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Virtual Screening for Novel Therapeutics Against Hepatocellular Carcinoma from Indian Ethnic Spices

Moumita Saha [a], Sirshendu Chatterjee [b]

[a, b] *Techno India University, Kolkata, West Bengal, India, 700091*

Abstract

Background:Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death worldwide.Nuclear factor E2-related factor 2 (Nrf2)/Kelch-like ECH-associated protein 1 (Keap1) is one of the most important signaling pathways for cell defence against xenobiotics and oxidative stress.Nrf2 is found to regulate the anticancer effect of a number of cytoprotective drugs, and also contributes to the growth of cancer.Therefore, it makes obvious that both of the Nrf2 agonists and antagonists could be utilised to treat cancer.Numerous spices and their bioactive components have been employed as healing herbs in folk medicine to cure a range of illnesses. Aim: Our aim of the study lies on searching potent anticancer therapeutic agent as a form of spice bioactive compound via analysing through various computational tools including molecular docking study. Methods: The docking process was carried out by retrieving and preparing human Nrf2 protein complex 2FLU from RCSB-PDB database, then the ligand molecules were downloaded from PubChem, databases. Furthermore, different toxicity software was used for the determination of toxicity and Pharmacokinetic properties. Lastly, docking was carried out by the AutoDock Vina, and lastly, the stability of the docked complexes was verified via dynamics simulation. Results: among all the bioactive compounds, piperine from black pepper show the highest binding affinity (-8.7 kCal/mol.) towards the target protein. All other compounds obey Lipinski's rule of 5 and have the potency to be applied as a future anti-cancer drug candidate. Conclusion: based on computational aspects, it can be concluded that Indian ethnic spices have enormous quality as a conventional therapeutic medium. Although more research is needed for better understanding.

Keywords: Gene interaction, Hepatocellular carcinoma, Molecular docking, Nrf2-Keap1 complex, Spice bioactive compounds, Toxicity analysis.

1. Introduction

Hepatocellular carcinoma (HCC), is the most common primary liver cancer, which is recommended as one of the most prevalent causes of cancer-related mortality worldwide. HCC is the ninth-leading cause of cancer-related death in the US. Despite improvements in screening, prevention, and new technology,incidence and mortality are increasing in treatment as well as diagnosis. Regardless of the cause, cirrhosis continues to be the predominant contributing factor to the occurrence of HCC. (Llovet JM. et al., 2016) (Villanueva A. 2019) Males developed HCC more frequently than females (2.4:1), and incidence rates were greater in Southern and Eastern Asia, Mid and Western Africa.

(Ferlay J., et al., 2010) Blacks, whites, and Hispanics are the groups with the highest age-adjusted prevalence of liver cancer (1.6 per 100,000 to 4.6 per 100,000), followed by American Indians and Alaskan Natives. (Altekruse SF. et al., 2009).

The interplay of environmental and genetic variables leads to the development of HCC. Important risk factors for the development of HCC include liver cirrhosis, infection with the hepatitis B and C viruses, excessive alcohol intake, ingestion of aflatoxin B1, and non-alcoholic steatohepatitis (NASH). (Gomes MA. et al., 2013) (Pimenta JR. et al., 2010) The stage of the malignancy at diagnosis affects the life expectancy of HCC patients. Although a few months are anticipated in the advanced stage, a five-year survival rate is achievable with early diagnosis and efficient treatment. (Forner A. et al., 2012) Early diagnosis allows for limited and successful treatment; nevertheless, when the disease progresses and standard chemotherapy is unable to produce adequate results, a poor outcome will be expected. (Liu Y-R. et al., 2015) The phrase "screening" describes the use of radiologic tests like magnetic resonance imaging (MRI), computerised tomography, and ultrasonography at intervals of six months, as well as the use of serological indicators like fetoprotein. A

variety of therapeutic approaches exist, but the two treatments that are curative, are surgical excision or orthotopic liver transplantation (OLT); it is open to anybody who meets the requirements of the University of San Francisco or falls below them. Additional treatments include molecularly targeted therapies, radiation therapy, systemic chemotherapy, cryoablation, percutaneous ethanol injection, trans-arterial chemoembolization and many more. Based on the tumour's size, location, extrahepatic spread, and underlying liver function, a therapeutic strategy is chosen. (Hosaka T. et al., 2013) Each hepatocellular carcinoma has a different set of somatic genetic mutations in its genome that contribute to the intricacy of the carcinogenesis processes in the liver. (Schulze K. et al., 2015) The maintenance of telomeres, cell cycle regulators, WNT/β-catenin, epigenetic modifiers, RAS/RAF MAP kinase, PI3K/AKT/mTOR, and NRF2/KEAP1 pathways are among the important signalling pathways targeted by somatic mutations that frequently affect a subset of 160 cancer driver genes. (Schulze K. et al., 2015) (Guichard C. et al., 2012) (Totoki Y. et al., 2014) One of the most crucial signalling pathways for cell defence against xenobiotics and oxidative stress is the nuclear factor E2-related factor 2 (Nrf2)/Kelch-like ECH-associated protein 1 (Keap1). (Yang Y. et al., 2020) (Robledinos-Anton N. et al., 2019) The NFE2L2 gene in humans encodes nuclear factor erythroid 2-related factor 2 (NRF2), often referred to as nuclear factor erythroid-derived 2-like 2.(Moi P. et al., 1994) In healthy cells, Nrf2 is an essential component of the cellular defence system that guards cells and encourages cell survival in stressful situations. (Subbiah V. et al., 2018) It is also a tumour suppressor that may eliminate ROS and carcinogenic substances. (Milkovic L. et al., 2017) However, it has been hypothesised that Nrf2's antioxidant defence mechanism additionally protects cells from radiation therapy, chemotherapeutic medicines, and anticancer medications. (Lisek K. et al., 2018) (Itoh K. et al., 1997) Elevated Nrf2 levels have been found in clinical studies of malignancies including lung, ovarian, melanoma, colorectal, endometrial carcinoma, breast, kidney, pancreatic, and hepatocellular carcinoma (Fig. 1).

Since the beginning of Nrf2 research, there has been substantial evidence highlighting the functions of Nrf2 in avoiding carcinogen-induced tumours. (Itoh K. et al., 2010) Nrf2, which was first identified as the transcription factor mediating the anticancer activity of several cytoprotective substances, has since been found to be involved in a variety of cellular activities. In contrast to the findings that led to its discovery, it is now evident that Nrf2 contributes to the development of cancer. It perhaps makes it possible for healthy cells to withstand the strain brought on by malignant transformation. (Cho HY. Et al., 2005) Nowadays, it is generally acknowledged that Nrf2 plays a crucial role in the ARE-mediated production of oxidative stress enzyme genes, such as antioxidants and detoxicants. Consequently, the Nrf2 route is crucial for preventing diseases that have inflammation and oxidative stress as their primary pathologic features. The pathogenic characteristic of the majority of liver disorders is oxidative stress. Because Nrf2 has a dual involvement in the redox adaptation of cancer cells and the defence of normal cells against oxidative stress, Therefore, it makes sense that both Nrf2 activators and inhibitors could be used in the treatment of cancer. (Sporn MB. Et al., 2012)

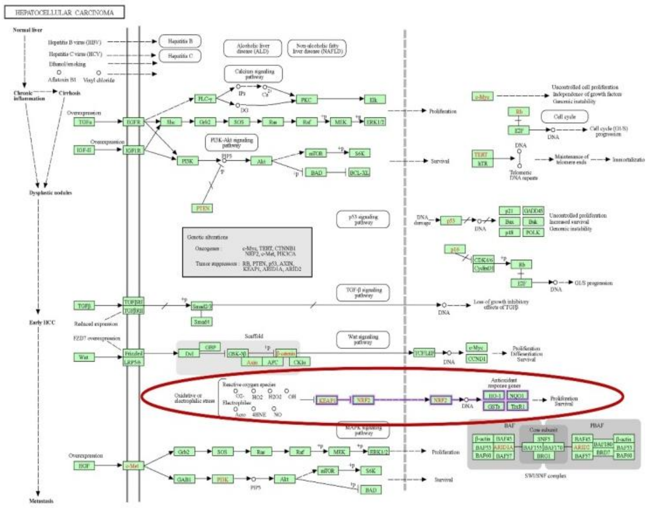

Fig.1. KEGG Pathway Showing the Mechanism of Action of Nrf2-KEAP1 Protein Complex

The flavour, taste, and colour of spices have made them popular seasonings for thousands of years. Because they contain a large number of bioactive chemicals and have a wealth of advantageous properties, many spices have been used as medicinal herbs in folk medicine to treat a variety of disorders effects on health. (Srinivasan K. 2014) (Rubio L. et al., 2013) The primary modes of action include triggering apoptosis, obstructing tumour growth, invasion, and migration, and making tumours more susceptible to radiation and chemotherapy. (Hegde PS. et al., 2016) (Zhou Y. et al., 2016) The effectiveness of already available chemotherapeutic drugs and radiotherapy improved, demonstrating that combination therapy is a feasible therapeutic approach for malignancies. In a nutshell, spices have potential as sources for adjuvant therapy for cancer. More antitumor bioactive

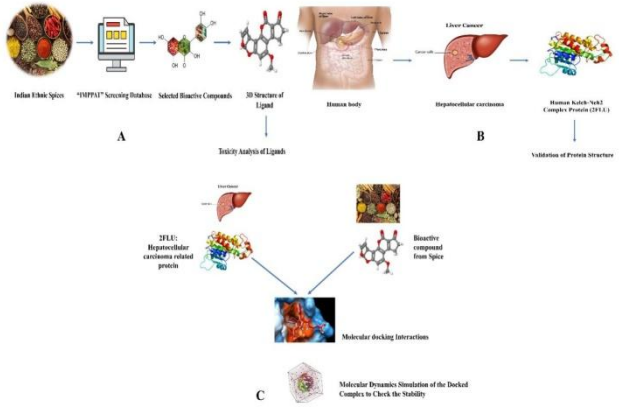

Fig. 2. The Schematic Workflow

components from spices need to be isolated and discovered in the future, and their pathways of action need to be studied in more detail.Therefore, using the Nrf2 pathway as a potential treatment for liver carcinoma has been a novel approach regarding the *in-silico* evaluation of herbal alternative therapy. The overall workflow is represented via Figure 2.

2. MATERIALS AND METHODS

2.1. Selection of Spice samples

For the purpose our *in-silico* computational study against hepatocellular carcinoma, we have primarily selected eight Indian ethnic spices: Green cardamom, White cumin, Black cumin, Black pepper, Black cardamom, Coriander, Wild celery and Mace (Figure 3). Most of these spices are regularly used in our daily household.

2.2. Selection and Preparation of the ligands

Chemical compounds, or more precisely, bioactive ligands (Table 1), were chosen using the "IMPPAT" online database support (https://cb.imsc.res.in/imppat/) (Figure 4). Upon selecting the name of spice sample in the search box of the server, data of several bioactive constituents will be generated Based upon literature reviewing, compounds specific to each spice has been chosen. 2D Structure Data Format (SDF) of all chemical compounds or ligand files were retrieved from PubChem (www.pubchem.ncbi.nlm.nih.gov/) and later translated to their 3D PDB format using Open Babel software. A PDBQT format file was created after adding (Rezaei-Seresht H. et al., 2019) hydrogenating atoms and the desired torsion to a PDB format file.

2.3. Selection of Receptor or Protein

For the purpose of protein selection, 2FLU protein has been selected. This protein denotes the structure of crystal structure of human Kelch-Neh2 Complex, belonging to ligase family. From Protein Databank (http://www.rcsb.org/), the 3D structure of 2FLU (Figure 5) has been obtained. Then to stabilize the receptor structures, the already attached ligands and water molecules were removed by BIOVIA Discovery Studio 2020 software https://discover.3ds.com/discovery-studio-visualizer-download/).(BIOVIA., 2020)

2.4. Validation of Protein Structure

The newly generated protein PDB structure was then undergone through a series of quality analyses including ERRAT, Procheck using SAVES 6.0 (https://saves.mbi.ucla.edu/), (Colovos C. et al., 1993) (De Oliveira CCS. et al., 2019) (Laskowski RA. et al., 1993) and ProSA-web (https://prosa.services.came.sbg.ac.at/prosa.php/). (Wiederstein M. et al., 2007)

2.5. Gene Interactions

Gene interaction of the protein human Kelch-Neh2 Complex was done using the flexible web interface and STRING server (https://string-db.org/). (Szklarczyk D. et al., 2023) The interactions come from computational prediction, knowledge transfer across species, and interactions gathered from other (primary) databases; they comprise immediate (physical) and oblique (functional) correlations.

Table 1: Table Showing the Name of Selected Compounds for *In-silico* Study Along with the Selection Criteria

Nature of Screening Criteria	Name of The Compound
"IMPPAT" Database	Green cardamom: Linalool White cumin: Cuminaldehyde Black cumin: Thymoquinone Black pepper: Piperine Black cardamom: α-Terpineol Coriander: Myrtenol Wild celery: Myrcene Mace: Myristicin

2.6. Molecular Docking Interaction Using AutoDock Vina

AutoDock Vina software (http://vina.scripps.edu/) (Trott O. et al., 2010) for molecular docking and virtual screening that significantly improves efficient binding mode predictions, thereafter gives more accuracy in protein-ligand interaction. AutoDock Vina works by calculating the grid maps and clusters. Before proceeding to final docking step, Kollman charges and other modifications were added to the purified form of protein and converted into a proper readable PDBQT file format. Similarly, ligand is also transformed into PDBQT file. A grid box on active residues of protein was generated with different grid dimensions and centres but with similar spacing i.e., 0.375. The exhaustiveness was set at 8 and binding energy affinity was predicted with AutoDock Vina software. The final visualization of docked structure was performed using BIOVIA Discovery Studio 2020 (https://discover.3ds.com/discovery-studio-visualizer-download/) and PYMOL software (https://pymol.org/). (Rehan M. et al., 2021)

2.7. Assessment of Structural Hotspots and Binding pockets on the Receptor Protein

An online server called CASTp 3.0 (http://sts.bioe.uic.edu/) (Binkowski TA. et al., 2003) is used to predict active amino acid residues, or alternatively structural hotspots on the receptor protein. A systematic quantitative characterisation of the surface topography of proteins is often provided by the Computer Atlas Surface Topography of Protein (CASTp).

2.8. iMod Server Prediction

iMod server (http://imods.chaconlab.org/) (López-Blanco JR. et al., 2014) even with huge macromolecules, enables the discovery of such modes and produces workable transition paths between two homologous structures.

Fig. 3 Selected Spice Samples

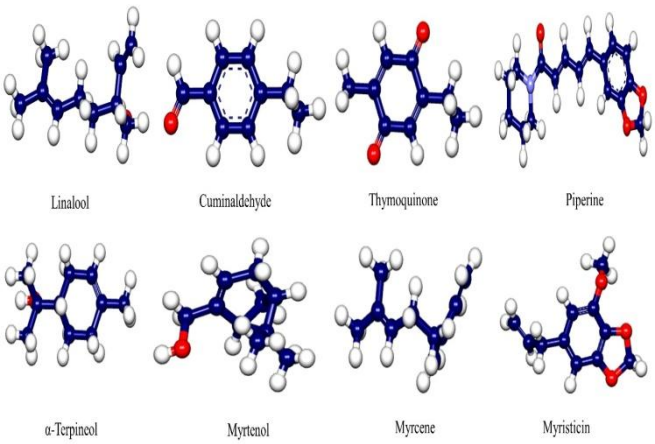

Fig. 4. Structures of Spice derived Major Bioactive compounds

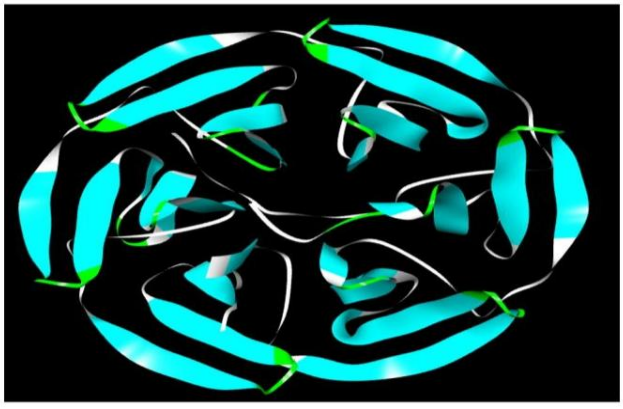

Fig. 5. 3D Structure of human Kelch- Neh2Complex [2FLU]

3. RESULTS AND DISCUSSIONS

3.1. Validation of Compound Structures

3.1.1. SwissADME Prediction of the Compounds

One of the primary challenges to the commercial use of natural compounds from spices as medicinal agents is the dearth of pharmacokinetic research. Therefore, it was intended for the study to apply a variety of in-silico techniques to assess the computational aspects of the spice's biologically active elements.Upon submission of ligand structure in SMILEs format, SwissADME results are generated based on ADME/toxicity analysis and Lipinski filter analysis upon submission of the ligand structure in SMILEs format. Here in our result, we have represented the outcome of the drug likeliness data in a tabulated manner. (Table 2)

According to the SwissADME server derived drug likeliness result, from Table 2, all the eight bioactive compounds of different spices show satisfactory result without any violations. That means, all of them follow Lipinski's rule 5 completely (Molecular weight must not exceed 500 Dalton; Hydrogen bond donors and acceptors must not exceed 5 and 10 respectively and last but not least, the octanol-water partition coefficient should not cross 5). (Lipinski CA. et al., 1997) In addition, bioavailability scores describe how quickly and how much a molecule enters the bloodstream after oral delivery, eventually reaching the desired areas, according to Table 2, all selected compounds show the similar score i.e., 0.55. This value implies that the compounds have 55% probability of being bioavailable. (Veber DF. et al., 2002)

3.1.2. Toxicity Prediction of the Compounds

Assessing the toxicity of small compounds is an essential stage in the drug discovery approach. The PreADMET server's toxicological prediction result, which includes the chemicals' hERG inhibition, mutagenicity, and carcinogenicity are shown in Table 3. According to the result, the negative prediction translates carcinogenic activity whereas positive means the compound possess no carcinogenic activity. (Mishra SS. et al., 2016) Talking about the mutagenic characteristics,all compounds are mutagenic in nature. In case of hERG inhibition, most of the compounds show medium to low probabilities of blogging hERG gene that often associated with sudden heart attacks in humans. (Yu HB. et al., 2016) The result obtained from pkCSM server is given in Table 4.The term "LD50" refers to the concentration of a test substance deemed to be lethal for 50% of the test subjects in the treated group; in this case, the lethal dose for rats has been determined and is stated in terms of mol/kg. It is one method of determining a compound's acute toxicity, or short-term poisoning potential. The highest dose or quantity of a medicine or test substance that does not manifest any undesirable side effects is referred to as the maximum tolerated dose. It is identified through multiple clinical trials unit of expression is log mg/kg/day. (Singh S. et al., 2013)

Table 2: Showing Results of Drug Likeliness of the Spice Bioactive Compounds Based on Lipinski's Rule of 5

Name of the Compound	Lipinski's Rule		
	Satisfactory	No. of Violations	Bioavailability Score
Linalool	Yes	0	0.55
Cuminaldehyde	Yes	0	0.55
Thymoquinone	Yes	0	0.55
Piperine	Yes	0	0.55
α-Terpineol	Yes	0	0.55
Myrtenol	Yes	0	0.55
Myrcene	Yes	0	0.55
Myristicin	Yes	0	0.55

Table 3: Mutagenicity and Carcinogenicity Analysis of the Spice Bioactive Compounds

Name of the compound	Toxicity			
	Mutagenicity	Carcinogenicity		
		Rat	Mouse	hERG inhibition
Linalool	Mutagen	Negative	Negative	Low risk
Cuminaldehyde	Mutagen	Negative	Negative	Medium risk
Thymoquinone	Mutagen	Positive	Positive	Low risk
Piperine	Mutagen	Negative	Positive	Medium risk
α-Terpineol	Mutagen	Negative	Negative	Low risk
Myrtenol	Mutagen	Negative	Positive	Low risk
Myrcene	Mutagen	Positive	Negative	Medium risk
Myristicin	Mutagen	Positive	Positive	Medium risk

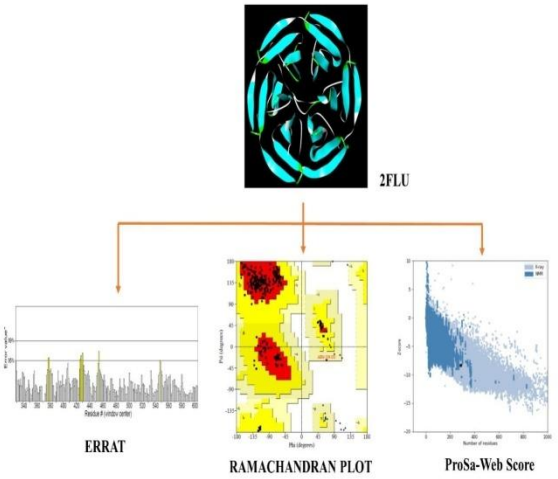

Fig. 6. Results of Protein Validation Parameters of 2FLU

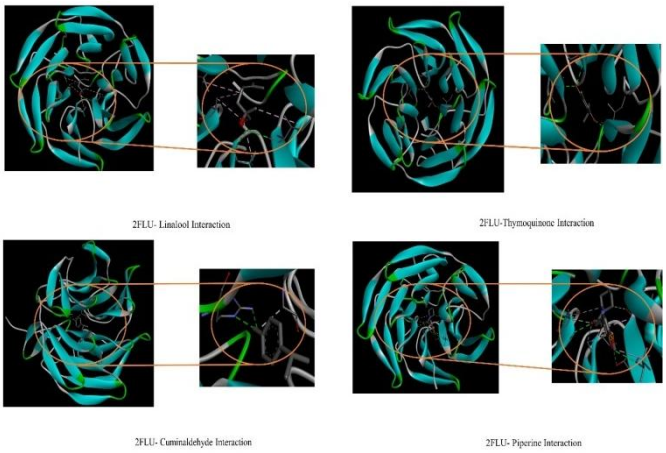

Fig. 9A1. Showing Results of Complete Representation and Close Insight of 2FLU Interaction with the Respective Ligands [Linalool; Cuminaldehyde; Thymoquinone;

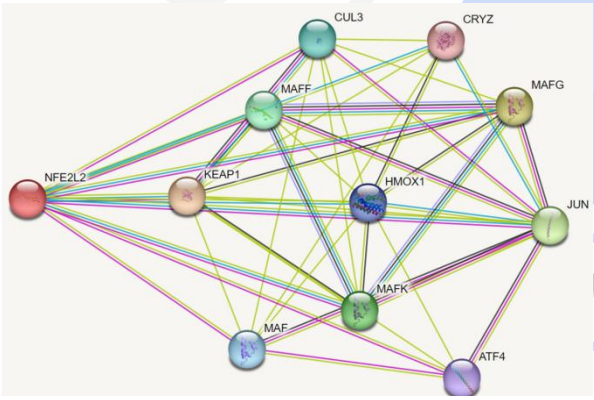

Fig. 7. Showing the Results of Gene-gene Interactions via STRING Database

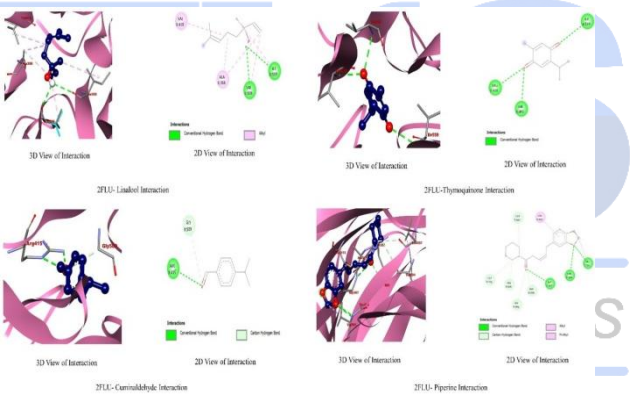

Fig. 9A2. Showing Results of 3D and 2D View of 2FLU Interaction with the Respective Ligands [Linalool; Cuminaldehyde; Thymoquinone; Piperine]

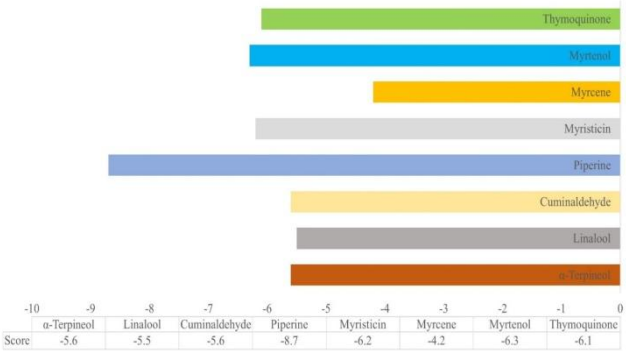

Fig. 8. Graphical Representation of Binding Affinity along with the Score (kCal/ mol.) towards the Target Protein 2FLU by the Respective Compounds/ Ligands

3.1.3. Validation of Protein Structure

According to the predictions made by the aforementioned web tools, Figure 6 depicts the overall quality of recognition of the 3D protein PDB structure 2FLU. The process of approving an ideal protein structure involves confirming the protein PDB model using a number of quality control metrics. According to ERRAT's results, the protein exhibits a quality score greater than 97.143%, indicating that the protein is well-modelled. The "overall quality factor" for non-bonded atomic interactions is displayed, with higher values denoting higher quality. Moving on, the ProSA-web result displayed the protein's total z score. In this case, the score is -8.29, indicating that the structure is located within the X-ray region. The

Ramachandran plot of the protein model then showed that, in accordance with the PROCHECK result, 91.6% of residues were found in the most preferred regions, followed by 8.0% in additional allowed and 0.4% in generously allowed. The collective outcomes derived from the previously mentioned attributes indicate that 2FLU protein is of high quality and appropriate for additional molecular interaction analysis.

3.1.4. Gene Interactions

The string database analysis results show the following scores: Number of nodes:11; Number of edges: 43; Average node degree:7.82; Average local clustering coefficient: 0.873; Expected number of edges: 12; PPI enrichment p-value: 2.36e-12. Basically, NFE2L2 gene that encodes for Nrf2 protein, shows various modes of interactions (Figure 7) with other genes as well which on the other aspects

Table 5: Table Showing Active Amino Acid Residues Obtained via Molecular Docking Interaction for Each of the Ligand Along with the Receptor Protein 2FLU

encode for different proteins of distinct pathways. According to the result from Figure 7, it can be observed that from NFE2L2 gene, lines of different colour arise and they get connected to a variety of other genes via single or multiple lines of interactions. Each colour represents a distinct mode of interaction for e.g., from a meticulously curated database, as well as experimentally found protein homology, text mining, gene fusions, gene co-occurrence, and gene neighbourhood. Each gene that interacts with NFE2L2 gene generates a specific score. Here from our interaction status, the score underlies between 0.999 to 0.972.

3.1.5. Molecular Docking Interaction Using AutoDock Vina

The binding affinity of various spice bioactive substances with the necessary protein crystal structure of the human Kelch-Neh2 Complex (2FLU) is ascertained based on the docking analysis performed by AutoDock Vina. The ligands with the highest binding affinity to the proteins are those with greater negative binding energy. We have chosen eight ligands based on our research, and each ligand exhibits a unique set of results due to variations in its binding capacity with the target protein receptors. As reported by the docking result, Piperine shows the maximum binding affinity i.e., -8.7 kcal/ mol. followed by Myrtenol (-6.3 kcal/mol.), Myristicin (-6.2 kcal/mol), Thymoquinone (-6.1 kcal/mol), α-terpineol and Cuminaldehyde (-5.6 kcal/mol), Linalool (-5.5 kcal/mol) and lastly it is Myrcene that shows the minimum affinity energy -4.2 kcal/mol. Figure 8 shows the overall outcome of the various binding energy affinities, with each docking interaction displayed in both 3D and 2D (Figure 9A1-9B2).

3.1.6. Assessment of Structural Hotspots and Binding pockets on the Receptor Protein

Table 5 displays the findings for the 3D protein PDB structure (2FLU) obtained from the CASTp 3.0 online server. This outcome demonstrates the key amino acids involved in the particular protein-ligand interaction.

Figure 10 shows the active binding pockets which are present in the 3D protein (2FLU). Each binding pockets represent the ligand attachment region on the protein. Binding pockets (1-6) are differentiated on the basis of their size and volume.

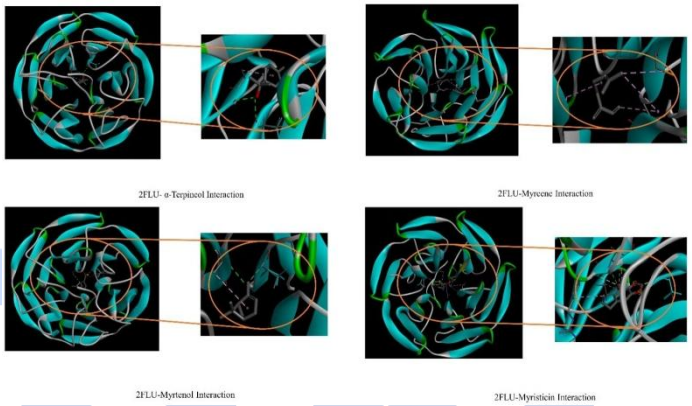

Fig.9B1. Showing Results of Complete Representation and Close Insight of 2FLU Interaction with the Respective Ligands [α-Terpineol; Myrtenol; Myrcene; Myristicin]

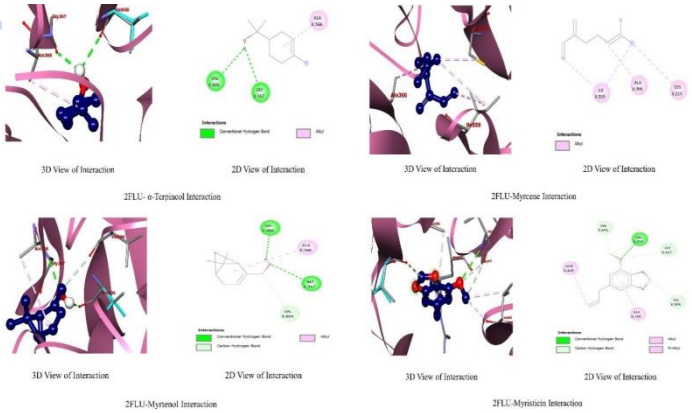

Fig. 9B2. Showing Results of 3D and 2D View of 2FLU Interaction with the Respective Ligands [α-Terpineol; Myrtenol; Myrcene; Myristicin]

3.1.7. iMod Server Prediction

Normal mode analysis is represented by the iMod Server prediction in internal coordinates, and Figure

11 typically displays the results as various factor analyses. The docked structure of the iMod interaction

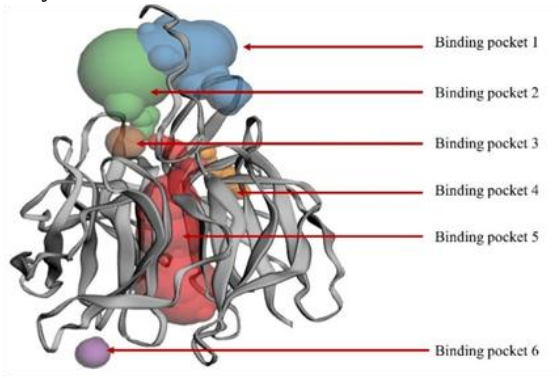

Fig. 10. Showing the Binding Pockets inside the Protein Structure [2FLU]

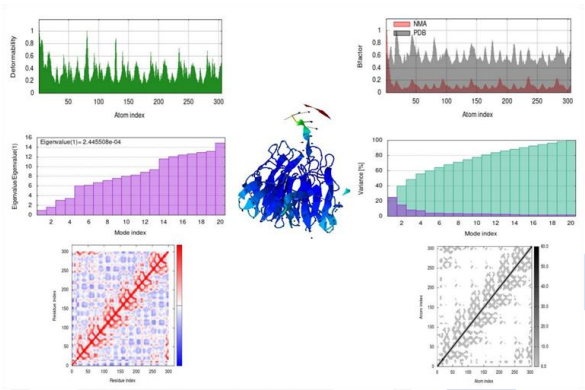

Fig. 11. Results of iMod Server Prediction of 2FLU-Ligand Complex

status between protein and ligand displays the mode of interaction between the receptor and ligand through black arrows. The overall protein-ligand interaction mode is further represented by the definitions of elastic network, deformability, experimental B factor, eigenvalue, and covariance map, in addition to the interaction status.

Table 4: LD 50 Value (Rat) and Maximum Tolerated Dose (Human) of the Spice Bioactive Compounds

Name of the Compound	Oral Rat Acute Toxicity (LD 50)	Maximum Tolerated Dose (Human)
Linalool	1.704	0.774
Cuminaldehyde	1.7	0.839
Thymoquinone	1.743	0.89
Piperine	2.811	0.38
α-Terpineol	1.923	0.886
Myrtenol	1.746	0.439
Myrcene	1.643	0.617
Myristicin	2.166	0.841

Table 5: Table Showing Active Amino Acid Residues Obtained via Molecular Docking Interaction for Each of the Ligand Along with the Receptor Protein 2FLU

Name of the Protein	Name of the Compound/ Ligand	Active Amino Acid Residues
1H2M	Linalool	ALA 366, VAL 418, ILE 559, VAL 606
	Cuminaldehyde	ARG 415, GLY 509
	Thymoquinone	VAL 418, VAL 465, ILE 559
	Piperine	LEU 365, ALA 366, GLY 367, CYS 513, LEU 557, THR 560, VAL 561, VAL 604, GLY 605
	α-Terpineol	ALA 366, GLY 367, VAL 606
	Myrtenol	ALA 366, GLY 367, VAL 604, VAL 606
	Myrcene	ALA 366, CYS 513, ILE 559
	Myristicin	ALA 366, ARG 415, GLY 417, VAL 418, VAL 465, VAL 606

4. Conclusion

In this study, we used virtual screening to examine how several spice bioactive components affected the development of hepatocellular carcinoma. Numerous herbs and spices are important for their ability to have positive effects on human health. These effects include stimulating digestion as well as having a number of pharmacological properties like those that are anti-inflammatory, antimicrobial, and anticarcinogenic, which are primarily attributable to the bioactive compounds. One of the most prevalent causes of death in the entire world is hepatocellular carcinoma. Although there are many helpful remedies and tools against this terrible ailment, most of them are hazardous to health. As a solution to this issue, conventional anti-cancer treatments utilising plant-based ingredients come to mind. By reviewing the anticarcinogenic potential of Indian culinary spices,

eight major Indian spices and their most potent bioactive component which we use in our kitchen almost on daily basis have been chosen. Upon selection, all the eight bioactive compounds obey Lipinski's rule of 5, that allows them to be further proceeded towards molecular docking process. According to the results of docking, out of all compounds, Piperine show the maximum binding affinity towards the protein that signifies human Kelch-Neh2 Complex, one of the responsible receptor proteins in hepatocellular carcinoma pathway. Other compounds also proven to have possible therapeutic prospects. From this it can be concluded that this group of spice bioactive compounds can be used as chemotherapeutic agents against hepatocellular carcinoma proteins. The normal mode analysis confirmed the stability of the drug and protein interaction. By repurposing, coupled with an algorithmic learning and intelligence-based approach, we can expand this sort of medicine. Moreover, toxicological studies conducted in-vivo and in-vitro needed to be employed to validate these prediction outcomes.

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Conflict of Interest

There are no conflicts of interest, the authors declare.

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