

PRELIMINARY SCREENING OF SPROUT OF CURCUMA LONGA RHIZOME AND IN- SILICO ANALYSIS OF BIOACTIVE COMPOUNDS FOR PROPHYLAXIS TREATMENT OF CORONA

Submitted in partial fulfilment of the requirements for the award of
Bachelor of Technology degree in Biotechnology

By

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**DEPARTMENT OF BIOTECHNOLOGY
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INSTITUTE OF SCIENCE AND TECHNOLOGY

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(DEEMED TO BE UNIVERSITY)

Accredited with Grade "A" by NAAC | 12B STATUS BY UGC |

APPROVED BY AICTE

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CHENNAI - 600119
MARCH-2021**



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DEPARTMENT OF BIOTECHNOLOGY

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Pamujula Nava Shubhasre (37230053)**, **Vikas Netha P. (37230095)**, who carried out the project entitled Preliminary Screening of Sprout of Curcuma Longa Rhizome And In-Silico Analysis Of Bioactive Compounds For Prophylaxis Treatment Of Corona under my supervision from December 2020 to March 2021

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Internal Guide

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Head of the Department

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Submitted for Viva voce Examination held on _____

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DECLARATION

We **Pamujula Nava Shubhasre (37230053),Vikas Netha P. (37230095)**hereby declare that the Project Report entitled Preliminary Screening of Sprout of Curcuma Longa Rhizome And In-Silico Analysis Of Bioactive Compounds For Prophylaxis Treatment Of Corona done by me under the guidance of **Dr. .Prakash P**, M.Tech., Ph.D. Assistant Professor, Department of Biotechnology, Sathyabama Institute of Science and Technology. Is submitted in partial fulfilment of the requirements for the award of Bachelor of Engineering / Technology degree 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.

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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

India is currently fighting against the deadly contagious, *corona virus* in all possible means. Though many antiviral drugs are available in mainstream of treatment for *respiratory viral infections*, so far no targeted specific antiviral has been identified and moreover vaccine trials are ongoing. Traditional system of medicine possesses a broad range of scope to bridge the gaps in treatment methods and the time lag between vaccine developments. There may be chance of mutation of virus, drug resistance etc. So far there is scarcity of research evidence for herbal medicines which is been the reason for non-acceptance by the scientific community. The aim of this study is to extract *secondary metabolites* from sprout shoot of *Curcuma longa* and identify by GCMS. The binding site of identified significant compounds will be computed through *secondary metabolites*

Key words: Respiratory viral infections, Secondary metabolites, Curcuma longa, Corona virus

Introduction

The recent outbreak of COVID-19, primarily in Wuhan, China, resulted in an international concern and was declared a public health emergency. Pneumonia associated with COVID-19 has raised global concern. The lack of proven antiviral drugs, vaccines, and asymptomatic carriers has made long-standing public health intervention procedures not effective in controlling the disease's spread [Sun J et al., 2020]. The novel coronavirus 2019, also called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is caused by the infection of SARS-CoV-2 that prevailed from Wuhan. Currently, it is almost more than 200 countries. Primarily, the efforts were made to focus more on clinical courses, identify and treat critically ill cases, and symptomatically treat the sick as the spread of coronavirus progresses with outbursts of pandemic influenza, Middle East respiratory syndrome (MERS), and other symptoms. This had necessitated understanding the public health activities to eradicate the incidence, prevalence, and spread of the novel Coronavirus and depict the possible effects. The epidemiology is solemnly dependent on the persons infected, their transmissibility, and their severity [2].

1.1. COVID-19 pandemic:

WHO termed the disease COVID-19, acronym for “coronavirus disease 2019” on February 11th, 2020, by adopting the norms for specifying WHO's human infectious diseases. This practice of naming contagious diseases in humans was established in consultation and association with the World Organisation for Animal Health (OIE) and the Food and Agriculture Organization of the United Nations (FAO). Egypt was the second country in the WHO EMRO region to record the first confirmed case on February 15th, 2020 [3].

1.2. WHO Risk Assessment

WHO has updated the risk assessment of the danger of spread and risk of effect on COVID-19 to be inflated at the global level. From February 20th, 2020 to February 28th, 2020 nearly 25 new countries have reported COVID-19 prevalence. The global figure of registered COVID-19 patients has raised to 3,435,894 cases by May 04th, 2020 causing 239,604 deaths by affecting 212 countries and territories. The spread of this novel coronavirus has been brought under control in China [4].

The European territory becomes the epicenter for a pandemic on March 14th, 2020 with more reported cases and death than the rest of the world combined other than China (WHO, 2020) [5,6].

1.2. Coronavirus: an overview of the virus

Coronaviruses are single-strand RNA viruses causing enzootic diseases, categorized into four subgroups as alpha, Beta, Gamma, and Delta. Human coronavirus 229E (HCoV-229E), Human coronavirus NL63 (HCoV-NL63), Transmissible gastroenteritis coronavirus (TGEV), Canine coronavirus (CCoV), Porcine respiratory coronavirus (PRCoV), Feline coronavirus (FeCoV), Porcine epidemic diarrhea coronavirus (PEDV) are categorized under α -CoVs. Human coronavirus 4408 (HCoV-4408), Human coronavirus OC43 (HCoV-OC43), Human coronavirus HKU1 (HCoV-HKU1), Severe acute respiratory syndrome coronavirus (SARS-CoV), Middle Eastern respiratory syndrome coronavirus (MERS-CoV), Bat coronavirus (BCoV), Porcine hemagglutinating encephalomyelitis virus (HEV), Murine Hepatitis Virus (MHV) are further characterized under β -CoVs. γ -CoVs include Avian infectious bronchitis virus (IBV) and Turkey coronavirus (TCoV) [7,8]. So far, there are six Coronaviruses infecting humans, and among them, only the following viruses HCoV-NL63, HCoV-229E, HKU1, and HCoV-OC43 have been infected humans during the past two decades.

In late 2019, there were several outbreaks of novel Pneumonia in a Seafood market in Wuhan, Hubei province, China, caused by a novel beta coronavirus-Wuhan coronavirus sharing 79.5% of the genome similarity with SARS-CoV, which caused 2002-2003 pandemic and renamed as SARS-CoV-2 by International committee on taxonomy of viruses [9]. Later by January 30th, 2020, WHO declared the COVID-19 as the sixth major international public health emergency.

Human coronaviruses are transmitted from animals, either rodents or bats, and this particular SARS-CoV-2 has reportedly been spread from pangolins in China [10]. These viruses are enveloped with a lipid layer containing spikes-like structure resembling crown structure and hence the name Coronavirus (Crown in Latin - Corona). These spikes include several unique diversified surfaces receptor-binding domain (RBD), which bind with the angiotensin-converting enzyme-2 (ACE-2) receptor present in lungs, heart, gastrointestinal tract, and kidneys [11] which forms the viral admission into the host cell [9].

After the viral entry, the SARS-CoV-2 fix to the ACE-2 receptors in the type II pneumocytes found in the lungs. This attachment triggers a cascade of inflammatory processes in the lower respiratory system. Further, it activates the spike protein through a process of type 2 transmembrane protease TMPRSS-2 which further leads to the cleavage of ACE 2. [12,13,14]. This route of cellular infection triggers the immune response of the host and activates the inflammatory cascade through the initiation of antigen-presenting cells (APC), which performs two significant functions viz., exposing the antigen to CD_4^+ Th1 cells and liberating IL-12 to stimulate the T helper cell further and followed by stimulation of CD_8^+ T_k cells. This, in turn, targets any cells with the extraneous antigen, followed by B-cells' stimulation to yield antigen-specific antibodies by activating Th1 cells [9]. The immunocompromised host becomes more susceptible to this virus and increases the mortality rate.

WHO reported the transmission rate of this virus *i.e.*, R_0 , to be between 1.4 and 2.5 and the incubation period after the viral entry into the host organism ranging from 2 to 14 days, making them more vulnerable to transmit through respiratory droplets than airborne

transmission. Asymptomatic transmission made them to isolate the infected patients and cure them effectively. Also, age-related risks and pre-existing diseases increase the fatality rate [9].

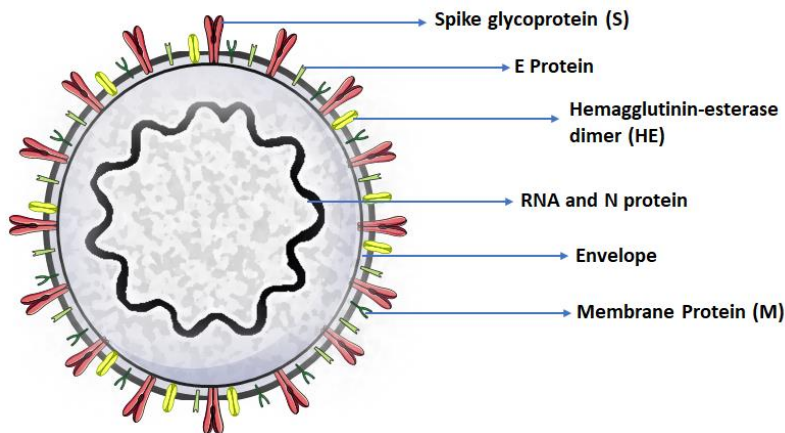


Figure 1: Structure of Coronavirus (COVID-19)

In the current scenario, the management of novel coronavirus infection is focused on supportive care and testing the prevailing anti-corona viral drugs efficacy. Antiviral drugs used during the past epidemics of beta coronavirus, such as interferon, darunavir, ribavirin, and lopinavir, are currently being evaluated for their potential through in vitro tests [15]. Initially, Adenosine analog, Remdesivir, which was earlier used against RNA viruses like SARS, MERS, and Ebola treatment, was used to treat the SARS-CoV-2 infection [16]. Also, Camostat mesylate, Chloroquine, and Hydroxy Chloroquine are being used as potent drugs to fight the SARS-CoV-2. Chloroquine and Hydroxy Chloroquine block the viral cell fusion and inhibit RBD glycosylation and cellular receptors of ACE-2, limiting the interaction and inhibiting the viral attachment to the host cells [9].

A long-term plan of developing mRNA-based vaccines is underway for the future method of prophylaxis. The recent finding focuses on targeting ACE-2 receptor interaction for which the convalescent sera are also used to treat the disease [9].

1.4. Coronavirus drug discovery:

In the alarming situation, drug development, proving the efficacy of a particular drug remains an arduous task for AYUSH professionals. Researchers around the world are striving hard to bring out a functional antiviral for COVID-19. In the current lockdown period, various computation-based analyses are initiated, taking the researchers on the right path to proceed further.

1.4.1. Computation - based analysis:

Several studies have been initiated to analyze the various aspects of the COVID-19 treatment computationally. The whole-genome sequence of different strains of Human Coronaviruses was compared with other strains to understand the phylogenetic relationship between 2019-nCoV/SARS-CoV-2. The studies found that it had a 79.7% similarity with SARS-CoV among the pathogens. This showed similarity in terms of whole-genome sequence and shared similarity with protein sequences, specifically the envelope protein and nucleocapsid protein of 2019-nCoV/SARS-CoV-2, conserved evolutionarily and they displayed a sequence identity of 96% and 89.6%, respectively, when compared with SARS-CoV. When analyzed on the interaction of this virus with the host proteins, it was identified that the proteins, namely JUN, XPO1, NPM1, and HNRNPA1, were shown to exhibit maximum interactions with HCoV. Also, the druggable targets were identified by exploring the HCoV - host interactome network. It has been observed that 47 proteins can be targeted by a minimum of one approved drug or drug that is under clinical trials, with DPP4, GSK3B, SMAD3, PARP1, and IKBKB being the most targetable proteins. *In silico* studies predicted 135 drugs that were connected to HCoV–host interactome ($Z < -1.5$ and $P < 0.05$, permutation test). These were further analyzed to obtain 16 repurposable drugs with high confidence (e.g., melatonin, mercaptopurine, and sirolimus) [17].

Another study reported using some anti-polymerase drugs currently on the market as potential inhibitors of the RNA-dependent RNA polymerase (RdRp) of the newly emerged coronavirus. Docking studies have revealed that Ribavirin, Sofosbuvir, Remdesivir, Galidesivir, and Tenofovir have shown good binding efficiency with RdRp, guanosine derivative (IDX-184), Setrobuvir, and YAK as most effective antiviral agents with high potential to fight, specifically the SARS CoV-2 strain. The advantage of using these drugs is that they are already in the market and hence non-toxic, thereby making drug repurposing a suitable option to manage the disease in a short time [18].

The target-based screening performed by Wu *et al.* (2020) initially identified the targets, namely, 3-chymotrypsin-like protease (3CLpro), Spike, RNA-dependent RNA polymerase (RdRp), and papain-like protease (PLpro) for which virtual screening using ZINC drug database, 78 antiviral drugs and their database which provides a cue to the treatment and management of COVID-19 was carried out [19].

The research fraternity is looking into the ability of natural products as potential inhibitors of viral growth. A study by Qamar *et al.* (2020) attempted to find medicinal plant products that can inhibit the viral enzyme viral 3-chymotrypsin-like cysteine protease 3 (3CLpro) that is crucial for the replication of coronavirus [20]. In SARS-CoV and MERS-CoV, this enzyme is a proven drug target. Hence, it was used as a target to screen libraries of medicinal plants containing 32,297 potential antiviral phytochemicals/traditional Chinese therapeutic compounds.

Another report by Zhang *et al.* (2020) suggested the role of natural compounds from traditional Chinese medicines. Initial screening of 125 herbs revealed that 26 were used for

treating viral infections related to immune/inflammation reactions and hypoxia response [21].

An immunoinformatic approach is also adopted to identify important epitopes of cytotoxic T Lymphocytes and B cells for 2019-nCoV surface glycoprotein. The surface glycoprotein of 2019-nCoV was found to have a resemblance with the spike glycoprotein of SARS-CoV (identity: 76.3% and similarity: 87.3%). Five CTL epitopes were identified in the surface glycoprotein. These five epitopes showed efficient binding with their corresponding MHC class I supertype representative based on molecular simulation studies. These interactions signify a potentially successful presentation of the epitopes by MHC class I molecules, thereby activating CTLs and generating immune responses [22].

AIM AND OBJECTIVE

- To Extract and characterize the volatile bioactive compounds from the turmeric shoot involved.
- To estimate phytochemical analysis of Alkaloid, Tannins, saponins, flavonoids, Quinines, Glycosides, Terpinoids, phenols, steroids and phlobatannins.
- To evaluate bioactive compounds by GCMS and LCMS
- In silico interaction studies of spike protein with active compounds

METHODOLOGY:

Sample preparation:

Collected samples were washed with running tap water to remove the dust and other epiphytes from the lichens. The lichens were transported to the laboratory in sterile polythene bags. In laboratory the samples were again rinsed with tap water to remove the residual dust and dried.

Solvent extraction:

5gms of dried sample was taken in two conical flasks. 50 ml of methanol and 50 ml of distilled water are added to the two flasks respectively. The soaked sample is placed in shaker and incubated for three days. Then the sample was taken out from shaker and is centrifuged for 10 min. The supernatant from the methanolic extract was taken and evaporated in a petri plate and 1- 2 ml of methanol was added and crude sample was prepared to 1-2 ml. The aqueous sample was taken and the same process is repeated with distilled water replacing methanol.

Phytochemical screening:

Phytochemical examinations were carried out for all the extracts as per the standard methods.

Detection of alkaloids:

Mayer`s Test:

Take few ml of extract, add a drop mayers reagent. Appearance of white creamy precipitate indicates the presence of alkaloids.

Detection of Glycoside:

Take few ml of sample, add concentrated HCl, add 2ml of glacial acetic acid and now add 1ml of H₂SO₄. Appearance of violet ring below the brown ring and green ring confirms the presence of glycosides.

Detection of saponins:

1ml of sample is added with 2ml of distilled water and mix well. Development of stable foam suggests the presence of saponins.

Detection of Terpenoids:

To a 2ml of extract, few drops of saturated solution of trichloro acetic acid are used formation of coloured precipitate confirms the presence of terpenoids.

GCMS ANALYSIS:

Analysis of Volatile compounds by GC MS: GC-MS of Varian, Saturn model 2000, equipped with ion trap detector (ITD) will be used for the identification of different components of volatile extracts. Sample will be injected on a DB-5MS (30 m × 0.25 mm id, 0.25 μ film thickness) column. Helium will be used as a carrier gas with a flow rate of 7.0-9.5 psi and split ratio 1:5. The column temperature was maintained at 75 °C for 5 min with a 2.5 °C rise/min to 250 °C (Shaguta naz et al (2010)).

Liquid Chromatography-Mass Sample spectrometry Analysis:

The extracted sample will be analyzed by using Agilent 6530 Accurate-Mass Q-TOF liquid chromatography-mass sample spectrometry (LC-MS) system with Agilent Zorbax Eclipse XDB-C18 column (2.1×50 mm, 1.8 micron), eluted with acetonitrile and water as gradient system. The mobile phase started with 99.5% of water and decreased to 50% of water over 10 min, followed by 50% of water and decreased to 0% over 25 min and then held at 0% of water for 5 min and finally increased to 99.5% of water over 3 min at a flow rate of 0.25 mL/min. The experiments will be performed in the positive ion mode. The flow rate of the drying gas will be set at 8 L/min at the temperature of 350 °C. The nebulizer pressure will be set at 35 PSIG with the capillary and injection volume set at 3000 V and 5 μL resamplelectively (Ke-Xin Yu et al, 2015).

Insilica analysis:

In silico interaction studies of spike protein with plant based natural compounds The plant based natural compounds from the selected sources will be retrieved using plant secondary metabolite databases and their three-dimensional structure would be retrieved from PUBCHEM. These molecules will be docked with Spike protein using Auto dock and the various parameters of binding like the binding energy, inhibition constant etc based on which efficiently bound plant molecules will be selected. The interactions will be observed using Discovery studio BIOVIA client. The selected plant molecules will be analyzed for their pharmacological properties (ADMET) using SWISSADME. This step would initially provide data regarding the type of plant molecules involved in interactions like alkaloids, flavanoids etc and gives cue for further experimental studies and validation.

REVIEW OF LITERATURE

The COVID-19 virus is exclusive among human coronaviruses since it has high transmissibility, uncontrollable mortalities in high-risk age groups, and has ability to cause huge social tension and economic loss. The present situation where there is increasing Covid19 infections and number of mortality per day shows that the world population seems to be susceptible to this virus. The development of scientifically validated safe drug for COVID 19 infection is the need of the hour globally. The duty of researcher is to identify potent antiviral drugs in their respective fields to bring back the country to normalcy. Most of the ingredients of the proposed formulation for steam therapy possess anti-microbial, antiviral, antioxidant, antipyretic effect as per Siddha classics and other research publications. The compound formulation is expected to be effective against combatting COVID 19 as per the concept of synergism. As research is an integral part of fight against COVID 19, this proposed project will give a lead to conduct Randomized Controlled Clinical trials which is the gold standard for establishing clinical evidence. If the proposed formulation was proved to be a potent antiviral it can be prescribed widely for COVID 19.

Since 2003, about three new pathogenic human coronaviruses have been emerged from animal reservoirs (Paules et al. 2020). These are Middle East respiratory syndrome- related coronavirus (MERS-CoV), severe acute respiratory syndrome coronavirus (SARS-CoV), and, severe acute respiratory syndrome coronavirus 2 (referred to as COVID-19, SARS-CoV-2, or 2019-nCoV) leading to global health emergencies, with high morbidity and mortality (Mark and Paul, 2020).

The novel corona virus has created a pandemic by infecting around 32,00,000 humans in more than 200 countries leading to around 2, 18, 000 death globally (WHO, 2020). Corona viruses primarily infect birds and mammals, increasing the mortality and causes deleterious impact on the human and animal health. In human these viruses cause few respiratory diseases viz common cold to server acute respiratory syndrome after pneumonia and bronchitis (Dewald and Burtram, 2020).

The recent history of infection of human corona viruses are seen in both upper and lower respiratory tract infections. Majority of CoVs cause enzootic infections, limited only to their natural animal hosts, but they have mutated to cross transmit among humans as zoonotic diseases (Rest and Mindell, 2003).

These viruses are enveloped with lipid layer containing spikes like structure resembling crown like structure and being the reason behind the name Corona virus (Crown in Latin - Corona). These spikes include several unique diversified receptor-binding domain (RBD) which are compatible to bind with the angiotensin-converting enzyme-2 (ACE-2) receptor found in lungs, heart, kidneys and gastrointestinal tract (Ksiazek et al., 2003) which pave way for the viral entry into the host cell (Firas et al., 2020).

After the entry of the virus, the SARS-CoV-2 bind to the ACE-2 receptors in the type II pneumocytes in the lungs and triggers a cascade of inflammation in the lower respiratory tract and activates the spike protein through process of type 2 transmembrane protease TMPRSS-2 which further leads to the cleavage of ACE 2 (Kuba et al., 2003; Glowacka et al., 2011 and Heurich et al., 2014). This route of cell infection triggers the host's immune response, and the inflammatory cascade through initiation of antigen-presenting cells (APC) which performs the two major functions viz. exposing the foreign antigen to CD4 + -T-helper (Th1) cells, and releasing interleukin-12 to further stimulate the Th1 cell and followed by stimulation of CD8 + - T-killer (Tk) cells that will target any cells containing the foreign antigen. Followed by the stimulation of B-cells to produce antigen-specific antibodies by activating Th1 cells (Firas et al., 2020).

Whereas the immunocompromised host fails to become more susceptible to this virus and increase the mortality and highly contagious. The coronaviral genome encodes four major structural proteins: the spike (S) protein, nucleocapsid (N) protein, membrane (M) protein, and the envelope (E) protein, all of which are required to produce a structurally complete viral particle. The envelope protein Structure The CoV E protein is a short, integral membrane protein of 76–109 amino acids, ranging from 8.4 to 12 kDa in size. The primary and secondary structure reveals that E has a short, hydrophilic amino terminus consisting of 7–12 amino acids, followed by a large hydrophobic transmembrane domain (TMD) of 25 amino acids, and ends with a long, hydrophilic carboxyl terminus, which comprises the majority of the protein (Mark and Paul, 2020).

Coronaviruses are unique as they bind with the Golgi complex by localizing with the E protein and the Golgi complex causing intracellular trafficking of viral particles (Nieto-Torres et al., 2011) .

The current scenario in the treatment of COVID 19 is supportive and to test the existing anti corona viral drugs efficacy. During the past epidemics of betacorona virus most of the employed antiviral drugs such as interferon, darunavir, ribavirin and lopinavir are being evaluated for its potential through Invitro tests (Chu, 2004).

Adenosine analog, Remdesivir was also used to treat SARS-CoV-2 as it was used against RNA viruses such as SARS, MERS and Ebola treatment (Sheahan et al., 2017).

In addition, Camostat mesylate, Chloroquine and Hydroxy Chloroquine are being used as potent drug against SARS-CoV-2. Chloroquine and Hydroxy Chloroquine are found to block the viral cell fusion and interfere with glycosylation of RBD and ACE-2 cellular receptors leading to the less interaction and inhibition of the virus for attachment to the host cells (Firas et al., 2020).

Long term plan of developing mRNA based vaccines are also underway for the future method of prophylaxis. The recent finding focuses on the targeting of ACE-2 receptors interaction for which the convalescent sera are also being used to treat the disease (Firas et al., 2020).

In this research an in silico approach was employed to find a suitable bioactive molecule from the formulation to target blockage of S-Protein – ACE-2 receptor interaction to inhibit the attachment and subsequent entry of the coronavirus onto the host cells.

Novel corona virus outbreak was realized in India in the month of March 2020. The Central Government has taken measures to curb the pandemic COVID 19 by implementing social distancing, ensuring frequent hand washing, using face masks etc., The AYUSH Ministry has been propagating the AYUSH medicines for boosting the immunity. The Government of Kerala started using Ayurvedic medicines along with the routine protocol. With a vision, In Tamil Nadu, the Government issued a G.O. (Ms).No. 201 for implementing a special programme “AAROKYAM” with AYUSH interventions for COVID -19 patients. The Siddha formulations Kabasura kudineer/Nilavembu kudineer is recommended for immune enhancement and Ammukara chooranam, Nellikai lehyam in convalescence period. In addition to this as per the Ministry of AYUSH, Corona Advisory– D.O.No. S. 16030/18/2019– NAM; dated: 06th March, 2020 Visha sura kudineer and Kaba sura kudineer has been mentioned in add ons to conventional care.

A molecular docking study was conducted by Rane et al (2020) using 10 potential naturally occurring flavonoids/non-flavonoids against the SARS-CoV-2 spike protein which was compared with the FDA approved drug hydroxychloroquine (HCQ). Interestingly, the docking analysis suggested that C-terminal of S1 domain and S2 domain of the spike protein are important for binding with these compounds. Kamferol, curcumin, pterostilbene, and HCQ interact with the C-terminal of S1 domain with binding energies of -7.4, -7.1, -6.7 and -5.6 Kcal/mol, respectively. Fisetin, quercetin, isorhamnetin, genistein, luteolin, resveratrol and apigenin on the other hand, interact with the S2 domain of spike protein with the binding energies of -8.5, -8.5, -8.3, -8.2, -8.2, -7.9, -7.7 Kcal/mol, respectively.

From this study, it is very well understood that these flavonoid and non-flavonoid moieties have significantly high binding affinity for the two main important domains of the spike protein which is responsible for the attachment and internalization of the virus in the host cell and their binding affinities are much higher compared to that of HCQ. In addition, ADME (absorption, distribution, metabolism and excretion) analysis also suggested that these compounds consist of drug likeness property which may help for further explore as antiSARS-CoV-2 agents.

Further, in vitro and in vivo study of these compounds will provide a clear path for the development of novel compounds that can interact with spike protein. Results obtained by Anrag et al (2020) indicated that 10 herbal molecules were docked against SARS-CoV 2 protease where in Oxyacanthine and Hypericin (-10.990 and -9.05 and kcal/mol respectively) have shown good binding efficacy among others but Oxyacanthine was the only natural product which made some of necessary interactions with residues in the enzyme require for target inhibition. Therefore, Oxyacanthine may be considered to be potential inhibitor of main protease enzyme of virus but need to be explored for further drug development process.

Another study reported the inhibitory effect of 17 organosulphur compounds in garlic essential oils on the host receptor angiotensin-converting enzyme 2 (ACE2) protein in the

human body that leads to a crucial foundation about coronavirus resistance of individual compounds on the main protease (PDB6LU7) protein of SARS-CoV-2. The results show that the 17 organosulfur compounds, accounting for 99.4% contents of the garlic essential oil, have strong interactions with the amino acids of the ACE2 protein and the main protease PDB6LU7 of SARS-CoV-2.

The strongest anticoronavirus activity is expressed in allyl disulfide and allyl trisulfide, which account for the highest content in the garlic essential oil (51.3%) (Thuy et al 2020).

Pitchiah et al 2019 has attempted to establish the antiviral activity of traditional Siddha formulations Kabasura kudineer and Thonthasura Sura kudineer for corona virus infection through in silico molecular docking. Importance of the proposed project in the context of Current status The COVID-19 virus is exclusive among human coronaviruses since it has high transmissibility, uncontrollable mortalities in high-risk age groups, and has ability to cause huge social tension and economic loss. The present situation where there is increasing Covid19 infections and number of mortality per day shows that the world population seems to be susceptible to this virus.

The development of scientifically validated safe drug for COVID 19 infection is the need of the hour globally. The duty of researcher is to identify potent antiviral drugs in their respective fields to bring back the country to normalcy. Most of the ingredients of the proposed formulation for steam therapy possess anti-microbial, antiviral, antioxidant, antipyretic effect as per Siddha classics and other research publications.

In silico studies on phytochemicals as inhibitors of Spike protein

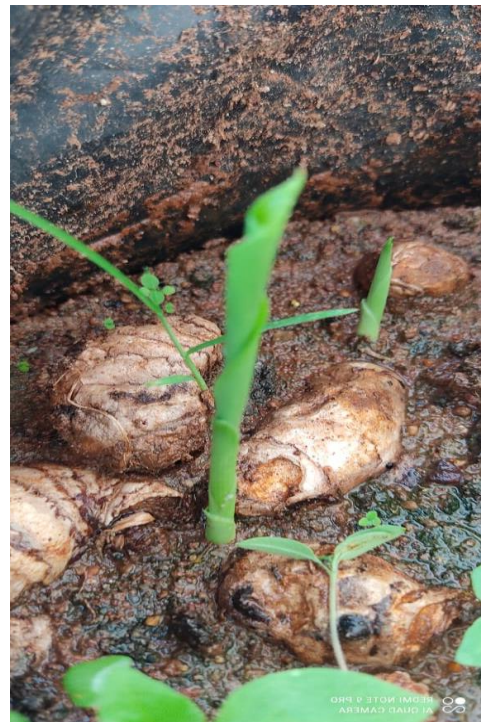
In combating Covid 19, several computational studies have been carried out to identify the potential natural plant compounds that can efficiently bind to spike protein of SARS-CoV-2. A study by Rane et al (2020) performed a molecular docking study using 10 potential naturally occurring flavonoids/non-flavonoids against the SARS-CoV-2 spike protein and compared their affinity with the FDA approved drug hydroxychloroquine (HCQ). Interestingly, the docking analysis suggested that C-terminal of S1 domain and S2 domain of the spike protein are important for binding with these compounds. Kamferol, curcumin, pterostilbene, and HCQ interact with the C-terminal of S1 domain with binding energies of -7.4, -7.1, -6.7 and -5.6 Kcal/mol, respectively. Fisetin, quercetin, isorhamnetin, genistein, luteolin, resveratrol and apigenin on the other hand, interact with the S2 domain of spike protein with the binding energies of -8.5, -8.5, -8.3, -8.2, -8.2, -7.9, -7.7 Kcal/mol, respectively. From this study, it can be understood that these flavonoid and non-flavonoid moieties have significantly high binding affinity for the two main important domains of the spike protein which is responsible for the attachment and internalization of the virus in the host cell and their binding affinities are much higher compared to that of HCQ. In addition, ADME (absorption, distribution, metabolism and excretion) analysis also suggested that these compounds consist of drug likeness property which may help for further explore as anti-SARS-CoV-2 agents. Further, in vitro and in vivo study of these compounds will provide a clear path for the development of novel compounds that can interact with spike protein.

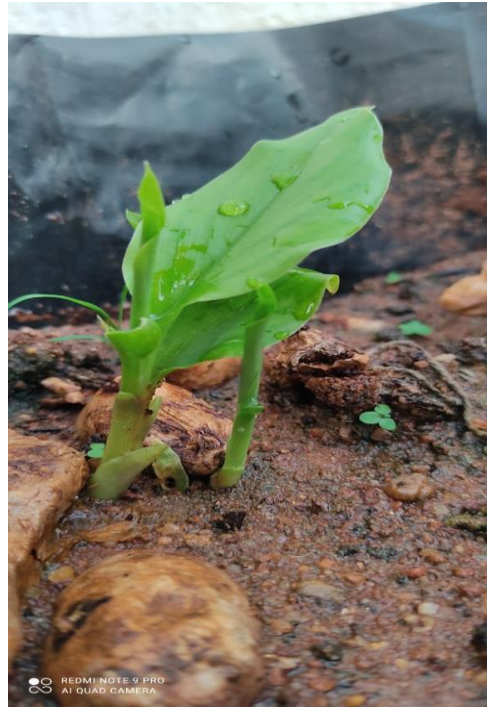
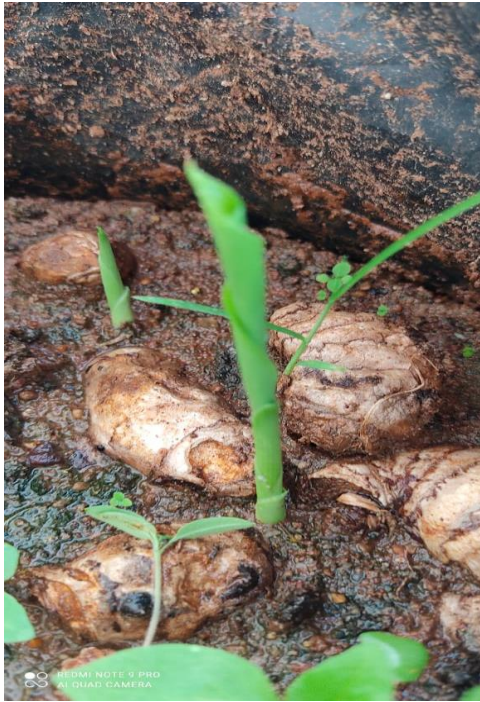
Results obtained by Anrag et al (2020) indicated that 10 herbal molecules were docked against SARS-CoV 2 protease where in Oxyacanthine and Hypericin (-10.990 and -9.05 and kcal/mol respectively) have shown good binding efficacy among others but Oxyacanthine was the only natural product which made some of necessary interactions with residues in the enzyme require for target inhibition. Therefore Oxyacanthine may be considered to be potential inhibitor of main protease enzyme of virus but need to be explored for further drug development process.

Another study reported the inhibitory effect of 17 organosulphur compounds in garlic essential oils on the host receptor angiotensin-converting enzyme 2 (ACE2) protein in the human body that leads to a crucial foundation about coronavirus resistance of individual compounds on the main protease (PDB[6LU7](#)) protein of SARS-CoV-2. The results show that the 17 organosulfur compounds, accounting for 99.4% contents of the garlic essential oil, have strong interactions with the amino acids of the ACE2 protein and the main protease PDB[6LU7](#) of SARS-CoV-2. The strongest anticoronavirus activity is expressed in allyl disulfide and allyl trisulfide, which account for the highest content in the garlic essential oil (51.3%) (Thuy et al 2020)

RESULTS

0.1 SHOOT GENERATION







0.2 Phytochemical Analysis

Table No.1: Phytochemical analysis of ethyl acetate crude extract

S.No.	Phytochemical analysis - Ethyl Acetate Extract	Inference
1.	Test for Alkaloid	+
2.	Test for Tannins	-
3.	Test for Saponins	-
4.	Test for Flavonoids	+
5.	Test for Quinines	+
6.	Test for Glycosides	-
7.	Test for Terpenoids	+
8.	Test for Phenols	-
9.	Test for Steroids	-
10.	Test for Phlobatannins	-



Figure No.1: Test for Alkaloid (Mayer's Test) with the inference of partial white precipitation indicates the presence of alkaloids at the bottom of tube that is by the low concentrated crude extract



Figure No.2: Test for Tannins



Figure No.3: Test for Saponins



Figure No.4: Test for Flavonoids (Shinoda Test) form partial yellowish green colour change that indicates the presence of flavonoids in the extract.

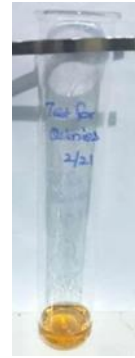


Figure No.5: Test for Quinines - concentrated sulfuric acid shows significant colour change into partial red color indicates the presence of quinines that is by the low concentrated crude extract



Figure No.6: Test for Glycosides (Molisch's Test)



Figure No.7: Test for Terpenoids (Salkowski Test) shows significant colour change into partial color change indicates the presence of terpenoids that is by the low concentrated crude extract



Figure No.8: Test for Phenols

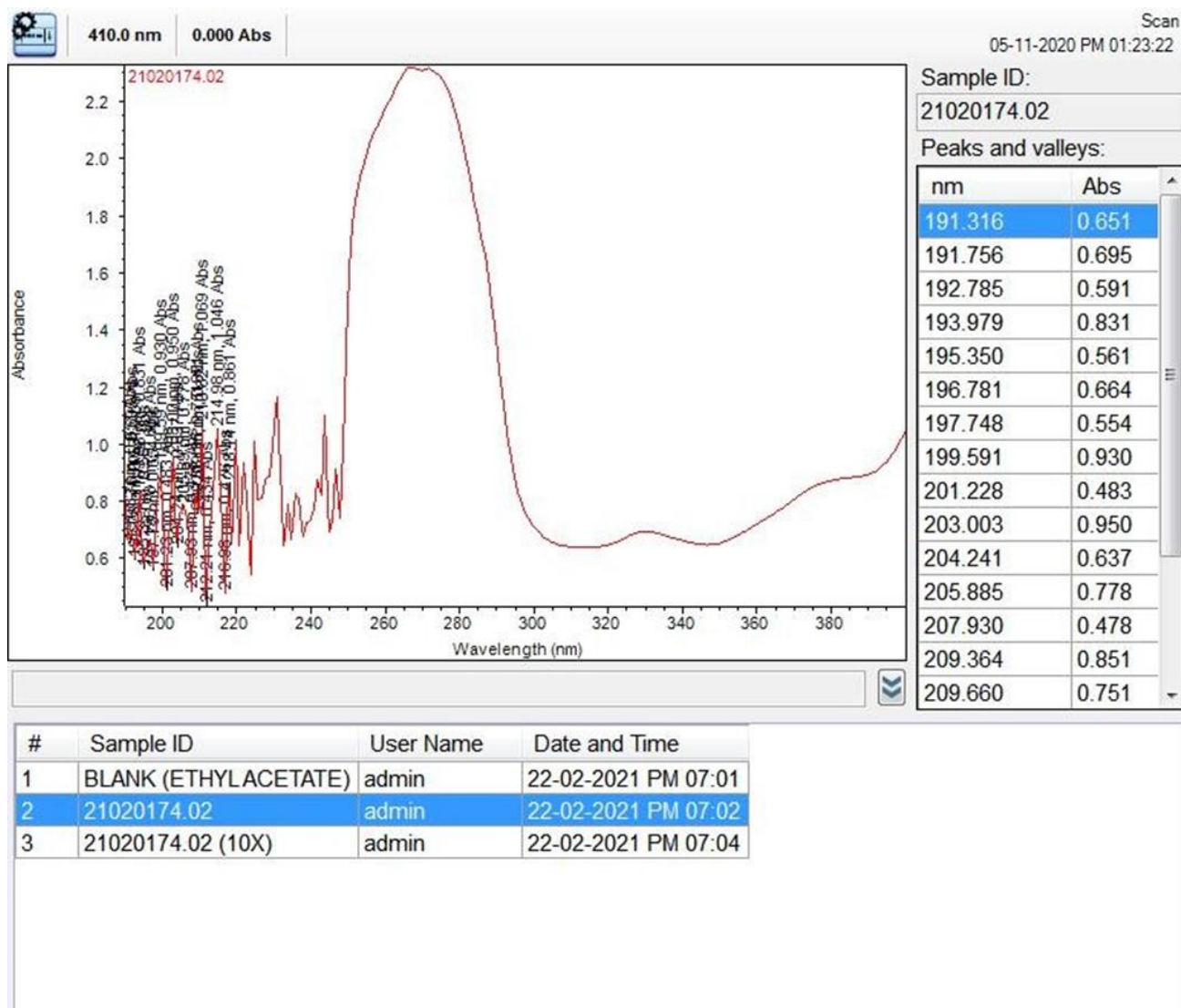


Figure No.9: Test for Steroids (LibermannBurchard Test)

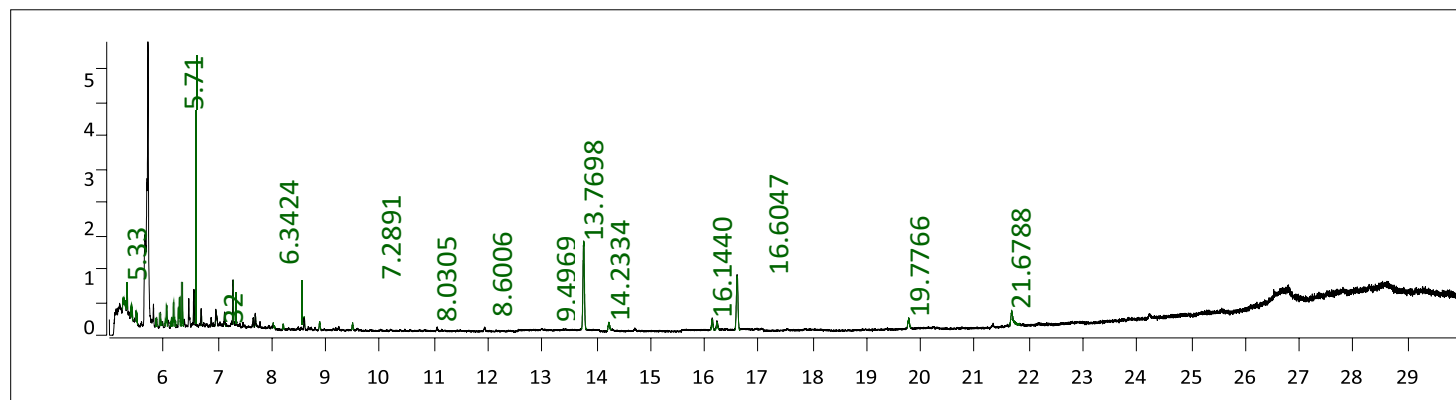


Figure No.10: Test for Phlobatannins

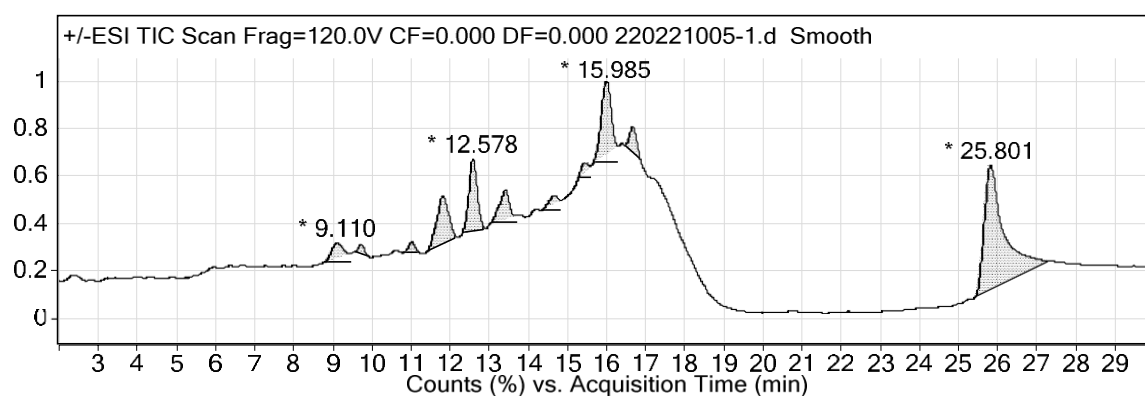
0.2 UV SPECTRUM



0.3 GCMS



0.4 LCMS



Peak	Start	RT	End	Height	Area	Area %
1	8.775	9.11	9.467	6472761	124930727	6.45
2	9.559	9.718	9.954	4231588	43216658	2.23
3	10.798	11.011	11.178	4608415	52562334	2.71
4	11.376	11.817	12.114	21528365	462499466	23.88
5	12.312	12.578	12.844	32227609	479491186	24.75
6	13.049	13.407	13.711	12791703	210414774	10.86
7	15.27	15.468	15.604	3338719	45580366	2.35
8	15.665	15.985	16.289	32634554	565031741	29.17
9	16.471	16.654	16.844	11272384	135588791	7
10	25.421	25.801	27.269	55480583	1936947121	100

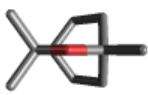
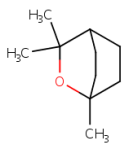
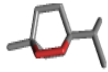
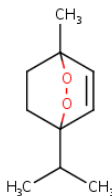
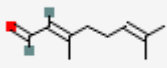
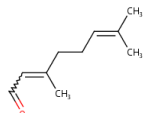
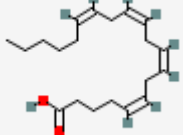
0.5 INSILICO ANALYSIS

In silico analysis of small molecules

The preliminary data for the following compounds obtained from GC MS were obtained from Pubchem and provided in Table 1.

The molecular weight for these compounds was less than 500 da and hence could be suitable as candidate drugs.

Table 1 Pubchem Data

	PubChem ID	Smiles	Molecular Weight	Molecular formula	2D image		
Eucalyptol	2758	<chem>CC1(C2CCC(O1)(CC2)C)C</chem>	154.25 g/mol	<u>C₁₀H₁₈O</u>			
Ascaridole	10545	<chem>CC(C)C12CCC(C=C1)(OO2)C</chem>	168.23 g/mol	<u>C₁₀H₁₆O₂</u>			
Citral	638011	<chem>CC(=CCCC(=CC=O)C)C</chem>	152.23 g/mol	<u>C₁₀H₁₆O</u>			
Arachidonic acid	444899	<chem>CCCCC=CCC=CCC=CCC=CCC(=O)O</chem>	304.5 g/mol	<u>C₂₀H₃₂O₂</u>			

Druglikeness properties

The compounds were analyzed for their druglike properties based on Lipinski's rule.

Lipinski's rule states that, in general, an orally active drug has no more than one violation of the following criteria:

- No more than 5 hydrogen bond donors (the total number of nitrogen–hydrogen and oxygen–hydrogen bonds)
- No more than 10 hydrogen bond acceptors (all nitrogen or oxygen atoms)
- A molecular mass less than 500 daltons
- An octanol-water partition coefficient^[6] ($\log P$) that does not exceed 5

Based on the parameters (Table 2), it can be concluded that the first three molecules were complying with the rules and hence can be considered as potential drug compounds. The compound Arachidonic acid showed two violations and hence may not be an ideal molecule as drug. However certain structural modifications can be carried out to optimize this compound.

Table 2 Druglikeness using SWISSADME

Molecule	#Rotatable bonds	#H-bond acceptors	#H-bond donors	TPSA	Consensus Log P	ESOL Class	GI absorption	BBB permeant	log Kp (cm/s)	Lipinski #violations	Bioavailability Score	Leadlikeness #violations
Eucalyptol	0	1	0	9.23	2.67	Soluble	High	Yes	-5.3	0	0.55	1
Ascaridole	1	2	0	18.46	2.34	Soluble	High	Yes	-5.73	0	0.55	1
Citral	4	1	0	17.07	2.71	Soluble	High	Yes	-5.08	0	0.55	1
Arachidonic acid	14	2	1	37.3	5.78	Moderately soluble	High	No	-3.2	1	0.85	2

Table 3 Graphical representation of druglikeness and violations

Eucalyptol	Radar chart for Eucalyptol showing druglikeness and violations across six parameters: LIPO, SIZE, POLAR, INSOLU, INSATU, and FLEX. The chart shows a red line connecting the values for each parameter, with a shaded pink area representing the overall druglikeness profile.
Ascaridole	Radar chart for Ascaridole showing druglikeness and violations across six parameters: LIPO, SIZE, POLAR, INSOLU, INSATU, and FLEX. The chart shows a red line connecting the values for each parameter, with a shaded pink area representing the overall druglikeness profile.
Citral	Radar chart for Citral showing druglikeness and violations across six parameters: LIPO, SIZE, POLAR, INSOLU, INSATU, and FLEX. The chart shows a red line connecting the values for each parameter, with a shaded pink area representing the overall druglikeness profile.
Arachidonic acid	Radar chart for Arachidonic acid showing druglikeness and violations across six parameters: LIPO, SIZE, POLAR, INSOLU, INSATU, and FLEX. The chart shows a red line connecting the values for each parameter, with a shaded pink area representing the overall druglikeness profile.

Adverse effect prediction

Small molecules from natural sources identified as probable drugs, can sometimes cause toxicity in the real time environment. Therefore an analysis is made on the possible side effects these molecules can cause in the body and the results are provided in Table 4. Compounds Eucalyptol and citral do not cause any side effects but Ascaridole and Arachidonic acid are likely to cause hepatotoxicity and nephrotoxicity which is given in terms of Pa (probability of active) and Pi (Probability of inactive). Though the results show some toxicity the values of Pa are very less indicating that they may be toxic at higher concentrations.

Table 4 Toxicity prediction

Molecule	Prediction		
Eucalyptol	No adverse effects		
Ascaridole	Pa	Pi	Side Effect Name
	0.384	0.270	Hepatotoxicity
	0.320	0.273	Arrhythmia
Citral	No adverse effects		
Arachidonic acid	Pa	Pi	Side effect Name
	0.304	0.152	Nephrotoxicity

Interaction studies

The PDB ID used for spike protein to study the interaction on these compounds is 6M0J. The protein is downloaded from PDB and studied for its structure (Fig 1). The structure was found to have two proteins which include spike protein S1 region of SARS-CoV 2 and its binding partner Angiotensin Converting Enzyme 2 (ACE2). This structure is resolved through X-RAY DIFFRACTION at 2.45 Å resolution. The ACE2 protein is of 603 aminoacids length and represented in green colour while S1 region of spike protein is of 229 aminoacids length and represented in orange color in Fig 1. This molecule is associated with three ligands namely 2-acetamido-2-deoxy-beta-D-glucopyranose (NAG), Zinc and Chloride ions.

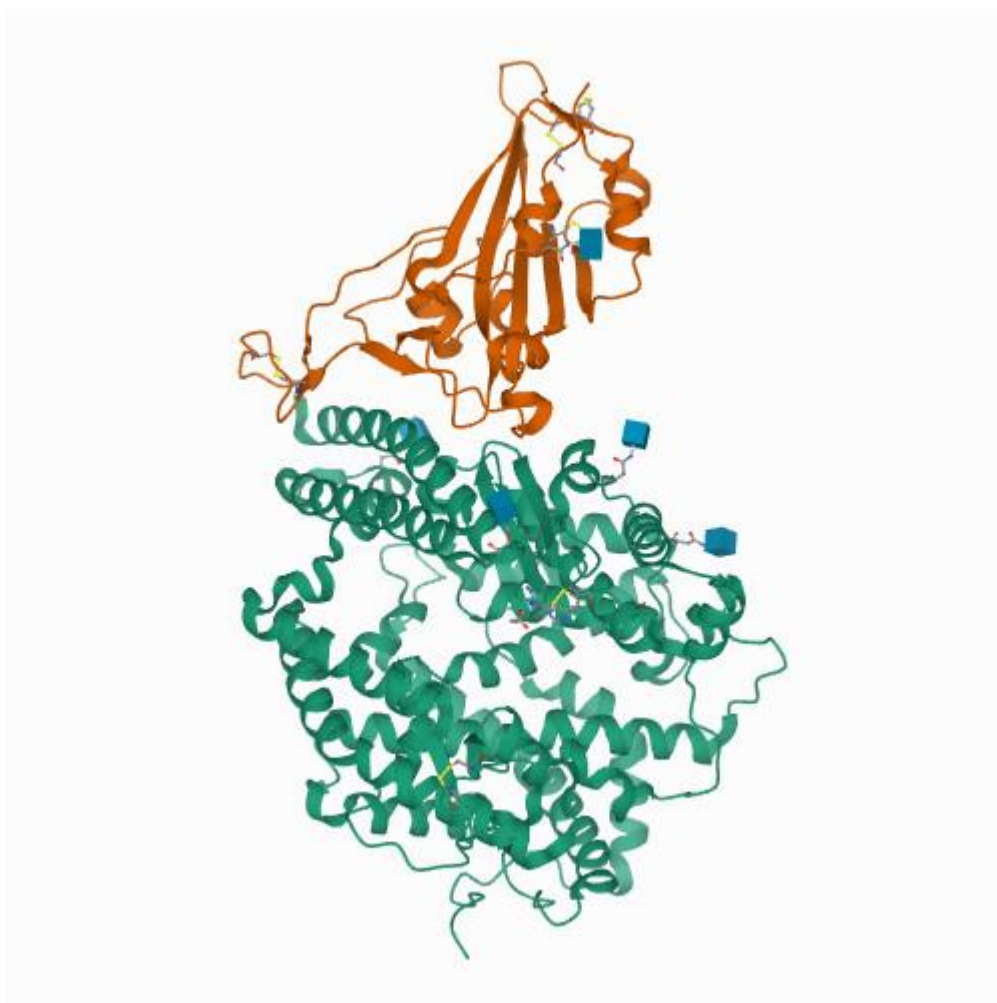


Fig 1 Crystal structure of SARS-CoV-2 spike receptor-binding domain bound with ACE2 (6M0J)

The interaction of spike protein with that of the compounds in the present study was carried out after removing the ACE2 protein from the structure as this would free the binding site for interaction.

Binding site determination

Based on the structure and literature study, it has been identified that the interface between the SARS-CoV RBD and ACE2 revealed a total of 16 residues of the SARS-CoV RBD in contact with 20 residues of ACE2. The aminoacids of RBD of spike protein involved in binding with ACE 2 are K417, G446, Y449, Y453, L455, F456, A475, F486, N487, Y489, Q493, G496, Q498, T500, N501, G502 and Y505.

The compounds taken in the present study were docked using Autodock software and the results are visualized using Biovia Discovery studio Client software and the 2D results are provided in Fig 2 – 5.

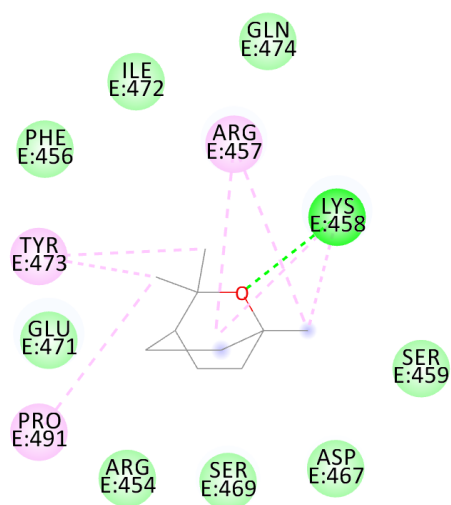


Fig 2 Interaction of Eucalyptol with Spike protein S1

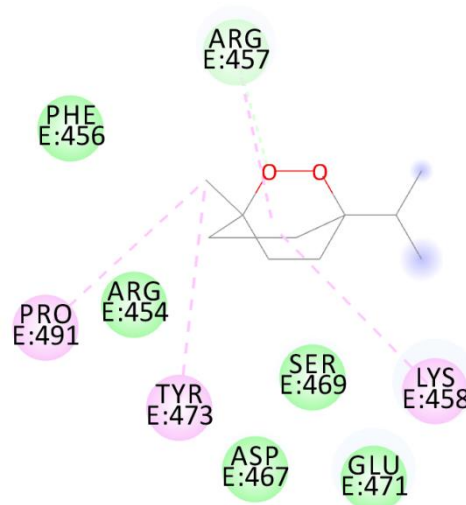


Fig 3 Interaction of Ascaridole with Spike protein S1

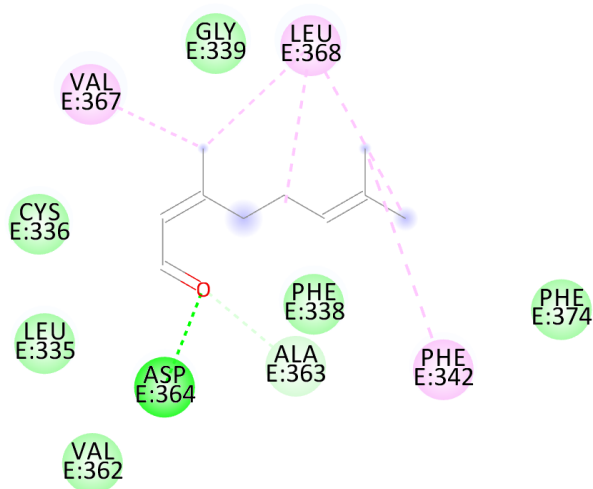


Fig 4 Interaction of Citral with Spike protein S1

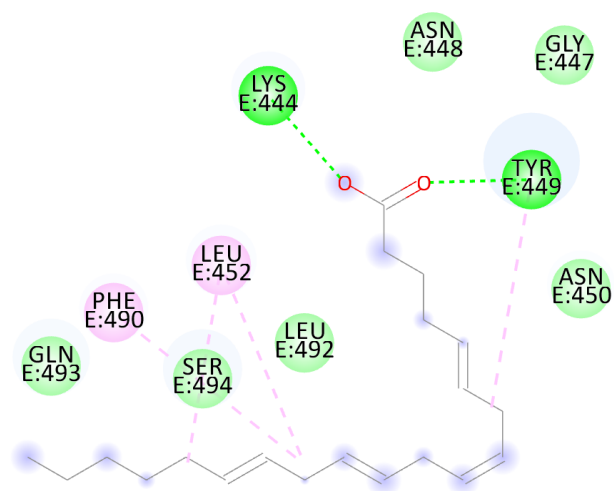


Fig 5 Interaction of Arachidonic acid with Spike protein S1

These results indicate that the compounds bound to Spike protein S1 in the same region as that of ACE2 though not with the same aminoacids. Except Ascaridole, all the three compounds formed one hydrogen bond with that of the binding site indicating a polar interaction. The binding energies of the compounds with the other parameters of interaction are provided in Table 5. The binding energies for Eucalyptol and Ascaridole were found to be lower compared to Citral and Arachidonic acid

Table 5 Binding energies

Pubchem Id	Binding Energy	Ligand Efficiency	Inhibition Constant (Um)	Intermol Energy	Vdw Hb Desolv Energy	Electrostatic Energy	Total Internal	Torsional Energy
Eucalyptol	-5.27	-0.48	136.21	-5.27	-5.15	-0.13	0	0
Ascaridole	-6	-0.5	39.87	-6.3	-6.07	-0.23	-0.2	0.3
Citral	-3.9	-0.35	1.38	-5.1	-5.02	-0.08	-0.17	1.19
Arachidonic acid	-3.07	-0.14	5.63	-7.54	-6.26	-1.29	-1.12	4.47

Conclusion

The results obtained in the present study clearly indicate that these compounds can bind to spike protein S1 in the same binding site like that of ACE2 and could possibly inhibit the binding of ACE 2 with that of the Spike protein.

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