

***Vitex negundo* Linn.- *In vitro* analysis and *In silico* approach towards SARS-CoV-2**

Submitted in partial fulfillment of the requirements for the award of

Bachelor of Technology degree in Biotechnology

by

ILMA MAJEED (Reg. No. 37230027)



**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO AND CHEMICAL ENGINEERING**

SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

**Accredited with Grade "A" by NAAC | 12B Status by UGC | Approved by AICTE
JEPPIAAR NAGAR, RAJIV GANDHI SALAI, CHENNAI - 600 119**

APRIL 2021



SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)

Accredited with "A" grade by NAAC | 12B Status by UGC | Approved by
AICTE

Jeppiaar Nagar, Rajiv Gandhi Salai, Chennai – 600 119

www.sathyabama.ac.in

DEPARTMENT OF BIOTECHNOLOGY

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of Ilma Majeed (37230027), who carried out the project entitled "*Vitex negundo* Linn.- *In vitro* analysis and *In silico* approach towards SARS-CoV-2" under my supervision from December 2020 to March 2021.

G. NARENDRA KUMAR

Internal Guide

DR RAMESH KUMAR V

Head of the Department

Submitted for Viva voce Examination held on _____

Internal Examiner

External Examiner

DECLARATION

I, Ilma Majeed, hereby declare that the Project Report entitled "*Vitex negundo* Linn.- *In Vitro Analysis* and *In Silico* approach towards SARS-CoV-2", done by me under the guidance of Mr. G. Narendrakumar, M.Tech, is submitted in partial fulfillment of the requirements for the award of Bachelor of Technology in Biotechnology.

DATE:



PLACE:

SIGNATURE OF THE CANDIDATE

ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to Board of Management of SATHYABAMA for their kind encouragement in doing this project and for completing it successfully. I am grateful to them.

I convey my thanks to Dr. V. Ramesh Kumar and Mr. G. Narendrakumar, Head of the Department, Department of Biotechnology for providing me the necessary support and details at the right time during the progressive reviews.

I would like to express my sincere and deep sense of gratitude to my Project Guide Mr. G. Narendra Kumar, M. Tech for his valuable guidance, suggestions and constant encouragement paved way for the successful completion of my project work.

I wish to express my thanks to all Teaching and Non-teaching staff members of the Department of Biotechnology who were helpful in many ways for the completion of the project.

ABSTRACT

The prime aim of this project is to study the remedial prospects in regard of SARS-CoV-2. Efforts have been directed towards excavating a therapeutic cure against the corona virus out of an array of medicinal compounds from *Vitex negundo* Linn., a large aromatic shrub, also famously called as the Chinese chaste tree. In order to obtain a potent plant isolate, rich in sanative values, the extraction conditions were optimized using Response Surface Methodology. The plant sap obtained via Soxhlet extraction using ethanol as the solvent was considered for a number of in-vitro studies. Firstly, the biomolecular assembly of the plant was analysed through the estimation of chlorophyll, carotenoid, protein, carbohydrate and lipid content. Following which, the anti-bacterial and anti-fungal properties were studied through various tests. In the light of its anti-pathogenic abilities, characterization of the plant extract has been carved out as an important step. FT-IR and GC-MS have been employed as the main techniques to elucidate the medicinal chemistry of the plant. The phytocompounds obtained thereby have been computationally optimized using iGEMDOCK and were then considered for docking against ten molecular targets of SARS-CoV-2. The results have prompted in the direction of very potent molecules against the virus which can be employed further in developing a promising cure.

Keywords: SARS-CoV-2, *Vitex negundo* Linn., Response Surface Methodology, biomolecular assembly, anti-pathogenic, FT-IR, GC-MS, iGEMDOCK

CONTENTS

CHAPTER NO.	TITLE	PAGE NO.
	ABSTRACT	i
	LIST OF ABBREVIATIONS	iv
	LIST OF FIGURES	v
	LIST OF TABLES	vii
1	INTRODUCTION	1
2	LITERATURE SURVEY	2
2.1	SARS-CoV-2 Global Update	2
2.2	Plant-Based Approach	3
2.3	<i>Vitex negundo</i> Linn.	4
2.4	Optimisation of Extraction Conditions	5
2.5	Evaluation of Phytochemical Activities	6
2.6	Characterization of Phytochemicals	6
2.7	ADME studies	7
2.8	Molecular Docking	8
3	AIM AND SCOPE OF THE PRESENT INVESTIGATION	9
3.1	Aim	9
3.2	Scope of the present investigation	9
4	EXPERIMENTAL MATERIALS AND METHODS USED	10
4.1	Materials required	10
4.2	Methodology	10
	4.2.1 Response Surface Methodology- Experimental design	10
	4.2.2 Sample collection	13
	4.2.3 Soxhlet Extraction	13
	4.2.4 Total Chlorophyll Content (TCH)	13
	4.2.5 Estimation of protein	14
	4.2.6 Test for carbohydrates – qualitative method	14

4.2.7	Estimation of lipids	14
4.2.8	Assessment of invitro antimicrobial activity	15
4.2.8.1	Microbial Source	15
4.2.8.2	Anti-bacterial activity	15
4.2.8.3	Anti-fungal activity	15
4.2.9	FT-IR	16
4.2.10	GC-MS	16
4.2.11	Docking	17
4.2.11.1	Ligand selection	17
4.2.11.2	Preparation of both enzymes and ligands	17
4.2.11.3	Molecular Docking	18
5	RESULTS AND DISCUSSION	19
5.1	Optimisation of the extraction process	19
5.1.1	Response 1	19
5.1.2	Response 2	23
5.1.3	Response 3	26
5.2	Phytochemistry	30
5.3	Antimicrobial Activity	33
5.3.1	Sensitivity Disc Pattern Using Standards	33
5.3.2	Antibacterial Activity – Using Extract	36
5.4	FT-IR	37
5.5	GC-MS	39
5.6	Swiss-ADME results	40
5.7	Docking	50
6	SUMMARY AND CONCLUSIONS	64
6.1	Summary	64
6.2	Conclusions	65
	REFERENCES	67

LIST OF ABBREVIATIONS

VN	-	<i>Vitex negundo</i>
SARS-CoV-2	-	Severe Acute Respiratory Syndrome Coronavirus-2
GI Abs	-	Gastro-intestinal Absorption
BBB Per	-	Blood Brain Barrier Permeant
P-gp Sub	-	P glycoprotein substrate
Cyt. Inh	-	Cytochrome P450 Inhibitor
LR Vio	-	Lipinski Rule Violation
RSM	-	Response surface methodology
DoE	-	Design of Experiments
ANOVA	-	Analysis of variance

LIST OF FIGURES

- Fig: 4.2.2.1 *Vitex negundo* Linn
- Fig: 5.1.1.1 Surface plot: Relationship between solvent concentration (A) and extraction time (B) for Antioxidant activity
- Fig: 5.1.1.2 Surface plot: Relationship between solvent concentration (A) and extraction temperature (C) for Antioxidant activity
- Fig: 5.1.1.3 Surface plot: Relationship between extraction time (B) and extraction temperature (C) for Antioxidant activity
- Fig: 5.1.2.1 Surface plot: Relationship between solvent concentration (A) and extraction time (B) for phenolic content
- Fig: 5.1.2.2 Surface plot: Relationship between solvent concentration (A) and extraction temperature (C) for phenolic content
- Fig: 5.1.2.3 Surface plot: Relationship between extraction time (B) and extraction temperature (C) for phenolic content
- Fig: 5.1.3.1 Surface plot: Relationship between solvent concentration (A) and extraction time (B) for flavanoids concentration
- Fig: 5.1.3.2 Surface plot: Relationship between solvent concentration (A) and extraction temperature (C) for flavanoids concentration
- Fig: 5.1.3.3 Surface plot: Relationship between extraction time (B) and extraction temperature (C) for flavanoids concentration
- Fig: 5.3.1.1 Antibacterial Activity
- Fig: 5.3.1.2 Antibiotic Sensitivity Pattern- Bacteria
- Fig: 5.3.1.3 *Aspergillus niger*
- Fig: 5.4.1 FT-IR Graph
- Fig: 5.5.1 GC-MS Graph
- Fig: 5.6.1 Benzoic acid, 2,4, dimethoxy 6 methyl (8,8 dimethoxy - 2-octyl)ester
- Fig: 5.6.2 Methoxyacetic acid, octadecyl ester
- Fig: 5.6.3: Methoxyacetic acid, heptadecyl ester
- Fig: 5.6.4 Despropionyl -3- methylfentanyl
- Fig: 5.6.5 Nonadecane -2,4 dione
- Fig: 5.6.6 Quinoxaline 2-isopropyl-3-phenyl 4-oxide
- Fig: 5.6.7 Piperidin 4-one, 1-(1-phenylcyclohexyl)

Fig: 5.6.8	2(1H) phenanthrenone, 3,4 4a tetrahydro-1,1 4a trimethyl
Fig: 5.6.9	Flavone
Fig: 5.6.10	2-(3-(N- aziridyl)propyl)aminomethyltetrahydropyran
Fig: 5.6.11	Tricyclo[4.4.0.0(3,9)]decane
Fig: 5.6.12	Tetrazolo(1,5-a) pyridine 5,6,7,8 tetrahydro
Fig: 5.7.1	6X6P
Fig: 5.7.2	6M2Q
Fig: 5.7.3	7BW4
Fig: 5.7.4	1R42
Fig: 5.7.5	6CS2
Fig: 5.7.6	6MOJ
Fig: 5.7.7	5X29
Fig: 5.7.8	3I6G
Fig: 5.7.9	3F9G
Fig: 5.7.10	6W75
Fig:5.7.11	Docking in progress on iGEM DOCK

LIST OF TABLES

Table 4.2.1.1	Experimental values and coded levels of the independent variables.
Table 4.2.1.2	Design Summary
Table 5.1.1.1	Experimental runs for Response 1
Table 5.1.1.2	ANOVA for Response Surface Quadratic Model
Table 5.1.2.1	Experimental runs for Response 2
Table 5.1.2.2	ANOVA for Response Surface Quadratic Model
Table 5.1.3.1	Experimental runs for Response 3
Table 5.1.3.2	ANOVA for Response Surface Quadratic Model
Table 5.2.1	Chlorophyll and carotenoid content- Standard values
Table 5.2.2	Total chlorophyll and carotenoid content (TCH)
Table 5.2.3	Estimation of protein – standard values
Table 5.2.4	Estimation of Protein - Sample
Table 5.2.5	Estimation of Carbohydrates– standard values
Table 5.2.6	Estimation of Carbohydrates - Sample
Table 5.2.7	Estimation of Lipid Content
Table 5.3.1.1	Antibiotic sensitivity pattern - Bacteria
Table 5.3.1.2	Antibiotic sensitivity pattern – Fungi
Table 5.3.2.1	Antimicrobial activity
Table 5.4.1	FT-IR
Table 5.5.1	GC-MS
Table 5.6.1	Phytochemicals – SMILES and PubChem CID
Table 5.6.2	Comparison of ADME profile of different phytochemicals
Table 5.6.3	Shortlisted phytochemicals as the final ligands for docking
Table 5.7.1	SARS-CoV-2 target proteins
Table 5.7.2	Docking results of SARS-CoV-2 spike protein (6X6P) with the ligand molecules
Table 5.7.3	Docking results of SARS-CoV-2 3CL protease (3CL pro) apo structure (6M2Q) with the ligand molecules
Table 5.7.4	Docking results of RNA-dependent RNA polymerase (7BW4) with the ligand molecules

Table 5.7.5	Docking results of Angiotensin Converting Enzyme 2 (1R42) with the ligand molecules
Table 5.7.6	Docking results of SARS Spike Glycoprotein – human ACE2 complex (6CS2) with the ligand molecules
Table 5.7.7	Docking results of Native Spike Protein (6MOJ) with the ligand molecules
Table 5.7.8	Docking results of Envelope Protein (5X29) with the ligand molecules
Table 5.7.9	Docking results of Membrane Protein (3I6G) with the ligand molecules
Table 5.7.10	Docking results of Chymotrypsin-like cysteine protease/ main protease (Mpro)/ 3CLike protease (3F9G) with the ligand molecules
Table 5.7.11	Docking results of Nonstructural protein 9 (6W75) with the ligand molecules
Table 5.7.12	Best docking results

CHAPTER 1

INTRODUCTION

As the brilliant brains across the world continue to further deepen the understanding of COVID-19, its epidemiological characteristics and possible avenues for treatments, a probable addendum might be useful in this pursuit. The current work is an attempt to analyze in-vitro results in the context of a statistical background. Detailed study on *Vitex negundo* Linn, an aromatic shrub, has helped in exploring the biomolecular scenery of the plant with respect to SARS-CoV-2. Though, plants have a multifarious combination of chemical ingredients but they vary significantly, due to many genetic and environmental factors, method of cultivation, time of collection, post-harvest processing, etc. (Khare, 2004). This inherent variability in the chemistry may adversely impact the efficacy of medicinal plants (Sivarajan and Balachandran, 1994). Therefore, RSM optimized *Vitex negundo* extract has been characterized to unravel the components harboring its chemical arena. Analysis of its protein, carbohydrate and lipid content has thrown immense light on the bulk organics while testing of anti-microbial and anti-fungal activities of the plant isolate has further proved its curing potential. Scientific reports avidly indicate the implications of *Vitex negundo* as a promising therapeutic candidate for a variety of disease conditions. All parts of the plant find usage in relation to a wide spectrum of health issues (Basri et al., 2014). It is a commercial crop in parts of Asia, Europe, North America and West Indies and is majorly used in the treatment of asthma, cancer (Najmi et al., 2015), liver disorders and many others (Muthuswamy et al., 2012). Recently, it has also been employed in order to prepare nano materials (Balasooriya et al., 2017; Menaka Thounaojam et al., 2012). In this study, efforts have been made to understand the feasibility of phytochemicals to be promising in regard of COVID-19. Listed biological targets of SARS-CoV-2 have been instrumental in providing information about the virus and its characteristic components. These targets have been docked against the characterized compounds of *Vitex negundo* Linn. to establish a picture of favorable outcomes. The result and discussion offer an encouraging milestone in this global fight against the pandemic.

CHAPTER 2

LITERATURE SURVEY

2.1 SARS-COV-2 GLOBAL UPDATE

Epidemiological updates from WHO have brought the cumulative numbers of the confirmed COVID-19 cases to 61.8 million till December 1, 2020. The pandemic has claimed upto 1.4 million deaths globally and the number is still on the rise. The vaccine development schemes employed by the scientists to curb the further spread of the disease are no doubt making use of all the latest techniques and procedures to better understand the disease and its causative agent. Studies reveal that SARS-CoV-2 belongs to the family of Coronaviruses. These pathogens are single-stranded positive-sense RNA viruses that possess large viral RNA genomes. Various research tactics employed recently show that SARS-CoV-2 has a genomic organization similar to that of other beta-coronaviruses (Mitra D et al., 2021). It consists of a 5'-untranslated region (UTR), a replicase complex (orf1ab) encoding non-structural proteins (nsps), envelope protein (E) gene, a spike protein (S) gene, a nucleocapsid protein (N) gene, a membrane protein (M) gene, 3'-UTR, and several unidentified non-structural open reading frames. Even though SARS-CoV-2 has been classified into the beta-coronaviruses group, it is different from MERS-CoV and SARS-CoV. Recent studies have highlighted that the genes of SARS-CoV-2 share less than 80% nucleotide identity and 89.10% nucleotide similarity with SARS-CoV genes. Beta-coronaviruses usually produce around 800 kDa polypeptide upon transcription of the genome. This polypeptide is proteolytically cleaved by an enzymes - papain-like protease (PL^{pro}) and 3-chymotrypsin-like protease (3CL^{pro}), which gives rise to various proteins. The 3CL^{pro} cleaves the polyprotein at 11 distinct sites to generate various non-structural proteins that play a role in viral replication. 3CL^{pro} plays a critical role in the replication of virus particles and is a potential target for anti-coronaviruses inhibitors screening because unlike structural and accessory protein-encoding genes, it is located at the 3' end which exhibits excessive variability. Structure-based activity analyses and high-throughput studies have identified potential inhibitors for SARS-CoV and MERS-CoV 3CL^{pro}.

The most eminent target proteins associated with the virus have been identified to be, SARS-CoV-2 spike (S) protein (Kothandan et al., 2021), SARS-CoV-2 3CL protease (3CL pro) apo structure 9 (Li et al., 2020), RNA-dependent RNA polymerase from SARS-CoV-2], Angiotensin Converting Enzyme 2 (ACE2), SARS Spike Glycoprotein – human ACE2 complex, Native Spike Protein (S), Envelope Protein (E), Membrane Protein (M), Chymotrypsin-like cysteine protease/ main protease (Mpro) / 3CLike protease, Nonstructural protein 9 (NSP 10) (Prajapat et al., 2020; Salvatori et al., 2020; Mitra et al., 2021). Studies are currently undertaken to study the individual function of these proteins and computationally evaluate the effects of various molecules to inhibit them.

2.2 PLANT BASED APPROACH

Nature acts as a reservoir of different resources of chemovar and pharmacophores. The naturally available resources provide an alternative to modern medicine in drug discovery which act as a suitable concomitant of that contrition which befits the current scenarios of our dependence on artificial medicinal regimes. Traditional medicine offers the most inexpensive and easily reachable source of the dealing in the primary healthcare with an astounding and ever-increasing number of plant species being already recognized for medical use globally, known to possess antimicrobial, anti-inflammatory, antioxidant, antiallergic, antidiabetic, antimalarial, anthelmintic, anticancer effects (Rampriya et al., 2017)

There is a global shift towards herbal formulations against a large repertoire of dreaded diseases and ailments. This scene can be reasoned well to the fact that the plants are known to cause notably no derogatory effects; and are readily available at affordable prices. But, to effectively use plant remedies without side-effects, they need to be selected appropriately and taken under proper medical supervision. The active component, which many a times is a secondary metabolite, varies in quality and quantity for a given plant species growing in different locations. The market value of such plants is dependent on their active content rather than merely their luxuriant growth.

2.3 *Vitex negundo* Linn.

To date, several molecules of natural origin, more specifically from the plants, have been screened in the quest of understanding their behavior with the target proteins of SARS-CoV-2. Medicinal plants, especially those mentioned in traditional Chinese medicine, have attracted noteworthy attention in the treatment prospects of SARS-CoV-2 because they include bioactive compounds that could be used to develop formal drugs against several known diseases with no or minimal side effects. The prime focus of my current study is about a plant which has been designated as the gift of nature to mankind owing to its multifarious medicinal uses. Five leaf caste tree (*Vitex negundo* Linn.) belongs to the Verbenaceae family, comprises 75 genera and approximately 2500 species and is commonly synonymized as Simali in Nepali, Nirgandi (Hindi), and Nirgundi (Sanskrit). It has been identified by various names in different states viz. Sambhaal in Himachal Pradesh; Somi, mala, nirgundi in Uttar Pradesh; notchi in Tamil Nadu. It is a deciduous shrub which mainly occurs in tropical to temperate regions (up to 2200 m from east to west). The plant has been studied to have originated from tropical Eastern and Southern Africa and Asia, it is also found in Afghanistan, Bangladesh, India, Bhutan, Pakistan, Myanmar, Malaysia and Nepal.

Ethnobotanically, it is believed that *Vitex negundo* (VN) is employed for the treatment of jaundice, wounds, body ache, toothache, asthma, eye pain, migraine amongst various others in line of research. *Vitex negundo* bark has found usage as verminosis and ophthalmopathy, while flowers are effective in case of cholera and the fruit has anthelmintic properties. The entire plant has been found to be effective against diuretic inflammations, fever and septic infections. Such diverse range of *Vitex negundo* applications are because of its rich biomolecular assembly of metabolites; polyphenolic compounds, terpenoids, glycosidic iridoids and alkaloids to name a few. Its leaves generally consisting of viridiflorol; β -caryophyllene; sabinene; 4-terpineol; gamma-terpinene; caryophyllene oxide; 1-octen-3-ol; globulol, angusid; casticin; vitamin-C; nishindine; gluco-nonitol; p-hydroxybenzoic acid; sitosterol. This plant also encompasses molecules like vetuginoside, negundoside, 5 hydroxy-7,4'-dimethoxy flavones. The seeds of VN have been studied to contain 3 β -acetoxylean-12-en-27-oic acid; 2 α , 3 α -dihydroxyleana-5,12-dien-28-oic acid; 2 β ,3 α diacetoxyleana-5,12-dien-28-oic

acid; 2 α , 3 β -diacetoxy-18-hydroxyoleana-5,12-dien-28-oic acid, vitedoin-A; vitedoin-B; a phenylanthralene-type lignan alkaloid, vitedoamine-A; five other lignan derivatives and many more. 2 β , 3 α -diacetoxyoleana-5,12-dien-28-oic acid; 2 α ,3 α -dihydroxyoleana-5,12-dien-28-oic acid; 2 α ,3 β -diacetoxy-18-hydroxyoleana-5,12-dien-28-oic acid; vitexin and isovitexin, acetyl oleanolic acid; sitosterol; 3-formyl-4,5-dimethyl-8-oxo-5H-6,7-dihydronaphtho (2,3-b) furan are important components of the roots of the plant. Essential oil of fresh leaves, flowers and dried fruits contain caryophyllene epoxide; (E)-nerolidol; β -selinene; α -cedrene; germacrene D; hexadecanoic acid; p-cymene and valencene among a lot more components. To the polyphenolic compounds present in this plant, a high antioxidant potential has been proposed which is investigated thoroughly by applying multifariously recognized in vitro systems. Besides the above referred bioactive effects, *Vitex negundo* has also been studied to show anti-HIV, larvicidal, and mosquito repellent activity. In this sense, the present study is aimed to characterize the phytochemical and nutritional composition of VN, and to assess their antimicrobial effects and to perform statistical study on the plant with respect to the most common targets of SARS-CoV-2 (Saklani et al., 2017).

2.4 OPTIMISATION OF EXTRACTION CONDITIONS

Extraction of various phytochemical compounds from *Vitex negundo* plant has been performed utilizing a variety of procedures (Kumar et al., 2010). Among them, Soxhlet extraction using ethanol as the solvent was found to be the best in regard of obtaining the maximum plant extract. The fixation of extraction conditions of phytochemical compounds is a highly crucial process for the evaluation and determination of the quantity and type of bioactive compounds (Zargar et al., 2012), each harbouring different therapeutic properties that will be extracted out. According to Aybastier et al. (2013), a lot of factors contribute to the efficiency of extractions, viz. the type of solvent, the concentration of solvent, time, temperature, solid–liquid ratios and pH. Response surface methodology (RSM) is a powerful mathematical technique being widely employed in many industries for a lot of technological operations to optimize the experimental conditions. RSM has also been found to be useful to maximize or minimize various independent

variables as it evaluates the effects and behaviour of multiple factors and their respective interactions on one or more than one response variables simultaneously. Also, RSM not only serves as a visual aid to obtain a clearer view about the effects of various factors on extraction but is also helpful in the location of the region where the extraction is optimized.

Therefore, optimizing the extraction conditions (solvent concentration, time and temperature) using RSM, to obtain the highest amount of phyto-compounds, more preferably the anti-oxidizing, phenolic, flavonoid compounds, because of their high medicinal value, could aid in obtaining better results.

2.5 EVALUATION OF PHYTOCHEMICAL ACTIVITIES

The plant extract obtained via Soxhlet extraction has to be subjected to a variety of in-vitro tests that could help in the analysis of the activity of the secondary metabolites. The various effects under consideration include the estimation of the chlorophyll content and the amount of biomolecules present in the extract. For this purpose, the standard methods introduced by eminent scientists such as estimation of the chlorophyll content using Arnon method (Arnon, 1949), and the implications of standardized reagents to test the amount of protein, carbohydrate and lipid contents could provide with better analysis results for further studies.

Following the content evaluation, the anti-bacterial and anti-fungal activity testing has been found to be correlated with their performance against other pathogens. For this purpose, all the assays were subjected to the necessary quality control procedures recommended by Clinical Laboratory Standard Institute (CLSI) as stated by Wikler et al. (2006).

2.6 CHARACTERIZATION OF PHYTOCHEMICALS

Anti-microbial analysis throws some light on the working ability of the plant metabolites which need to be further characterized (Rooban et al., 2012). The

characterization study of the biomolecules present in *Vitex negundo* could be performed via a number of techniques, including UV-visible spectroscopy, Scanning Electron microscopy (SEM), Fourier Transmission Infrared Spectroscopy (FTIR), X-ray Diffraction (XRD). According to Bunaciu et al. (2012), FTIR has been found to be the most feasible technique which eminently works towards providing information about the major functional groups present in the extract under study. Further, Gas Chromatography Mass Spectroscopy is a hyphenated system which is a very compatible technique and the most widely used one for the purpose of identification and quantification. The organics in a complex mixture consisting of unknown compounds can be determined by interpretation and also by matching the obtained spectra with reference spectra. GC-MS method used for the analysis of the obtained extracts can be an interesting tool for testing the amount of some active principles in herbs used in cosmetic, drugs, pharmaceutical or food industry (Monisha et al., 2016). The characterized biomolecules present an array of tools which if utilized effectively can pave way for novel therapeutics. Owing to the current developments, a lot of computational techniques are employed so that refined drug candidates can be obtained following which their interactions could be studied in relation to the target proteins of SARS-CoV-2 virus through molecular docking (Vora et al., 2019).

2.7 ADME STUDIES

In order to understand the working of the metabolites of *Vitex negundo* plant, it is essential to study the extracted compounds in detail. The profile of the pharmacokinetic parameters, which include absorption, distribution, metabolism, excretion and toxicity (ADMET) is of colossal importance in the drug industry and is commonly employed in Computer-aided Drug Designing procedures. to eventually help in mitigating unwanted effects. The ADME (Rolta et al., 2020) properties of the phyto-compounds of the plant extract calculated using Swiss ADME tool ([http://www. swissadme.ch/](http://www.swissadme.ch/)) can throw some light on the drug-like properties of the biomolecules under study. Through the understanding of ADME profile, a lot of important compounds could be short-listed, which can later be

evaluated on the lines of being potential ligand molecules against a variety of disease conditions including COVID-19.

2.8 MOLECULAR DOCKING

Molecular docking approach is a method to screen potential drug compounds among the phytochemicals or ligand molecules against disease causing target proteins. The important targets of SARS-CoV-2 which have been discussed so far include SARS-CoV-2 spike (S) protein (Kothandan et al., 2021), SARS-CoV-2 3CL protease (3CL pro) apo structure 9 (Li et al., 2020), RNA-dependent RNA polymerase from SARS-CoV-2], Angiotensin Converting Enzyme 2 (ACE2), SARS Spike Glycoprotein – human ACE2 complex, Native Spike Protein (S), Envelope Protein (E), Membrane Protein (M), Chymotrypsin-like cysteine protease/ main protease(Mpro)/ 3CLike protease, Nonstructural protein 9 (NSP 10). Once the phytochemicals have been docked against these target proteins, the data could be immensely valuable in the quest of finding effective cure against the most dreaded disease currently on calendar. The binding efficiency of the metabolites of *Vitex negundo* plant against the eminent proteins of SARS-CoV-2 when studied using iGEMDOCK module could be very beneficial in finding the appropriate orientation of the docked molecules. The total energy provided by the software is very useful in analyzing further the compatibility of the ligands with the target proteins (Balavignesh et al., 2013).

CHAPTER 3

AIM AND SCOPE OF THE PRESENT INVESTIGATION

3.1 AIM

To characterize various phytochemicals from *Vitex negundo* Linn. plant and perform in-silico studies with respect to SARS-CoV-2.

3.2 SCOPE OF THE PRESENT INVESTIGATION

The present study is conducted to gain structural insights into the SARS-CoV-2 marker proteins and to discover potent anti-COVID-19 natural compounds of *Vitex negundo* plant extract following a computational approach.

CHAPTER – 4

EXPERIMENTAL MATERIALS AND METHODS USED

4.1 MATERIALS

- 1) Leaves of *Vitex negundo* Linn.
- 2) Basic microbiological equipments-
 - Slides,
 - test tubes,
 - Petri dishes,
 - Growth mediums,
 - Inoculation loops,
 - Pipettes,
 - incubator,
 - autoclave,
 - laminar flow hoods
- 3) Soxhlet apparatus
- 4) UV Spectrophotometer
- 5) Softwares-
 - Design-Expert v.7
 - SwissADME,
 - iGEMDOCK,
 - UCSF Chimera

4.2 METHODS

4.2.1 Response Surface Methodology- Experimental design

A 2³-full factorial, central composite design (CCD) was employed in order to identify the relationship between the response functions and the process variables as well as to determine the conditions necessary to optimize the process of solvent extraction. The three independent factors or variables which were studied

include solvent extraction [X1, varying between 70 and 90% (v/v)], extraction time (X2, varying between 80 and 120 minutes) and extraction temperature (X3, varying between 40 and 60°C). Each variable to be optimized was coded at two levels, -1 and 1.

Table 4.2.1.1: Experimental values and coded levels of the independent variables.

Factor	Name	Units	Type	Low Actual	High Actual	Low Coded	High Coded
A	Solvent concentration	%	Numeric	70	90	-1	1
B	Extraction time	min	Numeric	80	120	-1	1
C	Extraction temperature	°C	Numeric	40	60	-1	1

The three independent variables were fitted into the given second-order polynomial model:

$$Y = b_0 + \sum b_i A_i + \sum b_{ii} A_i^2 + \sum b_{ij} A_i A_j$$

Where,

- Y represents response variable
- A_i represents the independent variables, and
- b_0 , b_i , b_{ii} and b_{ij} represent the regression coefficients for all the three terms (intercept, linear and quadratic)

Design-Expert v.7 version (Stat-Ease, Minneapolis, MN, USA) was used for performing CCD while ANOVA was applied for statistical testing of the obtained results at the significance level of 0.05. Three—dimensional plots were used in order to describe the relationship between the obtained results and the extraction parameters.

Data from the CCD were subjected to regression analysis using least-square regression methodology to obtain the parameters of the mathematical models. In order to check the statistical significance of the regression coefficients derived from the model, Student's t-test was employed. Analysis of variance (ANOVA) was applied to evaluate the statistical significance of the model. Surface plots that indicate the response as a function of the simultaneous variation of the independent variables were obtained using the fitted model.

Table 4.2.1.2: Design Summary

Study Type		Response Surface			Runs			17	
Initial Design		Box- Behnken			Blocks			No Blocks	
Design Model					Quadratic				
A	Solvent concentration	%	Numeric	70	90	-1	1	80	6.8599
B	Extraction time	min	Numeric	80	120	-1	1	100	13.72
C	Extraction temperature	C	Numeric	40	60	-1	1	50	6.8599

Y1	Antioxidant activity	IC50	17	Polynomial	5.8	20.9	12.164706	5.53778	3.6034	None	Quadratic
Y2	Phenolic content	GAE	17	Polynomial	49.8	182.5	106.94706	51.2941	3.6647	None	Quadratic
Y3	Flavonoids concentration	GAE	17	Polynomial	48.1	166.7	98.317647	45.6139	3.4657	None	Quadratic

4.2.2 Sample collection

Vitex negundo plants were collected from the Kukrail forests of Lucknow, Uttar Pradesh. The leaves were sun-dried for 3 days and the powdered form of the leaf samples were eventually obtained.



Fig: 4.2.2.1: *Vitex negundo* Linn.

4.2.3 Soxhlet Extraction

Exhaustive Soxhlet extraction was performed using classical apparatus with accurately weighed 50 g of the powdered leaf samples respectively (Chawla et al., 1992). Extraction was performed with 80% ethanol as the extraction solvent. After extraction, the ethanol solvent was evaporated by concentrating under vacuum with rotary evaporator at 50°C under reduced pressure. The extraction time was 100 minutes. These optimised parameters of solvent concentration, extraction temperature and extraction time have been obtained with the help of Response Surface Methodology in order to get the most efficient extract. The solvent free ethanol extract was thereafter evaluated (Stanley Irobekhan et al., 2016).

4.2.4 Total Chlorophyll Content (TCH)

This test carried out according to the method described by Arnon, (1949). 3g of fresh leaves were blended and then extracted with 10ml of 80% acetone and left for 15 minutes for thorough extraction. The liquid portion decanted into another test-tube and centrifuged at 2,500rpm for 3 minutes. The supernatant was then collected and the absorbance taken at 645nm and 663nm for chlorophyll a, b and 480, 510 nm for carotenoid using a spectrophotometer (Hanuman Prasad Sharma et al., 2014).

Calculations were done using the formula below.

Chlorophyll a (mg/ml) = 12.7 (A663) - 2.69 (A645) * final volume in ml

Chlorophyll b (mg/ml) = 22.9 (A645) - 4.68 (A663) * final volume in ml

Chlorophyll c (mg/ml) = 20.2 (A645) + 8.02 (A663)

$$\text{Tch: } 0.1 \text{ CT} \times \frac{\text{leaf dry weight}}{\text{leaf fresh weight}}$$

$$\text{Carotenoid} = 7.6 \times (\text{OD480}) - 1.49 \times$$

TCh = Chlorophyll a + b mg/g

Dx = Absorbance of the extract at the wavelength Xnm.

V = Total volume of the chlorophyll solution (ml)

W = Weight of the tissue extracted (g)

4.2.5 Estimation of protein

10µl of the sample and 190µl of working Biuret Reagent were added to 96 well plate. Then the plates were incubated for 20 minute at room temperature. In the meantime, the spectrophotometer was turned on to allow it to warm up and adjusted to the wavelength of 540nm. The readings are noted and the unknown concentration values are calculated using Microsoft excel work sheet.

4.2.6 Test for carbohydrates – qualitative method

To 200µl of plant extract, 100µl of Molisch's reagent and few drops of concentrated sulphuric acid were added. Presence of purple or reddish color indicates the presence of carbohydrates.

4.2.7 Estimation of lipids

The test samples were dried, powdered and 100 mg of each was macerated with 10 ml distilled water, transferred to a conical flask containing 30ml of Chloroform and Methanol (2/1; V/V). The mixture was thoroughly mixed and left overnight at room temperature in dark for complete extraction. Later, 20ml of Chloroform was mixed and 2ml of water was added and centrifuged. 2 layers separated, the lower layer of Chloroform which contained all the lipids, was carefully collected in the pre-weighed glass vials and the colored aqueous layer of Methanol which contained all the water-soluble substances and thick pasty interface layer were discarded in each test sample. The Chloroform layers were evaporated to dryness and weighed. Each treatment was replicated in duplicates and their mean values calculated.

Determination of the content of lipids in the sample by weight difference:

$$\text{Weight of lipid} = (\text{weight of container} + \text{extracted lipid}) - (\text{weight of}$$

Percentage lipid content:

$$\text{Lipid content (\%)} = \frac{\text{amount of lipid extracted (g)}}{\text{weight of original sample (g)}}$$

4.2.8 Assessment of invitro antimicrobial activity

4.2.8.1 Microbial Source

Staphylococcus aureus (ATCC 29213), *Streptococcus sp.* (ATCC 19615), *Escherichia coli* (ATCC 25922), *Candida sp.*, (ATCC 44097), *Aspergillus flavus* (ATCC 9643).

4.2.8.2 Anti-bacterial activity

The ethanol leaf extract from mother plant was used for antibacterial study (Arun Vijay et al., 2014; Prajapati et al., 2003). Different concentrations (5,2.5,1.25, 0.62,0.31mg/ml) of the concentrated ethanol leaf extract was tested for its antimicrobial activity against pathogenic bacterial strains such as *Staphylococcus aureus*, *Streptococcus sp.*, *Escherichia coli*. (Lopez et al.,2001).

Antibacterial activity was measured using the standard method of disc diffusion plates on agar (Erturk et al., 2003). For antimicrobial assay, all bacterial strains were grown in Mueller Hinton Broth Medium (Hi-media) for 24 hours at 37° C and plated on Mueller Hinton Agar (Hi-media) for agar diffusion experiments. Then 0.1ml of 0.5 McFarland standard culture of bacteria was spread on agar plate surfaces. Sterile disc (Hi Media, 6mm in diameter) were placed on the agar medium to load 20µl of different concentration (5-0.31mg/ml) of ethanoic leaf extracts were tested. Inhibition diameters were measured after incubation for 24 hours at 37° C. Blanks of solvent only (processed in the same way), were also tested for antibacterial activity

4.2.8.3 Anti-fungal activity

Three to five well-isolated colonies of the fungus were selected from an agar plate culture. The top of each colony was touched with a loop, and the growth was transferred into a tube containing 4-5 ml of PDA broth medium. The broth culture is incubated at 35°C until it achieves turbidity $1-2 \times 10^8$ CFU/ml. The turbidity of actively growing broth culture was adjusted with sterile saline. The inoculum of test plates was done by loopful of suspension inoculates into flask contains Agar. Mix it well and pour it into plate and rotate the plate for even distribution. On the dry PDA agar plate, 30µl of test compound suspensions were added to the well made on the plate (Suganthivan den and Sonal Dubey, 2016).

4.2.9 FT-IR

FT-IR measurement of sample was performed using Shimadzu FT-IR spectrophotometer.

4.2.10 GC-MS

Fid- GC was carried out using a Hewlett-Packard 6890 with HP-5 capillary column (phenyl methyl siloxane. 25 m, 0.25 mm i.d., ratio, 1:25, and flame ionization detector. Temperature programme: 60 °C (2 min) rising to 240 °C at 4 °C/min: injector temperature 250 °C, detector temperature, 260 °C. GC-MS was

performed using a Hewlett Packard 6859 with a quadrupole detector, on a HP-5 column, operating at 70 eV ionization energy, using the same temperature programme and carrier gas as above. Retention indices were calculated by using retention times of n-alkanes that were injected after the oil at the same chromatographic to Van Den Dool method (Zellner et al., 2008).

4.2.11 Docking

4.2.11.1 Ligand selection

From the characterized list of compounds obtained through GC-MS studies, the drug-likeness properties were evaluated and accordingly the short-listed candidates were taken further for docking against SARS-CoV-2 targets. For this step, SwissADME web tool was used. This tool gives free access to a pool of fast yet robust predictive models for physicochemical properties, pharmacokinetics, drug-likeness and medicinal chemistry friendliness, among which in-house proficient methods such as the BOILED-Egg, iLOGP and Bioavailability Radar. The canonical SMILES of the phytochemicals were obtained from PubChem and pasted on SwissADME page to obtain various ADMET parameters which were analysed to determine the drug-likeness of the compounds.

4.2.11.2 Preparation of both enzymes and ligands

The molecular docking analysis requires 3D structure of the protein and ligand. The 3D crystal structure of SARS-CoV-2 targets were downloaded from the protein data bank (PDB) that can be accessed through www.rcsb.org (The RCSB is a protein data bank that contains the crystal structure of the protein generated through X-Ray Diffraction or NMR (Nuclear Magnetic Resonance) method. The phytocompounds of *Vitex negundo*, obtained through GC-MS studies and short-listed via Swiss-ADME were considered as ligand molecule in the current study and were downloaded from NCBI PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) database. It is a database that contains information of chemicals along with their chemical structures. Both the structures were obtained in UCSF Chimera. The software aids in obtaining the 3D structure of the target proteins in PDB format and The 3D structure of ligand in Mol2 format. The molecules were then duly run in iGEM DOCK preparation.

4.2.11.3 Molecular Docking

iGEM DOCK prepares the target proteins and the ligands appropriately into forms which can be interacted to perform docking. Docking was eventually run on iGEM DOCK for each protein molecule against each ligand. The results were obtained for all the interactions and the energy values were provided by the software

CHAPTER 5

RESULTS AND DISCUSSION

5.1 OPTIMISATION OF THE EXTRACTION PROCESS

The factors namely solvent concentration, extraction time and extraction temperature were considered to study the response variables viz. anti-oxidant activity (IC₅₀), phenolic content (GAE) and flavanoid content (GAE) using Response Surface Methodology. The results obtained for each response have been incorporated as follows:

5.1.1 Response 1

Table 5.1.1.1: Experimental runs for Response 1

Std	A: Solvent concentration	B: Extraction time	C: Extraction temperature	Anti- oxidant Activity	Predicted Value	Residual value
	(%)	(min)	(C)	(IC ₅₀)		
9	80	80	40	5.8	5.7	0.15
13	80	100	50	19.3	20.1	-0.80
14	80	100	50	20.1	20.1	0.00
6	90	100	40	10.1	10.1	-0.01
3	70	120	50	11.5	11.4	0.14
8	90	100	60	6.9	6.7	0.16
2	90	80	50	10.6	10.7	-0.14
16	80	100	50	19.9	20.1	-0.20
12	80	120	60	6.3	6.5	-0.15
4	90	120	50	6.2	6.2	-0.01
11	80	80	60	12.3	12.3	-0.02
7	70	100	60	11.8	11.8	0.01
10	80	120	40	11.3	11.3	0.02
5	70	100	40	6.4	6.6	-0.16

17	80	100	50	20.9	20.1	0.80
1	70	80	50	7.1	7.1	0.01
15	80	100	50	20.3	20.1	0.20

Table 5.1.1.2: ANOVA for Response Surface Quadratic Model

Analysis of variance table [Partial sum of squares - Type III]						
Source	Sum of Squares	df	Mean Square	F Value	p-value	
					Prob > F	
Model	519.8413	9	57.76015	269.9973	< 0.0001	significant
A-Solvent concentration	1.125	1	1.125	5.258765	0.0555	
B-Extraction time	0.03125	1	0.03125	0.146077	0.7137	
C-Extraction temperature	1.71125	1	1.71125	7.999165	0.0255	
AB	19.36	1	19.36	90.4975	< 0.0001	
AC	18.49	1	18.49	86.43072	< 0.0001	
BC	33.0625	1	33.0625	154.5492	< 0.0001	
A ²	136.2007	1	136.2007	636.6642	< 0.0001	
B ²	130.2796	1	130.2796	608.9865	< 0.0001	
C ²	132.6322	1	132.6322	619.9837	< 0.0001	
Residual	1.4975	7	0.213929			
Lack of Fit	0.1375	3	0.045833	0.134804	0.9343	not significant
Pure Error	1.36	4	0.34			
Cor Total	521.3388	16				
Std. Dev.	0.462524			R-Squared	0.997127588	
Mean	12.16471			Adj R-Squared	0.993434486	
C.V. %	3.802181			Pred R-Squared	0.991704052	
PRESS	4.325			Adeq Precision	40.73408002	

According to the model for Response 1 which is about anti-oxidant activity, the "Pred R-Squared" of 0.9917 is in reasonable agreement with the "Adj R-Squared" of 0.9934. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Thus the ratio of 40.734 indicates an adequate signal. This model can be used to navigate the design space.

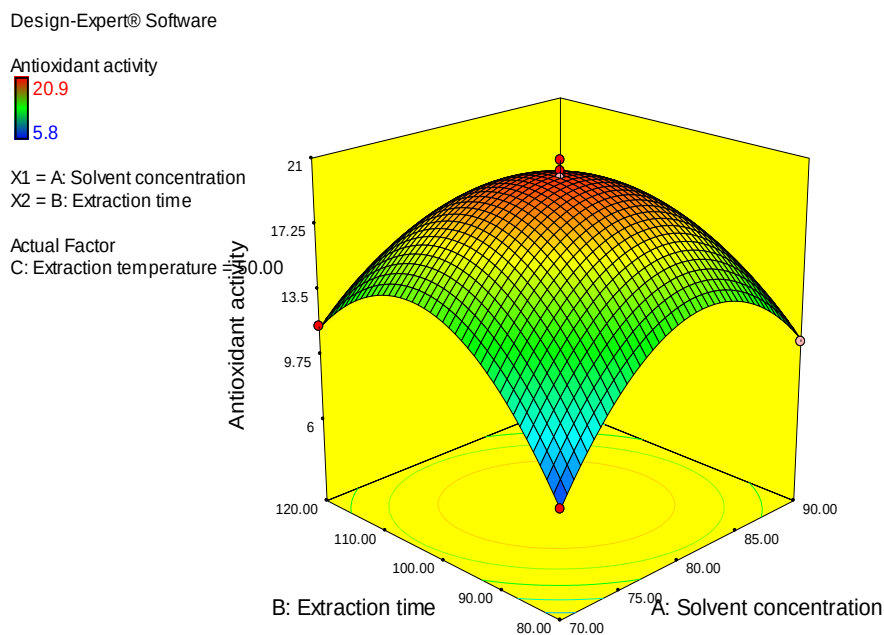


Fig:5.1.1.1 : Surface plot: Relationship between solvent concentration (A) and extraction time (B) for Antioxidant activity

Design-Expert® Software

Antioxidant activity



X1 = A: Solvent concentration
X2 = C: Extraction temperature

Actual Factor

B: Extraction time = 100.00

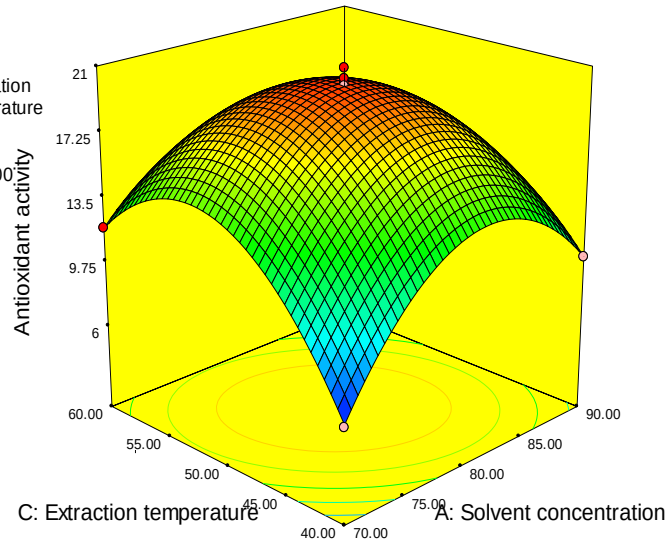


Fig:5.1.1.2 : Surface plot: Relationship between solvent concentration (A) and extraction temperature (C) for Antioxidant activity

Design-Expert® Software

Antioxidant activity



X1 = A: Solvent concentration
X2 = C: Extraction temperature

Actual Factor

B: Extraction time = 100.00

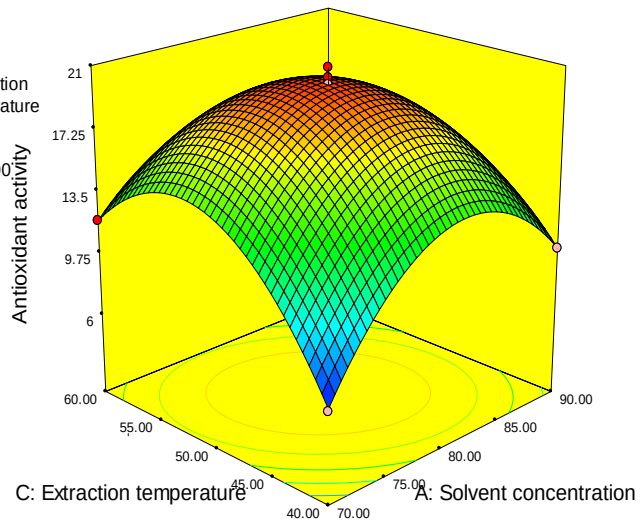


Fig:5.1.1.3 : Surface plot: Relationship between extraction time (B) and extraction temperature (C) for Antioxidant activity

Final Equation in Terms of Coded Factors:

$$\text{Antioxidant activity} = 20.1 - 0.375 * A - 0.0625 * B + 0.4625 * C - 2.2 * A * B - 2.15 * A * C - 2.875 * BC - 5.6875 * A^2 - 5.5625 * B^2 - 5.6125 * C^2$$

5.1.2 Response 2

Table 5.1.2.1: Experimental runs for Response 2

Std	A: Solvent concentration	B: Extraction time	C: Extraction temperature	Phenolic content	Predicted Value	Residual value
	(%)	(min)	(C)	(GAE)		
9	80	80	40	49.8	48.475	1.325
13	80	100	50	182.4	181.42	0.98
14	80	100	50	181.7	181.42	0.28
6	90	100	40	85.6	86.8	-1.2
3	70	120	50	99.6	99.475	0.125
8	90	100	60	58.2	56.9	1.3
2	90	80	50	92.5	92.625	-0.125
16	80	100	50	179.3	181.42	-2.12
12	80	120	60	52.3	53.625	-1.325
4	90	120	50	52.4	52.375	0.025
11	80	80	60	102.3	103.475	-1.175
7	70	100	60	102.3	101.1	1.2
10	80	120	40	97.2	96.025	1.175
5	70	100	40	57.3	58.6	-1.3
17	80	100	50	182.5	181.42	1.08
1	70	80	50	61.5	61.525	-0.025
15	80	100	50	181.2	181.42	-0.22

Table 5.1.2.2: ANOVA for Response Surface Quadratic Model

Analysis of variance table [Partial sum of squares - Type III]						
Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	44709.13	9	4967.681	1800.537	< 0.0001	significant
A-Solvent concentration	128	1	128	46.39362	0.0003	
B-Extraction time	2.645	1	2.645	0.958681	0.3601	
C-Extraction temperature	79.38	1	79.38	28.77129	0.0010	
AB	1528.81	1	1528.81	554.1174	< 0.0001	
AC	1310.44	1	1310.44	474.9692	< 0.0001	
BC	2371.69	1	2371.69	859.6194	< 0.0001	
A ²	11488.4	1	11488.4	4163.973	< 0.0001	
B ²	11687.2	1	11687.2	4236.026	< 0.0001	
C ²	11977.36	1	11977.36	4341.195	< 0.0001	
Residual	19.313	7	2.759			
Lack of Fit	12.565	3	4.188333	2.482711	0.2002	not significant
Pure Error	6.748	4	1.687			
Cor Total	44728.44	16				
Std. Dev.	1.661024			R-Squared	0.999568	
Mean	106.9471			Adj R-Squared	0.999013	
C.V. %	1.553127			Pred R-Squared	0.99527	
PRESS	211.5838			Adeq Precision	104.3568	

According to the model for Response 1 which is about anti-oxidant activity, the "Pred R-Squared" of 0.9953 is in reasonable agreement with the "Adj R-Squared" of 0.9990. "Adeq Precision" measures the signal to noise ratio. A ratio

greater than 4 is desirable. Thus the ratio 104.357 indicates an adequate signal. This model can be used to navigate the design space.

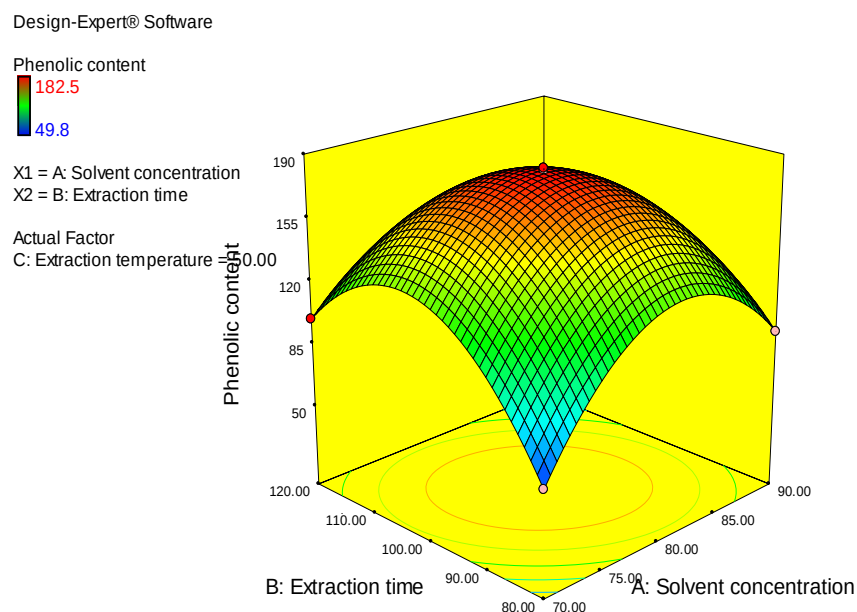


Fig:5.1.2.1 : Surface plot: Relationship between solvent concentration (A) and extraction time (B) for phenolic content

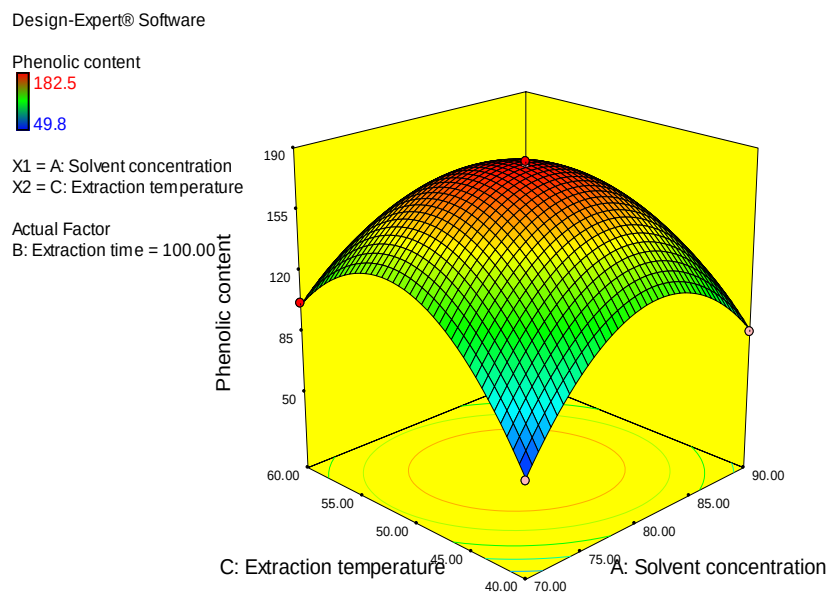


Fig:5.1.2.2 : Surface plot: Relationship between solvent concentration (A) and extraction temperature (C) for phenolic content

Design-Expert® Software

Phenolic content



X1 = B: Extraction time
X2 = C: Extraction temperature

Actual Factor

A: Solvent concentration = 80.00

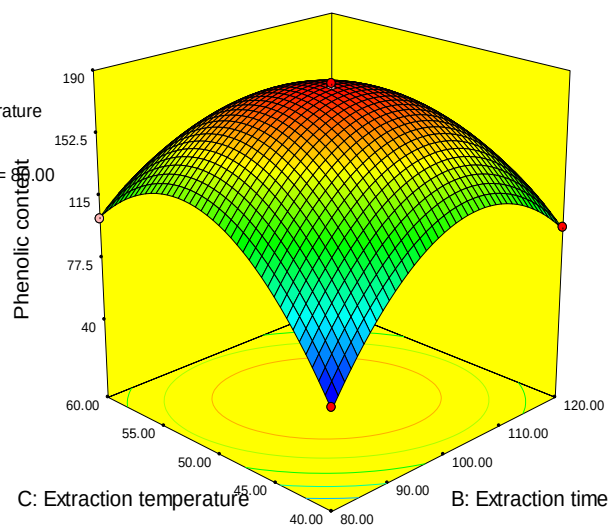


Fig:5.1.2.3 : Surface plot: Relationship between extraction time (B) and extraction temperature (C) for phenolic content

Final Equation in Terms of Coded Factors:

$$\text{Phenolic content} = 181.42 - 4 * A - 0.575 * B + 3.15 * C - 19.55 * A * B - 18.1 * A * C - 24.35 * B * C + 52.235 * A^2 - 52.685 * B^2 - 53.335 * C^2$$

5.1.3 Response 3

Table 5.1.3.1: Experimental runs for Response 3

Std	A: Solvent concentration	B: Extraction time	C: Extraction temperature	Flavanoids content	Predicted Value	Residual value
	(%)	(min)	(C)	(GAE)		
1	80	80	40	48.1	50.7125	-2.6125
2	80	100	50	166.7	163	3.7
3	80	100	50	165.4	163	2.4
4	90	100	40	92.3	92.0375	0.2625
5	70	120	50	92.6	94.95	-2.35

6	90	100	60	49.2	52.4875	-3.2875
7	90	80	50	102.3	99.95	2.35
8	80	100	50	159.9	163	-3.1
9	80	120	60	50.7	48.0875	2.6125
10	90	120	50	49.5	48.825	0.675
11	80	80	60	92.6	91.6625	0.9375
12	70	100	60	89.2	89.4625	-0.2625
13	80	120	40	88.3	89.2375	-0.9375
14	70	100	40	53.4	50.1125	3.2875
15	80	100	50	162.7	163	-0.3
16	70	80	50	48.2	48.875	-0.675
17	80	100	50	160.3	163	-2.7

Table 5.1.3.2: ANOVA for Response Surface Quadratic Model

Analysis of variance table [Partial sum of squares - Type III]						
Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	35285.19	9	3920.576	320.7671	< 0.0001	significant
A-Solvent concentration	12.25125	1	12.25125	1.002352	0.3501	
B-Extraction time	12.75125	1	12.75125	1.04326	0.3411	
C-Extraction temperature	0.02	1	0.02	0.001636	0.9689	
AB	2361.96	1	2361.96	193.2469	< 0.0001	
AC	1556.303	1	1556.303	127.3309	< 0.0001	
BC	1685.103	1	1685.103	137.8689	< 0.0001	
A ²	8291.118	1	8291.118	678.3488	< 0.0001	
B ²	8707.266	1	8707.266	712.3965	< 0.0001	

C ²	9540.042	1	9540.042	780.5312	< 0.0001	
Residual	85.5575	7	12.2225			
Lack of Fit	49.1175	3	16.3725	1.797201	0.2871	not significant
Pure Error	36.44	4	9.11			
Cor Total	35370.74	16				
Std. Dev.	3.496069			R-Squared		0.997581
Mean	98.31765			Adj R-Squared		0.994471
C.V. %	3.555892			Pred R-Squared		0.976172
PRESS	842.8175			Adeq Precision		42.85601

According to the model for Response 1 which is about anti-oxidant activity, the "Pred R-Squared" of 0.9762 is in reasonable agreement with the "Adj R-Squared" of 0.9945. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Thus the ratio 42.856 indicates an adequate signal. This model can be used to navigate the design space.

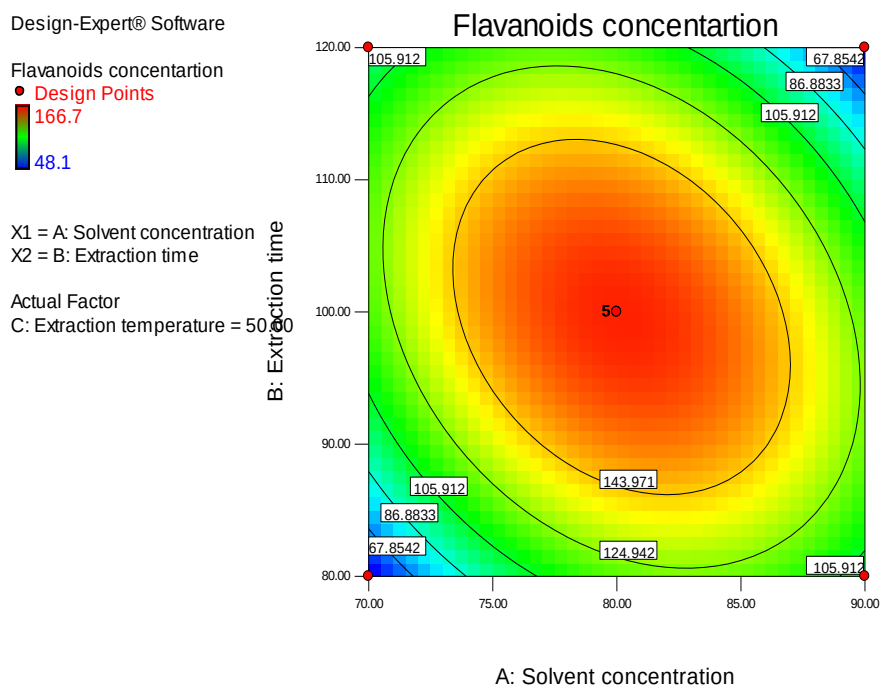


Fig:5.1.3.1: Surface plot: Relationship between solvent concentration (A) and extraction time (B) for flavanoids concentration

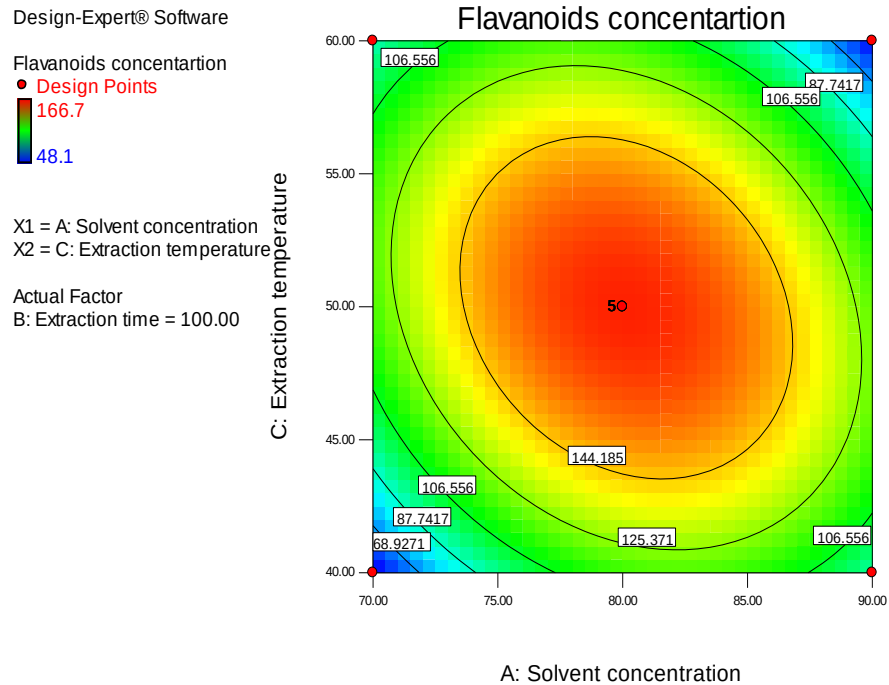


Fig:5.1.3.2: Surface plot: Relationship between solvent concentration (A) and extraction temperature (C) for flavanoids concentration

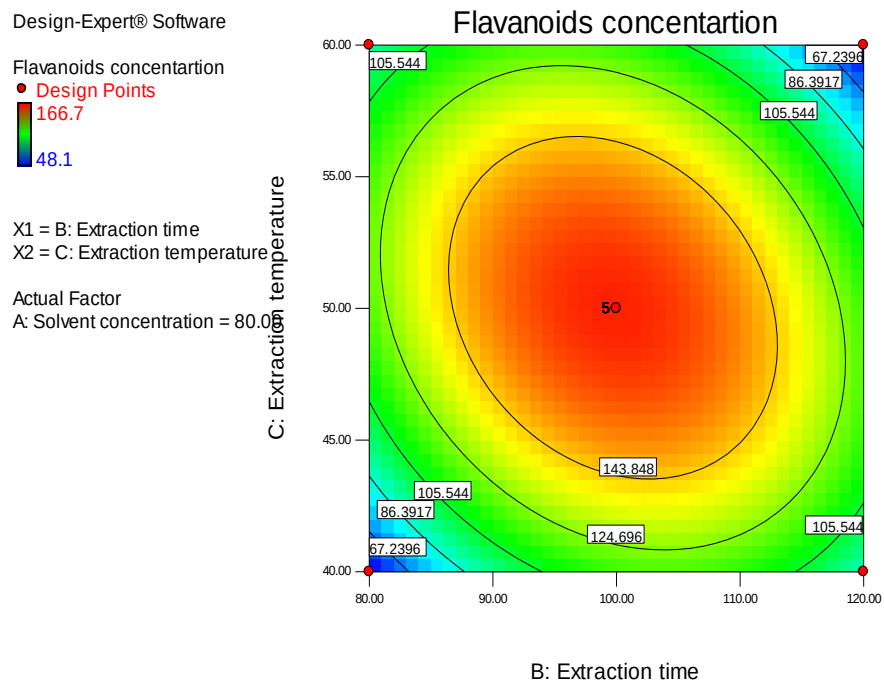


Fig:5.1.3.3: Surface plot: Relationship between extraction time (B)

and extraction temperature (C) for flavanoids concentration

Final Equation in Terms of Coded Factors:

$$\text{Flavanoids concentration} = 163 + 1.2375 * A - 1.2625 * B - 0.05 * C - 24.3 * A * B - 19.725 * A * C - 20.525 * B * C - 44.375 * A^2 - 45.475 * B^2 - 47.6 * C^2$$

The experimental screening performed was designed to assess the influence of three factors, that is, the ethanol concentration, extraction time and extraction temperature. In Table 1, it can be seen that the test values and coded levels of the three independent process variables used for the CCD. Values of the independent process variables (X1, values for all responses are analytically given in Table 2. The second order response surface was fitted to the above data using DoE version 7 with linear quadratic effects. The fitted models accounted for more than 99% of the responses variability with a non-significant lack-of-fit respectively.

At those recommended settings it was predicted with 99% confidence that the anti-oxidant activity would be 20.9 ± 10.4 (IC50 value), phenolic content would be 182.5 ± 11.5 mg GAE g⁻¹dw and flavanoid content would be 166.7 ± 10.2 when operated at optimum extraction conditions stated.

5.2 PHYTOCHEMISTRY

The amount of yield obtained from ethanolic extract was found to be 0.2g. The maximum ethanol yield was considered for various primary metabolite screening, in which chlorophyll and carotenoid was found to be 15.37 and 1.16 mg/g respectively.

Table. 5.2.1: Chlorophyll and carotenoid content- Standard values

OD (nm)	OD		Mean
663	0.144	0.137	0.141
645	0.118	0.123	0.121
480	0.212	0.248	0.230
510	0.388	0.396	0.392

Table. 5.2.2: Total chlorophyll and carotenoid content (TCH)

Tch	(mg/g)
Chlorophyll a	10.37
Chlorophyll b	1.44
Chlorophyll c	3.56
Total chlorophyll	15.37
Carotenoid	1.16

The protein concentration was found to be 1.250 mg/ml which is calculated from the based on the standard bovine serum albumin straight line equation $y = mx + C$ obtained as $y = 0.151x + 0.041$ where y is the unknown OD and x is the unknown concentration.

Table 5.2.3: Estimation of protein – standard values

Standard concentration	OD at 540nm		Mean
5	0.776	0.779	0.778
2.5	0.450	0.453	0.452
1.25	0.254	0.256	0.255
0.62	0.125	0.123	0.124
0.31	0.060	0.061	0.061
Blank	0.063	0.065	0.064

Table 5.2.4: Estimation of Protein - Sample

Sample name	OD at 540nm		Mean	Conc (mg/ml)	Conc (µg/ml)
Sample	0.225	0.235	0.230	1.250	1250.0

Similarly, the carbohydrates estimation using glucose as standard also obtained as 65.25g/l. The percentage of lipid content was calculated to be 62.65%.

Table 5.2.5. Estimation of Carbohydrates– standard values

Standard concentration	OD at 420nm		Mean
100	2.75	2.752	2.751
50	1.430	1.435	1.433
25	0.830	0.825	0.828
12.5	0.475	0.470	0.473
6.25	0.226	0.281	0.254
3.125	0.097	0.096	0.097
Blank	0.059	0.057	0.058

Table 5.2.6: Estimation of Carbohydrates - Sample

Sample name	OD at 420nm		Mean	Conc (mg/ml)	Conc (g/l)
Sample	1.836	1.850	1.843	65.25	65.25

Table 5.2.7. Estimation of Lipid Content

Weight of container	1.542
Extracted lipid	1.2
Weight of lipid (g)	1.2
Lipid content (%)	62.65

Biochemical analysis of extract of *Vitex negundo* contained more amount of carbohydrate, protein and lipids. Hence, there is a need for scientifically screening of the plants, which may help the pharmacologists and phytochemists in discovering innumerable therapeutic agents because the presence of rich medicinal properties likes all the secondary metabolites. Based on this ideology, the antibacterial and antifungal activity of the plant was tested in this current work. The plant is also reported to be used as a traditional medicine in alternative form

for human health and development. It also finds usage as a mosquito repellent. Plants aid us in improving the quality of our life and providing us a diseased free by offering protection against various illnesses. (Rampriya and Arul sheeba, 2017).

5.3 ANTIMICROBIAL ACTIVITY

5.3.1 Sensitivity Disc Pattern Using Standards

The 24 hour culture equivalent to 0.5 Mcfarland standard was swabbed on to Mueller hinton agar plates. After 16 to 18 hours of incubation, each plate is examined. The resulting zones of inhibition were uniformly circular and there was a confluent lawn of growth. There were no individual colonies apparent, indicating that the inoculum was not too light and dilution was right. The diameters of the zones of complete inhibition (as judged by the unaided eye, using scale) were measured by holding the back of the inverted plate, including the diameter of the disc. The petri plate was held a few inches above a black, nonreflecting background and illuminated with reflected light. Any discernible growth within zone of inhibition is indicative of resistance to the respective antibiotic (Vimal Arora, Lohar et al., 2011).

The zone margin should be taken as the area showing no obvious, visible growth that can be detected with the unaided eye. Faint growth of tiny colonies, which can be detected only with a magnifying lens at the edge of the zone of inhibited growth, is ignored. The sizes of the zones of inhibition are interpreted by referring through 2I (Zone Diameter Interpretative Standards and equivalent Minimum Inhibitory Concentration Breakpoints) of the CLSI M100-S12: Performance Standards for Antimicrobial Susceptibility Testing: Twelfth Informational Supplement, and the organisms reported as either susceptible, intermediate, or resistant to the agents that have been tested and shown in the tables below:

Table 5.3.1.1: Antibiotic sensitivity pattern - Bacteria

Antibiotic against bacterial species tested by disc diffusion Method			
S.No.	Agent	Code	Organism (zone diameter in mm)

			<i>Staphylococcus aureus</i>	<i>Streptococcus sp.</i>	<i>E coli</i>
1	Tetracycline	T	13	27	-
2	Ciprofloxacin	CP	31	37	31
3	Azithromycin	AZ	24	36	23
4	Ampicillin	A	-	32	-
5	Gentamicin	GEN	23	28	25
6	Amoxicillin + Clavulanic Acid	AU	-	-	-

Table 5.3.1.2: Antibiotic sensitivity pattern – Fungi

Antibiotic against fungal species tested by disc diffusion Method				
S.No.	Agent	Code	Organism zone diameter (in mm)	
			<i>Candida</i>	<i>Aspergillus flavus</i>
1	Flucanazole	F	31	37

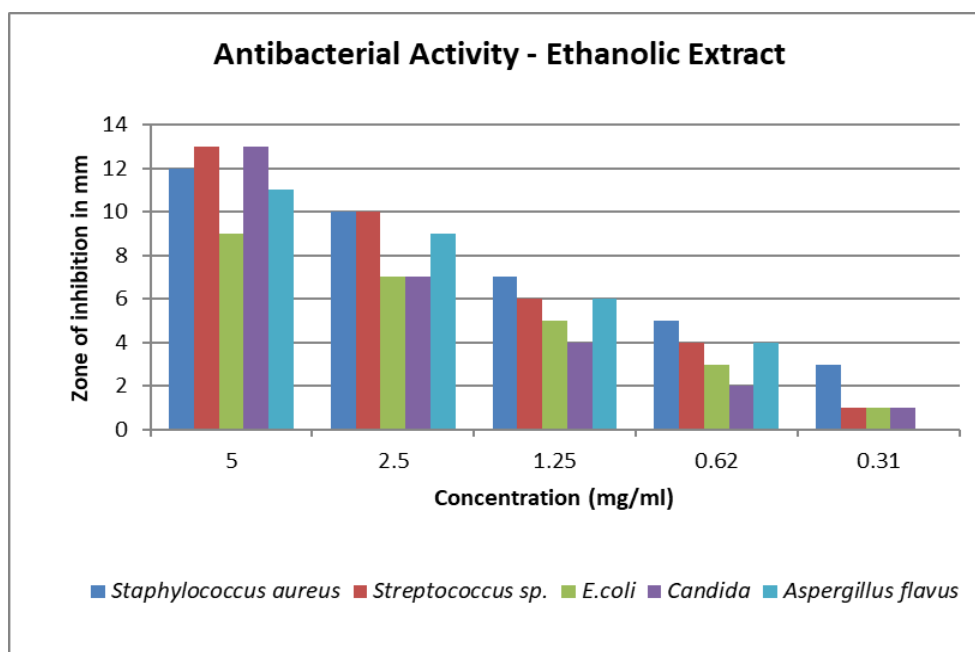


Fig: 5.3.1.1: Antibacterial Activity

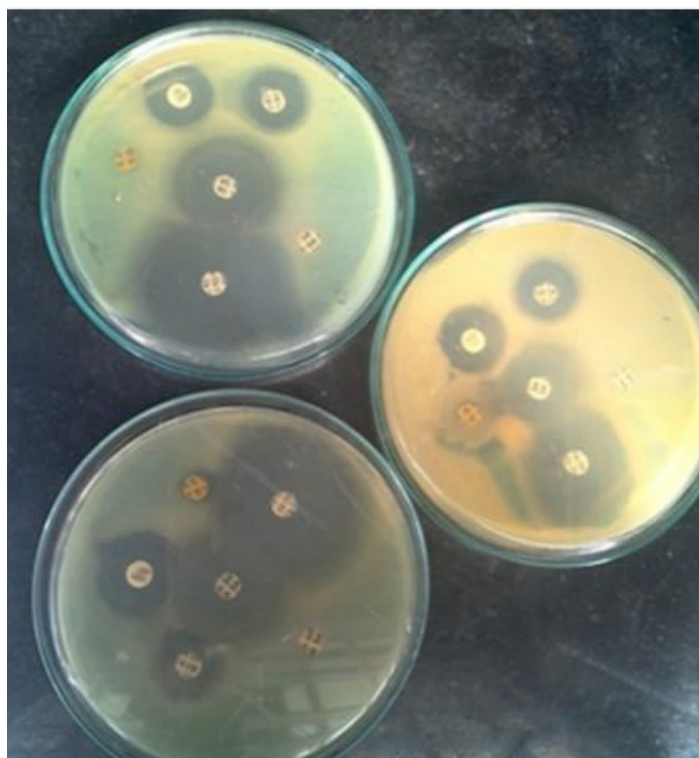


Fig: 5.3.1.2: Antibiotic Sensitivity Pattern- Bacteria

E. coli strains isolated is Gram-negative, motile, opportunistic bacterium belonging to the family *Enterobacteriaceae*. This was found to be highly resistant to Amoxicillin - clavulanic acid and ampicillin. While it was sensitive to other antibiotics with zone of inhibition nearly 18mm. *Streptococcus sp.*, isolated is Gram-positive, pathogenic bacterium belonging to the family *Streptococcaeae*. This was found to highly sensitive to ciprofloxacin, tetracycline, azithromycin, ampicillin, gentamicin and resistant to amoxicillin and clavulanic acid. The maximum sensitivity was seen in Ciprofloxacin with nearly 37 mm of zone of inhibition. All three strains isolated were also found to be sensitive to all the antibiotics except amoxicillin.



Fig: 5.3.1.3: *Aspergillus niger*

The fungal strains of *Candida sp.*, and *Aspergillus flavus* were found to sensitive for fluconazole (Saral, 1991).

5.3.2 Antibacterial Activity – Using Extract

The antibacterial activity was carried out for the various extracts of ethanol. It was found that ethanol extract at 5mg concentration, the three sample strains isolated were found to be less sensitive at 5mg for *E.coli* and major activity was shown by *Streptococcus sp.* and *Staphylococcus aureus* sample at higher concentration of 5mg as shown in Table 5.3.2.1. The extract was effective against candida sp., than *Aspergillus flavus* that showed activity only at 5mg and not in lesser concentration.

Table 5.3.2.1: Antimicrobial activity

Antibacterial activity - Ethanolic against microbial species tested by Well diffusion Method					
Microbes	Sample concentration (in mm)				
	5	4.5	3.25	3.62	4.31
<i>Staphylococcus aureus</i>	12	10	7	5	3
<i>Streptococcus sp.</i>	13	10	6	4	4
<i>E coli</i>	9	7	5	3	4

<i>Candida sp.</i>	13	7	4	2	3
<i>Aspergillus flavus</i>	11	-	-	-	3

5.4 FT-IR

FT-IR is one of the most widely used methods to identify the chemical constituents and elucidate the compounds structures and it has been used as a requisite method to identify medicines in Pharmacopoeia of many. FT-IR can be employed to determine the structure of unknown composition and the intensity of the absorption spectra associated with molecular composition or content of the chemical group. The FTIR method measures the vibrations of bonds within chemical functional groups and generates a spectrum that can be regarded as a biochemical or metabolic “fingerprint” of the sample. By attaining IR spectra from plant samples, it might possible to detect the minor changes of primary and secondary metabolites. At present, particularly in phytochemistry, FTIR has been exercised to identify the concrete structure of certain plant secondary metabolites.

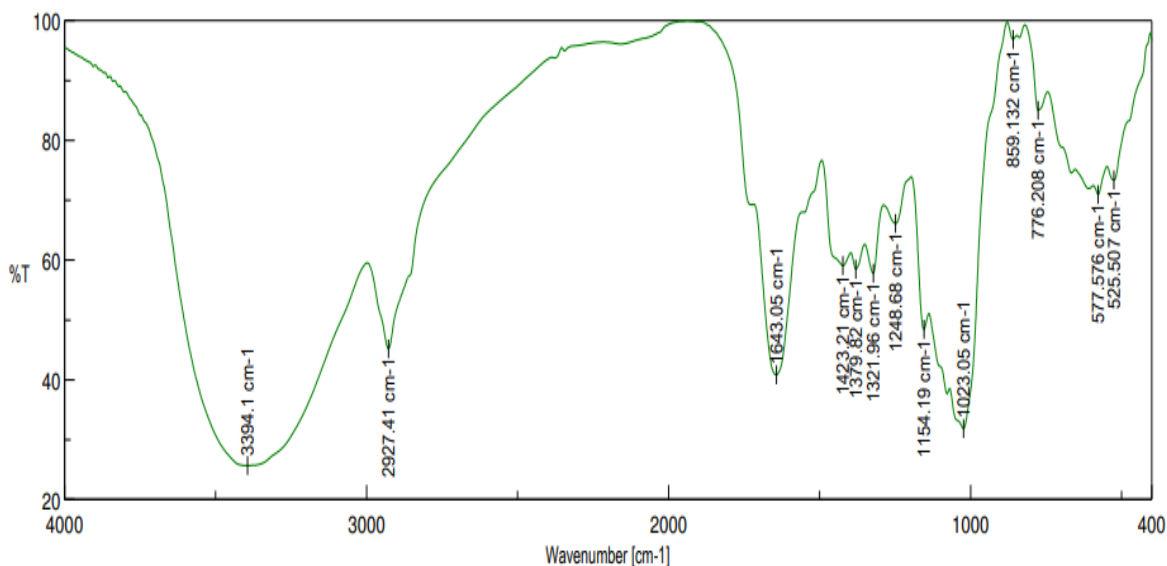


Fig:5.4.1: FT-IR Graph

By using FT-IR spectrum, the functional constituent's presence in the given leaf extract and even evaluate the qualities of medicinal materials can be confirmed. The results of the present study spectrum also revealed the functional constituents present in ethanolic leaf extracts of *Vitex negundo*. The results of the

present study confirms the presence of phenol, alkane, carboxylic acid, aromatic compound, nitro compound, alcohol, benzene compounds in ethanolic leaf extract of *Vitex negundo* (Sarika Khandare et al., 2012; Rajeshwari Sahu et al., 2014)

Table 5.4.1: FT-IR

S.No.	Peak values	Bond	Functional groups
1	3394.1	O-H	Alcohols, phenols
2	2927.41	C-H	Alkane
3	1643.05	C=O	Carboxylic acid
4	1423.21	C=C	Aromatic compounds
5	1379.82	N-O	Aliphatic nitro compounds
6	1248.68	C-O	Carboxylic acid
7	1023.05	C-O	Primary alcohol
8	859.132	C-H	Para benzene
9	776.208	C-H	Meta benzene

5.5 GC-MS

Gas Chromatography-Mass Spectrometry was performed to find out the chemical compounds present in the optimized plant extract and the following graph was obtained:

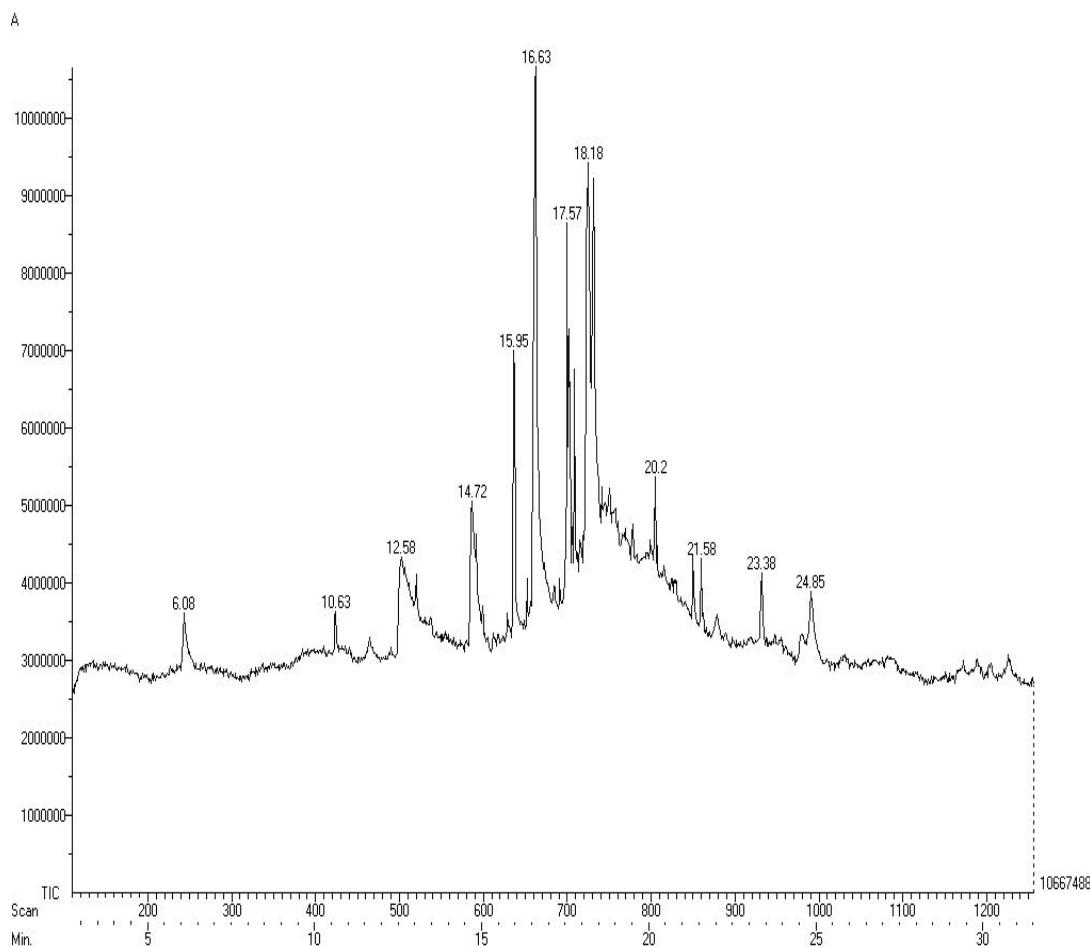


Fig: 5.5.1: GC-MS Graph

There were 12 peaks observed in the chromatogram. It has Retention Time (RT) of different values indicating the presence of different biochemical compounds (Table 5.5.1). The compound at 16.63 secs as retention time was Piperidin 4-one, 1-(1-phenylcyclohexyl) is the highest peak.

Piperidine are secondary amines and are members of saturated heterocyclic compounds which demands main cause of interest for chemists, biologist and pharmacist due to their significant and diverse range of promising biological activities like analgesic, anti-inflammatory (Telang et al., 1999), CNS, antioxidant, antihistaminic, antimicrobial and others from last few decades (Shazia et al., 2014; Tiwari et al., 2012).

Table 5.5.1: GC-MS

Peak No.	Peak RT time (in secs)	Details
1	24.85	Benzoic acid, 2,4, dimethoxy 6 methyl (8,8 dimethoxy - 2-octyl)ester
2	23.38	Methoxyacetic acid, octadecyl ester
3	21.58	Methoxyacetic acid, heptadecyl ester
4	20.2	Despropionyl -3- methylfentanyl
5	18.18	Nonadecane -2,4 dione
6	17.57	Quinoxaline 2-isopropyl-3-phenyl 4-oxide
7	16.63	Piperidin 4-one, 1-(1-phenylcyclohexyl)
8	15.95	2(1H) phenanthrenone, 3,4,4a tetrahydro-1,1 4a trimethyl
9	14.72	Flavone
10	12.58	2-(3-(N- aziridyl)propyl)aminomethyltetrahydropyran
11	10.63	Tricyclo(4,4 O (3,9)decane
12	6.08	Tetrazolo(1,5-a) pyridine 5,6,7,8 tetrahydro

These compounds also have anticancer activity that may be the molecules that inhibit the growth of cancerous cells. There was also presence of Nonadecane -2,4-dione, which has anti-cancerous and also has safe food flavoring activity (Philip Ashurst, 2012). In our study, it was observed that the ethanoic extract has more antibacterial effect against *Streptococcus sp.*, *Staphylococcus aureus.*, *E.coli*, *Candida sp.*, *Aspergillus flavus* . This may be due to the presence of these compounds in larger amount.

The other compounds present in the sample such as benzoic acid, methoxyacetic acid, Flavone, Tricyclo and Tetrazolo were also said to have other pharmacological properties.

5.6 Swiss-ADME RESULTS

The compounds obtained from VN through GC-MS were studied for ADMET properties with the help of Swiss-ADME.

Table 5.6.1: Phytocompounds – SMILES and PubChem CID

S N o.	Details	Canonical SMILES from PubChem	PubCh em CI D
1	Benzoic acid, 2,4, dimethoxy 6 methyl (8,8 dimethoxy - 2-octyl)ester	<chem>CC1=CC(=CC(=C1C(=O)OC(C)CCCCC(OC)OC)OC)OC</chem>	601561
2	Methoxyacetic acid, octadecyl ester	<chem>CCCCCCCCCCCCCCCCCCCCOC(=O)COC</chem>	542201
3	Methoxyacetic acid, heptadecyl ester	<chem>CCCCCCCCCCCCCCCCCCCCOC(=O)COC</chem>	545730
4	Despropionyl -3- methylfentanyl	<chem>CC1CN(CCC1NC2=CC=CC=C2)CCC3=CC=CC=C3</chem>	608913
5	Nonadecane -2,4 dione	<chem>CCCCCCCCCCCCCCCCCCCC(=O)CC(=O)C</chem>	567637
6	Quinoxaline 2-isopropyl-3-phenyl 4-oxide Synonyms: 2-Isopropyl-3-phenylquinoxaline 4-oxide	<chem>CC(C)C1=NC2=CC=CC=C2[N+]([O-])=C1C3=CC=CC=C3</chem>	622033
7	Piperidin 4-one, 1-(1-phenylcyclohexyl)	<chem>C1CCC(CC1)(C2=CC=CC=C2)N3CCC(=O)CC3</chem>	425091
8	2(1H) phenanthrenone, 3,4,4a tetrahydro-1,1 4a trimethyl	<chem>CC1(C(=O)CCC2(C1=CCC3=CC=CC=C32)C)C</chem>	588210
9	Flavone	<chem>C1=CC=C(C=C1)C2=CC(=O)C3=CC=CC=C3O2</chem>	10680
10	2-(3-(N-aziridyl)propyl)aminomethyltetrahydropyran	<chem>C1CCOC(C1)CNCCCN2CC2</chem>	541736
11	Tricyclo[4.4.0.0(3,9)]decane	<chem>C1CC2CC3C1CCC2C3</chem>	565074
12	Tetrazolo(1,5-a) pyridine 5,6,7,8 tetrahydro	<chem>C1CCN2C(=NN=N2)C1</chem>	81973

Swiss-ADME results were obtained in order to short list the suitable ligand candidates for further computational analysis in regard of SARS-CoV-2

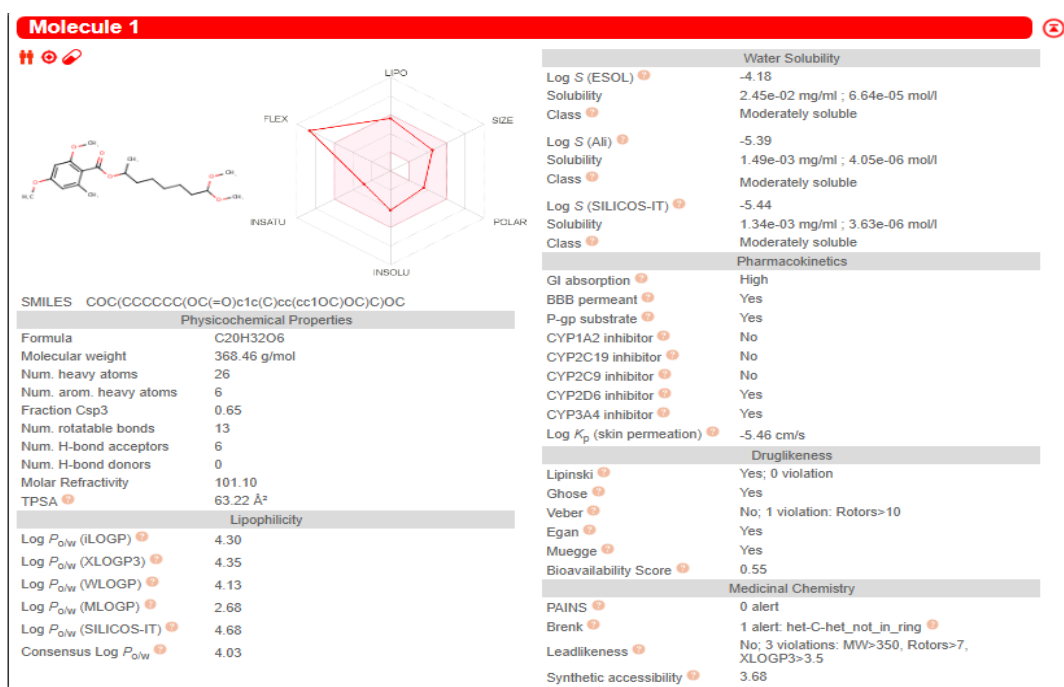


Fig:5.6.1: Benzoic acid, 2,4, dimethoxy 6 methyl (8,8 dimethoxy - 2-octyl)ester

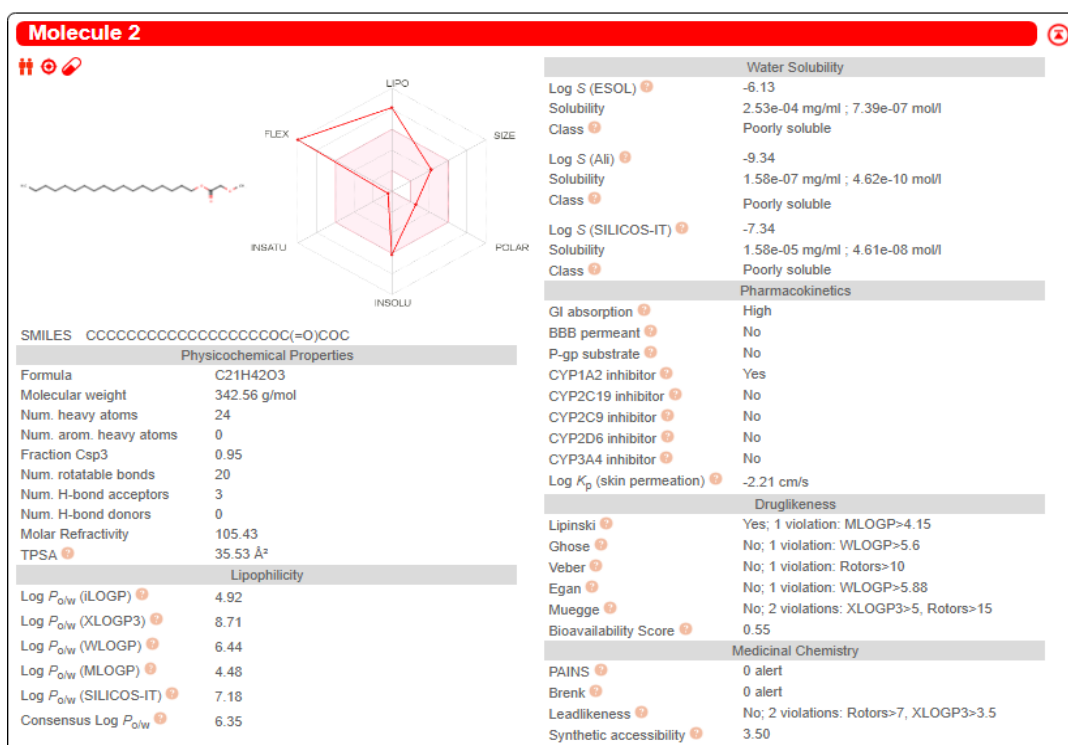


Fig:5.6.2: Methoxyacetic acid, octadecyl ester

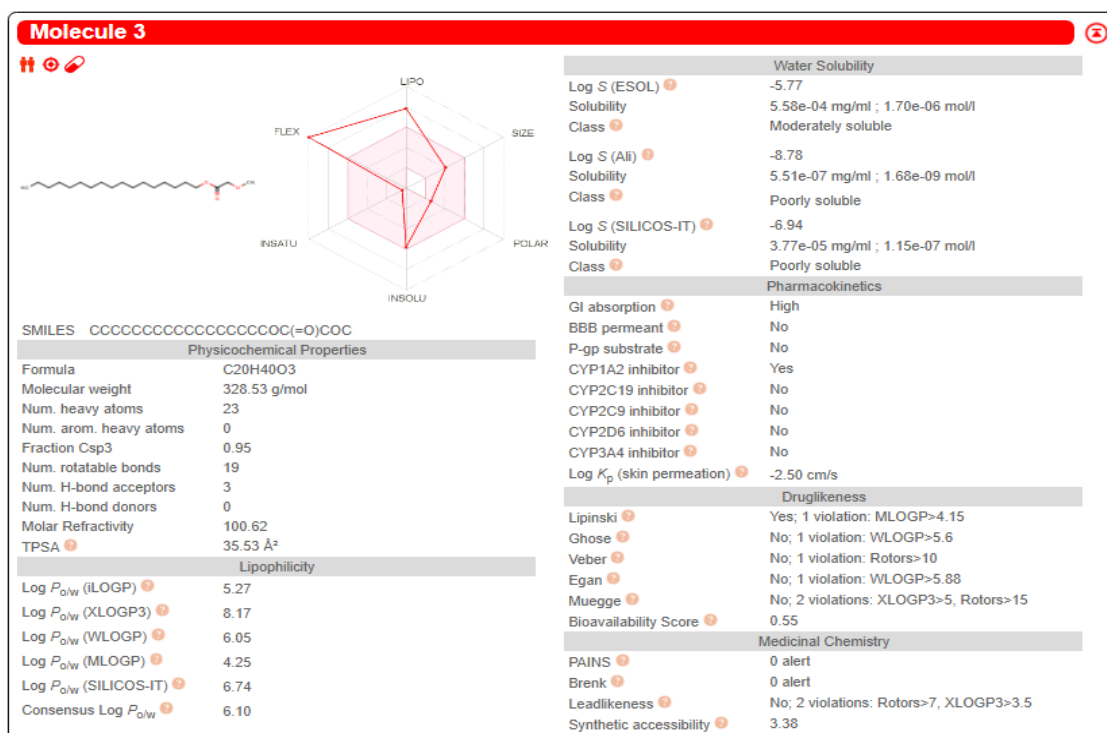


Fig:5.6.3: Methoxyacetic acid, heptadecyl ester

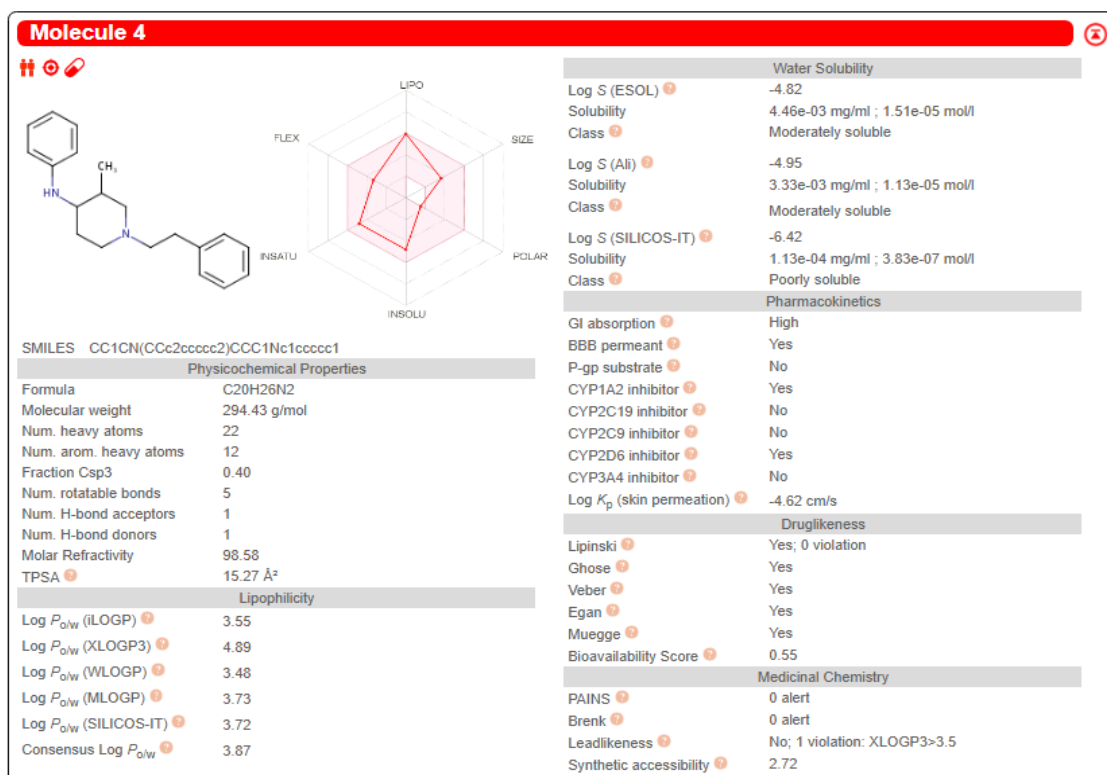


Fig:5.6.4: Despropionyl -3- methylfentanyl

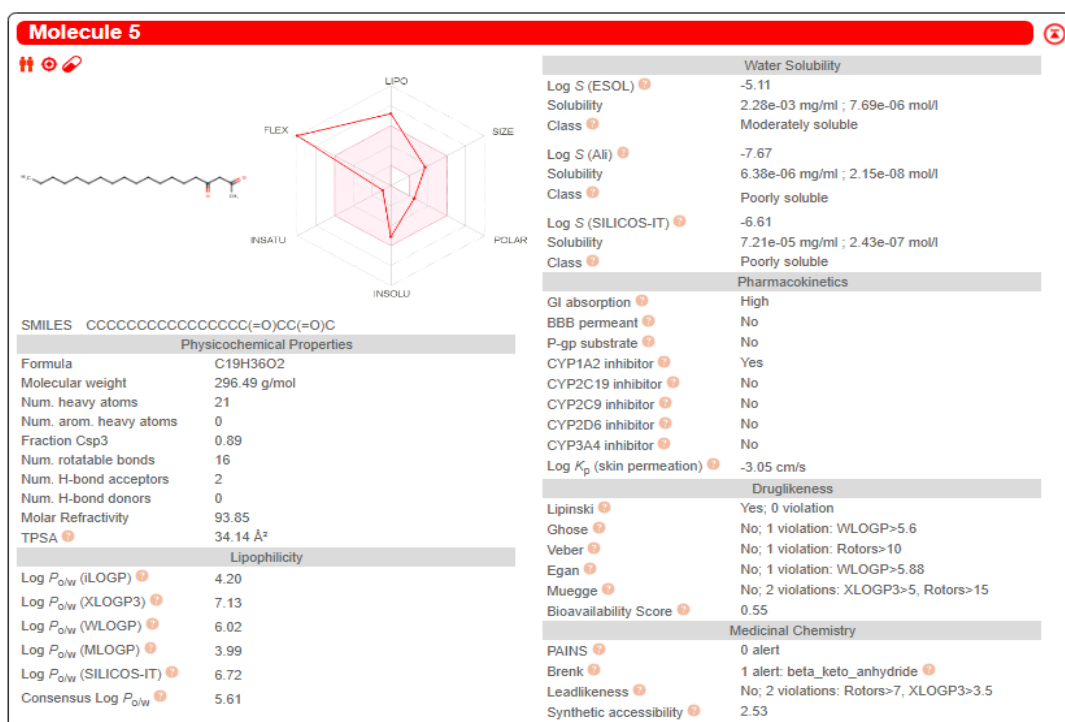


Fig:5.6.5: Nonadecane -2,4 dione

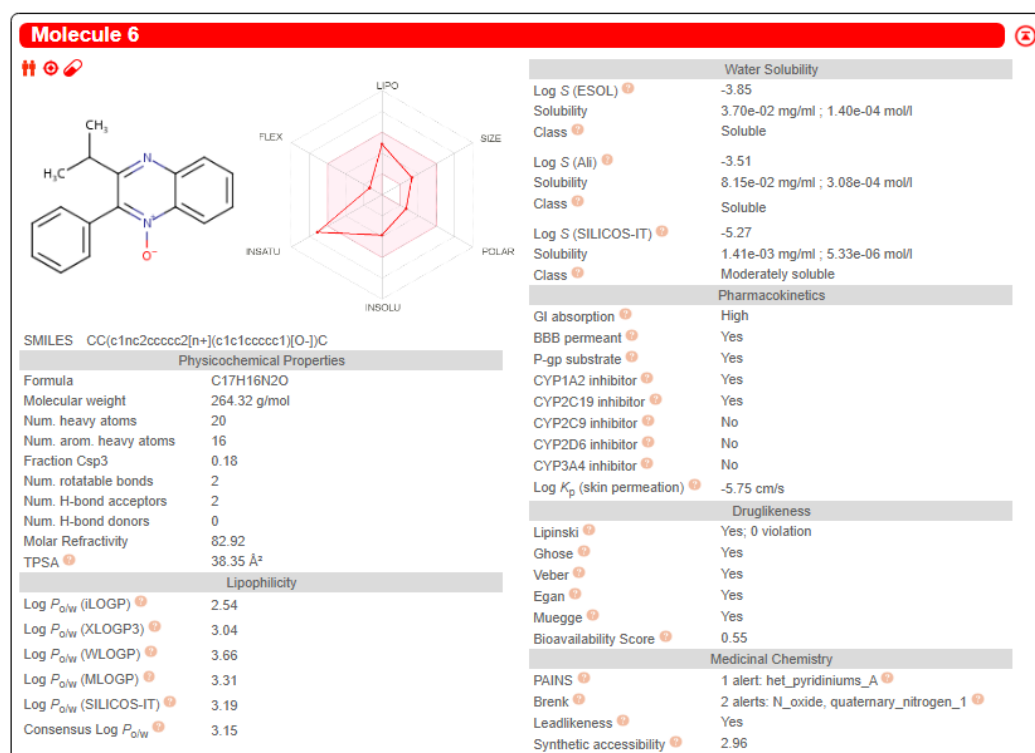


Fig:5.6.6: Quinoxaline 2-isopropyl-3-phenyl 4-oxide

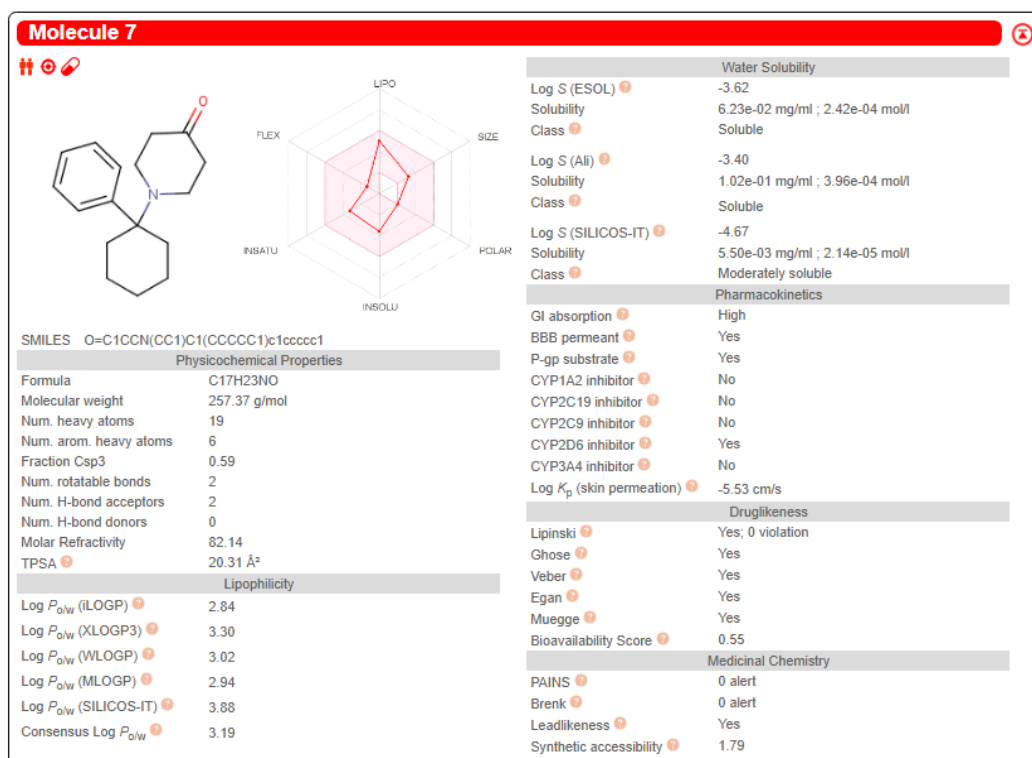


Fig:5.6.7: Piperidin 4-one, 1-(1-phenylcyclohexyl)

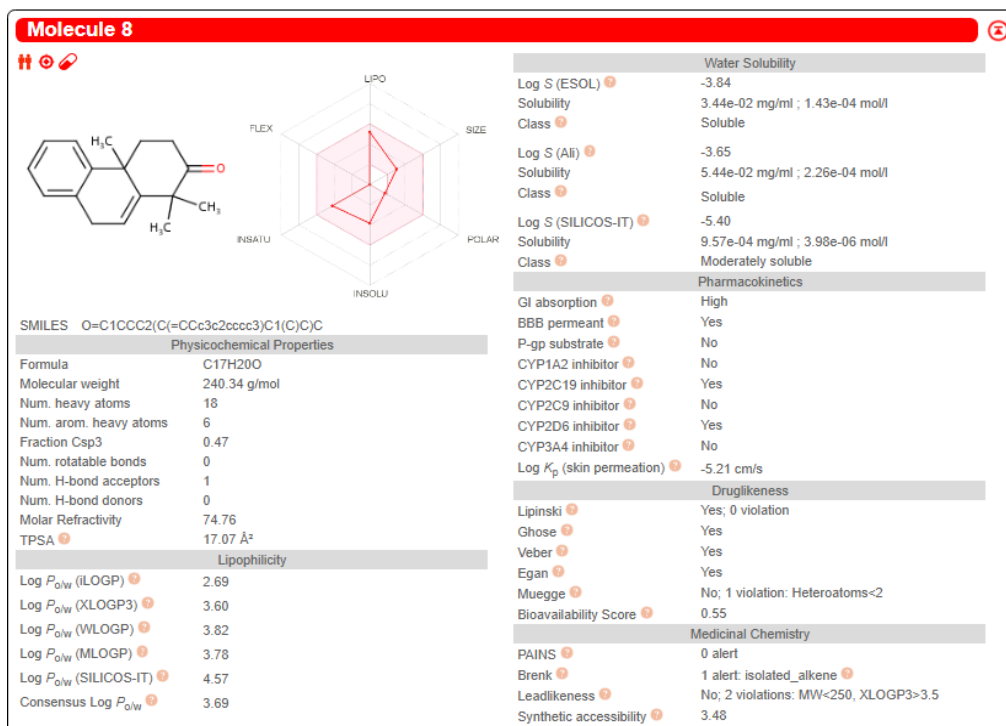


Fig:5.6.8: 2(1H) phenanthrenone, 3,4 4a tetrahydro-1,1 4a trimethyl

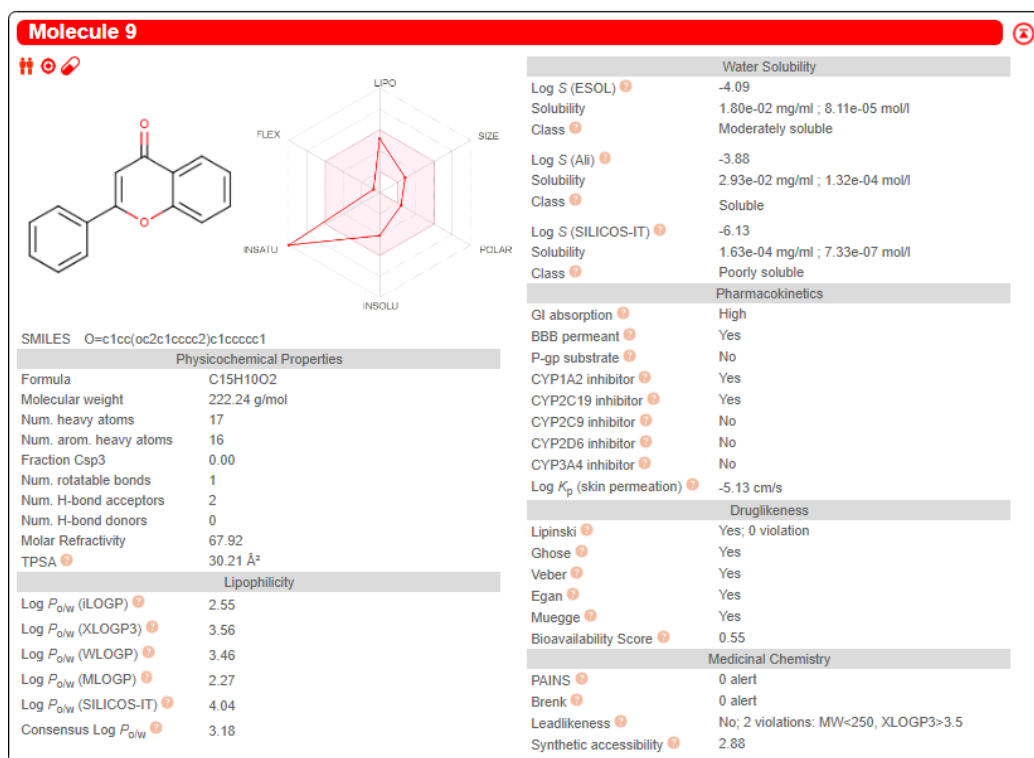


Fig:5.6.9: Flavone

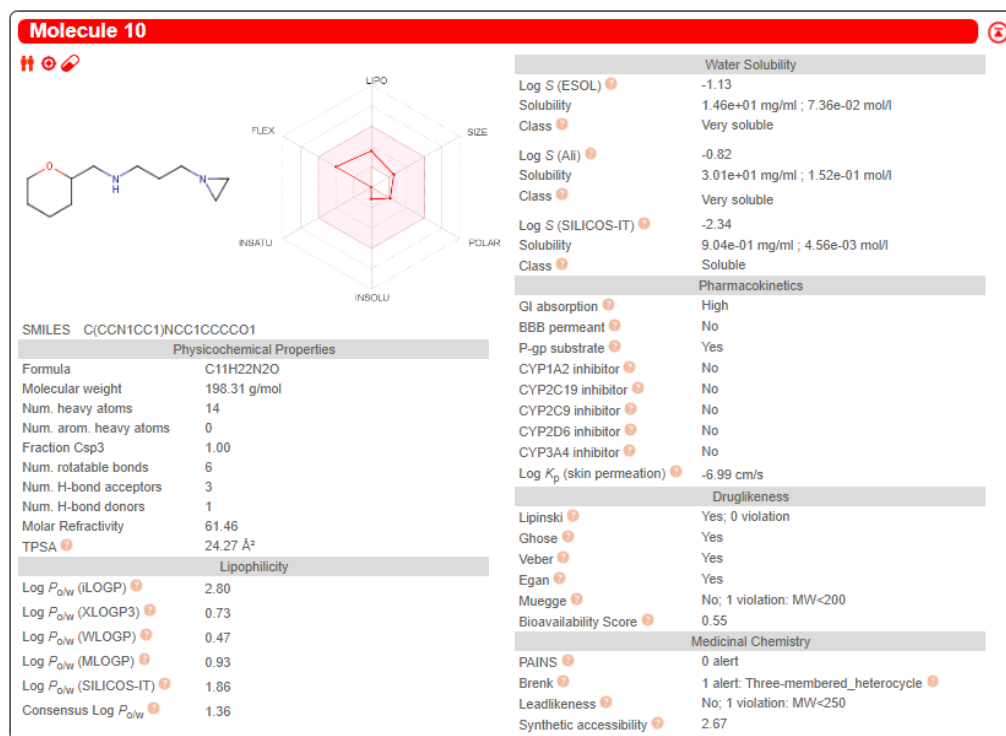


Fig:5.6.10: 2-(3-(N-aziridinyl)propyl)aminomethyltetrahydropyran

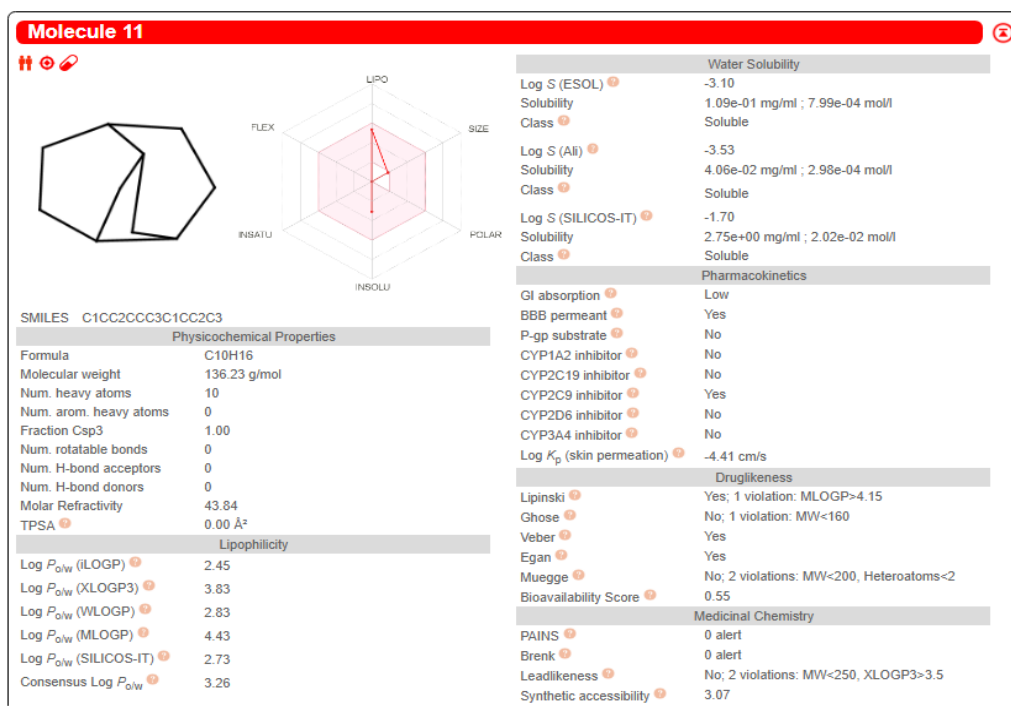


Fig:5.6.11: Tricyclo[4.4.0.0(3,9)]decane

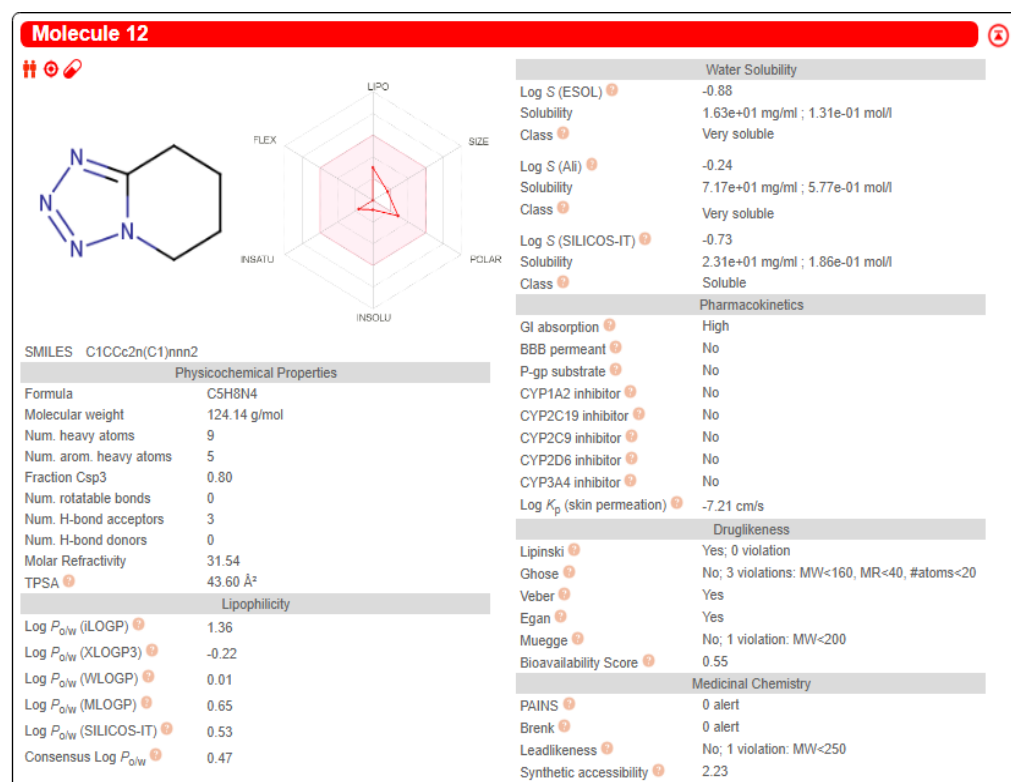


Fig:5.6.12: Tetrazolo(1,5-a)pyridine 5,6,7,8 tetrahydro

ADMET properties play an extremely important role in determining the drug likeliness to become a successful drug candidate. Every drug needs to overcome these parameters to become a successful drug candidate. More than 50% of drugs fail to overcome these parameters. The absorption of drugs is predicted by including factors such as membrane permeability, intestinal absorption, skin permeability levels, P-glycoprotein substrate or inhibitor. The distribution of drugs depends on factors that include the blood–brain barrier (logBB), CNS permeability, and the volume of distribution (VDss). Metabolism is predicted based on the CYP models for substrate or inhibition (CYP2D6, CYP3A4, CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4). Excretion is predicted through renal clearance property of the molecules and renal OCT2 substrate. The toxicity of drugs depends upon AMES toxicity, hERG inhibition, hepatotoxicity, and skin sensitization. These parameters were checked and analysed with their respective standard values. ADMET profiling of compounds was determined using SWISS ADME. Every administered drug pass through gastro-intestinal wall and then gets readily absorbed through gut wall and then passes the liver to get into blood stream. GI absorption is the parameter to predict gastro-intestinal absorption. Intestinal epithelial barrier and gut blood barriers (GBB) are vital barriers for any drug to pass. The distribution of compounds depends on the CNS and blood brain barrier permeability and if any compound is BBB permeable, it can be considered as lethal. P glycoprotein is a membrane transport channel with broad substrate specificity and plays a vital role in transporting of drugs to the organs. It plays a role of defense system against toxins entering the CNS. Another ADMET parameter is metabolism which includes the conversion of drug into metabolites through various enzymes. It depends upon the sets of enzymes name CYP450 (cytochrome 450) enzymes and its isoforms (CYP2D6, CYP3A4, CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4). Any compound that acts as an inhibitor of these enzymes can cause toxicity as these enzymes are responsible for the detoxification. Skin permeation is a parameter which represents the ability of skin to absorb certain phytochemicals it must be between -8.0 to -1.0 according to standard values. Compounds predicted to have higher Pka that means that they may not act as CNS drug but can be utilised for further purposes such as dermal remedies. The phytochemicals having skin penetration value higher than -1.0 which represents their easy penetration through the skin. Toxicity

of drug is generally expressed in LD50 (lethal dose 50%); drugs with more LD50 value are less perilous than drugs with higher LD50 values. There is an inverse relationship between the toxicity and LD50 value. According to the globally harmonized system of classification of labelling of chemicals (GHS) each drug falls into a class with respect to its LD50 value. Toxicity class is distributed into six classes starting from fatal to non-toxic.

The table below represents a comparison of various parameters amongst the extracted phytocompounds:

Table 5.6.2: Comparison of ADME profile of different phytocompounds

S.No	Phytocompounds/ Ligand	GI Abs.	BB B Per.	P-gp Sub.	Cyt . Inh.	Log Kp(cm/s)	LR Vio .
1	Benzoic acid, 2,4, dimethoxy 6 methyl (8,8 dimethoxy - 2- octyl) ester	High	Yes	Yes	Yes	-5.46	0
2	Methoxyacetic acid, octadecyl ester	High	No	No	Yes	-2.21	1
3	Methoxyacetic acid, heptadecyl ester	High	No	No	Yes	-2.50	1
4	Despropionyl -3- methylfentanyl	High	Yes	No	Yes	-4.62	0
5	Nonadecane -2,4 dione	High	No	No	Yes	-3.05	0
6	Quinoxaline 2-isopropyl-3-phenyl 4-oxide	High	Yes	Yes	Yes	-5.75	0
7	Piperidin 4-one, 1-(1-phenylcyclohexyl)	High	Yes	Yes	Yes	-5.53	0
8	2(1H) phenanthrenone, 3,4,4a tetrahydro-1,1 4a trimethyl	High	Yes	No	Yes	-5.21	0
9	Flavone	High	Yes	No	Yes	-5.13	0
10	2-(3-(N- aziridyl) propyl) aminomethyl tetrahydropyran	High	No	Yes	No	-6.99	0
11	Tricyclo[4.4.0.0(3,9)]decane	Low	Yes	No	No	-4.41	1
12	Tetrazolo(1,5-a) pyridine 5,6,7,8 tetrahydro	High	No	No	No	-7.21	0

Based upon the pharmacokinetic profile analysis using SwissADME and comparative study of various parameters, certain compounds present in the extract of *Vitex negundo* have been short-listed considering the status of their absorption in GIT and the ability to surpass the Blood Brain Barrier, interaction with P glycoprotein and Cytochrome P450 as well as whether they are following Lipinski's Rule or not. They have been tabulated as follows:

Table 5.6.3: Shortlisted phytocompounds as the final ligands for docking

S.No.	Name of the ligand	PubChem CID
1	Piperidin 4-one, 1-(1-phenylcyclohexyl)	425091
2	2-(3-(N- aziridyl)propyl)aminomethyltetrahydropyran	541736
3	2(1H) phenanthrenone, 3,4 4a tetrahydro-1,1 4a trimethyl	588210
4	Despropionyl -3- methylfentanyl	608913
5	Tetrazolo(1,5-a) pyridine 5,6,7,8 tetrahydro	81973

5.7 DOCKING

The structure of target molecules was taken from pdb and downloaded. The various targets of SARS-CoV-2 can be consolidated as:

Table 5.7.1: SARS CoV-2 target proteins

S No.	PROTEIN NAME	PDB ID
1	SARS-CoV-2 spike (S) protein	6X6P
2	SARS-CoV-2 3CL protease (3CL pro) apo structure	6M2Q
3	RNA-dependent RNA polymerase from SARS-CoV-2	7BW4
4	Angiotensin Converting Enzyme 2 (ACE2)	1R42
5	SARS Spike Glycoprotein – human ACE2 complex	6CS2
6	Native Spike Protein (S)	6MOJ

7	Envelope Protein (E)	5X29
8	Membrane Protein (M)	3I6G
9	Chymotrypsin-like cysteine protease/ main protease (Mpro)/ 3Cl-like protease	3F9G
10	Nonstructural protein 9 (NSP 10)	6W75

The 3D structure of the different protein molecules obtained from PDB have been included as follows:

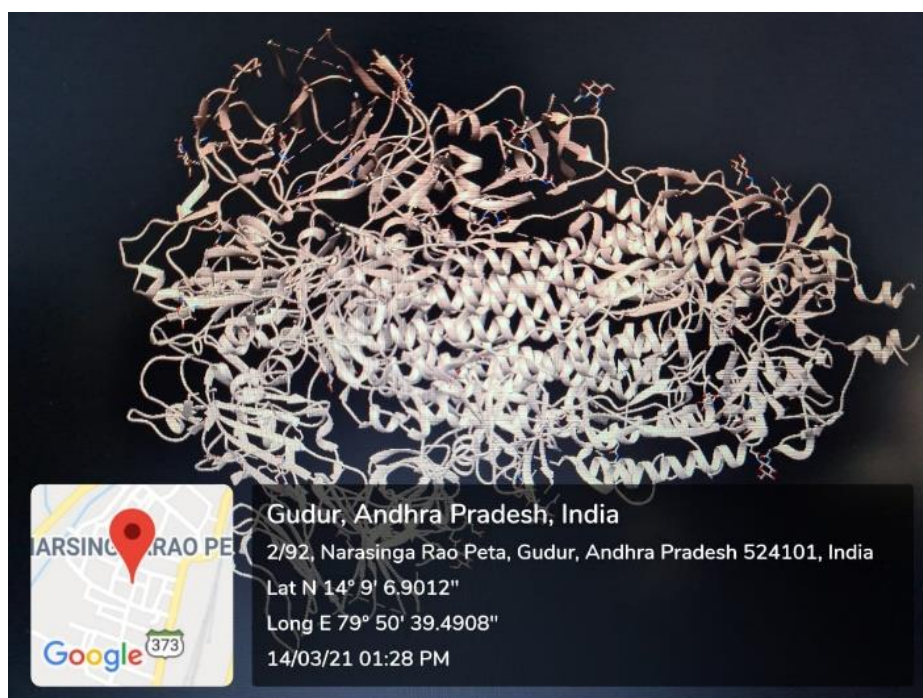


Fig:5.7.1: 6X6P



Fig:5.7.2:6M2Q



Fig:5.7.3: 7BW4

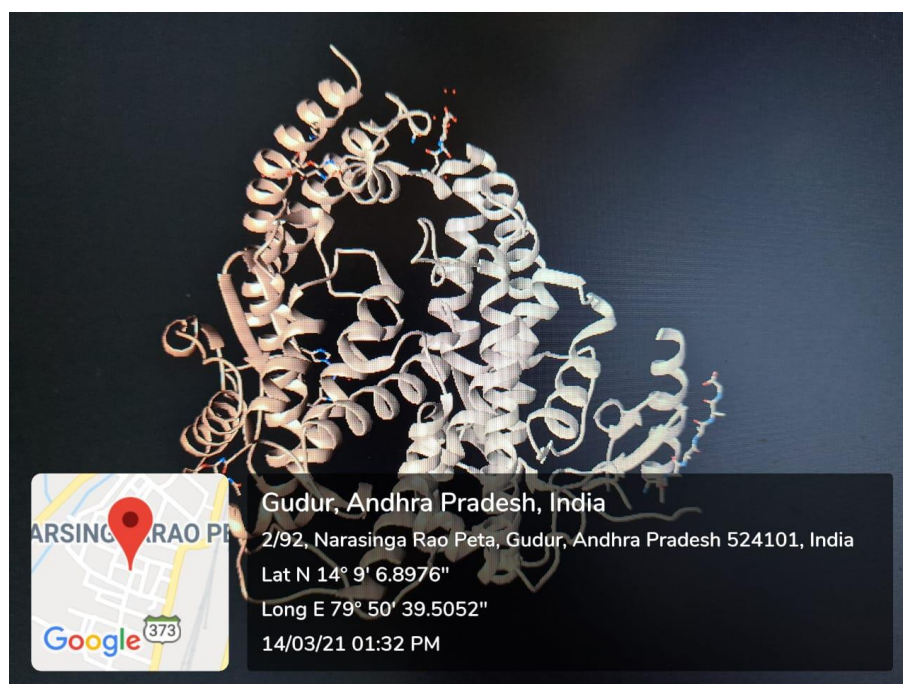


Fig:5.7.4: 1R42

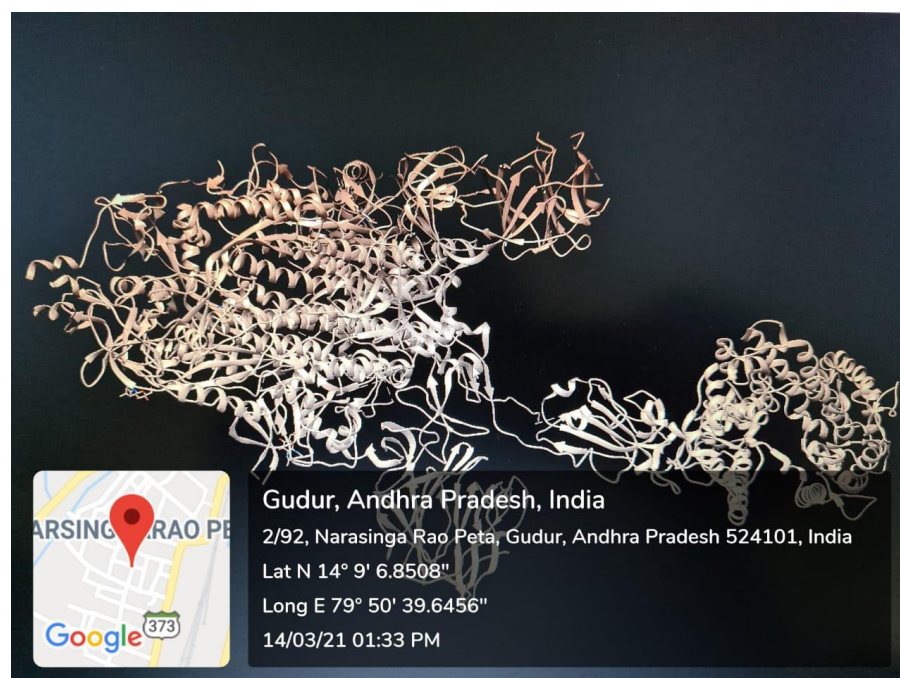


Fig:5.7.5: 6CS2

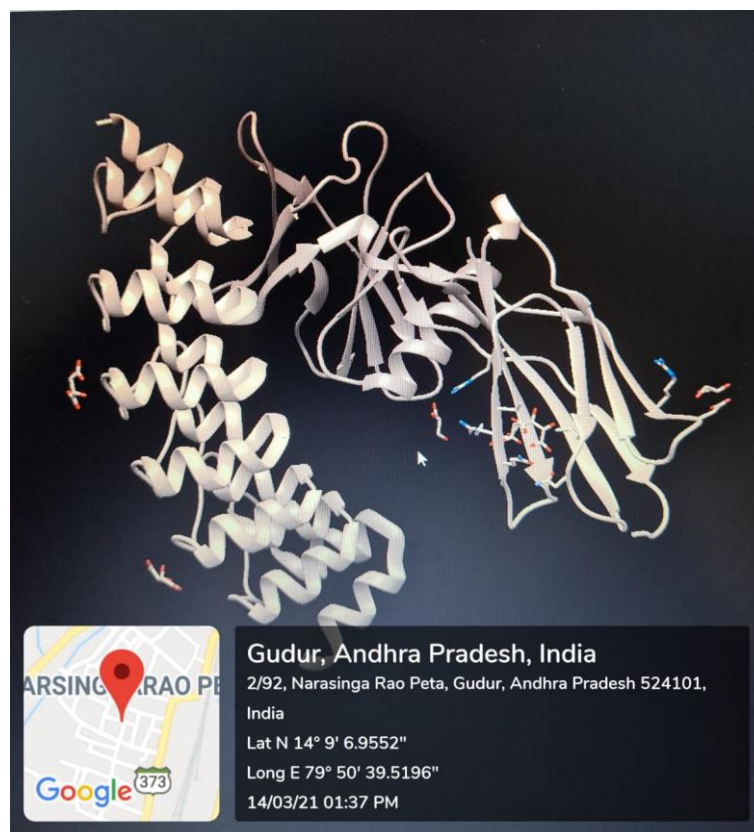


Fig:5.7.6: 6MOJ

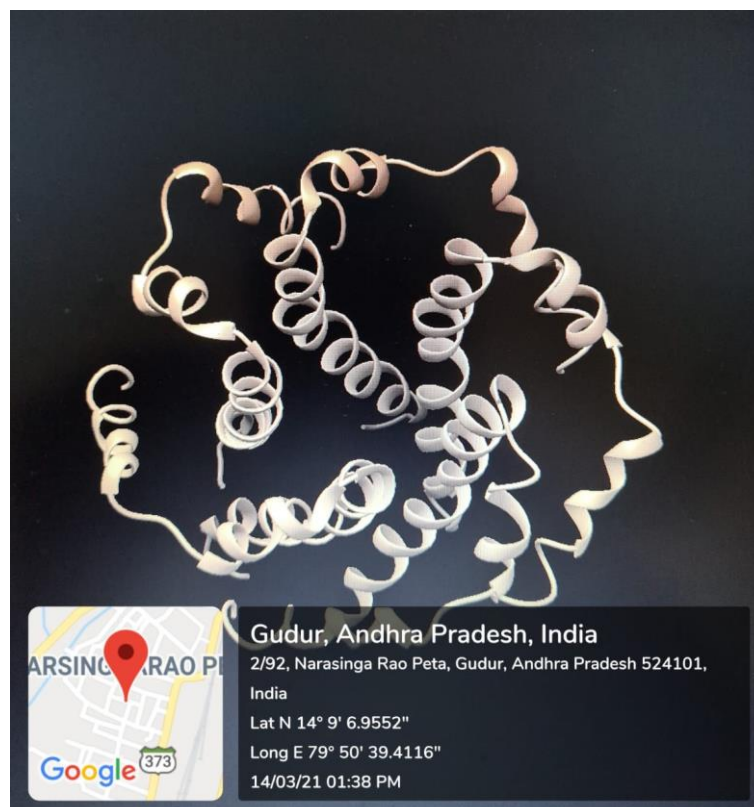


Fig:5.7.7: 5X29

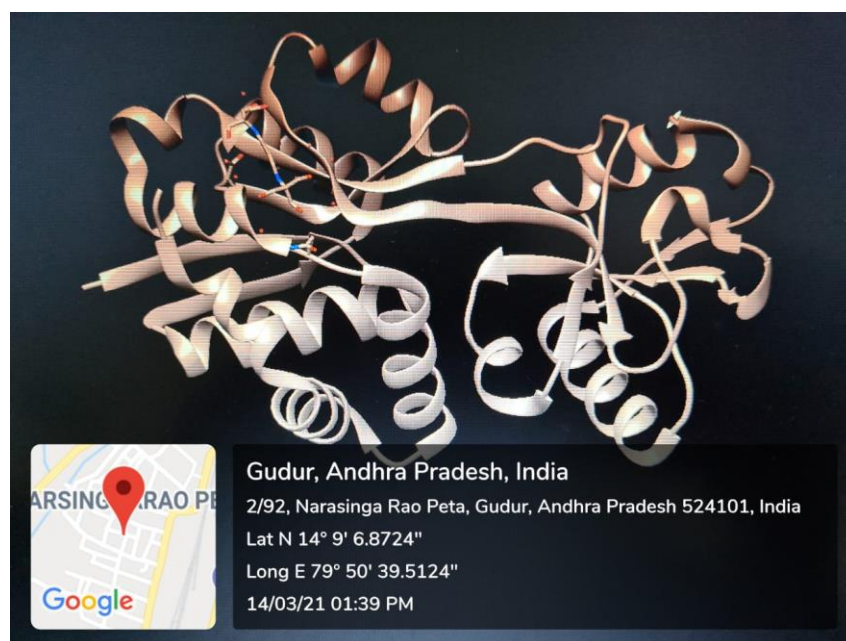


Fig:5.7.8: 3I6G

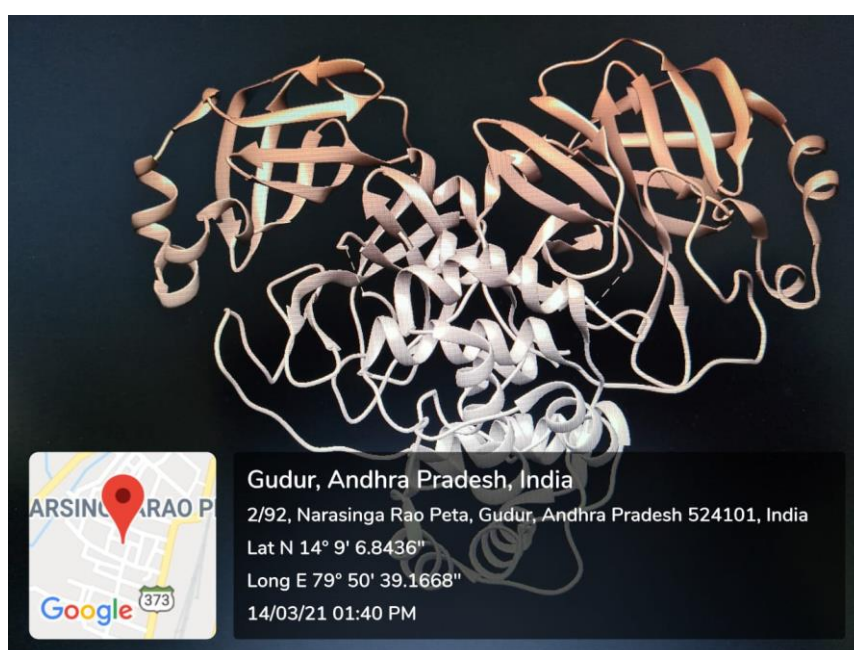


Fig:5.7.9: 3F9G



Fig:5.7.10: 6W75

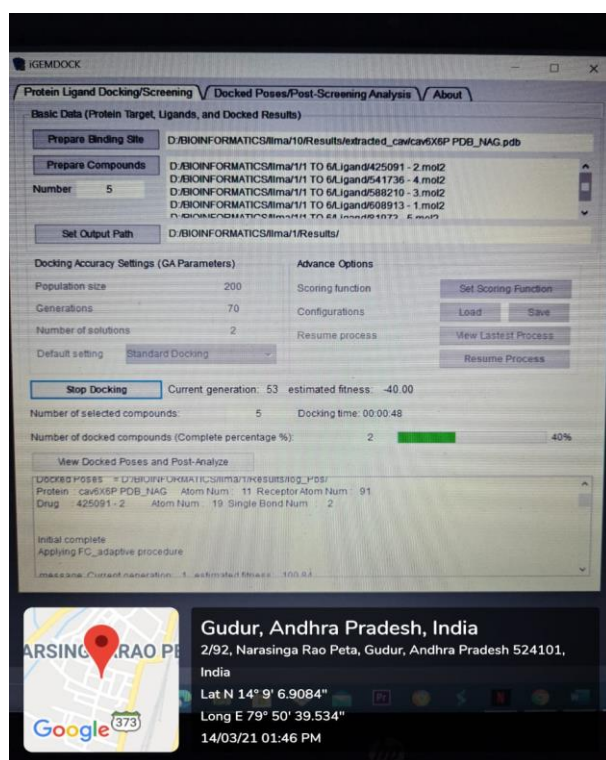
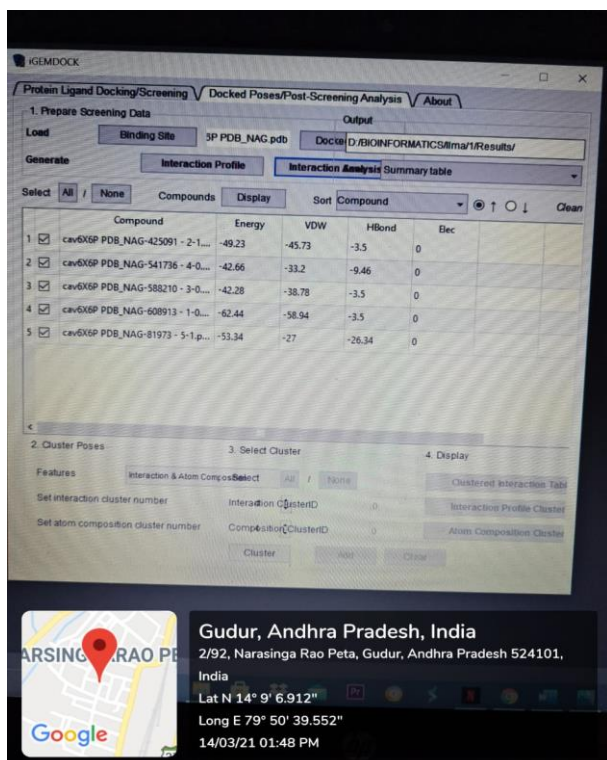


Fig:5.7.11: Docking in progress on iGEM DOCK

iGEM dock performs molecular docking and returns various kinds of energy values based on which the most feasible interaction is studied and finalized as the

most stable docked structure of the protein molecule and its respective ligand. The results of docking of all the ligands against the proteins have been tabulated as follows:

Table 5.7.2: Docking results of SARS-CoV-2 spike protein (6X6P) with the ligand molecules

Ligand (PubChem CID)	Total Energy	VDW	H Bond	Ele c	AverConPair
425091	-52.8691	-49.3691	-3.5	0	18.5263
541736	-46.6811	-40.6811	-6	0	18.9286
588210	-41.1123	-37.6123	-3.5	0	18.5
608913	-60.9069	-57.4069	-3.5	0	16.8636
81973	-53.1949	-26.879	-26.3159	0	19

As a result of docking of SARS-CoV-2 spike protein with the important drug-like compounds present in *Vitex negundo*, medium-level interaction is observed since the total energy values are particularly very less with -60.9069 being the highest and -41.1123 being the lowest.

Table 5.7.3: Docking results of SARS-CoV-2 3CL protease (3CL pro) apo structure (6M2Q) with the ligand molecules

#Ligand (PubChem CID)	Total Energy	VDW	H Bond	Elec	AverConPair
425091	-76.4288	-72.5186	-3.91026	0	27.0526
541736	-66.4108	-59.4108	-7	0	26.2143
588210	-76.5165	-61.1287	-15.3878	0	24.5556
608913	-83.9503	-80.4503	-3.5	0	22.4545
81973	-75.6792	-39.7027	-35.9765	0	30.8889

Docking of SARS-CoV-2 3CL protease (3CL pro) apo structure with the important drug-like compounds present in *Vitex negundo* reveals good amount of interaction among them. The total energy values include -83.9503 being the highest and -66.4108 being the lowest.

Table 5.7.4: Docking results of RNA-dependent RNA polymerase (7BW4) with the ligand molecules

#Ligand (PubChem CID)	Total Energy	VDW	H Bond	Elec	AverConPair
425091	-75.7049	-71.9607	-3.74423	0	23.4737
541736	-72.2263	-71.2916	-0.93468	0	32.3571
588210	-87.2183	-80.2183	-7	0	27.6667
608913	-91.0153	-91.0153	0	0	25.9091
81973	-73.6432	-46.2809	-27.3624	0	37.1111

The result of docking of RNA-dependent RNA polymerase with the important drug-like compounds present in *Vitex negundo* shows good amount of interaction among them. The total energy values include -91.0153 being the highest and --72.2263 being the lowest.

Table 5.7.5: Docking results of Angiotensin Converting Enzyme 2 (1R42) with the ligand molecules

#Ligand (PubChem CID)	Total Energy	VDW	H Bond	Elec	AverConPair
425091	-56.4099	-52.9099	-3.5	0	18.4737
541736	-48.3368	-41.1977	-7.13911	0	21
588210	-50.0285	-42.0272	-8.0013	0	18.5556
608913	-69.2845	-63.2845	-6	0	19.3182
81973	-47.5178	-29.5195	-17.9982	0	19

Docking results of Angiotensin Converting Enzyme 2 with the important drug-like molecules present in *Vitex negundo* show medium amount interaction among them. The total energy values include -69.2845 being the highest and -47.5178 being the lowest.

Table 5.7.6: Docking results of SARS Spike Glycoprotein – human ACE2 complex (6CS2) with the ligand molecules

#Ligand (PubChem CID)	Total Energy	VDW	H Bond	Elec	AverConPair
425091	-70.2528	-51.9866	-18.2662	0	19.7895
541736	-67.0314	-52.4895	-14.5419	0	24.6429
588210	-59.2945	-53.2945	-6	0	26.3889
608913	-79.2451	-72.2451	-7	0	20.4091
81973	-60.9123	-27.2786	-33.6337	0	22.1111

SARS Spike Glycoprotein – human ACE2 complex and the phytocompounds present in *Vitex negundo* show slightly good amount of interaction. The total energy values include -79.2451 being the highest and -59.2945 being the lowest.

Table 5.7.7: Docking results of Native Spike Protein (6MOJ) with the ligand molecules

#Ligand (PubChem CID)	Total Energy	VDW	H Bond	Elec	AverConPair
425091	-86.6494	-84.833	-1.81632	0	32.4737
541736	-67.3608	-64.6865	-2.67433	0	29.6429
588210	-76.8545	-72.1102	-4.74433	0	25.6667
608913	-92.1934	-88.6984	-3.495	0	32.3636
81973	-64.4204	-46.6896	-17.7308	0	40

Native Spike Protein of SARS-CoV-2 interacts very well with the important drug-like molecules present in *Vitex negundo*. The total energy values include -92.1934 being the highest and -64.4204 being the lowest.

Table 5.7.8: Docking results of Envelope Protein (5X29) with the ligand molecules

#Ligand (PubChem CID)	Total Energy	VDW	H Bond	Elec	AverConPair
425091	-79.1914	-78.5413	-0.6501	0	26.7895
541736	-75.2788	-72.0259	-3.25289	0	34.8571
588210	-73.6565	-71.1565	-2.5	0	26.5556
608913	-93.5503	-93.5503	0	0	25.9545
81973	-65.6766	-48.1976	-17.4789	0	33.3333

The important drug-like molecules present in *Vitex negundo* show very good docking results when studied against the Envelope Protein of SARS-CoV-2 virus. The maximum total energy is -93.5503 while the minimum energy is -65.6766.

Table 5.7.9: Docking results of Membrane Protein (3I6G) with the ligand molecules

#Ligand (PubChem CID)	Total Energy	VDW	H Bond	Elec	AverConPair
425091	-66.6249	-63.1249	-3.5	0	25.4211
541736	-61.5609	-56.2944	-5.26648	0	34.3571
588210	-56.9502	-56.9502	0	0	20.5556
608913	-75.8193	-65.3193	-10.5	0	24
81973	-68.0344	-44.1083	-23.9261	0	37.2222

Docking results of Membrane Protein with the important drug-like molecules present in *Vitex negundo* show medium amount interactions among them. The

total energy values include -75.8193 being the highest and -56.9502 being the lowest.

Table 5.7.10: Docking results of Chymotrypsin-like cysteine protease/ main protease (Mpro)/ 3CLike protease (3F9G) with the ligand molecules

#Ligand (PubChem CID)	Total Energy	VDW	H Bond	Elec	AverConPair
425091	-76.0669	-72.5669	-3.5	0	24.7895
541736	-72.7471	-66.7471	-6	0	33.7143
588210	-75.0883	-73.326	-1.76239	0	24.8333
608913	-81.8641	-81.8641	0	0	21.5455
81973	-80.246	-43.6039	-36.6421	0	34.7778

The result of docking of Chymotrypsin-like cysteine protease/ main protease (Mpro)/ 3CLike protease with the important drug-like compounds present in *Vitex negundo* shows a good amount of interaction among them. The total energy values include -81.8641 being the highest and -72.7471 being the lowest.

Table 5.7.11: Docking results of Nonstructural protein 9 (6W75) with the ligand molecules

#Ligand (PubChem CID)	Total Energy	VDW	H Bond	Elec	AverConPair
425091	-93.572	-89.401	-4.17094	0	31.0526
541736	-71.8329	-62.6914	-9.14148	0	28.2143
588210	-86.4354	-86.4354	0	0	30.5
608913	-98.3074	-91.9963	-6.31113	0	27.8636
81973	-64.4072	-30.6855	-33.7216	0	29

The important drug-like molecules present in *Vitex negundo* show extremely good amount of interaction when studied against the Nonstructural protein 9 of SARS-CoV-2 virus. The maximum total energy is -98.3074 which the highest for all docked structures while the minimum energy is -64.4072.

The various compounds characterized from the extract of *Vitex negundo* have displayed an array of promising candidates which can be utilized as drugs against the target proteins of SARS-CoV-2. Docking performed using iGEMDOCK has helped in obtaining various type of bond energies which provide an evidence of the amount of interaction between the ligands and the target proteins. From the obtained information, the total energy ranges from -41 to -98 for all the docked structures which throw ample amount of light in the direction of further studies. The structures possessing highest energy values could be considered as important milestones in the quest of developing novel drugs against the potential strains of SARS-CoV-2 virus. The top five interaction values have been tabulated below:

Table 5.7.12: Best docking results

Protein	Ligand	Total Energy	VDW	H Bond	AverConPair
6W75	608913	-98.3074	-91.9963	-6.31113	27.8636
6W75	425091	-93.572	-89.401	-4.17094	31.0526
5X29	608913	-93.5503	-93.5503	0	25.9545
6MOJ	608913	-92.1934	-88.6984	-3.495	32.3636
7BW4	608913	-91.0153	-91.0153	0	25.9091

From the above table, Despropionyl -3- methylfentanyl (608913) when docked against Nonstructural protein 9 (6W75), the total energy evaluated via computational analysis is -98.3074, which is the highest among all docked structures. Despropionyl -3- methylfentanyl is one of the compounds present in the extract obtained from *Vitex negundo* via RSM optimized Soxhlet extraction. This compound could be employed as a potential drug candidate against the most dreaded disease on calendar, COVID-19. The current study was designed to evaluate the various therapeutic possibilities of the medicinal plant, *Vitex negundo*

Linn., and the docking analysis against the various target proteins of SARS-CoV-2 has brought forth highly prominent phytochemicals which could prove to be eminent milestones in this quest of finding a cure for the pandemic.

CHAPTER 6

SUMMARY AND CONCLUSIONS

6.1 SUMMARY

- This project has been designed to identify the potential of phyto-compounds of *Vitex negundo* Linn. as a natural drug against the target proteins of COVID-19.
- As the initial step of the study, plant isolate was obtained by soxhlet extraction which has been optimised using Central Composite Design of Response Surface Methodology (RSM).
- Optimized extraction has been performed at the appropriate conditions of solvent concentration (80%), extraction temperature (50°C) and extraction time (100 min), using *Vitex negundo* leaves which have been sun-dried for 3 days.
- Some amount of the isolate has been used to study the phytochemistry of the plant.
- Estimation of total chlorophyll and carotenoid content has been done followed by the analysis of protein, carbohydrate and lipid content in the extract, using standard procedures.
- Fourier-transform infrared spectroscopy (FT-IR) has been performed using Shimadzu FTIR Spectrophotometer for the identification of various functional groups present in the phytochemical assembly of the plant.
- Gas chromatography-mass spectrometry (GC-MS) has further confirmed the presence of the chemicals with the stated functional groups. The instrument used was Hewett Packed 6890 with HP-5 Capillary Column.
- Twelve compounds have been confirmed through GC-MS studies which have been further studied through SwissADME software.
- Five phytocompounds with appropriate values of various pharmacokinetic parameters have been finalized as potential ligands to be studied against SARS-CoV-2 target proteins
- Docking has been performed between the potential ligand molecules screened from the plant isolate.

- iGEMDOCK has given energy values as well as other statistical data in regard of the docked structures.
- The results have been individually analysed and the energy values have been compared to understand the interactions between the molecules.
- Five best results have been tabulated state the potential ligands against the various target proteins of the virus

6.2 CONCLUSIONS

The current study is the amalgamation of in-vitro analysis with a computational background to foster better understanding of plant based-medicinal prospects in regard of SARS-CoV-2. A woody, aromatic shrub, famous by the name of Chinese chaste tree and scientifically termed as *Vitex negundo* Linn., has been employed to deliver an extract post optimization via RSM. Various wet-lab experiments have been performed to confer with the curative potential of the plant. The biomolecular design of *Vitex negundo* has been analyzed through various phytochemical techniques. Anti-microbial tests conducted on the extract, throw ample amount of light on the salubrious origin of the metabolites. To better elucidate the components which ornament the plant extract with therapeutic values, characterization has been performed. FT-IR and GC-MS have unveiled an elaborate array of chemical components present in the ethanolic plant extract. Each and every compound has been extensively studied against a picture of medicinal properties. The convoluted study plan has been made simple by the implication of bioinformatics tools at different stages. The SwissADME software has aided in understating the pharmacokinetics and druglikeness of the biochemicals and thereby offering an advantage to take only certain selected compounds for further studies. The biological targets of SARS-CoV-2 have been finalized based on an avid account through the literature survey. SARS-CoV-2 targets and the phytochemically derived ligands have been docked against each other to illustrate effective binding position which plays a significant role in structural based drug designing. The software employed for docking was iGEMDOCK. The results have clearly demonstrated that the approach utilized in this study is successful in finding novel anti-covid drug candidates from *Vitex*

negundo. The ligand, Despropionyl -3- methylfentanyl showed high binding affinity against Nonstructural protein 9 (PDB ID: 6W75). It has shown to exactly fit into the active site region and the ligand formed more number of H-bond interactions with hydrogrn bond energy being -6.31113 while the total energy is -98.3074. The current study has helped in stating the importance of *Vitex negundo* as an effective medicinal implication against the coronavirus and it can be a possible source for metabolites which can bind to SARS-CoV-2 target proteins and render them harmless to a great extent.

REFERENCES

1. Alara, Oluwaseun Ruth, Nour Hamid Abdurahman, and Olusegun Abayomi Olalere, "Ethanol extraction of bioactive compounds from *Vernonia amygdalina* leaf using response surface methodology as an optimization tool.", *Journal of Food Measurement and Characterization*, 12, no. 2, 2018, 1107-1122.
2. Amyrgialaki, Eleni, Dimitris P. Makris, Andy Mauromoustakos, and Panagiotis Kefalas, "Optimisation of the extraction of pomegranate (*Punica granatum*) husk phenolics using water/ethanol solvent systems and response surface methodology.", *Industrial Crops and Products*, 59, 2014, 216-222.
3. Arnon, Daniel I. "Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*." *Plant physiology* 24, no. 1, 1949, 1.
4. Arun Vijay, M., Natheer Hassan, Y., Srinivasa Kumar, K.P., "Evaluation of phytoconstituents and antibacterial activity of *Vitex negundo* var. *Negundos*.", *Int. J. Adv. Interdis. Res.* 1, 2014, 1–4.
5. Aybastier O, Isik E, Sahin S, Demir C, "Optimization of ultrasonic-assisted extraction of antioxidant compounds from blackberry leaves using response surface methodology.", *Ind Crop Prods*, 44, 2013, 558–565.
6. Azahar, Nur Fauwizah, Siti Salwa Abd Gani, and Nor Fadzillah Mohd Mokhtar. "Optimization of phenolics and flavonoids extraction conditions of *Curcuma Zedoaria* leaves using response surface methodology.", *Chemistry Central Journal* 11, no. 1, 2017, 1-10.
7. Balasooriya, E.R., Jayasinghe, C.D., Jayawardena, U.A., Ruwanthika, R.W.D., de Silva, R.M., Udagama, P.V., "Honey mediated green synthesis of nanoparticles: new era of safe nanotechnology.", *J. Nanomat.*, 5919836., 2017.
8. Balavignesh, V., E. Srinivasan, N. G. Ramesh Babu, and N. Saravanan. "Molecular docking study ON NS5B polymerase of hepatitis c virus by screening of volatile compounds from *Acacia concinna* and ADMET prediction.", *Int J Pharm Life Sci* 4, 2013, 2548-58.
9. Basri, F., Sharma, H.P., Firdaus, S., Jain, P. and Ranjan, A., "A review of ethnomedicinal plant-*Vitex negundo* Linn.", *International Journal of Advanced Research*, 2(3), 2014, 882-894.

10. Belwal, Tarun, Praveen Dhyani, Indra D. Bhatt, Ranbeer Singh Rawal, and Veena Pande, "Optimization extraction conditions for improving phenolic content and antioxidant activity in *Berberis asiatica* fruits using response surface methodology (RSM).", *Food chemistry* 207, 2016, 115-124.
11. Bunaciu, Andrei A., Hassan Y. Aboul-Enein, and Serban Fleschin, "FTIR spectrophotometric methods used for antioxidant activity assay in medicinal plants.", *Applied spectroscopy reviews* 47, no. 4, 2012, 245-255.
12. Chawla, A. S., Sharma, A. K., Handa, S. S. Dhar, K. L., "Tyrosinase inhibitory lignans from the methanol extract of the roots of *Vitex negundo* Linn. and their structure-activity relationship.", *J. Nat prod.* 55(2), 1992, 163-167.
13. Erturk, O., Kati, H., Yayli, N. and Demirbag, Z. "Antimicrobial activity of *Viscum album* L. subsp. *abietis* (Wiesb).", *Turk J. Biol.*, 27, 2007, 255 - 258.
14. Farshchi, Amir, Golbarg Ghiasi, Khatabi P. Malek, HOSSEIN FARZAEI, and Amin Niayesh, "Antinociceptive effect of promethazine in mice.", 2009, 140-145.
15. Gupta, Ankit, Madhu Naraniwal, and Vijay Kothari. "Modern extraction methods for preparation of bioactive plant extracts.", *International journal of applied and natural sciences* 1, no. 1, 2012, 8-26.
16. Hanuman Prasad Sharma and Paras Jain, "A review of Ethnomedicinal plant – *Vitex negundo*.", *International journal of advanced research* vol 2 (3), 2014, 882-894.
17. Khare CP. "Encyclopedia of India Medicinal plants.", Springer Verlange, Berline, Heidelberge, New York., 2004, 474-476.
18. Kim JB, Cho KJ, Ali HA, Elamin MH, Ki C, Kim SS. LC/PDA/ESI-MS, *The FASEB Journal*, 21, 2007, 1b76.
19. Kothandan, Ram, Cashlin Anna Suveetha Gnana Rajan, Janamitra Arjun, Rejoe Raymond Michael Raj, and Sowfia Syed, "Virtual screening of phytochemical compounds as potential inhibitors against SARS-CoV-2 infection.", *Beni-Suef University journal of basic and applied sciences* 10, no. 1, 2021, 1-7.
20. Kumar, P. Praveen, S. Kumaravel, and C. Lalitha, "Screening of antioxidant activity, total phenolics and GC-MS study of *Vitex negundo*.", *African Journal of Biochemistry Research* 4, no. 7, 2010, 191-195.

21. Lakshmanashetty, Raghavendra H., Vijayananda B. Nagaraj, Madhumathi G. Hiremath, and Vadlapudi Kumar, "In vitro antioxidant activity of *Vitex negundo* L. leaf extracts.", Chiang Mai J. Sci 37, no. 3, 2010, 489-497.
22. Lee, M.T., Lin, W.C., Yu, B., Lee, T.T., "Antioxidant capacity of phytochemicals and their potential effects on oxidative status in animals—a review. Asian Australas.J. Anim. Sci., 30, 2017, 299–308.
23. Li, Yan, Jinyong Zhang, Ning Wang, Yanjing Zhang, Yongjun Yang, Yue Yuan, Haiming Jing, "High-throughput Screening and Experimental Identification of Potent Drugs Targeting SARS-CoV-2 Main Protease.", 2020.
24. Lopez Fernandes S, Kim HS, Choi EC, Delgado M, Granja JR, Khasanov A, Kraehenbuehl K, Long G, Weinberger DA, Wilcoxon KM, Ghadiri MR., "Antibacterial agents based on the cyclic D,L-alpha-peptide architecture.", Nature, 412(6845), 452
25. Mylonaki, Stefania, Elias Kiassos, Dimitris P. Makris, and Panagiotis Kefalas, "Optimisation of the extraction of olive (*Olea europaea*) leaf phenolics using water/ethanol-based solvent systems and response surface methodology.", Analytical and Bioanalytical Chemistry 392, no. 5, 2008, 977-985.
26. Meena, A. K., Niranjana, U.S., Rao, M.M., Padhi, M.M. and Ramesh Babu., "A review of the important chemical constituents and medicinal uses of *Vitex* genus". Asian Journal of Traditional Medicines.6 (2), 2011.
27. Menaka Thounaojam, Valodkar, MayurRathore, Puran Singh Jadeja, Raviraj Singh, "Cytotoxicity evaluation and antimicrobial studies of starch capped water soluble copper nanoparticles.", FAO of the UN, 2012.
28. Mitra D, Verma D, Mahakur B, Kamboj A, Srivastava R, Gupta S, Pandey A, Arora B, Pant K, Panneerselvam P, Ghosh A., "Molecular docking and simulation studies of natural compounds of *Vitex negundo* L. against papain-like protease (PLpro) of SARS-CoV-2 (coronavirus) to conquer the pandemic situation in the world.", Journal of Biomolecular Structure and Dynamics, 2021, 1-22.
29. Monisha, S. Irudaya, G. Dayana Jeya Leela, A. Anitha Immaculate, and J. Rosaline Vimala, "GC-MS Analysis and Green Synthesis of Copper Nano particles Using *Vitex negundo*. L Leaf Extract.", International Journal of Scientific & Engineering Research, 2, 2016.

30. Muthuswamy, U., Kuppusamy, A., Nandagopi, U., Thirumalaisamy, S. and Varadharajan S., "Protective effect of the leaves of *Vitex negundo* against ethanol-induced cerebral oxidative stress in rats". Tanzania J Health Res., 14(1), 2012: 1-1.
31. Najmi, A.K., Pillai, K.K., Pal, S.N. and Aqil, M., "Free radical scavenging and hepatoprotective activity of jigrine against galactosamine induced hepatopathy in rats. Journal of ethnopharmacology, 97(3), 2005, 521-525.
32. Nallusamy, Saranya, Jayakanthan Mannu, Caroline Ravikumar, Kandavelmani Angamuthu, Bharathi Nathan, Kumaravadivel Nachimuthu, Gnanam Ramasamy, Raveendran Muthurajan, Mohankumar Subbarayalu, and Kumar Neelakandan, "Shortlisting Phytochemicals Exhibiting Inhibitory Activity against Major Proteins of SARS-CoV-2 through Virtual Screening.", 2010.
33. Nile, S.H., Nile, A.S., Keum, Y.-S., "Total phenolics, antioxidant, antitumor, and enzyme inhibitory activity of Indian medicinal and aromatic plants extracted with different extraction methods.", 3 Biotech, 2017, 7-76.
34. Perdue, R.E. Jr. "Procurement of plant materials for antitumor screening". Cancer Treat. Rpt., 60, 1976, 987-998.
35. Prajapati ND, Purohit SS, Sharma AK, Kumar TA. "Handbook of Medicinal Plants", AGROBIOS, (India) Jodhpur, 2003, 3
36. Prajapat, Manisha, Phulen Sarma, Nishant Shekhar, Ajay Prakash, Pramod Avti, Anusuya Bhattacharyya, Hardeep Kaur et al. "Update on the target structures of SARS-CoV-2: a systematic review." Indian journal of pharmacology 52, no. 2, 2020, 142
37. Rajeshwari Sahu et al., "Ultraviolet Visible and Fourier Transform Infrared Spectroscopic Studies on Non-conventional Species of Curcuma". IJACS., 2014, 2(4), 300-302.
38. Rampriya S, Arul Sheeba Rani M, "Preliminary phytochemical and biochemical studies on *Vitex negundo*, Linn.", International Journal of Advanced Science and Research, Vol 2 (2), 2017, 53-56.
39. Rolta, Rajan, Rohitash Yadav, Deeksha Salaria, Shubham Trivedi, Mohammad Imran, Anuradha Sourirajan, David J. Baumler, and Kamal Dev, "In silico screening of hundred phytochemicals of ten medicinal plants as potential inhibitors of nucleocapsid phosphoprotein of COVID-19: an

- approach to prevent virus assembly.", Journal of Biomolecular Structure and Dynamics, 2020), 1-18.
40. Rooban, B. N., V. Sasikala, V. Gayathri Devi, V. Sahasranamam, and Annie Abraham, "Prevention of selenite induced oxidative stress and cataractogenesis by luteolin isolated from *Vitex negundo*.", Chemico-biological interactions 196, no. 1-2, 2012, 30-38
 41. Saklani, Sarla, Abhay Prakash Mishra, Harish Chandra, Maria Stefanova Atanassova, Milan Stankovic, Bhawana Sati, Mohammad Ali Shariati, "Comparative evaluation of polyphenol contents and antioxidant activities between ethanol extracts of *Vitex negundo* and *Vitex trifolia* L. leaves by different methods." Plants 6, no. 4, 2017, 45.
 42. Salvatori, Giovanni, Laura Luberto, Mariano Maffei, Luigi Aurisicchio, Giuseppe Roscilli, Fabio Palombo, and Emanuele Marra, "SARS-CoV-2 SPIKE PROTEIN: an optimal immunological target for vaccines.", Journal of translational medicine 18, 2020, 1-3.
 43. Saral R., "Candida and Aspergillus infections in immunocompromised patients: an overview.", Rev. Infect Dis, Vol.13(3), 1991, 487-92.
 44. Sarika Khandare, "Genotoxic Potential Assessment, UV-Vis Spectrophotometric and FTIR Analysis of Leaf Extract of *Vitex negundo* L.", IJSID., 2(1), 2012, 244-252.
 45. Shazia Haider, Zafar Saeed Saify, Nasreen Begum, Seema Ashraf, Tabinda Zarreen, S.M.Ghufran Saeed, "Emerging Pharmaceutical Applications Of Piperidine, Pyrrolidine And It S Derivatives.", Vol. 3(7), 2014, 987-1024.
 46. Sivarajan, V.V, BalachandranI, "Ayurvedic drugs and their plant sources.", New Delhi, India, Oxford and IBH, 1994.
 47. Stanley Irobekhan Reuben Okoduwa, Ismaila A Umar, Dorcas B James, Hajara M Inuwa, James D Habila, "Evaluation of extraction protocols for anti-diabetic phytochemical substances from medicinal plants" World J Diabetes, Vol. 7(20), 605-614
 48. Suganthi N and Sonal Dubey, "Phytochemical constituents and Pharmacological activities of *Vitex negundo* Linn.," vol. 8(2), 2016, 800-807.
 49. Telang, R. S., S. Chatterjee, and C. Varshneya, "Study on analgesic and anti-inflammatory activities of *Vitex negundo* Linn.", Indian journal of pharmacology 31, no. 5, 1999, 363.

50. Tiwari. N, S.Luqman,N.Masood, "Validated high performance thin layer chromatographic method for simultaneous quantification of major iridoids in *Vitex trifolia* and their antioxidant studies.", 2012, 207-214.
51. Vora, Jaykant, Shivani Patel, Mohd Athar, Sonam Sinha, Mahesh T. Chhabria, Prakash C. Jha, and Neeta Shrivastava, "Pharmacophore modeling, molecular docking and molecular dynamics simulation for screening and identifying anti-dengue phytocompounds.", *Journal of Biomolecular Structure and Dynamics*, 2019.
52. Vimal Arora, Lohar, V., Singhal, S.and Bhandari, A.,"*Vitex negundo* – “A Chinese Chaste Tree”, *International Journal of Pharmaceutical Innovations.*, 1(5), 2011, 9- 20.
53. Wikler, Matthew A., "Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard." CLSI (NCCLS) 26, 2006, M7-A7.
54. Zargar, M., A. H. Azizah, A. M. Roheeyati, A. B. Fatimah, F. Jahanshiri, and M. S. Pak-Dek, "Bioactive compounds and antioxidant activity of different extracts from *Vitex negundo* leaf.", *Journal of Medicinal Plants Research* 5, no. 12, 2011, 2525-2532.
55. Zellner, Barbara d'Acampora, Carlo Bicchi, Paola Dugo, Patrizia Rubiolo, Giovanni Dugo, and Luigi Mondello, "Linear retention indices in gas chromatographic analysis: a review.", *Flavour and Fragrance Journal* 23, no. 5, 2008, 297-314.