

In a number of developing countries, including India, traditional medicine is an essential part of the health care system. There are a number of natural remedies that have been in use for ages but unfortunately lack systematic scientific evaluation and documentation. The world today is looking at these remedies for a number of ailments. These remedies, however, can find their place in mainstream medicine only if their claims are scientifically evaluated and systematically documented [12].

Thus, in the recent years, network-based approaches are commonly used to decipher new drug-target relationships in drug discovery [13]. The network-target-based network pharmacology is a promising strategy for the next generation mode of drug research and development for traditional medicine.

REVIEW OF LITERATURE

According to the World Health Organization (WHO), traditional medicine incorporates plant-based, mineral-based and animal-based health practices that are applied individually or in combination to treat and prevent diseases and maintain well-being [14]. The WHO estimates that around 80% of the world's population relies on traditional medicine for their health needs. In this connection, growing research is being carried out worldwide with regard to plant-based medicines. Shilajit is one such substance.

Shilajit is a blackish-brown exudate from high mountain rocks and is produced over a few mineral years from organic matter and plants that have been trapped between mountain rock layers in a few regions, including Tibet, China, India and Altay. It is found on the walls of caves embedded in rocks at high altitudes between 1000 and 5000 m or as rock exudates with specific weather conditions regarding summer and winter temperatures, sunshine duration and precipitation amount. With time, the pressure from the weight of the mountains transforms the materials into a rich mineral mass that then oozes out of the rocks [15].

Figure courtesy: Chirag Jindal, Wikipedia, 2008

Figure 1: Shilajit, a blackish brown exudation from the Atlai Mountains

There were many different beliefs regarding the origin of Shilajit. Some thought it was a plant fossil, a substance of mixed plant and animal origin whereas others believed that it was of vegetative origin. Many researchers claim that Shilajit exudes plant secondary metabolites from a layer or rocks of mountains. It has been mentioned that the sap or latex juice of the plant comes out as a sticky exudate from the rocks of mountains due to the intense heat from the sun. Claims are put forth that the mosses of species, such as Barbula, Fissidenc, Minium, Thuidium, and species of Liverworts, like Asterella, Dumortiera, Marchantia, Pellia, Plagiochasma, and Stephenrencellaa anthoceros, were present in the surrounding area of the Shilajit-exuding rocks, and these bryophytes are responsible for the formation of Shilajit [11]. The bryophytes reveal the presence of minerals and metals in their tissues such as silver, copper, iron, zinc and lead ore, which are similar to the elements present in Shilajit.

Shilajit is not a rock, but a complex mixture of humic organic substances, plant and microbial metabolites that occur in the rock rhizosphere [16]. Shilajit has been used in one form or another under indigenous medicine systems such as Ayurveda for thousands of years. Shilajit is one such remedy, which as a rejuvenator and adaptogen has been in use as a folk medicine for over 3000 years and has been ascribed a number of pharmacological activities and for treating a number of disease conditions. It has been said that with Shilajit's help there is hardly any curable disease that cannot be controlled or cured [17].

Shilajit has been extensively used by Hindu physicians to treat several various diseases. According to Ayurveda, Shilajit is known to arrest the aging process and produce rejuvenation, which are two important aspects of Ayurvedic rasayana [18]. According to traditional Indian learning, when given internally, Shilajit acts as a tonic, laxative, diuretic, expectorant, anti-bilious, lithotriptic, immuno-modulator and anti-hypertensive and when it is applied externally, it acts as analgesic, antiseptic, germicide and deobstruent [11].

Shilajit has also been known to be used in folk medicine to treat bone fractures, joint inflammation, stomach disorders, nervous and cardiovascular diseases, impotence and strains of muscles and tendons. It is also supposed to be an effective treatment for stomach ulcers, wounds, diabetes, and urinary tract infections. Moreover, considering the actions of fulvic acid (a component of Shilajit) in preventing tau self-aggregation into pathological filaments, this compound might be help in the prevention of Alzheimer's disease [19]. In India, it was also claimed that Shilajit was used as yogava [20], that is, as a synergistic enhancer of other drugs. Its organic components also play a role in transporting various minerals to their cellular targets.

Figure courtesy: E. Wilson et al., Journal of Ethnopharmacology, 2011

Figure 2: Purified bar of Shilajit

Shilajit's chemical content is controlled by multiple factors such as adjacent plant species, rock and environment, soil geological, moisture, temperature and altitude, etc. Shilajit obtained from India, for example, contains a higher percentage of fulvic acids (21.4 %) compared to Shilajit obtained from Nepal (15.4 %), Pakistan (15.5 %) and Russia (19.0%) [11].

Extensive research to determine the exact chemical nature of Shilajit has been conducted. Studies carried out in the 1980s showed that the major organic mass of Shilajit comprised humus (60–80%) along with other components such as benzoic acid, hippuric acid, fatty acid, ichthyol, ellagic acid, resin, triterpenes, sterol, aromatic carboxylic acid, 3, 4-benzocoumarins, amino acids and phenolic lipids [21]. The major physiological action of Shilajit was found to be due to the presence of the bioactive dibenzoalpha-pyrones along with humic and fulvic acids which acted as carrier molecules for the active ingredients [22]. On performing an HPLC analysis of Shilajit, some of the polyphenols determined were tannic acid, gallic acid, ferulic acid [23].

In general, Shilajit contains two classes of organic compounds, namely-Humic substances and organic non-humic metabolites. Humic substances are Shilajit's major organic constituents in an amount of about 80-85 percent. The humic substances are the result of degradation of organic matter, mainly vegetal substances, resulting from the action of many microorganisms. The humic substances in Shilajit can be further divided into three fractions— Fulvic acids (Fas), Humic acids (Has) and Humins (HMs).

Shilajit's Fas micropores are occupied by low molecular weight. Bioactive molecules such as oxygenated dibenzene-alpha-pyrones and their dimeric and oligomeric equivalents [18, 24, 25], other low molecular weight phenolic entities and Fas of Shilajit act as an efficient carrier of several classes of drug molecules for continuous systemic distribution and absorption [26].

Shilajit's non-humic substances are low molecular weight marine fossil, plant and microbial compounds that occur in and around Shilajit bearing rocks. Low molecular weight oxygenated dibenzo-alpha-pyrines (DBP) and hydroxy acetophenones (HAPS) are the remaining non-humic organic masses in Shilajit. These compounds comprise a mixture of low molecular weight aromatic, aliphatic alicylic and heterocylic (N- and S-containing) compounds. The two oxygenated dibenzo-alpha-pyrones, namely 3-hydroxydibenzo-alpha-pyrone and 3, 8-dihydoxy dibenzoalpha-pyrone, occurred both in the free form in Has and Fas [27] micropores as well as in conjugated forms in Shilajit's humus [28].

Due to the presence of some of its active components, Shilajit is said to be very good for health. Natural polyphenols are one such component. These can be used for the prevention and treatment of cancer. Potential mechanisms included antioxidant, anti-inflammation as well as the modulation of multiple molecular events involved in carcinogenesis [29]. Ellagic acid, an example of a natural polyphenol, has globally attracted increasing attention for its antoicarcinogenic properties. Over the past few decades, promising evidence of ellagic acid anti-tumor activity has shown that it is capable of preventing tumor growth and metastasis, inhibiting apoptosis-inducing tumor cell proliferation, breaking DNA-binding carcinogens, and hampering inflammation, angiogenesis, and drug resistance processes [30]. Ellagic acid has powerful preventive and therapeutic effects on various types of cancer, including colon cancer, breast cancer, prostate cancer, skin cancer, esophageal cancer, and osteogenic sarcoma [31, 32].

Among the many active principles in Shilajit, fulvic acids and humic substances are another such important component. They make up around 80-85% of the total organic mass [33]. In humic acid molecules, reactive groups such as carboxyl, phenol, quinone give the acids their mineral and antioxidant properties. Humic acids are mostly known for their anti-viral, anti-inflammatory, anti-oxidant, anti-tumor, anti-toxin activities. Many researchers have suggested that HA has the ability to protect against cancer and viruses that cause cancer. This suggests that HA has various biological activities such as cancer prophylaxis and treatment, reduced cell proliferation and reduced angiogenesis [34].

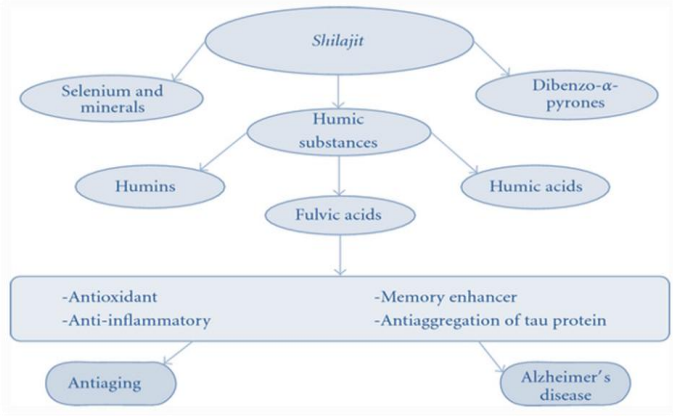


Figure courtesy: C. Carrasco-Gallardo et al., International Journal of Alzheimer's Disease, 2012.

Figure 3: Shilajit's main components and the potential uses of fulvic acids

Humic acids and fulvic acids are the two main natural acidic organic polymers that can be extracted from humus created in soil, aquatic environments or sediments. Humic acids are known to accumulate gradually over time as a residue from the metabolism of microorganisms. However, this process is not well understood as there is a lot of variability in its molecular features. Its structure is unlike those of proteins or carbohydrates, which are the two most common polymers found in a biological substance. Humic acids are characterized as a loose assembly of aromatic polymers of varying reactivity and acidity.

Shilajit is a naturally occurring complex formulation that is used in Ayurvedic Medicine. It is a mixture that contains several chemical ingredients with multiple potential targets. Compared with modern drugs with explicit mechanisms, the challenge for traditional medicine is to understand the molecular mechanism of multi-component therapies. Moreover, the lack of scientific facts of pharmacological action and the bioactive principle within multi-component therapies has already impeded the advancement of traditional medicine. The introduction of the emerging technique, Network Pharmacology, provides a novel strategy to understand the mechanisms of complex formulations in a holistic way and imply new applications of classic traditional medicines.

Network pharmacology, which integrates information science and systematic medicine, is evolving as a frontier research field of drug discovery and development. Network pharmacology intends to systematically reflect and disclose, with a network-based insight, the biological basis of complex diseases and drug effects. At the same time, a key concept derived from the multi-target nature of traditional medicine, "network target" has been proposed, shifting the current paradigm - "single target."

Systems biology is a recent trend in bioscience research that focuses from a holistic perspective on the complex interactions in biological systems rather than altering the single molecular component [35]. With the rapid increase of available biomedical data in the postgenomic era, systems biology and polypharmacology have provided fresh insight into drug discovery. A novel concept of network pharmacology was developed based on the core concept that many effective drugs act on multiple rather than single targets in therapeutic areas [7]. Network pharmacology can be reconstructed using molecular networks that combine multidisciplinary concepts including biochemistry, systems biology and bioinformatics [36].

Currently, due to the presence of polygenic syndromes and not just isolated diseases, a multi-target approach will be needed [5]. A multi-targeted approach can be used to manage lifestyle disorders such as obesity, diabetes, cardiovascular diseases and cancers. The network-based pharmacology network is a promising strategy for the next-generation approach of drug research and development in traditional medicine.

The network pharmacology also tries to repurpose existing drug molecules for several other therapeutic conditions. These efforts, however, require some guidance to select the right target type and new drug molecule scaffolds. Integrating systems biology and pharmacology networks can speed up the search for drug targets and help design new drugs that can modulate multiple biological targets [36]. A 'network target' concept was proposed to help design and predict the best treatments possible [37]. The built network system can predict the main active components and their targets, which can be useful for the therapeutic applications of traditional medicine [38]. Traditional knowledge can play the vital role in the process of discovering formulations and repurposing existing drugs [39].

Biological network approaches have proved useful in organizing high-dimensional biological datasets and extracting meaningful information. A network is a way to represent data sets highlighting node relationships. A graph is created in which these nodes, representing genes, proteins, small molecules or any other entity capable of interacting in the modeling system, are connected by edges representing the nature of the interaction [40]. Edges represent these interactions between the nodes and, when the information is available, edges can have directions, weights and other attributes that provide information about the hierarchy of effects. Different relationships between nodes which can be used to study drug are depicted in Figure 4. Depending on the nature of the study, interactions can be experimentally determined physical and chemical interactions, genetic regulatory interactions, relationships of higher order such as co-expression or some other common property linking the nodes.

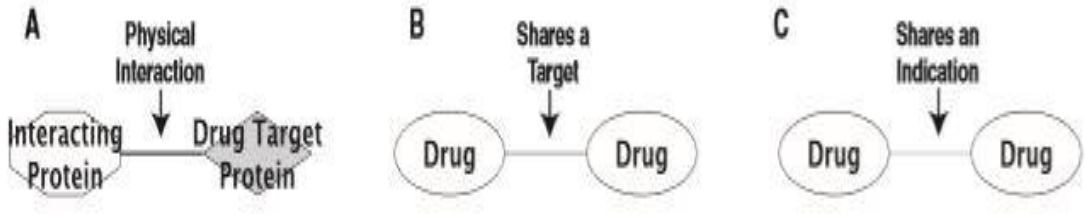


Figure courtesy: S. Berger et al., BMC Bioinformatics, 2007

Figure 4: Relationships between nodes in networks used in systems pharmacology.

Network data structures are compliant to many sophisticated forms of computational analysis that may reveal important, non-obvious node properties and their relationships. Networks enable different sources of experimental data and biological knowledge to be integrated into a framework that provides new insight into the systems. These approaches can combine datasets of the genome scale with specific gene and protein information [44, 45]. Recently, studies of metabolic networks, protein-protein interaction networks, gene regulatory networks and other biological networks have provided insight into the origins of overall cell behaviors and evolutionary design principles, as well as specific cell biological processes or diseases. From these analyzes, one can develop experimentally testable hypotheses ranging from predicting new functions of specific genes to the properties of human cellular networks on the scale of genomes [46].

It is possible to group network studies in pharmacology of systems into three broad categories- (A) Global studies on the drug network that incorporate information on many types of drugs and biological datasets such as data on protein-protein interaction may generate network properties of drug targets [41]. These properties provide information on historical trends in drug development and may suggest properties of what makes a target druggable (a term used in drug discovery to describe a biological target, such as a protein, that is known to or is predicted to bind with high affinity to a drug). (B) Disease-specific network studies use specific disease information to identify potentially new drug targets and therapeutic strategies [42]. (C) Studies integrating information on specific diseases and drugs may identify new drug indications, unknown drug targets and other potentially interesting drug properties [43].

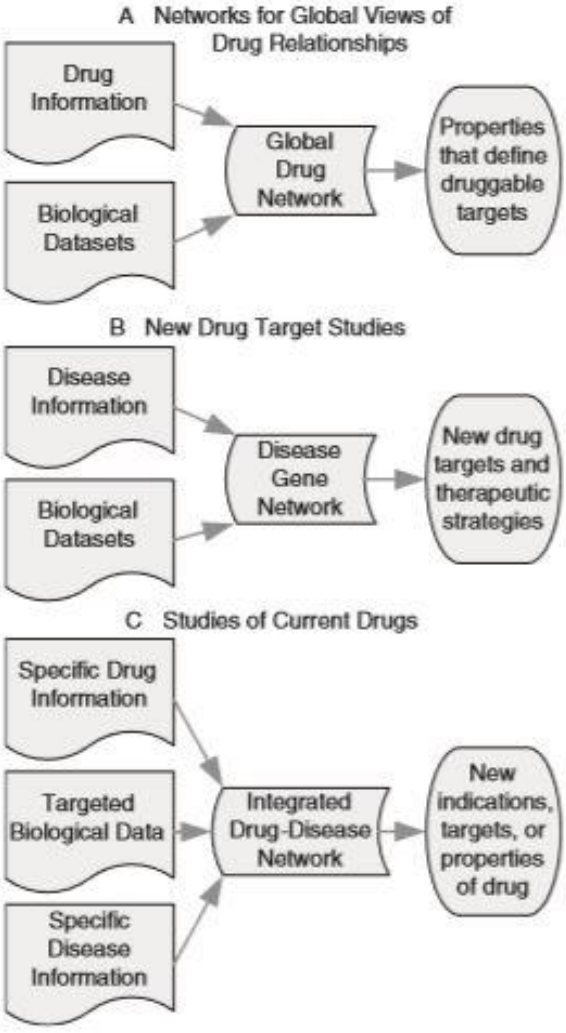


Figure courtesy: S. Berger et al., BMC Bioinformatics, 2007

Figure 5: Types of network studies in systems pharmacology

Network approaches enable biomedical researchers to organize current knowledge quickly by integrating various types of large datasets. Applying such approaches to pharmacology problems can enable system level descriptions of drug action, rapid identification of new therapeutic strategies, and the development and prescription of potentially safer and more effective drugs. It also makes it possible to predict and explain the unexpected effects of medicines and suggests factors that influence drug efficacy and safety. As of now, network approaches in pharmacology systems are still in their early stages. As more data is collected and generated, more detailed networks will be created.

To move quantitative drug-target analysis to high-throughput formats, a new wave of technology development is needed. In addition, the results of network studies need to find a path to clinical relevance in order to become a truly translational field. This could be done by using systems analysis of drug effects to improve post-market data on drug surveillance in order to better understand and predict adverse drug events. The true impact of network analysis will ultimately be measured by how information gathered through systems pharmacology analysis will lead to better drug discovery, safer and more effective drug prescriptions, which will ultimately lead to safe, cost-effective and effective patient care [47].

MATERIALS AND METHODS

1. Finding the bio-actives and targets of Shilajit:

All the available structures of the bio-actives were taken from PubChem and were queried in Binding database or Binding DB for identifying their targets using special tool 'Find my compounds' targets'. Binding DB is a database of experimental protein-small molecule interaction data that is publicly available. It searches for the exact or similar compounds in the database and retrieves the target information of those compounds. The search for similarity gives structurally similar compounds with the degree of similarity to the scores to the structure being queried, where 1 is the highest possible value. A score of 1 shows either that the exact compound being queried is present or that it gives a structural similarity of 100% to another compound in the database [48].

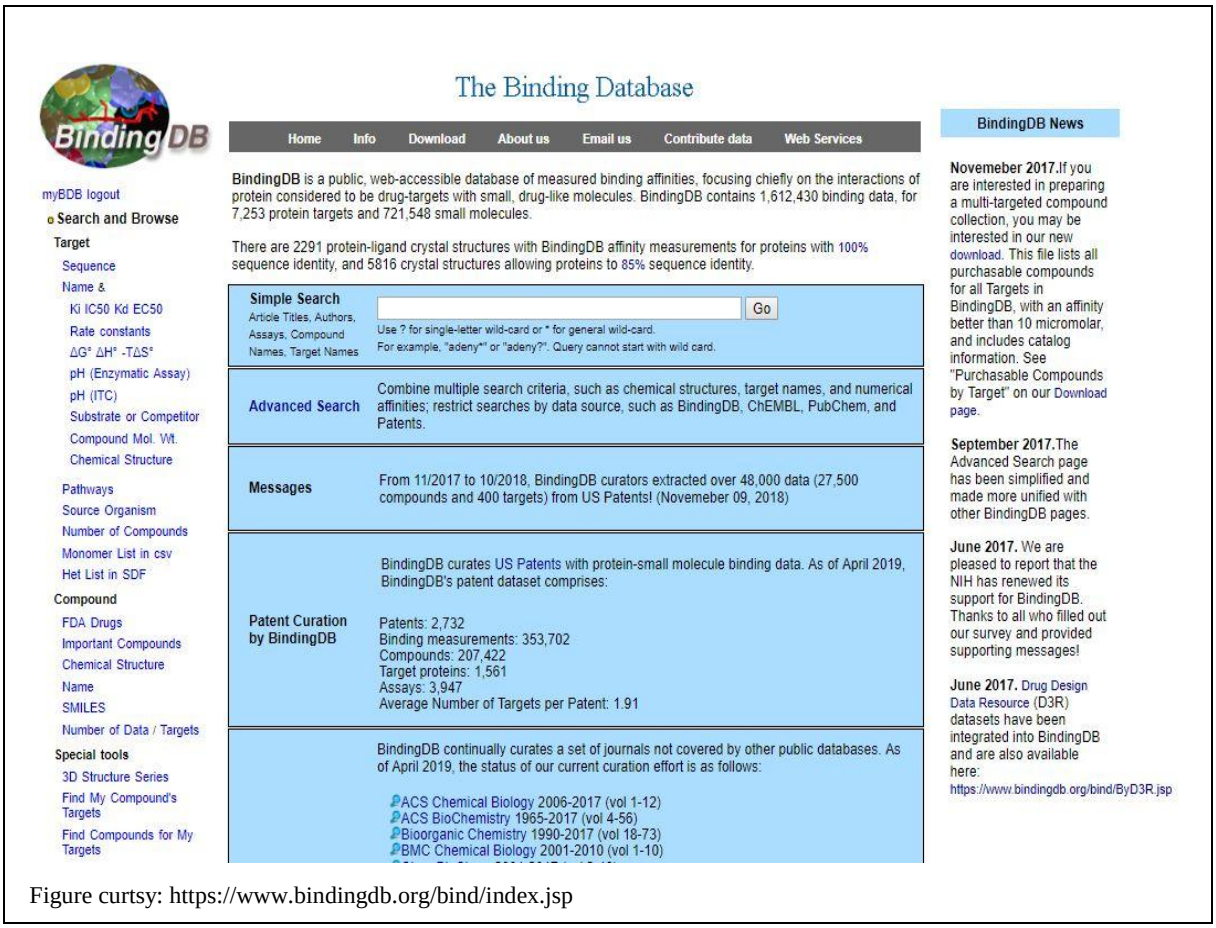


Figure curtsy: https://www.bindingdb.org/bind/index.jsp

Figure 6: Binding Database

Figure courtesy: Binding Database

Figure 7: Find my compounds' targets tool

Either the bioactive structure or canonical SMILES are taken from PubChem and queried in this tool. To improve the accuracy of the results, the similarity search is set to 0.70. One can even construct the compound structure in the ChemAxon viewer from scratch.

The bio-actives were also queried in the DrugBank database and STITCH database for identifying their targets.

DrugBank is a comprehensive online database with extensive pharmacological and biological information on drugs, their mechanisms and their targets. It is a unique chemoinformatics and bioinformatics database. DrugBank is widely used to enable drug discovery and target silico drug discovery. [49].

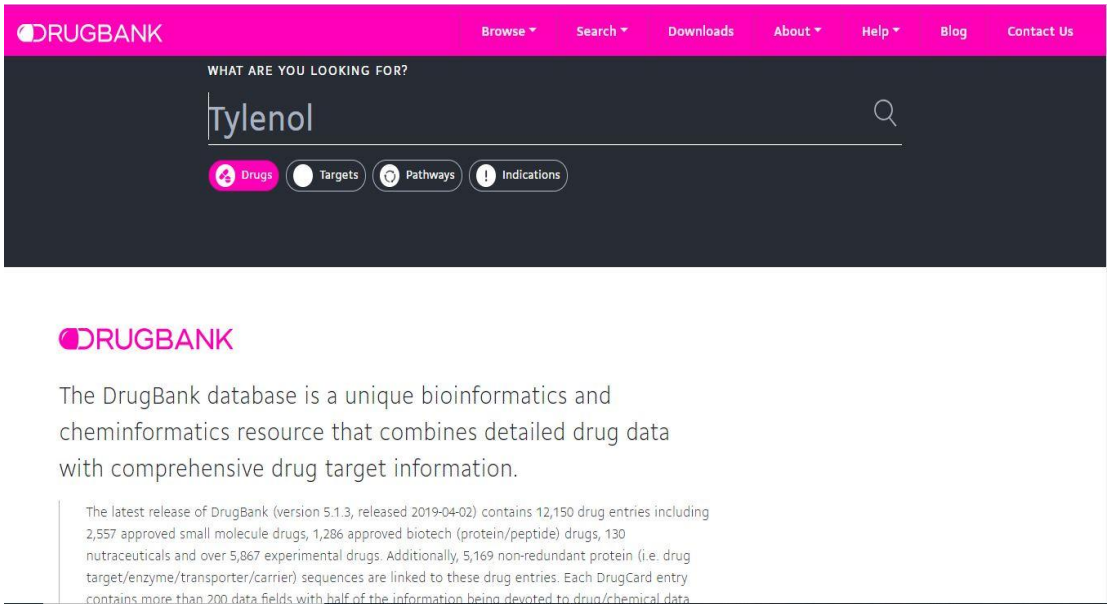


Figure courtesy: https://www.drugbank.ca/

Figure 8: DrugBank Database

In living organisms, interactions between proteins and small molecules are an integral part of biological processes. Many databases, texts and methods of prediction disperse information about these interactions, making it difficult to get a complete overview of the available evidence. To address this, the tool called STITCH ("Search Tool for Chemicals Interacting") was developed [50]. In addition to the increased scope of the database; a new network view has been implemented, which gives the user the ability to view binding chemical affinities within the interaction network. This allows the user to get a quick overview of the potential effects of the chemical on their interaction partners. STITCH provides a global network for each organism.

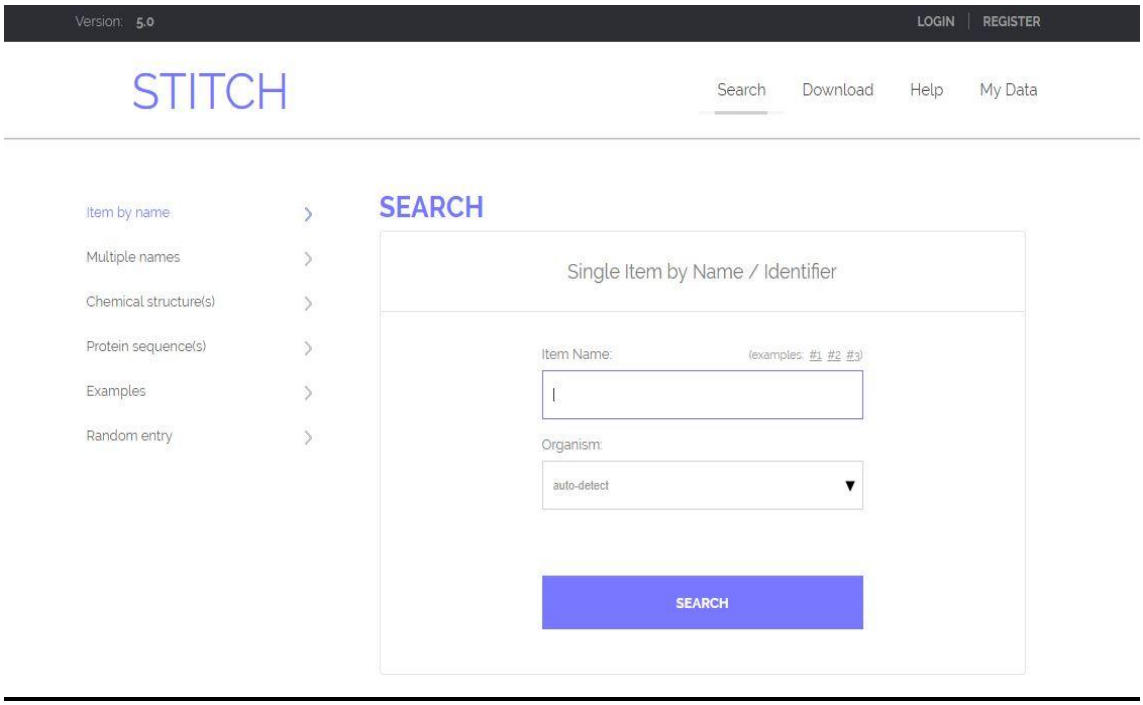


Figure courtesy : http://stitch.embl.de/cgi/input.pl?UserId=yxhiMY9lnwai&sessionId=aI9hi1pAIsuP

Figure 9: STITCH Database

2. Disease Indications:

The targets of the bioactives were searched in the Therapeutic Targets Database (TTD) for their association with any disease or indication. This database provides information on known and explored targets for therapeutic proteins and nucleic acids, targeted diseases, pathways and the corresponding drugs for each of these targets [51].

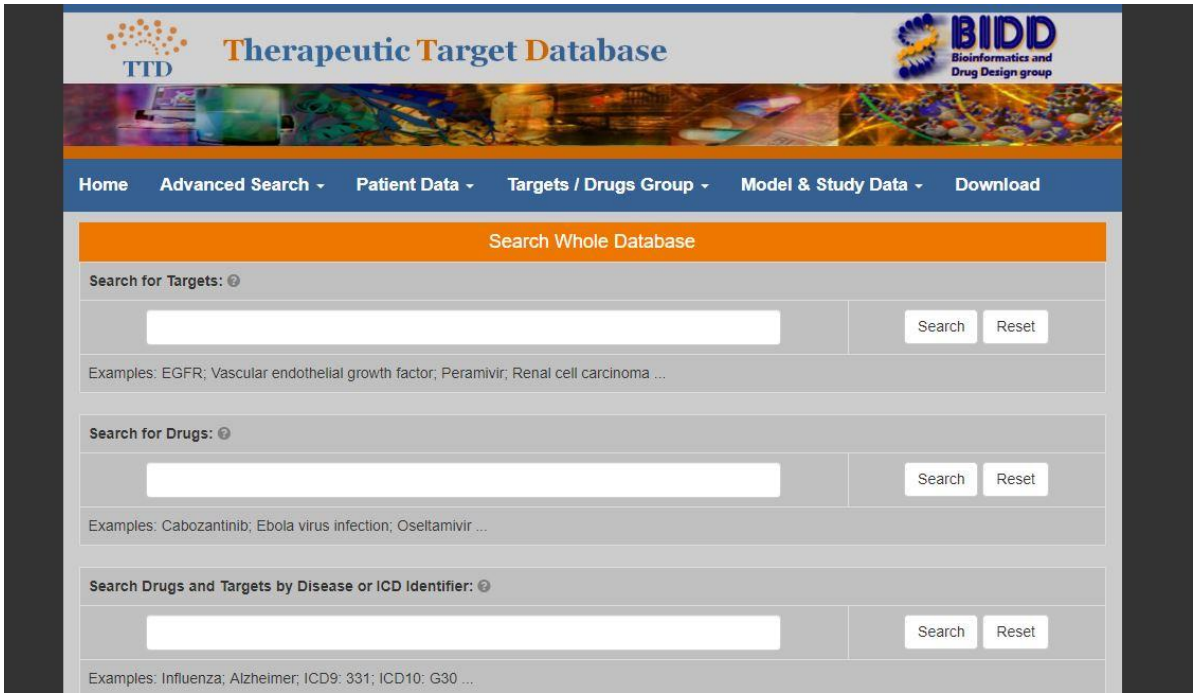


Figure courtesy: https://db.idrblab.org/ttd/

Figure 10: Therapeutic Target Database

3. Network Constructions:

A pharmacology network is made up of nodes, the points of communication or redistribution, and edges, the lines of communication joining the nodes. The entities that form the nodes of the network are the bio-actives of Shilajit networked with their related targets and their relevant diseases. The network is analyzed and visualized using Cytoscape 3.7.1; a java based open source software [52]. The interaction information files were imported into Cytoscape to create the networks.

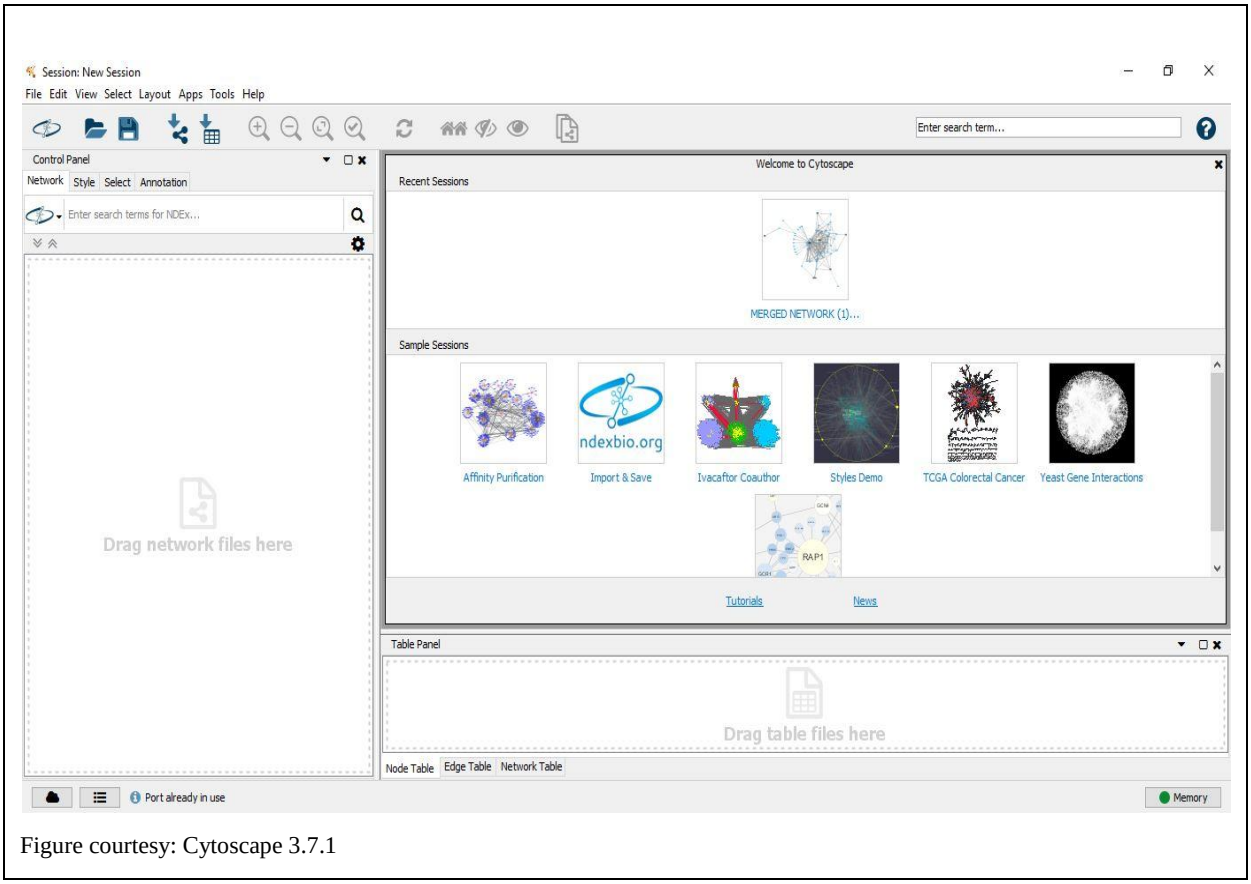


Figure courtesy: Cytoscape 3.7.1

Figure 11: Cytoscape 3.7.1

On importing the networks into Cytoscape, the Style tab for the node section displayed in the control panel for the node section was used to change the properties of the nodes. The following properties were changed- Fill color, Height, Label Color, Label Font Size, Shape, Transparency and Width.

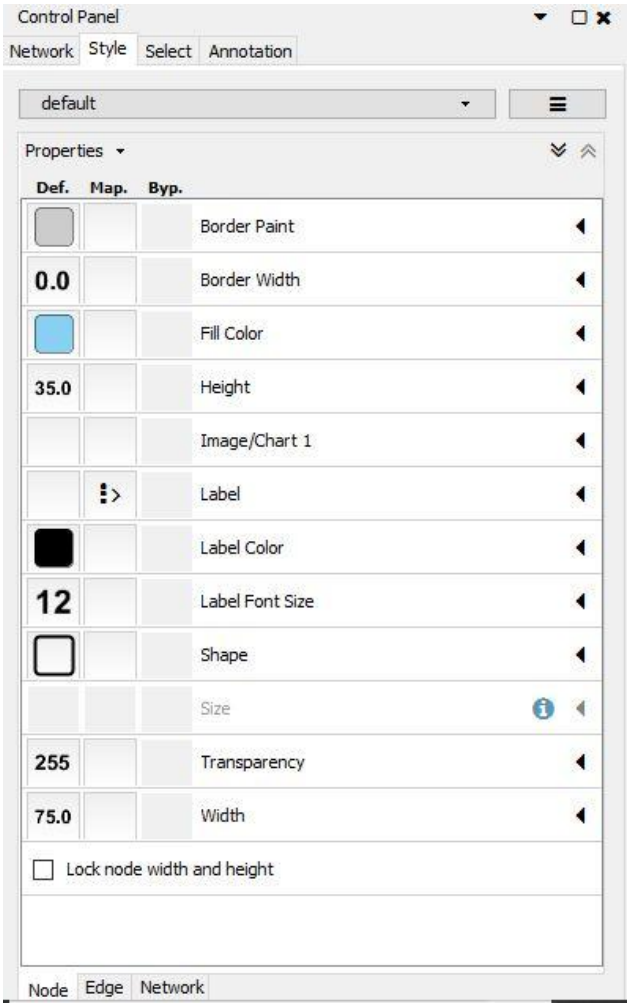


Figure courtesy: Cytoscape 3.7.1

Figure 12: Cytoscape control panel

All the individual imported networks are then merged using the ‘Merge’ option from the Tools drop down menu.

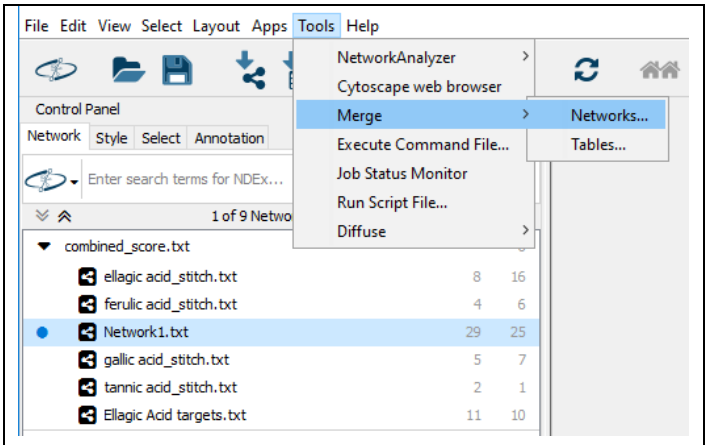


Figure courtesy: Cytoscape 3.7.1

Figure 13: Tools drop down menu

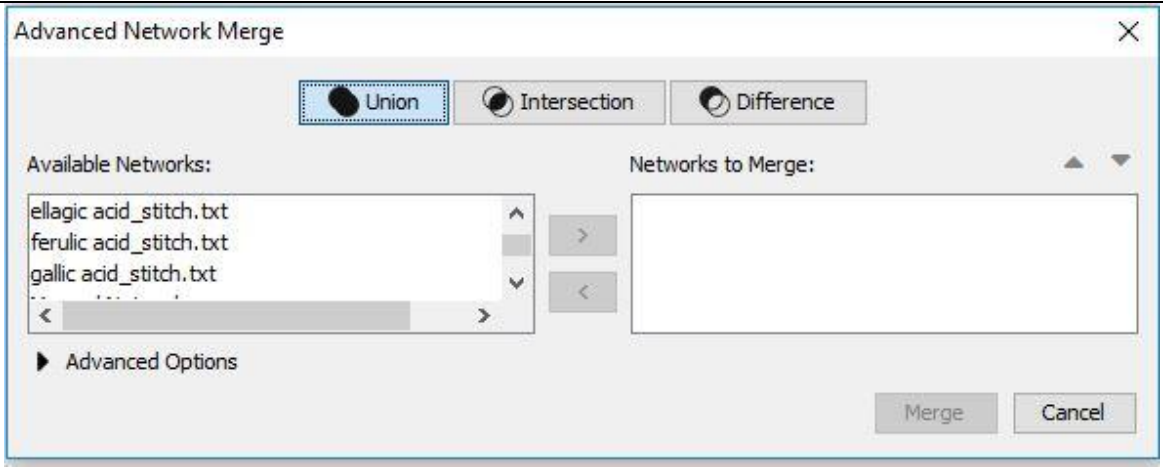


Figure courtesy: Cytoscape 3.7.1

Figure 14: Merge tool

The DisGeNET Cytoscape app is designed to visualize, query and analyze a representation of the gene-disease network and the variant-disease associations included in DisGeNET. The Gene Disease Network tab, displayed in the control panel, is used to identify various GDA networks by selecting different Data Sources, Association Types or Disease Classes from their respective drop-down menus [53]. In order to achieve a Shilajit-cancer network, the following parameters were set- Data Source: CTD_human; Association Types: All; Disease Classes: Neoplasms. The GDA networks may be also filtered using a cut-off value of the DisGeNET score, and/or Evidence Index (EI) and Evidence Level (EL).

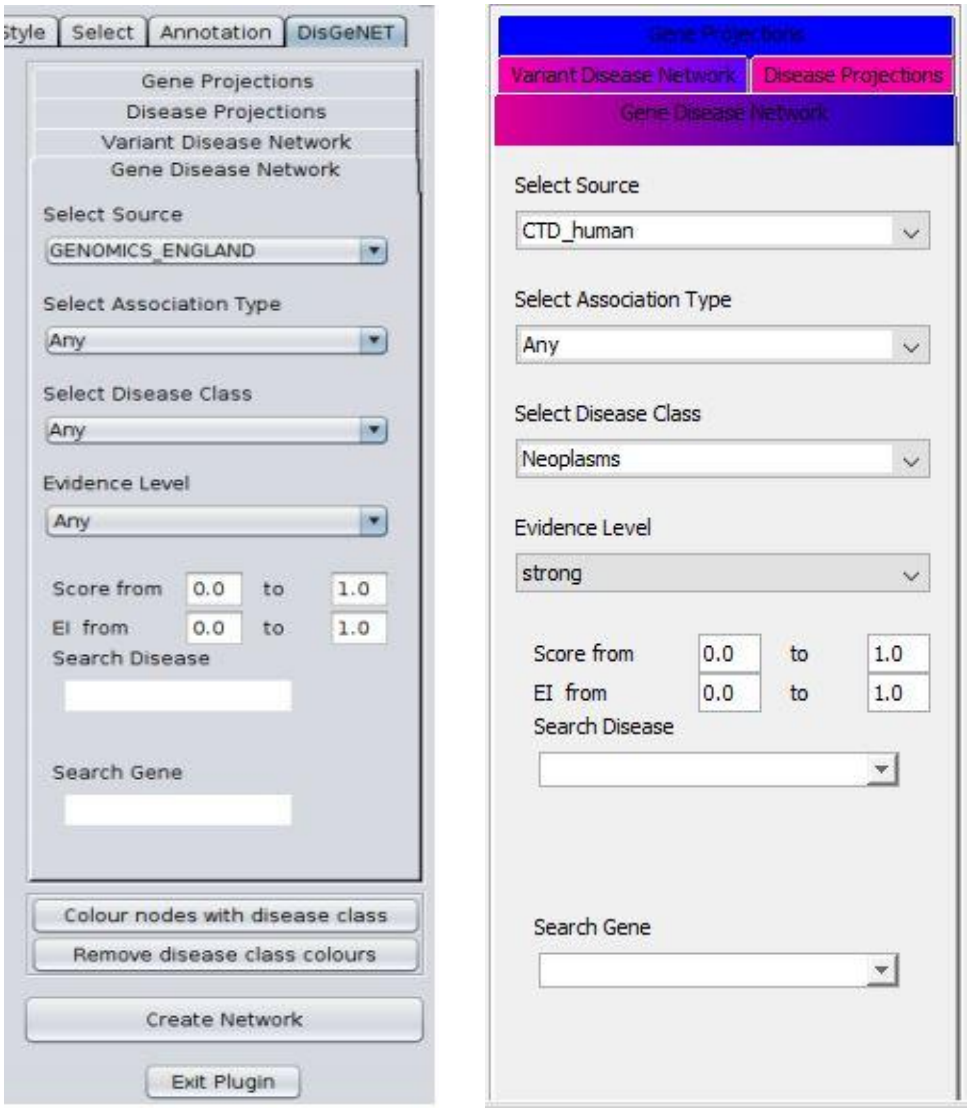


Figure courtesy: DisGeNET application 6.0

Figure 15: The DisGeNET app control panel

4. Validation – By Using Docking Method:

An online web service called SwissDock was used to predict the molecular interactions that may occur between the targets (protein) and the ligands (bioactive) [54]. It also predicts the preferred orientation between the two molecules to create a stable complex. Docking plays an important role in rational drug designing.

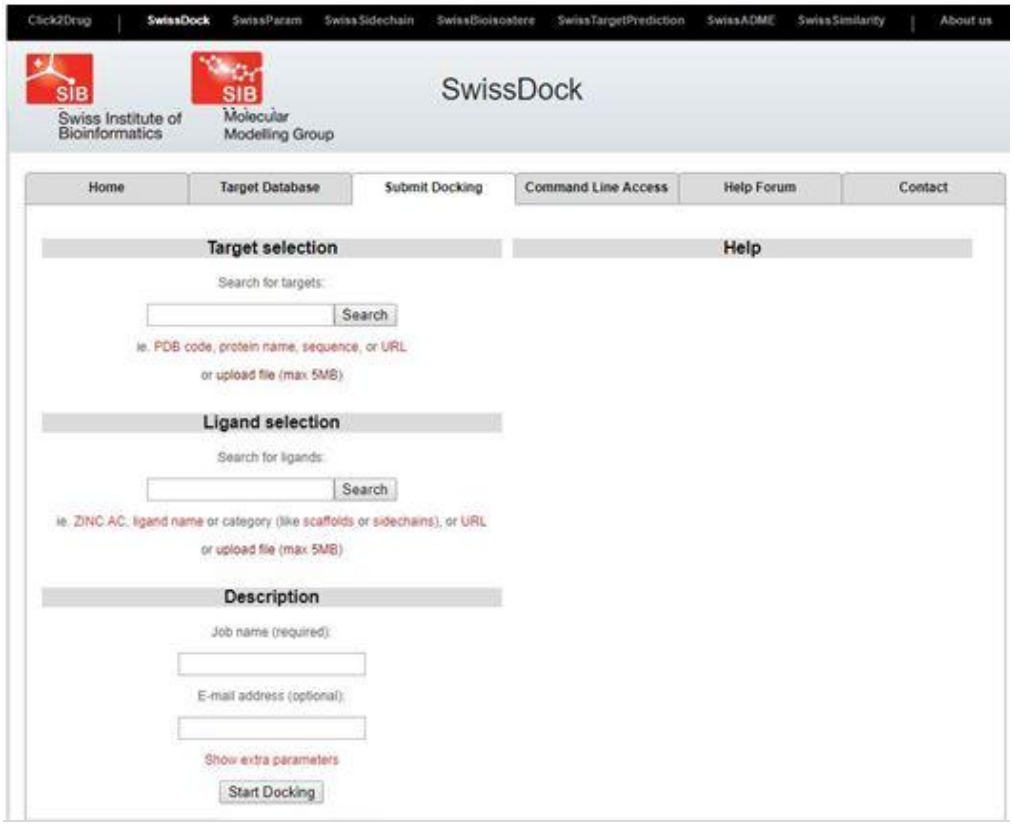


Figure courtesy: http://www.swissdock.ch/docking

Figure 16: SwissDock

5. Validation - By Using Cancer Cell Lines:

The MTT system uses mitochondrial dehydrogenases to measure the activity of living cells. The MTT method is simple, precise and generates reproducible results. The key component is (3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) or MTT [55]. When MTT is dissolved, it is converted from a yellow solution into an insoluble purple formazan by mitochondrial dehydrogenase enzymes of viable cells. The purple solution is spectrophotometrically measured.

RESULT AND DISCUSSION

Targets found through Binding Database:

Using the "Find my compounds' targets" tool in Binding DB, a list of predicted bioactive targets was obtained. Only targets with a search for similarity greater than or equal to 0.70 were selected. This has been done to improve the accuracy of the result.

My Compound's Targets

	Target Name	Uploaded compounds generating hits	Max Similarity	Hits (All Compounds)	Ki Data	IC50 Data	Kd Data	EC50 Data	Add to myBDB	Download
1	17-beta-hydroxysteroid dehydrogenase type 3	1	0.70	8	0	8	0	0	☐	2D 3D TSV
2	Aldose reductase	1	0.70	1	0	1	0	0	☐	2D 3D TSV
3	Aurora kinase B	1	0.70	1	0	1	0	0	☐	2D 3D TSV
4	Cannabinoid receptor	1	0.72	3	8	0	0	1	☐	2D 3D TSV
5	Cannabinoid receptor 1	1	0.72	1	1	0	0	0	☐	2D 3D TSV
6	Cannabinoid receptor 2	1	0.72	2	2	0	0	1	☐	2D 3D TSV
7	Carbonic anhydrase 9	1	0.79	3	4	0	0	0	☐	2D 3D TSV
8	DNA Gyrase	1	0.70	3	0	3	0	0	☐	2D 3D TSV
9	dTDP-4-dehydrorhamnose 3,5-epimerase RmlC	1	0.79	1	0	1	0	0	☐	2D 3D TSV
10	Estradiol receptor beta (ERβ)	1	0.70	22	0	23	0	1	☐	2D 3D TSV
11	Estrogen receptor	1	0.80	10	0	10	0	0	☐	2D 3D TSV
12	large T antigen	1	0.70	1	0	1	0	0	☐	2D 3D TSV
13	Monoamine oxidase	1	0.70	1	0	1	0	0	☐	2D 3D TSV
14	Monoamine Oxidase Type B (MAO-B)	1	0.70	1	0	1	0	0	☐	2D 3D TSV
15	Serine/threonine-protein kinase PIM	1	0.70	2	0	2	0	0	☐	2D 3D TSV
16	Vascular endothelial growth factor receptor 2	1	0.70	1	0	1	0	0	☐	2D 3D TSV

Figure courtesy: Binding DB

Figure 17: Targets of Dibenzoalpha-pyrone

My Compound's Targets

	Target Name	Uploaded compounds generating hits	Max Similarity	Hits (All Compounds)	Ki Data	IC50 Data	Kd Data	EC50 Data	Add to myBDB	Download
1	Carbonic anhydrase 9	1	0.74	1	1	0	0	0	☐	2D 3D TSV
2	Carbonic Anhydrase XIV	1	0.74	1	1	0	0	0	☐	2D 3D TSV
3	DNA Gyrase	1	0.70	1	0	1	0	0	☐	2D 3D TSV
4	Zn finger protein	1	0.74	1	1	0	0	0	☐	2D 3D TSV

Figure courtesy: Binding DB

Figure 18: Targets of 6-Amino-3, 4-benzocoumarin

My Compound's Targets

Target Name	Uploaded compounds generating hits	Max Similarity	Hits (All Compounds)	Ki Data	IC50 Data	Kd Data	EC50 Data	Add to myBDB	Download
Histone deacetylase	1	0.83	1	0	1	0	0	☐	2D 3D TSV
Transcriptional regulator MvfR	1	0.72	2	0	0	2	0	☐	2D 3D TSV

Figure courtesy: Binding DB

Figure 19: Targets of Benzamide

My Compound's Targets

	Target Name	Uploaded compounds generating hits	Max Similarity	Hits (All Compounds)	Ki Data	IC50 Data	Kd Data	EC50 Data	Add to myBDB	Download
1	Carbonic anhydrase	1	0.70	1	2	0	0	0	☐	2D 3D TSV
2	Carbonic anhydrase 12	1	0.70	1	1	0	0	0	☐	2D 3D TSV
3	Carbonic anhydrase 2	1	0.70	1	2	0	0	0	☐	2D 3D TSV
4	Carbonic anhydrase 9	1	0.70	1	1	0	0	0	☐	2D 3D TSV

Figure courtesy: Binding DB

Figure 20: Targets of Benzoic acid

My Compound's Targets

	Target Name	Uploaded compounds generating hits	Max Similarity	Hits (All Compounds)	Ki Data	IC50 Data	Kd Data	EC50 Data	Add to myBDB	Download
1	Aldose reductase (AR)	1	1.00	1	0	1	0	0		2D 3D TSV
2	cAMP-Dependent Protein Kinase (PKA)	1	1.00	1	0	1	0	0		2D 3D TSV
3	Casein Kinase II	1	1.00	1	0	1	0	0		2D 3D TSV
4	DNA Gyrase	1	1.00	4	0	5	0	0		2D 3D TSV
5	DNA polymerase eta	1	1.00	1	0	1	0	0		2D 3D TSV
6	DNA polymerase iota	1	1.00	1	0	1	0	0		2D 3D TSV
7	Dual specificity protein phosphatase (VHR)	1	1.00	1	0	1	0	0		2D 3D TSV
8	Epidermal growth factor receptor	1	1.00	1	0	1	0	0		2D 3D TSV
9	G-protein coupled receptor 35	1	1.00	1	0	1	0	1		2D 3D TSV
10	Heat Shock 70kDa Protein 1	1	1.00	1	0	1	0	0		2D 3D TSV
11	Hepatocyte growth factor receptor	1	1.00	1	0	1	0	0		2D 3D TSV
12	Insulin receptor	1	1.00	1	0	1	0	0		2D 3D TSV
13	Insulin-like growth factor I receptor	1	1.00	1	0	1	0	0		2D 3D TSV
14	likely tRNA 2'-phosphotransferase	1	1.00	1	0	1	0	0		2D 3D TSV
15	mothers against decapentaplegic homolog 3	1	1.00	1	0	1	0	0		2D 3D TSV
16	NUAK family SNF1-like kinase 1	1	1.00	1	0	1	0	0		2D 3D TSV
17	Proto-oncogene tyrosine-protein kinase Src	1	1.00	1	0	2	0	0		2D 3D TSV
18	Solute carrier family 22 member 6	1	1.00	1	0	1	0	0		2D 3D TSV
19	Vascular endothelial growth factor receptor 2	1	1.00	1	0	1	0	0		2D 3D TSV
20	Vascular endothelial growth factor receptor 2 and tyrosine-protein kinase TIE-2 (KDR and TIE2)	1	1.00	1	0	1	0	0		2D 3D TSV

Figure courtesy: Binding DB

Figure 21: Targets of Ellagic acid

My Compound's Targets

	Target Name	Uploaded compounds generating hits	Max Similarity	Hits (All Compounds)	Ki Data	IC50 Data	Kd Data	EC50 Data	Add to myBDB	Download
1	β-Carbonic anhydrase 2 (CA 2)	1	0.88	1	1	0	0	0		2D 3D TSV
2	β-Carbonic anhydrase 3 (CA 3)	1	1.00	2	2	0	0	0		2D 3D TSV
3	Collagenase	1	0.88	1	0	3	0	0		2D 3D TSV
4	Epidermal growth factor receptor	1	0.88	1	0	1	0	0		2D 3D TSV
5	Matrix metalloproteinase-1 (MMP1)	1	0.88	1	0	1	0	0		2D 3D TSV

Figure courtesy: Binding DB

Figure 22: Targets of Ferulic acid

My Compound's Targets

	Target Name	Uploaded compounds generating hits	Max Similarity	Hits (All Compounds)	Ki Data	IC50 Data	Kd Data	EC50 Data	Add to myBDB	Download
1	Alpha-(1,3)-fucosyltransferase VII	1	1.00	1	0	1	0	0	☐	2D 3D TSV
2	Carbonic anhydrase	1	0.86	1	1	0	0	0	☐	2D 3D TSV
3	Carbonic anhydrase 2	1	0.85	3	3	0	0	0	☐	2D 3D TSV
4	Carbonic Anhydrase XIV	1	0.96	2	2	0	0	0	☐	2D 3D TSV
5	M18 aspartyl aminopeptidase	1	0.96	1	0	1	0	0	☐	2D 3D TSV

Figure courtesy: Binding DB

Figure 23: Targets of Gallic acid

My Compound's Targets

	Target Name	Uploaded compounds generating hits	Max Similarity	Hits (All Compounds)	Ki Data	IC50 Data	Kd Data	EC50 Data	Add to myBDB	Download
1	Plasminogen activator inhibitor-1	1	1.00	1	0	1	0	0	☐	2D 3D TSV
2	Pyruvate dehydrogenase (PDH)	1	1.00	1	0	1	0	0	☐	2D 3D TSV
3	Tissue-type plasminogen activator	1	1.00	1	0	1	0	0	☐	2D 3D TSV
4	Urokinase-type plasminogen activator	1	1.00	1	0	1	0	0	☐	2D 3D TSV
5	Urokinase-type plasminogen activator (uPA)	1	1.00	1	0	1	0	0	☐	2D 3D TSV

Figure courtesy: Binding DB

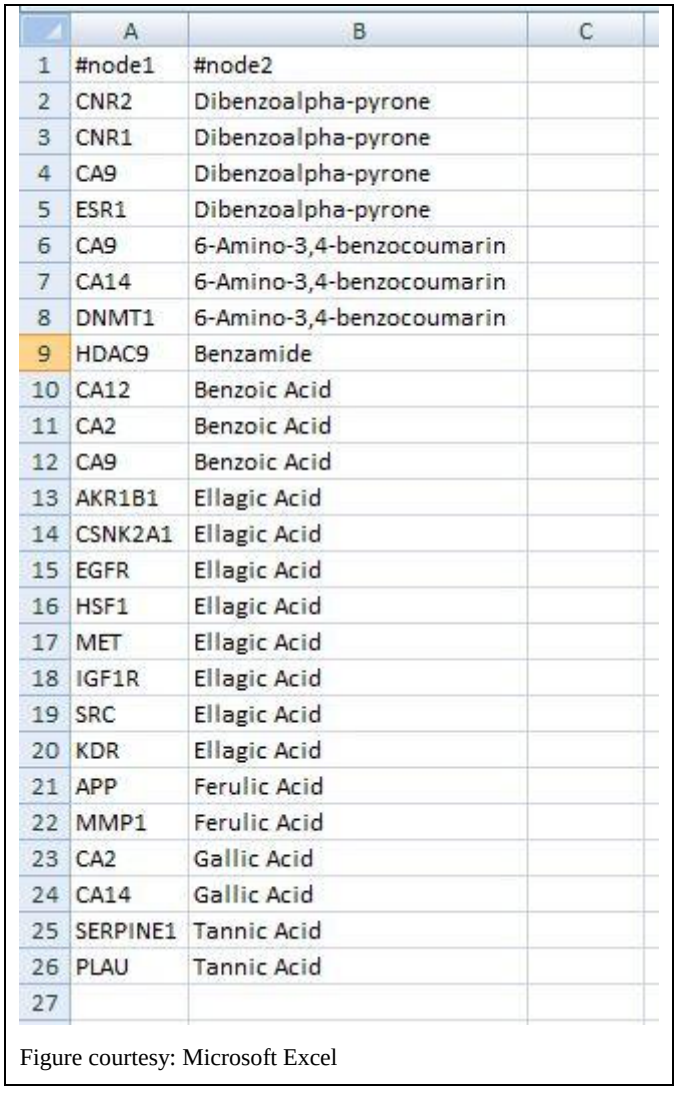
Figure 24: Targets of Tannic acid

Due to their structural complexity and sparse literature information, the targets of some of the bioactives such as humic acids, fulvic acids, etc. were not found.

Network Interaction Tables:

Once the targets of each bioactive were found, the targets were searched in the TTD to check for associations with any cancer-related disease. If there was no target entry or no target association with cancer, the target was ignored.

A list of all the cancer-related targets from Binding DB was created. An Excel sheet was used to create the predicted interaction network between targets and bioactives.



	A	B	C
1	#node1	#node2	
2	CNR2	Dibenzoalpha-pyrone	
3	CNR1	Dibenzoalpha-pyrone	
4	CA9	Dibenzoalpha-pyrone	
5	ESR1	Dibenzoalpha-pyrone	
6	CA9	6-Amino-3,4-benzocoumarin	
7	CA14	6-Amino-3,4-benzocoumarin	
8	DNMT1	6-Amino-3,4-benzocoumarin	
9	HDAC9	Benzamide	
10	CA12	Benzoic Acid	
11	CA2	Benzoic Acid	
12	CA9	Benzoic Acid	
13	AKR1B1	Ellagic Acid	
14	CSNK2A1	Ellagic Acid	
15	EGFR	Ellagic Acid	
16	HSF1	Ellagic Acid	
17	MET	Ellagic Acid	
18	IGF1R	Ellagic Acid	
19	SRC	Ellagic Acid	
20	KDR	Ellagic Acid	
21	APP	Ferulic Acid	
22	MMP1	Ferulic Acid	
23	CA2	Gallic Acid	
24	CA14	Gallic Acid	
25	SERPINE1	Tannic Acid	
26	PLAU	Tannic Acid	
27			

Figure courtesy: Microsoft Excel

Figure 25: Targets Found From Binding Database Network

On obtaining the predicted interaction network form STITCH and DrugBank, the interactions were downloaded in a tabular form. The individual networks were manually created using excel sheets. Tabular columns were made which contained the predicted targets of each bioactive. The targets, and their respective interactions, that had no connection to cancer were deleted from the table.

#node1	node2	node1_string_id	node2_string_id	combined_score
VEGF	CASP3	318507	317343	0.899
CASP3	PTGS2	317343	306292	0.768
TP53	ELLAGIC ACID	311901	-5281855	0.846
CASP3	ELLAGIC ACID	317343	-5281855	0.895
VEGF	ELLAGIC ACID	318507	-5281855	0.844
VEGF	TP53	318507	311901	0.916
VEGF	PTGS2	318507	306292	0.917
TP53	PTGS2	311901	306292	0.985
PRTN3	ELLAGIC ACID	307757	-5281855	0.817
NOS2A	CASP3	320175	317343	0.9
NOS2A	ELLAGIC ACID	320175	-5281855	0.888
CASP3	TP53	317343	311901	0.907
NOS2A	PTGS2	320175	306292	0.917
NOS2A	VEGF	320175	318507	0.912
PTGS2	ELLAGIC ACID	306292	-5281855	0.888
F3	ELLAGIC ACID	321929	-5281855	0.896

Figure courtesy: Microsoft Excel

Figure 26: Ellagic Acid (STITCH)

#node1	#node2
CA1	ELLAGIC ACID
CA2	ELLAGIC ACID
CA4	ELLAGIC ACID
CA6	ELLAGIC ACID
CA9	ELLAGIC ACID
CA12	ELLAGIC ACID
CA14	ELLAGIC ACID
CSNK2A1	ELLAGIC ACID
PRKCB	ELLAGIC ACID
SYK	ELLAGIC ACID

Figure courtesy : Microsoft Excel

Figure 27: Ellagic Acid (DrugBank)

#node1	node2	node1_string_id	node2_string_id	combined_score
PTGS2	FERULIC ACID	306292	-709	0.835
MAPK8	FERULIC ACID	327339	-709	0.8
MAPK8	PTGS2	327339	306292	0.78
MAPK8	MAPK3	327339	310698	0.997
MAPK3	FERULIC ACID	310698	-709	0.818
MAPK3	PTGS2	310698	306292	0.902

Figure courtesy: Microsoft Excel

Figure 28: Ferulic Acid (STITCH)

#node1	node2	node1_string_id	node2_string_id	combined_score
MAPK14	TNF	307530	307513	0.986
CASP3	MAPK14	317343	307530	0.929
CASP3	TNF	317343	307513	0.916
BACE1	GALLIC ACID	319144	-370	0.814
MAPK14	GALLIC ACID	307530	-370	0.661
CASP3	GALLIC ACID	317343	-370	0.889
TNF	GALLIC ACID	307513	-370	0.861

Figure courtesy: Microsoft Excel

Figure 29: Gallic Acid (STITCH)

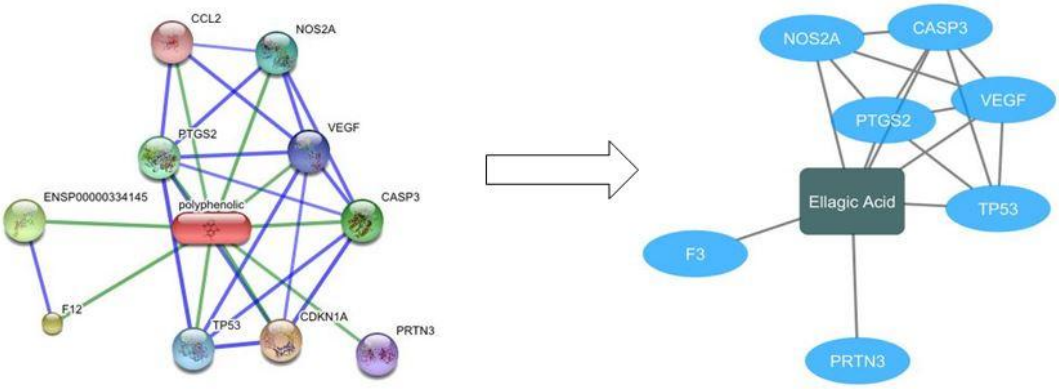
#node1	node2	node1_string_id	node2_string_id	combined_score
PLAU	TANNIC ACID	308226	-250395	0.479

Figure courtesy: Microsoft Excel

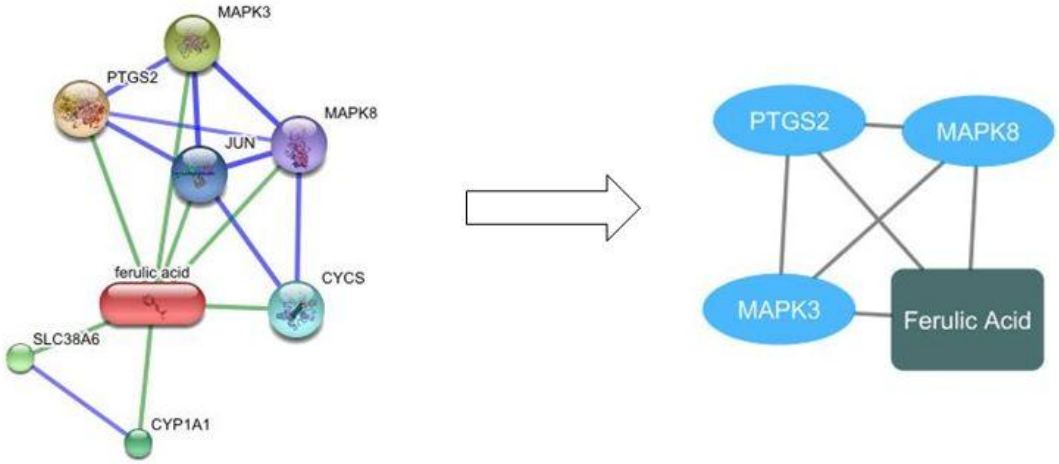
Figure 30: Tannic Acid (STITCH)

Networks Created Using Targets from STITCH:

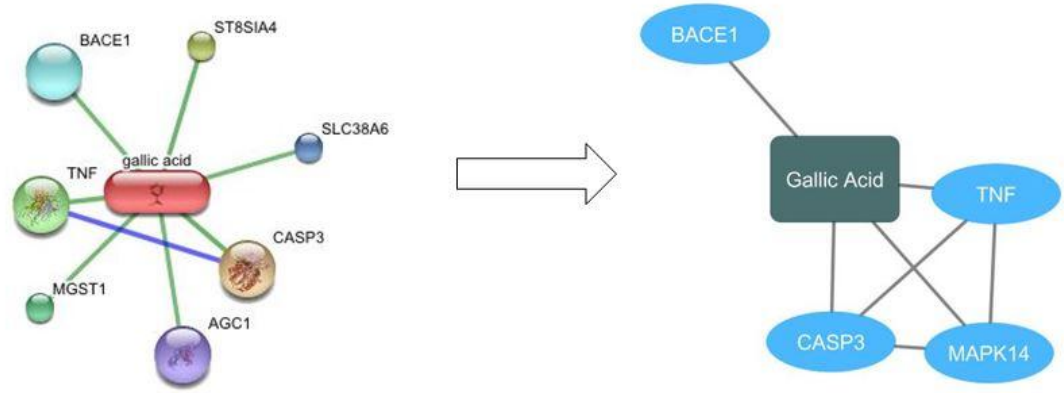
The edited interaction networks were imported into Cytoscape 3.7.1.



Ellagic Acid Target Network



Ferulic Acid Target Network



Gallic Acid Target Network

Figure Courtesy: Cytoscape 3.7.1

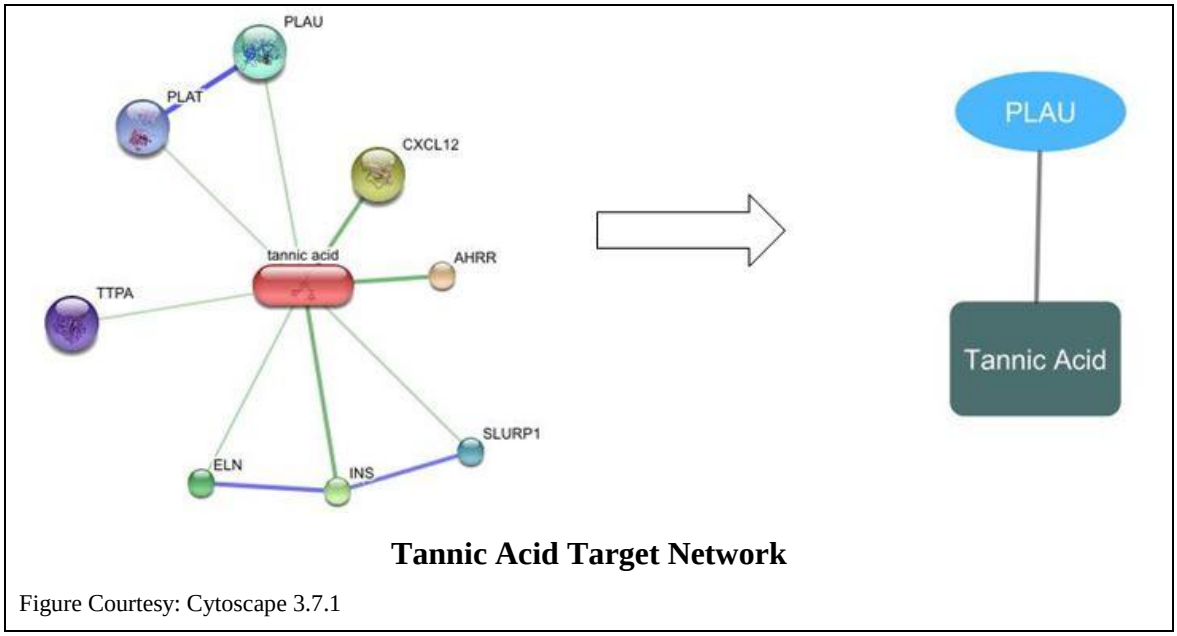


Figure 31: The edited bioactive interaction networks

Network Created Using Targets From DrugBank:

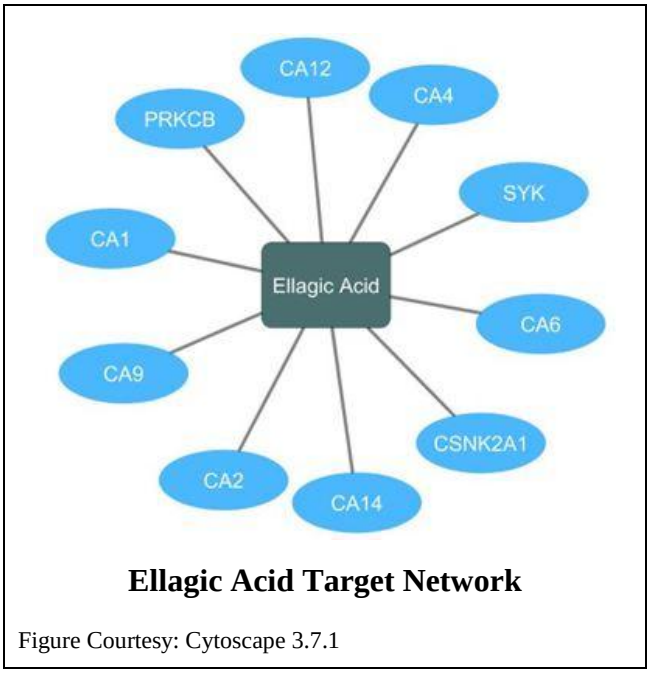


Figure 32: The edited ellagic acid network which was imported into Cytoscape

Network Created Using Targets From Binding DB:

The manually created interaction network, that included all the targets collected from binding DB, was imported into Cytoscape 3.7.1.

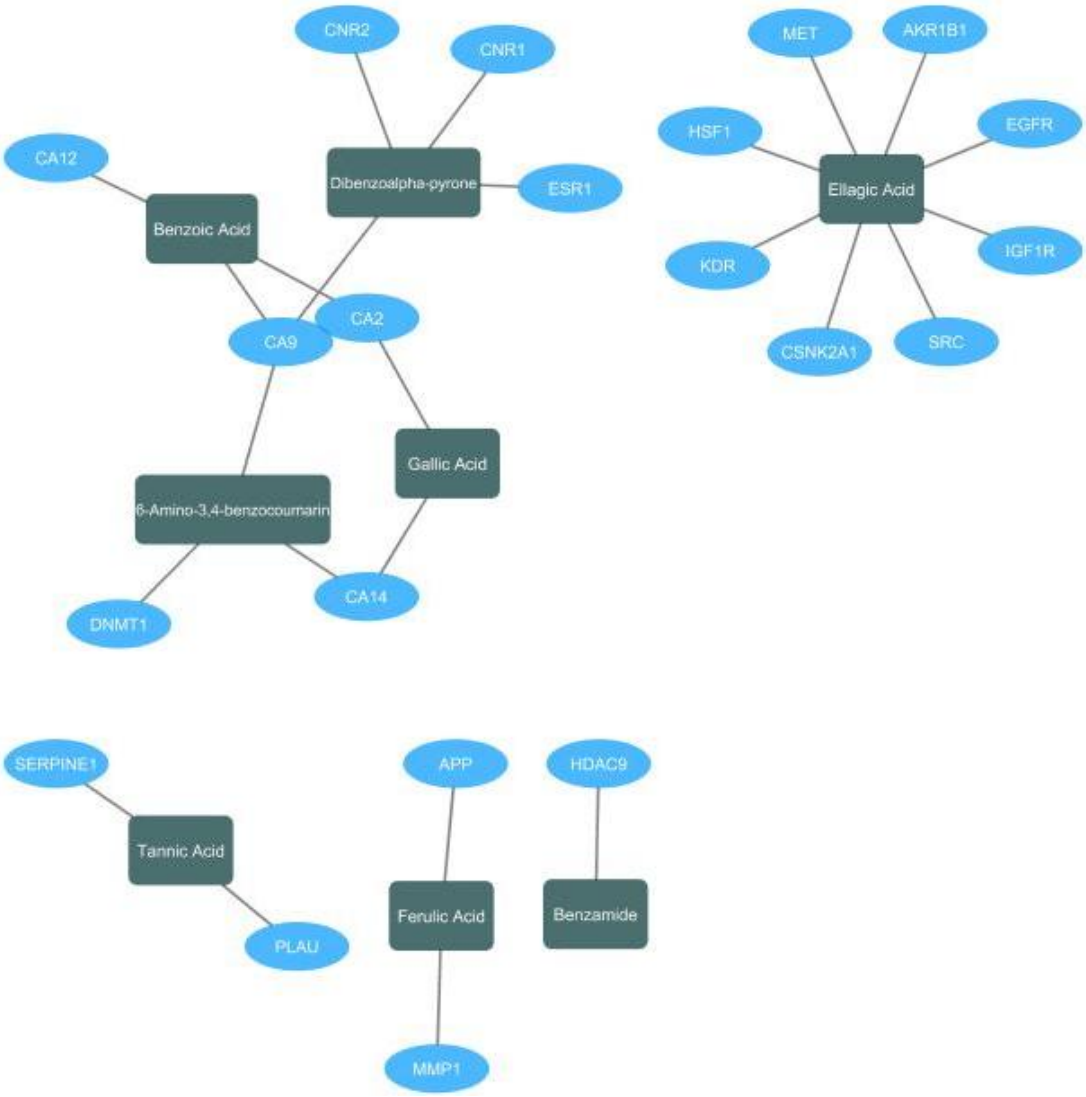


Figure Courtesy: Cytoscape 3.7.1

Figure 33: Interaction network of all the Binding DB targets

Shilajit Bioactive-Target Network:

On merging all the individual networks, the network looks like the following:

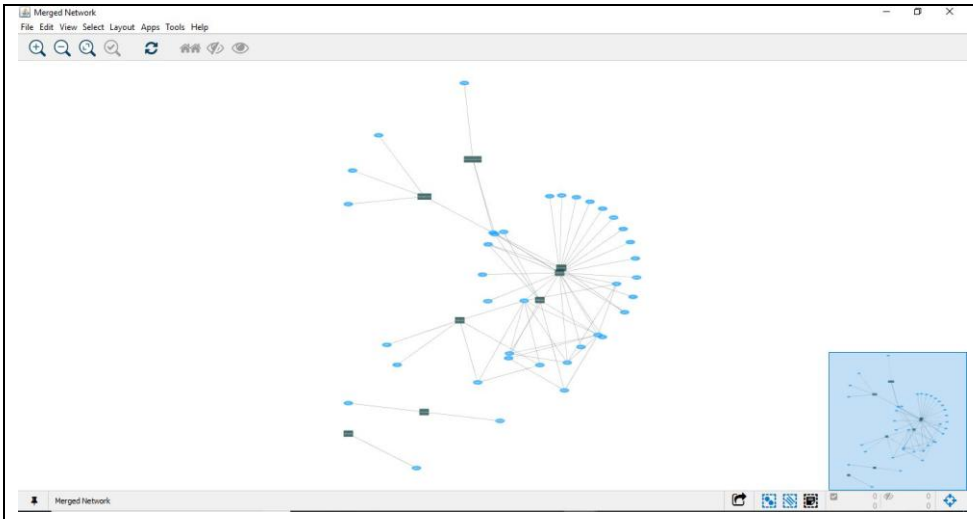


Figure Courtesy: Cytoscape 3.7.1

Figure 34: Merged Network

Shilajit-cancer Network:

In order to achieve a Shilajit-cancer network with the presence of all the associated neoplasm disease nodes, the Cytoscape plug in, DisGeNET, is used.

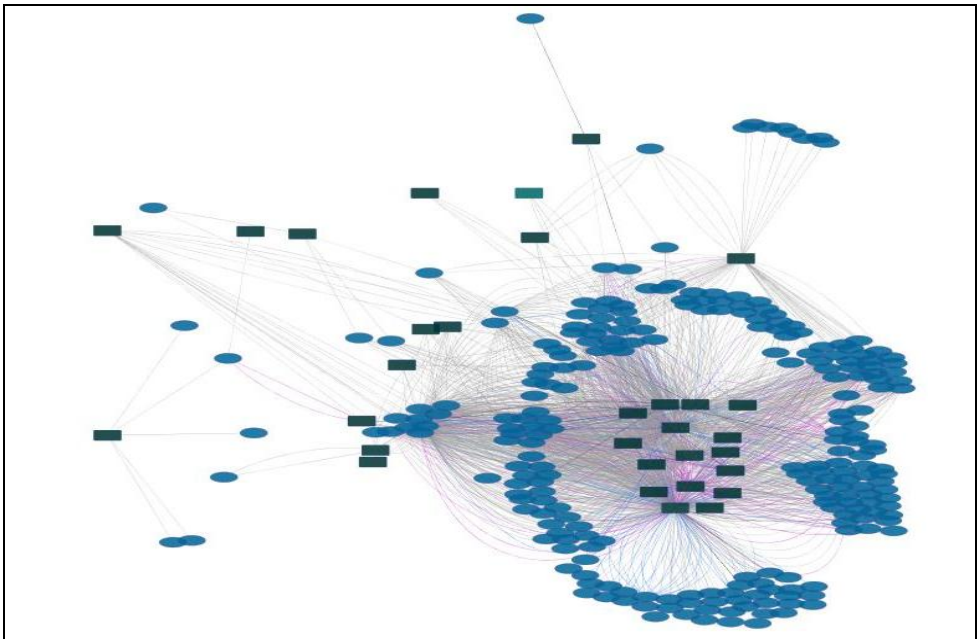


Figure Courtesy: Cytoscape 3.7.1

Figure 35: Shilajit-cancer Network

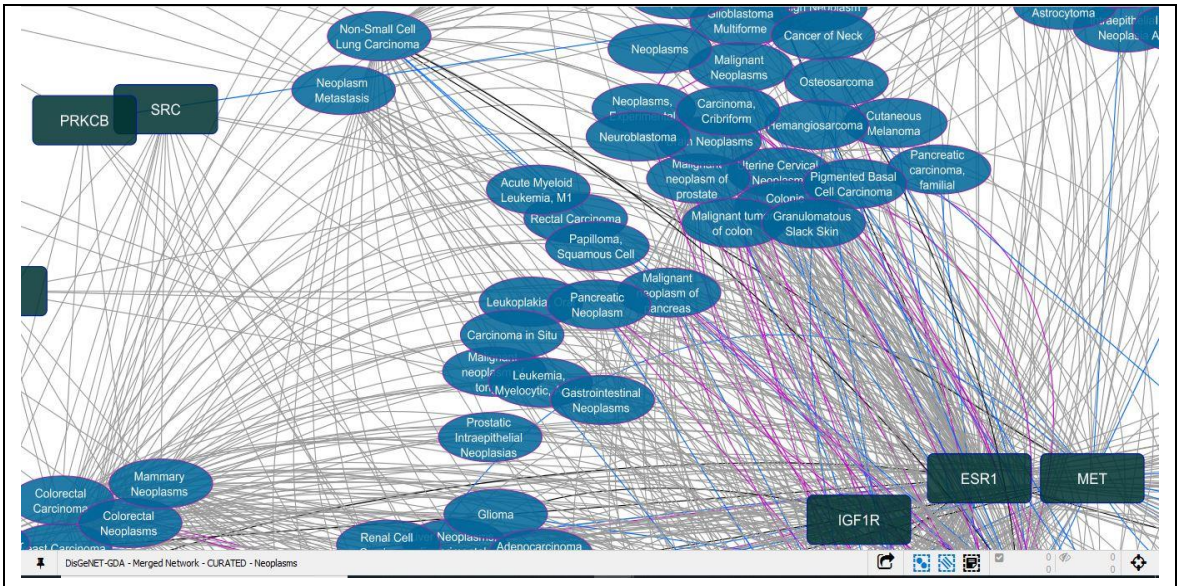


Figure Courtesy: Cytoscape 3.7.1

Figure 36: Zoomed in network

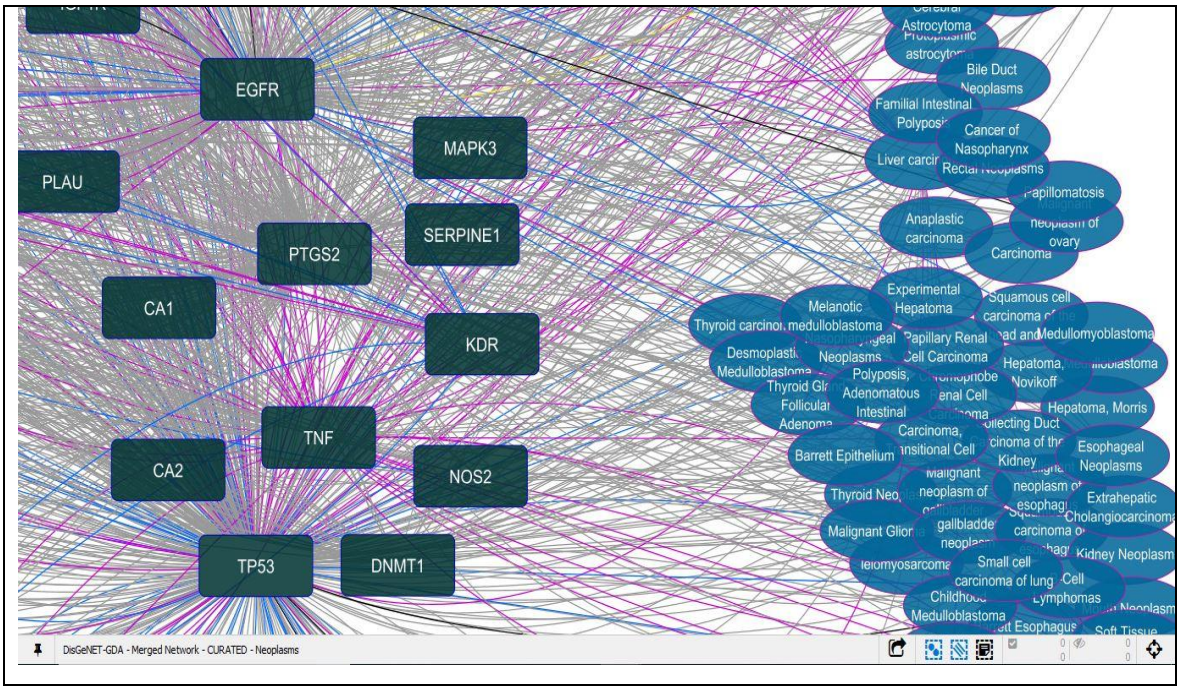


Figure 37: Zoomed in network (2)

On zooming into the Shilajit-cancer network, one can notice all the different diseases that the targets are associated to. For each of the targets, this information has been collected from various different literature sources. In figures 36 and 37, the various diseases associated to the targets SRC, PRKCB, EGFR, CA2, TP53, DNMT1, NOS2, TNF, MAPK3, PTGS2, etc. are shown.

styleName	nodeType	geneName	geneId	diseaseName	diseaseId	diseaseClassName	diseaseClass
HSF1	gene	HSF1	3297			[]	[]
F3	gene	F3	2152			[]	[]
ESR1	gene	ESR1	2099			[]	[]
EGFR	gene	EGFR	1956			[]	[]
DNMT1	gene	DNMT1	1786			[]	[]
CSNK2A1	gene	CSNK2A1	1457			[]	[]
CNR2	gene	CNR2	1269			[]	[]
CNR1	gene	CNR1	1268			[]	[]
CA9	gene	CA9	768			[]	[]
CA2	gene	CA2	760			[]	[]
CA12	gene	CA12	771			[]	[]
CA1	gene	CA1	759			[]	[]
AKR1B1	gene	AKR1B1	231			[]	[]
Mammary Carcinoma, Animal	disease			Mammary Carcinoma,...	C1257925	[Neoplasms, Animal Diseas...	[C04, C22]
Animal Mammary Neoplasms	disease			Animal Mammary Neo...	C0024667	[Neoplasms, Animal Diseas...	[C04, C22]
Li-Fraumeni-Like Syndrome	disease			Li-Fraumeni-Like Syn...	C2675080	[Neoplasms, Congenital, ...	[C04, C16, C18]
Li-Fraumeni Syndrome	disease			Li-Fraumeni Syndrome	C0085390	[Neoplasms, Congenital, ...	[C04, C16, C18]
LI-FRAUMENI SYNDROME 1	disease			LI-FRAUMENI SYNDR...	C1835398	[Neoplasms, Congenital, ...	[C04, C16, C18]
HEMANGIOMA, CAPILLARY INFA...	disease			HEMANGIOMA, CAPI...	C1865871	[Neoplasms, Congenital, ...	[C04, C16]
Polyposis, Adenomatous Intestinal	disease			Polyposis, Adenomat...	C2713442	[Neoplasms, Digestive Sys...	[C04, C06, C16]
Familial Intestinal Polyposis	disease			Familial Intestinal Pol...	C2713443	[Neoplasms, Digestive Sys...	[C04, C06, C16]
Adenomatous Polyposis Coli	disease			Adenomatous Polypo...	C0032580	[Neoplasms, Digestive Sys...	[C04, C06, C16]
Pancreatic carcinoma, familial	disease			Pancreatic carcinoma...	C2931038	[Neoplasms, Digestive Sys...	[C04, C06, C19]
Pancreatic carcinoma	disease			Pancreatic carcinoma	C0235974	[Neoplasms, Digestive Sys...	[C04, C06, C19]
Pancreatic Neoplasm	disease			Pancreatic Neoplasm	C0030297	[Neoplasms, Digestive Sys...	[C04, C06, C19]
Malignant neoplasm of pancreas	disease			Malignant neoplasm o...	C0346647	[Neoplasms, Digestive Sys...	[C04, C06, C19]
Carcinoma, Pancreatic Ductal	disease			Carcinoma, Pancreati...	C0887833	[Neoplasms, Digestive Sys...	[C04, C06, C19]
gallbladder neoplasm	disease			gallbladder neoplasm	C0016978	[Neoplasms, Digestive Sys...	[C04, C06]
Stomach Neoplasms	disease			Stomach Neoplasms	C0038356	[Neoplasms, Digestive Sys...	[C04, C06]
Squamous cell carcinoma of esop...	disease			Squamous cell carcin...	C0279626	[Neoplasms, Digestive Sys...	[C04, C06]
Rectal Neoplasms	disease			Rectal Neoplasms	C0034885	[Neoplasms, Digestive Sys...	[C04, C06]
Rectal Carcinoma	disease			Rectal Carcinoma	C0007113	[Neoplasms, Digestive Sys...	[C04, C06]
Malignant tumor of colon	disease			Malignant tumor of c...	C0007102	[Neoplasms, Digestive Sys...	[C04, C06]
Malignant neoplasm of stomach	disease			Malignant neoplasm o...	C0024623	[Neoplasms, Digestive Sys...	[C04, C06]
Malignant neoplasm of liver	disease			Malignant neoplasm o...	C0345904	[Neoplasms, Digestive Sys...	[C04, C06]
Malignant neoplasm of gallbladder	disease			Malignant neoplasm o...	C0153452	[Neoplasms, Digestive Sys...	[C04, C06]

Node Table | Edge Table | Network Table

Figure 38: Network Nodes Table

In order to see which bioactive had the most associations with neoplasm diseases, each bioactive node was selected and the number of diseases associated to it was counted. This was noted down in the form of a table.

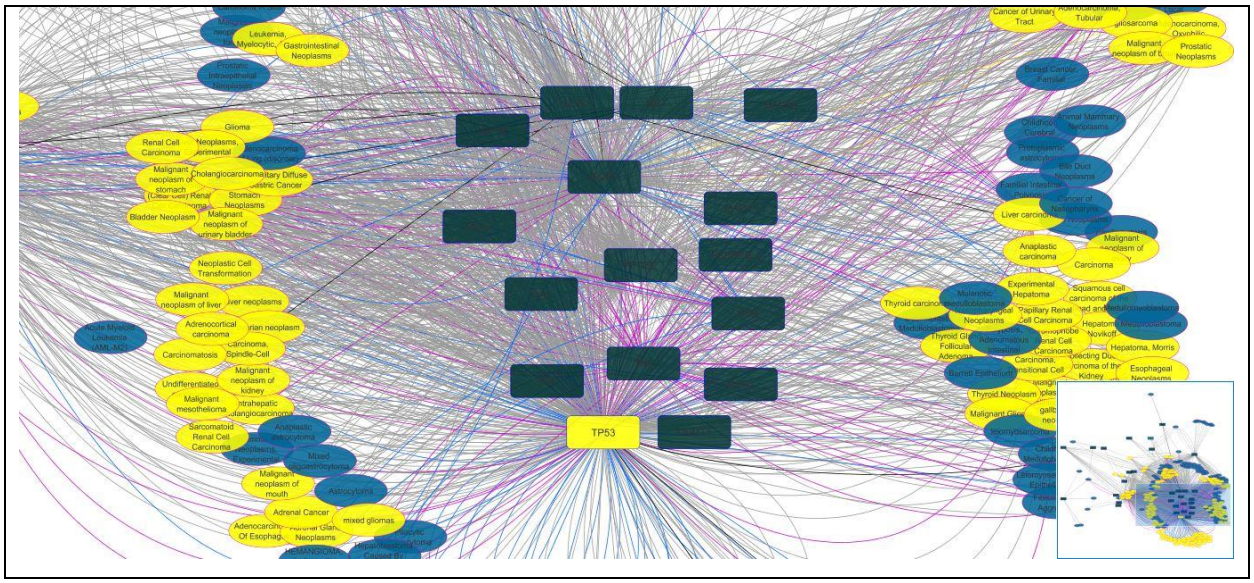


Figure 39: Diseases associated to TP53

geneName	No. of Interactions	Bio-active
TP53	146	Ellagic Acid
PTGS2	90	Ellagic Acid + Ferulic Acid
EGFR	87	Ellagic Acid
MET	67	Ellagic Acid
ESR1	57	Dibenzoalpha-pyrone
VEGFA	51	Ellagic Acid
TNF	47	Gallic Acid
DNMT1	31	6-Amino-3,4-benzocoumarin
KDR	27	Ellagic Acid
MAPK3	25	Ferulic Acid
PLAU	19	Tannic Acid
NOS2	14	Ellagic Acid
SRC	13	Ellagic Acid
MMP1	12	Ferulic Acid
IGF1R	11	Ellagic Acid
CA2	11	Ellagic Acid + Benzoic Acid
CA1	10	Ellagic Acid
CNR2	9	Dibenzoalpha-pyrone
PRKCB	8	Ellagic Acid
MAPK8	8	Ferulic Acid
SERPINE1	7	Tannic Acid
MAPK14	6	Gallic Acid
F3	5	Ellagic Acid

Figure 40: Interactions Table

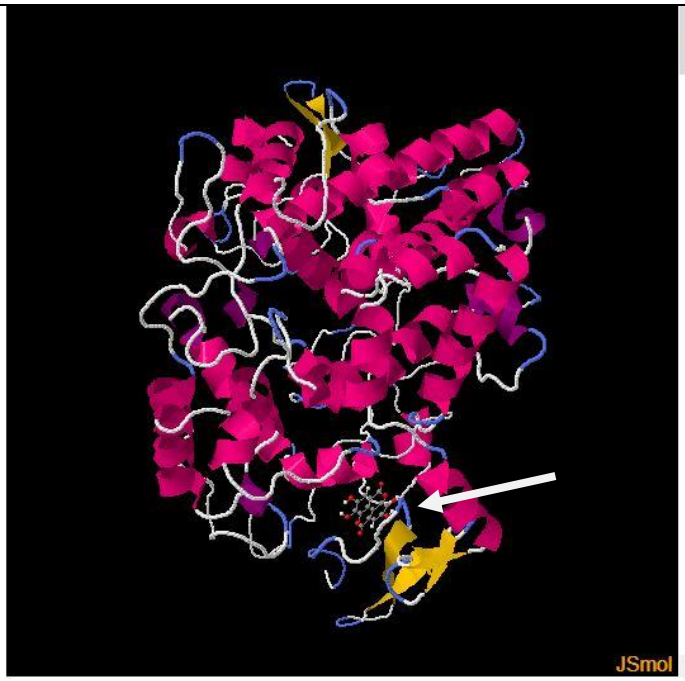
From the table, it was observed that ellagic acid had the most associations. This is because of the targets TP53, PTGS2, EGFR, MET, etc. The sum total of all the interactions of ellagic acid's targets with cancer related diseases is the highest compared to the others.

Validation by Docking:

Based on the data collected, it was observed that ellagic acid had the most associations with cancer diseases due to the targets TP53, PTGS2 and EGFR. Hence, docking is used to predict the target-bioactive complex structure. It is done in two interrelated steps- (1) sampling the conformations of the ligand (bioactive) in the active site of the target, (2) ranking these confirms via a scoring function.

The docking studies of ellagic aid with TP53 were not done due to the sparse literature information present on its 3D structure.

Results of interactions between ellagic acid and PTGS2, obtained through SwissDock are shown below:



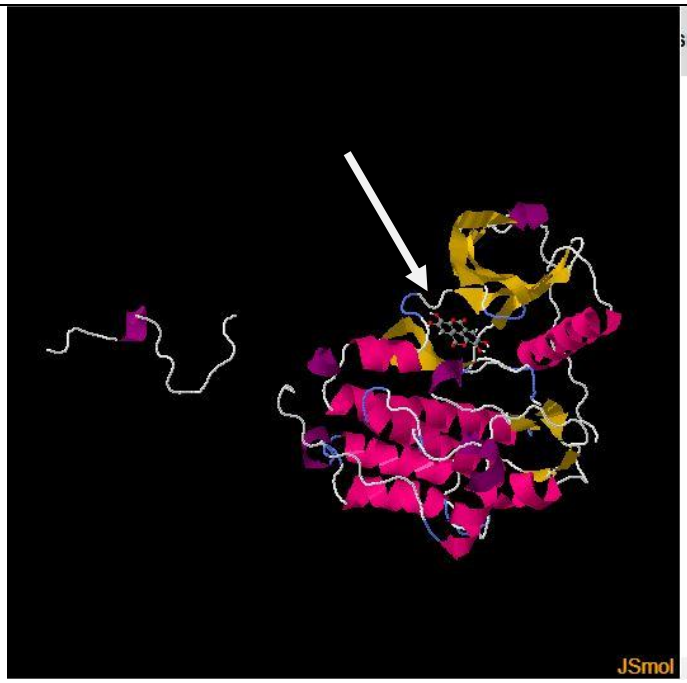
Show	Cluster	Element	FullFitness (kcal/mol)	Estimated ΔG (kcal/mol)
○	2	0	-2295.65	-8.34
◉	2	1	-2295.63	-8.34
○	0	0	-2295.73	-8.11
○	0	1	-2295.73	-8.11
○	0	2	-2295.71	-8.11
○	0	3	-2295.70	-8.11
○	1	0	-2295.68	-8.10
○	1	1	-2295.68	-8.10
○	1	2	-2295.09	-7.99
○	2	4	-2289.93	-7.96
○	0	4	-2289.33	-7.71
○	4	0	-2293.94	-7.71
○	4	1	-2293.94	-7.71
○	4	2	-2293.94	-7.71
○	4	3	-2293.94	-7.71
○	4	4	-2293.94	-7.71
○	4	5	-2293.93	-7.71
○	4	6	-2293.93	-7.71
○	4	7	-2293.93	-7.71
○	0	5	-2289.25	-7.69
○	3	4	-2290.26	-7.69

Figure courtesy: http://www.swissdock.ch/docking/view/swissdockd_lcaMrJ_36TU14PM7QZS8DP3NGT7

Figure 41: Docking ellagic acid with PTGS2

It was observed that cluster 2 had the lowest gibbs free energy of -8.34 kcal/mol.

Results of interactions between ellagic acid and EGFR, obtained through SwissDock are shown below:



Show	Cluster	Element	FullFitness (kcal/mol)	Estimated ΔG (kcal/mol)
◉	1	2	-2158.85	-7.84
○	1	1	-2158.85	-7.84
○	1	0	-2158.85	-7.84
○	1	4	-2158.43	-7.81
○	1	3	-2158.54	-7.80
○	7	0	-2156.43	-7.53
○	6	5	-2156.54	-7.46
○	6	3	-2156.74	-7.45
○	6	2	-2156.74	-7.45
○	6	1	-2156.75	-7.45
○	6	4	-2156.55	-7.41
○	1	6	-2155.89	-7.41
○	1	5	-2155.89	-7.41
○	6	13	-2154.66	-7.38
○	6	0	-2157.19	-7.38
○	6	8	-2156.07	-7.34
○	6	7	-2156.07	-7.34
○	6	6	-2156.07	-7.34
○	6	12	-2155.79	-7.31
○	6	11	-2155.79	-7.31
○	6	10	-2155.79	-7.31

Figure courtesy: http://www.swissdock.ch/docking/view/swissdockd_4CqOq5_QM942QF3MUSGJH3Q39NG

Figure 42: Docking ellagic acid with EGFR

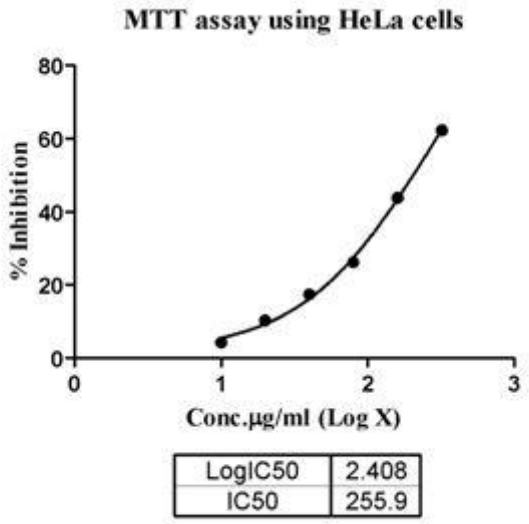
It was observed that cluster 1 has the lowest gibbs free energy of -7.84 kcal/mol.

Validation Using Cancer Cell Line:

The crude extract of Dabour Shilajit was tested against HeLa cell lines.

Compound Name	Concentration	OD at 590 nm	% Inhibition	IC50 µg/ml
Control	0	0.752	0.00	255.90
Shilajit	10	0.719	4.32	
	20	0.6175	10.28	
	40	0.621	17.42	
	80	0.555	26.17	
	160	0.423	43.75	
	320	0.284	62.23	

Table 1: IC50 value of Shilajit



Graph 1: % Inhibition vs Concentration of Shilajit

Sample tested against HeLa cell, has showed IC50 value of 255.9µg/ml with a % inhibition of 62.23.

CONCLUSION

Ellagic acid is a natural polyphenol component that is present in Shilait and has the most associations with cancer related diseases. Docking studies done on ellagic acid, a component of Shilajit shows that it binds with the cancer targets PTGS2 and EGFR in a stable conformation. The crude extract of Shilajit shows that when tested on cancer cell lines, it has a % inhibition value of 62.23. The present study indicates that Shilajit might be of value as sources or leads for novel anti-cancer drugs.

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