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AN *IN- SILICO* STUDIES FOR IMMUNOMODULATORY POTENTIAL OF PHYTOCONSTITUENTS FROM A NATURALLY OCCURRING HERB *NIGELLA SATIVA*

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ABSTRACT

Nigella sativa L. belongs to the family of Ranunculaceae, commonly known as Black Cumin or Kalonji. It is an annual herb used as a food ingredient and spice. *N. Sativa* is approved by the US Food and Drug Administration (FDA) as food and by the Flavor and Extract Manufacturers Association (FEMA) as generally recognized as safe (GRAS). We aimed to identify the potent immunomodulatory phytoconstituents of *N. Sativa* using the in-silico studies like molecular docking. For docking assessment, two proteins 1M48, and 1P9M were used for ten active constituents (Carvacrol, p-Cymene, Dithymoquinone, Gamma himachalene, Limonene, Nigellamine C, Nigellicine, Nigellidine, alpha-Pinene, and Thymoquinone) are selected. *In-silico* ADME studies were also performed. The molecular docking study is done using AutodockVina 1.1.2 and visualized by BIOVIA Discovery Studio. Curcumin is chosen as the standard of natural origin, while dexamethasone and salazosulfapyridine are synthetic standards. All the phytoconstituents showed good binding affinities with 1M48 (IL-2) protein in the range of -4.3 to -7.3 kcal/mol, while on 1P9M (IL-6) protein, the binding affinities observed to be in the range of -5.2 to -9.8 kcal/mol. The *in-silico* ADME studies showed that these phytoconstituents have better druggability and no cytotoxicity. The current study showed that the immunomodulatory properties of *N. Sativa* are from Nigellamine C.

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Introduction

Defending the host from the pathogenic destructive microorganism in vertebrates is the immune system. This protective mechanism of the body is extremely necessary for the investigation of host tissue integrity. The chief distinctive feature of immunomodulatory agents is to augment or diminution the immune response generated by host immune cells [1, 2]. Natural ingredients can control and cure acute, chronic disease infectious diseases, etc. the major advantage of using natural constituents is they have a novel structure, multi-target, safe and effective remedy [3, 4]. A notable rise in the occurrence and austerity of adverse drug reactions observed with several synthetic drugs ultimately boosted substantial interest in natural remedies [5]. From antique times, various traditional systems of medicine naming Ayurveda, Siddha, Tibb, Unani, and the Chinese system (TCM) substantiate several natural products, plants, and extracts possessing the ability to modulate the immune system [6, 7]. Considerable innovation and research were carried out on establishing and developing novel immunomodulatory agents. Amongst the few top-ranked evidence-based herbal medicines, *Nigella sativa* (Ranunculaceae)

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has a special place [8, 9]. It is native to Middle Europe, and Western Asia. *N. Sativa* is immersed in a rich historical and religious context. *N.Sativa* is an annual blossoming herb with erect stems,20-30 cm in height and green to dark green [10, 11]. The flowers are delicate white and turns blue at the time of maturity with 5 to 10 petals [11]. The fruits are inflated capsulesenclosed black colored, oval seeds with an approximate size of 1-3 mm [12]. *Nigella sativa* consists of fixed oil, volatile oil, alkaloids, proteins, saponin, flavonoids, phenolic acids, and tannins [6, 13, 14]. There are different active phytoconstituents present in *N. Sativa*, such as thymoquinone, thymohydroquinone, dithymoquinone, cymene, carvacrol, pinene, terpineol, limonene, anethol, hederin, citronellol, nigellicine, nigellidine, nigellamines, etc. [6, 15-18]. Seeds of *Nigella sativa* have been increasingly reported as a successful therapeutic agent. It showed antimicrobial, antibacterial, antifungal, antiparasitic, anticancer, anti-inflammatory, and immunomodulatory activities, helpful in atherosclerosis, asthma, cough, bronchitis, influenza, and eczema. Traditionally used as a diuretic, for headache, fever, bronchial disorders, insomnia, and titanus [19-24].

In recent years molecular docking becoming the key tool in computer-aided drug design for identifying novel ligands, binding affinity, and behavior of small molecules at the binding site of the target protein. With the help of molecular docking, we can be predicting the orientation of ligand bound to protein, protein-ligand, or protein-protein binding affinities, via various interactions [25-27]. The sum of these interactions between protein-ligand or protein-protein approximately represents the binding potential in the form of a docking score [26, 27]. The present study aimed to explore in silico interaction of them phytoconstituents with protein 1M48 (IL-2) and 1P9M (IL-6). 1M48 possesses a total structure weight of about 31.77 kDa while 1P9M has a total structure weight of 78.25 kDa.

Materials and Methods

Molecular Docking

Molecular docking is an important computational tool to identify the binding interaction at the target site and develop the pharmacophore [28].

Software and Database

Autodock_vina version 4.2.6 (https://vina.scripps.edu) software approaches were used to predict the in-silico interaction between active compounds of *N. Sativa* and immunomodulatory proteins like 1M48 (Human interleukin-2 or IL-2 protein) and 1P9M (Human interleukin-6 or IL-6 protein) observed in **Figure 1** [29]. BIOVIA Discovery Studio was downloaded from https://discover.3ds.com/ for visualization [30]. Open bable 3.1.1 was downloaded from http://openbabel.organd used to convert SDF to PDB format [31]. The calculations were performed on Windows 10 operating system.

Ligand Selection

Ten phytoconstituents i.e. Carvacrol, p-Cymene, Dithymoquinone, Gamma himachalene, Limonene, Nigellamine C, Nigellicine, Nigellidine, alpha-Pinene, and Thymoquinone are selected as the ligand **(Figure 2)**. The 2D and 3D structures of these ligands in SDF format were downloaded from https://pubchem.ncbi.nlm.nih.govto dock against Interleukins. Natural phytoconstituent curcumin while synthetic compounds dexamethasone and salazosulfapyridine were selected as standard. Ligand preparation was performed in open bable 3.1.1 (http://openbabel.org) [31].

Preparation of Protein

The three-dimensional (3D) structure of the protein was extracted from the protein data bank (PDB) (http://www.rcsb.org). The extracted protein was PDB ID: 1M48, PDB ID: 1P9M, and its chain were shown in **Figures 1a and 1b**, respectively. BIOVIA Discovery Studio was utilized for protein preparation [30]. All water molecules & het-atoms which responsible for increased resolution were deleted. Observe active sites, protein groups, and ligand groups.

Target and Ligand Optimization

In molecular docking, an optimization algorithm was utilized to find a suitable binding pose of a ligand against a protein target to show high binding affinity. This algorithm plays a dynamic role in determining the docking accuracy. A suitable binding pose was determined with autodock vina (version 4.2.6) and visualized with the help of BIOVIA Discovery Studio [29, 30].

Analysis of Target Active Binding Sites

The active site of protein comprised of amino acids was determined by the CASTp tool (Computed Atlas for Surface Topography of Proteins) **(Table 1)** (http://sts.bioe.uic.edu) [32]. Pharmacophore development of targeted protein PDB ID: 1M48 and PDB ID: 1P9M was analyzed with the help of BIOVIA Discovery Studio. After protein preparation, a single ligand is selected, and the receptor-ligand interaction is observed; the binding site and the expansion or SBD site sphere are edited. For PDB 1M48, the size of the sphere was 20 Å. On the contrary, for 1P9M, it was 80 Å. Afterward processing of protein, the ligand group was deleted, Polar hydrogen atoms and the Kollman charges were added, and the file was saved in PDB format [33, 34].

Molecular Docking Analysis

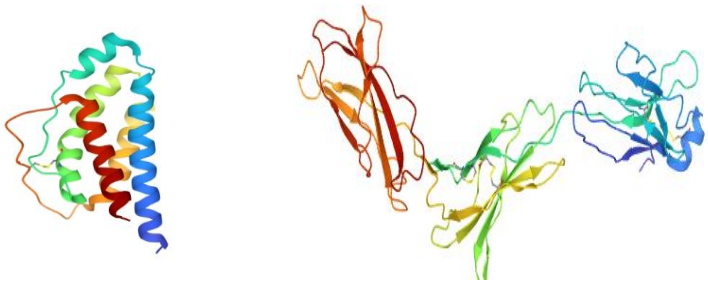
A docking study was employed to study the interaction between protein and ligand. The selected protein for docking plays roles with several intermediates, such as PDB ID: 1M48 (IL 2), and PDB ID: 1P9M (IL-6). Proteins were converted from PDB to PDBQT format with the help of the AutodockVina tool. The configuration files were prepared for both proteins Further, using a command prompt, docking was processed with a ligand file. The exhaustiveness of the global search was set to eight, and the maximum number of binding modes was nine. For the analysis of our ligand file, we used Biovia Discovery Studio to define ligand interaction [29, 30].

ADME Profiling

From the retrieved data of molecular docking of ten phytocompounds from *Nigella sativa* against target proteins, active constituents Carvacrol, p-Cymene, Dithymoquinone, Nigellicine, Nigellidine, alpha-Pinene, and Thymoquinone were considered for ADME properties using the Swiss ADME (http://www.swissadme.ch/index.php) to predict pharmacokinetic properties of phytoconstituents [35, 36].

Results and Discussion

Several protein structures were available for studying the immunomodulatory potential of the phytoconstituents from *N. Sativa.* We have selected the 1M48 (Human interleukin-2 or IL-2 protein) and 1P9M (Human interleukin-6 or IL-6 protein) and downloaded them from the RCSB protein data bank for the molecular docking study **(Figure 1)**. Molecular docking efficiently predicts binding affinities, amino acid involvements, and protein-ligand complexes and became one of the important strategies during drug discovery [37-41].



a) b)

Figure 1. 3D structure of 1M48 (a) and 1P9M (b) proteins

The protein code 1M48 is a crystal structure of human IL-2 complexed with (R)-N-[2-[1-(aminoiminomethyl)-3-piperidinyl]-1-oxoethyl]-4-(phenylethynyl)-L-phenylalanine methyl ester and 1P9M is a crystal structure of the hexameric human IL-6/IL-6 alpha-receptor/gp130 complex. Ten known phytoconstituents i.e., Carvacrol, p-Cymene, Dithymoquinone, Gamma himachalene, Limonene, Nigellamine C, Nigellicine, Nigellidine, alpha-Pinene, and Thymoquinone have used a ligand **(Figure 2)**. To compare them, natural standards like curcumin and synthesis standards like dexamethasone and salazosulfapyridine were used **(Figure 3)**. A flow of the process is selection, retrieving of proteins and ligands, molecular docking, and ADME study to find potential immunomodulatory phytoconstituent.

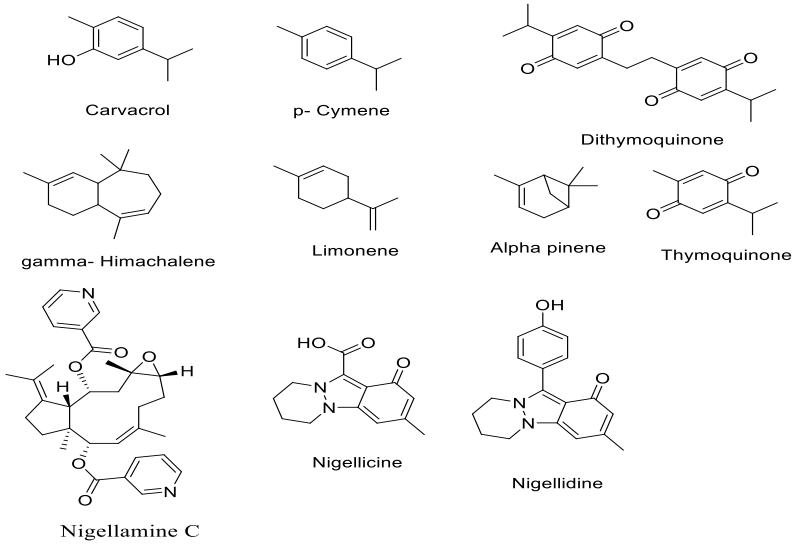
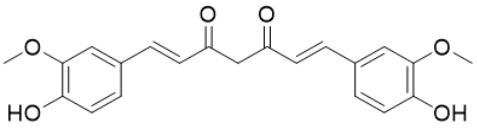
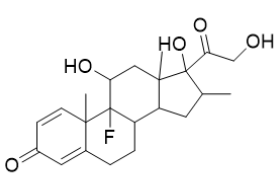


Figure 2. Selected phytoconstituents from *N. sativa*.

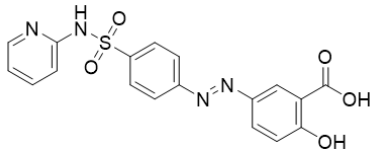


Curcumin

a) Herbal



Dexamethasone



Salazosulfapyridine

b) Synthetic

Figure 3. Structures of the standard used for comparison.

Table 1. Binding pockets of protein along with sequence

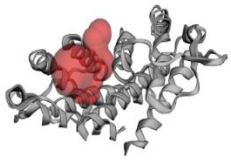
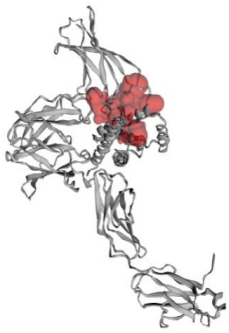
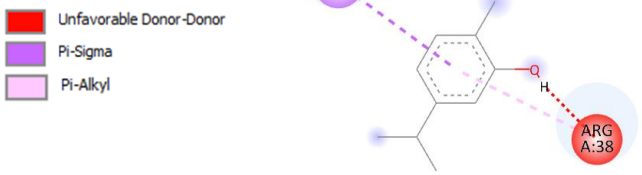
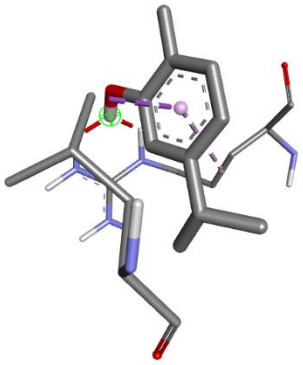
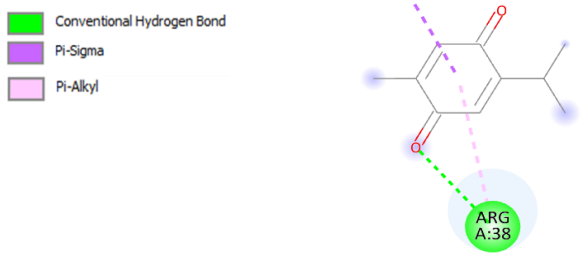
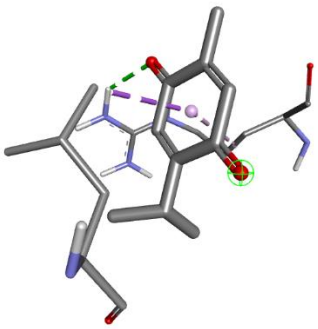
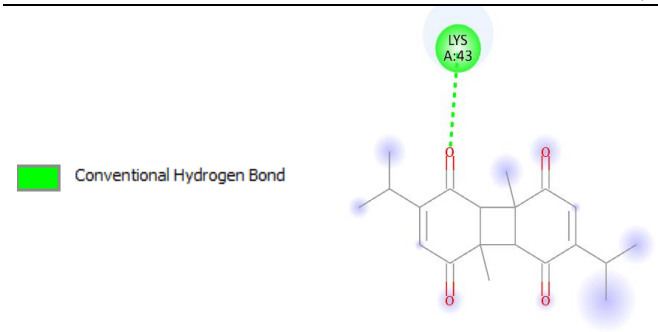
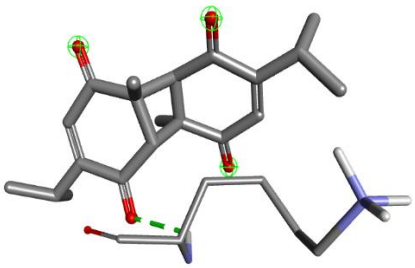
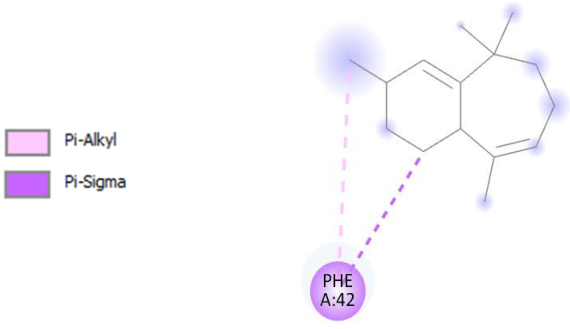
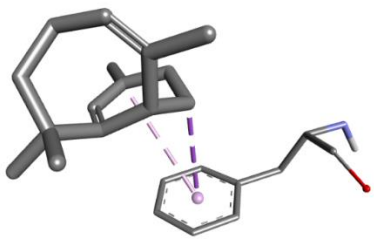
Binding pocket	Sequence
 **1M48**	Chain A APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKH LQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCEYADE TATIVEFLNRWITFCQSIISTLT Chain B APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKH LQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCEYADE TATIVEFLNRWITFCQSIISTLT
 **1P9M**	Chain A ELLDPCGYISPESPVVQLHSNFTAVCVLKEKCMDYFHVNANYIVWKTNHFTIPKE QYTIINRTASSVTFTDIASLNIQLTCNILTFGQLEQNVYGITIISGLPPEKPKNL SCIVNEGKKMRCEWDGGRETHLETNFTLKSEWATHKFADCKAKRDTPTSCTVDYS TVYFVNIEVWVEAENALGKVTSDHINFDPVYKVKPNPPHNLSVINSEELSSILKL TWTNPSIKSVIILKYNIQYRTKDASTWSQIPPEDTASTRSSFTVQDLKPFTEYVF RIRCMKEDGKGYWSDWSEEASGIT Chain B APPVPPGEDSKDVAAPHRQPLTSSERIDKQIRYILDGISALRKETCNKSNMCESS KEALAENNLNLPKMAEKDGCFQSGFNEETCLVKIITGLLEFEVYLEYLQNRFESS EEQARAVQMSTKVLIQFLQKKAKNLDAITTPDPTTNASLLTKLQAQNQWLQDMTT HLILRSFKEFLQSSLRALRQM Chain C EEPQLSCFRKSPLSNVVCEWGPRSTPSLTTKAVLLVRKFQNSPAEDFQEPCQYSQ ESQKFSCQLAVPEGDSSFYIVSMCVASSVGSKFSKTQTFQGCGILQPDPPANITV TAVARNPRWLSVTWQDPHSWNSSFYRLRFELRYRAERSKTFTTWMVKDLQHHCVI HDAWSGLRHVVQLRAQEEFGQGEWSEWSPEAMGTPW

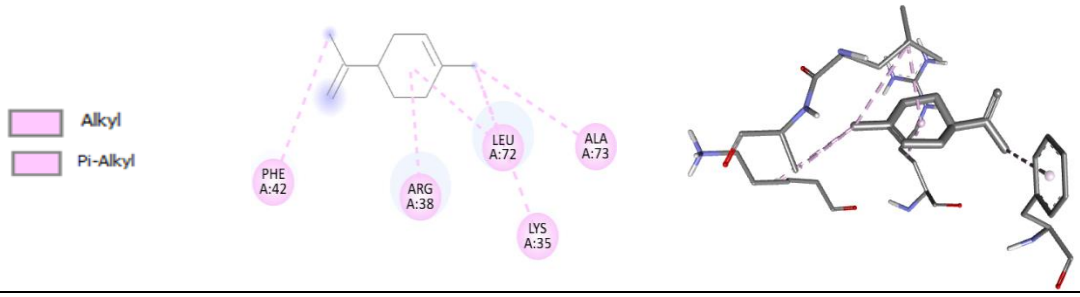
Table 1 Indicates the binding pockets in red color and highlighted residue sequence forming the binding pocket obtained from the CASTp tool [32].

The molecular docking results, including binding affinities and interactions of ten phytoconstituents and three standards, are presented in **Tables 2 and 3** to the targeted protein 1M48 and 1P9M respectively, with 2D and 3D presentation.

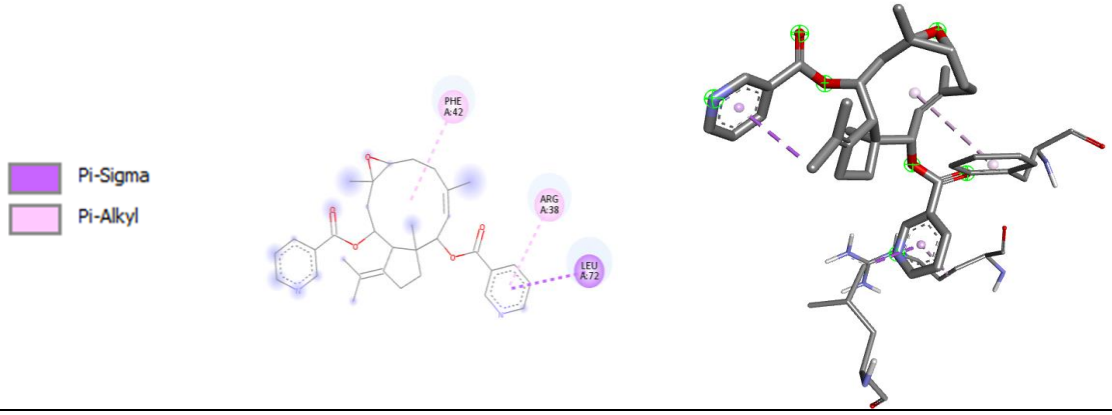
The ten phytoconstituents interacted individually with chain A 1M48 protein (presenting human IL-2) with LEU72, ARG38, ARG81, PRO82, ARG38, LYS43, PHE42, ALA73, LYS35, LYS35, MET39, and PRO65 amino acid residues. Among all the screened phytoconstituents, Nigellamine C showed a better binding affinity of -6.3 kcal/mol, which is close to the standard curcumin (-7.0 kcal/mol), and salazosulfapyridine (-7.3 kcal/mol). While alpha-pinene showed the lowest binding affinity of -4.3 kcal/mol.

Table 2. Interactions of different phytoconstituents with protein 1M48

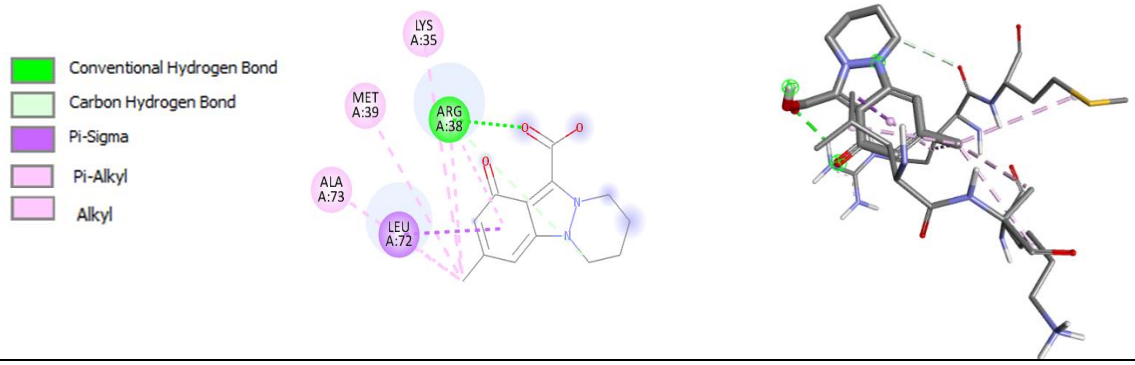
1M48 (Binding affinity)	2D PRESENTATION	3D PRESENTATION
CARVACROL (-5.6 kcal/mol)		
		
p-CYMENE (-6.0 kcal/mol)		
		
DITHYMOQUINONE (-5.8 kcal/mol)		
		
GAMMA HIMACHALENE (-5.2 kcal/mol)		
		
LIMONENE (-5.2 kcal/mol)		



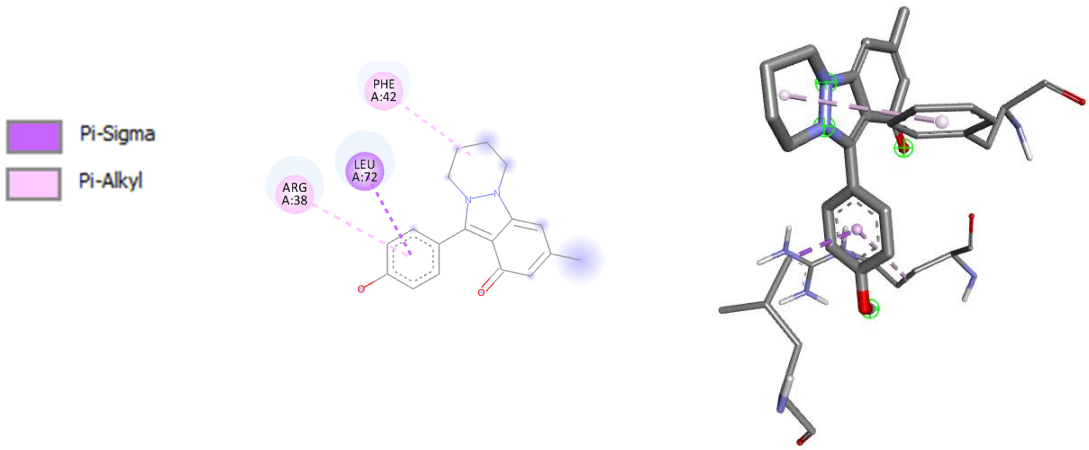
NIGELLAMINE C (-6.3kcal/mol)



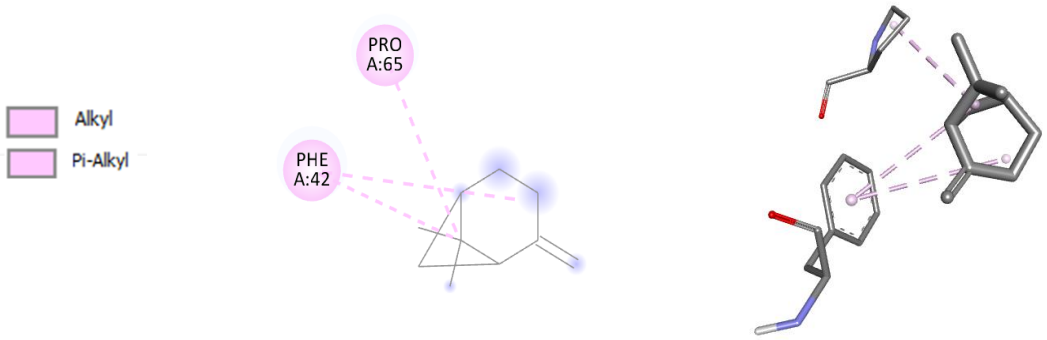
NIGELLICINE (-5.5 kcal/mol)



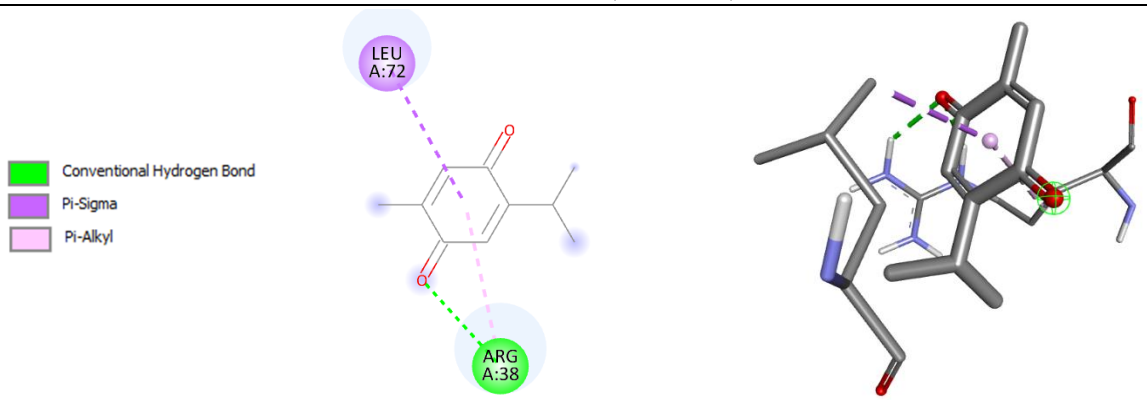
NIGELLIDINE (-5.8 kcal/mol)



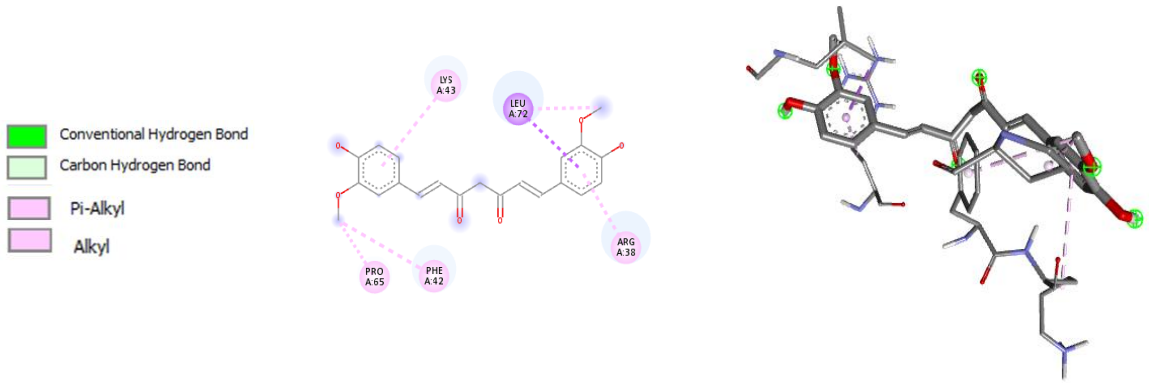
Alpha PINENE (-4.3 kcal/mol)



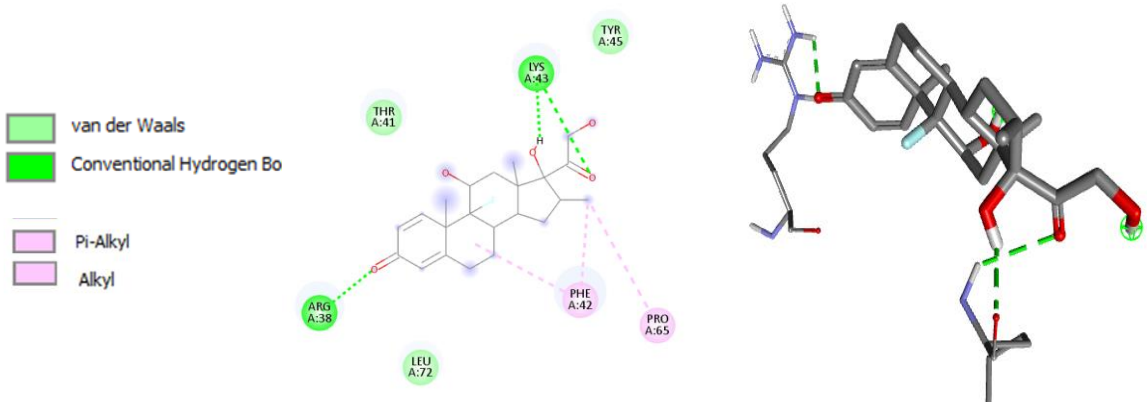
THYMOQUINONE (-6.0 kcal/mol)



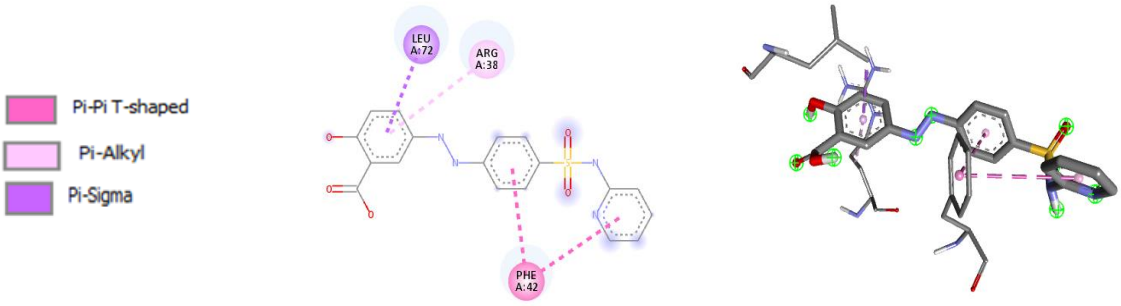
CURCUMIN (-7.0 kcal/mol)



DEXAMITHASONE (-6.2 kcal/mol)

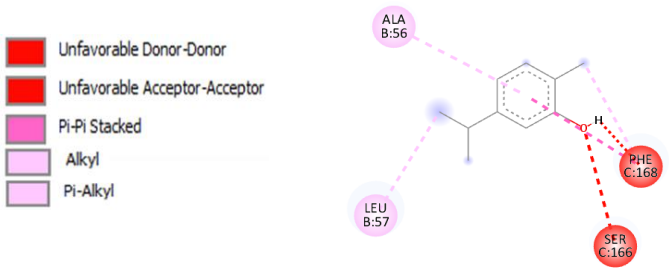
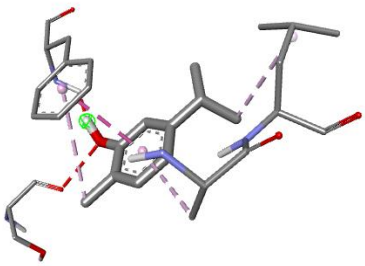
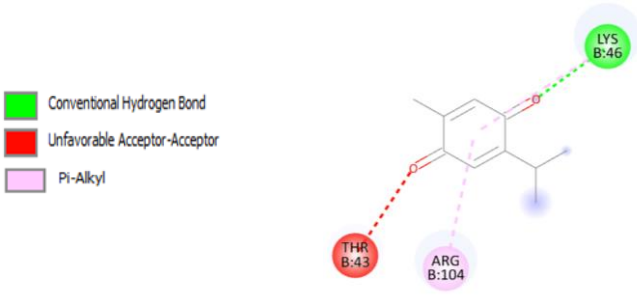
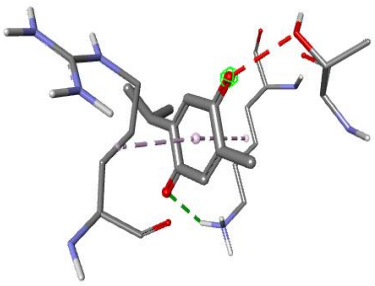
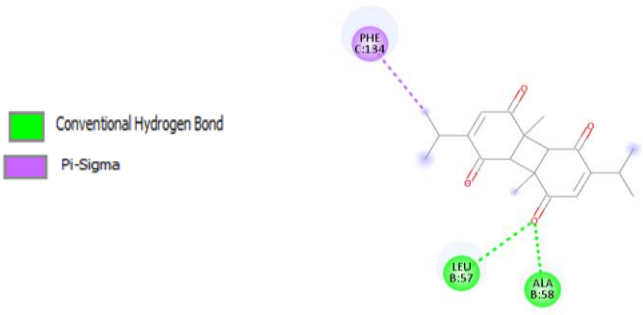
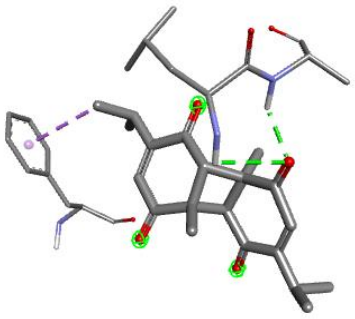


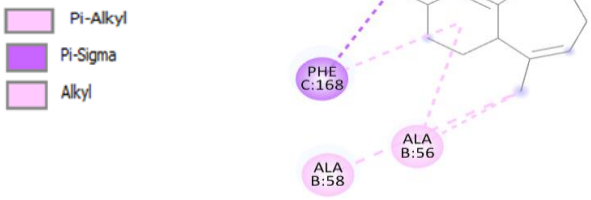
SALAZOSULFAPYRIDINE (-7.3 kcal/mol)

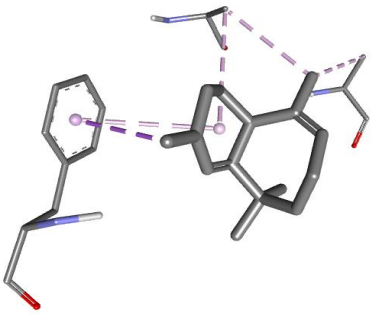


The study showed that these phytoconstituents interacted with the Chain B of 1P9M protein (presenting human IL-6) and bound with LEU57, ALA56, ALA58, LYS54, ARG168, ARG30, LEU33 amino acid residues. In addition to Chain B, these also interact with Chain C of 1P9M protein and are bound with PHE168, PHE134, GLN190, ASN136, ARG233, GLU278, and PHE279 amino acid residues. Among the all screened phytoconstituents, Nigellamine C shows the highest binding affinity of -9.8 kcal/mol, which is far better than standards like curcumin (-6.7 kcal/mol), dexamethasone (-8.1 kcal/mol) and Salazosulfapyridine (-7.7 kcal/mol). At the same time, Thymoquinone showed the lowest binding affinity of -5.1 kcal/mol **(Table 3)**.

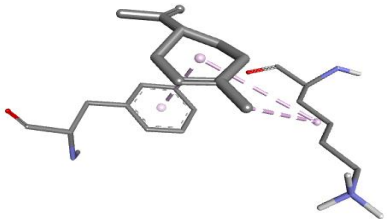
Table 3. Several ligand interactions against protein 1P9M

1P9M (Binding affinity)	2D PRESENTATION	3D PRESENTATION
CARVACROL (-5.8 kcal/mol)		
		
p-CYMENE (-5.2 kcal/mol)		
		
DITHYMOQUINONE (-7.8 kcal/mol)		
		
GAMMA HIMACHALENE (-6.8 kcal/mol)		

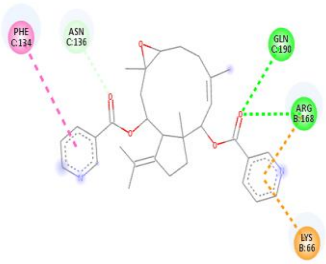


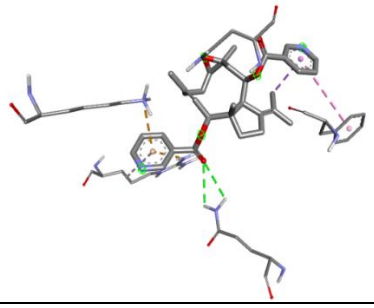


LIMONENE (-5.3 kcal/mol)

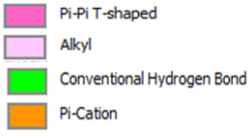


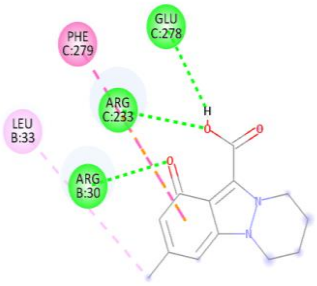
NIGELLAMINE C (-9.8 kcal/mol)

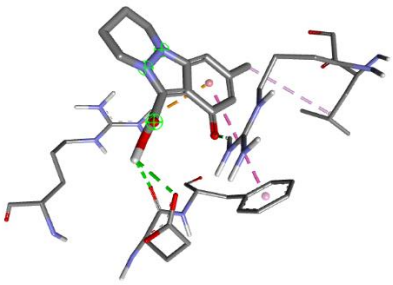




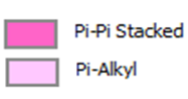
NIGELLICINE (-6.7 kcal/mol)

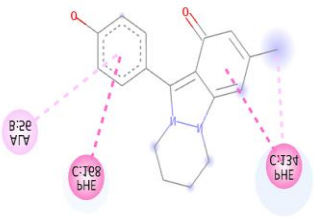


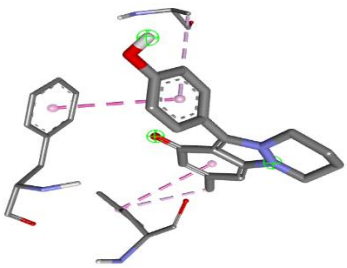




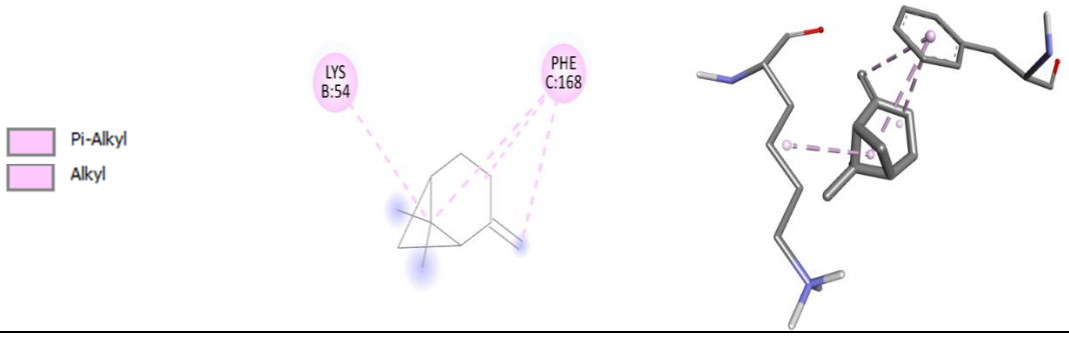
NIGELLIDINE (-7.6 kcal/mol)





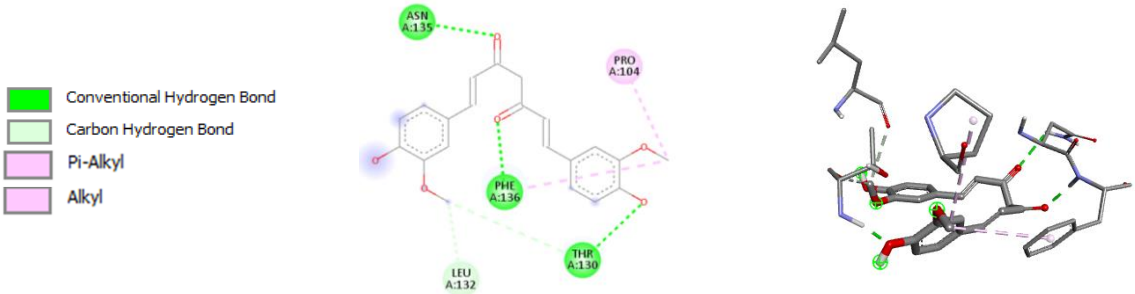


Alpha PINENE (-5.4 kcal/mol)

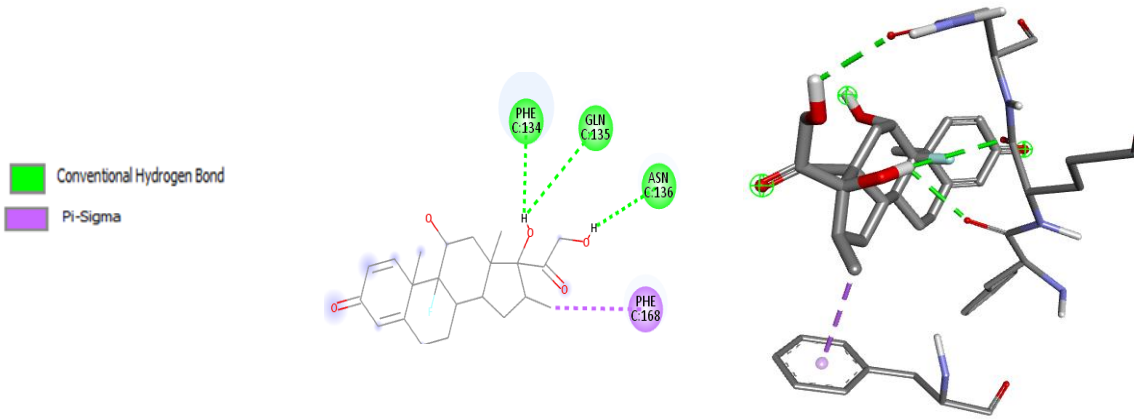


THYMOQUINONE (-5.1 kcal/mol)

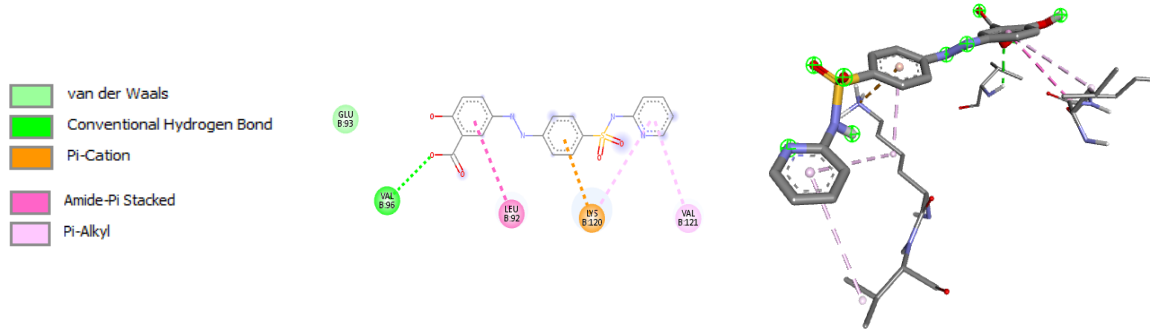
CURCUMIN (-6.7 kcal/mol)



DEXAMITHASONE (-8.1 kcal/mol)



SALAZOSULFAPYRIDINE (-7.7 kcal/mol)



A bar diagram **(Figure 4)** is drawn for a better comparison of the binding affinities of the different phytoconstituents and standards on both the IL-2 protein (1M48) and IL-6 protein (1P9M). This resulted in the most promising anti-inflammatory and immunomodulatorphytoconstituent, Nigellamine C, which could inhibit IL-2 and IL-6. Such correlation can be made from previous literature reports, suggesting the role of IL-2 and IL-6. The interleukin-2 (IL-2) formed by activated T cells through immune response helps transform naïve T cells into effector T cells [42]. It is synthesized as 153 amino acid polypeptide as a precursor and then matured IL2 which is composed of 133 amino acids with 15.5 kDa [43]. IL-2 promotes further growth and development of native immune cells and promotes survival and cell proliferation [42, 44, 45]. IL 2 is used in several inflammatory and autoimmune diseases recently, it was approved by US Food and Drug Administration (FDA) in treating melanoma and kidney cancer as immunotherapy [42, 46, 47].

Docking interactions of these phytoconstituents along with protein are presented below. **Tables 2 and 3** indicate the active site of protein 1M48 and 1P9M respectively, where a total of 10 bioactive constituents interact individually. Chain A residue of 1M48 bound with LEU72, ARG38, ARG81, PRO82, ARG38, LYS43, PHE42, ALA73, LYS35, LYS35, MET39, and PRO65. Among all the tested phytoconstituents salazosulfapyridine shows the highest (-7.3) docking score while pinene shows the lowest (-4.3) docking score presented in **Table 2**. The B chain of 1P9M bound with LEU57, ALA56, ALA58, LYS54, ARG168, ARG30, LEU33 additionally chain C bound with PHE168, PHE134, GLN190, ASN136, ARG233, GLU278, and PHE279, between all phytoconstituents nigellamine C shows the highest binding affinity (-9.8) on the other hand thymoquinone shows lowest binding affinity (-5.1) represented in **Table 3**.

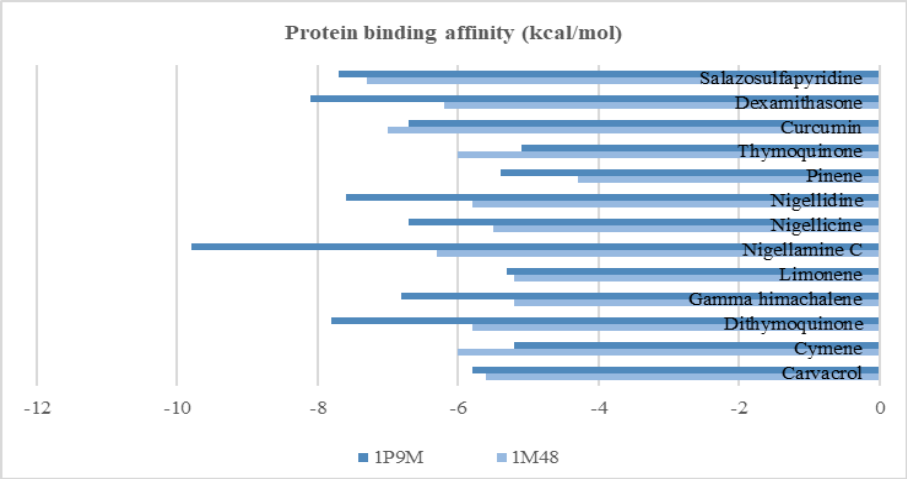


Figure 4. Protein binding affinity (kcal/mol) with active constituents of *Nigella sativa*

Majorly Interleukin-6 is secreted by leukocytes, monocytes, and macrophages. Human Interleukin 6 (IL-6) consists of 212 amino acids, 28-amino-acid signal peptides, and 21–26 kDa [48]. IL 6 is a cytokine with pleiotropic functions produced in response to tissue damage and regeneration, hematopoietic regulation, sustaining tumorigenesis, and infections [49]. IL6 acts as pro-inflammatory and anti-inflammatory as cytokine and myokine, respectively. Adding osteoblasts secrete IL-6 to stimulate bone formation while Smooth muscle cells from the middle layer of the artery secret IL6 as an anti-inflammatory myokine. Overproduction of IL6 causes the risk of inflammatory bowel disease, rheumatoid arthritis, and juvenile idiopathic arthritis [40, 41, 50].

Further after molecular docking studies, we performed the *in-silico* ADME studies of Carvacrol, p-Cymene, Dithymoquinone, Nigellicine, Nigellidine, alpha-Pinene, and Thymoquinone using the Swiss ADME (http://www.swissadme.ch/index.php) [35, 36]. The results showed that all these phytoconstituents showed no cytotoxicity and metabolic interferences with membrane receptors or Cyps. This suggests that these could be better drug candidates with good pharmacokinetic profiles. Most of these have good water solubility. Few have low GI absorption and BBB penetration. Also mostly followed Lipinski's rule of druggability.

In the present study, the phytoconstituents showed the interaction only with Chain A of 1M48 (IL-2) protein while involving Chain B and C of 1P9M (IL-6) protein. IL-2 interacts via LEU72, PHE42, and ARG38 through a hydrophobic bond, although IL-6 is bound via ARG168, GLN190, and ASN136, forming a hydrogen bond. The most promising Nigellamine C showed binding affinities of -6.3 kcal/mol and -9.8 kcal/mol with IL-2 and IL-6, respectively.

Conclusion

The present study examines the immunomodulatory potential of ten phytoconstituent of *Nigella sativa* on two immunomodulatory target proteins through *in-silico* studies, including molecular docking by AutodockVina and ADME by Swiss AMDE. These phytoconstituents were Carvacrol, p-Cymene, Dithymoquinone, Gamma himachalene, Limonene, Nigellamine C, Nigellicine, Nigellidine, alpha-Pinene, and Thymoquinone. The molecular docking and ADME studies suggested that Nigellamine C would be the most promising immunomodulator. However, further studies on these

phytoconstituents must establish a novel, safe and effective immunomodulators.

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Conflict of interest: None

Financial support: None

Ethics statement: Authers were personally involved in presented research work and take whole responsibility for its content.

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