DEVELOPMENT AND EVALUATION OF HERBAL FORMULATION FOR IMMUNOMODULATORY ACTIVITY

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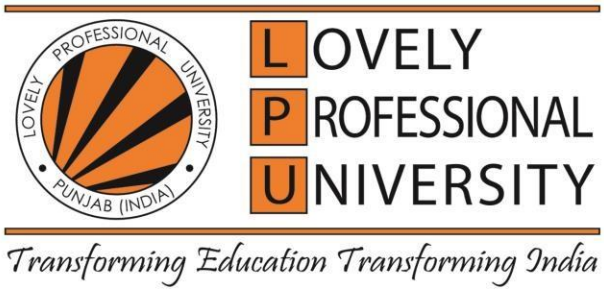

LOVELY PROFESSIONAL UNIVERSITY
PUNJAB

2024

DECLARATION

I, hereby declared that the presented work in the thesis entitled "**Development and evaluation of herbal formulation for immunomodulatory activity"** in fulfilment of degree of **Doctor of Philosophy (Ph.D.)** is outcome of research work carried out by me under the supervision**Dr. Ashish Suttee**, working as associate professor, in the department of pharmacognosy, school of pharmaceutical sciences, of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

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CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled "**Development and evaluation of herbal formulation for immunomodulatory activity**" submitted in fulfillment of the requirement for the reward of degree of **Doctor of Philosophy (Ph.D.)** in the department of pharmacognosy, school of pharmaceutical sciences, of Lovely Professional University, Punjab, India, is a research work carried out by **Kashid Snehal Uttam,** Registration No.**41900806**, is bonafide record of her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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ABSTRACT

Black cumin seed (*Nigella sativa*) the miracle herb has a historic and religious background. The sacred books Quran and Bible allusion this plant that, it can be remedy to most of the diseases. This medicinal plant is widely used for food or traditional medicine and is abundant in bioactive components. While *Pimpinella anisum* commonly known as aniseed is the oldest spices and a significant medicinal herb that the Unani, Greek, and Roman physicians mentioned. A distinct database such as Science Direct, Medline, PubMed, Scopus, EBSCO, and SID reported its phytochemical and pharmacological importance.

The aim of research work was to formulate and evaluate the immunomodulatory formulation using *Nigella sativa* and *Pimpinella anisum*. The qualitative as well as quantitative investigation of *N. sativa* and *P. anisum* was carried out for standardization of crude drugs. Additionally to valorize the species by phytochemical analysis GC- MS investigation carried out. Characterization of extract and volatile oil from *Nigella sativa* and *Pimpinella anisum* are considered necessary and several chief constituents were observed in both the plants. *In silico* approach using molecular docking tool will exploit to predicting its pharmacophore. *In silico* immunomodulatory potential of isolated compounds were explored using five proteins and 25 ligands from both the plant. The research work critically emphasize on antioxidant potential using various solvents and Bio-guided immunomodulatory potential. The research work would spotlight on pharmacological assessment of *P. anisum* and *N. sativa* for its immunomodulatory potential *in-vitro* where LPS- induced monocytic (THP-1) cell lines were utilized. Furthermore cocoa granules were formulated and its immunomodulatory potential were estimated using lymphocytic proliferation assay (e*x-vivo).* The analytical method development, validation and quantification was carried out. P-anisaldehyde and anethole in ethanolic extract of *P. anisum, N. sativa* and their formulations was quantified utilizing gas chromatography. Considering this research work it focus on multidisciplinary approach such as pharmacognostic approach, phytochemistry and applied medicinal chemistry approach, formulation and quality assurance technique approach, pharmacological approach and analytical chemistry approach.

Formulation of cocoa granules utilizing *Nigella sativa* and *Pimpinella anisum* will set the path food as medicine that can be consume easily with water, milk, curd or in form of smoothie. From the results it was concluded that both the plant are excellent immunomodulatory drugs furthermore this formulation can be serving in market as extraordinary nutraceutical formulation.

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ABBREVATIONS

ANOVA - Analysis of variance

BBB – Blood brain barrier

BSI - Botanical Survey of India

CM- Centimetre

2D – Two dimensional

3D – Three dimensional

DMSO - Dimethyl sulfoxide

DPPH – 2,2-diphenylpicrylhydrazyl

DOE – design of experiments

ELISA- Enzyme-linked immune sorbent assay

FBS –Fetal bovine serum

FID – Flame Ionization Detector

GC- Gas Chromatography

GC-MS- Gas Chromatography Mass Spectroscopy

GI – gastrointestinal track

Gm – Grams

HCL – Hydrochloric acid

Hrs – Hours

IFN-α -Interferon Alfa

IL-2 -Interleukin 2

IL-4 - Interleukin 4

LPS- Lipo-polysaccharide

LOD – loss on drying

LOQ – limit of quantification

Min - Minutes

µg/ml - Microgram/millilitre

MM – Millimetre

Mol. Wt - Molecular weight

MTT- 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide)

NS – *Nigella sativa*

N. sativa – Nigella sativa

NSE –*Nigella sativa* ethanolic extract

NSEF - *Nigella sativa* ethanolic extract formulation

NSF- *Nigella sativa* formulation

OECD - Organisation for Economic Co-operation and Development

PA - *Pimpinella anisum*

P. anisum –Pimpinella anisum

PAE - *Pimpinella anisum* ethanolic extract

PAEF- *Pimpinella anisum* ethanolic extract formulation

PAF – *Pimpinella anisum* formulation

PDB – Protein data bank

PDBQT - Protein data bank with partial charge Q and atom type T

PMA-Phorbol 12-myristate 13-acetate

RPM –Revolution per minute

RSA – Radical Scavenging Activity

R.T. – Retention time

SDF –Structural data file

WST - Water-soluble tetrazolium salt

CHAPTER - 1

INTRODUCTION

1. INTRODUCTION

1.1 Immunity

The body's ability to identify and combat both infectious and destructive microorganisms enables it to fend off illness and avert organ and tissue damage (1, 2). The term "immune system" refers to a group of cells, substances, and defense mechanisms that work to safeguard the host body against foreign antigens, including viruses, malignant cells, toxins, and several other microbes like bacteria, fungi, and parasites. Beyond the structural and chemical barrier against infection, the lymphatic system is the leading part of the immune system, which is a network of lymphatic vesicles and lymph nodes that contain immune cells. During stable body conditions, lymph nodes uphold peripheral tolerance. Bone marrow generates the majority of immune cells after early childhood (3–8).

1.2 Types of Immunity

Two subtypes of immunity exist: the innate immune system and the adaptive immune system. The innate immunity is considered the earliest immunological mechanism for a struggle against an obtrusive microorganism. After a few minutes or hours of hostility, it begins a rapid immunological reaction with no immunologic memory. On the other hand, adaptive immunity, which is made up of humoral and cellular immunity, is specific to antigens and depends on them. It can also remember things (3, 9). The cells involving the immune system are monocytes, lymphocytes, mast cells, macrophages, neutrophils, eosinophils, and basophils. Fig. no 1.1 summarizes the functions of these cells.

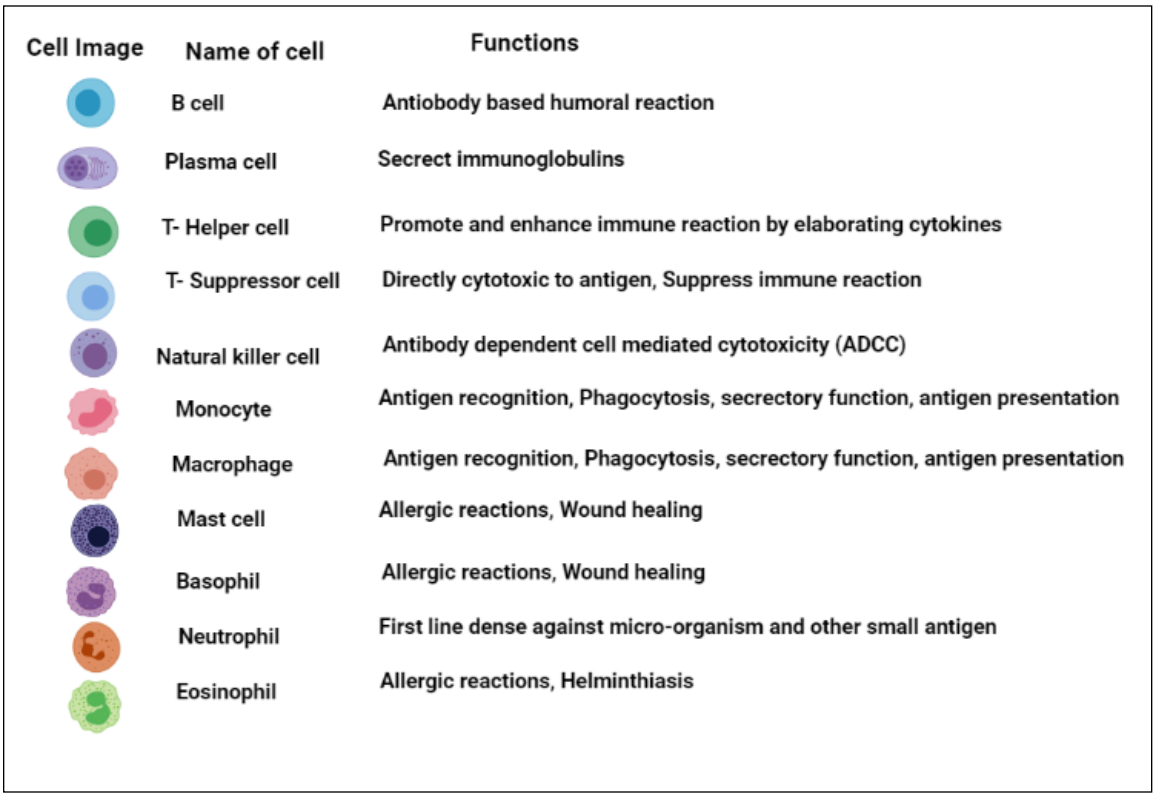


Fig. no 1.1 Immune system cell and their functions

1.2.1 Innate Immunity

This is a nonspecific response to the pathogen, particularly a relay group of proteins and phagocytic cells, which can quickly activate in response to the pathogen after recognition. The innate immune system consists of four types of defensive barriers: anatomical, physiologic, endocytic, phagocytic, and inflammatory. Macrophages, dendritic cells, histiocytes, Kupffer cells, and mast cells initiate the process of inflammation. Innate immunity plays a crucial role in recruiting immune cells, which in turn releases cytokines and chemokines at the site of infection and inflammation. TNF, IL-1, and IL-6 are among the important inflammatory cytokines generated during the early response to bacterial infection. Numerous cells, including innate lymphoid cells, NK cells, mast cells, baso-phils, eosino-phils, neutro-phils, and dendritic cells, participate in the initial phase of the immune response; their function is shown in fig no. 1.1 (4-9).

1.2.2 Adaptive Immunity

The adaptive immune system shows a specific response to pathogens, also called acquired immunity. Exposure to pathogens, which recognize foreign non-self-antigens and generate pathogen-specific immunological responses, activates this immunity. Adaptive immunity takes time to respond to antigens. For an antigen-specific response, T cells and B cells are involved with adaptive immunity and produce antibodies (4, 11). Diagrammatic representation (Fig. 1.2) summarizes the features and functions of T, B, and NK cells.

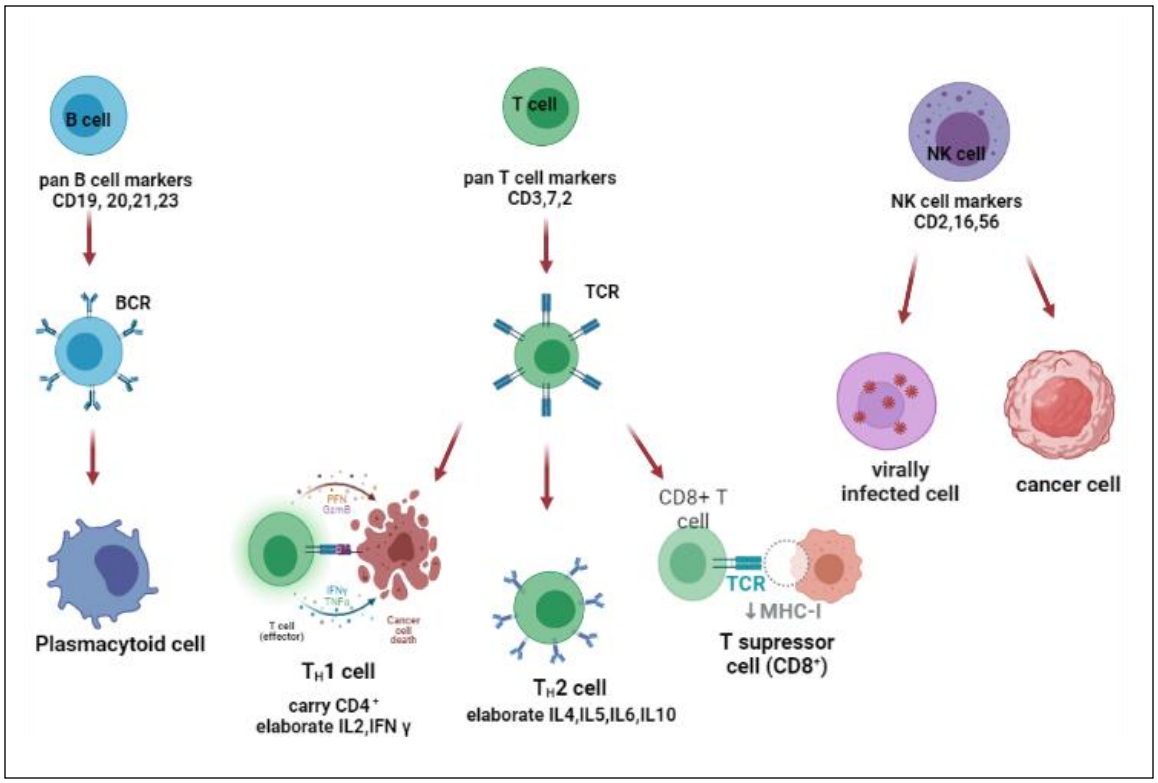


Fig no. 1. 2 Schematic representation of T, B lymphocytes and NK cells

1.3 Mechanism of immune system

The innate immune system provides preliminary defense against infections through a variety of barriers. At the time of acute/chronic inflammations, exposure to microorganisms, tissue damage, etc., the innate immune system initiates and sustains for up to 24 hrs. Conversely, the adaptive immune system necessitates a certain amount of time to activate and produce antibodies when lymphocytes are exposed to pathogens. The

lymphatic system may control the immune response by guiding dendritic cell entry at the periphery, promoting antigen or dendritic cell transfer, and departure from lymph nodes (7).

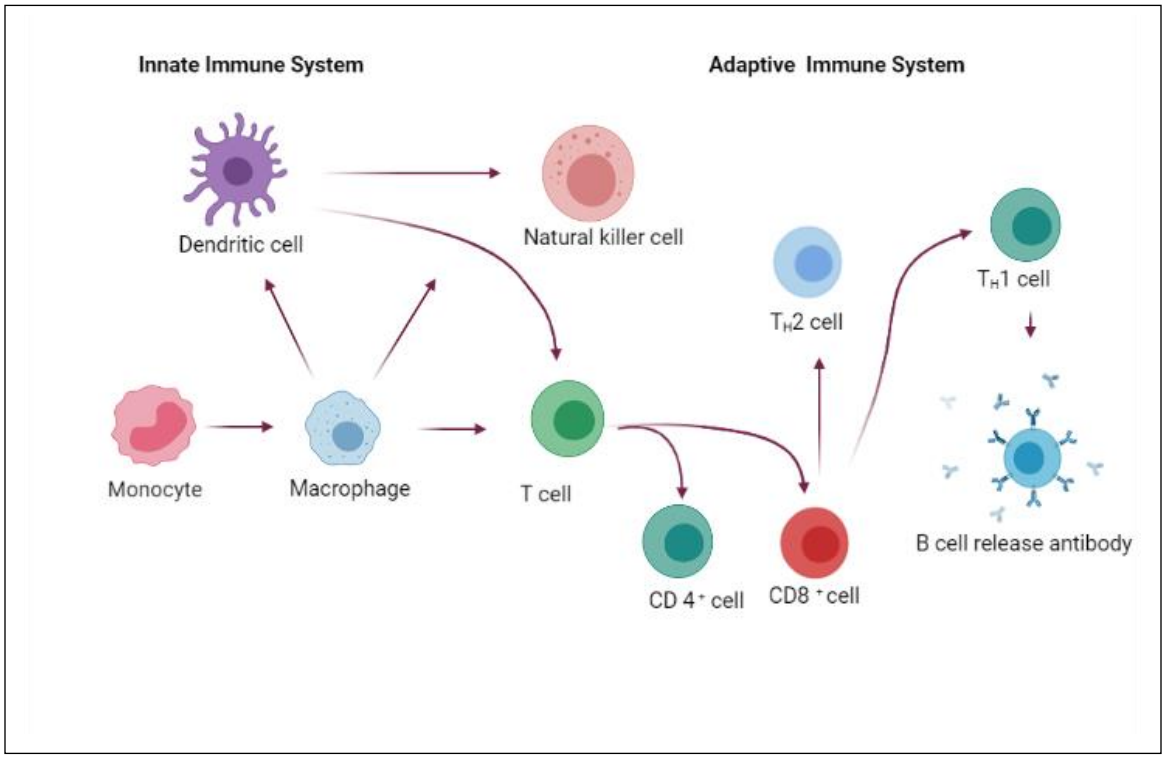


Fig no. 1.3 Mechanism of innate and adaptive immune system

1.4 Diseases of immunity

Deficiencies or failures in the innate or adaptive immune response can cause disease or illness. Such illnesses cause a variety of symptoms, including hypersensitivity due to overactivation of the immune system, autoimmune diseases due to body attacks on normal host cells, and immunodeficiency due to an ineffective immune response.

1.4.1 Hypersensitivity reactions

It is an inappropriate response of the normal immune system with adverse effects on the body, known as a hypersensitivity reaction. The table below categorizes these reactions into four distinct types.

Table no. 1.1 comparative feature of hypersensitivity reactions

Type	Action time	Mediator	Etiology
Type I (Anaphylactic)	15-30 min	IgE	Genetic, viral infection, pollutants
Type II (Cytotoxic)	15-30 min	IgG, IgM	HLA-lined, exposure to foreign tissue or cell
Type III (Immune complex, arthus reaction)	Within 6 hrs	IgG, IgM	Perseverance of low grade infection, environmental antigen, autoimmune process
Type IV (Delayed hypersensitivity)	After 24 hrs	Cell mediated	CD8+ T cells, cutaneous antigens

1.4.1.1 Type I hypersensitivity

This is the most typical reaction that occurs due to antigen re-exposure. Hay fever, bronchial asthma, food allergy, allergic rhinitis, and cutaneous angioedema are the well-known instances of type I hypersensitivity reactions. IgE attaching to mast cells primarily drives these disorders. It interacts with antigen, causing mass cells to degranulate and release histamine, leukotrienes, and other mediators, ultimately leading to allergic airway disease (4, 9, 12–13).

1.4.1.2 Type II hypersensitivity

It is a rare type of hypersensitivity reaction that occurs when IgG and IgM bind to the host cell surface and activate complexes. Erythroblastosis fetalis, autoimmune anemia, transfusion reaction, graves disease, myasthenia gravis, type I diabetes mellitus, and male sterility are the classic examples of cytotoxicity (4, 9, 13).

1.4.1.3 Type III hypersensitivity

Blood forms an antigen-antibody complex that deposits on tissue, either glomerular or Cell injury results in the subsequent activation of inflammatory reactions in the pulmonary basement, neutrophil influx, and degranulation of mast cells.

Glomerulonephritis, goodpasture syndrome, SLE, serum sickness, rheumatoid arthritis, and reaction-sensitive arthritis are classic examples of type III hypersensitivity (4, 9, 12–13).

1.4.1.4 Type IV hypersensitivity

This is the second most common type of reaction, a cell-mediated and antibody-dependent type of hypersensitivity. Overactivation of T cells, monocytes, or macrophages leads to the release of cytokines, which in turn cause inflammation, tissue damage, and cell death. Tuberculin reaction, tuberculosis, tuberculoid leprosy, reaction against virus cells, and tumor cells are classic examples of type IV hypersensitivity (9, 13).

1.4.2 Autoimmune disorders

Due to an immune system overreaction, the body's immune system fails to recognize itself and foreign cells, resulting in an 'autoimmune attack.' Auto-antibodies, inflammation, and self-reactive T cells are evidence of autoimmunity. Systemic sclerosis, rheumatoid arthritis, Lupus, and familial Mediterranean fever are classic examples of autoimmune disorders (14–17).

1.4.2.1 SLE

It is a multi-system autoimmune disease attacking its own tissues, including skin, joints, kidneys, brain, and heart, and several other vital organs, via producing antibodies that cause widespread inflammation and tissue damage. A lupus patient's blood was found to have a variety of auto-antibodies (14–15).

1.4.2.2 Rheumatoid arthritis

It is a chronic inflammatory auto-immune disease categorized by swelling and deformities of the joints. Some sufferers of rheumatoid arthritis have rheumatoid factor auto-antibodies in their blood; women’s are more prone to this disease with a ratio 3:1 (14, 16).

1.4.2.3Systemic sclerosis

It is a complex autoimmune illness that causes tissue fibrosis and vasculopathy in the skin and other internal organs (17).

1.4.3 Inflammation

Pathogens, damaged cells, and noxious substances can trigger this biological reaction in the immune system. Immunopathological features frequently include improperly controlled inflammatory responses and tissue damage brought on by inflammation. Psoriasis, inflammatory bowel disease, and asthma are classic examples of inflammations.

1.4.3.1 Asthma

This illness is characterized by chronic inflammation of the respiratory airways, which may be caused by exposure to allergens like dust or pollen or by an irritant like tobacco smoke, including potentially autoimmune ones. The reaction to immunosuppressive medications provides additional indirect support for the autoimmune theory (18).

1.4.3.2 Psoriasis

Psoriasis vulgaris is an inflammatory skin disease that lasts for a long time and is caused by the immune system. It shows up as red plaques covered in silvery scales, mostly on the skin's surface, scalp, and lower back (19–20).

1.4.3.3 Crohn's disease

Several genetic and environmental factors that affect the immune system cause this chronic, reversible inflammatory bowel disease. It is mainly characterized by inflammation in the gastrointestinal tract. Immunosuppressive therapy is required in this disease (21-22).

1.4.4 Immunodeficiency

The term "immunodeficiency" describes a condition where the immune system's fighting ability to counter infectious diseases considerably diminishes or is nonexistent. We categorize them into primary and secondary immune deficiencies. Primary immune deficiencies stem from immune system failure, typically inherited, while secondary immune deficiencies arise from environmental factors, viral or bacterial infections, malnutrition, and immunosuppressive drug therapy. B cell immune deficiencies, T cell immune deficiencies, severe combined immune deficiencies, phagocyte disorders, leukemia, multiple myeloma, lymphomas, and AIDS are the classic examples of immunodeficiency disorders (3, 23-24).

1.5 Immunomodulation

All medical treatments targeted at modulating the immune response are included in immunomodulation. In immune-deficient conditions, immune response amplification can be beneficial to fight established infections, prevent cancer, and avoid infection. It is crucial to treat the underlying cause of immunodeficiency. (25). The term immunomodulation refers to immunostimulants and immunosuppressants. Immunostimulants may operate through innate and adaptive immune responses that improve the immune system's resistance to infection. Immunostimulants act by augmenting the basic immune response in healthy people. Often administered in combination regimens during various forms of organ transplant rejection and autoimmune diseases, immunosuppressants are structurally and functionally heterogeneous groups of drugs (26). Various disease scenarios implicate the immune system. The immune system's overreaction causes asthma, eczema, and allergic rhinitis, while the immune system's self-attack causes some life-threatening diseases like myositis and lupus. Other auto-immune diseases are diabetes type 1, rheumatoid arthritis, IBD (inflammatory bowel disease), multiple sclerosis, etc. Bacterial and viral infections weaken the immune system. In the treatment of AIDs, infections and immunostimulants are obligatory, while in organ transplants, autoimmune disease, and cancer, immunosuppressants are necessary.

1.6 Role of spices in immunomodulation

The traditional medicines comprise the use of herbs, nutrition, and spices that are extensively available and used in day-to-day life in Asian culture (27). Diverse parts of the world, primarily in Asia, cultivate around 80 species. India is the origin of several spices that are widely used in traditional medicine and currently use as immunomodulation to treat a wide range of illnesses, including incurable ones like cancer, autoimmune diseases, malignancies, and viral infections. Because of their high antioxidant potential, spices are a cost-effective and promising choice for the consumer due to their high antioxidant potential. In addition, the widespread biological activity and safe status of spices not only inspire admiration in developed countries but also garner

consideration in the developing world. People added spices to food in ancient times to enhance its aroma and flavor (29–30). Numerous studies have suggested the use of spices for their valuable effects on human health through their anti-mutagenic, anti-inflammatory action, anti-oxidative, and immune-modulatory potential (31). Recent research has highlighted the extraordinary immunomodulatory potential of spices, particularly in the context of the COVID-19 pandemic. The AYUSH ministry has also promoted the use of spices for a COVID-19 patient. As the guidelines put up by the WHO and ICMR show, exploitation of herbs, spices, and nutrients can be obliged to manage this COVID via raising the immune system in patients (27). The immunomodulatory and anti-neoplastic effect of the spices is due to the presence of phenolic and flavonoids moiety, which can contest oxidative stress allied with cancer, as well as relatively high antioxidant potential (32).

1.7 Immunomodulation mechanism

Traditional herbs modulate the immune system via stimulation, suppression or by immunoadjuvant therapy. The schematic representation of same where given below

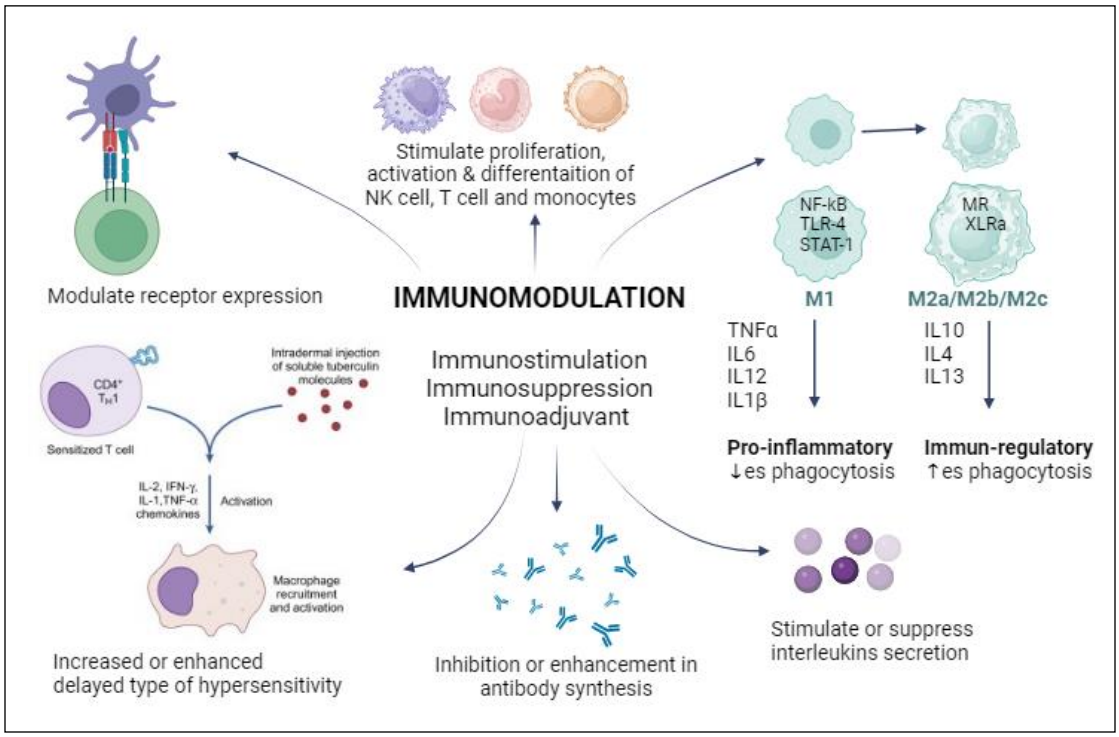


Fig no. 1.4 Mechanism of Immunomodulation

Immunostimulants act via-

- Phagocytosis
- The release of α- and γ-interferon
- The stimulation of T- and B-lymphocytes
- The production and release of cytokines
- The activation of the cell-mediated immune response, and the production of pulmonary surfactant (33).

Immunosuppressants act via-

- Prevention of T and B cell proliferation
- Decrease interleukins generation
- Hinder the cell-mediated immune response
- Alleviate phagocytosis
- Suppress the release of mediators and inflammatory cytokines
- Reduce prostaglandin formation (34).

Immunoadjuvants are chemicals that boost immune responses specific to antigens by stimulating and regulating both the innate and acquired immunity (35).

1.8 Food for Immunomodulation: Auspicious Theory

There is significant proof that food intake regulates immune function and that the immune system demands an adequate quantity of nutrients to function efficiently (36). Over generations, it has become glaringly evident just how crucial a healthy immune system is for maintaining one's health (37). The rise in frequency of infectious, autoimmune, and allergic diseases in the 21st century may be attributed to the immunomodulatory capacity of synthetic food preservatives (38). The immune system's complexity reinforces the concept of food as medicine, as it depends on multiple biological processes for proper operation, such as cell division, cell proliferation, energy consumption, and protein synthesis (36). Consumption of micronutrients has fallen as a result of the rise in dietary products that contain highly refined compounds, which promote immunological maturation (37). Now a day's, natural products with the purpose

of immunomodulation are, to a greater extent, beloved by many people for healthcare (39).

1.9 Role of anti-oxidants in Immunomodulation

Reactive oxygen, nitrogen, and other species generate themselves when the immune system functions properly, such as during phagocytosis. If they are unregulated, they may cause oxidative damage during inflammation, which may affect the immune system's components. There is an excess generation of reactive species that can be taken care of via antioxidants (40). Antioxidants are essential for maintaining cellular integrity and, as a consequence, for the homeostasis of the host's immune system. By maintaining the redox state of the cells, equilibrium between the quantities of pro-oxidants and antioxidants describes the cellular defense against genetic integrity. A deviation in this balance transforms host immunity, which affects normal cellular signaling pathways, causing uncontrolled proliferation of cells (41). The use of immunomodulators in the management of an assortment of disease conditions is receiving acceptance globally. Since, its wide scope of managing disease by the means of altering the immune or oxidant-antioxidant status. This therapy is currently recommended for the management of several chronic disease conditions (37).

1.10 Problem identification

According to WHO, COVID-2019 was a pandemic event that took place globally, resulting in 775,731,698 cases and 7,054,891 deaths until 2024. During this pandemic, the majority of COVID-19 virus-infected individuals will recover from mild to moderate respiratory infections without the need for extra medical attention. (42).

In addition, a number of other illnesses that required immunomodulatory treatment included hypersensitivity reactions, autoimmune diseases, asthma, inflammatory disorders, immunodeficiency, cancer, postoperative treatments for pulmonary diseases, and several infectious infections. A strong immune system is crucial for human well-being. Along with adverse effects, a number of chemically or biologically synthesized compounds that function as immunostimulants or immunosuppressants are available on the market (43). Fewer examples of these are cytokine inhibitors, corticosteroids,

histamine antagonists, monoclonal antibodies, cellular signaling, and non-steroidal anti-inflammatory medications. Nevertheless, immune-modulating agents obtained from traditional or medicinal plants with less severe side effects are now emerging as leading treatment options for cancer, infectious diseases, and autoimmune disorders (44).

1.11 Justification for Topic Selection

The herbal formulation is considered less toxic than synthetic medicines and helps in the management of immune disorders. Several traditional systems of medicine, including Ayurveda, Siddha, Tibb, Unani, and the Chinese system (TCM), have existed around since ancient times and have described the use of natural products, herbs, and extracts that have the capacity to modulate the immune system (45-46). The *Nigella sativa*, which is considered a curative measure for all kinds of diseases except death, additionally Locals use *Pimpinella anisum* as an indigenous remedy to cure ailments like asthma, bronchitis, cancer, sleeplessness, and nausea. Both plants have decent pharmacological claims in terms of immunomodulation. Considering this fact, effective and safe treatments for immunomodulation in the field of nutraceuticals can be screened using these two medicinal plants.

Earlier reports claimed *in vitro* immunomodulatory activity of oil, aqueous extract, or a specific isolated compound from *Nigella sativa*, while ethanolic extract along with its cytokine estimation using ELISA can be screened further. Additionally, the lymphocytic proliferation assay using formulation can be explored (47-49). Moreover limited data related to the formulation development using extract (targeting immunomodulation), *in vitro* and *ex vivo* immunomodulatory screening of *Pimpinella anisum* were found; hence, the safety profile of the drug can be explored further (50-51).

Therefore, this research primarily focuses on exploring the pharmacognostic evaluation, characterization of constituents, *in silico* screening of selected compounds, *in vitro* THP-1 immunomodulatory assays, formulation of cocoa granules, and *ex vivo* pharmacological and analytical screening. This developed formulation can be further commercialized as a nutritional supplement.

1.12 Motivation for a ready-to-mix formulation

The fast-paced lifestyles, increased affluence, growing urbanization, and traveling have all contributed to India's acceptance of convenience foods. Making the appropriate food choices is important for customers in the modern world, where both time and wellness are essential assets. But food consumers frequently make poor food choices and wind up eating readily available but harmful meals, which leads to the emergence of lifestyle diseases including infections, stomach and digestive system related issues, diabetes, obesity, cardiovascular disease, constipation, kidney disorders, etc. The emergence of these cuisines was facilitated by swift urbanization, industrial development, and changes in public eating patterns globally (52). The market is satisfied with the wide range of ready-to-eat and ready-to-cook formulations available. Research on ready-to-mix formulations with immunomodulatory potential remains limited to date. Adding value to medicinal herbs such as *N. sativa* and *P. anisum* can boost production and sales while also providing farmers with good chances to increase their revenue. Additionally, the acceptance of flavorful products comprising cocoa powder as beverages is higher, and hence ready-to-mix cocoa granules comprising *N. sativa* and *P. anisum* were formulated and evaluated.

CHAPTER – 2

LITERATURE REVIEW

2. REVIEW OF LITERATURE

2.1 *Nigella sativa*

2.1.1 Description-

Nigella is a genusof around 20 varieties of yearly plants belonging to the Ranunculaceae family, which comprises certain general species owing to their culinary and medicinal uses (53-54). These 20 species were indigenous to the Middle East, Southern Europe, North Africa, South Asia, and Southwest Asia. (55). The taxonomic situation of *Nigella* has experienced certain changes over the past few years *Nigella* section comprises *Nigella damascene, Nigella sativa, Nigella arvensis* L., *N. fumariifola* Kotschy, *N. hispanica* L., *N. segetalis* M. Bieb., *N. stellaris* Boiss., *N. elata* Boiss., *N. ciliaris* DC., *N. orientalis* L., *N. oxypetala*Boiss., and *N. turcica* Dönmez and Mutlu. *Nigella sativa* maintains high commercial attention in the food, cosmetics, and pharmaceutical industries (55-58).

2.1.1.1 Biological source

These are the dried seeds of *Nigella sativa, which* belongs to the family Ranunculaceae (58-59).

2.1.1.2 *Nigella sativa*

The annual herbaceous plant is c frequently known as "black cumin" or "black seeds," having around 60 cm in height fig no. 2.1. The plant has upright, branching stems that age to take on a green to dark green tint. Age causes its leaves green tint to change to red. *Nigella sativa* blooms from April to August and has five petals that turn green to blue with age and a diameter of 20 to 35 mm. The fruits are made up of 3-6 carpels, and each contains ovoid, black seeds (sizes 2 to 3.5 mm) within observed in Fig no 2.2 (58-61).

Fig. 2.1 *Nigella sativa* plant

Fig no. 2.2 *Nigella sativa* seeds

Nigella sativa Seeds (morphology)

Shape - Flattened, oblong, angular, and small

Size –varies between 2-3 mm. long and 1 mm. wide

Colour – dark Black in colour

Odour - Slightly aromatic in odour

Taste – Bitter in taste

2.1.1.3 Taxonomic Classification (62-65)

Kingdom: Plantae

Subkingdom: Tracheophytes

Superdivision: Spermatophyta

Division: Angiosperm

Subdivision: Spermatophytina

Class: Magnoliopsida

Order: Ranunculales

Family: Ranunculaceae

Genus: *Nigella*

Species: *Nigella sativa* (Linn.)

2.1.1.4 Vernacular Names (65-66)

Sanskrit:Krishnajira

Marathi: Kalonji Jire

Hindi: Kalaunji

English: black cumin

Punjabi: Kalaunji

Bengali: Kalojira

Malayam: karinjirakam

Kannada:karijirige

Tamil:Karunjeeragam

Urdu: Kalaunji

Italian: nigella

German: Scharzkummel

Spanish: neguilla

2.1.2 Phytochemistry

Alkaloids

Numerous alkaloids were identified in *Nigella sativa* seeds: the indazole ring found in nigellicine and nigellidine; the isoquinoline ring in nigellimine and nigellimine N-oxide. While dolabellane-type diterpene alkaloids such as nigellamines A1–A5,nigellamine B,C, D and magnoflorine were observed in the aerial part and seeds of the plant. Additionally Nigeglanine, 4-O-Methylnigeglanine, 4-O-methylnigellidine, 17-O-(β-D-glcp)-4-O-Methylnigellidine and Nigelanoid were also observed in seeds. (59, 65, 67-69).

Fatty Acids

The polyunsaturated fatty acids (PUFA) in *Nigella sativa* have been generally recognized as harmless by the United States FDA (Food and Drug Administration).The chief fatty acid was linoleic acid, followed by oleic acid, palmitic acid, stearic acid, lauric acid, myristic acid, eicosadienoic acid, and linolenic acid (65, 70-71).

Phenolic acids and flavonoids

Several polyphenols were observed in the seed, roots, and shoots of kalonji. Ferulic acid and sinapinic acid are major phenolic acids, while vanillic acid is observed in the least concentration. Quercetin and kaempferol (seed) and catechin (root) are major flavonoids in *Nigella sativa*. P-coumaric acid, chlorogenic acid, apigenin, rutin, nigelflavonoside B, and flavones were also found in the least concentration.Quercetin-3-O-β-D-glcp-(1⟶2)-β-D-galp-(1⟶2)-glcp, kaempferol 3-O-β-D-glucopyranoside and Quercetin-3-O-(6-O)-feroyl-β-D-glcp-(1⟶2)-β-D-galp-(1⟶2)-glucopyranoside are flavonoids were also observed in seeds of *N. sativa* (59,69, 72-74).

Triterpenoids

The presence of Triterpenoids such as 3-O-α-L-rha-(1-2)α-L-ara-28-O-α-Lrha(1-4)-β-D-glu(1-6)-β-D-gluhederagenine, 3-O-β-D-xyl(1-3)-α-L-rha-(1-2)-α-L-ara-hederagenine were identified in a methanolic extract of kalonji (74). The aerial part of the plant was

found.11-Methoxy-16-hydroxy-17-acetoxy-3-O-[β-D-xylp(1⟶3)-α-L-rhap(1⟶4)-β-D-glcp]-hederagenin, and lanosterol were observed in *N. sativa* seeds (69, 75).

Terpenes and terpenoids

The main active constituent responsible for *Nigella sativa* pharmacological potential is thymoquinone, but it also contains thymohydroquinone, dithymoquinone, monoterpenes such as α-Thujene, α-Pinene, α-Phellandrene, β-Pinene, α-Terpinene, O-cymene, Limonene, Linalool, 4-terpineol, and carvacrol were observed in *N. sativa* (seed) oil. Sesquiterpenes naming longifolene and widdrol were observed in minor concentrations (76-78).

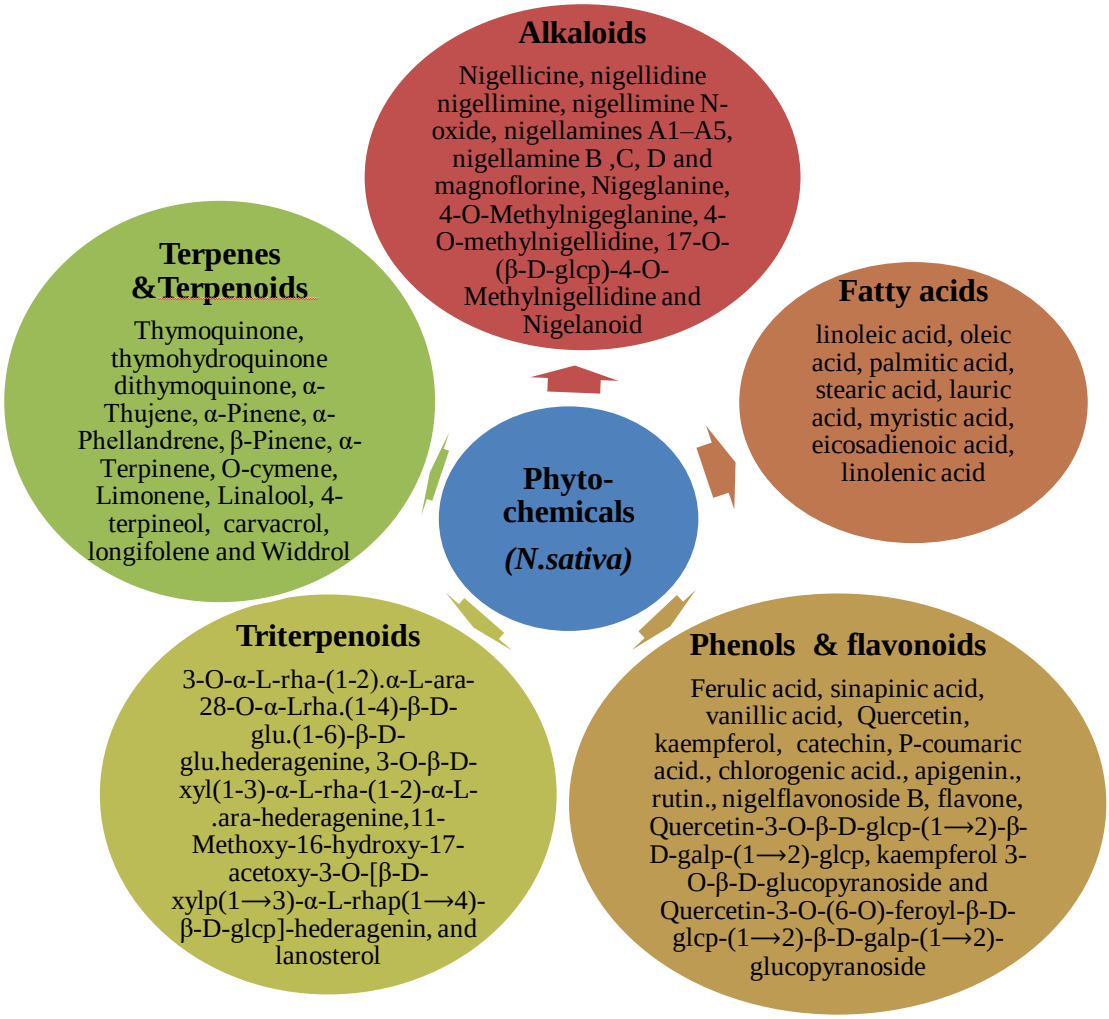
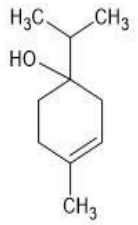
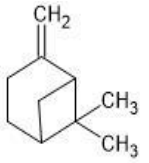


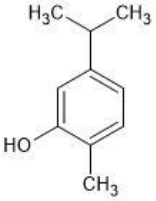
Fig. 2.3 Phytochemistry of *Nigella sativa*



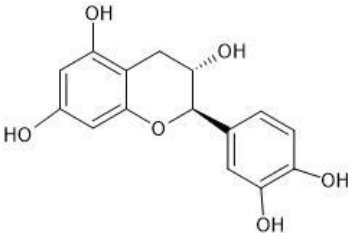
4-methyl-1-(propan-2-yl)cyclohex-3-en-1-ol



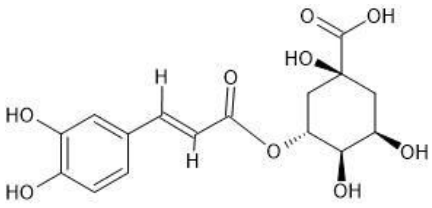
6,6-dimethyl-2-methylidenebicyclo [3.1.1]heptane



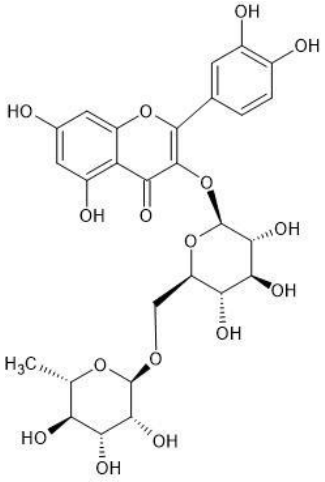
2-methyl-5-(propan-2-yl)phenol



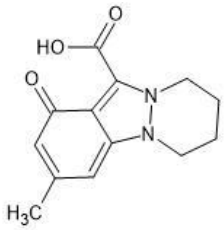
(2*R*,3*S*)-2-(3,4-dihydroxyphenyl)-3, 4-dihydro-2*H*-1-benzopyran-3,5,7-triol



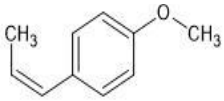
chlorogenic acid



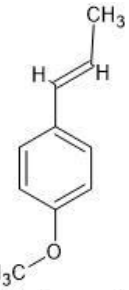
Rutin



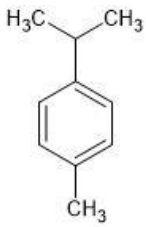
3-methyl-1-oxo-6,7,8,9-tetrahydro-1*H*-pyridazino[1,2-*a*]indazole-11-carboxylic acid



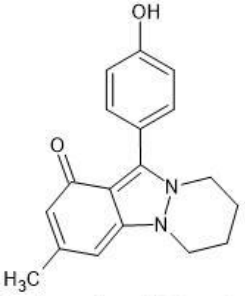
1-methoxy-4-[(1*Z*)-prop-1-en-1-yl]benzene



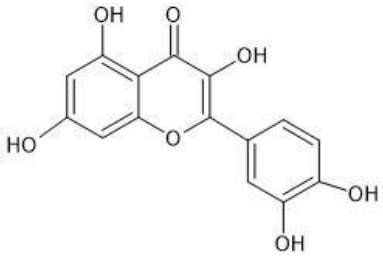
1-methoxy-4-[(1*E*)-prop-1-en-1-yl]benzene



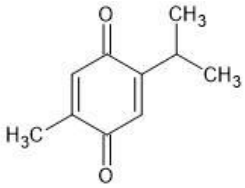
1-methyl-4-(propan-2-yl)benzene



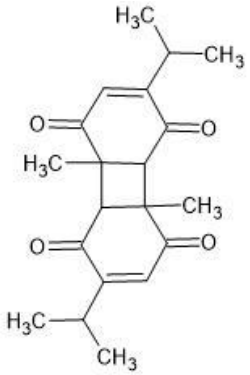
11-(4-hydroxyphenyl)-3-methyl-6,7,8,9-tetrahydro-1*H*-pyridazino[1,2-*a*]indaz



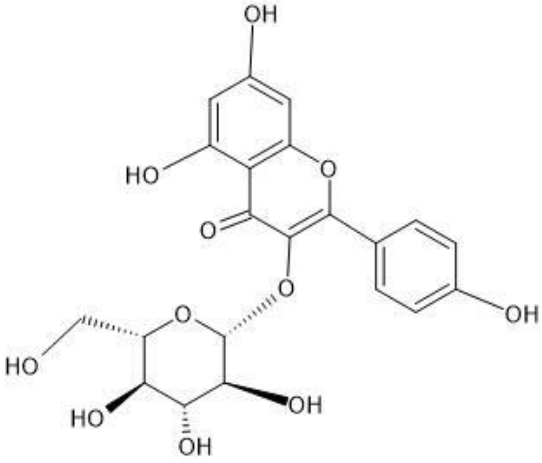
2-(3,4-dihydroxyphenyl)-3, 5,7-trihydroxy-4*H*-1-benzopyran-4-one



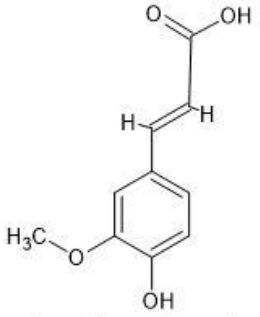
2-methyl-5-(propan-2-yl)cyclohexa-2,5-diene-1,4-dione



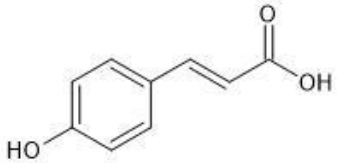
4a,8a-dimethyl-2,6-di(propan-2-yl)-4a,4b,8a,
8b-tetrahydrobiphenylene-1,4,5,8-tetrone



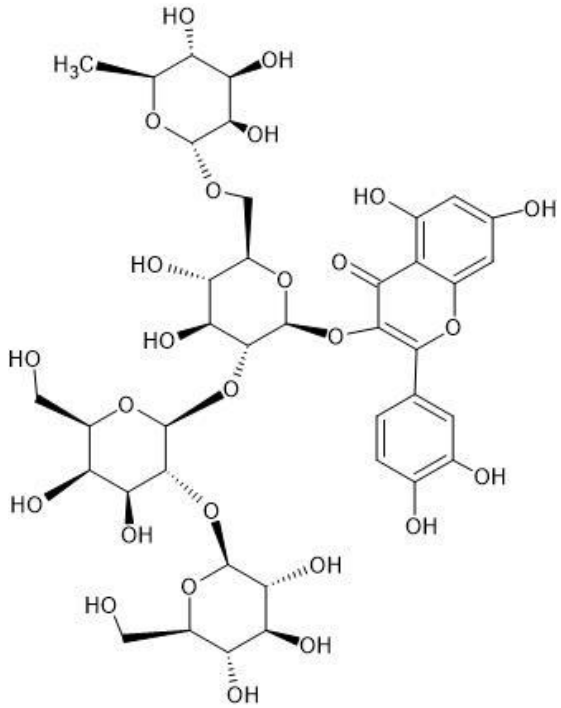
kaempferol 3-O-β-D-glucopyranoside



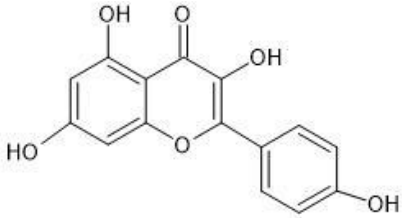
(2*E*)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoic acid



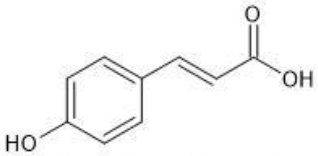
(2*E*)-3-(4-hydroxyphenyl)prop-2-enoic acid



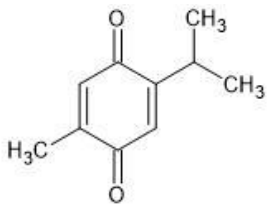
nigelflavonoside B



3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4
H-1-benzopyran-4-one



(2*E*)-3-(4-hydroxyphenyl)prop-2-enoic acid



2-methyl-5-(propan-2-yl)cyclohexa-2,5-diene-1,4-dione

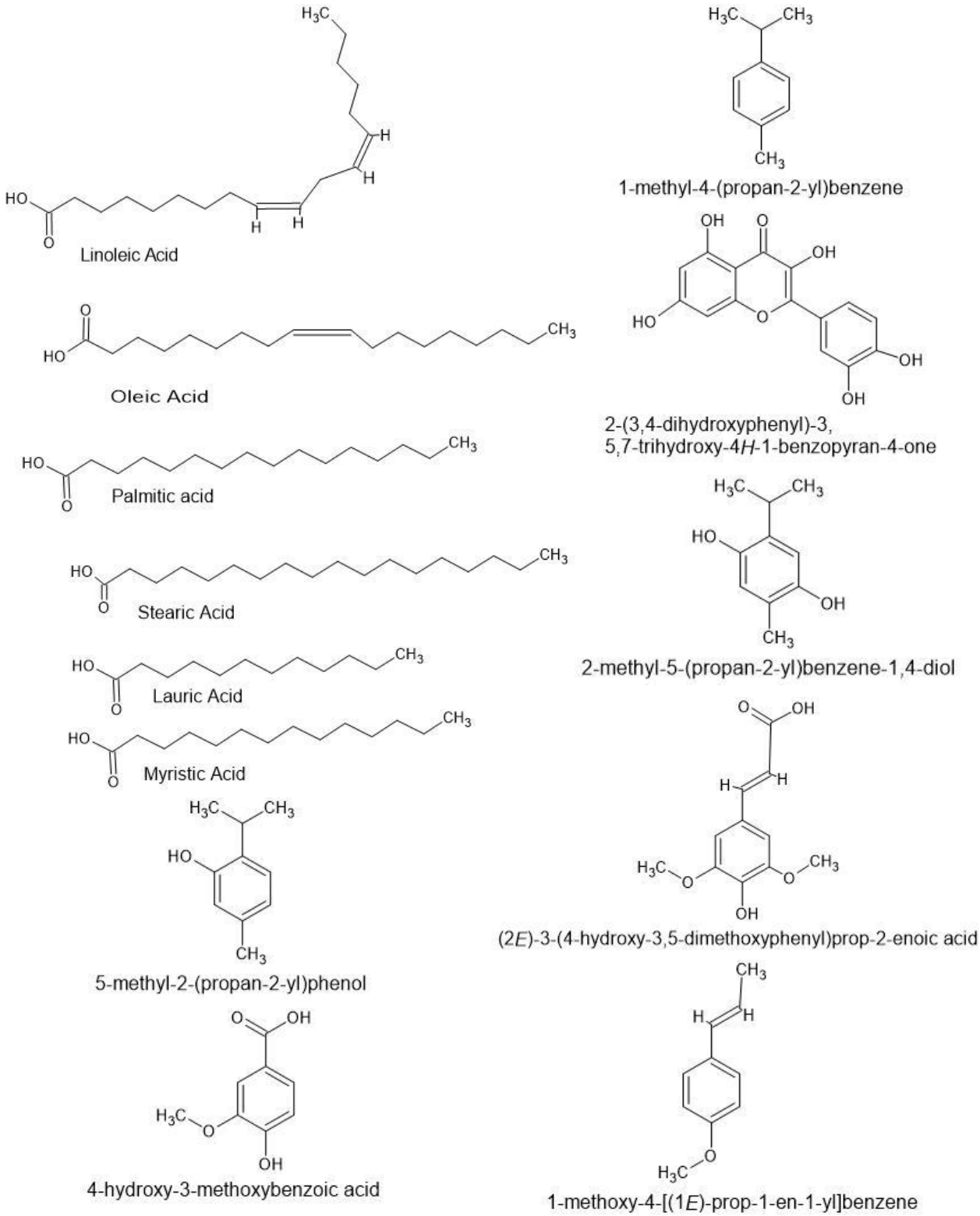


Fig. 2.4 Structures of major phytoconstituents present in *Nigella sativa*

2.1.3 Ethno-botanical uses

Nigella sativa, a well-known plant for its medicinal and culinary uses, has had a religious history since ancient times. Several ancient documents mention the use of kalonji, including Ayurveda, Unani, and the Bible. Prophet Mohammad (PBUH) mentioned that kalonji seeds can heal anything except death; in the Holy Bible, they were narrated as "curative black cumin," while Hippocrates and Dioscorides described them as. "melanthion," and Pliny. cited them as "gith" (79-82). In Tibb.-e-Nabwi., *Nigella sativa* is suggested on a daily basis (82).

It was employed in Ayurveda to expel kidney stones, stabilize vata and kapha, boost pitta, and lessen headaches, coughs, and asthma. It was employed as a stomachic, laxative, carminative, and galactagogues in unani literature as well as to treat inflammatory diseases, ascites, jaundice, piles, and tertian fever, paralysis, and eye conditions (59, 65). *Nigella sativa* is popular as a spice, condiment, and flavoring agent used in pickles, breads, salads, etc. It is also referred to as a liver tonic, diaphoretic, diuretic, emmenagogue, etc. (83). Black cumin oil is useful in several skin diseases such as eczema, psoriasis, skin infections, burns, and boils (84-85).

2.1.4 Pharmacological activity

2.1.4.1 Immunomodulatory activity

Macrophages play an essential role in innate immunity to initiate specific acquired immunity. When *Nigella sativa* seed (aqueous) extract was mixed with different types of macrophages, it increased their phagocytic and killing abilities *in vitro* under electron microscopy and fluorescence (86). Aqueous extract of black cumin seeds was used to study BALB/c mice and BL6 primary cells, and the results show indications of immunomodulatory activity by amplifying the number of WBCs and amplifying the weight of spleen in mice with improved splenocyte proliferation (87). According to a review article, thymoquinone is important as an immunomodulatory component. Sensitized guinea pig BAL fluid and lung tissue assays decreased IL-4 levels while increasing IFN- levels and decreasing endothelin-1 levels (88). An immunomodulatory model was performed using fish (Nile tilapia), which shows an overall augmented

immune response, specified as a significant boost of the WBC count, globulin proteins, and the phagocytic activities of fish phagocytes (89). *Nigella sativa* can modify cellular and humoral immune responses. *N. sativa* seed extracts and oil can suppress humoral immune responses while boosting cellular immune responses (90). Subjects treated for four weeks with oil of black cumin revealed a 55% rise in the CD4 to CD8 T-cell ratio as well as a 30% improvement in NK cell activity (91). A study was performed where the effects of thymoquinone were observed in rheumatoid arthritis using a rat model. Data shows black cumin seed possesses beneficial immunomodulatory potential via boosting T-cell and NK cell-mediated immune reactions (92).

2.1.4.2 Anti-oxidant activity

An antioxidant study of black cumin (seed) acetone and hexane extract was performed using the DPPH assay method and concluded that hexane extract shows the highest IC50 value than acetone extract (93). *Nigella sativa* (seeds) methanolic and water extracts were considered for determination of DPPH radical-scavenging activity and showed that methanolic extract had 40% higher antioxidant DPPH activity compared with aqueous extract (94). The hydro-alcoholic extract of black cumin was fractionated with hexane, chloroform, and ethyl acetate and water, and its antioxidant activity was determined *in vitro* and *in vivo*. *In vitro* antioxidant potential was determined using DPPH assay. superoxide anion scavenging assay, ferrous ion chelating activity, and β-carotene bleaching assay. While *in vivo* antioxidant potential is determined by using blood total antioxidant capacity and plasma antioxidant capacity. From results, it was concluded that seeds of *N. sativa* show considerable antioxidant activity *in vitro* and *in vivo*. Chloroform and ethyl acetate fractions show high antioxidant capacity due to phenolic compounds, whereas hexane fractions show activity due to essential oils (95).

2.1.4.3 Anti-cancer

Nigella sativa shows anti-proliferative, pro-apoptotic, cytotoxic, and anti-mutagenic activity. The *N. sativa* essential oil nano-emulsion reduces the viability of the MCF-7 breast cancer cell line. The results indicate that nano-emulsion encouraged apoptosis in MCF-7 cells; these findings suggest that nano-emulsion may be used to treat breast

cancer (96). Thymoquinone extracted from the *N. sativa* screen against the LL/2 lung cancer cell line at concentration 100 µM showed significant anti-cancer potential by inhibiting 90% cell proliferation (97). An anti-cancer study using thymoquinone on the HCT-116 cell line was performed to determine anticancer potential in colon cancer. The results conclude that thymoquinone is effective against colon cancer by promoting apoptosis in cancer cells (dose dependent) (98). On the contrary, thymoquinone was effective against the HT-29 (human colon carcinoma) cell line (99). *In vivo* anticancer potential of methanolic extract (*N. sativa* seeds) was determined utilizing a Fe-NTA-induced renal carcinogenesis model in wistar rats. Results show that 50 and 100 mg seeds prevent cancerous effects (100).

2.1.4.4 Anti-inflammatory activity

Determination of the anti-inflammatory response of black cumin (seed) sequential extract in the *in vivo* carrageenan-induced rat paw edema model was utilized, suggesting paw edema volume reduction as the dose increases (101). Another study was performed to determine the anti-inflammatory potential of *Nigella sativa* essential oil using a paw edema model using rats (carrageenan-induced) and ear edema in mice (croton oil-induced). In the carrageenan-induced paw edema model, no significant results were observed at doses of 100, 200, and 400 µL /kg. In the Croton oil-induced ear edema model, however, at doses of 10-20 micro L/ear there is less edema (102).

2.1.4.5 Analgesic activity

The analgesic potential of *Nigella sativa* was determined. *In vivo*, using the acetic acid-induced writhing test model, findings suggested that after black cumin (seed) sequential extract administration, a dose-dependent reduction of writhing number was observed with the acetic acid-induced model (101). Another study was reported using acetic acid-induced writhing, formalin, and light-tail flick tests model for determination of the analgesic potential of *N. sativa* oil, which possesses remarkable analgesic activity (102).

2.1.4.6 Anti-arthritic activity

The preventive effect of black cumin oil was evaluated *in vivo* using a rat model of arthritis for 25 days. Results concluded that a dose of 1.82 mL/kg prohibited the

development of arthritis (103). *In vitro* anti-arthritic activity was performed, and from the results, it was observed that aqueous and hydro-alcoholic extracts of *Nigella sativa* (seeds) show prominent activity (104).

2.1.4.7 Neuro-protective activity

The anxiolytic and locomotor activity of *Nigella sativa* seed methanolic extract were assessed using a stressed and unstressed animal model at a dose of 1 g/kg of body weight and concluded with a significant anxiolytic effect, while the extract reduced locomotor activity in both unstressed and stressed animals (105). An electroshock seizure model was used to determine the anti-epileptic effect, which revealed a reduction in several phases of epileptic seizure while using *Nigella sativa* methanolic extract (105). Forced swim test and tail suspension test models were exploited to estimate the antidepressant effect of *Nigella sativa* methanolic extract, which showed a minor reduction in the rats immobility. The study found that *Nigella sativa* had strong neuroprotective benefits during germination compared to non-germinated seed (105). Rat stroke models were employed in the assessment of the neuroprotective efficacy of petroleum ether and chloroform extracts from *N. sativa* seeds (in cerebral ischemia). As per the study, when extract is taken orally for seven days at a dose of 400 mg/kg, it enhances grip strength and locomotor activity while also showing a decrease in infarct volume (106). Hydro-alcoholic extract of kalonji seeds shortened oxidative stress; AChE activity, on the contrary, improved learning and memory impairment in a scopolamine-induced spatial memory impairment rat model (107).

2.1.4.8 Gastro-protective activity

The gastro-protective potential of *Nigella sativa* seed extract was evaluated using an acute gastric ulcer model induced by indomethacin. Results suggested that there was no effect on gastric acid secretion, a reduction in ulcer index, malondialdehyde, and protein content while an increase in total thiol, total hexose, and mucus content on oral administration of the extract (107). Essential oil of *N. sativa* was screened for its anti-ulcer property using aspirin-induced ulcer models and showed remarkable gastric protection (108). A stress gastritis model was employed to evaluate the gastro-protective

potential of *N. sativa* oil. Results concluded stress gastritis can be reduced on treatment with 10 ml/kg of body weight *N. sativa* oil for 15 days (109).

2.1.4.9 Anti-asthmatic activity

Mast cells of rats were examined for histamine release when treated with an ethanolic extract of *Nigella sativa* seed. Results highlight inhibition of histamine secretion from mast cells and show significant anti-inflammatory potential, hence useful in asthma (110). Bronchodilatory action of *N. sativa* seed extract in asthmatic patients was investigated. At the beginning, the bronchodilatory effect was similar to standard, and results conclude elevation in pulmonary function; however, it can be used as an anti-asthmatic agent (111). A clinical trial was carried out for the investigation of the anti-asthmatic potential of *Nigella sativa* (whole seed capsule) supplement in partially controlled asthma patients. On the basis of results, it was concluded that *N. sativa* somewhat progresses pulmonary function and inflammation in asthmatic patients with inhalation therapy (112).

2.1.4.10 Anti-diabetic activity

A streptozotocin-induced model using male Albino rats was employed to investigate the anti-diabetic effect of black cumin seed, which was concluded to be less effective as compared to Propolis (113). According to the review, *Nigella sativa* seeds have anti-diabetic potential by decreasing insulin resistance, increasing cell proliferation rate, increasing insulin secretion, increasing glucose uptake, and decreasing hepatic gluconeogenesis (114).

2.1.4.11 Inflammatory bowel disease (IBD)

Efficiency of black cumin oil was investigated against TNBS-induced ulcerative colitis model in rats. Results concluded that it partially protects colonic tissue by preventing anti-inflammatory responses in blood (115). A clinical trial was performed to examine the effect of *Nigella sativa* supplement on 46 patients with ulcerative colitis for 6 weeks. Results concluded that *Nigella sativa* seed powder cannot be considered as the main therapy in mild to moderate ulcerative colitis, but with dose variation, several clinical trials can be performed to study its potential (116).

2.1.4.12 Anti-microbial activity

Nigella sativa seed methanolic extract fraction has a significant inhibitory effect on *Staphylococcus saprophyticus* and *Staphylococcus epidermis* (59, 117). *Nigella sativa* oil was screened against methicillin-resistant *Staphylococcus aureus*, and it showed synergic effects along with antibiotics (118). The decoction of *Nigella sativa* seeds shows anti-bacterial potential against gram-positive as well as gram-negative bacteria at a concentration 100µg/mL (119). *Nigella sativa* seed oil utilized in cheese manufacturing shows an antimicrobial effect; hence, it is concluded that it can be used as a natural antibiotic in food (120).

2.1.4.13 Anti-fungal activity

Nigella sativa seed methanolic extract was tested against *Fusarium oxysporum* and *Macrophomina phaseolina* (soil-born fungi) and found to be a potential anti-fungal agent (121).

2.1.4.14 Larvicidal activity

Nigella sativa oil was screened for larvicidal activity in contradiction of the fourth in star larvae of *Aedesaegypti*, *Anopheles stephensi*, and *Culexquinque fasciatus* and shows remarkable larvicidal potential (122).

2.1.4.15 Diuretic activity

The diuretic potential of aq. extract of *N. sativa* seed was examined using doses 10, 30, and 50 mg/kg (intraperitoneal) in Albino. Rats possess notable diuretic activity (123).

2.1.5 Acute toxicity

An acute toxicity study performed on albino mice using an aqueous extract of *Nigella sativa* seed concluded no lethal effects were estimated at a dose of 5000 mg/kg (123). Mice were given dosages of 28.8 ml/kg body weight orally and 2.06 ml/kg. body weight intra-peritoneally to test the acute toxicity of *Nigella sativa* fixed oil. While a chronic toxicity model was employed in rats using 2 ml/kg body weight for. 12 weeks. The findings concluded that *N. sativa* fixed oil is not toxic (124). A diazinon-induced cardiotoxicity model was employed to determine thymoquinone toxicity in male Wistar rats. Results show decreased cardiotoxicity in animals consuming thymoquinone (125).

2.2 *Pimpinella anisum*

2.2.1 Description-

Pimpinella is one of the largest genus comprising around 150 species that belong to the family Apiaceae; most of them have been exploited as condiments and traditional therapeutic benefits. These species were diversified all over the world, including Western Asia, East Asia, the Eastern Mediterranean region, Southwest Asia, European countries, etc. (126-127). Among these species, *Pimpinella anisum* shares a significant position in the *Pimpinella* genus.

2.2.1.1 Biological source

These are the dried seeds of *Pimpinella anisum*, which belongs to the family Apiaceae (126–127).

2.2.1.2 Pimpinella anisum

Pimpinella anisum is an herbaceous annual plant with a strong flavor and aroma, also known as "anise or anise seed," having around 60-90 centimeters in height. The plant has upright, branching stems consisting of simple leaves with 1–5 cm long, pinnate, and divided into multiple leaflets. Flowers are white or yellow in color, roughly 3 millimeters in diameter. The fruits are 3-5 mm long, usually attached to a slender pedicel consisting of 8–12 primary ridges with uniform width observed in Fig. 2.5 (128).

Fig. 2.5 *Pimpinella anisum* plant

Fig. 2.6 *Pimpinella anisum* seeds

Pimpinella anisum seeds (morphology) (128)

Shape - ovoid

Size - 0.3 to 0. 5 cm long and 0.1 to 0.2 cm wide

Colour – greenish yellow or greenish-brown

Odour - characteristic

Taste - sweet and aromatic

2.2.1.3 Taxonomic Classification (129)

Kingdom: Plantae.

Subkingdom: Viridiplantae.

Superdivision: Embryophyta.

Division: Tracheophyta.

Subdivision: Spermatophytina.

Class: Magnoliopsida.

Order: Apiales.

Family: Apiaceae.

Genus: *Pimpinella.*

Species: *Pimpinella anisum (linn).*

2.2.1.4 Vernacular Names (128)

Sanskrit: avetapuap

English: Anise

Hindi: BadiyanRumee, Sauph, Anisoon

Marathi: AnisunaShopa

Punjabi: Valaitisounf

Bengali: Muhuri

Tamil:Shombu

Gujarati:Anisi, Sowa

Kannada: sompu

Malayalam:Shombu

Oriya: Sop

Telugu: Kuppisoptu

2.2.2 Phytochemistry

Terpenes and terpenoids

Pimpinella anisum consist of Terpene hydrocarbons, Monoterpene, Sesquiterpene and Phenylpropanoids, found in prominent amount. They are Linalool, α-terpinene, anisole, estragole, transanethole, p-anisaldehyde, Cisisoeugenol, β-elemene, limonene, γ-himachalene, Zingiberene, β-himachalene, β-Bisabolene, isolongifolene, neophytadiene and Diepi-α-cedrene (130-131).

Fatty Acids

Saturated Fatty acids including ascapric acid, lauric acid, myristic acid**,** palmitic acid, stearic acid, arachidic acid, and unsaturated fatty acids including petroselinic acid, oleic acid, linoleic acid, and linolenic acid were found in aniseed (131).

Phenolic acid

Phenolic acid for instance chlorogenicacid and rosmarinic acid are found in prominent amount while gallic acid, syringic acid, p-coumaricacid, ellargic acid, caffeicacid, and 4-(β-d-gluco-pyranosyloxy) benzoic acid observed in minor amount (131).

Flavonoids

Major flavonoids were observed in *Pimpinella anisum* were naringin, coumarin followed by rutin, quercetin, apigenin, cirsimartin, luteolin7-glucoside, Kaempferol-O-rutinoside isoorientin, and isovitexin (130-131).

Glucosides, alkyl glucoside and glucide

(E)-10-(2-hydroxy-5-methoxyphenyl) and (E)-3-hydroxy-anethole-d-glucopyranoside propane 3-hydroxyestragole 3-d-glucopyranoside 3-d-glucopyranoside, methyl syringateHexane-1,5-diol, 4-O-d-glucopyranoside, and The methanolic extract of anise fruit yielded 1-O-d-glucopyranoside and 1-deoxy-l-erythritol3-O-dglucopyranoside, two novel glucosidic compounds.(131-132).

Coumarin

Coumarins such as umbelliferone, umbelliprenine, bergapten, and scopoletin were found in *Pimpinella anisum* (133).

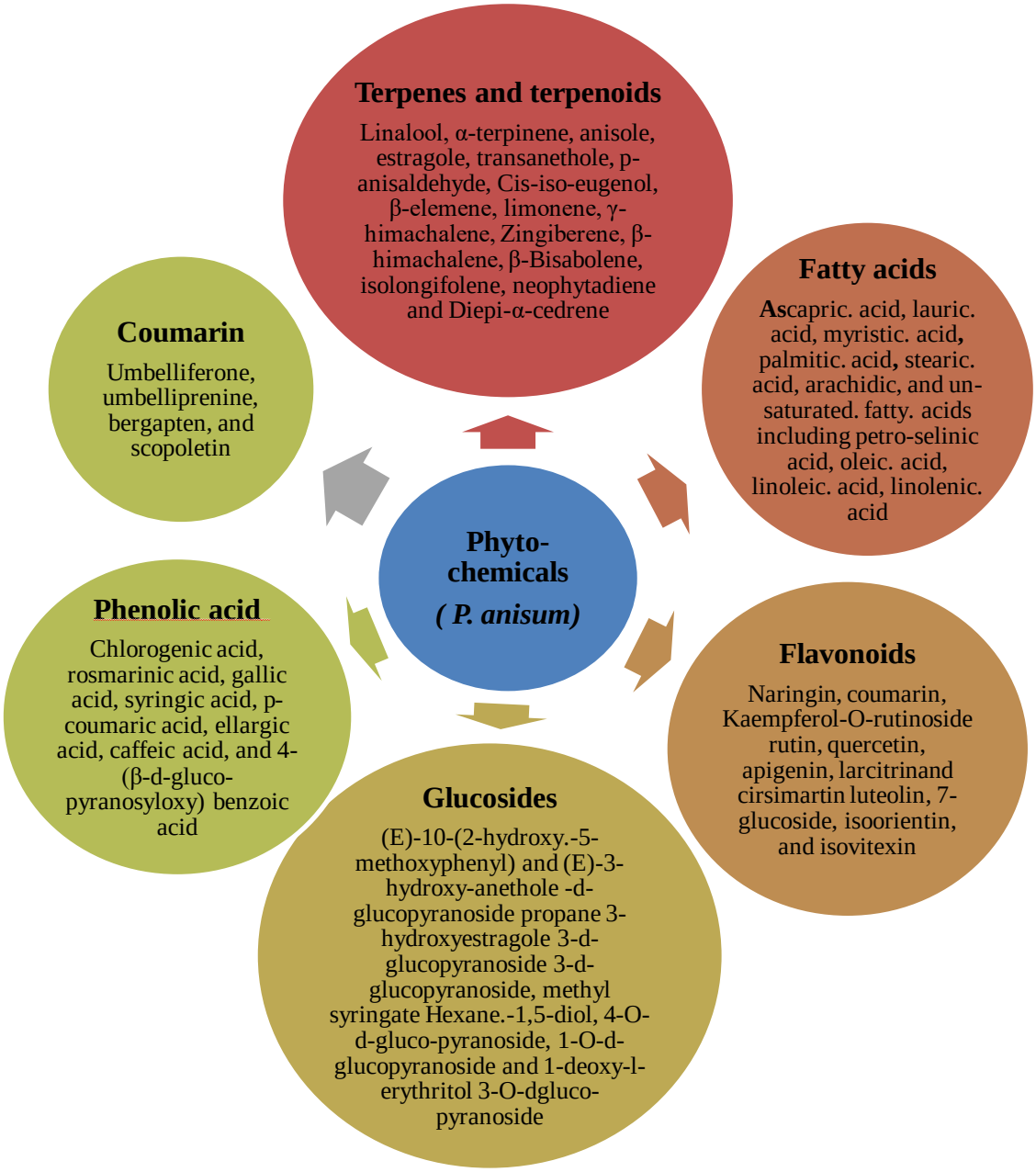
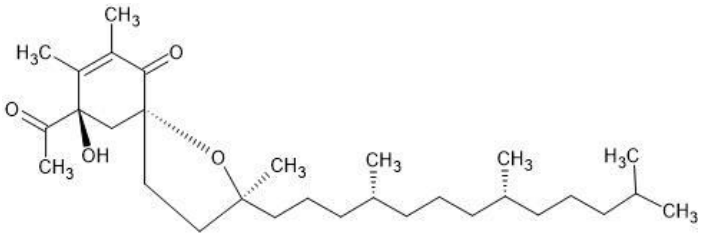
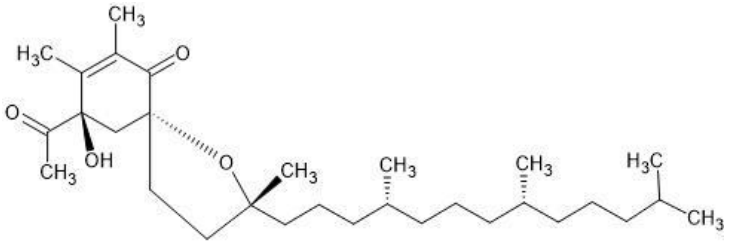


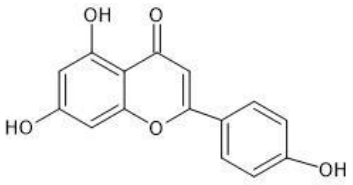
Fig. 2.7 Phytochemistry of *Pimpinella anisum*



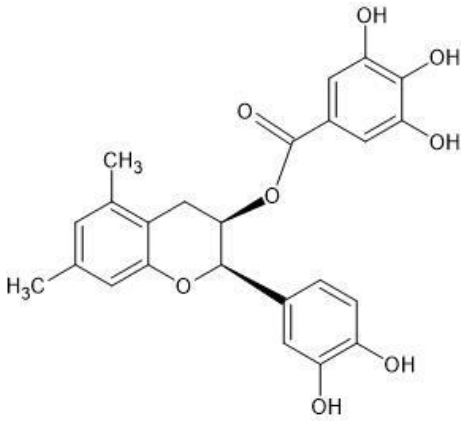
Alpha. -Tocospiro A



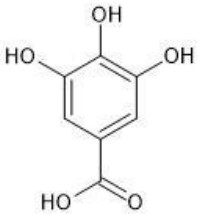
Alpha. -Tocospiro B



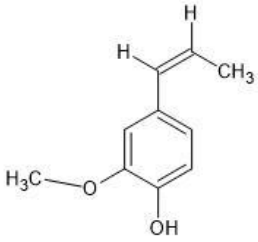
5,7-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one



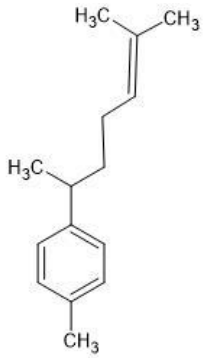
epicatechin-3-ΘE-gallate



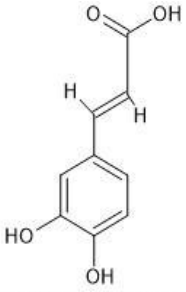
3,4,5-trihydroxybenzoic acid



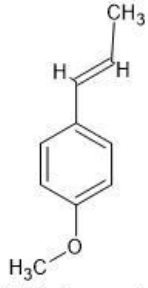
2-methoxy-4-[(1*Z*)-prop-1-en-1-yl]phenol



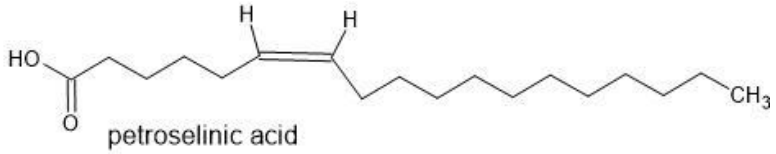
1-methyl-4-(6-methylhept-5-en-2-yl)benzene



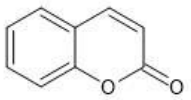
(2*E*)-3-(3,4-dihydroxyphenyl)prop-2-enoic acid



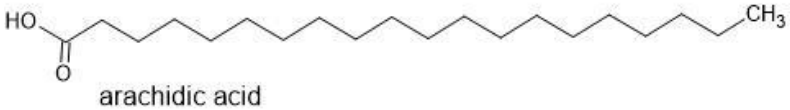
1-methoxy-4-[(1*E*)-prop-1-en-1-yl]benzene



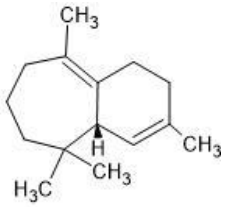
petroselinic acid



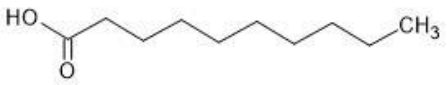
2*H*-1-benzopyran-2-one



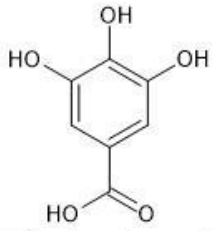
arachidic acid



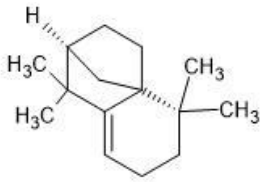
Beta-himachalene



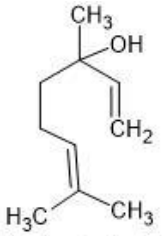
decanoic acid



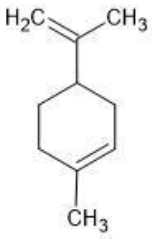
3,4,5-trihydroxybenzoic acid



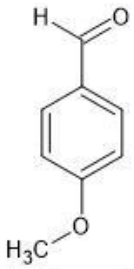
(2*S*)-1,1,5,5-tetramethyl-1,3,4,5,6,7-hexa hydro-2*H*-2,4a-methanonaphthalene



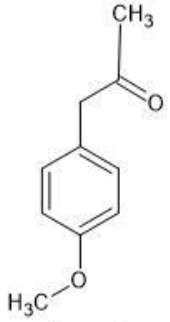
3,7-dimethylocta-1,6-dien-3-ol



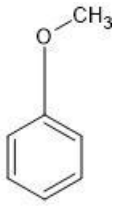
1-methyl-4-(prop-1-en-2-yl) cyclohex-1-ene



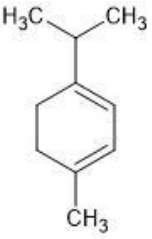
4-methoxybenzaldehyde



1-(4-methoxyphenyl)propan-2-one



anisole



1-methyl-4-(propan-2-yl)cyclohexa-1,3-diene

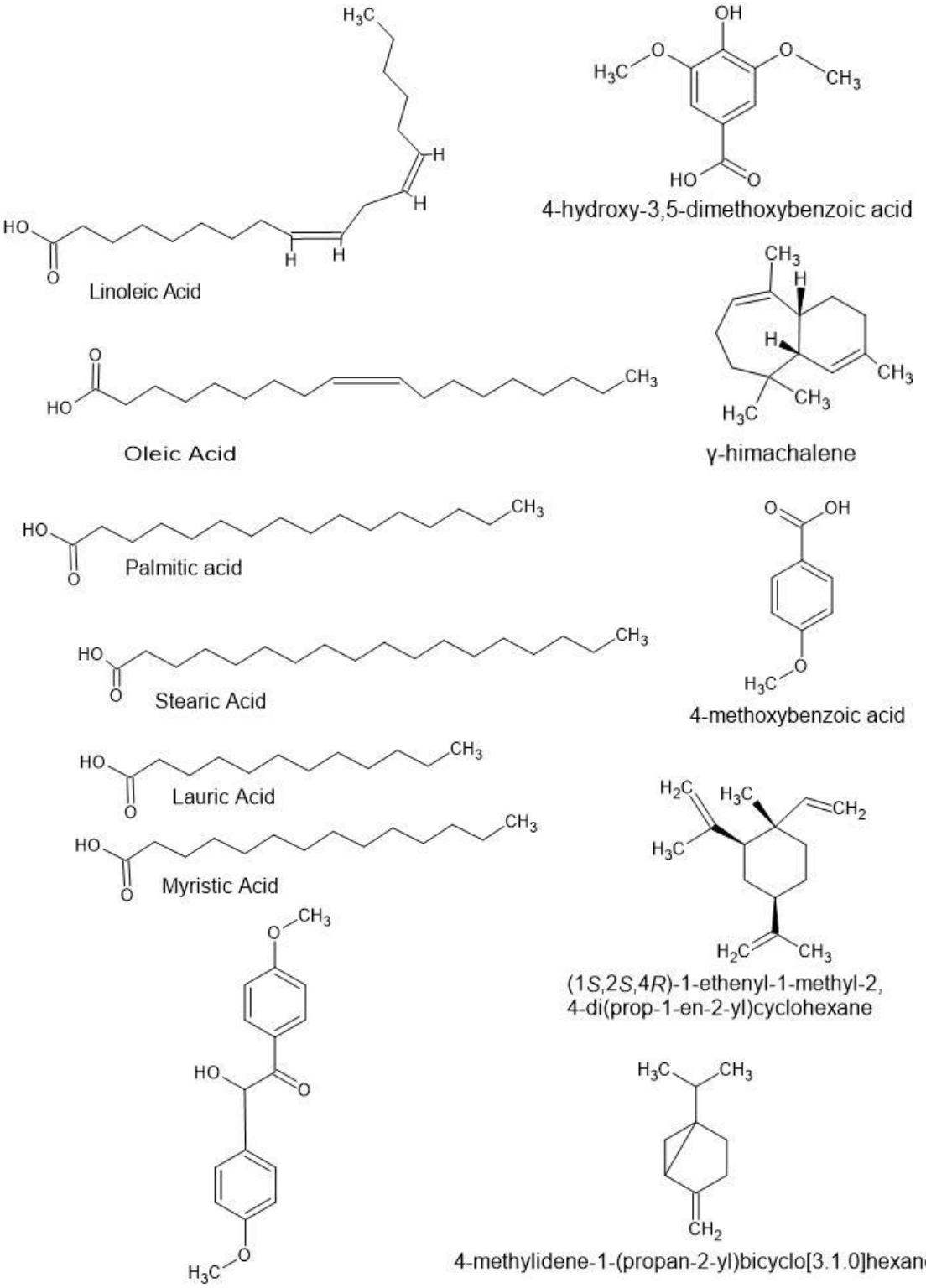


Fig. 2.8 Structures of major phytoconstituents present in *Pimpinella anisum*

2.2.3 Ethno-botanical uses

Traditional medicine uses the seeds of Pimpinella anisum as a carminative, fragrant, disinfectant, diuretic, insomnia, appetite stimulant, tranquilizer, and migraine analgesic. Aniseed is useful in polishing teeth and can enhance milk production, menstrual flow, urine, and perspiration output. Anise is listed in various ancient manuscripts as a remedy for epilepsy and seizures, as well as for sadness and nightmares (128, 132).

2.2.4 Pharmacological activity

2.2.4.1 Immunomodulatory activity

An immunomodulatory study performed on mice offers preclinical evidence that *Pimpinella anisum* possesses immunomodulatory potential when administered orally. The mechanism behind immunomodulation is the inhibitory impact on nitric oxide (NO) production and augmented cell-mediated immune responses (134). Another study performs to conclude the immunomodulatory consequence of *Pimpinella anisum* against ND (Newcastle Disease) as well as IBD (infectious bursal disease) viruses. The investigator found that aniseed had the best humoral and cellular immune responses, which ultimately proves its immunomodulatory potential at dose 0.5g/kg & 1g/kg in Broiler Chicks (135). Immunosuppressed mice were given cyclophosphamide dosages of 50 mg/kg IP and oral trans-anethole 500 mg/kg as part of an animal experiment. Trans-anethole increases WBCs (White Blood Cell) count as well as antibody levels to a to a near-normal value. It elevates not only IL-10 production but also diminishes TH1-type cytokines (IL-2) levels (136). An additional study was performed in which the 3 moieties isolated from hot water extract of aniseed show a lignin-carbohydrate complex for exhibiting immunomodulatory and antiviral potential (137).

2.2.4.2 Anti-oxidant activity

The antioxidant potential of ethanolic and aqueous extracts of *Pimpinella anisum* was evaluated in vitro (DPPH). Results concluded that ethanolic extract showed high radical-scavenging activity compared to aqueous extract (138). Antioxidant activity of metal nanoparticles of *Pimpinella anisum* was evaluated using the DPPH method, and excellent antioxidant potential was concluded (139).

2.2.4.3Anti-cancer activity

The PC-3 cell line was used to test the cytotoxic activity (prostate cancer). Results concluded that *Pimpinella anisum* ethanolic extract has anti-proliferative and anti-apoptotic properties; it is used to prevent cancer (140). Anti-proliferative activity of anise oil was screened against human cancer cells HepG2 (hepatoma), Caco2 (colon cancer), MCF-7 (breast cancer), and THP1 (monocytic cell) using the SRB assay. Results concluded that amounts of all screened cell lines showed that anise was highly cytotoxic against THP-1 cells (141). Cytotoxic activity of anise seed extract screened against epidermoid carcinoma using KB cell line. Results concluded that it possesses significant cytotoxic activity compared with cisplatin (142).

2.2.4.4 Anti-inflammatory activity

Researchers isolated polysaccharides from aniseed and screened them for their anti-inflammatory potential in mice using the paw edema model, yielding positive results (143). We determined the anti-inflammatory potential of *P. anisum* oil using a COX-2 inhibition assay, concluding that it reduces the production of COX-2 (144).

2.2.4.5 Analgesic activity

Researchers determined the efficacy of *P. anisum* oil against migraine headaches through a randomized clinical trial. The findings suggest that, compared to a placebo, *P. anisum* oil-based cream reduces the frequency and duration of migraine attacks (145).

2.2.4.6 Wound healing potential

Researchers isolated and screened polysaccharides to assess their ability to heal wounds in laser-burn mice. Applying a polysaccharide gel-base formulation to the wound after 7 days improves epithelium regeneration and accelerates wound healing (143).

2.2.4.7 Neuro-protective activity

Rat models used in both *in vitro* and *in vivo* testing were used to assess the anti-seizure and anti-hypoxia effects of *P. anisum* oil. Findings showed that it extends seizure attacks and significantly increases ATN latency at concentrations of 10–20 µg/l (146). Researchers used Swiss albino mice for a 21-day investigation on the effects of *P. anisum* extract on anxiety, depression, and memory. Findings indicate that the extract has

considerable antidepressant-like and anxiolytic properties (147). *P. anisum* extract results show potential antidepressant effects similar to fluoxetine when tested using antidepressant models such as the forced swimming test and tail suspension test.

2.2.4.8 Gastro-protective activity

Scientists tested an aqueous suspension of *P. anisum* for its ability to protect against stomach ulcers in rats and against an effect that stops gastric acid production in Shay rats with the pylorus tied off. The findings concluded that the suspension exhibits a gastro-protective effect by inhibiting gastric mucosal damage and basal gastric acid secretion (148-149).

2.2.4.9 Anti-asthmatic activity

The anti-asthmatic potential of an aqueous *P. anisum* L. seed extract was evaluated using an ovalbumin-induced asthma model. The treatment group has delayed ovalbumin-stimulated asthmatic difficulties and suppressed inflammatory responses, according to the findings (150).

2.2.4.10Anti-diabetic activity

A methanolic extract of *Pimpinella anisum* was used to create fractions of hexane, benzene, ethyl acetate, n-butanol, and water for an in vitro anti-diabetic study. According to the findings, the ethyl acetate fraction has the greatest anti-diabetic activity (151).

2.2.4.11 Polycystic Ovary Syndrome (PCOS)

A randomized, triple-blind clinical trial was performed on 72 women for 15 days using anise capsules (dose 4.5 g/day). After this treatment, 58% of women observed an increase in menstrual regularity (152).

2.2.4.12Anti-Menopausal activity

A randomized, triple-blind clinical trial was implemented on 72 women in 2009 to investigate the effect of *Pimpinella anisum* on hot flashes. 330 mg of *Pimpinella anisum* capsules were given 3 times a day for 4 weeks. Finding concluded that the occurrence and severity of hot flashes decreased after treatment in postmenopausal women (153).

2.2.4.13Anti-microbial activity

The antimicrobial potential of several extracts P. anisum was tested against *S. aureus., S. pyogenes., E. coli.,* and *K. pneumoniae,* whereas the aqueous and methanolic extracts showed zones of inhibition (154). Silver and gold nanoparticles of *P. anisum* were tested against *S. aureus, E. coli, A. flavus.,* and *C. albicans* and showed remarkable anti-microbial potential (155).

2.2.4.14 Anti-fungal activity

The anti-fungal potential of *P. anisum* seed against *Trichophyton rubrum* was assessed. At a concentration of 100 µg/ml methanolic extract of anise shows an excellent zone of inhibition (156). Additionally, the antifungal potential of *P. anisum* volatile oil was evaluated against *Rhizopus stolonifer* at the concentration of 625 µL/L it completely inhibits the growth of *Rhizopus* throughout the 7 days of cultivation (157).

2.2.4.15 Renoprotective activity

The effects of aspartame on renal and hepatic function were evaluated using male albino rats. Consumption of aspartame at a dose of 250 mg/kg/day for 2 months causes several changes in the structure of the kidney and liver. While the group receiving *P. anisum* oil at a dose of 0.5 ml/kg/day followed by aspartame for 2 months decreased the toxicity of aspartame (158). Another study was performed to evaluate the effect of *P. anisum* (ethanolic extract) using 40 male wistar rats against Gentamicin-induced nephrotoxicity at a dose of 300 mg/kg for 8 consecutive days. Results concluded that the group receiving *P. anisum* ethanolic extracts improved tubule damage (159).

2.2.4.16 Larvicidal activity

The effect of *P. anisum* essential oil on *Leptinotarsa decemlineata* was evaluated. For analysis, two formulations, one conventional and another encapsulated, were prepared using *P. anisum* oil. Both the formulations showed high acute mortality at low concentrations and concluded that anise possessed larvicidal potential (160).

2.2.5 Acute toxicity

Acute oral toxicity of an aqueous extract of aniseed was carried out in mice. Results concluded that no deaths were observed at doses up to 100 mg/kg, but all animals died at 200 mg/kg (161). Acute oral toxicity in male mice was examined for 14 days using 175,

550, 1750, and 5000 mg/kg of an aqueous extract of aniseed. Results concluded that no death was observed (162).

2.3 Formulation and development of *Nigella sativa*

Table no. 2.1 Previously developed formulations of *Nigella sativa*

Sr. no	Formulation	Pharmacological action	study on	Model/method	Dose	Year	Ref.
1.	Cookies	Dietary supplementation	Albino rats (male)	*In vivo* screening, protein quality evaluation	5%-25%	2024	163
2.	Magnetic nanoparticles	Antifungal activity	*Candida albicans*	*In vitro* (broth microdilution test)	100 µL	2023	164
3.	Dental nanoemulgel using *N. sativa* oil	Antimicrobial activity	*Staphylococcus aureus*	*In vitro* (agar well diffusion method)	0.5 g	2022	165
4.	Film-forming polymeric solution	Antibacterial activity	*S. aureus* and *S. epidermidis.*	Agar well difusion method	9.2% extract	2022	166
5.	Cream	Analgesic, wound healing activity	Rat	Formalin test, *in vivo* wound healing	-	2022	167
6.	Nanoemulsion Loaded with Pioglitazone	Hypo-glycemic action	Wistar rats (male)	*In vivo* anti-diabetic model	30 mg/kg	2022	168
7.	Transdermal Patches	Leishmanicidal Activities	Human Being	*In vivo* Anti-Lieshmanial Study	-	2021	169
8.	Nanoemulsion	Ice-cream industry	-	Zeta potential, creaming test	3%, 5%,10 %	2020	170
9.	Capsules	Amelioration of oxidative stress	Hashimoto's thyroiditis patients	Clinical trial	1 g/day	2020	171
10.	Capsules	Gastro-protective	H. pylori-	Helicobacter pylori	2	2020	172

			infected patients	eradication	g/day		
11.	Self-nano-emulsifying drug delivery system	hepatocellular carcinoma	HepG-2 cell line	*In vitro* MTT assay	1, 2 and 5 µg/ ml	2019	173
12.	Topical gels	Antimicrobial Activity	*S. aureus* suspension of bacteria	Mueller Hinton Agar (MHA) plates	15% of the seed extrac t	2019	174
13.	Ethosomal vesicles	anti-psoriatic activity	dorsal skin of Albino rat	*Ex vivo* skin permeation studies,	2% w/w	2019	175
14.	Ethosomal vesicles	anti-psoriatic activity	albino mice	Anti-psoriatic activity in mouse-tail	20 mg /kg	2019	175
15.	Oral alginate microcapsules	Inflammatory bowel disease,antioxidan t activity	-	DPPH	-	2019	176
16.	Emulgel	Anti-Microbial	*Staphylococcus aureus*	Agar plates	-	2019	177
17.	Cream	Vitiligo	Human Being	dermatological examination and Wood's lamp examination	-	2019	178
18.	Balm Sticks	Anti-Inflammatory Activity	rats	carrageenan-induced paw oedema and granuloma pouch	10%	2019	179
19.	Capsules	Anti-diabetic activity	Human Being	Type 2 Diabetes Mellitus patients	1.35 g/day	2019	180
20.	Capsules	Renal protective	Human Being	Patients with renal stones	1 g/day	2019	181

21.	Topical nanoemulsion	Anti-inflammatory activity	Wistar rats	arrageenan-induced hind paw edema method	-	2018	182
22.	Nanoparticles	antileishmanial activity	J774 macrophage cell	J774 macrophage cell infect with L. tropicaamastigotes	20, 30 and 50 mg/ ml	2017	183
23.	Polyherbal Tablet	anti-diabetic	3T3 Cell line	Glucose uptake assay	-	2017	184
24.	anticancer preparation, anti-human immunodeficiency virus preparation, anti-hepatitis preparation, and anti-hepatitis B virus preparation	Immunomodulatory property	human peripheral blood mononuclear cells	trypan blue dye exclusion method.	-	2017	185
25.	Alpha-zam	anti-HCV activity	1b HCV replicon cells	luciferase expression, viral RNA synthesis, and cytotoxicity.	-	2016	186
26.	Nanoemulsion	Anticancer activity (breast cancer)	MCF-7 cell line	*In vitro* MTT assay	20-100 µl/mL	2016	96
27.	Ointment	*wound healing activity*	Rat	Excision and incision wound healing models	10% w/w	2016	187
28.	Emulsion	*wound healing activity*	Rat	dead space wound model	500 mg/kg	2016	187
29.	Co-encapsulation	Alzheimer's disease	N2a cell	neuronal model murine neuroblastoma (N2a)	-	2016	188
30.	PHYTOVAG	vaginal fungal	toxicity study	Chinese hamster	12.5-	2016	189

	EX Suppository	infection	on pregnant rats	ovary (Cho) cells	400 µg/ml		
31.	Capsules	Anti-inflammation	Human Being	Rheumatoid arthritis patients	1 g/day	2016	190
32.	Cream	atopic eczema	-	In-vitro occlusion test, drug release	-	2015	191
33.	Microemulsion	Antibacterial Activity	*S. aureus, B. cereus and S. typhimurium,L. monocytogenes and P. aeruginosa* E. coli	agar well diffusion method	100.0, 400.0, 500.0 µg/well.	2015	192
34.	Proniosome	Neuroprotective	Wistar albino rats	Behavioral model	-	2014	193
35.	Lozenges	Antibacterial Activity	*Streptococcus pyogenes*	broth dilution assay	-	2012	194
36.	Soft gelatin capsules	Immunomodulatory	Male Albino mice	ELISA for TNF- α, IL-1β, and IFN- γ	200 mg	2011	195
37.	Polyherbal formulation	Anti-hyperlipidemic activity	Male Wistar albino rats	Streptozotocin-induced diabetes model	200 mg/kg	2010	196

2.4 Formulation and development of *Pimpinella anisum*

Table no. 2.2 Previously developed formulations of *Pimpinella anisum*

Sr.no	**Formulation**	**Pharmacological action**	**study on**	**Model/method**	**Dose**	**Year**	**Ref.**
1.	Fruit Juice	PCOD	Wistar rat (female)	*In vivo* model, Ovarian Histopathology	200 mg/kg	2024	197
2.	Traditional formulation	Anxiety, depression	IBS-C patients	double-blind randomized clinical	500 mg herbal	2024	198

				trial	formula		
3.	Herbal tea	Effect on Human milk volume and weight gain in infant	Preterm infant	randomized clinical trial	2:1gm anise:tea	2023	199
4.	Drinking water(aniseed: ginger extract)	Immuno-modulatory	Broiler chicks	Mean antibody titer against castle disease, infectious bronchitis and infectious bursal disease	(2 + 4gm), (2.5 + 5gm) and (3+ 6gm)	2023	200
5.	Emulgel (essential oil)	Anti-bacterial	*E. coli*	Cell viability, Minimum inhibitory concentraction	20–60 µg/mL	2023	201
6.	Polysacchride	Immunostimulant	RAW264.7 and NK cells	*In vitro* Inflammatory mediator release	-	2022	202
7.	Isonitrogenous and isolipidic diet	Immunostimulant	*Dicentrarchuslabrax (* fish)	*In vivo* phagocytic function, blood analysis and intestinal antibacterial count	1.5-3.5 g per kg	2022	203
8.	Nano emulsion (co-encapsulation of essential	Antifungal and anti-aflatoxigenicity	*Aspergillusflavus*	*In vitro* Antifungal activity	0.75:0.25 aniseed:coriender oil	2022	204

	oil)				ratio		
9.	Encapsulation as food preservative (essential oil)	Antifungal and anti-aflatoxigenicity	*Aspergillusflavus*	minimum inhibitory concentration, minimum aflatoxin inhibitory concentration	-	2021	205
10.	Metal nanoparticles	Antimicrobial activity	*Aspergillusflavus, E. coli, C. albicans S. aureus,*	*In vitro* Disk diffusion method.	-	2020	139
11.	Nano-emulsions (essential oil)	Insecticidal	*Triboliumcastaneum*	Toxicity to species and its F1 progeny, morphological and histological study	LC50 = 9.3% v/v	2018	206
12.	Powder supplement	Physiological-stimulant	chicks	Hematological and biochemical evaluation	500-1000mg/L	2017	207
13.	Vaginal gel	Antibacterial, antifungal	*S. aureus, S. lutea, C. albicans, C. glabrata, C. Parapsilosis.*	Agar disc diffusion	-	2016	208
14.	Lignin-Carbohydrate-Protein Complexes	Antiviral (HSV-1, HSV-2, HCMV, measles virus) and immunostimulant	Vero and MRC-5 cell line	Virus adsorption assay, virus penetration assay and virucidal assay	-	2011	209

CHAPTER - 3

HYPOTHESIS

3. HYPOTHESIS

During our entire lives, we become exposed to a wide range of pathogens, yet very few of them are able to cause illness. Human lifestyle changes also enhance the pathogen's resistance level. New findings in this experimental system suggest recommendations for therapies that lower immunity, disrupt viral/bacterial immunomodulation, and engage enhanced host immunity to better avoid and combat infections. The ancient systems of medicine such as Ayurveda, Siddha, Tibb, Unani, and TCM all discussed the use of crude drugs, extracts, and herbs that could alter the immune system (42-43).

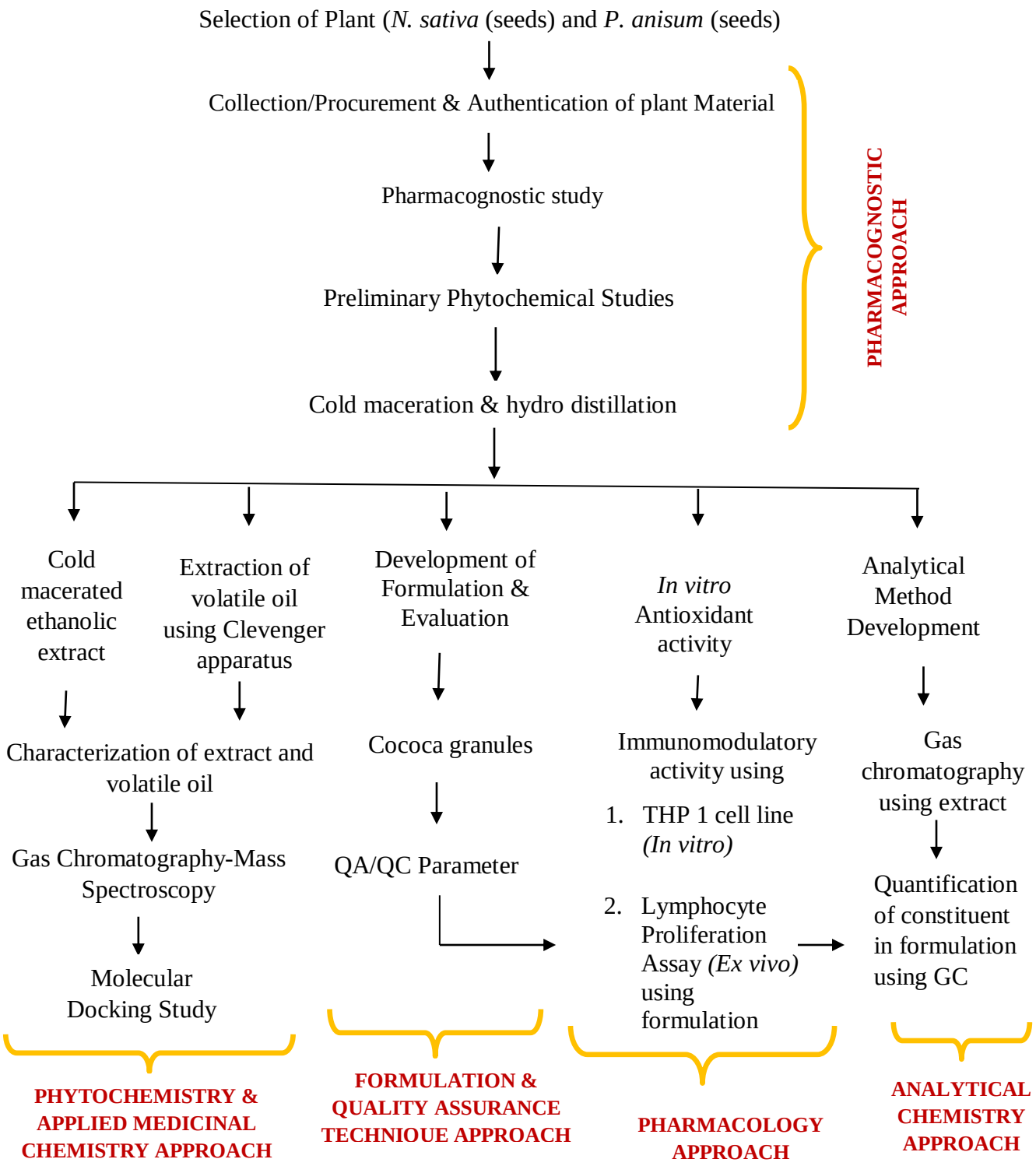
Nigella sativa is simply considered a miracle herb, as discussed in Traditional Arab and Islamic Medicine. Muhammad, the prophet of Islam, claims that *Nigella sativa* seeds can cure every disease except death. Islamic literature recognizes it as one of the most beneficial types of therapeutic medicine. Islamic literature advises the regular use of Tibb-e-Nabwi (prophetic medicine) (210). The Bible, homeopathy, Ayurveda, Unani, and Chinese systems of medicine all mention black cumin seeds. The FDA classifies *Nigella sativa* as GRAS for consumption (Generally Recognized as Safe) (211). Black cumin and anise are both the plants mentioned in books such as the Indian medicinal plants, Indian Material Medica and the Ayurvedic Pharmacopoeia of India.

Previous studies have reported the *in vitro* immunomodulatory activity of oil, aqueous extract, or a specific isolated compound from *Nigella sativa*. We can further screen ethanolic extract and its cytokine estimation using ELISA. We can also explore the lymphocytic proliferation assay using a specific formulation. (44-46). Furthermore, researchers discovered a scarcity of information about formulating extracts that target immunomodulation, as well as conducting *in vitro* and *ex vivo* immunomodulatory tests on *Pimpinella anisum* (47–48). This led to the development of immunomodulatory ready-to-mix granules, which could potentially become nutraceutical formulations with additional research.

Nigella sativa and *Pimpinella anisum* were the well-known drugs briefly mentioned in standard books and literature. The study integrates the standardization of crude drugs to confirm their authenticity through qualitative measures. We consider the extraction and

characterization of extracts and volatile oils from *Nigella sativa* and *Pimpinella anisum* necessary due to the high likelihood of discovering unknown phytoconstituents. The concept of food as an immune modulator needs exploring with well-known crude drugs. We need to use molecular docking to explore the specific phytoconstituents from *Nigella sativa* and *Pimpinella anisum* that contribute to their immunomodulatory effect. We will use an *in silico* approach and a molecular docking tool to predict the pharmacophore of these isolated phytoconstituents. Antioxidants are playing a crucial role in human health. The currently proposed works aim to critically emphasize antioxidant potential through the use of various solvents and bio-guided immunomodulatory techniques. Numerous research articles have conducted antioxidant studies on oil, but a study on extracts using various *in vitro* methods has been sought. The formulation of cocoa granules using *Nigella sativa* and *Pimpinella anisum* will pave the way for the use of food as medicine, which can be easily consumed with water, milk, curd, or in the form of a smoothie. We will determine the extract's in vitro immunomodulatory potential using the THP-1 cell line. We will study the modulation of macrophages and monocytes using a novel approach to immunomodulation: cytokine estimation by ELISA. According to ethno-medicinal claims, both drugs were used for respiratory and inflammatory diseases. Considering the thirst area, we chose the ex vivo model lymphocyte proliferation assay to analyze the immunomodulatory potential of these formulations. We will use gas chromatography to quantify P-anisaldehyde and anethole in the ethanolic extracts of *P. anisum, N. sativa*, and their formulations.

PLAN OF WORK


Selection of Plant (*N. sativa* (seeds) and *P. anisum* (seeds)
Collection/Procurement & Authentication of plant Material
Pharmacognostic study
Preliminary Phytochemical Studies
Cold maceration & hydro distillation
PHARMACOGNOSTIC APPROACH
Cold macerated ethanolic extract
Extraction of volatile oil using Clevenger apparatus
Development of Formulation & Evaluation
In vitro Antioxidant activity
Analytical Method Development
Characterization of extract and volatile oil
Gas Chromatography-Mass Spectroscopy
Molecular Docking Study
Cococa granules
QA/QC Parameter
Immunomodulatory activity using
1. THP 1 cell line *(In vitro)*
2. Lymphocyte Proliferation Assay *(Ex vivo)* using formulation
Gas chromatography using extract
Quantification of constituent in formulation using GC
PHYTOCHEMISTRY & APPLIED MEDICINAL CHEMISTRY APPROACH
FORMULATION & QUALITY ASSURANCE TECHNIOUE APPROACH
PHARMACOLOGY APPROACH
ANALYTICAL CHEMISTRY APPROACH

The proposed topic is divided into 5 phases

Phase-I: Pharmacognostic Approach

Phase-II: Phytochemistry & Applied Medicinal Chemistry Approach

Phase-III: Formulation & Quality Assurance Techniques Approach

Phase-IV: Pharmacological Approach

Phase-V: Analytical Chemistry Approach

Phase-I: Pharmacognostic Approach

- Identifications, procurements and authentications of *Nigella sativa* and *Pimpinella anisum*
- Phytochemical, proximate chemical analysis and physicochemical evaluation of plant material.
- Morphology and powder Microscopy of *Nigella sativa* and *Pimpinella anisum*
- Extractive values determination for selection of appropriate solvent for extraction.
- Extractions of crude drug using ethanol via cold maceration method.
- Extraction of volatile oil from *Nigella sativa* and *Pimpinella anisum* utilizing Hydro-Distillation method.

Phase-II: Phytochemistry & Applied Medicinal Chemistry Approach

After Phytochemical study the extract will further used for

- Chromatographic Characterization of volatile oil and ethanolic extract using GC-MS.
- Molecular Docking study for selected components using five target proteins (PDB ID:1M48,PDB ID: 1P9M, PDB ID: 1PW6, PDB ID: 5UO1, and PDB ID: 2AZ5)
- The retrieve data from molecular docking will help to know the pharmacological actions of phytoconstituents.

Phase-III: Formulation & Quality Assurance Techniques Approach

- Development and Optimization of cocoa granules for black cumin and aniseed
- QA & QC Evaluation of formulations for different parameters.
- Design of expert (DoE) for optimization of formulation

Phase-IV: Pharmacological Approach

- Anti-oxidant potential estimation of Extracts using DPPH Assay, Nitric oxide radical scavenging (NO) assay
- *In-vitro* Immunomodulatory potential of black cumin and aniseed extract were estimated using cell line THP 1 cell line and Cytokine Estimation by ELISA
- *Ex-vivo* immunomodulatory study of formulation perform with the Lymphocyte Proliferation Assay

Phase-V: Analytical Chemistry Approach

- Analytical Method Development was carried out for extracts and quantification of active constituent in cocoa granules using gas chromatography.

CHAPTER - 4

AIM AND OBJECTIVES

4. AIM AND OBJECTIVES

Aim

The research in this project is aimed at Development and Evaluation of Herbal Formulation which exclusively give emphasis to Immunomodulatory activity.

Objectives

1. Microscopy (powder microscopy), Macroscopy, Phytochemical and Physicochemical study of plant material.
2. Extraction, Analytical Characterization of Phytoconstituents present in extracts.
3. Characterization of volatile oil using chromatographic and spectroscopic methods
4. Molecular Docking study of selected components
5. Development and Evaluation of Conventional formulation for Immunomodulatory potential.
6. To study immunomodulatory potential *in-vitro* and *ex-vivo* (Pharmacological screening)
7. Analytical Method Development for Formulations by using Chromatographic method.

CHAPTER - 5

MATERIAL AND METHODS

5. MATERIAL AND METHODS

5.1 Phase-I: Pharmacognostic Approach

Material:

Instruments: Bunsen burner, water bath [Bio techniques], hot air oven [Lab hosp], hot plate [Lab hosp], heating mental [Lab hosp], digital microscope [SAGLO research equipment], muffle furnace [Bio Technics India], clevenger apparatus, etc.

Chemicals: Fehlings A, Fehlings B, Benedict's reagent, Wagner's reagent, Mayer's reagent, Hager's reagent, Dragandorff's reagent, Alpha naphthol solution, Concentrated Sulphuric Acid, Conc. Hydrochloric Acid, Dil. Acetic Acid, Dil. HCL, Dil. Sulphuric Acid, Glacial Acetic Acid, Ruthenium red, Sudan Red III, Alcoholic Picric acid, Potassium chloride solution [20%], Sodium hydroxide solution [2%, 4%, 10%], Tannic acid solution [20%], Ninhydrin solution, Phloroglucinol solution, Ferric chloride solution [5%, 10%], copper sulphate solution [1% 5%], Magnesium turning, chloroform, ethanol, solvent ether, Ammonia, methanol, distilled water, etc.

Method:

5.1.1 Procurement and authentication of plants

The seeds of *Nigella sativa* and *Pimpinella anisum* were collected from the Manikarnika, an Ayurvedic aushadalaya at Pimpari-Chindwad, Pune, India. These seeds are grown in rural areas of Solapur district and fully grown plants were collected and authenticated at the Botanical Survey of India (BSI), Pune.

5.1.2 Phytochemical evaluation of crude drugs

We conducted qualitative chemical tests to estimate primary and secondary metabolites, organic acids, and vitamins, following the test procedures outlined in official books (212-214).

We extracted drugs using a variety of solvents, including hexane, chloroform, ethanol, acetone, and water. We carried out further phytochemical screening of these extracts using procedures prescribed in official books to better understand the phytochemistry of a crude drug (213-214).

5.1.3 Physicochemical evaluation of plants

Several physical drug evaluations, such as foreign organic matter, moisture content, ash values, extractive values, and chemical drug evaluations, were performed according to the procedure described in the official books (212-214).

5.1.4 Morphological and microscopical drug evaluation

We evaluated the collected crude drugs for their organoleptic properties, including shape, size, color, odour, and taste. We conducted powder microscopies of plants for the microscopic drug evaluation (212-214).

5.1.5 Extraction by cold maceration

We cleaned and dried the seeds of *Nigella sativa* and *Pimpinella anisum*. We extracted the crude using a cold maceration process. For heat-sensitive constituents present in crude drugs, maceration is an appropriate method. We placed *Nigella sativa, Pimpinella anisum,* and ethanol in a separate, clear, air-dried container at room temperature for a continuous 7 days, occasionally stirring. The extract was filtered using muslin cloth; furthermore, to eliminate any residual moisture, the extracts were filtered using sodium sulfate and stored in air tight container at room temperature (215).

5.1.6 Extraction of volatile oil

We use the Clevenger apparatus to isolate volatile oils from *Nigella sativa* and *Pimpinella anisum*. The process involved moistening 100 gm of powdered crude drug with 400 ml of distilled water, placing it in a 1000 ml volume flask directly connected to the clevenger apparatus, and heating it for 3-5 hours (215).

5.2 Phase-II: Phytochemistry & Applied Medicinal Chemistry Approach

Material:

Instruments: GC-MS Shimadzu (GCMS-QP Series, Model GCMS-QP2020, with a Sh-Rxi-5Sil MS), Sonicator, etc.

Software: Autodock_vina version 4.2.6 & MGL tool, CASTp, BIOVIA Discovery Studio, Open bable 3.1.1, Pubchem, RCSB PDB, Swiss ADME, lazar Toxicity Predictions (version 1.4.2), Lipinski Rule of Five.

Method:

5.2.1 Gas chromatography of isolated volatile oils

We used chromatography for compound gas separation and analysis, and we performed GC on *Nigella sativa* and *Pimpinella anisum*. The GC specification is listed in the table below.

Table No. 5.1 Gas chromatography specification for volatile oils

Parameter	**Gas Chromatography Specification**
Detector	FID
Dilution Factor	1.0000
Sample Weight	1.0000
Solvent	grade acetone
Column Oven Temp.	40.0 °C
Injection Temp.	120.00 °C
Injection Mode	Split,
Flow Control Mode	Pressure,
Pressure	63.9 kPa.
Total Flow	22.2 mL/min.
Column Flow	1.20 mL/min.
Linear Velocity	39.5 cm/sec.
Purge Flow	3.0 mL/min.
Split Ratio	15.0
Washing Volume	8uL

5.2.2. GC-MS of extracts and volatile oils

We performed gas chromatography and mass spectroscopy of *Nigella sativa* and *Pimpinella anisum* for the separation and quantification of analytes. The table below provided the GC-MS specification. We coordinated the mass spectra using Wiley 9.0 and the National Institute of Standards and Technology libraries (216-221).

Table No. 5.2GC-MS specification for Extracts

Parameter	**GC-MS Specification**
Detector	Detector: MS
Column	TG-5MS silica column
Dimensions	30mm × 0.25mm, 0.25-µm film thickness
Detector	M.S
Solvent	grade acetone
IonSourceTemprature	280.00 ℃
Interface Temprature	280.00 ℃
Solvent Cut Time	3.00 min
Detector Gain Mode	Relative to the Tuning Result
Detector Gain	0.86 kV +0.00 kV
Threshold	0
[M.S Table]	**[MS Table]**
Start Time	3.00 min
End Time	46.00min
ACQ Mode	Scan
Event Time	0.30sec
Scan Speed	2000
Start m/z	40.00

Table No. 5.3GC-MS specification for volatile oils

Parameter	**GC-MS Specification**
Detector Temperature	270 ºC
Detector Range	1
Column Name	TG-5MS (Length – 30m) (Internal Diameter-0.25 µ)
Detector	MS

Column	T.G.-5M.S. silica column
Dimensions	30.mm × 0.25.mm, 0.25-µm film thickness
Solvent	Solvent : grade acetone
Ion-Source.Temprature	270.00 °C
Interface Temprature	270.00 °C
Solvent Cut Time	2.20 min
Detector. Gain Mode	Relative to the Tuning Result
Detector. Gain	0.84 kV +0.00 kV
Threshold	0
[MS Table]	**[MS Table]**
Start. Time	2.20 min
End. Time	60.00 min.
ACQ. Mode	Scan
Event. Time	0.30 sec
Scan. Speed	2500
Start m/z	35.00
End. m/z	700.00

5.2.3 Molecular docking

5.2.3.1 Selection of ligand

We selected a total of 25 phytoconstituents from Nigella sativa and Pimpinella anisum, including ethyl arachidate, ethyl docosanoate, ethyl linoleate, palmitic acid, ethyl sterate, ethyl palmitate, myristic acid, and alpha-Tocospiro A, alpha-Tocospiro B, alpha-Longipinene, Carvacrol, p-Cymene, Di-thymoquinone, Gamma-himachalene, Limonene, Nigellamine-C We downloaded the 2D and 3D structures of these ligands in SDF format from https://pubchem.ncbi.nlm.nih.gov to dock against several proteins. We used the natural active compound curcumin as a standard. We performed ligand preparations in open babel 3.1.1 (http://openbabel.org) (222).

5.2.3.2 Preparation of protein

We downloaded the 3D structures of the proteins from the Protein Data Bank at http://www.rcsb.org. We extracted proteins with PDB IDs 1M48, 1P9M, 1PW6, 5UO1, and 2AZ5, and observed their chains in Table 6.13. BIOVIA Discovery Studio was employed for the preparation of protein (223).

5.2.3.3. Target and Ligand Optimisation

With the use of BIOVIA Discovery Studio and autodock vina (version 4.2.6), a good binding pose was identified (223-224). The Computed Atlas for Surface Topography of Proteins (CASTp tool) found in table no. 6.14 (http://sts.bioe.uic.edu) (225) was used to identify the amino acid-rich active site of a protein. After producing the protein, we selected one ligand and examined its interaction with the receptor. We then modify the binding site and the expansion or SBD site sphere in accordance with the proteins listed in table no. 5.4. After further processing, we removed the ligand group from the protein, added Polar hydrogen atoms and Kollman charges, and kept the file in PDB format.

Table no 5.4 molecular docking configuration file specifications

PDB ID	**Sphere size**	**X co-ordinates**	**Y co-ordinates**	**Z co-ordinates**
1M48	20	1.051558	16.605492	-6.256933
1P9M	80	-57.152375	175.358185	45.212075
1PW6	20	88.691861	22.573681	9.981181
5UO1	20	114.808233	247.800419	358.411837
2AZ5	20	-19.409600	74.650750	33.849550

5.2.3.4 Molecular Docking Analysis

To ascertain how proteins and ligands interact, docking research was conducted. Using the Autodock Vina tool, proteins were transformed from the PDB format to the PDBQT format. The configuration files were ready, and docking was handled using a ligand file and a command prompt. The maximum number of binding modes was set to nine, while the global search's exhaustiveness was set to eight. Biovia Discovery Studio was used to define ligand interaction for the study of the ligand file (223-224).

5.2.4 ADME Profiling

Using Swiss ADME (http://www.swissadme.ch/index.php), 25 active phytoconstituents were evaluated for ADME features from molecular docking data in order to predict the pharmacokinetic parameters of the drug (226-227).

5.2.5 Toxicity prediction

For prediction of toxicity profiling, Lazar toxicity prediction software is used (https://lazar.in-silico.ch/predict). Lazar takes a chemical structure or a SMILE string as input and provides predictions for the carcinogenicity (mouse) and mutagenicity (*Salmonella typhimurium*) of a compound (228).

5.2.6 Lipinski rule of five for drug likeness

Lipinski rule of five predicts the probability of success or failure of any compound from natural or synthetic origin. The drug likeness of active compounds from *Nigella sativa* and *Pimpinella anisum* was analysed using the Lipinski rule of five. The input file of ligand in SDF format was added and submitted; ultimately, results were revealed and observed in table no. 6.25 (229).

5.3Phase-III: Formulation & Quality Assurance Techniques Approach

Material

Instruments: Tab density tester (Electrolab), sieve shaker, dissolution test apparatus (Electrolab), hot air oven [Lab hosp], stopwatch, mortar and pestle, granulating sieve, glasswares, etc.

Chemicals: extracts, cocoa powder, starch, sucrose, lactose, milk solid, barley malt extract, distilled water, etc.

Software: Design-Expert,

Method

5.3.1 Development and Optimisation of Cocoa Granules.

We referred to Formulation Table No. 5.4 for the optimization of the formulation, specifically the cocoa granules of *Nigella sativa* (NSEF) and *Pimpinella anisum* (PAEF). The key ingredients in this formulation were dried extracts of *Nigella sativa* or

Pimpinella anisum, as well as cocoa powder. Granulation aimed to create easily dissolved granules suitable for serving as food items, or nutraceuticals (230-231).

Table no 5.5 Optimization of granules formulation

Ingredients	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**
Sucrose %	7.5	9.5	11.5	7.5	9.5	11.5	7.5	9.5	11.5
Lactose %	2	2	2	2	2	2	2	2	2
Starch %	2	2	2	2	2	2	2	2	2
Cocoa powder %	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Extract %	5	5	5	5	5	5	5	5	5
Starch paste %	3	3	3	5	5	5	7	7	7
cereal malted extract %	44	44	44	44	44	44	42	42	42
milk solid:cocoa powder 4:1 (q.s.)	36	34	32	34	32	30	34	32	30
Total %	100	100	100	100	100	100	100	100	100

The cocoa granules were formulated using the wet granulation method. Mix the finely divided extract with sucrose, lactose, starch, and cocoa powder uniformly. Prepare 3%, 5%, or 7% starch paste in boiling distilled water and stir until it becomes translucent. Starch paste is added drop-wise in mortar to get cohesive mass. The final weight was makeup using cereal malted extract and milk solids. Screened prepared mass through granulating sieve (#22) and collected in tray. Granules were dried at a hot air oven temperature not exceeding 45 °C for 2–5 hours. The formulated cocoa granules were further evaluated and optimized (230-231).

5.3.2 QA & QC evaluation of formulations for different parameters.

The evaluation of prepared granules was carried out by the following parameters:

- Organoleptic evaluation

The colour, odour, and taste of granules were evaluated.

- Moisture content (LOD) (%)

The loss on drying assay was performed till a constant weight of the cocoa granules was fixed. The process was carried out at 100 °C while the weight of the granules was 5 g, and results were calculated.

- Bulk density (g/cm3)

Bulk density was determined using 100-mL glass cylinders per the procedure mentioned in the official books.

- Tapped density

Tapped density testers (Electrolab) with 100-mL glass cylinders were utilised to measure the tapped density of cocoa granules.

- Angle of repose

For determination, 100 ml of cocoa granules were introduced through a dry funnel having a nozzle that possesses 10 mm in diameter, and the angle of repose was determined as per the procedure mentioned in the official books.

- Flow rate (g/s)

The time required to pass 100 mL of cocoa granules through the orifice was measured using a funnel with a nozzle having a diameter of 10 mm and a stopwatch (232).

- Carr's index (%)

The percent compressibility index for cocoa granules was calculated according to pharmacopoeia equations via obtained tapped density and bulk density results.

- Hausner's ratio

Hausner's ratio for cocoa granules was calculated according to pharmacopoeia equations via obtained tapped density and bulk density results (233).

- Disintegration of granules (min)

The disintegration time of granules was determined by the procedure mentioned in US pharmacopoeia.

- Solubility time (min)

A conical flask containing 50.0 mL of distilled water and 1.0 g of cocoa granules was held at 37 °C with a magnetic stirrer set to 100 RPM. When there were no grains visible after the stopwatch-timed disintegration, the test was deemed successful (232).

5.3.3. Statistical analysis and optimization of formulation

Polynomial models were applied in statistical analysis and optimization using Design-Expert software. Several statistical parameters were compared in order to decide which model best suited the data, and an ANOVA was performed to assess whether any factors had a statistically significant impact on the replies. Utilizing response surface plots, the relationship between the two components and response was further clarified (234).

5.4 Phase-IV: Pharmacological Approach

Material

Instruments: microtiter plate, Elisa plate reader, micropipette, ELISA kit

Chemicals: 1, 1-Diphenyl-2, Picryl-Hydrazyl, ascorbic acid, methanol, sodium nitroprusside, phosphate buffer, DMSO, sulfanilic acid reagent, 20% glacial acetic acid, naphthylethylene diamine dichloride, PMA, RPMI-1640, LPS, MTT solution, WST, 10% FBS,

Software: graph pad prism

Method

5.4.1 *In vitro* antioxidant activity

Several models were available for studying the anti-oxidant properties of *Nigella sativa* and *Pimpinella anisum* ethanolic extract in the lab. The DPPH assay and the nitric oxide radical scavenging (NO) assay were chosen for screening.

5.4.1.1 Anti-oxidant activity by the DPPH method

We used the 96-well method to determine the antioxidant potential of *Nigella sativa* and *Pimpinella anisum*. 100µL of NSE and PAE were taken in the microtiter plate, 100µL of 0.1% methanolic. We added DPPH over the NSE and PAE and incubated in the dark for at least 30 minutes. Ascorbic acid was used as a standard; the control, standard, *Nigella sativa* extract (NSE), and *Pimpinella anisum* (PAE) were examined for discoloration. If the colour changes from purple to yellow, it is considered a strong positive response,

while pale pink is considered a weak positive response. Plates were read on Elisa's plate reader at 490 nm (235-236).

We calculated the radical scavenging activity using the formula in the official books.

5.4.1.2 Nitric oxide radical scavenging (NO) assay

200, 400, 600, 800, and 1000 (µg/ml) of extracts were combined with one mL of 10 mM sodium nitroprusside, which had been mixed in 0.5 mL of phosphate buffer saline. (pH 7.4) and incubate the mixture for 150 minutes at 25°C. After incubating the reaction mixture, we added 1.0 mL of pre-prepared Griess reagent. We added 1.0 mL of naphthylethylene diamine dichloride (0.1% w/v) and 1 mL of sulfanilic acid reagent to the reaction mixture. We measure the mixture's absorbance at 546 nm after a 30-minute incubation period at room temperature. The decreased absorbance indicates a high level of nitric oxide scavenging activity, and the calculation of radical inhibition confirms this (237-239).

5.4.2 In-vitro THP 1 cell line study

5.4.2.1 Preparation of test system

A human monocytic cell line commonly known as the THP-1 cell line was obtained from N.C.C.S. (National Centre for Cell Sciences), Pune, India. Once the THP-1 cell 80% confluence is acquired, the cells from the tissue culture flask will be collected and centrifuged. The supernatant will be decanted and the cell pellet will be re-suspended by adding fresh culture medium. Cell counts for the suspension will be performed by the tryphan blue method. The set-I cells will be seeded in 96-well plates at a density of 1 x 104 cells/well in 200µl of complete medium with 50 ng/mL of PMA. Whereas in Set II, 6 well plates, the cell density seeded will be 2 x 105 cells/ml with 50 ng/ml PMA. Plates will be incubated overnight in a CO_2 incubator at 37 °C and 5% CO_2. Cells will be washed with RPMI-1640 serum-free medium prior to each experiment to remove undifferentiated cells (240-244).

5.4.2.2 MTT assay

For analysing the cell viability of THP-1 cells, the MTT assay as described by Mosmann (1983) was used. The study was performed in the presence of various concentrations of

extracts of *Nigella sativa* (NSE) and *Pimpinella anisum* (PAE). The differentiated macrophages in Set I will be treated with varying concentrations (5 – 500 µg/mL) of NSE and PAE in the presence and absence of LPS (50 ng/ml) and then incubated for 24 h in CO_2 incubator at 37 ℃ and 5% CO_2. After incubation, plate 20µl of 5 mg/mL MTT solution will be added and incubated for an additional 4 hours under similar conditions. The crystals are solubilised in 200µl of dimethyl sulfoxide (DMSO.) with agitation. After solubilising the crystals, the optical density was acquired by a microtiterplate reader. (BioTek, Powerwave XS2, USA) at 570 nm. The blue formazon crystal dissolved by DMSO will be measured by the absorbance at 570 nm. Absorbance of water, reagent blanks, and standards will also be measured in the same manner (240-243).

5.4.2.3 Cytokine estimation by ELISA

For evaluation of the immunomodulatory effect of NSE and PAE, three non-cytotoxic concentrations will be determined from the MTT assay. After 24 h incubation with LPS + different concentrations of the test item, the supernatant will be collected, and TNF-α, IL-2, and IL-4 levels in the supernatant will be determined by using commercial enzyme-linked immune sorbent assay (ELISA) kits as per the instructions of the manufacturer (Krishgen) (240-244).

5.4.3 Acute Toxicity

Acute toxicity assay was accomplished by using 6–8-week-old male BALB/c mice according to the OECD guideline 423 (244). Nine mice were sorted into three groups of 3 mice each. Formulation NSEF and Formulation PAEF were orally administered with a single dose of 2,000 mg/kg body weight in each group of mice, while in the case of the control group, normal drinking water was orally administered to establish the comparative. Oral administrations at the rate of 10 ml/kg body weight of Formulation NSEF and Formulation PAEF were given to the BALB/c mice by using 18 Gauzebent oral gavaging needles. The mice were observed after administration, and during the 7-day study period, various parameters were examined.

5.4.4 Lymphocyte Proliferation Assay

IAEC approval was taken for conducting study (approval no. 1197/PO/Re/S/08/CCSEA).

5.4.4.1 Preparation of mouse splenocytes

All animals were divided into 4 groups (n = 6), (group 1: blank), (group 2: anti-CD3 cells), (group 3: formulation NSEF), and (group 4: formulation PAEF). All mice were sacrificed by cervical dislocation and splenocytes were isolated from Balb/c mice under aseptic conditions in Hank's balanced salt solution. For the purpose of splenocyte preparation, spleens were ruptured between glass slides in complete medium (RPMI 1640 (3.5 ml) with 1.0 percent FBS (350 l) and 1 percent antibiotic-antimycotic (42 l). Splenocytes have been separated from debris by centrifugation at 8000 RPM for 10 min (245-247).

5.4.4.2. Determination of splenocyte proliferation

An anti-CD3 monoclonal antibody (mAb; 2 g/ml) was used to activate splenocytes that were seeded in 96-well plates. The cells were treated with herbal formulations of NSEF and PAEF at various concentrations for 48 h at 37 ℃ in 5% CO_2. WST assays were employed to determine the proliferative effects of the herbal formulations on the splenocytes. The absorbance was read at 450 nm with a microplate reader (245-247).

5.4.4.3 WST Assay Procedure

Mix all components and reagents have prepared to room temperature. Phenol red is used as a culture medium.

Blank control wells: 100 µL culture medium + 10 µL WST-1

Anti-CD3 wells: Anti-CD3 monoclonal antibody (mAb; 2µg/ml) + 100 µL culture medium + 10 µL WST (245-247).

Herbal Formulations: 0.5 x 104 cells/well were stimulated with an anti-CD3 monoclonal. Antibody (mAb;2µg/ml) in a 96-well microtiter plate. The cells were treated with Herbal Formulation NSEF & PAEF at various concentrations (50 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml and 1600 µg/ml respectively) in a final volume of 100 µL culture medium. For 24 to 96 hours, incubate the cells. 3 to each well, add 10 L of the WST-1 reagent.

Cells should be incubated for 0.5 to 4 hours under typical culture conditions. Shake the dish on the shaker for a minute and absorbance was measured at 450 nm utilising a microplate reader (245-247).

5.4 Phase-V: Analytical Chemistry Approach

Material

Instruments: Thermo Scientific Trace 1110,

Chemicals: P-anisaldehyde, anethole, methanol, nitrogen all the chemicals were analytical grade.

Method

5.5.1 Analytical Method Development using Gas Chromatography

Gas chromatography was utilized for the method development of pure P-anisaldehyde and anethole. These are used as markers in *Pimpinella anisum* and *Nigella sativa,* respectively (248-251).

Sample preparation:

A standard solution of P-anisaldehyde was prepared in series dilutions of 2, 4, 6, 8, and 10 PPM. While the series dilutions such as 2, 3, 4, 5, and 6PPM were prepared using an anethole.

5.5.2 Validation of the analytical method

GC method has been developed and validated using P-anisaldehyde and anethole for *P. anisum* and *N. sativa*, respectively. The method was developed using GC-MS; accuracy, precision, and repeatability were evaluated; additionally, OLD and LOQ were calculated using the regression method (251).

5.5.3 Quantification of standard in formulation using gas chromatography

For the determination of standard P-anisaldehyde and anethole concentractions in *Pimpinella anisum* and *Nigella sativa* extracts, respectively, gas chromatography was utilized. We carried out the quantification using the area under curve method.

The preparation of the sample (formulation) involved accurately weighing 1gm of smashed granules and dissolving them in 100 ml of methanol while stirring. Use Whatman filter paper to filter the solution, and then use it as the stock solution. From this stock solution, 50 µl was taken and diluted up to 1 ml with methanol.

Preparation of sample (extracts): solutions were prepared by accurately weighing 10 mg of dried extract and dissolved in 5 ml of methanol by means of stirring. Filter the solution

with Whatman filter paper and use it as a stock solution. From this sto.ck solution, 50 µl was diluted up to 1 ml with methanol (248-251).

The table below contains the specification for GC.

Table no 5.6 Gas chromatography specification for method development

Parameter	**Specification**
Injector Inlet Temperature	100
Injector Flow	1.2 ml/min
Carrier Gas	Nitrogen
Injector Mode	Split (Spilt Flow 5ml)
Oven Mode	Ramped
Oven Method	Start: 70 ºC Hold-Time 2 min
	Rate of Heating 5 ºC up to 150 ºC (Hold-Time2 min)
	Rate of Heating 5 ºC up to 200 ºC (Hold-Time 3 min)
	Rate of Heating 5 ºC up to 260 ºC (Hold-Time 5 min)
Total Run Time	50 min
Detector Mode	FID
Detector Temperature	270 ºC
Detector Range	1
Column Name	TG-5MS (Length – 30 m) (Internal Diameter-0.25 µ)

CHAPTER - 6

RESULT AND DISCUSSION

6. RESULTS AND DISCUSSION

6.1 Phase-I: Pharmacognostic Approach

6.1.1 Procurement and authentication of plants

The seeds of *Nigella sativa* and *Pimpinella anisum* were authenticated from the Botanical Survey of India (BSI), has specimens no. BSI WRC den.Cer./ 2022/2705220030778 and BSI/WRC/ADEN.CER./2021/H1 respectively. Their certificates were attached in annexure I and II respectively.

6.1.2 Phytochemical evaluation of crude drugs

The phytochemical screening of crude drugs was carried out using the chemical tests mentioned in official books.

Table no. 6.1 Phytochemical Screening of *Nigella sativa* in different solvents

Phytoconstituents	**NS Hexane**	**NS Chloroform**	**NS Ethanol**	**NS Acetone**	**NS water**
Steroids & terpenes	+	+	+	+	-
Alkaloids	+	+	+	+	-
Glycosides	-	+	+	+	+
Tannins	+	+	+	+	-
Flavonoids	+	+	+	+	+

Note-+ indicated positive and – indicates absent

Table no. 6.2 Phytochemical Screening of *Pimpinella anisum* in different solvents

Phytoconstituents	**PA Hexane**	**PA Chloroform**	**PA Ethanol**	**PA Acetone**	**PA water**
Steroids & terpenes	+	+	+	-	+
Alkaloids	-	+	+	+	+
Glycosides	-	+	+	+	+
Tannins	-	-	+	+	+
Flavonoids	-	+	+	+	+

Note-+ indicated positive and – indicates absent

From the data received via Phytochemical screening methanol consists of most of the primary and secondary metabolites hence for cold maceration ethanolic were used as a solvent.

6.1.3 Physical evaluation of plants

The physicochemical evaluations of crude drugs were carried out as per the ayurvedic pharmacopoeia and results are within the limit

Table no 6.3 Physical evaluation of *Nigella sativa*

Parameters	**Mean %**	**Standard deviation**	**Range %**
Foreign matter	0.55	0.01	0.54 -0.56
Total ash value	4.734	0.09	4.64 – 4.82
Acid in-soluble ash value	0.325	0.003	0.322- 0.328
Water soluble ash value	3.413	0.03	3.383 – 3.443
Loss on drying	3.104	0.02	3.84 – 3.124

Table no 6.4 Physical evaluation of *Pimpinella anisum*

Parameters	**Mean**	**Standard deviation**	**Range**
Foreign matter	0.537	0.007	0.530 – 0.544
Total ash value	6.037	0.04	5.997 – 6.077
Acid in-soluble ash value	0.514	0.004	0.510 – 0.518
Water soluble ash value	2.272	0.01	2.262 – 2.282
Loss on drying	5.433	0.01	5.423 – 5.443

All the parameters are testing as per the ayurvedic pharmacopoeia of India and results are within the limit.

Table no 6.5 Extractive value determination of *Nigella sativa*

Parameters	**Mean %**	**Standard deviation**	**Range%**
Alcohol soluble extractive value	24.92	0.02	24.90 -24.94
Water soluble extractive value	15.86	0.05	15.91 – 15.81

Table no 6.6 Extractive value determination of *Pimpinella anisum*

Parameters	Mean %	Standard deviation	Range%
Alcohol soluble extractive value	16.06	0.1	16.16 – 15.96
Water soluble extractive value	32.15	0.3	31.85– 32.45

6.1.4 Morphological and Microscopical drug evaluation

6.1.4.1 Morphological evaluation

Table no 6.7 Seed Morphology of *Nigella sativa* and *Pimpinella anisum*

Charateristics	*Nigella sativa*	*Pimpinella anisum*
Shape	Flattened, oblong, small	Ovoid
Size	2 - 3 mm long and 1-2 mm wide	0.3 -0.5 cm long and 0.1 –0.2 cm wide
Colour	Black	Greenish yellow or greenish-brown
Odour	Slightly aromatic	Characteristic
Taste	Bitter	Sweet and aromatic
Image		

6.1.4.2 Microscopic drug evaluation

Powder microscopy of *Nigella sativa*

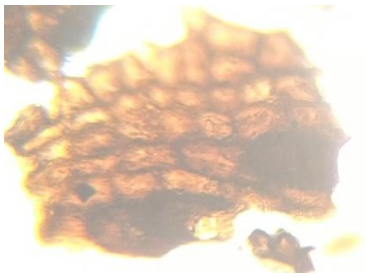


Fig. 6.1 Papillose cells

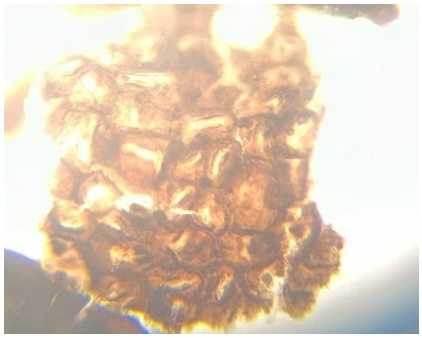


Fig. 6.2 Endosperm

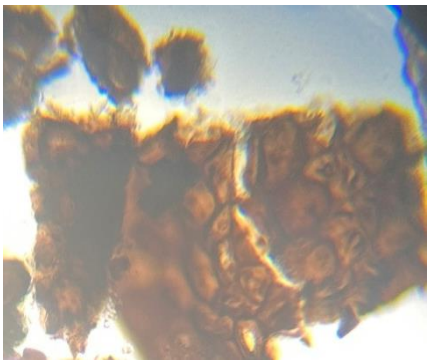


Fig. 6.3 Papillae with brown pigment

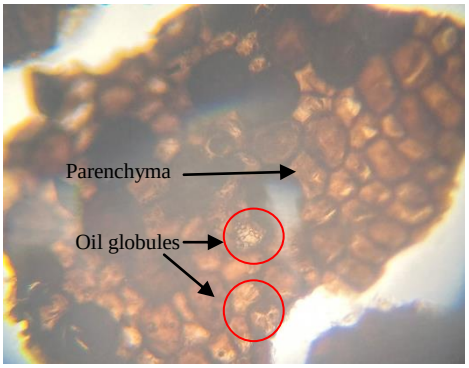


Fig. 6.4 Parenchymatous cell with oil globules

Powder microscopy of *Pimpinella anisum*

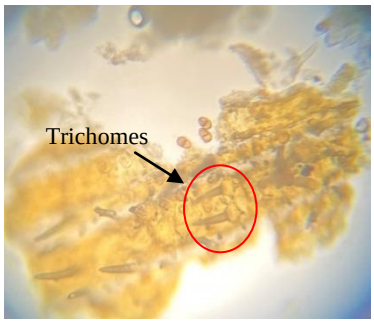


Fig. 6.5 Epidermis with covering trichomes

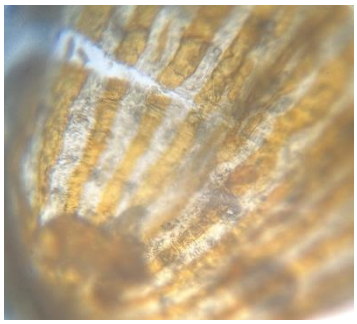


Fig. 6.6 Epidermal layer

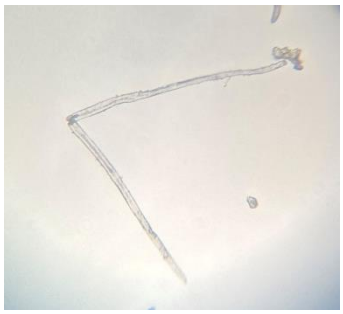


Fig. 6.7 Fibers

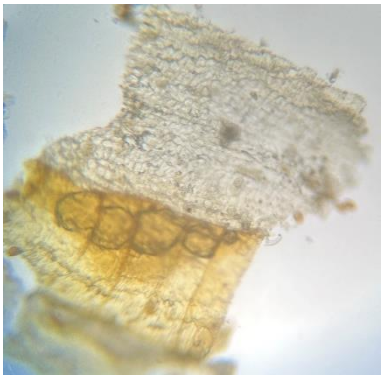


Fig. 6.8 Parenchyma cell with ridges

Fig. 6.9 Fibro-vascular tissue

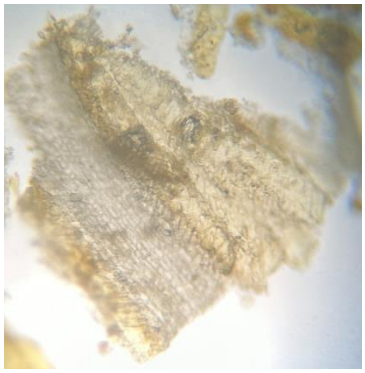


Fig. 6.10 Mesocarp

6.1.4 Extraction by cold maceration

18.3 g of *Nigella sativa* and 14 g *Pimpinella anisum* extracts were obtained from 100 g of powder through cold maceration.

6.1.6 Isolation of volatile oil

Clevenger apparatus is used for isolation of volatile oils from *Nigella sativa* and *Pimpinella anisum* 2.1% and 2.3% of volatile oil obtained respectively. As per literature review most of the active constituents are present in oil and possess remarkable pharmacological actions.

6.2 Phase-II: Phytochemistry & Applied Medicinal Chemistry Approach

6.2.1 Gas chromatography of isolated volatile oils

In the operation of gas chromatography, compounds are separated via conversion into gases. Most of the plant pigments and sugars are non-volatile, so they degrade at high temperatures instead of converting into gases. On the contrary, essential oils convert into gas and are easily separated on a chromatogram, making it a rational choice to study essential oils. The chromatogram given below in Fig. 6.11 indicates the active compound present in the isolated volatile oil of *N. sativa* while in Fig.6.12 indicates compound present in isolated volatile oil of *Pimpinella anisum.*

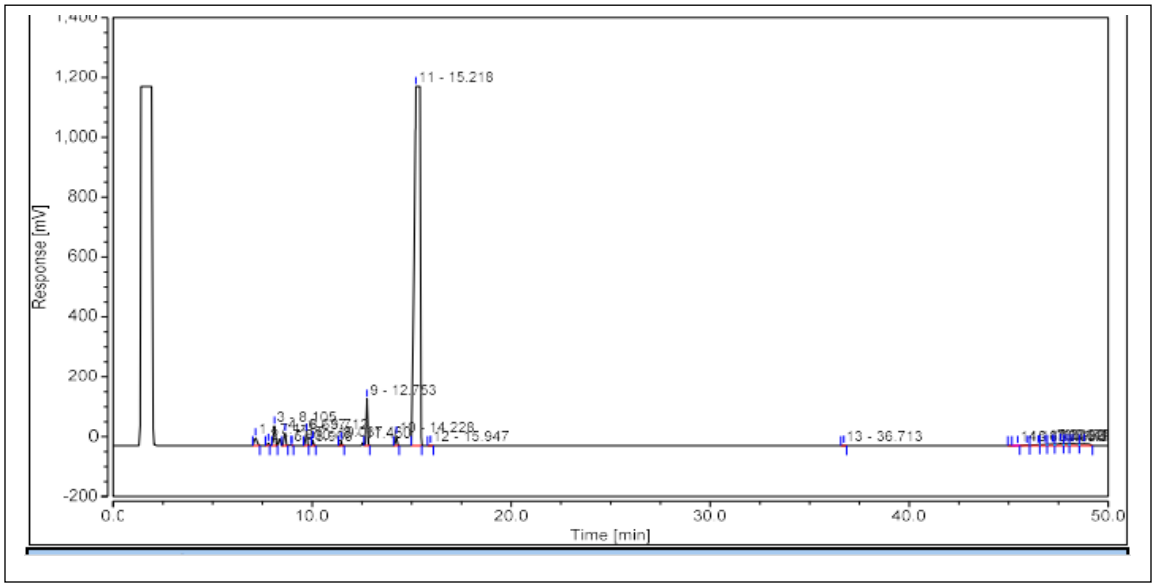


Fig. 6.11 Gas chromatogram for *Nigella sativa* volatile oil

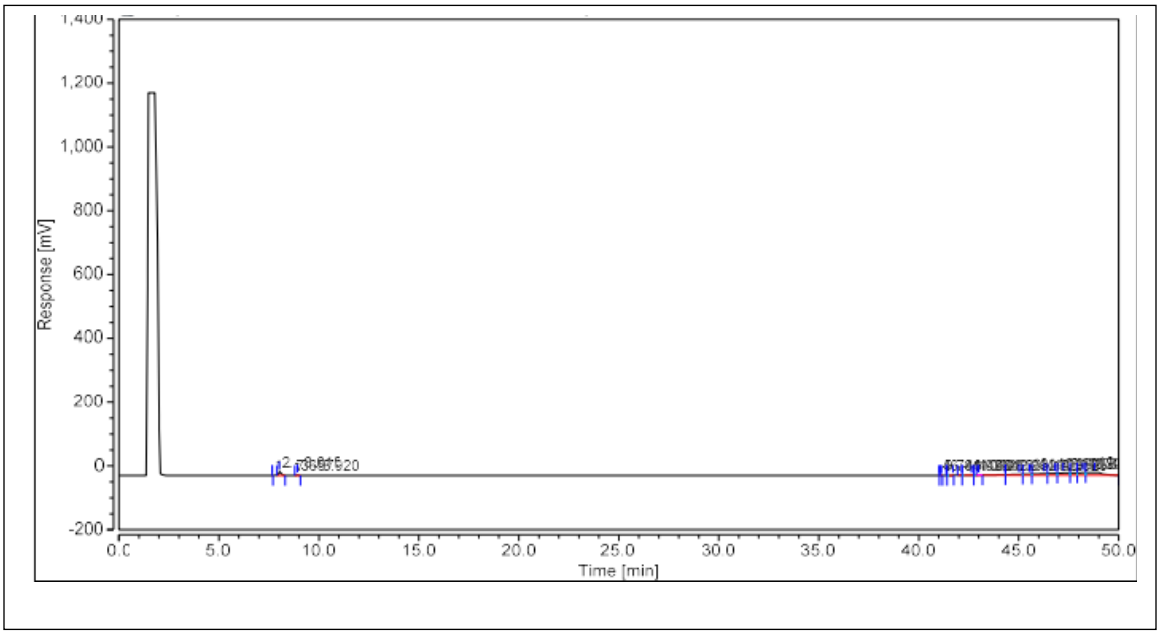


Fig. 6.12 Gas chromatogram for *Pimpinella anisum* volatile oil

6.2.2. GC-MS of extracts and volatile oils

6.2.2.1 GC-MS of extracts

GC–MS analysis was carried out for the ethanolic extracts of *N. sativa* and *P. anisum.* Forty constituents were recognized from *N. sativa*, while 130 compounds were observed in *P. anisum* extract. Amongst which mostly fatty acids, esters, flavonoids, and terpenoids are present in higher concentrations n-Hexadecanoic acid, tetradecanoic acid were the most common components in both extracts. In previous qualitative research on *N. sativa* seeds, sterols, triterpenes, tannins, flavornoids, and saponins were found. [13]. Cis-9-Octadecenoic acid, propyl ester was present in the highest concentration with a 27.98 A/H ratio (area/height) shows highest peak in GC-MS graph. Followed by n-Hexadecanoic acid with a 14.02 A/H ratio, beta -Monolinolein with A/H ratio was 8.91, Oxacyclononadec-10-en-2-one with a 7.80 A/H 7.80, Octadecanoic acid, and ethyl ester with a 4.84 A/H 4.84. Hydro-thymoquinone, methyl chavicol, 3-carene, and thymoquinone were present in the least contraction. The detailed GC-MS spectra of *N. sativa* was observed in figure no 6.13, while the detail concentration of the compound present in black cumin were indicated in table no. 6.8

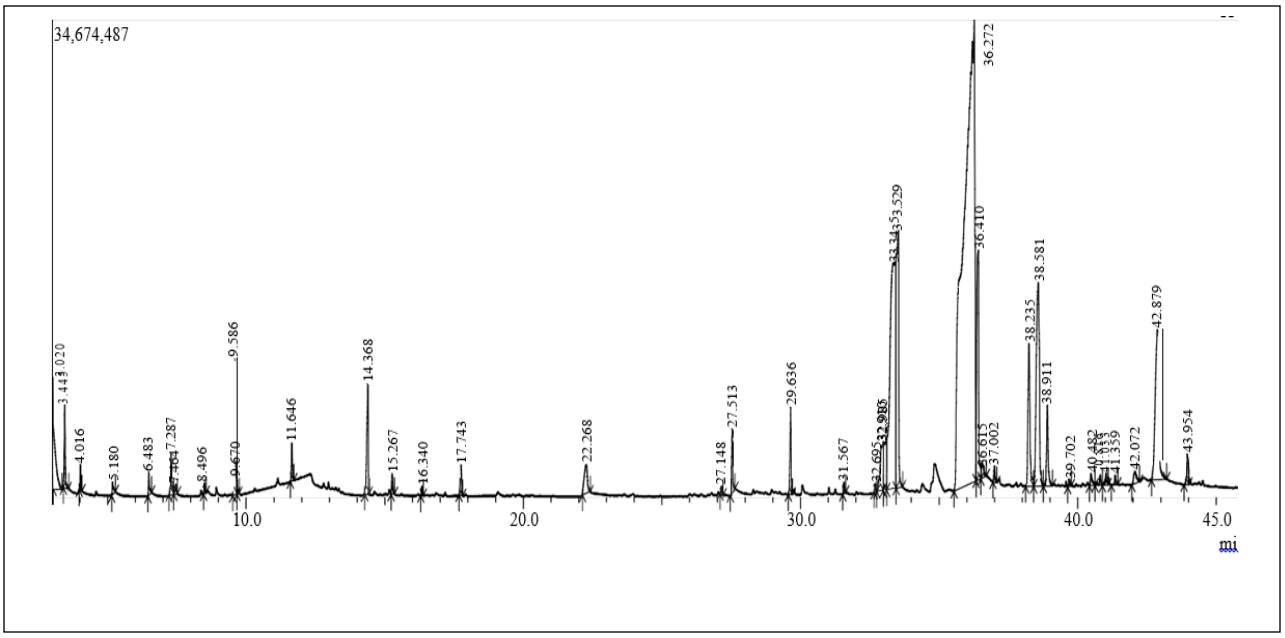


Fig. 6.13 GC-MS chromatogram for *Nigella sativa* extract

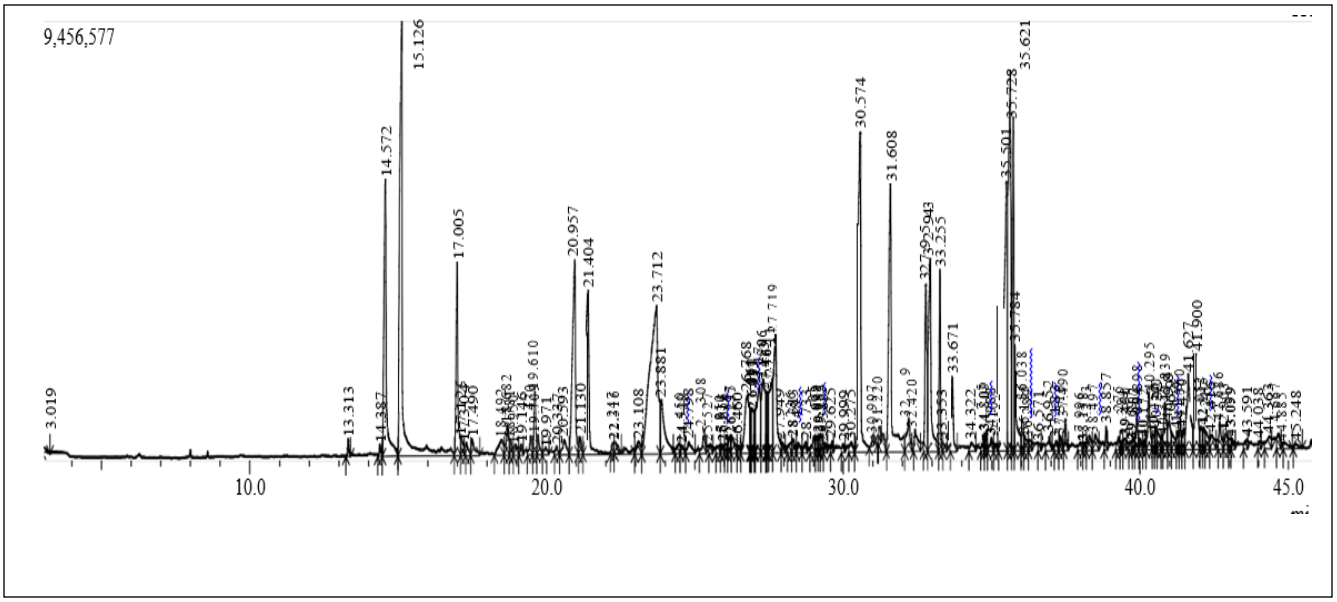


Fig. 6.14 GC-MS chromatogram for *Pimpinella anisum* extract

Table no 6.8 Compounds present in GC–MS Investigation of *Nigella sativa* extract

Name of compound	Mol. wt.	Mol. Formula	Peak area %	R.T. (min)
Alpha.-Longipinene	204	C15H24	0.08	16.340
Beta.-Pinene	136	C10H16	0.09	8.496
Thujene	136	C10H16	0.52	7.287
Oxalic acid	368	C22H40O4	0.09	27.148
Dimethyl myristamine	241	C16H35N	0.86	27.513
Tetradecanoic acid	256	C16H32O2	1.02	29.636
Pentadecanoic acid	270	C17H34O2	0.11	31.567
n-Hexadecanoic acid	256	C16H32O2	11.68	33.345
Octadecanoic acid, ethyl ester	312	C20H40O2	4.11	36.410
Linoleic acid, ethyl ester	308	C20H36O2	0.35 0.19	36.615 37.002
Eicosanoic acid, ethyl ester	340	C22H44O2	1.17	38.911
Docosanoic acid, ethyl ester	368	C24H48O2	0.10	41.359
Oxacyclononadec-10-en-2-one	280	C18H32O2	5.86	38.581
Podocarp-8(14)-ene	272	C20H32	0.32	32.695
Thymol	150	C10H14O	0.21	15.267
Erucylamide	337	C22H43NO	0.44	43.954
Beta.-Monolinolein	354	C21H38O4	4.95	42.879
o-Cymene	134	C10H14	1.81	9.586
D-Limonene	136	C10H16	0.10	9.670
Car-3-ene-2,5-dione	164	C10H12O2	1.63	14.368
Acetaldehyde	118	C6H14O2	0.62	3.445
Longifolene	204	C15H24	0.35	17.743
Thymoquinone	164	C10H12O2	-	R index 1340

Alpha-Pinene	136	C10H16	0.13	7.464
4-Terpinenyl acetate	196	C12H20O2	0.05	19.225
Carvacrol	150	C10H14O	0.23	23.591
Alpha.-Longipinene	204	C15H24	0.07	1403
Estragole/ methyl Chavicol	148	C10H12O	0.02	1172

In case of *Pimpinella anisum* anethole was present in the highest concentration with a 7.57% area and 6.91% height shows7.09 A/H ratio (area/height) shows highest peak in GC-MS graph, followed by 1-(4-Methoxyphenyl) propane-1, 2-diol with a 19.44 A/H ratio, anisic acid with A/H ratio was11.69. eicosanoic acid, octadecanoic acid, with an 8.93 and 4.49A/H ratio respectively. Squalene, alpha-tocospiro A, and alpha-tocospiro B with A/H ratio 10.48, 11.91 and 10.53 respectively. 9-cyclohexylheptadecane, beta-bisabolene, trans-sesquisabinene hydrate, and 2-hexenoic acid, 3, 4, 4-trimethyl-5-oxo-(Z) were present in the least contraction.

The detailed GC-MS spectra of *P. anisum* was observed in figure no 6.14, while the detail concentration of the compound present in aniseed were indicated in table no. 6.9

Table no 6.9 Compounds present in GC–MS Investigation of *Pimpinella anisum* extract

Name of compound	**Mol. wt.**	**Mol. Formula**	**Peak area %**	**R.T. (min)**
Methyl chavichol (Estragole)	148	C10H12O	0.14	13.313
Anethole	148	C10H12O	7.57	15.126
gamma.-Elemene	204	C15H24	0.27	17.129
:5-Hydroxy-6-methoxy-8-[(4-amino-1-methylbutyl)amino]quinolinetrihydrobromide	204	C15H24	0.68	19.610
2-Allyl-1,4-dimethoxybenzene	204	C15H24	0.28	19.709
1-Buten-4-ol, 3-methyl-4-(4methoxyphenyl)	192	C12H16O2	0.16	19.971
Isolongifolene	202	C15H22	0.16	19.146

Squalene	410	C30H50	0.33	44.363
P-Anisoin	272	C16H16O4	1.53	41.900
Cis anethole	148	C10H12O	0.12	14.387
P-Anisaldehyde	136	C8H8O2	3.96	14.572
P-Acetonylanisole	164	C10H12O2	2.11	17.005
P-Anisic acid	152	C8H8O3	0.45	18.492
Benzeneacetic acid	180	C9H8O4	0.23	18.950
Alpha.-Curcumene	202	C15H22	0.25	19.470
Beta.-Bisabolene	204	C15H24	0.12	20.337
Para-Anisaldehyde diethyl acetal	210	C12H18O3	3.36	21.404
R-Turmerol	218	C15H22O	0.31	23.108
Beta.-Asarone	208	C12H16O3	0.15	24.549
Beta.-Himachalene oxide	220	C15H24O	0.30	24.798
Isospathulenol	220	C15H24O	0.37	25.308
Tetradecanoic acid	228	C14H28O2	0.23	29.005
Ethyl anisate	180	C10H12O3	0.19	29.098
Flurenol butyl ester	282	C18H18O3	1.75	32.795
10(E),12(Z)-Conjugated linoleic acid	280	C18H32O2	5.80	35.501
n-Hexadecanoic acid	256	C16H32O2	3.21	32.943
Palmitic acid, ethyl ester	284	C18H36O2	1.27	33.255
Octadecanoic acid, ethyl ester	312	C20H40O2	0.41	6.038
			0.14	40.172
			0.11	44.038
Butanoic acid, 2-methyl-, 4-methoxy-2-(3-methyloxiranyl)phenyl ester	264	C15H20O4	0.13	30.997
			4.56	31.608
			0.48	32.420
Cis,cis-Linoleic acid	280	C18H32O2	0.15	34.705
Linoleic acid, phenylmethyl ester	370	C25H38O2	0.15	43.591

E,E,Z-1,3,12-Nonadecatriene-5,14-diol	294	C19H34O2	5.95	35.621
Methyl petroselinate	296	C19H36O2	0.15	34.809
Octadecanoic acid (steric acid)	284	C18H36O2	1.17	35.784
Eicosanoic acid	312	C20H40O2	0.20	34.322
Heneicosane	296	C21H44	0.26	36.115
Ethyl arachidate	340	C22H44O2	0.22	38.857
9-Cyclohexylheptadecane	322	C23H46	0.12	38.138
Ethyl docosanoate	368	C24H48O2	0.32	41.069
Alpha.-Tocospiro A	462	C29H50O4	0.16	44.885
Alpha.-Tocospiro B	462	C29H50O4	0.12	45.248
Phytol	296	C20H40O	0.23	34.988
Ascorbic acid 2,6-dihexadecanoate	652	C38H68O8	0.35	38.497
2-Hexenoic acid, 3,4,4-trimethyl-5-oxo-, (Z)	170	C9H14O3	0.11	29.999
Cetyl bromide	304	C16H33Br	0.39	42.686
1,2-Propanediol, 1-(p-methoxyphenyl)	182	C10H14O3	7.10	23.712
	182	C10H14O3	1.14	23.881
	182	C10H14O3	1.57	27.306
P- anisic acid ethyl ester	180	C10H12O3	0.19	29.098
1-Hydroxy-1-(4-methoxyphenyl)propan-2-one $$ 2-Propanone, 1-hydroxy-1-(4-methoxyphenyl)	180	C10H12O3	1.53 0.20 0.16 0.28 0.16	26.768 26.860 26.891 27.017 27.949
Pseudoisoeugenol 2-methylbutanoate	248	C15H20O3	0.18	28.733
	248	C15H20O3	6.19	30.574
Ethyl Oleate	310	C20H38O2	2.77	35.728
Heneicosane	296	C21H44	0.26	36.115

6.2.2.2 GC-MS of isolated volatile oil

Gas chromatography mass spectroscopy of isolated volatile oil was performed. 23 compounds were identified in *N. sativa* volatile oil while 34 compounds were observed in *P. anisum* volatile oil. Amongst them o-Cymene present in predominant amount though gamma.-Terpinene, 4-Terpinenyl acetate and cis-4-methoxy thujane were observes in least concentration in *Nigella sativa*. In the GC MS of *P. anisum* volatile oil beta-Terpineol, anethole were serving as chief constituent while Citronellol present in minimum concentration. The GC-MS spectra of volatile oils were given in fig no. 6.15, and 6.16 respectively.

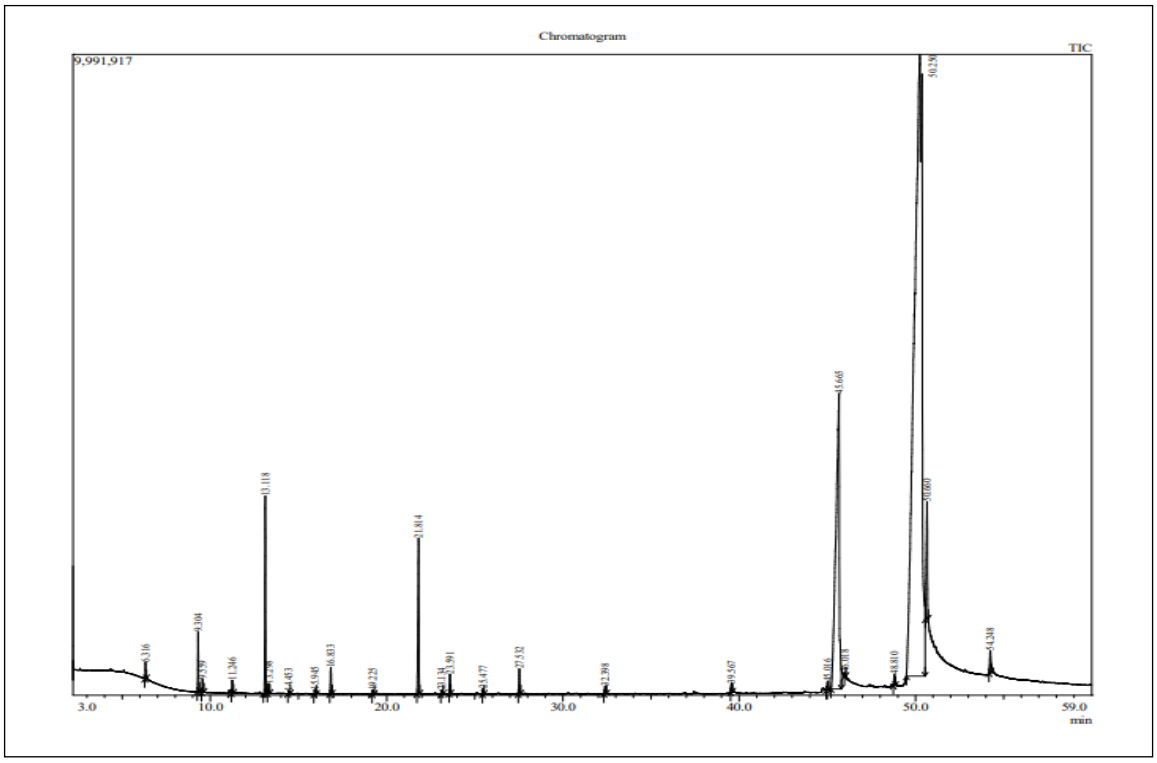


Fig. 6.15 GC-MS chromatogram for volatile oil of *Nigella sativa*

Table no. 6.10 Compounds in GC–MS Investigation of *Nigella sativa* volatile oil

Name of compound	Mol. wt.	Mol. Formula	Peak area %	R. T. (min)
o-Cymene	134	C10H14	2.23	13.118
D-Limonene	136	C10H16	0.11	13.298

Gamma.-Terpinene	136.23	C10H16	0.03	14.453
Anethole	148	C10H12O	0.05	23.134
Longifolene	204	C15H24	0.32	27.532
Thymoquinone	164	C10H12O2		1340
Alpha-Thujene	136.23	C10H16	0.57	9.304
Alpha-Pinene	136	C10H16	0.14	9.490
Cis-4-methoxy thujane	168	C11H20O	0.06	15.945
Linalool methyl ether	168	C11H20O	0.31	16.833
4-Terpinenyl acetate	196	C12H20O2	0.05	19.225
3-Carene	136	C10H16	2.17	21.814
Carvacrol	150	C10H14O	0.23	23.591
Alpha.-Longipinene	204	C15H24	0.07	25.477
Hydro thymoquinone	166	C10H14O2	0.12	32.398
Pinene	136	C10H16	0.15	11.246
Tetradecyl butyrate	284	C18H36O2	0.05	46.018

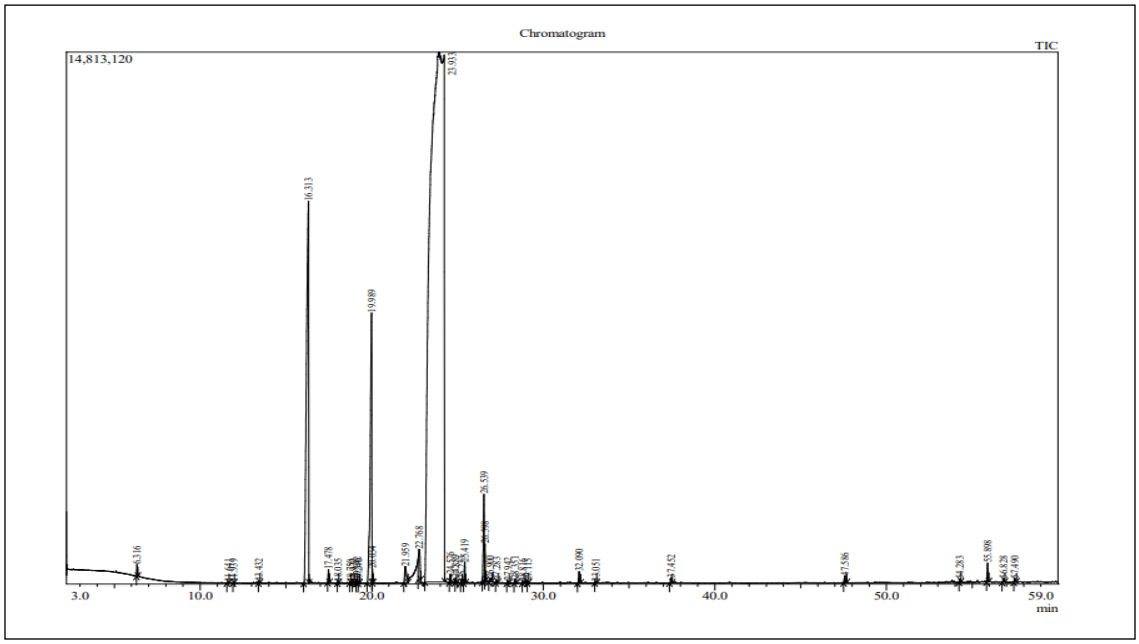


Fig. 6.16 GC-MS chromatogram for volatile oil of *Pimpinella anisum*

Table no. 6.11 Compounds in GC–MS Investigation of *Pimpinella anisum* volatile oil

Name of compound	Mol. wt.	Mol. Formula	Peak area %	R. T. (min)
Beta.-Terpineol	154	C10H18O	0.05	20.034
			9.42	16.313
Ethyllinalool	168	C11H20O	0.01	18.035
Citronellol	156	C10H20O	0.03	19.142
Anethole	148	C10H12O	4.66	19.989
Gamma-Terpineol	154	C10H18O	0.05	20.034
4-Methoxybenzoyl isothiocyanate	193	C9H7NO2S	0.58	22.768
6-Allyl-2-cresol	148	C10H12O	82.33	23.933
1,3,5-triethylbenzene	162	C12H18	0.06	24.576
Anisole	178	C12H18O	0.97	26.539
P-Anisaldehyde	152	C8H8O3	0.08	26.900
Trans- alpha-Bergamotene	204	C15H24	0.04	28.351
Methyl chavichol (Estragole)	148	C10H12O	0.05	19.999

6.2.3 Molecular docking

6.2.3.1 Selection of ligand

Pubchem was used to extrude a total of 25 phytoconstituents from *Nigella sativa* and *Pimpinella anisum*. Table No. 6.12 listed selected compounds for molecular docking and their details

Table no. 6.12 List of selected compounds for molecular docking and their details

Name	PubChem CID	Molecular formula	Molecular wt (g/mol)	Canonical smiles
Carvacrol	10364	$C_{10}H_{14}O$	150.22	CC1=C(C=C(C=C1)C(C)

				C)O
P- Cymene	7463	$C_{10}H_{14}$	134.22	CC1=CC=C(C=C1)C(C)C
Dithymoquinone	398941	$C_{20}H_{24}O_4$	328.4	CC(C)C1=CC(=O)C2(C(C1=O)C3(C2C(=O)C(=CC3=O)C(C)C)C)C
Limonene	22311	$C_{10}H_{16}$	136.23	CC1=CCC(CC1)C(=C)C
Nigellamine C	101341399	$C_{32}H_{38}N_2O_5$	530.7	CC1=CC(C2(CCC(=C(C)C)C2C(CC3(C(O3)CC1)C)OC(=O)C4=CN=CC=C4)C)OC(=O)C5=CN=CC=C5
Nigellicine	11402337	$C_{13}H_{14}N_2O_3$	246.26	CC1=CC(=O)C2=C(N3CCCCN3C2=C1)C(=O)O
Nigellidine	136828302	$C_{18}H_{18}N_2O_2$	294.3	CC1=CC(=O)C2=C(N3CCCCN3C2=C1)C4=CC=C(C=C4)O
Alpha pinene	6654	$C_{10}H_{16}$	136.23	CC1=CCC2CC1C2(C)C
Thymoquinone	10281	$C_{10}H_{12}O_2$	164.20	CC1=CC(=O)C(=CC1=O)C(C)C
P- Anisaldehyde	31244	$C_8H_8O_2$	136.15	COC1=CC=C(C=C1)C=O
Cis-anethole	1549040	$C_{10}H_{12}O$	148.20	CC=CC1=CC=C(C=C1)OC
Gamma himachalene	577062	$C_{15}H_{24}$	204.35	CC1=CC2C(CC1)C(=CCCC2(C)C)C
Linolool	6549	$C_{10}H_{18}O$	154.25	CC(=CCCC(C)(C=C)O)C
Estragole	8815	$C_{10}H_{12}O$	148.20	COC1=CC=C(C=C1)CC=C
Trans-anethole	637563	$C_{10}H_{12}O$	148.20	CC=CC1=CC=C(C=C1)O

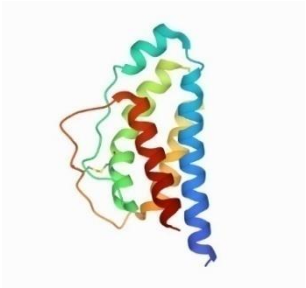
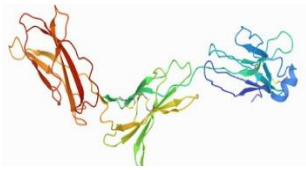
				C
Eicosanoic acid, ethyl ester (Ethyl arachidate)	29009	$C_{22}H_{44}O_2$	340	CCCCCCCCCCCCCCCCCCCC(=O)OCC
Ethyl docosanoate	22199	$C_{24}H_{48}O_2$	368	CCCCCCCCCCCCCCCCCCCCCC(=O)OCC
Linoleic acid, ethyl ester (ethyl linoleate)	5282184	$C_{20}H_{36}O_2$	308.5	CCCCCC=CCC=CCCCCCCCC(=O)OCC
n-Hexadecanoic acid (palmitic acid)	985	$C_{16}H_{32}O_2$	256.5	CCCCCCCCCCCCCCCC(=O)O
Octadecanoic acid, ethyl ester (ethyl sterate)	8122	$C_{20}H_{40}O_2$	312.5	CCCCCCCCCCCCCCCCCCCC(=O)OCC
Palmitic acid, ethyl ester (ethyl palmitate)	12366	$C_{18}H_{36}O_2$	284.5	CCCCCCCCCCCCCCCCC(=O)OCC
Tetradecanoic acid (myristic acid)	11005	$C_{14}H_{28}O_2$	228.37	CCCCCCCCCCCCCC(=O)O
Alpha.-Tocospiro A	21674156	$C_{29}H_{50}O_4$	462.7	CC1=C(C(C2(C1=O)CCC(O2)(C)CCCC(C)CCCC(C)CCCC(C)C)(C(=O)C)O)C
Alpha.-Tocospiro B	21674157	$C_{29}H_{50}O_4$	462.7	CC1=C(C(C2(C1=O)CCC(O2)(C)CCCC(C)CCCC(C)CCCC(C)C)(C(=O)C)O)C

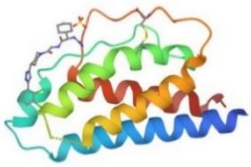
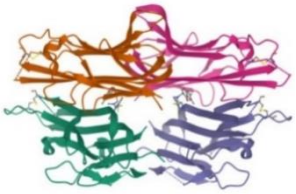
Alpha.-Longipinene	12311396	$C_{15}H_{24}$	204.35	CC1=CCC2C3C1C2(CCCC3(C)C)C
Curcumin	969516	$C_{21}H_{20}O_6$	368.40	COC1=C(C=CC(=C1)C=CC(=O)CC(=O)C=CC2=CC(=C(C=C2)O)OC)O

6.2.3.2 Preparation of protein

The selected proteins, its name and specifications were mentioned in table no. 6.13 obtained from Protein data bank.

Table no. 6.13 Selected protein for Immunomodulation screening and its details

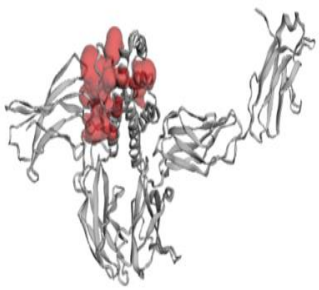
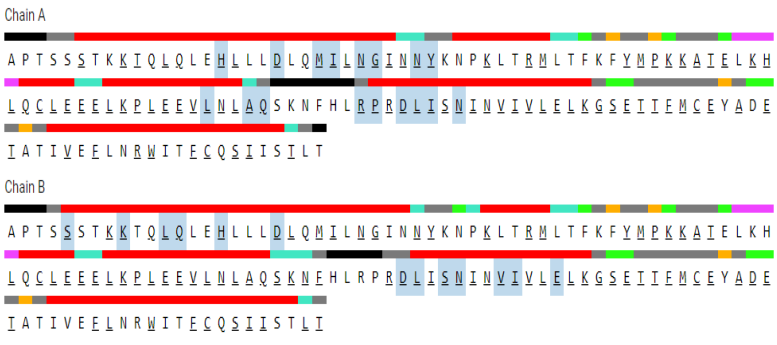
PDB ID	**Name of protein**	**Binding pocket**	**Area (SA) Å2**	**Volume (SA) Å3**
1M48	Crystal Structureof Human IL-2 Complexed with (R)-N-[2-[1-(Amino-iminomethyl)-3-piperidinyl]-1-oxoethyl]-4-(phenylethynyl)-L-phenylalanine methylester.		4126.492	4577.551
1P9M	Crystal structure of the hexameric human IL-6/IL-6, alpha- receptor/gp130 complex.		1562.214	917.876

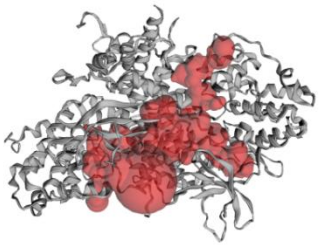
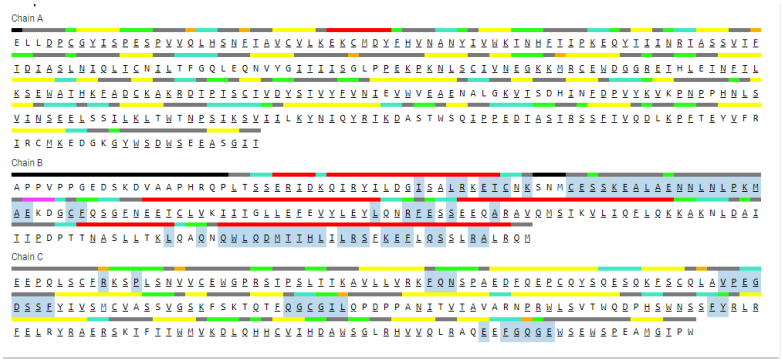
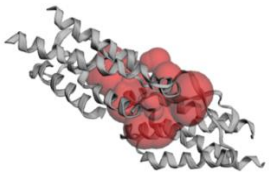
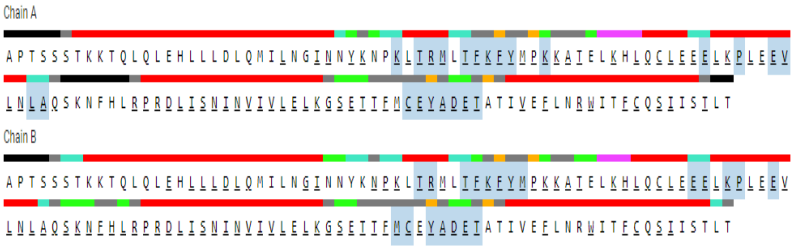
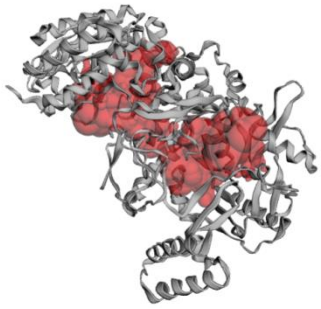
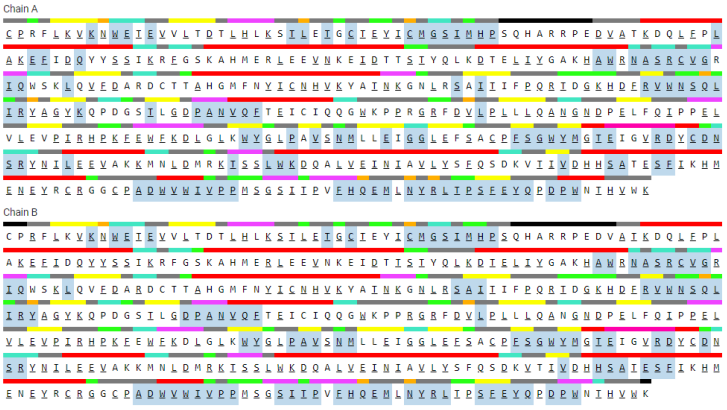
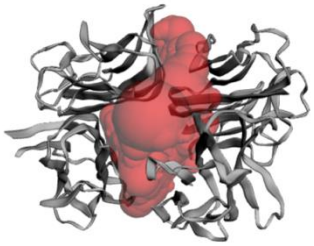
1PW6	Low Micro-molar Small MoleculeInhibitor of IL-2.		1088.862	1712.182
5UO1	Structure of human neuronal nitric oxide synthase heme domainin complex with 3-[(2-amino-quinolin-7-yl)methoxy]-5-((methyl-amino)methyl)benzonitrile.		3859.237	3296.295
2AZ5	Crystal Structure of TNF-α with a small molecule inhibitor.		2743.256	4514.068

6.2.3.3. Target and Ligand Optimization

The binding pocket sites of selected proteins and sequence were observed in table no. 6.14.

Table no. 6.14 binding pockets of protein and its sequence

1M48 	

1P9M 	
1PW6 	
5UO1 	
2AZ5 	

6.2.3.4 Molecular Docking Analysis

Table no. 6.15 Interactions of selected ligand with protein 1M48

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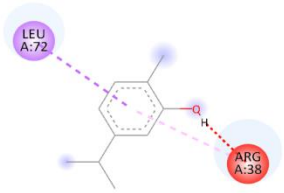
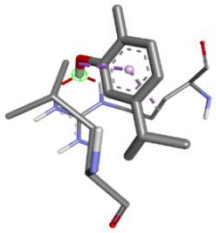
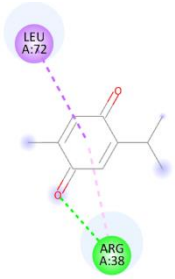
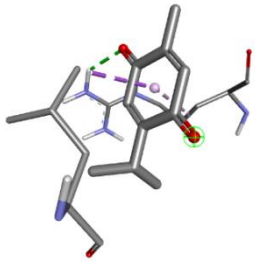
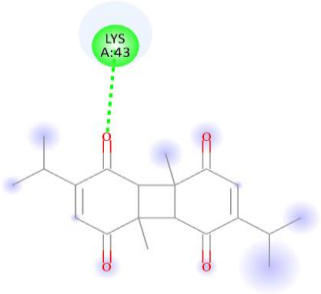
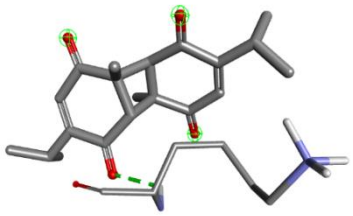
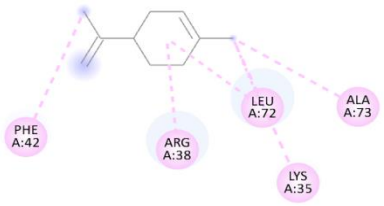
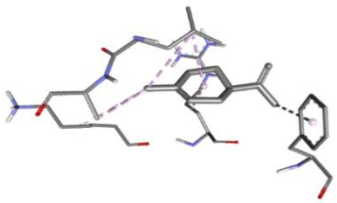
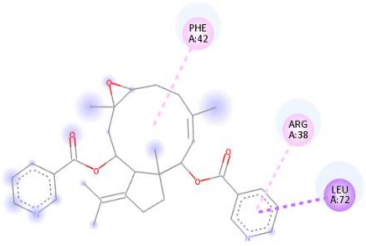
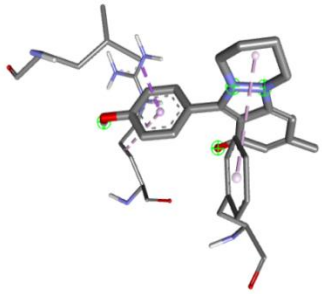
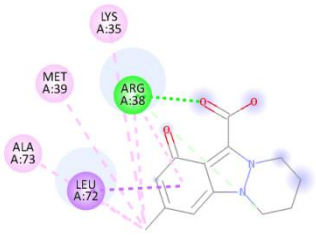
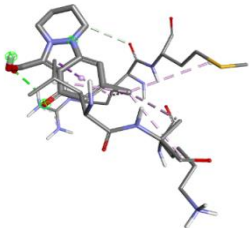
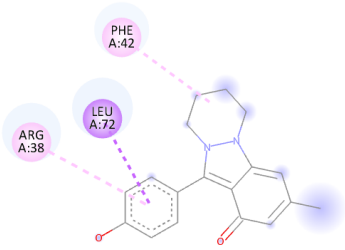
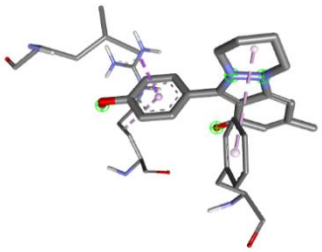
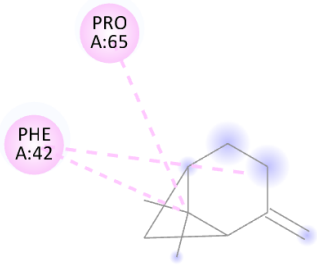
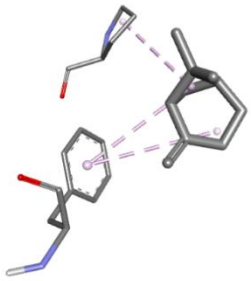
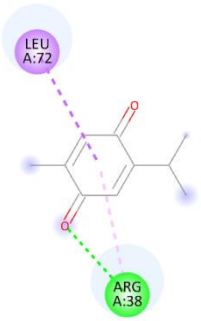
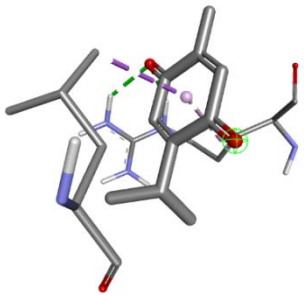
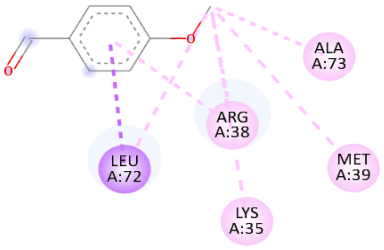
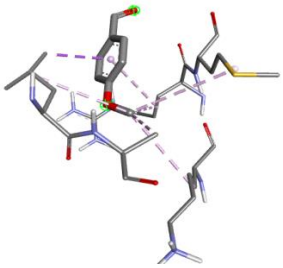
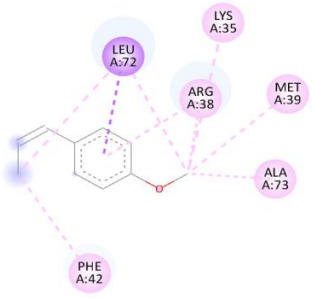
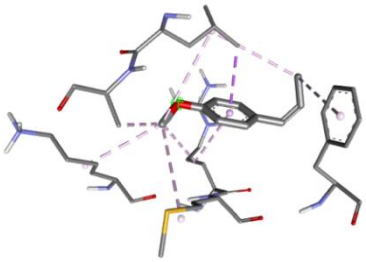
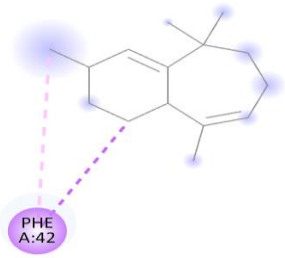
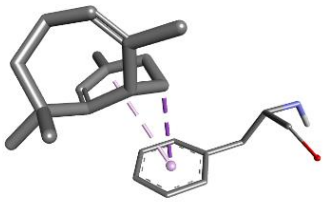
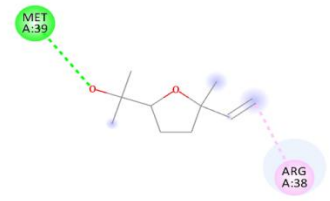
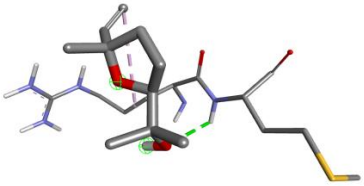
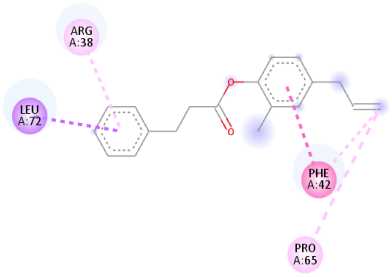
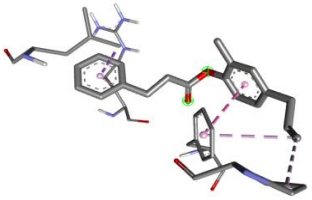
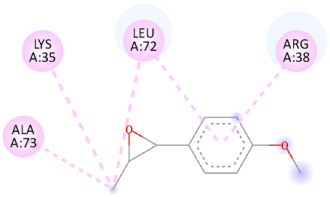
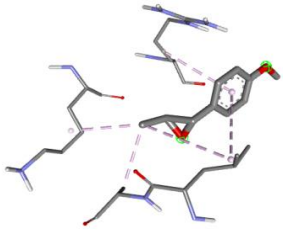
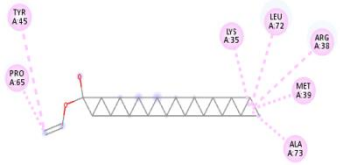
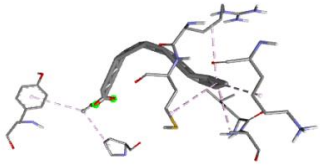
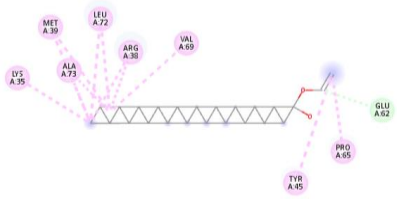
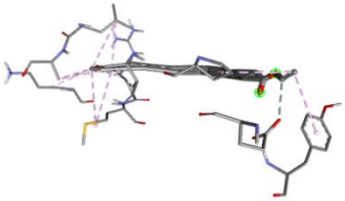
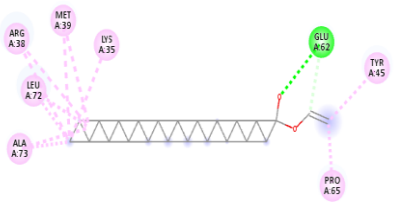
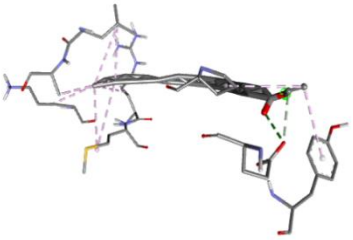
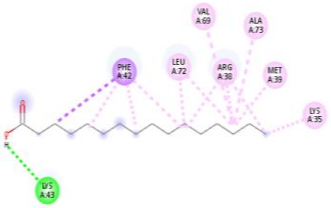
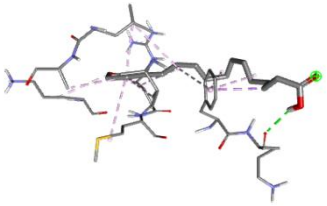
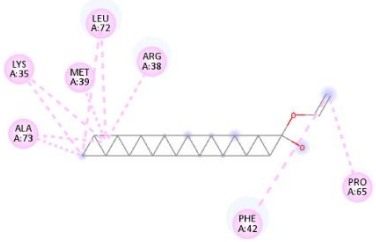
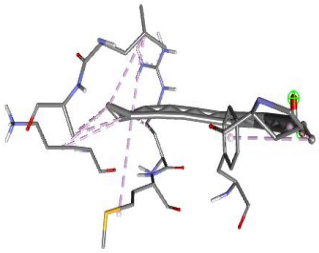
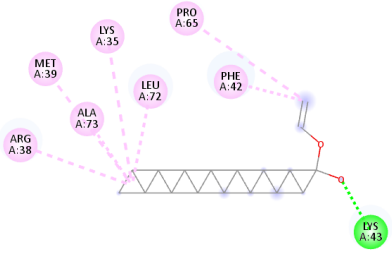
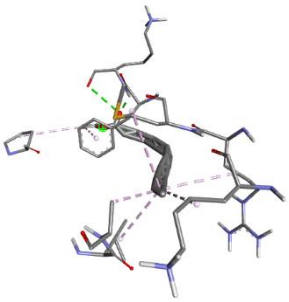
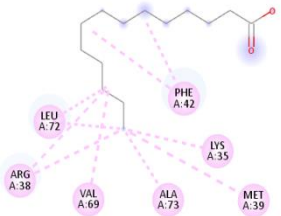
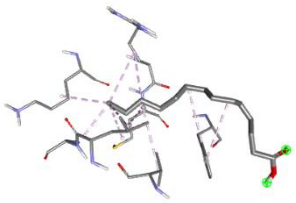
Carvacrol		
P- Cymene		
Dithymoquinone		
Limonene		

Nigellamine C		
Nigellicine		
Nigellidine		
Alpha pinene		

Thymoquinone		
P- Anisaldehyde		
Cis-anethole		
Gamma himachalene		

Linolool		
Estragole		
Trans-anethole		
Eicosanoic acid, ethyl ester (Ethyl arachidate)		
Ethyl docosanoate		

Linoleic acid, ethyl ester (ethyl linoleate)		
n-Hexadecanoic acid (palmitic acid)		
Octadecanoic acid, ethyl ester (ethyl sterate)		
Palmitic acid, ethyl ester (ethyl palmitate)		
Tetradecanoic acid (myristic acid)		

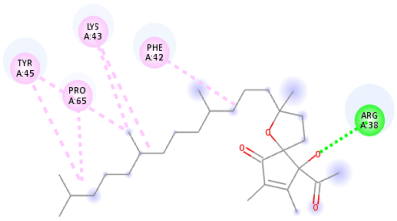
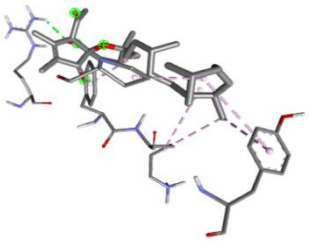
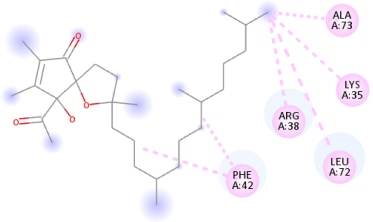
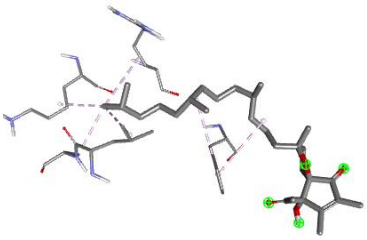
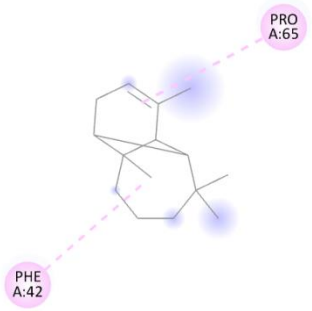
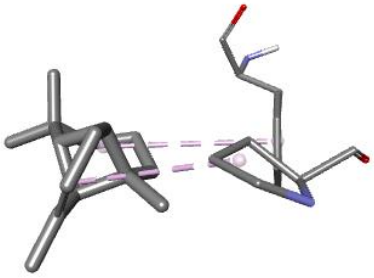
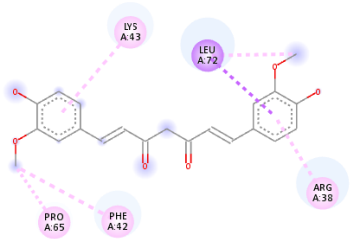
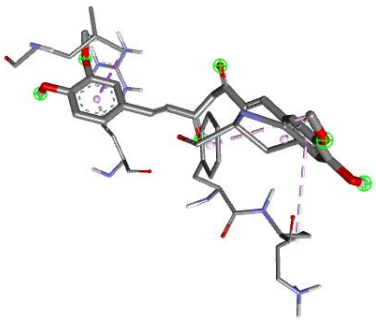
Alpha.-Tocospiro A		
Alpha.-Tocospiro B		
Alpha.-Longipinene		
Curcumin		

Table no. 6.16 Interactions of selected ligand with protein 1P9M

1P9M		
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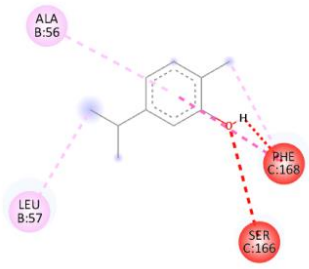
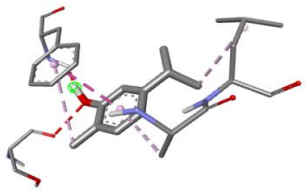
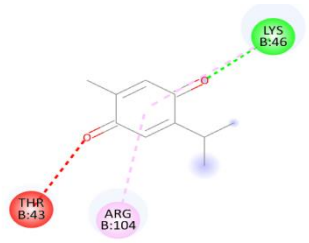
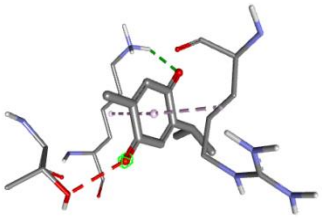
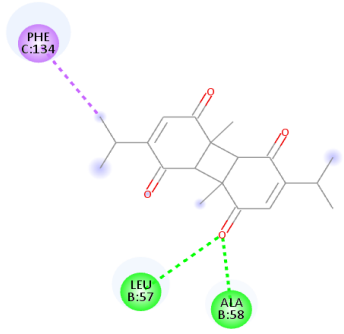
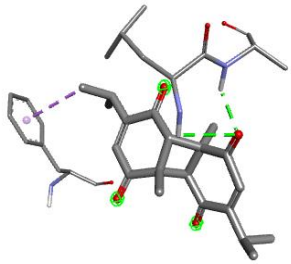
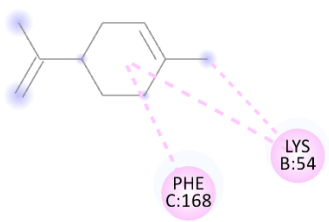
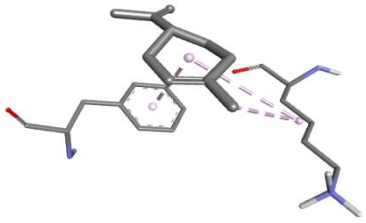
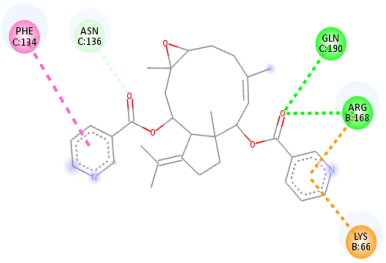
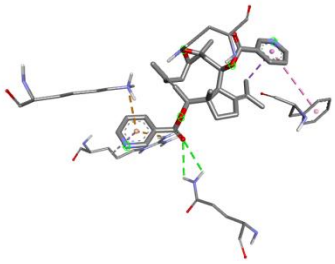
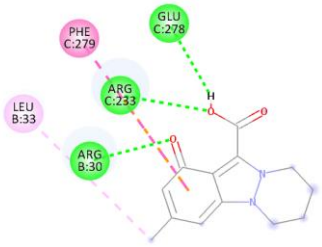
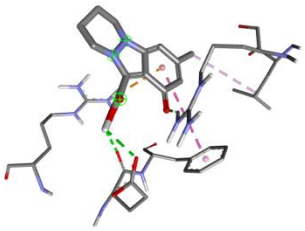
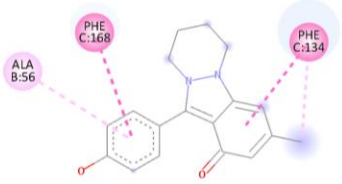
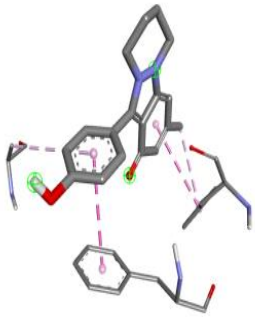
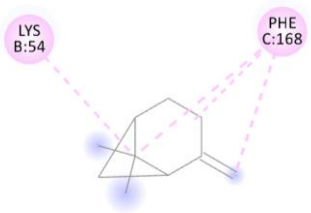
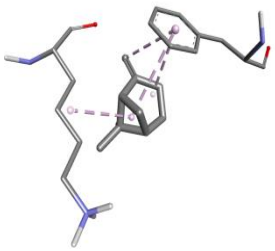
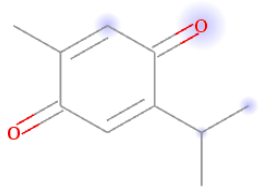
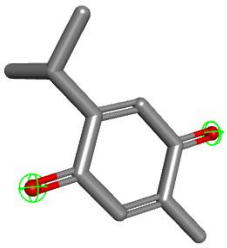
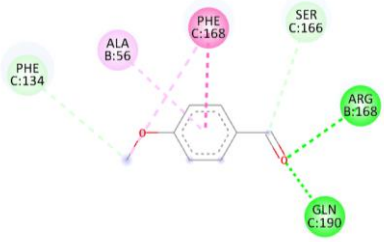
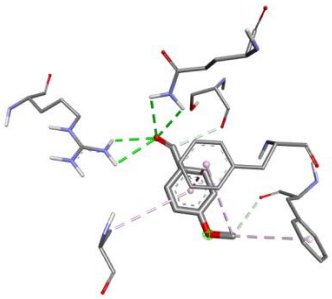
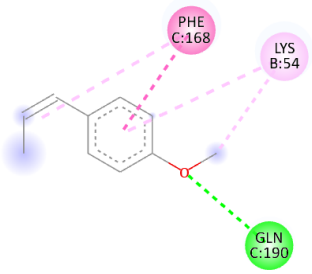
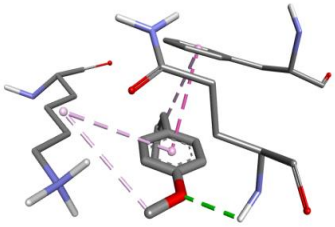
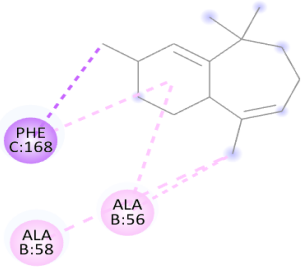
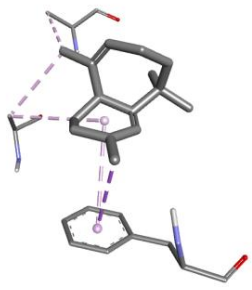
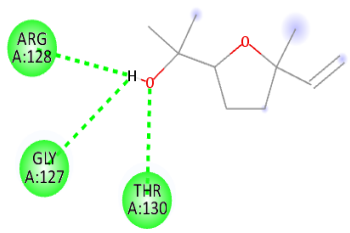
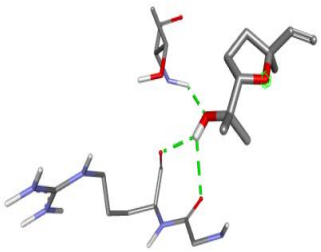
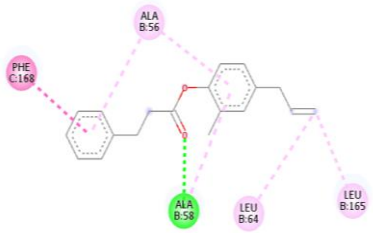
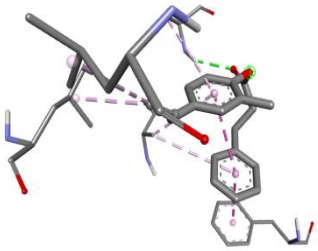
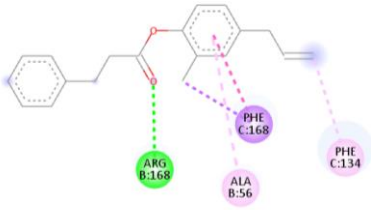
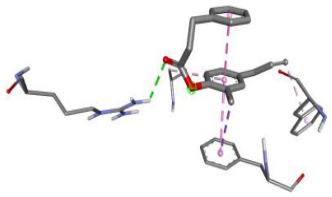
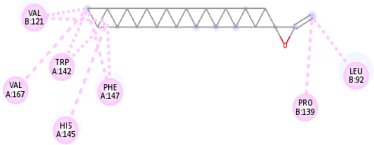
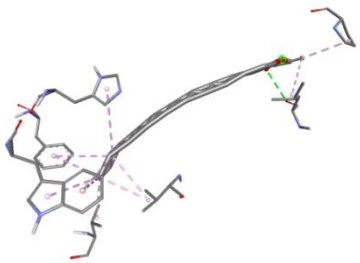
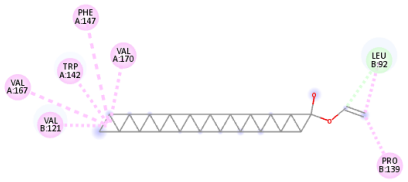
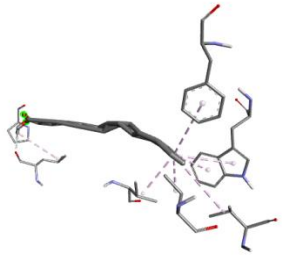
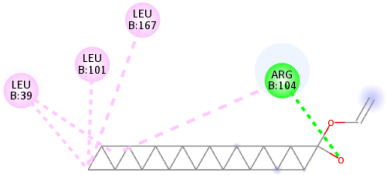
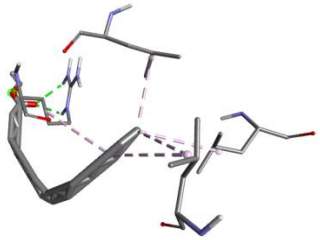
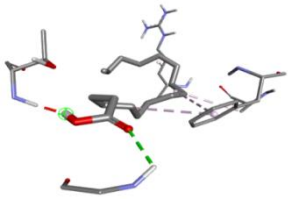
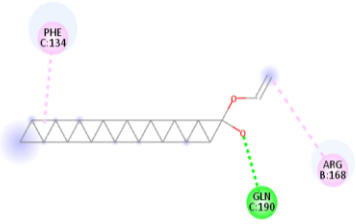
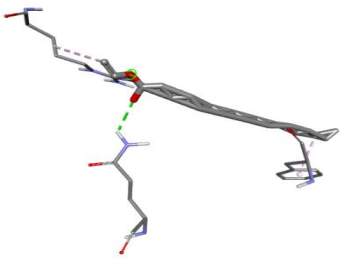
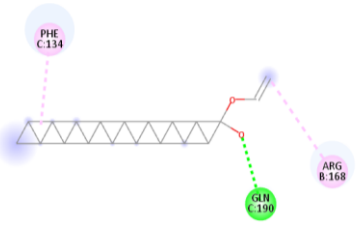
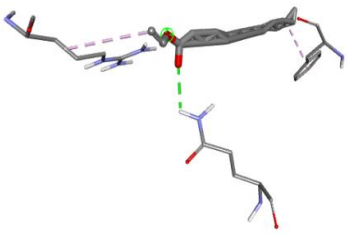
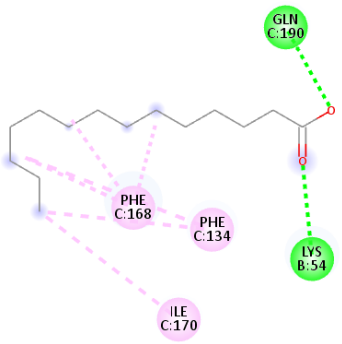
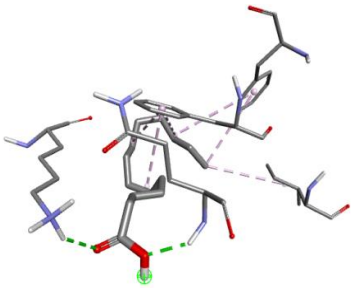
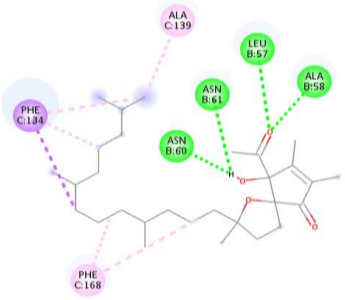
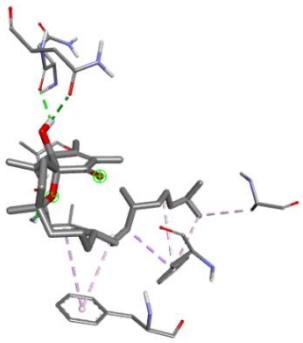
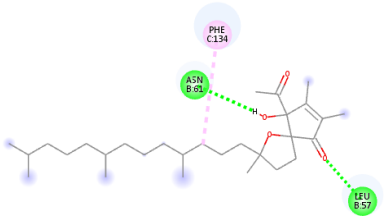
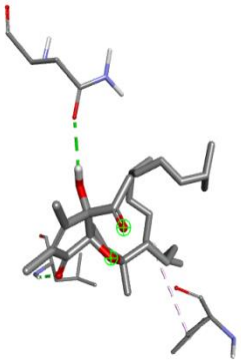
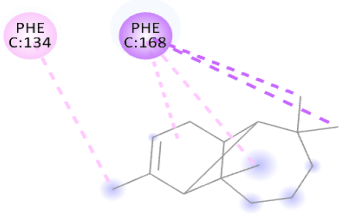
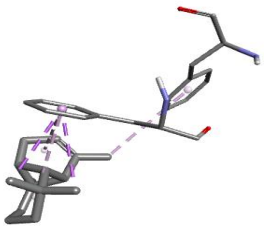
Carvacrol		
P- Cymene		
Dithymoquinone		
Limonene		

Nigellamine C		
Nigellicine		
Nigellidine		
Alpha pinene		

Thymoquinone		
P-Anisaldehyde		
Cis-anethole		
Gamma himachalene		

Linolool		
Estragole		
Trans-anethole		
Eicosanoic acid, ethyl ester (Ethyl arachidate)		

Ethyl docosanoate		
Linoleic acid, ethyl ester (ethyl linoleate)		
n-Hexadecanoic acid (palmitic acid)		
Octadecanoic acid, ethyl ester (ethyl sterate)		
Palmitic acid, ethyl ester (ethyl palmitate)		

Tetradecanoic acid (myristic acid)		
Alpha-Tocospiro A		
Alpha-Tocospiro B		
alpha-Longipinene		

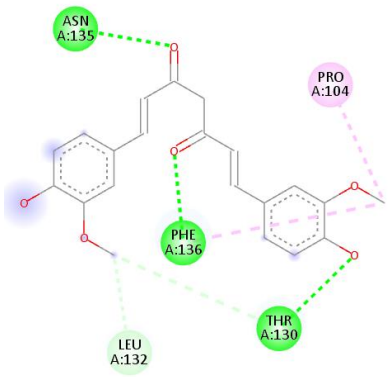
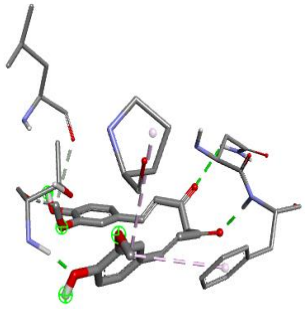
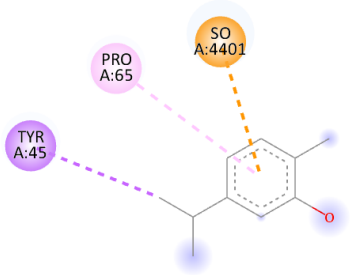
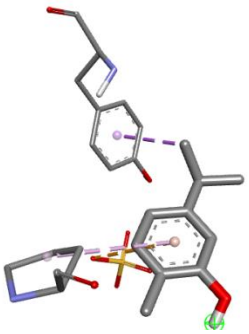
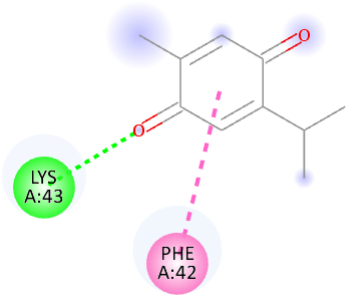
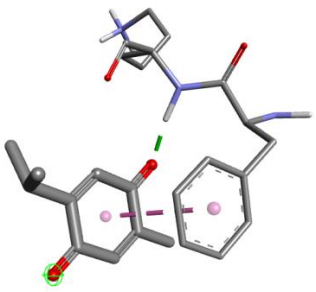
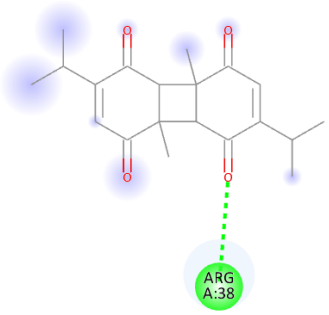
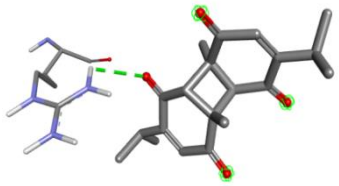
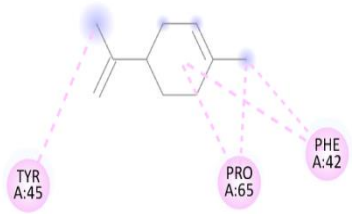
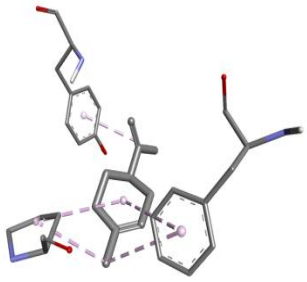
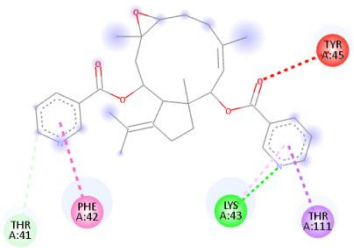
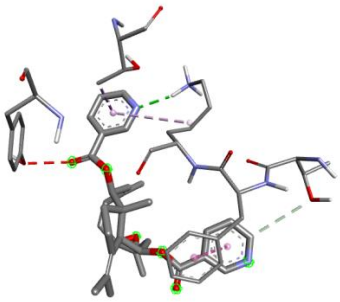
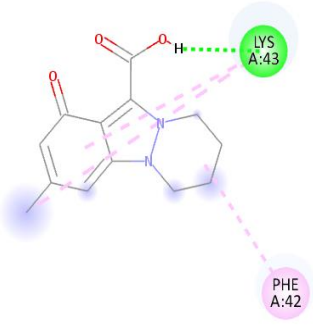
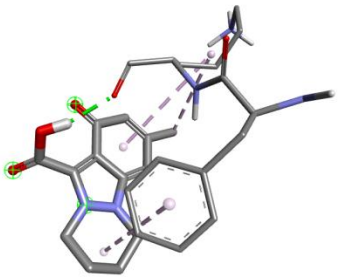
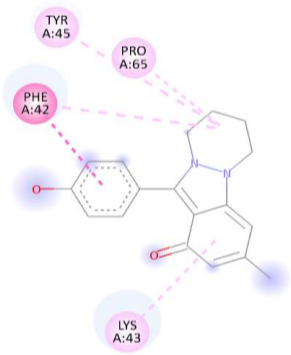
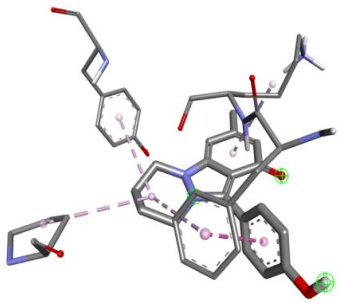
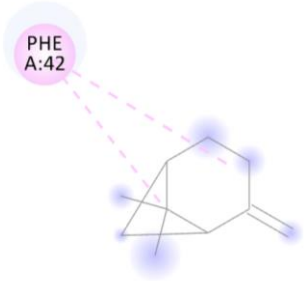
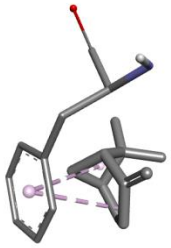
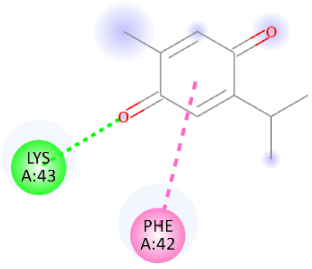
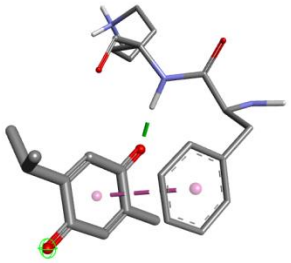
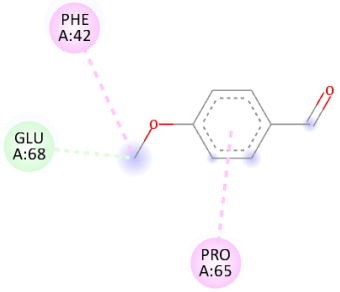
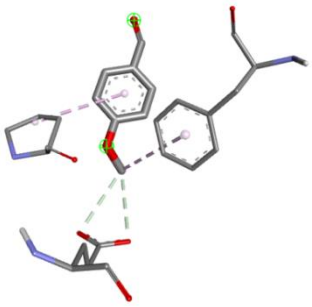
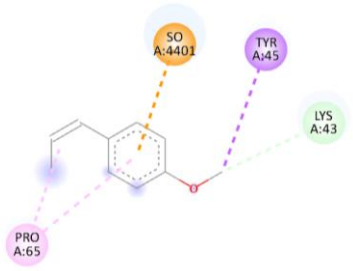
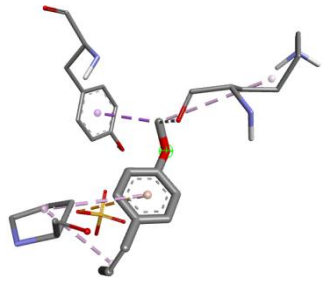
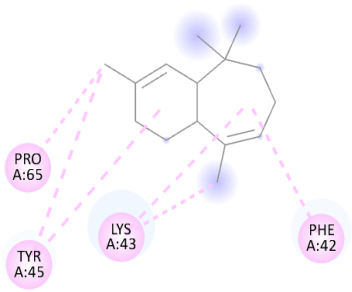
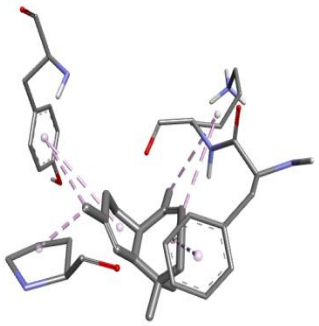
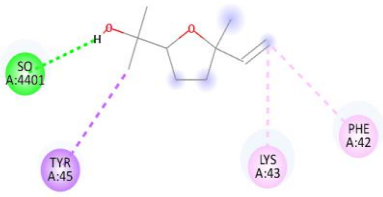
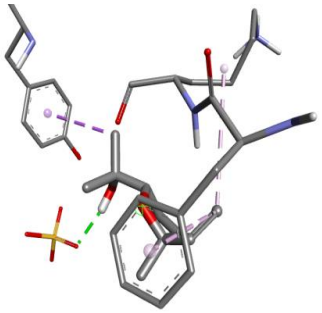
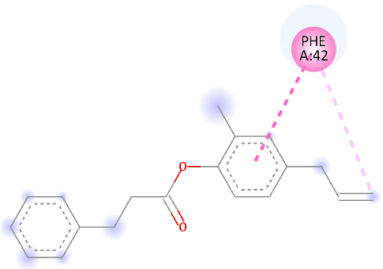
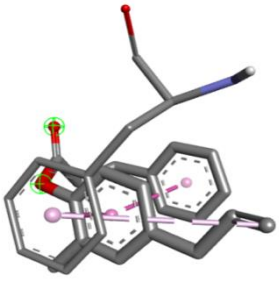
Curcumin		

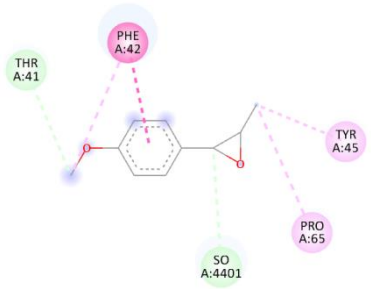
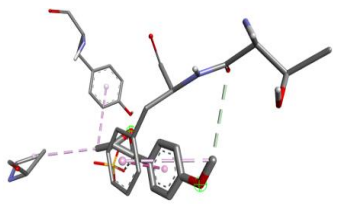
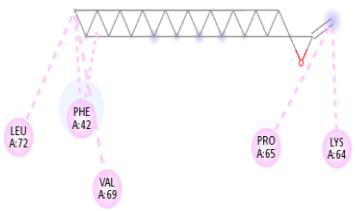
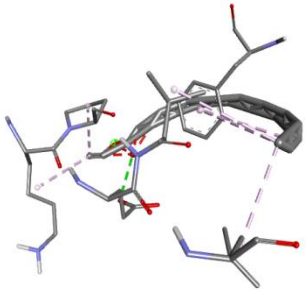
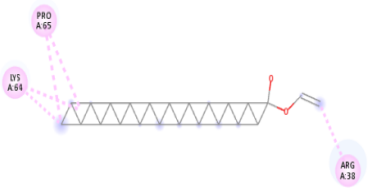
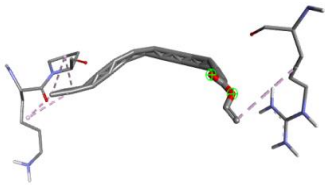
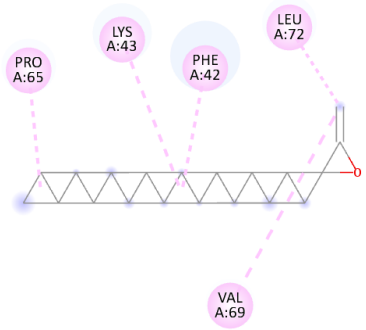
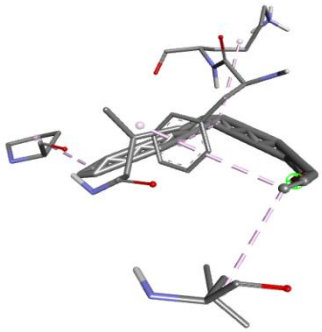
Table no. 6.17 Interactions of selected ligand with protein 1PW6

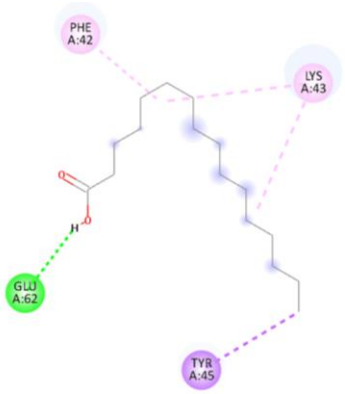
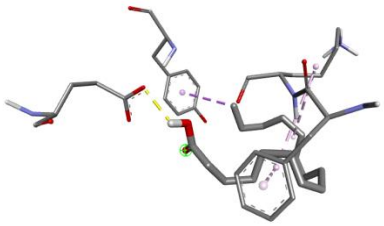
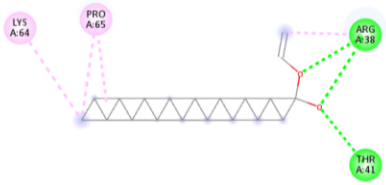
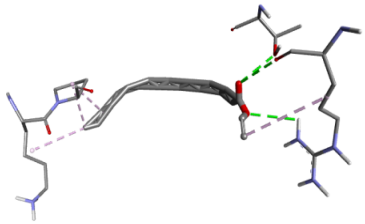
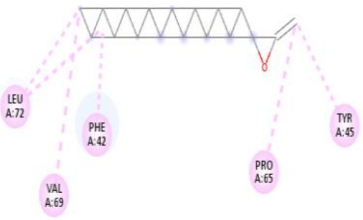
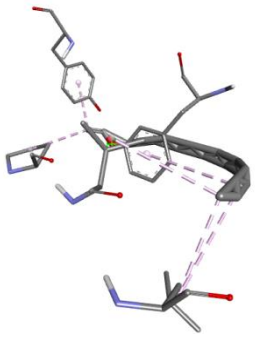
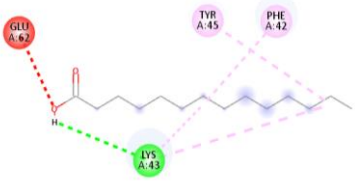
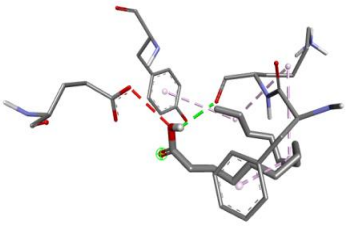
1PW6		
COMPOUND	**2 D STRUCTURE**	**3 D STRUCTURE**
Carvacrol		
P- Cymene		

Dithymoquinone		
Limonene		
Nigellamine C		
Nigellicine		

Nigellidine		
Alpha pinene		
Thymoquinone		
P- Anisaldehyde		

Cis-anethole		
Gamma himachalene		
Linolool		
Estragole		

Trans-anethole		
Eicosanoic acid, ethyl ester (Ethyl arachidate)		
Ethyl docosanoate		
Linoleic acid, ethyl ester (ethyl linoleate)		

n-Hexadecanoic acid (palmitic acid)		
Octadecanoic acid, ethyl ester (ethyl sterate)		
Palmitic acid, ethyl ester (ethyl palmitate)		
Tetradecanoic acid (myristic acid)		

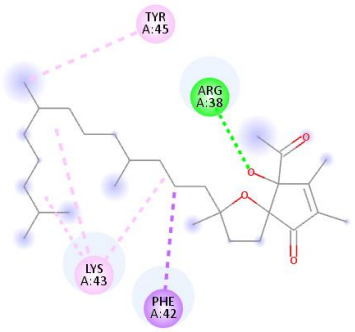
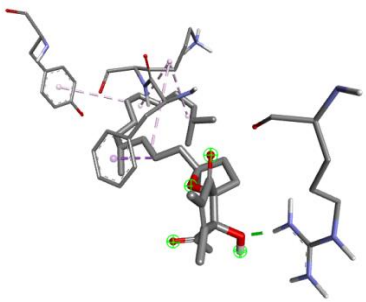
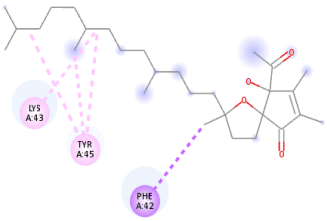
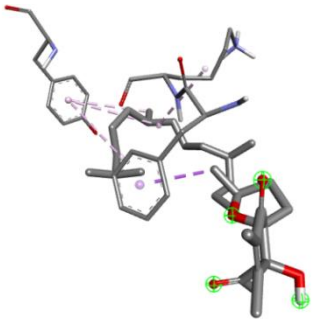
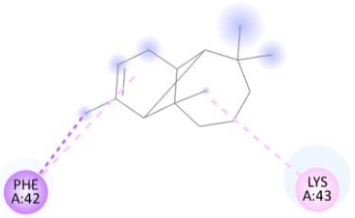
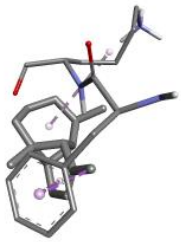
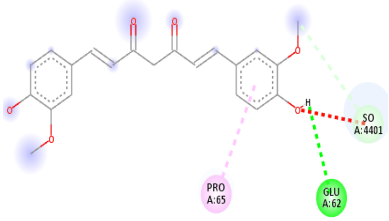
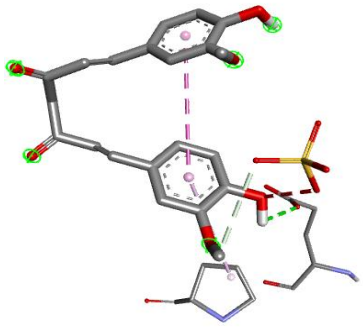
alpha-Tocospiro A		
alpha-Tocospiro B		
alpha-Longipinene		
Curcumin		

Table no. 6.18 Interactions of selected ligand with protein 5UO1

5UO1		
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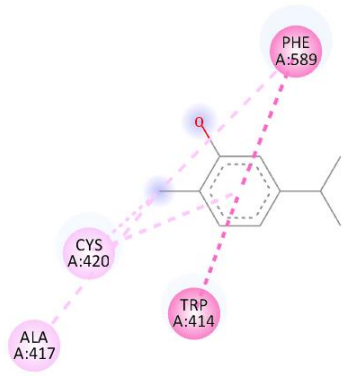
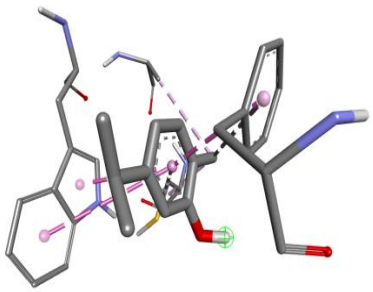
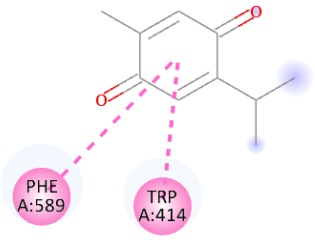
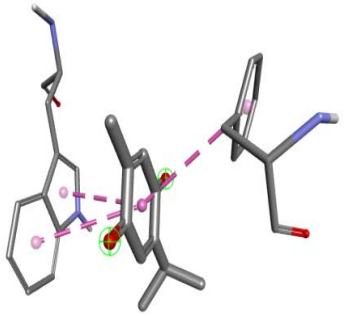
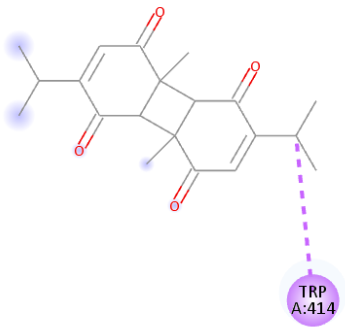
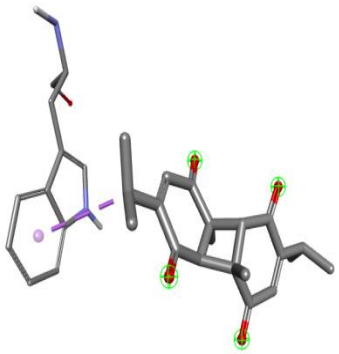
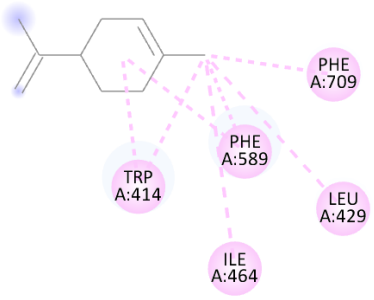
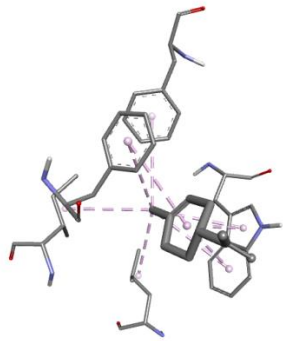
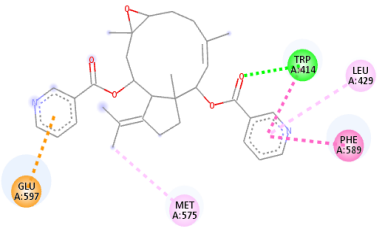
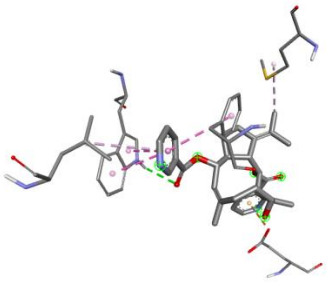
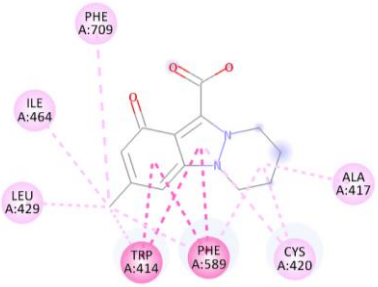
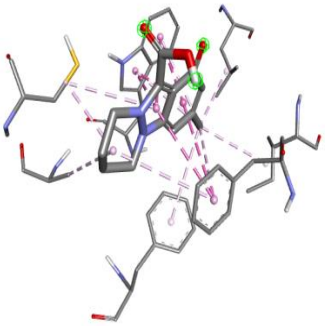
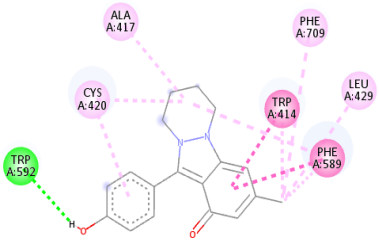
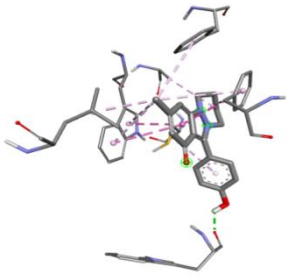
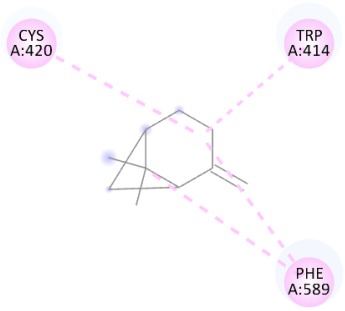
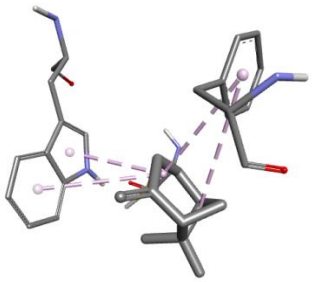
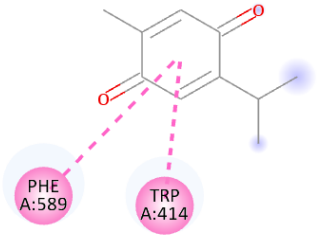
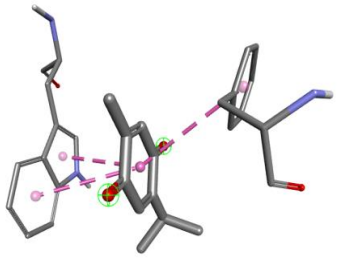
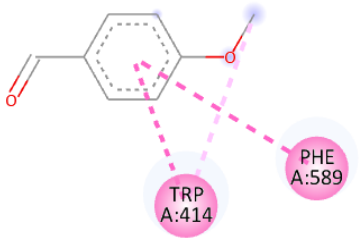
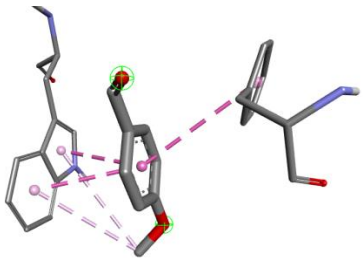
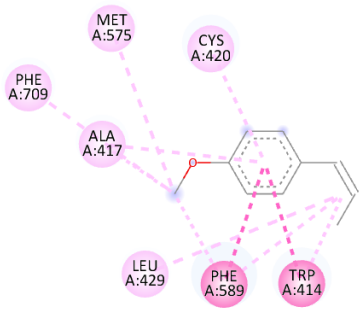
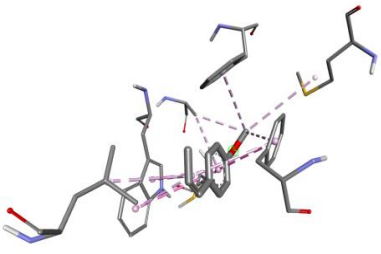
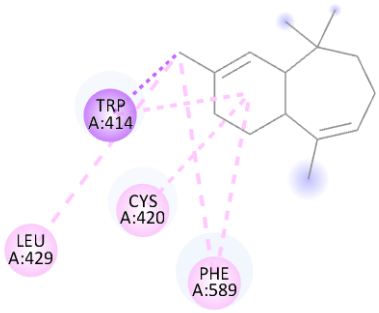
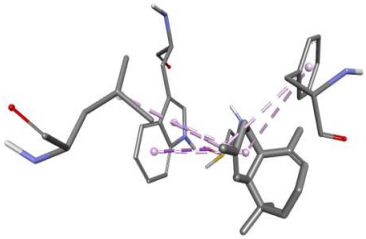
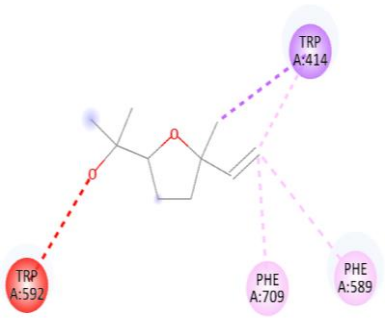
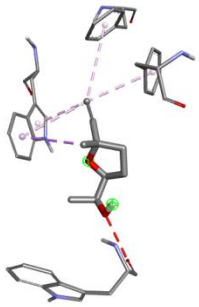
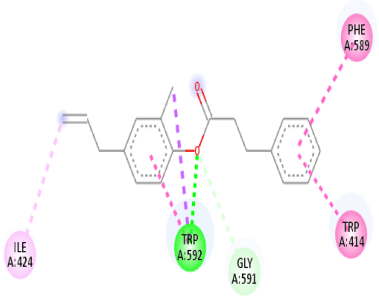
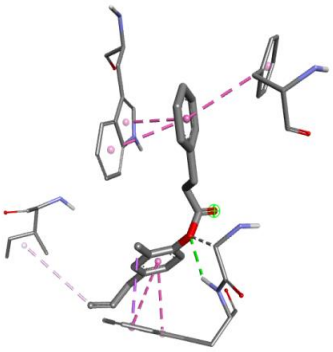
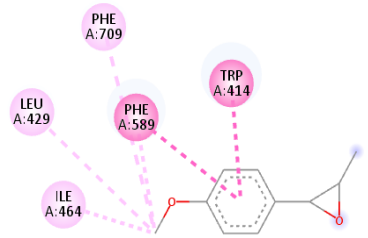
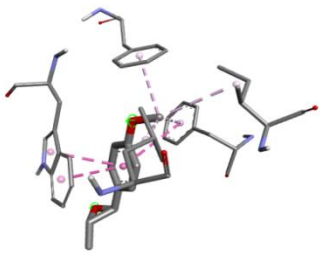
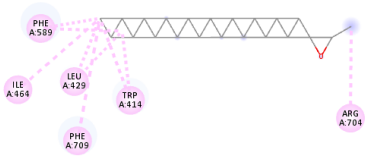
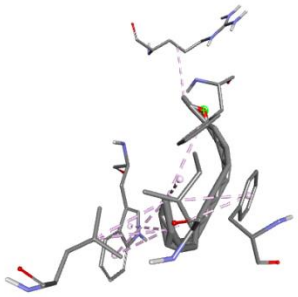
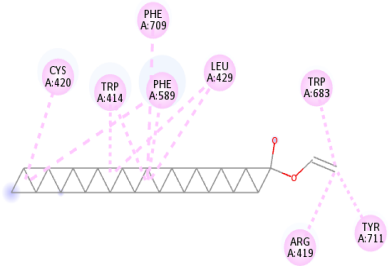
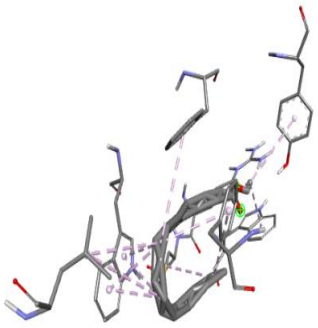
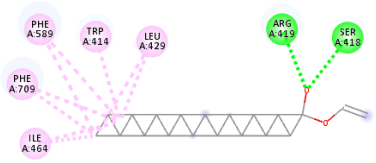
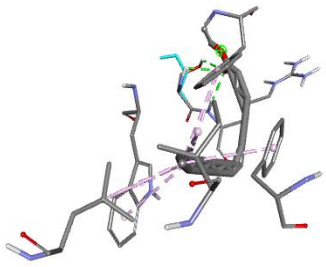
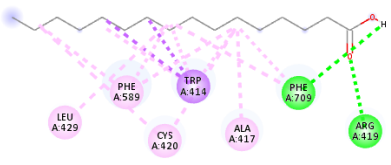
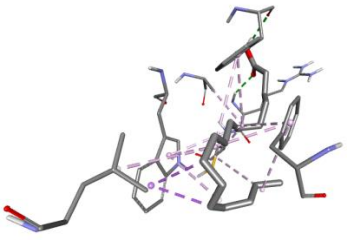
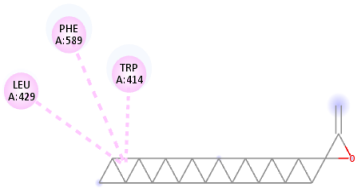
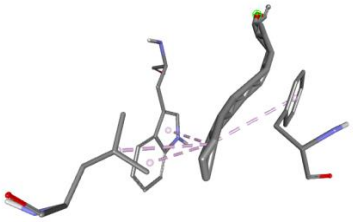
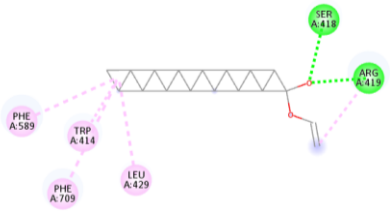
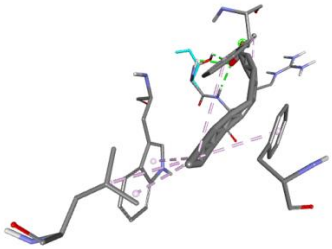
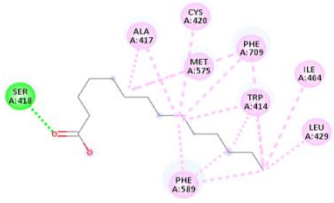
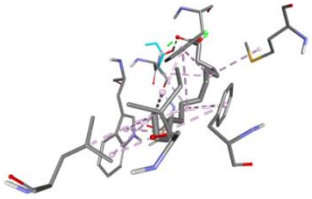
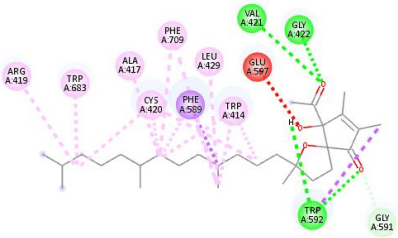
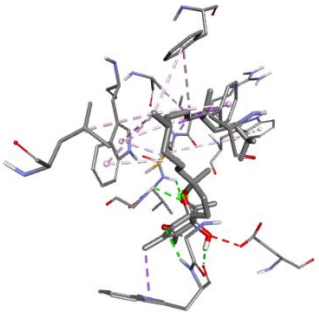
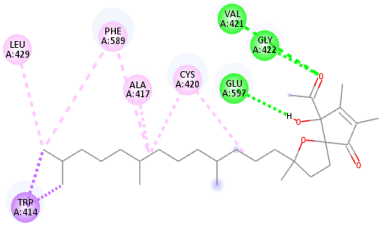
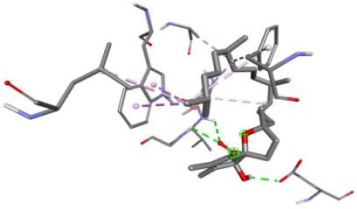
Carvacrol		
P- Cymene		
Dithymoquinone		

Limonene		
NigellamIne C		
Nigellicine		
Nigellidine		

Alpha pinene		
Thymoquinone		
P- Anisaldehyde		
Cis-anethole		

Gamma himachalene		
Linolool		
Estragole		
Trans-anethole		

Eicosanoic acid, ethyl ester (Ethyl arachidate)		
Ethyl docosanoate		
Linoleic acid, ethyl ester (ethyl linoleate)		
n-Hexadecanoic acid (palmitic acid)		

Octadecanoic acid, ethyl ester (ethyl sterate)		
Palmitic acid, ethyl ester (ethyl palmitate)		
Tetradecanoic acid (myristic acid)		
Alpha -Tocospiro A		
Alpha -Tocospiro B		

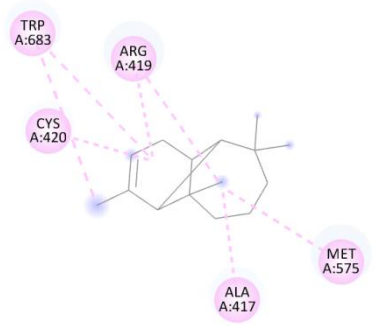
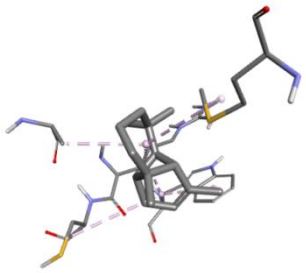
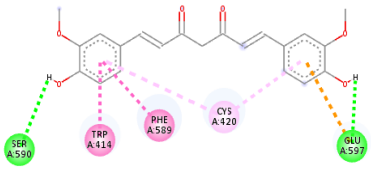
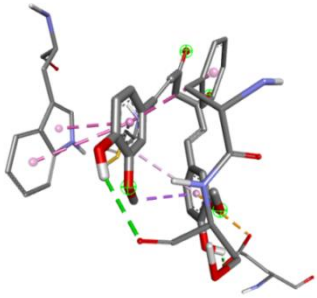
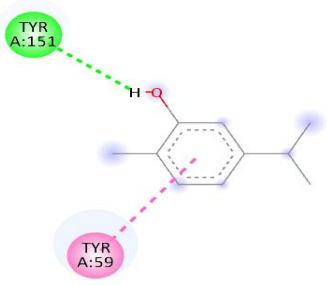
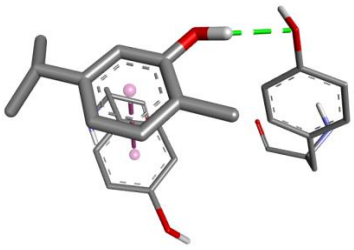
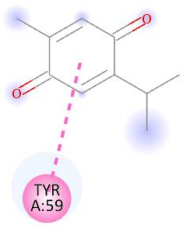
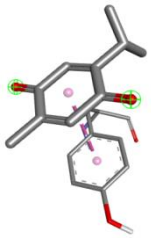
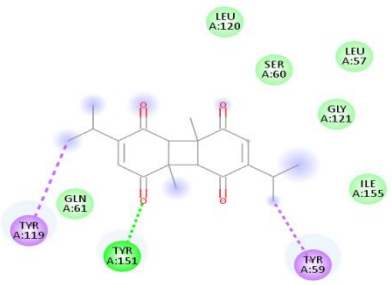
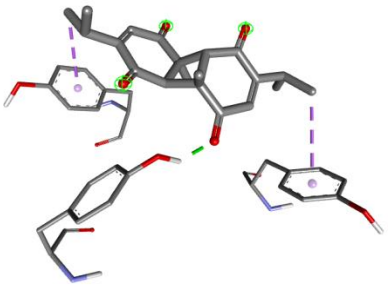
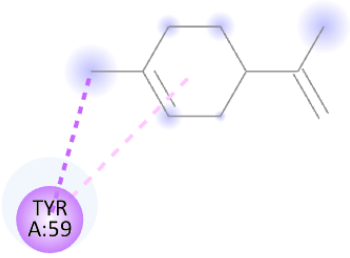
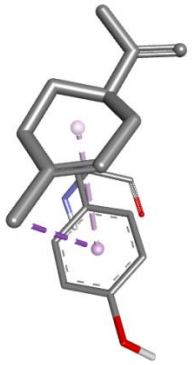
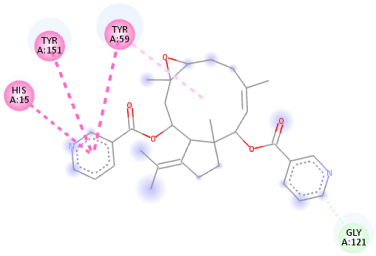
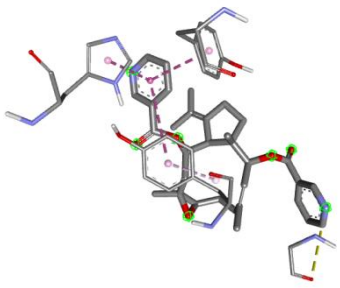
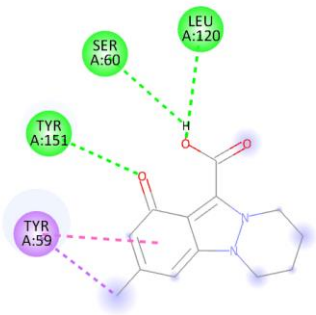
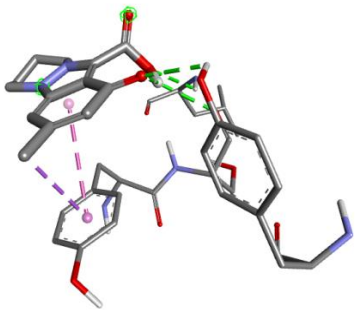
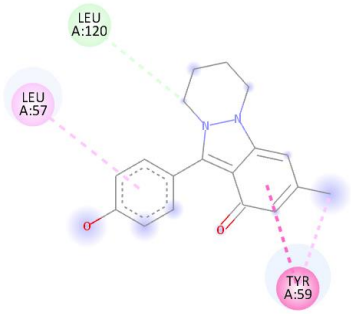
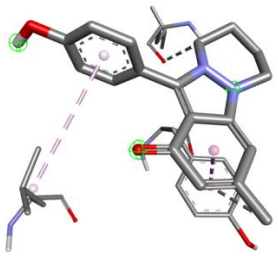
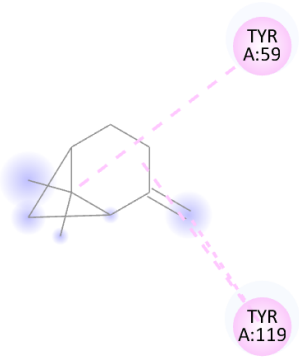
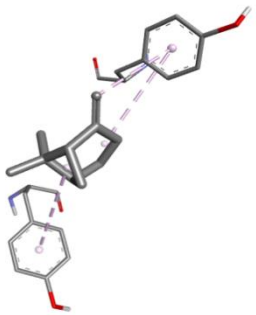
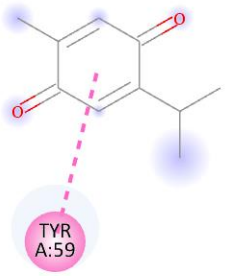
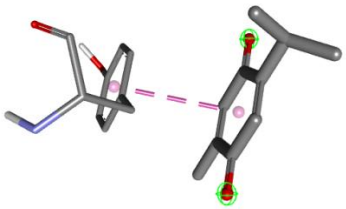
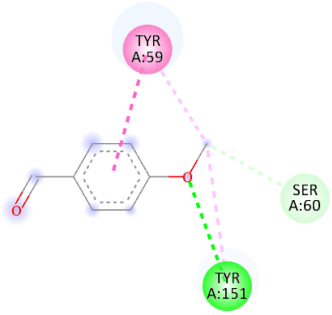
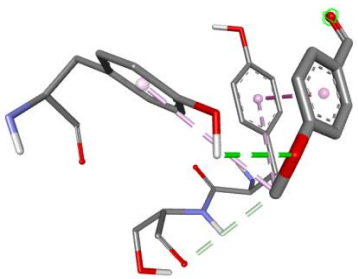
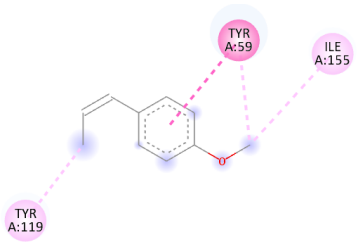
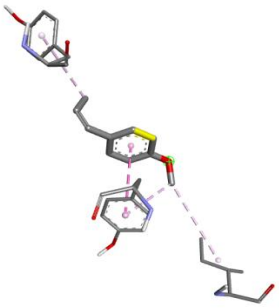
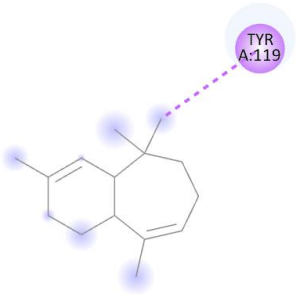
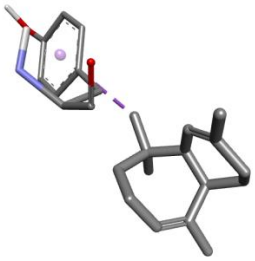
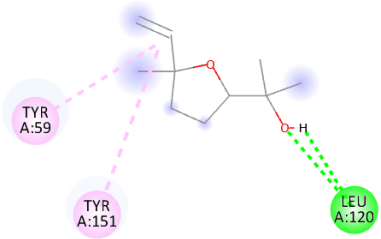
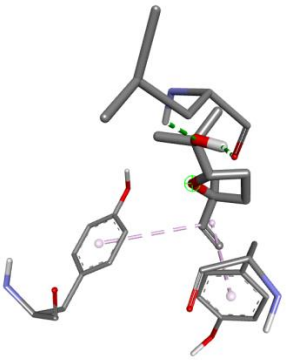
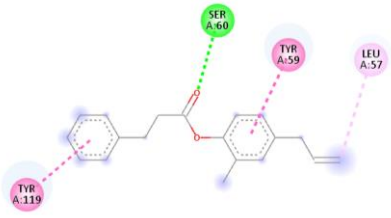
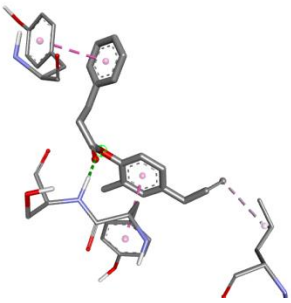
Alpha-Longipinene		
Curcumin		

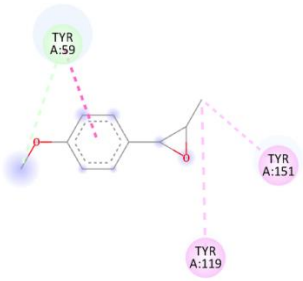
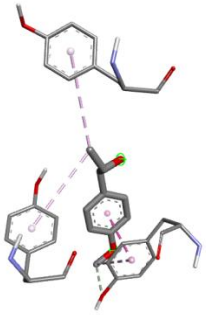
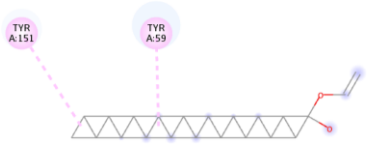
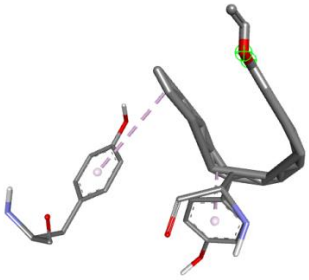
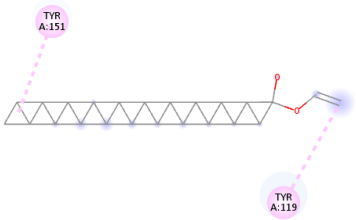
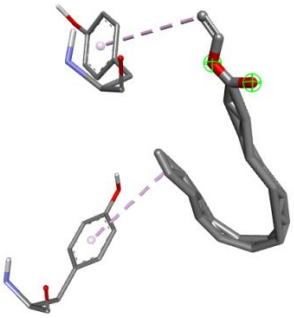
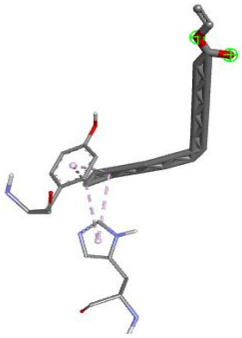
Table no. 6.19 Interactions of selected ligand with protein 2AZ5

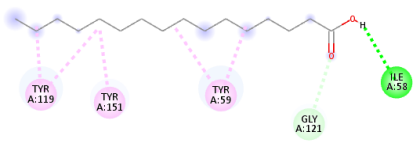
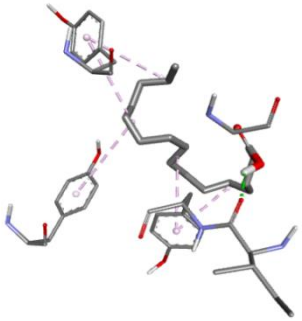
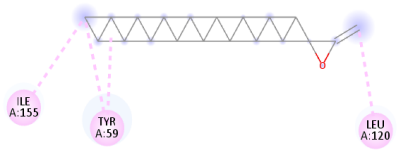
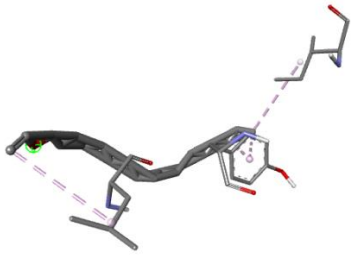
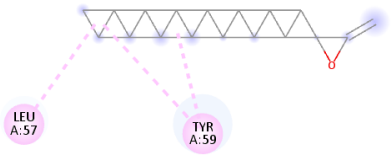
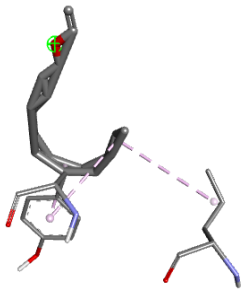
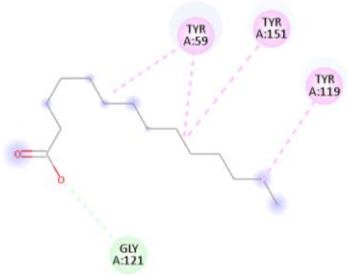
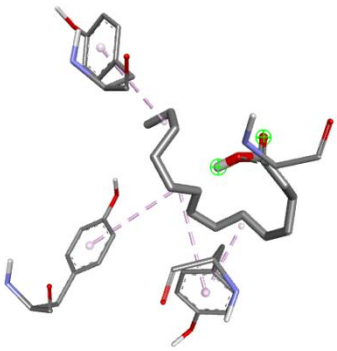
2AZ5		
COMPOUND	**2 D STRUCTURE**	**3 D STRUCTURE**
Carvacrol		
p- Cymene		

Dithymoquinone		
Limonene		
NigellamIne C		
Nigellicine		

Nigellidine		
Alpha pinene		
Thymoquinone		
P - Anisaldehyde		

Cis-anethole		
Gamma himachalene		
Linolool		
Estragole		

Trans-anethole		
Eicosanoic acid, ethyl ester (Ethyl arachidate)		
Ethyl docosanoate		
Linoleic acid, ethyl ester (ethyl linoleate)		

n-Hexadecanoic acid (palmitic acid)		
Octadecanoic acid, ethyl ester (ethyl sterate)		
Palmitic acid, ethyl ester (ethyl palmitate)		
Tetradecanoic acid (myristic acid)		

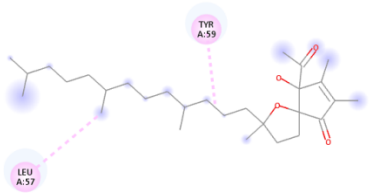
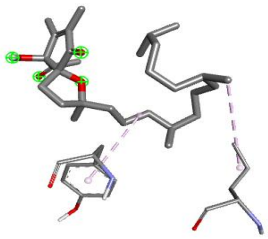
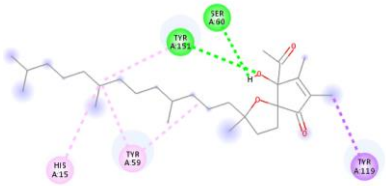
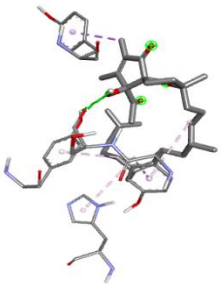
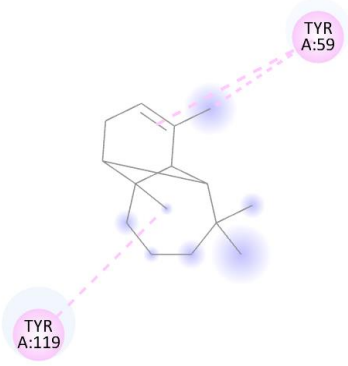
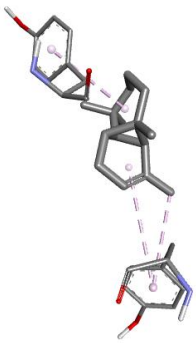
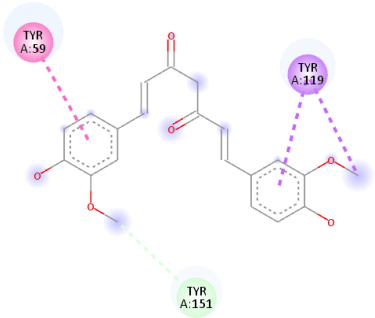
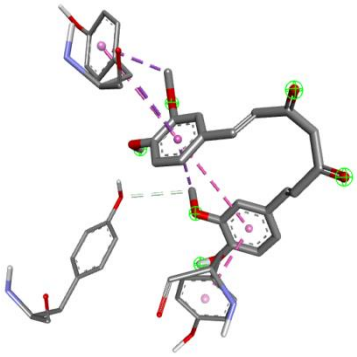
Alpha -Tocospiro A		
Alpha -Tocospiro B		
Alpha-Longipinene		
Curcumin		

Table no. 6.20 Type of interactions and interacting amino acid residues of 1M48

Compound Name	**Binding energy (Kcal/mol)**	**Bond**	**Amino acid residues**
Carvacrol	-5.6	Hydrophobic Hydrogen Bond	LEU72, ARG38, ARG81, PRO82
P- Cymene	-6.0	Hydrophobic Hydrogen Bond	ARG38, LEU72
Dithymoquinone	-5.8	Hydrogen Bond	LYS43
Limonene	-5.2	Hydrophobic	ARG38, LEU72, ALA73, LYS35, PHE42
Nigellamine C	-6.3	Hydrophobic	LEU72, PHE42, ARG38
Nigellicine	-5.5	Hydrogen Bond Hydrophobic	ARG38, LEU72 ALA73, LYS35, MET39, LEU72, ARG38
Nigellidine	-5.8	Hydrophobic	LEU72, PHE42, ARG38
Alpha pinene	-4.3	Hydrophobic	PRO65 PHE42
Thymoquinone	-6.0	Hydrogen Bond Hydrophobic	ARG38, LEU72
P- Anisaldehyde	-4.7	Hydrophobic	LEU72, ALA73, LYS35, ARG38, MET39
Cis-anethole	-4.9	Hydrophobic	LEU72, ALA73, LYS35, ARG38, MET39, PHE42
Gamma	-5.2	Hydrophobic	PHE42

himachalene			
Linolool	-4.2	Hydrogen Bond Hydrophobic	MET39, ARG38
Estragole	-6.9	Hydrophobic	A:LEU72, PHE42, PRO65, ARG38
Trans-anethole	-4.9	Hydrophobic	ALA73, LYS35, LEU72, ARG38
Eicosanoic acid, ethyl ester	-7.6	Hydrophobic	LYS35, ARG38, ALA73, MET39, LEU72, PRO65, TYR45
Ethyl docosanoate	-6.8	Hydrogen Bond Hydrophobic	GLU62, ARG38, VAL69, ALA73, MET39, LEU72, LYS35, PRO65, TYR45
Linoleic acid, ethyl ester	-6.8	Hydrogen Bond Hydrophobic	GLU62, ARG38, ALA73, ALA73, MET39, LEU72, LYS35, PRO65, TYR45
n-Hexadecanoic acid	-4.7	Hydrogen Bond Hydrophobic	GLU62, ARG38, ALA73, MET39, LEU72, LYS35, PRO65, TYR45
Octadecanoic acid, ethyl ester	-6.8	Hydrophobic	LYS35, ARG38, ALA73, MET39, LEU72, PRO65, PHE42
Palmitic acid, ethyl ester	-6.3	Hydrophobic	LYS35, ARG38, ALA73, MET39, LEU72, PRO65, PHE42
Tetradecanoic acid	-4.5	Hydrophobic	ARG38, VAL69, ALA73, LEU72, LYS35, MET39, PHE42
Alpha.-	-5.8	Hydrogen Bond	ARG38, LYS43, PRO65,

TocospiroA		Hydrophobic	LYS43, PHE42, TYR45, TYR45
Alpha.-Tocospiro B	-6.2	Hydrophobic	ALA73, LYS35, ARG38, LEU72, PHE42
Alpha.-Longipinene	-5.1	Hydrophobic	PRO65, PHE42
Curcumin	-7.0	Hydrophobic	LEU72, PRO65, LEU72, PHE42, LYS43, ARG38

Table no. 6.21 Type of interactions and interacting amino acid residues of 1P9M

Compound Name	**Binding energy (Kcal/mol)**	**Bond**	**Amino acid residues**
Carvacrol	-5.8	Hydrophobic	PHE168, LEU57, ALA56
P- Cymene	-5.2	Hydrogen Bond Hydrophobic	LYS46, ARG104
Dithymoquinone	-7.8	Hydrogen Bond Hydrophobic	LEU57, ALA58, PHE134
Limonene	-5.3	Hydrophobic	LYS54, LYS54, PHE168
Nigellamine C	-9.8	Hydrogen Bond	ARG168, GLN190
Nigellicine	-6.7	Hydrogen Bond Hydrophobic Electrostatic	ARG30, ARG233, GLU278, PHE279, LEU33
Nigellidine	-7.6	Hydrophobic	PHE134PHE168, PHE134, ALA56
Alpha pinene	-5.4	Hydrophobic	LYS54, PHE168

Thymoquinone	-5.1	Unsatisfactory	Nil
P- Anisaldehyde	-4.7	Hydrogen Bond Hydrophobic	ARG168, GLN190, PHE134, SER166, PHE168, ALA56
Cis-anethole	-5.2	Hydrogen Bond Hydrophobic	GLN190, PHE168, LYS54
Gamma himachalene	-6.8	Hydrophobic	PHE168, ALA56, ALA58
Linolool	-5.3	Hydrogen Bond	THR130, GLY127, ARG128
Estragole	-6.2	Hydrogen Bond Hydrophobic	ALA58, PHE168, LEU64, LEU165, ALA56
Trans-anethole	-6.1	Hydrogen Bond Hydrophobic	ARG168, PHE168, PHE134, ALA56
Eicosanoic acid, ethyl ester	-7.3	Hydrogen Bond Hydrophobic	LEU92, VAL121, VAL167, PRO139, TRP142, HIS145, PHE147
Ethyl docosanoate	-6.8	Hydrogen Bond Hydrophobic	LEU92, VAL167, VAL170, VAL121, PRO139, TRP142, PHE147
Linoleic acid, ethyl ester	-8.6	Hydrogen Bond Hydrophobic	ARG104, LEU39, LEU101, LEU167
n-Hexadecanoic acid	-5.1	Hydrogen Bond Hydrophobic	GLY127, ALA152, ARG154, PHE136
Octadecanoic acid, ethyl ester	-6.5	Hydrogen Bond Hydrophobic	GLN190, ARG168, PHE134
Palmitic acid, ethyl ester	-6.2	Hydrogen Bond Hydrophobic	GLN190, ARG168, PHE134
Tetradecanoic acid	-4.5	Hydrogen Bond Hydrophobic	LYS54, GLN190, ILE170, PHE134, PHE168

Alpha.-Tocospiro A	-6.9	Hydrogen Bond Hydrophobic	LEU57, ALA58, ASN60, ASN61, ALA139, PHE134, PHE168
Alpha.-Tocospiro B	-5.7	Hydrogen Bond Hydrophobic	LEU57, ASN61, PHE134
Alpha.-Longipinene	-6.5	Hydrogen Bond Hydrophobic	PHE168, PHE134
Curcumin	-6.7	Hydrogen Bond Hydrophobic	THR130,ASN135, PHE136, LEU132, PRO104

Table no. 6.22 Type of interactions and interacting amino acid residues of 1PW6

Compound Name	**Binding energy (Kcal/mol)**	**Bond**	**Amino acid residues**
Carvacrol	-4.5	Electrostatic Hydrophobic	SO4401, TYR45, PRO65
P- Cymene	-4.5	Hydrogen Bond Hydrophobic	LYS43, PHE42
Dithymoquinone	-5.3	Hydrogen Bond	ARG38
Limonene	-4.7	Hydrophobic	PRO65, PHE42, TYR45
Nigellamine C	-6.4	Hydrogen Bond Hydrophobic	LYS43, THR41, THR111, PHE42
Nigellicine	-5.3	Hydrogen Bond Hydrophobic	LYS43, PHE42, LYS43
Nigellidine	-6.2	Hydrophobic	PHE42, PRO65, TYR45, LYS43

Alpha pinene	-3.7	Hydrophobic	PHE42
Thymoquinone	-4.5	Hydrogen Bond Hydrophobic	LYS43, PHE42
P- Anisaldehyde	-3.7	Hydrogen Bond Hydrophobic	GLU68, PHE42, PRO65
Cis-anethole	-4.0	Hydrogen Bond Hydrophobic	LYS43, TYR45, PRO65
Gamma himachalene	-5.1	Hydrophobic	LYS43, LYS43, PRO65, PHE42, TYR45
Linolool	-4.1	Hydrophobic	TYR45, LYS43, PHE42
Estragole	-4.5	Hydrophobic	PHE42
Trans-anethole	-4.1	Hydrogen Bond Hydrophobic	THR41, PHE421, PRO65, PHE42, TYR45
Eicosanoic acid, ethyl ester	-6.5	Hydrogen Bond Hydrophobic	GLU68, VAL69, LEU72, LYS64, PRO65, PHE42
Ethyl docosanoate	-5.8	Hydrophobic	LYS64, PRO65, ARG38
Linoleic acid, ethyl ester	-7.6	Hydrophobic	LYS43, PRO65, VAL69, LEU72, PHE42
n-Hexadecanoic acid	-3.7	Hydrogen Bond Hydrophobic	GLU62, TYR45, LYS43, PHE42
Octadecanoic acid, ethyl ester	-6.0	Hydrogen Bond Hydrophobic	ARG38, THR41, PRO65, LYS64
Palmitic acid, ethyl ester	-5.5	Hydrophobic	LEU72, VAL69, PRO65, PHE42, TYR45
Tetradecanoic acid	-3.8	Hydrogen Bond Hydrophobic	LYS43, PHE42, TYR45
Alpha.-TocospiroA	-5.6	Hydrogen Bond Hydrophobic	ARG38, PHE42, LYS43, TYR45

Alpha.-Tocospiro B	-5.5	Hydrophobic	PHE42, LYS43, TYR45
Alpha.-Longipinene	-4.7	Hydrophobic	PHE42, LYS43, PHE42
Curcumin	-5.5	Hydrogen Bond Hydrophobic	GLU62, SO4401, PRO65

Table no. 6.23 Type of interactions and interacting amino acid residues of 5UO1

Compound Name	**Binding energy (Kcal/mol)**	**Bond**	**Amino acid residues**
Carvacrol	-7.5	Hydrophobic	TRP414, PHE589, ALA417, CYS420
P- Cymene	-7.2	Hydrophobic	TRP414, PHE589
Dithymoquinone	-9.5	Hydrophobic	TRP414
Limonene	-7.4	Hydrophobic	LEU429, ILE464, TRP414, PHE589, PHE709
Nigellamine C	-10.8	Hydrogen Bond Hydrophobic Electrostatic	TRP414, GLU597, PHE589, MET575, LEU429
Nigellicine	-8.6	Hydrophobic	TRP414, PHE589, ALA417, CYS420, LEU429, ILE464, PHE709
Nigellidine	-10.1	Hydrogen Bond Hydrophobic	TRP592, TRP414, PHE589, ALA417, CYS420, LEU429, PHE709, CYS420
Alpha pinene	-5.4	Hydrophobic	CYS420, TRP414, PHE589
Thymoquinone	-7.2	Hydrophobic	TRP414, PHE589
P-Anisaldehyde	-6.2	Hydrophobic	TRP414, PHE589

Cis-Anethole	-7.2	Hydrophobic	TRP414, PHE589, ALA417, LEU429, MET575, PHE709, CYS420
Gamma himachalene	-7.3	Hydrophobic	TRP414, CYS420, LEU429, PHE589
Linalool	-7.4	Hydrophobic	TRP414, PHE589, PHE589, PHE709
Estragole	-9.4	Hydrogen Bond Hydrophobic	TRP592, GLY591, TRP414, PHE589, ILE424
Trans- Anethole	-6.5	Hydrophobic	TRP414, PHE589, LEU429, ILE464, PHE709
Eicosanoic acid, ethyl ester	-9.9	Hydrophobic	LEU429, ILE464, ARG704, TRP414, PHE589, PHE709
Ethyl docosanoate	-8.0	Hydrophobic	CYS420, LEU429, ARG419, TRP414, PHE589, TRP683, PHE709, TYR711
Linoleic acid, ethyl ester	-11.1	Hydrogen Bond Hydrophobic	SER418, ARG419, LEU429, ILE464, LEU429, TRP414, PHE589, PHE709
n-Hexadecanoic acid	-6.4	Hydrogen Bond Hydrophobic	ARG419, PHE709, TRP414, ALA417, CYS420, LEU429, PHE589
Octadecanoic acid, ethyl ester	-8.7	Hydrophobic	LEU429, TRP414, PHE589
Palmitic acid, ethyl ester	-8.1	Hydrogen Bond Hydrophobic	SER418, ARG419, LEU429, TRP414, PHE589, PHE709
Tetradecanoic acid	-6.5	Hydrogen Bond Hydrophobic	SER418, ALA417, CYS420, MET575, LEU429, TRP414,

			PHE589, PHE709
Plpha.-Tocospiro A	-8.8	Hydrophobic	VAL421, GLY422, TRP592, GLY591, PHE589, ALA417, ARG419, CYS420, LEU429, TRP414, TRP683, PHE709
Alpha.-Tocospiro B	-8.5	Hydrogen Bond Hydrophobic	VAL421, GLY422, GLU597, TRP414, ALA417, CYS420, LEU429, PHE589
Alpha.-Longipinene	-6.7	Hydrophobic	ALA417, ARG419, CYS420, MET575, TRP683
Curcumin	-8.4	Hydrophobic	GLU597, SER590, TRP414

Table no. 6.24 Type of interactions and interacting amino acid residues of 2AZ5

Compound Name	**Binding energy (Kcal/mol)**	**Bond**	**Amino acid residues**
Carvacrol	-4.8	Hydrogen Bond Hydrophobic	TYR151, TYR59
P- Cymene	-4.6	Hydrophobic	TYR59
Dithymoquinone	-6.8	Hydrogen Bond Hydrophobic	TYR151, TYR59, TYR119
Limonene	-4.5	Hydrophobic	TYR59
Nigellamine C	-6.3	Hydrogen Bond Hydrophobic	GLY121, TYR59, HIS15, TYR151
Nigellicine	-5.9	Hydrogen Bond Hydrophobic	LEU120, TYR151, SER60, TYR59
Nigellidine	-6.2	Hydrogen Bond	LEU120, TYR59, LEU57

		Hydrophobic	
Alpha pinene	-4.2	Hydrophobic	TYR59, TYR119
Thymoquinone	-4.6	Hydrophobic	TYR59
p-Anisaldehyde	-4.0	Hydrogen Bond Hydrophobic	TYR151, SER60, TYR59
Cis-Anethole	-4.3	Hydrophobic	TYR59, ILE155, TYR119
Gamma himachalene	-5.5	Hydrophobic	TYR119
Linalool	-4.8	Hydrogen Bond Hydrophobic	LEU120, TYR59, TYR151
Estragole	-6.7	Hydrogen Bond Hydrophobic	SER60, TYR59, TYR119, LEU57
Trans- Anethole	-4.5	Hydrogen Bond Hydrophobic	TYR59, TYR119, TYR151
Eicosanoic acid, ethyl ester	-6.2	Hydrophobic	TYR59, TYR151
Ethyl docosanoate	-5.4	Hydrophobic	TYR119, TYR151
Linoleic acid, ethyl ester	-6.9	Hydrophobic	HIS15, TYR151
n-Hexadecanoic acid	-4.3	Hydrogen Bond Hydrophobic	ILE58, GLY121, TYR59, TYR119, TYR151
Octadecanoic acid, ethyl ester	-5.4	Hydrophobic	ILE155, LEU120, TYR59
Palmitic acid, ethyl ester	-5.0	Hydrophobic	LEU57, TYR59
Tetradecanoic acid	-4.2	Hydrogen Bond Hydrophobic	GLY121, TYR59, TYR119, TYR151

Alpha.-Tocospiro A	-5.3	Hydrophobic	LEU57, TYR59
alpha.-Tocospiro B	-5.7	Hydrogen Bond Hydrophobic	TYR151, SER60, TYR119, HIS15, TYR59
Alpha.-Longipinene	-5.3	Hydrophobic	TYR59, TYR119
Curcumin	-6.0	Hydrogen Bond Hydrophobic	TYR151, TYR119, TYR59

6.2.4 ADME Profiling

The absorption, distribution, metabolism, and excretion of selected compound were predicted via Swiss ADME and its data table mentioned below. All the compounds passes Lipinski rule and recommended that these components can be better drug choice with decent pharmacokinetic profile.

Table no. 6.25 ADME profiling of compounds

Query	**iLOGP**	**ESOL Class**	**GI absorption**	**BBB permeant**	**Liver Toxicity**			**Metabolism Cyp Inhibitors**					**Membrane Transporters**			**Lipinski violations**
					DILI	Cyto- toxicity	HLM	1A2	3A4	2D6	2C9	2C19	BBB	P-gp Inhibitor	P-gp Subs	
Carvacrol	2.24	Soluble	High	Yes	No	No	Yes	No	No	No	No	No	Yes	No	No	0
P-Cymene	2.51	Soluble	Low.	Yes.	Yes.	No.	No.	No	No	No	No	No	Yes	No	No	1

Gamma himachalone	Cis-Anethole	P-Anisaldehyde	Thymoquinone	Alpha pinene	Nigellidine	Nigellicine	Nigellamine C	Limonene	Dithymoquinone
3.26	2.58	1.68	1.99	2.63	2.57	1.71	3.55	2.72	2.46
Soluble	Soluble	Soluble	Soluble	Soluble	Soluble	Soluble	Poorly Soluble	Soluble	Soluble
Low	High	High	High	Low	High.	High.	Low.	Low.	High.
No.	Yes	Yes.	Yes.	Yes.	Yes.	Yes.	No.	Yes.	Yes.
Yes.	No	No.	No.	No.	No.	No.	No.	Yes.	Yes.
No.	No	No.	No.	No.	No.	No.	No.	No.	No.
Yes.	Yes	Yes.	Yes.	Yes.	Yes.	Yes	Yes.	Yes.	Yes.
No	Yes	Yes.	No	No	No	No	No	No	No
No	No	No	No	No	No	No	Yes	No	No
No	No	No	No	No	No	No	No	No	No
No	Yes	No	No	No	No	No	No	No	No
No	No	No	No	No	No	No	No	No	No
No	Yes	Yes	Yes	No	No	No	Yes	Yes	Yes
No	No	No	No	No	No	No	Yes	Yes	No
No	No	No	No	No	Yes	No	Yes	Yes	No
1	0	0	0	1	0	0	1	0	0

Octadecanoic acid, ethyl ester	n-Hexadecanoic acid	Linoleic acid, ethyl ester	Ethyl docosanoate	Eicosanoic acid, ethyl ester	Trans-Anethole	Estragole	Linalool
5.47	3.85	5.03	6.34	5.59	2.51	2.47	2.44
Poorly soluble	Moderately soluble	Moderately soluble	Poorly soluble	Poorly soluble	Soluble	Soluble	Soluble
Low	High	High	Low	Low	High	High	High
No.	Yes.	No.	No.	No.	Yes.	Yes.	Yes.
No.	No.	No.	No.	No.	No.	No.	No.
No.	No.	No.	No.	No.	No.	No.	No.
Yes.	Yes.	Yes.	Yes.	Yes.	Yes.	Yes.	Yes.
Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
No	No	No	No	No	No	No	No
No	Yes	Yes	No	No	No	No	No
No	No	No	No	No	No	No	No
No	No	No	No	No	No	No	No
No	Yes	No	No	No	Yes	Yes	Yes
No	No	No	No	No	No	No	No
No	No	No	No	No	No	No	No
1	1	1	1	1	0	0	0

Curcumin	Alpha.-Longipinene	Alpha.-Tocospir	Alpha.-Tocospiro A	Tetradecanoic acid	Palmitic acid, ethyl ester
3.27	3.25	5.18	5.18	3.32	4.65
Soluble	Moderately soluble	Poorly soluble	Poorly soluble	Moderately soluble	Moderately soluble
High	Low	Low	Low	High	High
No.	No.	No.	No.	Yes.	No.
No.	No.	No.	No.	No.	No.
No.	No.	No.	No.	No.	No.
Yes.	No.	Yes.	Yes.	Yes.	Yes.
No	No	No	No	Yes	Yes
No	Yes	No	No	No	No
Yes	Yes	No	No	No	No
No	No	No	No	No	No
No	No	No	No	Yes	No
No	No	No	No	No	No
No	No	Yes	Yes	No	No
No	No	Yes	Yes	No	No
0	1	0	0	0	1

6.2.5 Toxicity prediction

The entire compounds were found to be non-carcinogenic and non-mutagenic in nature.

6.3 Phase-III: Formulation & Quality Assurance Techniques Approach

6.3.1 Development and Optimization of cocoa granules.

13 formulations were developed and optimization was performed utilizing DOE

6.3.2 QA & QC Evaluation of formulations for different parameters.

The prepared cocoa granules were evaluated and data were enclosed in table no 6.26 and table no 6.27 for N*igella sativa* and *Pimpinella anisum* respectively. This information made it abundantly evident that the evaluation parameters for every batch were within acceptable limits.

Table no. 6.26 Evaluation of cocoa granules of *Nigella sativa*

Parameter	F1	F2	F3	F4	F5	F6	F7	F8	F9
Moisture content (LOD) (%)	2.39±0.036	2.80±0.015	3.00±0.265	1.26±0.015	1.37±0.025	1.57±0.025	1.78±0.021	1.81±0.047	1.89±0.040
Bulk density (gm/ml)	0.60±0.021	0.51±0.015	0.57±0.015	0.54±0.020	0.55±0.032	0.57±0.015	0.42±0.026	0.60±0.010	0.60±0.100
Tapped density (gm/ml)	0.70±0.025	0.54±0.040	0.61±0.038	0.55±0.021	0.59±0.035	0.60±0.010	0.34±0.021	0.65±0.026	0.64±0.031
Angle of repose (degrees)	00.29±0.068	20.80±0.100	16.69±0.042	28.36±0.050	21.33±0.351	37.23±0.051	23.43±0.150	19-79±0.045	17.74±0.042
Flow rate (g/s)	10.20±0.040	6.25±0.012	2.22±0.026	7.30±0.066	7.69±0.089	9.01±0.015	5.26±0.035	7.30±0.200	7.14±0.026
Hausner's ratio	1.19±0.015	1.06±0.006	1.08±0.015	1.02±0.025	1.04±0.023	1.09±0.046	1.06±0.042	1.09±0.025	1.06±0.006
% compressibility index (%)	15.50±0.265	5.60±0.100	6.60±0.032	1.80±0.040	6.80±0.100	5.00±0.044	5.90±0.080	7.70±0.100	6.30±0.015

Time for solubility (min)	0.51±0.006	1.10±0.040	1.54±0.021	0.58±0.006	1.22±0.012	1.58±0.031	1.03±0.006	1.38±0.023	1.68±0.025
Disintegration time (min)	0.53±0.020	1.00±0.007	1.29±0.049	1.00±0.012	1.27±0.019	1.46±0.006	1.04±0.040	1.34±0.049	1.57±0.015

Table no. 6.27 Evaluation of cocoa granules of *Pimpinella anisum*

Parameter	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**
Moisture content (LOD) (%)	2.5±0.074	2.8±0.030	3.17±0.025	1.22±0.020	1.34±0.021	1.48±0.021	1.68±0.036	1.74±0.021	1.83±0.021
Bulk density (gm/ml)	0.62±0.021	0.53±0.025	0.58±0.010	0.5±0.015	0.52±0.025	0.58±0.026	0.49±0.035	0.60±0.015	0.59±0.026
Tapped density (gm/ml)	0.72±0.030	0.58±0.010	0.65±0.006	0.57±0.010	0.58±0.015	0.61±0.010	0.53±0.032	0.65±0.025	0.63±0.025
Angle of repose (degrees)	23.01±0.055	21.8±0.026	16.88±0.056	28.34±0.038	22.05±0.031	36.89±0.036	24.16±0.072	20.99±0.095	18.12±0.045
Flow rate (g/s)	10.39±0.053	7.04±0.021	3.42±0.010	7.37±0.021	7.74±0.044	8.79±0.030	5.26±0.026	6.81±0.021	7.18±0.038
Hausner's ratio	1.17±0.006	1.05±0.010	1.09±0.017	1.01±0.015	1.04±0.031	1.1±0.021	1.07±0.025	1.07±0.010	1.06±0.032

% compressibility index (%)	14.2±0.038	5.7±0.021	6.9±0.040	1.7±0.021	7.1±0.038	5.4±0.031	6.10±0.021	7.40±0.021	6.0±0.032
Time for solubility (min)	0.49±0.015	1.12±0.025	1.58±0.032	0.59±0.015	1.22±0.015	1.57±0.012	1.01±0.015	1.36±0.021	1.69±0.026
Disintegration time (min)	0.40±0.006	0.59±0.071	1.49±0.029	0.53±0.012	1.17±0.034	1.43±0.001	57±0.005	1.26±0.014	1.54±0.028

6.3.3. Statistical analysis and optimization of formulation

6.3.3.1 Data analysis

Thirteen formulations of *Nigella sativa* and *Pimpinella anisum* were prepared separately as per the central composite design utilizing Design-Expert version 13 (DOE) software. The loss on drying and time of solubility were considered as dependent variable responses formulations. From fit summary using Design-Expert software, It was clear that and quadratic model for both loss on drying and time of solubility were suggested for *Nigella sativa* cocoa granules (table no. 6.28) whereas for *Pimpinella anisum* formulation quadratic model for both loss on drying and time of solubility were suggested (Table no 6.29).

Table no. 6.28 Experimental design and observed response of cocoa granules of *Nigella sativa* in central composite design

Sr. no	**Factor- 1**	**Factor2**	**Response 1**	**Response 2**
	A:Sucrose	**B:Starch paste**	**Loss on drying**	**Time of solubility**
	gm	%	%	Min
1	11.5	7	1.89	1.68

2	11.5	5	1.57	1.58
3	11.5	3	3	1.54
4	9.5	7	1.81	1.38
5	9.5	5	1.34	1.19
6	9.5	5	1.3	1.18
7	9.5	5	1.42	1.21
8	9.5	5	1.36	1.2
9	9.5	5	1.37	1.22
10	9.5	3	2.8	1.1
11	7.5	7	1.78	1.03
12	7.5	5	1.26	0.58
13	7.5	3	2.4	0.51

Table no. 6.29 Experimental design and observed response of cocoa granules of *Pimpinella anisum* in central composite design

Sr.no	**Factor- 1**	**Factor 2**	**Response 1**	**Response 2**
	A:Sucrose	**B:Starch paste**	**Loss on drying**	**Time of solubility**
	gm	%	%	Min
1	11.5	7	1.83	1.69
2	11.5	5	1.48	1.57
3	11.5	3	3.17	1.58
4	9.5	7	1.74	1.36
5	9.5	5	1.37	1.2
6	9.5	5	1.33	1.19
7	9.5	5	1.4	1.21

8	9.5	5	1.34	1.22
9	9.5	5	1.39	1.17
10	9.5	3	2.8	1.12
11	7.5	7	1.68	1.01
12	7.5	5	1.22	0.59
13	7.5	3	2.5	0.49

In case of *Nigella sativa* formulation, statistical summary of response shown in table no 6.30 indicates R^2 value 99.52%, 99.03% for loss on drying and time of solubility respectively. In case of loss on drying response the model F-value of 292.26 implies the model was significant Furthermore, the Lack of Fit F-value was 2.09. Additionally in case of time of solubility model F-value of 143.24 implies the model was significant. Furthermore, the Lack of Fit F-value was 16.99 suggests the Lack of Fit was not significant relative to the pure error of the above two parameters.

Table no. 6.30 Statistical summary of the response *(Nigella sativa)*

Fit statistics	Loss on drying	Time of solubility
Std. Dev.	0.0531	0.0443
Mean	1.79	1.18
C.V. %	2.96	3.74
R^2	0.9952	0.9903
Adjusted R^2	0.9918	0.9834
Predicted R^2	0.9727	0.9156
Adeq Precision	50.3934	40.0816

Table no. 6.31 ANOVA for Quadratic model Response 1: loss on drying *(N. sativa)*

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	4.12	5	0.8234	292.26	< 0.0001	Significant
A:Sucrose	0.1734	1	0.1734	61.55	0.0001	
B:Starch paste	1.23	1	1.23	437.65	< 0.0001	
AB	0.0600	1	0.0600	21.30	0.0024	
A^2	0.0004	1	0.0004	0.1269	0.7321	
B^2	2.24	1	2.24	796.47	< 0.0001	
Residual	0.0197	7	0.0028			
Lack of Fit	0.0120	3	0.0040	2.09	0.2442	not significant
Pure Error	0.0077	4	0.0019			
Cor Total	4.14	12				

Table no. 6.32 ANOVA for Quadratic model Response 2: time of solubility *(N. sativa)*

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Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.41	5	0.2813	143.24	< 0.0001	Significant
A:Sucrose	1.20	1	1.20	609.59	< 0.0001	
B:Starch paste	0.1473	1	0.1473	74.99	< 0.0001	
AB	0.0361	1	0.0361	18.38	0.0036	
A^2	0.0205	1	0.0205	10.45	0.0144	
B^2	0.0150	1	0.0150	7.66	0.0278	
Residual	0.0137	7	0.0020			

Lack of Fit	0.0127	3	0.0042	16.99	0.0097	Significant
Pure Error	0.0010	4	0.0003			
Cor Total	1.42	12				

The figure given below indicates actual loss on drying, Predicted VS actual loss on drying and predicted VS actual time of solubility for *N. sativa* cocoa granules.

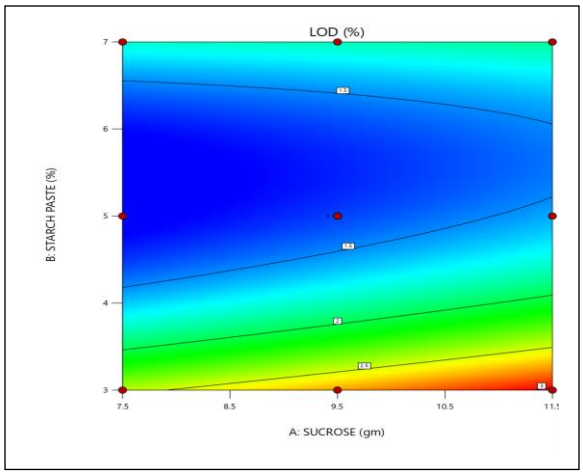

Fig no. 6.17 Actual loss on drying (*N.sativa*)

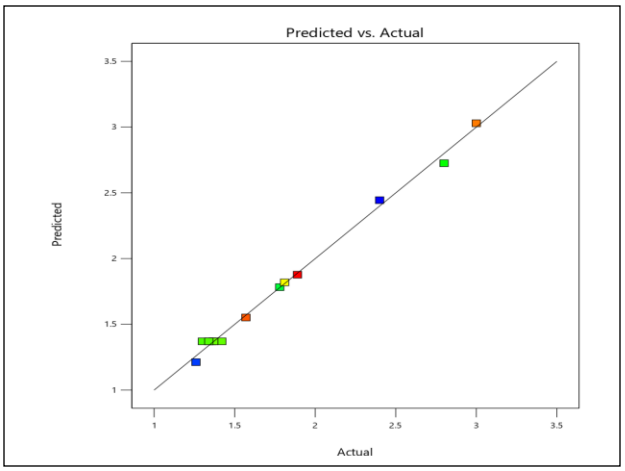

Fig no. 6.18 Predicted VS actual loss on drying (*N.sativa*)

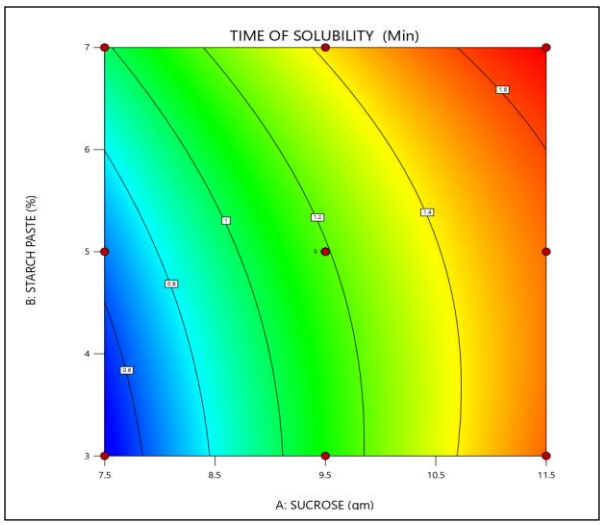

Fig no. 6.19 Actual time of solubility (*N.sativa*)

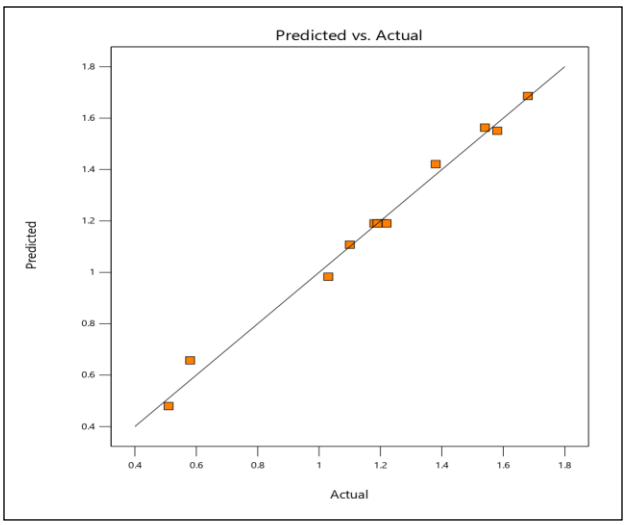

Fig no. 6.20 Predicted VS actual time of solubility (*N.sativa*)

3D surface plot of *Nigella sativa* cocoa granules for loss on drying and time of solubility were shown in figure given below

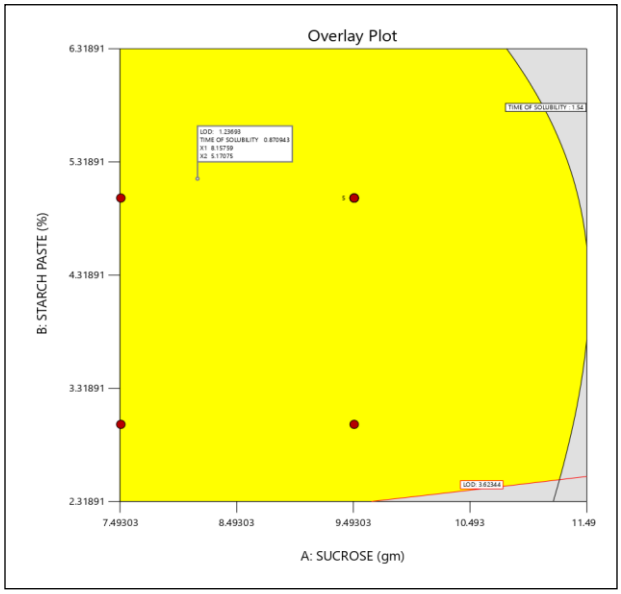

Fig no. 6.21 Optimization of *N. sativa* overlay plot

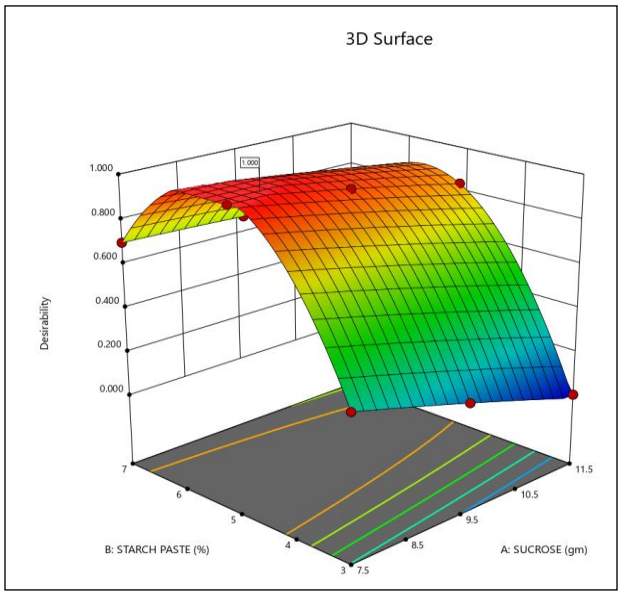

Fig no. 6.22 Desirability 3D surface response (*N. sativa*)

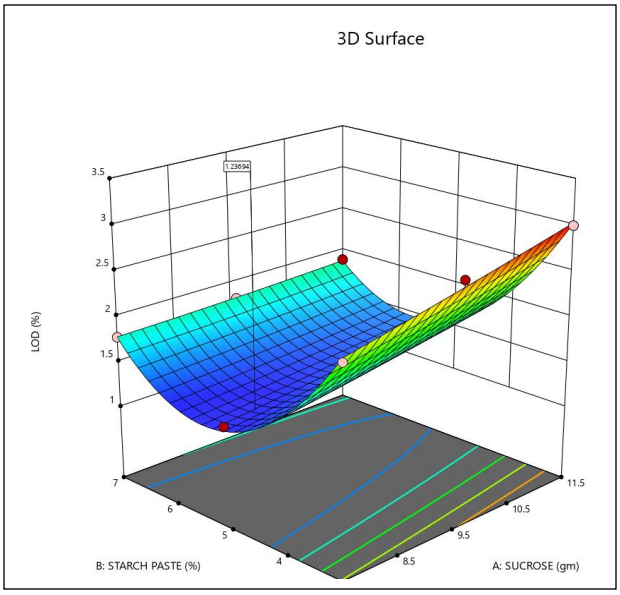

Fig no. 6.23 Loss on drying 3D surface response (*N. sativa*)

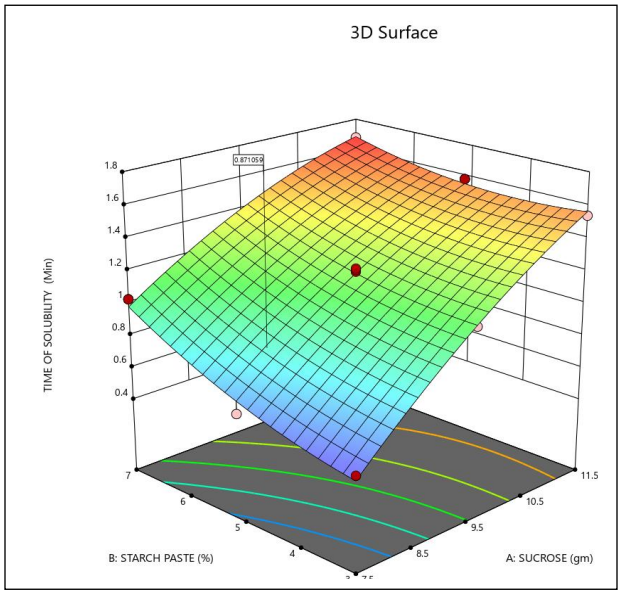

Fig no. 6.24 Time of solubility 3D surface response (*N. sativa*)

In case of *Pimpinella anisum* formulation, statistical summary of response shown in table no 6.33 indicates R2 value 99.74%, 99.33% for loss on drying and time of solubility respectively. In case of loss on drying response the model F-value of 532.12 implies the model was significant. Furthermore, the Lack of Fit F-value was 3.15. Additionally in case of time of solubility model F-value of 207.95 implies the model was significant. Furthermore, the Lack of fit F-value was 7.49 suggests the lack of fit was not sigificant relative to the pure error of the above two parameters.

Table no. 6.33 Statistical summary of the response *(Pimpinella anisum)*

Fit statistics	Loss on drying	Time of solubility
Std. Dev.	0.0423	0.0374
Mean	1.79	1.18
C.V. %	2.36	3.16
R^2	0.9974	0.9933
Adjusted R^2	0.9955	0.9885
Predicted R^2	0.9809	0.9486
Adeq Precision	67.9059	47.4916

Table no. 6.34 ANOVA for Quadratic model Response 1: loss on drying *(P. anisum)*

Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	
Model	4.76	5	0.9512	532.12	< 0.0001	significant
A:Sucrose	0.1944	1	0.1944	108.75	< 0.0001	
B:Starch paste	1.73	1	1.73	966.74	< 0.0001	
AB	0.0676	1	0.0676	37.82	0.0005	
A^2	0.0000	1	0.0000	0.0222	0.8857	

B^2	2.36	1	2.36	1318.58	< 0.0001	
Residual	0.0125	7	0.0018			
Lack of Fit	0.0088	3	0.0029	3.15	0.1483	not significant
Pure Error	0.0037	4	0.0009			
Cor Total	4.77	12				

Table no. 6.35 ANOVA for Quadratic model Response 2: time of solubility *(P. anisum)*

Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	
Model	1.45	5	0.2909	207.95	< 0.0001	significant
A:Sucrose	1.26	1	1.26	901.12	< 0.0001	
B:Starch paste	0.1261	1	0.1261	90.19	< 0.0001	
AB	0.0420	1	0.0420	30.05	0.0009	
A^2	0.0195	1	0.0195	13.92	0.0073	
B^2	0.0160	1	0.0160	11.42	0.0118	
Residual	0.0098	7	0.0014			
Lack of Fit	0.0083	3	0.0028	7.49	0.0406	significant
Pure Error	0.0015	4	0.0004			
Cor Total	1.46	12				

6.3.3.2. Response surface analysis

3D surface plot of *Pimpinella anisum* cocoa granules for loss on drying and time of solubility were shown in figure given below

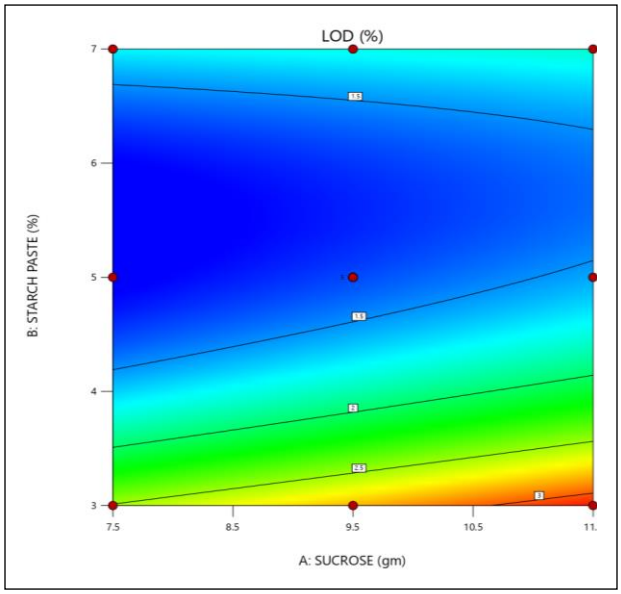

Fig no. 6.25 Actual loss on drying (*P. anisum*)

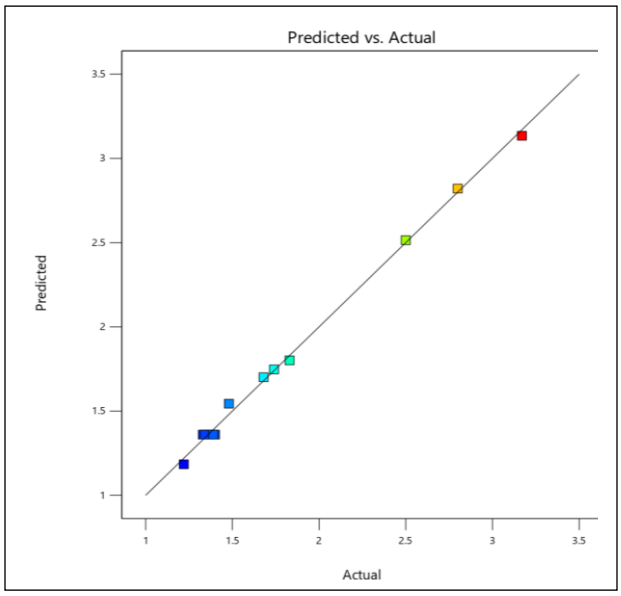

Fig no. 6.26 Predicted VS actual loss on drying *(P. anisum)*

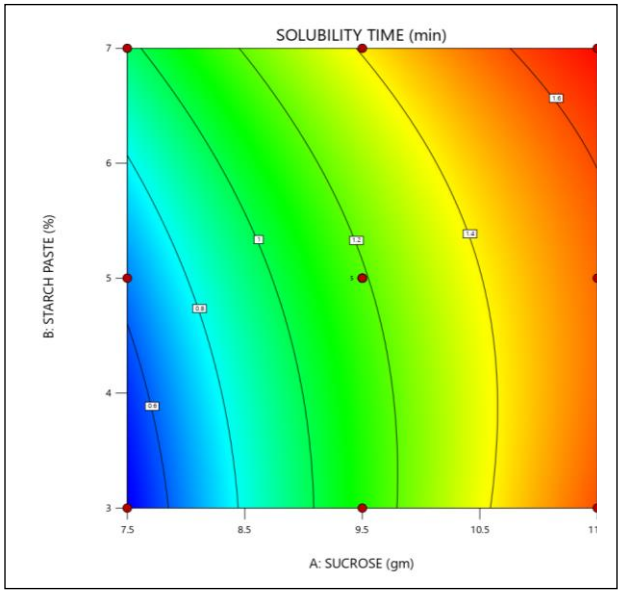

Fig no. 6.27 Actual time of solubility (*P. anisum*)

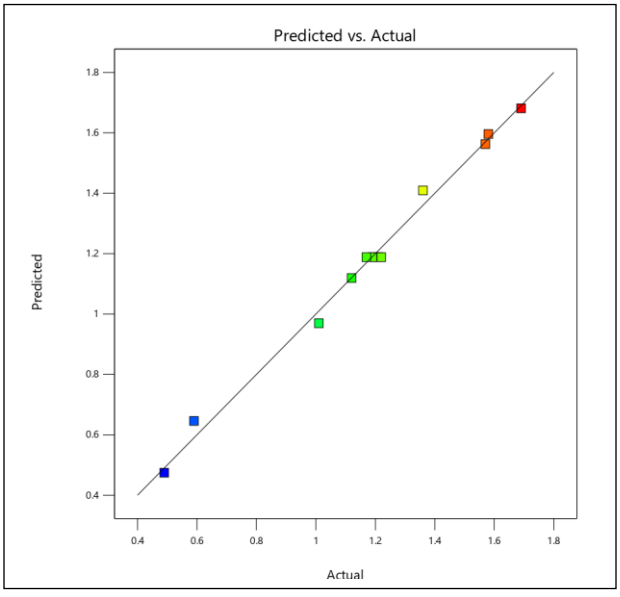

Fig no. 6.28 Predicted VS actual time of solubility (*P. anisum*)

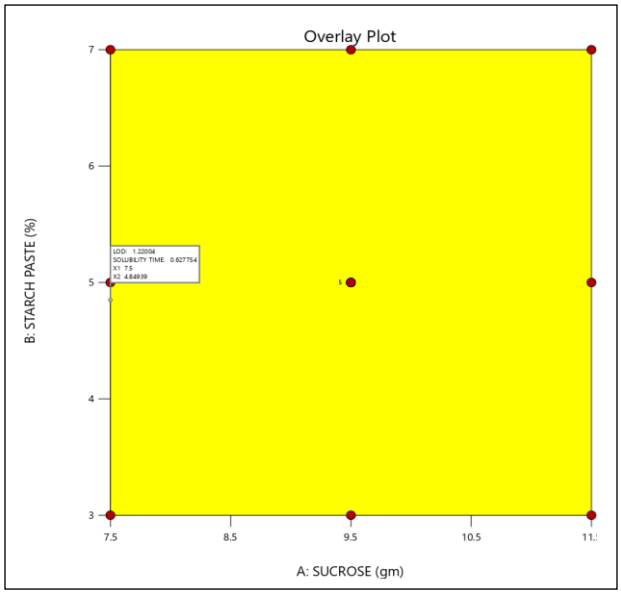

Fig no. 6.29 Optimization of *P. anisum* overlay plot

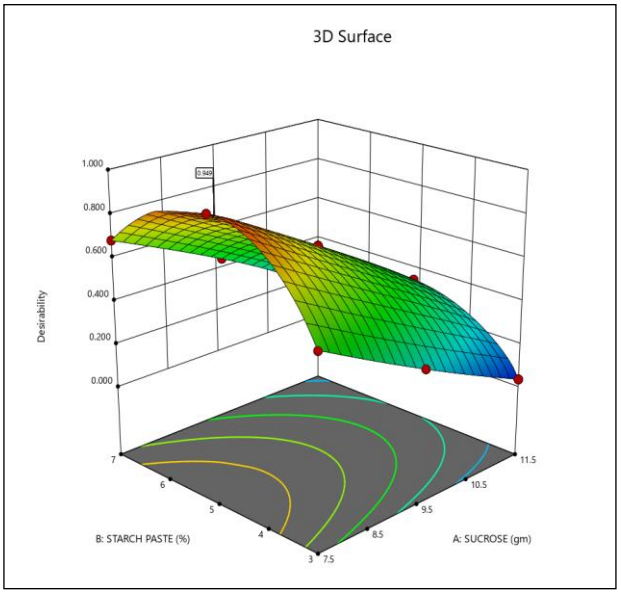

Fig no. 6.30 Desirability 3D surface response (*P. anisum*)

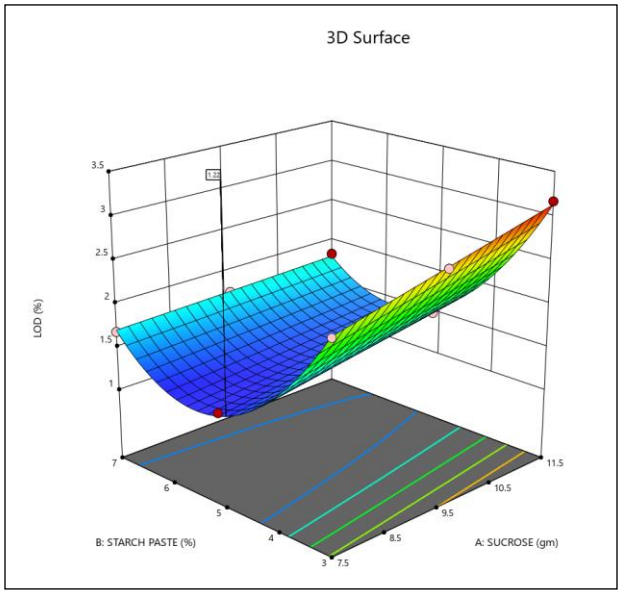

Fig no. 6.31 Loss on drying 3D surface response (*P. anisum*)

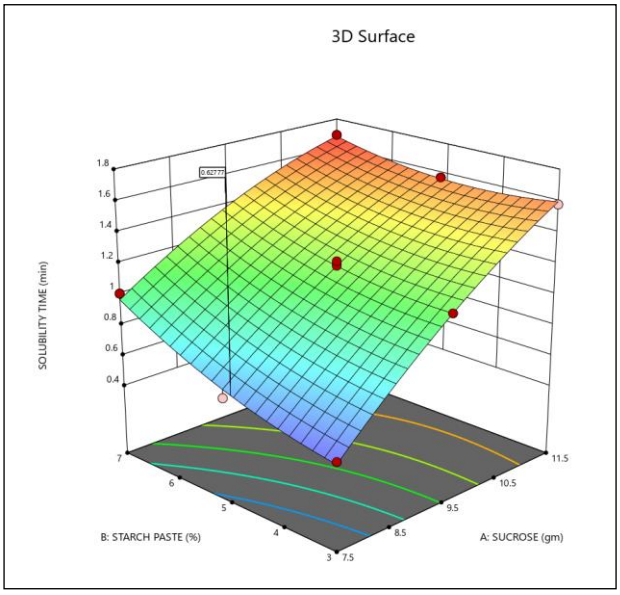

Fig no. 6.32 Time of solubility 3D surface response (*P. anisum*)

Table no. 6.36 Solutions suggested using Design-expert

	Nigella sativa		***Pimpinella anisum***	
	Actual (F4)	Suggested	Actual (F4)	Suggested
sucrose	7.5	8.158	7.5	7.500
Starch paste	5	5.171	5	4.849
Response 1: Loss on drying	1.26	1.237	1.22	1.220
Response 2: time of solubility	0.58	0.871	0.59	0.628
Desirability	-	1.000	-	0.949

6.4 Phase-IV: Pharmacological Approach

6.4.1 *In-vitro* antioxidant activity

6.4.1.1 Anti-oxidant activity by DPPH method

Table no. 6.37 *In-vitro* Antioxidant study by DPPH assay

Sr. no.	**Sample (1mg/ml)**	**Absorbance**	**Mean**	**Percentage of DPPH radical scavenging**
1	Control	1.201	1.267	-
		1.307		
		1.293		
2	Standard (Ascorbic acid)	0.492	0.492	61.16
		0.489		
		0.496		
3	*Pimpinella anisum* extract	0.559	0.557	56.04
		0.549		
		0.565		
4	*Nigella sativa* extract	0.473	0.468	63.06
		0.483		
		0.449		

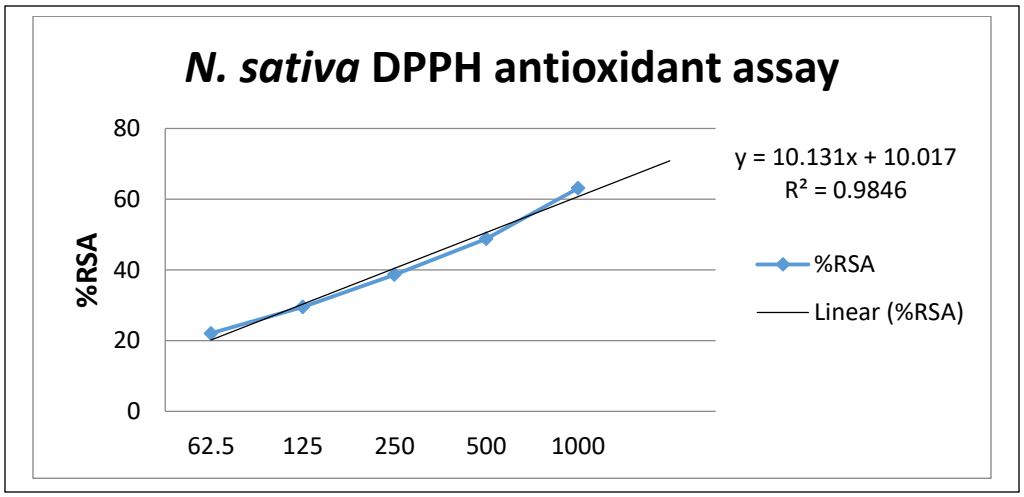

Fig no. 6.33 *In vitro* antioxidant activity of *N. sativa* (DPPH)

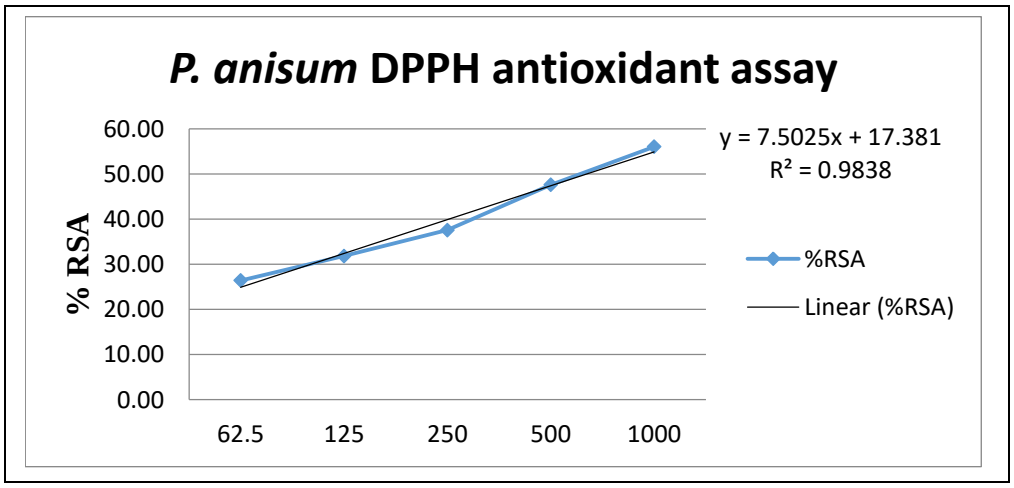

Fig no. 6.34 *In vitro* antioxidant activity of *P. anisum* (DPPH)

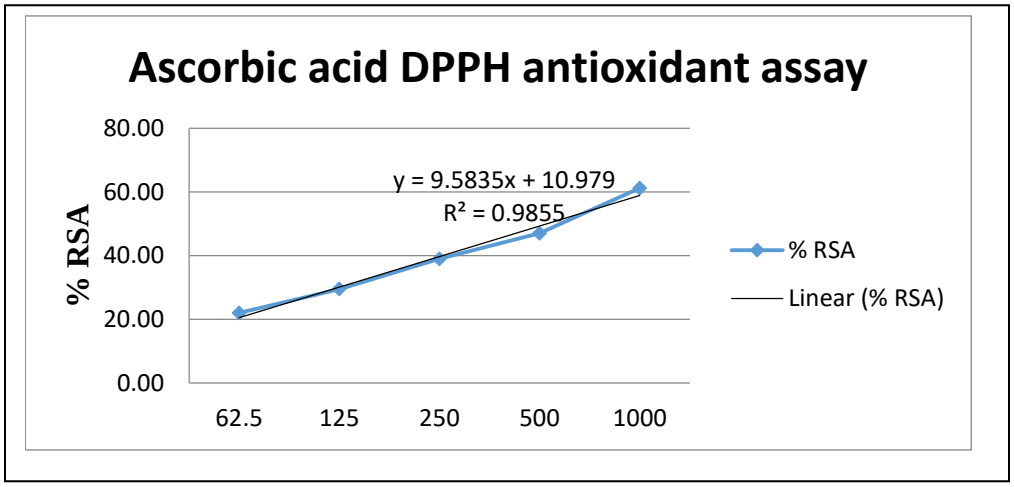

Fig no. 6.35 *In vitro* antioxidant activity of ascorbic acid (DPPH)

6.4.1.2 Nitric oxide radical scavenging (NO) assay

From the *In vitro* antioxidant assay it was clear that both the drug possess good antioxidant potential. *Nigella sativa* shows higher antioxidant potential as compared to *Pimpinella anisum.* The % Nitric oxide radical scavenging activity was observed in table no. 6.38, while the graphs (Fig no. 6.36 and 6.37) indicate the increase in radical scavenging activity as concentration increases for *Pimpinella anisum* and *Nigella sativa* respectively.

Table no. 6.38 *In-vitro* Antioxidant study by Nitric oxide assay

Sr.no	Sample	Concentration (µg/ml)	Mean	%Nitric oxide radical scavenging activity
	control	-	0.75	-
1	PAE	200	0.57	24.00
		400	0.47	37.33
		600	0.39	48.00
		800	0.33	56.00
		1000	0.26	65.33
2	NSE	200	0.24	68.00
		400	0.22	70.66
		600	0.20	73.33
		800	0.18	76.00
		1000	0.17	77.33

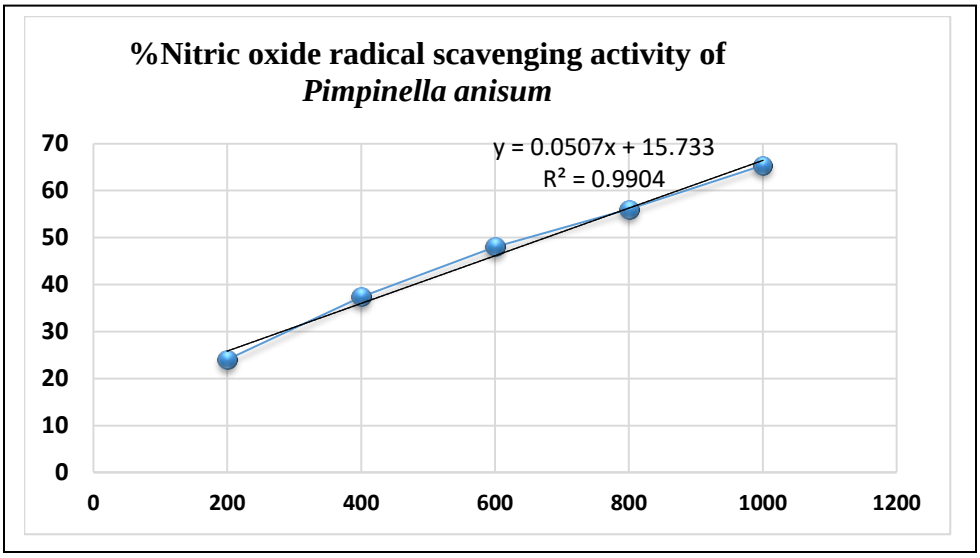

Fig no. 6.36 *Pimpinella anisum* antioxidant activity by Nitric oxide assay

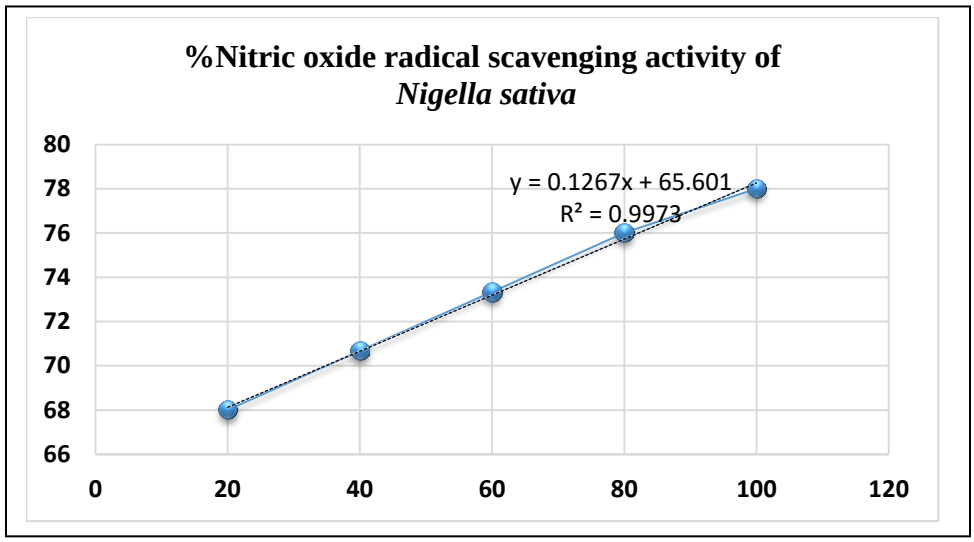

Fig no. 6.37 *Nigella sativa* antioxidant activity by Nitric oxide assay

6.4.2 *In-vitro* THP 1 cell line study

6.4.2.1 MTT assay

Initial investigations were carried out using the MTT assay to exclude out any harmful effect of NSE and PAE on THP-1-derived macrophages and to determine its non-lethal doses. It emerged how NSE and PAE affected the viability of THP-1-derived

macrophages when LPS was present. Cell viability was assessed using the MTT test after various quantities of NSE and PAE were added to the culture medium along with the administration of LPS (50 ng/mL for 24 h). The proportion of viable cells in treated cultures compared to untreated cultures is used to express the results. According to the assay's findings, pre-treating THP-1-derived macrophages with varying doses of NSE and PAE (5-500 g/mL) for 24 hours while simultaneously exposing them to LPS for 24 hours did not negatively impact cell viability at concentrations of 5 to 25 g/mL. Fig no 6.38. However, from 50 to 500 g/mL, cell viability drastically dropped. These findings show that NSE and PAE are not hazardous up to a concentration of 25 g/mL, hence these values were employed for further experiments.

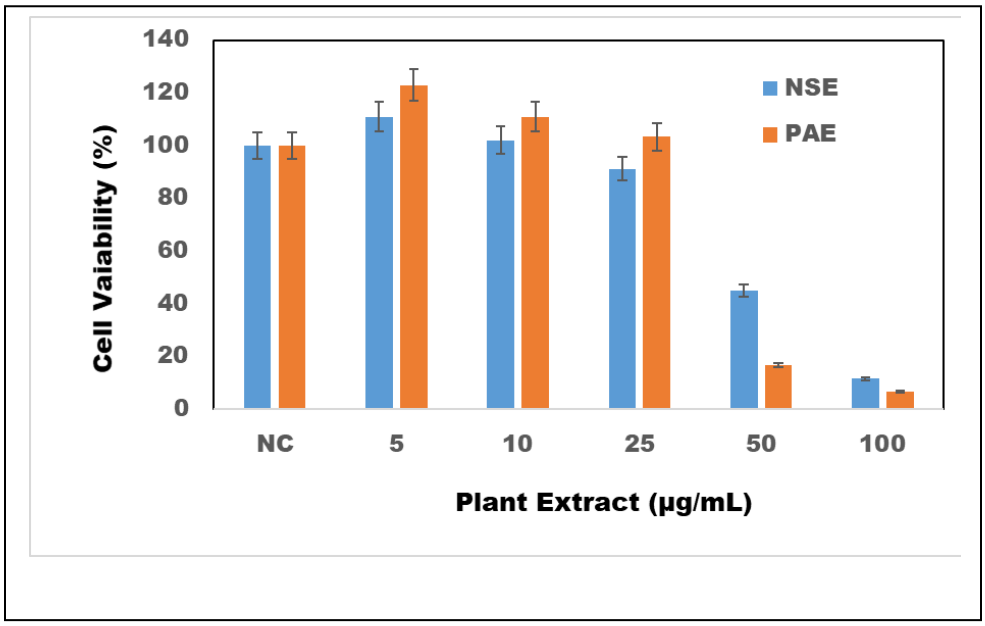

Fig no. 6.38 THP 1 cell viability along with NSE and PAE

6.4.2.2 Cytokine estimation by ELISA

For 24 hours, the cells were exposed to LPS along with NSE, and PAE in escalating concentrations. Then, cytokine secretion in the culture media was assessed using an ELISA technique.

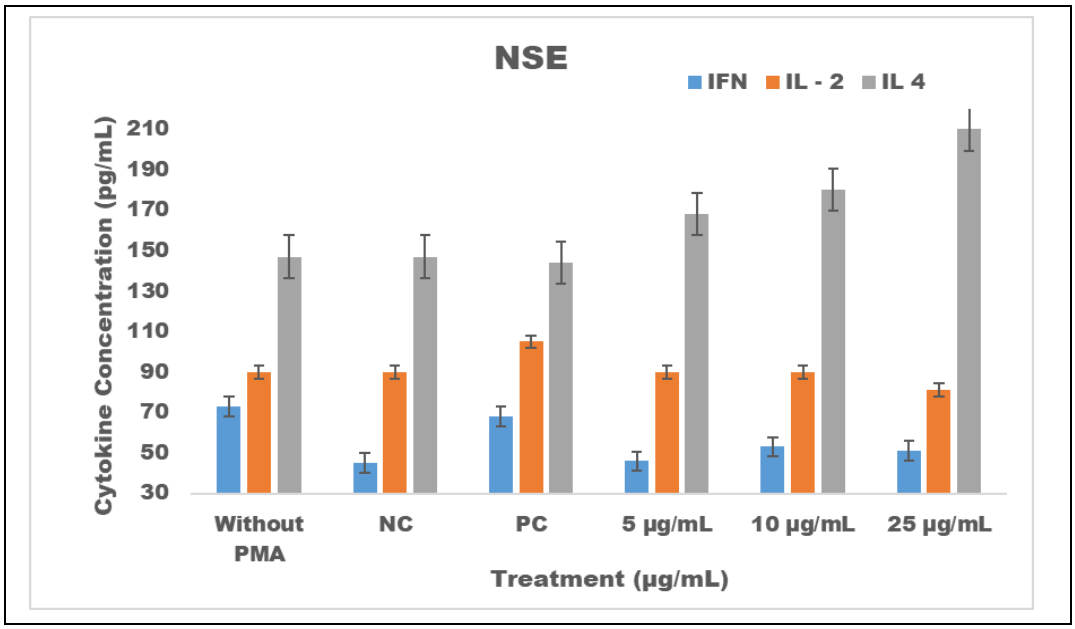

Fig no. 6.39 THP 1 cell treated with NSE at different concentrations for 24hrs

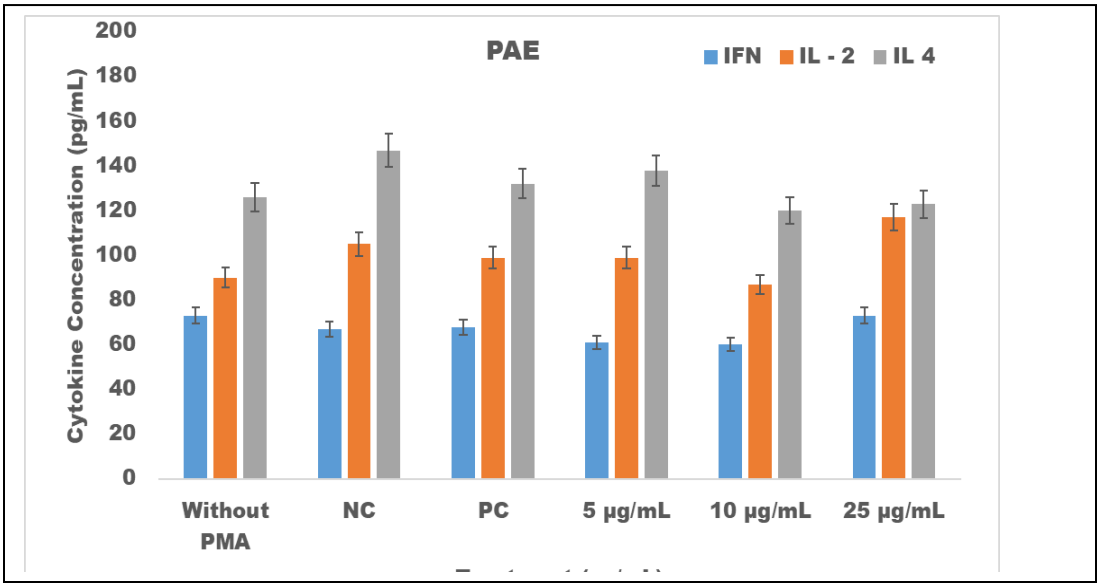

Fig no. 6.40 THP 1 cell treated with PAE at different concentrations for 24hrs

To understand the effect of NSE and PAE on LPS-stimulated IL-2, IL-4 and IFN-α production were assed using ELISA. As shown in Fig no. 6.39, Compared to control cells, NSE treating THP-1-derived macrophages with LPS alone for 24 h increased the production of IL-4 and decreased the release of IL-2 and IFN- relative to control cells.

The treatment with PMA and LPS increased the overproduction of IL- 2 and IFN – α in THP-1-derived macrophages. On the other hand, after THP-1 exposure to NSE, the anti-inflammatory cytokine IL-4 generation was dramatically enhanced. Whereas the effect was reverse in case of PAE treatment Fig no. 6.40 Where IL- 4 production decreased and IL – 2 increase in comparison to control cells. IFN – α in PAE did not show any varied results.

6.4.3 Acute toxicity study

The acute Toxicity study of Formulation 1 NSEF & Formulation 2 PAEF revealed that all of the BALB/c mice given 2000 mg/kg of the drug were healthy, active, and did not exhibit any adverse effects.

6.4.4Lymphocyte Proliferation Assay

The dose was significantly increased for instance 50µg/ml to 100µg/ml, 200µg/ml, 400µg/ml and 1600µg/ml. Lymphocytes proliferation was significantly high at 400µg/ml with NSEF and PAEF. Additionally, Lymphocytes proliferation was significantly high for both formulations at the dose of 1600µg/ml as compared to anti- CD3. NSEF shows higher significance compared to PAEF.

Table no. 6.39 Absorbance of control and test samples recorded at 450nm

Test sample	**Dose (µg/ml)**	**Mean**	**Mean**±SD
Blank	-	0.1899	0.1899±0.0018
Anti-CD3	2	0.5447	0.5447±0.0021
NSEF 50	50	0.3919	0.3919±0.2591
NSEF 100	100	0.5253	0.5253±0.0031
NSEF 200	200	0.5345	0.5345±0.0042
NSEF 400	400	0.5650	0.5650±0.0035
NSEF 1600	1600	0.6101	0.6101±0.0033
PAEF 50	50	0.5251	0.5251±0.0045

PAEF 100	100	0.5293	0.5293±0.0015
PAEF 200	200	0.5619	0.5619±0.0057
PAEF 400	400	0.5751	0.5751±0.0024
PAEF 1600	1600	0.6190	0.6190±0.0041

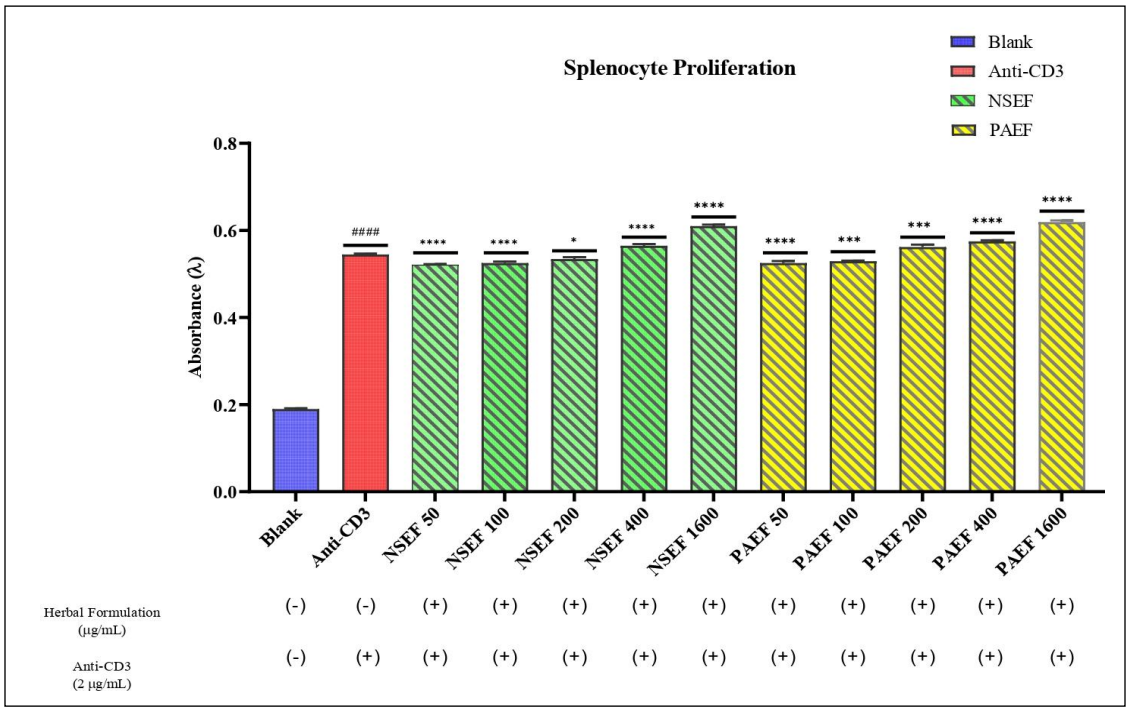

Fig no. 6.41 the proliferative effects of the Herbal formulations on the splenocytes

Data presented as Mean ± SEM one way ANOVA followed by Tukey's multiple comparisons test were applied, DFn= 2, *P<0.0001 as compared to NSEF and PAEF.

6.5 Phase-V: Analytical Chemistry Approach

6.5.1 Analytical Method Development using gas chromatography.

6.5.1.1 Calibration curve of P- anisaldehyde using gas chromatography

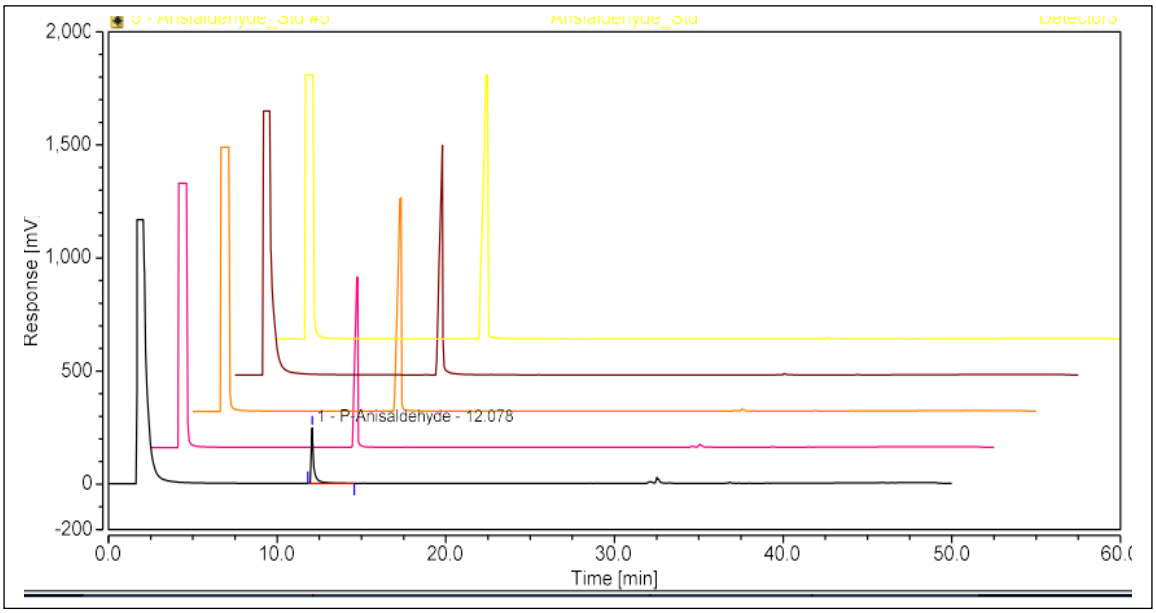

Fig no. 6.42 Overlay graph of standard P-anisaldehyde

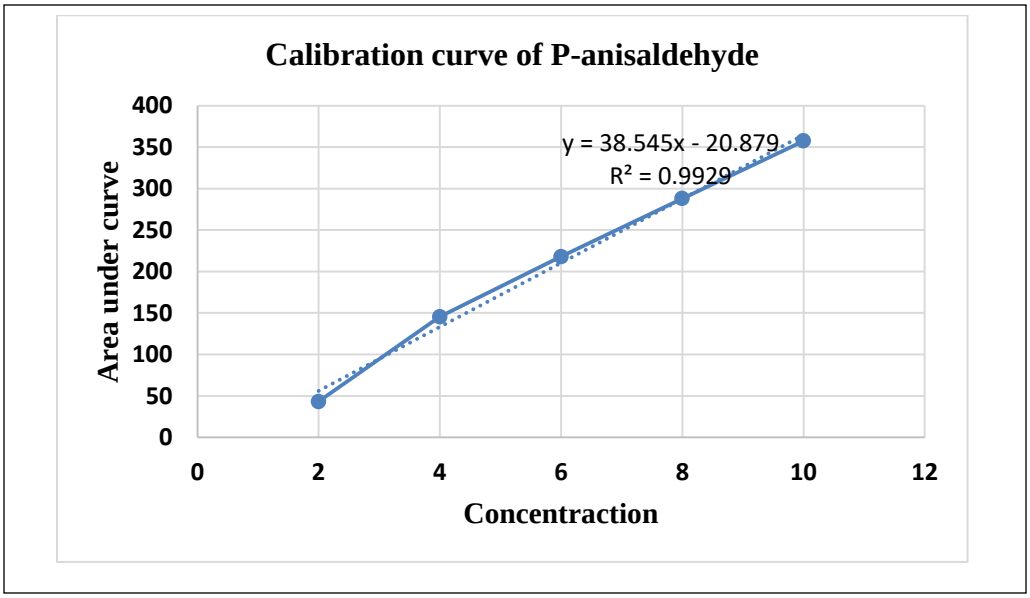

Fig no. 6.43 Calibration curve of standard P-anisaldehyde

The overlay graph of P-anisaldehyde was observed in fig. no 6.42 while, the standard P-anisaldehyde calibration curve (Y = 38.545x -20.879, R^2 = 0.9929) was plotted in fig no. 6.43

6.5.1.2 Calibration curve of anethole using gas chromatography

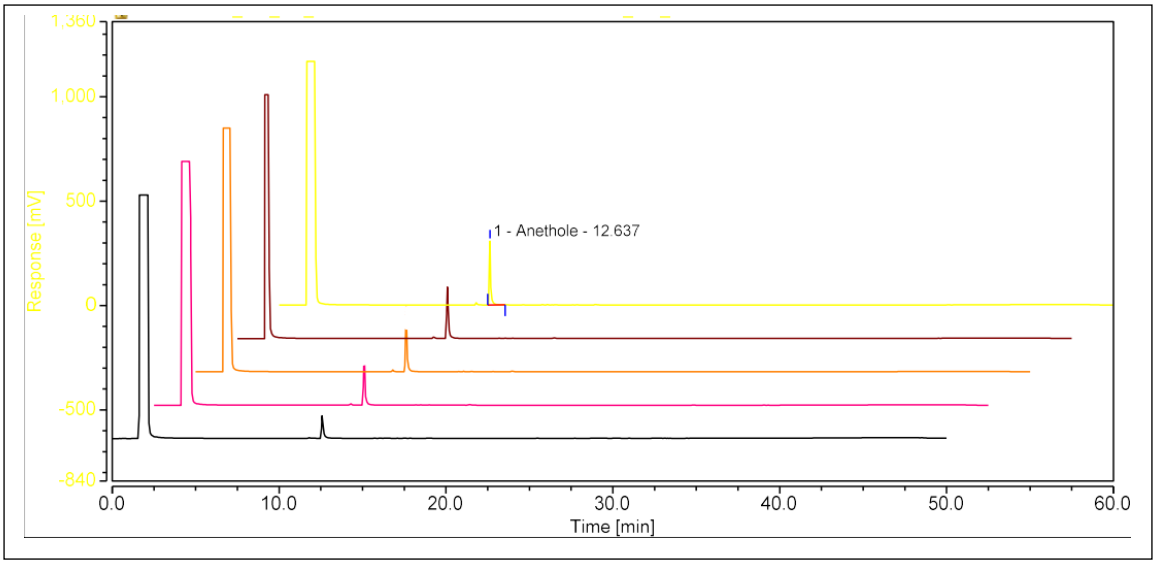

Fig no. 6.44 Overlay graph of standard anethole

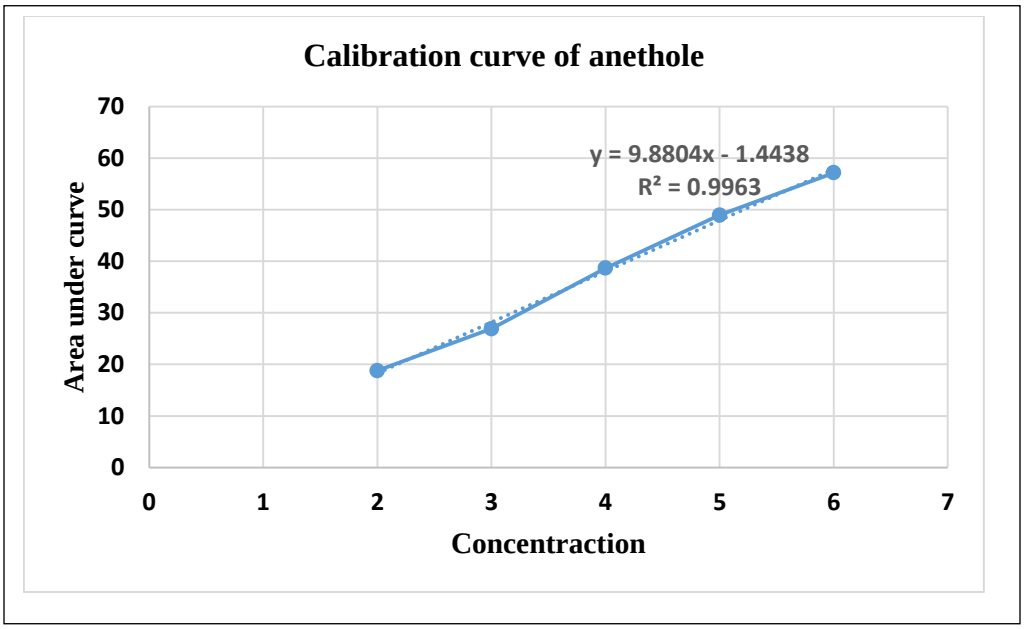

Fig no. 6.45 Calibration curve of standard anethole

The overlay graph of anethole was observed in fig. no 6.44 while, the standard anethole calibration curve (Y = 0.9674x -0.5156, R^2 = 0.998) was plotted in fig no. 6.45

6.5.2 Validation of the analytical method

6.5.2.1 Validation of the analytical method for P-anisaldehyde determination

GC method has been developed and validated using P-anisaldehyde. The active constituent can be easily found using this analytical approach. This developed method shows good linearity, accuracy, precision and repeatability with P-anisaldehyde. The intraday and interday readings of P-anisaldehyde were mentioned in table no. 6.40.Additionally the LOD (lower limit of detection) was 0.9 PPM, while the LOQ (limit of quantification) was determined as 2 PPM.

Table no. 6.40 Accuracy and Precision of P-anisaldehyde (Intra and Interday)

Concentration (PPM)	**INTRADAY (P-anisaldehyde)**			**INTERDAY(P-anisaldehyde)**		
	Calculated conc.	Precision (% RSD)	Accuracy (% Error)	Calculated conc.	Precision (% RSD)	Accuracy (% Error)
2 PPM	1.89 ± 0.003	0.000371	-5.5	1.68 ± 0.01	0.00037	-16
6 PPM	6.01 ± 0.002	0.002092	0.16666	6.18 ± 0.04	0.00022	3
10 PPM	9.87 ± 0.0001	0.000125	-1.3	9.81 ± 0.04	0.00012	-1.9

6.5.2.2 Validation of the analytical method for Anethole determination

GC method has been developed and validated using Anethole. The active constituent can be easily found using this analytical approach. This developed method shows good linearity, accuracy, precision and repeatability with anethole. The intraday and interday readings of anethole were mentioned in table no. 6.41. Additionally the LOD (lower limit of detection) was 0.5 PPM, while the LOQ (limit of quantification was determined as 1.7 PPM

Table no. 6.41 Accuracy and Precision of Anethole (Intra and Interday)

Concentration	INTRADAY (Anethole)			INTERDAY(Anethole)		
	Calculated conc.	Precision (% RSD)	Accuracy (% Error)	Calculated conc.	Precision (% RSD)	Accuracy (% Error)
2	2.04 ± 0.059	0.00316	2.272	2.01 ± 0.0041	0.00022	0.947
4	4.05 ± 0.003	0.00009	1.450	4.03 ± 0.0046	0.00012	0.809
6	5.93 ± 0.015	0.00028	-1.151	5.96± 0.0124	0.00022	-0.648

6.5.3 Quantification

6.5.3.1Quantification of P-anisaldehyde in cocoa granules using gas chromatography

For the purpose of quantification of P-anisaldehyde in *Pimpinella anisum* extract and formulation gas chromatography were utilized. By using the percent assay calculation method the unknown concentration of standard was determined in extract and formulation.

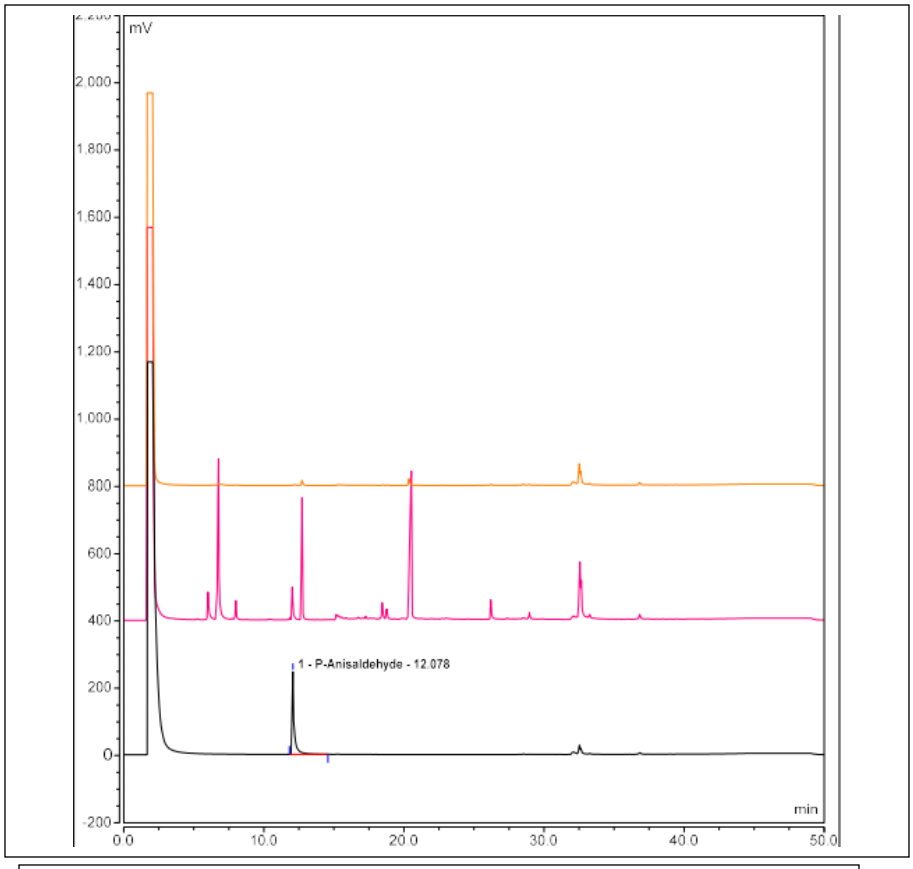

Fig no. 6.46 Overlay graph of PAF, extract and standard

Table no. 6.42 Quantification of P-anisaldehyde in extract and PAF

Sr. no	Sample	RT	Area	P-anisaldehyde concentration (%)
1	*P. anisum* extract	12.728	29.93	27.42
2	Cocoa granules of *P. anisum*	12.717	1.669	1.53

6.5.2.2 Quantification of anethole in cocoa granules using gas chromatography

For the purpose of quantification of anethole in *Nigella sativa* extract and formulation gas chromatography were utilized. Using the percent assay calculation method the unknown concentration of standard was determined in extract and formulation.

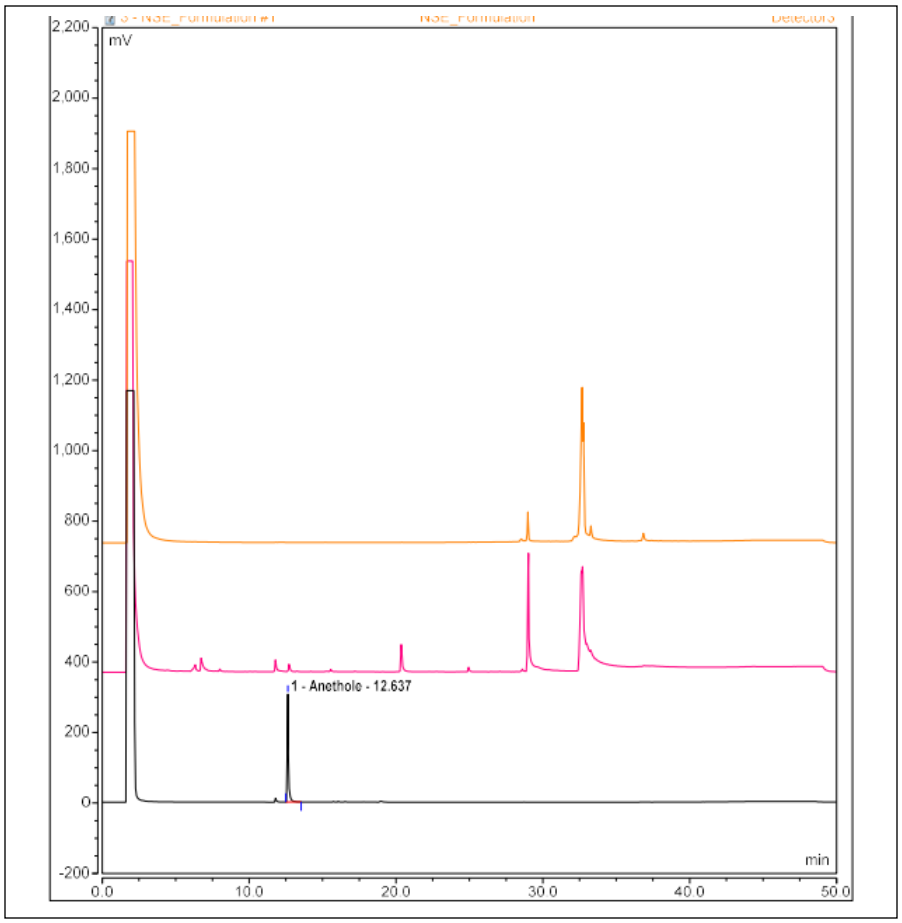

Fig no. 6.47 Overlay graph of NSF, extract and standard

Table no. 6.43 Quantification of anethole in extract and NSF

Sr. no	Sample	RT	Area	Anethole concentration (%)
1	*N. sativa*extract	12.715	2.268	4.35
2	Cocoa granules of *N. sativa*	12.808	0.047	0.09

From the analytical method quantification data it was found that 1.53 % of P-anisaldehyde present in cocoa granules of *Pimpinella anisum* while 27.42% present in ethanolic extract. While in case of *Nigella sativa* 0.09% anethole were quantified in cocoa granules of *N. sativa* and 4.35% in ethanolic extract.

6. DISCUSSION

Medical plants are crucial in the curing of several diseases. As a result, there is a need to certify the superiority of natural medicine. The standardization of herbs is extremely significant. In the current era of medication, the acceptability and use of herbal remedies are significant in terms of extending life by enhancing the immune system (247). Drug research will further explore the extraction of crude drugs for their standardisation, formulation, pharmacological activity, and other aspects. We used the conventional method, cold maceration, to extract *Nigella sativa* and *Pimpinella anisum*, thereby protecting the thermostable components present in crude drugs. As solvents, we used hexane, chloroform, ethanol, acetone, and water. Then, we did phytochemical screening and found that the *Nigella sativa* and *Pimpinella anisum* ethanolic extracts had the highest concentrations of phytoconstituents. To obtain pure components, we used the clevenger apparatus to extract volatile oils from *Nigella sativa* and *Pimpinella anisum*. The crude drugs were evaluated morphologically, microscopically, and physicochemically. The morphological and microscopical properties were the same as those specified in the ayurvedic pharmacopoeia. Furthermore, the extractive values, foreign matter, total insoluble acid, and water-soluble ash value ranges meet the quality standards specified in the ayurvedic pharmacopoeia (252-253).

Nutritious elements may impact the immune system either directly or indirectly, causing changes in immunological function (254). Phytochemicals are plant-derived components that have a dynamic function in immune response modulation and disease management (255). Gas chromatography was utilised to study the phytochemistry of isolated volatile oils. The Hyphen technique connects different analytical technologies for separation and quantification (256). To screen the phytoconstituents from *N. sativa* and *P. anisum* that will contribute as immunomodulating agents, gas chromatography, mass spectroscopy, and a hyphen technique were utilized. We used TG-5MS silica columns and graded acetone solvent for the GC-MS analysis of the extracts.

For GC-MS analysis, researchers earlier used a cold macerated ethanolic extract of *N. sativa* and discovered 33 active constituents, with octadecadienoic acid methyl ester

as a major constituent (27.8%) (257). The current study observed several other phytoconstituents. The ethanolic extract of *N. sativa* was observed using GC-MS, including n-hexadecanoic acid, octadecanoic acid, ethyl ester, oxycyclononadec-10-en-2-one, and beta. Monolinolein was present in higher concentrations, whereas thymoquinone, longifolene, o-cymene, thymol, thujene, etc. were observed in average amounts. Using supercritical carbon dioxide to extract the oil from *N. sativa*, p-cymene and thymoquinone were discovered to be the main constituents (258). The GC-MS analysis of the volatile oil from *N. sativa* in this study shows that it contains the highest amounts of o-cymene, alpha-thujene, and alpha-pinene, among other chemicals.

However, the ethanolic extract of *P. anisum* shows a number of phytoconstituents, such as anethole, P-Anisaldehyde, p-Anisoin, 10(E), 12(Z)-conjugated linoleic acid, n-Hexadecanoic acid, E, E, Z-1,3, 12-Nona.decatri.ene-5,14-diol, and more. We observed anethole, anisole, methyl chavichol, and beta-terpineolin volatile oil of *P. anisum.* However, environmental variables during cultivation, post-harvest storage, and processing can all impact the phytochemical concentration (259).

Molecular docking is a computer-aided drug discovery tool useful for structure-based drug design, molecular stimulation, and quantitative structure-activity relationships (260-261). In the current investigation, we performed molecular docking studies using five proteins, named 1M48, 1P9M, 1PW6, 5UO1, and 2AZ5, and screened them against 25 ligands. These proteins were selected based upon literature claims for immunomodulatory potential (262-263). PDB ID: 1M48 is the crystal structure of human IL-2; 1P9M is the crystal structure of hexameric human IL-6; 1PW6 is a small molecular inhibitor of IL-2; 5UO1 is the crystal structure of human neuronal nitric oxide synthase; and 2AZ5 is the crystal structure of a TNF-alpha inhibitor. Considering the mechanism of action, activated T cells generate interleukin-2, which transforms native T cells into effector T cells through an immune reaction with pleiotropic effects, making it the optimal choice for kidney cancer (264). Leukocytoses, monocytoses, and macrophages make interleukin-6. Most of the time, macrophages and Th1 cells release TNF-α. And nitric oxide synthase is a backwards-firing neurotransmitter that is changed by bacterial lipopolysaccharide, cytokines, and

other things. Results showed 2D and 3D interactions with different amino acid residues.

All five proteins interact with 25 phytoconstituents from *N. sativa* and *P. anisum* and show different binding affinities. PDB ID: 1M48 binds to ligands through both hydrophobic and hydrogen bonds, and its binding strengths ranged from -4.2 to -7.6 Kcal/mol. Eicosanoic acid, ethyl ester, shows the highest binding affinity, while linolool shows comparatively low binding affinity. PDB ID: 1PW6 shows binding affinity between -3.7 to -7.6 Kcal/mol and binds via electrostatic, hydrophobic, and hydrogen Bonding to ligands n-Hexadecanoic acid, p-anisaldehyde, and alpha-pinene shows comparatively low binding affinity; on the contrary, linoleic acid, ethyl ester, shows the highest binding affinity. PDB ID: 5UO1 shows binding affinity between -5.4 to -11.1(Kcal/mol) and binds to ligands via hydrogen, hydrophobic, and electrostatic bonds. Additionally, linoleic acid, ethyl ester, nigellidine, estragole, eicosanoic acid, and ethyl ester show remarkable binding affinity, while alpha-pinene shows comparatively low binding affinity. PDB ID: 2AZ5 binds to ligands via hydrophobic and hydrogen bonds, and the binding affinities vary between -4.0 to -6.9 kcal/mol. Linoleic acid ethyl ester, estragole, and dithymoquinone show excellent binding affinity, while p-anisaldehyde and tetradecanoic acid show comparatively low binding affinity. PDB ID: 1P9M shows binding affinities between -4.7 to -9.8 kcal/mol bind to ligands via hydrogen, hydrophobic, and electrostatic bonds. Nigellamine C, linoleic acid, and ethyl ester exhibit remarkable binding affinity, whereas p-anisaldehyde demonstrates comparatively low binding affinity. Furthermore, thymoquinone shows unsatisfactory bonding with proteins. We determined ADME profiling and drug likeness, and found compounds to be non-carcinogenic and non-mutagenic in nature. *In silico* molecular docking, ADME and Lipinski rules revealed that several phytoconstituents have a higher binding affinity.

Molecular docking data revealed that the isolated volatile oil of *N. sativa* exhibited binding affinity for PDB ID:1M48 between -4.3 and -6.0, PDB ID:1P9M between -5.1 and -5.8, PDB ID:1PW6 between -3.7 and -4.5, PDB ID:5UO1 between -5.4 and -7.5, and PDB ID:2AZ5 between -4.2 and -4.8 kcal/mol.

On top of that, the binding affinities for the volatile oil of *P. anisum* vary from -4.2 to -5.2 kcal/mol for PDB ID: 1M48 to -4.7 to -6.1 kcal/mol for PDB ID: 1P9M to -3.7 to

-4.1 kcal/mol for PDB ID: 1PW6 to -6.2 to -7.4 kcal/mol for PDB ID: 5UO1 to -4.0 to -4.8 kcal/mol for PDB ID: 2AZ5. In contrast, the binding affinities of both extracts are higher than those of volatile oils. Additionally, since extracts contain the key components of oil as well as other constituents such as PUFAs, the molecular docking study came to the conclusion that the extracts are a superior option for formulation and development than volatile oils or any one phytoconstituent.

India's present scenario of circumstances refers to a move in the direction of prepared foods that are high in nutrients and ready to eat. Furthermore, the growing awareness among customers about immunomodulation and the possible health benefits of herbal extracts motivates the development of nutraceutical formulations (49). Granulation is the most important unit process for transforming powder into free-flowing particles. Granules improve density, simplify storage and shipping, and reduce hazardous exposure and process-related risks (265). Granulation is a cost-effective treatment when all other benefits are considered. We prepared cocoa granules using *Nigella sativa* ethanolic extract (NSE) and *Pimpinella anisum* ethanolic extract (PAE). We used the DOE software to optimize the 13 formulations created through central composite design, incorporating two factors and three levels.

We suggested a quadratic model for the NSEF (*Nigella sativa* ethanolic extract formulation), considering loss on drying and time of solubility as the dependent variables. For NSEF, the F-value of 292.26 for LOD and 143.24 for time of solubility and model is significant. In NSEF, the predicted R^2 of 0.9727 for LOD and 0.9156 for time of solubility is in rational conformity with the adjusted R^2 of 0.9918 and 0.9834, respectively. In *Nigella sativa* formulations, a signal-to-noise ratio of 50.393 for LOD and 40.082 for time of solubility suggests a sufficient signal.

For the PAEF (*Pimpinella anisum* ethanolic extract formulation), we proposed a quadratic model to account for the dependent variables of loss on drying and time of solubility. Moreover, the PAEF F-value of 532.12 for LOD and 207.95 for time of solubility and model is significant. In NSEF, the predicted R^2 of 0.9809 for LOD and 0.9486 for time of solubility is in rational conformity with the adjusted R^2 of 0.9955 and 0.9885, respectively. In the *Pimpinella anisum* formulation, a signal-to-noise ratio of 67.906 for LOD and 47.492 in the case of time of solubility suggests a sufficient

signal. DOE's solution was implemented, and granules were analysed; additionally, an *ex vivo* lymphocytic proliferation assay was conducted using NSEF and PAEF.

Oxygen stress almost certainly causes senescent deterioration in immune cells if it disrupts the equilibrium between the generation of free radicals and protective antioxidants (266). We investigated the antioxidant capacity of NSE and PAE using an *in vitro* antioxidant model based on DPPH and nitric oxide. In the case of the DPPH assay, ascorbic acid is considered a standard. We observed the discolouration of standard *Nigella sativa* extract (NSE) and *Pimpinella anisum* (PAE) from purple to yellow. If the color shifts from purple to yellow, it indicates a strong positive response, whereas a pale color indicates a weak positive response. Pink was classified as a weak positive response. Elisa's plate reader measured the plates at 490 nm. Their DPPH radical scavenging activity was found to be 61.16 for ascorbic acid, 63.06 for *Nigella sativa* and 56.04 for *Pimpinella anisum* at 1000 µg/ml. while the concentrations were tested within a range of 62.5 -1000 µg/ml and their percentage radical scavenging activity was plotted in a graph. Furthermore, in the nitric oxide assay, NSE has a higher antioxidant potential than PAE, and its concentrations range from 200 to 1000 g/ml. We further extended the study to evaluate THP1 cell lines *in vitro* and estimate IL-2, IL-4, and INF-using ELISA techniques.

A human monocyte leukemia cell line THP-1 undergoes differentiation into macrophage-like cells when treated with phorbol esters. THP-1 cells exhibit native monocyte-derived macrophage behaviour when differentiated (267). LPS activates them through the activation of NF-B, a crucial transcription factor that triggers the production of effector genes, and TLR-4 (268). The cytokines interleukin-2 (IL2) and interleukin-4 (IL4) belong to the four-helix bundle family, and their receptors resemble one another (269). These proteins are critical in immune system regulation because they aid in regulating the pace of lymphocyte clonal proliferation, among other things. They are therefore of interest as trans-membrane signalling proteins, as well as potential pharmaceutical targets. The current study looked at how NSE (an ethanolic extract from *Nigella sativa*) and PAE (an ethanolic extract from *Pimpinella anisum*) affected the THP1 cell line. IL-4 controls the production of antibodies, the growth of effector T-cell responses, blood cell production, and inflammation (270). Interleukin 2 (IL-2) was originally thought to be a significant pro-inflammatory agent

since it not only encourages T cell growth and NK cell function but also strengthens the body's antitumor immune response. INF-α is important for host defence as it shows antiviral potential. Based on the results, it was clear that NSE reduced the release of IL-2 and INF-1 and improved IL-4 in LPS-stimulated THP-1-derived macrophages. In the case of PAE, ELISA estimates showed a decrease in IL-4 production and an increase in IL-2 and INF-production in THP-1 cells.

Additionally, we conducted an *ex vivo* lymphocytic proliferation assay to bolster the pharmacological study. We conducted the acute oral toxicity study in accordance with OECD 423 guidelines for NSEF and PAEF at doses of 2000 mg/kg, which resulted in a healthy outcome without any adverse effects. Furthermore, we screened the prepared cocoa granules using the more stable WST (water-soluble tetrazolium salt) assay. The dose was significantly increased from 50 µg/ml to 100 µg/ml, 200 µg/ml, and 400 µg/ml to 1600 µg/ml. The data was statistically analysed using one-way ANOVA followed by Tukey's multiple comparisons test, DFn = 2, *P<0.0001 as compared to NSEF and PAEF. Lymphocyte proliferation was significantly high at 400µg/ml with NSEF and PAEF, as further shows graded results. Furthermore, lymphocyte proliferation was significantly higher for both formulations at a dose of 1600µg/ml as compared to anti-CD3. NSEF shows higher significance compared to PAEF.

The ethanolic extract of *N. sativa* and *P. anisum* contains several kinds of phytoconstituents. Current research uses anethole and P-anisaldehyde as standards to quantify these compounds in formulations and extracts, taking into account the data retrieved from GC-MS analysis. We developed and validated analytical methods based on linearity, accuracy, precision, LOD, and LOQ. Analytical estimation of NSEF and PAEF was carried out for quantification, and gas chromatography was utilized. We plotted the calibration curve of the anethole at concentrations of 2, 3, 4, 5, and 6 PPM, yielding the equation Y = 0.9674x -0.5156 and R^2 = 0.998. Conversely, we plot the calibration curve of P-anisaldehyde using concentrations of 2, 4, 6, 8, and 10 PPM, yielding an equation Y = 38.545x -20.879, R^2 = 0.9929. We determined the unknown concentration of the standard in the extract and formulation using the area under curve method. The findings suggested that cocoa granule formulation (PAEF) contains 1.53% P-anisaldehyde, whereas the ethanolic extract of *P anisum* contains

27.42%. Additionally, the formulation of *N. sativa* NSEF includes 0.09% anethole, and the ethanolic extract has 4.35% anethole.

CHAPTER – 7

SUMMARY AND CONCLUSION

7. SUMMARY AND CONCLUSION

This section summarises the results obtained after performing thorough research work. Both the plant *Nigella sativa* and *Pimpinella anisum* possess numerous healthy benefits hence in present research work sticks to multidisciplinary approach and work up on Pharmacognostic, Molecular docking, Pharmaceutical, Pharmacological, and Analytical approach. In current research work, seed part of both the plants *Nigella sativa* and *Pimpinella anisum* were standardised using several qualitative and quantitative investigation methods as per official books and the results are as per specified in The ayurvedic pharmacopoeia of India. The ethanolic extract and volatile oil of both the plant were collected and quantified using gas chromatography mass spectroscopy (GC-MS). The result indicates that *Nigella sativa* and *Pimpinella anisum* contains several Terpenoids, flavonoids, polyphenols, MUFAs and PUFAs.

Additionally to claim its Immunomodulatory potential molecular docking studies were performed using five proteins PDB ID: 1M48 (IL-2), 1P9M (IL-6), 1PW6 (IL-2 inhibitor), 5UO1 (NO synthase), and 2AZ5 (TNF-α inhibitor) and 25 ligands were performed. These ligands were selected based on Phytochemistry of *N. sativa* and *P. anisum* and its GC-MS findings. As the protein 1M48 represents IL-2 and 1PW6 represents IL-2 inhibitors the results were far differ from each other. Nigellamine C, and linoleic acid, ethyl ester were shows excellent binding affinity as IL-2 inhibitors on contrary estragole, eicosanoic acid, ethyl ester and curcumin were shows excellent binding affinity as IL-2 stimulant. Nigellamine C and linoleic acid, ethyl ester were admirable IL6 stimulant. Amounts 25 ligands nigellamine C, nigellidine, eicosanoic acid ethyl ester, linoleic acid ethyl ester, dithymoquinone and estragole shows highest binding affinity and can be significant NO synthase stimulant. Dithymoquinone, estragole and linoleic acid ethyl ester shows highest binding affinity and can be decent TNF-α inhibitor. *In silico* screening of 25 ligands for its pharmacokinetic, pharmacodynamics and toxicity prediction were carried out. Findings suggested that all ligands were found to be non-carcinogenic, non-mutagenic in nature and can be serve as drug. Molecular docking studies showed that the volatile oil from *N. sativa* had binding affinities for five different proteins that ranged from -3.7 to -7.5 kcal/mol. Although *P. anisum's* volatile oil has binding affinities that range from -3.7 to -7.4 kcal/mol, these values are quite low when compared to extracts. Additionally, since

extracts contain the key components of oil as well as other constituents such as PUFAs, the molecular docking study came to the conclusion that the extracts are a superior option for formulation and development than volatile oils or any one phytoconstituents.

The cocoa granules incorporating 5% of ethanolic extract were prepared using *Nigella sativa* and *Pimpinella anisum* naming NSEF and PAEF respectively. Both the formulations complies all the evaluation parameters. DOE software was used to optimise the formulation using central composite design. The primary advantage of central composite design over factorial design is that it is more accurate, and no three-level factorial experiment is required. The final solutions suggested by DOE were prepared, and granules were analysed; furthermore, an *Ex vivo* lymphocytic proliferation assay was conducted using NSEF and PAEF.

Using an *in vitro* antioxidant model based on DPPH and nitric oxide, the antioxidant capacity of NSE and PAE was investigated. Ascorbic acid was considered as standard in DPPH assay. *Pimpinella anisum* (PAE), and *Nigella sativa* extract (NSE) were examined for discoloration from purple to yellow using Elisa's plate reader at 490 nm. The IC50 values were calculated using the concentrations range varying in between 62.5 to 1000 µg/ml. The results for IC50 value were found to be 7.67 to 105.39 for ascorbic acid which is serving as standard, 7.16 to 99.70 for *Nigella sativa*, and 10.65 to 140.98 for *Pimpinella anisum* ethanolic extract. Additionally nitric oxide assay of NSE shows higher antioxidant potential compared to PAE and its concentrations varies between 200-1000µg/ml and results indicates that both the extract shows graded antioxidant response.

Additionally, the acute toxicity profile of formulations NSEF and PAEF was tested in albino mice in accordance with OECD 423 criteria in order to define the safety profile and LD_{50} value. The acute oral toxicity study showed no signs of toxicity at doses of 2000 mg/kg and mice were healthy and active. The *in-vitro* immunomodulatory studies of both extracts were carried out using THP 1 cell line study and its estimation was carried out by ELISA. The MTT assay was performed to determine cell viability and both the extracts i.e. NSE and PAE shows viability up to 25 µg /mL. To comprehend the impact of NSE and PAE on LPS Stimulated THP-1 cells the estimation of IL-2, IL-4 and IFN-α production were studied. As THP-1-derived

macrophages were treated with LPS separately for 24 hours, the production of IL-4 was increased but their release of IL-2 and IFN was decreased in comparison to control cells. The treatment with PMA and LPS increased the overproduction of IL- 2 and INF – α in THP-1-derived macrophages. On the other hand, after THP-1 exposure to NSE, the anti-inflammatory cytokine IL-4 production was dramatically amplified. Whereas the effect was reverse in case of PAE treatment Where IL- 4 production decreased and IL – 2 increase in comparison to control cells. IFN – α in PAE did not show any varied results. The *in-vitro* outcomes suggest that both the extracts were found to be highly potent immunomodulatory potential. The *N. sativa* was found to have opposite results in comparison to the *P. anisum* in *in-vitro* THP 1 cell line study. The *ex-vivo* immunomodulatory potential of cocoa granules of *N. sativa* and *P. anisum* were estimated using Lymphocytic proliferation assay. Findings suggested that formulation PAEF and NSEF shows Lymphocytes proliferation was significantly high at 400µg/ml. Lymphocytes proliferation was significantly high for both formulations at the dose of 1600µg/ml as compared to anti- CD3. NSEF shows higher significance compared to PAEF.

For analytical estimation of P-anisaldehyde and anethole in *P anisum* and *N. sativa* respectively gas chromatography were employed. Analytical method were developed and validated via linearity, accuracy, precision, LOD and LOQ. Calibration curve of P-anisaldehyde and anethole were plotted and analytical estimation of NSEF and PAEF were carried out for quantification gas chromatography was utilized. Using the area under curve method the unknown concentration of standard was determined in extract and formulation. The finding suggested that cocoa granules formulation (PAEF) contains 1.53% P-anisaldehyde while the ethanolic extract of *P anisum* has 27.42%. Additionally, the formulation of *N. sativa* NSEF includes 0.09% anethole and the ethanolic extract has 4.35% anethole.

CONCLUSION

In conclusion, both ethanolic extracts of *N. sativa* and *P. anisum* have numerous phytoconstituents, while with the cold maceration method, fewer PUFAs, MUFAs, terpenoids, and polyphenols were identified using GC-MS. From *in silico* prediction, we can conclude that estragole and eicosanoic acid ethyl ester show significant binding affinity against IL2 and hence can be further screened for metastatic renal cell carcinoma. IL-6 is excellent as a host defence by producing acute inflammation; hence, nigellamine C and linoleic acid ethyl ester can be screened further for their inflammatory properties as they show good binding affinity. NO synthase was known to regulate vasomotor tone, decrease platelet aggregation, and promote vascular smooth muscle cell proliferation. Nigellamine C, nigellidine, eicosanoic acid ethyl ester, linoleic acid ethyl ester, dithymoquinone, and estragole show tremendous binding affinity; in the future, they can be screened against cardiovascular disease. The major function of TNF-α inhibitors is to reduce inflammation. The phytoconstituents such as dithymoquinone, estragole, and linoleic acid ethyl ester show decent binding affinity; hence, these constituents can be screened against arthritis, rheumatoid arthritis, psoriasis, ulcerative colitis, Crohn's disease, etc. Considering their anti-oxidant activity, both NSE and PAE show good antioxidant potential. The pharmacological evaluation of *N. sativa* and *P. anisum* suggested that both extracts screened for their *in vitro*THP 1 cell line study. MTT assay shows both extracts NSE and PAE shows viability up to 25 µg /mL.Concluding the *in vitro* THP 1 cell line study NSE produces anti-inflammatory response by producing IL4 and productions of antibody were enhanced. Additionally IL2 production is reduce hence minimum or no antiviral and anticancer potential may be present in NSE. On contrary PAE have antiviral and anticancer potential due to increase in IL2 and INF-α production. The cocoa granules were prepared using 5% of *N. sativa* and *P. anisum* extract and optimized with the help of DOE using centre composite design. The formulations (NSEF and PAEF) containing 5% of extract were tested for its acute oral toxicity study and results concluded that, there was no signs of toxicity at dose 2000µg/ml and mice were healthy and active. Hence, concluding that the prepared cocoa granules of NSEF and PAEF were safe for consumption. This data of toxicity

study support for further *ex vivo* lymphocytic proliferation assay. However in the case of *ex vivo* lymphocytic proliferation assay NSEF and PAEF, both formulations show extraordinary proliferation rate at 400µg/ml with graded response and can be used as nutraceutical formulations to boost immune function.

IL2 has been used for autoimmune diseases and systemic lupus erythematosus at low dose hence talking about its future scope PAE can be a candidate for autoimmune disease screening. Additionally it may possess antiviral and anticancer potential hence PAE can be screen as anticancer drug using several cell line, while NSE can be screen for its anti-inflammatory, anti-asthmatic potential. Considering the molecular docking results and phytochemical prolife from GC-MS the PUFAs were shows good binding ability and they can screen further for immunomodulatory potential.

CHAPTER - 8

BIBLIOGRAPHY

8. BIBLIOGRAPHY

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Outward No. : SPCOP/2023-24/07 Date : 09/06/2023

CERTIFICATE

This is to certify that the project proposal entitled **"Evaluation of Anti-Inflammatory Activity of Medicinal Plant Extract and Isolated Compound on Thioglycollate Induced Balb/C Mice."** Part of dissertation entitled **"Development and Evaluation of Herbal Formulation for Immunomodulatory Activity"** Submitted by **Mrs. Snehal Kashid** under the Guidance of **Dr. Sumit Ashok Joshi** has been approved by the IAEC having IAEC approval No- **1197/PO/Re/S/08/CCSEA**

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प्रमाणपत्र / CERTIFICATE

यह प्रमाणित किया जाता है कि/ This is to certify that Ms. Snehal Uttam Kashid Research student from L P U, Punjab. Asstt. Prof. at SPMS College of Pharmacy, Akluj, Maharashtra के द्वारा प्रस्तुत कि ये गये नमुने, हमारे विशेषज्ञ द्वारा निम्नलिखित अनुसार पहचाना गया/ पहचाने गये है।/ the specimen/ specimens submitted by aforesaid is/ are identified by our expert/s as:

नमुना नंबर Specimen No.	वनस्पति का नाम Plant Name	कुल Family
SUK 01	**Pimpinella anisum** L.	Apiaceae

06.07.2022

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प्रमाणपत्र/CERTIFICATE

यह प्रमाणित किया जाता है कि/This is to certify that Ms. Snehal Uttam Kashid, Ph.D Researcher from LPU, Punjab. Asstt. Prof. at SPM's College of Pharamacy, Akluj. के द्वारा प्रस्तुत किया गया नमुना/किये गये नमुने, हमारे विशेषज्ञ एवं वैज्ञानिक द्वारा निम्नलिखित अनुसार पहचाना गया/पहचाने गये है।/the specimen/specimens submitted by aforesaid is/are identified and authenticated by our expert's & Scientist as :

नमुना संख्या Specimen No.	वनस्पति का नाम Plant Name	वानस्पतिक कुल Family
SUKNS-1	**Nigella sativa L.**	Ranunculaceae

30.05.2022.

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