IN SILICO SCREENING OF *PUNICA GRANATUM* AND CITRUS FRUIT (CITRUS *SINENSIS, CITRUS RETICULATA*) PEEL PHYTOCHEMICALS FOR ANTIFUNGAL ACTIVITY AGAINST SOIL PATHOGEN PROTEINS.

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CANDIDATE'S DECLARATION

I, NISHI NIKITA HEMBROM, (23/MSCBIO/33), hereby declare that the project Dissertation titled "In silico screening of *Punica granatum* and citrus fruit (*Citrus sinensis*, *Citrus reticulata)* peel phytochemicals for antifungal activity against soil pathogen proteins" in partial fulfilment of the requirements for the award of the Degree Master of Science in the Department of Biotechnology, Delhi Technological University is an authentic record of my own work carried out during the period from January to June Under the supervision of Prof. Jai Gopal Sharma.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

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CERTIFICATE BY SUPERVISIOR

I, hereby certify that the project dissertation titled, "In silico screening of *Punica Granatum* and citrus fruit (*Citrus Sinensis, Citrus Reticulata*) peel phytochemicals for antifungal activity against soil pathogen proteins" which is submitted by, Nishi Nikita Hembrom (23/MSCBIO/33). Department of Biotechnology, Delhi Technological University, Delhi in partial fulfilment of the requirement for the award of the degree of Master of Science, is a record of the project work carried out by the student under my supervision. To the best of my knowledge this work has not been submitted in part or full for any Degree or Diploma to this university or elsewhere.

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ABSTRACT

Fungal infection in plants causes abundant amount of crop losses and affect soil health. Synthetic fungicides are the way to prevent fungal infection to the plants. However, the excess use of fungicide has led to the development of fungicide resistance and environmental pollution. Residue of fungicide persist on the edible part of the plant which could be harmful for human consumption. Natural fruit derived bioactive compounds have showed the potential to be used as an antifungal agent to be used as an alternative to the synthetic fungicides. This study focuses on the in-silico screening of the bioactive compounds from Punica granatum (pomegranate) and citrus fruit (Citrus sinensis, Citrus reticulata) peels against fungal proteins. Sterol 14-alpha demethylase from Aspergillus fumigatus and chitin synthase export chaperone from *Fusarium oxysporum* f. sp. *Lycopersici* 4287 were selected as the target protein for molecular docking. These proteins are responsible for the fungal membrane and cell wall synthesis hence were selected as possible target for antifungal activity.

Molecular docking is done with the PyRx software and the result were visualized in the BIOVA discovery studio. The Binding affinities and different conformation of docking position were evaluated. The findings of molecular docking show the ability of phytochemicals from pomegranate and citrus fruit to have significant antifungal potential. This study suggests that phytochemicals can be used as safer alternative for synthetic fungicides and provides foundation for future studies for validating agro-industrial waste as useful for managing fungal diseases.

Keywords: Fusarium, Pomegranate peel, Citrus fruit peel, antifungal, Bioactive compounds, molecular docking.

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CHAPTER 1

INTRODUCTION

Soil pathogens cause various diseases in plants and crops causing food insecurity as well as impact soil health tremendously. chemical based fungicides have protected the plant from pathogens and improved the crop production. It has reduced the infection from soil pathogens at considerable rate. However, chemical-based fungicide cause soil pollution and is harmful for soil and environment as well as to humans. Intensive screening and processing are done to the harvested crops for humans to be able to consume. (1)

Agro-Industrial food waste, especially fruit peels which are thrown down the bins are very good source of bioactive compounds which have excellent antimicrobial, antifungal, antibiotic properties against pathogens. Plants contains phytochemicals like Alkaloids, glycosides, flavonoids etc. which is responsible for antioxidants, anti-inflammatory, antimicrobial product of plants. (2) Agro-Industrial food waste can potentially be used as a natural antifungal agent which could mitigate soil pollution and be sustainable and economical (1).

Fusarium oxysporum, A soil-borne fungal species that affects the plants growth and can cause various plant diseases. *F.oxysporum* causes vascular wilts. Although it causes disease in very narrow range of plants, application of Pomegranate peel extract shows reduction in fusarium population in the soil (3). *R.solani* pathogen causes root and stem rot in plants (4).

This review studies the phytochemical profiles of pomegranate peel and citrus fruit peel and its effects on soil and foodborne pathogens (5). Pomegranate is the highly cultivated in Asian countries especially India, Pakistan and Iran. Over time Pomegranate has been cultivated so much for its medicinal and Therapeutic properties and also to meet demands from all over the world. With increase in demand, the by-product of pomegranate such as seeds, peels are also discarded in tones. Peel of the pomegranate comprise 20-30% of the fruit which contains major bioactive compounds. Bioactive compounds in pomegranate peels include ellagic acid, punicalagin which are the main component for antimicrobial property. Pomegranate having abundance of bioactive compounds result in various antimicrobial and antifungal properties which are very important for improving human health and fighting pathogens. Pomegranate peel are used in industries for various purposes such as composting, Nutritional supplements, Animal feed, Biogas production etc. Pomegranate peel plays very important role in composting.it helps with the plant growth and protects plant from pathogens. Pomegranate peel aqueous extract (PPE) is used as antifungal in agricultural industries. This property of pomegranate is extremely helpful for reducing the use of fertilizers, hence reduce soil pollution and help retain soil health. It promotes sustainable waste management of fruit peels which is very good for the environment. Also, it can be used as a potential substitute for synthetic fungicides which can help combat soil pollution and good for soil health (6)

Citrus (*genus Citrus L.*) is the most important crop which provides fresh produce as well as many foods made from this fruit like juice, jams, flavoring agents. Most of the by-products are made from citrus fruits and contribute so much to the peels. The peels could be used as a cheap sustainable and less pollution to the soil contradicting synthetic pesticides. Citrus fruits contain bioactive compounds which is responsible for resistance to pathogens. Naringin and hesperidin are the most abundant flavonoids in citrus fruits (7). *Aspergillus flavus,* fungi cause spoilage of food and also produce toxic mycotoxins which causes serious health problems in humans. Many citrus fruits are effective against various *Fusarium sp., Rhizopus sp.*, Candida *sp*. Etc. salas et al showed purified flavanones (naringin, hesperidin) could inhibit the growth of *Aspergillus flavus* by 30-40%. Studies show the potential to use citrus fruit peel extract as antifungal agent in industry. Among citrus fruits mandarin and pomelo are considered to be most effective against pathogens (7)(8).

Molecular Docking plays an important role in the Drug Discovery to analyze the interaction between protein and ligand. This project aims to explore the antifungal property of pomegranate peel phytochemicals (ellagic acid, Gallic acid) and citrus fruit peel bioactive (Limonene, Naringin) against soil pathogens. Molecular docking help identify the bioactive compound from pomegranate to be effective as an antifungal. Docking is done against the fungal protein- sterol 14 α- demethylase, chitin synthase. The aim is to promote sustainable and eco-friendly alternative to chemical or synthetic fungicide which could mitigate soil toxicity.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Soil Pathogen

About 70% of plant disease are caused by the soil pathogens. Most developing countries depend on the food crops for human consumption as well as exporting the harvested food. Many fungal pathogens produce mycotoxins which are detrimental to human health causing chronic illness.

Many *Fusarium spp.* are pathogenic to the important crops which are consumed by humans on a daily basis like wheat, maize, barley etc. which could result in a food shortage and causing huge economic impact. Cereal crops are mostly attacked by the fungal pathogens. Diseases are often pernicious so it is difficult to control. Most fusarium species cause vascular wilts, root rot, stem rot, seed rot and many plant diseases. *Fusarium* consist of large group of fungi which are ubiquitous and are threat mostly to plants and sometimes humans. Large number of economically valuable crops are at risk from *fusarium spp.* Around 70 species are recognized under the *Fusarium* genus. *Fusarium* are described to have septate, hyaline and produce colored mycelia which appear cottony. Certain species produce either microconidia or macroconidia whereas some species produce both as a reproductive system in *Fusarium*. Some also produce chlamydospores. Fusarium produce different types of toxins which negatively impact plants and humans. Disease caused by fusarium are quite difficult to control because it doesn't show any symptoms until and unless infected to the point of no recovery.

Fusarium largely affect economical crops which impacts food security in a country. The main way Fusarium cause vascular wilts in many plants is by building up mycotoxins in the plant vascular system. Because fusarium spp. is capable of developing resistance to the fungicides easily *Fusariums* are detrimental to plants. The most infectious *Fusariums* are- F.solani, *F.Graminearum, F.oxysporum, F. fujikuroi*.(13)

F.graminearum is mostly known to infect cash crops like wheat barley etc. it causes a disease called Fusarium head blight which generally affects the grains in term of quality size and affected by mycotoxins which could be harmful for the market sale (9). *F.oxysporum* is known to cause vascular wilts in plants where the vascular system of plants is accumulated by mycotoxins produced by fusarium causing blockage as a result shortage of water hence the wilt. F.oxysporum causes Tomato wilt, banana wilt. Banana wilt also known as panama disease is a major disease caused by fusarium in most South African countries (10). It is also known to cause wilt in garden flowers as well. *F. fujikuroi* mainly cause rot to the different plant part such as root, stem, seed rot. Studies have shown it cause stem end rot in potato plant seen in Korea (11), stem end rot in avocado plant was discovered in Sri Lanka (12). *F.solani* has resistance to typical antifungal present in the market such as Azole.

Historically there has been many cases of famine where soil pathogens were responsible like the Irish potato famine caused by *P.infestants.*

Aspergillus species are associated with corn ear rot, peanut crown and root rot, and yellow mold. They are also reported to be associated with cotton ball rot, black mold on onion and garlic. Aspergillus sp. is also very prominent in fruit rot during harvest or post-harvest because of fruit maturation, high sugar concentration. Grape rot, citrus fruit rot, strawberry rot, pomegranate rot etc. is also the infection caused by Aspergillus sp.

2.2 Plant infection

The process of infecting plants involves a process by which fungi enter the plant and cause various disease.

Adhesion

The infection starts when the hyphae adhere to the root system of plants. The fungal pathogen can detect the host plant even before touching the host plant, the mechanism is still (unknown).

Penetration

The fungus enters the plant cell by degrading the defence system of plant by secreting some enzymes like protease, cellulase etc. The fungus enters the vascular system of the plant and tolerate all the antifungal property of the plant. Eventually hacks the plant vascular system and cause infection. Some fungus also releases mycotoxins which accumulate in the vascular system and cause wilts in plants.

Colonization

After entry, the pathogen colonizes the plant system. The fungus reaches the vascular system and hacks the plant. The fungus starts reproduction and starts growing in the whole plant.

Disease Development

Fungus starts to draw nutrient from the plants. It starts releasing mycotoxins in xylems causing occlusion hence the wilts seen in plants. Various symptom such as rot, wilt, unproductive and eventually plant die. Spores are produced which will can be dispersed by wind, water etc. (13)

2.3 Economic and ecological impacts

The economic burden of fungal disease is substantial. The Major setback is the loss of crops for the trade, export, human consumption. Crops destruction led to crop loss, economic loss and significant threat to the food security. Panama disease in south Africa caused them huge loss in the banana global market (10). Avocado is very commercial fruit and has high value, but the post-harvest stem rot caused by fusarium caused great loss in Sri Lanka effecting the marketability of the fruit. (12)

Mycotoxins are produced by fungi which are hazardous to plants and human health. Penicillium, Aspergillus, Fusarium are the three genera of fungus which majorly produce mycotoxins. Mycotoxins majorly affect fruits and plants post-harvest affecting its marketability and detrimental to human health (14). Aflatoxin contamination in peanut and groundnuts often leads to product loss and not suitable for exports.

According to FAO the global fungicide market was 22.34 billion USD in 2024, largely due to soil borne fungi. Soil borne fungi is required to treated with repeated fungicide to protect the crop every season which increases the production cost. Apart from that most fungi develop resistance against existing fungicide requiring more research and development of new fungicide imposing economic burden. (15)

Fungicides help reduce the plant disease significantly but it inadvertently also increases soil pollution. Fungicides are also greatly responsible for water body pollution by leaching into freshwater. Fungicides decrease soil respiration and impact soil health causing damage to the beneficial soil microorganisms affecting soil diversity. Increasing application of fungicides hinder the soil-enzyme activity.

Sometimes Residue of fungicide persist on edible part of the plant that makes it unfit for the marketability. The foods need to go through major processing before being fit to be sold. However, if the produce still has some amount of fungal residue (mycotoxin) it can be harmful for human health.

2.4 Conventional Methods Against Soil Pathogens

Conventional methods used to protect plants from pathogens are crop rotation, Soil solarization, soil burning/steaming, soil amendments. Chemical Fungicides are the current best method to tackle plant diseases. It has showed significant improvement in crop yield and farmer's income. Benzimidazole, Pentachlorophenol, phenyl amides, Azoles, fumigates etc. are some of the most used fungicides. These fungicides are used in different modes likes seed treatment, soil drenching, soil fumigation etc.

Various modern technologies and composition of fungicides have been developed which are very effective in controlling disease in question. Nowadays lower use rate fungicides are being used which less harmful for environment and humans.

However, Resistance to this fungicide has been the problem for centuries. Pathogens develop resistance to the fungicides which are in use which arise the need to modify fungicides constantly as well as inventing novel fungicides. America faced fungal resistance for benzimidazole within 2 years of use which was concerning. since 1970s reports of various pathogen resistance increased toward benomyl, dodine, phenyltin (16).

Biological control Agents are the naturally occurring organisms which suppress pathogens. They are used as an alternative to the chemical fungicides. These agents are Safer for human and soil microbiota. These originate from non-toxic microbes hence they are eco-friendly and doesn't require huge resources as they are natural.

BCA competes with the fungal pathogens for the nutrients inhibiting the growth of the pathogens. They release antifungal metabolites like lytic enzymes which harm the fungus. Biological control agents induce or enhance antifungal resistance on plants.

Acknowledging BCA as a good alternative for the chemical fungicides however there is still massive use of chemical fertilizers to outdo the use of BCA. There is a need to focus research on producing alternatives for synthetic fungicides (17).

Many fruit peels like pomegranate and citrus fruit peels have phytochemicals which can be used as alternatives too. These phytochemicals can enhance the actions of BCAs. It can also provide nutrients to BCAs and form synergism against soil pathogens.

2.5 Antifungal Properties of pomegranate and citrus fruits

Historically, Pomegranate and citrus fruits like orange, mandarin, lemon has been used as a medicine due to the presence of rich phytochemicals in them. Traditionally these has been used as an antimicrobial for human use. Punicalagin, ellagic acid, gallic acid, naringin, Limonene, Hesperidin are some of the bioactive compounds found in fruits which have antifungal, antibacterial, antioxidant properties. Other than human health, Bio-actives found in Plants are found to be very effective against soil borne pathogens inhibiting the plant disease. In the context of agriculture, fruit peels are being studied to find alternatives to the synthetic fungicides. It is found that phytochemicals are also effective in controlling post-harvest decay. Studies done by A. Hernandez et al shows Citrus fruit peel has the property to control post-harvest fruit decay (18). Thus, the use of fruit peel has the capacity to be used as an alternative for sustainable agriculture practice.

2.5.1*Punica granatum*

Punica granatum (Pomegranate) cultivated majorly in central Asia, Southeast Asia, northern part of India (Himalayas) and some Regions of US. For years pomegranate fruit leaves peels flowers have been used for medicinal purpose. Studies show that extracts from pomegranate have high antifungal activity. Presence of high polyphenolic compounds in peel extract from pomegranate such as ellagic acid, gallic acid, punicalin, punicalagin are responsible for high antifungal property in pomegranate. Various soil-borne and post-harvest fungal species such as Fusarium, candida etc. have shown inhibition towards the pomegranate peel extracts. These extracts inhibit plant disease by disrupting the cell wall of fungi. Study by Liberato et al. states Gallic acid found in the pomegranate peel inhibits the fungi by change in membrane integrity (19).

Tayel et al in his research found that Pomegranate peel extract containing punicalagin, gallic acid, ellagic acid, catechin, granatin had significant effect in reducing the disease such as mold in citrus fruit which is mainly caused by P.digitatum during post-harvest. The efficacy of PPE was increased by synergistic activity of these compounds. Varying result was found according to the PPE concentration and period of application (dipping fruits in PPE) (20). Nawaz et al. stated in his study n-hexane fraction of the PPE has the highest inhibitory effect towards fungi which causes post-harvest infection. M. fructicola and B. dothidea were the major fungi which where inhibited during the study done (21). These studies promote the use of pomegranate as natural source for antifungal for development of preservatives, natural fungicides etc.

Some pathogenic species like *Fusarium sambucinum* and *Fusarium solani* which causes fusarium dry rot on potato tuber. E.A. Elsherbiny et al studies showed that methanolic peel extracts from pomegranate inhibited the mycelial growth and spore formation of Fusarium wilt causing cause dry rot in potato. It also showed the potential to control the dry rot post-harvest infection on potato tubers. It has the ability for curative as well as preventative application. The methanol extract consisted phenol derived components such as chlorogenic acid, ellagic acid, gallic acid etc. These compounds cause the anti-fungal property in the alcoholic extract inhibiting the pathogen (22).

Tomato wilt is a major tomato disease caused by soil borne pathogen F.oxysporum. rongai et al. studied to find the pomegranate peel aqueous extract efficacy in the tomato wilt. Water was used to extract the antifungal components from the fruit waste. The filtrate had bioactive components. Soil treated with the extract showed reduced fusarium numbers. The extract had high number of flavonoids and tannins which along with organic acids worked significantly in reducing the fusarium wilt in tomato plants. The extract reduced the severity as well as prevalence of tomato wilt. It is a great method to reduce tomato wilt as it is economically viable due to its source being agro-industrial waste products (3).

Silage is the feed made from crops for the domestic animals like cattle, cow, ox etc. silage is mostly seen to have mycotoxins deposits from fungi which affects the health of dairy cattle. Fusarium, Aspergillus, Penicillium are the major fungi which release mycotoxins and spoil the silage. Mycotoxins remain in the silage process without undergoing any change because of its stability. Study done by S. Sadhasivam et al. FB1 and FB2 mycotoxins production were significantly inhibited by the addition of pomegranate peel extract during the silage process. This study provides the potential for usage of PPE as an additive for the antifungal activity in the agriculture commodities to prevent mycotoxin contamination (23).

In other study done by Sadhasivam et al. he showed the synergistic effect of natural antifungal component from the pomegranate peel and conventional fungicide increases the effect of fungicide remarkably. This study proposed that combination of these products could be very helpful in inhibiting fungal growth and mycotoxin production controlling plant disease and crop contamination (24).

2.5.2 Mechanisms of pomegranate peels as antifungal

Although the exact mechanism of the bioactive compound action on fungi is not fully understood but there are studies which shows the general mode of action include degrading the cell membrane integrity leading to the cell lysis. Studies suggested that phenolic compounds from pomegranate peel seem to have various mechanism which affect the fungi such as destruction of cell membrane, disruption in nucleic acid synthesis, disruption of molecular functions etc. These changes inhibit the growth of fungus and hence the antifungal property.

Glazer et al. suggested Punicalagin is present in the higher content as antifungal agent. Punicalagin is a tannin which cause disruption in the metabolism of the cell, affecting the essential enzyme action, disturbing the cell content affecting metabolism. All these actions inhibit the growth of fungi preventing plant diseases (21)(25)(26).

N.S. Ferreira et al. investigated the pomegranate peel as antifungal against candida species. DCPPE (Dry crude pomegranate peel extract) was used as the extract, Gallic acid, punicalagin, ellagic acid identified major phenolic compounds. Gallic acid is considered as the significant bioactive as it inhibits fungi by various mechanism like disrupting the metabolism and cellular processes, disrupting membrane integrity, inducing oxidative stress in the cell. DCPPE significantly reduced the formation of biofilms which are responsible for the pathogenesis of the candida sp. Ellagic acid is an important player in diminishing the formation of biofilm (27).

Moreover, antifungal activity against fungi is not a single mechanism rather various activity combination which causes different result in inhibiting the fungi. Different results may be due to different concentration, composition of the extract, extract solvent etc.

Study by Kulkarni et al. showed the possible mechanism by which punicalagin acts as protective agent as antioxidant is by scavenging the ROS, and inhibiting lipid peroxidation. These studies provide potential for further investigation for the use of natural ingredients to replace synthetic material uses (26).

2.5.3 Citrus fruit (lemon, mandarin)

Citrus fruits belong to the family *Rutaceae* under the genus Citrus. It is one of the most important crops grown in tropical regions. From the earlier days citrus fruits have been used for agricultural, industrial and medicinal purposes. Citrus fruits are used in industrial sector for canned juices, fresh produce, jams etc. citrus fruit has been used as an antifungal, antimicrobial for human health for many years. Essential oils are the most common method of use.

Citrus fruit peels from lemon, orange etc. have bioactive compounds such as flavonoids, essential oil like naringin, hesperidin, limonene etc. which accounts for the antifungal property of the citrus peel. Studies have shown that essential oil inhibits the growth of soil borne pathogens such as Fusarium sp., Aspergillus sp., Candida sp., Penicillium sp. Etc.

Y. Liu et al studied citrus fruits- orange, lemon, and mandarin ethanolic extract for its antifungal activity against *Aspergillus flavus*. Mandarin showed the most inhibiting activity against *A.flavus* followed by orange and lemon. Naringin and hesperidin was the most effective bioactive compound found in the citrus extracts (7).

M. Saleem et al. in his research he studied the byproduct of fruit extract from citrus fruit for the antimicrobial effect on the wide range of microorganisms. He used the different solvents for the peel extract like- distilled water and organic solvents. The different solvent resulted in different inhibition capacity of the peel. It also dependent on the source of the extract, yellow lemon had the most inhibition capacity among the peels used followed by orange and banana. The microorganism used in the research project are G- positive bacteria, G- negative bacteria, club and sac fungi and yeast. The data report revealed the peel extract with the distilled water solvent showed the most inhibition activity than other solution in the order alcohols> ester. The order of effectiveness of peel extract from yellow lemon>orange> banana is as follows. The gram-negative bacteria showed the most inhibition between all the microorganisms examined. This study essentially showed the importance of all the factors which can improve the efficacy of fruit peels like type of fruit peel, extraction solvent and the presence of trace metals in the peel concentration. (2)

Anwar et al. studied *Citrus sinensis* peel oil extract to evaluate the antifungal and antimicrobial activity. The fungal strain used were *Aspergillus flavus, Aspergillus niger, Alternaria alternata*. The essential oil extraction was done Soxhlet method. The major constituent of the peel consisted of limonene (96-98%). The antifungal activity observed among the three fungal strains. The volatile oil is shown to inhibit the fungal growth of the three fungi, Aspergillus flavus showed the most inhibitory effect with the highest zone of inhibition (28).

The research done by Widaryuni et al. for the antifungal activity of the citrus peels extract against *Fusarium oxysporum* causing fusarium wilt in the plants. The study conducted the application of different concentration of ethanolic lime peel extract on the *Fusarium oxysporum*. The result was obtained by observing the diameter growth of Fusarium mycelium in different concentration of peel. The concentration taken were 15%, 30%, 45%, 60%. The minimum concentration at which inhibition starts is 15% and the maximum inhibition activity was seen at 60%. Higher the concentration of peel extracts higher the antifungal activity (29).

D. E. OKWU et al. carried out the study of antifungal activity of citrus fruit from peels as well as leaf against *Fusarium oxysporum* of okra plants. sweet orange, lime, grape, tangerine and lemon were the fruit peels and leaves were used. *Citrus sinensis* was the most effective from the peels than leaves. This may be due to the availability of high number of phenol components present in the Orange. Both Orange and benomyl have inhibition of 83%. This shows that *C.sinesis* waste by product can be utilized as another option for synthetic fungicides (30).

Ahmed Mshari et al. studies show pomelo to be most effective among orange, lime, lemon and mandarin against the fungi *Rhizoctonia solani*. Furfural was the key compound found in the volatile oil extract (31).

The studies show the potential to use citrus fruit peel from agro-industrial waste as an antifungal agent/ natural fungicide for plants to mitigate plant disease and limit the use of synthetic fungicides and improve the soil health. Citrus fruits should be studied for the use in the fungicide industry because it would be economically beneficial due to the use of waste materials and be environment friendly.

2.5.4 **Effects of citrus peel on fungal proteins**

The Bioactive compounds in the fruit peels effect the pathogen by the mechanisms such as it affects the enzymes important for metabolism of the pathogen, affects cell walls and cell membrane integrity, accumulating reactive oxygen due to mitochondrial membrane damage, inhibition of nucleic acid synthesis (31) (32).

Studies show that the compound citral found in *C.limon* can inhibit the cell cycle in *Candida albicans* which inhibits replication hence no proliferation. The citral also causes imbalance in the membrane integrity of *C.albicans* harming cytoplasmic proteins eventually causing prevention in cyto-morphometric destruction of the pathogen.

Limonene found in *C.limon* are responsible for damaging the chitin bond in 1,3-β glucan inhibiting chitin synthase causing destruction in membrane integrity and preventing hyphae formation (33).

The peel extract from lime shows white color instead of purple showing the effect of extract inhibiting the formation of polyketide synthase gene. The lime peel inhibits the production of nucleic acid and protein synthesis in the *Fusarium oxysporum*.

The general mechanism by which antifungal agents work is by affecting cell membrane integrity affecting its permeability and cause damage to the cell wall. Destruction of nucleic acid synthesis and affecting cell metabolism is also a major antifungal mechanism. Flavonoid and saponins found in fruit peels binds with ergosterol and forms pore on the cell membrane and damages the cytoplasmic content in the fungi cell. Tannins found in fruit peel can also damage cell membrane of the fungi inhibiting the fungi growth (34).

Phenolic compounds in citrus fruits can cause hyphal swelling, plasma leaking, cell wall deformity, and eventually causing hyphal wrinkling disrupting its functionality (35).

2.6 Studies on fruit waste-based Fungicides

Research for sustainable fungicides has boost the study into fruit waste-based fungicide which is a significant alternative for synthetic fungicide. While there is major use of synthetic fungicides which provide good protection to the plants, it is very harmful for the environment causing soil instability, on occasions fungicide leeches into the edible part of the crop causing health problems in humans and affects marketability of the produce.

Natural bioactive films have been studied which are approved for the protective use in fruit coating for preventing post-harvest infection. Chitosan films are studied to combine with the natural antifungal agents from fruit waste. Lemon peel extract was used in developing antifungal chitosan biofilm which enhanced the properties of inhibiting the fungal agents to a great extent (36).

Studies show oil palm fruit bunch as the potential for novel fungicide against *F.oxysporum*. 2-amino-5,6-dimethyl 3 H-pyrimidine-4-one was found as one of the major components in inhibiting the fusarium wilt disease and one of the promising sources for the natural and sustainable fungicide as well as economical. (37)

Valorization of food waste is a great step towards sustainable development and mitigate pollution. Incorporating fruit waste powders into the chitosan biofilm enhances the shelf life of fruits coated with the same. The coating with chitosan inhibited the microbial activity. These studies offer to explore the possibility of using the agro-industrial waste in the development of novel sustainable fungicides. (38)

The growing interest in developing sustainable, safer, cheaper alternatives for synthetic fungicide highlights the need for deep research into the fruit or plant waste-based practices in the agricultural industry. The present study provides the effectiveness of fruit peel towards the fungal species. Future research needs to be on commercializing the fruit peels to organic fungicides replacing synthetic fungicides. Additionally, synergistic effects of fruits with the various other agents such as BCA also holds promise to switching into environmentally responsible fungicides.

CHAPTER 3

METHODOLOGY

Molecular docking is the process of evaluating the interaction between the 3D structure of protein and ligand predicting their binding affinity and binding conformation. Molecular docking is very significant in drug discovery study. This study employed an in-silico approach to study the antifungal phytochemicals from the fruit peels against soil pathogens. Some of the bioactive compounds like gallic acid, ellagic acid (from pomegranate peel), naringin, limonene (citrus fruit peel) was docked with the fungal protein chitin synthase, 1 4 -alpha demethylase.

3.1 Selection of ligand

Bioactive compounds from fruit peels from pomegranate and citrus fruits were selected. Literature have shown to them having effective antifungal properties. Gallic acid and ellagic acid were used from the pomegranate peel extract as ligand. Naringin and limonene was used as a ligand from the citrus fruit peel.

3.2 Ligand Preparation

The 3D structure of ligands was obtained from PubChem database in SDF format. PubChem is an open chemical database maintained by the National Center for Biotechnology Interference (NCBI) at National Institutes of health (NIH). It provides information on small molecules including structure, properties, biological activity, toxicity, health and safety reports. other than small molecules it also contains information on large molecules such as nucleic acids, peptides, macromolecules. PubChem is freely accessible for students, professionals, scientists to get information on the required molecule. The resource on PubChem is submitted by various government agencies, Journal publishers and many more. One can retrieve 2D and 3D structure of the ligand in SDF, JSON, XML, ASNT format. For the molecular docking process SDF format is downloaded and further converted into PDB format.

For the present study ellagic acid, gallic acid, naringin, limonene was downloaded from the PubChem database in the 3D form in SDF format. The ligands were converted into PDBQT format by the open babel inbuilt in the pyRx-Python prescription software. Energy minimization was done to obtain stable conformations of the ligands.

Ligands downloaded from PubChem

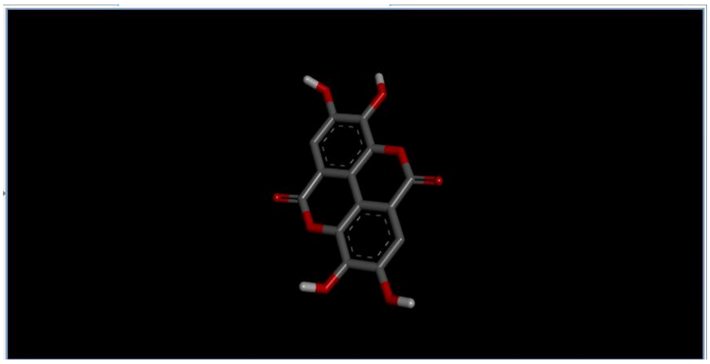

Fig 3.2.1-ellagic acid

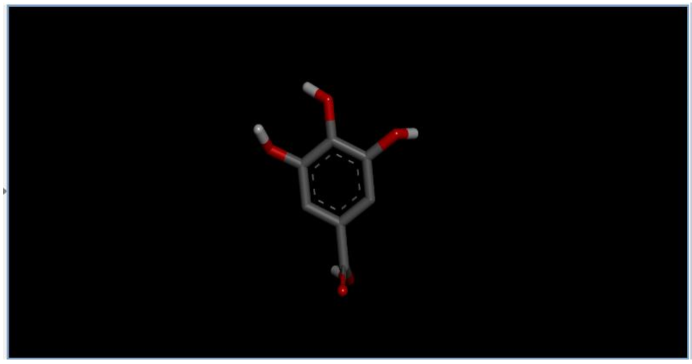

Fig-3.2.2 gallic acid

Fig-3.2.3 Naringin

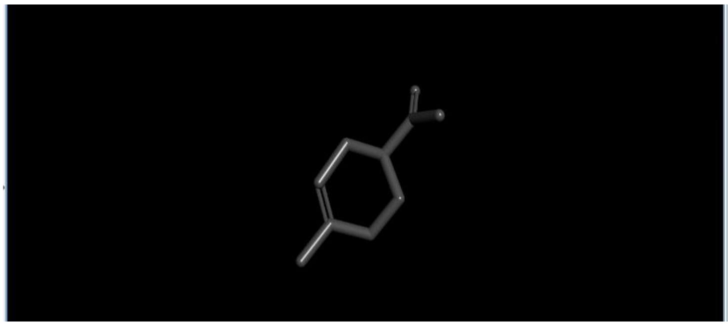

Fig-3.2.4 limonene

3.3 Target Preparation

The target protein selected were 14-alpha demethylase and chitin synthase. These proteins are majorly involved with the fungal cell membrane integrity. RCSB protein data bank was used to download the protein structure in PDB format.

Using BIOVA discovery studio the PDB protein is cleaned by deleting the water molecules, co-crystallized ligands if not required. Hetatoms are deleted from the protein in order to bind the desired ligands. In case of multimeric protein only one chain is kept usually chain A. Polar hydrogen atoms are added. After all this step the protein is saved in PDB file as protein.pdb.

Two target proteins were downloaded for the present study from the RCSB PDB and the protein was cleaned and prepared in the BIOVA discovery studio. Two proteins are – sterol 14-alpha demethylase (CYP51B) from fungus *Aspergillus fumigatus* and computed structure model of chitin synthase export chaperone (Due to absence of crystal structure of chitin synthase from Fusarium sp., a predicted 3D model from AlphaFold protein structure database is used for the molecular docking).

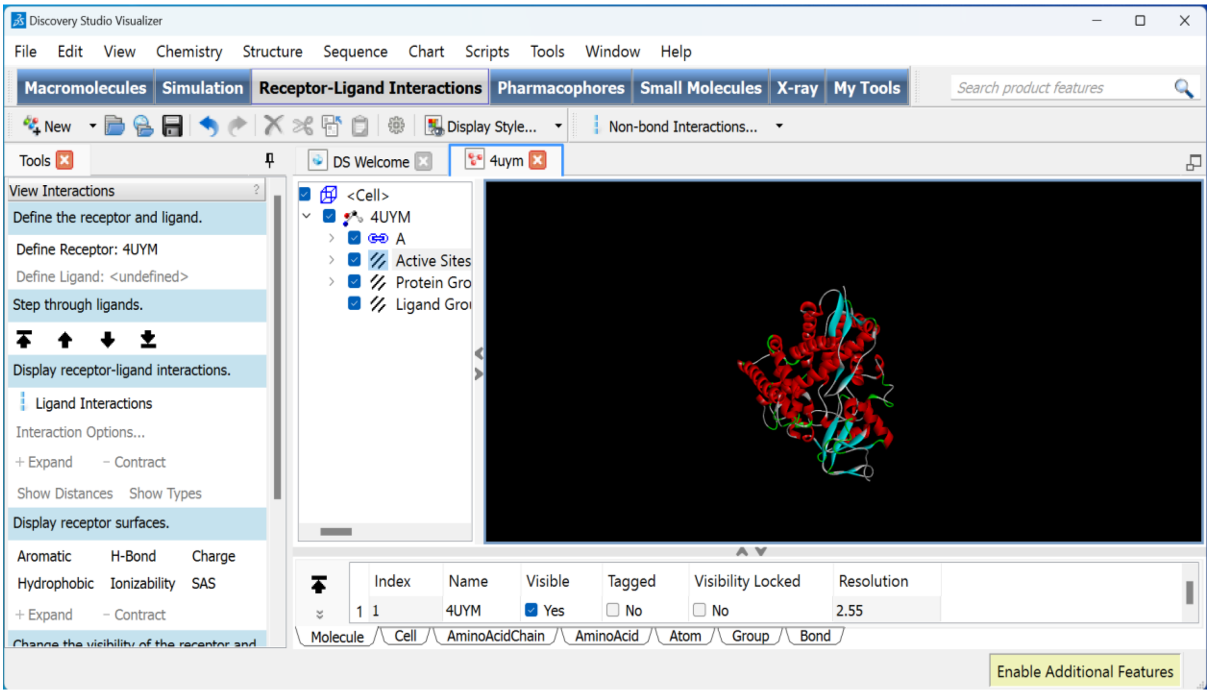

Fig- 3.3.1 Screenshot of protein sterol 14-alpha demethylase from *Aspergillus fumigatus*.

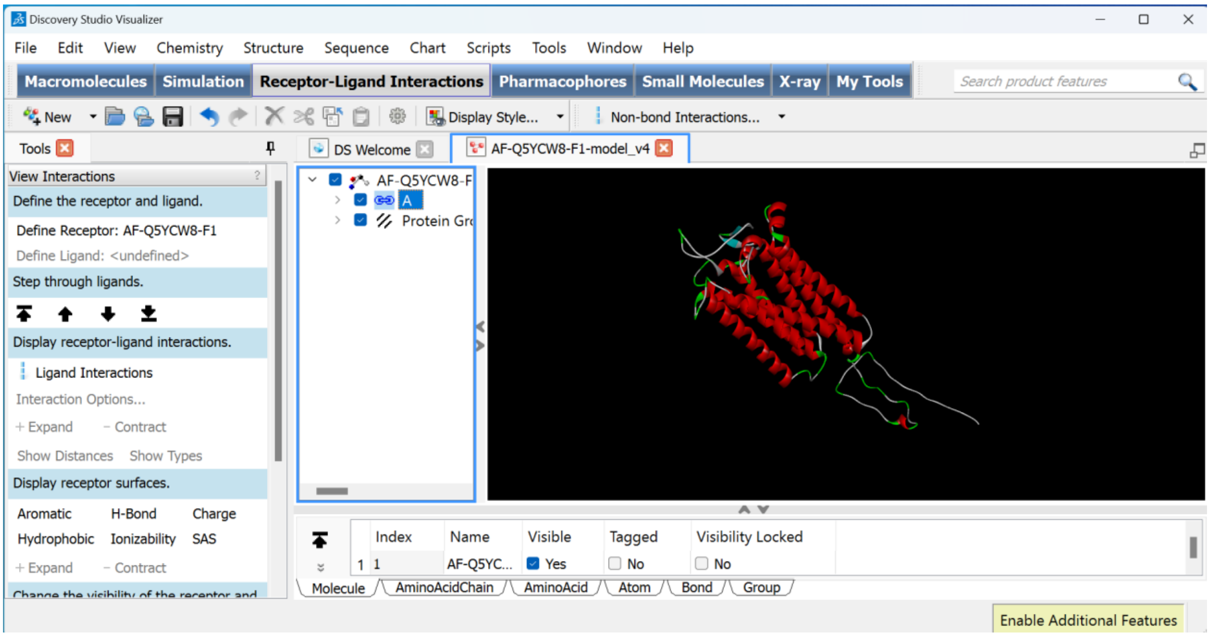

Fig-3.3.2 Screenshot of chitin synthase export chaperone from *Fusarium oxysporum f.sp. lycopersici* 4287.

3.4 Molecular Docking in PyRx

PyRx is the software used for the molecular docking. Pyrx has vina wizard, open Babel inbuilt in the pyrx software. Multiple ligands can be used in a single docking process making the docking easier and faster.

For the docking process, launch pyrx software and then load the prepared molecule, next convert the protein into macromolecule which saves the protein in the pdbqt format. Next the ligand is loaded by openBabel present in the right corner of the interface. The Energy of the ligand is minimised and converted into pdbqt format. Next using vina wizard select the macromolecule and ligand and click on forward. Next define the grid box, in this study blind docking is done so the whole protein is covered with the grid box and then click on forward. The docking process starts and when docking is done a table with binding affinity and RMSD value file is presented which is saved in the excel sheet. Next save the pdb file of the docked ligand on the protein. Next open the BIOVA discovery studio, open the protein molecule and click on hierarchy next open the docked ligand conformation and paste it on the hierarchy of the protein molecule. The docking position of the ligand and protein will be displayed. Analyse the interaction between the protein and ligand. Analyse the binding affinities, evaluate docked positions.

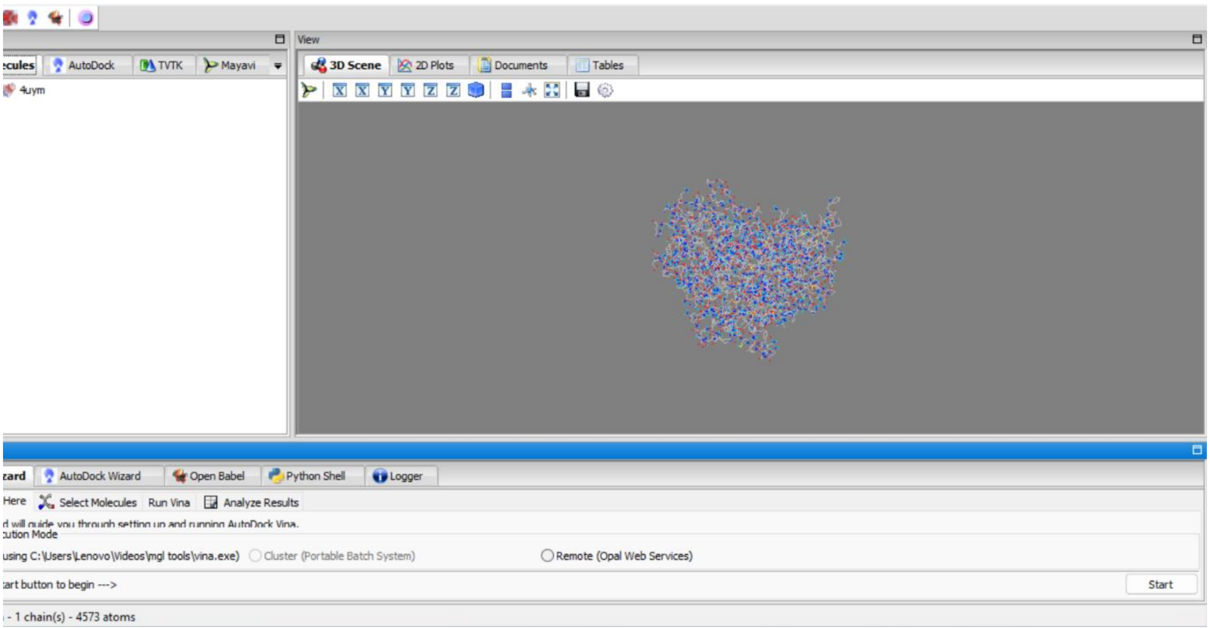

Fig 3.4.1- screenshot showing the protein molecule on the pyrx.

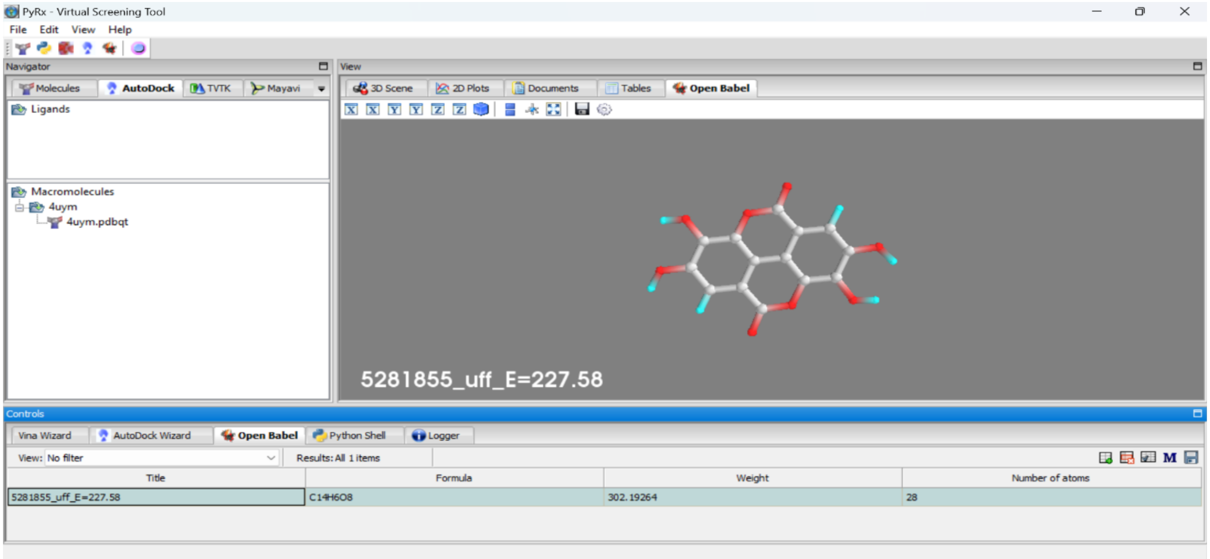

Fig 3.4.2- screenshot showing ligand energy minimised.

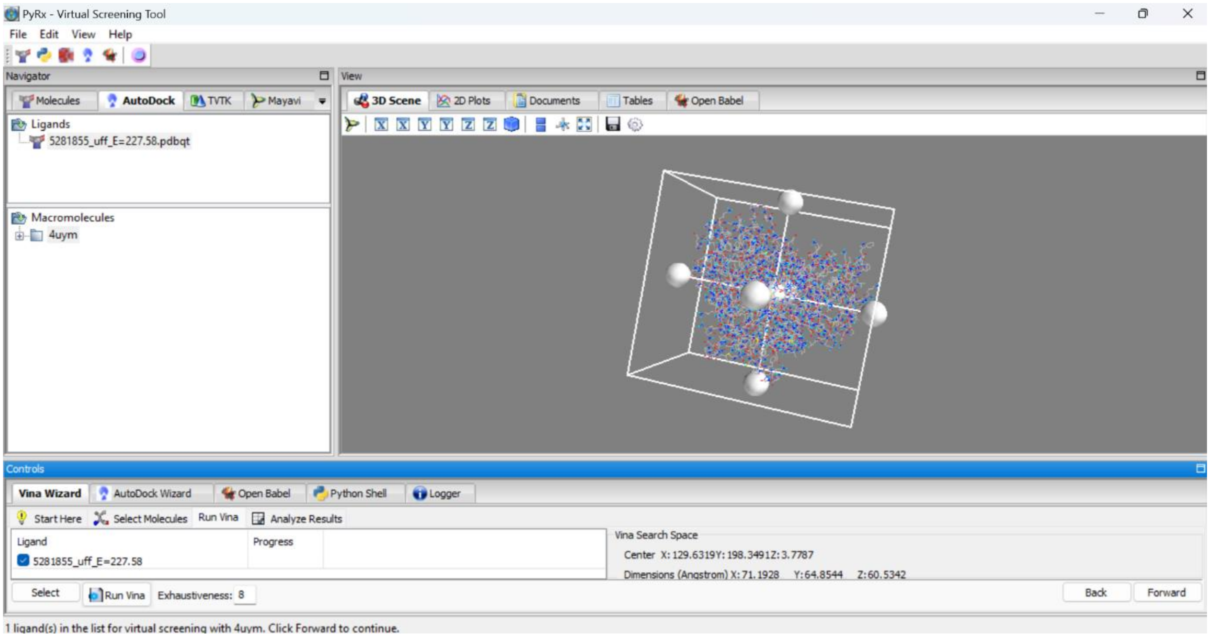

Fig 3.4.3- screenshot of grid box covering the protein macromolecule.

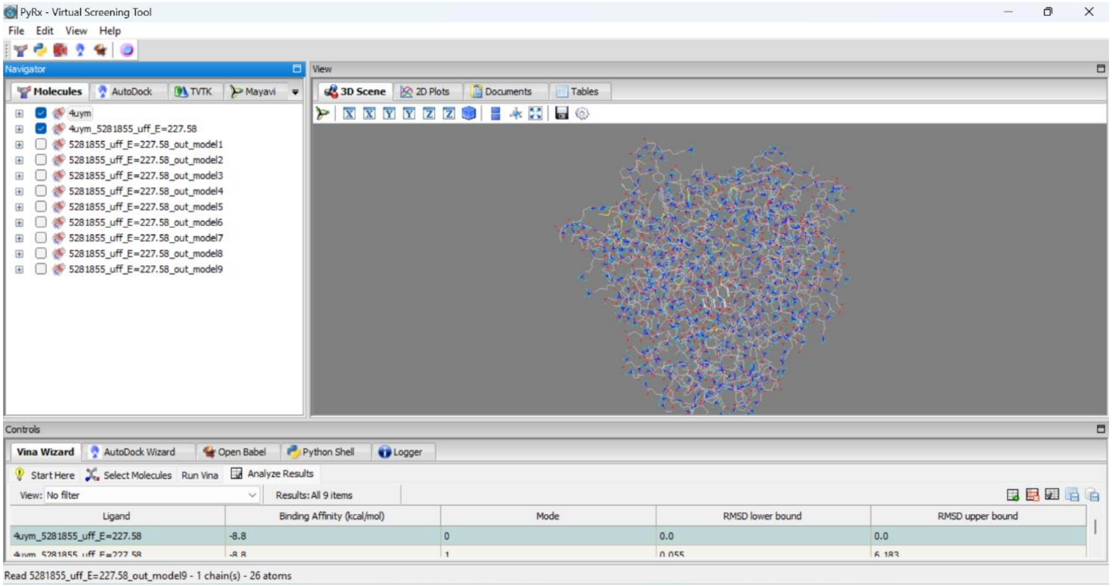

Fig 3.4.4- screenshot of protein macromolecule with ligand.

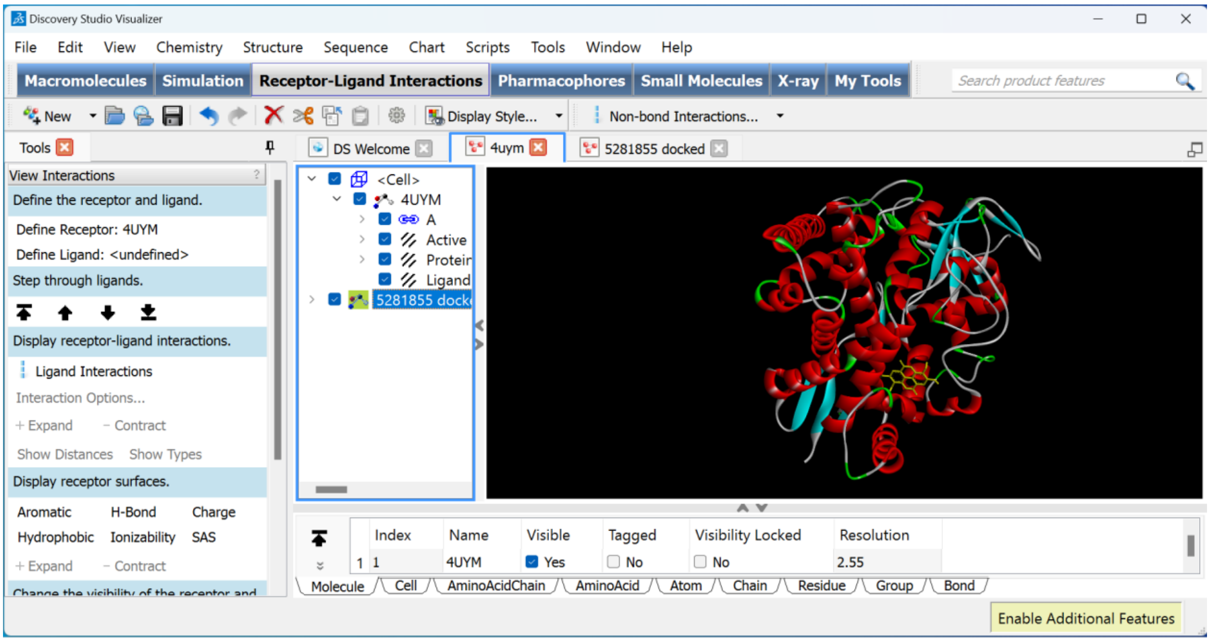

Fig 3.4.5- screenshot of Docked protein and ligand in the BIOVA discovery studio.

CHAPTER 4

RESULT

Molecular docking was carried out using pyrx to analyse the interaction between protein and ligand. The phytochemicals from pomegranate peel and citrus fruit peel were docked with the fungal proteins involved in the fungal ergosterol biosynthesis and cell wall formation. Ellagic acid and gallic acid were used as ligands from pomegranate peel and Naringin (*Citrus reticulata*) and limonene (*Citrus sinensis*) were used as a ligand from citrus fruit peel. For Protein, sterol 14-alpha Demethylase from *Aspergillus fumigates* and chitin synthase export chaperone from *Fusarium oxysporum f. sp. lycopersici* 4287 were used for molecular docking. The binding affinity of phytochemicals were analysed against the proteins.

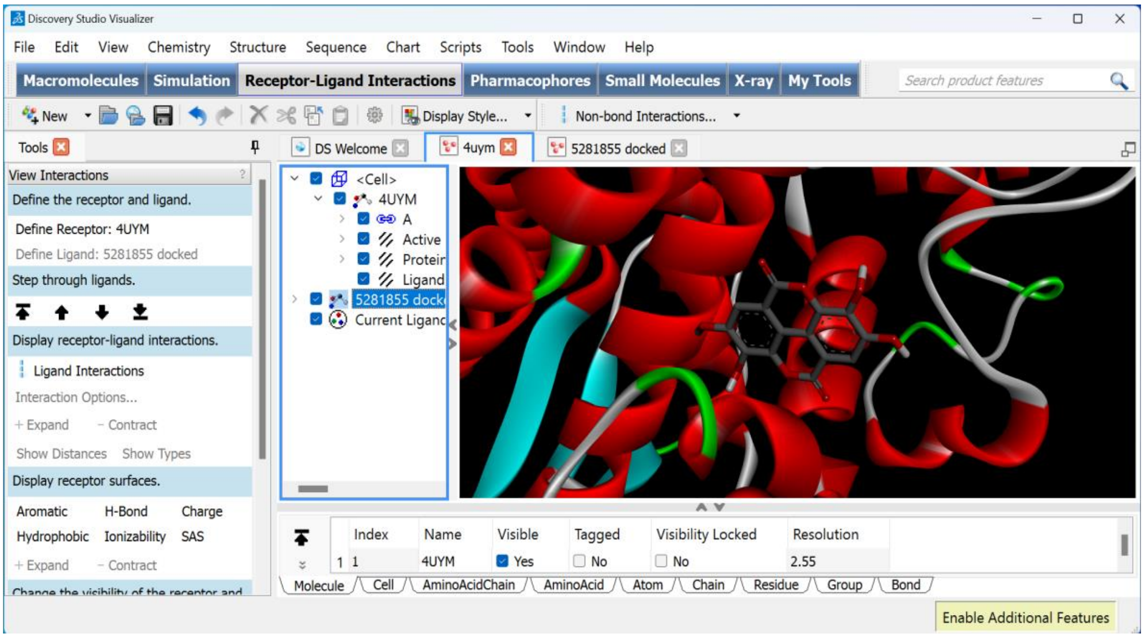

Fig 4.1- ellagic acid docked with sterol 14-alpha demethylase

	A	B	C	D
1	Ligand	Binding Afi	rmsd/ub	rmsd/lb
2	4uym_528	-8.8	0	0
3	4uym_528	-8.8	6.183	0.055
4	4uym_528	-8.7	32.991	31.998
5	4uym_528	-8.7	4.357	1.239
6	4uym_528	-8.7	4.515	1.287
7	4uym_528	-8.6	4.804	1.566
8	4uym_528	-8.6	4.368	1.572
9	4uym_528	-8.2	3.798	1.157
10	4uym_528	-8.2	3.671	1.113

Fig 4.2- Binding affinity of ellagic acid with sterol 14-alpha demethylase

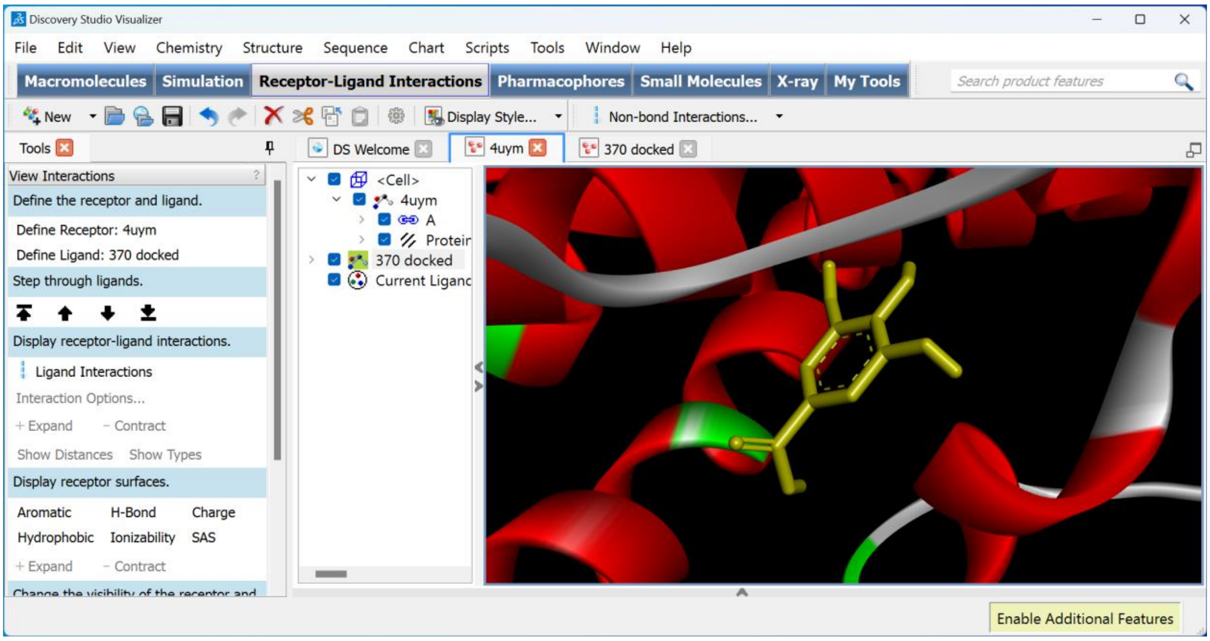

Fig4.3- gallic acid docked with sterol 14-alpha demethylase

	A	B	C	D
1	Ligand	Binding Af	rmsd/ub	rmsd/lb
2	4uym_370	-6.3	0	0
3	4uym_370	-6.3	2.405	0.018
4	4uym_370	-5.9	15.417	14.459
5	4uym_370	-5.9	14.899	14.109
6	4uym_370	-5.7	4.462	2.186
7	4uym_370	-5.6	22.75	21.601
8	4uym_370	-5.6	2.341	2.03
9	4uym_370	-5.5	31.741	30.803
10	4uym_370	-5.5	32.132	31.093

Fig4.4- Binding affinity of gallic acid with sterol 14-alpha demethylase.

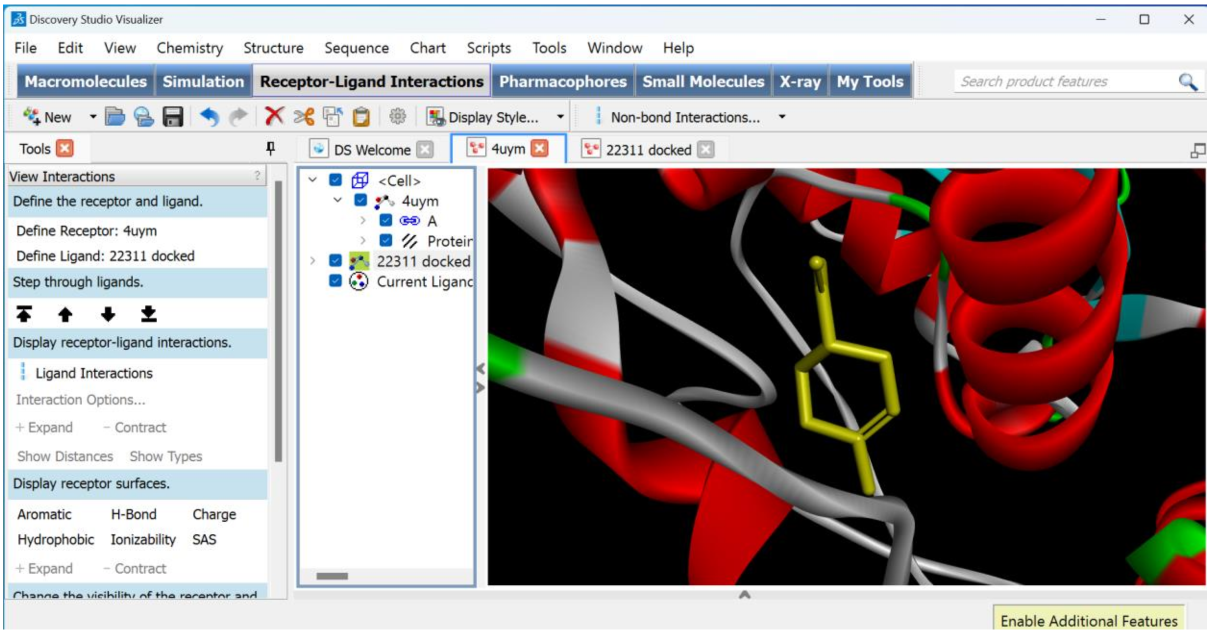

Fig 4.5- limonene docked with sterol 14-alpha demethylase

	A	B	C	D
1	Ligand	Binding Aff	rmsd/ub	rmsd/lb
2	4uym_223	-6.4	0	0
3	4uym_223	-6.2	4.356	1.034
4	4uym_223	-6	2.108	0.752
5	4uym_223	-6	4.677	0.971
6	4uym_223	-5.9	3.395	1.961
7	4uym_223	-5.9	33.93	32.294
8	4uym_223	-5.9	5.027	2.476
9	4uym_223	-5.9	33.637	31.957
10	4uym_223	-5.8	33.568	32.272

Fig4.6- Binding affinity of limonene with sterol 14-alpha demethylase

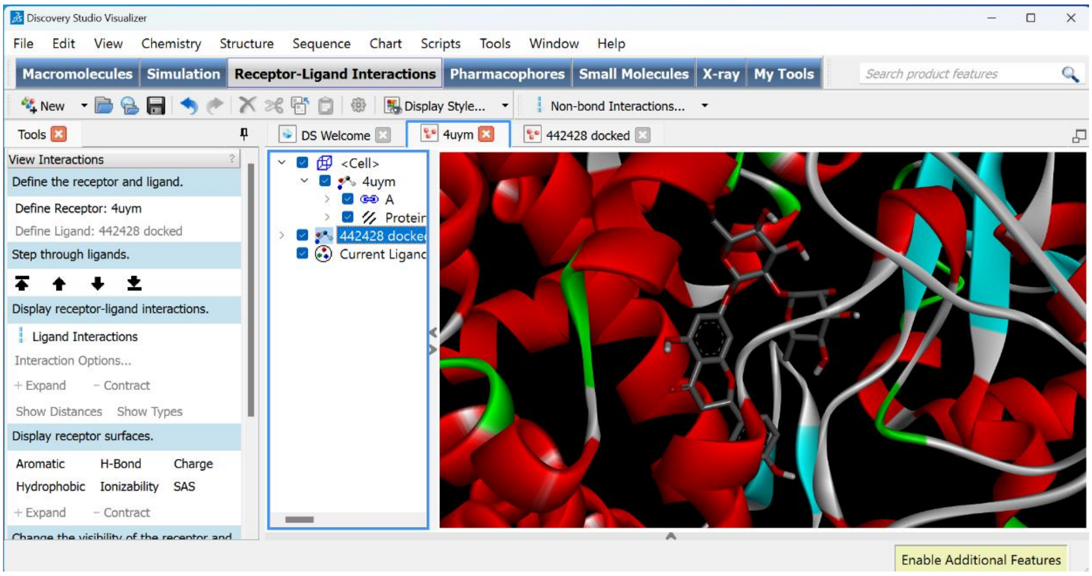

Fig 4.7- Naringin docked with sterol 14-alpha demethylase

	A	B	C	D
1	Ligand	Binding Aff	rmsd/ub	rmsd/lb
2	4uym_442	-9.4	0	0
3	4uym_442	-9.3	8.456	2.582
4	4uym_442	-9.3	8.331	2.212
5	4uym_442	-8.7	19.802	16.393
6	4uym_442	-8.7	7.078	3.351
7	4uym_442	-8.7	7.815	2.87
8	4uym_442	-8.7	18.096	15.401
9	4uym_442	-8.6	6.597	4.152
10	4uym_442	-8.6	17.828	15.177

Fig 4.8- Binding affinity of Naringin with sterol 14-alpha demethylase

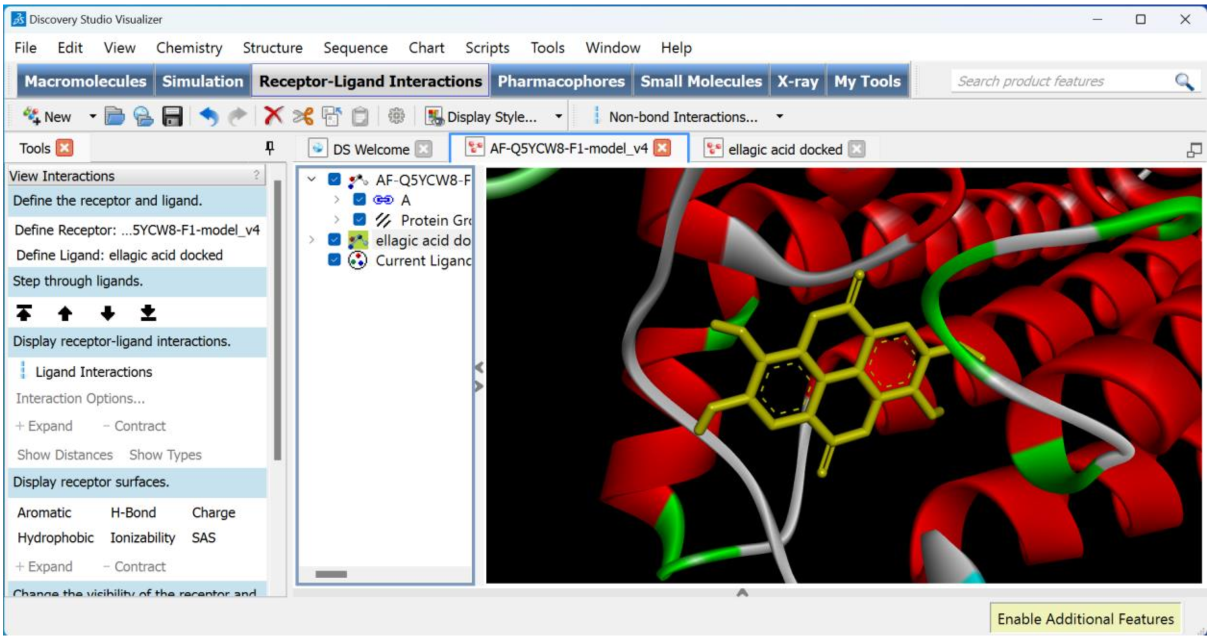

Fig 4.9- ellagic acid docked with chitin synthase export chaperone

	A	B	C	D
1	Ligand	Binding Aff	rmsd/ub	rmsd/lb
2	AF-Q5YCW	-8.4	0	0
3	AF-Q5YCW	-8.3	4.853	1.784
4	AF-Q5YCW	-8.3	6.183	0.046
5	AF-Q5YCW	-7.9	15.486	12.428
6	AF-Q5YCW	-7.9	15.649	12.464
7	AF-Q5YCW	-7.7	14.466	12.65
8	AF-Q5YCW	-7.6	14.887	11.844
9	AF-Q5YCW	-7.4	16.301	13.066
10	AF-Q5YCW	-6.8	15.74	13.369
11				

Fig 4.10 - Binding affinity of ellagic acid with chitin synthase export chaperone

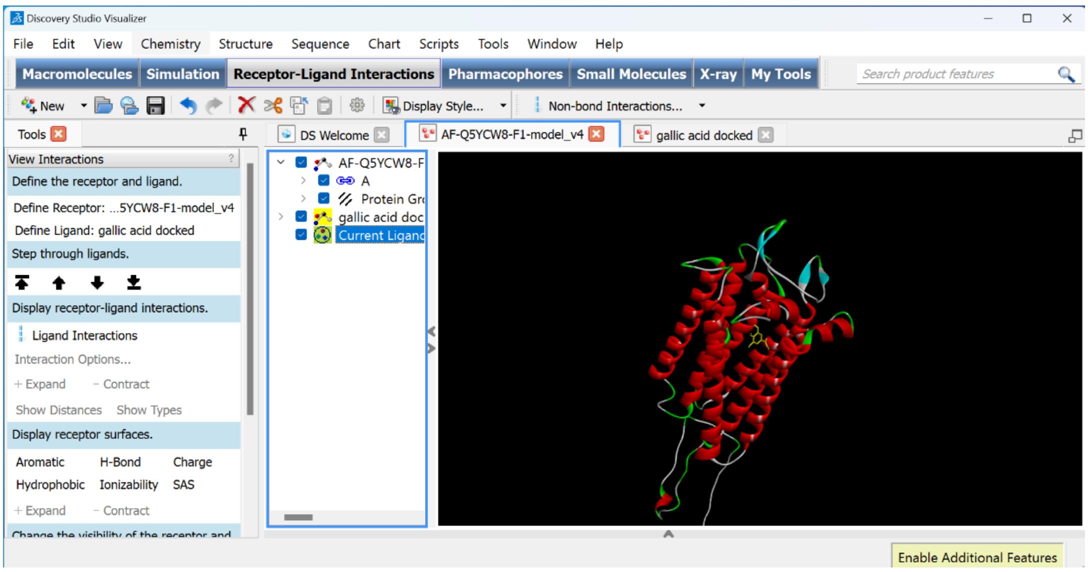

Fig- 4.11 gallic acid docked with chitin synthase export chaperone

	A	B	C	D
1	Ligand	Binding Afi	rmsd/ub	rmsd/lb
2	AF-Q5YCW	-5.2	0	0
3	AF-Q5YCW	-5	10.347	8.848
4	AF-Q5YCW	-5	7.032	5.315
5	AF-Q5YCW	-4.9	28.084	26.793
6	AF-Q5YCW	-4.8	28.145	26.803
7	AF-Q5YCW	-4.8	17.984	17.073
8	AF-Q5YCW	-4.6	28.4	27.029
9	AF-Q5YCW	-4.6	28.593	27.291
10	AF-Q5YCW	-4.6	28.736	27.474

Fig-4.12 Binding affinity of gallic acid with chitin synthase export chaperone

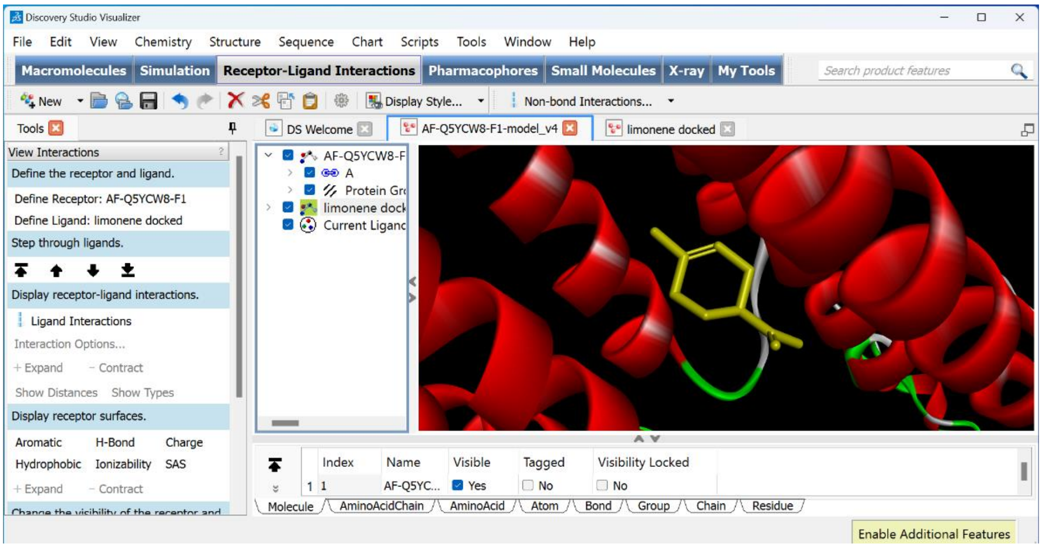

Fig-4.13 limonene docked with chitin synthase export chaperone

	A	B	C	D
1	Ligand	Binding Afi	rmsd/ub	rmsd/lb
2	AF-Q5YCW	-6.4	0	0
3	AF-Q5YCW	-6.3	4.737	0.682
4	AF-Q5YCW	-6.3	2.604	1.341
5	AF-Q5YCW	-6.3	2.696	1.565
6	AF-Q5YCW	-6.2	2.502	1.418
7	AF-Q5YCW	-6.1	4.919	1.16
8	AF-Q5YCW	-5.9	5.763	2.192
9	AF-Q5YCW	-5.8	19.251	18.324
10	AF-Q5YCW	-5.8	20.664	19.736

Fig-4.14 Binding affinity of limonene with chitin synthase export chaperone

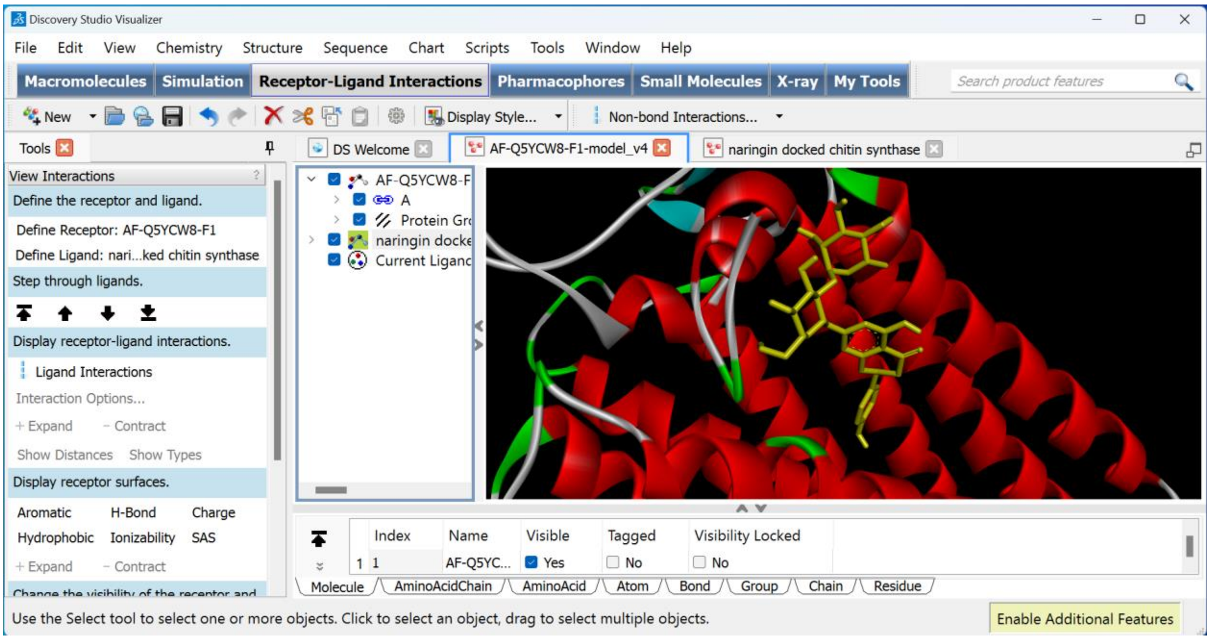

Fig-4.15 Naringin docked to chitin synthase export chaperone

	A	B	C	D	E
1	Ligand	Binding Afi	rmsd/ub	rmsd/lb	
2	AF-Q5YCW	-8.2	0	0	
3	AF-Q5YCW	-8.1	9.445	4.373	
4	AF-Q5YCW	-8	12.185	7.362	
5	AF-Q5YCW	-7.9	10.563	5.844	
6	AF-Q5YCW	-7.8	10.582	2.583	
7	AF-Q5YCW	-7.6	10.967	7.205	
8	AF-Q5YCW	-7.5	21.844	19.783	
9	AF-Q5YCW	-7.4	22.643	20.228	
10	AF-Q5YCW	-7.2	7.098	3.519	

Fig- 4.16 Binding affinity of Naringin to chitin synthase export chaperone

Naringin showed the strongest binding affinity with sterol 14-alpha demethylase at -9.4 kcal/mol and ellagic acid showed the binding affinity at -8.4 with the chitin synthase export chaperone.

CONCLUSION

The present study explored the antifungal activity of bioactive compounds from the specific fruit peels against the fungi protein using in silico molecular docking. The bioactive compounds from pomegranate peel-ellagic acid and gallic acid, citrus fruit peel-naringin and limonene were used in molecular docking against the fungal proteins- sterol 14-alpha demethylase from *Aspergillus fumigatus* and CSM chitin synthase export chaperone from *Fusarium oxysporum f.sp. lycopersici* 4287. Soil borne pathogens cause severe damage to the crop and consequently there is significant use of synthetic fungicides to prevent and treat plant diseases impacting soil health and increasing soil pollution. The excessive use of synthetic fungicide impacts environment and sometimes the residue of synthetic fungicide persist on the produce which could impact human health. The increasing environmental impact highlights the need of an alternative to the synthetic fungicide.

In this study naringin and ellagic acid showed the effective binding affinity against CYP51B and chitin synthase export chaperone respectively. These proteins play significant role in fungal cell wall formation and fungal growth.

The findings of the molecular docking suggest the potential of using fruit peel wastes in the production of organic fungicides to mitigate the soil pollution due to synthetic fungicides.

While this study was in silico method, further in vitro and in vivo studies could show potential in commercial production of safer fungicide to replace chemical fungicides and integrate the use of organic fungicides in the agricultural industry.

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