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ORIGINAL ARTICLE

An Integrated Approach of Network Based System Pharmacology Approach and Molecular Docking to Explore Multiscale Role of *Pinus roxburghii* and Investigation into its Mechanism

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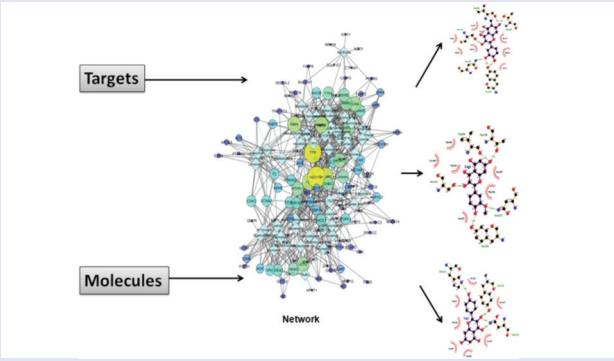
ABSTRACT

Background: *Pinus roxburghii* extract is a multi composed mixture of constituent compounds known to be used as an alternative medicine to treat various diseases. However, due to complex nature the knowledge about its therapeutic range and mechanism of action is still in its infant state. By interpreting the behaviour pattern of complex regulatory networks during various states such as healthy, diseased etc the chances are increased for new target identification, and disease prediction. **Objective:** Therefore, it is necessary to exploit the regulatory network of Pinus plant to elucidate its mechanism of action and prospective roles. **Materials and Methods:** In the present work, chemical prediction, target prediction, network biology, virtual screening, binding affinity and molecular docking approaches have been integrated to unravel the multiple potentials of the Pinus plant. **Results:** The dataset contained constituent molecules and 121 targets that are mapped to drug-target and drug-target-pathway networks to elucidate the relationships amongst the constituent molecules and the targets. The HSD17B1 target with the central role in Pinus regulatory network was screened as the aspiring target; the predicted association between the target and the molecules was validated with the help of molecular docking to obtain *Pinus* plant constituents (Rutin, Cedeodarin and Isorhamnetin) to treat Cancer and Epilepsy. **Conclusion:** The Pinus plant shows potential to show anti-cancer activity although the experimental validation will definitely be required but the study shows the path to work in the similar area

Key words: Alternative medicine, cancer, phytocompounds, *Pinus roxburghii*, system biology

SUMMARY

Pinus roxburghii is traditionally an important plant with medicinal values, however its anti-cancer role is yet unexplored. The network biology approach and molecular docking approach was used to explore the anti-cancer role of multiple constituents of the pinus plant. Rutin, Cedeodarin and Isorhamnetin were found to show potential anti-cancer role against the HSD17B1 target.



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INTRODUCTION

Health is one of the most required sectors of investment that motivates a number of research studies to save the potentially very precious life of humans. Last few decades of systematic drug discovery have contributed significantly to establish a fruitful source of chemotherapeutic agents in the treatment of various human diseases. The non availability of the economic primary health care systems make herbal medicines the prime source of therapeutic agents, especially amongst the rural communities. According to WHO, 80% people uses medicinal plants throughout the world.[1-3] Natural products though complex, have been great source of potential drugs since ancient times. These natural products are not only having "drug-like" pharmacological qualities but also possess "biological friendly" molecular properties. Herbal medicines suffer from unample modern research owing to the paucity of scientific approaches. Despite unexplored mechanism of action, herbal drugs are getting popular and over the last decade medicinal plants are being re-evaluated as a candidate for future alternative medicines.[4-6] *Pinus roxburghii,* common name 'chir pine', ayurvedic name 'saralkul' is longly being used for its medicinal properties. It is enriched in various terpenoids, steroids, xanthones, flavonoids, etc apart from its commercial applications for turpentine, paper, timber, etc it is traditionally also known to possess activity against various medical ailments. Ayurveda has prescribed it as an antidyslipidemic, antioxidant, antiseptic and spasmolytic in action; literature also suggests its role in treating ulcer, diseases of the eyes, skin, etc.[7] It is also an important Indian food source,[8] at colder places the leaves are used in tea which is a rich source of vitamin C,[9] the Pinus stems

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are also a rich source of vitamin C; a sweet edible manna is obtained from the bark, which is actually a gum moreover a flavouring vanillin is obtained as a by-product of other resins.[10] Its roasted seeds are taken as a galactagogue and are rich source of oil.[11,12]

The human body is a composite system that involves myriad interconnected metabolic pathways. Each function under living conditions involves a complex series of regulatory pathways, which integrates all environmental input signals into an output. With the improved understanding of the complex diseases in the recent period, there is a shift in the orthodox paradigm of "one drug one target" to the polypharmacology approach of "one drug many targets".[13] On an estimate around 35% of the known drugs were found to be active against multiple (more than one) targets. Such promiscuous behaviour presents various opportunities for modern drug discovery.[14,15] Computational drug discovery approach is an emerging interdisciplinary approach and an effective strategy for accelerating and economizing drug discovery and development. Systems biology is an interdisciplinary and an extremely efficient approach to study the complex behaviour of signalling pathways. It has become a powerful tool to study the mechanism of action and to screen the multicomposed mixture of herbal constituents.[16]

Chemiinformatics approaches have been successfully used to study traditional phytoconstituents. The applied hypothesis has been evidenced in many of the recent studies. Li *et al.* 2012, have performed a computational investigation to study the mechanism of Traditional Chinese Medicine (TCM) to treat cardiovascular disease.[17] Xie *et al.* 2009, performed network based systems biology approach to study the side effects of CETP inhibitors.[14] Li *et al.* 2014, investigated the mechanism of TCM *Eucommia ulmoides* using systems pharmacology.[18] Liu *et al.* 2013, used the similar approach for drug discovery using Licorice as an example.[19] Shi *et al.* utilize Bu-shen-Huo-xue (BSHX) formula used for treating chronic kidney diseases (CKD) to understand the mechanisms of action of traditional medicine using molecular docking and network pharmacology based approaches.[16] Motivated from these studies we used systems pharmacology approach to explore clearly the interesting associations, the new role for Pinus plant and mechanism of action.

The therapeutic potential of *Pinus roxburghii* has already been reported against various diseases.[7,20] However, several questions still needs to be addressed. We tried to figure out the extended therapeutic potential of the plant to treat multiple serious diseases. Further, we investigated the relationships between targets, phytocompounds and diseases to propose novel key target and mechanism of action. At the end the identified active chemical compounds, targets and biological relevance were compared with the available experimental studies.

MATERIALS AND METHODS

Our protocol involved following main steps: (1) Downloading phytochemical constituents of the Pinus plant from various online repositories (2) Finding targets for the phytoconstituents (3) Constructing drug-target and drug target pathway network (4) Molecular docking and interaction analyses (5) Biological function analyses.

Ligand structures and dataset preparation

A Literature search was performed for constructing the data set of molecules. Finally, 55 compound structures were downloaded from online chemical repositories like PubChem (http://pubchem.ncbi.nlm.nih.gov/) and DrugBank in their SDF file format,[21] the list of all the constituents of *P. roxburghii* were obtained from Kaushik *et al.*[7,22] All the structures of these compounds were optimized by MMFF94 Force Field through Chemdraw v12.[21,23]

Target identification

The identification of novel targets for the already known compounds is the urgent requirement for exploring novel functions of the existing molecules and finding novel targets against already existing diseases. As the wet techniques are quite time consuming and expensive to mine novel targets from the genome-wide scale, application of computational techniques can overcome these problems.[17,24] A pharmacophore modelling technique based on the compounds of the data set was applied to extract the potential targets of each molecule. Pharmmapper server was utilised for target fetching that extracts all the possible targets based upon the pharmacophoric sites of the chemical and ranks them accordingly. Combined algorithms of triangle hashing and genetic algorithm optimization were used sequentially to solve the ranking task of molecule. The target was set to the human targets only, while rest of the parameters were kept default.[25] Each compound based on different conformations resulted in 300 targets, from which top 15 targets were selected. Out of 55 ligands, three failed to fetch any target and thus deleted. 52 left molecules resulted into 15x52= 780 search combinations resulting into 121 unique targets. [Supplementary material Table S2].

Network construction and analysis

Modern drug discovery aims to inhibit the main target responsible for the disease; however for a complex disease a number of targets are responsible and therefore targeting individual target can be insufficient for restoring the healthy state. This multiple target approach require their activity modulation to achieve optimal therapeutic response.[26] The network construction and analysis approach will be helpful in exploring complex mechanism of plant based chemicals and novel interconnections between the existing chemicals and targets.

Drug target (DT) and drug target pathway (DTP) network

A D-T network with 52 molecules (D) and 121 targets (T) was constructed. In the network diagram, diamond shaped and circular shaped nodes depicts compounds and targets, respectively.

Cells communicate to each other with the help of chemical signals; these signals are responsible for their growth, division and death. These information are passed to each other through a process called signal transduction. This signifies the connection between two nodes of any signaling network, represented by edges in our network. Rectangular shaped green colored boxes depicts pathways. Cytoscape 2.8.1, a software package for biological network visualization and analysis was used to generate all types of network.[27] Network Analyzer plugin was used to analyse the quantitative properties of these networks [Figure 1].[28]

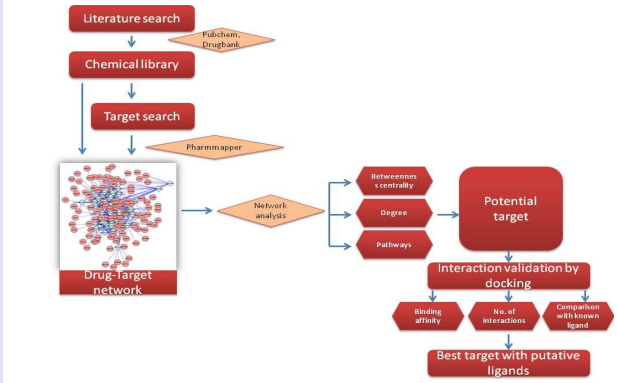

Figure 1: Flow chart showing step wise work flow of the study

Molecular docking

It is an important method that has a significant share in rational drug designing. The technique can predict binding orientation and binding affinty of the chemical ligand in the active site of their target quickly and efficiently. Autodock vina software was used to perform molecular docking. The three dimensional protein structure was downloaded from the online RCSB protein data bank. AutoDock tools (ADT) (version 1.4.5) was used to prepare receptor file. All the molecules in the library were in two dimensional SDF format, which were converted to PDBQT format using openBabel software. Autogrid, an auxiliary program was utilised to generate the grid around the active site of target protein. A 68x52x62 3D grid with a default 0.375 Å grid space was centered around the ligand binding site.[29,30]

Druglikeness evaluation

The development of high throughput screening techniques has raised the capacity for screening large number of compounds against particular biological target. To check attrition rate in drug discovery process a filter of druglikeness can be used to eliminate non-drug like molecules.[31] Online chemical modelling environment (Ochem) server was used to calculate the druglikeness parameter to check if the screened molecules are following the drug like behaviour.[32]

RESULTS AND DISCUSSION

Phytocompounds are becoming popular worldwide. Investigating herbal components is a potential way to explore the possibilities for herbal origin based drug discovery.

Dataset preparation

With reference to literature search the chemical constituents of the plant *P. roxburghii* were searched and their structures were downloaded from various online repositories. Similarly, the target dataset of 121 unique targets was prepared by using Pharmmapper server.

Network construction, analysis and target identification

Drug Target Network: identifying hubs of P. roxburghii for its action

As the therapeutic efficacy of the herbal product depends upon multiple components, their targets and their pathways, the elucidation of the mechanism of this complex interplay can seed a new path to establish the exact therapeutic role of the herbal extract. A network was constructed between 52 constituent molecules of *P. roxburghii* and 121 targets obtained through Pharmmapper server. Figure 2(a) and 2(b) shows the random layout and organic layout of the network.

A basic parameter, degree was considered to screen five best targets. Degree is one of the topological index, that corresponds to the number of nodes directly connected to a given node. It allows the direct estimation of the regulatory relevance of any particular node. The node with the highest degree score can be the regulatory hub and suggests the central and influential role of that node in signalling network.[28] HSD17B1 is the most central target as is connected to the highest number of compounds 34, followed by TTR with 32 associations, CRABP2 with 26 associations, VDR (22) and RXRA (22) targets. [Figure 2(c)] represents the organic view of the network showing the shades of colors depending upon the degree of respective nodes. The brightest and biggest node is the most connected node that is, HSD17B1.

Another fundamental parameter, betweenness was considered to assess the relevance of the position of the node in a network. Notably, HSD17B1 also showed the highest score for the betweenness centrality parameter, around 0.15. Betweenness parameter of a network in biological context indicates the functional relevance of protein to hold together communicating proteins. A higher value depicts the capacity of a protein to be in communication with distant proteins. The parameter is crucial to judge and maintain functionality and coherence of signalling mechanisms.[28] [Figure 3] represents the graph between parameters betweenness centrality and number of neighbours of the nodes of network shown in Figure 2.

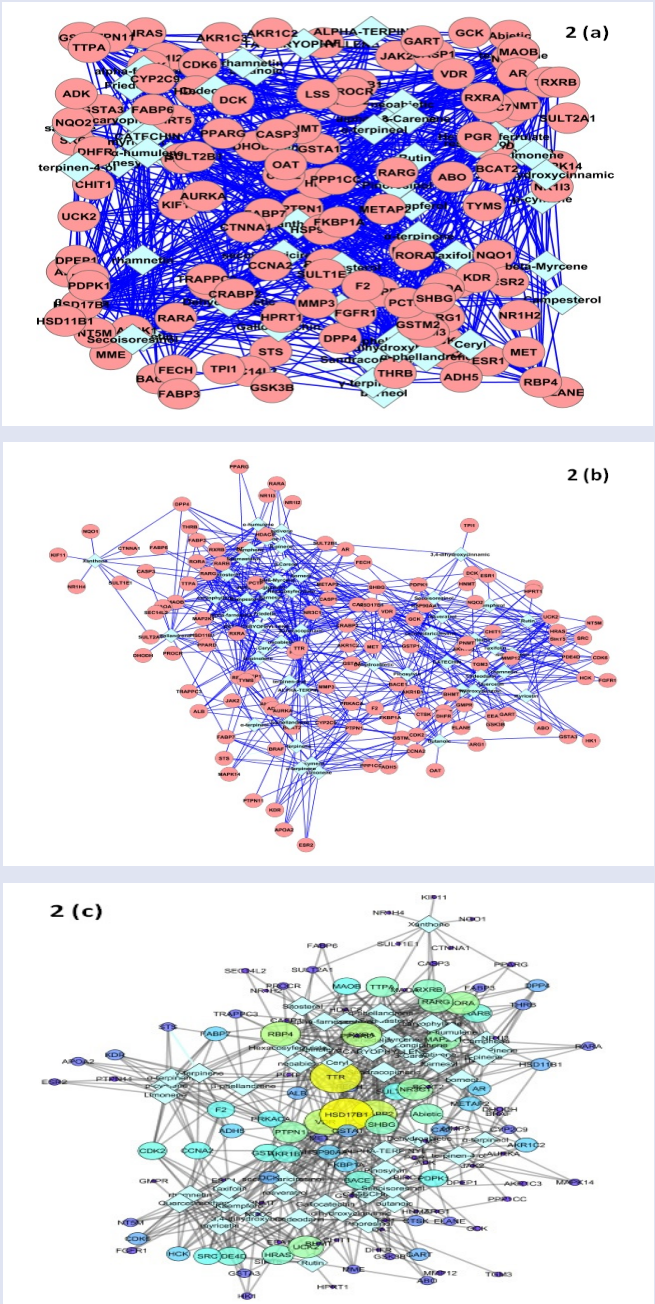

Figure 2: The bipartite drug-target network association of *Pinus roxburghii* plant: The light colored, square shaped blocks represents 52 molecules and circular block represents their 121 targets, 2 (a) showing the Random view; 2 (b) showing the Organic view; 2 (c) showing the Preferred layout; the most connected target is brightest and biggest.

The target with the highest degree and betweenness score would be the central and influential players of *Pinus roxburghii*.[33] The screened targets were found to be associated with most of the compounds (92%) and thus depict their central position in network.

Drug target pathway network: Investigating the mechanism

Based on [Figure 2 and 3], five targets were screened and to explore further their mechanism of action PharmGKB server was utilised to check the number of pathways each target is associated. The HSD17B1 belonged to four pathways (Aromatase Inhibitor Pathway (Breast Cell), Aromatase Inhibitor Pathway (Multiple Tissues), Estrogen Metabolism Pathway, Valproic Acid Pathway, VDR target belonged to Etoposide Pathway while "no curated pathways" were shown by TTR, CRABP2 and RXRA targets. Thus, on the basis of degree, betweenness centrality scores and associated pathways, HSD17B1 was found to be the most influential target against the Pinus constituents.

HSD17B1 is a protein coding gene. Aromatase inhibitor pathway from PharmGKB server depicts the important role of HSD17B1 in breast cancer.[34,35] The hormone 17beta-estradiol plays an important role in genesis and development of breast cancer. 17beta-hydroxysteroid dehydrogenase is responsible for the reversible reduction of estrone to 17beta-estradiol. In ER (Estrogen receptor) positive patients, amplification of HSD17B1 was associated with the decreased breast cancer survival.[35,36] The Valproic acid pathway is central to the function of Valproic acid that is used in the treatment of epilepsy and seizures.[37] Glister *et al.* 2012, reported the decreased expression of HSD17B1 mRNA when treated with Valproic acid to combat epilepsy. The estrogen metabolism pathway happens in liver, which is a site of biotransformation of estrogens depicting the major role of the pathway in physiological mechanisms.[34,38] This enzyme can be an important target for drug discovery.[39] The possibly predicted function of *Pinus roxburghii* plant in the treatment of Epilepsy and Cancer is supported by the studies of Kaushik D *et al.* 2012 and Kaushik P *et al.* 2015, respectively.[40,41] [Figure 4] represents drug-target-pathway (DTP) network and represents complex association between central targets, their associated molecules and pathways.

Molecular docking: Investigating the potential compounds

On the strong basis of 'multiple targets, multiple drug' theory the potential compounds that would be interacting with the HSD17B1 target were explored. The strength of interaction is the most effective parameter to rank different target-drug complexes. For this, the constituents of the Pinus were docked with the HSD17B1 target. The crystal structure of HSD17B1 was downloaded from Protein Data Bank (PDB) with PDB ID: 1FDS. The structure clearly shows that estradiol (known ligand) interacts with the target active site through three hydrogen bonds Ser142, Tyr 155 and His221.[39]

Energy involvement in interactions

A binding affinity cutoff score of -8.0 kcal/mol was used as the first filter to screen the ligands. Binding affinity is the measure of the strength of interactions between the two molecules, and is used to judge whether any molecule can be considered as potential drug candidate. The affinity score of the known ligand Estradiol was -9.2 kcal/mol, compared to this some of the ligands showed better score. We have kept the affinity window wider so as to include more ligands as there may be some cases where the energy value is not big but the interactions are good. A total of 15 molecules were found to be stable and having the threshold binding affinity value. Table 1 contains the values of binding affinities and the average of the two values were considered for final screening.

Atomic interactions between target and ligand

The interaction between the ligand and target complex was studied with the help of Ligplot software and the total hydrogen bonds (H bonds) and hydrophobic bonds (Hpb bonds) were analysed.[42] The last column of Table 1 represents the number of bonds common between the Estradiol and the respective ligand. Finally, four ligands Cedeoderin, Rutin, Pinoresinol diglucoside and Isorhamanetin were screened; these compounds showed 6, 8, 11 and 5 hydrogen bonds with HSD17B1 target respectively in comparison to 3 hydrogen bonds between the Estradiol and target. Rutin is a well known anti-cancer agent,[43-45] but in BindingDB no particular target is present against it. Isorhamnetin also possesses potential anticancer activity, suppresses skin cancer by direct inhibition of PI3-K and MEK1[46] and colon cancer cell growth,[47] BindingDB showed Cytochrome P450 1A1 (CYP1A1) to be its target. There were no reported targets against Cedeodarin and Pinoresinol diglucoside in BindingDB.[48]

It was followed that Ser 142 and Tyr 218 were found to be most important residues as were found to form hydrogen bond with most of the screened ligands [Figure 5]. Ser 142 was one of the residues involved in hydrogen bond formation by the Estradiol.

Druglikeness evaluation

Druglikeness (DL) is a complex balance of molecular properties, which is considered to determine the drug like properties of the molecule. The compounds were further evaluated for their drug like properties. The Ochem server was used to evaluate the DL value of each compound. The

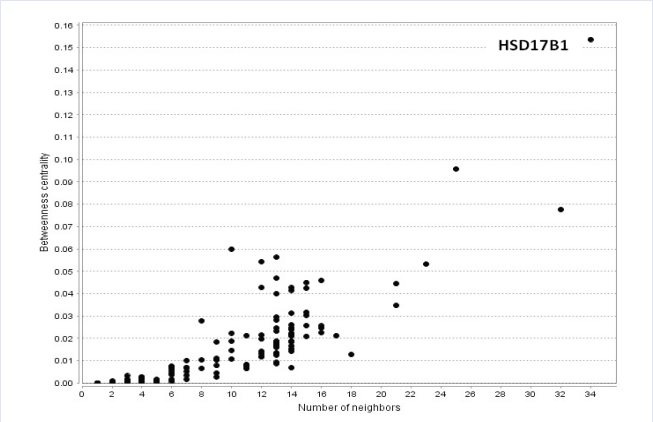

Figure 3: The graph of drug target network between Number of neighbours and Betweenness centrality parameters. HSD17B1 is the target with highest values for both the parameters.

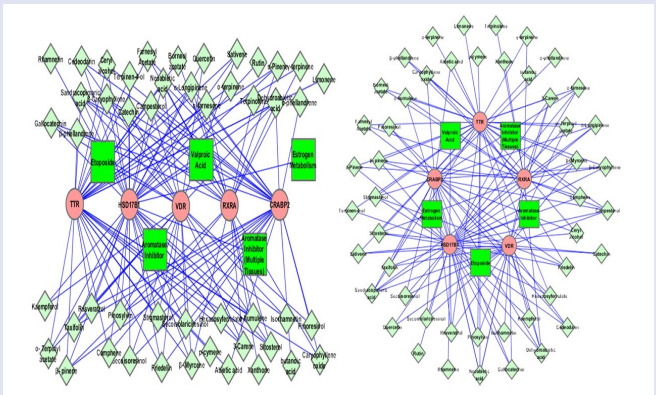

Figure 4: Drug-Target-Pathway Network: Five screened targets (circular and pink), with their 48 associated molecules (diamond shaped), and 5 pathways (rectangular and green) interconnected with edges to show their complex associations.

Table 1: Represents binding affinity, interactions of the 15 screened molecules along with the Estradiol (last row) known ligand of HSD17B1 target. Second last column provides the interactions formed by the molecules with the target and last column provide information regarding the number of bonds common between the Estradiol and the respective ligand.

S. No.	Ligands	Binding affinity	Interactions	Interactions Common to Estradiol
1	Campesterol	-9.6	3H+6HPB	1H+1Hpb
2	Cedeodarin	-8.8	6H+7HPB	1H+5Hpb
3	Dehydroabietic acid	-8.7	6HPB	2Hpb
4	Friedelin	-10.9	1H+12HPB	5Hpb
5	Isorhamnetin	-8.2	5H+6HPB	1H+5Hpb
6	Kaempferol	-8.2	6H+4HPB	5Hpb
7	Myricetin	-8.6	6H+7HPB	No bonds
8	Neoabietic acid	-8.9	6HPB	2Hpb
9	Pinoresinol diglucoside	-9.8	11H+15HPB	1H+7Hpb
10	Quercetin	-8.2	5H+6HPB	1H+4Hpb
11	Rhamnetin	-8.6	5H+7HPB	No bonds
12	Rutin	-10.0	8H+12HPB	1H+5Hpb
13	Sandracopimaric acid	-8.6	5HPB	2Hpb
14	Sitosterol	-9.5	3H+6HPB	1H+2Hpb
15	Stigmasterol	-10.3	11HPB	4Hpb
16	Estradiol	-9.2	3H+9Hpb	

DL value of the Estradiol was 0.77 and of the screened molecules was 1.10 for Rutin, 0.28 for Pinoresinol diglucoside, 0.67 for Isorhamnetin and 1.24 for Cedeodarin. Figure 6 explains the drug like behaviour of known ligand molecule (Estradiol) and screened compounds. The [Figure 6] clearly shows Rutin and Cedeodarin having DL greater than the Estradiol, Isorhamnetin with value 0.67 is close to Estradiol DL while Pinoresinol diglucoside is having a low DL; low DL value corresponds to the less probability of the molecule to be drug. The druglikeness evaluation as a parameter to judge screened ligands provides a strong support to the potential candidature of the ligands; similar criteria was also used by Selvaraj *et al.* 2011.[49]

CONCLUSION

Even after the development of modern medicines the utility and application of plant based medicines is still growing due to its ease in availability, non toxic and friendly properties. The polypharmacology approach "multiple target multiple drug" was applied with the help of network pharmacology approach to focus on predicting the most influential target of Pinus network, investigating its mechanism of action, association with deadly diseases and the plant constituents responsible for its therapeutic role.[50] The D-T and D-T-P networks were utilised to study the topological features to identify the target playing the central role, HSD17B1. The knowledge based approaches for drug discovery like interaction study, binding affinity and druglikeness were utilised to obtain the potential molecules against the HSD17B1 target. Combined study of virtual screening, network biology, binding affinity, interaction evaluation showed a great power to express the anticancer and anti-epileptic mechanism of action for Pinus plant hovering around screened ligands and pathways associated with the central hub of its drug-target network the HSD17B1 target.

The docking approach, interaction studies and druglikeness evaluation predicted some of the ligands to be better than the known ligand. Tyr 218 and Ser 142 were found to be the most important residues, as they are involved in most of the hydrogen bonds formed by the screened molecules. The importance of Ser 142 is supported by the experimentally reported hydrogen bonds with known ligand. For the first time, we have established the role of Pinus plant against cancer and epilepsy computationally; three potential chemical constituents from the extract of Pinus plant have been screened, which might be putative

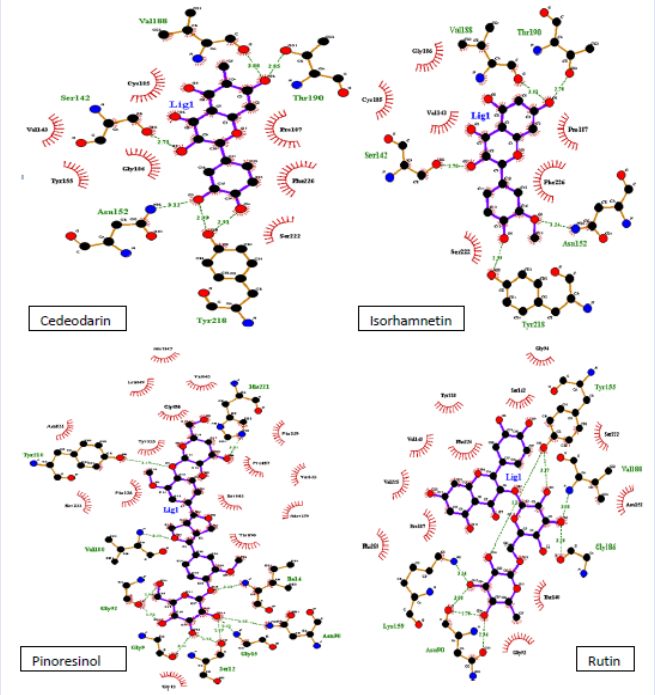

Figure 5: Docking and Interaction:Binding mode of, Isorhamnetin (top right), Cedeodarin (top left), Rutin (lower right), Pinoresinol diglucoside (lower left) in HSD17B1 (PDID:1FDS) active site.

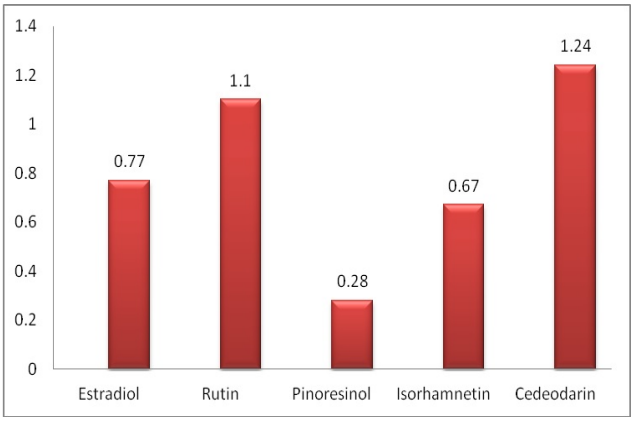

Figure 6: Druglikeness property of the screened compounds in comparison to the know

anti-cancer or anti-epileptic drugs. These plant based compounds were found to be participating in inhibiting the HSD17B1 target in a better way than the known ligand as evidenced by the binding affinity scores and number of interactions.

Conclusively, we suggest a novel target HSD17B1 against Pinus plant constituents; its mechanism of action and three compounds Rutin, Isorhamnetin, and Cedeodarin against HSD17B1. The method will provide an alternative computational strategy to investigate and understand the pharmacological basis of traditional medicine to explore novel drugs, targets and role of different plants.

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Conflicts of interest

There are no conflicts of interest

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